BIOPROCCESS TECHNOLOGY PRACTICAL

S.No	Name of the Practical
1.	Growth Curve of a Bacteria
2.	Determination of thermal death point (TDP) of an organism
3.	Production and analysis of Ethanol
4.	Production and analysis of Lactic acid
5.	Production and analysis of amylase
6.	Isolation of industrially important microorganisms from natural resources

Exp No: 1 **GROWTH CURVE OF BACTERIA**

Introduction

If a microorganism is inoculated into a flask of medium and the growth rate of this organism reproducing by binary fission is plotted as the logarithm of cell number versus incubation time, a growth curve consisting of four distinct phases results:

1. The initial phase, lag phase, refers to the fact that cell division does not occur immediately; the microorganisms must adjust to their new medium. The length of this stage will depend on the condition of the cells at the time of inoculation and the type of medium into which they were inoculated. If a young culture is transferred into the same medium under the same conditions, no lag time is seen.

2. During the next phase, the exponential phase, microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium and environmental conditions. The population is most uniform in terms of chemical and physiological properties. During this stage the generation time (doubling time) can be determined directly from a graph of log cell number versus time. See the MB 302 textbook for more details.

3. In the stationary phase, growth ceases. Normally this occurs once the population level has reached 109 cells/ml. This stage is reached because available nutrients are depleted, toxic waste products have accumulated, physical space is limited, and/or quorum sensing has occurred.

4. If incubation continues, the culture will enter a fourth phase, the death or decline phase, whereby the number of viable cells decreases exponentially.

Growth curves are typically determined using the viable plate method. This method employs spreading a diluted sample of bacteria over a solid medium and determining the number of colonies that arise. The number of viable microorganisms in the sample is calculated from the number of colonies formed multiplied by the dilution factor (inverse of the dilution). This method works well as long as microbes are well dispersed so that you increase the chance that each colony arises from an individual cell. On the Petri plates used, this method is only

statistically valid if the sample yields between 25-250 colonies. Plates with higher or lower counts are not used normally, although the data should still be recorded.

Bacterial cell numbers can also be approximated by measuring cell mass by means of optical density (O.D.) or turbidity, once the population has reached about 107 cells/ml. For exponentially growing cells this method is fairly reliable and provides an almost instantaneous estimation of the bacterial concentration for a culture. If cell number versus O.D. are graphed a straight line should result, indicating correlation between the two values. This follows the Beer-Lambert Law of Solutes, which typically applies to molecules in a solution but which has been shown to also apply for bacteria in suspension.

A microbiologist can produce a standard curve by measuring both O.D. and cell numbers by plate count for a bacterial population. The resulting graph can be used to extrapolate cell concentration by O.D. in future experiments, thus eliminating the delay caused by incubation. Cell size influences the results obtained by spectrophotometry so the same bacterium must be used and the values are only valid for cells in the exponential phase of growth.

In this experiment, we will determine the growth curve of E. coli under various nutritional and environmental conditions.

Objectives

Lab objectives are not required in the lab notebook for this experiment. The results for this experiment will be written up separately from the lab notebook.

Materials and Methods

E. coli culture grown overnight

50 ml of minimal salts broth in side arm flask

Tube of BHI or minimal salts broth as blank

Spectronic 21or 20D with black velvet cloth

TSA plates (2 for each sampling time)

1 can of sterile 1 ml pipettes

% peptone dilution blanks: 9.0 ml (clear liquid, blue cap)

% peptone dilution blanks: 9.9 ml (clear liquid, silver cap)

package of Kimwipes

hockey stick, ethanol

Procedure

Overview: each group will inoculate a flask with a specified amount of an overnight culture of E.coli. Immediately after inoculation, each group will take both an initial O.D. and a viable cell count sample. Additional O.D. measurements will be taken every 15 minutes over a 3 hour period, while additional viable plate count samples will be taken every 30 minutes over a 3 hour period. The dilution schemes are shown in Figure 12.1. For an O.D. measurement at any time point: if the O.D. is below 0.5, use dilution scheme A. If the O.D. is over 0.5, use dilution scheme B.

1. Blank your spectrophotometer with an appropriate blank (uninoculated BHI or minimal salts broth) at 600 nm. Use the black velvet cloth to prevent light from entering while you are measuring and use Kimwipes to wipe your tubes before measuring. Re-blank before every reading.

2. Vortex the overnight E. coli culture for a few seconds, to get all the cells in suspension. Immediately inoculate the side-arm flask with the specified amount of culture, for an O.D. value of ~ 0.07. (Note: this is less than 0.5, so every group should start with Dilution Scheme A shown on Figure 12.1)

3. Immediately after inoculating flask with E. coli, measure and record the time zero O.D. reading of the culture in the flask at 600 nm. Fill out the data chart provided as you take readings. It may take two people to measure the O.D. with the slightly cumbersome side arm flasks. Fill side arm with culture, wipe glass with Kimwipe. Place the arm into the chamber, in the same direction each time you take a measurement. Make sure the side arm is straight upright and snugly fit against the right side of the measurement chamber, otherwise the O.D. will be drastically off. Cover the flask with black velvet cloth during the reading. If you are having trouble, ask TA for help.

4. After measuring the O.D. at time zero, pour side arm contents back into main flask and then pull sample for the time zero dilution scheme. Remove 1 ml of culture from the flask (using aseptic technique), dispense into a labeled 9.0 ml dilution blank and then set it aside.

5. Place your flask into the appropriate shaker incubator (if your group is treatment 2, leave the flask on the lab bench at room temperature, no shaking). Be sure to use caution when handling and removing flask from shaker. These flasks break easily and are very expensive. The time the flask is out of the shaker should be kept to a minimum.

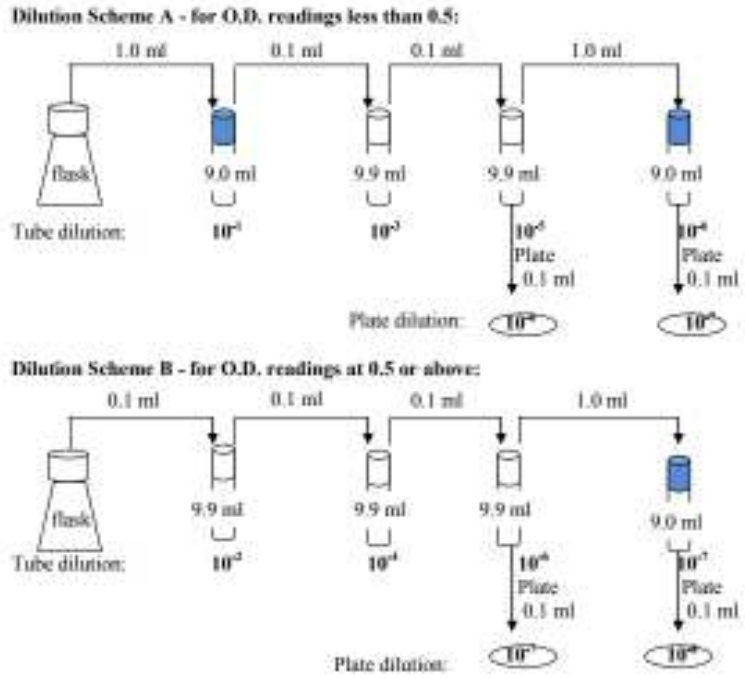
6. Vortex the dilution tube that you set aside in Step 4 for several seconds. This is to separate and homogenize the bacteria. Finish Dilution Scheme A on Fig. 12.1. Vortex subsequent dilution tubes thoroughly before transferring to next dilution. Make sure to use a new sterile pipette for each dilution. The same pipette can be used to make a dilution and a plate if both samples come from the same dilution tube. Remember to sterilize hockey sticks by dipping in alcohol and flaming. Plates should be spread immediately after sample is deposited onto them. Do NOT allow the plates to sit before spreading them. Be sure to mark each plate with group ID, treatment #, time of plating, and final dilution. Every group should be preparing 2 plates for each sampling time (i.e. every 30 minutes). Once the 2 plates are prepared, it is recommended that used dilution blanks be discarded before the next sampling time, to prevent confusion.

7. Groups should continue to take O.D. readings of the culture every 15 minutes for 3 hours, filling out the data sheet provided as readings are obtained. Dilutions/platings should be performed every 30 minutes for 3 hours. The OD reading will determine which dilution scheme (A or B) is appropriate. Perform dilution scheme A if the O.D. is less than 0.5. Perform dilution scheme B if the O.D. is at or greater than 0.5.

8. After the last reading, place a rubber band around all the plates for your group and place in the appropriate incubation tub. The plates will be incubated at 37°C for 48 h. Remove the tape from your side arm flask and carefully place it into a metal discard coffin.

9. Hand in the group data sheet to your TA with the names & seat #s of all the group members. 2.5 inclass points.

Figure 12.1 Flow chart of dilutions for viable cell count



Next Lab Period (after plates have been incubated)

Count the colonies on the plates that your group prepared. If the count goes above 250 colonieson a single plate, record the colony count for that plate as TNTC. Record the information on the data sheet provided and hand in to your TA at the end of lab. 2.5 in-class points.

A complete set of data for all treatments will be posted on Canvas. Use this information tocomplete the assignment described on the following pages.

Note: it is not required to record anything in your notebook for this experiment. However, each student should make note of the treatment that their group was assigned.

Exp No: 2 DETERMINATION OF THERMAL DEATH POINT (TDP) OF AN ORGANISM

Principle

Thermal death point is the temperature at which an organism is killed in 10 min and thermal death time is the time required to kill the organism/spore at a given temperature. Actually, it is necessary to compare susceptibility of different organisms to rising temperatures. However, some factors such as pH, moisture, composition of media and age of cells influence the TDP. Enzymatic activities of any organism operate well at their optimum temperature. Increase/decrease in temperature influences the microbial growth and survival leading to death also. The degree of tolerance is measured by exposing the microbe to gradually increasing the temperature for a given period e.g. 10 min and determining the survival.

REQIUREMENTS

Broth cultures of *E.coli* and *Bacillus cereus*
Nutrient broth tubes
Thermometer
Glass marker
Pipettes
Spreader
Water bath

PROCEDURE

- In order to determine the thermal endurance, heat the culture and separate tube containing medium in water bath held constantly at 40^0C.
- Place the thermometer in broth tube.
- Record the time when the reaches the test temperature.
- Use the broth cultures of *E.coli* and *B. cereus*.
- Label five plates, first plate as control, second with 10 min, third with 40 min and fourth with 40 min.

- Shake the culture and dispense 0.1 ml of organisms with a 1ml pipette to the control plate note the time as soon as the temperature of the nutrient broth reaches the desired temperature.
- After every 10 min, transfer 0.1 ml culture to the plates already labelled as 10min., 20 min., 30 min.,40 min as show in figure.
- Thereafter, pour the liquefied nutrient agar into each plate, rotate the plate for uniform spreading and keep on incubation for 24 to 48 hours.
- Tabulate your result as given in table.

Microorganisms	**Control(^{0}C)**	**Temperature regime (^{0}C)** **40 50 60 80**	**TDP**
E.coli			
B. cereus			
Any other bacteria			

(c) RESULTS

Highest mortality (% death cells) will be corresponded with that of incubation time, hence maximum TDP will be recorded at 40 minutes in E.coli, whereas B. cereus may show spore formation at this stage.

EX.NO: 3 Production and Analysis of Ethanol

Aim: To produce wine using Saccharomyces fermentation

Principle:

Fermentation is the anaerobic catabolism of a single chemical compound using a series of redox transformations with the goal of generating ATP by substrate-level phosphorylation. Saccharomyces is one of the most studied organisms in science and the major producer of commercial ethanol.

Many microorganisms (micro = small), notably yeasts and bacteria, extract energy from their food (glucose) by fermentation. One of the best-known types of fermentation is alcohol fermentation in which the overall chemical reaction is:

$C_6H_{12}O_6$ (glucose) → $2CO_2 + 2CH_3CH_2OH$ (ethyl alcohol) or, starting from sucrose or maltose,

$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CO_2 + 4CH_3CH_2OH$ (ethyl alcohol) Various fruits, especially grapes, could also be fermented to produce alcoholic beverages. Thus, alcoholic fermentation is the process which is responsible for the production of wine, beer, and other fermented products. It is the toxic nature of ethanol which acts to preserve these brews, and which leads to intoxication upon consumption. In fact, yeasts cannot generally survive in alcohol concentrations in excess of approximately 12 to 14%.

Materials required: Grapes, yeast culture, sugar etc..

Procedure:

1. Harvesting- This is the most critical stage of the process. The grapes must be harvested when the sugar, acid, phenol and aroma compounds are optimised for the style of wine desired.
2. Crushing and destemming- The grapes are removed from the stems and gently crushed to break the skins. Sulfur dioxide is added to the grapes at this stage to prevent oxidation and inhibit microbial activity. Enzymes may also be added to break down the cell walls and aid the release of juice.

3. Pressing- The juice extraction process depends on the type of wines to be used, but always involves squeezing the berries. After pressing the juice is allowed to stand to separate the solids. If necessary the juice may be clarified by filtration or centrifugation.
4. Fermentation- The juice is innoculated with live yeast, which then carries out the fermentation reaction:

   $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

   This reaction occurs through many intermediary biochemical steps. The process is carried out under a blanket of carbon dioxide as in the presence of oxygen the phenols are oxidised and the sugar and ethanol are converted to carbon dioxide and water.
5. Purification- Unwanted solids, salts and microorganisms are removed through a variety of physical processes, then the wine is bottled and sold.

Exp No: 6 Isolation of industrial important microbes from natural resources

Aim : to isolated industrially important microorganisms from the natural resources

Principal

Isolation of Microorganisms:

There are over a million species of microorganisms widely distributed in nature. Less than 1% of the world's microorganisms have been studied. In fact, only a few hundred species are important for industrial use. A selected list of organisms along with their products is given in Table 19.3.

TABLE 19.3 A selected list of important microorganisms and their products

Microorganism	*Product*
Algae	
Chlorella sorokiniana	Single-cell protein
Spirulina maxima	Single-cell protein
Bacteria	
Acetobacter aceti	Acetic acid
Acetobacter woodii	Acetic acid
Bacillus subtilis	Bacitracin
B. brevis	Gramicidin
B. thuringiensis	Endotoxin
Clostridium aceticum	Acetic acid
Methylophilus methylotrophus	Glutamic acid
Pseudomonas denitrificans	Vitamin B_{12}
Actinomycetes	
Streptomyces aureofaciens	Tetracycline
S. griseus	Streptomycin
S. tradiae	Neomycin
Nocardia mediterranei	Rifamycin
Micromonospora purpurea	Gentamycin
Fungi	
Aspergillus niger	Citric acid
A. oryzae	Amylase, cellulase, single-cell protein
Candida lipolytica	Lipase
C. utilis	Single–cell protein
Penicillium chrysogenum	Penicillin
Saccharomyces cerevisiae	Ethanol, wine, single-cell protein
S. lipolytica	Citric acid, single-cell protein
Rhizopus nigricans	Steroids
Gibberella fujikuroi	Gibberellin
Trichoderma viride	Cellulase

The good sources for the isolation of microorganisms are soils, lakes and river muds. It is estimated that a gram of soil contains 10^6—10^8 bacteria, 10^4—10^6 actinomycete spores and 10^2—10^4 fungal spores.

The common techniques employed for the isolation of microorganisms are given below:

1. Direct sponge of the soil
2. Soil dilution
3. Gradient plate method (Pour plate and streak plate technique)
4. Aerosol dilution
5. Flotation
6. Centrifugation.

The actual technique for the isolation of microorganisms depends on the source and the physiological properties of microorganisms.

The general scheme adopted for isolating microorganisms from soil or water source is given below:

i. The sample (soil or water) is diluted with sterile water to which an emulsifying agent (Tween) is added.

ii. Sample is thoroughly mixed and allowed to stand at room temperature.

iii. Supernatant is diluted, 10-1 to 10-10

iv. Various culture media are inoculated with diluted samples and incubated.

v. Colonies from the plates are isolated and identified.

vi. The required pure strains are maintained and preserved.

Enrichment Methods for Isolation of Microorganisms:

The culture conditions can be appropriately modified to isolate certain types of microorganisms. The types of organisms that can be isolated by use of enrichment methods is given in Table 19.4. For instance, thermophiles can be isolated by using high temperature while acidophilus can be isolated in acidic pH. Enrichment methods are certainly useful for quick isolation of specific types of organisms.

Strains of Microorganisms from Unusual Environments:

Biotechnologists often prefer to isolate microorganisms from very extreme and unusual environments. This is done with a hope that such strains may be capable of producing new products of industrial importance. The unusual environments such as cold habitats, high altitudes, deserts, deep sea and petroleum fields are constantly being tried for this purpose. The enrichment methods described above (Table 19.4) will be very useful for isolating unusual strains.

TABLE 19.4 Types of microorganisms that can be isolated by enrichment methods

Type of organisms	*Enrichment method*
Thermophiles	High temperature (42–100°C)
Psychrotrophs	Low temperature (5–15°C)
Acidophiles	Low pH (2-4)
Halophiles	High NaCl concentration
Anaerobes	N_2 atmosphere
Actinoplanes	Pollen grains
Myxobacteria	Wood bark

Screening of Metabolites for Isolation of Microorganisms:

The microorganisms can be tested directly for the product formation, and isolated. In fact, the water or soil samples can be directly used or suitably diluted for metabolite screening. Agar plates can be used for screening metabolites formed from the microorganisms. For instance, if the required product is an antibiotic, then the test system consists of the strains of organisms which inhibit the zones, on the agar plates.

The inhibitory activity indicates the possible presence of some antibiotic being produced by the microorganisms. Another example is the isolation of microorganisms producing amylases. When grown on agar plates containing starch, and then stained with iodine, amylase-producing organisms can be identified and isolated.

Screening for New Metabolites, and Isolation of Microorganisms:

Industrial microbiologists continue their search for newer metabolites produced by microorganisms. Research work is particularly directed for identifying chemotherapeutically important products for the treatment of tumors, bacterial diseases (newer antibiotics against resistant strains) and viral diseases, besides several other substances (e.g. hormones, enzyme inhibitors). In addition, isolation of microorganisms for improvement of food industry, and for efficient degradation of the environmental pollutants and hazardous chemicals also assumes significance.

Preservation of Microorganisms:

There are distinct methods for preservation of microorganisms. The most important being storage by refrigeration, freezing and lyophilization.

Materials Required:

Procedure

- Soil samples near to sewage of SIDCO industrial waste, were collected with the help of sterile spatula. Collected samples were transferred to sterile plastic bags in aseptic conditions.
- One gram of the above collected soil sample was weighed and mixed to 9 ml of sterile distilled water.
- Serial dilution was done up to 10^{-5}. Serial dilution of 10^{-5} of mixture was introduced into a sterile petri plates using the pour plate method into nutrient agar HIMEDIA fortified with 2% starch.
- The poured plates were incubated at 37°C for 24 hrs.
- .The bacterial isolates were further sub cultured to obtain pure culture. Pure isolates on starch agar slants were maintained at 4ºC.
- Screening of potent amylase producing bacteria by starch hydrolysis test The isolated pure strains were screened for the production of extracellular amylase using starch agar.
- Bacterial isolates were screened for amyl lytic activity by starch hydrolysis test on starch agar plate.

- The microbial isolates were streaked on the starch agar plate and incubated at 37°C for 48 hours.
- After incubation 1% iodine solution was flooded with dropper for 30 seconds on the starch agar plate. The isolates produced clear zones of hydrolysis were considered as amylase producing bacteria.

Result:

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Staining	Result
Gram staining	Gram +ve
. Endospore staining	Spore forming