20BTC503J - Bioprocess Technology

SEMESTER- I

YEAR:

NAME:

REG NO:

SCHOOL OF BIOENGINEERING
DEPARTMENT OF BIOTECHNOLOGY
SRM INSTITUTE OF SCIENCE AND TECHNOLOGY
(Under Section 3 of UGC Act, 1956)
S.R.M. NAGAR, KATTANKULATHUR- 603 203.
KANCHEEPURAM DISTRICT

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BONAFIDE CERTIFICATE

Register No.

*Certified to be the bonafide record of work done by*_____*of* **Biotechnology M. Tech.** *Degree course in the practical*_____*in* **SRM Institute of Science and Technology, Kattankulathur** *during the academic year*_____.

Lab Incharge

Date: **Head of the Department**

Submitted by University Examination held in _____*at* **SRM Institute of Science and Technology, Kattankulathur.**

Date: **Examiner 1** **Examiner 2**

Name: Class:

Reg. No.: Branch:

Exp. No.	Date of Performance	Title of Experiment	Date of submission	Signature
1		Effect of pH and temperature on □-amylase enzyme activity		
2		Enzyme kinetics		
3		Preparation of immobilized enzymes		
4		Medium optimization by PBD		
5		Medium optimization by RSM - BBD		
6		Medium optimization by RSM - CCD		
7		Batch growth kinetics of bacteria		
8		K_La determination by sulphite oxidation method		
9		Cell disruption by Sonication		
10		Cell disruption by high pressure homogenizer		
11		Extraction of protein by aqueous two phase extraction		
12		Protein concentration by salting out method		

Exp. No. 1a. Estimation of glucose by dinitro salicylic acid (DNS) assay method

Aim:

To estimate the concentration of glucose by using Dinitro Salicylic Acid assay method.

Theory:

The DNS method for estimating the concentration of reducing sugars in a sample was originally invented by G. Miller in 1959. Reducing sugars have the property to reduce many of the reagents. A reducing sugar is one that in a basic solution forms an aldehyde or ketone.

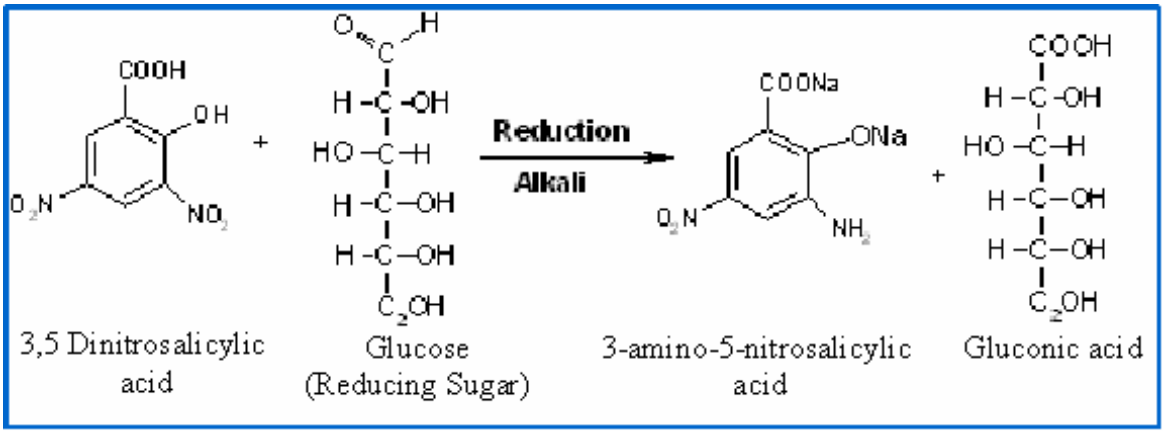
This method tests for the presence of free carbonyl group in reducing sugars. This involves the oxidation of the aldehyde functional group present in glucose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

Oxidation

Aldehyde group ----------> carboxyl group

Reduction

3, 5-dinitrosalicylic acid ----------> 3-amino, 5-nitrosalicylic acid



Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction is more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. The absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar.

The chemistry of the reaction is complicated since standard curves do not always go through the origin and different sugars give different color yields. The method is therefore not suitable for the determination of a complex mixture of reducing sugar.

List of reagents required:

- Glucose stock solution (10 mg /ml of deoionized water)
- Dinitrosalicylic Acid Reagent Solution
    - Dinitrosalicylic acid: 10 g
    - Sodium sulfite: 0.5 g
    - Sodium hydroxide: 10 g
    - Deionized water : 1 liter
- Potassium sodium tartrate solution, 40%

Procedure:

- Prepare glucose standards ranging from 0.1 to 1 ml by using dry clean and labelled test tubes (Refer table)
- Make it up to 3 ml with deionized water
- 3 ml of deionized water alone serves as a blank
- Add 3 ml of Dinitro Salicylic Acid reagent to all the test tubes
- Place all the test tubes in water bath at 90º C for 10-15 minutes to develop red-brown color
- Add 1 ml of 40% potassium sodium tartrate (Rochelle salt) solution to all the test tubes

- Cool the test tubes thoroughly and read the extinction (Optical density) of the colored solutions at 540 nm using the solution in tube 1 as a blank (control).

  (Note: All the tubes must be cooled to room temperature before reading since the extinction is sensitive to temperature change)

- Plot the standard curve of the absorbance (Y- axis) against the glucose concentration(mg/ml) (X-axis)

Tabulation:

S.No	Vol. of standard solution (ml)	Conc. Of standard solution (mg/ml)	Vol. of Distilled water (ml)	Vol. of DNS reagent (ml)		Vol. of 40% Na-K Tartrate (ml)	O.D at 540 nm
1	Blank	-	3	3		1	
2	0.1		2.9	3		1	
3	0.2		2.8	3	**Incubate the tubes at 90ºC for 10 - 15 minutes.**	1	
4	0.3		2.7	3		1	
5	0.4		2.6	3		1	
6	0.5		2.5	3		1	
7	0.6		2.4	3		1	

8	0.7		2.3	3		1	
9	0.8		2.2	3		1	
10	0.9		2.1	3		1	
11	1.0		2.0	3		1	

Result and Interpretation:

Graph: Concentration of Glucose (mg/ml) vs Optical Density (@540 nm)

Significance of the experiment:

Exp. No. 2. Effect of pH on a-amylase enzyme activity

Aim:

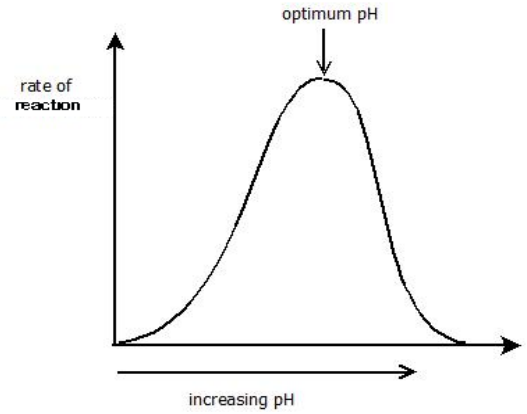
To find out the optimum pH for alpha amylase enzyme activity

Theory:

Enzymes are amphoteric molecules containing a large acid and basic groups, mainly situated on their surface. The charges on this group will vary according to their acid dissociation constants and with the pH of their environment. This will affect the total net charge of the enzyme and their distribution of charge on their surface, in addition to the reactivity of the catalytic active group. Certain enzymes have ionic groups on their active sites and these ionic groups must be in a suitable form (acid or base form) for the enzyme to function. Variations in the pH values of the medium results in changes in the ionic form of the active site, and changes in the activity of an enzyme and hence the reaction rates. The changes in the pH value may also alter the three dimensional shape of an enzyme. For these reasons, enzymes are only active over a certain pH range. The pH of the medium may affect the maximum reaction V_{max}, K_m and the stability of an enzyme. In some cases, the substrate may contain ionic groups, and the pH of the medium affects the affinity of the substrate to the enzyme. Take together the changes in the charges with pH effect at the activity, structural stability and solubility of the enzyme.

There will be pH characteristics of each enzyme at which the net charge on the molecule is zero. This is called the isoelectric point [PI] at which enzyme generally has minimum (stability) solubility in aqueous solution. In a similar manner to effect on enzyme, the charge and charge distributions on substrates products and coenzymes will also be affected by pH changes. An enzyme molecule will be cationic (Positively charged), when the environment's pH is lower than its isoelectric pH and anionic in nature vice versa. Changes in the pH or acidity of the environment can alter or totally inhibit the enzyme from catalyzing a reaction.

This change in the pH will affect the polar and non-polar intra-molecular attractive and repulsive interaction and thereby the shape of the enzyme and the active site can be altered, where the substrate molecule could no longer fit. Each enzyme has an optimum pH at which the velocity is maximum. Below and above this pH, the enzyme activity is much lower and at the extreme pH the enzyme is totally inactive.



Materials Required:

Starch Solution (1%), DNS reagent solution, Rochelle salt (40%), α-amylase solution (1%) and pH buffers ranging from 4 to 9.

Preparation of pH buffers:

- **Sodium acetate buffer, 0.1 M**

  Solution A: 11.55 ml glacial acetic acid/liter (0.2 M)

  Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2$ $3H_2O$)/liter (0.2 M)

Referring to table 1 for desired pH, mix the indicated volumes of solutions A and B, then dilute with H_2O to 100 ml

Table1.

pH	Solution A (ml)	Solution B(ml)
4	41.0	9.0
5	14.8	35.2

- **Sodium phosphate buffer, 0.1 M**

  Solution A: 27.6 g NaH_2PO_4 H_2O per liter (0.2 M)

  Solution B: 53.65 g Na_2HPO_4 $7H_2O$ per liter (0.2 M)

Referring to table 2 for desired pH, mix the indicated volumes of Solutions A and B, then dilute with H_2O to 100 ml.

Table2.

pH	Solution A (ml)	Solution B(ml)
6	**43.85**	**6.15**
7	**19.5**	**30.5**
8	**2.65**	**47.35**

- **TrisHcl buffer 0.1 M pH 9:** Prepare 0.1 M TrisHCl buffer by using Tris Base and adjust with 0.1 N HCl

Procedure:

- Prepare 0.1M pH buffer solutions ranging from pH 4 to pH 9
- Add aliquots of 1 ml of 1% starch solution to the dry clean labeled test tubes
- Prepare blanks for pH 4-9 by using distilled water
- Add 1 ml of different pH buffer solution (pH 4-9) to all the test tubes
- Mix well and incubate the test tubes for 5 minutes after adding the buffer solution
- Add 1.0 ml of α-amylase enzyme solution to all the test tubes
- Mix well and incubate the test tubes for **optimum time calculated from theExp. No. 3** at 40ºC.
- Remove the test tube according to the time of incubation and add 3 ml of DNS reagent
- Place the test tubes in boiling water bath (90° C) for 10 -15 minutes.
- Cool the test tubes and add 1 ml of sodium potassium tartrate.
- Measure the O.D at 540 nm using spectrophotometer.
- Plot the graph between time of incubation (min.) and enzyme activity V (U), (mmol/ml.min) to determine the optimum pH.

Tabulation:

Sl. No.	pH	Vol. of 1 % starch soln. (ml)	Vol. of 0.1 M pH buffer (ml)	Vol. of 1% alpha amylase solution (ml)		Vol. of DNS reagent solution (ml)		Vol. of 1%Na-K Tartrate (ml)	O.D at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] mmol/ml	V mmol/ml.min
1	4	1	1	1	Incubate the test tubes @ 40°C	3	Place the test tubes at 90°C for 10min.	1				
2	5	1	1	1		3		1				
3	6	1	1	1		3		1				
4	7	1	1	1		3		1				
5	8	1	1	1		3		1				
6	9	1	1	1		3		1				

***One unit (U)of alpha-amylase was defined as the amount of enzyme releasing one mmolof glucose equivalent per minute under the assay conditions and expressed as U/ml.**

Calculation:

Result:

Significance of the experiment:

Exp. No. 3. Effect of Temperature on enzyme activity

Aim:

To study the effect of temperature on activity of α-amylase enzyme and to find optimum temperature (T_{opt}), Activation energy (Ea) and deactivation rate constant (Ka).

Theory:

The rate of enzyme-catalyzed reaction gradually increases with increase in temperature within a certain range in which the enzyme is stable and retains full activity after which it decreases due to enzyme denaturation. The enzyme and substrate concentrations are kept constant and only temperature of enzymatic reaction is increased.

The overall rate of reaction (the rate of formation of product) must be limited by the amount of enzyme available and by the rate of breakdown of the enzyme-substrate complex.

$$V_{max} = K_2 [E_0] \quad \text{---------- (1)}$$

According to Arrhenius equation, effect of temperature on enzyme activity (reaction rate) is given by

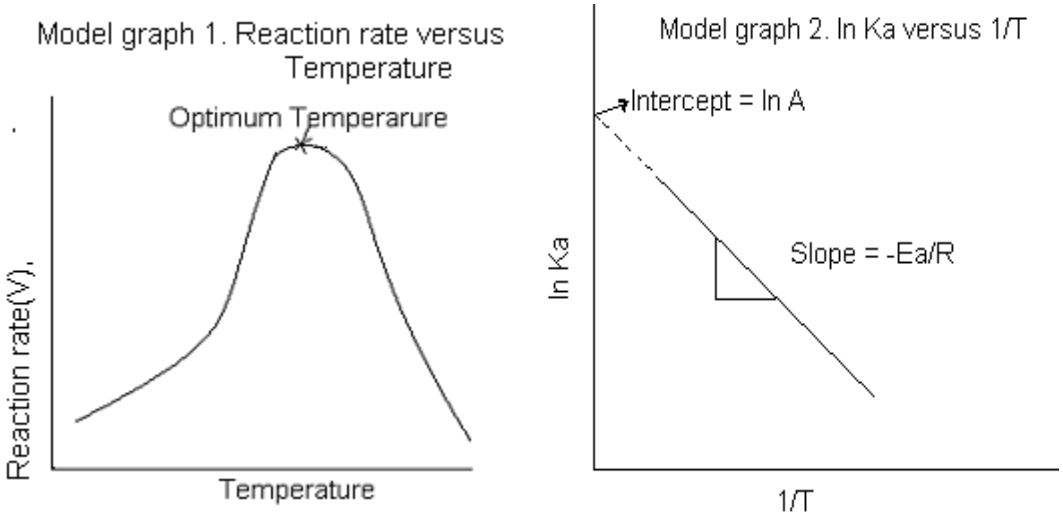
$$Ka = Ae^{-Ea/RT} \quad \text{---------- (2)}$$

Where, A is Arrhenius constant; Ea is the activation energy in joule/mmol; Ka is deactivation rate constant in min^{-}; R is gas constant ($8.314*10^{-6}$ joule/mmol K); T is temperature in K.

Variations in temperature may affect both K_m and V_{max} values. In general, the reaction velocity increases with increase in the temperature up to a maximum and then declines, resulting in a bell-shaped curve. Overall when enzymes are exposed to a temperature above 50°C, denaturation starts leading to the derangement in the native structure of protein and the active site. A majority of enzymes become inactive at higher temperatures greater than 70°C except thermophilic enzymes.

Materials required:

- Starch Solution (1 %), α-amylase solution (1 %), DNS solution, Rochelle salt (40%), 0.2 M Sodium phosphate buffer (pH 7).



Procedure:

- Add aliquots of 1 ml of 1% starch solution to dry clean labeled test tubes
- Prepare blank with 1 ml of distilled water
- Add 1 ml of 0.1 M **pH (Optimum pH calculated from Exp. No. 4) buffer** to all the test tubes
- Add 1ml of α-amylase enzyme solution to all the test tubes
- Incubate the test tubes with reaction mixture at various temperature ranges from 20 ºC to 80 ºC with 10 ºC intervals for **optimum time calculated from Exp. No. 3**
- Remove the test tube according to the time of incubation and add 3 ml of DNS reagent
- Place the test tubes in boiling water bath (90° C) for 10 -15 minutes.
- Cool the test tubes and add 1 ml of sodium potassium tartarate
- Measure the O.D at 540 nm using spectrophotometer.
- Estimate the amount of glucose produced [P] (mmol/ml).
- Find reaction rate mmol/ml.min ($V = V_{max}$) which is equal to d[P]/dt and activation rate constant (K_a), which is equal to ($V_{max}/[E_o]$), where [Eo] is initial enzyme concentration in mmol/ml.
- Plot a graph between reaction rates (V) versus temperature and find optimum temperature at which maximum reaction rate is observed.
- Plot a graph between 'ln Ka' versus (1/T) and find slope ($-E_a/R$) and intercept on Y-axis (ln A).

- Find deactivation rate constant (K_a) by substituting Ea, T_{opt} and Arrhenius constant (A) in equation (2).

Tabulation1:

Sl. No.	Temperature (°C)	Vol. of 1 % starch soln. (ml)	Vol. of 0.1 M pH buffer (ml)	Vol. of 1% alpha amylase solution (ml)	Incubate the test tubes at specified temperature	Vol. of DNS reagent solution (ml)	Place the test tubes at 90°C for 10min.	Vol. of 1%Na-K Tartrate (ml)	O.D at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] mmol/ml	V mmol/ml.min
1	20	1	1	1		3		1				
2	30	1	1	1		3		1				
3	40	1	1	1		3		1				
4	50	1	1	1		3		1				
5	60	1	1	1		3		1				
6	70	1	1	1		3		1				
7	80	1	1	1		3						

***One unit (U) of alpha-amylase was defined as the amount of enzyme releasing one mmol of glucose equivalent per minute under the assay conditions and expressed as U/ml.**

Tabulation 2:

Sl. No.	**Temperature** ^{o}K	**1/T**	**K** min^{-1}	**ln K** min^{-1}
1				
2				
3				
4				
5				
6				
7				

Calcultaion:

For calculating V from $V_{max} = K_2 [E_0]$

$$K_2 (K) = V_{max}(V)/ [E_0] \quad \text{---------} (3)$$

Molecular weight of alpha amylase = 55,000 Daltons = 9.1329×10^{-17} mg

Initial enzyme concentration [E_0] = 10 mg/ml = 1.09 x 10^{17} mmol/ml.

For calculating K and Ea from equation (2),

$$\ln K = \ln A - Ea/RT \quad \text{--------- (4)}$$

Result:

Optimum temperature (T_{opt}) = ……...ºC,

Activation energy (Ea) = …….. Joule/mmol.

Deactivation rate constant (K) = …….. min^{-1}

Arrhenius constant (A) = …… min^{-1}

Significance of the experiment:

Exp. No. 4. Enzyme Kinetics

Aim:

To study the kinetics of alpha amylase enzyme and determination of the kinetic parameters Vmax and Km.

Principle:

Enzyme kinetics represents the study of the rate at which an enzyme works usually as a function of the substrate parameter available to the enzyme. Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. They achieve their effect by temporarily binding to the substrate and, in doing so, lowering the activation energy needed to convert it to a product. Kinetics of simple enzyme catalyzed reactions is referred to as Michelis Menten kinetics or saturation kinetics. These models are based on data from batch reaction in which the initial substrate (So) and enzyme (Eo) concentration are known. In this experiment, the glucose formed can be estimated calorimetrically using DNS method.

One of the important parameters affecting the rate of a reaction catalyzed by an enzyme is the substrate concentration, [S]. During enzyme substrate reaction, the initial velocity V_0 gradually increases with increasing concentration of the substrate. Finally a point is reached, beyond which the increase in V will not depend on the [S]. It can be observed from the S Vs V graph that as the concentration of the substrate increases, there is a corresponding increase in the V. However beyond a particular substrate concentration, the velocity remains constant without any further increase. This maximum velocity of an enzyme catalysed reaction under substrate saturation is called the V_{max} , Maximum velocity.

Michelis-Menten equation for steady state kinetics approximation is

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

Where Vmax - velocity of enzyme reaction of saturating substrate concentration

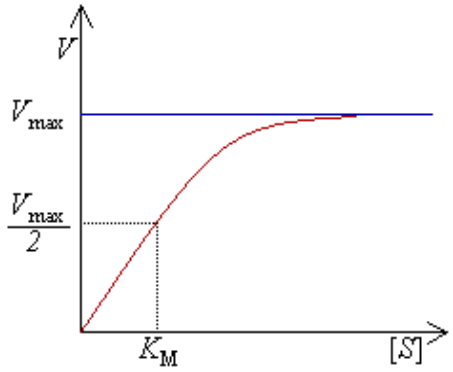
S - Substrate concentration

Km -Michelis Menten constant, measure of affinity of enzyme for substrate

Km = [S] at V = Vmax /2 from the graph V versus [S].

- A small Km indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
- A large Km indicates the need for high substrate concentrations to achieve maximum reaction velocity.

Michaelis-Menten Kinetics:

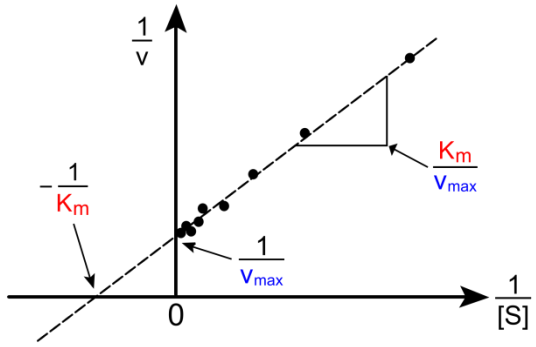


The plot of V versus [S] above is not linear; although initially linear at low [S], it bends over to saturate at high [S]. Before the modern era of nonlinear curve-fitting on computers, this nonlinearity could make it difficult to estimate K_M and V_{max}accurately. Therefore, several researchers developed linearisations of the Michaelis–Menten equation, such as the Lineweaver–Burk plot, the Eadie–Hofstee diagram and the Hanes–Woolf plot

Lineweaver - Burk plot:

A plot of 1/V versus 1/[S] gives slope of Km/Vmax; and Y-intercept of 1/Vmax and X intercept of -1/Km.

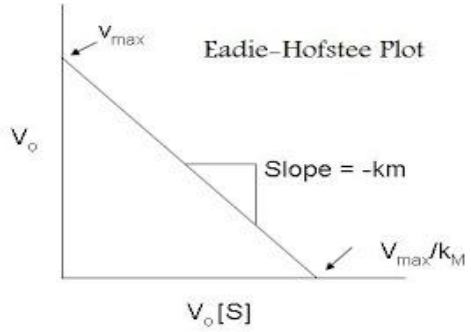
$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$



Eadie - Hofstee plot:

A plot ofV/[S] versus V results in a line of slope -Km and y-intercept of Vmax and X intercept of Vmax /Km.

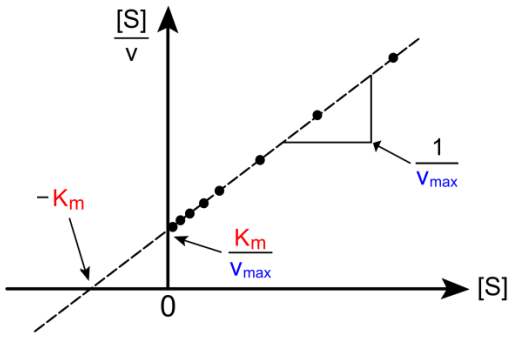
$$V = -K_m\left(\frac{V}{[S]}\right) + V_{Max}$$



Hanes - Woolf plot:

A plot of [S]/V versus [S] results in a line of slope 1/ Vmax ; Y intercept of Km/ Vmax ; X intercept of -Km.

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$$



Materials Required:

1. Stock starch solution (1%),
2. DNS reagent
3. Rochelle salt (40%),
4. α-amylase solution (1%).
5. 0.2 M Buffer

Procedure:

- Pipette out 0.1 to 1 ml of 1% substrate starch solution to dry clean labeled test tubes
- Add varying volume deionized water to make up the volume to 1 ml in all test tubes. Mix well.
- Add 1 ml of buffer **(Optimum pH determined from Exp. No. 4)**
- Add 1.0 ml of α-amylase enzyme solution to all the test tubes. Mix well
- Incubate the test tubes at **optimum temperature determined from Exp. No. 5** for **optimum time determined from Exp. No. 3**
- Remove the test tube according to the time of incubation and add 3 ml of DNS reagent
- Place the test tubes in boiling water bath (90° C) for 10 minutes.
- Cool the test tubes and add 1 ml of sodium potassium tartarate.
- Measure the O.D at 540 nm using spectrophotometer.

- Calculate the enzyme activity, V (mmol/ml.min.)
- Plot Michelis- Menten ((V)vs. [S]), Lineweaver- Burk ((1/[S]) vs (1/V)), Eadie - Hofstee (V vs V/[S]) and Hanes-Woolf ([S]/V vs [S])
- Determine the values of Vmax and Km from graphs.

Tabulation 1:

Sl. No	**Vol. of 1% starch soln. (ml)**	**Vol. of deionized water (ml)**	**Vol. of 0.1 M buffer (ml)**	**Vol. of 1% alpha amylase solution (ml)**	**Incubate the test tubes at optimum temperature for optimum time**	**Vol. of DNS reagent solution (ml)**	**Place the test tubes at 90°C for 10min.**	**Vol. of 1%Na-K Tartrate (ml)**	**O.D at 540 nm**
1	0.1	0.9	1	1		3			
2	0.2	0.8	1	1		3			
3	0.3	0.7	1	1		3			
4	0.4	0.6	1	1		3			

5	0.5	0.5	1	1		3			
6	0.6	0.4	1	1		3			
7	0.7	0.3	1	1		3			
8	0.8	0.2	1	1		3			
9	0.9	0.1	1	1		3			
10	1.0	-	1	1		3			

*One unit (U) of alpha-amylase was defined as the amount of enzyme releasing one mmol of glucose equivalent per minute under the assay conditions and expressed as U/ml.

Tabulation 2:

Sl. No	**Conc. of substrate [S] mg/ml**	**Conc. of glucose [P] mg/ml**	**Conc. of glucose [P] mmol/ml**	**V mmol/ml.min**	**1/V**	**1/[S]**	**V/[S]**	**[S]/V**
1								
2								

3								
4								
5								
6								
7								
8								
9								
10								

Result:

The values of Vmax and Km are calculated as follows,

	Name of the plot	**V_{max}**	**K_m**
	Michaelis-Menten		
	Lineweaver-Burk		
	Hanes-Woolf		
	Eadie-Hofstee		

Significance of the experiment:

EXP. No. 5. Enzyme Immobilization

Aim:

To immobilize the alpha amylase enzyme by gel entrapment method and to determine the radius of the immobilized bead.

Principle:

Alginate, commercially available as sodium salt of alginic acid, commonly called sodium alginate, is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae. The copolymer consists of two uronic acids: D-mannuronic acid (M) and L-gulucuronic acid (G). Because it is the skeletal component of the algae it has the nice property of being strong and yet flexible.

Alginic acid can be either water soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium. Polyvalent cations bind to the polymer whenever there are two neighboring gulucuronic acid residues. Thus, polyvalent cations are responsible for the cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions.

$$2\ Na(Alginate) + Ca^{++} \text{-------} > Ca(Alginate)_2 + 2\ Na^{+}$$

The ionically linked gel structure is thermostable over the range of 0-100ºC; therefore heating will not liquefy the gel. However, the gel can be easily redissolved by immersing the alginate.

Alginate is currently widely used in food, pharmaceutical, textile, and paper products. The properties of alginate utilized in these products are thickening, stabilizing, gel-forming, and film-forming. Alginate polymers isolated from different alginate sources vary in properties. Different algae, or for that matter different part of the same algae, yield alginate of different monomer composition and arrangement.

There may be sections of homopolymeric blocks of only one type of monomer (-M-M-M-) (-G-G-G-), or there may be sections of alternating monomers (-M-G-M-G-M-). Different types of alginate are selected for each application on the basis of the molecular weight and the relative

composition of mannuronic and gulucuronic acids. For example, the thickening function (viscosity property) depends mainly on the molecular weight of the polymer; whereas, gelation (affinity for cation) is closely related to the gulucuronic acid content. Thus, high gulucuronic acid content results in a stronger gel.

Materials required:

- Sodium Alginate (3%)
- 0.2 M $CaCl_2$
- Alpha amylase enzyme (1%)

Procedure:

Immobilization of enzyme

- Add 3g of sodium alginate slowly to 100 ml of distilled water by employing magnetic stirrer to prepare sodium alginate solution.
- Mix approximately 50 mg of enzyme with 5 ml of 3% (wt.) sodium alginate solution.
- Dispense the enzyme and sodium alginate solution into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution by using peristaltic pump or a syringe and needle at room temperature.
- The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter.
- Leave the beads in the calcium chloride solution to cure for 0.5-3 hours.

Determination of bead radius:

- Take known number of immobilized beads (N) in the measuring jar up to a certain height (for ex. 10 ml).
- Add water to the same height in the measuring jar to find out the void volume
- Decant the water completely into another measuring jar, and this volume is void volume, Vv
- Volume of each bead = $(V_{total} – V_v)/N$
- From the volume of the bead, radius can be calculated.

Calculation:

- Number of beads taken in the measuring jar (N) =
- Height of the immobilized beads = x ml
- Volume of water added to the same height = y ml
- Volume of N beads = (x-y) ml
- Volume of 1 bead = (x-Y)/N ml = z

$4/3\ \pi\ r^3 = z\ cm^3$

r = ?

- Calculate the radius of the bead

Result:

The enzyme α – amylase was immobilized and radius of the immobilized bead was calculated.

Radius of the immobilized bead =…… cm

Exp. No. 6 Medium Optimization by Plackett and Burman Design

Aim:

To screen the significant medium components for optimization by using the Plackett and Burman method.

THEORY:

A detailed investigation is needed to establish the most suitable medium for an individual fermentation process, but certain basic requirements must be met by any such medium. All micro-organisms require water, sources of energy, carbon, nitrogen, minerals elements and possibly vitamins plus oxygen (if aerobic). On a small scale it is relatively simple to devise a medium containing pure compounds, but the resulting medium, although supporting satisfactory growth may be unsuitable for use in a large scale process. On a large scale one must normally use sources of nutrients to create a medium which will meet as many possible of the following criteria:

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of consistent quality and be readily available throughout the year.
6. It will cause minimal problems during media making and sterilization.
7. It will cause a minimal problem in other aspects of the production process particularly aeration and agitation, extraction, purification, and waste treatment.

Medium optimization by the classical method of changing one independent variable (nutrient, antifoam, pH, temperature, etc.) while fixing all the other at a certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which will examine each possible combination of independent variable at appropriate levels will require a large number of experiments X^n, where X is the number of levels and n is the number of variables. This may be quite approximate for the three nutrients at two concentrations (2" trails), but not for six nutrients at three concentrations, in this instance, 3' (729) trials would be needed. Industrially the aim is to perform a minimum number of experiments to determine optimal conditions. Other alternative strategies must be considered which allow more than one variable to be changed at a time.

When more than one variable is to be investigated, the **Plackett-Burman** design may be used to find the most important variable in the systems, which are then optimized in further studies, (Plackett and Burman, 1946). These authors gave a series of design for up to a hundred experiments using an experimental rationale known as balanced incomplete blocks. This technique allows all the evaluation of X – 1 variable by X experiments. X must be a multiple of 4, e.g., 8, 12, 16, 20, 24, etc. Normally one determines how many experimental variables needed to be included in an investigation and then selects the Plackett-Burman design which meets the requirements in most closely in a multiple of 4. Any factors not assigned to a variable can be designated as a dummy variable. Alternatively, factors known to not affect may be included and designated as dummy variables. The incorporation of dummy variables into an experiment makes it possible to estimate the variance of an effect (experimental error).

Table -1 shows a Plackett-Burman design for seven variables (A-G) at high and low levels in which two factors, E and G, are designed as dummy variables. These can be used in design to obtain an estimate of error. However, more can be studied if fewer real variables need to be studied in an investigation (Stowe and Mayer, 1966). Each horizontal represents a trail, and each vertical column represents an L (low) and H (high) values of one variable in all the trails. This design (Table) requires that the frequency of each level of a variable in a given column should be equal. Consider the variable A; for the trials in which A is high, B is high in two of the trials and low in the other two.

Similarly, C will be high in either two trials and low in the two as will all the remaining variables. For those trials in which A is low B will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of A. The same logic then applies to each variable. However, no changes are made to the high and low values for the E and G columns. Gresham and Inamine (1986) state that although the difference between the levels of each variable must be large enough to ensure that the optimum response will be included, caution must be taken when setting the level differential for sensitive variables, since a differential that is too large could mask the other variables. These trails are carried out in a randomized sequence.

The effects of the dummy variables are calculated in the same way as the effects of the experimental variables. If there are no interactions and no error in measuring the response, the effect shown by a dummy variable should be 0. If the effect is not equal to 0, it is assumed to be a measure of lack of experimental precision plus any analytical error in measuring the response (Stowe and Mayer, 1966).

The stages of analyzing the data using Nelson's (1982) example are as follows:

1. **Determine the difference between the average of H (high) and L (low) responses for each independent and dummy variable.**

   Therefore the difference = ∑ A (H) - ∑ A (L)

The effect of an independent variable on the response is the difference between the average response for the four experiments at the high level and the average value for the four experiments at the low levels.

Analysis of the yield shown in Plackett-Burman Table

	Factor						
	A	B	C	D	E	F	G
∑ (H)							
∑ (L)							
Difference effect							
Mean square							
Mean square for error							
F- Test							

Thus the effect of

$$\frac{A}{4} = \frac{\sum A(H) - \sum A(L)}{4} = \frac{2\sum A(H) - \sum A(L)}{8}$$

This value should be near zero for the dummy variables.

2. **Estimate the mean square of each variable (the variance of effect).**

$$For\ A\ the\ mean\ square\ will\ be\ = \frac{(\sum A(H) - \sum A(L))^2}{8}$$

3. **The experimental error can be calculated by averaging the mean square of the dummy effects of E and G.**

$$mean\ square\ for\ error = \frac{\sum(\text{Mean square of dummy variables})}{\text{Number of dummy variables}}$$

4. **The final stage is to identify the factors which are showing large effects. This is done using an F-Test which is given by :**

$$\frac{\text{Factor mean square}}{\text{Error mean square}}$$

The factor which is having the highest F-Test value is identified as the most important factor.

PROCEDURE:

Prepare the medium according to the Plackett-Burman design for each trial of appropriate high and low concentrations.

Medium variables	**Low (L) g/l**	**High (H) g/l**
A. Glucose	0.5	4.0
B. Yeast extract	0.1	1.0
C. Casamino acid	0.1	0.5
D. Ammonium Chloride	0.5	2.0
E. KH_2PO_4	3.0	3.0
F. Glycine	0.1	1.0
G $MgSO_4$ (1M)	1 ml	1 ml

Where E and G are dummy variables.

1. Prepare 100 ml of medium as per the design table and adjust the pH to 7
2. Inoculate the sterilized medium by using 5ml of 24 h culture of *E.coli.*
3. Measure the initial O.D at 600 nm
4. Incubate the flasks at 37 ºC, 120 rpm for 2 h
5. Measure the optical density after 2h at 600 nm.

TABLE 2: PLACKETT-BURMAN RESPONSE TABLE

Trial	A	B	C	D	E	F	G	Response(O.D) at 600 nm
1	H	H	H	L	H	L	L	
2	L	H	H	H	L	H	L	
3	L	L	H	H	H	L	H	
4	H	L	L	H	H	H	L	
5	L	H	L	L	H	H	H	
6	H	L	H	L	L	H	H	
7	H	H	L	H	L	L	H	
8	L	L	L	L	L	L	L	

TABLE 3: ANALYSIS

variables / Calculated values	A	B	C	D	E	F	G
∑H							
∑L							

Difference							
Difference effect							
Mean square							
Mean square for error							
F- test							

RESULT:

The significant medium components based on F-test: 1.

2.

3.

4.

5.

6.

7.

8.

INFERENCE:

Exp. O. 7. BATCH GROWTH KINETICS OF BACTERIA

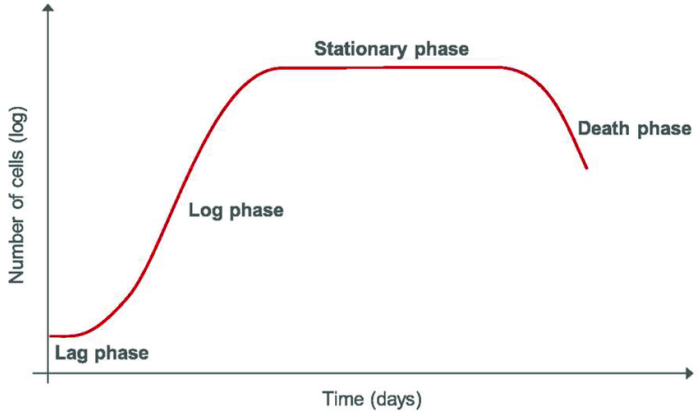
AIM

To study the growth of *E.coli* strain (bacteria) in batch culture using Glucose yeast extract medium and to find the specific growth rate and yield coefficient.

Principle

Batch culture systems represent growth in a closed system. This can either use a flask or fermentor containing a suitable growth supporting medium operated under optimum conditions of temperature, pH and redox potential, which is inoculated with the cells grown until some essential component of the medium is exhausted or the environment changes because of the accumulation of a toxic product, pH change etc. In general microbial growth is determined by cell dry weight measurement. The growth curve can be divided into three phases:

1. **Lag phase:** During this period the cell adapts to the new environment by synthesizing necessary enzymes for the utilization of available substrates.
2. **Exponential phase:** The cell constituents in this phase increase at a constant rate so that the cell population doubles and continues to double at regular intervals.
3. **Stationary phase:** In this phase, cell death occurs because of depletion of essential the rate of growth, hence there is no net growth or increase in cell number. This is followed by a death phase.



The growth rate typically changes in a hyperbolic fashion, if the concentration of the essential medium component is varied while the concentration of the other medium components are kept constant and it follows the Monod growth kinetics.

$$\mu = \frac{\mu max \, . \, S}{\square\square + \square}$$

Where S- concentration of the essential medium component, μ - specific growth rate hr^{-1}, μMax- Maximum specific growth rate achievable when $S>>K_S$, K_S - Monod constant and is equal to the concentration of the essential medium component at which the specific growth rate is half of its maximum value. Specific growth rate is linearly dependent on the concentration of the essential medium component at lower concentration and it is independent at higher the concentration of the essential medium component.

The growth of the microbial cells is autocatalytic.

The General mass balance is

Input + Formation = Output + Accumulation + Disappearance

The Cell mass Balance is

Cell mass Input + Cell Growth = Cell mass Output + Cell mass Accumulation + Cell death Here we neglect cell death and no cells are removed in a batch reactor.

$$\frac{\square\square}{\square\square} = \mu x \ldots \ldots (1)$$

where, X = biomass conc, t = time On

integrating eqn (1)

$\square_{\square} = \square_o . \square^{\mu\square}$ …..(2)

This is the equation for microbial growth in the exponential phase,

Where, X_o = initial biomass concentration, X_t = biomass concentration after time t

On taking "ln",

$$= \ln \square_o + \mu\square \ldots\ldots\ldots 3$$

A plot of ln x versus t gives straight line with slope μ.

Specific growth rate during the exponential can also be calculated by using the formula

$$\mu = \frac{\ln \square\square 1 - \ln \square\square 2}{\square 2 - \square 1}$$

where ,OD1 is the optical density at time t_1 and OD2 is optical density at time t_2.

Doubling time of the strain,

$$t_d = \frac{\ln 2}{\mu}$$

Following log phase is the decelerating phase and stationary phase, where the growth is almost constant with respect to time. Depletion of nutrients leads to declining growth phase where growth occurs but the death rate is greater.

The yield coefficient:

$$Y_{x/s} = \frac{\Delta x}{\Delta s} = \frac{x - x_o}{s_o - s} \quad \text{----------(5)}$$

X_o - initial concentration of biomass X_t = concentration of biomass at time, t", S_0 = initial substrate concentration, S_t = Residual substrate concentration at time, t".

Materials required

Shake flasks, Shaker, pipette, spectrophotometer and *E. coli* strain.

Glucose Yeast extract medium:

Glucose	3- 15 g/1
Yeast extract	5 g/1
NH_4Cl	1 g/1
NaCl	0.5 g/l
K_2HPO_4	5g/l
$MgSO_4$	0.5g/l

Trace metal solution: (1ml / liter of solution)

$FeSO_4$	50mg/l
$Al_2(SO_4)_3.7H_2O$	10 mg/l
$CuSO_4\ 4H_2O$	2 mg/l
H_3BO_3	l mg/1
$MnCl_3.4H_2O$	20 mg/l
$NiCl_2.6H_2O$	1 mg/1
$Na_2MoO_4.2H_2O$	50 mg/l
$ZnSO_4.7H_2O$	5 mg/l

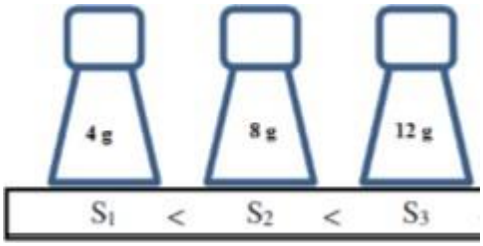
pH : 7.0

Temp: 37º C

After inoculation, readings were taken at regular time intervals and tabulated. The values of O.D, residual glucose, and dry weight were thus recorded.

Procedure

- Glucose-yeast extract medium was prepared with varying concentration of glucose.



- 5% of overnight grown inoculum was added to a 50 ml of Glucose-yeast extract medium.
- The culture medium was incubated at 37 ºC (optimum growth temperature of *E. coli*) in a shaker at 150 rpm.
- Bacterial growth was monitored at every 20-30 min (starting from t= 0), by withdrawing a 4 ml of sample from the culture and optical density was measured at 600 nm with a spectrophotometer.
- 1 ml sample was transferred to eppendorf tube and centrifuged at 4000 rpm for 10 min. Supernatant was transferred to another eppendrof tube and cell mass pellet weight was noted down.
- Residual glucose concentration in the above supernatant was estimated by DNS Assay.
- Above steps were repeated for different samples taken at different time intervals.
- Plot a graph between ln OD *versus* time and find the slope, which is equal to the specific growth rate μ.
- Doubling time and yield coefficient were calculated using the formula.

Glucose estimation protocol - DNS assay

- Take supernatant solution in the test tubes label it
- 3 ml of distilled water alone serves as blank.

- Add 3 ml of DNS reagent to all test tubes. Incubate at 90°C for 15 minutes in water bath.
- Add 1 ml 40 % Sodium Potassium tartrate solution.
- Read the OD at 575 nm.

Time (min)	**OD for cell mass at 600 nm**				**ln OD**				**Specific growth rate (μ) min $^{-1}$**			
	3 g/L	**6 g/L**	**9 g/L**	**12 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**
0	0.0	0.0	0.0	0.0								
15	0.004	0.005	0.07	0.009								
30	0.007	0.009	0.015	0.041								
45	0.011	0.014	0.030	0.067								
60	0.024	0.029	0.060	0.113								
75	0.043	0.051	0.077	0.196								
90	0.056	0.067	0.094	0.254								
105	0.073	0.094	0.113	0.331								
120	0.090	0.110	0.121	0.411								
150	0.121	0.147	0.176	0.502								
180	0.143	0.169	0.204	0.623								
240	0.177	0.191	0.276	0.703								
300	0.261	0.281	0.389	0.811								
360	0.421	0.530	0.660	0.921								
420	0.540	0.810	0.810	1.102								
480	0.730	0.934	1.103	1.310								
540	0.886	1.121	1.453	1.490								
600	0.970	1.414	1.823	1.670								
660	1.140	1.702	1.877	1.910								
720	1.152	1.771	1.914	2.091								

840	1.182	1.841	2.013	2.320								
1000	1.201	1.925	2.210	2.560								

Time (min)	**OD for residual substrate at 575 nm**				**Residual substrate (S), mg/ml**				**Yield coefficient (Yx/s) mg cell / mg sub.**			
	3 g/L	**6 g/L**	**9 g/L**	**12 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**
0	0.0	0.0	0.0	0.0								
15	0.004	0.005	0.07	0.009								
30	0.007	0.009	0.015	0.041								
45	0.011	0.014	0.030	0.067								
60	0.024	0.029	0.060	0.113								
75	0.043	0.051	0.077	0.196								
90	0.056	0.067	0.094	0.254								
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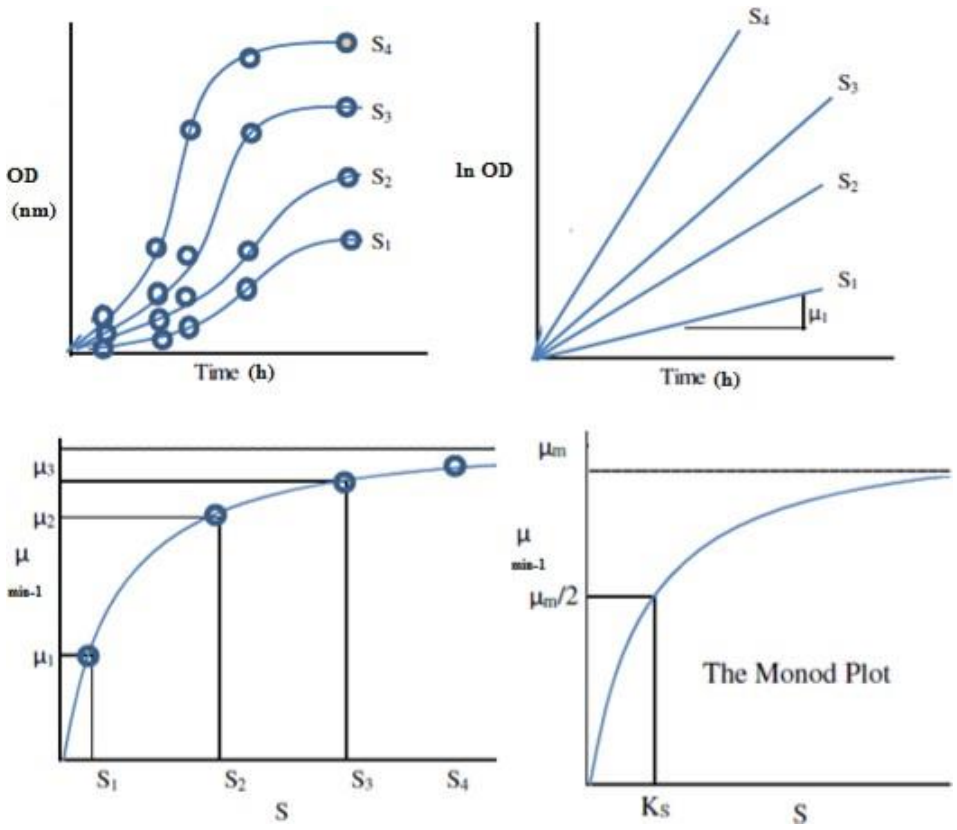
720	1.152	1.771	1.914	2.091								
840	1.182	1.841	2.013	2.320								
1000	1.201	1.925	2.210	2.560								

Model graph



Result

The batch growth characteristics of the given strain was studied and the following parameters were found:

Specific growth rate (from graph) μ = ...sec

Doubling time (from graph) t_d =.....sec

Yield coefficient $Y_{x\backslash s}$ =… ..... mg of dry cell mass/mg of substrate

Exp. No. 8 Determination of K_La by Sulphite Oxidation Method

AIM:

To determine the oxygen mass transfer coefficient, K_La using sodium sulfite oxidation method.

THEORY:

The volumetric liquid mass transfer coefficient (K_La) is a useful parameter to characterize bioreactors capacity for aeration. This helps the reactor design, optimization of technologies and scaling up or scaling down processes. The sodium sulfite combines with oxygen to give sodium sulfate, with CuSO4 as a catalyst in the reactor. The concentration of sodium sulfite at various time points is inversely proportional to the oxygen transfer rate.

$$Na_2SO_3 + \tfrac{1}{2} O_2 \xrightarrow{Cu^{2+}} Na_2SO_4$$

The above reaction is

A. Independent of sodium sulfite concentration within the range of 0.04N to 1.0N

B. The rate of the reaction is much faster than O_2 transfer rate.

Thus, the rate of oxidation is controlled by the rate of mass transfer alone.

The reaction consumes oxygen at a rate that is sufficiently fast so that transport of O_2 from gas to a liquid through the liquid film is the rate-limiting step. The rate of the reaction is zeroth order in Na_2SO_3. If the reaction is not fast enough, the reaction occurs in the liquid film around the gas bubbles. This would decrease apparent film thickness and give incorrectly high values of K_La. Concentrations of unreacted sulfite are determined by reacting the sulfite with excess iodine and then back titration of the iodine with thiosulphate. It is important to note that the dissolved oxygen is zero through the reaction.

By titrating sodium sulfite present in the reactor (by taking a sample at fixed intervals of time) against sodium thiosulphate, the quantity of sodium sulfite that would have reacted according to the equation can be measured as the difference between successive rate instants of time. Then based on stoichiometry, the corresponding number of moles of O_2 that would have been consumed can be determined.

The Oxygen transfer rate

$$\mathbf{OTR = K_La\ (C^*-C_L)}$$

Where $C^* = 8.43X10^{-3}$g/l at 25^oC

C_L = Dissolved Oxygen content.

In this case, $C_L = 0$, since the DO is maintained such that it is not saturated.

$$\mathbf{OTR = K_La\ (C^*)} \text{ -------- (1)}$$

$$\mathbf{K_La = OTR/\ C^*}$$

Thus K_La can be determined.

PROCEDURE:

- Take 100 ml of 0.5M Sodium sulphite & 0.002M copper sulphate solutions in a 250ml conical flask.
- Keep the conical flask with sodium sulfite solution in a magnetic stirrer for mixing and take '0^{th} h' sample for titration.
- Add 15 mL of Iodine solution and few drops of starch indicator to the 1 ml of sample withdrawn from the flask.
- Titrate the sample against 0.5N Sodium thiosulphate. (It becomes straw yellow color and then turn into dark blue color)
- Repeat the analysis for 5,10,15,20…….minutes samples (till constant titer value reached to continue the sampling)
- Plot the graph between titer volume vs. time
- Calculate the OTR by using the slope of the graph
- Calculate the Kla by using the given formula

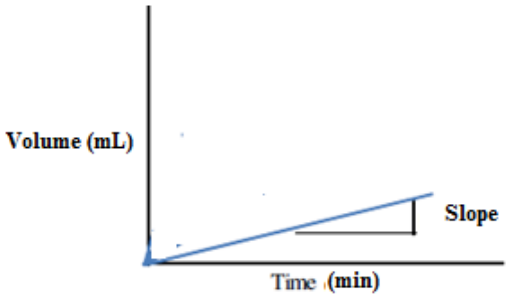
REACTION:

$O_2 + 2\ Na_2SO_3 \longrightarrow 2\ Na_2SO_4$

$2\ Na_2SO_3 + 2I + 2\ H_2O \longrightarrow 2\ Na_2SO_4 + 4HI$

$4\ Na_2SO_3 + 2I_2 \longrightarrow 2Na_2S_4O_6 + 4NaI$

MODEL GRAPH:



CALCULATION:

$$OTR = \frac{\text{Slope x Molarity of } Na_2S_2O_7 \text{ x Molecular weight of } O_2}{\text{Sample volume x Molecular weight of } Na_2S_2O_7}$$

$$K_la = OTR / C^*$$

TABULATION:

Sample time (min)	Titer volume (ml)

RESULT:

INFERENCE:

Ex. No. 9. CELL DISRUPTION BY SONICATION

Aim:

To disrupt microbial cells at different time intervals and to measure the amount of protein released and rate constant (k).

Principle:

The treatment of microbial cells in suspension with inaudible ultra sound (greater than 18000 Hz) results in their inactivation and disruption. Ultrasonication utilizes the rapid sinusoidal movement of a probe within the liquid. It is characterized by high frequency (18 kHz -1 MHz), small displacements (less than 50µm); moderate velocities (a few ms^{-1}), steep transverse velocity gradient (up to 4000 s^{1}) and very high acceleration (up to 80,000g). In Ultrasonication phenomena, when acoustic power input is sufficiently high will allow the multiple productions of micro bubbles, at nucleation sites in the fluid. The bubbles grow during the rarefying phase of the sound wave and then are collapsed during the compression phase. On collapse, a violent shock wave passes through the medium. The whole process of gas bubble nucleation, growth, and collapse due to the action of the intense sound wave is called cavitation. The collapse of the bubble converts sonic energy into mechanical energy in the form of shock waves equivalent to several atmospheric (300MPa) pressures. This energy input imparts motions to parts of cells which disintegrate when their kinetic energy content exceeds the cell wall strength. An additional factor which increases cell breakage is the micro streaming (very high-velocity gradient causing shear stress) which occur, near radically vibrating bubbles of gas, caused by the ultrasound. Much of the energy absorbed by all suspensions is converted to heat effectively, so cooling is necessary. The rate of protein released by mechanical cell disruption usually proportional to the amount of releasable protein.

$$\frac{dP}{dt} = -KP$$

Where P = protein content remaining in associated cells

t = time

K= release constant dependent on the system.

Integrating from P = Pm (maximum possible protein release at time zero) to P = Pt at time

't' gives

$$\int_{P_m}^{P_t} \frac{dP}{P} = -K \int_0^t dt$$

$$\Rightarrow \ln\left(\frac{P_m}{P_t}\right) = Kt$$

As protein (Pr) released from the cells is given by Pr =Pm-Pt, the following equation for cell breakage is obtained

$$\ln Pm/Pm - Pr = Kt$$

$$K = \ln (1\text{-}R)^{-1}/t,$$

Where R- Fractional release of protein(The protein release at a specific point divided by the maximum release attainable)

The constant (K) is independent of cell concentration up to high levels approximately proportional to the acoustic power above the threshold necessary for cavitations.

Procedure:

- Take six samples of given culture (2 ml) in Eppendorf tubes and centrifuge at 10000 rpm for 10 minutes at 4°C
- Discard the supernatant and resuspend the pellet in 1ml of distilled water.
- Place the samples under ice flakes during sonication. Set the power at 50W and frequency at 60 MHz.
- Sonicate the samples for different time intervals (30,60, 90, 120 and 150 s
- After sonication, centrifuge the samples at 10000 rpm for 10 minutes at 4°C.
- Take a required volume of supernatant and determine the amount of protein in the sample
- Plot the graph between sonication time(seconds) Vs. concentration of protein (mg/ml)
- Determine the rate constant (K)

Result:

The optimum sonication time : …….

The rate constant (k): …….....

Discussion:

Tabulation:

S.No.	Sonication time (sec)	O.D at 595 nm	Conc. of released protein (mg/ml)	R= Pr/Pm	ln (1-R)	K
1	30					
2	60					
3	90					
4	120					
5	150					
6	180					

Calculation:

Ex. No. 10. Cell Disruption by High Pressure Homogeniser

Aim:

To disrupt the cells by using high pressure homogenizer at different pressures and to measure the amount of protein released and rate constant (K)

Principle:

A high-pressure homogenizer consists of a high pressure positive displacement pump coupled to an adjustable discharge valve with a restricted orifice. The cell suspension is pumped through the homogenizing valve at 200-1000 atm atmospheric pressure depending on the type of microorganism and concentration of the cell suspension. The disrupted cell suspension is cooled as it exits the valve to minimize thermal denaturation of sensitive products. Cell disruption occurs due to various stresses developed in the fluid. The primary mechanism seems to be the stress developed due to the impingement of high velocity jet suspended cells on the stationary surface. The homogenizer including normal and shear stresses generates other stresses. The normal stress is generated as the fluid passes through the narrow channel of the orifice and the shear stress as the pressure rapidly reduces to almost atmospheric pressure as the cell suspension passes out of the orifice. Different parameters influence the degree of cell disruption and rate of release of the product. These include the nature of microorganism, its size, cell wall composition and thickness, and concentration of microbial cells, product location within the cell, type of homogenizer, operating pressure, temperature, and number of passes of the cell suspension through the homogenizer.

Cell disruption in homogenizer may also be described by first order kinetic expression as given by,

$$\ln(Pm/Pm-Pr) = KN P^a$$

$$\ln (1-R) = -KN P^a$$

Where N=number of passes through the valve

K is the first order rate constant depending on operating pressure

a= 1(Range is 0.9 – 2.9)

Where R- Fractional release of protein(The protein release at a specific point divided by the maximum release attainable).

Procedure:

- Set initial pressure as 100 bar
- Take 500 ml of given culture sample
- Homogenize the culture sample for different pressure (100- 500 bar)
- Repeat the homogenization for two cycles
- After homogenization, centrifuge the samples at 10000 rpm for 10 minutes at 4°C.
- Estimate the protein concentration in the supernatant by using Bradford's method.
- Plot the graph between pressure(bar) Vs. concentration of protein (mg/ml)
- Estimate the rate constant (K)

Result:

The optimal pressure and number of passes: ----------

The rate constant (K): ----------

Discussion:

Tabulation:

S.No.	Pressure(bar)	No. of passes	O.D at 595 nm	Conc. of released protein (mg/ml)	R= Pr/Pm	ln (1-R)	K
1	100	1					
		2					
2	200	1					
		2					
3	300	1					

		2					
4	**400**	**1**					
		2					
5	**500**	**1**					
		2					

Calculation:

Ex. No. 11. BATCH FILTRATION

Aim:

To filter the given slurry and to determine specific cake resistance α and medium resistance Rm.

Principle:

Filtration is the conventional unit operation aimed at the separation of particulate matter. Filtration is defined as the separation of solid in a slurry consisting of the solid and fluid by passing the slurry through a septum called the filter medium. The filter medium allows the fluid to pass through and retains the solids. The separated solid called the filter cake forms a bed of particles on the filter medium. The thickness of the cake increases from an initial value of zero to a final thickness at the end of filtration. During filtration the filtrate passes first through the cake and then the filter medium.

The rate of filtration is defined as the volume of filtrate collected per unit time per unit area of the filter medium. The resistance of the medium is constant and is independent of the cake.

$$\mathbf{At/V = K\ V/A + \beta}$$

$$\mathbf{K = \mu\alpha\rho_0/2\ \Delta p}$$

$$\mathbf{\alpha = K\ 2\ \Delta p/\mu\rho_0}$$

$$\mathbf{\beta = R_m\ \mu/\Delta p}$$

Materials required:

- $CaCO_3$ solution
- Filter paper
- Funnel
- Measuring cylinder

Procedure:

- Take 50 ml of 10% $CaCO_3$ and filter it through filter paper.
- Note down the volume of filtrate collected in measuring cylinder every minute until the end of filtration.
- Calculate the area of the funnel.
- Plot the graph between At/V vs. V/A and find out the slope and intercept.
- From the values of slope and intercept calculate α and R_m.

Result:

Discussion:

Tabulation:

Time (sec)	Volume of filtrate collected (ml)	At/V (s/cm)	V/A (cm)

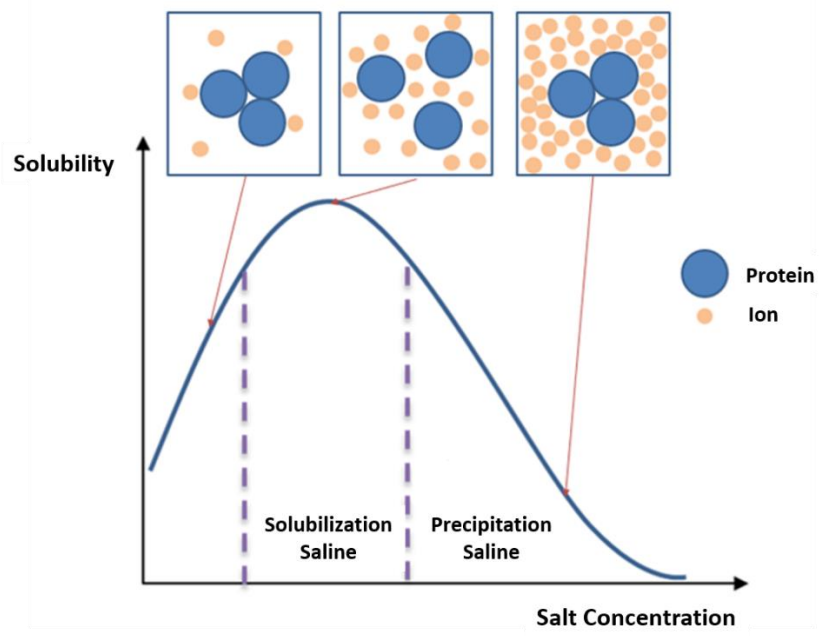
Exp. No. 12. Protein recovery by ammonium sulphate precipitation

Aim:

To recover the proteins from the given solution by using ammonium sulfate

Theory:

Ammonium sulfate precipitation is a method of protein purification by altering the solubility of the protein. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. Two distinct effects are observed: (1) Salting in: At low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e., increasing ionic strength). As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. (2) Salting out: At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution. The commonly used salt is ammonium sulfate, as it is very water soluble and has no adverse effects upon enzyme activity.



List of reagents required:

Ammonium sulphate solution: Weight 20%, 40%, 60%, 80%, 90%, 100% of saturated $(NH_4)_2SO_4$ in a centrifuge tubes as per standard chart.

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulphate to add (grams) per liter of solution at +20 °C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

No.	Ammonium sulfate (%)	g/l
1	10	57
2	20	113
3	30	176
4	40	242
5	50	314
6	60	390
7	70	472
8	80	561
9	90	657
10	100	761

- **BSA protein solution**: 0.2 % of Bovine serum albumin in phosphate buffer solution.
- **Bradford reagent :**
    - Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid.
    - Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

Materials and Methods

Step 1: Add 10ml of BSA solution to the each ammonium sulphate containing centrifuge tube.

Step 2: Mix the solution and incubate for 20—30 minutes to equilibrate.

Step 3: Centrifuge the solution for 10,000 rpm for 10 mins and Collect the precipitate by carefully discarding as much supernatant as possible.

Step 4: Add 100 µl of PBS buffer in the precipitate to dissolve the pellet.

Step 5: Measure the protein concentration by Bradford method at 540nm

S. No	Ammonium sulfate (%)	O.D at 540 nm	Protein concentration (mg/ml)	Protein recovery (%)
1	0	1.234		
2	20	1.3025		
3	40	1.349		
4	60	1.395		
5	80	1.426		

6	100	1.447		

$$\% \text{ Protein Recovery} = \frac{\text{Final protein content (mg/ml)}}{\text{Initial protein content (mg/ml)}} \times 100$$

Calculation:

Result:

- The maximum amount of protein recovered by using -------- % of ammonium sulfate
- Percentage of protein recovery at 37^0C is found to be ------

Discussion:

Ex. No. 13. Aqueous Two Phase Extraction

Aim:

To separate the given protein by aqueous two phase extraction and to find the partition coefficient.

Principle:

Downstream processing is an integral part of any product development, and the final cost of the product depends largely on the cost incurred during extraction and purification techniques. The conventional techniques used for product recovery, for example, precipitation and column chromatography, are not only expensive but also result in lower yields. Furthermore since solid–liquid separation by centrifugation or filtration results in some technical difficulties, for example, filter fouling and viscous slurries, therefore, there is an ongoing need for new, fast, cost effective, ecofriendly simple separation techniques. Thus, for separation of biomolecules, aqueous two phase systems (ATPS) offer an attractive alternative that meets the abovementioned requirements as well as the criteria for industrially compatible procedures. Hence, it is increasingly gaining importance in biotechnological industries. The advantage of using this technique is that it substantially reduces the number of initial downstream steps and clarification, concentration, and partial purification can be integrated into one unit. Furthermore, scale-up processes based on aqueous two phase systems are simple, and a continuous steady state is possible

An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which is obtained either by a mixture of aqueous solution of two polymers, or a polymer and salt. Generally, the former is comprised of PEG and polymers like dextran, starch, polyvinylalcohol, etc. In contrast, the latter is composed of PEG and phosphate or sulphate salts. This polymer-salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in the first extraction step

Partitioning of the two phases is a complex phenomenon, taking into account the interaction between the partitioned substance and the component of each phase. A number of different chemical and physical interactions are involved, for example, hydrogen bond, charge interaction, van der Waals' forces, hydrophobic interaction and steric effects11. Moreover, the

distribution of molecules between the two phases depends upon the molecular weight and chemical properties of the polymers and the partitioned molecules14 of both the phases.

Thus, the distribution of molecules between the two phases is characterized by the partition coefficient, *K*part, defined as the ratio of the concentrate in the top (*C*t) and bottom (*C*b) phase, respectively.

$$\mathbf{Kpart = Ct/Cb}$$

The yield (Y) was calculated as follows,

$$\mathbf{Yield\ of\ protein\ (\%) = (Ct\ or\ b\ x\ Vt\ or\ b) / (C_{CE}\ x\ V_{CE})\ X\ 100}$$

where, C_{CE} and C t or b are the protein contents (mg/ml) in crude extract and top or bottom phase respectively, V_{CE} and V t or b or b are the volumes of crude extract and top or bottom phase respectively.

Procedure:

1. Prepare 45% (w/w) of Sodium phosphate (i.e., 45 g of salt in 65 g of water and 50% of PEG 6000 – 50 g of PEG 6000 in 50 g of water)
2. Prepare the protein solution (1 g BSA/ 10 ml of water)
3. Add 5 ml of salt solution to the protein solution
4. Then drop by drop add 5 ml of PEG 6000
5. Allow the mixture to stand for some time to obtain the two phases.
6. Thoroughly mix the contents
7. Allow to settle for 20 minutes and separate the two phases by careful pipetting and note down the volume of top and bottom phase
8. Calculate the partition coefficient and yield by using the given formula.

Tabulation:

Phase	**Volume (ml)**	**O.D at 595 nm**	**Concentration of protein (mg/ml)**

Result:

The partition coefficient ----------

The yield of protein in top phase ----------

The yield of protein in bottom phase ----------

Discussion: