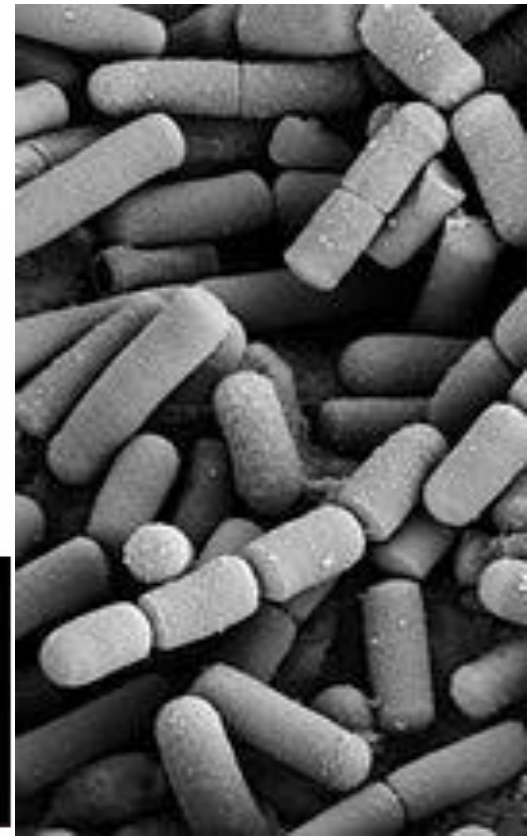




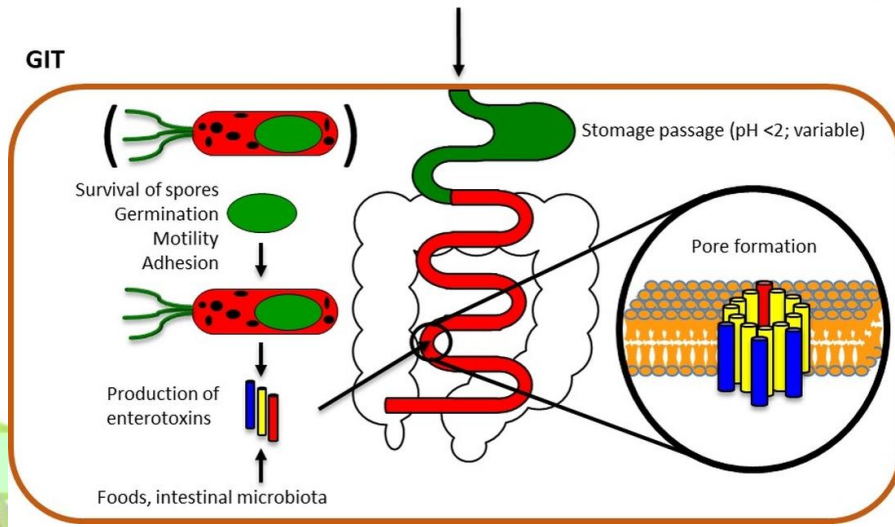
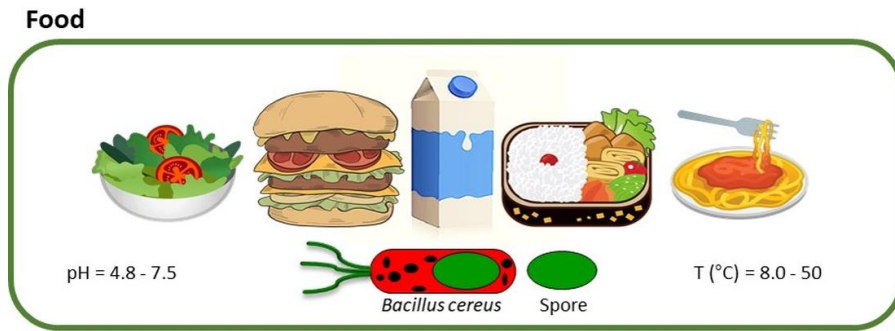
Enumeration of *Bacillus cereus*, *Clostridium botulinum* and *Clostridium perfringens*



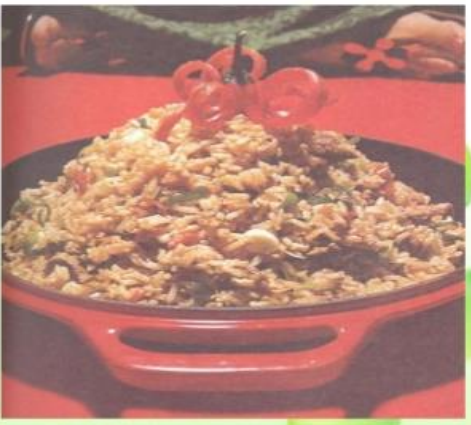
- ✓ ***Bacillus cereus***: Rods, aerobic, spore forming Gram-positive with square ends in short to long chains, spores are ellipsoidal, central to sub-terminal, thin-walled and do not swell.
- ✓ Pinkish color colonies on MYPA medium.
- ✓ Food poisoning: Inadequate refrigeration of food before serving.
- ✓ $>10^6$ *B. cereus*/g may result in food poisoning.
- ✓ European laboratories in collaborative study validated general 1993 ISO 7932 standard for enumeration of *B. cereus* in foods.



- ✓ **Outbreaks:** Cooked meat and vegetables, boiled/fried rice, vanilla sauce, custards, soups and raw vegetable sprouts.
- ✓ **Abdominal pain and diarrhoea** (incubation period of 4-16 h and symptoms last for 12-24 h). **Acute nausea and vomiting**, occurs within 1-5 h after consumption of contaminated food.



EMETIC FORM



Incubation period < 6 hours
Severe vomiting
Lasts 8-10 hours

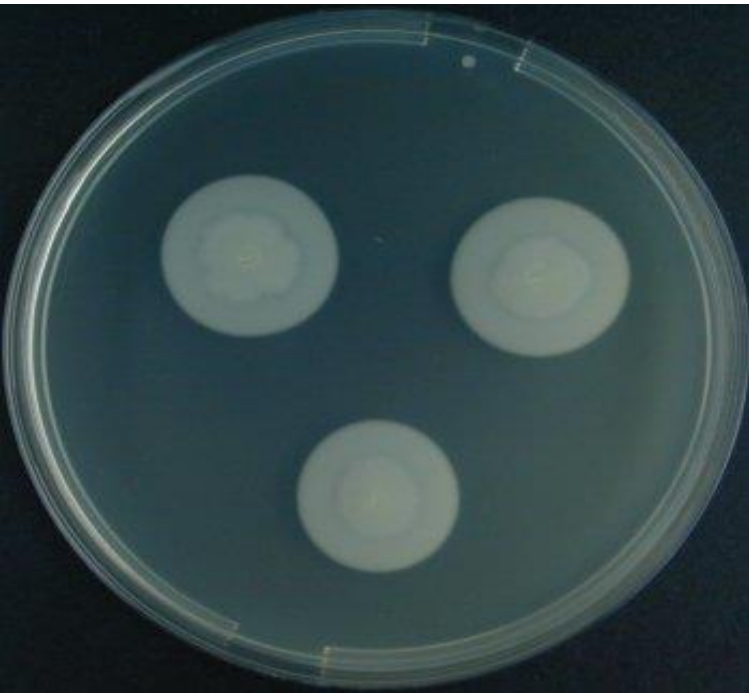
DIARRHEAL FORM



Incubation period > 6 hours
Diarrhea
Lasts 20-36 hours

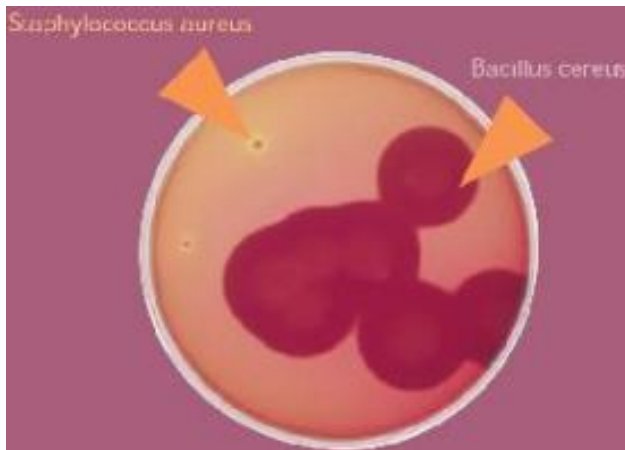
Enumeration Principle of *B. cereus*

- Mannitol-negative. Mannitol content of medium, allows differentiation of accompanying mannitol-positive microbial flora which is identified by change in colour of indicator phenol red to yellow.
- Not affected by concentrations of polymyxin which inhibit common accompanying microbial flora.
- Produces lecithinase. Insoluble degradation products of egg-yolk lecithin accumulate around colonies to form a white precipitate.
- *B. cereus* selective supplement contains polymyxin-B in lyophilized form.



Mannitol-Egg-yolk-Polymyxin-Agar (MYP Agar)

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.

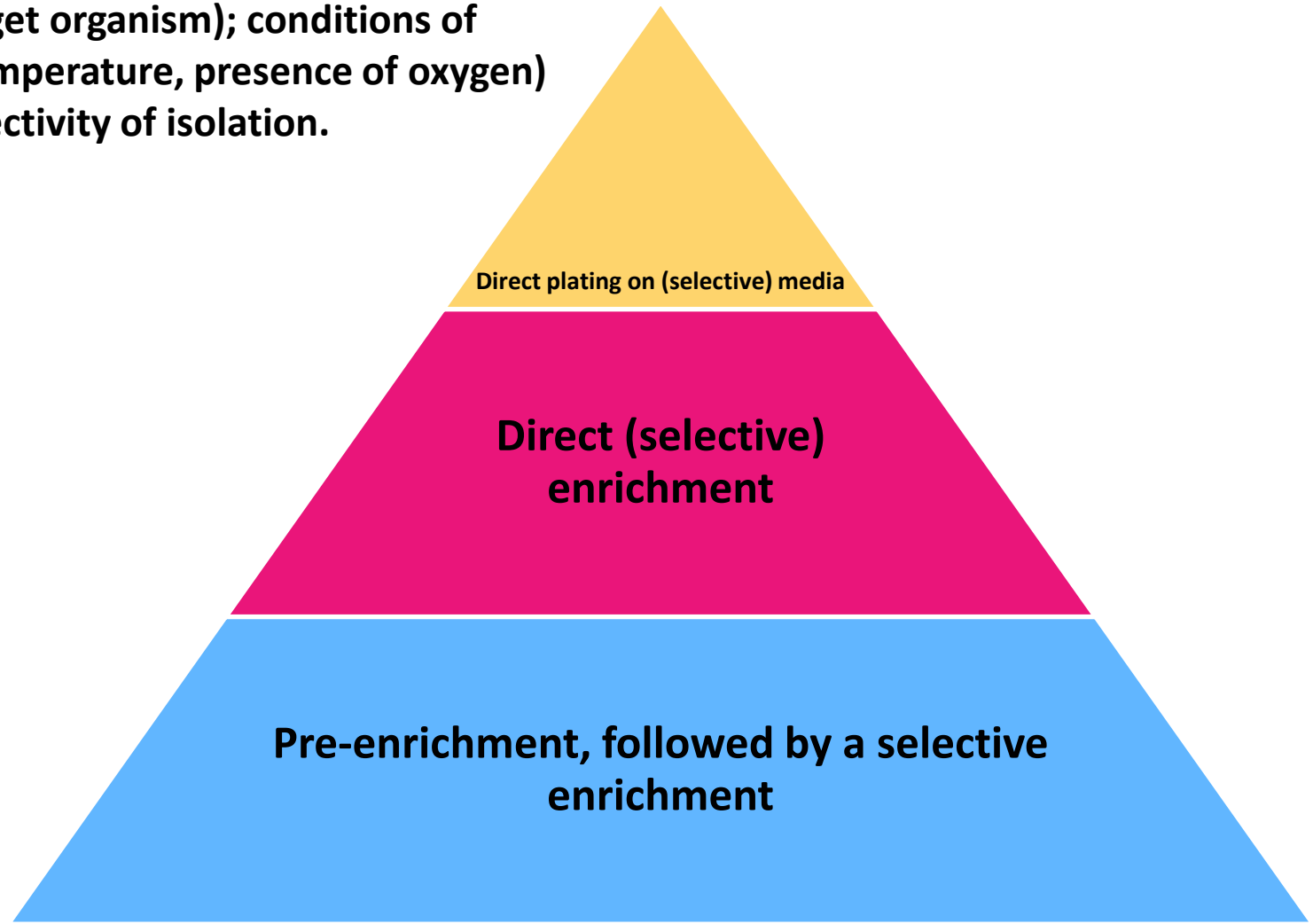


B. cereus appears as rough, dry colonies with pink to purple base which are surrounded by ring of dense precipitate. Colonies surrounded by yellow/clear zone are not *B. cereus*. Further tests confirm *B. cereus* (anaerobic degradation of D (+) glucose, degradation of gelatin, positive nitrate reduction)

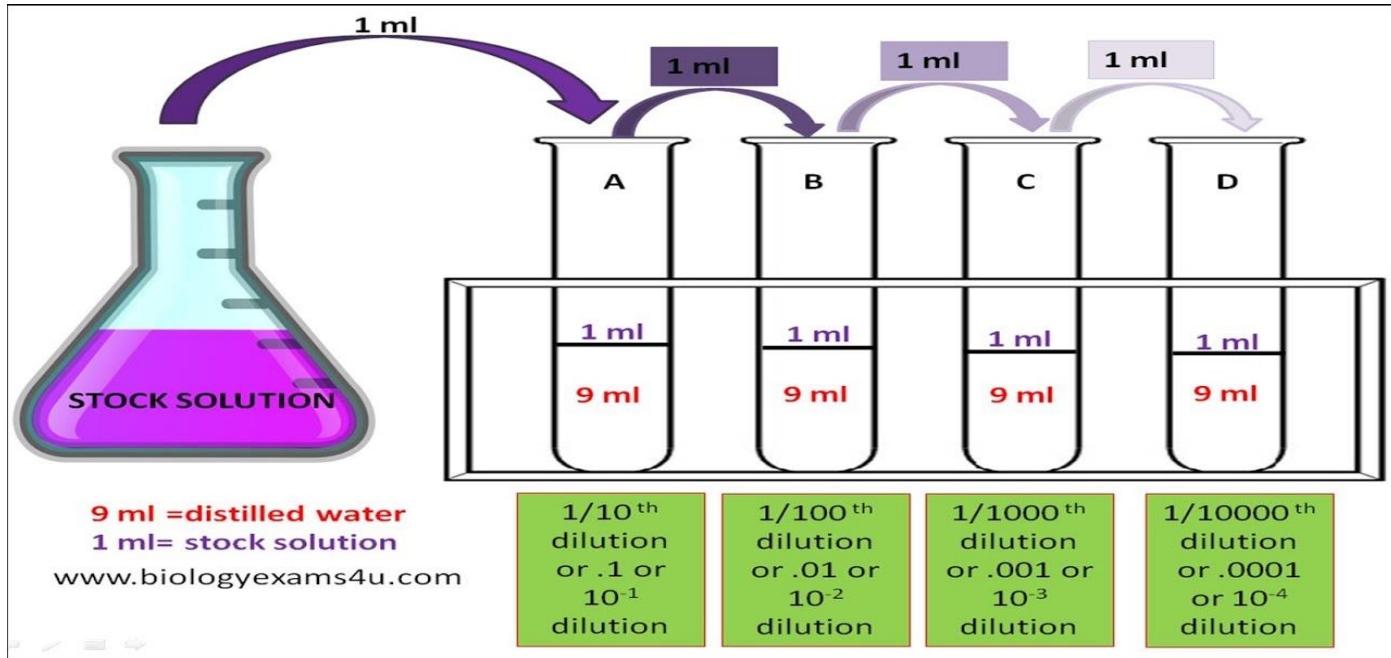
Preparation: Suspend 21.5 g in 450 ml de-mineralized water, autoclave (15 min at 121°C). Cool to 45-50 °C add 50 ml (volume can be varied depending on degree of turbidity) of sterile egg-yolk emulsion and contents of one vial *B. cereus* Selective Supplement, mix. pH: 7.2 ± 0.2 at 25 °C. Pour plates. Plates (including egg-yolk) are evenly turbid and slightly orange (red without egg-yolk).

Detection of *B. cereus*

Basic protocols depends: number and state of microorganisms in sample; selectivity of media (a balance between inhibition of competitors and inhibition of target organism); conditions of incubation (time, temperature, presence of oxygen) and selectivity of isolation.



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.

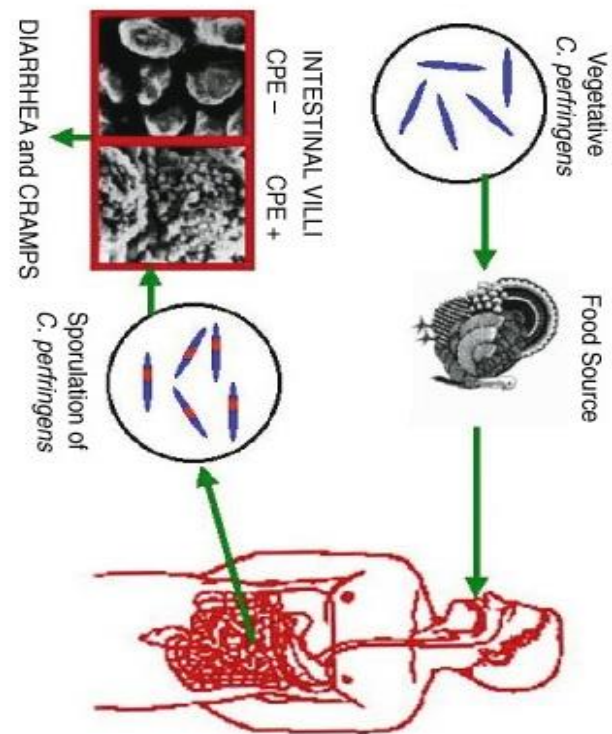


Sample preparation: Using aseptic technique, weigh 25 g of sample into sterile blender jar or macerate with sterile mortar pestel. Add 225 ml Butterfield's phosphate-buffered dilution water (1:10 dilution) and mix well for 2 min.

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 h at 30°C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced.

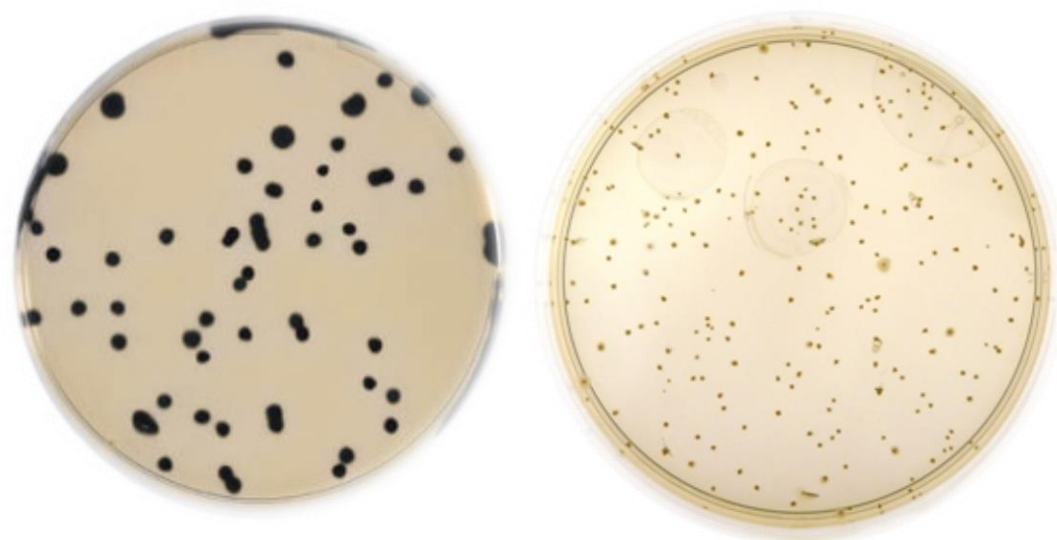
Confirmation: Pick 5 or more eosin pink, lecithinase-positive colonies from MYPA plates and transfer to nutrient agar slants. Incubate slants 24 h at 30°C . Prepare Gram-stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to sub terminal.

- **Food poisoning caused by *Clostridium perfringens*:** Meat or poultry are cooked and held without maintaining adequate heating or refrigeration before serving.
- Spores of some strains are resistant to temperatures as high as 100°C for more than one hour.
- Oxygen level sufficiently reduced during cooking to permit growth.
- Illness occurs 8-15 h after ingestion of contaminated food.
- **Symptoms:** Intense abdominal cramps, gas, and diarrhea (nausea and vomiting are rare) due to a protein enterotoxin produced during sporulation of organism in intestine.



- High correlation has been established between ability of *C. perfringens* strains to produce enterotoxin and their ability to cause food poisoning.
- *C. perfringens* cells lose their viability when foods are frozen or held under prolonged refrigeration.





Detection of Clostridia:

- Conventional methods for detection of clostridia is by heat killing of vegetative cells of clostridia and contaminating bacteria.
- Meat broth or blood agars promote spore germination.
- Addition of starch in many media is to facilitate germination.
- **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. Clostridia reduce sulphide to sulphite which gives black precipitate with iron present in medium.

- **Sodium sulphite and ferric citrate may be added to RCM to become differential RCM (DRCM), recommended for detection of sulphite reducing clostridia in drinking water and is specified in ISO standard 6461-1(1986) liquid enrichment method for water.**
- **Tryptose sulphite cycloserine agar (TSC) is 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 colonies. In addition to sulphite and iron, this medium utilizes sulphadiazine, oleandomycin phosphate and polymyxin B sulphate to give high degree of selectivity/specificity for *C. perfringens*.**

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.**
- Using 1:10 dilution, 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 center of duplicate agar plates.**
- Pour additional 15 ml TSC agar without egg yolk into dish and mix with inoculum by gently rotating dish.**

Presumptive confirmation test:

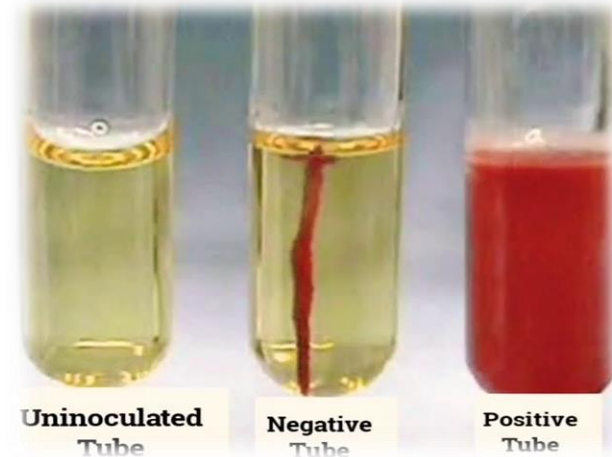
- Select 10 typical *C. perfringens* colonies from TSC or TSC-egg yolk agars plates and inoculate each into tube of freshly de-aerated and cooled fluid thioglycollate broth.
- Incubate in standard incubator 18-24 h at 35°C.
- Examine each culture by Gram stain and check for purity.
- *C. perfringens* is a short, thick, Gram positive bacillus.
- If there is contamination, streak contaminated culture on TSC agar containing egg yolk and incubate in anaerobic jar 24 h at 35°C.
- Surface colonies of *C. perfringens* are yellowish gray with 2-4 mm opaque zones caused by lecithinase activity.
- Used for isolating *C. perfringens* from chopped liver broth whenever the organism is not detected by direct plating on TSC agar.

Completed confirmation test

- **Gelatin liquefaction:**
- Stab-inoculate motility-nitrate (buffered) and lactose-gelatin media with 2 mm loopful 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 h 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 1 h at 5°C and examine for gelatin liquefaction.
- If medium gels, incubate an additional 24 h at 35°C and examine for gelatin liquefaction.

- **Gram staining:**
- Inoculate sporulation broth with 1 ml fluid thioglycollate medium culture and incubate 24 h at 35°C.
- Prepare Gram stain of sporulation broth and examine microscopically for spores.
- Store sporulated cultures.

- **Motility:**
- *C. perfringens* is non-motile.
- Examine tubes of motility-nitrate medium for growth along stab line.
- Non-motile organisms produce growth only in and along stab.
- Motile organisms usually produce diffuse growth out into medium, away from stab.



Nitrate reduction:

- C. perfringens* reduces nitrates to nitrites.

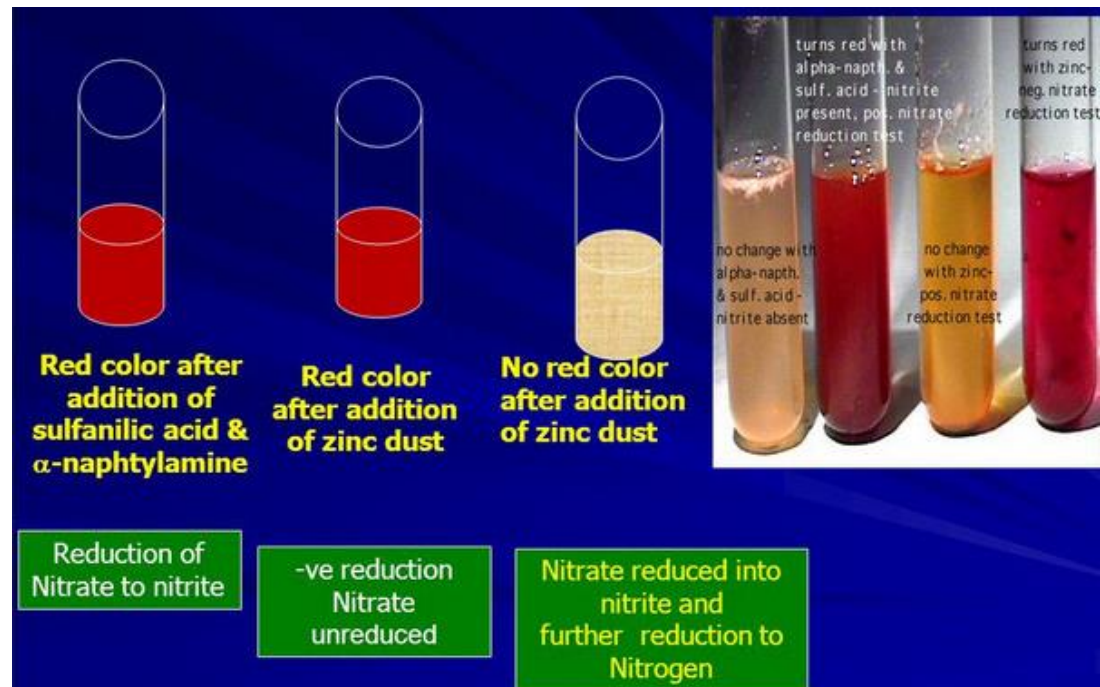
- To test for nitrate reduction, add 0.5 ml reagent A and 0.2 ml reagent B (R48) to culture in buffered motility-nitrate medium.

- Violet colour which develops within 5 min indicates presence of nitrites.

- If no color develops, add few grains of powdered zinc metal and let stand a few minutes.

- Negative test (no violet color) after zinc dust is added indicates that nitrates were completely reduced.

- Positive test after addition of zinc dust indicates that organism is incapable of reducing nitrates.



Clostridium botulinum

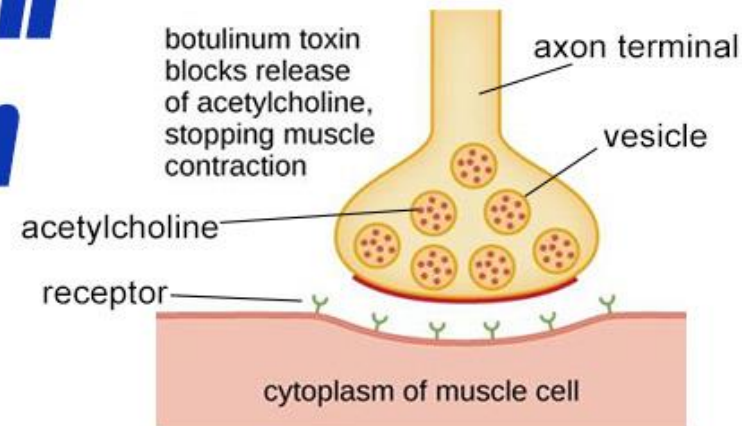
1. Anaerobic, rod-shaped spore forming bacterium that produces protein with neurotoxicity.
2. Under certain conditions, these organisms may grow in foods producing toxin(s).
3. Botulism, a severe form of food poisoning results when toxin-containing foods are ingested.
4. Mortality rate is high.
5. Toxins are not readily absorbed in human intestine.
6. Seven recognized antigenic types: A through G.
7. All cultures that produce type A toxin and some produce B and F toxins are proteolytic.
8. All type E strains and remaining B and F strains are non-proteolytic, with carbohydrate metabolic patterns differing from C and D non-proteolytic groups.
9. *C. botulinum* is widely distributed in soils and in sediments of oceans and lakes.
10. Types A and B are commonly encountered in foods associated with soil contamination.
11. Properly processed canned foods will not contain viable *C. botulinum*.
12. Home-canned foods are source of botulism than commercially canned foods.



Gram-positive bacilli with 'Tennis Racket' appearance

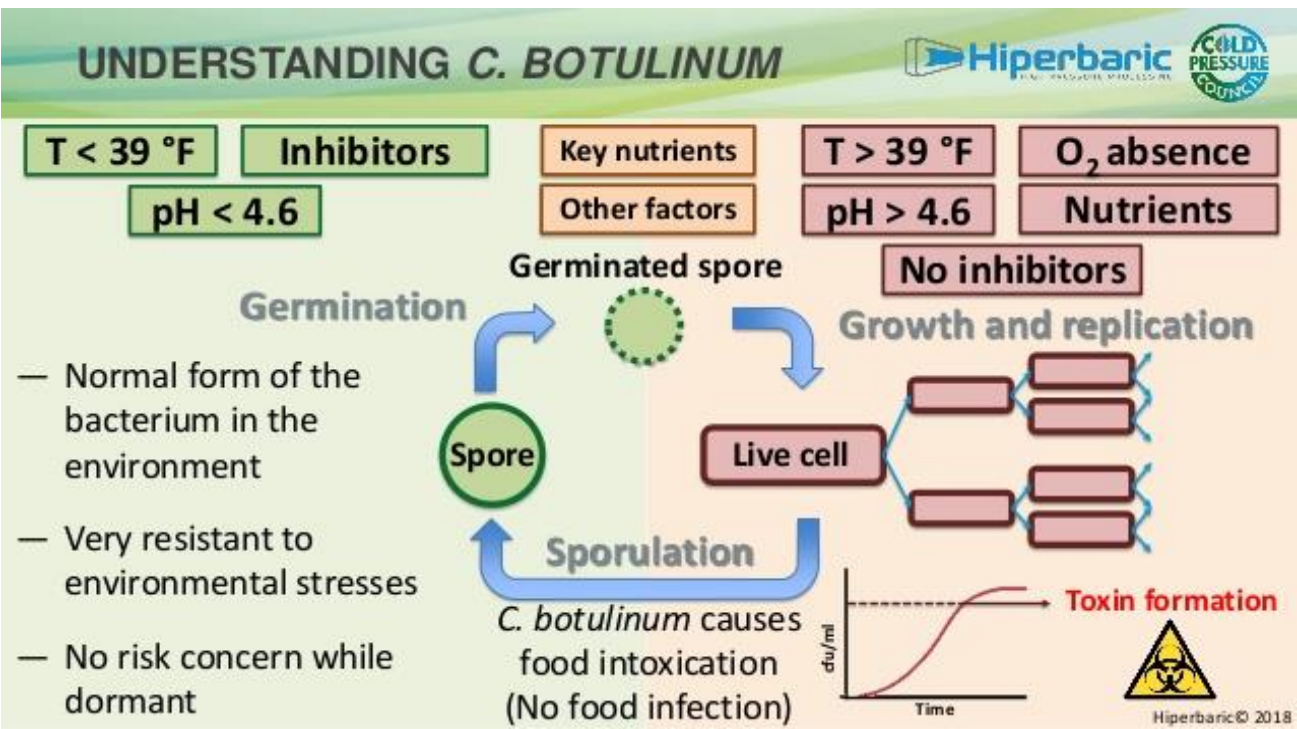


Clostridium botulinum



Growth of *Clostridium*:

1. If organisms do not grow, no toxin is produced.
2. Both nutritional and anaerobic requirements are supplied by many canned foods and various meat and fish products.
3. Growth in suitable foods can be prevented if product, naturally or by design, is acidic, low water activity, high concentration of NaCl, an inhibitory concentration of NaNO₂.
4. Refrigeration will not prevent growth and toxin formation by non-proteolytic strains.
5. 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 3-4°C.



Enumeration and detection:

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. Refrigerate reserve sample.**
- 3. Opening of canned foods: Examine product for appearance and odour.**

Enrichment:

- 1. Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation.**
- 2. Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth.**
- 3. Incubate at 35°C.**
- 4. Inoculate two tubes of TPGY broth as above. Incubate at 28°C.**
- 5. 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.**
- 6. Observe morphology of organisms and note existence of typical clostridia cells, occurrence and relative extent of sporulation, and location of spores within cells.**
- 7. Typical clostridia cell resembles a tennis racket.**
- 8. Usually, a 5-day incubation is the period of active growth giving the highest concentration of botulinal toxin. If enrichment culture shows no growth at 5 days, incubate an additional 10 days to detect possible delayed germination of injured spores.**

Isolation of pure cultures:

- 1. *C. botulinum* is more readily isolated from the mixed flora of an enrichment culture or original specimen if sporulation is good.**

Pre-treatment of specimens for streaking:

- 1. Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube.**
- 2. Mix well and incubate 1 h at room temperature.**
- 3. 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.**
- 4. Mix well and incubate 1 h at room temperature.**
- 5. Alternatively, heat (80°C for 10-15 min) 1 or 2 ml of enrichment culture or sample to destroy vegetative cells.**

Plating of treated cultures:

- 1. 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.**
- 2. If necessary, dilute culture to obtain well-separated colonies.**
- 3. Dry agar plates well before use to prevent spreading of colonies.**
- 4. Incubate streaked plates at 35°C for about 48 h under anaerobic conditions.**
- 5. Anaerobic jar or the GasPak system is adequate to obtain anaerobiosis.**

Selection of typical *C. botulinum* colonies:

- 1. Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough, some show spreading and have an irregular edge.**
- 2. On egg yolk medium, usually exhibit surface iridescence when examined by oblique light.**
- 3. This luster zone, often referred as pearly layer, usually extends beyond and follows irregular contour of colony.**
- 4. Besides pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate.**
- 5. Colonies of types A and B generally show smaller zone of precipitation.**

Isolation of pure cultures:

1. Re-streak toxic culture in duplicate on egg yolk agar medium.
2. Incubate one plate anaerobically at 35°C.
3. Incubate second plate aerobically at 35°C.
4. Culture may be pure if colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate).
5. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation.
6. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.

