

# Activity, Purity and Preservation of Starters



# Properties of ideal starters

1. A good starter should have the ability to produce lactic acid at vigorous and steady rate.
2. A starter should be pure without any contaminants.
3. It should be able to grow rapidly in suitable organic substances.
4. It can be easily cultivable in large quantities.
5. It should be able to maintain physiological constancy.
6. It should be able to produce necessary enzymes readily and profusely in order to bring about the desired chemical changes.
7. It should have the ability to carry out transformation under comparatively simple and workable modifications of environmental conditions.

# Activity And Purity Tests Of Starter Cultures

**Organoleptic tests:** Organoleptic tests require high degree of training and experience. These tests measure the qualities i.e appearance, flavour and consistency of product.

- **Body:** The body should be like firm custard. The texture should be such that the curd be able to retain its shape during gentle tapping of culture and on pronounced tapping it should break clearly and smoothly.
- **Texture:** It should not be lumpy and should not have whey pockets and should be smooth and viscous after thorough agitation.
- **Taste:** The taste should be clean and acidic, without bitterness and saltiness.
- **Flavour:** Should have typical aroma; free from off flavours and free from aroma like malty, bitter, rancid, unclean, yeasty, fruity and putrid etc.

## **Chemical tests:**

Various chemical parameters used for assessing the activity of starters include:

- Titratable acidity
- Volatile fatty acids
- Tests for diacetyl and acetyl methyl carbinol
- Tests for acetaldehyde

## **Microbiological tests:**

The microbiological methods used for determining the activity and purity of starter microorganism:

- Plate count test
- Direct microscopic count test: The ratio between rods and cocci
- Selective plating techniques
- Tests for the contaminants like coliforms, yeasts and molds (both should be absent in 1 ml of culture). Starter must be free from foreign bacteria, yeasts and molds. Depending upon the type and number of contaminants, the starter bacteria could be edged out in the competition for essential nutrients.

## Purity tests:

**Microscopic examination:** This is performed by Gram's staining or Newman's stain. Gram -ve bacteria, spore formers, yeasts and molds and staphylococci etc should be absent. The lactic bacteria should appear as Gram +ve cocci or thin rods.

**Catalase test:** Add few drops of 3% hydrogen peroxide to starter culture. Presence of effervescence indicates possible contamination of the given culture. Lactic acid bacteria are negative for catalase and thus catalase positive test indicates contamination of starters.

## **Activity tests:**

The starters are evaluated for their activity using methods:

**Titratable acidity test:** The cultures lose their activity due to acid injury if they are allowed to over ripen. A developed acidity in the range of 0.7 to 0.85% LA is optimal at 30C within 16 h.

**Horrell Elliker's test:** It is used mainly for mesophilic type of cultures. Prepare and sterilize the reconstituted milk. With sterile pipettes, transfer 0.3 ml of starter culture to be tested into each tube with 10 ml portions of milk. Place tubes of milk in a water bath and adjust temperature to 37.7°C and incubate 3.5 h. At end of incubation period, the entire contents of each tube are titrated with N/10 sodium hydroxide and phenolphthalein indicator. A titration value of 0.4% LA or higher indicates that the culture is well suited for cheese making. A titration value of 0.30% to 0.35 % LA indicates a slow culture. Starter developing acidity of less than 0.30 % LA invariably graded as inactive as it produces little or no acid during cheese manufacture.

**Whitehead-Cox Activity Test:** Whitehead and Cox recommended an activity test simulating some basic conditions of starter development in cheddar cheese and have used their method extensively for determining how well suited cultures are for this purpose.

**Creatine test:** The creatine test secure general information on the comparative amounts of acetyl methyl carbinol plus diacetyl. It is a common method used for indicating diacetyl content in flavour producing mesophilic cultures (dahi, lassi, buttermilk, cultured cream, cottage cheese). The cultures form a pink coloured complex with creatine.

**Dye reduction test:** An excellent culture reduces the resazurin dye or methylene blue dye in 35 min and a fairly good culture takes 50 to 60 min. Whereas; control milk without added culture does not reduce resazurin dye even after 4 h.

## **Preservation methods of starters**

Starters are preserved in liquid, frozen and dried forms. Liquid subcultures for routine use, while frozen and freeze dried forms are for long term preservation.

### **Forms of Starters:**

**Liquid:** Most popular and widely used where starters are normally preserved in small quantities. Working cultures are maintained with either weekly or daily sub-culturing. Starters can be preserved in liquid form using one of the following two different growth media viz., Reconstituted skim milk, litmus milk.

Reconstitute the Non-fat dry milk which is free from antibiotics to a total solid content of 10-12%. It is sterilized by autoclaving at 10-15 psi/10-15 mins. Check the sterility by incubating at 30°C for one week. Factors affecting the starter culture activity:

1. The rate of cooling after incubation
2. Level of acidity at the end of incubation period.
3. The temperature of storage
4. Duration of storage

In Litmus milk, the cultures can be reactivated once in every 3 months but disadvantages are: labour intensive, expensive, induces mutations and requires trained and skilled personnel. *L. lactis* ssp. *cremoris* and *leuconostocs* can be sub-cultured up to 50 times without fear of mutation and they can be incubated at 22°C for 16-18 h with 1% inoculum. The yoghurt cultures can be sub-cultured up to 15-20 times. Yoghurt cultures are incubated at 42°C for 3 -4 h with 1-2% inoculum. It can be stored for one week without loss of activity.

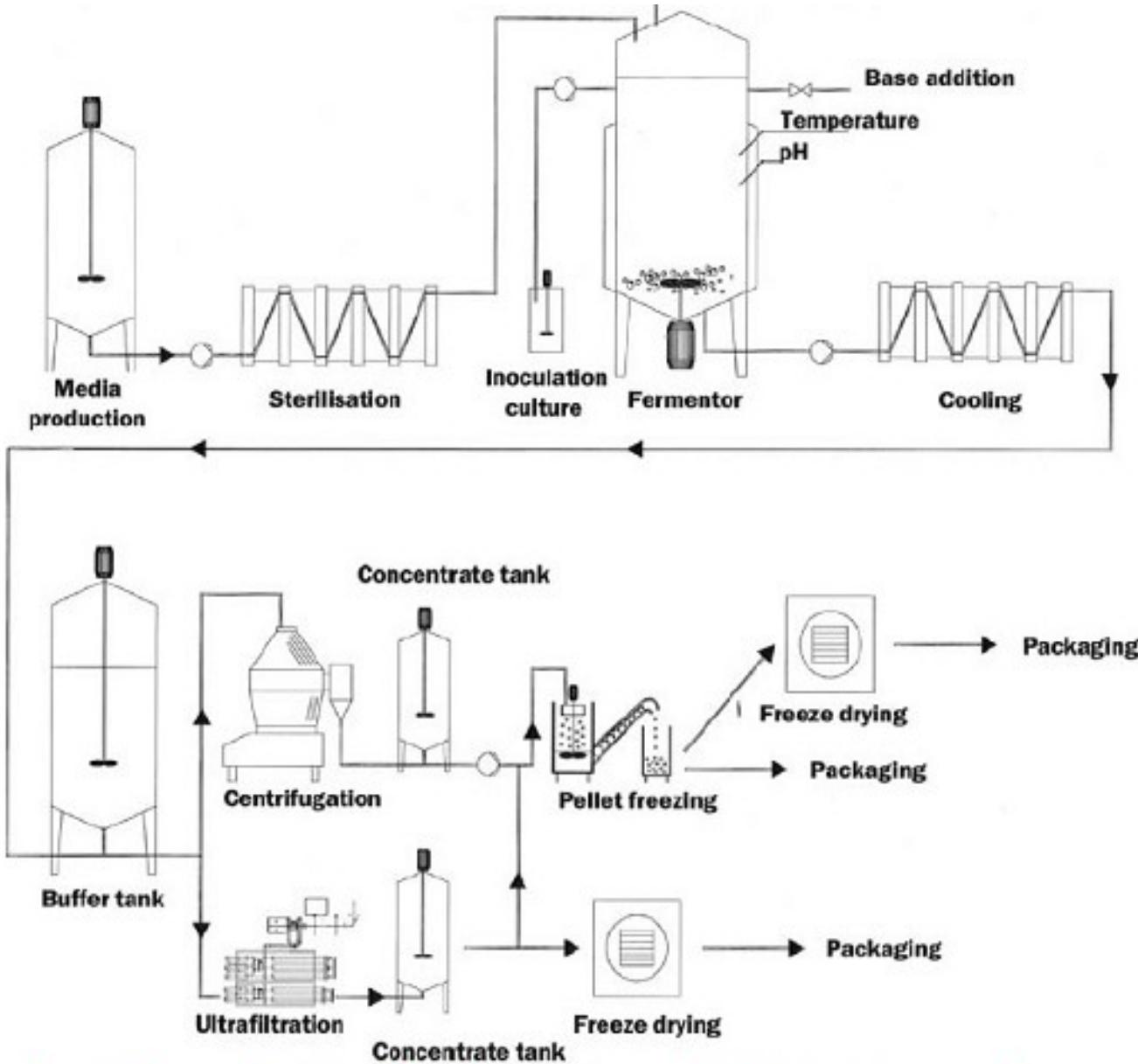
## Frozen, deep frozen and frozen concentrates:

**Frozen:** The cultures are frozen to -20°C. Liquid starters (Mother and Feeder cultures) can be preserved for few months.

**Deep freezing:** Propagate cultures continuously in neutralized growth medium to optimize cell population. Cell mass is then separated and re-suspended in sterile growth medium and protective agent is added prior to packing and freezing. Preserved cultures are stored at -40C. The cryoprotective agents are sodium citrate, glycerol and sodium  $\beta$ -glycerophosphate etc., are used. In the absence of the cryoprotective agents, there may be destruction of bacterial cells due to an increased concentration of electrolytes and other solutes inside the cell and in the outside suspending medium. The increased concentration of electrolytes and other solutes inside the cell results in denaturation of protein components and enzymes of bacterial cells, while the concentration of electrolytes outside the cell results in the dehydration of the protoplasm due to diffusion of the water through the cell wall membrane. Mechanical damage also may be caused as result of ice crystal formation.

**Ultra low temperature freezing:** This is carried out with help of liquid nitrogen at -196°C using only sterile reconstituted SMP containing 16% solids. Single strain inoculated at 2% v/v, dispensed in 4 ml quantities in sterile polypropylene, screw top tubes and frozen at -196 °C in liquid nitrogen. In this method, water molecules do not form large ice crystals. The biochemical processes inside the cell will cease and cultures can be stored for 2 years. The method is convenient, improved performance, culture reliability etc.

**Frozen concentrated:** Frozen concentrated cultures contain  $10^{10}$ - $10^{11}$  cfu/g, a sufficient concentration to allow 70 mL to inoculate 1000 L of medium for bulk culture preparation. Preparation of frozen concentrated cultures involves (1) growing cultures under optimal conditions using pH control, (2) harvesting the cells via centrifugation or ultrafiltration, (3) standardizing the cell suspension to a specific activity, (4) adding a cryoprotectant, (5) packaging, and (6) rapid freezing using liquid nitrogen.



**Production of a typical commercial starter culture.**

**Dried cultures:** There are different methods of drying the cultures.

They are: Spray drying, Vacuum drying, Freeze drying/ lyophilization, Concentrated freeze dried cultures (CFDC).

**Advantages of dried cultures:** alternative method for culture retention, overcome the work involved in maintaining liquid stock culture, facilitates the dispatch of dried cultures by post without any loss in activity and can be stored for a very long time.

**Disadvantages** are dried starter contains less number of viable cells and require several subcultures before regaining maximum activity.

**The viability of preserved culture depends on:**

- Basic growth medium
- Presence of cryoprotective agents
- Rapid removal of metabolic compounds (lactic acid)
- Nature of suspending medium
- Conditions of freezing/drying
- Rate of thawing of deep frozen cultures
- Method of concentration (biomass concentration)

## **Vacuum drying:**

**Survival rate is very poor (1-2%).**

**Several sub-cultures are required to obtain maximum activity.**

### **Method of preparation:**

- a. Mix the liquid culture with lactose**
- b. Neutralize the excessive acid with calcium carbonate**
- c. Concentrate the mixture partially by separation**
- d. Partially concentrated granules are vacuum dried**

## **Spray drying:**

**In spray drying, the survival rate with mesophilic lactic acid bacteria is 10% and with *L. lactis* ssp. *lactis* biovar *diacetylactis* is 44%. Survival rate can be improved by the addition of ascorbic acid and monosodium glutamate and propagation of cultures in buffered medium to increase viable cells per volume of sample or by neutralizing certain metabolites like lactic acid. The activity of spray dried cultures can be retained for six months when stored at 21°C.**

## Freeze drying:

This is also called as lyophilization where survival rate is very high. In this method the cultures are dried in frozen state i.e. the organisms are frozen and then water is removed by sublimation i.e. from ice as vapour and thus preserving the structure and preventing the damage. Only a small quantity is required to inoculate the mother culture.

The process damages the bacterial cell membrane by the formation of ice crystals which can be minimized by the addition of cryoprotective agents to the cell suspension.

The cells are also subjected to lethal effect due to the high presence of electrolytes as the ice crystals separate out. Both these processes can remove water from proteins and DNA and damage them. The protective solutes are of hydrogen bonding and or ionizing in nature which stabilize the cell membrane and prevent cellular injury during preservation.

Starter culture is propagated in sterilized

Reconstituted SMP (16 %) at 2 % inoculation

(pH adjusted to 6-6.5)

+

Cryoprotective agents and/or additives

(Ascorbic acid, Mono Sodium glutamate, aspartic acid

Inositol, sorbitol, mannitol, glucose, sucrose,

maltose.)

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The starters are held at 5 °C for 18 hrs

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Two to three ml is dispensed in sterilized glass vials.

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Vials are frozen at -40 °C for 2-3 hrs.

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They are freeze dried at -10 °C to 30 °C to final moisture of less than 3%

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Freeze dried vials are vacuum sealed and stored at 5 °C.

**Starter culture is propagated in sterilized reconstituted SMP (16%) at 2% inoculation. The starters are held at 5°C for 18 h. 2-3 ml is dispensed in sterilized glass vials. Vials are frozen at -40 °C for 2-3 hrs. They are freeze dried and vacuum sealed and stored at 5°C. *L. bulgaricus* is sensitive for freeze drying.**

- 1. Growth medium:** Milk fortified with yeast extract and hydrolyzed protein. Neutralize the growth medium to pH range of 5-6 and propagate organisms at optimum growth temperature.
- 2. Cell biomass and suspending medium:** Cell biomass has to be  $10^{10}$ cfu/ml and neutralize the suspending medium to 5-6 pH.
- 3. Freeze Drying:** Freeze the cultures at -20 °C to -30°C and dry at -10 °C to 30 °C to a final moisture of less than 3%
- 4. Packaging:** The cultures are vacuum packed as the presence of oxygen damages bacterial cells. Pack in glass ampoules, vials, laminated aluminum foil sachets.
- 5. Storage:** 5-10°C
- 6. Reactivation:** Follow the instructions of the manufactures. Re-hydrate at 20-25 °C to prevent leakage of cellular ribonucleotides from damaged cells of *L. bulgaricus*.

## **Direct-to-Vat Cultures: Advantages**

**Convenience:** The new generation of highly concentrated direct-vat inoculation cultures have the strength to go for inoculating much more milk with much less culture. The result is a narrowing of the cost-in-use gap between batch-set and direct-vat culture

**Culture reliability:** More reliable cultures can be obtained in direct-vat culture method.

**Improved daily performance and strain balance:** Use of carefully selected bacterial strains along with innovative fermentation and blending technology allow cultures to provide a fast fermentation at a very low inoculation rate. A rotation of six culture is available in convenient frozen pellet form.

**Greater flexibility:** The cultures can be chosen depending upon the requirement.

**Better control of phage:** Direct vat cultures help to overcome phage issues, one of cheese makers' biggest nightmares. Phage viruses only pose a threat when the Direct vat cultures are growing.

**Possible improvement in quality:** The direct-to-the-vat cultures can neutralize the lack of experience to a large degree if lot of experienced staff is not available.

Training the staff regarding bacteriophage, sanitation, equipment operation, calibration etc., is a tough task but the direct-to-the-vat cultures streamline the educational curve.

# Direct-to-Vat Cultures: Disadvantages

1. Difficulties in providing liquid nitrogen facilities
2. Higher cost
3. Greater dependence on starter suppliers
4. Distributing of responsibility in case of starter failure

Over the past few decades, the use of starter cell concentrates designated as either DVS or DVI cultures have increased being used, particularly in small plants, to replace bulk starter in cheese manufacture. Starter concentrates used in DVI cultures are concentrated cell preparations containing in the order of  $10^{11}$ - $10^{13}$  cfu/g. They are available as frozen pellets or in freeze-dried granular form. Commercial DVS frozen cultures are in pellet form.

# Factors affecting the survival of cultures during preservation

- 1. Growth:** Although continuous culture appears to be inexpensive and economical method for the cultivation of starters, it requires sterilized media and there is possibility of development of some variations in the strain. Hence, batch culture is best for all starter cultures. The starter concentrates prepared from batch cultures of starters give better results as compared to conventional cultures during the manufacture of cheese and other fermented foods.
- 2. Storage and transport:** Concentrated cultures must remain fairly stable during storage and transport for use directly in vat. Storage in liquid nitrogen at -196C is best means for preservation. However, the stability of starter concentrates between -20 to -40C exists for limited period of time. Viability of starter concentrates during storage at low temperatures can be improved with the help of lactose, glycerol etc.
- 3. Age of cells:** Cells in late log phase or early stationary phase appear to show better survival than young cells during freezing starter concentrates.

**4. Nature of microorganisms:** Aerobic Gram positive organisms show higher survival rates in the starter concentrates during storage at low temperature than anaerobic and Gram negative organisms. Different types of starters have different rates of viability.

**5. Cryoprotective agents:** During freezing, the stability of all strains is considerably improved by supplementing the medium with Tween-80. Other cryoprotective agents like 10% sucrose, 5% sodium citrate, 6% barley extract and glycerol are very effective. During freeze drying, the addition of horse serum and 8% glucose in the medium increases the survival rates of the starter concentrates during freeze drying.

**6. Packaging materials:** The common practice of carrying the starter concentrates in either liquid state or freeze dried form is by using glass ampoules. However, glass vials with rubber stoppers, nylon sachets, aluminium foil sachets and metallized polyesters are commonly used packaging materials for carrying starter concentrates.