Quality control of the preserved stock culture

Whichever technique is used for the preservation and maintenance of industrially important organisms it is essential to check the quality of the preserved organisms stocks. Each batch of newly preserved cultures should be routinely checked to ensure their quality. A single colony is transferred into a shake-flask to ensure growth of particular kind of microorganism; further shake-flask subculture is used for the preparation of huge quantity of vials. For the assessment of purity, viability and productivity of cultures few vials are tested. If samples fail any one of these tests the entire batch should be destroyed. Thus, by the use of such a quality-control system stock cultures many be retained, and used, with confidence.

Inoculum preparation

Industrial fermentations utilizing yeasts are the brewing of beer, the production of Baker's Yeast (biomass) and recent processes have also been established for the production of recombinant products.

Brewing

- Yeast can be used to inoculate a fresh batch of wort from previous fermentation or from propagator.
- It is common practice in the British brewing industry to use the yeast from the previous fermentation.
- The brewing terms used to describe this process and 'crop', referring to the harvested yeast from the previous fermentation, and 'pitch', meaning to inoculate.
- One of the major factors contributing to the continuation of this practice is the wort-based excise laws in the United Kingdom where duty is charged on the sugar consumed rather than the alcohol produced.
- Thus, dedicated yeast propagation systems are expensive to operate because duty is charged on the sugar consumed by the yeast during growth.

- The problems with this technique are chances of contamination and degeneration of strains, the most common problem with the degenerated cell is the change in the degree of flocculence and weakening of abilities of the yeast.

- In breweries employing top fermentations in open fermenters these dangers are minimized by collecting yeast to be used for future pitching from 'middle skimmings''.

- As the head of yeast develops, the surface layer (the most flocculent and highly contaminated yeasts) is removed and discarded and the underlying cells (the 'middle skimmings') are harvested and used for subsequent pitching.

- Therefore, the 'middle skimmings' contain cells which have the desired flocculence and which have been protected from contamination by the surface layer of the yeast head.

- The pitching yeast may be treated to reduce the level of contaminating bacteria and remove protein and dead yeast cells by such treatments as reducing the pH of the slurry to 2.5 to 3, washing with water, washing with ammonium persulphate and treatment with antibiotics such as polymixin, penicillin and neomycin.

- However, traditional open vessels are becoming rare and the bulk of beer is brewed using cylindro-conical fermenters.

- In these systems the yeast flocculates and collects in the cone at the bottom of the fermenter where it is subject to the stresses of nutrient starvation, high ethanol concentration, low water activity, high carbon dioxide concentration and high pressure, which decreases the viability and physiological state of the yeast crop, would not be ideal for an inoculum.

- The situation is further complicated by the fact that the harvested yeast is stored rapidly to about 1°, before it is used as inoculum suspending in beer and storing in the absence of oxygen.

- One of the key physiological features of yeast inoculum is the level of sterol in the cells. Sterols are required for membrane synthesis but they are only produced in the presence of oxygen.

- Thus, we have the irregularity of oxygen being required for sterol synthesis; yet anaerobic conditions are required for ethanol production.

- This irregularity is resolved traditionally by aerating the wort before inoculation.

- The difficulties outlined above and the likelihood of strain degeneration and contamination mean that are rarely used for more than five to ten consecutive fermentations which necessitates the periodical production of a pure inoculum.

- Pure inocula can be prepared by a yeast propagation scheme utilizing a 10% inoculum volume at each stage in the programme and employing conditions similar to those used during brewing.

- Continuous aeration may be used during the propagation stage which seems to have little effect on the beer produced in the subsequent fermentation.

- Yeast inoculum produced in this way would also be sterol rich, obviating the need for aerated wort.

- The simplest type of propagator is a single stage system resembling an unstirred, aerated fermenter which is inoculated with a shake-flask culture developed from a single colony.

- Two-stage systems propagator could be operated semi-continuously. It consisted of two linked vessels, 1.5 and 150 dm^3 respectively.

- The smaller vessel is filled with wort, sterilized, cooled, aerated and inoculated with a flask-grown culture. After growth for 3 to 4 days the culture was forced by air pressure into the second vessel which had been filled with sterilized, cooled wort and aerated.

- An aliquot of 1.5 dm^3 was forced back into the first vessel after mixing. In a further 3 to 4 days the larger vessel contained sufficient biomass to pitch a 1000 dm^3 fermenter and the first vessel contained sufficient inoculum for another second stage.

- However, although this procedure should produce a pure inoculum there is a danger of strain degeneration occurring in such a semi-continuous system.

Baker's Yeast

- The commercial production of bakers' yeast involves the development of an inoculum through a large number of aerobic stages.

- Although the production stages of the process may not be operated under strictly aseptic conditions a pure culture is used for the initial inoculum, thereby keeping contamination to a minimum in the early stages of growth.

- The development of inoculum for the production of bakers' yeast involve eight stages, the first three being aseptic while the remaining stages were carried out in open vessels.

- The yeast may be pumped from one stage to the next or the seed cultures may be centrifuged and washed before transfer, which reduces the level of contamination.

- The yields obtained in the first five stages are relatively low because they are not fed-batch systems, whereas the last three stages are fed-batch.

The Development of Inocula for Bacterial Processes

Introduction

- The main objective of inoculum development for traditional bacterial fermentations is to decrease lag phase.

- A long lag phase is not only is wastage of time but also medium is consumed in maintaining a viable culture prior to growth.

- The length of the lag phase is affected by the size of the inoculum and its physiological condition.

- Bacterial inocula should be transferred, when the cells are still metabolically active.

- The age of the inoculum is particularly important in the growth of sporulating bacteria, for sporulation is induced at the end of the logarithmic phase and the use of an inoculum containing a high percentage of spores would result in a long lag phase in a successive fermentation.

- 5% inoculum of thermophilic Bacillus in logarithm phase is used for the commercial production of proteases.

- A two-stage inoculum development programme is used for the production of proteases by Bacillus subtilis. Inoculum for a seed fermenter was grown for 1 to 2 days on a solid or liquid medium and then transferred to a seed vessel where the organism was allowed to grow for a further ten generations before transfer to the production stage.

- The lag phase in plant fermenters could be almost completely eliminated by using inoculum medium of the same composition as used in the production fermenter and employing large inocula of actively growing seed cultures in the production of bacterial enzymes.

- The inoculum development programme for a pilot-plant scale process for the production of vitamin B12 from ***Pseudomonas denitrificans*** is shown below (Spalla et al., 1989).

STOCK CULTURE

Lyophilised with skim milk

↓

MAINTENANCE CULTURE

Agar slope incubated 4 days at 28°

↓

SEED CULTURE - FIRST STAGE

2 dm^3 flask containing 0.6 dm^3 medium inoculated with culture from one slope; incubated with shaking for 48h at 28°

↓

SEED CULTURE - SECOND STAGE

40 - 80 dm^3 fermenter containing 25 - 50 dm^3 medium inoculated with 1 - 1.2% first stage seed culture. Incubated 25 - 30h at 32°

↓

PRODUCTION CULTURE

- 500 dm^3 fermenter with 300 dm^3 medium inoculated with 5% second stage seed culture. Incubated at 32° for 140 - 160 h
- The acetic-acid bacteria used in the vinegar process are extremely sensitive to oxygen starvation therefore it is essential to use an inoculum in an active physiological state.
- The cells at the end of fermentation are used as inoculum for the next batch by removing approximately 60% of the culture and restoring the original level with fresh medium.
- In this process there are enough chances of strain degeneration and contaminant accumulation.
- However, strain stability is a major concern in inoculum development for fermentations employing recombinant bacteria.

- Plasmid stability and productivity in ***E.coli*** biotin fermentation was improved if stationary, rather than exponential phase, cells were used as inoculum due to loss of plasmid in fermentation.

- In the lactic-acid fermentation the producing organism may be inhibited by lactic acid. Thus, production of lactic acid in the seed fermentation may result in generation of poor quality inoculum.

- High quality inoculum of ***Lactococcus lactis*** 10^{-1} on a laboratory scale is obtained using electrodialysis which reduced the lactate in the inoculum and reduced the length of the lag phase in the production fermentation.

Development of Inocula for Anaerobic Bacterial Processes

- Clostridial Acetone-Butanol fermentation is anaerobic process.

- Though the process was outcompeted by the petrochemical industry but there is still considerable interest in reestablishing the fermentation.

- The inoculum development programme described by McNeil and Kristiansen (1986) is given as below

Heat-shocked spore suspension inoculated

into 150 cm^3 of potato glucose medium

↓

Stage 1 culture used as inoculum for 500 cm^3molasses medium

↓

Stage 2 culture used as inoculum for 9 dm^3 molasses medium

↓

Stage 3 culture used as inoculum for 90,000 dm^3 molasses medium

- The stock culture is heat shocked to stimulate spore germination and to eliminate the weaker spores.
- The production stage is inoculated with a very low volume.
- The use of such small inocula necessitates the achievement of as near perfect conditions as possible to prevent contamination and to avoid an abnormally long lag phase.

Batch and Continuous sterilization of medium

Media may be sterilized by

1) Filtration,

2) Radiation,

3) Ultrasonic treatment,

4) Chemical treatment

5) Heat

Out of these methods, heat or steam is the most useful method for the sterilization of fermentation media.

A number of factors influence the success of heat sterilization

1. The number and types of microorganisms present
2. The composition of the culture medium
3. The pH value and the size of the suspended particle

Filtration is used for the sterilization of medium which is exception for the medium containing heat labile components

Sterilization process i.e. killing of microbes by steam under pressure is a first-order chemical reaction and, thus, may be written as

$$-dN/dt = kN \quad (1)$$

Where

N is the number of viable organisms present,

t is sterilization treatment time,

K is the reaction rate constant of the reaction, or the specific death rate

regardless of the volume of the batch, the minimum number of organisms to contaminate a batch is one.

On integration of equation (5.1) the following expression is obtained….

$$N_t / N_o = e^{-kt} \quad (2)$$

N_o is the number of viable organisms present initially

N_t is the number of viable organisms present after a period treatment, t

On taking natural logarithms, equation (5.2) is reduced to

$$\ln(N_t / N_o) = -kt \quad (3)$$

$$kt = \ln (N_0 / Nt) \quad (4)$$

A plot of the natural logarithm of N_t / N_o against time yields a straight line, the slope of which equals $-k$

This kinetic description makes two predictions which appear abnormal

1. Sterile condition is achieved in An infinite time (i.e. $N_t = 0$)

2. After a definite time number of cells present will be less than one

The relationship displayed in Fig. 5.1 would be observed only for pure culture in certain metabolic state, under ideal sterilization conditions

The value of k also indicates physiological state of organisms with kind of species, but dependent on the physiological form of the cell; for example, the endospores of the genus Bacillus are far more heat resistant than the vegetative cells

Richards (1968) produced a various graphs illustrating the deviation from theory which may be experienced in practice

The deviation in the above graph is due to the induction of spore germination by the heat and moisture of the initial period of the sterilization process.

Time for the sterilization is dependent on the type of the population. If the sensitive organisms are more in number than whole culture sterilization will be equal to that of the sensitive culture. But if the number of the resistant organisms is more than the sterilization of whole culture is equal to that of the sterilization of the resistant organisms.

Now by considering that contaminant may be by not a single type of organism but by different types of organisms. Sterilization of media required destruction of all types of organisms. The destruction of organisms in sterilization process is given by the factor called Del factor.

As first order reaction, as temperature increases, reaction rate increases due to an increase in the reaction rate constant, which, in case of the destruction of microorganisms is the specific death rate (k)? Thus k is true constant only under constant temperature conditions.

The relationship between temperature and reaction rate constant was demonstrated by Arhenius and may be represented by the equation

$$\frac{D \ln k}{dT} = \frac{E}{RT^2}$$

E – Activation energy

R – gas constant

T – Absolute temperature

On Integration

$K = A. e^{-E/RT}$

Therefore $kt = A. t. e^{-E/RT}$

On taking natural logarithm

$\ln k = \ln A - E/RT$

Plot of ln k versus 1/T gives straight line

Such a plot is Arhenius plot and enable calculation of activation energy and prediction of the reaction rate for any temperature.

Now $\ln Nt/No = -kt$

So, $\ln No/Nt = kt$

Therefore $\ln No/Nt = A. t. e^{-E/RT}$

Deindoerfer and Humphrey (1959) used the term In N_o / N_t as a design criterion for sterilization, which has been variously called the Del factor, Nabla factor and sterilization criterion represented by the term ▼

Thus, the Del factor gives idea about fractional reduction in viable organism count produced by a definite heat in particular time

Now ▼= ln No/Nt

But from above equation ln No/Nt = A. t. $e^{-E/RT}$

Thus ▼= A. t. $e^{-E/RT}$

On rearranging equation

ln t = E/RT + ln (▼/A)

This graph is used to obtain definite ▼ value with certain absolute temperature.

If we plot the graph from above line equation then we will get idea about time and absolute temperature required to achieve sterilization.

According to Deindoerfer and Humphrey, Richards Banks and Corbett a risk factor of one batch in a thousand being contaminated is frequently used in fermentation industry – that is, the final microbial count in the medium after sterilization should be 10^{-3} viable cells.

To apply kinetics it is necessary to know the thermal death characteristics of all the taxa contaminating the fermenter and unsterile medium, this is an impossibile and, therefore, the assumption may be made that the only microbial contaminants present are spores of *Bacillus stearothelmophilus* - that is, one of the most heat-resistant microbial types known

Thus, by adopting *B. stearothennophilus* as the design organism a considerable safety factor should be built into the calculations

It should be remembered that *B. stearothennophilus* is not always adopted as the design organism

If the most heat-resistant organism contaminating the medium ingredients is known, then it may be advantageous to base the sterilization process on this organism

The initial rise in yield is due to some components of the medium being made more available to the process micro-organism by the 'cooking effect' of a brief sterilization period (Richards, 1966).

Reaction contributing the decrease in nutrient value during sterilization

Interactions between nutrient components of the medium

- Maillard-type browning reaction discoloration of the medium as well as deterioration of nutrient value caused by the reaction of carbonyl groups and amino groups from reducing sugars, and amino acids and proteins respectively