Maintenance of Industrially Important Microorganisms

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Introduction

- Industrial microbiological establishments usually keep a collection of the microorganisms which possess the gene pools for producing the goods manufactured by the establishment.
- This stock of organisms is known as a *culture collection* and ensures a regular supply of organisms to be used in the manufacturing process.
- Organisms in a culture collection are maintained in a low metabolic state in which replication of the cells is kept to a minimum or even entirely restricted.
- Industrially important microorganisms are often mutants, and the condition of low metabolism in which they are kept, limits their tendency to revert to their low-yielding ancestors.
- In some circumstances organisms are maintained for comparatively short periods of days in an active state in which they are immediately ready for use in fermentations; such organisms are called *working stock.*
- *In many breweries, for example, the producing yeasts* are reused sometimes for up to eight runs or more before being discarded.
- In the interval between inoculations such yeasts are regarded by some workers as working stocks.
- It must be borne in mind that working stocks stand the chance of contamination and/or mutation, two serious problems inherent in industrial fermentations.

TYPES OF CULTURE COLLECTIONS

- Culture collections maintained by industrial establishments are usually specialized and store mainly those used in that particular organization.
- Some national culture collections handle a wide variety of org., of whatever kind.
  - The best known in this category is the American Type Culture Collection (ATCC).
- Other collections are specialized and may handle:
  - only pathogenic microorganisms, such as:
    - the National Collection of Type Cultures (NCTC) in Colindale, London, UK
  - industrial microorganisms, such as:
    - National Collection of Industrial Bacteria (NCIB) in Aberdeen, Scotland.
- Still others almost exclusively handle one type of organism such as:
  - Center vor Braunsveitzer (CBS) in Holland, which handles fungi exclusively.
- Many universities all over the world have culture collections which reflect their range of microbiological interests.
- Culture Collections around the world are linked by the World Federation of Culture Collections (WFCC).
- The WFCC is an affiliate of the International Union of Microbiological Societies (IUMS) the organization which links national microbiological societies world wide.

... TYPES OF CULTURE COLLECTIONS

- Culture Collections are organized on regional and international basis for the exchange of cultures and ideas and include the:
  - National Agriculllturally Important Microbial Culture Collection (NAIMCC, India),
  - BCCCM (Belgium Co-ordinated Collections of Microorganisms),
  - ECCO (European Culture Collection Organization),
  - JFCC (Japanese Federation of Culture Collections),
  - MICRO-NET (Microbial Information Network of China),
  - MSDN (Microbial Strain Data Network, UK),
  - UKNCC (United Kingdom National Culture Collection),
  - USFCC (United States Federation of Culture Collections, USA)
- Culture collections may be specialized and in-house such as those in industrial establishments.
- Others are public and have the function of acquiring, identifying, preserving and distributing microorganisms and for a fee will supply cultures for in teaching, research or to industry.
- Such culture collections receive cultures from all over the world and thus serve the overall purpose of maintaining worldwide microbiological biodiversity.
- In addition to making available organisms for industrial use, the major culture collections serve the important function of acting as depositories for microorganisms mentioned in the patenting of microbiological processes.

HANDLING CULTURE COLLECTIONS

- An industrial process may be initiated with organisms obtained through the Patent Office in connection with a patent.
- Often only one vial of such an organism is usually available.
- Once growth has been obtained from that vial the organism should be multiplied and stored in one or more of the several manners described later for the preservation of primary stock organisms in a Culture Collection.
- Several replicates are stored immediately for fear of contamination while tests are carried out to ascertain its potential for fulfilling the expected activity.
- If the tests show that the expected antibiotic or other desired metabolite is being produced in the expected quantity then stored organisms are retained.
- The stocks of those organisms which proved negative at first sampling should not be discarded in a hurry because further examination may show that poor productivity was due to factors extrinsic to the organism such as an inadequate medium.
- Date of transfer, the medium and the temperature of growth, etc., must all be carefully recorded to afford a means of assessing the effect of the preservation method.

METHODS OF PRESERVING MICROORGANISMS

- Methods employed in the preservation of microorganisms all involve some limitation on the rate of metabolism of the organism.
- A low rate of spontaneous mutation exists during the growth of microorganisms, about once in every 10^9 division.
- Lowering the metabolic rate of the organism will further reduce the chances of occurrence of mutations.
- The principles involved in preserving microorganisms are:

  (a) reduction in the temperature of growth of the organism;

  (b) dehydration or desiccation of the medium of growth;

  (c) limitation of nutrients available to the organism.
- All three principles lead to a reduction in the organisms metabolism.

Microbial Preservation Methods Based on the Reduction of the Temperature of Growth

Preservation on agar with ordinary refrigeration (4 – 10°C)

- Organisms growing on suitable agar at normal growth temperatures attain the stationary phase and begin to die because of the release of toxic materials and the exhaustion of the nutrients.
- Agar-grown organisms are therefore refrigerated as soon as adequate growth is attained as to preserve them.

a) Aerobic organisms

- *Agar slants:* Aerobic organisms may be grown on agar slants and refrigerated at 4 – 10°C as soon as they have shown growth.
- *Petri dishes: Aerobic organisms may also be stored on Petri dishes.*
- *The plates may be* sealed with electrical tapes to prevent the plates from drying out on account of evaporation.

b) Anaerobic organisms: Anaerobic organisms may be stored on agar stabs which are then sealed with sterile molten petroleum jelly

… Preservation on agar with ordinary refrigeration (4 – 10°C)

- The **advantage is** that agar storage methods are inexpensive because they do not require any specialized equipment.
- The **disadvantages are:**

(a) The organisms must be sub-cultured at intervals which have to be worked for each organism, medium used, laboratory practice, etc.

- This is because the temp. of refrigeration is not low enough to limit growth completely.

(b) Consequent on regular sub-culturing is the possibility that contaminations and or mutations may occur.

(c) The third disadvantage is that Petri dishes occupies a lot of space in the refrigerator when compared with agar slants.

- But even agar slants are too bulky in comparison with the small vials in which lyophilized (freeze-dried) cultures are stored.
- Since plates occupy a lot of space, test tubes are usually preferred for storage in refrigerators.

(d) Process of sub-culturing is tedious apart from possibility of contamination and mutation.

(e) When petroleum jelly is used as a seal, the arrangement can be messy.

... Microbial Preservation Methods Based on the Reduction of the Temperature of Growth

Oil overlay

- With the method of oil overlay whose function is to limit oxygen diffusion many bacteria, especially anaerobes and facultatives, and fungi survive for up to three years, and most of them for at least one year.
- ***Medium for storing organisms on agar***
- The nature of the medium on which organisms are stored is of importance.
- A medium prepared from natural components rather than a chemically defined material is preferable, since a defined medium may, because it lacks some components present in the natural components, select for organisms specifically capable of growing on it.
- A stock culture medium should also not be unduly rich in carbohydrates such as glucose which will lead to early production of acid and hence possible early microbial death.
- Where glucose is used, such as for lactic bacteria, the medium should be buffered with calcium carbonate.

Preservation in Deep Freezers at about -20°C, or between -60°C and -80°C

Preservation on glass beads

- The bacteria to be preserved are placed in broth containing cryoprotective compounds such as glycerol, raffinose, lactose, or trehalose.
- Sterile glass beads are placed in the glass vials containing the bacterial cultures.
- The vials are gently shaken before being put in the deep freezers.

Storage of agar cores with microbial growth

- It consists of placing agar plugs of confluent growth of bacteria and yeasts and hyphe of moulds or actinomycete in glass vials containing a suitable cryoprotectant and freezing the vials in deep freezers as above.
- To initiate growth a plug is placed in warm broth and plated out.

Advantages of the above freezing methods

(a) Its storage effectiveness for up to three years. It is useful for a wide range of organisms, and survival rates have been shown to be as good as freeze-drying in many organisms.

(b) the methods are simple to use and require a minimum of equipment;

(c) they save space as many hundreds of cultures can be stored in a small space;

(d) beads thaw rapidly and hence the method saves time,

(e) the methods can be adapted for both aerobic and anaerobic organisms;

(f) the methods are suitable for situations or countries where power outages occur, as the freezer can remain cold for some time during power failures.

Storage in low temperature liquid or vapor phase nitrogen (-156°C to -196°C)

- The liquid or vapor phase of nitrogen at -156°C to -196°C is widely used for preserving microorganisms and cultured cells.
- The period of survival and the number of surviving organisms are higher for most organisms than when freeze drying is used.
- Some organisms are prone to losing numbers with this method, but the loss is reduced with the use of cryoprotectants.
- Some of the most commonly used cryoprotectants are (vol/vol) 10-20% glycerol and 5-10% dimethyl sulfoxide (DMSO) in broth culture of the organism in vials which are then frozen in liquid nitrogen.
- Vials for storing organisms in low temperature nitrogen may be made of glass or fashioned from ordinary polypropylene (plastic) drinking straws.
- Straws (4 mm diameter) are usually cut into pieces 40 mm long and made into ampoules by sealing the ends with heat.

... Storage in low temperature liquid or vapor phase nitrogen (-156°C to -196°C)

Freezing at –156°C to -196°C has the following disadvantages:

(a) As liquid nitrogen evaporates, it has to be replenished regularly; if not replenished the cultures may be lost.

(b) A risk of explosion exists when cultures are frozen in liquid nitrogen in improperly sealed glass vials which permit entry of liquid nitrogen into the vials.

- Such vials may explode when warmed to thaw them.
- Vapor phase storage removes such dangers.

(c) Although it is not labor intensive the equipment is expensive.

(d) Finally it is not a convenient method for transporting organisms.

Microbial Preservation Methods Based on Dehydration

- Just as reduction in temperature limits the metabolism of the organism, dehydration removes water a necessity for the metabolism of the organism.
- **Drying on sterile silica gel**
- Many organisms including actinomycetes and fungi are dried by this method.
- Screw-cap tubes half-filled silica gel are sterilized in an oven.
- On cooling a skim-milk suspension of spores and the cells of the fungus or actinomycetes is placed over the silica gel and cooled.
- Dried at 25°C, cooled and stored in closed containers containing desiccants.
- **Preservation on sterile filter paper**
- Spore-forming microorganisms such as fungi, actinomycetes, or *Bacillus* spp. may be preserved on sterile filter paper by placing drops of broth containing the spores on sterile filter paper in a Petri dish and drying in a low temperature oven or in a dessicator.
- After drying the filter paper may be placed in sterile screw caps bottles and stored either at room temperature or in the refrigerator.

... Microbial Preservation Methods Based on Dehydration

- **Preservation in sterile dry soil**
- The most commonly used form of storage in a dry state is the use of dry sterile soil.
- In this method dry soil is sterilized by autoclaving.
- It is then inoculated with a broth or agar culture of the organism.
- The soil is protected from contamination and allowed to dry over a period of time.
- Subsequently it may be refrigerated.
- The method has been widely and successfully used to store sporulating organisms especially clostridia and fungi.
- It has also been used for bacilli and *Azotobacter* sp., some non-sporulating bacteria which do not survive well under Lyophilization, may be stored in soil.

... Microbial Preservation Methods Based on Dehydration

- **Freeze-drying (drying with freezing), lyophilization**
- The principle of the method is that the organism is first frozen.
- Subsequently, water is removed by direct vaporization of the ice with the introduction of a vacuum.
- As the suspension is not in the liquid state, distortion of shape and consequent cell damage is minimized.
- At the end of the drying the ampoule containing the organism may be stored under refrigeration although survival for many years has also been obtained by storage at RT.
- The suspending medium must be carefully chosen, because of differences in the cryoprotection properties of different substrates.
- Horse blood is usually used; others which have been successfully used are inositol, various disaccharides, and polyalcohols.
- The ampoule is usually evacuated after freeze drying.
- Lyophilization is preferred for the preservation of most organisms because of its:
  - success with a large number of organisms,
  - the relatively inexpensive equipment,
  - The scant demand on space made by ampoules,
  - the longevity (up to 10 years or more in some organisms) of most organisms stored by lyophilization.

... Microbial Preservation Methods Based on Dehydration

- **L-drying (liquid drying, drying without refrigeration)**
- Unlike freeze-drying, the organisms are not frozen, but dried from the liquid state.
- It has been used to preserve nonspore formers sensitive to freeze-drying, such as *Cytophaga, Spirillum* and *Vibrio.*
- Liquid drying has been effectively used to preserve organisms such as anaerobes that are damaged by freezing.
- Small vials made of glass are filled with a mixture of skim milk, medical grade activated charcoal and myo-inositol , autoclaved and thereafter frozen at about -40°C for a few hours.
- The vials are then freeze-dried and this leads to a disc of freeze-dried carrier material in the vials.
- The broth of the organism to be dried is placed on the disc and the material is subjected to a vacuum in the liquid unfrozen state at 20°C.

Microbial Preservation Methods Based on the Reduction of Nutrients

- **Storage in distilled water**
- Many organisms die in distilled water because of water absorption by osmosis.
- However some have been known to survive for long periods in sterile distilled water.
- Usually such storage is accompanied by refrigeration; some organisms are however, harmed by refrigeration.
- Among organisms which have been stored for long periods with this method are *Pseudomonas solanaceanum, Saccharomyces cerevisiae*, and *Sarcina lutea.*
- *The* attractiveness of this method is its simplicity and inexpensiveness; since so few organisms seem to be storable in this manner, it should not, for fear of losing the organism, be adopted as the sole method for storing a newly acquired or isolated organism until it has been shown to be suitable.

The Need for Experimentation to Determine the Most Appropriate Method of Preserving an Organism

- No one method can be said to suitable for the preservation of all and every organism.
- The appropriate method must be determined for each organism unless prior literature information exists.
- The preservation method must retain the characteristics which are desirable in the organism and this is crucial for industrial microorganisms.
- For example, the characteristic brick-red color of *Sarcina lutea* was lost in some preservation methods.
- While the production of rennet by *Rhizomucor* sp. affected by some methods.
- Antibiotics production by some actinomycetes were respectively affected by the method used for their preservation.