LABORATORY MANUAL

BIOPROCESS TECHNOLOGY - 17BTU411

SEMESTER: IV/ EVEN SEMESTER

BATCH: 2017-2020

Experiment-1

Bacterial growth curve

Aim:

To study different growth phases of bacterial population and plot a bacterial growth curve.

Introduction:

Most bacteria reproduce by an asexual process called binary fission, which results in doubling of the number of viable bacterial cells. In binary fission, events occur in sequences i.e. the chromosome duplicates, the cell elongates, and the plasma membrane pinches inward to the center of the cell. The time required for a cell population to divide and double in number is called the generation time or the doubling time. Generation time varies with organism and environment and can range from 20 minutes for a fast growing bacterium under ideal conditions, to hours in not so ideal conditions. The standard bacterial growth curve describes various stages of growth of pure culture of bacteria, beginning with the addition of cells in sterile media to the death of the cells.

The phases of growth typically observed include:

- Lag phase
- Exponential (log, logarithmic) phase
- Stationary phase
- Death phase (exponential or logarithmic decline)

In standard bacterial growth curve one keeps track of cell growth by some measure or estimation of cell number.

Principle:

Bacterial growth refers to an increase in the number of cells. When bacteria are inoculated into a liquid medium and the cell population is counted at intervals, it is possible to plot a typical bacterial growth curve that shows the growth of cells over time. It shows four distinct phases of growth.

Lag phase: During the lag phase, the bacterial cells adapt themselves to the new environment as they are introduced into a new medium. The bacterial population increase in size but show no change in number as they prepare themselves for cell division. They synthesize new enzymes to utilize new nutrients. The length of the lag phase is determined by the characteristics of the bacterial species and by conditions in the media, both the medium from which the organisms are taken and the one to which they are transferred. The bacterial population then enters an active phase of growth called the logarithmic phase.

Log or exponential phase: During this phase, the bacteria divide exponentially. The mass of each cell increases rapidly followed by reproduction. The number of bacteria doubles during each generation time. The growth curve is linear during this phase and the cells are metabolically most active. As the amount of nutrients decrease, the vigor of the population changes and the rate of reproduction and death equalizes. The bacterial population then enters a plateau called as stationary phase.

Stationary phase: During the stationary phase, as the nutrients get exhausted, and there is a buildup of waste and secondary metabolic products, the growth rate decreases to a point where it

almost equals to the death rate, hence viable cell number remains same. If incubation is still continued the population then enters the death phase.

Decline or death phase: During the death phase, viable cell population decreases exponentially as the amount of waste products increases. The death of cells in the population exceeds the formation of new cells. This continues until the population is diminished to a tiny fraction of the more resistant cells or the population might die out entirely.

Materials Required

Glass wares: Conical flasks, Measuring cylinder, Sterile test tubes, Sterile Petriplates

Reagents: Distilled water

Other requirements: Incubator, Shaker, Spectrophotometer, Micropipettes, Tips, Sterile Loops

Day 1:

1. Using sterile flexi loop, streak a loopful of *E.coli* culture from the stab onto the LB agar plate. Incubate at 37oC for 18-24 hours.

Day 2:

1. Pick up a single colony of each strain from the LB agar plate and inoculate it into a test tube containing 10 ml of autoclaved LB broth. Incubate the test tube overnight at 37oC.

Day 3:

1. Take 250 ml of autoclaved LB Broth in a sterile 500 ml conical flask.

2. Inoculate 5 ml of the overnight grown culture in above flask.

3. Take OD at zero hour. Incubate the flask at 37oC.

4. Aliquot 1 ml of the culture suspension at an interval of every 30 minutes and take the optical density

(OD) at a wavelength of 600 nm using spectrophotometer, till the reading becomes static.

Note: Alternatively, 50-100 µl of formaldehyde can be added to all the 1 ml aliquots of culture suspension taken after every 30 minutes. Optical density of all the aliquots can be taken at the end of the experiment.

5. At the end of experiment, plot a graph of time in minutes on X axis versus optical density at 600nm on Y axis to obtain a growth curve of Escherichia coli.

Formula for calculating growth rate constant (µ), generation time (g) and growth rate:

1. Growth rate constant (µ)= slope x 2.303

2. Generation time (doubling time) g= 0.693/ µ

Generation time is expressed in hours.

3. Growth rate= 1/generation time

Interpretation:

By performing this experiment, the different stages of bacterial growth curve can be observed. In the lag phase the OD remains constant. With time the cells enter the log phase where the OD increases until the cells reach the stationary phase, where the OD remains more or less constant. If OD is taken for longer period of time (7-8 hours) one can observe a decrease in the value. The generation time and growth rate of the organism in the given media can also be measured from the growth curve.

Experiment-II

Calculation of thermal death point of microbial samples

DETERMINATION OF THERMAL DEATH POINT (TDP) OF AN ORGANISM

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.

(a) REQIUREMENTS

Broth cultures of *E.coli* and *Bacillus cereus*

Nutrient broth tubes

Thermometer

Glass marker

Pipettes

Spreader

Water bath

(b)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			

RESULTS

Highest mortality (% death cells) will be corresponded with that of incubation time, hence maximum TDP will be recorded at ____ minutes in_____, whereas _____ may show spore formation at this stage.

Experiment-III

Production and analysis of ethanol

Introduction

The overall reaction in fermentation of hexose by Yeast particularly *S. cereviceae,* can be expressed as under

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

This reaction is considered as basic formula for calculation of alcohol fermentation. However, other than alcohol certain byproducts like glycerol, succinic acid, and acetic acid are produced along with ethyl alcohol. Haiden and Young discovered that free inorganic phosphate disappear during fermentation usually through EMP pathway. Ethyl alcohol is widely applicable as organic solvent, which is efficiently produced by growing Yeast. *S. cereviceae* obtained from cheap sources like corn syrup can be used in preparation of inoculum media.

Principle of Alcohol Estimation

Alcohol reacts with oxidizing agent i.e. potassium dichromate and gets oxidized to acetic acid. Remaining of the potassium dichromate will react with iodine. This is a light sensitive process, so incubation is carried out in dark. Liberated iodine is titrated with 0.1N sodium thiosulphate. In this process starch is used as an indicator.

Reaction

$$K_2CrO_7 + H_2SO_4 + 2C_2H_5OH \rightarrow 3CH\ 3COOH + 2K_2SO_4 + 4H_2O + 2Cr_2\ (SO_4)_3$$

Requirements

1. 500ml Production medium & 50ml inoculum medium
2. pH meter & Water bath
3. Methylene blue(1% w/v)
4. $NaHCO_3$ (Powder)
5. Concentrated HCl
6. 0.025 Molar K_2CrO7
7. 25%(W/V) KI
8. 1% (w/v) starch as indicator
9. 0.1N $Na_2S_2O_3$
10. 25% $ZnSO_4$

Composition of Media

Inoculum medium

Ingredients g/100ml	
Jaggery	5.00
(NH4)2SO4	0.15
KH2PO4	0.15
Yeast Extract	0.50
pH	4.5

Production medium

Ingredients g/1000ml	
Jaggery	150.00
KH2PO4	5.00
Yeast Extract	1.50
pH	4.50

Protocol

Transfer aseptically 10% (50ml) of Inoculum in 500ml Production medium and Mix it well

From Production medium aseptically withdraw approx 20 – 25 ml (Measure pH and Calculate G % sugar from sample withdrawn)

Acid Hydrolysis for 30 minutes (20ml of Broth +1ml of Conc. HCl in boiling water bath for 30 minutes+ neutralize with NaHCO3

Estimate the Gram percent of sugar by Cole's method

After 5 days take broth Recover the alcohol as per procedure

Procedure for Cole's method

Take 20 ml of K3 (FeCN) 6 in 250 ml flask to that add 5 ml of NaOH

Keep it for boiling under direct flame over the wire gauze add 2 drops of Methylene Blue

Titrate with hydrolyzed sugar (Note that Reading as PR)

From Pilot Reading Dilute the sample for TR (If PR is 2.0 then, we have to take 2 ml of neutralized sample and make the volume up to 10 with D/W) (For 3 TR reading total volume should be more than 30 ml)

Again repeat the same procedure from step 1 and note down the titration reading as TR (TR reading should be between 9 – 11)

Calculate Gram% sugar with the help of Sucrose Factor

Purity of the culture is checked by Gram's staining

pH is measured pH meter

Gram (w/v)% sugar is estimated

Experiment-IV

Production and analysis of amylase

Aim: To isolate amylase secreting bacteria for the production and analysis

Principle:

Starch is an insoluble polymer of glucose which act as a source of carbon and nitrogen for microorganism which have an ability to degrade them. Starch degrading microorganism transport the degraded from across the cytoplasmic membrane of the cell. Some bacteria possess the ability to produce amylase that breaks starch into maltose. The amylase is an extracellular enzyme which is released from the cell of microorganism.

Isolation and Screening of Bacterial Strains for Amylase Activity

- The bacteria were isolated from soil.
- Serial dilution is a process of diluting a sample several times to get isolated colonies.
- During serial dilution, 9 mL of sterile water was transferred into 7 tubes using sterile pipettes (10 mL) under aseptic conditions. By using a sterile pipette (1mL), 1mL of the sludge sample was transferred into 10^{-1} tube. The test tube was mixed properly. The dilution was continued under aseptic conditions from 10^{-2} to 10^{-7}.
- Then the diluted samples of about 1 mL from each test tube were transferred to sterile petriplates containing starch agar medium.

The starch agar medium comprised the following:

- Soluble starch 5 g,

- Yeast extract 1 g,
- Tryptone 2 g,
- $CaCl_2$ 0.003 g,
- $MgCl_2$ 0.1 g,
- KH_2PO_4 0.36 g,
- Na_2HPO_4 1.3 g,
- Agar powder 20 g,
- Distilled water 1 L.

- The plate was then incubated at 37°C for 42 h until the colonies appear.
- It is important that the surface of the plate be fairly dry so that the spread liquid soaks in.
- Pour iodine solution in the plate.
- The blue-black colour appears due to the formation starch-iodine complex.

Amylase assay

Amylase is another essential enzyme that contributes a major role in sludge hydrolysis. It acts on polysaccharides (sludge component) and convert them to simple sugar residues. These residues in turn reacts with 3,5 Dinitrosalycylic acid (DNS) reagent and results in the formation of Yellow brown coloured chromophore. The reaction mechanism of amylase is illustrated in Figure 2.7. For amylase activity 1 mL sample (enzyme source) was mixed with 1 mL of 1% soluble starch in citrate-phosphate buffer

(pH 6.5). The reaction mixture was incubated in a water bath at 40°C for 30 min. After incubation, 2mL DNS reagent was added to terminate the reaction. Then the mixture was boiled for 5 min, cooled and mixed with 20 mL of distilled water. The absorbance was measured at 540nm. One unit of absorbance is expressed as 1U/mL amylase activity.

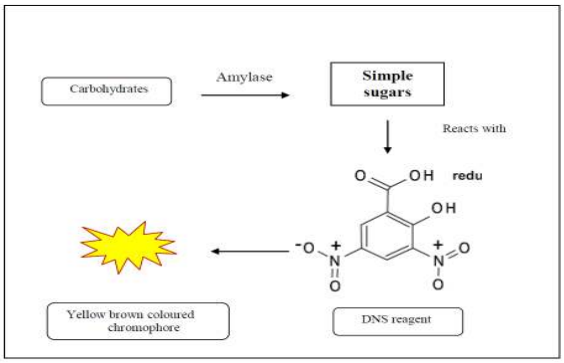

Figure 2.7 Schematic representation of amylase assay

Result:

The amylase activity of the isolated bacteria was _____ U/mL.

Experiment-V

Production and analysis of Lactic acid

Lactic acid production and analysis

INTRODUCTION

Fermented milks are products prepared by controlled fermentation of milk to produce acidity and flavour to desired level. Fermented milks are the most common products from which other products are also made. Starter culture organisms used in fermentations belongs to a family of bacteria collectively known as the Lactic Acid Bacteria (LAB). These LABS are united by a constellation of morphological, metabolic and physiological characteristics. There are several factors, which influence the quality of yoghurt. These include type of milk, processing conditions, storage conditions etc., however, quality of starter culture is the most important factor that influence the development of quality yoghurt. Lactic Acid Bacteria (LAB) are widely distributed in nature and occur naturally as indigenous microflora in raw milk, drinking yoghurt, etc. They are gram positive bacteria that play an important role in many food fermentation processes. Some species of the genus *Lactobacillus* (*Lb*.), *Lactococcus* (*Lc*.) and *Leuconostoc* (*Ln*.) are included in this group. The lactic acid fermentation has long been known and applied

by humans for making different food stuffs. For many centuries, LAB have been an effective form of natural preservation.

Isolation of lactic acid bacteria: The samples were aseptically weighed and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. The 0.1 mL from each dilution was then sub cultured, in duplicate, into the MRS agars used for isolating LAB. To prevent the growing of yeasts, the media were then supplemented with 100 mgL^{-1} of cycloheximide before being incubated at the appropriate temperatures (30°C) for 2-3 days. The MRS agar plates were incubated anaerobically at 42, 35 and 30°C for 3 days, in order to provide an optimal temperature for growing lactobacilli. Lactobacilli are microaerophillic and generally require layer plates for aerobic cultivation on solid media. When the medium is set, another layer of un-inoculated MRS Agar is poured over the surface to produce a layer plate. Lactobacilli isolated on MRS Agar should be further confirmed biochemically.

Ingredients Gms / Litre

Proteose peptone	10.000
Beef extract	10.000
Yeast extract	5.000
Dextrose	20.000
Polysorbate	1.000
Ammonium citrate	2.000
Sodium acetate	5.000

Magnesium sulphate 0.100

Manganese sulphate 0.050

Dipotassium phosphate 2.000

Agar 12.000

Final pH (at 25°C) 6.5±0.2

Analysis of lactic acid

Construction of calibration curve

Lactic acid (1.2 g) with the know concentration (89%, ρ = 1.2 g/mL) was placed in a 10-mL volumetric f lask and diluted with water. A stock solution with the x concentration of lactic acid 89 g/L was obtained. A series of lactic acid solutions was prepared from the stock solution using two-fold dilutions. A solution of iron(III) chloride (0.2%) was prepared. Iron(III) chloride (0.3 g) was placed in a 100 mL volumetric flask, diluted to the mark with water and stirred to the complete dissolution of the salt. The solution must be of room temperature 25 ± 5 ° C. A solution of lactic acid (50 μL) of a corresponding concentration was added to 2 mL of a 0.2% solution of iron(III) chloride and stirred. The absorbance of the obtained colored solutions was measured at 390 nm.

The reference solution contained 2 mL of a 0.2% solution of iron(III) chloride. The dependence of the absorbance of colored solutions on the concentration of lactic acid taken for the reaction was used for the calculation of the parameters of linear equation corresponding to the linearity range of the calibration curve. The processing of the results and the construction of the calibration curve were done using the Statistica 6.0 software.

Determination of lactic acid in cultural liquid

Cultural liquid was separated from the cells by centrifuging. The supernatant was diluted 20-fold with deionized water. A supernatant (50 μL) containing lactic acid was added to 2 mL of a 0.2% solution of iron(III) chloride and stirred and absorbance was measured at 390 nm against the reference solution (2 mL of a 0.2% FeCl3 solution). The concentration of lactic acid was calculated using a calibration curve taking into account the 20-fold dilution of the test sample.

Result:

Lactic acid producing bacteria was isolated from curd and lactic acid concentration was _____ at 390 nm.

Experiment-VI

Isolation of industrially important microorganism 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.

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:

o 1 Erlenmeyer flask containing 50ml of sterile agar (0.1%)

o 1 cup containing 0.5g of soil

o 4 small vials containing 4.5ml of sterile agar (0.1%)

o 5 sterile 1ml pipettes

o 1 sterile glass stirring rod

o 6 Petri plates containing about 10ml of PDA (potato dextrose agar)

o 6 strips of Parafilm

o paper towel

o disinfectant

o marking pens

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:

Staining	Result
Gram staining	Gram +ve
Endospore staining	Spore forming