

Microbiological Quality & Safety in Dairy Industry



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Microbiological Quality And Safety In Dairy Industry

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Lesson 1

CONSUMER AWARENESS ABOUT QUALITY AND SAFETY OF DAIRY FOODS: THE CHANGING SCENARIO

1.1 Introduction

India has a rich cultural heritage and the people believe in consuming home-made foods. Such foods are considered more nutritious and safe compared to processed foods as currently consumed in the western world. The food which we eat everyday has tremendous impact on our physical, mental and spiritual health. Currently, food consumption pattern, ever increasing demands on quality and safety are under continuous transformation and need critical appraisal to overview and timely adoption of corrective and preventive action in food supply chain. Consumer's preference of processed food with new requirements especially minimally processed/ cost effective foods without chemical preservatives with enhanced functional and therapeutic features has put up pressure on manufacturer to adopt all ways and means to ensure quality and safe food to the consumers. These consumer demands are forcing the manufacturer to implement new quality (ISO 9001:2000) and food safety system (HACCP) during various stages of supply chain from milch animal to consumer.

Further, consumer movement across the globe has also increased in recent past and as a result export/ import of food products has become more vulnerable in complying safety requirements of the consumers. Globalization of the food supply chain, the increasing importance of the Codex Alimentarius Commission and the obligations emerging from the World Trade Organization (WTO) agreements have resulted in unprecedented interest in the development of food standards and regulation, and the strengthening of food control infrastructure at the country level. The challenges for food control authorities include:

1. increasing burden of food borne illness and emergence of new food borne hazards;
2. rapidly changing technologies in food production, processing and marketing;
3. developing science-based food control systems with a focus on consumer protection;
4. international food trade and need for harmonization of food safety and quality standards;
5. changes in lifestyles, including rapid urbanization and
6. growing consumer awareness of food safety and quality issues and increasing demand for better information.

1.2 National Food Control System

Effective national food control systems are essential to protect the health and safety of domestic consumers. These are also critical in enabling countries to assure the safety and quality of their foods entering international trade and to ensure that imported foods conform to national requirements. The new global environment for food trade places considerable obligations on both importing and exporting countries to strengthen their food control systems and to implement and enforce risk-based food control strategies. To comply with these international requirements Food Safety and Standard Act 2006 has been enacted by the government of India to ensure quality and safe food to the consumers. Food Safety and Standard Authority of India (FSSAI) has been mandated by the FSS Act 2006 for performing functions like to consolidate various acts and orders that have previously handled food related issues in various ministries and departments. FSSAI has been created for laying down science based standards for articles of food and to regulate their manufacture, storage, distribution, sale and import to ensure availability of safe and wholesome food for human consumption. The development of national food control system based on scientific principles and guidelines and which address all sectors of the food chain are urgently required particularly in developing and under-developed countries to achieve improved food safety, quality and nutrition.

1.2.1 Food control

Food control is defined as a mandatory regulatory activity of enforcement by national or local authorities to provide consumer protection and ensure that all foods during production, handling, storage, processing, and distribution are safe, wholesome and fit for human consumption; conform to safety and quality requirements; and are honestly and accurately labelled as prescribed by law.

The foremost responsibility of food control is to enforce the food law(s) protecting the consumer against unsafe, impure and fraudulently presented food by prohibiting the sale of food not of the nature, substance or quality demanded by the purchaser.

Confidence in the safety and integrity of the food supply is an important requirement for consumers. Food borne disease outbreaks, involving agents such as *Escherichia coli*, *Salmonella* and chemical contaminants, highlight problems with food safety and increase public anxiety that modern farming systems, food processing and marketing do not provide adequate safeguards for public health. Factors which contribute to potential hazards in foods include improper agricultural practices; poor hygiene at all stages of the food chain; lack of preventive controls in food processing and preparation operations; misuse of chemicals; contaminated raw materials, ingredients and water; inadequate or improper storage, etc.

Specific concerns about food hazards have usually focused on:

1. Microbiological hazards;
2. Xenobiotic residues including synthetic (manmade) pesticides, drugs, antibiotics, plastics etc.
3. Misuse of food additives
4. Chemical contaminants, including biological toxin; and
5. Adulteration, artificial foods
6. The list has been further extended to cover genetically modified organisms, allergens, veterinary drug residues, radionuclides and growth promoting hormones used in the preparation of animal products

Consumers expect protection from hazards occurring along the entire food chain, from primary producer through consumer (often described as the farm-to-table continuum). Protection will only occur if all sectors in the chain operate in an integrated way and food control systems address all stages of this chain. As no mandatory activity of this nature can achieve its objectives fully without the cooperation and active participation of all stakeholders e.g. farmers, industry and consumers, the term Food Control System is used to describe the integration of a mandatory regulatory approach with preventive and educational strategies that protect the whole food chain.

Thus an ideal food control system should include effective enforcement of mandatory requirements, along with training and education, community outreach programmes and promotion of voluntary compliance. The introduction of preventive approaches such as the Hazard Analysis Critical and Control Point (HACCP) system have resulted in industry taking greater responsibility for and control of food safety risks. Such an integrated approach facilitates improved consumer protection, effectively stimulates agriculture and the food processing industry and promotes domestic and international food trade.

1.3 Global Considerations

With an expanding world economy, liberalization of food trade, growing consumer demand, developments in food science and technology and improvements in transport and communication international trade in fresh and processed food will continue to increase. Access of countries to food export markets will continue to depend on their capacity to meet the regulatory requirements of importing countries. Creating and sustaining demand for their food products in world markets relies on building the trust and confidence of importers and consumers in the integrity of their food systems. Such food protection measures are essential in view of agricultural production being the focal point of the economies of most under developing countries.

1.3.1 Codex Alimentarius Commission

The Codex Alimentarius Commission (CAC) is an inter-governmental body that coordinates food standards at the international level. Its main objectives are to protect the health of consumers and ensure fair practices in food trade. The CAC has proved to be most successful in achieving international harmonization in food quality and safety requirements. It has formulated international standards for a wide range of food products and specific requirements covering pesticide residues, food additives, veterinary drug residues, hygiene, food contaminants, labelling etc. These Codex recommendations are used by governments to determine and refine policies and programmes under their national food control system. More recently, Codex has embarked on a series of activities based on risk assessment to address microbiological hazards in food an area previously unattended. Codex work has created worldwide awareness of food safety, quality and consumer protection issues and has achieved international consensus on how to deal with these scientifically through a risk-based approach. As a result, there has been a continuous appraisal of the principles of food safety and quality at the international level. There is increasing pressure for the adoption of these principles at the national level.

1.3.2 SPS and TBT Agreements

The conclusion of the Uruguay Round of Multilateral Trade Negotiations in Marrakech led to the establishment of the WTO on 1 January 1995 and to the coming into force of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) and the Agreement on Technical Barriers to Trade (TBT). Both these Agreements are relevant in understanding the requirements for food protection measures at the national level, and the rules under which food is traded internationally. The SPS Agreement confirms the right of WTO member countries to apply measures to protect human, animal and plant life and health. The Agreement covers all relevant laws, decrees, regulations; testing, inspection, certification and approval procedures; and packaging and labelling requirements directly related to food safety. Member States countries are asked to apply only those measures for protection that are based on scientific principles, only to the extent necessary, and not in a manner which may constitute a disguised restriction on international trade. The agreement encourages use of international standards, guidelines or recommendations where they exist and identifies those from Codex (relating to food additives, veterinary drugs and pesticide residues, contaminants, methods of analysis and sampling and codes and guidelines of hygienic practices) to be consistent with provisions of SPS. Thus, the Codex standards serve as a benchmark for comparison of national sanitary and Phytosanitary measures. While it is not compulsory for member states to apply Codex standards, it is in their best interests to harmonize their national food standards with those elaborated by Codex. The TBT Agreement requires that technical regulations on traditional quality factors, fraudulent practices, packaging, labelling etc. imposed by various countries will not be more restrictive on imported products than they are on products produced domestically. It also encourages use of international standards.



Lesson 2
CONCEPTS OF QUALITY CONTROL, QUALITY ASSURANCE AND FOOD SAFETY

2.1 Introduction

With the rising liberalization of agro-industrial markets and thus the world-wide integration of food supply chains, the assurance of food quality and safety has become a major concern. Following serious and repeated incidents such as mad cow disease (Bovine Spongiform Encephalitis–BSE), Dioxin, Aflatoxin and most recently, Sudan Red consumer protection has become a priority in policy making in the large consumer markets. The recent occurrence of serious food scares and food contamination events – such as *Salmonella* contagion of peanut butter in the US, melamine contamination of milk in China and high pesticide content of aerated drinks manufactured in India – has significantly enhanced the concern for food safety and its impact on health, marketing and foreign trade. Protecting consumer health from food borne hazards has become a compelling duty for policy makers across the globe. Consequently, regulatory frameworks and standards are being developed wherein trade and health issues are being addressed by prioritizing consumer protection over freedom of trade. Thus, it has become imperative for the Indian industry and policy makers to adopt strong practices of food safety so as to remain sustainably competitive both in domestic and export markets. In this context, it is essential to have a close look at the recent changes in food safety regulations adopted in India which if effectively implemented will not only protect domestic consumers from food contamination hazards, but also become instrumental in making India meet international standards of food safety.

Hence, legal requirements for quality assurance systems and food control along the entire food chain, from seed and agricultural production, through food processing and the distribution system, up to the consumers’ table, are increasing considerably. Major prerequisite for ensuring food quality and safety is that all stakeholders in the food supply chain recognize that primary responsibility lies with those who produce, process and trade food and that public control should be based on scientific risk assessment (Fig. 2.1).

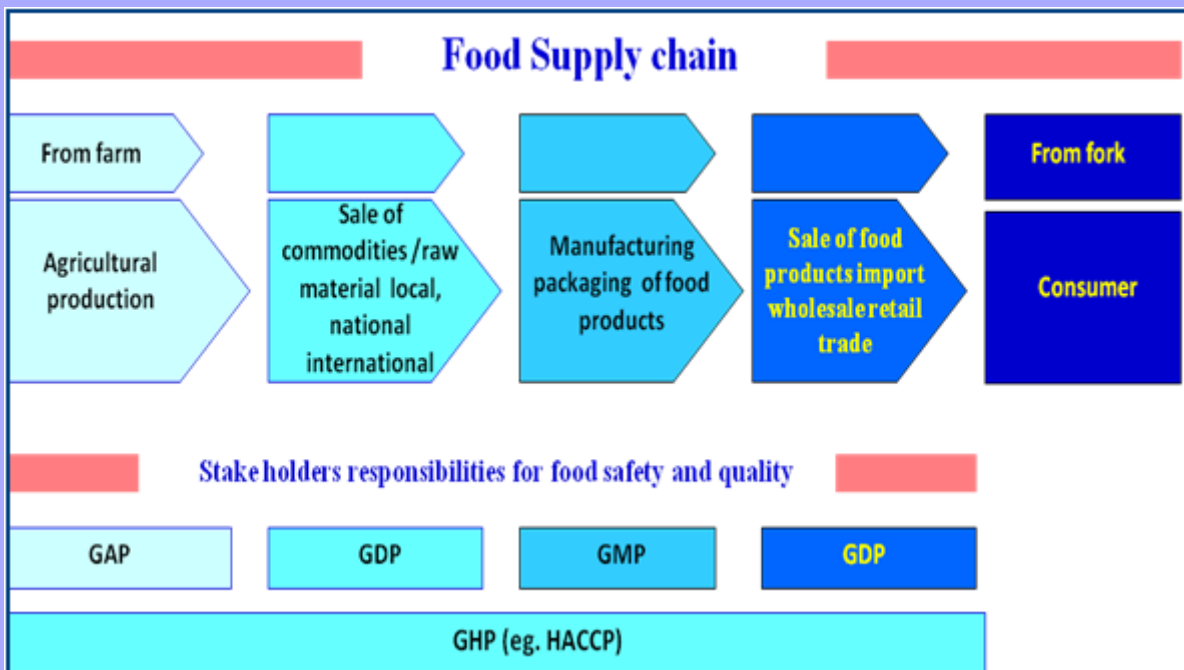


Fig. 2.1 Food Supply Chain and operators’ responsibility for food quality and safety (Source Will and Guenther, 2007)

GAP = Good Agricultural Practices
GDP = Good Distribution Practices
GMP = Good Manufacturing Practices
GHP = Good Hygienic Practices

Operators' responsibilities cover the whole food supply and marketing chain from primary production to final consumption and encompass all actors in exporting and importing countries. Public and private standards are subject to continuous changes as a result of on-going process of liberalization of the world trade to establish cost-effective supplier-buyer linkages and to gain a competitive edge. Globalization of the food supply chain, the increasing importance of the Codex Alimentarius Commission and the obligations emerging from the World Trade Organization (WTO) agreements have resulted in unprecedented interest in the development of food standards and regulations and the strengthening of food control infrastructure at the country level.

2.2 Food Safety, Quality and Consumer protection

Food safety provides an assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. Safety is a component of quality. In fact, many experts have argued that safety is the most important component of quality since a lack of safety can result in serious injury and even death for the consumer of the product. Safety differs from many other quality attributes since it is a quality attribute that is difficult to observe. A product can appear to be of high quality, i.e. well colored, appetizing, flavorful, etc. and yet be unsafe because it is contaminated with undetected pathogenic organisms, toxic chemicals, or physical hazards. On the other hand, product that seems to lack many of the visible quality attributes can be safe. Obvious quality defects can result in consumer rejection and lower sales, while safety hazards may be hidden and go undetected until the product is consumed. Since assuring safety is vital to public health, achieving safety must always take precedence over achieving high levels of other quality attributes. Food safety is not limited to microbiological safety. As recent history has demonstrated with bovine spongiform encephalitis (BSE) and Variant Creutzfeldt-Jakob disease (vCJD), anaphylactic shock from eating peanuts, dioxins entering the human food chain via animal feedstuffs, benzene in mineral water and glass fragments in baby food, food safety also includes chemical contamination and foreign bodies. Prions, the cause of BSE and vCJD, are an entirely new source of food-borne disease. Food-borne viruses are becoming recognized as significant to public health. As the examples of benzene and dioxins demonstrate, food safety is not necessarily about real risk to public health, but also about perceived risk.

2.2.1 New quality and food safety approaches

The aspects of liberalization of the global trade and the fact that the consumers in the industrialized countries are more and more demanding food to be not only economical, but also healthy, tasty, safe and sound in respect to animal welfare and the environment, are changing the so far quantity-oriented food production, guaranteeing the nutrient supply for a nation, into an international quality-oriented food market where commodities, production areas, production chains and brands compete with one another. The competitiveness of food production will soon be more dependent on the reliability of the safety and the quality of the food and acceptability of the production procedures than on quantity and price. In contrast to the quantity-oriented markets that are often subsidized and producers can always sell everything they produce, quality-oriented markets are market-driven or demand. Thus, apart from the steady increase of the national and international standards for food safety and public health, there is a growing influence of the consumer's demands on the production, its allied industries, advisers, consultants and marketing bodies. All of this means that the agricultural supply of food production is facing remarkable changes in the years to come, which is both challenge and opportunity for food producers, packing plants and processors as well as for the dairy and food profession.

2.2.2 Necessities for new approach

There are five major reasons for this need:

1. Despite the generally recognized achievements in making food safer over the decades with the mandatory inspection and the principles of food hygiene being the most successful means in protecting

the consumer against food-borne health risks, there are still deaths due to food-borne disease in man. Furthermore, the consumer's confidence in the safety of food is getting sceptical;

2. Modern agriculture is contributing to the increase of drug-resistant pathogens in humans, and, thus often being attacked by the medical society and consequently by the public;
3. Food safety issues can easily become non-tariff trade barriers and are increasingly used as marketing tools, nationally and internationally;
4. The consumer has the tendency to ask more and more for fresh and naturally raised products;
5. The traditional mandatory inspection still is indispensable, but unable to control and prevent the emerging food-borne pathogens that nowadays pose risks to human health.

2.3 Quality Control and Quality Assurance Concepts

Quality is defined by the International Organization for Standardization (ISO) as —the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs/ or the operational techniques and activities that are used to satisfy quality requirements. A food industry quality management system is an integrated set of documented food quality and food safety activities with clearly established inter relationships among the various activities. The objective of a quality system is to provide a food company with the capability to produce a food that fulfills all quality and safety requirements (Fig 2.2).

Time	Early 1900s	1940s	1960s	1980s and beyond
Focus	Inspection	Statistical sampling	Organisational quality focus	Customer driven quality
Concept	Old concept of quality: Inspect for quality after production.			New concept of quality: Build quality into the process.
Approach	Reactive approach – Quality control (QC) (QC-set of activities intended to ensure that quality requirements are actually being met)			Proactive approach – Quality Assurance (QA) (QA-set of activities intended to establish confidence that quality requirements will be met)

Fig. 2.2 Quality assurance concept in relation to changes in global food safety standards

2.3.1 Quality control

Quality control is the evaluation of a final product prior to its marketing, i.e. it is based on quality checks at the end of a production chain aiming at assigning the final product to quality categories such as "high quality", "regular quality", "low quality" and "non-marketable". Since, at the end of the production chain, there is no way to correct production failures or upgrade the quality of the final product, the low-quality products can only be sold at lower prices and the non-marketable products have to be discarded. Their production costs, however, had been as high as those of the high and regular quality products. Thus, quality control has only a limited potential to increase the quality and efficiency of a multi-step production procedure.

2.3.1.1 Rules of quality control

1. The dominant raw material(s) are selected for priority of attention
2. The selected raw materials are tested in relation to their contribution to product quality.
3. The raw materials tested are released from the stores only after the test results have been properly recorded.
4. Process control must relate the processing results to the raw materials test.
5. Define the critical points in the process and concentrate on these.
6. Finished product inspection should be reduced to the minimum level compatible with the confidence justified by the raw materials and process control.
7. Quality control is effective in proportion to its degree of integration into the overall organization of the factory.

2.3.2 Quality assurance

The ISO definition reads: "the assembly of all planned and systematic actions necessary to provide adequate confidence that a product, process, or service will satisfy given quality requirements." Quality Assurance, in contrast to quality control (Table 2.1), is the implementation of quality checks and procedures to immediately correct any failure and mistake that is able to reduce the quality of the interim products at every production step. Thus, the desired high quality of the final product is planned and obtained.

2.3.3 Quality control versus Quality assurance

Table 2.1 Comparison between quality control and quality assurance

Quality Control	Quality Assurance
Product oriented	Process oriented
Reactive approach	Proactive approach
Corrective action	Preventive action
Focuses on testing for quality	Focuses on building in quality
Detects defects	Prevents defects
Meant for implementing the process developed by a team	Meant for developing and organizing the best quality process
Makes sure that the results of what you have done are what you expected	Makes sure that you are doing the right thing the right way



Lesson 3

GLOBAL QUALITY AND FOOD SAFETY STANDARDS: AN OVERVIEW

3.1 Introduction

With India being a member of the Codex Alimentarius Committee since 1970, the Ministry of Health and Family Welfare (FSSAI acting as the National Codex Contact Point), has the primary responsibility for determination of government policy relating to food standards and enforcement of food control including national position on various issues relating to Codex. With the global food industry looking towards India as a food hot-spot, it is about time the national food legislation is aligned with Codex, encouraging innovation and facilitating trade without compromising consumer safety. Whilst formulating and implementing a single unified standard is a prodigious task, one of the major concerns of the industry which needs to be addressed by the government while finalizing the Food Safety and Standards Regulations, 2010 is tuning of international best practices with the domestic ground realities. Both the domestic and international industry is looking forward to FSSAI for the harmonization of Indian food standards for all food categories with the Codex Alimentarius Commission (CAC) standards. CAC is regarded as the 'World Authority' on food standards (Joint FAO/ WHO Food Standard Programme). Codex's focused objectives of (1) protecting consumers and (2) facilitating trade are shared by member countries and its standards based on scientific evidence and risk analysis principles are followed and/or adopted partially or in totality by countries around the world. The WTO in its Sanitary and Phytosanitary (SPS) Agreement recognizes the Codex standards as the global reference standards for consumers, food producers, processors, national food control agencies and all others involved in international food trade. The Agreement on the Application of SPS Measures and the Agreement on Technical Barriers to Trade (TBT) also encourage the international harmonization of food standards. Codex standards have thus become the benchmarks against which national food control measures and regulations are evaluated under the relevant provisions of the WTO Agreements.

3.2 Setting public health goals

3.2.1 Concept of appropriate level of protection

During the past decade, there has been increased interest and effort in developing tools to more effectively link the requirements of food safety programs with their expected public health impact. An appropriate level of protection (ALOP), expressed in terms of a desired reduction in the current level of risk, could be defined as the food safety goal. An ALOP is currently defined as 'a statement of the degree of public health protection that is to be achieved by the food safety system'. An explicit description of an ALOP may be in terms of the probability of an adverse public health consequence or the incidence of disease (e.g. the number of cases per 1,00,000 populations per year). Translation of an ALOP into a Food Safety Objective (FSO), expressed in terms of the required level of hazard control in food, provides a measurable target for producers, manufacturers and control authorities. An FSO is defined as 'the maximum frequency and/or concentration of a microbiological hazard in a food at the time of consumption that provides an appropriate level of protection'. An alternative definition of an FSO might be a limit to the prevalence and the average concentration of a microbial hazard in food, at an appropriate step in the food chain at or near the point of consumption that provides the appropriate level of protection. This ALOP has also been called 'acceptable level of risk' (ALR). This term is similar to the expression 'tolerable level of risk' (TLR) preferred by the ICMSF, because it recognizes that risks related to the consumption of food are seldom accepted, but at best tolerated. One of the tasks of governmental risk managers is thus to decide upon what is adequate, appropriate or tolerable in terms of food safety or health risk. How they have to do this is not described in detail by the WTO or the Codex. However, the determination of ALOP/ TLR should be science based, should include economic and social factors and should minimize negative trade effects. Integral to the agreement is that imported food should not compromise the ALOP. An exporting country can contest an importing country's judgment that a food is not meeting the ALOP by using scientific methods such as risk assessment, Codex standards, codes and guidelines. A country cannot demand that imported foods are 'safer' than similar domestically produced foods.

3.3 Concept of Risk Analysis

Risk analysis (RA) and its component parts (risk assessment, risk management and risk communication) should be used as a tool in evaluating and controlling microbiological hazards. A risk-management based approach (Fig. 3.1) is required to develop recommendations to ensure consumer protection and facilitate fair practices in the food trade. This structured approach may employ microbiological risk assessment and may utilize a spectrum of risk communication products including guidance documents, codes of hygiene practice, food safety objectives (FSO) and microbiological criteria. Some general guidelines used to manage pathogens in foods have been described by ICMSF (2002), indicating the respective roles of industry and government. A series of steps is described, including:

1. analysis of epidemiological data which may give rise to concern for public health or a need for improved controls;
2. risk evaluation by an expert panel or through quantitative risk assessment;
3. establishment of an FSO when necessary;
4. assessing whether the FSO is technologically achievable through preliminary process and/or product formulation criteria and
5. if the FSO is achievable, establishment of process/ product requirements.



Fig. 3.1 Principle of Risk assessment described by Codex Alimentarius Commission

3.3.1. Risk assessment

Risk assessment is the characterization and estimation of potential adverse health effects associated with exposure of individuals or populations to hazardous materials or situations. Risk assessment of microbiological hazards in foods has been identified as a priority area of work by the CAC. Risk assessment for microbiological hazards in foods is defined by the CAC as a scientifically based process consisting of four components: hazard identification, exposure assessment, hazard characterization, and risk characterization.

I. Hazard identification is predominantly a qualitative process intended to identify microorganisms or microbial toxins of concern in food or water. It can include information on the hazard of concern as well as relevant related data, such as clinical and surveillance data.

II. Exposure assessment should provide an estimate, with associated uncertainty, of the occurrence and level of the pathogen in a specified portion of food at the time of consumption, or in a specified volume of water using a production-to-consumption approach. While a mean value may be used, more accurate estimates will include an estimate of the distribution of exposures. This will typically include identification of the annual food and water consumption frequencies and weights or volumes for a given population or sub-populations(s), and should combine the information to estimate the population exposure to pathogens through a certain food or water commodity.

III. Hazard characterization provides a description of the adverse health effects that may result from ingestion of a microorganism. When data are available, the hazard characterization should present quantitative information in terms of a dose-response relationship and the probability of adverse outcomes.

IV. Risk characterization is the integration of the three previous steps to obtain a risk estimate (i.e. an estimate of the likelihood and severity of the adverse health effects that would occur in a given population, with associated uncertainties).

The goal of a risk assessment may be to provide an estimate of the level of illness from a pathogen in a given population, but may also be limited to evaluation of one or several step(s) in a food production or processing system. When requesting a risk assessment, the risk manager should be specific with regard to the problem with which the risk manager needs to deal, the questions to be addressed by the risk assessment, and an indication of the measures the manager would consider or has available for the reduction of illness.

3.3.2. Risk management

The process, distinct from risk assessment, of weighing policy alternatives in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control options.

3.3.3. Risk communication

The interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions.

3.4 Codex Alimentarius Commission

The CAC is a body of United Nations (UN) established by FAO in 1961 and is an inter-governmental organization that coordinates food standards at the international level. Its main objectives are to protect the health of consumers and ensure fair practices in food trade. The Codex Food Code (CFC) attempts to create harmonized standards. Prior to the SPS Agreement, the CFC could be adopted, applied and/ or ignored at the discretion of a government. However, the CFC has now been adopted within the SPS Agreement as the benchmark. The CAC has proved to be most successful in achieving international harmonization in food quality and safety requirements. It has formulated international standards for a wide range of food products and specific requirements covering pesticide residues, food additives, veterinary drug residues, hygiene, food contaminants, labelling, etc. These codex recommendations are used by governments to determine and refine policies and programmes under their national food control system. More recently, Codex has embarked on a series of activities based on risk assessment to address microbiological hazards in foods, an area previously unattended. Codex work has created worldwide awareness of food safety, quality and consumer protection issues, and has achieved international consensus on how to deal with them scientifically, through a risk-based approach. As a result, there has been a continuous appraisal of the principles of food safety and quality at the international level. There is increasing pressure for the adoption of these principles at the national level. Quality assurance systems have become a focal point for inclusion in the work of Codex. As an example, the CAC has recently adopted guidelines for the application of the HACCP system. The HACCP approach, along with the use of GMPs, is strongly recognized and recommended by Codex. The principal consideration behind the development of any Codex standard, guideline or other recommendation is the protection of consumer's health.

3.5 HACCP system

HACCP stands for Hazard Analysis Critical Control Point. HACCP is a systematic approach to the identification, evaluation, and control of food safety hazards. It is a proactive strategy where hazards are identified and assessed, and control measures are developed to prevent, reduce, or eliminate a hazard. The HACCP system, which is science based and systematic, identifies specific hazards and measures for their control to ensure the safety of food. HACCP is a tool to assess hazards and establish control systems that focus on prevention rather than relying mainly on end-product testing. Any HACCP system is capable of accommodating change, such as advances in equipment design, processing procedures or technological developments.

HACCP can be applied throughout the food chain from primary production to final consumption and its implementation should be guided by scientific evidence of risks to human health. As well as enhancing food safety, implementation of HACCP can provide other significant benefits. In addition, the application of HACCP systems can aid inspection by regulatory authorities and promote international trade by increasing confidence in food safety. The successful application of HACCP requires the full commitment and involvement of management and the work force. It also requires a multidisciplinary approach; this multidisciplinary approach should include, when appropriate, expertise in agronomy, veterinary health, production, microbiology, medicine, public health, food technology, environmental health, chemistry and engineering, according to the particular study. The application of HACCP is compatible with the implementation of quality management systems, such as the ISO 9000 series, and is the system of choice in the management of food safety within such systems.

3.6 Management Systems for Quality and Food Safety

Excellence in food quality and safety has taken a tangible form with the advent of ISO 9000 Quality Management System and HACCP standards. ISO 9000 encompasses all the activities of a company to ensure that it meets its quality objectives, while HACCP is directed towards ensuring food safety. The ISO 9000 standards were brought by the International Organization for Standardization (ISO) and the HACCP standards by the CAC. These standards have assumed importance worldwide both as an essential requirement to tap the market potential and as a marketable feature of the company. Since the global market has become more demanding in terms of quality, safety and timely delivery, installation of the ISO 9000 Quality Management System and HACCP by the food industry is essential for getting a competitive international edge. Food Safety Programs may need to be implemented to meet regulatory requirements, retailer requirements or manufacturer's requirements.

ISO 9000 Quality Management Systems: The ISO 9000 system is looked at as a system with minimum quality requirements. It builds a baseline system for managing quality. The focus, therefore, is on designing a total quality management system, one that complies with external standards, but includes the specific requirement of industry and integrates elements of competitiveness.



Lesson 4 CONSIDERATIONS IN FOOD LAWS AND REGULATIONS

4.1 Introduction

The development of relevant and enforceable food laws and regulations is an essential component of a modern food control system. Food laws traditionally consist of legal definitions of unsafe food and the prescription of enforcement tools for removing unsafe food from commerce and punishing responsible parties for the lapse. Food law generally does not provide punitive powers to the control agencies with a clear mandate and authority to prevent food safety problems. Hence, food safety programmes are reactive and enforcement-oriented rather than preventive and holistic in their approach to reduce the risk of food-borne illness. To the extent possible, modern food laws should not only be permitted with necessary legal powers and prescriptions to ensure food safety, but also allow the competent food authority or authorities to build preventive approaches into the system.

4.2 Definition of Food Standards

In addition to legislation governments need updated food standards. In recent years, many highly prescriptive standards have been replaced by horizontal standards that address the broad issues involved in achieving food safety objectives. While horizontal standards are a viable approach to delivering food safety goals, these require a food chain that is highly controlled and supplied with good data on food safety risks and risk management strategies and as such may not be feasible for many developing countries. The horizontal approach makes it possible to take a general overview of a particular situation, and facilitates implementation, particularly for food businesses working in many sectors, including not only manufacturers, but also for commerce and distribution. Similarly, many standards on food quality issues have been cancelled and replaced by labelling requirements. In preparing food regulations and standards countries should take full advantage of Codex standards and food safety lessons learned in other countries. Taking into account the experiences in other countries while tailoring the information concepts and requirements to the national context is the only sure way to develop a modern regulatory framework that will both satisfy national needs and meet the demands of the SPS Agreement and trading partners.

4.3 Food Legislation

Food legislation should include the following aspects:

- it must provide a high level of health protection;
- it should be based on high quality, transparent and independent scientific advice following risk assessment, risk management and risk communication;
- it should include provision for the use of precaution and the adoption of provisional measures where an unacceptable level of risk to health has been identified and where full risk assessment could not be performed;
- it should include provisions for labelling the product holistically and for the right of consumers to have access to accurate and sufficient information;
- it should provide for tracing of food products and for their recall in case of problems;
- it should include clear provisions indicating that primary responsibility for food safety and quality rests with producers and processors;
- it should include obligation to ensure that only safe and fairly presented food is placed on the market;
- it should also recognize the country's international obligations particularly in relation to trade and
- it should ensure transparency in the development of food law and access to information.

4.4 General Food Law

Humanity has imbibed certain hygienic practices since man started understanding the value of food. The unstinted quest made the man to have some control over production, processing, supply and consumption of food. The well-established practices took the form of accepted laws. The present day governments enacted these laws legally enforceable through various administrative rules and regulations.

4.4.1 Definition of food

Food or food stuff means any substance or products whether processed, partially processed or unprocessed that will provide sufficient nutrient and is intended to be, or reasonably expected to be ingested by humans. Food includes drink, chewing gum and any substance, including water, intentionally incorporated into the food during its manufacture, preparation or treatment.

4.4.2 The main objective of food law

It is to protect both the health and interests of the consumer. Consumer health is assured by the provision of safe food produced with due regard for plant health and the environment. All legislation must aid the free flow of food within the internal market for the general interests of all consumers. Generally, it applies to all stages of production, processing and distribution of food. All food, which is placed on the market, must be fit for human consumption and must not injure human health according to its intended use. The focus on potential injury to health must be both short- and long-term with due regard to subsequent generations and cumulative effects. If any food within any one lot, batch or consignment is unsafe, the entire lot, batch or consignment must be withdrawn from the market. At all times consumer safety being paramount is focused to ensure a high level of protection of human health and consumers' interests in relation to food, taking into account diversity, including traditional products, whilst ensuring the effective functioning of the internal market. If food complies with community provisions but is otherwise deemed unsafe, it should not be placed on the market.

The integrated 'farm to fork' approach is considered to be a general principle for food and feed safety policy. It guarantees a high level of safety for foodstuffs and food products marketed within all the stages of production and distribution chains. It involves both food and feed products produced and those imported from other countries.

4.4.3 General principles of food law

The regulation (official order) establishes several basic principles for food to be marketed. Food regulation is principally composed of three distinct areas: general principles of food law; the creation of the Food Safety Authority and the rapid alert system, crisis management and emergencies. The basic principles are outlined in the text below:

4.4.3.1 Food safety

Food is not allowed to be placed on the market if it is unsafe. Food is considered unsafe if it is injurious to health or unfit for human consumption. This general food safety requirement implies that although a product that complies with all specific requirements of food legislation (e.g. contaminants in food, etc.); it is not allowed on the market if a new hazard is found for which no requirements yet exist.

The following assumptions should be checked in order to establish food safety:

- a. Unsafe: the normal conditions of use of the food by the consumer at each stage of production, processing and distribution are taken into account as well as the information provided to the consumer.
- b. Injurious to health: probable immediate, short term and long term effects (including future generations) of the food to health, probable cumulative toxic effects and particular health sensitivities of a specific category of consumers should be considered.
- c. Unfit for human consumption: if the food is unacceptable for human consumption according to its intended use, for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay.

If food that is found to be unsafe is part of a batch, lot or consignment, it is presumed that all the food is unsafe, unless a detailed assessment proves that there is no evidence that the rest of the batch is unsafe. Food that complies with specific requirements on food safety is considered safe in so far as it concerns specific issues for which the requirements are set. If however, despite compliance to these requirements, the competent authorities have reasons to suspect that the food is not safe, they can still require its withdrawal from the market.

4.4.3.2 Responsibilities

Food business operators at all stages of production, processing and distribution of food are primarily responsible for safe food. They have to ensure that food under their control meets all the safety requirements of the law. If the food is unsafe, they cannot place it on the market. Further, they are obliged to withdraw and report to the competent authority if they have suspicion that their food products do not meet the standards.

4.4.3.3 Traceability

The Regulation includes provisions on the traceability of food in the food chain. At all times the origin of food products must be retrieved. Strictly speaking the requirements apply to food and feed businesses (including importers). These people are obliged to:

- know and document from whom they have bought their food (ingredients)
- know and document to whom they supply their products
- label at length their products so that they can establish traceability in case of a food safety problem.

However, importers are likely to be requested to trace faults in the food chain. Importers are legally responsible for marketing the food and therefore must be able to guarantee that the food brought onto the market meets all requirements.

4.4.3.4 Precautionary principle

The General Food Law establishes that food legislation is based on scientific risk analysis. This analysis consists of risk assessment, risk management and risk communication.

Risk assessments are based on the available scientific evidence and must be undertaken in an independent, objective and transparent manner. Risk management takes the results of the risk assessment into account, but also the opinions of the Food Safety Authority. If after an assessment of available information the possibility of harmful effects on health is identified, but scientific uncertainty persists, provisional measures may be necessary. The findings need to be communicated to the retailer, manufacturer and notified to the consumer. This whole exercise is called the precautionary principle. Such measures need to be proportionate and no more restrictive of trade than is required to achieve the high level of health protection. The measures are reviewed within a reasonable period of time. This period depends on the nature of the risk to health and the type of scientific information needed to clarify uncertainty.

4.4.3.5 Presentation

Food law aims at the protection of the interests of consumers in relation to the foods they consume. Provision of food law about the food labelling, advertising and presentation of food including their shape, appearance, packaging and packaging materials used cannot mislead consumers.

4.4.3.6 Rapid alert system for food and feed (RASFF)

The regulation gives legal effect to RASFF. The regulatory system deals with the obligatory notification of any direct or indirect risk to human health, animal health or the environment within a network consisting of national competent authorities, Food Regulatory Authority of India (FRAI). The regulation also confers special powers on taking emergency measures. Where it is evident that food originating in the country, or

imported from a another country, is likely to constitute a serious risk to human health, animal health or the environment and that such a risk cannot be contained satisfactorily by means of measures taken by the Member States.

4.4.3.7 Emergency measures

Where it is evident that food originating in the community or imported from another country is likely to constitute a serious risk to human health, animal health or the environment and when such a risk cannot be contained satisfactorily by means of measures taken by the Member States concerned, the Commission can on its own initiative or at the request of a Member State (and following the procedure set out in the regulation), immediately adopt certain measures set out in the regulation. Depending on the gravity of the situation, emergency measures can take the form (1) of the suspension of the marketing or use of the food in question (2) to subjecting the use and marketing of the food to special conditions.

4.4.3.8 Crisis management

It provides for the creation of a crisis unit. This crisis unit will be set up by the FRAI and will provide scientific and technical assistance if necessary.



Lesson 5 INTEGRATED FOOD LAW, ITS MAIN FEATURES AND FUNCTIONS

5.1 Introduction

India has many laws regulating the food safety and other activities of food industry. The requirement of food regulation may be based on several factors such as whether a country adopts international norms developed by the CAC of the Food and Agriculture Organization (FAO) of the United Nations (UN) and the World Health Organization (WHO); good agricultural and manufacturing practices; or a country may also has its own suite of food regulations. Each country regulates food differently and has its own food regulatory framework.

The introduction of integrated food law provides the much required 'one law-one regulator' platform for raising the food safety standards of India to match global standards. Its speedy and effective implementation is quickly warranted to put India onto the global food map. This would require an enabling implementation environment focused on creation of transparency, awareness creation, capacity building, product traceability, developing right infrastructure and extensive R&D capacity so as to match the dynamically changing requirements of food safety and standards. The initiative would also require a wide spread awareness and promotion campaign focused on changing the mindset of food producers so as to encourage adherence to food safety standards.

5.2 Integrated Food Law in India

Until the recent past, the Prevention of Food Adulteration Act, 1955 (PFA) was the main legislation concerning food safety and protection of consumer health. In addition, there were a number of other standards and acts administered by different governmental agencies. India has plethora of laws regulating the food safety and other activities of food industry. The requirement of food regulation may be based on several factors such as whether a country adopts international norms developed by the CAC of the FAO of the United Nations and the WHO; good agricultural and manufacturing practices; or a country may also have its own suite of food regulations. In order to give a boost to the food industry the need of the hour is to harmonize not only the various food laws but also the agencies. According to apex industrial bodies there should be only one national food safety code, which should cover all aspects of Indian food safety under a unified system. Realizing the importance of introducing an integrated, contemporary and comprehensive law, the Food Safety and Standards Act (FSSA), 2006, that overrides all earlier food laws.

This integrated law provides safe and wholesome food to the consumer, creates an enabling-environment for value addition to primary agricultural produce and encourage innovation and creativity for rapid development of food processing industries in an integrated manner thus ensuring a high degree of objectivity and transparency. The law intends to ensure better consumer safety through the introduction of food safety management systems based on science and transparency.

5.3 Main Features of the Law Include

- Single reference point for all issues related to food safety and standards
- Harmonization with international standards such as CODEX and, hence, responsive to international trade requirements
- Responsive to dynamic issues such as genetically modified food
- Clear procedures for food recall.
- Shift from a regulatory regime to self compliance through food safety management systems
- In addition, the Food Safety and Standards Authority of India (FSSAI) was set up in 2008 to lay down scientific standards and ensure availability of safe food for human consumption.

This Act covers all articles of food or drink for human consumption except drugs, tobacco, alcoholic beverages and natural agricultural/ horticulture/ marine produce. Measures to regulate the natural agricultural/ horticultural/ marine produce need to be notified separately. It will specifically repeal eight laws

- The Prevention of Food Adulteration Act, 1954
- The Fruit Products Order, 1955
- The Meat Food Products Order, 1973
- The Vegetable Oil Products (Control) Order, 1947
- The Edible Oils Packaging (Regulation) Order, 1998
- The Solvent Extracted Oil, De oiled Meal, and Edible Flour (Control) Order, 1967
- The Milk and Milk Products Order, 1992
- Essential Commodities Act, 1955 relating to food

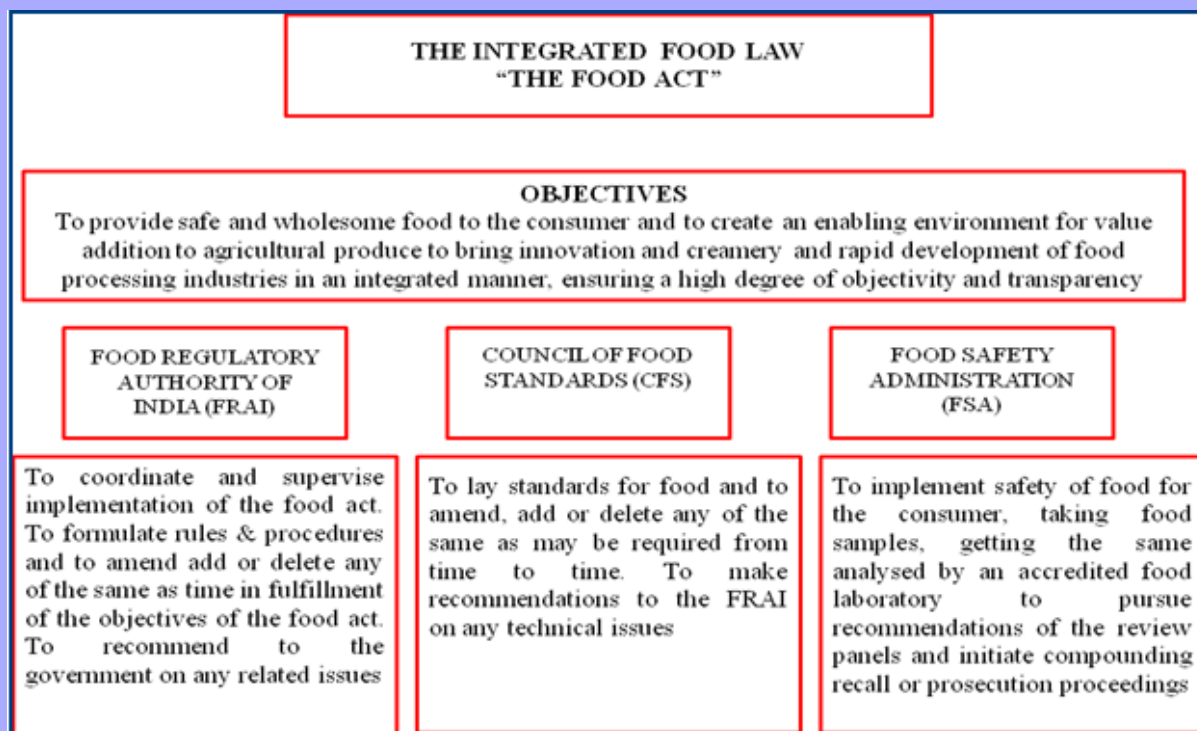


Fig. 5.1 Outline of integrated food law

5.3.1 Duties and functions of the authority

This Act established as new national regulatory body, the Food Safety and Standards Authority of India, to develop science based standards for food and to regulate and monitor the manufacture, processing, storage, distribution, sale and import of food so as to ensure the availability of safe and wholesome food for human consumption. All food imports will therefore be subject to the provisions of the Act and any rules and regulations made under the Act.

FSSAI has been mandated by the FSS act, 2006 for performing the following functions

1. Framing of regulations to lay down the standards and guidelines in relation to articles of food and specifying appropriate system of enforcing various standards thus notified.
2. Laying down mechanisms and guidelines for accreditation bodies engaged in certification of food safety management system for food businesses
3. Laying down procedure and guidelines for accreditation of laboratories and notification of the accredited laboratories.

4. To provide scientific advice and technical support to central government and state government in the matters of framing the policy and rules in areas which have a direct or indirect bearing of food safety and nutrition
5. Collect and collate data regarding food consumption, incidence and prevalence of biological risk contaminants in food, residues of various, contaminants in foods products, identification of emerging risks and introduction of rapid alert system
6. Creating an information network across the country so that the public, consumers, panchayats (village parliaments), etc. receive rapid, reliable and objective information about food safety and issues of concern
7. Provide training programmes for persons who are involved in or intend to get involved in food business
8. Contribute to the development of international technical standards for food, sanitary and phyto-sanitary standards
9. Promote general awareness about food safety and food standards
10. Bakery manufacturer needs to follow up with food authority for labelling, adulterants, additives and their permissible levels, etc.

5.3.2 New changes in integrated food law

One of the primary changes that FSSA would bring about is imposing the responsibility of food safety on the manufacturer for the first time (under the PFA regime, it was the Food Inspector responsible for examination and prosecution). With the introduction of the supply chain concept under the FSSA, the focus will not be on inspection, but on each person in the chain-sourcing, manufacturing, storing, distributing- assessed by Food Safety Officers (FSOs). FSSA prohibits the manufacture, import, storage, sale or distribution of any such article of food which is unsafe, i.e. food whose nature, substance or quality is injurious to health. There are specific responsibilities laid down under the FSSA for the Food Business Operators (FBO) for ensuring the safety of food articles. FSSA extends its jurisdiction to all persons by whom food business is carried on or owned under the definition of FBOs. The FBOs are strictly liable for any article of food which is unsafe under the FSSA. The FSSA also provides for food recall procedures whereby an FBO is required to immediately inform the competent authorities and co-operate with them, if the food which he has placed in the market is unsafe for the consumers. FSSAI has recently notified the draft Regulations on Food Recall Procedure to provide guidance to the FBOs for carrying out food recall for food that does not adhere to the food safety standards. It is also aimed to establish an effective and efficient follow-up action/ post-recall report system.

The FSSA has also provided for appointment of a Designated Officer (rank of a Sub Divisional Officer) by the Commissioner of Food Safety to be in charge of food safety administration for a specific district whose duties shall include issuing or cancelling licenses of FBOs, serving 'improvement notices', prohibiting sale of food articles violating prescribed standards, receiving reports and samples of food articles from FSOs and getting them analysed, sanction or launch prosecutions and get investigated any complaint against any contravention of the Act or against the FSOs. Other major reforms in the pipeline to ensure safe food articles by FBOs are mandatory compliance with Good Agricultural Practices (GAP) for big retailers, organic food certification, stricter labelling and claims regulations covering the entire range of health foods, such as health drinks, packaged food products as well as nutraceuticals including dietary supplements, etc.

5.3.3 Implementation challenges

Introduction of the FSSA could be instrumental in boosting both domestic and international consumer confidence, and making India match the international standards of food safety. However, its effective implementation is fraught with numerous challenges such as:

1. Poor general awareness towards the hazards associated with unsafe food practices and the best practices to be followed
2. Lack of basic supporting infrastructure such as testing labs
3. Insufficient technical expertise and skilled manpower for implementation of legislation at the grass root level

4. Exclusion of primary producers from the purview of the law thus putting the onus of preventing food hazards on the manufacturers/processors
5. Problems in traceability of product especially in the upstream processors of the food chain – from the farm gate to the processing unit
6. Creating an enabling environment

5.4 Key Challenges for Speedy and Effective Implementation of Food Safety and Standard Act 2006

In a vast and diverse country such as India, the efforts to harmonize food safety regulations to match international standards are bound to face implementation challenges. It is thus imperative to create an enabling environment so that a brilliant strategy does not fail at the grass roots level. Some of the key enablers for speedy and effective implementation include

5.4.1 Transparency and inclusive development of framework

During the initial phases of the implementation of the FSSA, a high degree of transparency needs to be maintained in the process of framing rules and regulations. Involvement of industry and other stakeholders during the preparation, evaluation and revision of food law is essential for comprehensive review and examination of the issues that could hamper the implementation on field.

5.4.2 Awareness creation

Effective awareness creation programs need to be carried out by the Governmental agencies for smooth transition from the current food safety laws to the proposed system, specifically by keeping the small and medium enterprises abreast of the salient features of the law and practical issues that are likely to be faced by the manufacturers and their solutions.

5.4.3 Capacity building

Massive efforts are required for capacity building in order to successfully implement the proposed FSSA at the grass root level. Well evolved training programs need to be conducted for the state, district and block level enforcement agencies. The programs would have to equip the implementing officers with knowledge on international standards of food safety and quality thus enabling regulators to make judicious decisions relating to food contamination.

5.4.4 Infrastructure creation

One of the critical links in the successful implementation of FSSA is food testing laboratories. Under the new law the manufacturers need to get their products tested every month and keep a certificate. Hence, building up a sufficient number of accredited laboratories is of paramount importance.

5.4.5 Building research & development capacity

There is also a need to build a strong R&D base in areas of food safety, quality control, food toxicity and related scientific risk assessment systems.

5.4.6 Certification of raw material

One of the major sources of contamination in food systems occurs during the primary production stage - which is kept out of the ambit of the FSSA. Successful and holistic implementation of food safety system would require an extensive campaign that encourages implementation of GAP at the farm level. Further, organized manufacturers should be encouraged to take pro-active steps to ensure that GAP is adhered to their suppliers, and a traceability system including geographic application put in place at the back-end thus reducing the risk of food contamination.

Lesson 6

CONCEPTS OF QUALITY MANAGEMENT SYSTEM (QMS) – ISO 9000:2000

6.1 Introduction

The concept of 'quality' has existed for many years, though the meaning has changed and evolved over time. In the early twentieth century, quality management meant inspecting products to ensure that they met specifications. In the 1940s, during World War II, quality became more statistical in nature. Statistical sampling techniques were used to evaluate quality, and quality control charts were used to monitor the production process. In the 1960s, with the help of so-called 'quality gurus', the concept took on a broader meaning. Quality began to be viewed as something that encompassed the entire organization, not only the production process. Since all functions were responsible for product quality and all shared the costs of poor quality and finally quality was considered as a concept that affected the entire organization.

Today, successful companies understand that quality provides a competitive advantage. They put the customer first and define quality as meeting or exceeding customer expectations. Since the 1970s, competition based on quality has grown in importance and has generated tremendous interest, concern, and enthusiasm. Companies in every line of business are focusing on improving quality in order to be more competitive. In many industries quality excellence has become a standard for doing business. Companies that do not meet this standard simply will not survive. The term used for today's new concept of quality is total quality management or TQM. One can see that the old concept is reactive, designed to correct quality problems after they occur. The new concept is proactive, designed to build quality into the product and process.

6.2 Basic Definition and Terminology

Quality: The quality of something can be determined by comparing a set of inherent characteristics with a set of requirements. If those inherent characteristics meet all requirements, high or excellent quality is achieved. If those characteristics do not meet all requirements, a low or poor level of quality is achieved. Quality is, therefore, a question of degree. As a result the central quality question is: how well does this set of inherent characteristics comply with this set of requirements? In short, the quality of something depends on a set of inherent characteristics and a set of requirements and how well the former complies with the latter. According to this definition quality is a relative concept. By linking quality to requirements, ISO 9000 argues that the quality of something cannot be established in a vacuum. Quality is always relative to a set of requirements. Thus, quality is the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs/ or the operational techniques and activities that are used to satisfy quality requirements.

Quality characteristic: A quality characteristic is tied to a requirement and is an inherent feature or property of a product, process, or system. A requirement is a need, expectation, or obligation. It can be stated or implied by an organization, its customers, or other interested parties. An inherent feature or property exists in something or is a permanent characteristic of something.

Quality assurance (QA): Quality assurance is a set of activities intended to establish confidence that quality requirements will be met. QA is one part of quality management.

Quality control (QC): Quality control is a set of activities intended to ensure that quality requirements are actually being met. QC is one part of quality management.

Quality improvement: Quality improvement refers to anything that enhances an organization's ability to meet quality requirements. Quality improvement is one part of quality management.

Quality management: Quality management includes all the activities that organizations use to direct, control, and coordinate quality. These activities include formulating a quality policy and setting quality objectives.

They also include quality planning, quality control, quality assurance and quality improvement.

Quality management system (QMS): QMS may be defined as ‘a set of coordinated activities to direct and control an organization in order to continually improve the effectiveness and efficiency of its performance’. It comprises of collective policies, plans, practices and the supporting infrastructure by which an organization aims to reduce and eventually eliminate non-conformance to specifications, standards and customer expectations in the most cost effective and efficient manner.

Quality planning: Quality planning involves setting quality objectives and then specifying the operational processes and resources that will be needed to achieve those objectives. Quality planning is one part of quality management.

Quality plan: A quality plan is a document that is used to specify the procedures and resources that will be needed to carry out a project, perform a process, realize a product, or manage a contract. Quality plans also specify who will do what and when.

Quality policy: An organization’s quality policy defines top management’s commitment to quality. A quality policy statement should describe an organization’s general quality orientation and clarify its basic intentions. Quality policies should be used to generate quality objectives and should serve as a general framework for action. Quality policies can be based on the ISO 9000 Quality Management Principles and should be consistent with the organization’s other policies.

Quality objectives: A quality objective is a quality oriented goal. A quality objective is something aim for or tries to achieve. Quality objectives are generally based on or derived from organization’s quality policy and must be consistent with it. These are usually formulated at all relevant levels within the organization and for all relevant functions.

Continual improvement: Continual improvement is a set of activities that an organization periodically carries out in order to enhance its ability to meet requirements. Continual improvements can be achieved by carrying out audits (and using audit findings and conclusions), performing management reviews, analysing data, setting objectives and implementing corrective and preventive actions.

Customer: A customer is anyone who receives products or services from a supplier organization. Customer can be people or organization and can be either external or internal to the supplier organization. For example, a factory may supply products or services to another factory (customer) within the same organization. According to ISO 9000, examples of customers include clients, customers, end-users, purchasers, retailers and beneficiaries.

Customer satisfaction: It is a perception. It is also a question of degree. It can vary from high satisfaction to low satisfaction. If customers believe that their requirements have been met, they experience high satisfaction. If they believe that their requirements have not been met, they experience low satisfaction. Since satisfaction is a perception, customers may not be satisfied even though all the contractual requirements have been met. Just because one hasn’t received any complaints doesn’t mean that customers are satisfied. There are many ways to monitor and measure customer satisfaction. One can use customer satisfaction and opinion survey, can collect product quality data (post-delivery), track warranty claims, examine dealer reports, study customer compliments and criticisms and analyse lost business opportunities.

Management: The term management refers to all the activities that are used to coordinate, direct and control an organization. In this context the term management does not refer to people. It refers to activities. ISO 9000 uses the term Top Management to refer to people.

Management system: A management system is a set of inter-related or interacting elements that organizations use to implement policy and achieve objectives. There are many types of management systems. Some of these include quality management, emergency management, food safety management, occupational health and safety management, information security management systems and business continuity management systems.

Nonconforming product: When one or more characteristics of a product fall to meet specified requirements, it is referred to as a nonconformity product. When a product deviates from specified product requirements, it fails to conform. Nonconformity products must be identified and controlled to prevent uninterrupted delivery.

Nonconformity: It refers to failure to comply with requirements. A requirement is a need, expectation, or obligation. It can be stated or implied by an organization, its customers, or others interested parties.

Procedure: A procedure is a way of carrying out a process or activity. According to ISO 9000, procedures may or may not be documented. However, in most cases, ISO 9001 expects documentation of procedures. Documented procedures can be very general or very detailed, or anywhere in between. While a general procedure could take the form of a simple flow diagram, a detailed procedure could be a one page form or it could be several pages of text. A detailed procedure defines and controls the work that should be done, and explains how it should be done, who should do it and under what circumstances. In addition, it explains what authority and what responsibility has been allocated, which inputs should be used and what outputs should be generated.

Process: A process is a set of activities that are interrelated or that interact with one another. Processes use resources to transform inputs into outputs. Processes are interconnected because the output from one process becomes the input for another process. In effect, processes are “glued” together by means of such input output relationships. Organizational processes should be planned and carried out under controlled conditions. An effective process is one that realizes planned activities and achieves planned results.

Process approach: The process approach is a management strategy. When managers use a process approach, it means that they manage the processes that make up their organization, the interaction between these processes and the inputs and outputs that tie these processes together.

6.3 QMS concept

6.3.1 Process-based quality management system (QMS)

A quality management system is a set or network of many of interrelated or interacting and interconnected processes (elements) that organizations use to direct and control how quality policies are implemented and quality objectives are achieved. A process-based quality management system uses a process approach to manage and control how its quality policy is implemented and how its quality objectives are achieved. A process-based QMS is a network of interrelated and interconnected processes. Each process uses resources to transform inputs into outputs. Since the output of one process becomes the input of another process, processes interact and are interrelated by means of such input-output relationships. These process interactions create a single integrated process-based QMS. The concept of a “process-based quality management system” is briefly mentioned in the introduction to ISO 9001 (Fig. 6.1). However, ISO 9000 does not formally define this important term. Since the output of one process becomes the input of another process, processes interact and are interrelated by means of such input-output relationships. These process interactions create a single process-based QMS. Top management is called to establish a customer oriented organization:

- By defining the systems and processes that can be managed and improved in effectiveness and efficiency,
- Acquiring and using process data and information on a continuing basis,
- Directing progress towards continual improvement and
- Using suitable methods to evaluate process improvement.

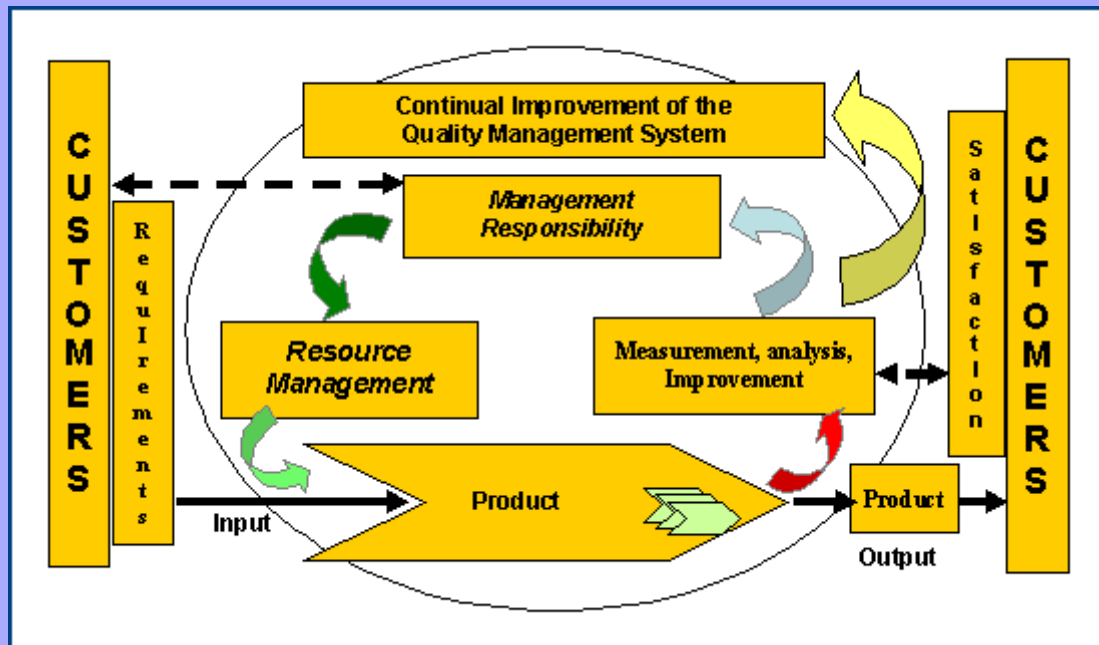


Fig. 6.1 Process module of the standard ISO 9000:2000

6.3.2 Interpretation of basic quality concepts and processes

Two significant attributes, among others which mark management processes at the beginning of the third millennium are: constant changes and the process approach to management. Changes, constant improvement and process approach are attributes of the Total Quality Management (TQM) concept and the characteristic of the new edition of the ISO 9000:2000 standard. The definition of the TQM and the process approach definition points that TQM is a concept or a philosophy for management operations where as quality is a function of management. It is a way of how to manage processes in order to achieve success and has become a movement and an approach, a religion of how to live. TQM concept is in literature and it is widely and thoroughly considered as the unity of several approaches such as:

1. Approaches that act on the removal of the burden created by the traditional way of work,
2. Approaches that allow science method usage in working process,
3. Approaches that allow equal distribution of work functions,
4. Approaches that provide the engineering of the process,
5. Approaches that allow transparency of the organization and
6. Group of approaches to TQM that enable competing ability.

ISO 9000:2000 standard defines process as the "system of activities that uses resources to transform inputs into outputs". This definition has a strong point in two major rules (1) inputs of one process are mainly outputs of another and (2) processes are managed in order to create new values that correspond to requirements and expectations of customers. So, cybernetic approach to management is at use today, an approach that establishes connection between inputs and outputs, during which process outputs must be verified according to input requirements in order to satisfy customer requirements and requirements of other interested sides. Also, process inputs must be defined and recorded in order to provide a base for demand formulation that is to be used for output validation and verification. Input requirements that are crucial for product or process must be identified in order to assign proper responsibilities and resources (ISO 9000:2000). Production process represents a flow that begins with external requirements of buyers and ends with the product that is used by buyers. Buyer makes judgment about realization or non-realization of his requirements. ISO 9000:2000 standards recommend that:

1. Desired results can be more efficiently achieved if proper resources and activities are managed as processes and
2. System approach to management: identification, understanding and system management of related processes to achieve goal that are set. Thus QMS make the organization efficient.

6.3.3 Process network - network architecture

Considering the definition that the process is "a system of activities..." then, every process can be structured as the unity of activities or chain of activities, and any activity can be structured as the chain of elementary tasks. For both definitions, for activities and tasks, the second part of the definition is the same "...that uses resources in order to transform inputs into outputs". ISO 9000:2000 standards explain the consistency of such structure as follows: "any activity that transforms inputs into outputs can be considered as the process". In order for its efficient functioning the organization should identify and manage inter related process.

6.3.4 The benefits of a QMS

A fully documented QMS will ensure that two important requirements as follow:

1. Customers' requirements: confidence in the ability of the organization to deliver the desired product and service consistently meeting their needs and expectations.
2. The organization's requirements: both internally and externally and at an optimum cost with efficient use of the available resources – materials, human, technology and information.

These requirements can be met truly only if objective evidence is provided, in the form of information and data, to support the system activities from the ultimate supplier to the ultimate customer. A QMS enables an organization to achieve the goals and objectives set out in its policy and strategy. It provides consistency and satisfaction in terms of methods, materials, equipment, etc., and interacts with all activities of the organization, beginning with the identification of customer requirements and ending with their satisfaction, at every transaction interface. Management systems are needed in all areas of activity whether large or small businesses, manufacturing, service or public sector. A good QMS will ensure the following in organization:

- i) Set direction and meet customers' expectations
- ii) Improve process control
- iii) Reduce wastage
- iv) Lower costs
- v) Increase market share
- vi) Facilitate training
- vii) Involve staff
- viii) Raise morale

The Quality Management System has been developed to comply with regulatory, industry and customer quality requirements imposed by customers or regulatory agencies for items and services. The QMS describes commitments to the quality assurance requirements of ISO 9001; ISO 9000-3. It defines the basic requirements applicable to customer contracts and is a commitment to the customers. It serves as a directive for all functions in establishing necessary policies and procedures that comply with the requirements of ISO 9001:2000 and ISO 9000-3:1997. Employees' involvement is most vital for effective quality management system. If we enforce quality management system following benefits of strategic quality planning could be availed by dairy industry:

A	Product quality	Improved product design; Reduced liability risk; New product introduction; Improved reaction to competition; Enhanced reputation; Improved after sales service; Better advertising strategies
B	Production quality	(i) Reduction of rework and other operating losses; (ii) Decreased labor/ material cost; (iii) Improved managerial control of operations; (iv) Better employee morale; (v) Smoother production flow, (vi) Improved reliability.
C	Business for performance	(i) Improved delivery times; (ii) Reduced order processing times; (iii) Reduced cycle times for new product introduction; (iv) Improved return on investment; (v) Higher market share; (vi) Customer relation



Lesson 7 PRINCIPLES OF QUALITY MANAGEMENT SYSTEM (QMS) – ISO 9000:2000

7.1 Introduction

Increases in international trade during the 1980s created a need for the development of universal standards of quality to enable one to objectively document quality practices around the world. The ISO 9000 standards were first published in 1987 and subsequently revised in the year 1994 and 2000. Standards are reviewed every five years to ensure that they are current and satisfy the needs of users.

ISO 9000 is a starting point for understanding the standards, as it defines the fundamental terms used in the ISO 9000 'family', or set of standards relating to quality management. ISO 9001 specifies requirements for a QMS to ensure that products fulfil customer requirements as well as applicable regulatory requirements; it also aims to enhance customer satisfaction. ISO 9004 provides guidance on continual improvement of QMS so that the needs and expectations of all interested parties are met. These interested parties include customers and end-users directors and staff in the organization; owners/ investors, suppliers and partners and society, at large.

ISO 9001 and ISO 9004 make a 'consistent pair' of standards that relate modern quality management to the processes and activities of an organization, and emphasize the promotion of continual improvement and achievement of customer satisfaction. ISO 9001, which focuses on the effectiveness of the QMS in meeting customer requirements, is used for certification or for contractual agreements between suppliers and buyers. On the other hand, ISO 9004 cannot be used for certification as it does not prescribe requirements but provides guidance for the continual improvement of an organization's performance. ISO 9001 focuses on 'effectiveness', i.e. doing the right things, whereas ISO 9004 emphasizes both 'effectiveness' and 'efficiency', i.e., doing the right thing in the right way.

7.2 Quality Management System (QMS) Principles

There are eight quality management principles on which the QMS standards of the ISO 9000:2000 and ISO 9000:2008 series are based. These principles can be used by senior management as a framework to guide their organizations towards improved performance. The principles are derived from the collective experience and knowledge of the international experts who participate in ISO Technical Committee ISO/ TC 176, Quality management and quality assurance, which is responsible for developing and maintaining the ISO 9000 standards. The eight quality management principles are defined in ISO 9000:2005, Quality management systems Fundamentals and vocabulary and in ISO 9004:2000, Quality management systems Guidelines for performance improvements. This document gives the standardized descriptions of the principles as they appear in ISO 9000:2005 and ISO 9004:2000. In addition, it provides examples of the benefits derived from their use and of actions that managers typically take in applying the principles to improve their organizations' performance.

7.2.1 Principle 1: Customer focus

Organizations depend on their customers and therefore should understand current and future needs of customer. The organization should not only meet customer requirements but also strive to exceed customer expectations.

7.2.1.1 Key benefits

- Increased revenue and market share obtained through flexible and fast responses to market opportunities.
- Increased effectiveness in the use of the organization's resources to enhance customer satisfaction.
- Improved customer loyalty leading to repeat business.

7.2.1.2 Applying the principle of customer focus typically leads to

- Researching and understanding customer needs and expectations.
- Ensuring that the objectives of the organization are linked to customer needs and expectations.
- Communicating customer needs and expectations throughout the organization.
- Measuring customer satisfaction and acting on the results.
- Systematically managing customer relationships.
- Ensuring a balanced approach between satisfying customers and other interested parties (such as owners, employees, suppliers, financiers, local communities and society as a whole).

7.2.2 Principle 2: Leadership

Leaders establish unity of purpose and direction of the organization. They should create and maintain the internal environment in which people can become fully involved in achieving the organization's objectives.

7.2.2.1 Key benefits

- People will understand and be motivated towards the organization's goals and objectives.
- Activities are evaluated, aligned and implemented in a unified way.
- Mis-communication between levels of an organization will be minimized.

7.2.2.2 Applying the principle of leadership typically leads to

- Considering the needs of all interested parties including customers, owners, employees, suppliers, financiers, local communities and society as a whole.
- Establishing a clear vision of the organization's future.
- Setting challenging goals and targets.
- Creating and sustaining shared values, fairness and ethical role models at all levels of the organization.
- Establishing trust and eliminating fear.
- Providing people with the required resources, training and freedom to act with responsibility and accountability.
- Inspiring, encouraging and recognizing people's contributions.

7.2.3 Principle 3: Involvement of people

People at all levels are the essence of an organization and their full involvement enables their abilities to be used for the organization's benefit.

7.2.3.1 Key benefits

- Motivated, committed and involved people within the organization.
- Innovation and creativity in furthering the organization's objectives.
- People being accountable for their own performance.
- People eager to participate in and contribute to continual improvement.

7.2.3.2 *Applying the principle of involvement of people typically leads to*

- People understanding the importance of their contribution and role in the organization.
- People identifying constraints to their performance.
- People accepting ownership of problems and their responsibility for solving them.
- People evaluating their performance against their personal goals and objectives.
- People actively seeking opportunities to enhance their competence, knowledge and experience.
- People freely sharing knowledge and experience.
- People openly discussing problems and issues.

7.2.4 Principle 4: Process approach

The process approach is a management strategy. When managers use a process approach, it means that they manage the processes that make up their organization, the interaction between these processes and the inputs and outputs that tie these processes together. A desired result is achieved more efficiently when activities and related resources are managed as a process.

7.2.4.1 *Key benefits*

- Lower costs and shorter cycle times through effective use of resources.
- Improved, consistent and predictable results.
- Focused and prioritized improvement opportunities.

7.2.4.2 *Applying the principle of process approach typically leads to*

- Systematically defining the activities necessary to obtain a desired result.
- Establishing clear responsibility and accountability for managing key activities.
- Analysing and measuring of the capability of key activities.
- Identifying the interfaces of key activities within and between the functions of the organization.
- Focusing on the factors such as resources, methods, and materials that will improve key activities of the organization.
- Evaluating risks, consequences and impacts of activities on customers, suppliers and other interested parties.

7.2.5 Principle 5: System approach to management

It relates to identifying, understanding and managing interrelated processes as a system contributes to the organization's effectiveness and efficiency in achieving its objectives.

7.2.5.1 *Key benefits*

- Integration and alignment of the processes that will best achieve the desired results.
- Ability to focus effort on the key processes.
- Providing confidence to interested parties as to the consistency, effectiveness and efficiency of the organization.

7.2.5.2 *Applying the principle of system approach to management typically leads to*

- Structuring a system to achieve the organization's objectives in the most effective and efficient way.
- Understanding the inter-dependencies between the processes of the system.
- Structured approaches that harmonize and integrate processes.
- Providing a better understanding of the roles and responsibilities necessary for achieving common objectives and thereby reducing cross-functional barriers.
- Understanding organizational capabilities and establishing resource constraints prior to action.
- Targeting and defining how specific activities within a system should operate.
- Continually improving the system through measurement and evaluation.

7.2.6 Principle 6: Continual improvement

Continual improvement is a set of activities that an organization periodically carries out in order to enhance its ability to meet requirements. Continual improvements can be achieved by carrying out audits (and using audit findings and conclusions), performing management reviews, analysing data, setting objectives and implementing corrective and preventive actions. Continual improvement of the organization's overall performance should be a permanent objective of the organization.

7.2.6.1 Key benefits

- Performance advantage through improved organizational capabilities.
- Alignment of improvement activities at all levels to an organization's strategic intent.
- Flexibility to react quickly to opportunities.

7.2.6.2 Applying the principle of continual improvement typically leads to

- Employing a consistent organization-wide approach to continual improvement of the organization's performance.
- Providing people with training in the methods and tools of continual improvement.
- Making continual improvement of products, processes and systems an objective for every individual in the organization.
- Establishing goals to guide and measures to track continual improvement.
- Recognizing and acknowledging improvements.

7.2.7 Principle 7: Factual approach to decision making

Effective decisions are based on the analysis of data and information

7.2.7.1 Key benefits

- Informed decisions.
- An increased ability to demonstrate the effectiveness of past decisions through reference to factual records.
- Increased ability to review, challenge and change opinions and decisions.

7.2.7.2 Applying the principle of factual approach to decision making typically leads to

- Ensuring that data and information are sufficiently accurate and reliable.
- Making data accessible to those who need it.
- Analysing data and information using valid methods.
- Making decisions and taking action based on factual analysis, balanced with experience and intuition.

7.2.8 Principle 8: Mutually beneficial supplier relationships

An organization and its suppliers are interdependent and a mutually beneficial relationship enhances the ability of both to create value.

7.2.8.1 Key benefits

- Increased ability to create value for both parties.
- Flexibility and speed of joint responses to changing market or customer needs and expectations.
- Optimization of costs and resources.

7.2.8.2 Applying the principles of mutually beneficial supplier relationships typically leads to

- Establishing relationships that balance short-term gains with long-term considerations.

- Pooling of expertise and resources with partners.
- Identifying and selecting key suppliers.
- Clear and open communication.
- Sharing information and future plans.
- Establishing joint development and improvement activities.
- Inspiring, encouraging and recognizing improvements and achievements by suppliers.



Lesson 8

STANDARD REQUIREMENTS FOR QUALITY MANAGEMENT SYSTEM (QMS) – ISO 9001:2000

8.1 Introduction

ISO 9000 consists of a set of standards and a certification process for companies. By receiving ISO 9000 certification, companies demonstrate that they have met the standards specified by the ISO. The standards are applicable to all types of companies and have gained global acceptance. In many industries ISO certification has become a requirement for doing business. Also, ISO 9000 standards have been adopted by the European Community as a standard for companies doing business in Europe. In December 2000 the first major changes to ISO 9000 were made, introducing three new standards as given in the following paragraphs.

8.2 ISO 9000:2000–QMS: Fundamentals and Standards

The ISO 9000:2000 set provides the terminology and definitions used in the standards. It is the starting point for understanding the system of standards. This standard describes the concepts of a quality management system (QMS) and defines the fundamental terms used in the ISO 9000 family. The standard also includes the eight quality management principles which were used to develop ISO 9001 and ISO 9004. This standard replaces ISO 8402:1994 and ISO 9000-1:1994.

8.3 ISO 9001:2000–QMS: Standard Requirements

This is the standard used for the certification of organization's QMS. It is used to demonstrate the conformity of quality management systems to meet customer requirements. This standard specifies the requirements for a QMS, whereby an organization needs to assess and demonstrate its ability to provide products that meet customer and applicable regulatory requirements and thereby enhance customer satisfaction. This standard replaces ISO 9001:1994, ISO 9002:1994 and ISO 9003:1994.

8.4 ISO 9004:2000–QMS: Guidelines for performance

ISO 9004:2000 provides guidelines for establishing a quality management system. It focuses not only on meeting customer requirements but also on improving performance. This standard provides guidance for continual improvement and can be used for performance improvement of an organization. While ISO 9001 aims to give quality assurance to the manufacturing processes for products and to enhance customer satisfaction, ISO 9004 takes in a broader perspective of quality management and gives guidance for future improvement. This standard replaces ISO 9004-1:1994. Guidelines for self-assessment have been included in Annexure A of ISO 9004:2000. This annex provides a simple, easy-to-use approach to determine the relative degree of maturity of an organization's QMS and to identify the main areas for improvement.

8.5 ISO 9001:2000–QMS: Standard requirements for application in a dairy plant

An ISO 9001:2000 quality management system can be implemented through the following steps:

Step-1 Evaluate the organization's need/goals for implementing a QMS

Need for implementing QMS may arise from repeated customer complaints, frequent warranty returns, delayed deliveries, high inventories, frequent production hold-ups and high level of rework or rejection of products or services. At this stage one should identify the goals which one would like to achieve through a QMS such as customer satisfaction, increased market share, improved communications and morale in the organization, greater efficiency and profitability, etc. Another objective in implementing a QMS may be a demonstration of compliance through third party certification, which may be requested by an important client or required for enlisting as a supplier to large companies, e.g. original equipment manufacturers (OEMs).

Step-2 Obtain information about the ISO 9000 family:

The persons identified for initiating the development of an ISO 9000 QMS need to understand the requirements of ISO 9001:2000 as read with ISO 9000:2000 and ISO 9004:2000. Supporting information such as quality management principles, frequently asked questions (FAQs), guidance on clause 1.2 (application) of ISO 9001:2000, guidance on documentation requirements of ISO 9001:2000 and other brochures are available free of charge on the ISO web site at <http://www.iso.org>

Step-3 Appoint a consultant, if necessary

If, within the organization, one does not have adequate competence to develop a QMS, one may appoint a consultant. Before doing so, it is good to check his/her background; knowledge about the product realization processes of the organization and experience in helping other organizations to achieve their stated goals, including certification. One should carry out a cost-benefit analysis of hiring a consultant and agree the scope of his/her work in writing. It is also possible to appoint a consultant only for the training of key staff; the latter can then carry out further training and development of the system.

Step-4 Awareness and training

Awareness about QMS requirements should be raised amongst all personnel performing activities that affect quality. One may plan for and provide specific training on how to develop Quality Manuals, on procedures, on QMS planning, on how to identify and implement improvement processes and on how to audit compliance with the QMS, etc. The Institute of Quality Assurance (IQA), the American Society for Quality (ASQ) and the International Auditor and Training Certification Association (IATCA) can provide lists of training organizations.

Step-5 Gap analysis

Gaps between existing quality management system and the QMS requirements of ISO 9001 need to be evaluated. Preparations should be made how to bridge these gaps, including by planning for any additional resources required. Gap analysis may be carried out through self-assessment or by the external consultant.

Step-6 Product realization processes: review clause 7 of ISO 9001: 2000

Relating to 'Product realization' to determine how the requirements apply or do not apply to the company's QMS.

The processes covered by this clause include

- Customer-related processes
- Design and development
- Purchasing
- Production and service provision
- Control of measuring and monitoring devices

Note that if the company is not responsible for preparing the design of the product, one can exclude the requirement for 'design and development' from the QMS and explain the reasons for doing so in the company's Quality Manual.

Step-7 Staffing

The organization should decide on the responsibilities of the persons who will be involved in developing and documenting the QMS, including the appointment of a management representative who will oversee the implementation of the QMS. Establishing a project steering committee may also prove useful to oversee progress and provide resources wherever required.

Step-8 Plan a time frame

A complete plan should be prepared to close the gaps identified in step 5 to develop the QMS processes. The plan should include activities to be performed, resources required, responsibilities and an estimated completion time for each activity. Clauses 4.1 and 7.1 of ISO 9001:2000 provide information that should be used when developing the plan. The total time required for each phase (planning, documentation, implementation and evaluation) depends on the extent of the gaps in the existing QMS.

Step-9 Draft a quality manual

- Include how the QMS applies to the products, processes, locations and departments of the organization;
- Exclude any requirement with justification for doing so as decided in step 6 above;
- Refer to or include: documented procedures for QMS;
- Describe the interaction between the processes of the QMS, e.g. the interaction between product realization processes and other management, measurement and improvement processes and
- Draft the quality policy and quality objectives for the organization.

The staff concerned in the organization should review the Quality Manual and the documented procedures so that their comments and suggestions can be taken into account before the Quality Manual and procedures are approved for issue and use. The effective date of implementation should also be decided.

Step-10 Carry out internal audits

During the phase of implementation of some three to six months after the documentation has been written, the trained auditors should carry out one or two internal audits covering all activities for the QMS and concerned management should take corrective action on the audit findings without delay. Wherever required, the manuals, procedures and objectives should be revised. After each internal audit, the top management should review the effectiveness of the system and provide necessary resources for corrective actions and improvements.

Step-11 Apply for certification

If the company decides to obtain third party certification, it can make an application for certification to an accredited certification body. The certification audit process is explained in step 7.

Step-12 Conduct periodic evaluations

After certification, the organization should periodically conduct internal audits to review the effectiveness of the QMS and see how it can be 'continually improved'. The organization should evaluate periodically if the purpose and goals (step 1) for which the QMS was developed are being achieved, including its continual improvement.

Step-13 Certification

The process of becoming certified to ISO 9001 and how to maintain this status once one has achieved it, are given in the steps below

1. How to select a certification body Organizations that desire to obtain a certificate need to submit an application to the certification body of their choice. The issues to consider when selecting a certification body include:
 - Whether the nature of accreditation of the certification body is acceptable in the market to which the organization wants to export;
 - The market image of the certification body;
 - Quotations for the certification and audit fees, etc.

It is advisable that one selects a certification body which is accredited. Accreditation is ‘a procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks,’ as per ISO/IEC Guide 2:1996. Thus an accredited certification body has been given formal recognition of its competence to carry out ISO 9000 certification/ registration.

Step-14 Prepare for assessment

The first requirement under ISO 9001:2000 is to define the organization's processes that affect quality, so that the first step is that the auditor from the certification body meets with the organization's management to gain an understanding of its processes. Normally speaking, then, the certification audit process starts with a review of the organization's Quality Manual and procedures by the certification body's auditor, to ensure that the Manual covers the requirements of the standard. This is known as an ‘adequacy audit’ or ‘document review audit’.

Step-15 Preparing for assessment

The auditor conveys any gaps (non-conformity) found in the documents to the organization for necessary actions and re-submission of the documents, if required. The certification body also examines, where relevant, the justification included in the Quality Manual for not including certain product realization processes (e.g. if a company does not design a product, it can exclude the requirements of Clause 7.3 of ISO 9001, but this would need to be explained in the Manual). Such exclusions should be acceptable to the certification body.

Step-16 Auditing

After satisfactory completion of the document review audit, the auditors undertake the second part of the audit process at the organization's location at a mutually agreed time and date(s) – certification audits are not surprise visits. The audit at location begins with an ‘opening meeting’. During this meeting, the auditors explain to the management how the audit will be conducted and when and how the findings will be conveyed to the management. The auditors collect evidence of conformity/ non-conformity through observation of activities, examination of procedures/ records, observations of conditions of house-keeping, through interviews with the concerned managers/ personnel of the organization, etc. on a sampling basis. The information gathered through interviews is verified/ tested by the auditors by acquiring the same information from other sources such as physical observations/ measurements performed on the product and their related records. The auditors visit and verify compliance with the QMS in all the departments and functions within the scope of the QMS.

Step-17 Non-conformity

The evidence collected by the auditors is compared with the audit criteria (Company's policies and objectives, manuals, procedures, instructions, contracts, regulations. etc.) and audit findings including non-conformity, if any, are clarified and reported to the management at the end of the site audit in a formal meeting with the management called ‘closing meeting’. The non-conformities (NCs) are graded by the auditors as ‘major’ or ‘minor’. ‘Observations’ are also noted. A ‘major’ NC indicates that

- The company has failed to implement any one part of or the full QMS; or
- Any specific department of the company has failed to implement the QMS as applicable to the department; or
- A number of ‘minor’ non-conformity in the same QMS requirements are found.

A ‘minor’ NC means an isolated incident of a failure to comply with a defined process or QMS requirement. An ‘observation’ indicates that if the situation as found during the audit is not addressed it may lead to an ‘NC’ in future.

Where a major nonconformity is found, the recommendation for certification is deferred until corrective action on the same is verified through a follow-up audit. After obtaining the organization's timetable for corrective action, recommendations for certification are decided by the Lead Auditor (the leader of the audit

team) and these recommendations are conveyed to the organization in the closing meeting itself.

Step-18 Award of the ISO 9000 certificate

The certification body issues a certificate to the organization, based upon the recommendations of the Lead Auditor and independent review of these recommendations. The certificate is issued for the specific scope of the business and the products or services for which the organization has implemented a QMS.

Step-19 Surveillance audits

The certificate is initially awarded for a period of three years. During this time, periodic surveillance audits (once or twice a year) are carried out by the certification body on mutually agreed dates. An audit plan for three years indicating the scope of audit in each surveillance audit is transmitted to the organization in advance by the certification body. These audits are planned in such a manner that all aspects of the QMS are audited over a period of three years. A re-certification audit is carried out after three years using steps 2-5 above. During the period of certification, the certification body may examine records relating to the quality complaints made by customers either directly to the organization, or to the certification body, to check if the organization is taking appropriate action (s) to eliminate the cause of such complaints. The certification body also examines any misleading use of the logo of the certification body and/ or the accreditation body or incorrect references to the certification, if any, made by the organization.



Lesson 9

INTRODUCTION TO FOOD SAFETY MANAGEMENT SYSTEM (FSMS) –ISO: 22000

9.1 Introduction

Food safety is a global concern, not only because of the importance for public health but also because of its impact on international trade. Globalisation of food production and procurement makes food chains longer and more complex and increases the risk of food safety incidents. Effective and harmonized food safety systems shall manage and ensure the safety and suitability of food in each link of the supply chain. For this reason ISO developed the standard for food safety management systems ISO 22000 which applies to all organizations in the food chain and thus ensures integrity of the chain. ISO 22000 is a generic food safety management system standard. It defines a set of general food safety requirements that apply to all organizations in the food chain. If an organization is part of the food chain, ISO 22000 wants it to establish a food safety management system (FSMS). It then wants it to use this system to ensure that food products do not cause adverse human health effects. Since ISO 22000 is a generic food safety management standard, it can be used by any organization directly or indirectly involved in the food chain. It applies to all organizations in the food chain. It doesn't matter how complex the organization is or what size it is, ISO 22000 can help ensure the safety of its food products.

9.2 Definition and Terminology

9.2.1 Control (noun)

To state wherein correct procedures are being followed and criteria are being met.

9.2.2 Control (verb)

To take all necessary actions to ensure and maintain compliance with criteria established in the HACCP plan.

9.2.3 Control measure

Any action and activity that can be used to prevent or eliminate a food safety hazard or reduce it to an acceptable level.

9.2.4 Corrective action

Any action to be taken when the results of monitoring at the CCP indicate a loss of control.

9.2.5 Critical control point (CCP)

A step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level.

9.2.6 Critical limit

A criterion which separates acceptability from unacceptability.

9.2.7 Deviation

Failure to meet a critical limit.

9.2.8 End products

Product that will undergo no further processing or transformation by the organization.

9.2.9 Flow diagram

A systematic representation of the sequence of steps or operations used in the production or manufacture of a particular food item.

9.2.10 Food safety hazard

Biological, chemical or physical agents in food, or condition of food, with potential to cause an adverse health effect.

9.2.11 Food safety policy

Overall intentions and direction of an organization related to food safety as formally expressed by top management.

9.2.12 HACCP plan

A document prepared in accordance with the principles of HACCP to ensure control of hazards which are significant for food safety in the segment of the food chain under consideration.

9.2.13 HACCP

A system which identifies, evaluates and controls hazards which are significant for food safety.

9.2.14 Hazard analysis

The process of collecting and evaluating information on hazards and conditions leading to their presence to decide which are significant for food safety and therefore should be addressed in the HACCP plan.

9.2.15 Hazard

A biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect. It is the potential to cause harm; risk on the other hand is the likelihood of harm (in defined circumstances, and usually qualified by some statement of the severity of the harm).

9.2.16 Monitor

The act of conducting a planned sequence of observations or measurements of control parameters to assess whether a CCP is under control.

9.2.17 Operating limits

Criteria more stringent than critical limits that are used by an operator to reduce that risk of contamination, eg., if a certain chemical concentration is required to control a hazard, the operating limit is generally set above the minimum concentration needed to ensure effective treatment.

9.2.18 Operational prerequisite programme (OPRP)

Identified by the hazard analysis as essential in order to control the likelihood of introducing food safety hazards (and/ or the contamination or proliferation of food safety hazards in the product (s) or in the processing environment.

9.2.19 Prerequisite programme (PRP)

Basic condition and activities that is necessary to maintain a hygienic environment throughout the food chain suitable for production, handling and provision of safe end products and safe food for human consumption.

9.2.20 Risk

An estimate of the likely occurrence of a hazard.

9.2.21 Severity

The seriousness of a hazard (if not properly controlled)

9.2.22 Step

A point, procedure, operation or stage in the food chain including raw materials, from primary production to final consumption.

9.2.23 Validation

Verification focused on collecting and evaluating scientific and technical information to determine if the HACCP plan, when properly implemented, will effectively control the hazards.

9.2.24 Verification

The use of methods, procedures or test, in addition to those used in monitoring, those determine if the HACCP system complies with the HACCP plan and/ or whether the plan needs modification.

9.3 ISO 22000, Food safety management systems – Requirements

ISO 22000, Food safety management systems – Requirements for any organization in the food chain, was first published in 2005. The standard provides international harmonization in the field of food safety standards, offering a tool to implement HACCP (Hazard Analysis and Critical Control Point) throughout the food supply chain. The process module of ISO 22000:2005 has been shown in Fig. 9.1.

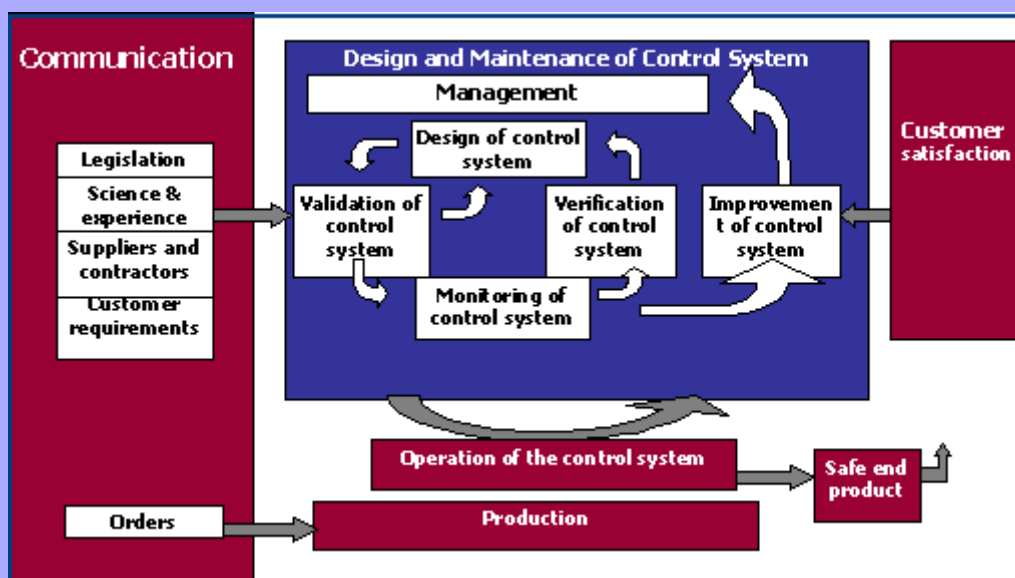


Fig. 9.1 Process module of the standard ISO 22000:2005

9.4 Goal of ISO 22000

The goal of ISO 22000 is to control and reduce to an acceptable level, any safety hazards identified for the end products delivered to the next step of the food chain. An end product is defined as a product that will not undergo any further processing or transformation by the organization. The standard combines the following generally-recognized key elements to ensure food safety at all points of the food chain:

- Requirements for good manufacturing practices or prerequisite programs
- Requirements for HACCP according to the principles of the Codex Alimentarius (an international commission established to develop food safety standards and guidelines)
- Requirements for a management system
- Interactive communication between suppliers, customers and regulatory authorities.

ISO 22000:2005 specifies requirements for a food safety management system where an organization in the food chain needs to demonstrate its ability to control food safety hazards in order to ensure that food is safe at the time of human consumption. It is applicable to all organizations, regardless of size, which are involved in any aspect of the food chain and want to implement systems that consistently provide safe products. The means of meeting any requirements of ISO 22000:2005 can be accomplished through the use of internal and/ or external resources. ISO 22000: 2005 specifies requirements to enable an organization

- To plan, implement, operate, maintain and update a food safety management system aimed at providing products those, according to their intended use, are safe for the consumer,
- To demonstrate compliance with applicable statutory and regulatory food safety requirements,
- To evaluate and assess customer requirements and demonstrate conformity with those mutually agreed customer requirements that relate to food safety in order to enhance customer satisfaction,
- To effectively communicate food safety issues to their suppliers, customers and relevant interested parties in the food chain,
- To ensure that the organization conforms to its stated food safety policy,
- To demonstrate such conformity to relevant interested parties and
- To seek certification or registration of its food safety management system by an external organization, or make a self-assessment or self-declaration of conformity to ISO 22000:2005.

ISO 22000:2005 is fully compatible with ISO 9001:2000, so it is suitable for the development of a fully integrated, risk-based management system. This also means that organizations with an existing management system should find it fairly easy to expand its scope to include ISO 22000. ISO 22000 was the first in a new family of standards related to food safety namely:

- ISO/ TS 22004:2005 provides guidance on the application of ISO 22000:2005.
- ISO 22005:2007 provides requirements for the design and implementation of a feed and food traceability system.
- ISO/ TS 22003:2007 set requirements for bodies providing audit and certification of ISO 22000.

9.5 Use of FSMS (ISO 22000)

- Primary producers: Dairy Farms, Ranches, Fisheries, Dairies
- Processors: Dairy, Fish, Meat, Poultry, Feed
- Manufacturers of Soups, Snacks, Breads, Cereals, Dressings, Beverages, Seasonings, Packaging, Frozen food, Canned food, Confectionery, Dietary supplements
- Food service providers: Grocery stores, Restaurants, Cafeterias, Hospitals, Hotels, Resorts, Airlines, Cruise ships, Seniors lodges, Nursing homes
- Other service providers for Storage service providers, Catering service providers, Logistics service providers, Transportation, Distribution, Sanitation, Cleaning
- Product suppliers: Suppliers of tools, utensils, equipment, additives, ingredients, raw materials, cleaning agents, sanitizing agents, packaging materials, other food contact materials

9.6 Methodology for Developing an ISO 22000 and HACCP

ISO 22000 uses HACCP. It was developed by the CAC. HACCP is a methodology and a management system. It is used to identify, prevent and control food safety hazards. HACCP management systems apply the following methodology:

1. Conducting a food safety hazard analysis.
2. Identifying your critical control points (CCP's).
3. Establishing critical limits for each critical control point.
4. Developing procedures to monitor critical control points.
5. Designing corrective actions to handle critical limit violations.
6. Creating a food safety record keeping system.
7. Validating and verifying the system.

An HACCP plan is a document that describes how an organization plans to manage and control its food safety hazards. An HACCP plan contains at least the following information:

1. Critical control points (CCP's)
2. Hazards that will be controlled at each CCP
3. Control measures that will be used at each CCP
4. Critical limits that will be applied at each CCP
5. Procedures that will be used to monitor CCPs
6. Actions that will be taken when limits are violated

ISO 22000 shows organizations how to combine the HACCP plan with pre-requisite programs (or programmes) and operational pre-requisite programs into a single integrated food safety management strategy.

9.6.1 Prerequisite programs (PRPs)

There are the conditions that must be established throughout the food chain and the activities and practices that must be performed in order to establish and maintain a hygienic environment. PRPs must be suitable and be capable of providing food that is safe for human consumption. PRPs are also referred to as good hygienic practices, good agricultural practices, good production practices, good manufacturing practices, good distribution practices and good trading practices.

9.6.2 Operational pre-requisite programs (OPRPs)

These are pre-requisite programs (PRPs) that are essential. They are essential because a hazard analysis has shown that they are necessary in order to control specific food safety hazards. OPRPs are used to reduce the likelihood that products will be exposed to hazards, that they will be contaminated and that hazards will proliferate. PRPs are also used to reduce the likelihood that the processing environment will be exposed to hazards.

9.7 Comparison of ISO 22000 with HACCP and ISO 9000:2000

Developed with the participation of food sector experts, ISO 22000 incorporates the principles of HACCP and covers the requirements of key standards developed by various global food retailer syndicates, in a single document. The prerequisite programmes (PRPs) are the main difference between ISO 22000 and HACCP. The incorporation of PRPs in the ISO 22000 made the system more flexible as a smaller number of CCPs was introduced. ISO 22000 strengthens the HACCP system in several ways. It is a management standard, therefore, it shares the following common elements with other management system standards

- Policy
- Planning
- Implementation and operation
- Performance assessment
- Improvement
- Management review

The main changes of ISO 22000 compared with HACCP are the following:

1. Extension of the scope to include all the food businesses from feed and primary production as well as the organizations indirectly involved in the food chain.
2. The hazards that require control are those managed not only by CCPs but also through prerequisite programmes (PRPs).
3. There is provision of crisis management procedures in the case that external dangers turn up.
4. Exist additional requirements for external communication between the food organizations and the relevant authorities involved in food safety beyond the internal communication requirements.
5. ISO 22000 uses a systems approach to prevent new hazards from occurring in the food products and recognize the new technologies to control food safety hazards. On the other side, HACCP is inherently a system to prevent food safety hazards.
6. ISO 22000 strengthens HACCP by linking the plan to PRPs and defining management's responsibilities.
7. ISO 22000 is implemented through the whole supply chain and not only in this final stage. The ISO 22000 standard is fully compatible with other ISO management system standards such as ISO 9001. However, there are differences between the two standards.
8. The focus of ISO 9001 is quality while the focus of ISO 22000 is food safety.
9. ISO 22000 extends the successful management system approach of the ISO 9001:2000 quality management system standard which is widely implemented in all sectors but does not itself specifically address food safety.
10. The standard ISO 22000 can be applied on its own, or in combination with other management system standards such as ISO 9001:2000 with or without independent (third party) certification of conformity. Companies already certified to ISO 9001 will find it easy to extend this to certification to ISO 22000.



Lesson 10 HACCP CONCEPT AND PRINCIPLE

10.1 Introduction

HACCP is a system that relies on process controls to minimize food safety risks in the food processing industry. It is useful to think of HACCP as a preventative food safety system and not a traditional quality control inspection system. HACCP is an international food safety system that deals with the control of factors affecting the ingredients, product and processing of food. It is widely recognized by scientific authorities and international organizations as the most effective approach available for producing safe food. The goal of HACCP is to identify biological, chemical and physical hazards and to include preventive measures throughout the process which would stop these hazards before they begin. These measures would, in turn, prevent unsafe food from reaching the consumer. Significant hazards for a particular food product are identified after a review of all the processing steps and use of scientific information. The steps at which these hazards can be controlled are identified and critical limits, such as process temperatures and hold times, at key process steps are set. Monitoring procedures are implemented to evaluate conformance with these critical limits. Should the process fall outside these limits, pre-planned corrective actions are taken to prevent the potentially defective product from entering the commerce stream. In addition, the HACCP system relies on extensive verification and documentation to assure that food safety has not been compromised during any step. Thus, HACCP provides a structure for assessing risks or what could go wrong and for putting the controls in place to minimize such risks.

10.2 History of HACCP

The Pillsbury Company encountered this dilemma in the 1960's in its attempts to fulfill several food production contracts with the US Army and the National Aeronautics and Space Administration (NASA). NASA in particular had very stringent microbiological acceptance criteria, not wanting to risk the illness of an astronaut during a space mission. In essence, nothing short of 100% product testing could assure NASA that a particular packet of food was safe to consume. It was obvious to all involved that product testing could not be used to guarantee food safety. A much better system of food safety assurance was required. HACCP is a preventive system in which food safety can be designed into the product and the process by which it is produced. It is a system of product design and process control. The HACCP system of food safety is very effective at controlling identified hazards. Most importantly, it does not rely upon product testing to assure food safety. HACCP system used to assure 100% safety of the food to be used in space was developed in 1960 it was published and documented in 1971 in USA. HACCP is in use worldwide and has been endorsed by the joint FAO/ WHO Codex Alimentarius Commission in 1985 based on its successful industrial application; HACCP became mandatory programs in all meat and poultry plants in 1994. The HACCP program has been implemented in the nation's processing plants with January 2000 as deadline.

10.3 HACCP and Food Regulation

The US Food and Drug Administration (FDA) used HACCP-based principles when setting up their low-acid food canning regulations in the 1970s. In 1995 the FDA issued regulations that made HACCP mandatory for fish and seafood products and in 2001 they issued regulations for mandatory HACCP in juice processing and packaging plants. In addition, a voluntary HACCP program was implemented in 2001 for Grade A fluid milk and milk products under the cooperative federal/ state National Conference on Interstate Milk Shipments (NCIMS) program. The FDA has also implemented pilot HACCP programs for a variety of other food processing segments as well as for retail foods. HACCP has also been implemented by the USDA. In 1998, USDA's Food Safety and Inspection Service (FSIS) mandated HACCP for the nation's meat and poultry processing plants. The US food processing industry will inevitably be faced with more mandatory HACCP programs under FDA and USDA/FSIS regulations in future. The HACCP system has also been implemented under regulations in other countries (e.g., Europe, Canada, Australia and New Zealand) and is a high priority program under Codex Alimentarius, the world food standards authority. In India, quality control with regard

to food products are being enforced through various regulatory mechanisms like the Prevention of Food Adulteration Act (PFA), Agriculture Grading and Marketing (AGMARK), Fruit Products Order (FPO), etc. The Bureau of Indian Standards (BIS) has recently launched a HACCP certification programme for the food industry. The Mother Dairy of Delhi and the Punjab Cooperative Milk Federation have received HACCP certificates. The Agriculture and Processed Food Export Development Agency (APEDA) has helped mango processing units in Andhra Pradesh in implementation of HACCP. While efforts are being made to implement HACCP in the organised sector of the food industry, there is a need to implement HACCP in the unorganised sector also as it accounts for 70-80% of food produced and processed in India.

10.4 Steps and Principles of HACCP Concepts

The HACCP concept is based on the seven principles of the Codex Alimentarius as laid down in Article 5 of the Regulation (EC) 852/ 2004 on the hygiene of foodstuffs. The HACCP concept must be regularly reviewed to ensure that it is always up to date. The HACCP concept must be updated according to reflect any changes to the product or the manufacturing process as well as new scientific discoveries concerning potential risks.

Prior to application of HACCP to any sector of the food chain, that sector should be operating according to the Codex General Principles of Food Hygiene, the appropriate Codex Codes of Practice and appropriate food safety legislation. Management commitment is necessary for implementation of an effective HACCP system. During hazard identification, evaluation and subsequent operations in designing and applying HACCP systems, consideration must be given to the impact of raw materials, ingredients, food manufacturing practices, role of manufacturing processes to control hazards, likely end-use of the product, categories of consumers of concern and epidemiological evidence relative to food safety. The intent of the HACCP system is to focus control at CCP's. Redesigning of the operation should be considered if a hazard which must be controlled is identified but no CCP's are found. HACCP should be applied to each specific operation separately. CCP's identified in any given example in any Codex Code of Hygienic Practice might not be the only ones identified for a specific application or might be of a different nature. The HACCP application (Fig. 10.1) should be reviewed and necessary changes made when any modification is made in the product, process, or any step. It is important when applying HACCP to be flexible where appropriate, given the context of the application taking into account the nature and the size of the operation. The development of an HACCP concept involves the following steps:

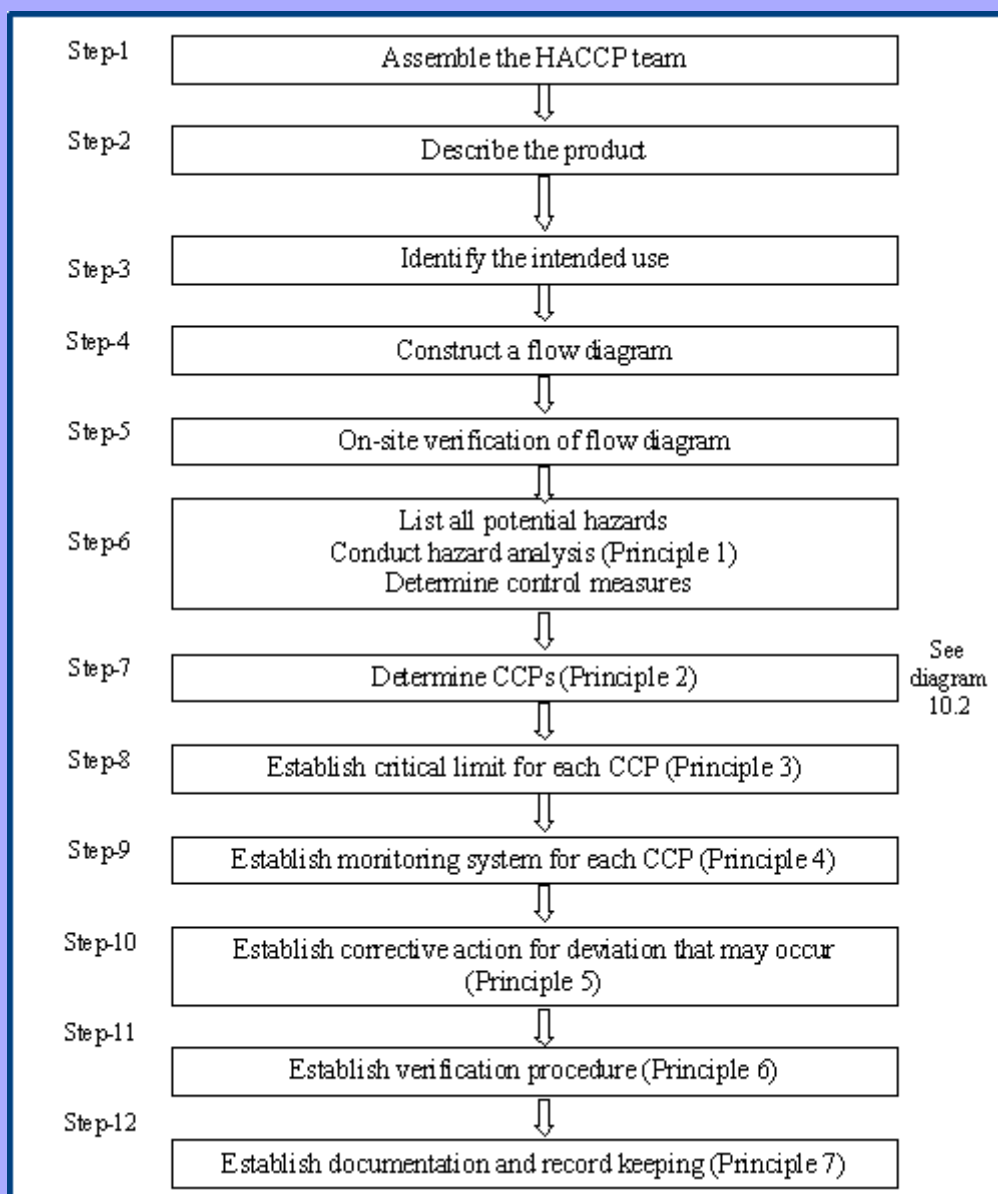


Fig. 10.1 Codex Alimentarius logic sequence

10.4.1 Establishment of a multi-disciplinary HACCP team

The HACCP team is formed of specialists from the areas of production, quality assurance, food law and development. The team members have appropriate training and knowledge of the application of HACCP principles in practice. The HACCP team should report directly to the company's management. When a dairy embarks upon implementing HACCP system, it becomes necessary to gather together as much expertise and experience on the discussion table as possible including involvement of top management. As development of HACCP based food safety management programme requires a multi-disciplinary team effort, it should include expertise in veterinary health, production, microbiology, toxicology, public health, food technology, environmental health, chemistry and engineering according to the particular study. This cross-functional expertise is necessary to adequately analyze all physical, chemical and biological hazards through the food chain. If the people drawn in the team are not specialists and are not properly trained and experienced, HACCP system is unlikely to be effective.

The HACCP team should have knowledge, experience and attributes to correctly:

- Identify potential food hazards.
- Evaluate the existing system and data in a logical manner,
- Assign levels of severity and risk to identified hazards.
- Analyze problems and recommend controls, criteria and procedures for monitoring and verification to bring lasting solutions to recurring problems, recommend appropriate corrective actions when deviations occur.
- Communicate both within the team and with people across all levels of the dairy.
- Predict the success of the HACCP plan.
- It is necessary to have right blend of people in the HACCP team. The team should normally have five to eight people depending on the size of the organization and complexity of operations.

10.4.2 Product description

The product description contains such information as product characteristics, product composition, Storage requirements, placing on the market. The dairy should describe its product(s). The description should include the major raw materials, food ingredients, preservation and packing materials used and their impact on food safety. This can also include a brief description of how the process occurs and/ products are made and stored. It would be useful if hazards that may exist either in ingredients or in packing material are identified. A description of the method of distribution includes type of transport and any special consideration to maintain product safety. For example, ice cream is described as a frozen ready to eat product containing both pasteurized and unpasteurized components. The skim milk powder, butter, sugar and water are pasteurized while the flavourings, nuts and chocolate are added without further heat processing. Air is also whipped into the product at freezing. Separate HACCP plan should be made for each product. But if two or more products have the identical raw material, ingredients, process operations, packaging, storage and distribution, they can be clubbed together in one HACCP plan.

10.4.3 Identify intended use

This intended use should be based on the expected uses of the product by the end user or consumer. It should be indicated how the product is to be used including if it is to be fully cooked before consumption, what preparations will be needed, what will be serving requirements, shelf life, etc. If consumer has special consideration such as infant or geriatrics it should be made clear so that necessary emphasis may be given to safeguard their special interest. For instance ice cream is consumed without further processing by general population including high risk groups but infant milk food is meant for infants and is given special consideration.

10.4.4 Construct a process flow diagram

The HACCP team constructs a detailed process flow diagram for each product indicating critical steps of control. Each step within the specified area of operation is analyzed for the particular part of the operation under consideration to produce the flow diagram. When applying HACCP system to a given operation, consideration is given to steps preceding and following the specified operation. The process flow diagram is used as the basis of the hazard analysis and should therefore contain sufficient technical detail for the study to progress. Each step within the specified area of operation should be analyzed for the particular part of the operation under consideration to produce the flow diagram.

10.4.5 On-site verification of process flow diagram

When the process flow diagram is complete, it is verified by the HACCP team at site to confirm the processing operation against the flow diagram during all stages and hours of operation and amend the flow diagram where appropriate. This is partly an in office exercise and partly on site activity. In office exercise includes dissecting the process stage and discussing the implications of process parameters and then they are verified at the site. The verification of the flow diagram at site is done by actually walking through the plant to check the accuracy and completeness and make sure that the steps listed on the diagram describe what really occurs in producing the product. It would be useful if a plant layout is also available because a bad layout may provide avenues for cross contamination from raw material to products, facilities to products and persons to

product. This should also form part of on-site verification.

10.4.6 Conduct hazards analysis (principle 1)

When the process flow diagram is completed and verified, the HACCP team conducts a hazard analysis and lists all the biological, chemical and physical hazards that may be reasonably expected to occur at each step from primary production, processing, manufacture and distribution until the point of consumption. When conducting the hazard analysis, consideration must be given to the impact of raw materials, ingredients, manufacturing practices, role of manufacturing processes to control hazards, likely end-use of the product, consumer populations at risk and epidemiological evidence relative to food safety. The team should then identify in the HACCP plan which hazards are of such nature that their elimination or reduction to acceptable levels is essential to the production of safe food. The team must then consider what preventative measures, if any, exist which can be applied for each hazard. Preventive measures are those action and activities that are required to eliminate hazards or reduce their impact or occurrence to acceptable levels. More than one preventive measure may be required to control a specific hazard(s) and more than one hazard may be controlled by a specified preventative measure.

10.4.7 Identify the critical control points (principle 2)

A critical control point is a point/procedure where a food safety hazard can be prevented, eliminated or reduced to acceptable levels. The identification of a CCP in the HACCP system is facilitated by the application of a decision tree. All hazards that may be reasonably expected to occur, at each step, should be considered. If a hazard has been identified at a step where control is necessary for safety and no preventive measure exists at that step, or any other, then the product or process should be modified at that step, or at any earlier or later stage, to include a preventive measure. Application of the decision tree determines whether the step is a CCP for the identified hazard. For the identification of CCP's, a decision tree can be used. Each process step and each identified hazard must be considered, in sequence, using the decision tree (Fig. 10.2)

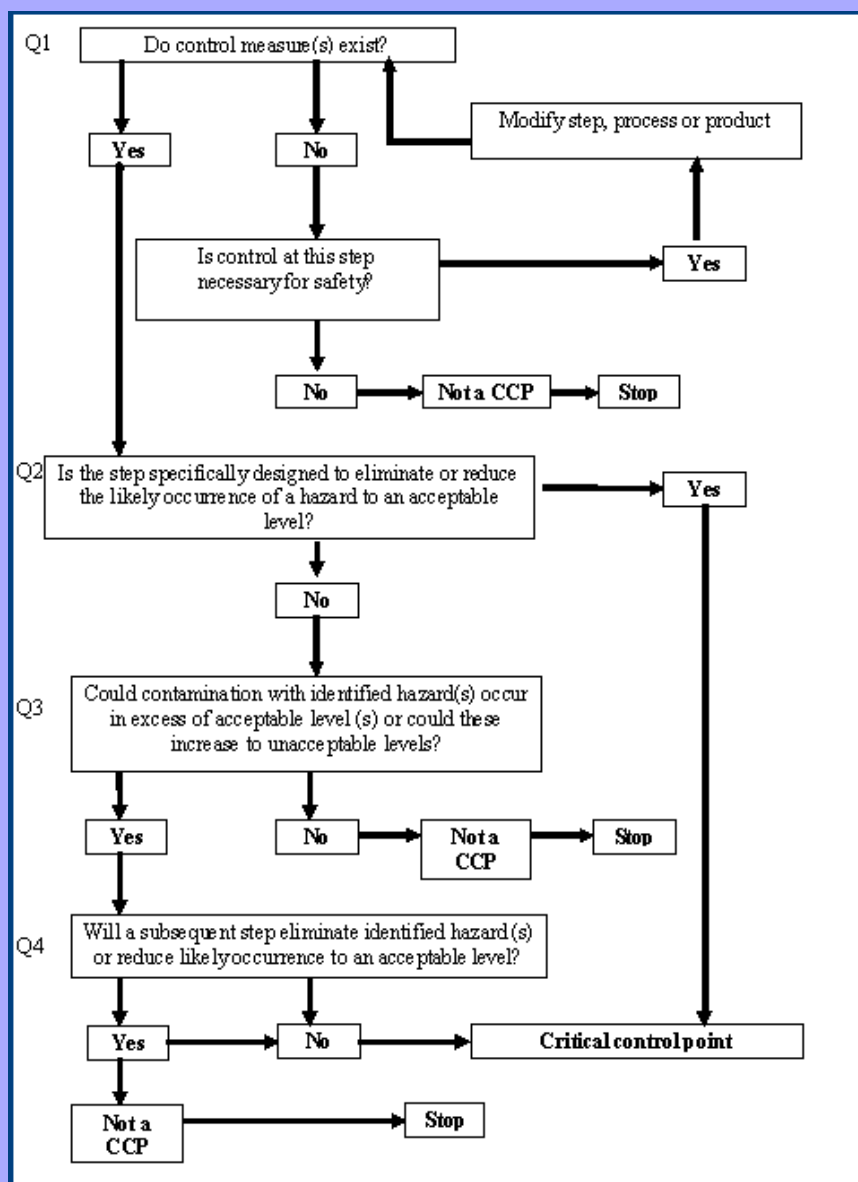


Fig. 10.2 Example of decision tree to identify CCPs (answer questions in sequence)

10.4.8 Establish critical limits for each CCP (principle 3)

Since the critical control points define the boundaries between safe and unsafe products, it is vital that they are specified at the correct levels and validated at each criterion. The HACCP team should therefore fully understand the criteria governing safety at each CCP in order to set the appropriate critical limits. Critical limits must be specified for each preventive measure. In some cases more than one critical limit will be elaborated at a particular step. Criteria often used include measurements of temperature, time, moisture level, pH, and available chlorine and sensory parameters such as visual appearance and texture.

10.4.9 Establish a monitoring system for each CCP (principle 4)

Monitoring is one of the most important aspects of the HACCP system. It is the scheduled measurement of a CCP relative to its critical limits. The monitoring procedures must be able to detect loss of control at the CCP and provide information in time for corrective action to regain control of the process. Data derived from monitoring must be evaluated by a designated person with knowledge and authority to carry out corrective actions when indicated. If monitoring is not continuous, then the frequency of monitoring must be sufficient to ensure that the CCP is under control. Most monitoring procedures for CCPs will need to be done rapidly because they relate to on line processes and there will not be time for lengthy analytical testing. Physical and

chemical measurements are often preferred to microbiological testing because they may be done rapidly and can often indicate the microbiological control of the product. All records and documents associated with monitoring CCPs must be signed by the person(s) doing the monitoring and by a responsible reviewing official of the company.

10.4.10 Establish corrective actions (principle 5)

Specific corrective actions must be developed for each CCP in the HACCP system in order to deal with deviations when they occur. The actions must ensure that the CCP has been brought under control. Actions taken must also include proper disposition of the non conforming product. Deviation and product disposition procedures must be documented in the HACCP record keeping. Corrective action should also be taken when monitoring results indicate a trend towards loss of control at a CCP. Action should be taken to bring the process back into control before the deviation leads to a safety hazard.

10.4.11 Establish verification procedures (principle 6)

The HACCP system should include verification procedures to provide assurance that HACCP system is being complied with on day to day basis. This can be done most effectively by using audit method. Monitoring and auditing methods, procedures and tests, including random sampling and analysis, can be used to determine if the HACCP system is working correctly. The frequency of verification should be sufficient to confirm that the HACCP system is working effectively. For examples of verification activities include

- Review of the HACCP system and its records,
- Review of deviations and product dispositions,
- Confirmation if CCPs are under control,
- Validation of established critical limits.

10.4.12 Establish record keeping and documentation (principle 7)

Efficient and accurate record keeping is essential to the application of a HACCP system. Records need to be kept of all areas which are critical to product safety to demonstrate that the HACCP system is in compliance with the documented system. Documentation of HACCP operation at all steps should be included and assembled in a HACCP plan. Records are useful in providing a basis for analysis of trends as well as for internal investigation of any food safety incidents which may occur. It is extremely useful to allocate a unique reference number to each HACCP record. The types of records that might be retained are as follows:

- HACCP plan,
- Modification to HACCP plan,
- CCP monitoring records,
- Deviations and associated corrective action,
- Training records,
- Audit records,
- HACCP system

10.5 Example of a worksheet

1	Describe product																
2	Diagram process flow																
3	List																
	<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Step</th> <th style="width: 15%;">Hazard(s)</th> <th style="width: 15%;">Control measure(s)</th> <th style="width: 10%;">CCP's</th> <th style="width: 10%;">Critical limits</th> <th style="width: 15%;">Monitoring procedure(s)</th> <th style="width: 15%;">Corrective action(s)</th> <th style="width: 10%;">Records</th> </tr> </thead> <tbody> <tr> <td style="height: 200px;"></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> </tbody> </table>	Step	Hazard(s)	Control measure(s)	CCP's	Critical limits	Monitoring procedure(s)	Corrective action(s)	Records								
Step	Hazard(s)	Control measure(s)	CCP's	Critical limits	Monitoring procedure(s)	Corrective action(s)	Records										
4	Verification																

The Codex General Principles of Food Hygiene has recommended a Hazard Analysis and Critical Control Point (HACCP) based approach as a means to enhance food safety and has indicated how to implement the principle 1. The HACCP concept was developed in the 1960's as a system to ensure the safety of food products. The HACCP can be defined as a system which identifies, evaluates and controls hazards which are significant for food safety. Its introduction signaled a shift in emphasis from end product testing to preventive control at all stages of food production. The HACCP system was initially developed for use by food processors for preventing food-borne hazards. However, the application of the HACCP system has been expanding to form a basis for regulated food control and as a standard for international food trade. It is being promoted internationally as a preventive system of hazard control that is considered to be the most effective and efficient way to ensure food safety. It is an action oriented programme to identify and reduce food-borne diseases. The Principles of the HACCP system set the basis for the requirements for the application of HACCP, while the guidelines for the application provide general guidance for practical application. Since the publication of the decision tree by Codex, its use has been implemented many times for training purposes. In many instances, while this tree has been useful to explain the logic and depth of understanding needed to determine CCPs, it is not specific to all food operations, e.g., slaughter and therefore it should be used in conjunction with professional judgment and modified in some cases.

Lesson 11 TQM TOOLS AND TECHNIQUES

11.1 Introduction

Total Quality Management (TQM) tools and techniques have been formulated since last 60 years. These tools and techniques are used to identify the potential problems, frequency of their occurrences and method to control these problems for adopting best class practices. From results of different surveys conducted, it is estimated that more than 100 TQM tools are being used so far. But in different books different tools are described. This lesson describes most commonly used tools under the umbrella of TQM.

Total Quality Management (TQM) is an integrated system of principles, methods, and best practices that provide a framework for organizations to strive for excellence in everything they do. The roots of Total Quality Management (TQM) go back to the teachings of Drucker, Juran, Deming, Ishikawa, Crosby, Feigenbaum and countless other people that have studied, practiced, and tried to refine the process of organizational management. TQM is a collection of principles, techniques, processes, and best practices that over time have been proven effective. Most all world-class organizations exhibit the majority of behaviours that are typically identified with TQM.

No two organizations have the same TQM implementation. There is no recipe for organization success, however, there are a number of great TQM models that organizations can use. These include the Deming Application Prize, the Malcolm Baldrige Criteria for Performance Excellence, the European Foundation for Quality Management, and the ISO quality management standards. Any organization that wants to improve its performance would be well served by selecting one of these models and conducting a self-assessment.

The model begins with understanding customer needs. TQM organizations have processes that continuously collect, analyze, and act on customer information. Activities are often extended to understanding competitor's customers. Developing an intimate understanding of customer needs allows TQM organizations to predict future customer behavior.

TQM organizations integrate customer knowledge with other information and use the planning process to orchestrate action throughout the organization to manage day to day activities and achieve future goals. Plans are reviewed at periodic intervals and adjusted as necessary. The planning process is the glue that holds together all TQM activity.

TQM organizations understand that customers will only be satisfied if they consistently receive products and services that meet their needs, are delivered when expected, and are priced for value. TQM organizations use the techniques of process management to develop cost-controlled processes that are stable and capable of meeting customer expectations.

TQM organizations also understand that exceptional performance today may be unacceptable performance in the future so they use the concepts of process improvement to achieve both breakthrough gains and incremental continuous improvement. Process improvement is even applied to the TQM system itself!

The final element of the TQM model is total participation. TQM organizations understand that all work is performed through people. This begins with leadership. In TQM organizations, top management takes personal responsibility for implementing, nurturing, and refining all TQM activities. They make sure people are properly trained, capable, and actively participate in achieving organizational success. Management and employees work together to create an empowered environment where people are valued.

11.2 Six Sigma

It is a business strategy to achieve excellence by applying different statistical, TQM and Project Management tools. Six Sigma originated from the field of statistics as a set of practices designed to improve manufacturing

processes and limited defects, but its application was subsequently extended to other types of business processes as well. In Six Sigma, a defect is defined as any process output that does not meet customer specifications, or that could lead to creating an output that does not meet customer specifications. The term 'Six Sigma' referred to the ability of manufacturing processes to produce a very high proportion of output within specification. Processes that operate with 'six sigma quality' over the short term are assumed to produce long-term defect levels below 3.4 defects per million opportunities (DPMO). Six Sigma's implicit goal is to improve all processes to that level of quality or better. Bill Smith first formulated the particulars of the methodology at Motorola in 1986. Six Sigma was heavily inspired by six preceding decades of quality improvement methodologies such as quality control, TQM, and 'Zero Defects', based on the work of pioneers such as Shewhart, Deming, Juran, Ishikawa, Taguchi and others.

Six Sigma doctrines assert that

- Continuous efforts to achieve stable and predictable process results (i.e. reduce process variation) are of vital importance to business success.
- Manufacturing and business processes have characteristics that can be measured, analyzed, improved and controlled.
- Achieving sustained quality improvement requires commitment from the entire organization, particularly from top-level management.

11.2.1 Methods

Six Sigma projects follow two project methodologies inspired by Deming's Plan-Do-Check-Act Cycle. These methodologies, composed of five phases each, bear the acronyms DMAIC (Define Measure Analysis Improve Control) and DMADV (Define Measure Analyze Design and Verify).

11.2.2 DMAIC

It is used for projects aimed at improving an existing business process. DMAIC is pronounced as 'duh-may-ick'.

11.2.3 DMADV

It is used for projects aimed at creating new product or process designs. DMADV is pronounced as 'duh-mad-vee'.

Table 11.1 Differences between DMAIC and DMADV

DMAIC	DMADV
<i>Define</i> the problem, the voice of the customer, and the project goals, specifically	<i>Define</i> design goals that are consistent with customer demands and the enterprise strategy.
<i>Measure</i> key aspects of the current process and collect relevant data.	<i>Measure</i> and identify CTQs (characteristics that are Critical To Quality), product capabilities, production process capability, and risks.
<i>Analyze</i> the data to investigate and verify cause-and-effect relationships. Determine what the relationships are, and attempt to ensure that all factors have been considered. Seek out root cause of the defect under investigation.	<i>Analyze</i> to develop and design alternatives, create a high-level design and evaluate design capability to select the best design.
<i>Improve</i> or optimize the current process based upon data analysis using techniques such as design of experiments, <i>poka yoke</i> or mistake proofing, and standard work to create a new, future state process. Set up pilot runs to establish process capability.	<i>Design</i> details, optimize the design, and plan for design verification. This phase may require simulations.
<i>Control</i> the future state process to ensure that any deviations from target are corrected before they result in defects. Implement control systems such as statistical process control, production boards and visual workplaces and continuously monitor the process.	<i>Verify</i> the design, set up pilot runs, implement the production process and hand it over to the process owner(s).

11.3 Lean Manufacturing

It is a way of manufacturing that increases speed and reduces unnecessary wastes. Lean Manufacturing (also called Toyota Production System, TPS) is a production system inspired by the Japanese concept of kaizen (the strategy of continuous improvement). Unlike kaizen, Lean has a focus not only on quality control but also on quantity control to eliminate waste and reduce costs. There are many definitions and viewpoints for ‘Lean Manufacturing’, but the main goal of ‘Lean Manufacturing’ is to eliminate waste. ‘Waste’ refers especially to excess input materials and useless processing steps. The goal of ‘eliminating waste’ may also be stated as providing exactly what the customer values for the lowest cost of production. This will maximize profit per unit. The word ‘Lean’ implies ‘cutting the fat’ or ‘trimming waste’, where ‘fat’ or ‘waste’ refer to whatever is not valued by the customer. So another way of expressing the ‘Lean Manufacturing’ goal is to only use materials and processes that add value for the customer.

11.4 Kaizen

Kaizen (Japanese for ‘improvement’ or ‘change for the better’) refers to philosophy or practices that focus upon continuous improvement of processes in manufacturing, engineering, supporting business processes and management. It has been applied in health-care, psychotherapy, life-coaching, government, banking and many other industries. When used in the business sense and applied to the workplace, kaizen refers to activities that continually improve all functions and involves all employees from the CEO to the assembly line workers. It also applies to processes such as purchasing and logistics that cross organizational boundaries into the supply chain. By improving standardized activities and processes, kaizen aims to eliminate waste (see lean manufacturing). Kaizen was first implemented in several Japanese businesses after the Second World War,

influenced in part by American business and quality management teachers who visited the country. It has since spread throughout the world and is now being implemented in many other venues besides just business and productivity.

The cycle of kaizen activity can be defined as:

- Standardize an operation
- Measure the standardized operation (find cycle time and amount of in-process inventory)
- Gauge measurements against requirements
- Innovate to meet requirements and increase productivity
- Standardize the new, improved operations
- Continue cycle ad infinitum

This is also known as the Shewhart cycle, Deming cycle, or PDCA. Masaaki Imai made the term famous in his book *Kaizen: The Key to Japan's Competitive Success*.

11.4.1 The five main elements of kaizen

- Teamwork
- Personal discipline
- Improved morale
- Quality circles
- Suggestions for improvement

11.5 Lean Six Sigma

It is combination of Six Sigma and Lean Manufacturing concept to reduce errors and increase productivity side by side. ‘Lean’ and ‘Six Sigma’ methodologies seem quite different but a company can benefit by wisely uniting these approaches. Based on the above simplified lists, a Six Sigma project would concentrate on analysis and finish by implementing rigid controls. A ‘Lean’ project seems to assume the analysis is complete, since it does not explain how to minimize or maximize. Also, the ‘Lean’ project gives more autonomy to workers, at least to make suggestions for improvements (Table 11.1).

Table 11.2 Comparison between six sigma project and lean project methodology

Six Sigma Project Methodology	Lean Project Methodology
Select projects to maximize financial improvement but define a limited project scope	Minimize inputs and wasted outputs
Look at the problem from the customer’s point of view	Maximize throughput of any process
Investigate and document the business process, including inputs and outputs	Start a process to meet the customer’s request, rather than to build inventory
Use statistics: take samples and analyze for variations	Produce what the customer requires, correctly, the first time
Minimize variations to ensure repeatable quality	Build ‘continuous improvement’ into every process, by bringing suppliers and front-line workers ‘into the loop’

11.6 Total Productive Maintenance (TPM)

It is a new way of carrying maintenance activities and invented by the Japanese. TPM is a manufacturing-led initiative that creates a collaborative approach among all stakeholders within an organization — particularly between operations and maintenance — in an effort to achieve production efficiency, uninterrupted operations and ensure a quick, proactive maintenance response to prevent equipment-specific problems. The goal of TPM is to create a production environment free from mechanical breakdowns and technical disturbances by involving everybody in maintenance duties without heavily relying on mechanics or engineers. Modernization and the ongoing automation in different industries have noticeably amplified the gap between operators and their machines. Years ago, machine operators were limited to manning their respective posts. Whenever there is a mechanical trouble, operators would stop working and would call in the mechanics to fix the problem. Even with the slightest snag, operators would leave everything to maintenance for fear of making the problem worse and besides they don't want to take on the mechanics' jobs. On the other hand, the traditional mechanics could love the smell of a breakdown. They know that they have become indispensable specialists in the trade — they are assured of a stable job every time a fix is needed. So, the vicious cycle goes on and on and the aftermath of which is immense amount of waste: man hours, production time, opportunity lost and ballooning maintenance expense. But with the adoption and adaptation of Total Productive Maintenance, the vicious cycle has come to an end. Today, TPM builds on the classical Japanese concepts of autonomous maintenance with process mapping for cross-functional duties. Coupled with the right tools and training, TPM equips the operators the necessary skills to address mechanical or equipment-related issues. Calling the engineers and mechanics is no longer necessary since operators are already prepared and confident in dealing with the problems. Undoubtedly, Total Productive Maintenance is one of the most effective ways to create a lean organization with reduced cycle time and improved operational efficiency.

11.7 Environmental Management System (ISO 14001)

It is standard given by ISO to facilitate organization to reduce their wastes that are harmful for the environment. ISO 14001 was first published in 1996 and specifies the actual requirements for an environmental management system. It applies to those environmental aspects which the organization has control and over which it can be expected to have an influence. ISO 14001 is often seen as the corner stone standard of the ISO 14000 series. However, it is not only the most well known but is the only ISO 14000 standard against which it is currently possible to be certified by an external certification authority. Having stated this, it does not itself state specific environmental performance criteria.

This standard is applicable to any organization that wishes to:

- Implement, maintain and improve an environmental management system
- Assure itself of its conformance with its own stated environmental policy (those policy commitments of course must be made)
- Demonstrate conformance
- Ensure compliance with environmental laws and regulations
- Seek certification of its environmental management system by an external third party organization
- Make a self-determination of conformance

11.8 Laboratory Management System (ISO/ IEC 17025)

It is a standard for laboratories for their accreditation. ISO/ IEC 17025:2005 specifies the general requirements for the competence to carry out tests and/ or calibrations, including sampling. It covers testing and calibration performed using standard methods, non-standard methods, and laboratory-developed methods. It is applicable to all organizations performing tests and/ or calibrations. These include, for example, first-, second- and third-party laboratories, and laboratories where testing and/ or calibration forms part of inspection and product certification. ISO/ IEC 17025:2005 is applicable to all laboratories regardless of the number of personnel or the extent of the scope of testing and/ or calibration activities. ISO/ IEC 17025:2005 is for use by laboratories in developing their management system for quality, administrative and technical operations. Laboratory customers, regulatory authorities and accreditation bodies may also use it in confirming or

recognizing the competence of laboratories. ISO/ IEC 17025:2005 is not intended to be used as the basis for certification of laboratories. Compliance with regulatory and safety requirements on the operation of laboratories is not covered by ISO/ IEC 17025:2005.

11.9 5S

It is methodology for improvement in daily and business life in five steps. The 5S are pre-requisites for any improvement program. As waste is potential gain, so eliminating waste is a gain. 5S philosophy focuses on effective work place organization, implies work environment, reduction in waste while improving quality and safety. There is no hope for efficiency or quality improvement with dirty work place, waste of time and scrap. The 5S philosophy is becoming a hallmark in the world of manufacturing. The system hinges on five Japanese concepts: seiri, seiton, seison, seiketsu, and shitsuke. This good housekeeping approach can lead to a smoother running operation. It is a program that can be adapted to other business models as well, bringing the Kaizen way to a variety of workplaces. Kaizen is a process of improvement that requires efforts for the entire team, from management to the workers. It is a series of improvement models they come together to create a faster, better way of working without high cost and maximizes efficiency. The five S stands for the five first letters of these Japanese words (Table 11.2)

Table 11.3 5S Principle

Principle	Meaning
<i>Seiri</i>	Sorting Out
<i>Seiton</i>	Systematic Arrangement
<i>Seison</i>	Spic and Span
<i>Seiketsu</i>	Standardizing
<i>Shitsuke</i>	Self-discipline

11.10 The PDCA Cycles

Imai stated the Japanese executives recast the Deming wheel from the 1950 JUSE seminar into the Plan-Do-Check-Act (PDCA) cycle.

1. Design

Plan: Product design corresponds to the planning phase of management

2. Production

Do: Production corresponds to doing-making, or working on the product that was designed

3. Sales

Check: Sales figures confirm whether the customer is satisfied

4. Research

Action: In case of a complaint being filed, it has to be incorporated into the Planning phase, and action taken for the next round of efforts

The resulting PDCA cycle is shown in Fig. 11.1. The four step cycle for problem solving includes planning (definition of a problem and a hypothesis about possible causes and solutions), doing (implementing), checking (evaluating the results) and action (back to plan if the results are unsatisfactory or standardization if the results are satisfactory). The PDCA cycle emphasized the prevention of error recurrence by establishing standards and the ongoing modification of those standards. Even before the PDCA cycle is employed, it is essential that the current standards be stabilized. The process of stabilization is often called the SDCA

(standardize-do-check-action) cycle. Ishikawa stated: 'If standards and regulations are not revised in six months, it is proof that no one is seriously using them.'

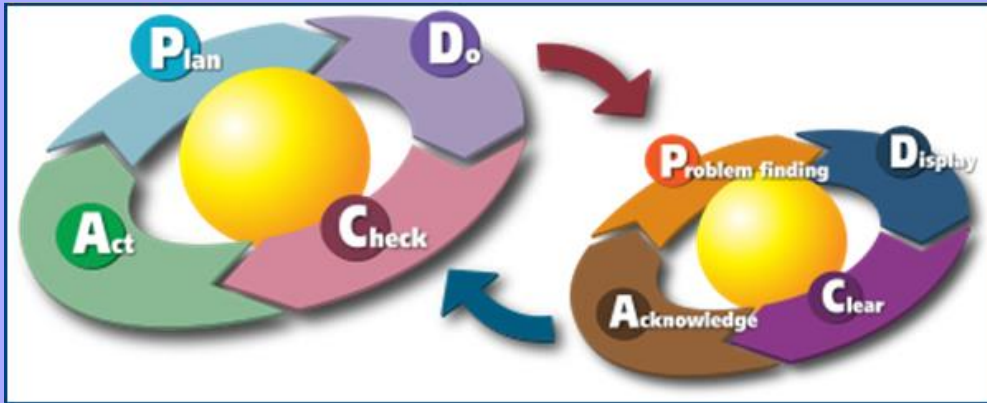


Fig. 11.1 PDCA cycle

11.11 Quality circles

It is group of people, who identify problems and give their solution for improvement in daily life and business. Quality circles were first seen in the United States in the 1950's and later developed by Dr. Kaoru Ishikawa in Japan in the 1960's. Circles were re-exported to the US in the early 1970's and brought total quality management in 1980's resulting in reduction in the use of quality circles and can be a useful tool if used properly. Voluntary groups of employees who work on similar tasks or share an area of responsibility can participate in quality circle. They agree to meet on a regular basis to discuss and solve problems related to work. They operate on the principle that employee participation in decision-making and problem-solving improves the quality of work. It enhances productivity, improve quality and boost employee morale.



Lesson 12
STATISTICAL QUALITY CONTROL

12.1 Introduction

Product wholesomeness and uniformity can be more effectively maintained through a QA program that incorporates available scientific and mechanical tools. Quality is considered to be the degree of acceptability by the user. These characteristics are both measurable and controllable. The major ingredients needed for a successful QA program are education and cooperation. The HACCP approach can be incorporated in a QA program because it applies to a zero defects concept in food production. Effective surveillance of a QA program can detect unsanitary products and variations in production.

Statistical QC techniques make inspection more reliable and eliminate the cost of 100% inspection. The principal tool of a statistical QC system is the control chart. Trends of control charts provide more information than do individual values. Values outside the control limits indicate that the production process should be closely observed and possibly modified.

12.2 Role of Statistical Quality Control

Statistical quality control is the application of statistics in controlling a process for the betterment. Measurements of acceptability attributes are taken at periodic intervals during production and are used to determine whether or not the particular process in question is under control-that is, within certain predetermined limits. A statistical QA program enables management to control a product. This program also furnishes an audit of products as they are manufactured. The samples taken for analysis are destroyed; thus, only SQC is practical for monitoring food safety. The greatest advantage of an SQC program is that it enables management to monitor an operation continuously and to make operating a closely controlled production process.

Sample selection and sampling techniques are the critical factors in any QC system. Because only small amounts (usually less than 10 g) of a product are used in the final analysis, it is imperative that this sample be representative of the lot from which it was selected. Statistical quality control also referred to as operations research, operations analysis, or reliability, is the use of scientific principles of probability and statistics as a foundation for decisions concerning the overall acceptability of a product. Its use provides a formal set of procedures in order to conclude what is important, and how to perform appropriate evaluations. Various statistical methods can determine which outcomes are most probable and how much confidence can be placed in decisions.

12.3 Central Tendency Measurements

Three measurements are commonly used to describe data collected from a process or lot. These are the arithmetic mean or average, mode or modal average and median. The mean is the sum of the individual observations divided by the total number of observations. The mode is the value of observations that occurs most frequently in a data set. The median is the middle value present in collected data. By using these values, the manufacturer can represent characteristics of central tendencies of the measurements taken. Table 12.1 illustrates calculated values for the mean, mode and median from a collection of sample data.

Table 12.1 Central Tendency Values

Data	Mean	Mode	Median
13, 15, 17, 19, 21, 23, 25, 29, 32, 35, 39, 43	26.9	25	27

12.4 Variability

There must be a uniformity and minimal variation in microbial load or other characteristics between the products manufactured. Two measures of variation are the range and standard deviation. Measuring variability by means of the range, R, is accomplished by subtracting the lowest observation from the highest.

$$R = X_{\max} - X_{\min}$$

From Table 12.1 the calculation would be:

$$R = 43 - 13 = 30$$

Because the range is based on just two observations, it does not provide a very accurate picture of variation. As the number of samples increases, the range tends to increase because there is an increased chance of selecting an extremely high or low sample observation. Standard deviation is a more accurate measurement of how data is dispersed as it considers all the values in the data set. The formula for calculating the standard deviation, S, is:

$$S = \sqrt{\frac{(x_1 - \bar{x})^2 + (x_2 - \bar{x})^2 + \dots + (x_n - \bar{x})^2}{n - 1}}$$

Although this formula is more complicated than the range calculation, it can be determined easily by using a personal computer. As the standard deviation increases, it reflects increased variability of the data. To maintain uniformity, the standard deviation should be kept to a minimum.

12.5 Displaying Data

It is beneficial to represent data in a frequency table, especially when a large sample of numbers must be analyzed. A frequency table displays numerical classes that cover the data range of sampling and list the frequency of occurrence of values within each class. Class limitations are selected to make the table easy to read and graph. The frequency table of microbial load from raw materials (Table 12.2) displays how data is divided into each class. To help visualize how these data is arranged, one can graph it in the form of a histogram. Fig. 12.1 takes the information from Table 12.2 and displays it graphically. The histogram in Figure 12.2 depicts an important curve common to statistical analysis — the normal curve or normal probability density function. Many events that occur in nature approximate the normal curve. The normal curve has the easily recognizable bell shape and is symmetrical about the center. The area underneath the curve represents all the events described by the frequency distribution. From Figure 12.2, the mean is the highest point on the curve. The variation of the curve is represented by the standard deviation. It can be used to determine various portions underneath the curve. This is illustrated in the figure where one standard deviation to the right mean represents roughly 34% of the sample values. Consequently, 68.27% of the values fall within ±1 standard deviation from the mean. Similarly 95.45% fall within ± 2 standard deviations. Virtually all of the area (99.75) is represented by ± 3 standard deviations. The information thus far can be used to establish control limits in order to determine whether a process is in a state of statistical control.

Table 12.2. Frequency Table for Microbial Load (CFU/ g)

CFU/ g or ml	Frequency
0-100	7
100-1000	17
1000-10000	25
10000-100000	8
100000-1000000	4

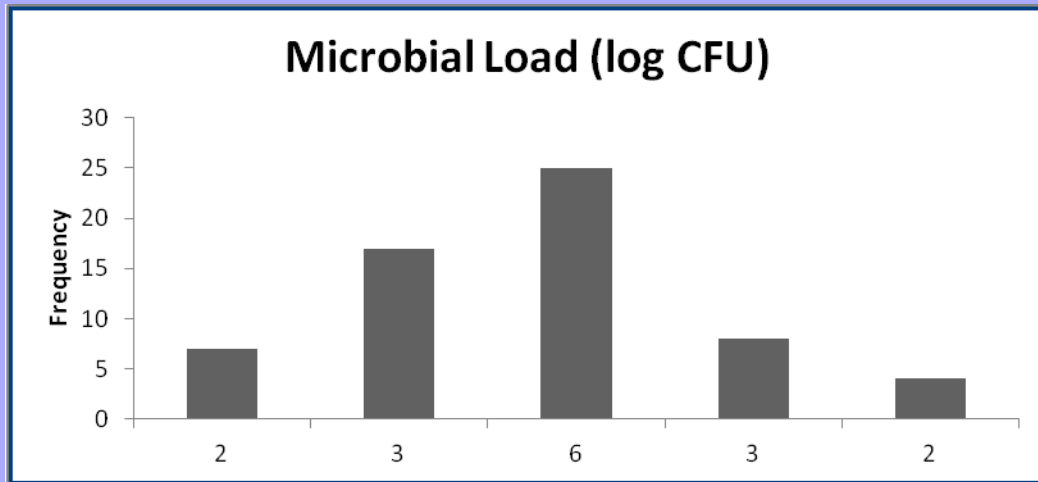


Fig. 12.1 Histogram of Microbial Load

12.6 Control Charts

Control charts offer an excellent method of attaining and maintaining a satisfactory level of acceptability. The control chart is a widely used industry technique for on-line examination of materials produced. In addition to providing a desired safety level, it can be useful in improving sanitation and in providing a sign of impending trouble. The primary objective is to determine the best methodology, given the available resources, then to monitor control points. This variation can be classified as either chance-cause variation or assignable-cause variation. In chance-cause variation, the end products are different because of random occurrences.

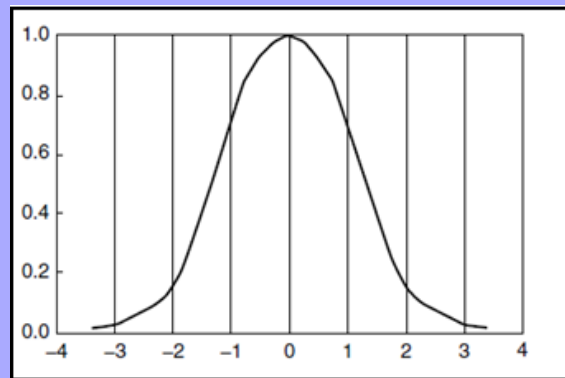


Fig. 12.2 Normal curve

They are relatively small and are unpredictable in occurrence. There is a certain degree of chance-cause variation present. Assignable-cause variation is just what the name implies. Cause can be ‘assigned’ to a contributing factor, such as a difference in microbial load of raw materials, process, and machine aberration, environmental factors, or operational characteristics of individuals involved along the production line. This variation, once determined, is controlled through appropriate corrective action. When a process shows only variation due to chance causes, it is “under control.” Quality control charts were developed in order to differentiate between the two types of variation and to provide a method to determine whether a system is under control. Fig. 12.3 illustrates a typical control chart for a quality characteristic. The y-axis represents the characteristic of interest plotted against the x-axis, which can be a sample number or time interval. The center line represents the average or mean value of the quality trait established by the manufactured product when the process is under control. The two horizontal lines above and below the center line are labeled so that as long as the process is in a state of control, all sample points should fall between them.

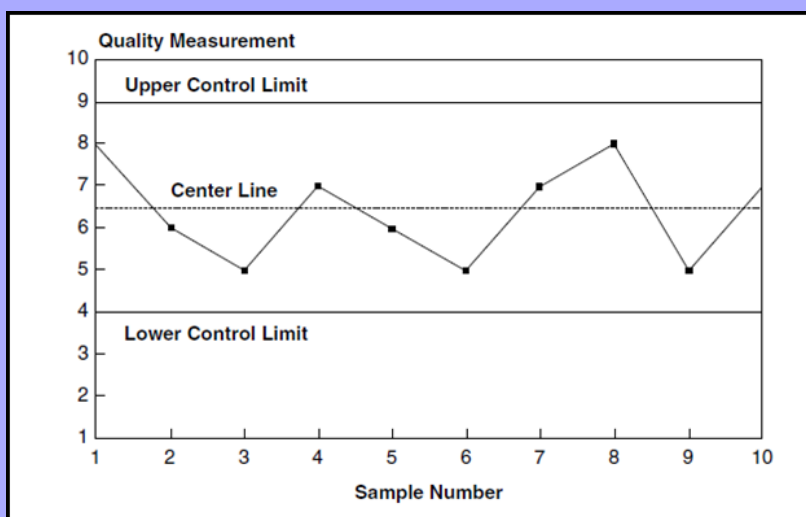


Fig. 12.3 Typical control Chart for a quality characteristic.

The variation of the points within the control limits can be attributed to chance cause and no action is required. An exception to this rule would apply if a substantial number of data points fall above or below the center line instead of being randomly scattered. This would indicate a condition that is possibly out of control and would warrant further investigation.

If a point falls above or below the out-of bounds lines, one can assume that a factor has been introduced that has placed the process in an out-of-control state, and appropriate action is required. Control charts can be divided into two types:

1. Control charts for measurement
2. Control charts for attributes

12.7 Measurement Control Charts

Measurement of variable control charts can be applied to any characteristic that can be measured. The X chart is the most widely used chart for monitoring central tendencies, whereas the R chart is used for controlling process variation. The following examples show how both of these control charts are used in a manufacturing environment.

A food manufacturer may monitor the microbial load of raw milk satisfy safety concerns. Five samples may be pulled after every shift during an 8-hour shift and analyzed for total bacterial count Table 12.3.

Table 12.3 X and R Values for microbial load Measurements

Sample	Microbial load of raw milk (log CFU)					X	R
1	5.1	4.8	4.6	5	4.9	4.88	0.5
2	4.9	5.3	4.6	5.3	4.6	4.94	0.7
3	4.8	4.9	4.7	5.4	4.8	4.92	0.7
4	5.3	5.2	5.3	5.1	4.7	5.12	0.6
5	4.6	5.1	5.2	4.9	4.9	4.94	0.6
6	4.7	5	4.9	4.9	5	4.9	0.3
7	4.8	4.9	4.8	4.8	5.1	4.88	0.3
8	4.4	4.8	4.7	4.7	4.9	4.7	0.5
Average						4.91	0.525

First calculate the average (X) and range (R) for each inspection sample. For example, sample calculations for sample 1 are:

$$X = \frac{5.1 + 4.8 + 4.6 + 5.0 + 4.9}{5} = 4.88$$

R is the highest value minus the smallest value of the five samples.

After all of the sample Xs and Rs are calculated, take the average of the Xs and Rs to obtain Average X and Average R.

$$\text{Ave. } X = \frac{\text{sum of all } X}{\text{Number of sample lots}} = 4.91$$

$$\text{Ave. } R = \frac{\text{Sum of all } R\text{s}}{\text{no of lots}} = 0.525$$

From the calculated, the center line for the X and R chart can be defined to be:

X Chart center line = 4.91
 R Chart center line = 0.525

In order to calculate the upper control limits (UCL) and lower control limits (LCL), the standard deviation for each sample lot must be determined. Rather than perform the lengthy calculation needed for this value, another method can be used to determine these values. The control limits for the previous charts were represented by:

$$\text{UCL} = \bar{X} + 3\delta$$

$$\text{LCL} = \bar{X} - 3\delta$$

By substituting a factor (A₂) from a statistical table into the above equation for UCL and LCL, the needed values for the control point can be obtained. In this example, the value for (A₂) for a sample size of 5 is 0.525.

The new equation becomes:

$$\text{UCL} = \text{Ave. } X + A_2 \text{ Ave. } R$$

$$\text{LCL} = \text{Ave. } X - A_2 \text{ Ave. } R$$

Substituting,

$$\text{UCL} = 4.91 + 0.25(0.252) = 4.9735$$

$$\text{LCL} = 4.91 - 0.25(0.252) = 4.8464$$

The control limits for the R chart are determined similarly, using factors D₄ and D₃ from the statistical reference table.

$$D_4 = 2.11, D_3 = 0$$

$$\text{UCL} = D_4 R = 2.11 (0.252) = 0.53172$$

$$\text{LCL} = D_3 R = 0 (0.252) = 0$$

Once these calculations are complete, the values can be plotted on an X-Y chart to obtain the X and R charts (Figures 12.4 and 12.5) for microbial load detection. Figures 12.4 and 12.5 illustrate complete control charts from the sample data. Both graphs show a process currently under control, with all data points lying within the boundaries of the control limits and an equal number of points above and below the center line.

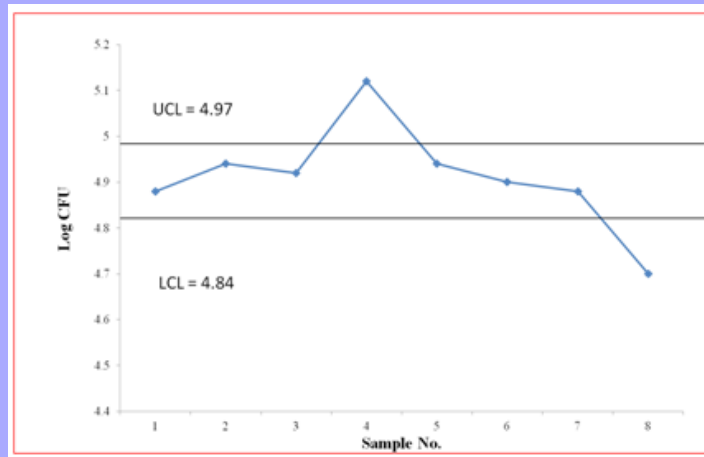


Fig. 12.4 X chart for Microbial Load

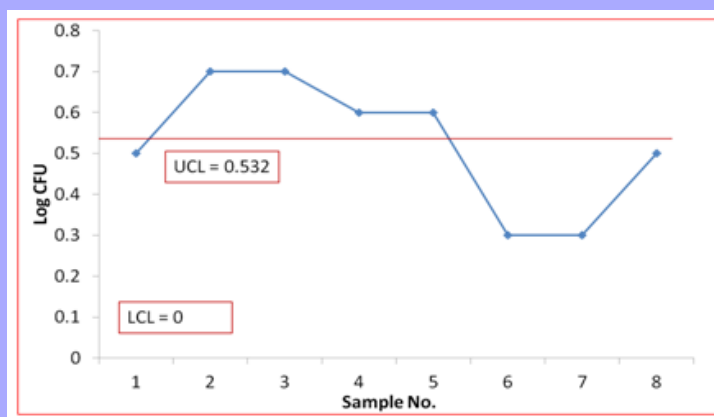


Fig. 12.5 R chart for Microbial Load

12.8 Attribute Control Charts

Attribute control charts differ from measurements charts in that one is interested in an acceptable or unacceptable classification of products. The following charts are commonly used for attribute testing:

- p charts
- np charts
- c charts
- u charts

12.8.1 p Charts

The p chart, one of the more useful attribute control charts, is used for determining the unacceptable (p) fraction. It is defined as the number of unacceptable items divided by the total number of items inspected. For example, if a producer examines five samples per hour (for an 8-hour shift) from the production line and finds a total of eight unacceptable units, p would be calculated as follows:

Total number of unacceptable (dy) = 9
 Total number of inspected (dx) = 5(8) = 40

$$p = \frac{dy}{dx} = \frac{9}{40} = 0.225$$

Sometimes this value is represented as percentage unacceptable. In this example, percentage defective would be:

$$0.225 \times 100 = 22.5\%$$

An attribute control chart can be constructed from a sampling schedule by obtaining an average fraction unacceptable (ρ) value from a data set and using the formula, or the desired control limits. Because attribute testing follows a binomial distribution, the standard deviation would be calculated:

$$\delta = \frac{\sqrt{\text{ave. } \rho(1 - \text{ave. } \rho)}}{n}$$

Where n is the number of items in a sample.
Control limits would be obtained by:

$$\text{UCL} = \rho + 3\delta$$

$$\text{LCL} = \rho - 3\delta$$

When these data are plotted and no points are outside of the control limits, it can be assumed that the process is in a state of statistical control, and any variation can be attributed to natural occurrences.

12.8.2 np Charts

np charts can be used to determine the number of unacceptable instead of the fraction defective, and the sampling lots are constant. The formula for the number of unacceptable (np) is:

$$\text{Number of unacceptable (np)} = n \times p,$$

Where n is the sample size and p is the unacceptable fraction defective. If one value is known, the other can be easily calculated. For example, if a sample lot of 50 is known to be 5% unacceptable, the number of unacceptable should be:

$$np = 50 \times 0.05 = 2.5$$

The calculation for determining the control limits would be the same as for the p chart, except that the standard deviation would be:

$$d = np(1 - p)$$

12.8.3 c Charts

These charts are used when the concern is the number of defects per unit of product. They are not as frequently incorporated as the p and np charts but can be effective if applied correctly. Assume that a manufacturer examines 10 lots and discovers 320 defects. The equations for the average (c) and standard deviation required for a c chart is:

$$\text{Ave. } c = \frac{500}{15} = 33.3$$

$$\delta = \sqrt{c} = \sqrt{33.3} = 5.77$$

The control limits would be:

$$\text{UCL} = \text{Ave. } c + 3\sqrt{c} = 32 + 3(5.77) = 50.65$$

$$\text{LCL} = \text{Ave. } c - 3\sqrt{c} = 32 - 3(5.77) = 16.01$$

12.8.4 u Charts

Sometimes, a constant lot size may not be attainable when examining for defects per unit area. The u chart is used to test for statistical control. By establishing a common unit in terms of a basic lot size, one can determine equivalent inspection sample lot sizes from unequal inspection samples. The number of equivalent common basic lot sizes (k) can be calculated as:

$$k = \text{size of sample lot} / \text{size of common lot}$$

The u statistic can be determined from c, the number of defects of a sample lot, and the k value defined in the above equation.

$$\mu = c/k$$

From these values, the upper and lower control limits for the u chart can be defined as:

$$\text{UCL} = \text{Ave. } u + 3\sqrt{\text{ave. } u/k}$$

$$\text{LCL} = \text{Ave. } u - 3\sqrt{\text{ave. } u/k}$$

In addition to charting, a manufacturer may introduce other statistical analyses, such as modeling, variable correlations, regression, analysis of variance, and forecasting to the production area. These techniques provide additional statistical methods for examining processes in order to ensure maximum production efficiency.

12.9 Explanation and definition of statistical quality control program standards

The following terms apply to maintenance of standards:

- Standard: The level or amount of a specific attribute desired in the product.
- Quality attribute: A specific factor or characteristic of the food product that determines a proportionate part of the acceptability of the product. Attributes are measured by a predetermined method, and the results are compared against an established standard and lower and upper control limits to determine if the product attribute is at the desired level in the food product.
- Retained product: A product that is not to be used in production or sold until corrective action has been taken to meet the established standards. Retained products should not be released for production or sales use until the problem is corrected.

12.10 Rating Scales

Two rating scales have been devised for evaluation of attributes:

1. Exact measurement: For attributes that can be measured in precise units (bacterial load, percentage, parts per million, etc.).

2. Subjective evaluation: Used when no exact method of measurement has been developed. The evaluation must be conducted through sensory judgment (taste, feel, sight, smell). This is usually described numerically. Two scales have been developed for evaluating acceptability:

<i>Scale 1</i>	<i>Scale 2</i>
7—Excellent	4—Extreme
6—Very good	3—Moderate
5—Good	2—Slight
4—Average	1—None
3—Fair	
2—Poor	
1—Very poor	

The number of samples to conduct at any point during production to evaluate the sanitation operation also depends on the variations of analysis of the samples. A minimum of three to five samples of approximately two kg each should be selected and pooled from each lot of incoming raw material. After a sufficient number of samples have been analyzed, control charts can be constructed for each raw material.

Sampling of the finished product should be conducted at a special step in the production sequence, such as at the time of packaging. Sampling at this stage does not need to be done on individual products for inspection or regulatory purposes because it is directed at monitoring process control, not individual product analysis. However, to be familiar with the wholesomeness and overall acceptability of each product, the preferred procedure is to analyze and maintain control charts on all products.

Sample size usually consists of three to five specimens that serve as a representative of the population sampled. Another guideline for sample size is the square root of the total units, and, for large lots, an acceptable size may be the square root of the total units divided by 2. Daily sampling is necessary to monitor process control effectively. Action limits for finished products should be as outlined under the analysis program and should be used in determining whether the process conforms to the designated specifications. If three consecutive samples exceed the maximum limit for contamination, production should cease, with further cleaning.

12.11 Cumulative Sum (CUSUM) Control Charts

Data can be plotted where greater sensitivity in detecting small process changes is required by use of the CUSUM chart. This chart is a graphic plot of the running summation of deviation from a control value. These differences are totaled with each subsequent sampling time to provide the CUSUM values. This monitoring technique can be incorporated in sanitation operations that require a higher degree of precision than obtained from a regular statistical QC chart.

The CUSUM chart gives a more accurate account of real changes, faster detection and correction of deviation, and a graphical estimation of trends. It enhances an optimum process control for various applications. Webb and Price (1987) suggested that the CUSUM chart was not developed for multiple levels and is not practical for use on production processes that drift over an extended period of time. If used, it is important that the results of the CUSUM system be kept current so that immediate corrective action may be taken.

A personal computer can rapidly perform the statistical computations and identify the points that require corrective action, thus reducing the burden of processing large quantities of data. This data can be available to promptly expedite corrective actions, project future performances and determine when and where preventive QC procedures are necessary.

Lesson 13 CONCEPTS OF HYGIENE AND SANITATION

13.1. Introduction

In history medicine and hygiene have always been counterparts in healing and preventing diseases. However, both disciplines have mostly gone hand in hand in improving human health. After the recognition of germs as causing agent of diseases the significance of hygiene developed rapidly and is now considered as the corner stone of safe food production.

In the mid nineteenth century, two persons lay the foundation of modern hygiene. It was Hungarian physician Semmelweis and the British surgeon Lister. Both introduced hygienic methods which still appear to be essential in modern society. Ignác Fülöp Semmelweis (1818 - 1865) was a Hungarian physician who demonstrated that puerperal fever 1 (also known as "childbed fever") was contagious and that its incidence could be drastically reduced by enforcing appropriate hand-washing behavior by medical care-givers. Since the cadaverous matter could not be removed from hands merely by washing them with soap and water and therefore experiments with different chemicals were started where he recommended the additional use of chlorinated lime resulting in reduction of death rate caused by puerperal fever to zero level.

In 1865, Louis Pasteur suggested that decay in wounds was caused by living organisms in the air, which on entering matter caused it to ferment. He considered that microbes in the air were likely causing the putrefaction and need to be destroyed before they entered the wound. Joseph Lister (1827-1912) made a triumphal tour of the leading surgical centers in Germany in 1875 where he met Robert Koch who demonstrated in 1878 the usefulness of steam for sterilizing surgical instruments and dressings.

Public health concern with food borne diseases emerged around 1880's. This was after micro-organisms were recovered as infectious agents. Koch and his assistants devised the techniques for culturing bacteria outside the body, and formulated the rules for showing whether or not a bacterium is the cause of a disease. Identification of agents involved in food borne diseases and the etiological research of food-borne diseases began at the end of the 19th century when the work of Van Ermengem served to clarify the etiology of botulism in man (Van Ermengem, 1897). Later milestones in this category included the recognition of *Clostridium perfringens* as a food-borne pathogen in 1943 (McClane, 1979) and *Bacillus cereus* in the 1950's (Kramer and Gilbert, 1989). Human infections with *Listeria monocytogenes* were well known by the 1940's and food borne transmission was suspected (Rocourt and Cossart, 1997), but it was not until the occurrence of an outbreak in Canada in 1981 that proper evidence was obtained. Since then, numerous food-borne outbreaks have been reported in different countries, and prevention of listeriosis has become a major challenge for the Food Industry. Around 1980 – 1985 *S. enteritidis* re-emerged via the internal contamination of chicken eggs. At the same time a new emerging pathogenic started to emerge: *Escherichia coli* O157:H7 (Willshaw et al., 2000). This organism causes hemorrhagic colitis. In some victims, particularly the very young, may develop the hemolytic uremic syndrome (HUS) which is characterized by renal failure and hemolytic anaemia.

13.2. Hygiene and food sanitation

Following the discovery, around 1880, that food can be an important source of disease-causing organisms, investigations started to concentrate on the reservoirs and routes of transmission of pathogens. At the same time, attention began to focus on the presence of pathogenic bacteria in the intestines of animals, as a source of food contamination, and foods of animal origin, as routes of transmission to humans. Savage (1909) observed that faecal contamination of food must be very common and milk, in particular, was suspected to be a vehicle of infection. Theodor Escherich, a German paediatrician, who devoted his efforts to improving childcare, particularly in relation to infant hygiene and nutrition, was the first to make a plea for heat-

processing of milk to prevent infant diarrhoea (Escherich, 1890). After that time, the heating processes used for food began to improve. Real progress was made when Esty and Meyer (1922) developed the concept of process-performance criteria for heat treatment of low-acid, canned food-products to reduce the risk of botulism. An outstanding example is the work of Enright et al. (1956, 1957), who established performance criteria for the pasteurization of raw milk that provided an appropriate level of protection (ALOP) against *Coxiella burnetii*, the causative agent of Q fever. Studies on the agent responsible for tuberculosis had been carried out earlier. Based on the need to improve hygiene in slaughter plants, the USA was one of the first countries to introduce a Meat Inspection Act in 1906. This brought the following reforms to the processing of cattle, sheep, horses, swine and goats destined for human consumption:

- All animals were required to pass an inspection by the US Drug Administration prior to slaughter;
- All carcasses were subject to a post-mortem inspection;
- Standards of cleanliness were established for slaughterhouses and processing plants.

In the UK, it was recognized that legislation alone was not sufficient to protect consumers against food borne diseases, and the health authorities became aware of the need for public education to achieve cleaner food supplies. Food handling practices were very poor. A new Food and Drug Act was introduced in the UK in 1938, and it became necessary to use hygienic conditions and practices in handling, wrapping and delivering food, and adequate hand-washing facilities for food handlers. A clear breakthrough in public health was the processing and disposal of domestic and sewage wastes, in conjunction with the purification of water supplies to ensure that any pathogens present were not passed to consumers via drinking water. Also, sanitary microbiologists were appointed to inspect food-processing and eating establishments to ensure that proper food-handling procedures were followed. These made a significant contribution to the development of appropriate hygiene standards.

13.2.1 Definitions of hygiene

In ancient times, it was clear that diseases could be overcome, either by actively curing (Asclepius) or through the power of cleanliness (Hygeia). The first definitions of 'hygiene' are derived from the work of the Goddess Hygeia:

'Healing through cleanliness'

'The science dealing with the preservation and promotion of health'

By the beginning of the 20th century, it had become clear that preventive measures were the only way to produce safe food, and the discipline of food hygiene was born. Current definitions of 'food hygiene' are presented in Table 12.1.

Table 12.1 Definitions of food hygiene in current use

Definition	Reference
Conditions and practices that preserve the quality of food to prevent contamination and food-borne illnesses.	http://www.nlm.nih.gov/medlineplus/ency/article/002434.htm
All measures necessary to ensure the safety and wholesomeness of foodstuffs. EU's General Food Hygiene Directive	Anonymous, 1993
All conditions and measures necessary to ensure the safety and suitability of food at all stages of the food chain.	Codex Alimentarius Commission (Anon., 1997) CAC/RCP 1-1969, Rev. 3 (1997), Amended 1999
The measures and conditions necessary to control hazards and ensure fitness for human consumption of a foodstuff, taking into account its intended use	Environmental Health Journal, 2000, 108/9 http://www.ehj-online.com/archive/2000/september/sept10.html COM (2000) 438. Final. Brussels, 14 July 2000.

13.2.2 Food sanitation

Sanitation is a term for the hygienic disposal or recycling of waste materials, particularly human excrement. In consequence, sanitation is an important public health measure that is essential for the prevention of disease. In the USA, there is a particular focus on the concept of food sanitation, which may be defined as 'the hygienic practices designed to maintain a clean and wholesome environment for food production, preparation and storage' (Marriot, 1999). This second definition links hygiene more specifically with maintaining a clean working environment for food processing. Even here, hygiene requirements extend beyond the practice of cleaning itself to incorporate those elements that make effective cleaning possible and allow control of insects and other pests. In the microbiological sense, sanitation is defined as 'a cleaning and disinfection process that results in a 99 – 99.9 % reduction in the number of vegetative bacteria present'.

13.2.3 Personal hygiene

Personal hygiene is of great importance for the maintenance of health in general. Human beings are natural carriers of many micro-organisms and sources include the hair, skin, mucous membranes, digestive tract, wounds, infections and clothing. Good personal hygiene is primarily directed towards preventing both disease and discomfort. Hand washing, dental care, avoidance of spitting, daily showering, etc, as well as clean living play an important part. Disposal of waste is also important. All these measures are preventive in character and are readily carried out.

13.2.4 Hygienic design of facilities and equipments

Hygienic design of food-production facilities, processing equipment etc. is a most important factor in ensuring that food is safe and wholesome. Poorly designed farms, factories, and equipment can easily result in contamination of food products and lead to food-poisoning incidents. Furthermore, design deficiencies may result in losses of product due to spoilage increased cleaning costs and reduced production time. These aspects are also of possible environmental concern. Therefore, it is essential that both manufacturers and users of food-processing equipment are aware of hygienic design principles and requirements such as those described in EU Directives 98/ 37/ EC and 93/ 43/ EEC, and Hygienic Design DIN EN 1672/ 2 (1997). Hygienic production of food thus depends upon a combination of food-processing procedures and hygienic design of buildings and equipment, in full compliance with legislation.

13.2.5. Hygiene control measures in food processing

Hygiene in food processing started with the introduction of general measures, including cleaning and disinfection, prevention of re-contamination and treatment of food products to kill any microbial pathogens present. Research carried out in the earlier 19th century resulted in predictions relating to many processes, such as heating, acidification, drying and the use of curing agents and its effect on both pathogenic and spoilage organisms. Such knowledge ushered in a new era in safe food production. This era is characterized by the division of hygiene measures into specific practices that are controllable and other general measures.

13.3 GHP concept

One of the first safety systems developed by the food industry was that involving the application of Good Manufacturing Practices (GMP), as a supplement to end-product testing. GMP covers all aspects of production, from starting materials, premises and equipment to the training of staff and the WHO has established detailed guidelines. GMP also provides a framework for hygienic food production, which is often referred to as Good Hygienic Practice (GHP). The GHP concept is largely subjective and its benefits tend to be qualitative rather than quantitative. It has no direct relationship to the safety status of the product, but its application is considered to be a necessary preventive measure in producing safe food. Those hygiene measures that have a predictable outcome and are subject to control can be incorporated in the Hazard Analysis Critical Control Point (HACCP) concept. This concept seeks, among other things, to avoid reliance on microbiological testing of the end-product as a means of controlling food safety. Such testing may fail to distinguish between safe and unsafe batches of food and is both time-consuming and relatively costly. However, effective application of the HACCP concept depends upon GHP being used. The establishment of GHP is the outcome of long practical experience and major components of the system are

13.3.1 Design of premises and equipment

This includes the location and layout of the premises to avoid hygiene hazards and facilitate safe food production. Food processing and handling equipment should always be designed with hygiene in mind, including ease of cleaning.

13.3.2 Control of the production process

Control measures are applied throughout the supply chain and cover factors such as raw materials, packaging and process water, as well as the product itself. Key aspects include management and supervision of the process as a whole, as well as appropriate recording systems.

13.3.3 Plant maintenance and cleaning

Both processing equipment and the fabric of the building should be maintained in good order. Suitable programmes need to be developed for plant cleaning and disinfection, and their effectiveness monitored routinely. Systems are also needed for pest control and management of waste.

13.3.4 Personal hygiene

Staffs are required to maintain high standards of personal hygiene in relation to wearing of protective clothing, hand washing and general behaviour. Visitors must also be strictly controlled in these respects. The health status of personnel should be monitored regularly and any illness or injuries recorded.

13.3.5 Transportation

Requirements should be established for the use and maintenance of transport vehicles, including their cleaning and disinfection. Vehicle usage should be managed and supervised.

13.3.6 Product information and consumer awareness

It is important that the final product is suitably labeled and that the consumer is provided with all relevant information on product handling and storage, including a 'use-by' date. Labelling should also indicate the batch and origin of the product, so that full traceability is possible.

13.3.7 Staff training

In relation to food hygiene and safety, all personnel should receive appropriate training and be made fully aware of their individual responsibilities. Such training should be repeated and updated as required.

13.4 Future aspects

13.4.1 Further development of hygiene control

From long experience, it has become clear that certain hygiene controls are very effective in reducing food borne disease, and the effects of certain measures, like heating the product, have a predictable outcome. Thus, they have been incorporated eventually in the HACCP system. However, there are still a large number of important measures that contribute to food safety but their effects are neither quantifiable nor properly understood. Examples include the effects of cleaning and disinfection, steps to prevent cross-contamination in food processing and hand washing and other aspects of personal hygiene. On the other hand, micro-organisms may sometimes become established unexpectedly in processing equipment and food-production facilities, thus increasing contamination of the product. In this case, the usual process parameters are controlled, but other, unknown factors are having an effect. Clearly, more information is needed on the factors that affect product safety and those that have little or no effect.

13.4.2 Building hygiene into the system

A new research area that aims to improve general hygiene involves nano-technology. This technology is a promising means of developing processes that are inherently hygienic. For example, coatings based on nano-technology can make the environment more hygienic by preventing bacterial attachment to surfaces (ceilings, floors and walls of processing facilities, conveyor belts etc.) and/ or bacterial proliferation on these surfaces. Coatings have already been developed and successfully applied to prevent fouling of, for example, windows, water-closets and tiles. Thus, modern technology can do much to protect society from pathogenic agents, but this takes no account of one important factor: natural disease-resistance. Without such resistance, human beings will continue to be highly vulnerable and require even-more protection from pathogens.

13.4.3 Changing pattern of microbial hazards

Society is increasingly confronted with microbial problems that are not susceptible to control by traditional measures. This may involve new hazards, including viral contamination of food and the occurrence of bacteria resistant to antibiotics and disinfectants. Many of these problems arise from the introduction of new technologies, new methods of producing raw food-materials and socio-economic changes in society, including overcrowding, increased traveling and global food-production and trade. Food borne disease continues at a high level, despite increasing attention to food hygiene, and with no alternative strategy available. This situation is an important challenge to modern society and requires a degree of foresight that goes well beyond present concepts of hygiene control.



Lesson 14 PERSONNEL HYGIENE

14.1 Introduction

Everyone has the right to expect the food he/ she consumes is safe and suitable for consumption. Food poisoning is a serious health problem and can cause severe illness and even death. In addition, outbreaks of food borne illness damage trade and tourism, lead to loss of earnings, unemployment and litigation. Food spoilage is wasteful, costly and adversely affects trade and consumer confidence. Increase in international food trade and foreign travel have brought important social and economic benefits. Eating habits have undergone a major change worldwide during the past three decades. These opportunities have also made the world population vulnerable for spread of diseases over the globe very fast, if not handled hygienically. Hence, effective hygiene control is vital to avoid adverse effects on human health and economy due to food borne illness and spoilage.

Personnel handling milk and milk products constitute one of the most important sources of microbial contamination including potential pathogens in these products. Hence, personnel hygiene plays a very significant role in influencing the microbiological quality of the milk and milk products which, in turn, may affect the health of the consumers. If the personnel handling of these delicate food stuffs are not well acquainted with the codes of hygiene practices, they can inadvertently introduce lots of undesirable microorganisms merely by touching. This can be achieved by adopting food hygiene principles throughout the food chain and HACCP based approach. All stakeholders including producers, manufacturers, food handlers and consumers have a responsibility to assure that food is safe and suitable for consumption. Both government and industry have the responsibility to provide safe and suitable milk and milk products for consumers practicing principles of food hygiene.

14.2 Food handlers

Any person who directly handles packaged or unpackaged food, equipment and utensils or food contact surfaces and is therefore expected to comply with food hygiene requirements. Food handlers are potential sources of microorganisms that cause illness and food spoilage. Parts of the body that contribute to the contamination of food include the skin, hands, hair, eyes, mouth, nose, naso-pharynx, respiratory tract, and excretory organs. These parts are contamination sources as carriers, through direct or indirect transmission, of detrimental microorganisms.

14.2.1 Carrier

A carrier is a person who harbours and discharges pathogens but does not exhibit the symptoms of the disease. Carriers are divided into three groups:

14.2.2 Convalescent carriers

After recovering from an infectious disease, continue to harbour the causative organism for a variable length of time, usually less than 10 weeks.

14.2.3 Chronic carriers

People continue to harbour the infectious organism indefinitely, although they do not show symptoms of the disease.

14.2.4 Contact carriers

People acquire and harbour a pathogen through close contact with an infected person but do not acquire the disease.

14.2.1 Pathogenic bacteria associated with food handlers

14.2.1 *Streptococci*

These organisms, commonly harboured in the human throat and intestines, are responsible for a wider variety of diseases than other bacteria. They are also frequently responsible for the development of secondary infections.

14.2.2 *Staphylococci*

The most important single reservoir of *Staphylococci* infection of humans is the nasal cavity. Equally important to the food industries are those who possess the pathogenic varieties of the organism as part of their natural skin flora. These people are a constant threat to consumer safety if they are permitted to handle food products.

14.2.3 Intestinal microorganisms

This group of organisms includes *Salmonella*, *Shigella*, *Escherichia coli*, cholera, infectious hepatitis and infectious intestinal amoebas. These microorganisms are of public health concern because they can contribute to serious illness.

14.4. Personal Contamination of Food Products

The intrinsic factors that affect microbial contamination by people are as follows.

14.4.1 Body location

The composition of the normal microbial flora varies depending on the body area. The face, neck, hands and hair contain a higher proportion of transient microorganisms and a higher bacterial density. The exposed areas of the body are more vulnerable to contamination from environmental sources. When environmental conditions change, the microbial flora adapt to the new environment.

14.4.2 Age

The microbial population changes as a person matures. This trend is especially true for adolescents entering puberty. They produce large quantities of lipids known as sebum, which promotes the formation of acne caused by *Propionibacterium acnes*.

14.4.3 Hair

Because of the density and oil production, the hair on the scalp enhances the growth of microbes such as *S. aureus* and *Pityrosporum*.

14.4.4 pH

The pH of the skin is affected through the secretion of lactic acid from the sweat glands, bacterial production of fatty acids, and diffusion of carbon dioxide through the skin. The approximate pH (5.5) for the skin is more selective against transient microorganisms than it is against the resident flora. Factors that change the pH of the skin (soap, creams, etc.) alter the normal microbial flora.

14.4.5 Nutrients

Perspiration contains water soluble nutrients (i.e., inorganic ions and some acids), whereas sebum contains lipid (oil)-soluble materials such as triglycerides, esters, and cholesterol. The role of perspiration and sebum in the growth of microorganisms is not fully understood.

14.5. Methods of Disease Transmission

14.5.1 Direct transmission

Many diseases are transmitted through direct transfer of the microorganisms to another person through close contact. Examples are diphtheria, scarlet fever, influenza, pneumonia, smallpox, tuberculosis, typhoid fever, dysentery and venereal diseases. Respiratory diseases may be transferred via atomized particles extruded from the nose and mouth when a person talks, sneezes, or coughs. When these particles become attached to dust, they may remain suspended in the air for an indefinite length of time. Other people may then become infected upon inhaling these particles. Wall mounted no-touch hand sanitizer may be a good alternative to prevent such incidences of direct bodily touch.

14.5.2 Indirect Transmission

The host of an infectious disease may transfer organisms to vehicles such as water, food, and soil. Lifeless objects, other than food, capable of transmitting infections are doorknobs, telephones, pencils, books, washroom fittings, clothing, money, and knives. Intestinal and respiratory diseases such as salmonellosis, dysentery and diphtheria may be spread by indirect transmission. To reduce the transfer of microorganisms by indirect transmission, wash basins should have foot-operated controls instead of hand-operated faucets, and doors should be self-closing.

14.6. Precautions

14.6.1. Hand Washing

14.6.1.1 Transient or resident bacteria

Microorganisms found on the hand surfaces may be transient or resident bacteria. Transient bacteria are picked up accidentally by food handlers and are transient in that they reside on the hands only temporarily (e.g., *E. coli*). Residual microorganisms permanently reside on the hand surfaces and are the normal or resident microflora of the skin (e.g., *Staphylococcus epidermidis*). The first line of defense against disease is frequent and effective hand washing by food handlers. Approximately 38% of food contamination is attributable to improper hand washing. Few examples are illustrated below:

1. On a 4-day Caribbean cruise, 72 passengers and 12 crew members has diarrhoea, and 13 people had to be hospitalized. Stool samples of 19 of the passengers and 2 of the crew contained *Shigella flexneri* bacteria. The illness was traced to German potato salad prepared by a crew member that carried these bacteria. The disease spread easily because the toilet facilities for the galley crew were limited.
2. Over 3,000 women who attended a 5-day outdoor music festival in Michigan became ill with gastroenteritis caused by *S. sonnei*. The illness began 2 days after the festival ended, and patients were spread all over the United States before the outbreak was recognized. An uncooked tofu salad served on the last day caused the outbreak. Over 2,000 volunteer food handlers prepared the communal meals served during the festival. Before the festival, the staff had a smaller outbreak of shigellosis. Sanitation at the festival was mostly acceptable, but access to soap and running water for hand washing was limited. Appropriate hand washing facilities could have prevented this explosive outbreak of food borne illness.
3. *Shigella sonnei* caused an outbreak of food borne illness in 240 airline passengers on 219 flights to 24 states, the District of Columbia, and four countries. The outbreak was identified only because it involved 21 of 65 football team players and coaches. Football players and coaches, airline passengers, and flight attendants with the illness all had the same strain of *S. sonnei*. The illness was caused by cold food items served on the flights that had been prepared by hand at the airline flight kitchen.

14.6.1.2 Procedures for hand washing

Hand washing is conducted to break the transmission route of the microorganisms from the hands to another source and to reduce resident bacteria.

14.6.1.2.1 Manual double hand washing method

Hand washing for 15 seconds (as opposed to the average of 7 seconds) with soap and water, which act as emulsifying agents to solubilise grease and oils on the hands, will remove transient bacteria. Increased friction through rubbing the hands together or by using a scrub brush with soap can reduce the number of transient and resident bacteria than is done by quick hand washing. Hand washing and drying efficacy against resident flora ranges from 35 to 60%. All hand washing agents, including water, are effective when the hands are dried with paper towels. Fig. 14.1 illustrates a manual double hand washing method.

14.6.1.2.2 Mechanized hand washers

Because proper hand washing is essential to attain a sanitary operation, mechanized hand washers are being used. Typical unit is located in the processing area as illustrated in figure 14.2. When workers enter the area, they must use the washing unit. This equipment is responsible for increased hand washing frequency by 300%. The user inserts the hands into two cylinders, passing a photo-optic sensor, which activates the cleansing action. High pressure jet sprays within each cylinder spray a mixture of antimicrobial cleansing solution and water on the hands followed by a portable water rinse. This 10 second cycle is 60 % more effective at removing pathogenic bacteria from the hands than the average manual hand washing and reduced water cost.

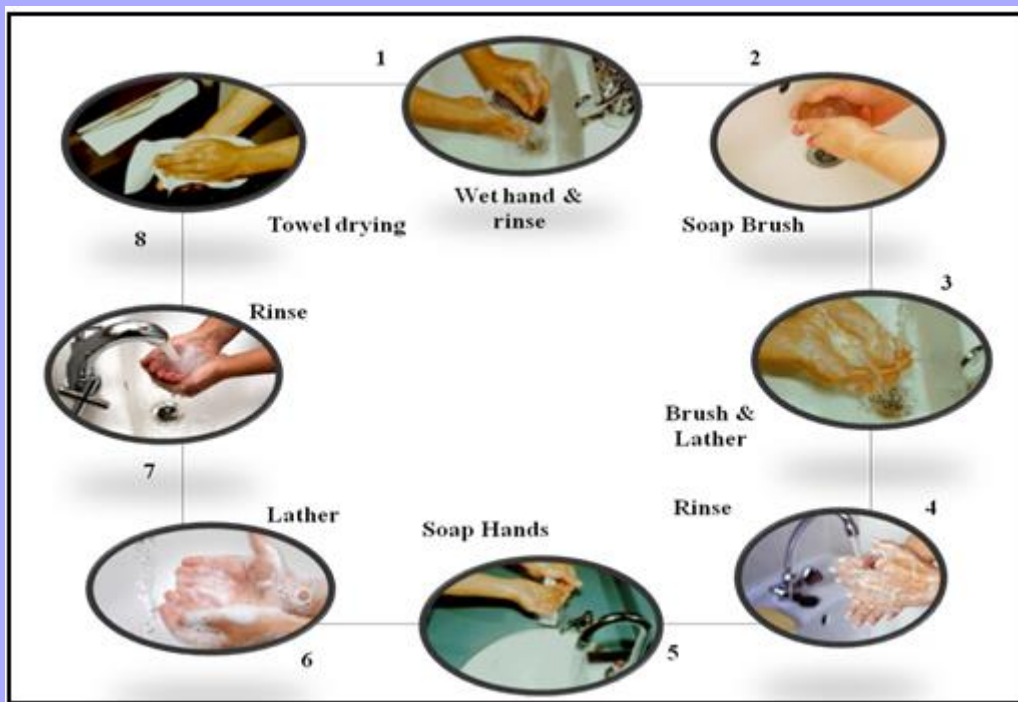


Fig. 14.1 Step wise procedure for Manual double hand washing



Fig. 14.2 Mechanized hand washers

Disinfection steps after hand washing: Alcohol hand rub, gel, or rinse sanitizers that contain at least 60% alcohol have been incorporated as a disinfection step after washing hands with soap and water. The alcohol present will evaporate in approximately 15 seconds. Alcohol-based instant hand sanitizers used after hand washing, provide an additional 10 - to 100 fold reduction. Instant hand sanitizers (protective creams and lotions) should be considered when washing is not possible but they do not have a lasting effect. Ethanol is more effective at destroying viruses than iso-propanol; however, both alcohols are effective for the destruction of bacteria, fungi, and viruses. The most effective regimen for antimicrobial control is the combination of an antibacterial soap hand wash followed by an alcohol gel application. Caution is necessary when alcohol is applied because it is flammable at the concentration found in hand sanitizers.

Other antiseptic hand washing compounds include iodine and iodophors (complexes of iodine with a carrier such as poly-vinyl-pyrrolidone). Although effective, iodine is irritating and may cause allergic reactions. Gloves should be put on after the hands are washed and dried. If the hands are not dry, residual moisture forms an incubation environment for bacteria under the gloves.

14.7 Requirements for Hygienic Practices

Management must establish a protocol to ensure hygienic practices by employees. Supervisors and managers should set an example for employees by their own high levels of hygiene and good health while conveying the importance of these practices to the employees. They should provide proper laundry facilities for maintenance of cleanliness through clean dressing rooms, services, and welfare facilities. Management should require employees to have a pre-employment physical examination to verify that they are in good physical, mental, and emotional health. This is an excellent opportunity to impress the importance of good hygienic habits on a new employee and to emphasize how employees “shed” *Salmonella and Shigella*. Furthermore, those with skin infections may be identified before they handle food. All employees who work with food should be checked regularly for signs of illness, infection, and other unhealthy conditions.

Several countries have a legal requirement for pre-employment health examinations and require that they be repeated at regular intervals. However, this regulation has been challenged because of the expense of routine medical examinations, the difficulty of administering these plans, and because a clear relationship between food handlers and food borne disease has not been established.

14.7.1 These practices should be conducted to ensure personal hygiene

1. Physical health should be maintained and protected through practice of proper nutrition and physical cleanliness.
2. Illness should be reported to the employer before working with food so that work adjustments can be made to protect food from the employee's illness or disease.
3. Hygienic work habits should be developed to eliminate potential food contamination.
4. During the work shift, hands should be washed after using the toilet; handling garbage or other soiled materials; handling uncooked muscle foods, egg products, or dairy products; handling money; smoking; coughing; or sneezing.
5. Personal cleanliness should be maintained by daily bathing and use of deodorants, washing hair at least twice a week, cleaning fingernails daily, using a hat or hair net while handling food, and wearing clean underclothing and uniforms.
6. Employee hands should not touch food service equipment and utensils. Disposable gloves should be used when contact is necessary.
7. Rules such as "no smoking" should be followed, and other precautions related to potential contamination should be taken.

14.7.2 Employers should emphasize hygienic practices of employees as follows

1. Employees should be provided training in food handling and personal hygiene.
2. A regular inspection of employees and their work habits should be conducted. Violations of practices should be handled as disciplinary violations.
3. Incentives for superior hygiene and sanitary practices should be provided.
4. Food handlers should be responsible for their own health and personal cleanliness. Employers should be responsible for making certain that the public is protected from unsanitary practices that could cause public illness. Personal hygiene is a basic step that should be taken to ensure the production of wholesome food.

14.8 Sanitary Food Handling

A protective sanitation barrier between food and the sources of contamination should be provided during food handling. Barriers include hairnets, disposable gloves, mouth guards, sneeze guards and food packaging and containers.

Role of Employees: Food processing and food service firms should protect their employees and consumers from workers with diseases or other microorganisms of public health concern that can affect the wholesomeness or sanitary quality of food. This precaution is important to maintain a good image and sound operating practices consistent with regulatory organizations. In most communities, local health codes prohibit employees having communicable diseases or those who are carriers of such diseases from handling foods or participating in activities that may result in contamination of food or food contact surfaces. Responsible employers should exercise caution in selecting employees by screening unhealthy individuals. Although some areas no longer require health cards because of the expense involved, many local health departments require all employees who handle food to be examined by a physician who will issue a health card only to healthy individuals. Selection of employees should be predicated upon these facts

1. Absence of communicable diseases should be verified through a county health card or a physician's report.
2. Applicants should not exhibit evidence of a sanitary hazard, such as open sores or presence of excessive skin infections or acne.
3. Applicants who display evidence of respiratory problems should not be hired to handle food or to work in food processing or food preparation areas.
4. Applicants should be clean and neatly groomed and should wear clothing free of unpleasant odour.

5. Applicants should successfully complete a sanitation course and examination such as that provided by the National Restaurant Association.

14.8.1 Required personal hygiene

Food organizations should establish personal hygiene rules that are clearly defined and uniformly and rigidly enforced. These regulations should be documented, posted, and/ or clearly spelled out in booklets. Policy should address personal cleanliness, working attire, acceptable food handling practices, and the use of tobacco and other prohibited practices.

14.8.2 Facilities

Hygienic food handling requires appropriate equipment and supplies. Food handling and food processing equipment should be constructed according to regulations of the appropriate regulatory agency. Welfare facilities should be clean, neat, well lighted, and conveniently located away from production areas. Restrooms (washrooms, lavatories) should have self-closing doors. It is also preferred that hand washing stations have foot- or knee operated faucets that supply water at 43°C to 50°C. Remotely operated liquid soap dispensers are recommended because bars of soap can increase the transfer of microorganisms. Disposable sanitary towels are best for drying hands. The consumption of snacks, beverages, and other foods, as well as smoking, should be confined to a specific area, which should be clean and free of insects and spills.

14.8.3 Employee supervision

Employees who handle food should be subjected to the same health standards used in screening prospective employees. Supervisors should observe employees daily for infected cuts, boils, respiratory complications and other evidence of infection. Many local health authorities require food service and food processing firms to report an employee who is suspected to have a contagious disease or to be a carrier.

14.8.4 Employee Responsibilities

Although the employer is responsible for the conduct and practices of employees, responsibilities should be assigned to employees at the time employment begins.

- Employees should maintain a healthy condition to reduce respiratory or gastrointestinal disorders and other physical ailments.
- Injuries, including cuts, burns, boils, and skin eruptions, should be reported to the employer.
- Abnormal conditions, such as respiratory system complications (e.g., head cold, sinus infection, and bronchial and lung disorder), and intestinal disorders, such as diarrhoea, should be reported to the employer.
- Personal cleanliness that should be practiced includes daily bathing, hair washing at least twice a week, daily changing of undergarments and maintenance of clean fingernails.
- Employees should tell a supervisor that items such as soap or towels in washrooms should be replenished.
- Habits such as scratching the head or other body parts should be stopped.
- The mouth and nose should be covered during coughing or sneezing.
- The hands should be washed after visiting the toilet, using a hand kerchief, smoking, handling soiled articles, or handling money.
- Hands should be kept out of food. Food should not be tasted from the hand, nor should it be consumed in food production areas.
- Food should be handled in utensils that are not touched with the mouth.
- Rules related to use of tobacco should be enforced.

14.9 Training

All workers seeking employment in dairy industry must undergo tailor made training programme "health minimum", which should covers foundations of fundamentals of nutrition, microbes, food poisoning and its prevention, personal and production hygiene.

Dairy plants must have an adequate program in place to monitor and control training programs and maintain appropriate documentation. The objective of the personnel training program must be to ensure safe food handling practices. The personnel training program must provide, on an ongoing basis, necessary training for production personnel. A procedure must be developed to verify effectiveness of the training program. Production personnel must be trained to understand critical elements for which they are responsible, what critical limits are, importance of monitoring limits, and actions they must take if limits are not met. Ongoing training in personal hygiene and hygienic handling of food must be provided to every food handler, and training in personal hygiene and hygienic handling of food must be provided to all persons entering food handling areas. Plants must demonstrate that personal hygiene is carried out and controlled. No person known to be suffering from or to be a carrier of a disease likely to be transmitted through food or afflicted with infected wounds, skin infections, sores, or diarrhoea is permitted to work in any food handling area in any capacity in which there is any likelihood of such a person contaminating food with pathogenic microorganisms. All persons having open cuts or wounds may not handle food or food contact surfaces unless the injury is completely protected by a secure, waterproof covering. All persons entering a dairy food production area must wash their hands thoroughly with soap under warm-running potable water. Hands must be washed after handling contaminated materials and after using toilet facilities. Where required, employees must use disinfectant hand dips. All persons working in dairy food handling areas must maintain personal cleanliness while on duty. Protective clothing, hair covering, and footwear functional to the operation in which the employee is engaged must be worn and maintained in a sanitary manner. Gloves, if worn, must be clean and sanitary.

All persons entering dairy food handling areas must remove objects from their person that may fall into or otherwise contaminate food. Tobacco, gum, and food are not permitted in dairy food handling areas. Jewelry must be removed before entering food handling areas. However, jewelry, including Medic Alerts that cannot be removed, must be covered. Personal effects and street clothing must not be kept in food handling areas and must be stored in a manner to prevent contamination of dairy foods. Access of personnel and visitors must be controlled to prevent contamination. All necessary precautions must be taken to prevent contamination, including use of foot baths and hand dips where required.

14.10 Hygiene Monitoring

Targets for hygiene are of little value unless they are monitored. Microbiological methods are available to monitor the total counts on items of plant, most methods require the establishment of a laboratory designed to handle live cultures. The cleanliness can be assessed by objective examination of hand. This is carried out in microbiological laboratory by taking washings from the hands by means of sterile cotton-wool pads and inoculating the washings on to nutrient media. For this the surfaces of palms of both hands and the fingers are rubbed at least five times with each pad and then used to rub the spaces between the fingers, the nails and the space underneath the nails. The results of the examination should be communicated to the workers. The importance of personnel hygiene must be discussed with them (Fig. 14.3).

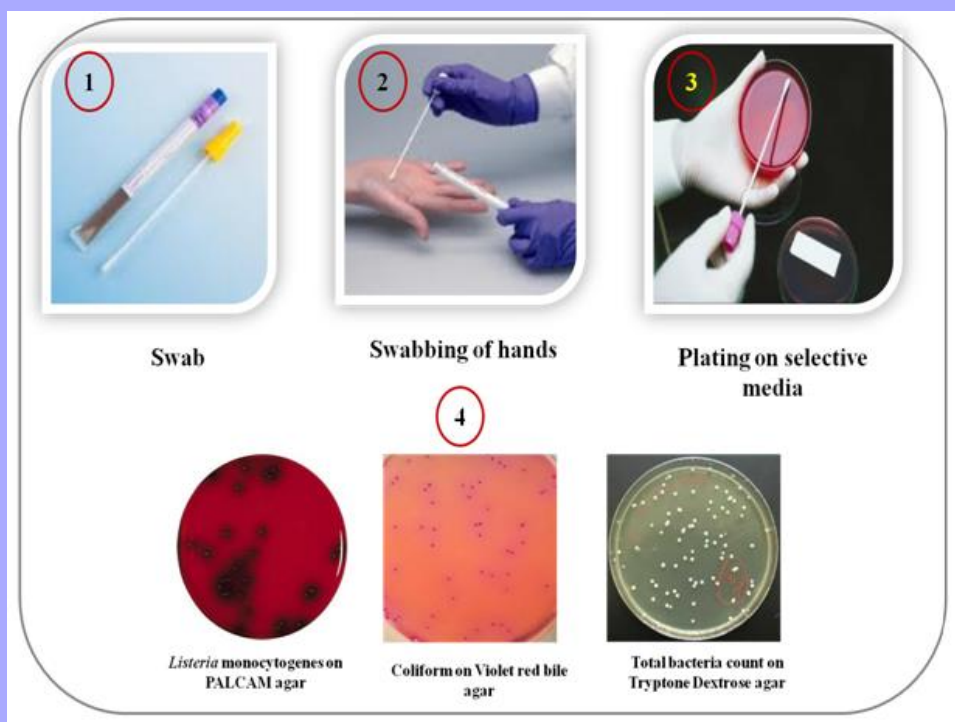


Fig. 14.3 Hygiene monitoring of Personnel

The bacterial cultures growing on Petri dishes should be shown to them. The scale of marks for judging the results of bacterial examination are "excellent" is given to the workers from whose hands washings show < 2000, "good"- 2000 to 5000 bacteria, "fair" - 5000 to 10 000 CFU/ ml. and >10,000 is "bad". If gas appears in the fermentation liquid a "bad" mark is given independently of the total number of bacteria.



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Lesson 15 EQUIPMENT HYGIENE

15.1 Introduction

Pathogenic microorganisms are the major safety concern for the food industry. The vast majority of outbreaks of food-related illness are due to microbial pathogens and their effective control depends on a thorough understanding of the growth conditions favoring particular organisms. This understanding can be used to minimize contamination of incoming raw materials, to inactivate bacteria during processing and prevent decontaminated food from becoming re-contaminated. It is also important to know where and how microorganisms can become established, if growth conditions are favorable.

Hygiene problems in equipment are caused when micro-organisms attach to the surfaces, survive on them and later become detached thus contaminating and reducing the quality of the product. This can be due to a poor hygienic design in cases where the machines cannot be cleaned properly. Constructions that cause problems include sites where soil, product debris and micro-organisms can accumulate, e.g. dead ends, sharp corners and low-quality seals and joints. Even if 'dead' areas have been 'designed out', some product will attach to equipment surfaces, despite the possibility of fast-moving liquids. Microbes may reside on such surfaces long enough to multiply, and contaminate the product. The problem is exacerbated when a process includes dead spaces where product can stagnate. Microbes may also penetrate through very small leaks. There is considerable evidence that they can pass through microscopic openings very rapidly and that pressure differences may retard, but not prevent, passage, even if the pressure-difference is as high as 0.5 bar. The bacterium *Serratia marcescens* may move at a speed of 160 mm per hour. Motile bacteria may propel themselves against the flow of liquid through a leak. Whether motile or not, they may also penetrate by forming a biofilm on the surface.

Good hygienic design of food processing equipment protects the product from contamination with substances harmful to consumer health and provides access for cleaning, maintenance and inspection. Factors reported to affect the hygiene level of food processing equipment, include hygienic design of the equipment, hygienic practices of personnel, cleaning and disinfection of the equipment, lubricants used in the equipment as well as lay-out of the processing, air-currents, type of food product and cleanliness of the processing environment.

15.2. General aspects of sanitary construction and design of food equipment

The surfaces of food equipment can be subdivided into two categories:

1. Food product contact surfaces, and
2. Non-product contact surfaces.

A food product contact surface is defined as a surface in "direct contact with food residue, or where food residue can drip, drain, diffuse, or be drawn". Because these surfaces, if contaminated, can directly result in food product contamination, rigid sanitary design criteria must be met. Non-product contact surfaces are those that are part of the equipment (e.g., legs, supports, housings) that do not directly contact food. As contamination of non-product contact surfaces can cause indirect contamination of the food product, these surfaces cannot be ignored with regard to sanitary design.

15.2.1 Food product contact surfaces

In terms of sanitary design, all food contact surfaces should be: Smooth, impervious, free of cracks and crevices, nonporous, nonabsorbent, non-contaminating, nonreactive, corrosion resistant, durable and maintenance free, nontoxic and cleanable. The food contact surfaces should comply with the following criteria:

15.2.1.1 Surface texture and/ or finish

If any surface is ground, polished, or textured in any way, it must be done so the final surface is smooth, durable, free of cracks and crevices and meets the other sanitary design requirements described above. Sanitary Standards require that ground or polished stainless steel surfaces meet ground surface, and unpolished surfaces meet a No. 2B or mill finish. The 3-A Sanitary Standards development group has recently adopted an industry recognized method for determining an acceptable food contact surface termed roughness average or Ra value. The Ra is determined using a sensitive instrument (termed a profilometer) which employs a diamond tipped stylus to measure peaks and valleys in a relatively smooth surface.

15.2.1.2 Construction and fabrication

Food equipment should be designed and fabricated in such a way that all food contact surfaces are free of sharp corners and crevices. All mating surfaces must also be continuous (e.g., substantially flush). Construction of all food handling or processing equipment should allow for easy disassembly for cleaning and inspection. Where appropriate (e.g., vessels, chambers, tanks), equipment should be self-draining and pitched to a drainable port with no potential hold up of food materials or solutions. Piping systems not designed for routine dis-assembly must be sloped to drain. Piping systems installed in modern food processing systems designed for cleaning-in-place (CIP), require special consideration and close monitoring with regard to drainage.

15.2.1.3 Internal angles

Internal angles should be coved or rounded with defined radii. Equipment standards specify appropriate radii for specific equipment applications and components. For example, radii requirements stated in the 3A sanitary standards

15.3 Functional requirements of equipments

Hygienic food processing equipment should be easy to maintain to ensure it will perform as expected to prevent microbiological problems. Therefore, the equipment must be easy to clean and protect the products from contamination. In the case of aseptic equipment, the equipment must be pasteurisable or sterilisable (depending on the application) and must prevent the ingress of micro-organisms (i.e. it must be bacteria tight). It must be possible to monitor and control all of its functions which are critical from a microbiological safety point of view.

15.3.1 Cleanability and decontamination

Cleanliness is a very important issue. Equipment which is difficult to clean will need procedures which are more severe, require more aggressive chemicals and longer cleaning and decontamination cycles. Results will be higher cost, reduced availability for production, reduced lifetime of the equipment, and more effluent.

15.3.2 Prevention of ingress of micro-organisms

Ingress of micro-organisms into products must be avoided in general. Usually, it is desirable to limit the number of micro-organisms in food products as much as possible to meet requirements of public health and required shelf life. Equipment intended for aseptic processes must additionally be impermeable to micro-organisms.

15.3.3 Prevention of growth of micro-organisms

Equipment that causes problems in milk processing and packaging include pipes (Joints & Bends), filling and packing machines, conveyors, plate heat exchangers and tanks with piping. *Listeria monocytogenes* has been found on equipment and process surfaces, which are difficult to clean. Such equipment can thus cause microbial contamination in food processing. Therefore the design of the equipment and process line in the food processing and packaging industry is important for preventing formation of the biofilm and so improving process and production hygiene. Furthermore, pathogens in biofilms have been found to be more resistant to

many types of disinfectants.

15.3.3.1 Biofilm on dairy equipments

Biofilms form in two stages. First, an electrostatic attraction occurs between the surface and the microbe. The process is reversible at this stage. The next phase occurs when the organism forms an extracellular polysaccharide, which firmly attaches the cell to the surface. The cell then multiplies, forming micro-colonies and, ultimately, the biofilm (Fig15.1). Biofilm development may take place on any type of surface and is difficult to prevent, if conditions sustain microbial growth. These films are very difficult to remove during cleaning operations. Microorganisms that appear to be more difficult to remove because of biofilm formation include the pathogens *Staphylococcus aureus* and *Listeria monocytogenes*. Current information suggests that heat treatment is more effective than the application of chemical sanitizers and Teflon appears to be easier to clear of biofilm than stainless steel.

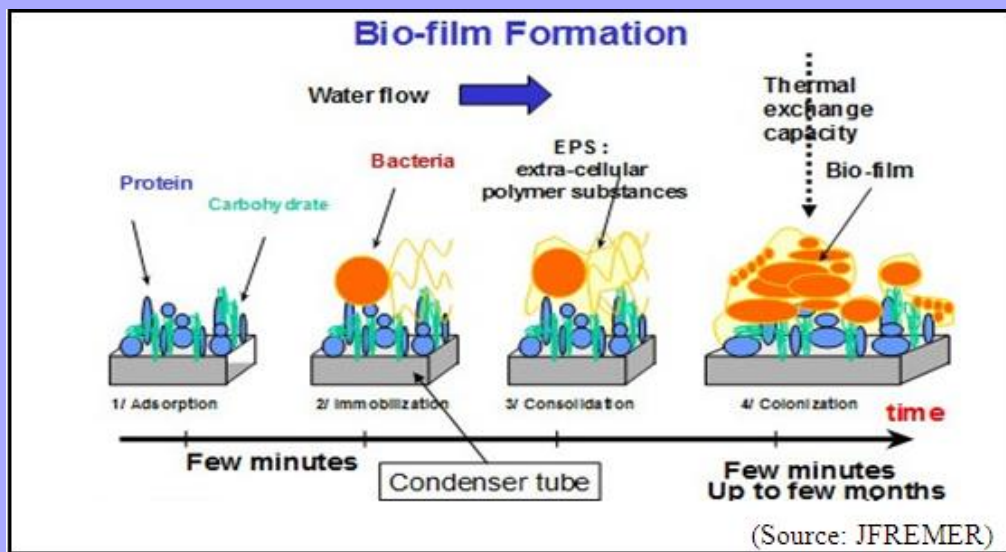


Fig. 15.1 Mechanism of biofilm formation

15.3.3.2 Microorganisms in biofilm formation

Many organisms, including a number of pathogens (*Klebsiella pneumoniae*, *Legionella pneumophila*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Yersinia enterocolitica*) form biofilms, even under hostile conditions, such as the presence of disinfectants. Adverse conditions even stimulate microorganisms to grow in biofilms. Thermophilic bacteria (such as *Streptococcus salivarius* spp. *thermophilus*) can form a biofilm in the cooling section of a milk pasteurizer, sometimes within five hours, resulting in massive contamination of the pasteurized product (up to 10^6 cells per mL). On metal (including stainless steel) surfaces, biofilms may also enhance corrosion, leading to the development of microscopic holes. Such pinholes allow the passage of microbes and thus may cause contamination of the product. Like other causes of fouling, biofilms will also affect heat-transfer in heat exchangers. On temperature probes, biofilms may seriously affect heat-transfer and thereby the accuracy of the measurement. Reducing the effectiveness of heat treatment may itself help to stimulate further bacterial growth.

15.3.3.3 Effect of cleaning and sanitation chemicals on biofilm microflora

Biofilms may be much more difficult to remove than ordinary soil. If the cleaning procedure used is not capable of removing the biofilm completely, decontamination of the surface by either heat or chemicals may fail, since a biofilm dramatically increases the resistance of the embedded organisms. It is therefore imperative that product contact-surfaces are well cleaned before disinfection. The effects of a variety of cleaning and sanitizing chemicals on *L. monocytogenes* which was allowed to attach to stainless steel and plastic material used in conveyor belts over a period of 24 hours. They found that sanitizers alone had little effect on the attached organisms, even when the exposure time was increased to 10 minutes. Unattached cells, on the other

hand, showed a 5-log reduction in numbers within 30 seconds. In general, acidic quaternary ammonium compounds, chlorine dioxide and peracetic acid were the most effective sanitizers for eliminating attached cells. Least effective were chlorine, iodophors and neutral quaternary ammonium compounds. When the attached organisms were exposed to cleaning compounds prior to treatment with sanitizers, the bacteria were readily inactivated.

15.3.4 Compatibility with other requirements

A design with excellent hygienic characteristics but lacking the ability to perform its functional duties is of no use; hence a designer may have to compromise. Such action, however, will have to be compensated by more intensive cleaning and decontamination procedures and these must be documented so that the users are aware of the nature of the compromise. The cleanability of the equipment, including the CIP where appropriate, must be demonstrated.

15.3.5 Validation of the hygienic design of equipment

Irrespective of the amount of know-how and experience with hygienic design which is applied when designing and fabricating, practice has shown that inspection, testing and validation of the resulting design to check if the requirements are met is very important. In critical cases it may be necessary to check the hygiene level as part of the maintenance procedures. The designer has to make sure that relevant areas are accessible for inspection and/or validation. Acceptance for food processing equipment used in some European countries is based upon "cleanability" testing performed in European Hygienic Design Group (EHEDG) laboratories. The organization like International standard organization (ISO), International Dairy Federation (IDF) and Codex Alimentarius commission (CAC) are generally involved in development of equipment hygiene standards. Some of these organizations have symbol or insignia use authorization programs that require third party verification of compliance with the appropriate standard or guideline.

15.4 Hygiene of personnel working with equipment

An important factor affecting on hygienic status of dairy processing equipment is the hygienic practices of people working with the equipment. Many researchers have published information on improper hygienic practices in food handling and outbreaks have been reported. Contamination of foods by food handlers has been identified as one of the most important causes of food borne outbreaks. Personnel are both reservoirs and vectors of micro-organisms. The level and risk of contamination from personnel is difficult to measure as it depends on various factors such as the different activities and the range of personnel movement patterns during the working day as well as the perceptions and attitudes of the personnel.

15.5 Equipment Hygiene Monitoring

Hygiene detection of the food processing equipment and surfaces is one important part of the quality control. By sampling the surfaces it can be possible to prevent the contamination of milk and milk products, helps in tracing the source of contamination and optimization of cleaning process. Quantitative estimation of micro-organisms from surfaces is very difficult because of strong microbial adherence and because the cells grow in layers, forming biofilms. The threshold of detection of adhering micro-organisms can vary according to the enumeration technique employed and some techniques underestimate the number of micro-organisms on a surface.

The problems associated with identifying biofilm microorganisms is repeatability and reproducibility. The methods currently available mostly used in surface hygiene monitoring are cultivation methods based on surface agar contact method (swab, rinse, adhesive tape method and Vacuum method) and agar contact method (Replicate organisms direct agar contact (RODAC), agar slice and direct surface agar plating method) the ATP bioluminescence method (measurements directly from the surface or from swabbed sample).

15.5.1 Monitoring techniques and microbiological criteria

15.5.1.1 Swab contact method

In swab contact method, a cotton swab moistened with the quarter strength ringers solution is applied over a known area of the equipment and in the process all the micro-organisms adhered to the surface of the equipments are transferred to the swab. After proper mixing, the swab suspension is diluted and plated on different agar plates containing tryptone dextrose agar, violet red bile agar and potato dextrose agar etc. followed by incubation at respective recommended temperature - time combination. The counts of the colony developed in the respective plates were counted. Generally, two specified areas of 50 cm² are randomly selected on the test equipment. This method can be applied to any type of equipment, the recovery of the surface bacteria is only moderate (52-90%).

This method has now been modified and simplified by combining with membrane filtration technique and place it in enriched broth for qualitative identification of microorganisms (Fig. 15.2). This swab suspension is filtered through cellulose acetate filter (0.45 µm) and then filter membrane is layered on a selective medium.

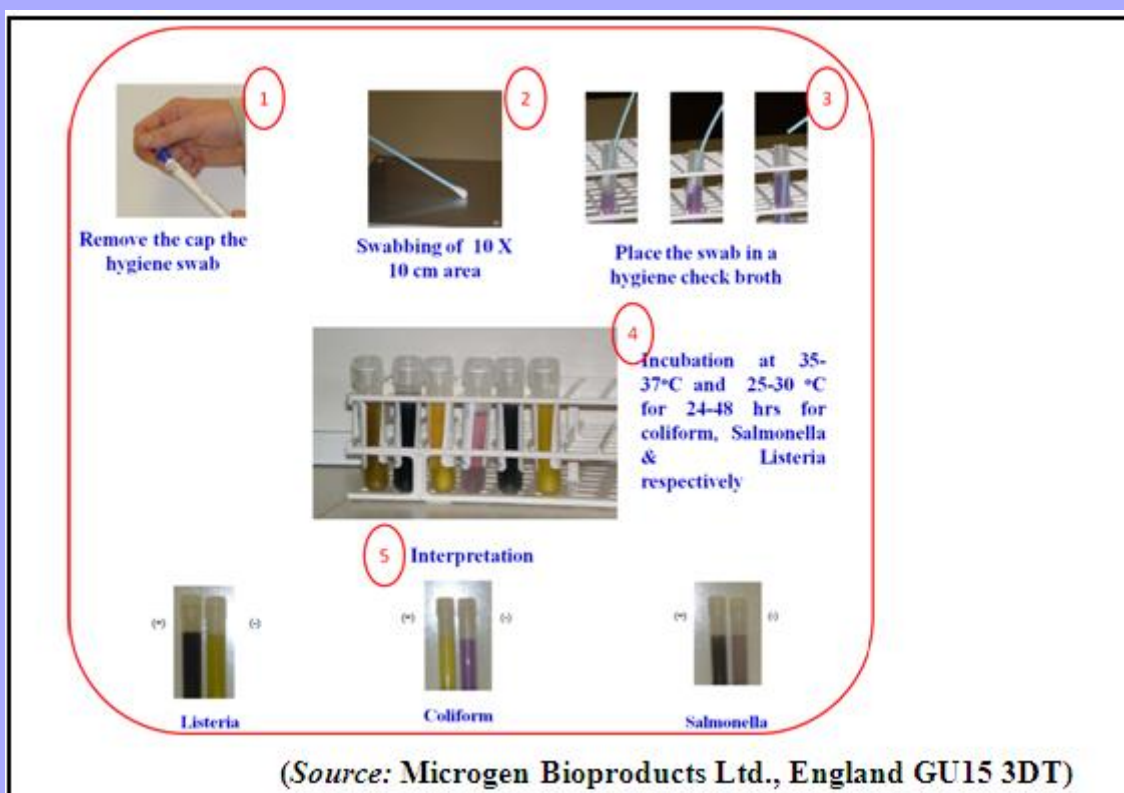


Fig. 15.2. Swab technique for qualitative identification of hygiene

15.5.1.2 Surface rinse method

It is a very effective and useful method for enumeration of microorganisms on the surface of the equipment. This method is mostly used for microbiological examination of bottles, cans, farm dairy equipment and pipelines of the processing machines. In this method, a standard amount of 20 mL for milk bottles and 500 mL for milk cans, of sterile quarter strength ringer's solution is poured into the containers, mixed or shaken several times so that the diluents or rinse comes in contact with whole surface of the container followed by colony count of rinse is determined on specific media. This is method has a moderate recovery (70%) and good repeatability.

15.5.1.3 Agar contact method

a) RODAC

This method is most popular as surface sampling technique and can give not only approximate number of the organisms but also their types. In this method, plastic strips of specific size (25cm²) are filled enough molten agar medium to allow a convex surface to form. After solidification of the medium, the agar surface is gently pressed against the test surface of the equipment, removed and covered with the lid and subsequently

incubated. This test is not suitable for heavily contaminated surfaces. Another drawback with this method is that it cannot distinguish single surface bacteria and clusters of microorganisms. The possible maximum accurate count is limited to 200 colonies/ plate. The recovery is very high (80%) and reproducibility is excellent.

b) Agar slice method

The efficacy of this method is comparable to RODAC method. The specified molten agar medium is filled in a sterile syringe and is allowed to solidify. The solidified medium is pushed out and allowed to make contact with the test surface. The portion of the agar that comes in contact with the surface is sliced off and transferred to petri dish or subsequent incubation and colonies are counted. The recovery with this method is 72-75%.

c) Direct surface agar plating method

This method, the molten agar medium directly poured onto the surface. Due to the direct contact of the surface to the medium, the colonies are developed after appropriate incubation and counted. The recovery is fairly high (80%). The main draw backs are that does not work with certain equipments like pipelines and cans.

15.5.1.4 Microbiological criteria for dairy equipment

The microbial count of the surface studied is calculated as per the following formula:

$$\text{Count/ ft-2} = 9.29 \times \text{count/ 100 cm}^2$$

Or

$$\text{Count/ 100 cm}^2 = 0.108 \times \text{count/ ft}^2$$

The results and their interpretation for finding the microbiological status of the contact surface in question have been summarized in Table 15.1 and 15.2

Table 15.1 Microbiological criteria for sanitary condition of dairy equipment

Grade	Total bacterial count/ 100 cm ²	
	For storage of raw products	For storage of pasteurized products
Good	< 200	< 10
Satisfactory	200 - < 1000	10-50
Unsatisfactory	1000 – 10000	>50
Heavily contaminated	>10000	-

(Source: Yadav *et al.* 1992; Luck, 1981)

The suggested microbiological standards for the surface counts of dairy equipment are give in Table 15.2. Where large surfaces are available the area swabbed (or rinsed or contacted) is 900 cm².

Table. 15.2 Bacteriological standards for sterility of dairy equipment

Equipment	Standards for satisfactory sterility	
	Max. SPC/l capacity (Rinse method)	Max. SPC/ 900 cm ² of surface (Swab Method)
Milk can/ pail/ milk bottle	1000	-
Milk receiving tank	-	5000
Weighing bowl/pan	-	5000
Storage/ holding/ transport tanks	-	5000
Batch pasteurizer	-	5000
Plate heat exchanger	-	5000
Surface cooler	-	5000
Cotton filter cloth evaporator	-	5000
Roller dryer	-	5000
Bottle filler	25000*	
HTST plate pasteurizer	25000*	
Homogenizer	25000*	
Cream separator/clarifier	25000*	
Spray dryer	25000*	
Ice cream freezer	25000*	
Walls, floors etc.	25000*	
Milk crates	25000*	

*Circulation method, SPC of first liter of rinse coming out

(Source: Yadav et al. 1992; Luck, 1981)

15.5.1.5 ATP bioluminescence test

ATP bioluminescence is biochemical which measures the presence of adenosine triphosphate (ATP) by its reaction with the luciferin-luciferase complex. It can be incorporated in the estimation of microbial load of a food sample, equipment surfaces and water sample. The bioluminescent reaction requires ATP, luciferin, and firefly luciferase - an enzyme that produces light in the tail of the firefly. During the reaction, luciferin is oxidized and emits light. A luminometer measures the light produced, which is proportional to the amount of ATP present in the sample. The ATP content of the sample can be correlated with the number of microorganisms present because all microbial cells have a specific amount of ATP. An automated luminometer can detect the presence of yeast, mould, or bacterial cells in liquid samples in three minutes (Figs. 15.3 and 15.4).

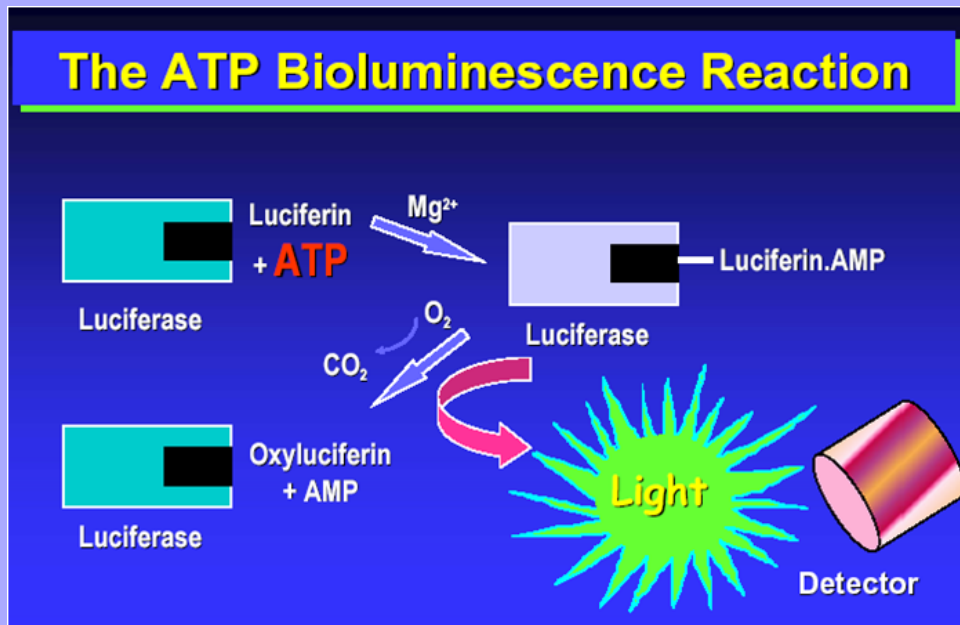


Fig. 15.3 ATP bioluminescence assay Principle

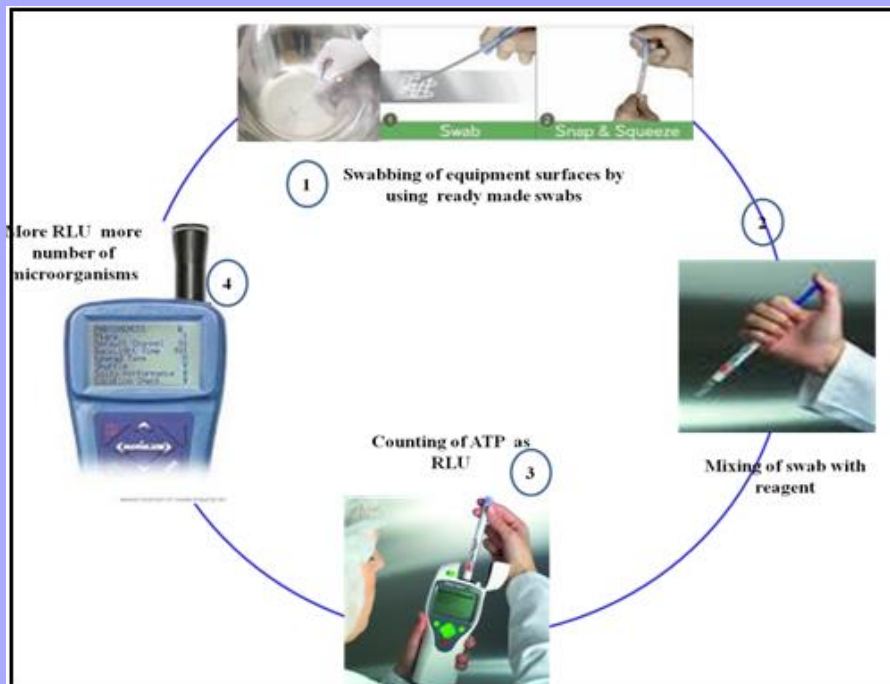


Fig. 15.4 Step wise procedure for ATP bioluminescence assay

A computer-interfaced luminometer, which employs customized software, a printer, and an automatic sampler, can analyze samples with a sensitivity of one microorganism per 200 mL. Use of this method has increased because ATP bioluminescence test can release product in less than 24 hours. A major advantage of this test is that ATP from tissue exudates can be detected, whereas other tests do not offer this feature. Furthermore, this test identifies dirty equipment. However, this test has also limitation in the sense that cleaning compound residues can quench the light reaction to prevent proper response from the assay system. The results were expressed as log₁₀ Relative Light Unit (RLU) released by the total ATP.

The higher the amount of ATP on the surface or food samples, the higher the light output expressed in RLU. According to recommendations of the ATP-bioluminescence equipment manufacturer, measurements lower than 150 RLU were considered clean, from 151 to 300 RLU were considered suspect, and values higher than 301 RLU were considered inadequate hygienic conditions.

15.6 Assessment of Efficacy of Detergent and Sanitizer by Capacity and suspension test

15.6.1 Capacity test

Capacity test involves taking a definite quantity of test solution (sanitizer) & definite number of cells. The action of sanitizer depends on its concentration & the period of contact with organism. The shorter the period to destroy the test organism in a particular concentration, the better would be the efficiency of detergent/sanitizer. Suspension test is based on the enumeration of survivors of the test culture after a definite period of contact with sanitizer.

Procedure

1. Take 20 mL Sanitizer , inoculate 1 mL inoculum (washed suspension of culture O.D.0.3)
2. After 1 min take out 0.1 mL of mixture & transfer in 5 mL nutrient broth(N.B.)
3. Add 0.1 mL inoculums immediately so that amount remain same, take 10 increments
4. Incubate all samples and one positive blank (from 21 mL add 1 mL in N. B.) at 37°C for 48 hrs
5. Observe growth in nutrient broth tubes
6. Plating and count colonies

15.6.2 Suspension test

In these tests, a sample of the bacterial culture is suspended into the disinfectant solution and after exposure it is verified by subculture whether this inoculum is killed or not. Suspension tests are preferred to carrier tests as the bacteria are uniformly exposed to the disinfectant. There are different kinds of suspension tests: the qualitative suspension tests, the test for the determination of the phenol coefficient and the quantitative suspension tests. Initially this was done in a qualitative way. A loopful of bacterial suspension was brought into contact with the disinfectant and again a loopful of this mixture was cultured for surviving organisms. Results were expressed as 'growth' or 'no growth'. In quantitative methods, the number of surviving organisms is counted and compared to the original inoculum size. By subtracting the logarithm of the former from the logarithm of the latter, the decimal log reduction or microbicidal effect (ME) is obtained. An ME of 1 equals to a killing of 90% of the initial number of bacteria, an ME of 2 means 99% killed. A generally accepted requirement is an ME that equals or is greater than 5: at least 99.999% of the germs are killed. Even though these tests are generally well standardized, their approach is less practical.

Procedure

1. Take 100 mL of disinfectant and inoculate 1 mL inoculums.
2. From step 1 take 1mL and inoculate in petriplate after 0, 1, 2, 5, 10, 15 minutes
3. Incubate plate at 37°C for 48 hrs and count colonies



Lesson 16 ENVIRONMENTAL HYGIENE

16.1 Introduction

Foods can become contaminated during growth and harvesting of raw materials, storage and transport to the factory, and processing into finished products. The final product may then become re-contaminated during subsequent storage and transport to shops, and during storage and preparation by the consumer. The main sources of contamination are the environment, animals and people. The main transmission routes (vectors) of contamination are contaminated surfaces, air, water, people and pests. Processing, packaging material and equipment and transport vehicles may also act as vectors. Contact between food material and an inert surface leaves residual food debris that favours the growth of microorganisms. Over time, these can multiply to significant numbers and become endemic in a processing plant. Lubricants, often unavoidable in equipment with moving parts, may also contribute to chemical contamination. Non-contact surfaces, such as floors, walls, ceilings, overhead beams and equipment supports, are potential reservoirs of microbial contamination and can also be a source of physical and partly based on chemical contaminants (e.g. from flaking plaster and its associated chemicals). They need to be designed so that they are durable and can be cleaned effectively.

Production animals are important reservoirs of microorganisms and slaughter animals introduce large numbers of microorganisms into the processing plant. Among them are many so-called zoonotic pathogens that are present on the skin and in the gastro-intestinal and respiratory tracts. Pathogens carried on hands are also a major source of contamination.

Air can be a significant medium for the transfer of contaminants to food products. Unless the air is filtered, microorganisms will be present, and air may also carry 'light' foreign bodies, such as dust, straw-type debris and insects. Water is used in the food industry as an ingredient, a processing aid and for cleaning. Its use as an ingredient or processing aid can give rise to both microbial and chemical contamination, so it is important to use water of a high microbiological and chemical quality (i.e. potable quality). Water used in hand washing facilities poses a potential problem, as does that from condensation of steam or water vapour, leaking pipes and drains, and rainwater. Stagnant water is particularly hazardous, since microbial levels can increase rapidly under favourable conditions. The water used in cleaning programmes also needs to be of adequate quality. Personnel can transfer enteric and respiratory pathogens to food, e.g. via aerosol droplets from coughing near the processing line. People can equally be vectors of physical contaminants, such as hair or fingernail fragments, earrings, plasters and small personal belongings.

Pests, such as birds, insects and rodents, are potentially a major contamination problem, and particular care needs to be taken to prevent their entry into food production areas. Buildings must be designed to keep them out. Floors, ceilings and walls should not allow insects and other invertebrates the chance to live and breed. This chapter will highlight the environmental hygiene issues aimed at improving or maintaining the standard of basic environmental conditions affecting the well being of people, clean and safe water supply, clean and safe ambient air, protection of food from biological and chemical contaminants, and adequate housing in clean and safe surroundings.

16.2 Air Control

Movement of people, products, packaging and machinery creates unavoidable airborne contamination within the factory and transfer contamination from outside the factory to inside. Although microorganisms do not multiply in air, this is an effective method of distributing bacteria to surfaces within a dairy plant. In chilled rooms, evaporator fans draw large quantities of air over the evaporator cooling coils and distribute it around the room. Any contaminants in the air are likely to pass over the evaporator surfaces, some will be deposited, and, if conditions are suitable, attachment, growth and further distribution of airborne contaminants may occur. In addition to being a potential source of contamination, the development of a microbial biofilm on evaporator cooling coils may affect heat transfer rates of the equipment.

Air-control systems are effective in controlling airborne contamination, such as endospores of *Bacillus* species, various species of non-spore-forming bacteria and yeasts, as well as a range of mould spores, transmitted by untreated air being drawn into the area, condensation, blow lines from equipment and compressed air lines from packaging equipment. These control systems are, however, not as effective in controlling contamination from other sources, for example, personnel, traffic, buildings and raw materials. Chemical taints can, for instance, enter the production area through airborne transmission.

For products where air quality does not limit shelf-life and safety, the air quality should be controlled so that it does not become a limiting factor. Air control will be important in controlling the risk of contamination for products that are minimally processed, and where growth of microorganisms is controlled by preservation systems. In high-care areas, air supply is critical, if the area can be physically isolated, air systems should provide a positive air pressure and further reduce the risk of casual microbial contaminants. This will prevent microbiological contamination from potentially contaminated areas to non-contaminated areas. Ducting of incoming air through a primary filter to remove gross contamination (5.0–10.0 μm diameter), followed by a filter capable of removing 90–99% of particles above 1.0 μm , is essential for areas containing open vats of food. Special consideration should also be given to airflow in cheese factories, especially where mould, smear-ripened or soft cheeses are produced. Routine monitoring to maintain air quality is essential. Temperature, humidity, airflow and pressure and microbiological monitoring are all aspects that should be included. The location of air intakes and outlets, the method and frequency of cleaning filter-holders, and routine for replacing filters are aspects that merit inclusion within the HACCP plan. Adequate means of natural or mechanical ventilation should be provided, in particular to: minimize air-borne contamination from aerosols and condensation droplets; control ambient temperatures, humidity and odours to ensure safety and suitability of food. Ventilation systems should be designed and constructed so that air does not flow from contaminated to clean areas.

16.2.1 Air-quality control

16.2.1.1 Dairy processing areas

The most practical approach to control microbial airborne contamination indoors is the removal of all potential contamination sources from sensitive areas where the product might be exposed to air. Good ventilation is necessary to remove moisture released during the processing of dairy products, and it will also prevent condensation and subsequent mould growth on surfaces. Attention is nowadays given to air cleaning in food plants, inter alia, by establishing air-flow barriers against cross-contamination from the environment. The air entering processing rooms is normally chilled and filtered to remove practically all bacteria, yeasts and moulds. It is essential that filtered sterilized air be supplied to areas where sterile operations are to be carried out. Rigid-frame filters or closely packed glass fibres are available to achieve contamination-free air for culture transfer, and manufacturing and packaging of sterilized milk and milk products. The use of high-efficiency particulate air (HEPA) filters will remove 99.99% of airborne particles 0.3 μm and larger, while ultra-low-penetration air (ULPA) filters remove 99.999% of particles as small as 0.12 μm . Passage of air through a combined HEPA/ ULPA filter is usually considered suitable for use where contamination-free work is to be carried out. Standard high-efficiency air filter systems allow more air into the room than normal, thereby establishing a positive air pressure. Upon opening a door, filtered air flows out, thus blocking the entry of untreated air and minimizing microbial contamination. For optimal ventilation, sufficient air changes have to be made to prevent the build-up of condensation on surfaces. Specialist advice should be sought to choose the correct filter type/ system on the basis of the air quality required for the specific operation in each controlled area. There is a difference between high-care areas, where the aim is to minimize air contamination, and high-risk areas that are designed to prevent recontamination.

Rooms in which direct exposure to outside air is inevitable can have air-flow barriers installed, mounted over open doorways to secure a significant downward velocity of air flow, preventing contamination from outside. Compressed air can also contribute to contamination of products by dust and microorganisms and, in the case of lubricated compression systems, by oil fumes. Whenever air under pressure comes into direct contact with the product (pneumatic filling, agitation or emptying of tanks) or is directed at milk contact surfaces it should be of the highest quality. Sterile compressed air can be obtained by drying the air after compression in adsorption filters (e.g. chemically pure cotton, polyester or polypropylene) and to install a series of 0.2 μm pore-size filters downstream, immediately preceding the equipment where the air is needed. Air sanitation

systems can also be applied to control airborne contamination and include

- Fogging to reduce the number of airborne microorganisms and also to disinfect surfaces that may be difficult to reach;
- UV light; and
- Ozone treatment.

The use of clean-room clothing, head covering, masks and gloves largely eliminates the release of microorganisms and skin particles into the processing environment. A good hygiene-training programme for factory personnel will also contribute to reducing contamination by workers.

16.2.1.2 Air filtration for indoor air quality

Air filtration is an important air process function which is one of the requirements for acceptable indoor air quality. Air which is filtered to remove particulate and gaseous products is required to maintain a wide range of processes and environmental conditions in good working order, and examples range from package air conditioning to electronics assembly, food processing and office buildings equipped with Heating, ventilating and air conditioning (HVAC) systems. The degree of air filtration is determined by the operational requirements such as:

- Primary coarse filtration for ventilation of warehouses and factories, and as pre-filters for secondary filters.
- Secondary 'fine' filtration for quality office and some processing environments.
- Semi-HEPA and HEPA filtration for food processing, electronics, hospitals, pharmaceutical manufacture and many more.

High efficiency filters are in great demand for a wide range of applications, and many office building HVAC systems now use filters which are very efficient down to one micron particles.

16.2.1.3 HVAC (Heating, ventilating and air conditioning)

It refers to technology of indoor or automotive environmental comfort. HVAC (Figure 16.2) system design is a major sub-discipline of mechanical engineering the principles of thermodynamics, heat transfer and fluid mechanics.

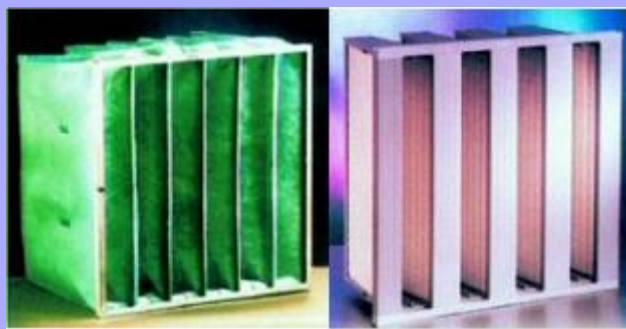


Fig. 16.1 HVAC system design

Gas phase filtration is usually installed in Britain where a particular odour or fume emanating from outside of the building must be removed. Some general examples would be the smell of refuse, sewage processing, high concentrations of engine exhaust and aircraft fumes. There is a limited application for an adsorption system to control odours generated within buildings, and the application of such filters requires careful consideration. In some cases a chemisorptions process may be an alternative solution.

16.2.1.4 Outdoor environment

The control of airborne microorganisms in the immediate surroundings of dairy premises is more difficult than in closed, indoor environments where more controlled measures can be taken (Figure 16.1). One aspect that could be helpful in reducing the microbial load in outdoors is the control of organic materials. UV light, humidity, temperature, wind direction and speed have a significant influence on the total number of airborne microorganisms in the outdoor atmosphere. Air quality in processing areas, the factory environment (e.g. walls, floors, drains) and air used in the manufacturing of dairy products should be monitored regularly. Future trends will be focused on the local air control of production lines, instead of controlling production areas and also on the type of clothing that is used by personnel in food production areas.



Fig. 16.2 Air filtration at outdoor environment

16.2.1.5 Clean room operations

Cleaning procedures should effectively remove food residues and other soils that may contain microorganisms or promote microbial growth. Most cleaning regimes include removal of loose soil with cold or warm water followed by the application of chemical agents, rinsing and sanitation. Cleaning can be accomplished by using chemicals or combination of chemical and physical force (water turbulence or scrubbing). High temperatures can reduce the need for physical force. Chemical cleaners suspend and dissolve food residues by decreasing surface tension, emulsifying fats and peptizing proteins.

16.2.3 Air quality monitoring

There are many techniques used for the determination of microorganisms in air such as settling plate techniques

16.2.3.1 Settling plate technique

Petri dishes containing 20 ml of culture media like PCA, PDA, VRBA or BPA distributed at the dairy processing area and exposed for about 15-30 minutes. The Petri dishes were closed and incubated at 35°C for 48 hours for aerobic plate count, 25°C/ 3-5 days for yeast and mould, 37°C/ 48 hours for *S. aureus* and total coliforms. The results were expressed as CFU. cm⁻² week⁻¹

16.2.3.2 Impaction technique

In this technique, a volume of 100, 500 or 1000 ml of air suctioned by using air sampler and impressed on solid medium surface contained on Petri dishes, according to APHAs recommendations. First the sampler's lid should be sterilized at 121°C for 15 minutes, was sanitized with alcohol (70%), before and after sampling. The Petri dishes were incubated in the same conditions as the settling plate technique. The results were expressed as CFU m⁻³ of air.

The numbers of CFU m⁻³ are determined by using a formula:

$$Pr = N [1/N+1/N-1+1/N-2+1/N-r+1]$$

Where

Pr = Portable number of CFU/ air volume; N= total number lid pores (400), r = lid pores that have already been crossed by viable particles.

16.3 Water Quality

A variety of microorganisms can gain access into water from different sources. The characteristic microflora of water consists of *Achromobacter*, *Alcaligenes*, *Bacillus* spores, *Flavobacterium*, *Pseudomonas* and to a certain extent *Chromobacterium* and *Serratia* depending upon the sources of water supply. *Micrococci* may also be present but their number varies considerably. Water contaminated by faecal sources may chiefly contain intestinal gram negative coccobacilli namely coliforms (*E. coli*), enterococci (*S. faecalis*) as well as anaerobic sporeformers as *Clostridium welchii* (*perfringens*). In addition to these some potential pathogens belonging to family Enterobacteriaceae (*Salmonella*, *Shigella*) and *Vibrio cholera* may also gain access into water sources.

There are mainly two types of microorganisms that might be found in water i.e. non-pathogens and pathogens. The non-pathogens are mainly spoilage type organisms that can cause off-flavours, odours and slimes but are little concern to the public health. The contamination of water by potential pathogens could, on the other hand, be of great public health significance because the consumption of such contaminated water may lead to outbreaks of diseases caused by *Salmonella*, *Shigella* and *Vibrio* such as typhoid fever, dysentery and cholera respectively.

16.3.1 Water in contact with food

Water is used as an ingredient, as a production-process aid and for cleaning, and may be supplied from external sources or recovered water. Consequently, the overall water requirements of a dairy plant are large, and water conservation and water management have become major issues. While not all supplies need to be of potable quality or better, if used as an ingredient, there exists the potential for microbial and chemical contamination. If a water company supplies water, the water will mostly comply with quality standards, suitable for dairy processing. Only occasionally, toxic algae or protozoa (e.g. *Cryptosporidium* spp.) in reservoirs cause problems. Furthermore, *Escherichia coli*, *Listeria monocytogenes* or *Salmonella* spp. cannot grow in water and are sensitive to the levels of chlorine found in drinking water. However, these organisms may survive in water, and so it is important that water used for rinsing equipment, or curd, or flavourings components of cottage cheese, should be of a higher quality than mains water. The level of chlorination in potable water is usually ineffective against spoilage groups like *Pseudomonas* spp. Since these bacteria cause taints under aerobic conditions, bacterial standards have been proposed for bacterial groups and are given in Table 16.1. Consequently, chlorinating plant water supplies to $>21 \text{ mg L}^{-1}$ and monitoring that the level is maintained throughout processing contributes significantly to assuring the safety and shelf-life of the products produced. If ground water is utilized, it must be pre-treated to comply with the mentioned safety and quality standards.

Table 16.1 Microbiological specifications of drinking and process waters

Parameter	Admissible limit		Test reference
	Maximum	WHO	
Total viable count/ 100 ml			
22°C (CFU/ ml)	<100	-	Please refer to lesson 24 on Enumeration of hygiene indicator organisms
37°C (CFU/ ml)	<10	-	
Coliforms (CFU/ 100 ml)	<1	0	Please refer to lesson 24 on 'Enumeration of hygiene indicator organisms'
Faecal coliforms	<1	0	
Faecal streptococci	<1	-	
Sulphite reducing			
<i>Clostridium</i> spp. (CFU/ 21 ml)	<1	-	Please refer to lesson 29 on 'Enumeration of <i>Bacillus cereus</i> , <i>Clostridium botulinum</i> and <i>Clostridium perfringens</i> '

According to IDF, water can be recovered from recycled, rinsing or cooling water. If the recovered water should be used for purposes where it comes into indirect or direct contact with food, it must not present a risk of microbiological contamination. Water from hand washing, unwanted water from steam and leaking pipes can all be vectors of contamination and stagnant water is especially hazardous. Water storage time should not exceed 24 hours and storage tanks should be constructed of stainless steel, easy to clean and closed.

Adequate supply of potable water with appropriate facilities for its storage, distribution and temperature control, should be available to ensure the safety and suitability. Potable water should be as per latest edition of WHO guidelines of high standard. Non-potable water (for use in, for example, fire control, steam production, refrigeration and other similar purposes where it would not contaminate food) shall have a separate system. Non-potable water systems shall be identified and shall not connect with, or allow reflux into, potable water systems.

15.3.2 Water treatment methods

Once water is analyzed for that unwanted constituents which are safety concern for using at dairy farm, processing. The water must be treated to remove anti-quality factors. This has been accomplished successfully and economically in some dairy farms. There are different methods available for treatment of water used in dairy plant as described below.

16.3.2.1 Activated carbon filters (ACF)

Activated carbon filters are used to filter water through carbon granules. Contaminants (constituents) attach to the granules and are removed. Chlorine, some compounds associated with coloration, odour and off-taste of water; mercury; some pesticides; radon gas; and volatile organic compounds can be removed by ACF. Depending upon the amount of water treated, the filters may have to be replaced frequently and regularly or in time contaminants will not be able to attach to the filter. Infrequent filter maintenance may result in bacterial growth on the filter and ineffectiveness.

16.3.2.2 Air stripping (AS)

It involves passing water down a tube while air is forced up through the tube. Contaminants are transferred from water to air and vented off. Whereas this method is effective to remove hydrogen sulphide, some odours and tastes, radon gas and some volatile organic chemicals; it typically is not recommended for household or small commercial use because of high energy costs and high noise generation. Bacterial growth also is a potential problem.

16.3.2.3 Chlorination

Chlorination is an effective and widely used method to kill many kinds of microorganisms in water. It also will aid in removal of unwanted colour, odour, or taste from water. This method also will remove hydrogen sulphide and dissolved iron and manganese, if followed by mechanical filtration or an ACF. Radon gas and volatile organic compounds also can be removed by chlorination. Chlorine is pumped directly into the water in proportion to water flow and it may have some residual effects in the system. If the chlorination is not properly operated, it can be expensive and potentially hazardous if chlorine by-products are allowed to escape.

In typical systems the chlorine content of the treated water should not be high enough to cause problems for cattle. From time to time, high concentrations of chlorine were released to the dairy water system when the city was cleaning its system; in this case (1,000 to 1,500 ppm chlorine in water at the dairy) water intake and performance of cows was reduced when chlorine content was high. This practice may affect water intake because spikes in chlorine content in the water tank may affect consumption; even with the slow-release tablets. Alternative methods (cleansers, brush and thorough rinsing) to keep tanks clean are recommended.

16.3.2.4 Ultraviolet radiation (UR)

Ultraviolet radiation, in which water is passed by a special light source, is another method to kill bacteria in water. There is no residual effect with UR. However, it is difficult to know if UR is working and it may not work if the water is too cloudy or water is passing by the light source too fast. Also as the penetration power of UV rays is quite low, only the surface layer of water can be sterilized.

16.3.2.5 Ozonation (O₃)

Ozonation in which water is exposed to ozone gas, also destroys microorganisms. The equipment typically is quite expensive; however there are no residual effects on the environment or treated water. This method also can be used to remove colour, off-taste, odours, hydrogen sulphide, solubilised iron and manganese; if the water is subsequently passed through a mechanical or ACF system.

16.4 Pest Control

Pests such as insects, birds have been recognized as important carriers of pathogens and other microorganisms. In one interesting case a Salmonella outbreak has been traced back to amphibians, which had accidentally entered the production facility. While massive direct recontamination can be excluded, sporadic cases may be attributed to these vectors. More important, however, is the transport and ingress of pathogens into food processing environments and their possible establishment in suitable niches. Pest control is therefore, essential in dairy food industries.

16.4.1 Risks posed by pests

1. The spread of pathogens are transferred from the gut or external surface of the pest
2. Damage to property
3. Contamination of work surfaces and foodstuffs
4. Adverse public opinion and loss of reputation
5. Prosecution and closure
6. Poor staff relations

16.4.2 Pests are the carriers of pathogens

Rodents can cause damage to food intended for humans, by consumption, contamination with faeces and urine, as well as other physical and microbiological contaminants (Table 16.2). Rodents have the capability to spread many human pathogens, such as *Cryptosporidium parvum*, *Escherichia coli*, *Leptospira* spp, *Listeria* spp, *Salmonella* spp, Hantaviruses, bubonic plague and toxoplasmosis. Sparrows, pigeons and gulls may also carry bacteria causing salmonellosis. Pigeons carry ornithosis, a disease similar to viral pneumonia that can be transmitted to man through infected droppings or respiratory droplets. Ornithosis is often mistaken for flu in humans and so is possibly far more common than is realised. Cockroaches also contaminate food directly as they move from filth to food indiscriminately and are therefore implicated in the mechanical transmission of many pathogens, such as those causing food poisoning and wound infections.

Table 16.2 Food borne Pathogens Isolated from Rodents

Food borne pathogens	Consequences to man	Source
<i>Salmonella</i> spp.: <i>Salmonella enteritidis</i> , <i>Salmonella typhimurium</i> , <i>Salmonella dublin</i>	Diarrhoea, vomiting, abdominal, cramps, fever, dehydration (20-30,000 cases per year)	Rats and Mice
<i>Listeria monocytogenes</i>	Flu-type symptoms, meningitis (septicemia, spontaneous abortion). Appr. 100 cases per year	Rats
<i>Yersinia enterocolitica</i>	Diarrhoea and/ or vomiting, fever, abdominal pain (mimics appendicitis). Appr. 200 reported cases per year	Rats & Mice
<i>Campylobacter jejuni</i>	Flu-like symptoms, diarrhoea (which may contain blood), abdominal cramps. Over 50,000 cases per year	Rats
<i>E. coli</i> O157: H7	Severe abdominal cramp, kidney failure, death in susceptible groups. Appr. 800 reported cases per year	Rats
<i>Leptospira icterohaemorrhagiae</i>	Fever, Abdominal Pain, Jaundice	Human infection usually occurs through contact with water, moist soil contaminated with rat urine or direct contact with rats.
<i>Cryptosporidium parvum</i>	Severe watery diarrhoea, Abdominal cramps (approx. 4,000 reported cases per year*)	Rats

16.4.3 Pest management in food-handling and other specialized facilities

16.4.3.1 Pest prevention

The objective of the pest management programme is the maintenance of pest-free conditions in all areas of the site. The following systematic approach should be taken to all pest control and pest prevention issues, that being:

16.4.3.2 Exclusion

This includes any measure used to prevent entry of organisms indoors through openings in the building structure, doors, windows, or on infested plant or food materials. Exclusion is often neglected or ignored with entire reliance being placed on destruction, in many cases after infestation has occurred. Some techniques include screening openings to prevent entry of flies, mosquitoes, and beetles; caulking cracks and crevices to remove existing or potential harbourages of pantry pests and cockroaches; and sealing or repairing exterior openings to prevent entry of bats, mice, bees, and wasps. The use of pesticides may then fail to achieve the desired result because building structure and conditions within are incompatible. Plants and food products must be carefully inspected for infestations at the time of purchase and before they are brought indoors.

16.4.3.3 Sanitation and inspection

Sanitation is the most important aspect of pest management in food-handling facilities. Food processing plants are subject to sanitation inspections, depending on the type of facility. The pest management professional should be aware of the problem areas. Pest control technicians must conduct a thorough inspection of the facility and notify the plant manager of potential or existing problems. This allows steps to be taken to prevent or correct problems before they are detected by regulatory inspectors or before complaints are received from customers. Sanitary measures include: disposing of garbage on a weekly basis during warm weather to control filth flies and cockroaches; discarding overripe fruits to control fruit flies and fungus beetles; removing bird nests as these harbour dermestids, clothes moths, mites and lice and vacuuming to reduce populations of fleas, carpet beetles, house dust mites, and several ground-dwelling insects and insect relatives. It is also important to keep kitchen areas clean to reduce incidence of pantry pests and cockroaches.

16.4.3.4 Habitat modification

This includes any method used to eliminate or disrupt areas where pests reside. For example, removing weeds and keeping well-mowed lawns reduces incidence of crickets and ticks. Removing debris and fallen leaves near foundations reduces sow bug and centipede populations. Wood or wooden piles, where carpenter ants, ground beetles, and spiders seek harbourage, must be stored away from structures. Creating a vegetation-free barrier around the perimeter of the building will reduce incidence of many ground-dwelling pests such as clover mites. The use of dehumidifiers is recommended, especially in basements, to create and maintain a dry environment to discourage incidence of sow bugs, centipedes, firebrats and house dust mites.

16.4.3.5 Temperature control

Artificially manipulating the temperature of substrates infested by pests or areas where pests reside is an inexpensive non chemical strategy. The time from treatment to death of a pest and numbers of the pest killed, may vary with the pest stage, temperature and duration of exposure. Pantry pests, clothes moths, and carpet beetles can be eliminated by subjecting infested foods, clothes, and carpets, respectively, to extremely hot or cold temperatures. In general, all developmental stages of pantry pests, clothes moths and carpet beetles can be killed within minutes to hours when exposed to temperatures below 0°C and above 40°C.

16.4.3.6 Mechanical control

A rolled newspaper or magazine and fly swatters are some tools used for killing visible and less mobile or immobile pests. On infested plants, hand-picking insects (e.g., hornworms) are a partially effective means of pest control. Infested leaves must be excised from plants, bagged, and discarded.

16.4.3.6.1 Traps

Traps are escape-proof devices that capture highly mobile and active pests. Live traps can be used for rabbits, pocket gophers and squirrels. Unbaited sticky traps such as red spheres, resembling apples, are useful for trapping apple maggot adults. Coloured (yellow) sticky traps are effective in capturing whiteflies and aphids. Sticky traps can be baited with commercial lures (pheromones and food attractants) to enhance trap catch. For example, sticky traps baited with lures for pantry pests, wasps, and flies are commercially available.

Traps are useful for early detection and continuous monitoring of infestations. They are not effective in reducing populations unless the pest population is isolated or confined to a small area. The chance of detecting the presence of pests in a given area is related to the number of traps used. Therefore, when pests are present in very low numbers, it is advantageous to use more than a few traps. Pests must be active or mobile to be captured in traps. Therefore, any environmental variable (temperature, humidity, wind, light, or food) or biological factor (age, sex, mating status, etc.) that influence pest activity, affects trap catch. Consequently, absence of pests in traps does not imply that the pests are not present in the sampled area.

16.4.3.6.2 Diatomaceous earth (DE)

Several DE formulations are commercially available. These products contain fossilized siliceous (silicon-

containing) skeletons of aquatic diatoms (algae) of various shapes and sizes (<1 to 34 microns). DE formulations predominantly are made up of non-crystalline or amorphous silicon dioxide. Although the exact mode of action of DE products is not known, it is believed that DE kills insects and insect relatives by absorbing and abrading the water-proofing, waxy outer covering of the insect-skin (i.e. cuticle). Absorption and abrasion to the waxy layer of the cuticle leads to water loss and subsequently death due to dehydration. DE products are most effective on soft-bodied insects or insect relatives. Because the mode of action is mechanical, insects and insect relatives may not develop resistance to this natural product.

16.4.3.6.3 Biological control agents

Parasitic and predatory insects, mites, and nematodes are now commercially available to control pests. For example, lacewing larvae and ladybird beetle larvae and adults are predators of aphids. Parasitic and predatory organisms should be used only where pesticides are discontinued or were not previously used, because these beneficial organisms are highly susceptible to pesticides. The degree of control achieved with the use of beneficial organisms is variable and the cost-effectiveness for many such beneficial organisms has not been well-documented. Three different varieties of the bacterium *Bacillus thuringiensis* are available to control larvae of moths and butterflies (caterpillars), mosquitoes and black flies (maggots) and beetles (grubs). The varieties *Kurstaki*, *israelensis*, and San Diego are effective against caterpillars, maggots and grubs, respectively. The larvae succumb to the bacterial toxin after ingesting or consuming the treated substrate. For controlling Japanese beetle grubs on lawns, the use of *Bacillus papillae* may offer some control. Recent evidence suggests that caterpillars can develop resistance to the *B. thuringiensis* toxin.

16.4.5 Insecticides in food-handling establishments

Insecticides applied in food-handling establishments must not come in contact with or possibly contaminate food products. For this reason, it is important to distinguish between food and non-food areas of these establishments. Non-food areas may include locker rooms, lavatories, machine rooms, boiler rooms, rubbish rooms and garages. These are areas where food is not normally present, except perhaps as it is being transported from one area to another. Food areas include any location where food is stored or processed. Certain restrictions apply to the types of insecticides and treatments that can be used in food or non-food areas. Some definitions and general guidelines follow. For more specific details on whether a product can be used in food or non-food areas, refer to the product label.

16.4.5.1 Residual insecticides

Residual insecticides are those products applied to obtain insecticidal effect lasting several hours or longer. There are four types of residual applications: general, barrier, spot and crack and crevice. Each may be used in certain areas of food-handling establishments as directed by the product label.

16.4.5.2 General treatment

General treatment is application to broad expanses of indoor surfaces such as walls, floors and ceilings, or outside treatments. This is permitted only in non-food areas using only those insecticides so registered.

16.4.5.3 Barrier treatment

Barrier treatment is usually considered the application of pesticides to thresholds and other entrances, the foundation, and the soil adjacent to the foundation. A barrier treatment with residual sprays, dusts, or granules may be beneficial in controlling outdoor pests that may become invaders or nuisances when populations build up.

16.4.5.4 Spot treatment

Spot treatment is application to limited areas on which insects are likely to walk but will not be in contact with food, utensils, or by workers. Such areas may occur on floors, walls, and the bases or undersides of equipment. Spot treatments should not exceed two square feet. In many cases, spot treatment is allowed only in non-food areas. Check the label to be certain of the proper use of spot treatments.

16.4.5.5 Crack and crevice treatment

Crack and crevice treatment is the application of small amounts of insecticides into cracks and crevices in which insects hide or through which they may enter a building. Such openings commonly occur at expansion joints, between different elements of construction, and between equipment and floors. The openings may lead to voids, such as hollow walls, equipment legs and bases, conduits, motor housings, or junction or switch boxes. The crack and crevice treatment may entail the use of sprays, dusts, or baits. It can be used in food areas as long as the insecticide is placed into cracks and crevices. Residual insecticides may be applied when food establishments are in operation unless the label of the product being used specifically indicates that all operations must be stopped at the time of application.

16.4.5.6 Non-residual insecticides

Non-residual insecticides defined as those applied to obtain insecticidal effects only during the time of treatment) used in space treatments (aerosol, ULF and fog treatments), the application should be made while the food-handling establishment is not in operation and exposed foods are removed or covered. Also, food-handling surfaces should be cleaned before use. However, the use of non-residual insecticides as contact treatments (which means hitting the target pest with a wet spray for immediate insecticidal effect) can be done while the establishment is in operation. Both space treatments and contact treatments are considered general insecticide applications.

16.4.6 Rodenticides in food-handling establishments

Rodenticides are usually applied in attractive food baits or as liquids. Such baits ordinarily require “tamper resistant” containers that are designed to protect animals and children as well as to avoid contamination of food. When placing bait stations, special attention is required to protect the containers from damage and from being stolen or tampered with. Rodenticides may be used outside the facility to intercept rodents before they gain entry. They may be used inside the facility as long as they do not come in contact with food.



Lesson 17

TREATMENT AND DISPOSAL OF DAIRY WASTE WATER

17.1 Introduction

With increase in demand for milk and milk products, many dairies of different capacities have come up in different places. These dairies collect milk from the producers and then either simply bottle it for marketing, or produce different milk products according to their capacities. Large quantities of waste water originate due to their different operations. The organic substances in the wastes comes either in the form in which they were present in milk or in a degraded form due to their processing. As such, the dairy waste, though biodegradable, are very strong in nature.

Dairy plants process a wide variety of products including milk, cheese, butter, ice cream, yogurt, non-fat dry milk, whey and lactose. The volume and composition of dairy wastes from each plant depends on the types of products produced, waste minimization practices, types of cleaners used, and water management in the plant. Because most dairy plants process several milk products, waste streams may vary widely from day to day.

17.2 Sources of Dairy Wastes

The liquid waste from a large dairy originates from the following sections or plants: receiving stations, bottling plant, cheese plant, casein plant, condensed milk plant, dried milk plant, and ice cream plant. The main sources of dairy effluents are those arising from the following:

1. Spills and leaks of products or by-products
2. Residual milk or milk products in piping and equipment before cleaning
3. Wash solutions from equipment and floors
4. Condensate from evaporation processes
5. Pressings and brines from cheese manufacture

Dairy plant operators may choose from a wide variety of methods for treating dairy wastes from their plants. This may range from land application for small plants to operation of biological waste-water treatment systems for larger plants. Some dairy plants may pre-treat the effluents and discharge them to a municipal waste-water treatment plant.

In addition to the wastes from all the above milk processing units, some amount of uncontaminated cooling water comes as waste; these are very often re-circulated.

17.3 Objectives of Treating Dairy Wastes

The objectives of treating dairy wastes are to

1. Reduce the organic content of the waste-water,
2. Remove or reduce nutrients that could cause pollution of receiving surface waters or groundwater, and
3. Remove or inactivate potential pathogenic microorganisms or parasites.

The level of treatment needed for dairy waste-water for each plant is dictated by the environmental regulations applicable to the location of the dairy plant. The Environmental Protection Agency (EPA) establishes general regulations concerning discharges to surface waters and groundwater. Each state environmental regulatory agency is responsible for ensuring compliance with those regulations. Each plant must have a discharge permit for each outfall discharging to surface waters. The limits within that permit depend on the flow and type of surface water into which the treated waste-water is discharged. If a plant discharges waste-water to municipal sewers for treatment, the municipal treatment system may require pre-treatment of high-strength wastes to bring the waste load down to domestic sewage strength. This allows for proper treatment of waste-water before it is discharged to surface water. For land applications, state regulatory agencies dictate hydraulic loadings and maximum levels of toxic substances that can be land spread on each unit of land.

17.4 Composition of Dairy Wastes

Because more than 95% of the waste load from dairy plants comes from milk or milk products, it is of value to know the average composition of these products. Milk solids are primarily composed of fats, proteins, and carbohydrates. Other constituents in dairy waste water may include sweeteners, gums, flavouring, salt, cleaners, and sanitizers. Biochemical oxygen demand (BOD) is the amount of dissolved oxygen (DO) consumed by microorganisms for biochemical oxidation of organic solids in waste water. The analytical procedure for determining BOD measures dissolved oxygen consumed by a seeded, diluted waste water sample incubated at 20°C for 5 days. One gram of milk fat has a BOD of 0.89 g, whereas milk protein, lactose, and lactic acid have BOD value of 1.03, 0.65, and 0.63 g, respectively. Roughly, one kg of BOD in dairy waste water represents 9 kg of whole milk. Chemical oxygen demand (COD) is the amount of oxygen necessary to oxidize the organic carbon completely to CO₂, H₂O and ammonia. The COD is measured calorimetrically after refluxing a sample of waste water in a mixture of chromic and sulphuric acid. If the BOD/ COD ratio of waste water is less than 0.5, then the organic solids in the waste are not easily biodegraded. The BOD/ COD ratio for dairy wastes has been reported to range from 0.50 to 0.78.

Some minor constituents, such as phosphorus and chloride, are also very important in the treatment of dairy wastes. Phosphorus is the element that limits plant and algal growth in surface waters. Discharge of any significant levels of phosphorus in waste effluents to surface waters can lead to decreased water quality in lakes and streams. Milk and milk by-products can contribute significant quantities of phosphorus to dairy wastes. The phosphorus content of milk is approximately 1000 mg/ l, whereas whey contains 450 to 575 mg/l. Salty whey and brines can contribute significant levels of chloride to dairy waste water. Chloride concentrations in excess of 400 mg/ l in effluents discharged to streams can result in chronic toxicity to sensitive water insects such as *Daphnia magna*. Because chloride cannot be removed with biological or chemical treatments, waste minimization is the only method for reducing chloride in dairy wastes. BOD₅ values and percentage contribution of various milk components to such values has been reported in Table 17.1.

Table 17.1 BOD₅ values and percentage contribution of milk components

Product	% contribution to BOD ₅ by			
	BOD ₅ (mg/l)	Milk fat	Milk protein	Lactose
Skim milk	67,000	6.3	49.3	44.5
Whole milk	104,000	17.8	43.3	39.0
Half and half	156,000	62.4	19.7	17.9
Heavy cream	399,000	89.2	5.7	5.0
Churned buttermilk	68,000	4.2	48.2	46.7
Evaporated milk	208,000	34.6	35.0	30.6
Ice cream	292,000	30.7	15.9	15.2
Whey	34,000	5.9	20.6	70.8

Source: Harper and Blaisdell (1971).

The dairy wastes are very often discharged intermittently the nature and composition of wastes also depend on the types of products produced, and the size of the plant. Characteristics of the wastes of a typical Indian dairy, handling about 3,00,000 to 4,00,000 litres of milk in a day have been compiled in Table 17.2.

Table 17.2 Composition of waste water of a typical dairy

Properties	Value
pH	7.2
Alkalinity	600 mg/l as CaCO ₃
Total dissolved solids	1060 mg/l
Suspended solids	760 mg/l
BOD	1260 mg/l
COD	84 mg/l
Total nitrogen	84 mg/l
Phosphorous	11.7 mg/l
Oil and grease	290 mg/l
Chloride	105 mg/l

17.5 Aerobic Treatment of Dairy Waste Water

Wastes from processing milk products are almost entirely composed of organic material in solution or colloidal suspension, although some larger suspended solids may be present in waste water from cheese or casein manufacturing plants. Sand and other foreign material is present in limited amounts as a result of floor or truck washes. Because milk waste contains very little suspended matter, preliminary settling of solids does not result in any appreciable reduction of BOD.

However, a screen and grit chamber with 0.95-cm mesh wire screen is recommended to remove large particles to prevent clogging of pipes and pumps in the treatment system. This is especially important if the waste is to be pumped with high-pressure pumps, as in spray irrigation. After preliminary treatment in the screen and grit chamber, the waste should be pumped to an equalization tank. With wide variations in waste water flow, strength, temperature, and pH, some reaction time is required to allow neutralization of acid and alkaline cleaning compounds and to allow for complete reaction of residual oxidants from cleaning solutions with organic solids of dairy waste. Ideally, a minimum of 6–12 hours of equalization should be provided to allow for waste stabilization. The equilibrated waste can then be treated with one of the following systems or a combination of treatment systems as explained below:

17.5.1 Treatment ponds or lagoons

Dairy plants in rural areas with insufficient farmland available for land application may be able to use ponds or lagoons for economical treatment of dairy wastes. A pond or lagoon normally consists of a shallow basin designed for treatment of dairy waste water without extensive equipment and controls. The three types of ponds used are aerobic, facultative, and anaerobic.

17.5.2 Aerobic ponds

Aerobic ponds are generally 0.5–2.0 meters deep, and contents are mechanically mixed and aerated to allow penetration of sunlight necessary for growth of algae. The algae produce oxygen through photosynthesis and use waste products from the bacteria involved in the biological breakdown of milk wastes. At 20°C, a BOD removal of 85% can be experienced with an aeration period of 5 days.

17.5.3 Anaerobic ponds

Anaerobic ponds are generally used to pre-treat dairy wastes with high protein and fat levels or for stabilizing settled solids. Organic matter is biodegraded and gases such as CH₄, CO₂, and H₂S are produced. To reduce effectively the BOD in anaerobic effluent, an aerobic process must follow to allow aerobic microorganisms to use up the residual breakdown products. The typical retention time for anaerobic treatment ponds ranges from 20 to 50 days.

17.5.4 Activated sludge

Activated sludge is one of the most popular methods for treating dairy wastes. The process consists of aerobic oxidation of organic matter to CO₂, H₂O, NH₃ and cell biomass followed by sedimentation of activated sludge. A portion of the activated sludge is returned to the aeration tank to continue the treatment cycle.

Activated sludge contains a large mass of various microorganisms plus organic and inorganic particles. The concentration of biomass in the aeration or contact tank is normally called the mixed liquor suspended solids (MLSS). Bacteria make up the largest portion of activated sludge in the aeration process. Bacteria are primarily responsible for oxidation of organic matter and formation of polysaccharides and other polymeric materials that aid in flocculation of the microbial biomass. Table 17.3 lists the bacterial genera found in activated sludge. Estimates of aerobic bacterial counts in activated sludge are approximately 10¹⁰/g of MLSS or 10⁷–10⁸/ ml. The active fraction of bacteria in activated sludge flocs represents only 1%–3% of total bacteria present. This indicates that the major portion of activated sludge is actually dead cells and extracellular material. Activated sludge does not normally favour growth of yeast, algae, or fungi. Protozoa may represent up to 5% of the MLSS. Protozoa are predators of bacteria in activated sludge; they help reduce effluent suspended solids and soluble BOD.

Table 17.3 Bacterial genera found in activated sludge

Major genera	Minor genera
<i>Zoogloea</i>	<i>Aeromonas</i>
<i>Pseudomonas</i>	<i>Aerobacter</i>
<i>Comomonas</i>	<i>Micrococcus</i>
<i>Flavobacterium</i>	<i>Spirillum</i>
<i>Alcaligenes</i>	<i>Acinetobacter</i>
<i>Brevibacterium</i>	<i>Gluconobacter</i>
<i>Bacillus</i>	<i>Hyphomicrobium</i>
<i>Achromobacter</i>	<i>Cytophaga</i>
<i>Corynebacterium</i>	
<i>Sphaerotilus</i>	

Source: Sterritt and Lester (1988).

17.5.4.1 Conventional process

In the conventional activated sludge process, dairy waste-water is introduced into the aeration tank along with a portion of activated sludge from the clarifier. Air is incorporated into the waste mixture with diffusers or mechanical aerators. The air serves two purposes in the aeration tank: first, to supply oxygen to aerobic microorganisms and, second, to keep the activated sludge floc thoroughly mixed with incoming waste-water to allow maximal efficiency in oxidation of organic matter. Key parameters controlling operation of the activated sludge process are rate of (a) aeration in the tank, (b) return of activated sludge to the aeration tank, and (c) waste or excess sludge discharged from the treatment system. Normal detention time for conventional activated sludge treatment of municipal or low strength waste-water is 4–8 hours. However, dairy waste-waters may require longer detention times, 15–40 hours, to reduce BOD₅ to an acceptable level. This type of process is called an extended aeration system.

17.5.4.2 Contact stabilization process

Another modification of the activated sludge treatment is a three-step process known as the contact stabilization process (Figure 17.1). This process allows for a 30 minutes detention time in the contact tank in which microorganisms obtain their food. Sludge containing the organisms and their food is separated in the clarifier. Sludge that is to be returned to the contact tank is first sent to an aerated stabilization tank for 4–8 hours during

which time microorganisms finish digesting their food. By aerating only sludge that is being returned to the initial contact tank, less tank space and less air are required. This system produces less sludge and is better suited for shock loading. The BOD of dairy waste-water could be reduced by 99% and total Kjeldahl nitrogen by 91% after a total detention time of 19.8 hours in this type of system.

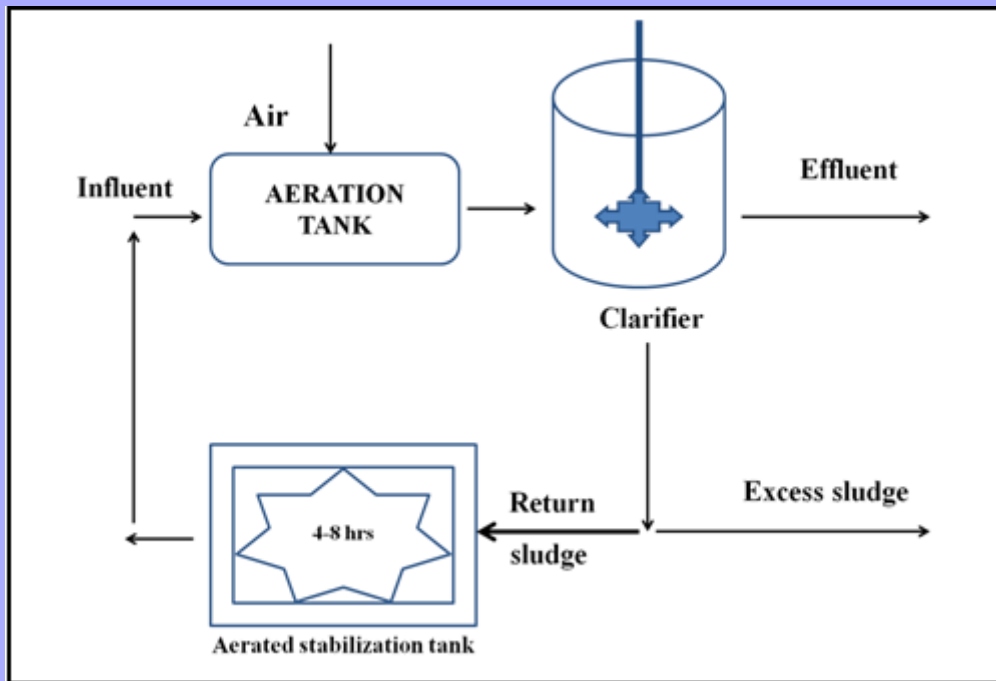


Fig. 17.1 Activated sludge system with contact stabilization

17.5.4.3 Flocculation

Settling of sludge in the clarifier usually proceeds best when the microbial growth rate is slow and nutrient concentrations are very low. Extracellular polysaccharides and slimes produced by *Zooglea ramigera* and other activated sludge organisms play a leading role in bacterial flocculation and floc formation. Good sludge settling and BOD removal occurs at high MLSS concentrations. Microbial flocculation can be enhanced with addition of poly-electrolytes, alum, or iron salts as coagulants. Poor settling of sludges may be observed if excess production of exo-polysaccharides by bacteria occurs in activated sludge. This non-filamentous bulking may be corrected with chlorination. Filamentous bulking may be caused by excessive growth of filamentous bacteria such as *Sphaerotilus* spp. or *Nostocoida limicola*. A low level of dissolved oxygen in the aeration tank is the primary factor contributing to growth of this filamentous bacterium in activated sludge.

17.5.5 Biological filtration

17.5.5.1 Trickling filters

Biological filters, such as trickling or percolating filters, are one of the earliest types of biological waste treatment. In a biological filter, the biofilm is attached to a support substance such as gravel, stones, or plastic materials. As wastewater is pumped over the biofilm, it oxidizes organic matter and removes nutrients such as nitrogen and phosphorus. A basic trickling filter is composed of a tank containing a filter medium to a depth of 1.0–2.5 metres, a wastewater distributor that applies the waste solution evenly over the medium bed, and a final clarifying tank to remove sludge and solids sloughing off the filter medium (Figure 17.2).

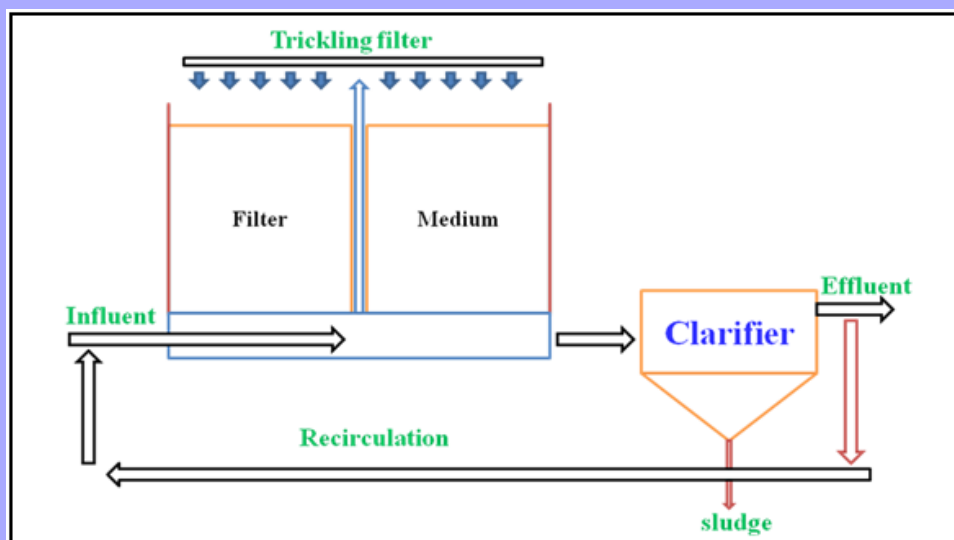


Fig. 17.2 trickling filtration process

In some instances, wastewater is re-circulated through the system to provide for added dissolved oxygen to primary influent and greater removal of BOD. The two most important factors affecting microbial growth on the support medium are flow rate of wastewater and size and geometrical configuration of support material. In the initial start-up of the filter, the medium surface is colonized by gram-negative bacteria followed by filamentous bacteria. The biofilm formed on support material is called a zoogeal film and is composed of bacteria, fungi, algae, protozoa, and other life forms such as rotifers, nematodes, snails, and insect larvae. Some of the bacterial genera active in trickling filters are *Achromobacter*, *Flavobacterium*, *Pseudomonas* and filamentous bacteria such as *Sphaerotilus*. Growth conditions on the outer surface of the biofilm are aerobic but the inner portion of the biofilm next to support material tends to be anaerobic. Trickling filters are categorized by the loading rate to the filter medium. Low-rate trickling filters (40 kg BOD/ 100 m³/ day) allow for nitrification and more complete removal of nutrients from wastewater. High-rate filters (60–160 kg BOD/ 100 m³/ day) rarely have nitrification take place and have lower treatment efficiencies. BOD removal by trickling filters is approximately 85% for low-rate filters and 65–75% for high rate filters.

17.6 Anaerobic Digestion

Anaerobic digestion has been used to stabilize waste treatment sludges for many years. However, in recent years, it has also been designed to treat high-strength dairy wastes. In anaerobic breakdown of dairy wastewater, lactose is first fermented to lactic acid and fats and proteins are hydrolyzed to organic acids, amino acids, aldehydes, and alcohols. Second, the intermediate organic compounds are converted to methane and CO₂. Because anaerobic digestion does not require oxygen for decomposition of organic material, operating costs for treatment are greatly reduced from that of aerobic treatments. However, it is a much slower treatment process that is more susceptible to toxic upsets.

17.6.1 Conventional process

The anaerobic digester is a large fermentation tank in which fermentation, sludge settling, sludge digestion, and gas collection take place simultaneously. Many dairy plants use a two-stage system in which the first stage is complete mixing of the contents of a fermentation tank and the second stage is a digester in which the contents are allowed to stratify. The two-stage anaerobic process allows for higher loading rates and shorter hydraulic retention times. In anaerobic treatment of wastewater, fermentation of sugars, amino acids, and fatty acids is primarily carried out by strict and facultative anaerobic bacteria such as *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus* and *Streptococcus*. Production of methane from fermentation intermediate compounds is accomplished by methanogenic bacteria, which are strict anaerobes. Approximately two-thirds of the methane is derived from acetate conversion by acetotrophic methanogens and the other one-third is the result of carbon dioxide reduction by hydrogen. Methanogens are difficult to grow in pure culture. The milk fat was inhibitory to methanogenic bacteria, and dairy effluents should be treated by anaerobic digestion only after the milk fat concentration was less than 100 mg/l. They also indicated that anaerobic cultures at the start-up of anaerobic digestion should be acclimatized to casein to ensure proper degradation of casein in the process.

Methanogenic bacteria are also sensitive to acidic conditions with complete inhibition below pH 6. With efficient operation of one- or two-stage anaerobic digesters, dairy plants should experience BOD reductions of 78–95%. Biogas from the digester contains up to 67– 75% methane.

17.7 BOD Measurement

Biological (or biochemical) oxygen demand (BOD) is an important parameter in water resource management. BOD is a parameter used to measure the quality of water and treatment results in wastewater. In addition, BOD analysis potential is used in the planning and design wastewater treatment facilities. In routine use BOD determination is used to check the wastewater in the inflow and discharge of wastewater treatment plants. The BOD determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of Waste waters, effluents, and polluted waters. The test has its widest application in measuring waste loadings to treatment plants and in evaluating the BOD-removal efficiency of such treatment systems. The test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron. It also may measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5.

The method (5-day BOD) consists of filling with sample, to overflowing, an airtight bottle of the specified size and incubating it at the specified temperature for 5 days. Dissolved oxygen is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the BOD measurement.

17.8 Treated Dairy Effluents and its Disposal

Dairy plants discharging waste waters directly to streams, bays, rivers, creeks and /or estuaries must have a permit for this discharge. Dairy plants that use non-discharge systems such as land disposal will also need a permit. Permits for discharge are usually obtained from the state government control agency. Effluents from waste treatment systems must be sufficiently reduced in BOD and biological nutrients (e.g., P, NH₃) that discharge to surface waters does not significantly affect aquatic life. Environmental regulatory agencies specify limits for composition of effluents discharged to each type of stream or watershed. To reduce the volume of dairy wastewater to be treated and reduce treatment costs, careful attention must be given to minimizing losses of milk and milk products in the dairy plant. With good product conservation and selection of an effective waste treatment process, dairy plant operators should be able to operate profitably and meet environmental requirements.



Module 4. Microbiological risk profile and safety criteria for dairy products

Lesson 18

MICROBIOLOGICAL RISK PROFILE: CONCEPTS

18.1 Introduction

Food Safety and Standards Authority of India has the responsibility for protecting the health and safety of consumers through the development of food standards. The development through the chain of Primary Production and Processing (PPP) standards require thorough assessment of risk to public health and safety. Codex defines the term risk as ‘a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard in food’. Risk profiling is defined by FAO/ WHO as the process of describing a food safety problem and its context in order to identify those elements of the hazard or risk relevant to various risk management decisions. Risk assessment is a scientific process undertaken to characterize the risk to public health and safety posed by food-borne hazards associated with a food commodity.

There are a number of tools to assess risks to public health and safety, including risk profiling, quantitative and qualitative risk assessments and scientific evaluations. Risk profile identifies and examines where biohazard may enter the dairy supply chain from the primary produce to processed food. The application of these tools to the assessment of the risks to public health and safety is dependent on the purpose of the assessment and on the availability, quality and quantity of relevant data. One needs to follow established international guidelines and incorporate elements of the CAC when undertaking risk profiles, risk assessment and other scientific evaluations. Guidance for undertaking risk assessments has been drafted internationally by the FAO and WHO.

In assessing risks to public health and safety available scientific data concerning the safety of the commodity under consideration and the properties of the hazard are evaluated. This requires utilization of relevant scientific data and includes procedures to address uncertainty and variability in the conclusions drawn from the data, i.e. consideration of the relevance and quality of data and the veracity of its source.

The outcome of any assessment of risks to public health and safety may include a statement on the probability and severity of an adverse health effect due to the consumption of a food containing a particular biological, chemical or physical agent. An assessment may also identify where in the production chain controls over hazards will have the greatest impact on minimizing risk, i.e. informing the risk managers where intervention will be most effective. The outcomes of the assessing risks to public health and safety for dairy products are used to inform risk management decisions.

18.2 Microbiological Risk Profile

The assessment of risks to public health and safety from microbiological hazards in milk and milk products has been undertaken in the form of a microbiological risk profile. It provides a broad overview of risks associated with consumption of dairy products. The risk profile identifies key food safety hazards and assesses where in the primary production and processing supply chain these hazards might be introduced, increased, reduced or eliminated.

18.2.1 Scope and purpose

The purpose of the risk profile in dairy products is to provide an objective analysis of relevant scientific data and information to identify the public health and safety risks associated with dairy products. This will enable risk managers to consider the risks associated with dairy products and the reductions in risk that may be

achieved with various production and process control options. The risk profile may also identify the need for more detailed microbiological risk assessments for specific dairy commodities like dairy products prepared from raw milk especially cheese. The microbiological risk profile identifies and examines hazards along the dairy supply chain from milk production through to consumption of dairy products and has considered the relevant inputs into the dairy primary production and processing chain. The risk profile encompasses the following elements:

1. Identification and description of the micro-organisms those may be associated with dairy products including key attributes of each organism and its public health impact (hazard identification/hazard characterization).
2. Examination of prevalence and concentration data on potential hazards from products along the entire dairy food chain.
3. Examination of epidemiological data (domestic and international) related to the consumption of dairy products.

This risk profile identifies the microbiological public health and safety risks associated with dairy products. In compiling the risk profile, a wide range of scientific literature, data and information should be reviewed and evaluated. A broad range of microorganisms need to be considered in this assessment. The microorganisms should be representative of those that may be present in raw milk, either directly transmitted via the mammary gland or via faecal/ environmental contamination. In addition, micro-organisms that originate from the milking environment and/ or post-pasteurization contamination should also be considered. When examining each dairy commodity category, only those potential pathogens relevant to the commodity being evaluated should be assessed. The estimate of the severity of adverse health effects caused by a food-borne agent may be based on the ranking scheme for food-borne pathogens and toxins described by the International Commission on Microbiological Specifications for Foods. The ICMSF ranking scheme categorizes hazards by the severity of the threat they pose to human health, taking into consideration the: i) likely duration of illness, ii) likelihood of death, and iii) potential for ongoing adverse health effects. The severity of adverse health effects caused by a hazard may be ranked as moderate, serious or severe according to the following definitions:

Severity	Description
Moderate	Not usually life threatening, no sequelae (morbid symptoms); normally short duration; symptoms are self-limiting; can be severe discomfort
Serious	Incapacitating but not life threatening, sequelae infrequent; moderate duration
Severe	Life threatening, or substantial sequelae, or long duration

Under the ICMSF ranking, severe hazards can further be divided into those applying to the general population and those applying to specific sub-populations, that is, susceptible individuals (for example, the very young and old, the immuno-compromised, and pregnant women and their unborn children). This takes into account those situations where a hazard considered to be of moderate or serious to the general population may cause a severe illness in certain susceptible sub-populations. A brief summary of the micro-organisms to be generally looked for their severity of associated illness linked with dairy products and the availability of epidemiological data is depicted in Table 18.1.

Table 18.1 Summary of microorganisms to be generally considered in the risk profile

Organism	Shed directly in milk#	Contaminant of raw milk##	Survives pasteurization	Severity of illness §	Dairy products implicated in food-borne illness
<i>Aeromonas</i> spp.	×	✓	×	Serious	+
<i>Bacillus cereus</i>	×	✓	✓	Moderate	++
<i>Brucella</i> spp.	✓	✓	×	Severe	+
<i>Campylobacter</i>	×	✓	×	Serious	++
<i>Clostridium botulinum</i>	×	✓	*	Severe	+
<i>Clostridium perfringens</i>	×	✓	✓	Moderate	+
<i>Corynebacterium</i> spp.	✓	✓	×	Serious	+
<i>Coxiella burnetii</i>	✓	✓	×	Serious	+
<i>Cryptosporidium</i>	×	✓	×	Severe	+
<i>Enterobacter sakazakii</i>	×	✓	×	Severe [^]	++
Pathogenic <i>E. coli</i>	×	✓	×	Severe	++
<i>Listeria monocytogenes</i>	✓	✓	×	Severe [^]	++
<i>Mycobacterium avium</i> subs. <i>paratuberculosis</i>	×	✓	×	-	-
<i>Mycobacterium bovis</i>	✓	✓	×	Severe	+
<i>Salmonella</i> spp	×	✓	×	Serious	++
<i>Shigella</i> spp	×	✓	×	Serious	+
<i>Staphylococcus aureus</i>	✓	✓	x**	Moderate	++
<i>Streptococcus</i> spp.	✓	✓	×	Serious	+
<i>Yersinia enterocolitica</i>	×	✓	×	Serious	+

(Source: ICMSF, 2002; FSANZ, 2006)

18.3 Grouping of Dairy Commodities

For the purpose of this profile, dairy commodities may be grouped into broad categories as follows:

1. Milk and cream
2. Butter and butter products
3. Concentrated milk products
4. Dried milk powders
5. Infant formulae
6. Colostrum
7. Ice-cream
8. Dairy deserts
9. Dairy-based dips
10. Cultured and fermented milk products
11. Cheese
12. Indigenous dairy products
13. Casein, whey products and other functional milk derivatives

18.4 Risk Ranking of Dairy Products

The actual ranking of the dairy products is quite variable. Once a shelf-stable UHT product is opened, it may become contaminated and when subjected to temperature abuse it could become a high-risk food. In contrast, the low pH and low water activity of extra hard cheese means it will be very robust and unlikely to support the growth of any pathogen that adventitiously contaminates the surface. Dried milk powders and infant formulae

are inherently stable products due to their low water activity, however, these products may be prone to contamination and upon reconstitution become higher risk, especially if improperly reconstituted and stored. Dairy products likely to support the growth of pathogens and prone to contamination after pasteurization, may be categorized as higher risk than other dairy products, while dairy products that are inherently stable with respect to pathogens, if correctly formulated, can be classified as low risk (Table 18.2 and Fig. 18.1).

Table 18.2 Risk ranking of dairy products

Risk ranking	Dairy product	Risk characterization
Higher risk	Unpasteurized	No pathogen reduction step
	Soft cheeses	Mild pH, long shelf life allowing growth of <i>Listeria monocytogenes</i>
	Dairy desserts	Mild pH, fermentable carbohydrate, long shelf life
	Fresh cheese	Dependent on variety - some have low acid, high moisture
	Dairy dips	Dependent on variety – some have low acid, high moisture, added ingredients
Intermediate risk	Unsalted butter	Absence of salt, high moisture content
	Low fat spreads	Absence of salt, high moisture content
	Pasteurized milk	Storage temperature only hurdle to control post-pasteurization contamination
	Ice-cream	Stored frozen, but soft serve may allow growth of <i>Listeria monocytogenes</i>
Low risk	Yoghurt	Low pH does not allow growth of pathogens
	Salted butter	High salt concentration
	Hard cheese	Low water activity, low pH
	Extra hard cheeses	Low water activity, low pH
	UHT milk	Commercially sterile
	Dried milk powder	Low water activity, however prone to contamination

(Source: FSANZ, 2006)

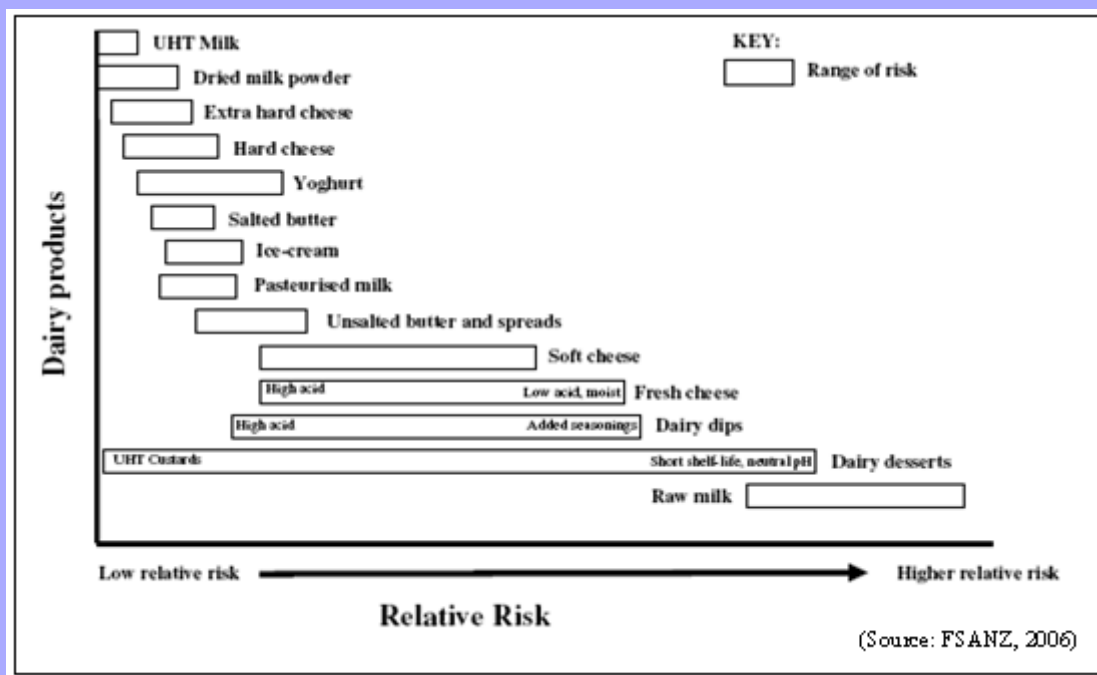


Fig. 18.1 Graphical presentation of relative risk ranking of dairy products

18.5 Risk Management Issues and Control Strategies for Dairy Products

The critical factors having the most significant impact on the safety of processed dairy products are as follows:

1. The quality of raw materials
2. Correct formulation
3. Effective processing
4. The prevention of recontamination of products
5. Maintenance of temperature control through the dairy supply chain

While pathogenic microorganisms may contaminate raw milk supplies, pasteurization is a very effective Critical Control Point (CCP) in eliminating pathogens; good manufacturing practices must also be employed to ensure that post-pasteurisation contamination does not occur. The effectiveness of pasteurization is dependent upon the microbiological status of the incoming raw milk. Control measures at the primary production level involve minimizing the likelihood of microbiological hazards contaminating the raw milk. This is achieved through the implementation of a food safety program incorporating GAP. These measures are effective in reducing the microbial load of milk being sent for processing.

However, should microbial contamination of raw milk occur, it is critical that milk is stored at a temperature that minimizes the opportunity for the bacteria to multiply. Temperature abuse of the milk may allow growth of pathogenic bacteria to the extent where the pasteurization process may not eliminate all pathogenic bacteria and/or toxins. Aflatoxins can be formed and ingested by dairy cattle during feeding eventually contaminating the milk. Aflatoxin contamination of milk is more common where intensive supplementary feeding of dairy herds is conducted.

18.5.1 Correct formulation

Ingredients used in the manufacture of dairy products that are added post pasteurization must be of a high microbiological standard. Many non-dairy ingredients added to ice-cream mix after heat treatment include fruits (canned, fresh, or frozen and usually in concentrated sugar syrups), nuts, chocolate, pieces of toffee and biscuit, colors and flavors. These ingredients and those added to other dairy products such as yoghurt, dairy desserts, dairy dips and cheese may introduce pathogens into the product.

The addition of ingredients added after pasteurization was identified as a high risk factor and was recommended that dairy products with these additions (e.g. ice-cream and cheeses) be moved into the high risk category and the finished product be subject to additional end product microbiological analysis. The microbial quality of dry-blended ingredients into infant formula was identified as a significant source of contamination as there is no heat treatment to destroy bacteria in the final product.

18.5.2 Effective processing (pasteurization)

Dairy processing facilities primarily use High Temperature Short Time (HTST) pasteurization (minimum 72°C for 15 seconds) or batch pasteurization (minimum 65°C for 30 minutes) and then rapid cooling to 7-8°C to eliminate the pathogens of concern in milk. However, most factories actually heat the milk to higher temperatures and hold it for a longer time period as an in-built safety margin. In most cases milk and dairy products are consumed as RTE foods and will readily support the growth of any contaminating microorganism. In the past, the dairy industry has been subjected to a high level of food safety regulation ensuring high levels of hygiene and sanitation are maintained. The pasteurization process eliminates all pathogenic bacteria found in raw milk, with the exception of the spore forming bacteria *B. cereus* and *C. perfringens*.

18.5.3 The prevention of recontamination of product

Post-pasteurization contamination can pose a major problem where good manufacturing practices are not employed. Pathogenic microorganisms can be introduced into a dairy processing environment with raw milk. Once these organisms gain access to the processing plant the presence of nutrients and moisture can allow not only for survival but also the multiplication of these organisms. The application of food safety programs including elements of GMP and GHP are critical to limit the potential for pathogens to contaminate dairy products after pasteurization. The primary organisms of concern are *Listeria monocytogenes* for most dairy products and *Salmonella* in dried milk products.

18.5.4 Maintenance of temperature control through the dairy supply chain

The intrinsic nature of many dairy products means they will support the growth of pathogenic bacteria that may contaminate the product. This categorizes these products as 'potentially hazardous foods'. Exception to this are products such as yoghurt and hard cheeses (low pH) and ice-cream (stored frozen). As these are potentially hazardous foods maintenance of temperature control through the dairy supply chain is critical to ensure these foods remain safe and suitable.

Notwithstanding the above there is need for ongoing vigilance and further development of safety control measures to counter the emergence of new pathogens and the re-emergence of traditional pathogens in various foods. These organisms often occupy specific environmental niches and may arise through changing technologies, methods of food handling and preparation, dietary habits and population. Post-processing contamination in plant and the maintenance of control over contamination and storage conditions during transport, retail display and home use remain major factors impacting on the safety of dairy products.



Lesson 19 MICROBIOLOGICAL CRITERIA AND SAMPLING GUIDELINES

19.1 Introduction

Microbiological standards have been set where risk assessment has shown that the risk of food-borne illness associated with the consumption of certain foods is relatively high and that a standard could contribute to the management of the risks identified. Where the justification for a standard was not found, guideline criteria have been developed. These guideline criteria are intended to complement other risk-management strategies undertaken by government and industry. These are mandatory criteria that provide benchmark levels against which unacceptable microbial contamination of food can be identified and remedial action initiated when limits are exceeded. Failure to meet guideline levels generally indicates a failure in the process or hygiene procedures and requires action to identify the cause and remedy the problem.

19.2 Microbiological Criteria

A criterion is a yardstick on which a judgment or decision can be made. A microbiological criterion will stipulate that a type of microorganisms, group of microorganisms or toxin produced by a microorganism must either be not present at all, be present in only a limited number of samples, or be present as less than a specified number or amount in a given quantity of a food or food ingredient.

19.3 Components of a Microbiological Criterion

A microbiological criterion should include the following:

1. A statement describing the identity of the food
2. A statement of the contaminant of concern, i.e. the microorganisms or group of microorganisms and/or toxin or other agent
3. The analytical method to be used for the detection, enumeration, or quantification of the contaminant of concern
4. The sampling plan
5. The microbiological limits considered appropriate to the food and commensurate with the sampling plan

19.4 Types of Microbiological Criteria

19.4.1 Mandatory and advisory criteria

A mandatory limit is one that the food cannot exceed. Food that does not meet the criterion must be subjected to some action e.g., it may be rejected by the purchaser, destroyed, reprocessed, sold as an inferior grade, or diverted to a use where the contaminant is not of concern. Certain mandatory criteria also may result in the loss of license to process food when limits are consistently exceeded. Advisory criteria often serve as an alert to deficiencies in processing, distribution, storage, or marketing. They are not mandatory but permit judgment to be made when limits are not met.

19.4.1.1 Standards

A microbiological standard is a microbiological criterion that is a part of a law, ordinance, or administrative regulation. A standard is a mandatory criterion. Failure to comply with it constitutes violation of the law, ordinance, or regulation and will be subject to the enforcement policy of the regulatory agency having jurisdiction.

19.4.1.2 Guidelines

A microbiological guideline is a criterion that often is used by the food industry or a regulatory agency to monitor a manufacturing process. Guidelines function as alert mechanisms to signal whether microbiological conditions prevailing at critical control points or in the finished product are within the normal range. Hence they are used to assess processing efficiency at critical control points with GMP. A microbiological guideline is an advisory criterion in that a given lot of food exceeding the limit for non-pathogenic organisms would not be taken of the market or even downgraded. Guidelines may be mandatory, however, in the sense that food company management and regulatory agencies may demand that the conditions responsible for persistent microbiological deficiencies be corrected without delay.

19.4.1.3 Specifications

A microbiological specification is a microbiological criterion that is used as a purchase requirement whereby conformance with it becomes a condition of purchase between buyer and vendor of a food or ingredient. A microbiological specification can be either mandatory or advisory.

19.5 Microbiological Criteria for Acceptance or Rejection of Sample Lots

1. the food which must comply with the microbiological limits set in relation to that food;
2. the microorganism or group of microorganisms of concern;
3. the number of sample units to be taken and tested;
4. the level of microorganisms considered acceptable, marginally acceptable or critical (depending on the sampling plan specified) and
5. The number of samples that should conform to these limits.

19.6 Sampling Plans and FSSAI Microbiological Standards for Milk and Milk Products

Sampling plan and interpretation: The following terms, as used by the International Commission on Microbiological Specifications for Foods (ICMSF) are defined and used in development of FSSAI standards for different milk and milk products.

n = the number of sample units which must be examined from the batch/lot of food to satisfy the requirements of a particular sampling plan.

c = the maximum allowable number of defective sample units. This is the number of sample units, which may exceed the microbiological limit specified by m . These are considered marginally acceptable results provided they did not exceed the limit specified by M . When more than this number is found; the lot is rejected by the sampling plan.

m = Represents an acceptable level and values above it are marginally acceptable in terms of the sampling plan.

M = A microbiological criterion which separates marginally acceptable quality from unsatisfactory/potentially hazardous quality. Values above M are unacceptable in terms of the sampling plan and detection of one or more samples exceeding this level would be cause for rejection of the lot.

When 5 or more units of the same variety from a lot or consignment are analyzed ($n = 5$), no more than 2 units ($c = 2$) should exceed the maximum tolerance (m) for microbiological levels stated in the reference criteria and no 1 unit should exceed the stated level for the maximum tolerance (M).

Microbiological criteria and their interpretation: Three categories of microbiological quality have been assigned in standard based on Total plate count, levels of indicator organisms (Coliform count and yeast & mold count) and the number or presence of pathogenic bacteria. These are satisfactory, unsatisfactory and potentially hazardous.

1. Satisfactory: if a maximum of c/n value are between m and M , and the rest of the values observed are $< m$ ---- means the results are within limits of acceptable microbiological quality and no action is required.
2. Unsatisfactory: If one or more of the values observed are $>M$ or more than c/n values are between m and M means the results are outside acceptable microbiological limits linked with hygiene indicators (Total plate count, Coliform count and Yeast and mold count) and are indicative of poor hygiene or poor handling practices. Under these conditions the premises producing such unsatisfactory product shall be stopped and will carry out the detailed investigations for nonconformity/ noncompliance during manufacturing. The manufacturing of such product will be re-started only after HACCP/ GMP audit clearance of the premises by the food safety authority and compliance of fresh product with the regulatory limits.
3. Potentially hazardous: If one or more of the values observed are $>M$ or more than c/n values are between m and M means the results are outside acceptable microbiological limits linked with pathogenic bacteria (*E. coli*, *Salmonella*, coagulase positive *S. aureus*, *B. cereus*, *C. perfringens*, *L. monocytogenes*) and are indicative of serious food safety concern and immediate remedial action should be initiated. Such results will attract enforcement/ prosecution by the concerned food safety authorities. Withdrawal of any of the food still available for sale or distribution and if applicable, recall action may be initiated. An investigation of food production or handling practices shall be investigated to determine the source/ cause of the potential of the problem so that remedial action can commence. A detail risk assessment shall also be done. Failure by an owner to either cease manufacture of product or withdraw/ recall product from sale when requested to do so shall result in seizure of that product where the officer has reason to believe that it is contaminated with pathogenic bacteria.

19.7 Sample Size, Storage and Transport Standards & Reference Methods of Testing

Each sample unit taken for submission to the laboratory should be of sufficient size to allow the appropriate analysis or analysis to be carried out and should, preferably, be much larger than the analytical size needed to allow for further testing if required. A minimum sample size of 100 g or 100 ml is usual. Food samples obtained by an authorized officer as a result of a consumer complaint, or as part of an investigation of a suspected food poisoning incident, may only be single samples. While this is less than the five sample units generally prescribed, the results of an analysis on such a sample is still considered valid. Food samples provided to the testing laboratory should have undergone only minimum change in their microbiological status since the point of sampling. It is very important, for example, that samples are not contaminated during the process. Knowledge of aseptic sampling techniques and the use of appropriate sampling tools may therefore be necessary.

It is also important that the temperature during transport and storage of the food samples is appropriate to minimize microbial growth. Chilled foods should, for example, be transported in an insulated container and frozen foods kept frozen. Every effort should be made to minimize the period between sampling and analysis. The sampling for microbiological testing as per the sampling plan in the standards is to be ensured at manufacturing units as per the guidelines given in IS: 11546:1999 (ISO 707:1997). The samples are to be stored, transported as per the guidelines proposed in Table 19.1 and processed for different microbiological parameters as per the approved test procedures given in Table 19.2 from an accredited laboratory within 24 hours of sampling. Otherwise even under cold storage conditions some psychrotrophic microorganisms can grow and change the microbial status of the food product. Preservatives shall not be added to samples intended for microbiological examination as being practiced for chemical testing. Product samples in duplicate (minimum) are to be tested from the different accredited laboratory. The final decision is to be drawn based on two test results obtained from the accredited lab.

Table 19.1 Guidelines for sample size, storage & transport temperature for milk & milk products

S. No	Product	Temperature before and during transport (°C)	Minimum sample size #
1	Pasteurized milk, Cream & Flavored milk	0 to 4	100 ml or g
2	Sterilized & UHT Milk, Cream, Flavored Milk, Evaporated Milk	Ambient, max. 30	100 ml or g
3	Sweetened Condensed Milk (SCM)	0 to 4	100 g
4	Pasteurized Butter	-18 or lower	50 g
5	Dried Products: Milk Powder, Whey, Edible Casein/Ice Cream Mix	Ambient, max. 30	100 g
6	Ice Cream, Frozen Desserts, Milk Lolly, Ice Candy	-18 or lower	100 g
7	Processed Cheese & Cheese Spreads	4 to 8	100 g
8	Other Types Of Cheeses	4 to 8	100 g
9	Yoghurt, Dahi, Chakka & Srikhand	0 to 4	100 g
10	Paneer	0 to 4	100 g
11	Khoa	0 to 4	100 g

a large sample size may be necessary according to the tests required and the type of product

Table 19.2 Reference test procedure for different microbiological parameters proposed in draft standards

Microbiological requirements		Reference test procedure	Reference
Total Plate Counts (TPC)		General guidance for the enumeration of microorganisms – Colony Count Technique at 30°C	IS 5402; 2002/ ISO:4833:1991
Coliform Count		General guidance for the enumeration of Coliforms	IS: 5401: PART-1; 2002/ ISO: 4831:1991
<i>E. coli</i>		Detection of bacteria responsible for food poisoning part – isolation, identification and enumeration of <i>E. coli</i>	IS:5887 (PART I) -1976 (Reaffirmed as 1995)
<i>Salmonella</i>		Methods for detection of bacteria responsible for food poisoning	IS:5887:PART-3; 1999/ ISO: 6579; 1993
<i>S. aureus</i>		Methods for detection of bacteria responsible for food poisoning – part - 8 horizontal method for enumeration of coagulase - positive staphylococci (<i>S. aureus</i> and other species) - section 1 technique using Baird – Parker agar medium)	IS:5887 :PART 8; SEC 1 :2002 /ISO 6888- 1:1999
Yeast & Mould Count		Method for Yeast and Mould Count of foodstuff and animal feeds.	IS:5403-1999
Spore Count	Aerobic	Specification for sterilized milk	IS:4238-1967 (Reaffirmed 1999)
	Anaerobic	Methods for detection of bacteria responsible for food poisoning part-IV Isolation and identification of <i>C. welchii</i> , <i>C. botulinum</i> and <i>B. cereus</i> and enumeration of <i>C. welchii</i> , <i>C. botulinum</i> and <i>B. cereus</i>	IS:5887 PART IV; 1976
<i>Listeria monocytogenes</i>		Microbiology of Food and Animal Feeding Stuffs –Horizontal Method for the Detection and Enumeration of <i>Listeria monocytogenes part-1 detection method</i>	IS:14988:PART1; 2001 ISO: 11290-1;1996

Note: ¹A lot means a quantity of food, which is prepared or packed under essentially the same conditions, usually:

- From a particular preparation or packing unit; and
- During a particular time ordinarily not exceeding 24hours

A lot of food does not comply with the standards if the number of defective samples units is greater than c, or the level of a microorganism in a food any one of the sample units exceeds M. sampling plans for different milk and milk products are presented in the format used by ICMSF.



Lesson 20

RISK PROFILE AND CRITERIA FOR MILK, FAT-RICH PRODUCTS AND CONCENTRATED AND DRIED MILKS

21.1 Introduction

In recent development consumers' protection from food borne hazards has become a compelling duty for policy makers across the globe. The recent occurrence of serious food scares and food contamination events- such as *Salmonella* contagion in peanut butter, *E.coli* O104:H4 through seed sprouts in Germany, *Listeria monocytogenes* outbreak through melons in Colorado farm, USA, milk contamination with melamine in China, aerated drinks contamination with pesticide in India has raised food safety concerns and its impact on health, marketing and foreign trade. New serious chemical hazards have emerged in the food chain, such as natural toxicants like mycotoxins and marine toxins, environmental contaminants, such as mercury lead, and naturally occurring substances in plants. Although, traditional approaches have proved largely successful, risk assessment now also needs to take account of susceptible populations, combined with low level of exposure to several chemicals and effects on development of the fatal neural system. Food borne diseases have a significant impact not only on health but also on development. Moreover, globalization of the food trade and development of international food standards have raised awareness of the interaction between food safety and export potential for developing countries. With India being a member of the CAC, the Ministry of Health and Family Welfare, has the primary responsibility for determination of Government policy relating to food standards and enforcement of food control including national position on various issues relating to Codex. With the global food industry looking towards India as a food hot-spot, it is about time the national food legislation is aligned with Codex, encouraging innovation and facilitating trade without compromising consumer safety. The introduction of integrated food law provides the much required "one law-one – regulator" platform for raising the food safety standards of India to much global standards. Its speedy and effective implementation is quickly warranted to put India onto the global map. This would require an enabling implementation environment focused on creation of transparency, awareness creation, capacity building, certification of raw material & traceability system , developing right infrastructure, extensive R&D capacity and compliance of milk and milk products for FSSAI standards for Microbiological criteria for hygiene and safety indicators and presence of non-microbial contaminants: antibiotic residues, aflatoxin M1, pesticides, heavy metals etc., so as to match the dynamically changing requirements of food safety and standards. The initiatives would also require a wide spread awareness and promotion campaign focused on changing the mindset of food producers so as to encourage adherence to food safety standards.

20.2 Risk Profile and Criteria for Milk and Milk Products

20.2.1 Milk and cream

Milk is defined as mammary secretion of milking animals, obtained from one or more milking for consumption as liquid milk or for further processing but excludes colostrums. Milk refers to the fluid form of milk derived from cows, sheep, goats, buffaloes, camels, mares and other mammalian animals and available for human consumption through retail sale. Milk may be sold in many forms, including whole milk, skim milk, low-fat milk, flavoured milk and in other modified forms. Some of these products require the removal of the fat portion as cream. Cream is defined as "a milk product comparatively rich in fat, in the form of an emulsion of fat-in-skim milk, which can be obtained by separation from milk". Cream is produced from whole milk by skimming or other separation means.

20.2.1.1 Microbial flora of milk

The microbial status of raw milk is influenced by various factors associated with milk production on farm. These factors impact on both the numbers of micro-organisms present in raw milk and the type of bacterial flora. Generally, few bacteria are present in milk drawn from the udder of a healthy animal, but bacteria may enter milk if it is drawn from an infected animal or if it is contaminated by unhygienic milking practices and poor milk handling. Main groups of microorganisms comprising the microflora of raw milk are shown in Table 20.1.

Table 20.1 Major microflora of raw milk

Group	Incidence
Micrococci e.g. <i>Micrococcus</i> , <i>Staphylococcus</i>	30-99%
Streptococci e.g. <i>Enterococcus</i>	0-50%
Asporogenous Gram +ve rods e.g. <i>Corynebacterium</i> , <i>Microbacterium</i> , etc.	<10
Gram -ve rods e.g. <i>Klebsiella</i> , <i>Escherichia</i> , <i>Enterobacter</i> , <i>Pseudomonas</i> , etc.	<10
Sporeformers e.g. <i>Bacillus</i> spores or vegetative cells	<10
Miscellaneous e.g. <i>Streptomyces</i> , yeasts, moulds, etc.	<10

(Source : FSANZ, 2006)

Various pathogenic micro-organisms may also be associated with raw milk. These include organisms shed by an infected animal (pathogens will predominate in milk from mastitis cows) or organisms that enter the milk from contaminated equipment and poor milking hygiene. Surveys of raw cow's milk, mainly conducted overseas, have detected *Aeromonas* spp., *B. cereus*, *Brucella* spp., *Campylobacter* spp., *Coxiella burnetii*, pathogenic *E. coli*, *L. monocytogenes*, *Mycobacterium* spp., *Salmonella* spp., *S. aureus*, *Streptococcus* spp. and *Y. enterocolitica*. Pasteurization involves heat treatment of milk with the aim of ensuring a microbiologically safe product as well as to extend the shelf-life during refrigerated storage. Milk pasteurization may be carried out either as a batch holding heat treatment or a high-temperature-short-time (HTST) heat treatment. The batch process involves low-temperature-holding for 30 minutes or longer at temperatures of approximately 63°C. This has been largely replaced by HTST treatment at temperatures of $\geq 72^\circ\text{C}$ for at least 15 seconds. The Code states: Milk must be pasteurized by

1. Heating to a temperature of no less than 72°C and retaining at such temperature for no less than 15 seconds and immediately shock cooling to a temperature of 4.5°C ; or
2. Heating using any other time and temperature combination of equal or greater lethal effect on bacteria; where dairy products contain elevated levels of fat or solids, the specified temperature is increased to compensate for the protective effect of these fat and solids on microorganisms.

These specifications are sufficient to reduce populations of vegetative bacterial pathogens to a level considered safe for public health. The pasteurization process used by processors of milk often employs temperatures and times in excess of 72°C for 15 seconds. This is to provide a higher margin of safety and to extend the shelf-life of liquid milk. Pasteurization processes for cream products utilize higher temperatures because of the protective effects of fat on micro-organisms. Internationally recognized heat treatments for pasteurization of cream is 65°C for 30 minutes for cream with 10-20% fat, and 80°C for 15 seconds for cream with $>20\%$ fat. Thickened cream has thickeners such as alginates and/or carragenans added before pasteurization. Pathogens such as *Salmonella*, *Campylobacter*, *Staphylococcus*, pathogenic *E. coli* (particularly enterohaemorrhagic *E. coli*), *Y. enterocolitica* and *Listeria monocytogenes* which may be present in raw milk are inactivated by pasteurization. However, pasteurization will not destroy heat stable enterotoxins such as those produced by *Staphylococcus aureus*, if the organism has grown and produced enterotoxin in raw milk prior to pasteurization. Inadequate chilling of raw milk is one of the key factors for the build-up of *Staphylococcus* enterotoxins. Pasteurization also has the advantage of destroying many of the spoilage micro-

organisms present in raw milk, especially psychrotrophic bacteria which may proliferate during low temperature storage of liquid milk products. Pasteurization, however, cannot be relied upon to destroy some of the more heat resistant bacteria (thermoduric) or bacterial spores produced by members of the genera *Bacillus* and *Clostridium*. After pasteurization, milk and milk products still contain low numbers of thermoduric microorganisms such as *Micrococcus* and *Enterococcus* species and some lactic acid bacteria. For this reason, pasteurized milk and milk products have a limited shelf-life even when stored at refrigeration temperatures.

To minimize growth of the surviving microbe, and to minimize post-process recontamination the steps of cooling pasteurized milk, filling and packaging and refrigerated storage of pasteurized milk and cream must be well managed. Pasteurized milk is particularly vulnerable to post-pasteurization contamination and asepsis and good hygiene is essential for preventing contamination by pathogenic micro-organisms and for defending its shelf-life.

The shelf life of milk is influenced by the number of psychrotrophic bacteria that survive pasteurization or subsequently contaminate the pasteurized product and grow at low temperatures in the liquid during storage. Although these contaminants are initially present in low numbers they can, under certain conditions, grow quickly and produce enzymes that break down protein and fat and generate off flavors and odours. The typical shelf life for pasteurized milk is from 7-14 days, although there are seasonal and regional variations.

UHT processing of milk involves heating milk at a temperature higher than 130°C with a holding period of 1-10 seconds with subsequent aseptic packaging. Usually the temperature and time combination is 138-145°C for 3-5 seconds. Sterilization treatment of milk is similar to that of UHT but at a higher temperature and is usually applied to condensed milk. The term 'sterilization', as used here, refers to commercial sterility of the milk or milk product. Milk and milk products of commercial sterility are not absolutely sterile in microbiological terms. However, those micro-organisms and spores that may survive the sterilization treatment are incapable of development under normal conditions of storage. Temperature and time combinations for the sterilization of milk and milk products range from 105-120°C for 10-40 minutes. Both UHT treatment and sterilization destroy bacterial endospores. Milk and milk products after UHT treatment or sterilization can be stored without refrigeration for extended periods of time.

20.2.1.2 Microbial pathogens of major concern in milk and cream

Available epidemiological data indicates that illness resulting from the consumption of pasteurized milk and cream is rare although outbreaks involving *Campylobacter* spp., *Salmonella* spp., *E. coli* O157:H7, *L. monocytogenes* and *Yersinia* spp. have been linked to consumption of pasteurized milk. These outbreaks have usually been traced to inadequate pasteurization and/or post-pasteurization contamination and/or temperature abuse and not to any failure of the pasteurization process.

Table 20.2 Microbiological Criteria for milk and cream

SI. No.	Requirements	Sampling Plan	Pasteurized milk/ Pasteurized cream
1	Total plate count	m	30,000/g
		M	50,000/g
2	Coliform Count ²	m	< 10 /g
		M	
3	<i>E. coli</i> ³	M	Absent/ g
4	<i>Salmonella</i> ⁴	M	Absent/ 25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	< 10/ g
		M	
6	Yeast and mould count ⁶	m	-
		M	-
7	Spore Count:		-
	(a) Aerobic ^{7a} (<i>B. cereus</i>)	m	
		M	-
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	-
M		-	
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	20
			0 ^{2-5,8}
		Storage & transport	0 to 4°C
Sample size	100ml or g		

20.2.2 Butter and butter products

Butter is produced from cream by churning or an equivalent process. Butter spreads are based on vegetable fats, a blend of vegetable and butter fat, or butterfat alone (light butter).

20.2.2.1 Microbial pathogens of major concern

While butter represents a dairy product of low risk to public health there have been incidents of food-borne illness attributed to this product. Staphylococcal food poisoning has been traced to whipped butter in the United States although temperature abuse was a contributory factor. There have also been two outbreaks of listeriosis linked to the consumption of butter. *L. monocytogenes* was isolated from several points in a production facility packaging small butter packages in an outbreak in Finland in 1998-1999. More recently a cluster of listeriosis cases implicating butter occurred in England. Mishandling may have been a contributing factor in this outbreak. In addition in the US, there have been several recalls issued for *L. monocytogenes* contaminated butter. Butter does not appear to be a good growth medium for *L. monocytogenes* as salt added during manufacture and distributed in the water phase is at or close to the limit for growth at refrigeration temperatures. However, growth has been demonstrated experimentally in butter during storage and it appears that *L. monocytogenes* favours the water rather than the lipid phase during butter

making. This is supported by the outbreaks and recalls that have been associated with *L. monocytogenes* in butter.

Table 20.3 Microbiological criteria for pasteurized butter

SI. No.	Requirements	Sampling Plan	Pasteurized Butter
1	Total plate count	m	10,000/g
		M	50,000/g
2	Coliform Count ²	m	10/g
		M	50/g
3	<i>E.coli</i> ³	M	Absent/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	10/g
		M	50/g
6	Yeast and mould count ⁶	m	20/g
		M	50/g
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	-
		M	-
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	-
		M	-
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	2 ^{1-2, 5-6}
			0 ^{3, 4, 8}
		Storage & transport	-18°C
Sample size	100ml or g		

20.2.3 Concentrated milk products

Concentrated milk products have reduced water content and include evaporated milks and sweetened condensed milks. Sweetened condensed milk is characterized by its high sugar content, which varies from 61-64% calculated as sucrose/ (sucrose + water) in the product. Unlike evaporated milk, which is preserved by heat treatment (UHT treatment or sterilization), sweetened condensed milk is preserved by its sugar content.

20.2.3.1 Microbial pathogens of major concern

No reported cases of food-borne disease outbreak have been attributed to the consumption of sweetened condensed milk or evaporated milk. These products generally do not support the growth of micro-organisms and are shelf stable. The main microbiological concern with evaporated milk is primarily non-pathogenic

thermophilic spore-forming bacteria such as *Bacillus stearothermophilus*, which spoil the product. The main concern for sweetened condensed milks is *S. aureus*. Sweetened condensed milk is not a sterile product; the low water activity (aw between 0.83-.85) makes it unlikely to support the growth of pathogenic bacteria. Likewise, spores of *Clostridium* and *Bacillus* spp. present in sweetened condensed milk will also not be able to grow. The exception is *S. aureus*, which can grow at a aw of around 0.85. However vegetative cells of the non-spore former *S. aureus* will not survive the pre-heat treatment given to sweetened condensed milk and growth and toxin production of any spores is severely limited because of the anaerobic environment of sweetened condensed milk.

Table 20.4 Microbiological criteria for sweetened condensed milk

Sl. No.	Requirements	Sampling Plan	Dried milks
1	Total plate count	m	40,000/g
		M	50,000/g
2	Coliform Count ²	m	10/g
		M	50/g
3	<i>E. coli</i> ³	M	Absent/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	-
		M	Less than 10/g
6	Yeast and mould count ⁶	m	-
		M	-
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	100/g
		M	1000/g
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	10/g
		M	100/g
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	2 ^{1&5}
			0 ^{2-4,6,7b,8}
		Storage & transport	0-4°C
Sample size	100ml or g		

20.2.4 Dried milks

Whole milk, skim milk, whey, buttermilk, cheese and cream may be dried into powders by the application of heat. The fluid is initially concentrated by evaporation, then spray dried to form a powder.

20.2.4.1 Microbial pathogens of major concern

Microbial pathogens of major concern in dried milk include *Salmonella*, *L. monocytogenes*, *B. cereus*, *C.*

perfringens, *S. aureus* and, more recently, *Enterobacter sakazakii*. While these organisms will not grow in powders, they may remain viable for long periods of time and resume growth when the powder is reconstituted and stored at favourable temperatures. Surveys conducted overseas have shown the presence of *B. cereus* and *E. sakazakii* (New name *Chronobacter sakazakii*) in dried milk, while Australian surveys have detected *Salmonella* spp. and *S. aureus*. Dried milk has been implicated in a number of food-borne disease outbreaks involving *Salmonella* and *C. perfringens*. In an outbreak due to consumption of milk powder contaminated with *S. aureus*, it was considered likely that illness was a result of preformed *S. aureus* enterotoxin surviving the heating process. Illness has also been attributed to *S. aureus* contamination and abuse of reconstituted non-fat dried milk. More recently a large outbreak of illness from *S. aureus* in Japan caused more than 13,000 cases and was due to preformed staphylococcal enterotoxin in the milk powder. This was traced back to poor hygienic and manufacturing practices during processing of liquid milk, in particular the storage conditions. Outbreaks demonstrate that failures in preventive systems, such as presence of water allowing multiplication or the presence of zones difficult to maintain and to clean (isolation from a drying tower) were the origin of contamination. In other cases illness has been due to contamination and abuse of reconstituted products. *S. aureus*, if present, may grow and produce enterotoxin when dry milk is rehydrated, if it is subject to time/ temperature abuse. *C. perfringens* and *B. cereus* are able to produce spores that can survive pasteurization and survive the manufacture of powdered milk production. They represent a problem when powdered milks are reconstituted and stored for prolonged periods at incorrect temperatures. Most *B. cereus* strains isolated from dairy products are able to grow and produce toxins below 10°C.

Although there have been no outbreaks of listeriosis linked to dry dairy products, the persistence of *Listeria* spp. in the dairy plant environment and the association of listeriosis with other dairy products indicate the potential for *Listeria* contamination of dry dairy products. There is evidence that *L. monocytogenes* can survive a typical spray-drying process in the manufacture of dried milk powders. Although dried milk powders will not support microbial growth due to their low water activity, *L. monocytogenes* is one of the few food-borne pathogens that can grow at refrigeration temperatures and, if present in the dried milk powders, it could possibly multiply when made up and stored in the refrigerator for a long period. Outbreaks due to *Salmonella* usually share a common factor, the accumulation of contaminated dust and powder deposits in the factory environment which are eventually transferred to the product by mechanical fault. The most common hazard reported is the accumulation of powder deposits in the drier insulation, which having become contaminated by environmental salmonellae, gains access to the product via stress cracks in the inner skin of the dryer. The second most important hazard is due to contaminated air and may occur during the secondary drier stages, transport of powder to silos or during filling and packing operations.

Table 20.5 Microbiological criteria for dried milks

SI. No.	Requirements	Sampling Plan	Dried milks
1	Total plate count	m	40,000/g
		M	50,000/g
2	Coliform Count ²	m	10/g
		M	50/g
3	<i>E. coli</i> ³	M	Absent/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	-
		M	Less than 10/g
6	Yeast and mould count ⁶	m	-
		M	
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	100/g
		M	1000/g
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	10/g
		M	100/g
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	2 ^{1&5}
			0 ^{2-4,6,7b,8}
		Storage & transport	0-4°C
		Sample size	100ml or g

20.2.5 Infant formulae

Powdered infant formula belongs to a special sub-set of powdered milks. These products are formulated to be as similar to human milk as is possible then concentrated and spray/or roller dried. In some cases, specific heat-labile ingredients are added after drying. Typically, infant formulae contain milk, whey proteins or soy proteins, or protein hydrolysate together with those forms of fat, carbohydrate, vitamins and minerals that are bio-available to the infant.

20.2.5.1 Microbial pathogens of major concern

Microbial pathogens of concern with powdered infant formulae are similar to those for dried milk powders, mainly *B. cereus*, *C. perfringens*, *E. sakazakii*, *L. monocytogenes*, *Salmonella*, *Shigella* and *S. aureus*. However, control over the microbiological status of these products is essential because of the vulnerable status of infants. Global surveys of infant formulas have indicated the presence of *B. cereus* and *E. sakazakii*. While *Salmonella* is rarely found in surveys of powdered infant formula, low-level contamination of powdered infant formula with *Salmonella* has been epidemiologically and microbiologically associated with infections in infants. Illness has also been attributed to *S. aureus* contamination and to abuse of reconstituted infant powdered milk.

More recently a growing number of reports has linked *E. sakazakii* infection in infants to powdered infant formula. In several investigations outbreaks of *E. sakazakii* infection has occurred among neonates in neonatal intensive care units. Mortality rates from *E. sakazakii* infection have been reported to be as high as 50% or

more, but this figure has declined to <20% in recent years (Codex). An outbreak involving *Salmonella bredeney* was traced to contamination of powdered milk-based infant formulae. Liquid, ready-to-feed infant formulae are commercially sterile, generally do not support the growth of micro-organisms and are shelf stable.

20.2.6 Colostrums

Bovine colostrum is the initial mammary secretion after the birth of a calf. It is produced for about 1-2 days (depleted usually within 4-5 days or 8-10 milking) and provides the newborn animal with a concentrated source of factors that boost its immune status and support physical and physiological development. Immediately post-partum the colostrum obtained from cows is excluded from bulk milk collection and was normally fed to farm animals. Until recently, it has not been widely commercially exploited although the high concentration of bioactive substances in colostrums has attracted increasing interest in the last few years because of their potential pharmaceutical and dietary uses. The sports food market is rapidly expanding, due to the perceived benefits of colostrum in providing an immune and performance boost to athletes. The use of colostrum as passive immune protection for humans has been reviewed recently. Important biologically active substances contained in colostrums include immune-globulins, leucocytes, lactoferrin, lysozyme, cytokines (interleukin (IL)-1 β , IL-6, IL-10, tumour necrosis factor- α & granulocyte-, macrophage- and granulocyte/macrophage colony- stimulating factors) and other hormones/ growth factors (e.g. insulin-like growth factors I and II). Some of the bioactive substances found in bovine colostrum provide specific (antibody) or non-specific (e.g. lactoferrin and lactoperoxidase) defences against infectious agents and foreign antigens.

20.2.6.1 Microflora of major concern

The microflora in powdered bovine colostrum is similar to that in other milk powder products and includes *Salmonella*, *L. monocytogenes*, *B. cereus*, *C. perfringens* and *S. aureus*. Post-pasteurization, colostrum may contain viable spores. Micro-organisms present in the dried product will also arise from post-processing contamination, and vegetative cells of pathogens might survive extended periods in the dried product although growth will not occur.

The final microbiological quality of colostrum powder will be influenced by the microbial load of the colostrum after milking, processing and the maintenance of good hygiene post- processing. *S. aureus* if present in the raw colostrum may grow and produce enterotoxin if the colostrum is subjected to temperature abuse prior to pasteurization. The persistence of *Listeria* spp. in the dairy plant environment and the association of listeriosis with other dairy products indicate the potential for contamination of dry dairy products such as colostrum powder. Both *C. perfringens* and *B. cereus* are able to produce spores that can survive pasteurization and even ultra-high temperature. If colostrum is inadequately stored when made up, the spores of *C. perfringens* and *B. cereus* can germinate and rapidly multiply, creating a potential health risk. The toxin of *S. aureus* is heat stable and, if poor sanitary conditions allow the organisms to proliferate and produce toxin in the pre-pasteurization stage, the toxin will carry over to the final product.

Lesson 21

RISK PROFILE AND CRITERIA FOR FROZEN, FERMENTED AND INDIGENOUS PRODUCTS AND DAIRY BY-PRODUCTS

21.1 Introduction

Food spoilage is an enormous economic problem worldwide. Through microbial activity alone, approximately one-fourth of the world's food supply is lost. Milk is a highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms. The microbiological quality of milk and dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatment contamination. Undesirable microbes that can cause spoilage of dairy products include Gram-negative psychrotrophs, coliforms, lactic acid bacteria, yeasts, and moulds. In addition, various bacteria of public health concern such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, pathogenic strains of *Escherichia coli* and enterotoxigenic strains of *Staphylococcus aureus* may also be found in milk and dairy products. For this reason, increased emphasis should be placed on the microbiological examination of milk and dairy foods. Microbiological analysis are critical for the assessment of quality and safety, conformation with standards and specifications, and regulatory compliance

The causative agent of an illness is usually determined through epidemiological studies, but confirming the identity of a key ingredient or the original source of product contamination, or critical factors contributing to their occurrence is problematic. This inability to attribute cases of food-borne illness to causal vehicles is a major issue internationally and is especially difficult where illness is linked to foods with multiple ingredients. Critical in this process is the capacity to link epidemiological data to animal and food monitoring data. The development of public health interventions requires accurate data defining the source from which humans are acquiring pathogens and how specific foods contribute to the total burden of food-borne illness. However, outbreak data represents only a small component of actual cases of food-borne illness, as many outbreaks go unrecognized. People do not always seek medical attention for mild forms of gastroenteritis and not all food-borne illnesses require notification to health authorities.

Following criteria in food matrix may be considered while characterizing the risk:

1. Intrinsic properties of the product (i.e. the impact of aw, pH, salt concentration, and their effect on the growth of contaminating microorganism)
2. Extent to which food is exposed to factory environment or handling after heat treatment
3. Hygiene and control during distribution and retail sale
4. Degree of reheating or cooking before consumption {many dairy products are RTE (Ready to Eat), so this is rarely a factor}.

21.2 Ice-Cream

Ice-cream is a frozen aerated emulsion made from cream or milk products or both and other food components. Manufacture of ice-cream involves the preparation of an ingredient mix comprising milk fat, milk solids, sweetener, water and other ingredients which are pasteurized and homogenized, aged, then whipped to incorporated air while being frozen. The final product is then packaging and hardened during frozen storage prior to distribution. Other types of ice-cream are available in many forms, flavors and packages. Different products prepared both from edible fats and milk or milk products include gelatin, soft serve, stick ice creams and confections, etc.

21.2.1 Microbial pathogens of major concern

While pathogenic bacteria will not grow in ice-cream, some pathogens, if present, may survive long periods of frozen storage. Therefore, any pathogens present in ice-cream as a result of post-process contamination may

pose a potential hazard to consumers. The major microbial pathogens of concern associated with ice-cream are *L. monocytogenes*, *Salmonella* spp. and *S. aureus*. Reported outbreaks of food-borne illness attributed to ice-cream have typically involved home-made ice-cream where raw milk and/or raw egg were used and the heat treatment was inadequate. Outbreaks involving consumption of commercially manufactured ice-cream have been found to be the result of post-processing contamination. In the 1994 outbreak of *S. enteritidis* food-borne illness a transport tanker previously used to transport unpasteurized raw egg was used to transport ice-cream mix that was not subsequently re-pasteurized. Several recalls of ice-cream due to contamination of *L. monocytogenes* have occurred in the US since 1985. However no direct link to listeriosis has been documented. Chocolate-coated ice-creams were recalled in 1995 because of *L. monocytogenes* contamination. An outbreak of *S. orangeburg* associated with gelatin was reported in 1998. Contamination of the gelatin most likely resulted from equipment contaminated with the *Salmonella*. Gelatin differs from ice-cream in that it has a very low (dairy) fat content (varying from 1.4 -8%). Milk based gelatin also has less air incorporated (approximately 35-40%) compared to ice cream which is approximately 50%. *Aeromonas* spp. and *B. abortus* have been detected in surveys of ice cream and ice cream products overseas, while *L. monocytogenes* has been detected in surveys of ice cream in Australia.

21.3 Dairy Desserts

In recent years there has been rapid proliferation in the range of dairy-based desserts available in the marketplace. These are typically branded, ready-to-eat products that are sold through retail outlets such as from supermarket cabinets with products ranging from medium to long shelf-life. These products often include probiotic bacteria, fiber, vitamins, minerals, and include flavors and colors that appeal to children and adults. Dairy-based desserts include acidified and non-acidified products. Examples of these types of products include custards, puddings, sachet desserts etc. Difficulties with differentiation are increasingly common with a blurring of the lines which differentiate yoghurt (fermented) products, from cream desserts, and products containing probiotics. Dairy-based desserts can be based on fresh milk (skim or full-fat), milk powder (skim or whole) or on milk protein concentrates. Flavors, colours and sweeteners may be added, along with a wide variety of hydrocolloid thickening agents to improve texture, of which, starches and carragenans are most common. Other additives used include emulsifiers and binding agents.

21.3.1 Microflora of major concern

The microbiological profile of these products is extremely varied reflecting the nature of the ingredients incorporated into these products and variations in the preservation and processing operations employed in their manufacture. Published microbiological data on these types of products is limited. Survey data typically indicates that these products conform to national regulations and are of acceptable microbiological quality. In determining the potential pathogens associated with these products the typical microflora associated with milk and creams are combined with microflora originating from ingredients that vary from fruit to flavorings. Of particular concern is the survival of spores from *B. cereus* in the milk or presence there in ingredients such as thickeners.

The major risk in chilled dairy desserts is that they will become contaminated with pathogens which could grow during the products' shelf-life. Components of this type of product such as cream and custards are by formulation (pH and aw) and method of manufacture (i.e. exposed to the factory environment) high risk. Custard and cream rely on proper heat treatment to eliminate pathogens that may be present in the raw materials used. Where these products are heat treated non-spore forming vegetative cells will be destroyed whereas spores of *B. cereus* may survive and become activated. The rapid cooling of products that have a heating step will help prevent growth of these spores. Another major public health concern can arise from post-pasteurization contamination, particularly from heat labile ingredients and during filling and packaging. Points in the process where product can become re-contaminated are during assembly of the final product. Items such as roasted nuts added as decorative toppings to desserts can also be a route of contamination. In addition, great care must be taken to avoid the addition of psychrotrophic bacteria such as *L. monocytogenes* which may grow during prolonged refrigerated storage. Another concern relates to spores of psychrotrophic *B. cereus* that may survive pasteurization and grow and elaborate toxin during the extended storage of some types of dairy desserts. Risks to the consumer from these products, if contaminated, are temperature abused and consumed at or beyond end of normal shelf-life.

Table 21.1 Microbiological criteria for Ice cream, frozen dessert

Sl. No.	Requirements	Sampling Plan	Ice cream, frozen dessert, milk lolly, ice candy
1	Total plate count	m	2,00,000/g
		M	2,50,000/g
2	Coliform Count ²	m	50/g
		M	100/g
3	<i>E. coli</i> ³	M	Absent/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	--
		M	Less than10/g
6	Yeast and mould count ⁶	m	-
		M	Less than10/g
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	--
		M	--
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	--
		M	--
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent /g
Sampling Guidelines ⁹		n 1-8	5
		c	2 ^{1&2}
			0 ^{3,4,5,6,8}
		Storage & transport	0 to 4 °C
		Sample size	100ml or g

21.4 Dairy-Based Dips

As with dairy desserts there has been an increase in the number of dairy-based dips in the marketplace. These products are very diverse and typically ready-to-eat commodities and are sold from cabinets in retail outlets. Dairy-based dips range from processed cheese-type products and starch-thickened bases flavoured with cheese solids to sour cream or yoghurt based and flavoured dips. A wide range of condiments can be added to the dairy dip base including herbs and spices, dehydrated vegetables and flavouring agents. These products range from medium to long shelf-life.

21.4.1 Microflora of major concern

The microbiological profile of these types of products is extremely varied reflecting the nature of the various components incorporated into these products and variations in the preservation and processing operations employed in their manufacture. Cold filling is frequently practiced and careful management is essential to avoid contamination. This is especially important where heat labile ingredients are added to the product after a terminal heat process. Published microbiological data on these types of products is limited. Survey data typically indicates these products conform to national regulations and is of acceptable microbiological quality. In determining the potential pathogens associated with these products the typical microflora associated with milk and cream is combined with microflora originating from ingredients that vary from vegetables and fruit to flavourings, herbs and spices. Where heat labile ingredients are added after a heat treatment steps, great

care must be taken to avoid the addition of psychrotrophic bacteria such as *L. monocytogenes*.

21.5 Cultured and Fermented Milk Products

Yoghurt and fermented milk products are prepared by fermentation of milk or milk products using specific micro-organisms that reduce the pH and coagulate milk proteins. Yoghurt is characterized by fermentation with thermophilic *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* with or without other lactic acid producing bacteria. Fermented milk products include yoghurt, cultured buttermilk, cream (sour cream) and acidophilus milk.

21.5.1 Microbial pathogens of major concern

Fermented products are rarely associated with food-borne disease as their pH is too low and the lactic acid concentration too high to permit growth of vegetative pathogens and death of non-growing cells is likely to be rapid. However, consumption of yoghurt containing large numbers of yeasts can lead to digestive disturbances. The limited outbreaks of food-borne illness that have been reported typically have involved *S. aureus*, *C. botulinum* and *E. coli* 0157:H7. Slow growth by the starter culture provides an opportunity for growth of pathogens that contaminate the milk or ingredients, for example, staphylococcal toxin may accumulate in the ingredients where too much sugar inhibited the growth of starters but not the growth of *S. aureus*, resulting in illness. In yoghurt outbreak, under processing of canned hazel-nut puree used to flavor the yoghurt caused growth and toxigenesis of *C. botulinum* spores in the puree. In addition the sugar in the ingredients was replaced by aspartame, leading to an increase in water activity to a level allowing growth of the pathogen. From a number of microbiological surveys of cultured and fermented milk products identified in the literature, only one reported the positive identification of a pathogen (*Y. enterocolitica* in fermented cow's milk)

Table 21.2 Microbiological criteria for fermented milks

SI. No.	Requirements	Sampling Plan	Fermented milks
1	Total plate count	m	--
		M	--
2	Coliform Count ²	m	10/g
		M	50/g
3	<i>E. coli</i> ³	M	Absent/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	50/g
		M	100/g
6	Yeast and mould count ⁶	m	50/g
		M	100/g
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	--
		M	--
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	10/g
		M	--
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n 1-8	5
		c	2 ^{2,5,6}
			0 ^{3,4,7b,8}
		Storage & transport	0 to 4 °C
Sample size	100ml or g		

21.6 Cheese

The term cheese covers over 1,000 varieties of fermented dairy products with significant variations in their flavors, texture and appearance. The process of converting liquid milk into cheese involves a series of steps that are modified to produce a cheese of the desired characteristics. Starter culture and rennet are added to milk resulting in the production of a cheese curd through a process of coagulation and acidification. The curds are usually cut and with mild (38-43°C) heating there is separation of the whey which is drained from the curds. The curds are salted before they are pressed into moulds and then stored under controlled conditions to ripen the cheese. Cheese may be grouped according to manufacturing or processing procedures, consistency or rheology, country of origin, general appearance, sources of milk and chemical analysis.

21.6.1 Microbial pathogens of major concern in cheeses

Cheese has been the vehicle in a number of outbreaks of food-borne illness, involving pathogenic micro-organisms such as *Bacillus* spp., *Brucella* spp., *C. botulinum*, *E. coli*, *L. monocytogenes*, *Salmonella*, *Shigella* and *S. aureus*. Evidence from outbreak investigations suggests that illness resulting from consumption of cheese is often the result of faulty controls in cheese production; use of contaminated starter cultures or contaminated ingredients; post-pasteurization contamination; or mishandling during transportation and/or distribution. In microbiological surveys conducted overseas and a number of potential pathogens have been detected in cheeses made from pasteurized milk, namely *L. monocytogenes* and *S. aureus*. Additional pathogens have been detected in raw milk cheese (*B. cereus*, *Brucella* spp., pathogenic *E. coli* and *Y. enterocolitica*). *Bacillus* spp. and *L. monocytogenes* have been detected in pasteurized milk cheeses.

Table 21.3 Microbiological criteria for All other cheeses

SI. No.	Requirements	Sampling Plan	All other cheeses
1	Total plate count	m	--
		M	--
2	Coliform Count ²	m	100/g
		M	500/g
3	<i>E. coli</i> ³	M	Less than 10/g
4	<i>Salmonella</i> ⁴	M	Absent /g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	100/g
		M	1000/g
6	Yeast and mould count ⁶	m	10/g
		M	100/g
7	Spore Count: (a) Aerobic ^{7a} (<i>B. cereus</i>)	m	--
		M	--
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	10/g
		M	100/g
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g Hard cheese Absent/ 25g other cheeses
Sampling Guidelines ⁹		n1-8	5
		c	2 ^{2,5,6,7b}
			0 ^{3,4,8}
		Storage & transport	4-8°C
Sample size	100ml or g		

21.7 Indigenous Dairy Products

21.7.1 Khoa

Khoa a partially dehydrated milk product is prepared from whole milk by continuous heating in an iron cauldron ('karahi') over a direct fire or in a steam kettle. It is consistently stirred and scraped with a spatula during heating till a semi-solid (doughy) consistency is obtained. The desirable consistency is achieved when the product shows sign of leaving the bottom and sides of karahi. After removing from the fire, the contents are worked up in a solid mass which is now called khoa or mawa. Depending on the method of preparation three varieties of khoa are available, viz. 1) pindi, 2) dhap and 3) danedar. These varieties differ in composition and texture and are made use of in preparing different sweets, viz., burfi and peda (from pindi); gulabjamun and pantoa (from dhap) and kalakand (from danedar).

21.7.1.1 Microbiological quality of Khoa

Khoa, like other indigenous products such as chhana, kheer, dahi, etc., can serve as a favourable medium for the growth of a variety of microorganisms because of high moisture content and good nutritive value. The market khoa keeps well for 48 hours under usual Indian conditions of handling and storage. However, storage beyond this period often results into deterioration due to microbial action. These microorganisms gain access into this product as contaminants from different sources. Various groups of bacteria (acid producers, proteolytic, chromogenic, lipolytic, aerobic sporeformers, psychrotrophs, thermophiles and pathogens), yeast and moulds grow profusely on khoa. The rapid spoilage of khoa is attributed to contamination with moulds from external sources.

21.7.1.2 Pathogens of major concern

A number of pathogens like *E. coli*, *S. typhi*, *S. dysenteriae* and *V. cholerae* are able to survive for long periods during storage of khoa. Subsequently, a number of related studies have revealed the occurrence of *staphylococci*, especially those of coagulase positive types in khoa. The *staphylococcus* has been known to produce heat stable enterotoxin in this product which causes food poisoning. Since the product is manufactured by traditional method without any regard to quality of raw material used and hygienic storage, the shelf life of the product is adversely affected by the thermophilic organisms and organisms acquired during storage. High nutritional value and high water activity ($a_w = 0.96$) of khoa is conducive to the growth of bacteria. Microbial content of heat dried dairy product is temperature dependent and time of preheating evaporation process is also a predisposing factor contamination and growth during storage also affect microbial population of heat dried dairy products. Psychrotrophic bacteria may also affect quality and flavour of heat-treated product, some heat tolerant enzymes produced by some psychrotrophic species cause spoilage both before and after heating. Microbes produce undesirable effects like change in odour, colour, taste and texture of food. Besides, this contamination of products with pathogenic bacteria can result into outbreaks of gastrointestinal infection and thus threat to consumer. A plethora of studies carried out in different part of India evidenced that pathogenic organism as *B. cereus*, *S. aureus* often contaminate khoa. Probably the microbe's access to khoa is mainly by improper handling of workers and contaminated utensils used during processing. In a study total fifty samples of khoa were brought from different localities of Chambal region at random and processed. Bacterial colony counts were also performed. *Staphylococcus* species and *Streptococcus* species were the predominant isolates. The viable counts obtained ranged from 1.3×10^4 to 2.1×10^6 CFU/g. Contamination of khoa by pathogenic bacteria could be an important factor of gastrointestinal infections including food poisoning and food borne illness. *E. coli* was isolated from milk products like mawa/ khoa, cream, dahi, cheese, butter and gulabjamun.

In 2002, Soomro et al. observed that 12 (60%) of mawa/ khoa samples were contaminated with *E. coli* in contrast to 11 (55%) of dahi followed by 8 (40%) gulabjamun samples. Jtakar et al. (1982) studied on microbiological quality of market milk sweets in twin cities of Hyderabad and Secunderabad and observed 90% of peda, 75% of kalakand and 100% of rasagolla samples were contaminated with yeasts and moulds. Garg and Mandokhot (1984) observed that peda in general had more bacterial contamination than burfi contaminated with *E. coli*, *P. aerogenes*, *S. flexneri*, *S. schottmuelleri* and hemolytic *streptococci*. Tamberkar and Butda (2010) investigated 50 samples of peda randomly collected from various shops of Amravati city and analysed for bacteriological quality. Out of 92 strains of bacteria identified, the prominent were *P.*

aerogenosa (23.91%), *S. aureus* (17.39%), *S. typhi* (16.30%), *E. coli* (14.13%), *E. aerogenes* (11.9%), *Shigella flexneri* (8.69%), *Proteus vulgaris*(7.6%), etc.

Table 21.4 Microbiological Criteria for Khoa

Sl. No.	Requirements	Sampling Plan	Khoa
1	Total plate count	m	50,000/g
		M	1,00,000/g
2	Coliform Count ²	m	50/g
		M	90/g
3	<i>E. coli</i> ³	M	Less than 10/g
4	<i>Salmonella</i> ⁴	M	Absent /25g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	50/g
		M	100/g
6	Yeast and mould count ⁶	m	50/g
		M	100/g
7	Spore Count:		
	(a) Aerobic ^{7a} (<i>B. cereus</i>)	m	--
		M	--
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	--
		M	--
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent /g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	2 ^{1,2,5,6}
			0 ^{3,4,8}
		Storage & transport	0 to 4 ⁰ C
		Sample size	100g

21.7.2 Paneer

Paneer is an important indigenous coagulated milk product prepared by the combined action of acid coagulation and heat treatment of cow or buffalo milk or a combination thereof (milk solids and suitably processed may be used). The phenomenon of precipitation involves the formation of large structural aggregates of proteins in which milk fat and other colloidal and soluble solids are entrapped along with whey. According to PFA Rules, 1955 paneer is a product obtained from cow or buffalo milk or a combination thereof by precipitation with sour milk, lactic acid or citric acid. It shall not contain more than 70% moisture and the milk fat content shall not be less than 50% of the dry matter. Skim milk is also recommended for the preparation of paneer where cow or buffalo milk is precipitated with sour milk, lactic acid or citric acid. The product shall not contain more than 70% moisture and milk fat content shall not be more than 13% of the dry matter.

21.7.2.1 Microbial pathogens of major concern

Microbiological quality of Paneer, like other indigenous milk products, chiefly depends on the condition of manufacture, subsequent handling, storage and sale of the product. The possible sources of contamination might be air, water, utensils, cutting knife, muslin cloth as well as persons handling the product. Hence the number and types of microorganisms and their distribution in the product may vary depending on the location of the sweetmeat maker (halwai) shop, extent of exposure of the product to the atmosphere, temperature and period of storage, etc. Despite a higher final temperature (62°C) the duration of heating employed in the manufacture of Indian cheese (Paneer) was not sufficient to inactivate *E. coli* O157:H7. In a study 60 market dairy food samples, one sample each of raw milk, paneer and ice cream were found to be positive for *E. coli* O157:H7 with respective RT-PCR counts of 6.7, 6.2 and 5.9 log CFU respectively. Paneer is used in the

preparation of certain curries and about 5% of the milk produced is converted to paneer. It may contain as high as 70% moisture which is conducive to microbial growth. Studies carried out on microbial quality of paneer have indicated that it is often contaminated with *S. aureus* and coliforms. The HACCP has been applied to identify the Critical Control Point for coliforms and *Staphylococcus* contamination.

21.7.3 Chhana

Chhana is one of the two chief bases (the other being khoa) for preparing a variety of indigenous sweetmeats. Chhana is also called paneer in certain parts of the country. However, the only difference between paneer and chhana preparation is that pressing is avoided in the latter which gives a loose texture to chhana unlike paneer. The PFA and ISI definitions of paneer also apply to chhana.

21.7.3.1 Microflora of major concern

Chhana samples showed an average bacterial count of 1.6×10^4 per gram. However, during storage at 37°C, the count increased to 31×10^6 and 110×10^6 at the end of 24 and 48 hours, respectively. The spoilage of product was chiefly due to thermophilic bacteria. Among the bacterial types isolated from chhana *micrococci* predominated and constituted 45% of the total microflora followed by sporeformers (34%).

21.8 Casein, Whey Products and Other Functional Milk Derivatives

An increasing awareness of the nutritional and health benefits of dairy products has driven the development of markets for a wide array of functional and nutritional ingredients derived from milk. Improvements in fractionation technologies have allowed the manufacture of these on a commercial basis from surplus milk and other dairy by-products.

21.8.1 Microflora of major concern

Pathogens of concern in the production of skim milk and skim milk powder include *Salmonella*, *L. monocytogenes*, *B. cereus*, *C. perfringens* and *E. sakazakii*. Casein and whey products produced from skim milk might contain spores of bacilli and *clostridia* and cells of other pathogens might survive extended periods in the dried products if present, although growth will not occur. A microbiological survey of dairy products conducted overseas detected *B. cereus* in whey powder. Products formed from severely temperature-abused milk might contain *S. aureus* enterotoxin, which is exceptionally heat stable, but this is unlikely to occur in a well-regulated processing environment. Fat-enriched milk fractions e.g. anhydrous milk-fat (AMF) may protect pathogenic microorganisms such as *E. coli*, *Salmonella* or *Listeria*, if present. However this is unlikely given the low moisture content of the product.

Table 21.5 Microbiological Criteria for Paneer/ Chhana

Sl. No.	Requirements	Sampling Plan	Paneer/ Chhana
1	Total plate count	m	3,00,000/g
		M	5,00,000/g
2	Coliform Count ²	m	50/g
		M	90/g
3	<i>E coli</i> ³	M	Less than 10/g
4	<i>Salmonella</i> ⁴	M	Absent /g
5	<i>Staphylococcus aureus</i> ⁵ (coagulase positive)	m	50/g
		M	100/g
6	Yeast and mould count ⁶	m	150/g
		M	250/g
7	Spore Count:		
	(a) Aerobic ^{7a} (<i>B. cereus</i>)	m	--
		M	--
	(b) Anaerobic ^{7b} (<i>Clostridium Perfringens</i>)	m	--
		M	--
8	<i>Listeria Monocytogenes</i> ⁸	M	Absent/g
Sampling Guidelines ⁹		n ¹⁻⁸	5
		c	2 ¹²⁵⁶ 0 ³⁴⁸
		Storage & transport	0 to 4 °C
		Sample size	100ml or g

Lesson 22

BIOSAFETY CONCEPTS IN HANDLING DAIRY PATHOGENS

22.1 Introduction

Biosafety concepts in quality control laboratories ensure that adequate safety conditions are implemented to avoid potential hazards associated with the handling of milk and milk products. The biosafety guidelines define the essential competencies needed by laboratory personnel to work safely in a laboratory. Competencies are measurable and include not only knowledge, skills, and abilities but also judgment and self-criticism.

22.2 Biohazardous Agent

A biohazard can be defined as any organism, or material produced by such an organism, that is known or suspected to cause human or animal disease. Exposure to bio-hazardous agents may occur via puncture, wounds or as a result of absorption through the respiratory tract, digestive system, skin and mucous membranes. Such exposures may result while handling samples, microbiological cultures and radioactive substances to be used for detection of various contaminants. Exposure to bio-hazardous agents is intended to be prevented or limited by establishing and following the appropriate bio-safety level practices and conditions.

22.3 Biosafety Level

The application of knowledge, techniques and equipment to prevent personal, laboratory and environmental exposure to potentially infectious agents or bio-hazards. Biosafety defines the containment conditions under which infectious agents can be safely manipulated. The objective of containment is to confine bio-hazards and to reduce the potential exposure of the laboratory worker, persons outside of the laboratory, and the environment to potentially infectious agents. The term 'bio-safety' is used in describing measures used to provide a barrier between the infectious/ pathogenic organisms being handled and the worker (and ultimately, the community at large). The bio-safety levels in microbiological laboratories can be designated as bio-safety level (BSL) 1, 2, 3 and 4. The levels are classified into four groups depending on the safety requirements, the highest safety standards (Level-4) are reserved for the most hazardous pathogens (Risk Group 4), and the least stringent (Level-1) for those which have minimal impact on health (Risk Group 1). Containment is achieved through the use of appropriate safety equipment, facility design and lab procedures and practices. Careful consideration must be given to both facility design and work practices to ensure protection of laboratory personnel, their colleagues and the community as a whole.

Table 22.1 Risks and characteristics associated with pathogens from Risk Groups 1 to 4, and recommended containment level and class of biological safety cabinet

Risk group	Risk assessment	Characteristics	Examples	Biosafety level	Bio safety cabinet
1	Low individual; low community.	Unlikely to cause disease in animals or humans	<i>Bacillus subtilis</i> , <i>E. coli</i> K12	1	Not required
2	Moderate individual; low community.	Rarely cause serious human or animal disease; effective prevention and treatment available; limited risk of spreading.	<i>Salmonella typhimurium</i>	2	Class I or Class II
3	High individual; low community	May cause serious disease in humans or animals; effective prevention and treatment available; unlikely to be spread by casual contact.	<i>Yersinia pestis</i> , <i>Bacillus anthracis</i> , cultured isolates of HIV	3	Class II
4	High individual; high community.	Likely to cause very serious disease in humans or animals; readily transmitted from one individual to another, or between animals and humans; preventative vaccines or effective treatment not available.	haemorrhagic fever (<i>Haemorrhagic colitis</i>)	4	Class III

22.3.1 Biological safety cabinets

Biological safety cabinets reduce the risk of airborne infection by reducing the escape of aerosolized infectious agents into the laboratory environment. In addition to protecting workers, some biological safety cabinets protect the work inside the cabinet from airborne contamination (product protection). Biological safety cabinets minimize contact between the operator and pathogens through the use of directional airflow, high efficiency particulate air (HEPA) filtration of supply and/ or exhaust air, and, in some cases, a physical barrier such as a plastic or glass shield (Fig. 22.1). Filters are an essential component of the biological safety cabinet, and have particle removal efficiencies of 99.97% or better for 0.3 micron diameter particles. This size particle is used as the basis for filter definition because it is considered the most difficult to remove. Thus, a filter that can trap 0.3 micron diameter particles can easily eliminate other sizes. HEPA filters consist of continuous sheets of glass fiber paper pleated over rigid corrugated separators and mounted in a wooden or metal frame.



Fig. 22.1 HEPA filters

22.3.2 Classes of biological safety cabinet

Horizontal and vertical clean benches are not biological safety cabinets. Air filtered through HEPA filters is directed over the work surface and then discharged directly into the room. Thus, these units provide product protection, but do not protect the operator from exposure to the materials being handled; they must not be used for work with potentially infectious or toxic materials. There are three basic types of biological safety cabinet, each providing different levels of containment:

22.3.2.1 Class I

(i) open-fronted (ii) protects operator and environment (iii) for work with low and moderate risk agents (Risk Groups 2 and 3) where product protection is not critical

General Principle of Operation

An inward flow of room air through the work opening, away from the operator, prevents the escape of airborne pathogens into the laboratory. Negative cabinet pressure is created by a blower that exhausts the air, either into the room or to the outside, through a HEPA filter. It is this HEPA filtration of exhaust air that provides environmental protection (Fig. 22.2). A disadvantage of this type of cabinet is that the product is exposed to contaminants that are pulled in from the room environment. In addition, internal air turbulence may result in cross-contamination within the cabinet.



Fig. 22.2 Biosafety Level 1 work with open bench tops and standard microbiological practices

22.3.2.2 Class II

(i) open-fronted (ii) protects operator, product and environment from particulate contamination (iii) for work with low to moderate risk agents (Risk Groups 2 and 3).

General Principle of Operation

Escape of pathogens into the worker's environment is prevented by an inward flow of room air which enters the front opening without crossing the work area and by HEPA filtration of exhaust air (this provides environmental protection), while downward flow of HEPA filtered air through the work area removes work zone contaminants and protects the product (Fig. 22.3). The amounts of room air drawn into the intake grille and the amount of air exhausted through the exhaust filter are equal. This balance is critical: positive pressure will allow the outflow of pathogens, while negative pressure will result in inflow of room contaminants. The different types of Class II cabinets (e.g. Type A, Type B or 100% exhaust) vary in: airflow velocities, amount of cabinet air recirculated (from 0 to 70%), amount of cabinet air exhausted (from 30 to 100%), destination of exhaust air (back to lab or outside), exhaust ducting (building system versus dedicated ducts). It should be kept in mind that toxic or radiolabelled chemicals must not be handled in cabinets that recirculate air within the cabinet or exhaust into the laboratory.



Fig. 22.3 Biosafety Cabinet Level-II and its working

22.3.2.3 Class III

(i) Totally enclosed, gas tight, with glove ports for manipulation of pathogens (ii) provides the greatest level of operator and product protection (iii) for work with high risk pathogens (Risk Group 4).

General Principle of Operation

These cabinets form a physical barrier between the operator and microbiological agent. Internal negative pressure confines any leaks to the inside of the cabinet. Supply and exhaust air is HEPA filtered; a dedicated exhaust fan, separate from that of the facility ventilation system, discharges directly to the outdoors. There is no recirculation of air within the cabinet. A Class III cabinet system must be designed to allow for safe introduction, handling and removal of all materials throughout the procedure. Equipment such as the incubator, refrigerator, centrifuge, autoclave and chemical dunk tank are connected to the cabinet system.

22.4 Working Safely in a Biological Safety Cabinet

Biological safety cabinets must be combined with good work practices for optimum safety and contamination control. Recommended practices when using a biological safety cabinet include – movement of arms into and out of the cabinet can disrupt airflow, adversely affecting cabinet performance. Whenever possible, place all materials needed for a procedure inside the cabinet before starting. Avoid bringing non-essential equipment and supplies into the cabinet. Place supplies, equipment and absorbent towels so that air intake or exhaust grilles are not obstructed. Keep opening and closing of lab doors and other personnel activity to a minimum. If

a burner is deemed to be indispensable (remember that burners contribute to the heat load, generate convection currents that interfere with airflow and may damage the filters), use one that has a pilot flame. Attach a HEPA filter cartridge between the vacuum trap and the source valve. Work at least 4-6 inches inside the cabinet window. Carry out work on an absorbent pad to contain small spills. Clean up spills as soon as they occur; remove and disinfect the grille if contaminated. Designate separate areas within the cabinet for contaminated and clean materials; place contaminated material at the rear of the work area.

22.4.1 Biosafety level-1

Biosafety Level 1 is used when working with agents (Risk Group 1) that pose no risk to healthy adults (Fig. 22.2). The laboratory may be near a public area but doors should be kept closed. Work may be carried out on an open bench top. Lab surfaces (walls, ceilings, furniture and floors) should be cleanable. Open windows should have insect screens. Eyewash stations and hand washing facilities should be available. Street clothes and lab coats should not be kept together. Disinfection should be carried out as required, using effective concentrations and contact times; solutions should be replaced regularly.

22.4.2 Biosafety Level-2

Biosafety Level 2 is appropriate for work with Risk Group 2 agents (Fig. 22.3). The following precautions, in addition to those for containment Level 1, are recommended.

22.4.2.1 Standard microbiological practices

1. The laboratory supervisor must enforce the institutional policies that control access to the laboratory.
2. Persons must wash their hands after working with potentially hazardous materials and before leaving the laboratory.
3. Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human consumption must not be permitted in laboratory areas. Food must be stored outside the laboratory area in cabinets or refrigerators designated and used for this purpose.
4. Mouth pipetting is prohibited; mechanical pipetting devices must be used.
5. Policies for the safe handling of sharps, such as needles, scalpels, pipettes, and broken glassware must be developed and implemented.
6. Perform all procedures to minimize the creation of splashes and/or aerosols.
7. Decontaminate work surfaces after completion of work and after any spill or splash of potentially infectious material with appropriate disinfectant.
8. Decontaminate all cultures, stocks, and other potentially infectious materials before disposal using an effective method.
9. A sign incorporating the universal bio-hazard symbol must be posted at the entrance to the laboratory when infectious agents are present.



Fig. 22.4 Biohazard sign

10. An effective integrated pest management program is required.
11. The laboratory supervisor must ensure that laboratory personnel receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures.

22.4.2.2 Special practices

None required.

22.4.2.3 Safety equipment (primary barriers and personal protective equipment)

1. Special containment devices or equipment, such as Biosafety Cabinet (BSCs) Level –II are required.
2. Protective laboratory coats, gowns, or uniforms are recommended to prevent contamination of personal clothing.
3. Wear protective eye wear when conducting procedures that have the potential to create splashes of microorganisms or other hazardous materials. Persons who wear contact lenses in laboratories should also wear eye protection.
4. Gloves must be worn to protect hands from exposure to hazardous materials.



Fig. 22.5 Personal protective equipment

22.4.2.4 Laboratory facilities (secondary barriers)

1. Laboratories should have doors for access control.
2. Laboratories must have a sink for hand washing.
3. The laboratory should be designed so that it can be easily cleaned. Carpets and rugs in laboratories are not appropriate and epoxy floorings are recommended.



Fig. 22.6 Epoxy floorings



Fig. 22.7 Doors for access control

22.4.3 Biosafety level -3

Biosafety Level - 3 is recommended for work with Risk Group 3 agents. Laboratory personnel must receive specific training in handling pathogenic and potentially lethal agents, and must be supervised by scientists competent in handling infectious agents and associated procedures. All procedures involving the manipulation of infectious materials must be conducted within BSCs or other physical containment devices. Measures should include the recommendations outlined for levels 1 and 2, plus the following standard and special safety practices, equipment, and facility requirements apply to BSL-3.

22.4.3.1 Standard microbiological practices

As per BSL-1 (22.4.2.1)

22.4.3.2 Special practices

In addition to special practices of BSL-2, all procedures involving the manipulation of infectious materials must be conducted within a BSC, or other physical containment devices. No work with open vessels is conducted on the bench. When a procedure cannot be performed within a BSC, a combination of personal protective equipment and other containment devices, such as a centrifuge safety cup or sealed rotor must be used.

22.4.3.3 Safety equipment (primary barriers and personal protective equipment)

1. All procedures involving the manipulation of infectious materials must be conducted within a BSC (preferably Class II or Class III), or other physical containment devices.
2. Workers in the laboratory where protective laboratory clothing with a solid-front, such as tie-back or wrap-around gowns, scrub suits, or coveralls. Protective clothing is not worn outside of the laboratory. Reusable clothing is decontaminated before being laundered. Clothing is changed when contaminated.
3. Eye and face protection (goggles, mask, face shield or other splash guard) is used for anticipated splashes or sprays of infectious or other hazardous materials. Eye and face protection must be disposed of with other contaminated laboratory waste or decontaminated before reuse. Persons who wear contact lenses in laboratories must also wear eye protection.
4. Gloves must be worn to protect hands from exposure to hazardous materials. Glove selection should be based on an appropriate risk assessment. Alternatives to latex gloves should be available. Gloves must not be worn outside the laboratory. In addition, BSL-3 laboratory workers:

- Changes gloves when contaminated, glove integrity is compromised, or when otherwise necessary. Wear two pairs of gloves when appropriate.
- Remove gloves and wash hands when work with hazardous materials has been completed and before leaving the laboratory.
- Do not wash or reuse disposable gloves. Dispose of used gloves with other contaminated laboratory waste. Hand washing protocols must be rigorously followed.
- Eye, face, and respiratory protection must be used in rooms containing infected animals.

22.4.3.4 Laboratory facilities (secondary barriers)

1. Laboratory doors must be self-closing and have locks in accordance with the institutional policies. The laboratory must be separated from areas that are open to unrestricted traffic flow within the building. Laboratory access is restricted. Access to the laboratory is through two self-closing doors. A clothing change room (ante-room) may be included in the passageway between the two self-closing doors.
2. Laboratories must have a sink for hand washing. The sink must be hands-free or automatically operated. It should be located near the exit door. If the laboratory is segregated into different laboratories, a sink must also be available for hand washing in each zone. Additional sinks may be required as determined by the risk assessment.
3. The laboratory must be designed so that it can be easily cleaned and decontaminated. Carpets and rugs are not permitted. Seams, floors, walls, and ceiling surfaces should be sealed. Spaces around doors and ventilation openings should be capable of being sealed to facilitate space decontamination.
4. Laboratory furniture must be capable of supporting anticipated loads and uses. Spaces between benches, cabinets, and equipment must be accessible for cleaning.
5. All windows in the laboratory must be sealed.
6. BSCs must be installed so that fluctuations of the room air supply and exhaust do not interfere with proper operations. BSCs should be located away from doors, heavily travelled laboratory areas, and other possible airflow disruptions.
7. Vacuum lines must be protected with HEPA filters, or their equivalent. Filters must be replaced as needed. Liquid disinfectant traps may be required.
8. An eyewash station must be readily available in the laboratory.
9. A ducted air ventilation system is required. This system must provide sustained directional airflow by drawing air into the laboratory from 'clean' areas toward 'potentially contaminated' areas. The laboratory shall be designed such that under failure conditions the airflow will not be reversed.
10. HEPA filter housings should have gas-tight isolation dampers, decontamination ports, and/ or bag-in/ bag-out (with appropriate decontamination procedures) capability. The HEPA filter housing should allow for leak testing of each filter and assembly. The filters and the housing should be certified at least annually.
11. HEPA filtered exhaust air from a Class II BSC can be safely re-circulated into the laboratory environment if the cabinet is tested and certified at least annually and operated according to manufacturer's recommendations. A method for decontaminating all laboratory wastes should be available in the facility, preferably within the laboratory (e.g. autoclave, chemical disinfection, or other validated decontamination method).
12. Equipment that may produce infectious aerosols must be contained in primary barrier devices that exhaust air through HEPA filtration or other equivalent technology before being discharged into the laboratory. These HEPA filters should be tested and/ or replaced at least annually.
13. Facility design consideration should be given to means of decontaminating large pieces of equipment before removal from the laboratory.
14. Enhanced environmental and personal protection may be required by the agent summary statement, risk assessment, or applicable local, state, or federal regulations. These laboratory enhancements may include, for example, one or more of the following: an anteroom for clean storage of equipment and supplies with dress-in, shower-out capabilities; gas tight dampers to facilitate laboratory isolation; final HEPA filtration of the laboratory exhaust air; laboratory effluent decontamination; and advanced access control devices, such as biometrics.
15. The BSL-3 facility design, operational parameters, and procedures must be verified and documented prior to operation. Facilities must be re-verified and documented at least annually.

22.4.4 Biosafety level-4

Level 4 containment is used for work with Risk Group 4 agents. Additional recommendations include: physical isolation of the laboratory, with an airlock for access, entry restricted to authorized personnel and recorded in a log book: no one should work alone, use of class III biological safety cabinets and/ or positive-pressure protective suits, additional safety measures for ventilation, waste treatment, and gas and water services.



Lesson 23 ENUMERATION OF HYGIENE INDICATOR ORGANISMS

23.1 Introduction

Indicator organisms are bacteria that are used as a sign of quality or hygienic status in a food, water or environment. The definition of the word ‘indicator,’ in fact, includes the concept of the indicator organism, i.e. something ‘so strictly associated with particular conditions that its presence is indicative of the existence of these conditions’. Historically, these conditions have been related to in-sanitation and public health concerns. Over the years, however, the use of indicator organisms has been extended to provide evaluations of the quality, in addition to the safety, of particular commodities.

Indicator organisms are important components of microbiological testing programs conducted both by regulatory agencies and the food industry. They may signify the potential presence of pathogens, a lapse in sanitation as required in good manufacturing practices (GMPs), or a process failure. They may reflect quality attributes that can influence con-an indicator organism alone is cause for concern; in other cases, it is the quantity that is significant. Many foods provide an environment conducive to microbial growth, and indicator counts in such foods may reflect the time and conditions of storage. The microbiological snapshot that is the indicator test must always be assessed in an appropriate context, taking into account the natural microbial ecology, intrinsic and extrinsic chemical and physical factors that might influence microbial growth, process history, and storage conditions of the product. The dual goals of safety and quality often overlap in the water, food, and environmental arenas, and it is important to choose the type of indicator organism that best fits a particular system. This is not an easy task, and the question of indicator selection has generated much discussion and debate. Perhaps adding confusion to the discussion are attempts that have been made over the years to apply various terms so as to distinguish the different functions of indicators, e.g. index, marker, model, sentinel, and surrogate organisms. It seems reasonable to view two general categories of indicators, i.e. safety and quality indicators. Safety indicators suggest that a microbial hazard may exist, and their use is intended to minimize the risk of exposure to the hazard. Quality indicators are used to assess issues important to product acceptability, e.g. shelf life, organoleptic characteristics, spoilage, etc. The International Commission on Microbiological Specifications for Foods (ICMSF) has noted that selection of an indicator must be considered carefully with an understanding of how to interpret the results of indicator testing. Indicators are a compromise, representing an analytical substitute for detection of the target hazard or concern directly. They can never be used to prove the presence or absence of the target.

23.2 Selection Criteria for Indicator Organisms

The ICMSF has listed the factors that should be considered when selecting an indicator organism for a particular purpose:

1. Presence of the indicator should suggest a faulty process or practice or a potential for spoilage.
2. Survival or stability of the indicator should be similar to or greater than the hazard or spoilage organism.
3. Growth characteristics of the indicator should be similar to or faster than the hazard or spoilage organism.
4. Identifiable characteristics of the indicator should be stable.
5. Method for detection and/ or quantitation should be easy, rapid, inexpensive, reliable, sensitive, and validated; does not risk analyst health; and is suitable for in-plant use.
6. Quantitative results should show a correlation between the concentration of the indicator and the level of the hazard or spoilage organism.
7. Results should be applicable to process control.

23.3 Hygiene Indicators

The microbial composition of a product significantly determines its quality. The types and number of microorganisms present influence the sensory properties (taste, aroma, texture, color) and shelf life of the product. Among these microbial populations, a particular one may be useful as an indicator to reflect quality changes in the product. Such quality indicators are often used to ensure that the product is microbiologically stable and aesthetically acceptable. The primary attribute of a quality indicator is that its growth and numbers should be inversely related to acceptable product quality. The indicator should be present in all products whose quality is to be assessed, its growth is unaffected by other microbial populations present and there should be relatively simple methods available for detection, differentiation, and quantization. A good example is the yeast and mould determination, which can serve as a quality indicator for cereal grains. Growth of yeasts and moulds in cereal grains is generally not influenced by the presence of many other microorganisms, which would be inhibited by the low water activity of the commodity.

23.4 Commonly Used Indicator Organisms

Many different types of indicators have been advocated for use in particular applications; however, this is limited to the most common indicators used for foods and drinking water, i.e. the aerobic plate count; coliforms; *E. coli*; Enterobacteriaceae, *enterococci* and the yeasts and moulds.

23.4.1 Aerobic plate count

The aerobic plate count (APC) is one of the most widely used indicator tests. Although the applications of the APC are diverse, on one thing there is agreement: it cannot be used as a safety indicator, as there is generally no correlation between APCs and the presence of pathogens or their toxins. The APC may be a quality indicator, and then only when used in an appropriate context. It has no indicator value for some products, for example, vegetable sprouts, which naturally have high APCs in the range of 10^8 – 10^9 CFU/g, or for fermented products, such as yogurt, which yield high APCs due to the starter cultures incorporated. The APC of a product may reflect the microbial load of raw materials and ingredients, or its age and storage history. Assays for specific spoilage microorganisms may be necessary and more reliable than APCs for determining the acceptability of certain products. Nevertheless, in the appropriate context, the APC can indicate adherence to sanitation GMPs and product acceptability.

23.4.1.1 Detection and enumeration

The method recommended by the International Organization for Standardization (ISO 4833) calls for aerobic incubation on plate count agar at 30°C for 72 hours. The FDA's BAM recommends 35°C for 48 hours for non-dairy foods. The Standard Plate Count, which is used for estimating bacterial populations in dairy products, strictly specifies 32°C for 48 hours. The 'pour plate' method for the APC is officially recognized (AOAC 966.23C; ISO 4833). The 'spread plate' technique is generally easier to perform and may have other advantages: different colony morphologies may be recognized, translucent media are not required, and microorganisms are not exposed to the heat of the molten agar. Other rapid methods have been officially recognized, including use of the hydrophobic grid membrane filter (HGMF; AOAC 986.32), pectin gel (AOAC 988.18), and dry rehydratable film (AOAC 990.12). SimPlate® Total Plate Count, which uses colorimetric detection of growth in micro wells to determine the most probable number (MPN) of the microorganisms, is the most recent method to receive official status (AOAC 2002.07).

23.4.2 Coliforms and *E. coli*

The coliform group is not a valid taxonomic distinction, but is defined functionally, i.e., by the fermentation of lactose in the coliform test. Coliforms may be defined as Gram-negative, oxidase-negative, aerobic or facultative anaerobic non-spore-forming rods, able to grow in the presence of bile salts, and which ferment lactose to produce acid and gas within 48 hours at 37°C. Genera that fit this description are *Citrobacter*,

Enterobacter, *Escherichia* and *Klebsiella*. However, *Citrobacter*, *Enterobacter*, *Klebsiella* and include species that are normal inhabitants of plants and the environment; thus, a positive coliform test does not necessarily indicate faecal contamination, as originally proposed by the PHS in 1914 for evaluation of drinking water. This realization discredited the coliform test as an indicator of faecal pollution and prompted development of the faecal coliform test. Sometimes referred to as thermotrophic, thermoduric, or thermotolerant coliforms, the faecal coliforms have the same properties as the coliform group, except that the fermentation is able to proceed at 44.5–45.5°C. However, species that have this capacity also are known to be present naturally in the environment; thus the faecal coliforms are not specific indicators of faecal pollution of water, either. *E. coli* is present in all mammalian faeces at high concentrations; it does not multiply appreciably but can survive in water for 4–12 weeks, and so it is useful as an indicator of faecal pollution of drinking water systems. The case for *E. coli* as an indicator in foods and the processing environment is not as clear, however. Certainly, the organism can survive, but it can also grow, in certain foods. It can become established in the food processing environment and contaminate foods in the facility; thus, recent faecal contamination cannot be concluded when it is detected in foods or food manufacturing plants. The coliform groups and *E. coli* are most widely applied in the food industry as sanitation and process integrity indicators and for Hazard Analysis Critical Control Point (HACCP) verification.

Both quantitative and presence/absence methods are described for determining total coliforms and faecal coliforms. Methods for coliform testing generally incorporate the distinguishing physiological characteristics of the group, i.e. lactose fermentation and resistance to bile salts (or a similar surfactant, such as Sodium Lauryl sulfate). Colony counts of the coliform group are obtained from violet red bile lactose (VRBL) agar (ISO 4832; ISO 5541/1). Injured coliform populations may be recovered by first inoculating the sample onto a non-selective agar medium, incubating for several hours to allow resuscitation, followed by a VRBL agar overlay for selection. The MPN method for enumeration of coliforms uses Lauryl sulfate tryptose (LST) broth as a first step, with confirmation of positive tubes, indicated by gas production, in brilliant green bile lactose broth (BGBLB).

Because certain strains of *E. coli* known to exist in some foods, e.g. meat products, do not produce gas in LST, the ISO method 4831 recommends transfer of all turbid LST tubes, regardless of gas production, to BGBLB for confirmation. Membrane filtration, which allows analysis of a larger sample volume than other methods, is recommended for coliform counts in 100 ml water (ISO 9308-1). Appropriate enzymatic treatments for foods are necessary to allow filtration of 0.5–2.0 ml sample volumes in the application of HGMF for coliform determinations (AOAC 983.25). The huge number of samples that are routinely tested for coliforms spurred development of rapid methods for these determinations.

23.4.3 Enterobacteriaceae

The family Enterobacteriaceae encompasses approximately 20 genera, including *E. coli* and the other members of the coliform group; food-borne pathogens *Salmonella*, *Shigella*, *Yersinia* and other related genera. The family was originally proposed as an indicator alternative to the coliform group, because testing for the entire family would be more inclusive for the pathogenic genera. Lactose, the carbohydrate specified in the coliform test, is not fermented by *Salmonella*, *Shigella*, or *Yersinia*, so their presence would not be detected by the test. But substituting glucose for the lactose in the test would allow detection of all members of the Enterobacteriaceae, including the pathogens, as well as variant strains that do not show the typical lactose fermentation trait. The rationale for the use of the Enterobacteriaceae as indicators was advanced by reports noting low or negative coliform test results despite detection of *Salmonella* in certain foods, by a shigellosis outbreak in a nursing home in which Enterobacteriaceae tests might have indicated a cause for concern, and by a cheese-associated outbreak caused by an enteropathogenic *E. coli* strain that was a slow lactose fermenter. These reports notwithstanding, the Enterobacteriaceae are no more indicative of faecal contamination in foods than are the coliforms, i.e. not indicative at all. Nevertheless, they are useful, like the coliforms, as process integrity indicators.

The Enterobacteriaceae may be superior to the coliforms as indicators of sanitation GMPs because they have

collectively greater resistance to the environment than the coliforms, can colonize where sanitation has been inadequate, and are sensitive to sanitizers. Thus, the Enterobacteriaceae are useful for monitoring sanitation in food manufacturing plants, although they are more widely used as indicators in Europe than in the United States.

23.3.1 Detection & enumeration

Like the coliforms, members of the Enterobacteriaceae family demonstrate bile resistance, but unlike the coliforms, they do not universally demonstrate lactose fermentation. However, they all ferment glucose. A simple switch of the carbohydrate from lactose to glucose in the coliform selective medium formulation provides a way to test for all of the members of the family, including the pathogens. The Enterobacteriaceae are enumerated on violet red bile glucose (VRBG) agar or by MPN determination using brilliant green bile glucose (BGBG) broth (ISO 7402). A petrifilm method for determining Enterobacteriaceae counts has recently received AOAC Official Method SM status (AOAC 2003.01). Given that the Enterobacteriaceae are commonly used as sanitation indicators, and as such may be subjected to various environmental stresses, a method for their recovery by pre-enrichment in non-selective buffered peptone water, followed by selective growth in BGBG broth and isolation on VRBG agar, has been validated (ISO 8523).

23.4.4 Enterococci

Monitoring the microbiological quality of milk relies largely on examination for indicator bacteria such as coliforms, *E. coli* and *Enterococci*. The presence of *Enterococcus* group, which is a subgroup of the faecal *Streptococci*, serves as a valuable bacterial indicator for determining the extent of faecal contamination and it is more specific than the detection of coliforms, which may originate from non-faecal. *Enterococci* have different useful applications in the dairy industry. Nevertheless, they also have been described as spoilage micro-organisms and cross-contaminants during food processing, when their initial numbers in raw milk are high, pasteurisation is poor, or the pasteurized milk is not stored properly indicates poor hygiene during milk handling and processing. There are no standards set for the minimum and maximum count of *enterococci* because their counts vary with product handling, time of storage and other factors and are not normally counted in microbiological analyses. But, being a severe problem in dried milk products and infant feed as *enterococcal* counts in milk-based infant foods are as high as 19×10^2 cfu/g, PFA Rules 1956 have given standards for malted and infant milk food, according to which faecal *streptococci* should be absent in 0.1 gram. *Enterococci* can enter the milk chain either primarily from human or animal faeces but also secondarily from contaminated water sources, the exterior of the animal or other contaminated milking equipment or bulk storage tanks handled in the processing plant. The HTST pasteurisation (72°C/15 seconds) followed by proper storing conditions ensures no *enterococcal* re-growth and thus *enterococcal* presence is extremely unlikely to occur in the pasteurised milk. In principal dairy products – milk powder and butter – their presence is unlikely, since their manufacture involves one or several heating stages that will effectively inactivate any *enterococcal* bacterium. Therefore, if final products are contaminated with *enterococci*, these would not have originated from the raw milk, but from post-heat re-contamination and growth.

23.4.4.1 The genus *Enterococcus*

The genus *Enterococcus* was carved out of the earlier larger genus, *Streptococcus*, and ‘faecal *streptococci*’ or ‘Lancefield’s group D *streptococci*’ are still maintained in this genus. Out of the 20 species of this genus, only two (*E. faecalis* and *E. faecium*) are suggested to be responsible for nosocomial infections.

The *Enterococcus* spp. is regular Gram-positive, on-spore forming, non motile, facultative anaerobic, gamma-haemolytic on blood agar, catalase negative, homofermentative ovoid cocci (pairs to short chains). These bacteria can grow between temperature ranges of 5 to 50°C with an optimum growth temperature of 30 to 37°C. Typical pH ranges for growth is 4.6 to 9.9 with an optimum growth pH at neutral condition i.e. at 6.0-7.0.

23.4.4.2. Enumeration of *enterococci* from dairy products

Principle: The enumeration of *enterococci* in dairy products is based on its extraordinary physiological characteristics which differentiates them from other microorganisms and include:

1. Bile tolerance up to 40%
2. Sodium chloride tolerance up to 6.5%
3. Survive heating at 60°C for 30 minutes
4. Hydrolyse esculin in the presence of bile salts by the enzymatic action of β -glucosidase/ esculinase
5. High level of endemic antibiotic resistance
6. Reduces 2,3,5-triphenyltetrazolium chloride (TTC)
7. Tolerance to sodium azide
8. Enzymatic action of β -glucosidase on various chromogenic and fluorogenic substrates.

23.4.4.3 Conventional methods

Exploiting above parameters several media have been devised for *enterococci* isolation and enumeration from dairy products. Even though several media have been advocated for the selective isolation and quantification of *enterococci*, several protocols have been published for diverse purposes. Till date, there is no single method alone that universally meets all requirements as all have one or more shortcomings. The typical culture media employed for the estimation of enterococci in water, foods, feeds and clinical specimens such as the Slanetz-Bartley (membrane *Enterococcus* agar), kanamycin esculin azide (KAA) medium, citrate azide agar (CAA) and bile esculin azide agar (BEA) are advantageously applied in the case of selective enumeration of *enterococci* as single components, i.e. if *enterococci* are the only microbial component in the product. However, like any other members of the LAB, *enterococci* are often found associated with a micro-flora of considerable diversity and this is reflected in a much more complicated situation when samples containing such a mixed micro-flora have to be examined for enterococcal recovery. Consequently, a number of selective agents, incubation conditions, and combinations and modifications thereof have to be used; taking into account various advantages but also drawbacks. The use of media containing either selective chromogenic dyes or selectively inhibitory substances (e.g. antibiotics) may, however, enable some differential bacteriological enumeration. In spite of the large variety of suggested media and methods with their modifications, the citrate azide agar and the bile esculin azide agar, are the most recommended media for enterococcal isolation in dairy products. It is always practical to bear in mind, when examining enterococci in dairy products a higher incubation temperature (45°C) may be necessary to suppress the growth of the background micro-flora.

23.4.4.4 List of various selective agents commonly used for enterococci and their mode of action

Selective Agents	Mode of action
Sodium azide	Inhibition of enzyme systems (catalase, cytochrome c oxidase) in electron transport
2,3,5-triphenyltetrazolium chloride (TTC)	Reduction of TTC leads to the insoluble red formazan compound and it depends on pH of the medium
Antibiotics (gentamicin, kanamycin)	Less selective than sodium azide, act on different sites (ribosomes, proteins, etc.)
Esculin	β -D-glucosidase enzyme hydrolyzes esculin to esculetin and dextrose; esculetin combines with ferric ammonium citrate to form black color complex
Chromogenic/ Fluorogenic substrates	β -D-glucosidase enzyme acts upon these substrates to form different colored/ fluorescent compounds

23.4.4.5 Various media used for selective isolation of *enterococci* and their mode of action

23.4.4.5.1 Citrate azide agar

It is a selective agar used for the identification of *enterococci* in meat, meat products, dairy products and other food stuffs. The high concentrations of citrate and azide almost completely inhibit the growth of the accompanying microbial flora. Enterococci reduce the colorless 2,3,5-triphenyltetrazolium chloride dye present in medium to a red formazan, their colonies thus become red in color (Fig. 23.1).

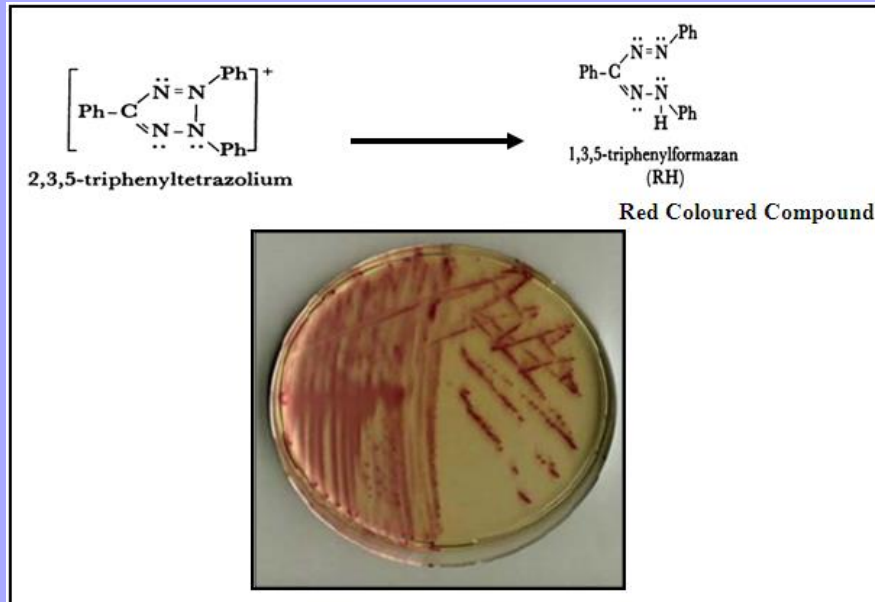


Fig. 23.1 Red coloured colonies on citrate azide agar

23.4.4.5.2. Bile esculin azide agar

Bile esculin azide agar is a modification of the earlier medium bile esculin agar. This formula modifies Bile Esculin Agar by adding sodium azide and reducing the concentration of bile. Organisms positive for esculin hydrolysis, hydrolyze esculin to esculetin and dextrose (glucose). The esculetin reacts with the ferric citrate to form a dark brown or black complex (Fig. 23.2 & 23.3). Oxbile is used to inhibit Gram-positive bacteria and other *streptococci*, while sodium azide inhibits Gram-negative bacteria. Enzymatic digest of casein and yeast enriched meat peptone are the carbon, nitrogen, and vitamin sources used for general growth requirements in bile esculin agar. Sodium chloride maintains the osmotic balance of the medium. Sodium citrate acts as a preservative.

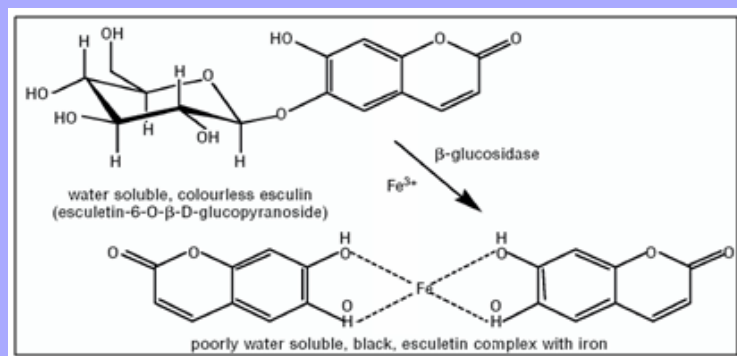


Fig. 23.2 Reaction catalysed



Fig. 23.3 Black color colonies on bile esculin azide agar

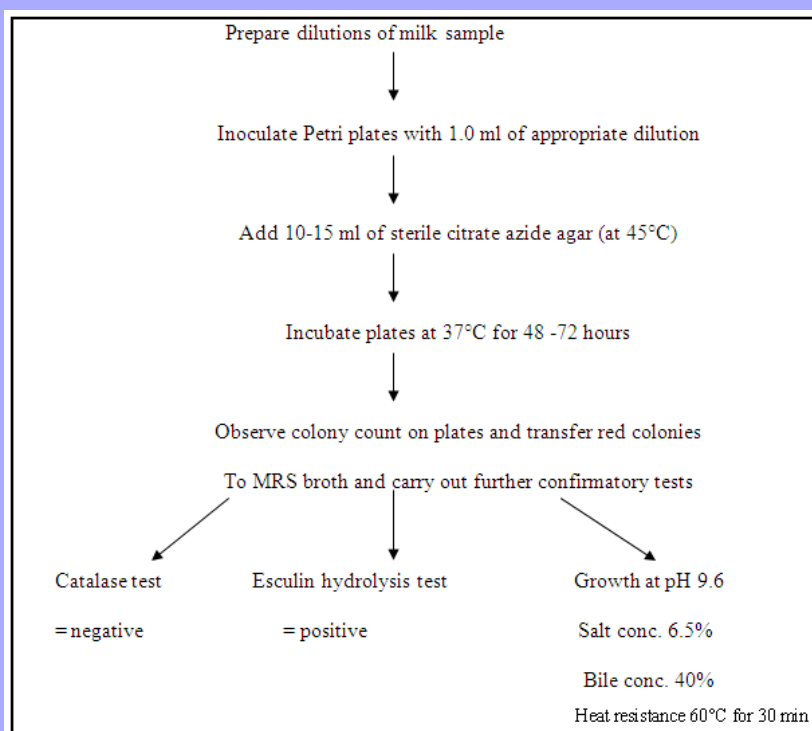
23.4.4.5.3 Kanamycin-esculin-azide (KAA) medium

It is a selective isolation and enumeration medium for enterococci in foods. Sodium azide and kanamycin provide the selective inhibition required whilst esculin and iron salts form an indicator system for the presumptive identification of *enterococci* as explained above. Incubation at 42°C will increase the medium's selectivity.

23.5.3.4 Slanetz-bartley (Membrane *enterococcus* agar)

Enterococci reduce 2, 3, 5-triphenyltetrazolium chloride to the insoluble red dye formazan, producing colonies which are dark red or maroon on the surface of the membrane or agar. This reaction is not exclusive to *enterococci*, and the count at this stage should be considered presumptive. Colonies may be confirmed as enterococci by demonstrating esculin hydrolysis using kanamycin esculin azide agar.

23.4.4.5 Conventional procedure for isolation and enumeration of *enterococci* in milk



23.4.4.6 Enzyme/ substrate based detection method

There are unique biochemical pathways that characterize each genus, species or strain and are based on unique key enzymes that participate in such metabolisms. These enzymes are referred to as ‘marker enzymes’. The chromogenic or fluorogenic substrate complex is hydrolyzed by marker enzyme and free chromogen or fluorogen is released which can be detected either colorimetrically or fluorometrically. It is therefore useful to assay directly for the activity of these enzymes for detection of micro organisms.

23.4.4.6.1 Hi chrome rapid *enterococcus* agar (Chromogenic based)

It is recommended for rapid detection of *enterococci* from water samples sources. The enzyme β -glucosidase which act as marker enzyme for enterococci cleaves the chromogenic substrate X-GLU (5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside), resulting in an intensive bluish green color colonies due to formation of indigo (Fig. 23.4 & 23.5). The medium contains special peptone, which provides nitrogenous compounds and other essential nutrients. Sodium chloride maintains the osmotic balance of the medium. Sodium azide inhibits the accompanying micro-flora, especially gram negative organisms. Tween 80 (Polysorbate80) acts as a source of fatty acids.

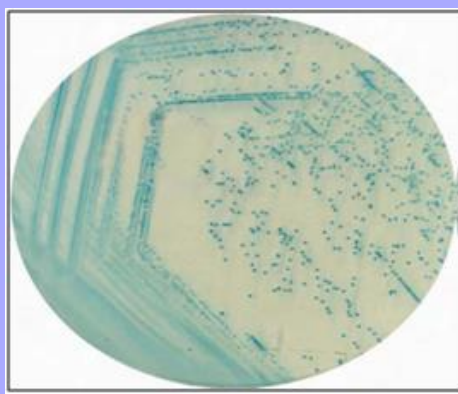


Fig. 23.4 Bluish green color colonies on Hi chrome rapid enterococcus agar

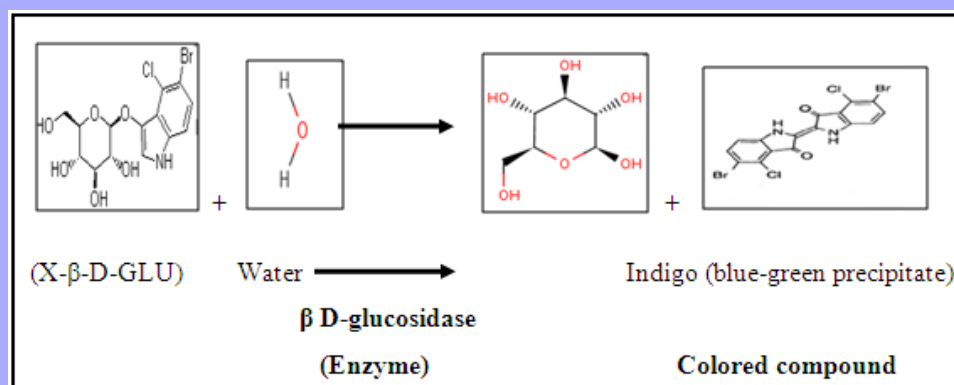


Fig. 23.5 Reaction catalysed

23.4.5 Yeasts and moulds

Yeasts and moulds are commonly enumerated in foods as quality indicators. They have no predictive value for the occurrence of toxigenic fungi or other pathogens. As a group, the yeasts and moulds are diverse and can grow on virtually any type of food. They survive a wide range of environmental conditions: pH 2–9; temperatures of 5°–35°C; and water activity (a_w) of 0.85 or less. As quality indicators, they can be used to assess ingredient acceptability, organoleptic characteristics, stability, and shelf life of a product. Osmophilic yeasts, commonly members of the genus *Zygosaccharomyces*, can grow down to A_w of 0.65 and are used as indicators in low a_w foods, e.g., jams, syrups, juice concentrates.

23.4.5.1 Detection and enumeration

Although they have a diverse growth habit, yeasts and moulds grow slowly in laboratory culture when compared with bacterial groups. Thus, yeasts and moulds are enumerated by a plate count procedure that uses agar supplemented with agents inhibitory to bacteria. Chloramphenicol, rose bengal, and dichloran are common selective agents. Spread or pour plates, incubated at 25°C for 3–7 days, are recommended (ISO 7954). If osmophilic types are suspected, care must be taken to decrease the A_w of both the plating media and diluents as appropriate and to allow extended incubation times (31). Rapid official methods using HGMF (AOAC 995.21) and dry rehydratable film (AOAC 997.02) recommend 50 hours or 5-day incubation, respectively. A method using the SimPlate colorimetric format determines yeast and mould counts in 56–72 hours (AOAC 2002.11). Despite the improvements provided by the rapid methods, a relatively lengthy time of analysis still is required for yeast and mould determinations, compared with other microbial groups. Significant economic consequences can result if product release is delayed until assay results are obtained. Clearly, there is a need for more research to improve methods for determining yeasts and moulds in foods.

Lesson 24

ENUMERATION OF *E. COLI*/ *E. COLI* O157:H7

24.1 Introduction

Escherichia coli, originally known as *Bacterium coli* commune, was identified in 1885 by the German paediatrician, the Theodor Escherich. *E. coli* is widely distributed in the intestine of humans and warm-blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host. *E. coli* is a member of the family Enterobacteriaceae, which includes many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immune-compromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans.

In 1892, Shardingger proposed the use of *E. coli* as an indicator of faecal contamination. This was based on the premise that *E. coli* is abundant in human and animal faeces and not usually found in other niches. Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent faecal contamination and the possible presence of frank pathogens. Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter*, *Enterobacter* and *Klebsiella* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics, so that they are not easily distinguished.

Although coliforms were easy to detect, their association with faecal contamination was questionable because some coliforms are found naturally in environmental samples. This led to the introduction of the faecal coliforms as an indicator of contamination. Faecal coliform, first defined based on the works of Eijkman is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermo tolerant coliforms. Faecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C. The faecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as faecal coliforms. The inclusion of *Klebsiella* spp. in the working definition of faecal coliforms diminished the correlation of this group with faecal contamination. As a result, *E. coli* has re-emerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*.

Currently, all 3 groups are used as indicators but in different applications. Detection of coliforms is used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Faecal coliforms remain the standard indicator of choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent faecal contamination or unsanitary processing. Almost all the methods used to detect *E. coli*, total coliforms or faecal coliforms are enumeration methods that are based on lactose fermentation.

24.2 Enumeration and Isolation of *E. coli*

The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other two phases of the assay are performed and then uses the combinations of positive results to consult a statistical tables, to estimate the number of organisms present. Typically only the first two phases are performed in coliform and faecal coliform analysis, while all three phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. The 5-tube MPN is used for water, shellfish and shellfish harvest water testing and there is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly

contaminated.

There is also a solid medium plating method for coliforms that uses violet red bile agar (VRBA) which contains neutral red pH indicator, so that lactose fermentation results in formation of pink colonies. There are also membrane filtration tests for coliform and faecal coliform that measure aldehyde formation due to fermentation of lactose.

24.2.1 Presumptive test for *E. coli*

Weigh 50 g food into sterile high-speed blender jar. Frozen samples can be softened by storing it for <18 h at 2-5°C, but do not thaw. Add 450 ml of Butterfield's phosphate-buffered water and blend for 2 min. If < 50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluents to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluents. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 seconds. Do not use pipettes to deliver <10% of their total volume. Transfer 1 ml portions to three LST (Lauryl Sulfate Tryptose) tubes for each dilution for at least three consecutive dilutions. Hold pipette at angle so that its lower edge rests against the tube. Let pipette drain 2-3 seconds. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

Incubate LST tubes at 35°C. Examine tubes and record reactions at 24 ± 2 hours for gas, i.e. displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 hours and examine and record reactions again at 48 ± 2 hours. Perform confirmed test on all presumptive positive (gas) tubes.

24.2.2 Confirmed test

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35°C and examine for gas production at 48 ± 2 hours. Calculate most probable number (MPN) (see Appendix 2) of coliforms based on proportion of confirmed gassing LST tubes for three consecutive dilutions.

24.2.3 Confirmed test for faecal coliforms and *E. coli* by eijkman test

From each gassing LST tube from the presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 hours at 45.5°C and examine for gas production. If negative, re-incubate and examine again at 48 ± 2 hours. Use results of this test to calculate faecal coliform MPN. The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures

NOTE: Faecal coliform analyses are done at $45.5 \pm 0.2^\circ\text{C}$ for all foods, except for water testing.

24.2.4 Completed test for *E. coli*

To perform the Completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 hours at 35°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to five suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 hours at 35°C and use for further testing.

NOTE: Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all five isolates may need to be tested.

24.2.4.1 Gram stain

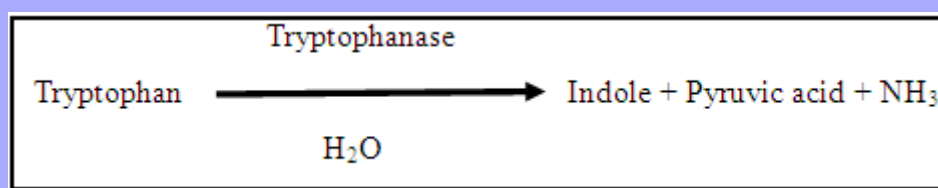
All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

24.2.4.2 IMViC tests

IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of family Enterobacteriaceae. The four reactions are: Indole production test, Methyl Red reduction test, Voges Proskauer test and Citrate utilization test.

Indole test

Some bacteria can produce indole from amino acid tryptophan using the enzyme tryptophanase. Production of indole is detected using Ehrlich's reagent or Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red colour. An alcoholic layer concentrates the red colour as a ring at the top.



Procedure

Inoculate tube of tryptone broth and incubate 24 ± 2 hours at 35°C . Test for indole by adding 0.2-0.3 ml of Kovacs' reagent. Appearance of distinct red colour in upper layer is positive test.

Methyl red-reactive compounds

This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in colour at a pH of 4.4 or less.

Procedure

Inoculate tube of Glucose peptone water broth and incubate 48 ± 2 hours at 35°C . Transfer 1 ml to 13 x 100 mm tube. Add 5 drops of methyl red solution to each tube. Distinct red colour is positive test. Yellow is negative reaction.

Voges Proskauer (VP) Test

VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In these test two reagents, 40% KOH and alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of α -naphthol to produce red colour. Role of α -naphthol is that of a catalyst and a colour intensifier.

Procedure

Inoculate tube of glucose peptone water broth and incubate 48 ± 2 hours at 35°C . Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml α -naphthol solution and 0.2 ml 40% KOH and shake. Add a few crystals of creatine. Shake and let stand 2 hours. Test is positive if eosin pink colour develops.

Citrate utilization test

This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromo-thymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO₂. Production of Na₂CO₃ as well as NH₃ from utilization of sodium citrate and ammonium salt respectively results in alkaline pH. This results in change of medium's colour from green to blue.

Procedure

Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 hours at 35°C. Development of distinct turbidity is positive reaction.

24.2.4.3 Gas from lactose

Inoculate a tube of LST and incubate 48 ± 2 hours at 35°C. Gas production or effervescence after gentle agitation is positive reaction.

24.2.5 Interpretation

All cultures that ferment lactose with gas production within 48 hours at 35°C, appear as Gram-negative non spore forming rods Calculate MPN of *E. coli* based on proportion of EC tubes in three successive dilutions that contain *E. coli*.

24.2.5 Various media used for the selective isolation of *E. coli*

24.2.5.1 HiCrome selective ECC base agar

Principle and interpretation

HiCrome ECC Selective Agar is a selective medium recommended for the simultaneous detection of *Escherichia coli* and total coliforms in water and food samples. The chromogenic mixture contains two chromogenic substrates. The enzyme β-galactosidase produced by coliforms cleaves the chromogen resulting in the salmon to red coloration. The enzyme β-glucuronidase produced by *Escherichia coli*, cleaves X-glucuronide. Colonies of *Escherichia coli* are dark blue to violet colored due to cleavage of both the chromogen (Fig. 24.1). The addition of L-tryptophan improves the indole reaction, thereby increasing detection reliability. Cefsulodin, when added inhibits *Pseudomonas* and *Aeromonas* species.

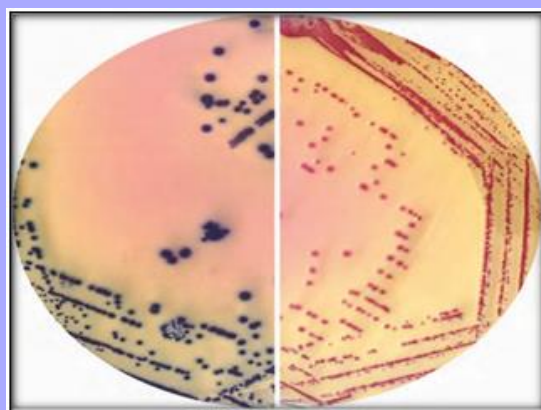


Fig. 24.1 Blue colour colonies of *E. coli* & red colour colonies of *E. aerogenes*

24.2.5.2 HiCrome M-lauryl sulphate agar

Principle and interpretation

HiCrome M-Lauryl Sulphate Agar is a modification of the Lauryl tryptose broth, formulated by Mallman and Darby. This Chromogenic medium is recommended for the presumptive identification and differentiation of *E. coli* and other coliforms by a single membrane filtration technique. The incorporation of chromogen X-glucuronide and the dye phenol red favors the differentiation of *E. coli* and other coliforms on the basis of colour. Peptic digest of animal tissue and yeast extract provide essential growth nutrients to the organisms. Lactose acts as a source of fermentable sugar Sodium Lauryl Sulphate inhibits organisms other than coliforms. The enzyme Beta-glucuronidase produced by *E. coli*, cleaves X-glucuronide, imparting a green colour to the colonies and along with phenol red indicator aids in detection of lactose fermenter (Fig. 24.2).

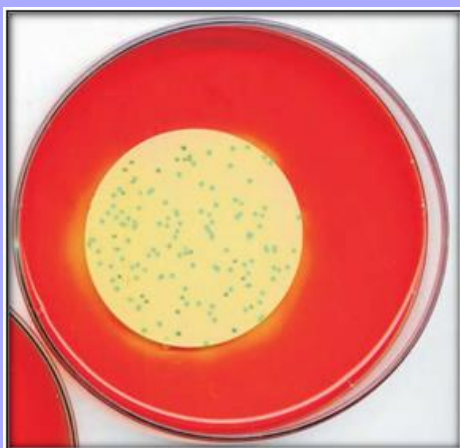


Fig. 24.2 Green colour colonies of *E. coli* are obtained

24.3 Pathogenic *E. coli*

Shiga toxin producing *E. coli* (STEC) are food-borne pathogens that may cause serious illness in humans. Among the food related zoonoses, they are the fourth most occurring group in Belgium, but as far as human symptoms are concerned, they are one of the most dreaded organisms. Bovine animals, which are asymptomatic carriers, are the most important reservoir. Infection of humans is mostly caused by the consumption of infected foodstuffs derived from bovine animals, such as milk and milk products.

A wide range of serogroups is capable of provoking these human infections, the most important of them being O26, O103, O111, O145 and O157. The STEC bacteria owes its infective capacity to a combination of virulence properties, the most important of which are: the production of type I and II shiga toxins, which are responsible for kidney failure, the proteins encoded on the LEE locus, responsible for the modification and the intimate adherence to the gastro-intestinal cells, and enterohemolysin, which plays a role in the destruction of blood. The combination of these manifestations results in a complex pathology known as the hemolytic uremic syndrome (HUS). In Belgium, 50 human STEC infections per year are reported on average, 50 percent of which are caused by serogroup O157 and some 20 patients develop HUS. The non-sorbitol-fermenting (NSF) O157 strains have been studied most frequently. In fact, an internationally standardized isolation method for food and feed (ISO 16654) is available for that group and is considered as the gold standard. The method is based upon phenotypic characteristics of the organism, such as increased resistance and enzymatic properties. The NSF O157 strain is isolated in approximately 4 days using conventional culture. First, the sample is selectively enriched by adding antibiotics (novobiocin) for 6 hours at a relatively high temperature (41.5°C). Then, immunomagnetic separation (IMS) is used on the enriched sample.

24.3.1 LST-MUG method for detecting *E. coli*/*E. coli* O157:H7

The LST-MUG assay is based on the enzymatic activity of β -glucuronidase (GUD), which cleaves the substrate 4-methylumbelliferyl β -D-glucuronide (MUG), to release 4-methylumbelliferone (MU). When

exposed to long wave (365 nm) UV light, MU exhibits a bluish fluorescence that is easily visualized in the medium or around the colonies. Over 95% of *E. coli* produces GUD, including anaerogenic (non-gas-producing) strains. One exception is enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7, which is consistently GUD negative. The lack of GUD phenotype in O157:H7 is often used to differentiate this serotype from other *E. coli*, although GUD positive variants of O157:H7 do exist. The production of GUD by other members of the family Enterobacteriaceae is rare, except for some *Shigella* (44-58%) and *salmonellae* (20-29%). However, the inadvertent detection of these pathogens by GUD-based assays is not considered a drawback from a public health perspective. Expression of GUD activity is affected by catabolite repression so on occasion, some *E. coli* are GUD-negative, even though they carry the *uidA* gene (*gusA*) that encodes for the enzyme (19). In most analyses however, about 96% of *E. coli* isolates tested are GUD-positive without the need for enzyme induction.

MUG can be incorporated into almost any medium for use in detecting *E. coli*. But some media such as EMB, which contain fluorescent components, are not suitable, as they will mask the fluorescence of MU. When MUG is incorporated into LST medium, coliforms can be enumerated on the basis of gas production from lactose and *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light, thus it is capable of providing a presumptive identification of *E. coli* within 24 h.

24.3.2 Presumptive LST-MUG test for *E. coli*

Prepare food samples and perform the MPN Presumptive test as described in section I.C. above, except use LST-MUG tubes instead of LST. Be sure to inoculate one tube of LST-MUG with a known GUD-positive *E. coli* isolate as positive control (ATCC 25922). In addition, inoculate another tube with a culture of *Enterobacter aerogenes* (ATCC 13048) as negative control, to facilitate differentiation of sample tubes that show only growth from those showing both growth and fluorescence. Incubate tubes for 24 to 48 ± 2 h at 35°C. Examine each tube for growth (turbidity, gas) then examine tubes in the dark under longwave UV lamp (365 nm). A bluish fluorescence is a positive presumptive test for *E. coli*. After 48 h of incubation, 96-100% of *E. coli*-positive tubes can be identified (28). Perform a confirmed test on all presumptive positive tubes by streaking a loopful of suspension from each fluorescing tube to L-EMB agar and incubate 24 ± 2 h at 35°C. Follow protocols outlined in 24.2.4, above, for Completed test for *E. coli*. Calculate MPN of *E. coli* based on combination of confirmed fluorescing tubes in 3 successive dilutions.



Lesson 25

ENUMERATION OF *SALMONELLA* AND *SHIGELLA***25.1 Introduction**

Salmonella is a group of bacteria that can cause diarrhoeal illness in people. Salmonellosis is a bacterial disease commonly manifested by an acute enterocolitis, with sudden onset of headache, abdominal pain, diarrhoea, nausea and sometimes vomiting. Deaths are uncommon, except in the very young, in the very old, the debilitated and immunosuppressed. However, morbidity and associated costs of salmonellosis may be high. Salmonellosis is classified as a food borne disease, because contaminated food, mainly of animal origin, is the predominant mode of transmission. Epidemiologically, *Salmonella* gastroenteritidis may occur in small outbreaks in the general population. However, large outbreaks in hospitals, institutions for children, restaurants are not uncommon and usually arise from food contaminated at its source, or, less often, during handling by an ill person or a carrier, but person-to-person spread can occur. They cause illnesses in humans and many animals, such as typhoid fever and enteritis. *Salmonella* (e.g. *Salmonella enterica* subsp. *enterica* serovar *enteritidis*) can cause diarrhoea. According to the World Health Organization over 16 million people worldwide are infected with typhoid fever each year, with 500,000 to 600,000 of these cases proving to be fatal.

A large outbreak of *Shigella sonnei* gastroenteritis occurred in Murcia Region (Southeast Spain) in the winter of 1995–1996. More than 200 people were affected. Epidemiological investigations implicated a regionally manufactured fresh pasteurised milk cheese as the vehicle of infection. The dispersed sale of the cheese resulted in a regional dissemination of the organism and people were affected in eight townships. Research suggested that an infected food handler at the cheese factory might have been the source of contamination and that the processing method might have allowed cross-contamination to occur. This study emphasises the importance of increasing the control of strict hygiene during the processing of fresh cheese, since legislation does not forbid direct contact by hand that could result in contamination of cheese even when the milk pasteurisation process was correctly performed. The higher susceptibility in young children of contracting Shigellosis and typhoid fever in addition to the high prevalence of *Salmonella* and *Shigella*—found to grow rapidly in liquid infant formula—has focused the attention of the scientific community to study the survival capabilities of these organisms in foods. In addition, the wide distribution of this commodity throughout the world creates the risk of a bioterrorism attack directed against the infant population. In this study, we investigated the survival of *S. Typhi* and *S. dysenteriae* over a 12-w period in dehydrated infant formula under ambient air or nitrogen atmospheric conditions.

25.2 Transmission and Source of Infection of *Salmonella*

Salmonella infections are zoonotic; they can be transmitted by humans to animals and vice versa. Infection via food is also possible. *Salmonella* is usually transmitted to humans by eating foods contaminated with animal faeces. Contaminated foods usually look and smell normal. Contaminated foods are often of animal origin, such as beef, poultry, milk, or eggs, but any food, including vegetables, may become contaminated. Thorough cooking kills *Salmonella*. Food may also become contaminated by the hands of an infected food handler who did not wash hands with soap after using the bathroom. *Salmonella* may also be found in the faeces of some pets, especially those with diarrhoea, and people can become infected if they do not wash their hands after contact with pets or pet faeces.

25.3 Isolation and Enumeration principles of *Salmonella* and *Shigella*

The examination of various products for the isolation of *Salmonella* often requires the use of methods, different from those used in clinical and public health laboratories. Many methods used for examining these foods are essentially similar in principle and employ the step of pre-enrichment, selective enrichment, differential and selective plating for isolation and identification of selected isolates.

25.3.1 Pre-enrichment

Twenty-five (25) grams or ml of sample is added to 225 ml of buffered peptone water and incubated at 37°C for 24 hours.

25.3.2 Selective enrichment

Transfer one ml portion from pre-enrichment step to each 10 ml of selenite eosine broth and tetrathionate broth and incubated at 37°C for overnight. Then the contents of both tubes were mixed and a loopful was streaked on to the xylose lysine deoxycolate agar (XLDA), and bismuth sulphite agar (BSA) plate and Hektoen enteric agar (HEA). These plates were incubated at 37°C for 24 hours. The incubation may be continued up to 72 hours before report as nil.

25.3.2.1 Typical salmonella and Shigella colonies on different selective media

a. Xylose lysine deoxycolate (XLDA) agar

XLDA was developed to improve the recovery of enteric pathogens, especially *Salmonella* and *Shigella* species. Lactose, Sucrose, and Xylose are the fermentable carbohydrates present and phenol red is used as the pH indicator. Bacteria that ferment none of these sugars, e.g., *Shigella*, appear as red, translucent colonies. Yellow colonies indicate a rapid fermentation of lactose and acid pH, as demonstrated by *Escherichia coli*. Since *Salmonella* ferment xylose as readily as coliforms, a second differential mechanism, lysine decarboxylase, is utilized. Those organisms that ferment xylose as well as decarboxylate lysine exhaust the xylose rapidly and the lysine reaction causes a pH reversal to the alkaline reaction similar to *Shigella*. Lactose and sucrose are added in excess to prohibit this same reversion by lysine-positive coliforms. Sodium thiosulfate and ferric ammonium citrate are indicators of H₂S production only when alkaline conditions exist; *Salmonella* will, therefore, form red colonies with black center in 24 hours. Sodium deoxycolate is added to inhibit gram-positive growth and to retard the growth of many strains of coliforms. Many cultures of *Salmonella* may produce pink colonies with or without glossy black centres or may appear as almost completely black colonies (Fig. 25.1).



Fig. 25.1 Pink and black color colony of *Salmonella* on XLD agar

b. Bismuth sulphite agar (BSA)

Brown, grey, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect. Bismuth Sulphite Agar is a modification of the original Wilson and Blair selective medium for the isolation and preliminary identification of *Salmonella typhi* and other *Salmonellae* from pathological material, sewage, water supplies, food and other products suspected of containing these pathogens. In this medium freshly precipitated bismuth sulphite acts together with brilliant green as a selective agent by suppressing the growth of coliforms, whilst permitting the growth of *Salmonellae*. Sulphur compounds provide a substrate for hydrogen sulphide production, whilst the metallic salts in the medium stain the colony and surrounding medium black or brown in the presence of hydrogen sulphide.

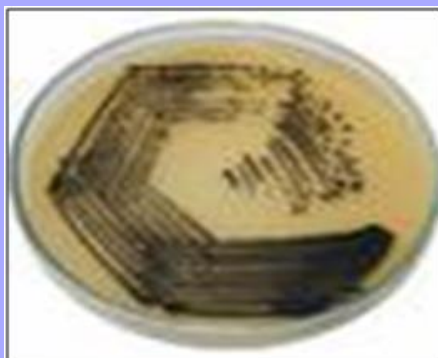


Fig. 25.2. Black color colony of *Salmonella* on BSA

c. Hektoen Enteric Agar (HEA)

HEA is used for isolating and differentiating enteric pathogens such as *Salmonella*, *Shigella* and other Gram-negative Enterobacteriaceae. It is used particularly in foods where multi-steps are followed to isolate the pathogens of gastroenteritis. The nutrients for growth are provided by the meat, peptone and yeast extract. The increased content of the peptone and the three fermentable carbohydrates (lactose, sucrose, salicin) as sources of carbon and energy reduce the inhibitory action of the bile salts on *Salmonella* and *Shigella* spp. Bromothymol blue and acid fuchsin are pH indicators. Sodium thiosulphate provides sulphur and ferric ammonium citrate is the indicator for H₂S production. H₂S positive colonies are blue-green to blue colonies with or without black center (Fig. 25.3).



Fig. 25.3. Blue-green to bluish colony of *Salmonella* on HEA

d. RAMBACH Agar (chromogenic medium) for *Salmonella*

RAMBACH Agar is a differential diagnostic culture medium for identifying non-typhi *Salmonella* in foodstuffs and clinical samples. The nutritive substrates in the RAMBACH Agar enable Enterobacteriaceae to multiply readily. Sodium deoxycolate inhibits the accompanying Gram-positive flora. RAMBACH Agar enables species of *Salmonella* to be differentiated unambiguously from other bacteria by means of a new procedure, for which a patent application has been submitted. This is made possible by adding propylene glycol to the culture medium. *Salmonellae* form acid with propylene glycol, so that, in combination with a pH indicator, the colonies have a characteristic red color (Fig. 25.4). In order to differentiate coliforms from *Salmonellae*, the medium contains a chromogen indicating the presence of β -galactosidase, a characteristic for coliforms. Coliform microorganisms grow as blue-green or blue-violet colonies. Other Enterobacteriaceae and Gram-negative bacteria, such as *Proteus*, *Pseudomonas*, *Shigella*, *S. typhi* and *S. paratyphi* grow as colorless to yellow colonies.



Fig. 25.4 Red color colony of *Salmonella* on RAMBACH agar

25.3.3 Biochemical tests for detection of *Salmonella* and *Shigella*

Lysine-decarboxylase broth, phenol red dulcitol broth, TSI Agar slant, tryptone broth, potassium cyanide (KCN) broth, malonate broth, indole production test, phenol red sucrose broth or purple sucrose broth, MR-VP broth, etc.

Indole and H₂S production

It is done to determine the ability of organisms to degrade the amino acid tryptophan to indole. Tryptophan is an essential amino acid that can undergo oxidation by some bacteria possessing the enzyme tryptophanase to indole. Presence of indole is detected by adding Kovac's reagent composed of p-dimethyl amino benzaldehyde, butanol & hydrochloric acid. Indole is extracted from the medium into the reagent layer by the acidified butanol and forms a complex with p-di methyl amino benzaldehyde to give cherry red color (Fig. 25.5). Some bacteria are capable of degrading cystine & certain other sulphur containing compounds with the formation of H₂S. Heavy metals are usually incorporated to detect the H₂S production. Addition of 3 – 4 drops of Kovac's Indole reagent on the surface after incubation indicated production of Indole from tryptophan amino acid.



Fig. 25.5 Indole test

Citrate Utilization

A slant of Simmons Citrate Agar was used & streaking was done from inoculum and incubated at 37°C for 24 hour, color change from green to blue shows citrate utilization positive. This test determines the ability of organism to use citrate as a source of carbon in the absence of glucose & lactose this ability depends upon citrate permeases that facilitates its transport into the cell. Citrate is the major intermediate of Kreb's cycle & produced by condensation of active acetyly CoA with oxaloacetic acid. During its conversion to pyruvic acid the medium becomes alkaline as CO₂ generated combines with sodium and water to form Na₂CO₃ whose presence is detected by the bromothymol blue indicator which undergoes color changes from green to deep blue.

A slant of Simmons Citrate Agar was used and streaking was done from inoculums and incubated at 37°C for 24 hours. Color change from green to blue shows citrate utilization positive.

Urease Test

It depends upon the ability of micro organism to degrade urea in the presence Urease enzyme produced by bacteria and is indicated by phenol red indicator which undergoes color change from peach to pink. If on streaking a Urease agar slant and on incubating it at 37°C for 24 hrs color changes to pink the bacteria is Urease positive (Fig. 25.6).

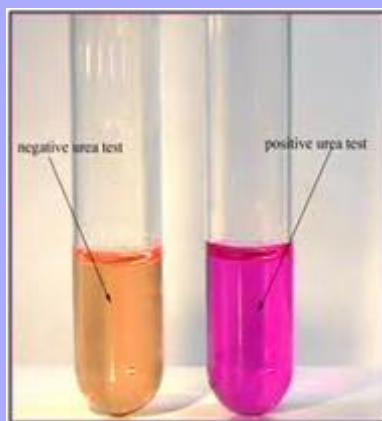


Fig. 25.6 Urease Test

Methyl red reduction (MR) Test

This test enables the microbiologist to determine the pathway being used to ferment glucose, and in the process helps to determine the species of bacteria that is most likely present. MR/ VP is actually two tests: The methyl red (MR) test determines whether or not large quantities of acid have been produced from mixed acid fermentation of glucose. End products of this pathway include lactic, acetic, formic and succinic acids. The Voges-Proskauer (VP) test determines whether a specific neutral metabolic intermediate, acetoin, has been produced instead of acid from glucose. Acetoin is the last intermediate in the butanediol pathway. The pH indicator methyl red detects the acidic end product by formation of red color (Fig. 25.7). A tube of MR-VP broth is inoculated with suspected colony incubated at 37°C for 24 hours. After incubation pour 2-3 drops of methyl red.



Fig. 25.7 Methyl red reduction test

Triple sugar iron (TSI) Test

Triple sugar iron agar is composed of three sugars viz. glucose, sucrose and lactose. Ferrous sulphate and phenol act as indicator, which is employed for detection of fermentation of sugars, indicated by the change in

color due to production of organic acid. The appearance of bubbles in the butt or pushing of the entire slant indicates production of gas from the fermentation of sugar by an organism. H₂S production by an organism is indicated by reduction of ferrous sulphate of medium to ferric sulphate, which is manifested as black precipitate (Fig. 25.8). TSI slant stabbed and streaked and incubated at 37°C for 24 hours. After incubation sugar fermenting bacteria has fermented the sugars and may produce H₂S.



Fig. 25.8 Triple sugar iron test

Reactions observed on TSI slants

Reaction	Fermentation
Acid butt (yellow), alkaline Slant (red)	Glucose fermented
Acid throughout medium, butt and slant yellow	Lactose or Sucrose or both fermented
Gas bubbles in butt, medium sometimes split	Aerogenic culture
Blackening of the butt	Hydrogen sulfide
Alkaline slant and butt (medium entirely red)	None of the three sugars fermented

Biochemical reactions of *Salmonella* and *Shigella*

Test or substrate	Result		<i>Salmonella</i> species reaction	<i>Shigella</i> species reaction
	Positive	Negative		
Glucose (TSI)	Yellow Butt	Red Butt	+	-
Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+	-
H ₂ S (TSI)	Blackening	No blackening	+	-
Urease	Purple-red color	No color change	-	-
Phenol red dulcitol broth	Yellow color and/ or gas	No gas; no color change	+	-
KCN broth	Growth	No growth	-	+
Malonate broth	Blue color	No color change	-	+
Indole test	Violet color at surface	Yellow color at surface	-	±
Phenol red lactose broth	Yellow color and/ or gas	No gas; no color change	-	-
Phenol red sucrose broth	Yellow color and/ or gas	No gas; no color change	-	-
Voges-Proskauer test	Pink-to-red color	No color change	-	-
Methyl red test	Diffuse red color	Diffuse yellow color	+	+
Citrate utilization			+	+
P-phenyl Pyruvic acid			-	-

Lesson 26

ENUMERATION OF *LISTERIA MONOCYTOGENES*

26.1 Introduction

Majority of illnesses and death each year affecting millions of people throughout the world are due to foodborne diseases. Pathogenic bacillus *Listeria monocytogenes* not only causes a severe human foodborne disease, but also has been linked to infections in more than 20 different animal species. Animal listeriosis has particularly been reported in sheep cattle, goats, and; symptoms include meningitis, abortions, and septicaemia as well as, less commonly, non-systemic infections such as uveitis and mastitis. Human listeriosis outbreaks have been traced to a variety of different dairy products (including pasteurized milk, butter, chocolate milk, Hispanic style cheeses) have been reported. According to the USDA/ FDA *Listeria* risk assessment, Hispanic style fresh cheeses represent a particular high risk food for acquiring listeriosis. Ability to survive outside a mammalian host and under a variety of stress conditions makes *L. monocytogenes* strains a particular concern for the dairy industry. *Listeria monocytogenes* is an intracellular pathogen and affects healthy as well as immuno-suppressed populations. In healthy individuals, this organism can cause gastroenteritis and fever. Overall, mortality rate due to infection is about 20–30%, with an annual death rate of about 2500 people. The infective dose for this pathogen is not known; however, it is estimated to be about 100 to 10⁶ cells depending on the immunological status of the host. It causes three forms of disease gastrointestinal form, systemic listeriosis, and abortion and neonatal listeriosis. While *L. monocytogenes* has been found in raw milk, current pasteurization time-temperature combinations effectively inactivate *L. monocytogenes*. Post-processing contamination from plant environments probably represents the most common source of *L. monocytogenes* contamination of pasteurized dairy products.

26.2 Some Characteristics of the Genus *Listeria*

All the seven species in the Gram-positive non-sporing genus *Listeria* are rods about 0.5 µm × 0.5-2.0 µm, produce catalase and are positive in the methyl red and Voges-Proskauer reactions. They are indole, oxidase, H₂S, urease, nitratase and gelatinase negative. The non-capsular facultative anaerobic *Listeria* spp. grows well at water activity (aw) of >0.95 but can multiply at aw of 0.90. They also grow well at 10% salt concentration but survive at 25.5%. *L. monocytogenes* can tolerate lower pH when kept at near refrigeration temperatures. Two species *L. monocytogenes* and *L. ivanovii* are clearly β-haemolytic and this ability has been linked to their pathogenicity. Some reactions useful in differentiating the pathogenic *Listeria* species are listed in Table 26.1.

Table -26.1 Differentiation between the two important pathogenic *Listeria* species - *L. monocytogenes* and *L. ivanovii*.

Species	β-Hemolysis ^a	Mannitol	Rhamnose	Xylose	Virulence ^b
<i>L. monocytogenes</i>	+	-	+	-	+
<i>L. ivanovii</i> ^c	+	-	-	+	+

Note

^a Sheep blood agar stab.

^b Mouse test.

^c Ribose fermenting strains are classified as *L. ivanovii subsp. ivanovii* and ribose non-fermenters as *L. ivanovii subsp. londiniensis*.

The CAMP test is considered by many to be the most important test for confirming pathogenic species. This test is performed by streaking a β -haemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrically opposite each other on a sheep blood agar plate. *Listeria* test cultures are then streaked at right angles to the two other cultures. After 48 hours of incubation at 35-37°C, β -haemolysis by *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak, whereas for *L. ivanovii* it becomes enhanced near the *R. equi* streak. The other *Listeria* species remain non-haemolytic.

26.3 Isolation and Enumeration Principle of *Listeria* Species from Dairy Products

Although *Listeria* grows rapidly on ordinary bacteriological media, direct isolation of the organism from infected and contaminated material is often unsuccessful. Difficulties in isolating *Listeria* date back to 1926 when *L. monocytogenes* was first described by Murray et al. Even when the presence of the pathogen had been established, the re-isolation of the organism by direct plating on nutritious agars frequently failed. It was soon clear that the reliability of any isolation method for *Listeria* intended for food analysis would depend on its ability to cope with low-level contamination ($<10^2$ cells per ml), among samples containing high-level competitive microflora, coupled with the ability to recover sub-lethally injured cells.

26.3.1 Enumeration principle

1. Enumeration of *Listeria* spp. is based on the principle that the organism show tolerance to the selective agent used in the isolation procedure listed in Table 26.2.
2. Ability to hydrolyse esculin by the enzyme β -glucosidase
3. Organism show weak β -haemolysis
4. Ability to produce phosphatidylinositol-specific phospholipase-C (PI-PLC)
5. Ability to ferment rhamnose

So based on various formulation and combination different chromogenic and fluorogenic culture media have been developed.

26.4 Conventional Method for Isolation of *Listeria* spp. From Dairy Products

26.4.1 Cold enrichment method

For many years the only method available was the cold enrichment technique first advocated by Gray et al. (1948) in which sample material was inoculated into a tryptose broth without selective agents and held at 4°C for long periods and has been considered a standard method for nearly 40 years. Cold enrichment is still an excellent isolation technique; however the need for prolonged incubation (up to several months or even a year) is a serious disadvantage. As shown in Figure 26.1, numerous methods have been developed over the years for isolating and detecting *Listeria* in food, clinical and environmental samples. Three general types can be distinguished:

- 1) Direct plating
- 2) Enrichment followed by plating
- 3) Enrichment followed by a rapid method

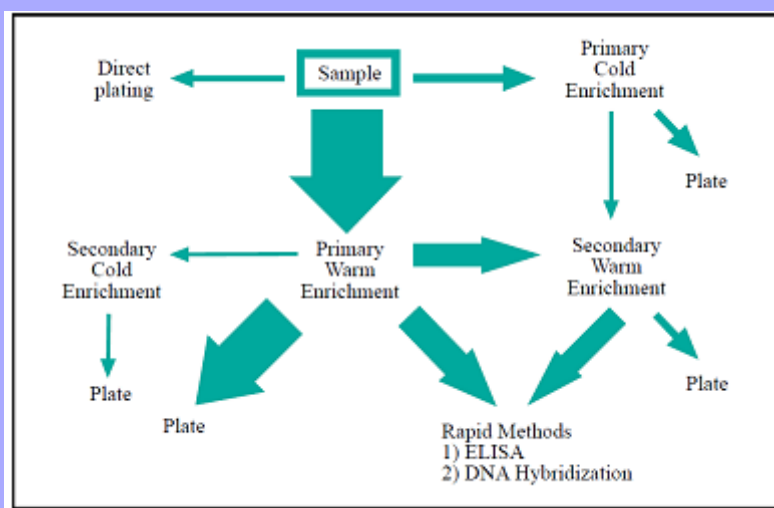


Fig. 26.1 (2) Scheme for isolation and detection methods of *Listeria*. The size of arrows denotes an estimated popularity of various methods

The first and least used is the direct plating of a food suspension onto a selective solid agar medium such as the LiCl-phenylethanol-moxalactam (LPM), PALCAM containing Polymyxin B, Acriflavin, Lithium chloride, Ceftazidime, phenylethanol, moxalactam medium with or without esculin and modified Oxford (MX) agar. Direct plating offers the advantage of allowing the analyst to quantify the populations of the *listeriae* cells in the food directly. Unfortunately, direct plating is rather insensitive and can detect only 100 CFU of *Listeria* per gram food. Thus, for smaller numbers of *listeriae*, some type of enrichment procedure must be employed, in order to resuscitate injured *listeriae* cells and to increase their numbers relative the background flora. The most widely used approach for isolating and detecting *Listeria* spp. uses one or two warm enrichment steps in nutritious broth, followed by plating onto a selective agar. Warm enrichment is based on the accelerated growth of *listeriae* at increased incubation temperature (30-37°C), with the addition of selective compounds to inhibit growth of competitive microflora. Table-26.2 summarise the most commonly used selective agent for isolation of *Listeria* spp.

Although the analysis time for these methods is dramatically reduced when compared to cold enrichment/plating methods (3-7 days versus several weeks), they are still too slow and laborious to be suited for routine applications. This has created a tremendous stimulus for developing alternative so-called rapid methods. Generally, these utilize a simplified warm enrichment procedure in combination with the latest developments in chromogenic and fluorogenic substrates, DNA and immuno-technology to reduce isolation and identifications times.

Table-26.2 Example of antimicrobial agents that are commonly used in *Listeria* selective broth and agar media

Antimicrobial Agent	mg/L	Organism inhibited
Lithium chloride	0.5	Gram positive bacteria
Acriflavin	5-25	gram-positives, including <i>Lactobacillus bulgaricus</i> and <i>Streptococcus thermophilus</i>
Nalidixic acid	20-40	gram-negatives except <i>Pseudomonas</i> and <i>Proteus</i>
Moxalactam, phenylethanol	20	Gram-negative bacteria
Ceftazidime	50	Gram-negative bacteria
Cycloheximide	50	Fungi
Fosfomycin	100	Inhibitory to <i>Staphylococcus</i> and <i>Bacillus</i>

26.4.2 Procedures for the isolation of *Listeria* spp. from dairy products

The FDA method employs a single enrichment step using FDA enrichment broth at 30°C.³² After 24 and 48 h the culture is streaked onto selective LiCl-phenylethanol-moxalactam (LPM) and Oxford/ PALCAM agars. The plates are then incubated at 30/35°C (LPM/ Oxford/ PALCAM) for 24-48 hours. Suspected plates are examined by using beamed white light striking the plate bottom at 45° angle. This technique is known as Henry's Light technique and shows *Listeria* colonies as sparkling blue or white. In the PALCAM agar, which contains the esculin/ ferric iron indicator system, *Listeria* colonies show up as black with a black halo under ordinary viewing and appears as light green colonies. Five or more typical *Listeria* colonies are picked and transferred to trypticase soy agar and incubated at 30 °C for 24-48 hours. Further identification is carried out by conventional morphological examination and biochemical tests.

The USDA method is a two-stage enrichment procedure involving primary enrichment in *Listeria* enrichment broth I (LEB I), secondary enrichment in Fraser broth and subsequent plating on modified Oxford agar (MXA). Typical *Listeria* colonies exhibit black halos from esculin hydrolysis. The ISO/ AFNOR method is similar to the USDA method, but uses 1/2 Fraser broth (with half the amount of selective agents) as the first enrichment step and complete Fraser broth as a second enrichment step. Both steps are followed by selective plating on PALCAM and/ or Oxford agars. Typical colonies on the Oxford agar show up as black due to esculin hydrolysis. The PALCAM agar contains two indicator systems: esculin/ ferric iron and D-mannitol with phenol red. This double diagnostic system allows for more easy distinction of *Listeria* from possible contaminating *enterococci* and *staphylococci*. On PALCAM agar *Listeria* colonies are green with black haloes against a pink-purple background. A summary of the ISO/ AFNOR method is presented in Figure 26.2.

26.4.3 Rapid enumeration of *Listeria* spp. using chromogenic media

Chromogenic and fluorogenic media are microbiological growth media that contain enzyme substrates linked to a chromogen (color reaction), fluorogen (light reaction) or a combination of both. The enzyme substrates, e.g. 5-Bromo-4-chloro-3-indolyl- β -D-glucoside, 5-Bromo-4-chloro-3-indoxyl myo-inositol-1 phosphate, ammonium salt, 4-Methylumbelliferyl myo-inositol-1-phosphate N-methyl-morpholine salt, are most commonly used chromogenic and fluorogenic substrate for detection of *L. monocytogenes*. The target population are characterized by enzyme systems that metabolize the substrate to release the chromogen/ fluorogen. This results in a colour change in the medium and/ or fluorescence under long wave UV light.

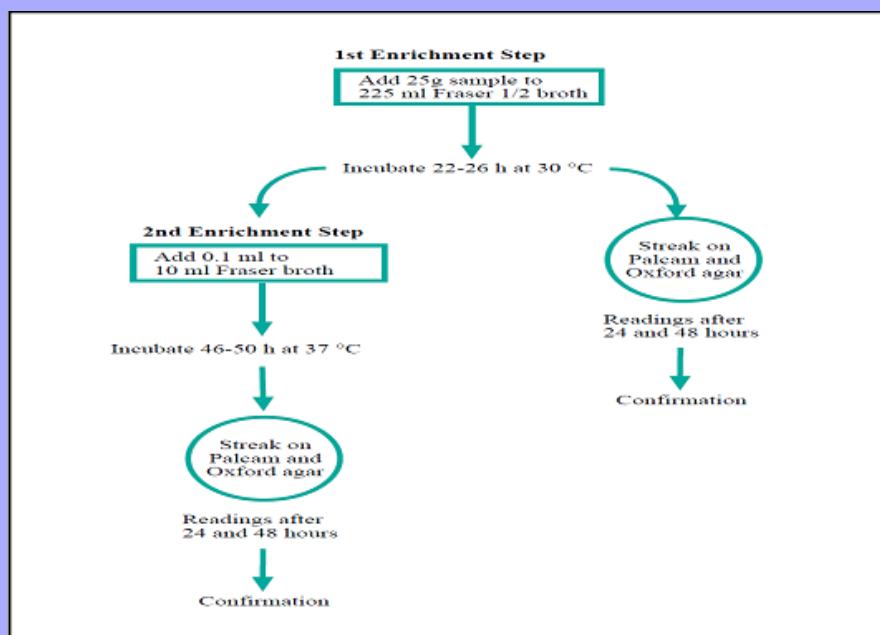
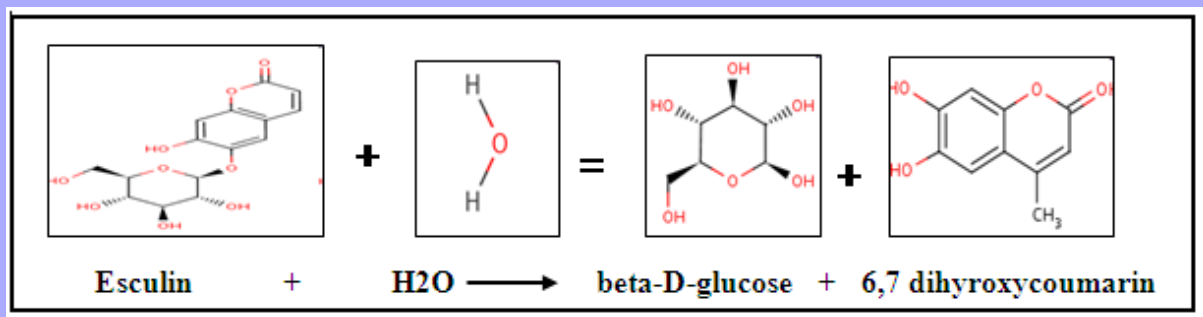


Fig. (26.2) Conventional enrichment method for the isolation and detection of *Listeria* according to the NF ISO 11290-1/ AFNOR standard

26.4.4 PALCAM agar base

26.4.4.1 Enumeration principle and interpretation

Differentiation on PALCAM Agar Base is based on esculin hydrolysis and mannitol fermentation. *Listeria* spp. hydrolyzes esculin, which appears as blackening in the medium. The blackening by esculin-hydrolyzing bacteria results from the formation of 6,7-dihydroxycoumarin, which reacts with ferric ions that are present in the medium as ferric ammonium citrate. Mannitol and the pH indicator, phenol red, have been added to differentiate mannitol-fermenting strains of possible contaminants, including *enterococci* and *staphylococci*. *Listeria* spp do not ferment mannitol, which is demonstrated by a color change in the colony and/or the surrounding medium from red or grey to yellow based on the production of acidic end products. Polymyxin B, acriflavin, ceftazidime, and lithium chloride are selective agents used to suppress Gram-negative and certain Gram-positive bacteria. Reaction catalysed by the enzyme beta-glucosidase which results in the formation of black color compound for detection of *Listeria* species



26.4.4.1.1 Colony characteristics of *listeria* species on PALCAM agar

Listeria spp. are presumptively indicated by grey-green colonies with a black precipitate following incubation for 24 - 48 hours at 35°C on PALCAM Agar Base

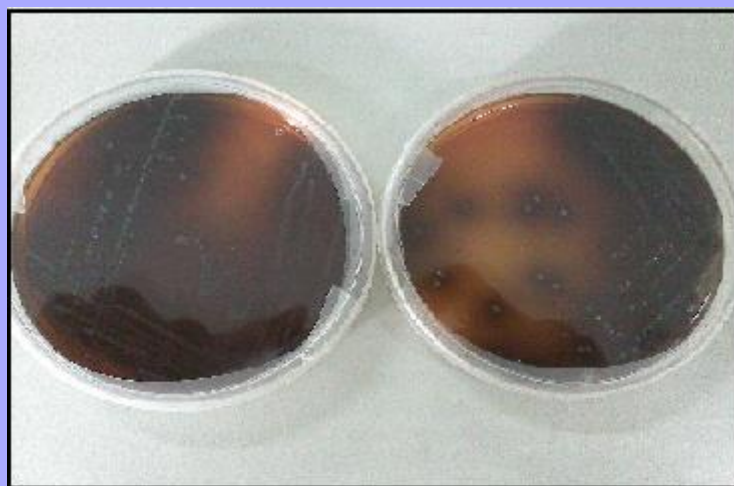


Fig. 26.3. PALCAM Agar

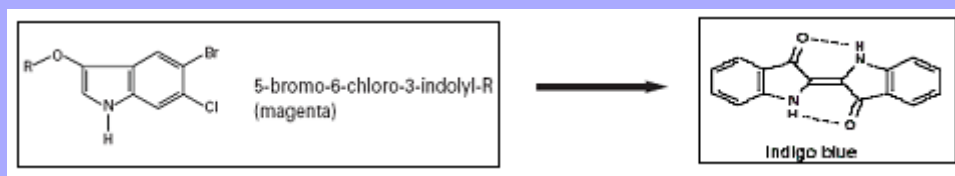
26.4.5 Chromocult *listeria* selective agar base

26.4.5.1 Enumeration principle and interpretation

The rich basis of Chromocult *Listeria* Selective Agar is the addition of 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside which makes it possible to differentiate between β-D-glucosidase positive and negative bacteria. *Listeria* is β-D-glucosidase-positive and grows on the medium in the form of blue-green colonies.

The addition of inhibitors results in a marked reduction in the growth of the majority of concomitant Gram-positive and Gram-negative pathogens, as well as of yeasts and fungi. To detect *L. monocytogenes* L- β -phosphatidylinositol is added to the medium. *L. monocytogenes* has the enzyme phosphatidylinositol phospholipase C (PI-PLC) described as a virulence factor. This phospholipase activity results in the formation of opaque haloes around *L. monocytogenes* colonies. Apart from *L. monocytogenes*, only *L. ivanovii* among the listeriae shows phospholipase C activity.

Beta-glucosidase forms indigo blur color by hydrolysing chromogenic substrate



26.4.5.1.1 Colony characteristics chromocult *listeria* selective agar base

All colonies which appear blue-green with an opaque halo on the medium are counted as suspect *L. monocytogenes* colonies (typical colonies) (Fig. 26.4).

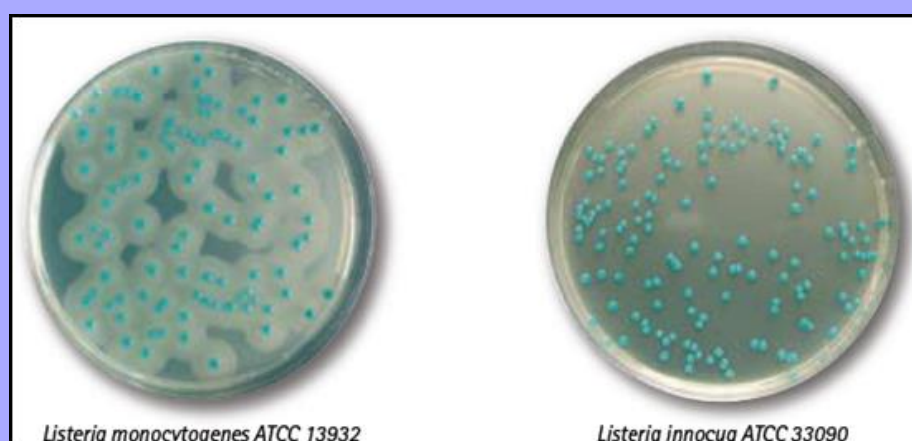


Fig. 26.4 Colony characteristics of *Listeria* spp. on Chromocult *Listeria* Selective Agar Base

26.4.6 HiCrome listeria agar base, modified

26.4.6.1 Enumeration principle and interpretation

HiCrome Listeria Agar Base, allows growth of only *Listeria* species and gives a specific and direct identification of *L. monocytogenes* within 24-48 hours after pre-enrichment. This medium is based on both, the specific beta-glucosidase activity detection and the rhamnose fermentation. The colonies of *L. ivanovii* appear blue without a yellow halo (rhamnose -ve) while the colonies of *L. monocytogenes* and *L. innocua* are blue with a yellow halo (rhamnose +ve). Peptone, yeast extract and meat extract provide nitrogenous substances, vitamin B complex and other essential growth nutrients. Rhamnose is the fermentable carbohydrate with phenol red as an indicator. Sodium chloride maintains the osmotic equilibrium. The added lithium chloride and Hi-Crome *Listeria* Selective Supplement inhibit growth of most Gram positive bacteria, Gram negative bacteria, yeasts and moulds.

26.4.6.1.1 Colony characteristics

The colonies of *L. ivanovii* appear blue without a yellow halo (rhamnose -ve) while the colonies of *L.*

monocytogenes and *L. innocua* are blue with a yellow halo (rhamnose +ve) (Fig. 26.5).

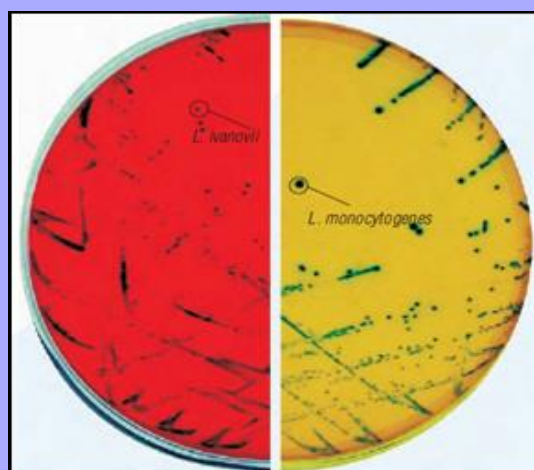


Fig. 26.5. *Listeria* species on hiCrome *listeria* agar base

26.4.7 ALOA chromogenic agar

26.4.7.1 Enumeration principle and interpretation

To minimise the growth of contaminating organisms, lithium chloride and a balanced antimicrobial mixture is employed. The incorporation of the chromogenic substrate X-glucoside for the detection of β -glucosidase demonstrates the presence of *Listeria* spp., whilst the detection of a specific phospholipase C enzyme produced by pathogenic *Listeria* spp. including *L. monocytogenes* is also achieved. *Listeria* spp. grow on this medium producing blue/ green colonies, with pathogenic species producing similar coloured colonies surrounded by a characteristic opaque halo after 24 hours incubation at 37°C. Non-*Listeria* spp. produces white colonies.

26.4.7.1.1. Colony characteristics

All *Listeria* spp. Examined produced typical green – blue coloured colonies 1.0 – 2.0 mm in diameter after 24 hours incubation, with all *L. monocytogenes* producing a distinctive opaque halo. Strains of *L. ivanovii* also showed an opaque halo.

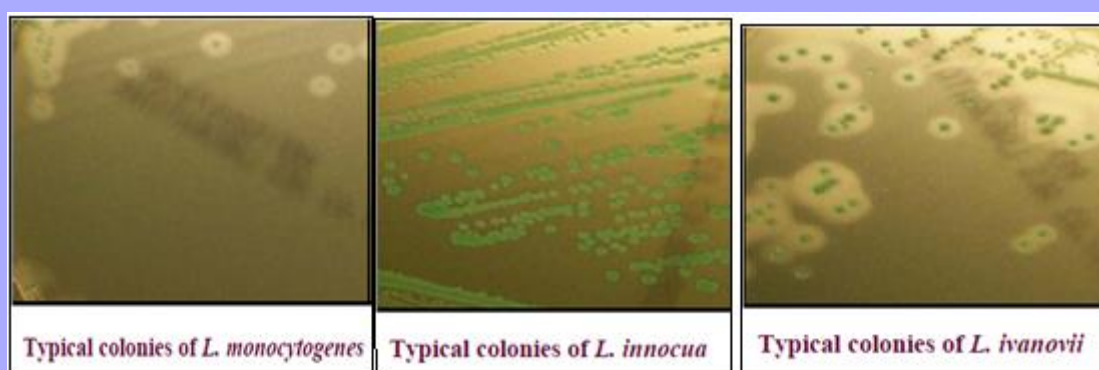


Fig. 26.6 *Listeria* species on ALOA chromogenic agar

26.4.8 3M™ petrifilm™ environmental listeria plates

The 3M Petrifilm Environmental *Listeria* Plate is a sample-ready culture medium used for the detection and/or
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enumeration of *Listeria* in environmental samples. Petrifilm Environmental *Listeria* Plate detects the following *Listeria* species as red-violet colonies: *Listeria monocytogenes*, *Listeria innocua* and *Listeria welshimeri*, but does not differentiate these organisms from one another. A repair broth (5 mL of buffered peptone water) is added to the collected environmental sample and allowed to remain at room temperature for 1 hour. This short repair step is not an enrichment step. Three millilitres of the sample is pipetted onto the 3M Petrifilm Environmental *Listeria* Plate and the plate is then incubated for 26-30 hours. The 3M Petrifilm Environmental *Listeria* Plate methods may be used as a qualitative, quantitative or semi-quantitative test:

- A qualitative interpretation may be desired if a yes/ no answer is sufficient and appropriate
- A quantitative interpretation may be desired if different actions will be taken based upon the number present
- A semi-quantitative interpretation may be desired if different actions will be taken depending on the relative level present, and/ or if recording actual numbers is not desirable.

26.4.8.1 Colony characteristics

Following *Listeria* species as red-violet colonies: *Listeria monocytogenes*, *Listeria innocua* and *Listeria welshimeri*

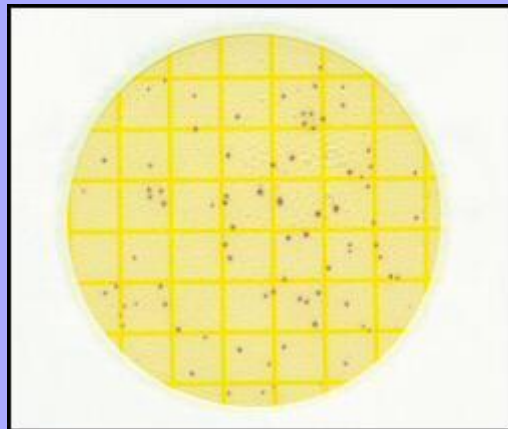
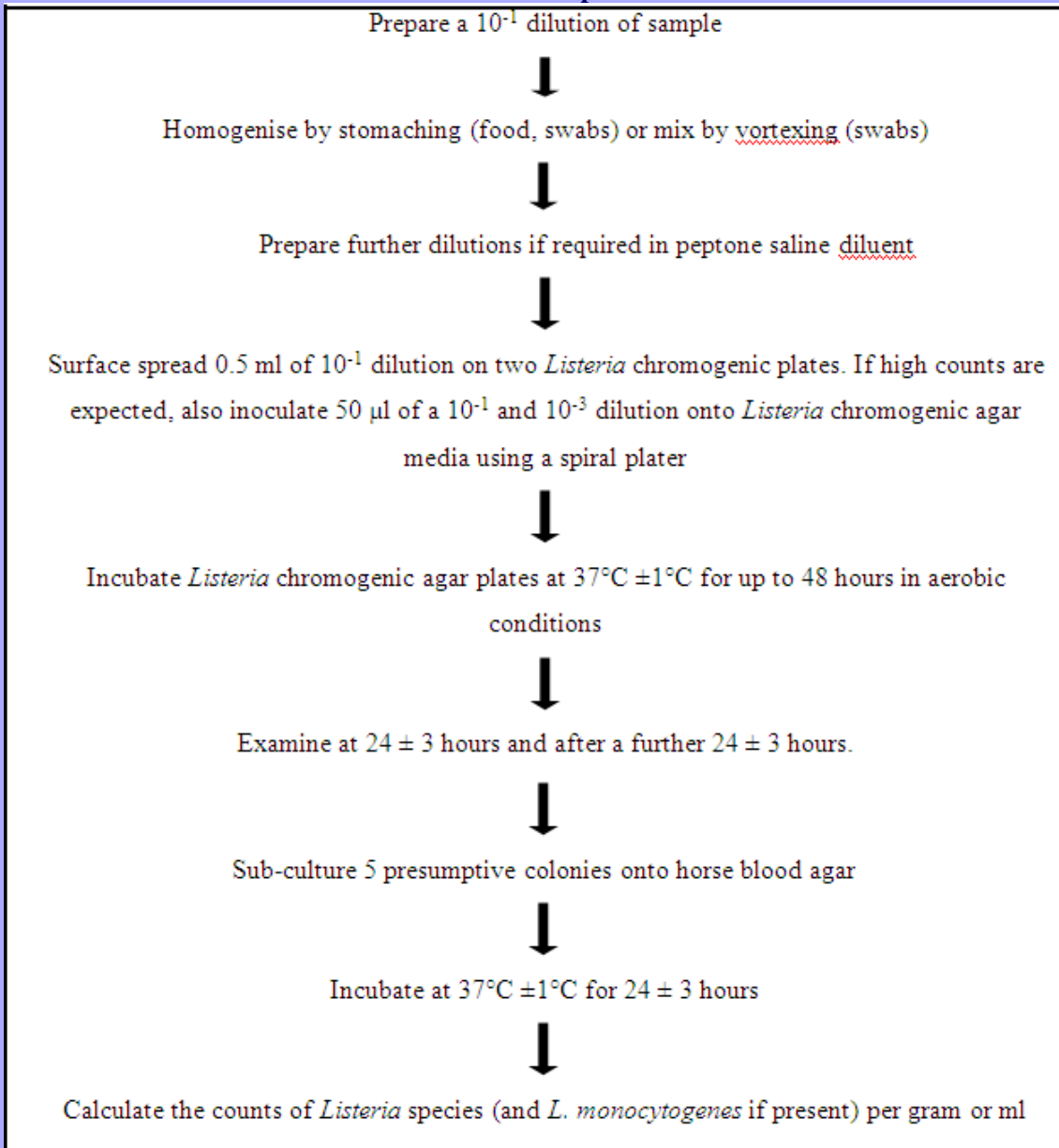


Fig 26.7 Colony characteristics of *listeria* species on 3M™ Petrifilm™

26.5 Enumeration procedure



Lesson 27

ENUMERATION OF *STAPHYLOCOCCUS AUREUS*

27.1. Introduction

Staphylococcus aureus is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation. *S. aureus* can cause severe food poisoning. It has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals and family groups than the records show. Foods are examined for the presence of *S. aureus* and/or its enterotoxins to confirm that *S. aureus* is the causative agent of food borne illness, to determine whether a food is a potential source of "staph" food poisoning, and to demonstrate post-processing contamination, which is generally due to human contact or contaminated food-contact surfaces. Conclusions regarding the significance of *S. aureus* in foods should be made with circumspection. The presence of a large number of *S. aureus* organisms in a food may indicate poor handling or sanitation; however, it is not sufficient evidence to incriminate a food as the cause of food poisoning. The isolated *S. aureus* must be shown to produce enterotoxins. Conversely, small staphylococcal populations at the time of testing may be remnants of large populations that produced enterotoxins in sufficient quantity to cause food poisoning. Therefore, the analyst should consider all possibilities when analyzing a food for *S. aureus*. Methods used to detect and enumerate *S. aureus* depend on the reasons for testing the food and on the past history of the test material. Studies of colonial morphology on Baird-Parker agar, lysostaphin sensitivity, coagulase and thermo-nuclease production, and glucose and mannitol fermentation were conducted on 100 enterotoxaemia and 51 non enterotoxaemia strains of *S. aureus*. In all cases, the reactions of enterotoxaemia and non enterotoxaemia strains varied by 12% or less. This research indicates that none of these tests can be relied upon to differentiate toxic and nontoxic staphylococci.

27.2. Enumeration Principle of *S. aureus*

- This medium contains lithium chloride and tellurite to inhibit the growth of accompanying microbial flora, whereas pyruvate and glycine selectively stimulate the growth of *staphylococci*
- *Staphylococcus* colonies show two characteristic features when grown in this opaque medium (opaque, because of its egg-yolk content)

- a) characteristic zones and rings are formed as a result of lipolysis and proteolysis,
- b) Reduction of tellurite to tellurium produces a black colouration.

- The egg-yolk reaction and tellurite reduction are usually found to occur together with a positive coagulase reaction recommend that egg-yolk should be replaced with blood plasma, if coagulase-positive *staphylococci* are to be detected directly recommend the addition of sulphamethazine to suppress the growth and swarming of *Proteus* species. Coagulase is an enzyme capable of coagulating blood plasma.
- The coagulase formation by *S. aureus* and its formation of enteric toxins are very closely related. The Coagulase test is an important indicator for the pathogenicity of *Staphylococcus* strains. *S. aureus* forms two kinds of coagulase. Free coagulase is an extracellular enzyme which reacts with a coagulase-reacting factor in plasma (pro-thrombin or its derivatives). Bound coagulase, also known as clumping factor, is localized on the surface of the cell wall and reacts with α - and β -chains of the plasma fibrinogens to form a coagulate. Because this coagulase test is carried out in a test tube both forms of coagulase are measured.

27.2.1 Typical composition (g/ litre)

Peptone from casein 10.0; meat extract 5.0; yeast extract 1.0; sodium pyruvate 10.0; glycine 12.0; lithium

chloride 5.0; agar-agar 15.0. Also to be added: egg-yolk tellurite emulsion 50 ml; if required, added sulphamethazine 0.05 g/l.

27.2.2 Preparation

Suspend 58 g in 0.95 litre, autoclave (15 min at 121°C). Cool to 45-50°C, mix in 50 ml egg yolk tellurite emulsion and, if required, 50 mg sulphamethazine/ litre. Pour plates. pH: 6.8 ±0.2 at 27°C. The plates are opalescent and yellowish-brown in colour. The ready-to-use culture medium can be stored in the refrigerator (approx. 4°C) for up to one month.

27.2.3 Typical Colony characteristics

Typical *S. aureus* colonies are convex, irregular shape, black, shiny 1-5 mm in diameter with a narrow, white edge surrounded by a clear zone 2-5 mm wide. Opaque rings within the clear zones only appear after 48 hours of incubation (Fig. 27.6). Opaque zone develop around the colonies after 24 hours. *S. epidermis* growth occurs sometimes: Very small, brown to neither black, non clear zones. *Micrococcus* with dark brown, dull, clear zones sometimes appear after 48 hours. *Bacillus* species are white; yeasts have no clear zones.

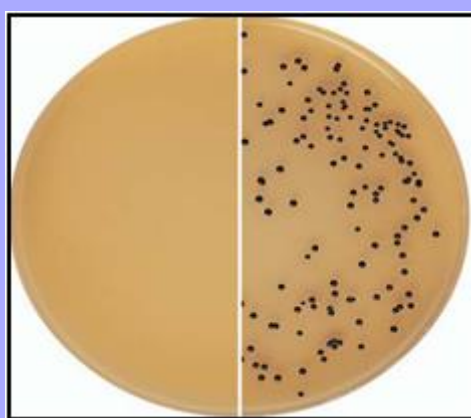


Fig. 27.1 *S. aureus* on Baird-Parkar agar

27.2.4 Enumeration, isolation and identification of *Staphylococcus aureus*

S. aureus is a facultative anaerobic, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. Some strains of *S. aureus* also produce an enterotoxin that is the causative agent of *S. aureus* gastroenteritis. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowned plates, gray to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone.

27.2.4.1 Media and reagents

Use hydrogen peroxide, Baird-Parker medium & Buffer peptone water for enrichment of sample.

27.2.4.2 Sample preparation

Using aseptic technique, weigh 25 g of sample into sterile blender jar or macerated with sterile mortar pestle. Add 225 ml Butterfield's phosphate-buffered dilution water (1:10 dilution) and mixed well for 2 min. using the 1:10 dilution, make serial dilutions of sample for enumeration of *S. aureus*.

27.2.4.3 Direct plate counts

For each dilution to be plated, aseptically transfer one ml sample suspension to 3 plates of Baird-Parker agar, distributing one ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is

absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily absorbed, place plates upright in incubator for about one hour. Invert plates and incubate 45-48 hours at 35°C. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test.

27.3 Coagulase Test (Clotting of dilute mammalian blood)

27.3.1 Principle

S. aureus is known to produce coagulase, which can clot plasma into gel in tube or agglutinate cocci on slide. This test is useful in differentiating *S. aureus* from other coagulase-negative *staphylococci*. Most strains of *S. aureus* produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellularly, bound coagulase is a cell wall associated protein. Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of *S. aureus* and tube coagulase may be used for confirmation. While there are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists. Free coagulase is heat labile while bound coagulase is heat stable.

27.3.2 Procedure

Dense suspensions of *Staphylococci* from culture are made on two ends of clean glass slide. One should be labelled as “test” and the other as “control”. The control suspension serves to rule out false positivity due to autoagglutination. The test suspension is treated with a drop of citrated plasma and mixed well. Agglutination or clumping of cocci within 5-10 seconds is taken as positive (Fig. 27.2). Some strains of *S. aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test.

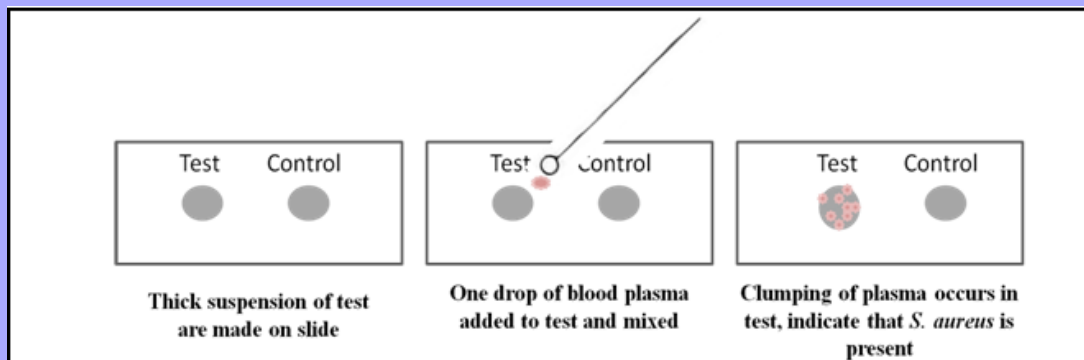


Fig. 27.2 Coagulase Test

27.4 DNase Test

27.4.1 Principle

Colonies producing DNase hydrolyse the deoxyribonucleic acid (DNA) content of this medium located in their immediate vicinity. If the medium is then flooded and acidified with 1 N HCl (acid kills the viable cells), the DNA precipitates out (turbidity) and clear zones appear around DNase-positive colonies. Some authors recommend instead flooding the medium with toluidine blue solution or the use of DNase test agars containing toluidine blue or methyl green. These dyes form coloured complexes with DNA. Presumptive pathogenic *S. aureus* shows colour changes on DNA hydrolysis with pink zone or almost colourless on media containing toluidine blue and methyl green respectively. The pathogenic *S. aureus* can also be differentiated by exploiting the fact that they metabolize mannitol to form acid, in this case mannitol and a pH indicator (phenol red) must be added to the culture medium.

27.4.2 Procedure

1. Inoculate by streaking a pure culture of the organism to be tested onto the surface of the test agar.
2. Incubate under optimal conditions (in the case of staphylococci, 24 hours at 35°C aerobically).
3. When necessary first check the plates for mannitol fermentation, and then carefully flood the surface of the plates with 1 N hydrochloric acid.

27.4.3 Result and interpretation

Before addition of 1 N HCl into the medium look for yellow surrounded by a yellow zone for by mannitol positive *S. aureus*. After addition of 1N HCl into the medium HCl appears yellow, surrounded by a yellow zone before addition of 1N HCl, it appears well defined, pink or clearer zones for coagulase positive *S. aureus* otherwise turbid culture medium



Lesson 28

ENUMERATION OF *BACILLUS CEREUS*, *CLOSTRIDIUM BOTULINUM* AND *CLOSTRIDIUM PERFRINGENS***28.1 Introduction**

Bacillus cereus rods are aerobic, spore forming Gram-positive with square ends in short to long chains. The spores are ellipsoidal, central to sub terminal, thin-walled and do not swell. *B. cereus* is responsible for a minority of food borne illnesses causing severe nausea, vomiting and diarrhoea. It produces pinkish color colonies on MYP medium. Food poisoning caused by *B. cereus* may occur when foods are prepared and held without adequate refrigeration for several hours before serving. *B. cereus* is commonly found in soil, on vegetables, and in many raw and processed foods. Consumption of foods that contain $>10^6$ *B. cereus*/g may result in food poisoning. Foods incriminated in past outbreaks include cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts. Two types of illness have been attributed to the consumption of foods contaminated with *B. cereus*. The first and better known type is characterized by abdominal pain and diarrhoea; it has an incubation period of 4-16 hours and symptoms that last for 12-24 hours. The second, which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of contaminated food; diarrhoea is not a common feature in this type of illness. Twenty, mostly European laboratories took part in a collaborative study to validate the general 1993 ISO 7932 standard for the enumeration of *B. cereus* in foods. The precision of the test method appeared to be unaffected by the type of food or the concentration of *B. cereus* present in the test sample.

28.2. Enumeration Principle of *B. cereus*

- *B. cereus* is mannitol-negative. The mannitol content of the medium thus allows differentiation of the accompanying mannitol-positive microbial flora which is identified by a change in colour of the indicator phenol red to yellow.
- *B. cereus* is not affected by concentrations of polymyxin which inhibit the common accompanying microbial flora. Addition of polymyxin is necessary, however, if the sample material is suspected to contain high numbers of accompanying microorganisms.
- *B. cereus* produces lecithinase. The insoluble degradation products of egg-yolk lecithin accumulate around the *B. cereus* colonies to form a white precipitate.
- A lecithinase reaction occurs very early in many strains, *B. cereus* colonies can, therefore, often be rapidly identified before accompanying polymyxin-resistant microorganisms have had a chance to fully develop.
- *B. cereus* selective supplement contains polymyxine-B in lyophilized form.
- It suppresses the growth of the accompanying bacterial flora during the culture of *B. cereus*.

28.3 Mannitol-Egg-yolk-Polymyxine-Agar (MYP Agar)**28.3.1 Typical composition (g/litre)**

Peptone from casein 10.0; meat extract 1.0; D (-) mannitol 10.0; sodium chloride 10.0; phenol red 0.025; agar-agar 12.0 Also to be added (per litre of medium); egg-yolk emulsion 100 ml; polymyxin B sulfate 100,000 IU *B. cereus* Selective Supplement.

28.3.2 Preparation

Suspend 21.5 g in 450 ml de-mineralized water, autoclave (15 min at 121°C). Cool to about 45 to 50 °C add 50 ml (this volume can be varied depending on the degree of turbidity desired) of sterile egg-yolk emulsion and

the contents of one vial *B. cereus* Selective Supplement, mix. pH: 7.2 ± 0.2 at 25 °C. Pour plates. The plates (including egg-yolk) are evenly turbid and slightly orange (red without egg-yolk).

28.3.3 Typical colony characteristics

B. cereus appears as rough, dry colonies with a pink to purple base which are surrounded by a ring of dense precipitate (Fig. 28.1). Colonies surrounded by yellow or a clear zone are not *B. cereus*. Further tests should be performed to confirm the identity of *B. cereus* (anaerobic degradation of D (+) glucose, degradation of gelatin, positive nitrate reduction)

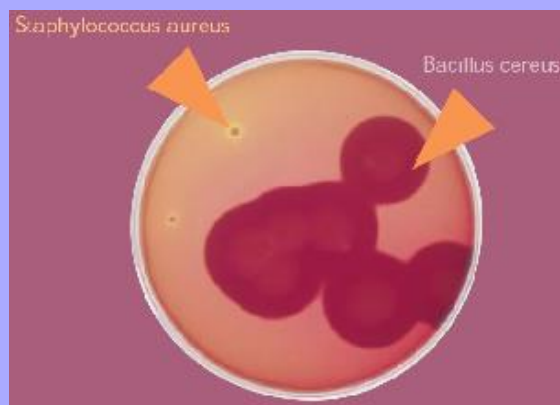


Fig. 28.1 *B. cereus* colonies on mannitol-egg-yolk-polymyxine-agar (MYP Agar)

28.4 Detection of *B. cereus*

Enumeration, isolation and identification of *B. cereus* has been described in three basic steps

1. Direct plating on (selective) media
2. Direct (selective) enrichment
3. Pre-enrichment, followed by a selective enrichment

Depending on the number of cells expected in a sample and/or the standards described, one or more of these procedures may be used for the detection or enumeration of bacteria. Often an enrichment procedure is required for the detection of pathogens, whereas, due to higher expected numbers, direct plating is possible for the detection of indicator and spoilage organisms. The success of the basic protocols depends on: 1) the number and the state of the micro-organisms in the sample; 2) the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism); 3) conditions of incubation (time, temperature, presence of oxygen) and 4) the selectivity of the isolation medium (the ease of distinguishing the target organism from competitive micro flora). Another improvement on the traditional method is the development of chromogenic isolation and enumeration media, which make it easy to distinguish the target organisms.

28.4.1 Media and reagent

Mannitol egg-yolk polymyxin agar (MYPA) base plates, polymyxin B solution for MYPA (0.1%), Voges-Proskauer medium & reagent, Nutrient agar for *B. cereus* & Butterfield's Phosphate-buffered dilution water.

28.4.2 Sample preparation

Using aseptic technique, weigh 25 g of sample into sterile blender jar or macerate with sterile mortar pestle. Add 225 ml Butterfield's phosphate-buffered dilution water (1:10 dilution) and mixed well for 2 min. Using the 1:10 dilution, make serial dilutions of sample for enumeration of *B. cereus*.

28.4.3 Plate count of *B. cereus*

Prepare serial dilutions from 10⁻² to 10⁻⁶ by transferring 10 ml homogenized sample (1:10 dilution) to 90 ml dilution blank, mixing well with vigorous shaking, and continuing until 10⁻⁶ dilution is reached. Inoculate duplicate MYPA plates with each dilution of sample (including 1:10) by spreading 0.1 ml evenly onto surface of each plate with sterile glass spreading rod. Incubate plates 24 hours at 30°C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced. *B. cereus* colonies are usually a pink color which becomes more intense after additional incubation.

28.4.4 Confirmation of *B. cereus*

Pick 5 or more eosin pink, lecithinase-positive colonies from MYPA plates and transfer to nutrient agar slants. Incubate slants 24 hours at 30°C. Prepare Gram-stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to sub terminal.

28.5 *Clostridium Perfringens*

Food poisoning caused by *Clostridium perfringens* may occur when foods such as meat or poultry are cooked and held without maintaining adequate heating or refrigeration before serving. The presence of small numbers of *C. perfringens* is not uncommon in raw meats, poultry, dehydrated soups and sauces, raw vegetables, and spices. Because the spores of some strains are resistant to temperatures as high as 100°C for more than one hour, their presence in foods may be unavoidable. Furthermore, the oxygen level may be sufficiently reduced during cooking to permit growth of the *Clostridia*. Spores that survive cooking may germinate and grow rapidly in foods that are inadequately refrigerated after cooking. Thus, when clinical and epidemiological evidence suggests that *C. perfringens* is the cause of a food poisoning outbreak, the presence of hundreds of thousands or more of these organisms per gram of food substantiates the diagnosis.

Illness typically occurs 8-15 hours after ingestion of the contaminated food. The symptoms, which include intense abdominal cramps, gas, and diarrhea (nausea and vomiting are rare), have been attributed to a protein enterotoxin produced during sporulation of the organism in the intestine. The enterotoxin can be detected in sporulating cultures, and a method for this purpose is included. A high correlation has been established between the ability of *C. perfringens* strains to produce enterotoxin and their ability to cause food poisoning. However, it is difficult to obtain consistent sporulation with some strains.

C. perfringens cells lose their viability when foods are frozen or held under prolonged refrigeration unless special precautions are taken. Such losses may make it difficult to establish *C. perfringens* as the specific cause of a food poisoning outbreak. It is recommended that samples which cannot be examined immediately be treated with buffered glycerin-salt solution and stored or shipped frozen to the laboratory as described below.

28.5.1 Principle

Conventional methods for the detection of clostridia have traditionally incorporated heat killing of vegetative cells of clostridia and contaminating bacteria (to identify the presence or quantify the clostridial spores present). This is followed by the use of a nutritionally rich base medium, e.g. meat broth or blood agars, to promote spore germination. The addition of starch in many media is to facilitate germination and in some methods gentle heating of the sample prior to inoculation is recommended.

Reinforced Clostridial Medium (RCM) is based on non-selective growth of contaminating bacteria so some media now contain inhibitors and other selective agents. Sulphide and an iron source are usually used as indicators. The clostridia reduce the sulphide to sulphite which gives a black precipitate with the iron present in the medium. Sulphite reducing clostridia are then enumerated as black colonies if solid media is used.

Sodium sulphite and ferric citrate may be added to RCM to become differential RCM (DRCM) which was recommended for the detection of sulphite reducing clostridia in drinking water and is specified in the ISO standard 6461-1(1986) liquid enrichment method for water.

Tryptose sulphite cycloserine agar (TSC) was a medium for detection of vegetative and spore forms of *C.*

perfringens in foodstuffs and clinical specimens, which contains cycloserine as an inhibitor of accompanying bacterial flora and causes the colonies which develop to remain smaller. In addition to incorporation of sulphite and iron, this medium utilizes sulphadiazine, oleandomycin phosphate and polymyxin B sulphate to give a high degree of selectivity and specificity for *C. perfringens*. Other *Clostridium* species, e.g. *C. bifermentans* and *C. butyricum*, are inhibited.

28.5.2 Plate count of viable *C. perfringens*.

Using aseptic technique, place 25 g food sample in sterile blender jar. Add 225 ml peptone dilution fluid (1:10 dilution). Homogenize 1-2 min at low speed. Obtain uniform homogenate with as little aeration as possible. Using 1:10 dilution prepared above, make serial dilutions from 10⁻¹ to 10⁻⁶ by transferring 10-90 ml peptone dilution fluid blanks. Mix each dilution thoroughly by gently shaking before each transfer. Pour 6-7 ml TSC agar without egg yolk into each of ten 100 × 15 mm petri dishes and spread evenly on bottom by rapidly rotating dish. When agar has solidified, label plates, and aseptically transfer one ml of each dilution of homogenate to the center of duplicate agar plates. Pour additional 15 ml TSC agar without egg yolk into dish and mix with inoculum by gently rotating dish.

28.5.2.1 TSC agar containing egg yolk emulsion

An alternative plating method preferred for foods containing other types of sulphite-reducing organisms is to spread 0.1 ml of each dilution with sterile glass rod spreader over previously poured plates of TSC agar containing egg yolk emulsion. After inoculum has been absorbed (about 5 min), overlay plates with 10 ml TSC agar without egg yolk emulsion. When agar has solidified, place plates in upright position in anaerobic jar. Establish anaerobic conditions and place jar in 35°C incubator for 20-24 hours. (TSC agar containing egg yolk is incubated 24 hours). After incubation, remove plates from anaerobic jar and select those containing 20-200 black colonies for counting. *C. perfringens* colonies in egg yolk medium are black with a 2-4 mm opaque white zone surrounding the colony as a result of lecithinase activity.

28.5.3 Presumptive confirmation test

Select 10 typical *C. perfringens* colonies from TSC or TSC-egg yolk agars plates and inoculate each into a tube of freshly deaerated and cooled fluid thioglycollate broth. Incubate in standard incubator 18-24 hours at 35°C. Examine each culture by Gram stain and check for purity. *C. perfringens* is a short, thick, Gram-positive bacillus. If there is evidence of contamination, streak contaminated culture on TSC agar containing egg yolk and incubate in anaerobic jar 24 hours at 35°C. Surface colonies of *C. perfringens* are yellowish gray with 2-4 mm opaque zones caused by lecithinase activity. This procedure is also used for isolating *C. perfringens* from chopped liver broth whenever the organism is not detected by direct plating on TSC agar.

28.5.4 Completed confirmation test

28.5.4.1 Gelatin liquefaction

Stab-inoculate motility-nitrate (buffered) and lactose-gelatin media with 2 mm loopfuls of pure fluid thioglycollate medium culture or portion of isolated colony from TSC agar plate. Stab lactose-gelatin repeatedly to ensure adequate inoculation, and then rinse loop in beaker of warm water before flaming to avoid splattering. Incubate inoculated media 24 hours at 35°C. Examine lactose-gelatin medium cultures for gas production and color change from red to yellow, which indicates acid production. Chill tubes for one hour at 5°C and examine for gelatin liquefaction. If medium gels, incubate an additional 24 hours at 35°C and examine for gelatin liquefaction.

28.5.4.2 Gram's staining

Inoculate sporulation broth with one ml fluid thioglycollate medium culture and incubate 24 hours at 35°C. Prepare Gram stain of sporulation broth and examine microscopically for spores. Store sporulated cultures.

28.5.4.3 Motility

C. perfringens is non-motile. Examine tubes of motility-nitrate medium for type of growth along stab line. Non-motile organisms produce growth only in and along stab. Motile organisms usually produce diffuse growth out into the medium, away from the stab.

28.5.4.4 Nitrate reduction

C. perfringens reduces nitrates to nitrites. To test for nitrate reduction, add 0.5 ml reagent A and 0.2 ml reagent B (R48) to culture in buffered motility-nitrate medium. Violet colour which develops within 5 min indicates presence of nitrites. If no color develops, add a few grains of powdered zinc metal and let stand a few minutes. A negative test (no violet color) after zinc dust is added indicates that nitrates were completely reduced. A positive test after addition of zinc dust indicates that the organism is incapable of reducing nitrates.

28.6 *Clostridium Botulinum*

Clostridium botulinum is an anaerobic, rod-shaped spore forming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism.

There are seven recognized antigenic types: A through G. Cultures of five of these types apparently produce only one type of toxin but all are given type designations corresponding to their toxin production. Types C and D cross-react with antitoxins to each other because each antigenic type produces more than one toxin and have at least one common toxin component. Type C produces predominantly C1 toxin with lesser amounts of D and C2, or only C2, and type D produces predominantly type D toxin along with smaller amounts of C1 and C2. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized. There is a slight reciprocal cross-neutralization with types E and F, and recently a strain of *C. botulinum* was shown to produce a mixture of predominantly type A toxin, with a small amount of type F.

28.6.1 Pathogenicity

Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic. All type E strains and the remaining B and F strains are non-proteolytic, with carbohydrate metabolic patterns differing from the C and D non-proteolytic groups. Strains those produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other sea-foods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of food-borne illness caused by these types. Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinum spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not contain viable *C. botulinum*. Home-canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners' great awareness and better control of the required heat treatment.

28.6.2 Growth characteristics

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms do not grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in otherwise suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity (aw), a high concentration of NaCl, an inhibitory concentration of NaNO₂ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by non-proteolytic strains unless the temperature is precisely controlled and kept below 3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for non-proteolytic strains it is 26-28°C. Non-proteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the non-proteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulin toxin in the blood, feces, or vomitus of the patient. Specimens must be collected before botulin antitoxin is administered to the patient. Identifying the causative food is most important in preventing additional cases of botulism.

28.6.3 Enumeration and detection

28.6.3.1 Sample preparation

1. Refrigerate samples until testing, except unopened canned foods, which need not be refrigerated unless badly swollen and in danger of bursting. Before testing, record product designation, manufacturer's name or home canner, source of sample, type of container and size, labeling, manufacturer's batch, lot or production code, and condition of container. Clean and mark container with laboratory identification codes.
2. Solid and liquid foods. Aseptically transfer foods with little or no free liquid to sterile mortar. Add equal amount of gel-phosphate buffer solution and grind with sterile pestle before inoculation. Alternatively, inoculate small pieces of product directly into enrichment broth with sterile forceps. Inoculate liquid foods directly into enrichment broth with sterile pipettes. Reserve sample; after culturing, aseptically remove reserve portion to sterile sample jar for tests which may be needed later. Refrigerate reserve sample.
3. Opening of canned foods. Examine product for appearance and odour. Note any evidence of decomposition. Do Not Taste the product under any circumstances. Record the findings.

28.6.3.2 Enrichment

Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation. Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth. Incubate at 35°C. Inoculate two tubes of TPGY broth as above. Incubate at 28°C. Use TPGYT (Trypticase-peptone-glucose-yeast extract) as alternative only when organism involved is strongly suspected of being a non-proteolytic strain of types B, E, or F. Introduce the inoculum slowly beneath surface of broth to bottom of tube. After 5 days of incubation, examine enrichment cultures. Check for turbidity, gas production, and digestion of meat particles. Note the odour.

Examine cultures microscopically by wet mount under high-power phase contrast, or a smear stained by Gram reagent, crystal violet, or methylene blue under bright-field illumination. Observe morphology of organisms and note existence of typical clostridia cells, occurrence and relative extent of sporulation, and location of spores within cells. A typical clostridia cell resembles a tennis racket. At this time test each enrichment culture for toxin, and if present, determine toxin type according to procedure in F, below. Usually, a 5-day incubation is the period of active growth giving the highest concentration of botulin toxin. If enrichment culture shows no growth at 5 days, incubate an additional 10 days to detect possible delayed germination of injured spores

before discarding sample as sterile. For pure culture isolation save enrichment culture at peak sporulation and keep under refrigeration.

29.6.3.3 Isolation of pure cultures

C. botulinum is more readily isolated from the mixed flora of an enrichment culture or original specimen if sporulation has been good.

29.6.3.4 Pre-treatment of specimens for streaking

Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube. Mix well and incubate 1 hour at room temperature. To isolate from sample, take 1 or 2 ml of retained portion, and add an equal volume of filter-sterilized absolute alcohol in sterile screw-cap tube. Mix well and incubate 1 hour at room temperature. Alternatively, heat (80°C for 10-15 min) 1 or 2 ml of enrichment culture or sample to destroy vegetative cells.

29.6.3.5 Plating of treated cultures

With inoculating loop, streak 1 or 2 loopfuls of ethanol or heat-treated cultures to either liver- veal-egg yolk agar or anaerobic egg yolk agar (or both) to obtain isolated colonies. If necessary, dilute culture to obtain well-separated colonies. Dry the agar plates well before use to prevent spreading of colonies. Incubate streaked plates at 35°C for about 48 hours under anaerobic conditions. A Case anaerobic jar or the GasPak system is adequate to obtain anaerobiosis; however, other systems may be used.

29.6.3.5.1 Selection of typical *C. botulinum* colonies

Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough. Colonies commonly show some spreading and have an irregular edge. On egg yolk medium, they usually exhibit surface iridescence when examined by oblique light. This luster zone, often referred to as a pearly layer, usually extends beyond and follows the irregular contour of the colony. Besides the pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate. Colonies of types A and B generally show a smaller zone of precipitation. Considerable difficulty may be experienced in picking toxic colonies since certain other members of the genus *Clostridium* produce colonies with similar morphological characteristics but do not produce toxins.

29.6.3.5.2 Isolation of pure culture

Re-streak toxic culture in duplicate on egg yolk agar medium. Incubate one plate anaerobically at 35°C. Incubate second plate aerobically at 35°C. The culture may be pure if colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate). Failure to isolate *C. botulinum* from at least one of the selected colonies means that its population in relation to the mixed flora is probably low. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.



Lesson 29

RAPID ENUMERATION OF HYGIENE AND SAFETY INDICATOR ORGANISMS

29.1 Introduction

Food spoilage is an enormous economic problem worldwide. Through microbial activity alone, approximately one-fourth of the world's food supply is lost. Milk is a highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms. The microbiological quality of milk and dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatment contamination. Undesirable microbes that can cause spoilage of dairy products include Gram-negative psychrotrophs, coliforms, lactic acid bacteria, yeasts, and molds. In addition, various bacteria of public health concern such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, pathogenic strains of *Escherichia coli* and enterotoxigenic strains of *Staphylococcus aureus* may also be found in milk and dairy products. For this reason, increased emphasis should be placed on the microbiological examination of milk and dairy foods. Microbiological analyses are critical for the assessment of quality and safety, conformation with standards and specifications, and regulatory compliance.

Although bacteria are the first type of microorganisms that come to mind when discussing microbial food safety, they are by no means the only pathogenic food borne microorganisms. Mycotoxin producing moulds, human enteric viruses, protozoan parasites and marine biotoxins are also of importance. However, since foods are only screened for bacteria routinely, in this article we will focus on the techniques used to detect bacterial contamination in food product by traditional methods.

Three basic principles for isolation and identification have been described for the detection of microorganisms are:

1. Direct plating on (selective) media
2. Direct (selective) enrichment
3. Pre-enrichment, followed by a selective enrichment

Depending on the number of cells expected in a sample and/or the standards described, one or more of these procedures may be used for the detection or enumeration of bacteria. Often an enrichment procedure is required for the detection of pathogens, whereas, due to higher expected numbers, direct plating is possible for the detection of indicator and spoilage organisms. The success of the basic protocols depends on: 1) the number and the state of the micro-organisms in the sample; 2) the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism); 3) conditions of incubation (time, temperature, presence of oxygen) and 4) the selectivity of the isolation medium (the ease of distinguishing the target organism from competitive micro flora). Since 1970s there has been considerable improvement in traditional methods: new apparatus for homogenizing samples (stomacher, pulsifier), spiral platers, (automated) colony counters and the TEMPO-method, based on the automated most probable number (MPN) techniques used in traditional microbiology for counting low numbers of micro-organisms. Another improvement on the traditional method is the development of chromogenic isolation and enumeration media, which make it easy to distinguish the target organisms.

29.2 Rapid Methods

Since traditional enumeration procedures often require rather long incubation times, there is a need for rapid methods to detect food borne pathogens, indicator and spoilage organisms. In most food legislation, microbiological criteria are stated for food borne pathogens, but to a lesser extent for indicator organisms. The laboratory needs to choose whether to use traditional or rapid methods. However, due to the very low numbers of some food borne pathogens present in a product (e.g. *Salmonella* in milk powder or *Cronobacter* in infant formula), time-consuming enrichment procedures are necessary (the time varying from 1-2 days, depending

on the type of enrichment). Many rapid methods, mainly immunological and/or DNA-based, are commercially available for the detection of food borne pathogens. However, traditional methods are still first choice for the enumeration of indicator and spoilage organisms.

29.3 Detection and Enumeration of Microorganisms

There are several methods for detection and enumeration of microorganisms in food. The method that is used depends on the purpose of the testing.

1. Direct Enumeration: Using direct microscopic counts (DMC), Coulter counter etc. allows a rapid estimation of all viable and nonviable cells. Identification through staining and observation of morphology also possible with DMC.
2. Viable Enumeration: The use of standard plate counts, most probable number (MPN), membrane filtration, plate loop methods, spiral plating etc., allows the estimation of only viable cells. As with direct enumeration, these methods can be used in the food industry to enumerate fermentation, spoilage, pathogenic, and indicator organisms.
3. Metabolic Activity Measurement: An estimation of metabolic activity of the total cell population is possible using resazurin, methylene blue dye reduction, acid production, electrical impedance etc. The level of bacterial activity can be used to assess the keeping quality and freshness of milk. Toxin levels can also be measured, indicating the presence of toxin producing pathogens.
4. Cellular Constituents Measurement: Using the luciferase test to measure ATP is one example of the rapid and sensitive tests available that will indicate the presence of even one pathogenic bacterial cell.
5. DNA/ RNA probes: A probe is a nucleic acid sequence typical of the organism of interest, used to detect homologous DNA or RNA sequences in the target organism. RNA as target sequence has an advantage in having 10⁴ copies per cell versus DNA which has only one or two copies per cell. Nucleic acid fragments for testing are prepared using restriction endo-nucleases.
6. Fluorescent antibody method: Fluorescent antibody (FA) reagent reacts with test antigen. Resultant antigen-antibody complex emits fluorescence and is detected using a fluorescence microscope. AOAC-approved method for detecting *Salmonella* yields results within 52 hours.
7. Immunodiffusion: Gel diffusion methods are widely used for detecting and quantifying bacterial toxins and enterotoxins, for example, *S. aureus* enterotoxin and *C. botulinum* toxins. Most widely used test is the Crowle modification of the Ouchterlony slide.
8. Immunomagnetic separation (IMS): The Dynal system uses Dynabeads, polystyrene beads coated with specific antibodies. The antibodies combine with the target organism in the test sample and the bead bacterial complexes are separated using a magnetic particle concentrator. Incorporation of the IMS step greatly reduces the isolation time for the target organism. IMS can be used for isolation of *E. coli* O157:H7, *Listeria*, and *Salmonella*.

29. 4 Direct Enumeration using Microscopic Technique

29.4.1 Breed-smear or DMC

The direct microscopic count (DMC) also known as Breed-smear is the method based on the microbiological examination of the milk sample. It is one the first method to be used for counting bacteria in milk. The basis of this method is that the representative sample of milk the average number of bacteria are counted to detect the quality of milk by comparing with the standard counts. The procedure involves preparing milk smear with breeds pipette on a grease free slide in a specified area with specific amount of milk is 0.01 ml milk in 1 cm² area. It is stained with Newman's stain after fixing the organisms and then washed. The numbers of organisms are counted in different fields.

The microscopic factor is the ratio of area of the smear to the area of the microscopic field and calculated as

$$MF = 100 * 100 / \pi r^2$$

Where r is the radius of the field

Counts = Average count x M.F. x Dilution factor

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Advantages

This method is one of the most popular and most rapid one to be used in dairy microbiology.

Disadvantage

- This method involve the failure to stain,
- Irregular distribution of bacteria.
- This method include the use of a very small volume which leads to increased errors
- Large microscopic factor which limits the sensitivity to about 5,00,000 bacteria/ ml
- Operator fatigue resulting from prolonged use of the microscope.

29.4.2 Electronic counting and fluorescence labelling

Fluorescent stains have been used to count bacteria in wet or dried preparation of milk. Bacteria often stain better with fluorochromes than with conventional stains.

29.4.2.1 Bactoscan

The basis of Bactoscan is the use of fluorescent microscopy. The somatic cells and the casein micelles are dissolved chemically. Later bacteria are separated by continuous centrifugation. Bacteria thus obtained are incubated with proteases to remove the residual protein and then stained with acridine orange. The treated sample is applied in a thin film on the surface of rotary disc and passed under a microscope objective the fluorescent impulses in a microscope are converted into electrical impulses and recorded. The instrument has capacity to analyze 68 samples per hour with analysis time of 7 minutes. The method correlates well with the SPC with correlation of up to 0.88. The Bactoscan can be used to rapidly detect the milk of poor bacteriological quality and could be used to grade milks at the 100,000 bacteria per ml.

29.4.2.2 Flow cytometry

This method is new to microbiological slides. In this method, the microbial suspension must first be subjected to an enzymatic treatment for the purpose of disrupting aggregates. The cells are treated with fluorochemicals which bind to nucleic acid, protein etc. To get the stoichiometric binding to the cell, the fluorochromes are used at saturation levels. The fluorescent marked suspended cells are focused into a narrow flow path delimited by a liquid sheath which enters the detection system at high speed. The detection system consists of a fine laser brush which spans the liquid stream. If a cell passes through this brush the bound fluorochrome produce detectable and measurable fluorescent emission. This method is capable of examining at least 1,000 cells/ sec and is therefore very rapid.

29.4.2.3 Direct epifluorescent filter technique (DEFT)

The Direct Epifluorescent Filter Technique is rapid method for counting bacteria in milk which uses membrane filtration and epifluorescent microscopy. Membranes can be made from nitrocellulose, cellulose acetate esters, nylon, polyvinyl chloride and polyesters. Membrane filters are used in modified conventional methods for a variety of purposes:

- To concentrate target organisms from a large volume to improve detection limit
- To remove growth inhibitors
- To transfer organisms between growth media without physical injury through resuspension

DEFT is a direct method used for enumeration of microbes based on binding properties of fluorochemical acridine orange. In this food samples are pretreated with detergents and proteolytic enzymes, filtered on to a polycarbonate membrane stained with acridine orange and examined under fluorescent microscope. The

number of viable cells is determined based on the count of orange cells on the filter and can be performed in 10 min. Before filtration the milk (2 ml) is treated with proteolytic enzyme (trypsin) and a non-ionic detergent (Triton X-100) for 10 minutes at 50°C. This treatment enables lysis of somatic cells and dispersion of fat globules so as to enable the treated milk to be filtered through a 0.6 µm pore size polycarbonate membrane filter. Bacteria remain intact and are retained on the surface of the membrane. Then the membrane along with bacteria is stained with the acridine orange a fluorechrome, mixed with Tinopal AN (fluorescent brighter), and then counted by means of an epifluorescent (incident fluorescent) microscope.

Acridine orange can be used to differentiate deoxyribonucleic acid (DNA) from ribonucleic acid (RNA) by green as opposed to orange fluorescent. In the DEFT actively growing bacteria because of higher RNA content fluoresce orange. The bacteria can easily be distinguished from the small amounts of fluorescent debris present on the filter. The DEFT count is rapid, taking less than 25 minutes to complete, inexpensive and is suitable for milks containing 6000-10 million bacteria/ ml. The DEFT count correlates well ($r = 0.91$) with the SPC.

29.5 Viable Enumeration

It is done by using standard plate counts, most probable number (MPN), membrane filtration, plate loop methods, spiral plating etc.,

29.5.1 Plate loop method

The standard plate count is time-consuming and requires a considerable amount of equipment-pipettes and dilution bottles. This procedure requires the use of standardized loops (0.01 and 0.001) for making serial dilutions instead of pipettes and dilution bottles. (1). It consists briefly of: (1) a 0.01 and a 0.001 ml calibrated loop, Luer-Lok hypodermic needle, Cornwall continuous-pipetting outfit: Becton-Dickerson & Co. No. 1251 (consisting of a metal pipetting holder, a Cornwall Luer-Lok syringe and a filling outfit), 2 ml capacity, adjusted to deliver 1.0 ml. An approximate 30° bend has been made about 3-4 mm from the loop.

The PLC method employs 0.01 and 0.001 ml loops. The PLC counts using the 0.01 ml loop was determined on a 1:10 dilution of the same sample, thus the 0.01 and 0.001 both represented a 1:1000 dilution. Incubation was at 30 °C.

29.5.2 Spiral plate

Spiral plater spreads continuously the sample using stylus (dispenser) moving away from centre to periphery of the petri-plate based on Archimedes principle and creating the dilution by means of difference caused in the distance of the deposition on the petri-plate containing the medium for a fixed volume of the sample delivered per unit time. Counts should be calculated, where possible, using dilutions giving 20 or more colonies on the plate.

29.5.3 Droplet technique

Standard Plate Count Agar (SPCA) is prepared in 9 ml amounts in screw capped test tubes. For each viable count, three test tubes of medium are to be melted and cooled to 45°C in a water bath. The bottom of a petridish should be marked with the sample number, and three equally spaced parallel lines on the outer surface.

One ml of the product suspension is to be pipetted out into the first test tube of cooled molten agar and well mixed. With a sterile dropping pipette delivering, 5 x 0.096 ml droplets were formed in the Petri dish along one of the marked parallel lines. Three drops (0.096 ml) were then added to the second test tube of agar. The contents of the test tube are thoroughly mixed using a fresh capillary pipette by repeatedly filling and emptying, and a second row of 5 droplets should be deposited along the second parallel line. A third dilution (0.096 ml in 9 ml) was made in the remaining test tube of cooled agar and droplets made in the same way above the third line in the Petri dish. The Petri dish then contained one total viable count (Fig. 29.1).

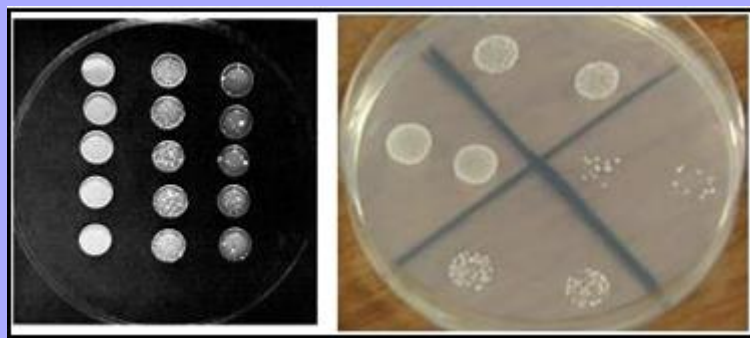


Fig. 29.1 Droplets in petridishes after incubation

A second count was placed in the lid of the same dish in exactly the same way. In some instances, droplets are overlaid with 1 or 2 drops of sterile agar to prevent growth of surface colonies. The dishes are to be counted after 24 and 48 hours at specified incubation temperatures.

Colonies were counted at 10x magnification using a bench lens, or stereomicroscope, or a specially designed projection viewer. The mean counts for up to 5 droplets are to be taken, depending on the number of colonies. For example, if the count exceeded 100/ droplet, only two are counted. Up to 200 colonies/ droplet could be conveniently counted (Fig. 29.2).

By using 9 ml quantities of agar and regarding the transferred volume as 0.1 ml, it was, in fact, 0.096 ml - the error introduced through the pipetted volume was insignificant. Possibility of error and the need for judgment in using dropping pipettes have been eliminated by a specially designed diluter/ dispenser.

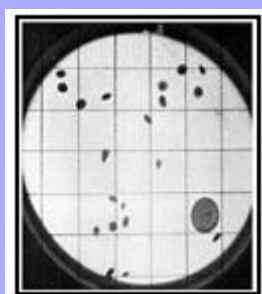


Fig. 29.2 A droplet photographed from the ground glass screen of the viewer

29.5.4 Hydrophobic grid membrane filter

It is a direct method for counting viable bacteria. The principle involved in this is that it uses a membrane filter imprinted with hydrophobic material in a grid pattern, the hydrophobic lines divide the filter surface into compartments of equal and known size and act as a barrier to the spread of colonies.

Square occupied by colonies are counted and converted to MPN estimate of the organisms by application of the formula.

$$MPN = \frac{[N \text{ Log } \{N/(N-P)\}] \times 1}{\text{Dilution Factor}}$$

Where,

N = Total number of Squares

Log = Natural logarithm

P = Number of positive squares.

In this method bacteria are retained on the membrane filters after the filtration of the sample. The protocol of the method involves the placing of filter on a pad soaked in nutrient medium and counting the colonies formed after 1-3 days of incubation. The variation of this method is available in form of Hydrophobic Grid Membrane

Filter (HGMF) given by Sharpe and Michand in 1974. The processing time is reduced as the dilution series is not required. Also efficiency is better as compared to conventional filters. This method has a square grid pattern printed in hydrophobic material likewise. On a conventional membrane filter dividing filter in 2000-4000 compartments depending on size of the grid. It functions as MPN technique and size variations and lateral species of colonies is prevented. Automated counting of colonies is done. The HGMF method has been used in the enumeration of coliforms in a variety of foods. The long incubation time is a disadvantage with this method.

29.5.5 Petrifilm

Other rapid method kits speed up standard microbiological methods by using special substrates, enzymes, or other apparatus. For example, a Petri film plate count card contains prepared media. One just adds one's sample at the appropriate dilution and incubates it. One can then count the bacteria present in the sample. It is disposable and eliminates the need to make agar plates.

The 3M™ Petrifilm™ Plate Reader (Fig. 29.3) provides consistent, automated reading and recording of results of 3M™ Petrifilm™ Plates (Aerobic, Coliform, *E. coli*/Coliform Count and Select *E. coli*) in 4 seconds, thereby increasing productivity, reducing costs and eliminating variation between lab techs. The included software displays results and colour images of the plate on the computer screen, marking the colonies for easy verification.

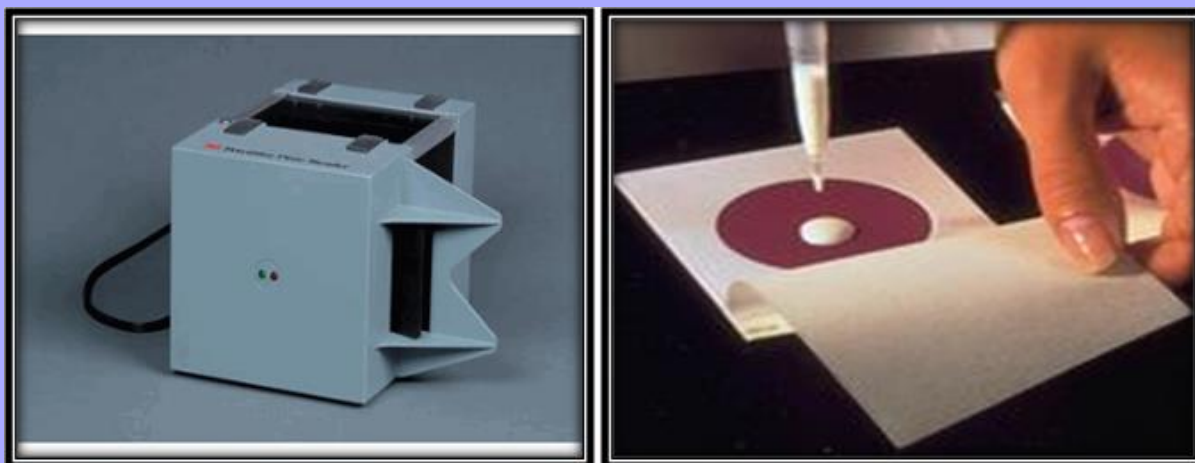


Fig. 29.3 3M™ petrifilm™ plate reader

29.6 Enzyme/Substrate Based Detection Method

With a positive MUG test kit a special chemical reaction alerts the microbiologist that the organism one is looking for is present. One type of MUG test kit is called a Coli complete test. The discs are impregnated with two chemicals that react in the presence of coliforms and *E. coli*. One inoculates the tube, adds one of the discs, and incubates it. If a blue colour develops, one has a presumptive positive for coliforms. One then shines an ultraviolet light on the tube. If the tube fluoresces, one has a presumptive positive for *E. coli* (Fig. 29.4).

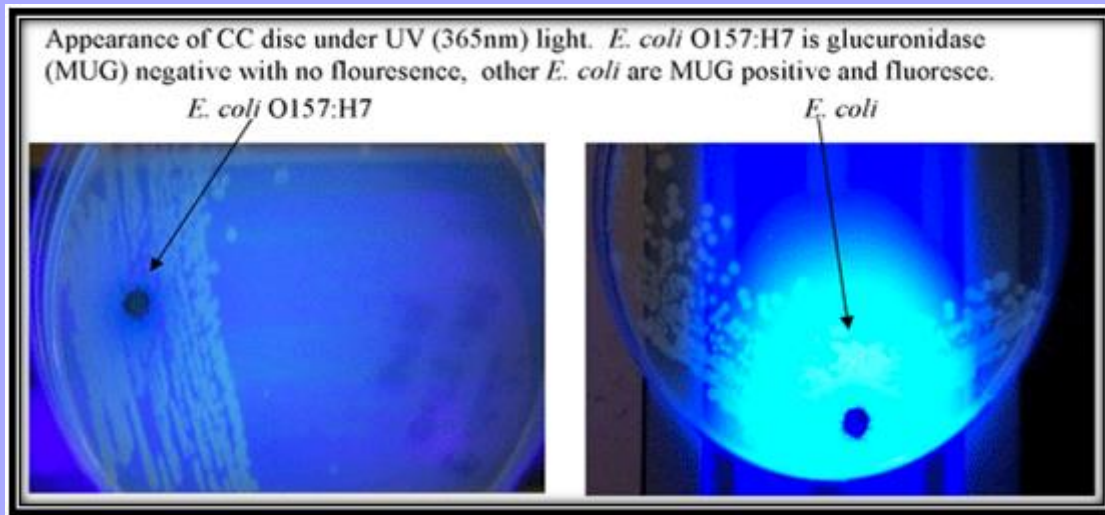


Fig 29.4 Enzymatic detection of *E. coli* and *E. coli* O157:H7

29.7 Metabolic Activity Measurement

An estimation of metabolic activity of the total cell population is possible using resazurin, methylene blue dye reduction, acid production, electrical impedance etc. The level of bacterial activity can be used to assess the keeping quality and freshness of milk. Toxin levels can also be measured, indicating the presence of toxin producing pathogens.

29.7.1 Dye reduction tests

The basis of the dye reduction tests is the ability to produce enzyme like dehydrogenases, which can transfer hydrogen from a substrate to a redox dye which undergoes change in color. The rate of reduction of color depends upon the enzyme activity which is taken as index of number of organisms present in milk. The reduction time is inversely related to the bacterial count of the sample. These tests are used for non-refrigerated, bulked raw milk.

29.7.2 Electrical methods

This method is based on the principle that the growth of micro organisms results in changes in the composition of the culture medium as nutrients are converted into metabolic end products. The complex uncharged molecules like carbohydrates or lipids are converted to simpler charged molecules like lactic acid and acetic acid. Charged proteins are poly peptides are converted to amino acids, bicarbonates etc. This overall conversion leads to the increase in conductance and capacitance of the medium in which they grow thus decreasing the Impedence.

These electrical changes in microbial cultures form the basis of detecting micro organisms and their metabolic effects. The impedance is defined as the resistant to flow of alternating current through a conducting material. A model with resistor and capacitor in series is formed completing the electrical circuit by placing the electrodes in the microbiological media. The following formula gives the total impedance in this model.

$$Z^2 = R^2 + \frac{1}{(2\pi fc)^2}$$

Where

Z -----> = <----- Impedance, R = -----> resistance
 C ---> = <----- Capacitor and f = ---> frequency of alternating current

The impedance changes are detected by passing a small alternating current through media and comparing impedance of the inoculated medium with that of uninoculated one.

29.7.3 Pyruvate test

Pyruvate is the key intermediate of the metabolism as the lipid, polysaccharides and protein degradation leads to pyruvate formation. Thus estimating the pyruvate content can give the amount of bacterial contamination. It is suitable because of its pooling nature and high solubility. It is not affected by pasteurization and UHT.

$$\text{Pyruvate (} \mu \text{ mole/ml)} = \frac{\text{Change in absorbance}}{E_{340\text{nm}} \times \text{NADH} \times \text{Dilution factor}}$$

The principle behind this method is an enzymatic reaction. In this presence of reduced form of co-enzyme NADPH₂, Pyruvate is completely converted to lactate by enzyme lactate dehydrogenase and NADH₂ is oxidized to NAD, Decrease in NADH₂ is proportional to amount of pyruvate at 340 nm. Pyruvate count of 2.25 ppm is correlated with 3 x 10⁵ cells/ml and 11.0 ppm with 4 x 10⁷ cells/ml.

29.7.4 Limulus lysate test

This test can be used rapidly and specifically to determine the cumulative content of Gram negative bacteria in foods. The G-ve bacteria produce a lipopolysaccharide (LPS) (endotoxin) which is a high molecular weight complex. This LPS is released into the surrounding medium after the death and lysis of cells, although some viable cells may also release. The horse shoe crab called amoebocyte, whose cytoplasm is packed with granules. In the presence of lipopolysaccharide the limulus blood clots.

This test is specific for LPS and very sensitive as little as 10⁻¹² g lipopolysaccharide per ml can be detected, occasionally even 10⁻¹⁵ g/ ml. A single G-ve organism contains approximately 10⁻¹⁴ g. Lipopolysaccharide, because of the extreme sensitivity of the test all utensils must be absolutely free from lipopolysaccharide (LPS).

In this test, a ten-fold dilution series of the sample is prepared and equal volume of limulus lysate is mixed in a test tube.

1. The tube is then incubated at 37°C for 4 h.
2. Before being inverted and read, if the mixture remains unchanged and runs down the wall of the tube then that dilution of the sample does not contain lipopolysaccharide
3. If a firm opaque gel is formed which sticks to the bottom of the tube that dilution of the sample contains lipopolysaccharide
4. Generally visual reading of 10-fold dilution will give sufficient information about the level of polysaccharide present in the sample. The accuracy of the sample can be increased by using a 2-fold dilution series.

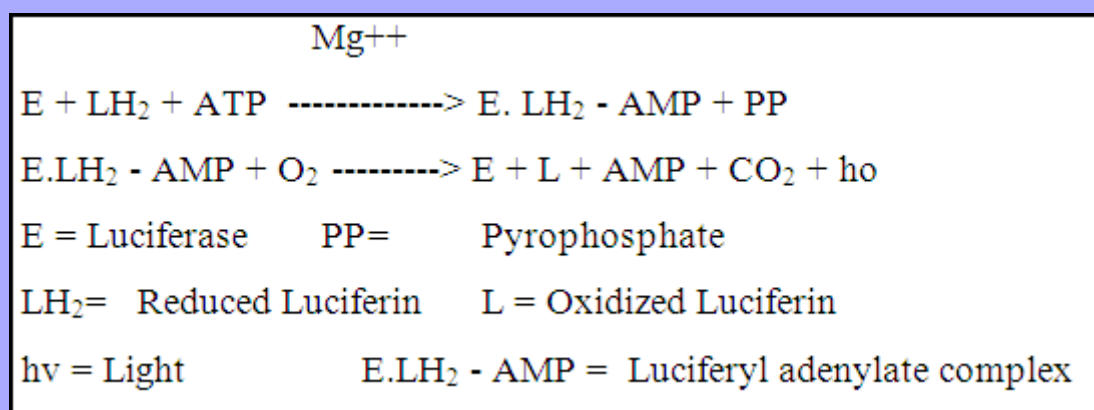
As the lipopolysaccharide is heat resistant, the limulus test has been used to assess the cumulative contamination of G-ve bacteria in dairy products. An estimate of the bacteriological quality of the raw milk is possible by applying the limulus test to UHT milk.

29.8 Cellular Constituents Measurement

29.8.1 Bioluminescence

The ATP is considered currency of energy for cell. The function and significance of this in the metabolism of living cells suggests that its assay should be an excellent monitor of biological activity in the sample. The firefly luciferase reaction, where light is produced by an enzymatic reaction, is frequently used as an assay as it is specific for ATP.

Following reactions occur here



The light is recorded by photometer and is proportional to the concentration of ATP. There are two factors which adversely affect the estimation of bacterial numbers by the measurement of ATP - first, non-bacterial ATP and second quenching of the emitted light. Many biological materials such as Urine, meat and milk contain non-bacterial ATP which must be destroyed before an accurate estimate of bacterial ATP can be made. In milk the somatic cells are lysed and then incubated with an enzyme. Apyrase to destroy the ATP released. Following this treatment, the bacteria are chemically disrupted and the released ATP measured. Failure to destroy the non-bacterial ATP will give a high estimate of bacterial ATP, and hence bacterial numbers. ATP has been used as an indicator of bacterial numbers in food. The bioluminescent method and SPC hold a 93% correlation for range of 10^4 - 10^7 cells/ml. These techniques are suitable for detecting raw milk of poor quality.

29.9 DNA/ RNA probes

Some of the rapid methods involve using antibodies, nucleic acids, or robotics to detect pathogens and toxins. Of these, the antibodies are most versatile and are used in various test kits. They take advantage of antibody-antigen interactions that are specific to a particular pathogen. A latex agglutination test works that way. If the reaction is positive, the latex beads cause the bacteria to clump.

The identification of bacteria by DNA probe hybridization methods is based on the presence or absence of particular genes. This is in contrast to most biochemical and immunological tests that are based on the detection of gene products such as antigens or chemical end products of a metabolic pathway.

The physical basis for gene probe tests stems from the structure of DNA molecules themselves. Usually, DNA is composed of two strands of nucleotide polymers wound around each other to form a double helix. These long nucleotide chains are held together by hydrogen bonds between specific pairs of nucleotides. Adenine (A) in one strand binds to thymine (T) in the complementary strand. Similarly, guanine (G) in one strand forms a hydrogen bond with cytosine (C) in the opposite strand. The hydrogen bonds holding the strands together can usually be broken by raising the pH above 12 or the temperature above 95°C. Single-stranded molecules result and the DNA is considered denatured. When the pH or temperature is lowered, the hydrogen bonds are reestablished between the AT and GC pairs, reforming double-stranded DNA. The source of the DNA strands is inconsequential as long as the strands are complementary. If the strands of the double helix are from different sources, the molecules are called hybrids and the process is termed hybridization.

A gene probe is composed of nucleic acid molecules, most often double-stranded DNA. It consists of either an entire gene or a fragment of a gene with a known function. Alternatively, short pieces of single-stranded DNA can be synthesized, based on the nucleotide sequence of the known gene. These are commonly referred to as oligonucleotides. Both natural and synthetic oligonucleotides are used to detect complementary DNA or RNA targets in samples. Double-stranded DNA probes must be denatured before the hybridization reaction; oligonucleotide and RNA probes, which are single-stranded, do not need to be denatured. Target nucleic acids are denatured by high temperature or high pH, and then the labeled gene probe is added. If the target nucleic acid in the sample contains the same nucleotide sequence as that of the gene probe, the probe will form hydrogen bonds with the target. Thus the labeled probe becomes specifically associated with the target.

The unreacted, labeled probe is removed by washing the solid support, and the presence of probe-target

complexes is signaled by the bound label. In addition to DNA, probes and/or their targets can be made of RNA. A number of commercially available gene probe kits use synthetic DNA probes specific for ribosomal RNA targets. DNA:RNA and RNA:RNA hybrids are somewhat more thermally stable than DNA:DNA duplexes, but RNA molecules are quite labile at alkaline pH.

29.10 Fluorescent Antibody Method

The use of fluorescently-labeled monoclonal antibodies, with detection by multi-parameter flow cytometry, was investigated for the rapid detection of *salmonellas*. Accurate detection of specific *Salmonella* serotypes was demonstrated down to levels of below 10^4 cells ml^{-1} (within 30 min) and 1 cell ml^{-1} (after 6 h non-selective pre-enrichment). This level of sensitivity was attained even in the presence of high levels of other bacterial species that would otherwise have interfered with the results. With combinations of different antibodies, each with a unique fluorescent label, simultaneous analysis for two species was possible

29.11 Immunomagnetic Separation (IMS)

The isolation stage can be shortened by replacing a selective enrichment stage with non growth related procedures. IMS uses super-paramagnetic particles, which are coated with antibodies against the target organisms to selectively isolate the organisms from a mixed population. IMS is analogous to selective cultural enrichment, whereby the growth of other bacteria is suppressed while the pathogen of interest is allowed to grow. The separation process consists of two fundamental steps, where the suspension containing target cells is mixed with immunomagnetic particles for incubation no longer than 60 min and finally, they are separated using an appropriate magnetic separator. In the second step, the magnetic complex is washed repeatedly to remove unwanted contaminants and the target cells with attached magnetic particles can be used for the further experiments. Polystyrene beads coated with iron oxide and antibodies are the most common magnetic carriers used for concentration and separation of selected microorganism from foods. The Immunomagnetic beads have been used for capture of *E. coli* O157:H7, *Salmonella* and *Listeria*. In recent years, applications of IMS coupled with PCR assays are showing very promising results for the detection of *E. coli* O157:H7, *Salmonella enteritis* and *Listeria monocytogenes*. The detection limit for IMS with PCR was 1 cfu/1-25 g of sample following enrichment for *L. monocytogenes*. The immune magnetic separation may be employed either directly or indirectly. However, in selective enrichment stage separation, chemical reagents are antibiotics are used to select pathogens,. Since reagents can be harsh and may cause cells stress or injury, LMS is a milder alternative to enrichment; also the elimination of selective enrichment step shortens analysis time. The major drawbacks of the IMS-based assays are the requirement of enrichment and a sample clean up step.



Lesson 30

MONITORING ANTIBIOTIC RESIDUES AND AFLATOXIN M1

30.1 Introduction

Antibiotics are extensively used in dairy cattle management for preventing and curing disease like mastitis, brucellosis etc. Based on chemical structure, the most commonly used antibiotics in dairy animals are β -lactam, tetracycline, amino glycoside, sulfonamide and macrolides. Among these, β -lactam antibiotic is one of the major groups of antibiotics used for treatment of dairy cattle. The substantial excretion of these residues in milk is attributed to indiscriminate use of antibiotics, lack of medication records, use of unapproved drugs, contaminated milking equipment, purchase of treated cows, failure to observe withdrawal period in lactation animals. These residues in milk are allergic, carcinogenic and cause development of antibiotic resistant pathogenic strains. The presence of antibiotics residues in milk supply can have adverse affects during processing of dairy foods in terms of starter failure, poor ripening of cheese and efficiency of dye reduction test. Maximum residual limit (MRL) has been recommended for a number of anti-microbial agents for their compliance in milk (Table 30.1 & 30.2).

Table-30.1 List of antibiotics used in dairy husbandry

Family	Example
Carbohydrate(amino glycoside) antibiotics	Streptomycin and neomycin
Macrocyclic lactone (Macrolides)	Erythromycin, oligomycin, amphotomycin
Amino acid peptide and β -Lactam antibiotics	Penicillin, polymixin, bacitracin, cephalosporin,
Acydic antibiotics	Streptovitacin
Aromatic antibiotics (polyenes, antifungal)	Chloramphenicol, griseofulvin
Aliphatic antibiotics	Variotin
Quinine and similar antibiotics	Tetracycline

Table 30.2 Maximum Residual Limit (MRL) recommended for milk

Anti-microbial agents	MRL in milk
Benzyl penicillin	0.004 mg/ kg
Cefiofur sodium	0.1 mg/ kg
Dihydro- streptomycin and streptomycin	0.2 mg/ kg
Gentamicin	0.1 mg/ kg
Neomycin	0.5 mg/ kg
Oxyteracyclin	0.1 mg/ kg
Pectinomycin	0.2 mg/ kg
Spiramycin	0.1 mg/ kg
Sulfadimidine	0.025 mg/ kg

30.2 Detection Methods for Antibiotics and Drug Residues in Milk

Various rapid antibiotic detection methods have been commercialized in last two decades. Currently, seven types of detection methods are commonly used for detection of antibiotic residues in milk i.e. microbial growth inhibitor assay, microbial receptor assay, enzyme-colorimetric assay, receptor binding assay, spectrophotometer assay, chromatographic methods and immunoassay. These methods are qualitative, quantitative or semi-quantitative. However, they have one or more limitations in terms of precision, accuracy, sensitivity, cost and infrastructural requirements. However, microbial inhibitor & immuno-receptor based tests have gained most popularity for dairy application globally.

30.2.1 Reference method

The EU reference method for the determination of antibiotic residues in raw milk and in heat-treated milk is the International Dairy Federation (IDF, 1991) microbial inhibition test. The IDF microbial inhibitor test uses *B.stearothermophilus var. calidolactis*, ATCC 10149 as the test organism due to its relatively high sensitivity to inhibitory substances. The IDF test procedure has been chosen as representative of similar procedures which in principle use *B. stearothermophilus* as the test organism. However, it is quite complex and lengthy to carry out as it involves the continual growth of large quantities of *B. stearothermophilus* spores. As the test involves a colour change, which is dependent on the growth of *B. stearothermophilus*, if the organism fails to grow then a false negative result may occur. Therefore, due to the afore-mentioned technical difficulties in carrying out the reference method, microbial inhibitor test kit assays based on the IDF method are the routine methods used for the determination of antibiotic residues in milk.

30.2.2 Microbial inhibitor test

The 'traditional' tests known as 'microbial inhibitor' tests, involve incubating a susceptible organism in the presence of the milk sample. In the absence of an antibiotic, the organism grows and can be detected visually either by opacity of the agar growth medium or by a color change resulting from acid production. In the presence of an antibiotic, or any other inhibitor, the organism fails to grow and a zone of inhibition or lack of a color change is observed (Fig.30.1). Such tests are exceptionally sensitive to β -lactam antibiotics. They are generally reliable and cost-effective but require incubation for several hours before the result can be visualized.

30.2.2.1 Delvotest SP

The Delvotest developed by Gist-brocades BV, The Netherlands is the best known microbial inhibitor test. Its first version was developed, in the 1970s as Delvotest P, to detect β -lactams. The target organism, *B. stearothermophilus*, is encapsulated in an agar medium containing a pH indicator, a nutrient tablet and milk sample both being dispensed onto the agar surface. The 'ampoule version' is designed for individual tests or small-scale testing whilst a micro-tire plate version is designed for mass testing where 96 tests can be undertaken simultaneously. A negative result is indicated by a color change from purple to yellow, due to acid development during incubation at 64°C for 2½ hours.

The Delvotest P has been used throughout the world and has sensitivity to penicillin G of 0.005 IU/ml. A more recent development, the Delvotest SP, is capable of detecting a wider spectrum of substances, notably sulphonamides, but also has increased sensitivity to tylosin, erythromycin, neomycin, gentamicin, trimethoprim and other antimicrobials. The Delvotest SP appears identical to the Delvotest P, the only difference being the need to incubate the Delvotest SP for 2¾ hours. The Delvotest SP is sold throughout the world and, universally, has sensitivity to penicillin G of 0.003-0.004 IU/ml.

Test procedure

The growth of *B. stearothermophilus* spores at 64°C initiates an acidification process which causes the turning of a pH indicator from purple to yellow. The presence of antibacterial substances will cause delay or inhibition of the spores, depending on the concentration of the residues. In the presence of residues the spores

will not multiply and the pH indicator will remain purple. Following steps are involved in procedure:

1. Add 1 nutrient tablet to each of the agar wells in the strip.
2. Inoculate 100 µl of milk into the agar well plus nutrient tablet.
3. Seal the wells for incubation
4. Incubate the strip of wells in a water bath at $64^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 2.50 hours (at the time the negative control has been changed to yellow)
5. Examine the strip for colour change from purple to yellow. A yellow reading indicates that no inhibitory substances are present; a purple reading indicates that antibiotic residues are present and a yellow/purple reading indicates a doubtful result.

30.2.2.2 Copen Test

The Copen test is also based on the IDF standard method for determination of antibiotic residues in milk. This method is very similar to Delvotest SP, however, in this test the nutrient tablet is already added to the agar medium.

30.2.2.3 Charm farm test

The Charm Farm test is a microbial inhibition test which uses a one step single service vial. It is a broad screening assay for five families of veterinary drugs, including beta-lactams, sulphonamides, tetracyclines, aminoglycosides and macrolides in raw, commingled, bovine milk. The results are stable upto 8 hours after assay completion and can be read by visual colour comparison or optionally with a pH meter. The Charm Auto-Farm Equipment is required to run this test. The test can be completed in approximately 3.5 hours. Up to 12 tests can be run simultaneously.

30.2.2.4 Charm AIM-96

The Charm AIM-96 Test is designed for high volume, broad spectrum screening of raw, pasteurised, and homogenised or skim milk. The results can be read by visual colour comparison or optionally with a microplate reader. The Charm AIM-96 detects beta-lactams, sulphonamides, tetracyclines, aminoglycosides and macrolides. 96 tests can be completed simultaneously in approximately 4 hours.

30.2.2.5 MDR test

An analytical process which involves sporulation & activation of dormant spores of *B. stearothermophilus* in newly developed medium & their germination/ outgrowth in presence of selective germinant mixture has been developed and is available commercially as microbial drug residue (MDR) test in India (Fig 30.2). The validated process is in line with AOAC approved charm 6602 assay and can be used effectively for semi-quantitative detection of antibiotic residues in different types of milk with results within 2.30-3.0 hours at MRL/ or above levels as recommended by the codex.

In addition, there are several other microbial inhibitor tests, produced by several companies. These include the Brilliant Black Reduction Test, the Valio T101 test, the Copen microbial inhibitor test, the Lumac rapid antibiotic test and the Biosys bioluminescence method. Microbial inhibitor tests are cheap and easy to perform, however, there are some limitations in the sense that they need longer incubation period and are not specific for antibiotics. There are occasional reports of positive reactions associated with other inhibitors such as lactoferrin, lysozyme or sanitizers.

30.2.3 Immune-receptor test

The desire for a more rapid and reliable result has promoted the development of tests that employ the 'immune receptor' test principle, which is a variation of the well-established enzyme-linked immunosorbent assay

(ELISA). Essentially, a specific target antibiotic group is captured by immobilized antibodies, or by a broader-spectrum receptor such as a bacterial cell. Most tests involve a competitive principle in which antibiotic in the sample competes with an internal antibiotic standard for the immune receptor. The antibody-antibiotic complex is then usually linked to an enzyme that catalyses a color or fluorescence reaction and a comparison of the intensity of the 'test' reaction with that of a 'control' determines whether the sample is positive or negative. Because of their competitive principle, a low intensity usually means 'positive' whilst a high intensity is regarded as 'negative'. Immune receptor tests can be made quantitative but are generally used to provide a 'pass/fail' result. They are generally more expensive than microbial inhibitor tests but only detect substances that react immunologically with the immobilized receptor and they provide a result in less than 10 min.

30.2.3.1 Commercially available immuno-receptor test

The commercially available immune receptor tests employ several variations of capture mechanism and color reaction but most possess the common features of an immunological reaction coupled with a change in color (or fluorescence). There are, however, two exceptions. The Penzym test (UCB Byproducts, Belgium) employs the inhibition of an enzyme reaction (DD-carboxypeptidases activity), instead of an immune reaction, to detect the presence of a β -lactam and it visualizes this by a color change. The test produces pink color when a sample contains no antibiotics while a yellow color is interpreted as positive. Conversely, the Charm II assay (Charm Sciences Inc., USA) employs an immune reaction to bind the antibiotic to a microbial receptor but detects this complex using a low-level ^3H or ^{14}C radio-label, instead of an enzyme reaction.

The Charm II assay (Charm Sciences Inc., USA) is not a single test but a family of separate tests for specific groups of antibiotics, notably β -lactams, sulphonamides, tetracycline, novobiocin, amino glycosides and macrolides, as well as various other substances such as chloramphenicol. The Charm II assay is an immune receptor test but is suitable for large laboratories only, requiring a range of laboratory equipment, including a centrifuge and sample mixers to prepare samples as well as a scintillation counter to detect the radio-label. Calibration curves need to be prepared for each group of antibiotics and a 'negative control' sample must be tested each day. The charm β -lactam test uses bacteria with specific receptor sites that bind all β -lactam drugs. The bacteria are added to a milk sample along with an exempt amount of [^{14}C] labeled penicillin G. Any β -lactam already in milk competes for the binding sites with the labeled penicillin G. The amount of [^{14}C]-penicillin G that binds to the receptor sites is measured compared to a previously determined control point or to a standard curve. The greater the amount of [^{14}C]-penicillin G measured, the lower the β -lactam concentration in the sample.

30.2.4 Novel iodometric test

The developed test is working on principle of spore germination and induction of β -lactamase enzyme in presence of inducer i.e. specific β -lactam group in milk. In case when specific group i.e. β -lactam is absent, the induction of β -lactamase enzyme during germination as well as production of penicilloic acid as a result of enzymatic action will be minimal resulting in no color change of starch & iodine mixture (Animation 5). However, in presence of inducer there will be reduction of starch iodine mixture as a result of significant induction of marker enzyme and production penicilloic acid (Fig. 30.3). The change in color of starch iodine mixture from blue to colourless will indicate the presence of β -lactam group in milk when incubated at $35 \pm 2^\circ\text{C}$ for 15-20 minutes.

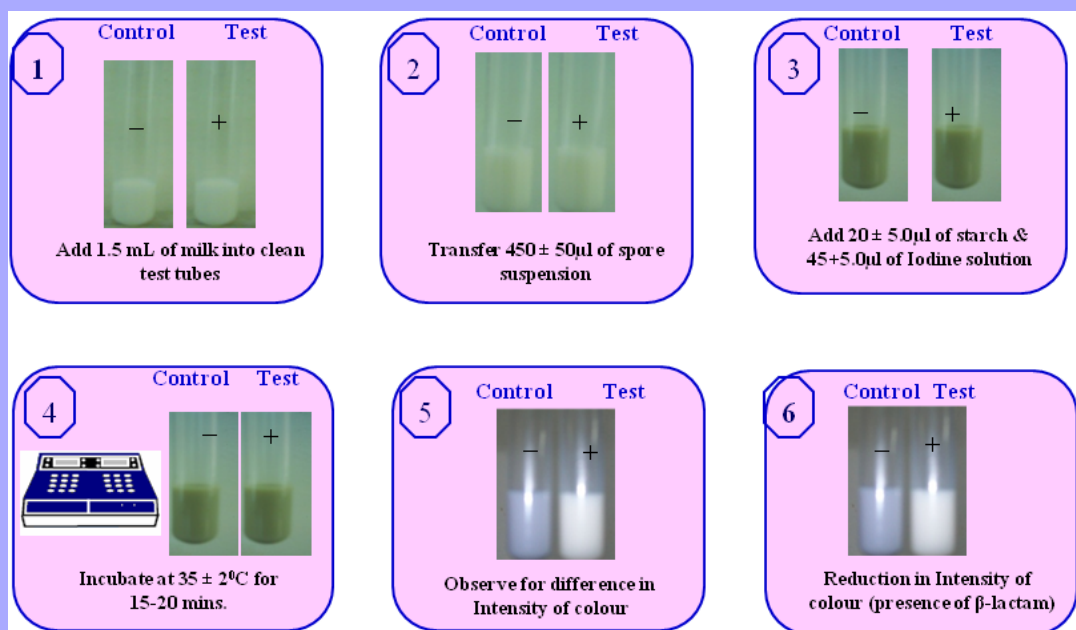


Fig.30.3 Detection of β -Lactam group in milk based on induction principle

30.2.4.1 Novel features

1. Real time test (Result within 15-20min)
2. Cost effective
3. Semi-quantitative detection at Codex MRL
4. No cross reactivity with non β -Lactam Groups
5. Validated with AOAC approved charm 6602 assay
6. Stability of test kit Up to 7-8 months under refrigeration storage
7. Wide spectrum of application with raw, pasteurized and dried milks.

30.3 Test Methods for Aflatoxin M1 in Milk

Aflatoxins are toxic metabolites produced by certain fungi in/on foods and feeds. They have been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans. Chemically they are defined as di-furano-cyclo-pentano-cumarines or di-furano-pentano-lido-cumarines, i.e. aflatoxins contain a dihydrofuran or a tetrahydrofuran ring, to which a substituted coumarin system is condensed. Out of about 20 known aflatoxins, the moulds *Aspergillus flavus* and *A. parasiticus* produce exclusively aflatoxin B1, B2, G1 and G2, and all the other aflatoxins are derivatives of these four aflatoxins.

Aflatoxin M1 was the first metabolite of Aflatoxin B1, which could unequivocally be detected in the milk. Out of this reason this first derivative was called Aflatoxin M1 (milk). When cows are fed contaminated feed, aflatoxin B1 is converted by hydroxylation to aflatoxin M1, which is subsequently secreted in the milk of lactating cows. Aflatoxin M1 is quite stable towards the normal milk processing methods such as pasteurization and if present in raw milk, it may persist into final products for human consumption. Most controlling government agencies worldwide have regulations regarding the amount of aflatoxins allowable in human and animal foodstuffs. Many countries have declared limits for the presence of aflatoxin M1 in milk and milk products. In the codex the limit for the presence of M1 in milk is set at 0.5 $\mu\text{g}/\text{L}$ or 0.5 parts per billion (ppb).

30.3.1 Source of aflatoxins in milk

Aflatoxin M1 contamination in milk results primarily from the conversion of aflatoxin B1 that is metabolized by enzymes found primarily in the liver. After aflatoxin M1 is formed, it is excreted in the urine and milk of the cow. The action level for aflatoxin B1 is 20 parts per billion (ppb) for feed fed to lactating dairy cows. As both aflatoxins B1 and M1 may cause cancer in humans, the action level of 0.5 ppb of aflatoxin M1 in milk is strictly enforced by the United States Food and Drug Administration (FDA). Aflatoxin B1 in feed is a mycotoxin produced by *Aspergillus* that grow on grain, especially corn, cotton seed and sometimes peanuts. Feed does not contain aflatoxin M1 as it is found only in milk.

30.3.2 Rapid screening methods

Rapid screening methods such as microbial inhibition assay, enzyme-linked immunosorbent assay, immunoaffinity, and lateral flow tests are used by industry and state laboratories for screening milk samples. Positive samples may require further analysis by validated methods such as the officially approved high-performance liquid chromatography (HPLC) for aflatoxin M1 in milk. With any methodology, there are concerns about the sensitivity, precision, and reproducibility of the method and the subsequent rate of false-positive, false-violative (positive test result with non-actionable levels in the sample), and false-negative results. Rapid screening methods need to provide detection at the action level but not be overly sensitive as to cause the loss of milk due to false violatives. The commercially available methods used in detection of Aflatoxin M1 in milk are given in Table. 30.2.

Table 30.3 Commercially available methods for aflatoxin M1

Detection Method	Mechanism	Reaction Type	Time (Minutes)	Sensitivity (ppb)
Charm-II (radioimmunoassay)	Binding of radio labeled Aflatoxin with microbial receptor sites	Color Change	16	0.25 – 0.5
ELISA (RIDA screen)	Ag-Ab reaction	Color Change	70	50
HPLC with fluorescence detector	Immunoaffinity columns containing antibodies against AFMI	Fluorescence	60	6
Electrochemical immunosensor	Ag-Ab with electrochemical transducer	Color Change	75	30-160
Flow injection immunoassay	Ag-Ab with amperometric transducer	Color Change	> 60	20-500
Chemiluminescent enzyme immunoassay	Ag-Ab	Luminescence	80	1
Long range surface Plasmon enhanced fluorescence spectroscopy	LRSPFS with inhibition immunoassay	Fluorescence	53	6

30.3.2.1 Spore based assay for aflatoxin M1

The bacterial spores have unique ability to sense environmental changes in response to specific “germinant” and transform rapidly into growing vegetative cells. This characteristic can be effectively used as biosensor

for tracking microbial and non-microbial contaminants. A test based on the specific spore germination and its inhibition in presence of specific analyte, i.e., aflatoxin M1 has been developed (Kumar, 2012). In case where analyte is absent in milk, specific indicator enzyme (s) are produced by active bio-sensing molecules which will act specifically on chromogenic/or fluorogenic substrate resulting in colored reaction/or fluorescence as end product which is measured semi-quantitatively by either visually/or using optical system at specific excitation/emission spectra {Fig. 30.4 (Animation 6, 7)}

30.3.2.2 Enzyme linked immune sorbent assay (ELISA)

ELISA is a widely used biochemical technique for the detection of an antigen in a sample. The sandwich ELISA utilizes two antigen specific antibodies, a capture antibody bound to a solid phase and an enzyme linked detection antibody. Direct enzyme conjugation of the detection antibody ensures an easy-to-use and sensitive assay with minimal background signal. A competitive assay in which there is a competitive binding of an antigen-specific, biotin-linked antibody to sample antigen or to antigen bound to the microtiter well. Bound antibody is detected with enzyme-linked streptavidin.

30.3.2.3 Lateral flow assay

The Charm Safe Level Aflatoxin M1 Quantitative (SLAFMQ) test is a colloidal gold lateral flow immunoassay. Aflatoxin M1 in a milk sample competes with the antibody gold beads for binding to 2 test lines. Remaining unbound binder forms on the control line. The test and control lines are compared with a reflectance reader, and a ppt concentration is determined with an algorithm. A negative interpretation with a reading of [less than or equal to] 400 ppt and a positive interpretation with a reading >400 ppt was designed to detect 500 ppt, the U.S. and Codex violative level at 90% positive with 95% confidence.

30.3.2.4 Charm II test for aflatoxin (competitive assay)

The test procedure is as follow:

- Add 300 μ L solution AF to test tube
- Fill tube 3/4 full with milk sample or standard
- Centrifuge the sample at 3400 rpm for 5 minutes
- Cool to 4 $^{\circ}$ C \pm 2 $^{\circ}$ C
- Add white tablet to empty test tube
- Add 300 \pm 100 μ L water
- Mix 10 seconds to break up tablet (take additional time if required to be sure tablet is broken up)
- Add 5.0 \pm 0.25 mL centrifuged sample or standard from below fat layer (new tips for each sample, milk temp. 4 $^{\circ}$ C \pm 2 $^{\circ}$ C)
- Immediately add purple tablet
- Immediately mix by swirling milk up and down 15 times for 15 seconds
- Incubate at 35 \pm 2 $^{\circ}$ C for 3 minutes
- Centrifuge at 3400 rpm for 5 minutes.
- Immediately remove from centrifuge and pour off milk
- Remove fat ring wipe dry with swabs. Do not disturb pellet
- Add 300 \pm 100 μ L water
- Mix thoroughly to break up pellet
- To one tube at a time, add 3.0 \pm 0.5 mL scintillation fluid. Cap, invert (or shake) until mixture has uniform cloudy appearance.
- Immediately count in analyzer for 60 seconds. Read CPM (count per minute) on (3 H) channel

Lesson 31 BIOSENSORS FOR MONITORING CONTAMINANTS

31.1 Introduction

In order to meet strict regulations on food safety issues and owing to greater public awareness of environmental contaminants there is a huge need to monitor wider range of food contaminants linked with supply chain so that quality and safe foods can be ensured to consumers. Analysts currently have a range of portable analytical techniques at their disposal for monitoring across a variety of contaminant namely pesticides, aflatoxin M1, drug residues, heavy metals and microbial pathogens in food matrixes. More recently, biosensors have emerged as another promising technology in the analyst's armoury, especially for applications requiring persistent monitoring. Biosensors are defined as analytical devices integrating biological elements and signal transducers. The biological elements such as enzymes, antibodies, receptors proteins, nucleic acids, cells, or tissue sections or bacterial spores interact specifically with an analyte, producing a signal that the transducer recognizes and converts into measurable parameters (Fig. 31.1). The amount of signal generated is proportional to the concentration of the analyte, allowing for both quantitative and qualitative measurements in time. Although biosensors are of the essence for detection of contaminants but still operation of biosensors is a challenging task for their utility owing to the cost and shelf life of bio-recognition molecule. The resolution to above challenges is spore based biosensor which has evolved as robust, easy to use, simple, and inexpensive method for long term preservation, storage and transport of biosensing element.

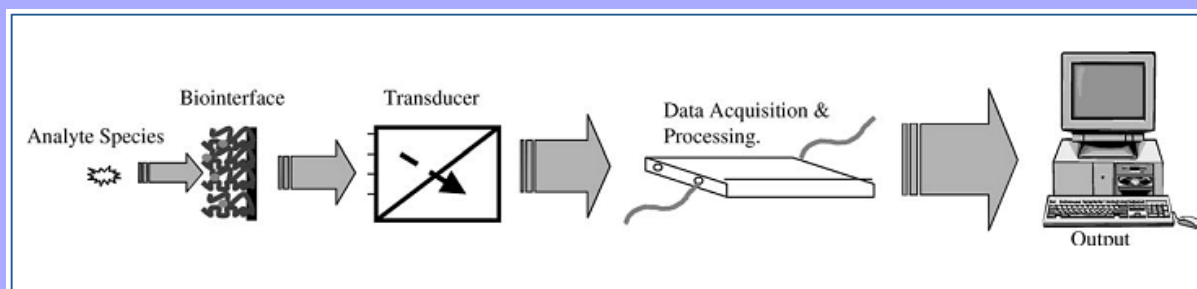


Fig.31.1 Schematic diagram showing the main components of a biosensor

The spore based biosensing systems are much superior in terms their activity, viability and analytical performance can be retained up to a period of 8 months when kept as dried spores at room temperature. The biosensors based on spore germination are real time sensing systems as germination process completes within minutes of sensing germinants in the environment. The spore production is a low priced process and its immobilization effortless process which curtails the cost of bio-recognition molecule employed in a biosensor.

31.2 Biosensor Technology

The two main elements in a biosensor are a biological recognition element or bio-receptor and a signal transducer. The bio-receptor is a bio-molecule that recognizes the target analyte and can be divided into three distinct groups: bio-catalytic, bio-affinity, and microbe-based systems. Biocatalysis-based biosensors depend on the use of pure or crude enzymes to moderate a biochemical reaction. For environmental applications, enzyme-based reactions involve enzymatic transformation of a pollutant or inhibition of enzyme activity by the pollutant. Enzyme inhibition approaches tend to cater for a larger number of environmental pollutants, usually of a particular chemical class such as antibiotic/ drug residues, aflatoxin M1, pesticides and heavy metals in food system. However, such methods requires the use of chromogen/ or fluorogens for measuring the presence of target contaminants in food matrix. A spore inhibition based enzyme substrate assay (SIB-ESA) for detection of aflatoxins M1 milk has been developed. Spores of *Bacillus* spp. have been lyophilized/ immobilized in micro centrifuge tube /sensor disk to which milk and substrate is added. In case where analyte

is absent in milk system, specific indicator enzyme(s) are produced by active bio-sensing molecules which will act specifically on chromogenic/or fluorogenic substrate resulting in colored reaction (Patent Reg # 3064/DEL/2010)/ or fluorescence as end product which is measured semi-quantitatively by either visually/ or using optical system at specific excitation/emission spectra.

31.3. Inhibition principle

Another system based on enzyme inhibition principle has been invented for monitoring of β -lactam antibiotics in milk. It is based on the principle of resistance mechanism of some β -lactamase generating *Bacillus* spp. Some spore forming bacteria such as *B. cereus* and *B. licheniformis* produce β -lactamase enzyme due to induction by β -lactam antibiotics and the enzyme production is proportional to the concentration of inducer present in milk. A real time microbial assay based on β -lactamase enzyme using starch iodine as colour indicator has been developed. The microbial assay is working on principle of non competitive enzyme action on inducer (β -lactam) resulting in indirect reduction of starch iodine mixture through penicilloic acid. A comparison of the intensity of the test reaction with that of a control was taken as criteria to determine whether the sample is positive or negative. The assay can detect specifically β - lactam groups in spiked milk within 15-20 min at regulatory codex limits with negligible sensitivity towards non β - lactam groups. The presence of Inhibitors other than antibiotic residues in milk did not interfere with the working principle of microbial assays (Patent Reg No. 115/DEL/2009).

31.4 Affinity Based Biosensors

Bioaffinity-based biosensors rely on the use of proteins, DNA or microbial receptor to recognize and bind a particular target. For environmental applications such systems depend primarily on the use of antibodies. This is due to the ready availability of monoclonal and polyclonal antibodies directed toward a wide range of environmental pollutants, as well as the relative affinity and selectivity of these recognition proteins for a specific compound or closely related groups of compounds. Nucleic acid-based affinity and electrochemical biosensors for potential environmental applications have recently been reported. Application areas for these include the detection of chemically induced DNA damage and the detection of microorganisms through the hybridization of species-specific sequences of DNA. Charm assay (Charm Sciences Inc., USA) is an example of bio-affinity biosensor which employs an immune reaction to bind the antibiotic to a microbial receptor and detects this complex using a low-level ^3H or ^{14}C radio-label. The Charm assay can detect a family of antibiotics, notably β -lactams, sulphonamides, tetracyclines, novobiocin, aminoglycosides and macrolides, as well as various other substances such as chloramphenicol. The Charm II assay is an immune receptor test but is suitable for large laboratories only, requiring a range of laboratory equipment, including a centrifuge and sample mixers to prepare samples as well as a scintillation counter to detect the radio-label.

31.5 Microbial Biosensors

Microbial biosensors involves application of microorganisms as such/ or their spores as biological recognition element. These generally involve the measurement of microbial respiration, or its inhibition, by the analyte of interest. Compared to enzyme-based approaches, microorganism-based biosensors are relatively inexpensive to construct and can operate over a wide range of pH and temperature. The broad specificity of microbial biosensors to environmental toxins make them particularly applicable for general toxicity screening like biological oxygen demand (BOD) or in situations where the toxic compounds are well defined, or where there is a desire of measure total toxicity through a common mode of action.

A signal transducer is the second essential component of a biosensor. It converts the recognition event into a measurable signal. The transducer can take many forms depending upon the parameters being measured. The most well developed classes of transducers are potentiometric, amperometric, conductometric, optical, acoustic or piezoelectric etc. These utilize various electrochemical responses to measure changes in the electrical properties of the biological recognition element. Most of the reported potentiometric biosensors for detection of environmental pollutants have used enzymes that catalyze the consumption or production of protons. Phosphoric and carbamate pesticides can be evaluated through the use of a pH electrode that measures the activity of acetyl cholinesterase. The activity of the enzyme is affected by the presence of pesticides.

Further application of spore as signal transducer application targets real time detection of bacterial contamination using the inhibition of enzyme acetyl esterase coupled to spore germination using optical device for measurement. The use of spore as signal transducer is feasible if an illustrative knowledge of spore germination process and germinants are required. It involves selective enrichment of target bacteria in a selective media. The enriched bacterial cells will produce specific marker enzymes which act on germinogenic substrate and produce specific germinant (sugars and amino acids). The germinants induce spore germination and germination mediated concomitant de novo acetyl esterase enzymatic activity. As a consequence germination derived product can be easily detected by quantification of fluorescent signal produced as result of DAF hydrolysis by acetyl esterase. Based on above principle of germinogenic substrate detection of *enterococci* detection system has been developed which targets specific marker enzyme β -D glucosidase of *enterococci* will and aesculin as germinogenic substrate which releases germinant β -D glucose. The sensitivity of spore based bioassay was 5.66 log counts of cells in 5-6 hrs in spiked milk.

31.6 Optical Biosensors

In the field of biosensors transducers based on optical detection techniques are also emerging. These may employ linear optical phenomenon, including fluorescence, phosphorescence, polarization, rotation, interference, surface plasmon resonance (SPR), total internal reflection fluorescence (TIRF), etc. or non-linear phenomena, such as second harmonic generation. Advantages of optical techniques involve the speed and reproducibility of the measurement. Microbial spore germination based optical biosensor for the detection of *enterococci* in milk is being developed in our laboratory (Fig. 31.2). The detection technique being used is electron multiple charged couple device (EMCCD), as optical transducer which improves the sensitivity as it equipped to detect germination of single spore (Patent file no. Ref. # IPR 119/DEL/2011).

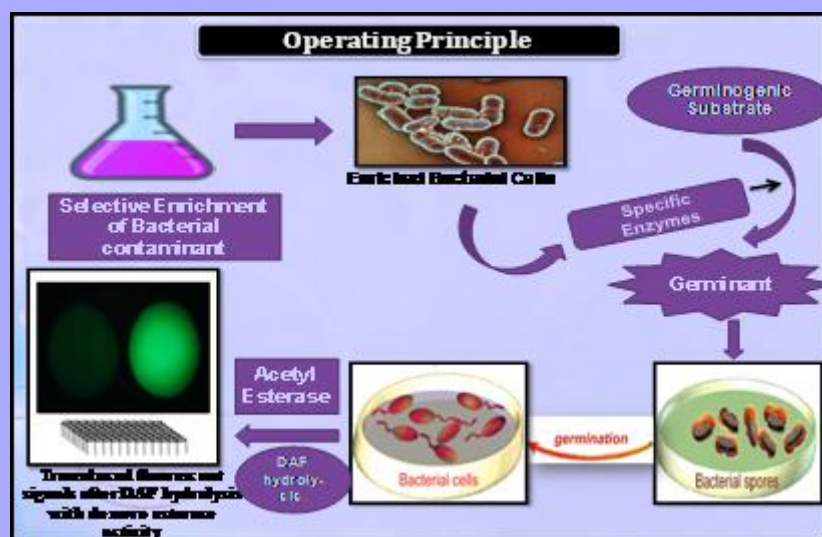


Fig. 31.2. Principle of Microbial spore germination based optical biosensor for the detection of bacterial contaminants

The basic requirement of a biosensor is that the biological material should bring the physico-chemical changes in close proximity of a transducer. In this direction immobilization technology has played a major role. Immobilization not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilizing it for reuse. The biological material is immobilized directly on the transducer or in most cases, in membranes, which can subsequently be mounted on the transducer. Selection of a technique and/or support would depend on the nature of the bio-material and the substrate and configuration of the transducer used.

31.7 Immobilization Techniques

Some of the widely used immobilization techniques include adsorption, entrapment, covalent binding and cross-linking. Immobilization of enzymes and whole cells through adsorption perhaps is the simplest of all the techniques and was achieved successfully in monitoring of aflatoxin M1 and *enterococci* on sensor disc/ or

bio-chip using EMCCD system and plate reader. Most of these techniques have the drawbacks of weak adhesion as well as complexity of the process. Novel techniques have been developed for immobilizing viable or non-viable cells through adhesion on a variety of polymeric surfaces including glass, cotton fabric and synthetic polymeric membranes using polyethylene-imine (PEI). This technique is gaining importance in the introduction of enzymes and microbes on transducer surfaces.

31.8 Commercial Biosensors

Although most biosensors systems have been tested only on non-real samples (such as in distilled water or buffer solutions), a few biosensors applied to real samples have appeared in recent years. Some representative examples of their application to the determination of different classes of key pollutants and environmental quality parameters, such as biological oxygen demand (BOD), toxicity or endocrine effects, in a variety of matrices are listed in Table 31.1. The application of biosensors to real samples must be a necessary step before their commercialization, which is, in general, the aim of the device development. Results must also be validated by comparison with those obtained with standard protocols in order to get the acceptance of end users. Most commercial biosensors developed are focused in clinical applications, such as for glucose and lactate. Prospective biosensor market for food, pharmaceutical, agriculture, military, veterinary and environment are still to be explored.

Table. 31.1 Biosensors applied to the determination of pollutants in real samples

Analyte	Sample source	Transducer, recognition element
Pesticides	River water	Optical, immunochemical
Phenols	Wastewater	Electrochemical, enzymatic
Linear alkyl benzene sulphonate (LAS)	River water	Electrochemical, bacteria
Toxicity	Wastewater	Electrochemical, bacteria
Toxicity	Wastewater	Optical, bacteria
Alkanes	Groundwater	Optical, bacteria
Estrogens and Xenoestrogens	Real water samples (lake and a sewage plant)	Optical, human estrogen receptor (EC)
BOD	River water	Optical, <i>Pseudomonas</i> sp.
Zinc dichromate chromate	Soil (extract)	Optical, bacteria
Mercury	Soil (extract)	Optical, <i>Pseudomonas</i> sp.
Arsenite Daunomycin, PCBs, aflatoxin	River water (pre-concentrated)	Electrochemical, DNA
<i>Chlamydia trachomatis</i> (DNA)	River water (pre-concentrated)	Electrochemical, DNA
Aflatoxin M1	Milk	Optical, Spore
Antibiotic (Broad Spectrum)	Milk	Optical, Spore
Antibiotic (β -lactam)	Milk	Optical, Spore

31.9 Future Prospects

The hurdles to application of biosensors include:

- Diversity and complexity of samples.
- Relatively high development costs for single analyte systems,
- Limited shelf and operational life

Nevertheless, there are a number of areas where the unique capabilities of biosensors might be exploited to meet the requirements of environmental monitoring. Advances in areas such as multi-pollutant-screening could allow these techniques to be more competitive. The present scenario demands for increased range of detectable analytes with portable device structure. Solving the resulting integration issues will require further convergence with associated technologies such as biochemistry, polymer chemistry, electronics, micro-fluidics and separation technology. Micro-electro-mechanical systems or MEMS technology is one of the promising areas that may be going to fulfill these demands in future. The technology is an integration of mechanical elements, sensors, actuators, and electronics on a common silicon substrate through micro fabrication technology. Bio-chips and sensor arrays for detection of a wide range of hazardous chemical and biological agents can be made out of these MEMS based devices, making it feasible for simultaneous detection of multiple analytes. This also brings the lab-on-chip concept. However, Immobilization and stabilization of bio-molecules on these nano-devices may be a greater challenge. Some of the works in these areas have already been initiated. Utilization of molecular recognition ability of bio-molecules like avidin-biotin or streptavidin-biotin in conjunction with a lithographic technique is being investigated for the micro immobilization of enzymes on silicon wafers for biosensor applications. Immobilization of enzymes on silicon supports has attracted attention in biosensor chip technology and a variety of classical techniques have been proposed.

There are interesting possibilities within the field of biosensors. Given the existing advances in biological sciences, coupled with advances in various other scientific and engineering disciplines, it is imminent that many analytical applications will be replaced by biosensors. A fruitful fusion between biological sciences and other disciplines will help to realize the full potential of this technology in the future.



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