

* Aim:- Understanding the different components of a bioreactor and general overview of laboratory equipments.

* Equipment 1:- Bioreactor

→ Introduction:-

- A bioreactor is defined as a closed system used for growing microorganisms (bacteria, fungi, algal), plant cells and animal cells in a culture medium where the growth of the same can be enhanced in a sterile condition by controlling environmental conditions e.g. temperature, dissolved oxygen etc.

→ Principle:-

- The fermentor apparatus is designed to contain all culture within the confines of the bioreactor vessel and to allow sampling without exposing the content of the tank to the open atmosphere which would allow any extraneous organism to enter. In addition, pH, temperature and DO are all controlled at user specified set points, so that the production of by-products will not have adverse effects on the culture environment. The vessel has to be adequately stirred and aerated so as to prevent any stagnant or oxygen poor zones.

→ Main components:-

• Glass vessel (and static seal):-

It is the main component of a bioreactor where an organism is grown and an aseptic culture medium, with all the probes like temperature, pH, DO etc is maintained at a desired temp., pH, DO etc for the particular organism being grown.

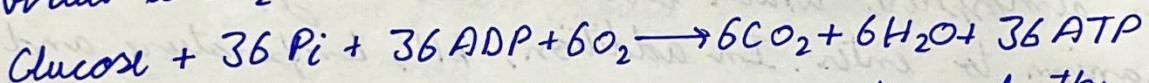
• Static seal:-

Sealing between the top plate and vessel is an important criterion to maintain airtight condition and aseptic environment. Sealing is done between 3 types of surfaces like glass-glass,

lip seal and 'O' ring type sealing.

DO probe:-

The concentration of dissolved oxygen in bioreactors is measured using a dissolved oxygen (DO) electrode. Oxygen has a very low solubility in water, only about 7 PPM at standard temperature and pressure. Hence, oxygen is usually the limiting nutrient for organism growth, ~~less~~ unless a feed strategy is designed to limit the growth rate via a different nutrient of choice. Above a critical O_2 concentration, growth rate is independent of DO concentration. Oxygen limitation ~~less~~ usually results in the production of by-products because of incomplete substrate oxidation to CO_2 . Molecular O_2 consumed by most organisms via respiration can only be used as a terminal electron acceptor and therefore, must be converted to CO_2 .



Air is fed to the vessel from an air compressor, through the DO controller and into the vessel through a tube with fine holes in it (the sparger). The mixing energy of the impeller causes the air bubbles to dissolve somewhat into the culture broth. The DO probe must be calibrated ~~to~~ prior to the fermentation run. Because DO probe are often unreliable, it is a good idea to calibrate the probe prior to autoclaving to ensure its proper function. Autoclaving the probe usually requires readjustment ~~to~~ prior to inoculation.

pH probe:-

The pH, i.e., concentration of the hydrogen ion in the media which is one of the crucial factors for the growth of the desired organism is ~~not~~ measured by the use of pH electrode.

All microorganism that grow in culture are sensitive to the pH of the medium. The pH in the cytoplasm of the organism is normally a neutral value, since proteins depend on hydrogen bonding for their proper folding and function. Most, but not all culturable organism grow best around neutral pH (4 to 5.5 and 8). High cell density in the culture results in the rapid depletion of one or more nutrients, which often results in the production of organic acids: acetic acid, succinate, formic and lactate. Likewise, the production of CO₂ results in the dissolution of CO₂ in the broth and the formation of bicarbonate. To maintain the pH in the vessel, base is added in response to a low offset from from a pH setpoint. The bases most often used for pH control are NH₄OH (which also contributes Nitrogen in a usable form to the bacteria), KOH, NaOH or bicarbonate.

Like the DO probe, the pH probe should be calibrated both before and after autoclaving.

Temperature probe:-

The temperature probe measures the prevailing temperature of the culture media. This temperature probe is inserted into the culture vessel such that the temperature in the center of the vessel is measured. Temperature may also be an important parameter for optimization of the production of a desired product. Heat produced is a direct result of metabolism, and in particular, of respiration. Hence, while it may be necessary to add heat to the bioreactor just after inoculation, once a critical culture density is reached, it is usually necessary to cool the reactor to maintain the optimum temperature for the organisms. Temperature control is easily achieved in most bioreactor with a cooling water or internal cooling coils, as is done in very large industrial bioreactors. A temperature probe (thermocouple) is inserted into the culture vessel such that the temperature in the center of the vessel is measured. The probe is connected to a control box which adjusts a heating (or)

The time taken by the temperature control system to stabilize the temperature (cooling element) until there is no offset from the set-point.

Sampling tube:-

Through this tube, using a syringe of generally 5 mL or 10 mL size, culture of the organism taken for its further analysis w.r.t. its growth stage, product formation (if any) etc.

Agitator (for culture mixing):-

In shake flasks, cultures are mixed through shaking, whereas in a bioreactor cultures are stirred with an impeller. The impeller is mounted on a rotor shaft which is in turn connected to a motor. The agitator is required to achieve a number of mixing objective like bulk fluid and gas phase mixing, air dispersion, oxygen transfer, heat transfer, suspension of solid particles and maintain homogenous environment throughout the vessel contents.

Agitators are classified as disk turbines, vaneless disks, open turbines of variable pitch and marine propellers. The disk turbine consists of a disk disc with series of ~~rectangular~~ rectangular vanes set in a vertical plane around the circumference. The vaneless disc has a series of rectangular vanes.

Sparger (for aeration):-

The oxygen demand for aerobic microbial culture is high. Bioreactor system needs to be supplying enough air or oxygen to maintain DO at desired levels. In this case, fine bubble aerators should be used. Large bubbles will have less surface area than small bubbles which will facilitate oxygen transfers to a great extent. Bubbles of small size with maximum surface area, take more time to reach the surface, increasing the residence time to each bubble in the water, allowing a better oxygen transfer rate. To maximise the dissolved oxygen concentration in the microbial culture air/oxygen are usually introduced to the culture medium through submerged gassing with the help of a sparger.

Three basic types of sparger being used and they are classified as porous sparger, orifice sparger and nozzle sparger. Porous sparger has been primarily used on lab-scale bioreactors where there is no greater mixing is required.

The throughput of air from this sparger is low due to pressure drop across the sparger and there is also problem of fine holes becoming blocked by growth of the microorganisms culture. Orifice sparger is a perforated pipe kept below the impeller in the form of crosses or rings. Nozzle sparger consists of a single opening to provide stream of air bubbles. Ideally, this sparger is placed centrally below the impeller and as far away as possible from it to ensure that the impeller is not flooded by the air stream.

• Condenser:-

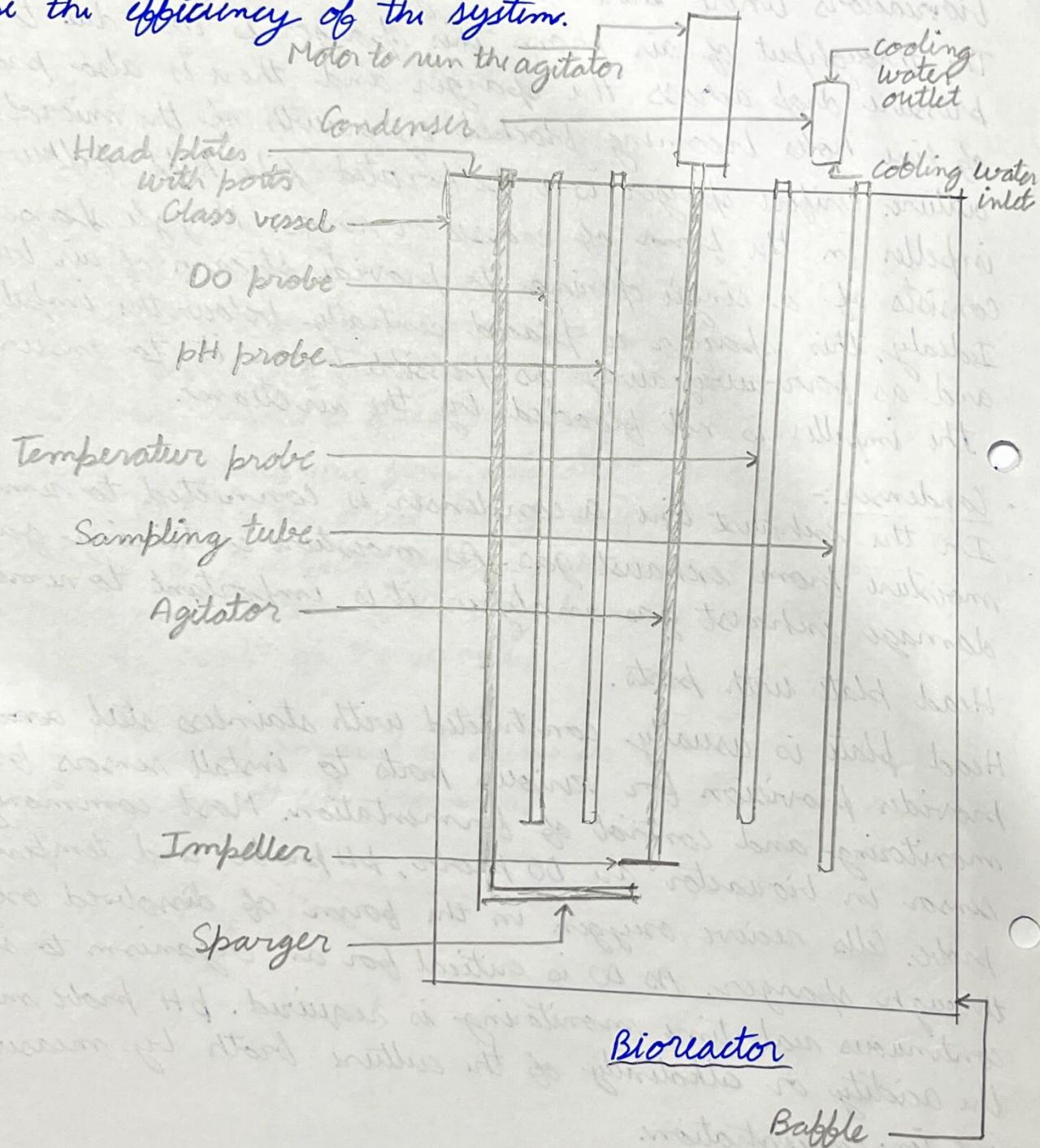
In the exhaust line a condenser is connected to remove the moisture from exhaust gas. As moisture containing gas will damage exhaust gas analyser it is important to remove moisture. Head plate with ports.

Head plate is usually constructed with stainless steel ~~and~~ and provides provision for various ports to install sensors for monitoring and control of fermentation. Most commonly used sensor in bioreactor are DO probe, pH probe and temperature probe. Cells receive oxygen in the form of dissolved oxygen through sparger. As DO is critical for an organism to survive continuous real-time monitoring is required. pH probe measures the acidity or alkalinity of the culture broth by measuring H^+ ions concentration.

• Baffles:-

Baffles are metal strips that prevent vortex formation around the walls of vessel. These metal strips are ~~safely~~ radially attached to the wall of vessel. ~~there~~ Usually 4 baffles are present. There should be enough gap b/w wall & baffle to minimize microbial growth on the baffle and walls of the vessel.

The above mentioned are the major parts of a typical bioreactor. However, depending upon various types of bioreactors, there could be more accessories which will serve to increase the efficiency of the system.



* Equipment 2:- Weighing balance / analytical balance

→ Introduction :-

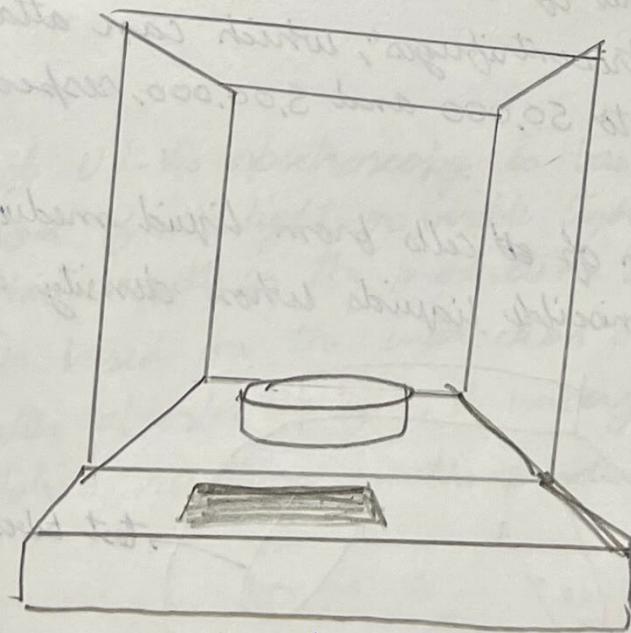
- An analytical balance is a form of a scale that measures mass to a high degree of precision. The readability range of a typical laboratory analytical balance lies b/w 0.1 mg to 220 gm. However, the readability range may vary depending on the instrument model and brand.

→ Principle :-

An analytical balance consists of a measuring pan, which is placed inside a transparent enclosure called the draft sheet, so as to ~~protect~~ prevent the air currents in the room from affecting the balance's operation. Analytical balances work on the principle of "magnetic force restoration"; which means that the force exerted by the object being weighed is lifted by an electromagnet. A detector measures the current required to oppose the downward motion of the weight in the magnetic field, and displays the weight of the sample.

→ Uses :-

1. To weigh small amount of chemicals.
2. To weigh dry & wet biomass.



Analytical balance

* Equipment 3 :- Refrigerated centrifuge

→ Introduction :-

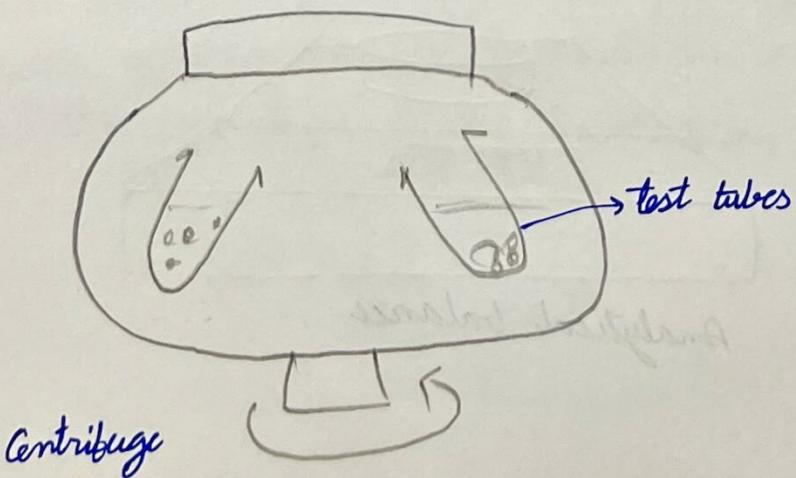
- Centrifuge is used to separate materials of different densities, especially when the difference in their densities is so small that they cannot be separated by gravitational force. In this equipment, temperature can be maintained from -20 to +40°C.

→ Principle :-

- It uses centrifugal force, which causes the denser particle to settle to the bottom, and the low-density particles to rise to the top.
- A centrifuge typically has 3 basic parts : a rotor, a drive shaft and a motor. The rotor holds the motor containing the liquids to be centrifuged. Rotor is mounted on the drive shaft, which connects to the motor. Motor provides power to run the rotor. Rotor comes in different sizes and can be chosen based on the volume of the tubes containing the sample to be centrifuged. The centrifuge allows the user to set the speed, time duration and temperature for centrifugation. The speed of the centrifuge can be set in terms of "revolution per minute (RPM)" or "relative centrifugation force (RCF)" or the g-number. The maximum RPM that can be reached in a typical centrifuge is 15,000 RPM. Depending on the maximum RCF limit of the instrument, there are "high-speed centrifuges" and "ultracentrifuges", which can attain very high RCF values of up to 50,000 and 5,00,000, respectively.

→ Uses :-

1. Used for separation of cells from liquid medium.
2. To separate 2 immiscible liquids whose density difference is small.



* Equipment 4 :- UV-visible spectrophotometer

→ Introduction :-

- UV-Vis spectrophotometer (or spectrophotometer) is a quantitative technique used to measure how much a chemical substance absorbs light. This is done by measuring the intensity of light that passes through a sample with respect to the intensity of light through a reference sample or blank. This technique can be used for multiple sample types including liquids, solids, thin-films and glass. When incident light strikes matter it can either be absorbed, reflected or transmitted. The σ absorbance of ∞ radiation in the UV-Vis range cause atomic excitation which refers to the transition of molecules from a low-energy ground state to an σ excited state.
- UV-Vis spectrophotometers are therefore able to determine the concentration of specific analytes in a microvolume by controlling the analysis wavelengths and the pathlengths. It uses light in the visible and adjacent (near UV and σ near-infrared ~~IR~~ (NIR)) ranges.

UV/Vis spectrophotometer is used in the quantitative determination of concentration of the absorber in the solutions of the transitional metal ions and highly conjugated organic compounds.

→ Principle:-

- The principle of UV-Vis spectroscopy is based on the ~~absorb~~ absorption of UV light or visible light by chemical compounds, which results in the production of distinct spectra. Spectroscopy is based on the interaction b/w light and matter. When the matter absorbs the light, it undergoes excitation and de-excitation, resulting in the production of spectrum.

- When matter absorbs UV radiation, the electrons present in it undergo excitation. This causes them to jump from a ground state (an energy state with a relatively small amount of energy associated with it) to an excited state (an energy state with a relatively large amount of energy associated with it). It is important to note that the difference in the energies of the ground state and the excited state of the electron is always equal to the amount of UV radiation or visible radiation absorbed by it.
- The statement of the Beer-Lambert Law can be written as follows: When a beam of monochromatic light is made incident on a solution that contains a substance that absorbs the monochromatic light, the rate at which the intensity of the beam decreases along the thickness of the solution is directly proportional to the concentration of the absorbing substance in the solution and is directly proportional to the intensity of the incident monochromatic radiation.
- As per the Beer-Lambert Law, the greater the number of absorbing molecules (that have the ability to absorb light of specific wavelength), the greater the extent of absorption of the radiation. The Beer-Lambert law states that:

$$A = \epsilon LC$$

where,

$A \rightarrow$ amount of light absorbed for a particular wavelength by sample

$\epsilon \rightarrow$ molar extinction coefficient

$L \rightarrow$ distance covered by the light through the solution

$C \rightarrow$ concentration of the absorption species

- Following is an equation to solve for molar extinction coefficient:

$$\epsilon = ALC$$

But Beer-Lambert law is a combination of 2 different laws: Beer's law and Lambert's law.

- What is Beer's Law?

Beer's Law was stated by August Beer which states that concentration and absorbance are directly proportional to each other.

- What is Lambert's Law?

Johann Heinrich Lambert stated Lambert law. It states that absorbance and path length are directly proportional.

Beer-Lambert Law Formula

$$I = I_0 e^{-\mu(x)}$$

Where,

$I \rightarrow$ Intensity

$I_0 \rightarrow$ Initial Intensity

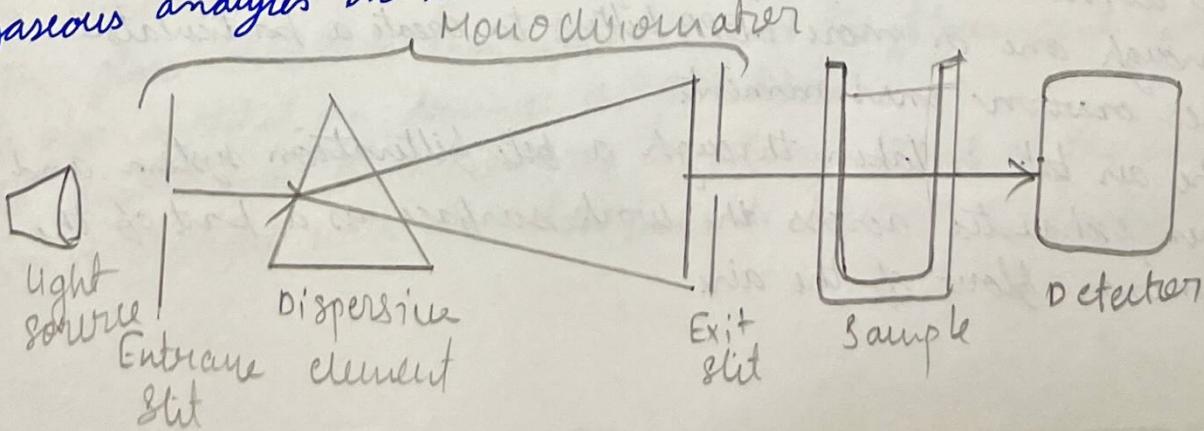
$x \rightarrow$ depth in meters

$\mu \rightarrow$ coefficient of absorption

- There are 4 basic components to a ~~single~~ single beam UV/VIS spectrophotometer: a light source, a ~~monochromator~~ monochromator, a sample and a detector.

→ Uses

UV-Visible spectroscopy is widely used in the field of analytical chemistry, especially during the quantitative analysis of a specific analyte. For example, the quantitative analysis of transition metal ions can be achieved with the help of UV-Visible spectroscopy. Furthermore, the quantitative analysis of conjugated organic compounds can also be done with the help of UV-Visible spectroscopy. It can also be noted that this type of spectroscopy can also be carried out solid and gaseous analytes in some conditions.



*Equipment 5 :- Høgi Horizontal laminar Air blow hood

→ Introduction :-

- A laminar flow cabinet is defined as enclosed workbench which is used to create a contamination free work environment through installed HEPA (High efficiency Particulate Air) filters that captures all the particles entering the cabinet. A laminar hood is used for work with substances which are not hazardous for the personnel health.
- A laminar flow unit has an industry-wide usage and can be applied in quite a lot of industries such as medical, research, pharmacy, educational and also in electronics, optics, micromechanics, plastic industries, etc. as all these need to be carried out in a clean and sterile environment. A laminar hood is used for work with substances which are not hazardous for personal health, as it does not provide personal health protection. The best usage of a clean bench is for working on certain specialized experiments in the lab that require a clean environment to design products that are non-toxic. It creates clean air for an individual environment inside a cabinet. It can also be tailor-made under the specific requirements of the laboratory.

→ Principle :-

- The principle of laminar flow cabinet is based on the laminar flow of air through the cabinet. The device works by the use of inward flow of air through one or more HEPA filters to create a particulate-free environment. The air that is taken through a filter filtration system and then exhausted across the work surface as a part of the laminar flow of the air.

- The air first passes through the filter pad or pre-filter that allows a streamline blow of air into the cabinet.
 - Next, the blower or fan directs the air towards the HEPA filters.
 - The HEPA filters then trap the bacteria, fungi and other particulate materials so that the air moving out of it is particulate-free air.
 - Some of the effluent air then passes through perforation present at the bottom rear end of the cabinet, but most of it passes over the working bench while coming out of the cabinet towards the back of the operator.
 - The laminar flow hood is enclosed on the sides, and constant positive air pressure is maintained to prevent the intrusion of contaminated external air into cabinet.
- The procedure to be followed while operating a laminar flow cabinet is given below:

1. Before running the laminar flow cabinet, the cabinet should be checked to ensure that nothing susceptible to UV rays is present inside the cabinet.
2. The glass shield of the hood is then closed, and the UV light is ~~switched~~ switched on. The UV light should be kept on for about 15-20 minutes to ensure the surface sterilization of the working bench.
3. The UV light is then switched off, and a time period of ~~around~~ around 10 minutes is spared before the airflow is ~~switched~~ switched on.
4. About 5 minutes before the operation begins, the airflow is switched on. ~~during the operation~~
5. The glass shield is then opened, and the fluorescent light is also switched on during the operation.
6. To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% ethanol.

7. Once the work is completed, the airflow and fluorescent lamp both are closed and glass shield is also closed.

→ Precautions :-

While operating the laminar ^{air} blow, the following things should be considered:

1. The laminar blow cabinet should be sterilized with the UV light before and after the operation.
2. The UV light and airflow should not be used at the same time.
3. No operations should be carried out when the UV light is switched on.
4. The operator should be dressed in lab coat and long gloves.
5. The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work with 70% ethanol.

The following are some common uses of a laminar blow cabinet in the laboratory:

1. Laminar blow cabinets are used in laboratories for contamination sensitive processes like plant tissue culture.
2. Other laboratories processes like media plate preparation and culture of microorganism can be performed inside the cabinet.
3. Operation of particle sensitive electronic sensitive devices are performed inside the cabinet.
4. Laminar blow cabinets can be made tailor-made for some specialized works and can also be used for general lab techniques in the microbiological as well as the industrial sectors.
5. In the pharmaceuticals industries, drug preparations techniques are also performed inside the cabinet to ensure a particulate-free environment during the operations.

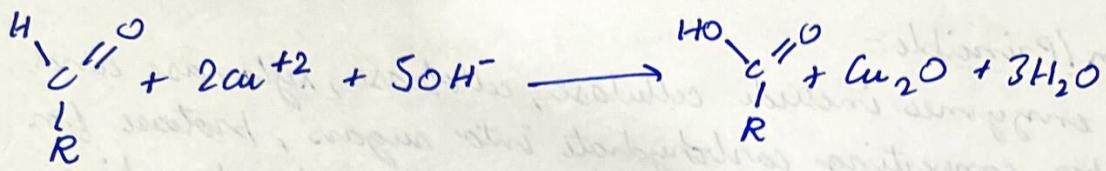
Experiment 2

→ Aim:- To produce hydrolytic enzymes (cellulase) in shake flasks and confirm using Benedict's test.

Introduction / Principle:-

- Hydrolytic enzymes include cellulase, cellobiase, xylanase and amylase for converting carbohydrate into sugars, protease for hydrolysing protein into amino acids and lipase for degrading lipids into glycerol and long chain fatty acids (LCFA). The hydrolytic enzyme, "cellulase", is an enzyme complex composed of endo-1,4- β -d-glucanases or endoglucanases, exo-1,4- β -d-glucanases or cellobiohydrolases and 1,4- β -d-glucosidases, which act on cellulose to produce glucose. Cellulose is one of the most abundant biodegradable materials on Earth which can be produced by many organisms, including bacteria and vascular plants. As a kind of important industrial enzyme, cellulase has been widely used in the feed industry, alcoholic fermentation and other fields. The utilisation of cellulase in animal feed has been reported widely.
- *Bacillus* is a kind of probiotics that can secrete high activity of protease, lipase, amylase and cellulase. *Bacillus amyloliquefaciens* is an important potential probiotic strain that has been found to secrete amylase and ~~has been known~~ to applied to many types of mammalian feed for improving their intestinal microenvironment. This same host organism will be used for harvesting cellulase in this experiment.
- Benedict's Test is used to test for a simple carbohydrate. The Benedict's test identifies reducing sugars, which have free ketone or aldehyde functional groups.
- Some sugars such as glucose are called reducing sugars because they are capable of transferring hydrogens (electrons) to other compounds, a process called reduction. When reducing sugars are mixed with Benedict's reagent and heated, a reduction reaction

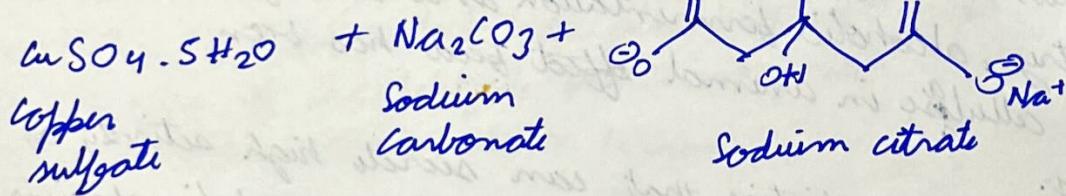
causes the Benedict's reagent to change colour. The colour varies from green to dark red (brick) or rusty-brown, depending on the amount of and type of sugar.



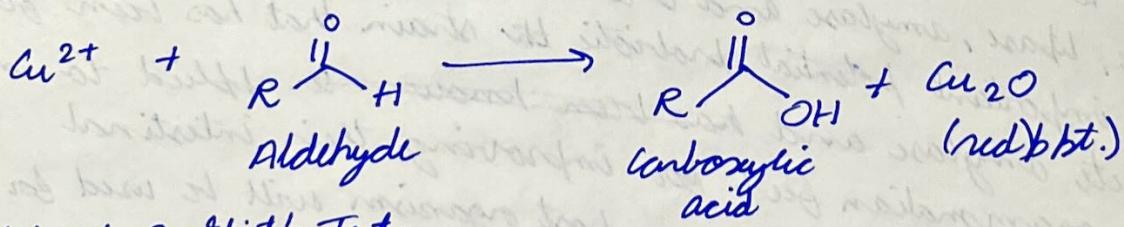
- Benedict's quantitative reagent contains potassium thiocyanate and is used to determine how much reducing sugar is present. This solution forms a copper thiocyanate precipitate which is white and can be used in titration. The titration should be repeated with 1% glucose soln instead of the sample for calibration.

- Benedict's solution (longer shelf life than Fehling's, solution)

i) Preparation of the solution:



ii) Use in oxidation reaction



Principle of Benedict's Test :-

- When Benedict's soln and simple carbohydrate are heated, the soln changes to orange/red/brick red colour. This reaction is caused by the reducing property of simple carbohydrates. The copper (II) ions in the Benedict's solution are reduced to copper (I) ion which causes the colour change.

The red copper(I) oxide formed is insoluble in water & is precipitated out of the soln. This accounts for the ppt formed. As the conc. of reducing sugar increases, the nearer the final

* colour is to brick red. and the greater the ppt. formed.
Sometimes, a brick red solid, copper oxide precipitates out of the solution and collects at the bottom of the test tubes.

Sodium carbonate provides the alkaline conditions which are required for the redox reacⁿ. Sodium citrate complexes with the copper (II) ions so that they do not deteriorate to copper (I) ions during storage.

- Complex carbohydrates such as starches do not react +ve to the Benedict's Test unless they are broken down using heat or digestion. Table sugar is a non-reducing sugar and does not ab react with iodine or with Benedict's reagent. Sugar needs to be decomposed into its components glucose and fructose then the glucose test would be +ve but the starch test would still be negative.

→ Composition & Preparation of Benedict's solution :-

- Benedict's solⁿ is a deep blue alkaline sol^m used to test for the presence of the aldehyde functional group, -CHO.
Anhydrous sodium carbonate → 100gm
Sodium citrate → 173gm
Copper (II) sulfate pentahydrate → 17.3gm
- One litre of Benedict's solution can be prepared from 100gm of anhydrous sodium carbonate, 173gm of sodium citrate and 17.3gm of copper (II) sulfate pentahydrate.

List of reagents and instruments:-

- Organism:-
E. coli - 21 (DE 3) pLys S cells harboring recombinant plasmids of Ba GHS-WT or Ba GHS-UV2.

- Equipment:-
Erlenmeyer flask, shake incubator, centrifuge, microcentrifuge tube, spectrophotometer.

Reagent :-

Benedict's reagent

Media composition :-

The medium composition used for *E. coli* BL-21 (DE3) bLYSS cells production was LB broth. (Luria Bertani Broth)

Procedure :-

1. Medium was prepared according to given composition and autoclaved. After this, the antibiotic kanamycin ($50\text{ }\mu\text{g/mL}$) was added to the medium.
2. A 250 mL Erlenmeyer flask containing 100 mL of media was inoculated with 7% (v/v) culture.
3. The cells were grown at 37°C and 180 rpm up to mid-exponential phase to cell absorbance at 600 nm is $0.6A$.
4. 1 mM final concentration of isopropyl- β -D-thiogalactopyranoside (IPTG) was added, and it was further incubated at 24°C , 180 rpm for 18 hrs for induction of protein expression.
5. The cells were then centrifuged at $8,000g$ at 4°C for 10 min. The cell pellets were resuspended in 4 mL of 20 mM sodium phosphate buffer, pH 7.0.
6. After this, the cell suspension was sonicated on ice for 15 minutes by using 5 sec on/ 5 sec off pulse and 33% amplitude.
7. Now, the sonicated cell suspension was again centrifuged at $13000g$ at 4°C for 50 minutes and the supernatant was collected.
8. Finally, the cell free supernatant containing the enzyme was analyzed for endoglucanase (CMCase) activity using Benedict test.

Note :- In case of mutant culture test tubes are inoculated incubated in dark (use boil).

Experiment. 7

Benedict's test procedure :-

- 3 test tubes were taken marked as Positive control (containing only glucose), Negative control (containing only CMC) and CMC + supernatant (contain enzyme) - 2 mL each.
- The 3 test tubes were incubated for 2 hours at 65°C temperature.
- After incubation, 2 mL of Benedict's reagent was added to each test tube.
- The test tube were kept in boiling bath for 10 minutes and the change in colour/precipitate formation was observed.

Result interpretation of Benedict's Test :-

- If colour upon boiling is changed to green, then there would be 0.1 to 0.5% sugar in soln.
If it changes colour to yellow, then 0.5 - 1% sugar is present.
If it changes to orange, then 1 - 1.5% sugar is present.
If colour changes to red, then 1.5 - 2% sugar is present.
If colour changes to brick red, then more than 2% of sugar is present in the soln.
- Positive Benedict's test:** Formation of a reddish ppt. within 3 minutes.
Reducing sugar present. Ex: Glucose.
- Negative Benedict's test:** No colour change (Remains blue). Reducing sugar absent.

Result observed from experiment

Cellulase enzyme was isolated successfully after 48 hours of culture period.

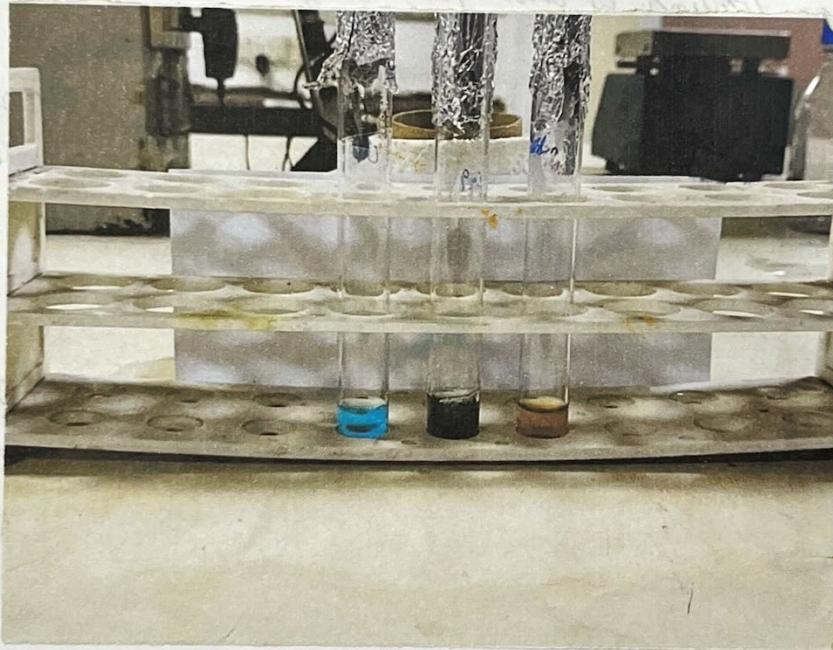
After performing the Benedict's test, the following observation were made:

- The first test tube (negative control) developed red-brick colour ppt. due to the presence of only glucose.
- The second test tube (negative control) developed blue colour due to the absence of only CMC and absence of glucose (reducing sugar).

3. The third test tube, colour slightly changed from blue to red, which confirmed the presence of the cellulase enzyme in the supernatant.

→ Conclusion :-

- The hydrolytic enzyme cellulase was successfully isolated from the culture of *Bacillus amyloliquefaciens* SS35 and confirmed using Benedict's test. The colour change from blue to red through Benedict's test confirmed the presence of cellulase enzyme from the supernatant harvested from the *ex* culture.



Experiment 3

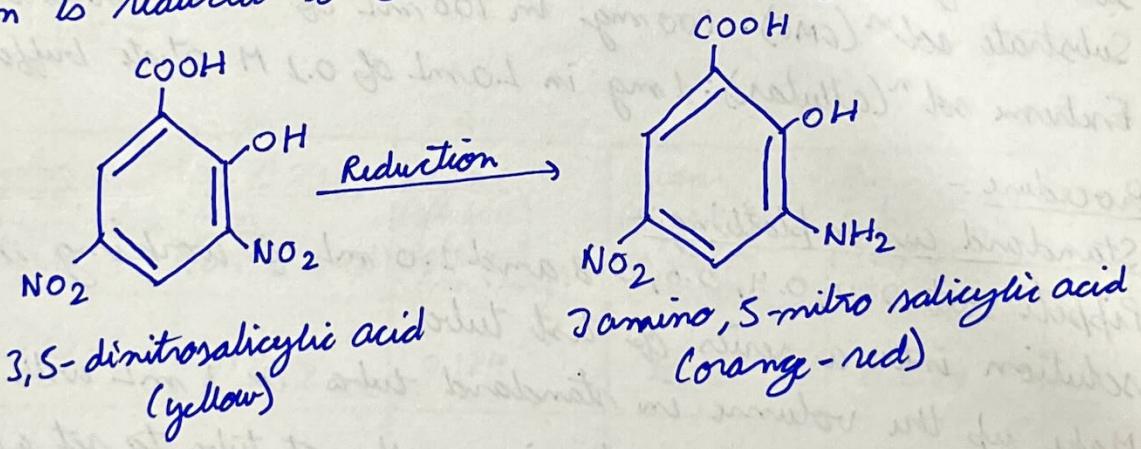
→ Aim:- To determine the activity of hydrolytic enzyme cellulase by kinetic characterization (K_m and V_{max}).

→ Introduction :-

- Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. Some of the reducing sugars are glucose, galactose, lactose and maltose. The DNS method is one of the classical and widely used methods for the quantitative determination of reducing sugar.

→ Principle:-

- Reducing sugars have the property to reduce many of the reagents. One such reagent is 3,5-dinitrosalicylic acid (DNS). 3,5-DNS in alkaline solution is reduced to 3-amino 5-nitro salicylic acid.



→ Reagents and Instruments required:-

* chemicals:-

- Tri-sodium citrate
- Citric acid
- Carboxymethyl cellulose
- D-Glucose
- 3,5-dinitrosalicylic acid
- Sodium potassium tartrate tetrahydrate
- Sodium hydroxide
- Hydrochloric acid

*Reagents:-

- Preparation of DNS reagent: 1gm of DNS is dissolved in 50mL of distilled water. Add 30gm of sodium ~~potta~~ potassium tartrate tetrahydrate. Then add 20mL of 2N NaOH, which turns the solution transparent orange yellow colour. The final volume is made to 100 mL with the distilled water.
- 0.1 M Citrate buffer (pH 4.8)
 - Prepare 80mL of distilled water in a suitable container.
 - Add 1.554 gm of ~~tri~~ sodium citrate to the solution.
 - Add 0.906 gm of citrate citric acid to the solution.
 - Adjust solution to final desired pH using HCl or NaOH.
 - Add distilled water until volume is 100 mL.
- Standard glucose solⁿ: Stock: 100 mg in 100 mL distilled water.
Substrate solⁿ (CMC): 100 mg in 100 mL of 0.1M citrate buffer
Extreme solⁿ (cellulase): 1 mg in 1.0mL of 0.1 M citrate buffer.

→ Procedure:-

*Standard curve plotting:-

- Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 mL of working standard solution into a series of test tubes.
- Make up the volume in standard tube to 1 mL with distilled water.
- Pipette out 1 mL distilled water in a separate tube to set a blank.
- Add 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent to each tube.
- Then place the tubes in a boiling water bath for 5 minutes.
- Then take the tubes out and cool the tubes to room temp.
- Then the absorbance was measured at 540 nm using UV-visible spectrophotometer.
- Plot the standard curve b/w concentration of the standard glucose solution (mg/mL) and optical density at 540 nm.

* Activity of hydrolytic enzyme on different substrate

- Follow steps 1-7 from standard curve plotting with the substrate solution (CMC).
- The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose.
- Enzyme activity (IU/mL) of enzyme is defined as the amount of enzyme required for the formation of 1 μmol of sugar from CMC per minute.
- A graph was drawn by plotting the substrate concentration on X-axis and optical density on Y-axis.
- Draw Lineweaver-Burk plot. From the graph, calculate the values of K_m and V_{MAX} .

→ Observations and Results :-

<u>Substrate concentration</u> (mg/mL)	<u>Enzyme concentration</u> (mg/mL)	<u>OD at 540 nm</u> (DNS method)	<u>Sugar (Glucose) released</u> (mg/mL)	<u>Sugar (Glucose) released</u> (μmol)	<u>Enzyme activity</u> (IU/mL of enzyme)
1. 0.2	1.0	0.562	0.184	1.02	0.034
2. 0.4	1.0	0.596	0.194	1.07	0.035
3. 0.6	1.0	0.642	0.210	1.165	0.038
4. 0.8	1.0	0.733	0.240	1.332	0.044
5. 1.0	1.0	0.724	0.276	1.310	0.043

• Standard plot eqⁿ :-

$$y = 3.062 x$$

$y \Rightarrow$ OD of soln at 540 nm

$x \Rightarrow$ conc. of glucose mg/mL

Eq. Eqⁿ of Lineweaver-Burk plot for enzyme kinetics:-

	V_0	$\frac{1}{V_0}$	[S] (μmol/mL)	$\frac{1}{[S]} \mu$ (mL/μmol)	$\frac{1}{[S]} (\text{mL}/\text{4 mol})$
1	0.034	29.4	200	0.005	1.25
2	0.035	28.57	400	0.0025	0.625
3	0.038	26.31	600	0.0016	0.416
4	0.044	22.72	800	0.0012	0.312
5	0.045	23.25	1000	0.0011	0.25

$$\text{Slope of the graph} \Rightarrow \frac{29.4 - 22.72}{0.005 - 0.0012} = \frac{28.57 - 22.72}{0.625 - 0.312} = \frac{\cancel{6.68}}{\cancel{0.0038}} = \frac{1.879}{0.312} = \frac{5.82}{0.312} = 18.654$$

- $y\text{-intercept} = \frac{1}{V_{MAX}} = 16$

$$\Rightarrow V_{MAX} = \cancel{0.005} 0.0625 \mu\text{mol/min. mL}$$

- $x\text{-intercept} \Rightarrow \frac{1}{K_m} = -0.75$

$$\Rightarrow K_m = 1.33 \mu\text{mol/mL}$$

Results:-

- UV-VIS spectrophotometry can be used to measure enzyme activity indirectly by measuring concentration of glucose released.

- $V_{MAX} = \cancel{0.005} 0.0625 \mu\text{mol/min. mL}$

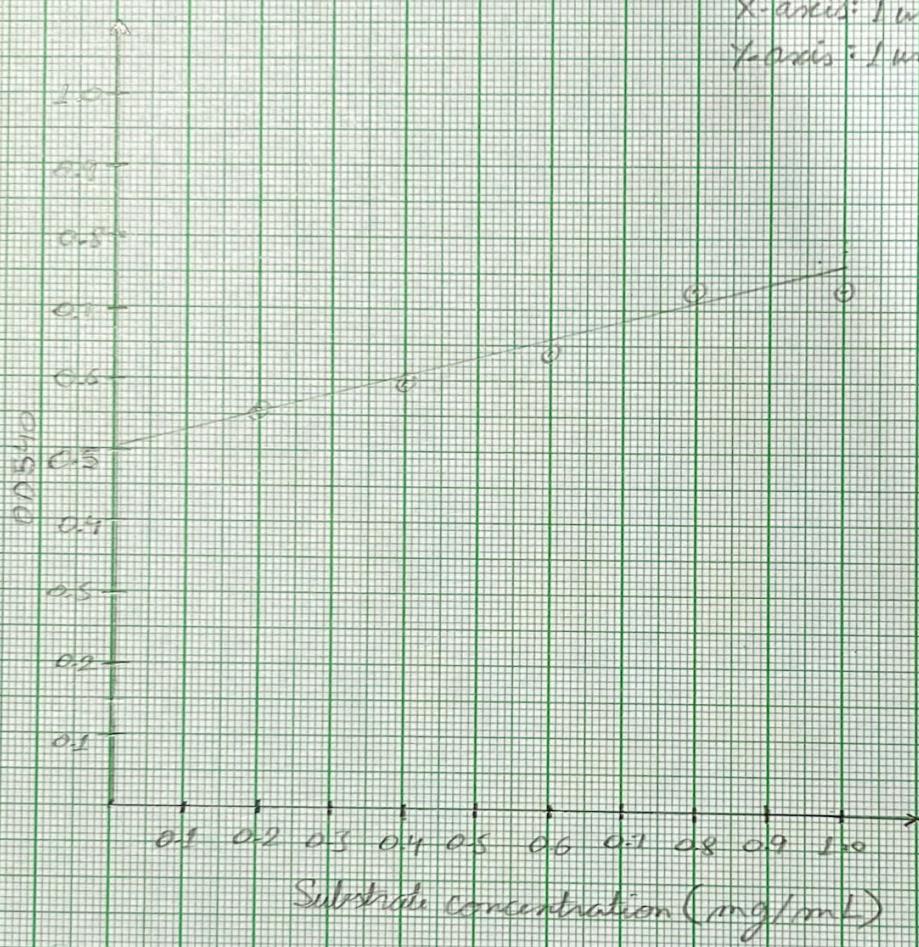
- $K_m = \cancel{0.005} 1.33 \mu\text{mol/mL}$

Standard Curve

Scale

X-axis: 1 unit = 0.1 mg/ml

Y-axis: 1 unit = 0.1



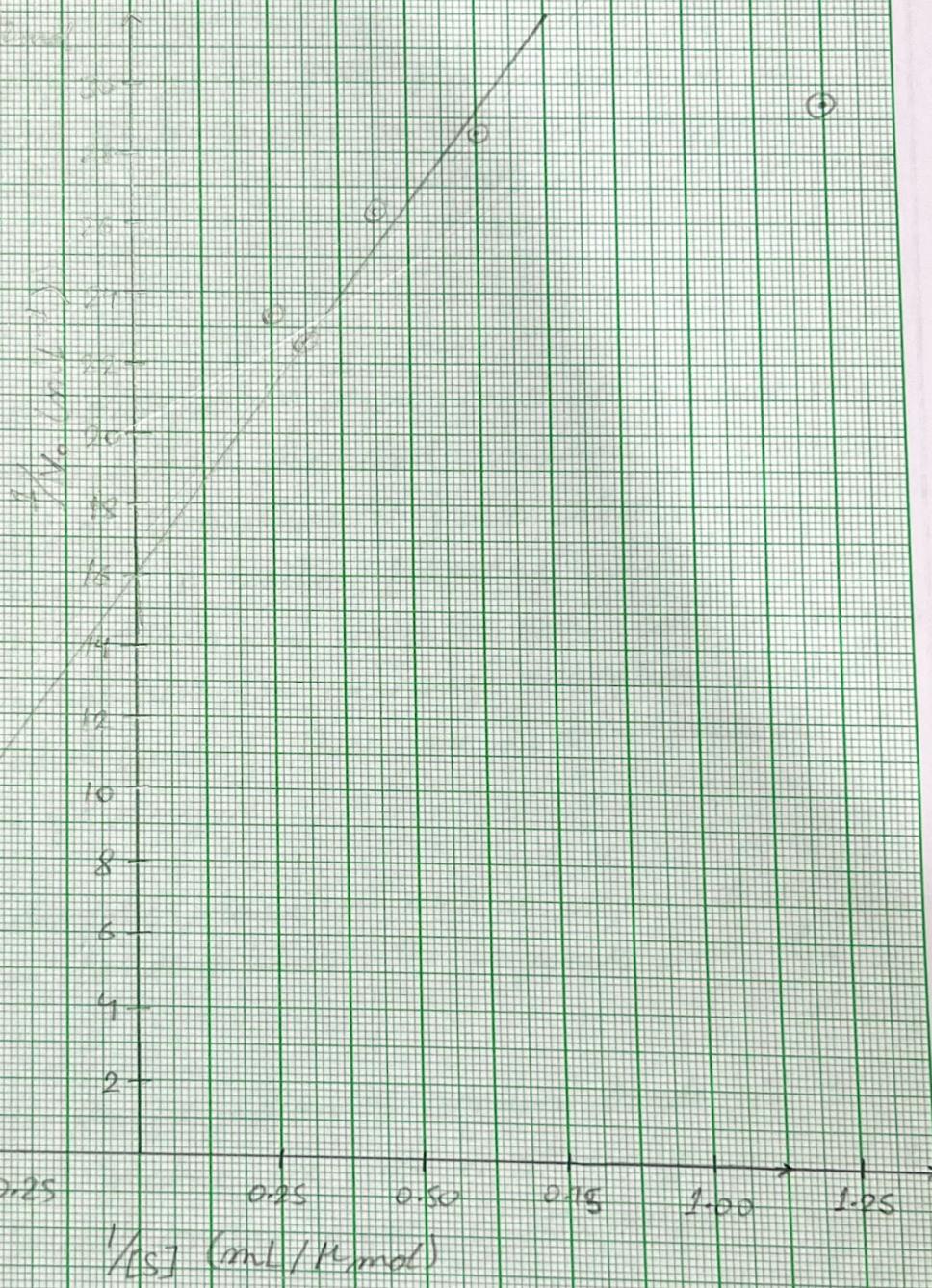
Graph

X-axis: $\text{I} / \text{V}_0 (\mu\text{l}/\mu\text{mol})$ = 0.125 ml / μmol

Y-axis: $\text{I} / \text{V}_0 (\mu\text{l}/\mu\text{mol})$ = 2 mmol

a

a



Experiment 4

Aim: To learn the technique of immobilizing enzyme in alginate beads and determine its enzymatic activity by assay.

Introduction:-

Immobilization is a technique for the combination of enzyme in an insoluble support matrix. The matrix is usually a high molecular weight polymer such as polyacrylamide, starch, cellulose, etc. The advantage of immobilizing enzymes over free enzyme is to increase their stability and efficiency. The immobilized enzymes can also be recovered at the end of the reaction and can be used repeatedly.

Principle:-

- Enzymes are often immobilized on inert, insoluble material like calcium alginate. These beads provide increased resistance to changes in conditions such as pH or temperature. They also allow enzymes to be held in place throughout a reaction, following which they are easily separated from the products and may be used again. The enzyme can be immobilized by entrapment, ion exchange adsorption, porous ceramics and even covalent bonding. There are several methods for the ~~and~~ immobilization of enzyme.
- Absorption: Involves electrostatic interaction such as Van Der Waal's forces, and ionic and hydrogen bonding between the enzymes and the support matrix.
- Covalent Binding: Involves the formation of covalent bonds b/w the enzymes and the support matrix. The bond is normally formed b/w the functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of enzymes.

- Entrapment: involves the mixing of enzyme molecules with a polyionic polymer material and then crosslinked by the polymer with multivalent cations in an ion exchange reaction to form a lattice structure that traps the enzymes.
- Encapsulation: can be achieved by ~~wood~~ enveloping the enzymes within various forms of semi-permeable membranes.
- Crosslinking: involves the joining of enzymes with each other to form large 3-dimensional complex structures and can be achieved by physical or chemical method without any support system.
- * External and internal mass transfer limitation:
Mass transfer significantly impacts the performance of immobilized enzymes, involving external (film) and internal (porous matrix) diffusion. Immobilized enzyme efficiency can be compromised by mass transfer limitations. Enhancing bulk solution velocity reduces external resistance. However, internal resistance influenced by factors like particle size, enzyme load and reactant concentration remains prominent. Effective diffusivity (D_{eff}) differs from bulk diffusivity (D_{ab}) due to pore space and matrix effects, vital for describing kinetics in immobilized enzyme reaction.

- Parameters can assess diffusion's impact on the transformation rate in support particles. Effectiveness factor (n) quantifies diffusional limitations by comparing actual reaction rate to one uniform substrate exposure. It gauges how diffusion affects the process rate.

$n = \frac{\text{reaction rate with intraparticle diffusion limitations}}{\text{reaction rate without diffusion limitations}}$

$= \frac{\text{observed reaction rate with immobilized enzyme}}{\text{reaction rate with free enzyme}}$

- The effectiveness factor is a dimensionless parameter that measures how effectively the catalyst is being used. For near unity, the entire volume of the particle is reacting with at the same high rate because the reactant is able to diffuse quickly through the support material. For near zero, the reaction is conducted at the lowest rate. The reactant is unable to penetrate significantly and the reaction rate is limited to a small portion of the particle volume. The diffusional resistance is predominant which lowers the overall reaction rate.

→ Reagent and instruments required:-

* Equipment :- Flasks, spectrophotometer, sample tube, micropipette

* Chemicals :-

- Tri-sodium citrate
- citric acid
- Carboxymethyl cellulose
- D-glucose
- 3,5-Dinitrosalicylic acid (DNS)
- Sodium potassium tartrate tetrahydrate
- Sodium hydroxide
- Hydrochloric acid

* Reagents :-

- Preparation of DNS reagent
- 0.1 M citrate buffer (pH 4.8)

- Prepare CaCl_2 solution (3% or 0.2 M)
- Substrate solⁿ (Tetra carboxy methyl cellulase)
- Enzyme solⁿ (Cellulase)

→ Procedure:-

* Day 1:-

• Enzyme immobilization :-

1. In a 2 mL micro-centrifuge tube take 500 μL of the enzyme (cellulase) and to it add 500 μL of the 5% sodium alginate solⁿ. Put the cap tightly & then mix. properly.
2. Take 100 mL of calcium chloride (0.2M) solution in a conical flask (150 mL size). Crush the ice and keep the flask on ice.
3. Take 200 μL from the alginate and enzyme mix in a microsyringe, and add drop-wise to the calcium chloride solⁿ. While adding make sure that the flask is swirled gently (keep on a magnetic stirrer).
4. Leave the immobilized enzyme beads to harden in the calcium chloride solⁿ overnight at 4°C for hardening.
5. The alginate will be ionically cross-linked by the calcium ions.

* Day 2:-

• Detection of Enzymatic Activity of immobilized enzymes by cellulase assay.

1. Test tubes and microcentrifuge tubes were marked T1, T2, T3, T4 & T5. Samples were prepared with the following compositions

S.No.	<u>Sample</u>	<u>CMC</u>	<u>dH₂O</u>	<u>Enzyme</u>	<u>Beads</u>
1	T0(Blank)	0.25 mL	0.75 mL	—	—
2	T1	0.25 mL	0.74 mL	10 μL	—
3	T2	0.25 mL	0.725 mL	25 μL	—
4	T3	0.25 mL	0.70 mL	50 μL	—
5	T4	0.25 mL	0.675 mL	75 μL	—
6	T5	0.25 mL	0.75 mL	—	10

2. These microcentrifuge tubes were incubated at 50°C for 30 min.
After incubation, TS tube was spun at 8000 rpm for 2 minutes.

• DNS Assay:-

T0(Blank)	— 1 mL of reaction mix	Add 1 mL of DNS to each tube
T1	— 1 mL of reaction mix	
T2	— 1 mL of reaction mix	
T3	— 1 mL of reaction mix	
T4	— 1 mL of reaction mix	
TS	— 1 mL of reaction mix	

Keb in boiling bath for 5 minutes



Then cool it down for 5-10 min, and
add 0.35 mL of Rochelle salt



cool it down



check OD at 540 nm

→ Observations:-

S.No.	Stock conc. of enzyme	Sample Vol. of No. enzym	Working conc. of enzyme (mg/mL)	Vol. of enzyme (mL)	OD ₅₄₀ 00540
1.	1 mg/mL	T0(Blank)	—	—	0.0055
2.	1 mg/mL	T1	0.01	10 mL	0.075
3.	1 mg/mL	T2	0.025	25 mL	0.0347
4.	1 mg/mL	T3	0.05	50 mL	0.0493
5.	1 mg/mL	T4	0.075	75 mL	0.1264
6.	1 mg/mL	TS	—	—	0.0480

Calculations and Results :-

$$x = \frac{y}{7.062}; \quad y = OD, \quad x = \text{glucose conc.}$$

$$T_1: x = 0.00179 \text{ mg/mL} = 0.01 \mu\text{mol/mol}$$

$$T_2: x = 0.01133 \text{ mg/mL} = 0.063 \mu\text{mol/mol}$$

$$T_3: x = 0.0161 \text{ mg/mL} = 0.090 \mu\text{mol/mol}$$

$$T_4: x = 0.0412 \text{ mg/mL} = 0.228 \mu\text{mol/mol}$$

$$T_5: x = 0.0143 \text{ mg/mL} = 0.080 \mu\text{mol/mol}$$

Sample	Enzyme Activity ($\mu\text{mol/min. mL}$)
T ₁	0.01
T ₂	0.063
T ₃	0.080
T ₄	0.228
T ₅	0.080

$$\text{Efficiency of immobilized enzyme} = \frac{\text{Activity of free enzyme} \times 100}{\text{Activity of immobilized enzyme}}$$

$$= \frac{\text{Activity of immobilized enzyme} \times 100}{\text{activity of free enzyme AVG}}$$

$$= \frac{0.08}{0.0978} \times 100$$

$$= 82\%$$

Calculation??

00540 →

0.02 0.04 0.06 0.08

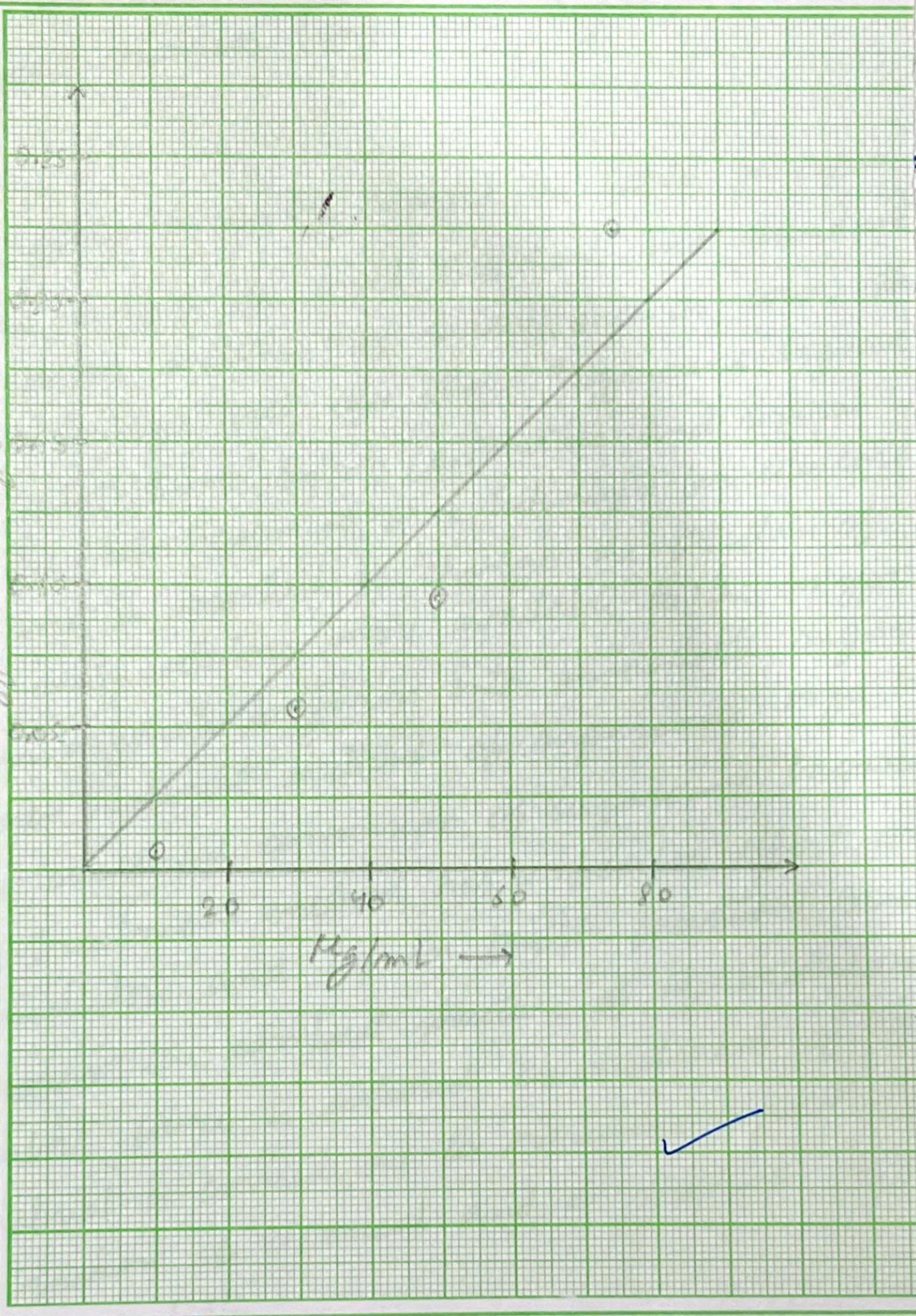
conc. of ammonia (Mg/mL) →

✓

Graph showing Density of blood (ml/ml) vs. time

Axis

Neelgagan



Experiment 5

Aim: - To perform the pre-treatment and enzymatic hydrolysis of the lignocellulosic biomass (sugarcane bagasse)

Introduction:

Biomass resources are readily accessible around the world as residual wastes and agricultural biomass. The most important and abundant renewable biomass resources include crop residue, such as corn starch, wheat straw, sugarcane bagasse and rice straw. Sugarcane bagasse is composed of cellulose, hemicellulose, lignin, extractives and several inorganic materials. Pre-treatment is a technique for cellulose conversion processes and is essential to change the structure of cellulosic biomass to make cellulose more available to the enzymes that converts the carbohydrates polymers into fermentable sugar. Different types of pre-treatment methods of sugarcane bagasse are necessary for successful conversion of sugarcane bagasse to ethanol. Each pre-treatment method has a specific effect on the cellulose, hemicellulose and lignin fraction. During the pre-treatment process the compact structure of lignocellulosic is disrupted and cellulose fibre is exposed. Pre-treatment of the lignocellulosic material is carried out to overcome recalcitrance through the combination of chemical and structural changes to the lignin and carbohydrate.

Enzymatic hydrolysis is the process in which cellulases are added to hydrolyse pre-treated lignocellulosic biomass into fermentable sugars. The process involved several key steps (1) transfer of enzymes from the bulk aqueous phase to the surface of the cellulose (2) Adsorption of the enzymes and formation of enzyme-substrate complexes, (3) hydrolysis of the cellulose, (4) transfer of the hydrolysis products from the surface of the cellulose particles to the bulk aqueous phase and (5) hydrolysis of cellobiose and cellulose to glucose in aqueous phase. The overall rate of the process

is influenced by the structural features of lignocellulosic biomass and the composition and source of cellulases. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because it is usually conducted at mild temp. conditions and does not cause corrosion problems. Both bacteria and fungi can produce cellulases for hydrolysis of lignocellulosic materials.

→ Principle:

Alkali pre-treatment is a widely studied chemical pre-treatment method which is based on the solubilization of lignin in the alkali solⁿ. The various alkaline reagents used commonly for alkali pre-treatment are the hydroxides of sodium, potassium, calcium and ammonium. Among these sodium hydroxide was found to be most effective. A saponification reaction takes place throughout the alkali pre-treatment process which causes cleavage of the intermolecular ester linkages b/w hemicellulose and lignin. This results in solubilisation of lignin and hemicellulose fragments in the alkali solⁿ and bring the cellulose in the interaction of enzymes. Also, alkali pre-treatment changes the lignocellulosic structure via cellulose swelling that leads to reduction in crystallinity and degree of polymerization thereby increasing internal surface area. In addition, removal of acetyl groups and uronic acid substitutions in hemicelluloses during alkali pre-treatment also increases the accessibility of the carbohydrates to enzymatic hydrolysis.

Procedure:-

1. Pre-treatment of lignocellulosic biomass (Sugarcane bagasse):-
 1. Take 100 mL of 1% (w/v) NaOH in a conical flask.
 2. Then add 10 g of sugarcane bagasse to the soln.
 3. Autoclave the flask for 30 min.
 4. After autoclaving filter the soln using filter paper/muslin cloth.
 5. Separate the solid biomass after filtration.
 6. Wash the solid biomass with water in order to remove unreacted NaOH.
 7. Dry the biomass for overnight at 60°C.

2. Enzymatic hydrolysis:-

1. Add 2 gram of untreated sugarcane bagasse (NSB) and 20 mL of citrate buffer to the conical flask named NSB+ Buffer and it serves as blank.
2. Add 2 gram of untreated sugarcane bagasse (NSB) and add 20mL of enzyme soln (1.5 mg/mL) to the conical flask named NSB+ Buffer. Enzyme.
3. Add 2g of pre-treated sugarcane bagasse (PSB) and 20mL of a citrate buffer to the conical flask named PSB+ buffer.
4. Add 1 g of pre-treated sugarcane bagasse (PSB) and add 20mL of enzyme soln to the conical flask named PPSB+ enzyme.
5. Inoculate conical flasks in shaking water bath at 55°C for 24 hours at 120 rpm.
6. Boil the above conical flasks at 100°C for 5 minutes for enzyme inactivation.
7. After this, transfer the liquid content of the conical flasks to the microcentrifuge tubes.
8. Now centrifuge these microcentrifuge tubes for 5 minutes at 10000 rpm. Collect the supernatant for enzyme assay.

9. Perform DNS assay for estimation of the amount of reducing sugar content and calculate the yield.

*DNS Assay:-

1. Take 0.1 mL of collected supernatant add 0.9 mL of distilled water for all the samples.
2. Add 1 mL of DNS reagent to each tube.
3. Keep it for 15 minutes.
4. Cool it down and check OD at 540 nm.

Observation and Results:-

S.No.	Amount of pre-treated biomass(g)	Enzyme conc. (mg/mL)	OD at 540 nm (DNS method)	Sugar released (glucose) (mg/mL)	Sugar released (glucose) (μmol)	Enzyme activity (IU/mL of enzyme)
2.	0	1.5	0.4966	0.162	18	0.0125
3.	2	0	-0.3658	0	0	NA
4.	2	1.5	0.98583	0.3129	74.76 1.778 139.08	0.024 0.096

↳ 4 times diluted

The yield of reducing sugar obtained for alkali pre-treatment is $\frac{0.00162}{0.00162} \times 100 = 100\%$ g/g of sugarcane bagasse and enzymatic pre-treatment is $\frac{0.01257}{0.003129} \times 100 = 400\%$ g/g of sugarcane bagasse.

Calculation?

Experiment No. 6

→ Objective: To study growth kinetics (specific growth rate, specific substrate uptake rate and specific product formation rate) of a bacterial culture in a bioreactor operated under batch mode.

→ Introduction/Principle:-

A bioreactor provides better control in terms of temperature, pH, antibiotic addition, DO etc. in successful fermentation processes. When microbial cells are inoculated into a fresh culture medium under batch condition and their increase in concentration is monitored, several distinct phases of growth can be observed. There is an initial lag phase, which is variable duration. This is then followed by the exponential growth phase, where cell numbers increases exponentially. This is also referred to as the logarithmic phase, the name arising from the common method of plotting the logarithm of cell number x against time. Following this is a short phase of declining growth, and then the stationary phase. Here the cell numbers are constant. Finally, the cell numbers decline during the death phase due to substrate limitation. During the growth the biomass growth rate (dX/dt) in batch reactor can be written as:

$$\boxed{\frac{dX}{dt} = \mu X}$$

$$\boxed{\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1}}$$

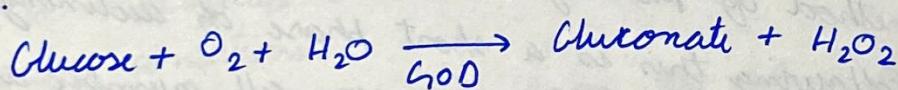
where 't' is time, 'X' is biomass concentration μ is the specific biomass growth rate.

During the exponential phase in batch, we can write:

$$\boxed{\frac{dS}{dt} = k S}$$

where 'S' is the substrate concentration at time 't' and 'k' is the specific substrate utilization rate constant. ^{Experiment}
 Rate of substrate utilization will be estimated by analyzing the glucose concentration through G.O.D (Glucose oxidase)-P.O.D (Peroxidase) method.

- Glucose oxidase enzyme (G.O.D) oxidizes the specific substrate beta-D-glucose to gluconic acid, and hydrogen peroxide is liberated. Peroxidase enzyme acts on hydrogen peroxide to liberate nascent oxygen (O_2). Nascent oxygen then couples with 4-aminoantipyrine and phenol to form red quinoneimine dye. The intensity of colour is directly proportional to conc. of glucose in the sample. The intensity of colour measured colorimetrically and compared with that of standard treated similarly.



Specific rate of product formation is defined as the amount of product formed per unit biomass per unit time, and is described by eqn:

$$q_{vp} = 1/X \times [dP/dt]$$

In this experiment, the product, fusion protein, will be estimated by SDS-PAGE.

- Procedure:-
1. The fermenter vessel is added with 1.5 L of the growth medium and autoclaved.
 2. The DO probe is calibrated according to the manufacturer's instruction.
 3. The temperature of the fermenter is set to 30°C.
 4. Under aseptic conditions, the fermenter is inoculated with 2-5% v/v of seed culture obtained earlier under shake flask conditions.
 5. The fermenter controller is set to proper settings of pH(5.8), agitator speed (400 rpm) and aeration (14pm).
 6. Samples are taken at regular interval of time for the measurement of biomass, glucose and fusion protein conc.

Day 1:-

1. Bioreactor is inoculated with 4-5% of seed bacterial culture under aseptic conditions.
2. 2 mL of sample is withdrawn from the bioreactor at following time intervals - 0 hr, 2 hr, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 12 hrs and 24 hrs.
3. After collecting each sample culture optical density (OD) has to be measured using spectrophotometer at 600 nm wavelength to get biomass density (X) using previously developed X vs OD relation.
4. The samples are centrifuged and the supernatant is stored for glucose (substrate) and fusion protein (product) estimation.
5. GOD-POD method is used to determine specific substrate utilization rate of the microorganism and strand is estimated using HPLC.

Day 2:-

1. Glucose concentration is measured by GOD-POD reagent.
2. Fusion protein concentration is determined by SDS-PAGE.

At first, 1 mL sample is divided into 2 equal volumes of 500 μL in 2 microcentrifuge tubes (Do it for all the 7 or 8 samples you have). One set is used for glucose estimation and the remaining one for product formation rate.

→ Procedure for dry cell weight estimation:

Dilute the over-night culture of *Pseudomonas fluorescens* in 50 mL flask as per the dilution chart provided



Take O.D. of different dilution at 600nm



Take the weight of dry and labelled microcentrifuge tubes up to 4 decimal points (As many no. as the dilutions) - @



Spin down the cell mass (in dilution tubes) at 10,000 rpm for 10 minutes



Suspend the cell pellets in 1 mL of milliQ water and transfer to labeled microcentrifuge tube



Spin down the cell mass at 10,000 rpm for 10 min



Discard the supernatant and incubate the microcentrifuge tube in an oven (80°C) for overnight drying



Next day, weigh the microcentrifuge tubes
(up to 4 decimal points) - ①



Take the difference ② and ① to get the cell
biomass dry weight

By plotting a calibration curve b/w cell biomass dry weight
and optical density and estimating the slope from the
calibration curve to convert OD data into ~~dry~~ dry cell
weight data using correlation provided in the eqn.

→ Calculations:-

Biomass OD at 600nm = 0.4 biomass dry cell weight (gm/L)

Procedure for AOD-POD method

(i) Reagents:-

1. Glucose reagent (L) = 150 mL

2. Glucose standard (S) = 5 mL (100 mg/dL)

(ii) Procedure:-

All samples are diluted 10 times with dH₂O.

1. 10 μL of diluted sample is pipetted out and mixed with

1000 μL of glucose.

2. Temp = 37°C for 15 min.

3. OD at 546 nm.

Addition sequence

	<u>B(mL)</u>	<u>S(mL)</u>	<u>T(mL)</u>
i. Glucose reagent	1.0	1.0	1.0
ii. distilled H ₂ O	0.01	-	-
iii. Glucose standard(S)	-	0.01	-
iv. Sample	-	-	0.01

→ Calculations :-

$$\text{Total glucose (gm/L)} = \frac{\text{Abs of test sample}}{\text{Abs of standard}} \times 100$$

Abs of glucose standard = 0.1598

(i) Chemicals/Buffers/Reagents

1. Stock acrylamide solution

2. Buffers

• Separating gel buffer

• Stacking gel buffer

3. 10% w/v ammonium persulfate

4. 10% w/v sodium dodecyl sulfate (SDS)

5. N,N,N',N'- tetramethylethylenediamine (TEMED)

6. Sample buffer → 0.6 tris-HCl, pH 6.6 → 5mL

• 10% SDS → 0.5gm

• Sucrose → 5gm

• β-mercaptoethanol → 0.25mL

• Bromophenol blue (0.5%) → 5.0mL

Make up to 50 mL with distilled water

7. Electrophoretic buffer: Tris (12 gm), glycine (57.6 gm), and SDS (2.0gm)

• Make up to 2L with water.

8. Protein stain → 0.1% Coomassie brilliant blue R250 in 50% methanol

9. Destaining solution: 10% methanol, 7% glacial acetic acid

10. Protein sample

11. Standard protein molecular weight markers.

Method

1. Clean internal surfaces of gel plates with spirits, dry and then join gel plates together, clamp it in vertical position.
2. Erlenmeyer flask or disposable plastic tube, prepare the separating gel by mixing the following:

	<u>for 15% gels</u>	<u>for 10% gels</u>
(i) 1.87S tris-HCl, pH 8.8	8 mL	8 mL
(ii) Water	11.4 mL	18.1 mL
(iii) Stock acrylamide	20 mL	13.3 mL
(iv) 10% SDS	0.4 mL	0.4 mL
(v) Ammonium persulfate	0.2 mL	0.2 mL

3. Degas this solution under vacuum for about 30 sec.
4. Add 14 mL TEMED and gently swirl the flask to insure even mixing.
5. While ~~reagent~~ separating gel is setting, prepare the 4% stacking gel solution. Mix in (100 mL ~~sterile~~ erlenmeyer flask) Degas this solution (vacuum for 30 sec)
6. When the separating gel has set, pour off the overlaying water.
7. Add 14 mL of TEMED to stacking gel.

Preparation of samples and running the gel:-

8. About 10 mL protein, 5 mL buffer, mixed (5 min, 95-100°C) to denature protein (kept sample in ice).
9. After polymerization is complete, remove the teflon comb. Rinse out any unpolymerized acrylamide solution from the well using electrophoretic buffer, and ~~assemble~~ assemble the cassette in tank. Add tris-glycine in buffer to top and bottom reservoirs.

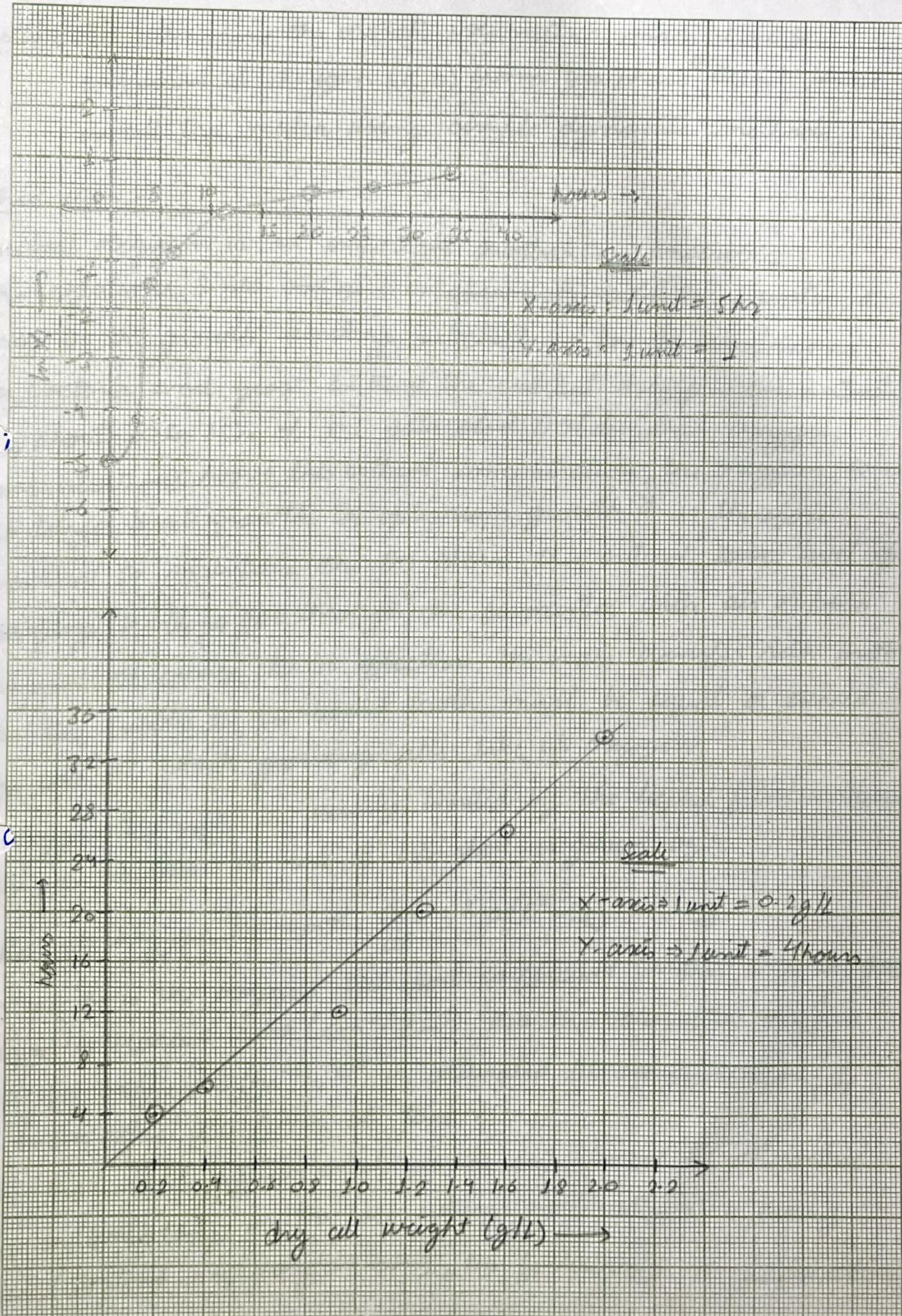
11. Load up to 5-10 ~~μl~~ μl of each sample in a predetermined into the wells.
12. Connect electrophoresis apparatus to the power (50mA current, 200V voltage) (2.5-3.0 hrs) for large format gels. ($16 \mu\text{m} \times 16 \mu\text{m}$).
13. Dismantle gel apparatus, by open the gel plates; Remove the gel; discard the staining gel and place the staining gel in stain solution (staining 2 hrs at room temperature).
14. After destaining, store gels in H_2O containing 20% glycerol.
15. The gel can now be used for immunoblotting to determine the protein sample.

t	$X(\text{g/L})$	$S(\text{g/L})$	$P(\text{g/L})$	Time interval	$\ln \frac{X_i}{X_{i-1}}$	μ	q_S	q_P
t_0 (6th hour)	x_0	s_0	p_0	-	-	-	$\left(\frac{q_S}{S_X} \right)$ time	$\left(\frac{q_P}{P_X} \right)$ time
t_1 (2nd)	x_1	s_1	p_1	t_1 to t .	$\ln(x_1/x_0)$	$\frac{\ln(x_1/x_0)}{t_1 - t_0}$	$\left(\frac{S_0 - S_1}{X_1 - X_0} \right)$ $\frac{S_0 - S_1}{X_1 - X_0}$	$\left(\frac{P_1 - P_0}{X_1 - X_0} \right)$ $\frac{P_1 - P_0}{X_1 - X_0}$
0	0.003	14.73	0	0	-	-	-	-
2	0.016	14.58	0	2	1.674	0.837	6.893	0
4	0.191	11.3	0	4	4.154	1.028	4.792	0
6	0.379	10.21	0	6	4.839	0.806	2.484	0
12	0.954	7.14	0.037	12	5.762	0.480	1.385	0.007
20	1.276	4.32	0.094	20	6.053	0.303	1.351	0.012
26	1.642	2.59	0.162	26	6.305	0.243	1.175	0.016
34	1.944	0.97	0.181	34	6.474	0.190	1.095	0.014

→ Results :-

q_S decreases with time and q_P increases with time, i.e., the substrate concn decrease as the biomass increases & the product concn increases. While observing the growth kinetics of pseudomonas bacterial culture.

Experiment 7



Experiment 7

Objective: To determine the overall volumetric mass transfer coefficient of oxygen (K_{La}) from gas phase to liquid phase in cell free media using simple dynamic method.

→ Introduction:-

Dissolved oxygen is an important substrate in anaerobic fermentations. Since oxygen is sparingly soluble in water, it may be the growth-limiting substrate in the fermentations.

For bacteria and yeast cultures, the critical oxygen concentration is about 10 to 15% of the saturated DO (dissolved oxygen concentration). Above this critical concentration, the oxygen conc. no longer limits growth. For optimum growth it is therefore important to maintain the DO above this critical level of sparging (bubbling gas through) the fermentor with air or pure oxygen. Of course, to be effective, the mass transfer rate from the gas bubbles to the liquid broth must be equal or exceed the rate at which growing cells take up oxygen.

Oxygen transfer is usually limited by the liquid film surrounding the gas bubbles. The rate of transport is given by:

$$\text{Rate of oxygen transport}, \frac{dc}{dt} = K_{La}(C^* - C_t) \quad \dots (1)$$

where K_L is the oxygen transport coefficient (cm/hr), a is the gas-liquid interfacial area (cm^2/cm^3), K_{La} the volumetric oxygen transfer coefficient (hr^{-1}), C^* is saturated DO concentration, C_t is the actual DO conc. at time t .

In the model system used for the experiment to determine K_{La} in water, i.e., in the case where no reaction is taking place (without microorganism), the following simplification are valid:

- Oxygen uptake rate ($OUR = q_{O_2} X$) = 0 because there is no O_2 sink in the system.
- Only data for oxygen transfer rate [$OTR' = k_{La}(C^* - C_L)$] will be considered in the following data:

$$\frac{dC_L}{dt} = OTR - OUR = k_{La}(C^* - C_L) - q_{O_2} X \\ = k_{La}(C^* - C_L)$$

Where q_{O_2} is the specific oxygen uptake rate, X is the biomass concentration, C^* is the saturation dissolved oxygen concentration and C_L is the dissolved oxygen concentration at any time t .

Solving the above differential equation with an initial concentration C_{L0} (at t_0) results equation:

$$\ln(C^* - C_{L0}) / (C^* - C_L) = k_{La}(t - t_0)$$

The concentration C_{L0} can be achieved by flushing nitrogen in the system. At time t_0 the degassing with nitrogen is stopped and from time t_0 there is a constant aeration. To determine the k_{La} value by the dynamic method, short bursts of measurements with an electrode are necessary so that measurement time with the electrode has no influence on the value of k_{La} .

We will perform the "gassing-in" method to dissolve O_2 . The fermentor should be filled with required amount of water/media. The dissolved oxygen would be first removed by sparging with nitrogen, and the air will be purged. The rate of gas transfer to the liquid is determined by using a DO probe that is mounted in the fermentor. This probe is connected to a DO meter, which in turn connected to a data acquisition (DAQ) system on the PC. Copy the collected data and export it to MS excel sheet to do the further analysis.

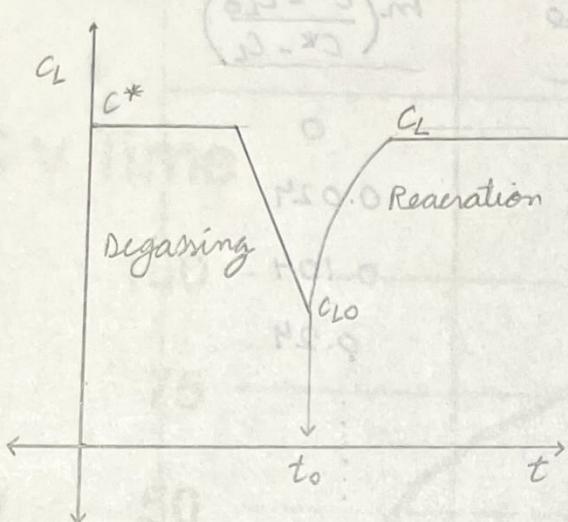


Fig ⇒ Schematic representation of Dynamic method

Here C^* = saturation DO,

C_{L0} = DO at time t_0 ,

C_L = steady state DO at time t .

→ Calculation :-

In MS excel sheet, prepare Time (sec) and C(DO) table.

Plot C vs Time plot. And identify C^* .

To the existing table add new columns:- $\left(\frac{C^* - C_{L0}}{C^* - C_L}\right)$ and $\ln\left(\frac{C^* - C_{L0}}{C^* - C_L}\right)$.

Plot $\ln\left(\frac{C^* - C_{L0}}{C^* - C_L}\right)$ vs time plot. Use the data only from the linear region of the first plot (C vs Time) to create this plot. Do linear regression to fit the data to a straight line ($y = mx$). Calculate k_{La} from this graph.

We will do the same experiment with different rate of stirring. For each case you have to calculate the k_{La} , as we have mentioned above.

Prepare the table Stirring speed vs k_{La} .

Plot k_{La} vs stirring speed plot to show the effect of agitation on oxygen transfer. Usually, there exist a linear relation b/w these two. If possible, fit your data to a straight line.

<u>Time (sec)</u>	<u>$C(00)$</u>	<u>$\frac{C^* - C_{L0}}{C^* - C_L}$</u>	<u>$m \left(\frac{C^* - C_{L0}}{C^* - C_L} \right)$</u>
5	0.8	1	0
10	3.2	1.025	0.024
15	10.5	1.10	0.104
20	22	1.27	0.24
⋮	⋮	⋮	⋮
145	98.8	491	6.19
150	98.9	982	6.88

$$RPM = 800$$

$$C^* = 98.8$$

$$C_{L0} = 0.8$$

$$\ln \left[\frac{C^* - C_{L0}}{C^* - C_L} \right] = k_{La}(t - t_0)$$

$$K_{La} = \frac{0.024}{5} = 0.0048 s^{-1}$$

From linear regression, we get the slope of $0.0489 s^{-1}$

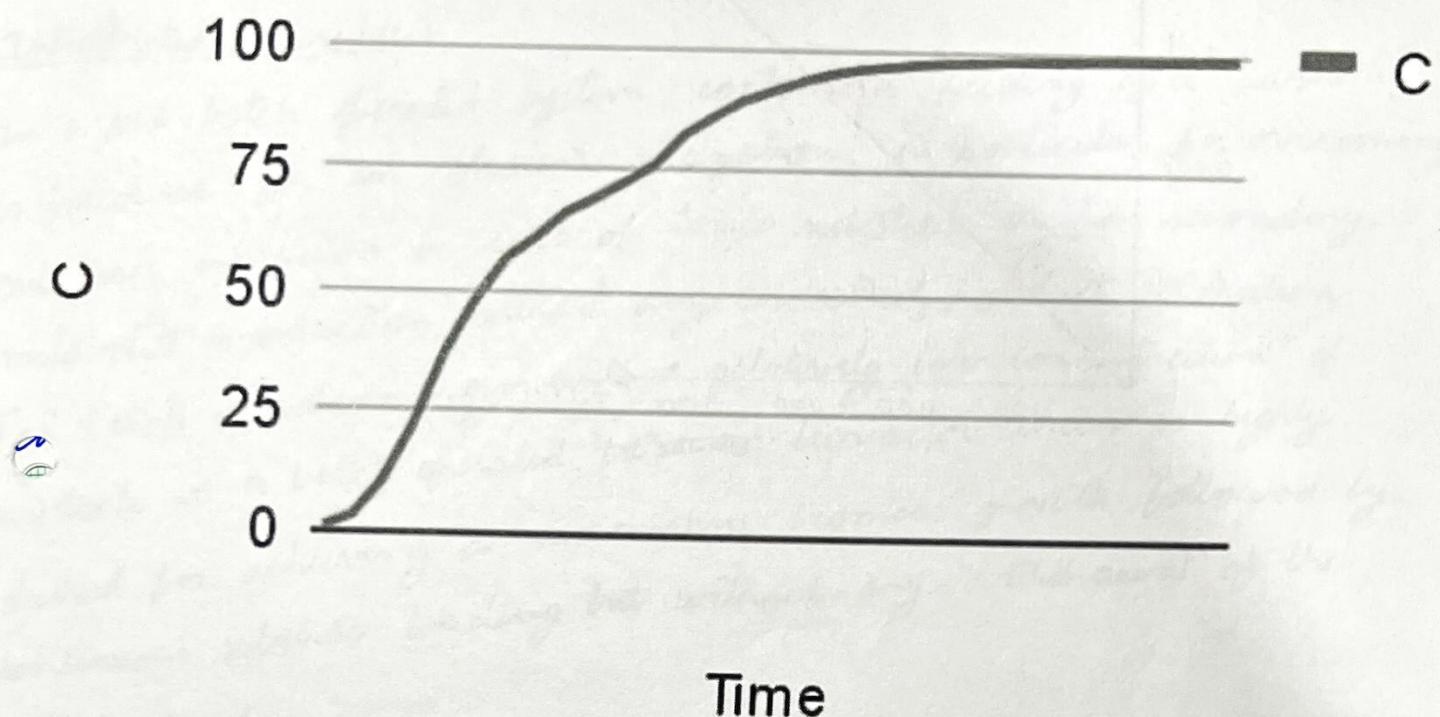
Result :-

The calculated k_{La} is $0.00489 s^{-1}$ RPM > 800

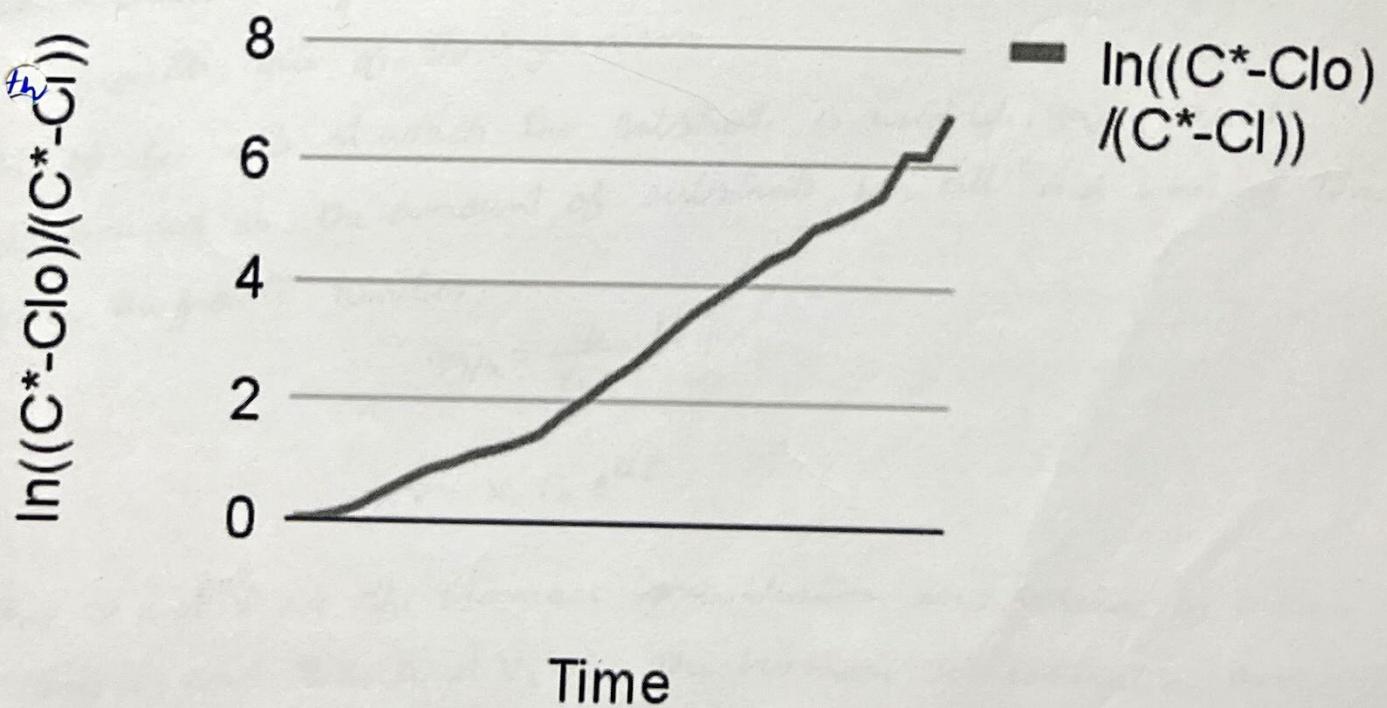
RPM	K_{La}
300	0.0012 0.0013
500	0.0012 0.0018 0.00128
600	0.0012 0.0015 0.006
800	0.0013 0.0019 0.005
1000	0.00102

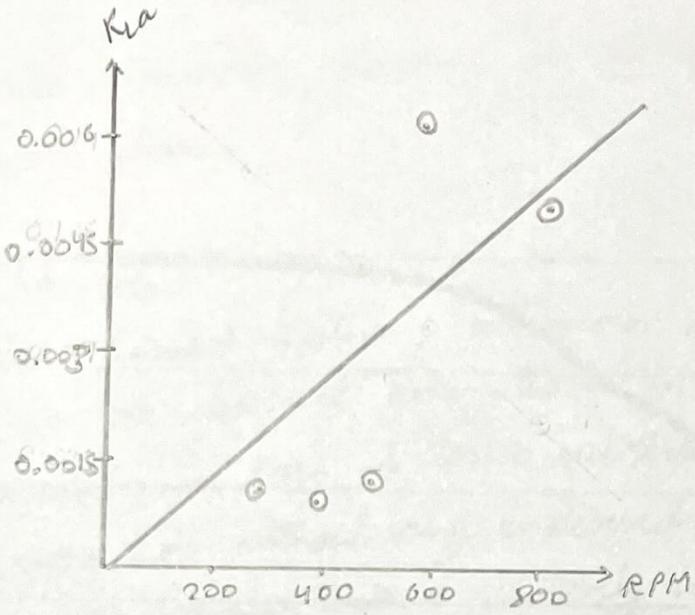
We can interpret K_{La} increases with RPM.

C v Time



$\ln((C^*-ClO)/(C^*-Cl))$





Experiment - 8

Aim: Fed batch bioreactor operation for utilization of glucose as carbon source.

Introduction/Principle:

In a fed batch operated system, continuous feeding of a substrate is followed for an efficient utilization, in particular for overcoming substrate inhibition in case of toxic substrate or for secondary metabolite production without any undesired product formation. Fed batch operation begins with a relatively low concentration of substrate in a batch operated ~~batch~~ bioreactor which is highly desired for achieving a maximum biomass growth, followed by continuous substrate loading but without any withdrawal of the contents inside.

The bioreactor is operated initially under batch for the growth of *Cordimia sp.*, followed by continuous feeding of glucose in mineral salt media. During the process, substrate is added as required by the growth rate of the organism.

The specific rate at which the substrate is used up, $q_{S/n}$, can be determined as the amount of substrate per cell and unit of time from the growth kinetics:

$$q_{S/n} = \frac{1}{Y_{n/S}} \mu$$

$$XV = X_0 V_0 e^{\mu t}$$

Where X and V are the biomass concentration and volume of culture at time t , and X_0 and V_0 are the biomass concentration and volume of growth medium in the reactor at time $t=0$. μ is the specific growth rate of the organism.

The specific rate of substrate uptake $q_{S/n}$ (g/Lh) in a fed-batch culture is satisfied by addition on demand. The required volumetric feeding rate, Q_S , consists of $q_{S/n}$ and the cell density X .

$$Q_S = q_{S/n} X$$

This must be identical to the feeding rate:

$$Q_S = \frac{FS_0}{V}$$

where F is the rate of feeding (L/h) at the given time, S_0 is the concentration of the input and V is the volume of the reaction. The method of feeding can be either constant, which results in linear growth or adjusted to increase exponentially so that S is maintained at an optimal level and results in exponential growth. The ~~gross~~ balance in a fed batch fermenter may be described as follows:

Biomass: $\frac{d(VX)}{dt} = \mu VX$

from which: $\frac{dX}{dt} = (\mu - D)X$, $\frac{dVS}{dt} = 0$

Since the volume increase as a result of the input is:

$$\frac{dV}{dt} = F$$

D is the dilution rate as a result of input: $D = \frac{F}{V}$.

For limiting substrate the following is valid:

$$\frac{d(VS)}{dt} = S_0 F - \frac{(\mu X V)}{Y_{n/S}} = 0$$

Hence,

$$\text{Feed rate } (F) = \frac{\mu X V}{Y_{n/S} S_0} = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{n/S} S_0}$$

Methodology:-

1. Preparation of mineral salt media (MSM):

Medium with the following composition along with glucose (20 g/L) is prepared. Initial pH of the medium is adjusted to 7 using NaOH/HCl.

Reagent	composition (g/L)
MgSO ₄ .7H ₂ O	0.409
CaCl ₂ .2H ₂ O	0.0265
KH ₂ PO ₄	1
NH ₄ NO ₃	1
Na ₂ HPO ₄ .12H ₂ O	6
FeCl ₃ .6H ₂ O	0.0833

2 Preparation of inoculum:-

Cordonia sp. used in this experiment is initially grown using 50 mL LB medium at 28°C and 150 rpm orbit shaking.

For seed culture preparation, the bacterium is grown in an Erlenmeyer flask (250 mL) containing 100 mL MSM and 20 g/L glucose as the sole carbon source.

The flask is then incubated at 28°C and 150 rpm in an orbital shaking incubator for 48 hrs.

The 48 hr grown culture is centrifuged at 5000×g and the pellet obtained is washed twice with 25 mL distilled water. The washed pellet is then suspended in 100 mL MSM and subsequently used as the inoculum in the fed-batch ~~bacterial~~ bioreactor experiment.

3. Experimental procedure:-

For fed-batch operation mode to achieve steady state utilisation of glucose by the bacterium, the reactor is filled with 1 L MSM containing glucose as the glucose media.

The prepared seed culture of the bacterium is then added as the inoculum to the media at the beginning of the experiment.

The reactor is run initially for 96 hr under batch mode for achieving maximum biomass growth. The agitation and temperature in reactor are maintained at 400 rpm and 28°C.

After the initial batch run, MSM containing glucose is continuously fed to the reactor so as to maintain simultaneous biomass growth and glucose utilization without any inhibition. Feeding starts at the end of exponential growth phase of the bacterium in the reactor. For the feeding, a feeding rate F according to equation (10) is obtained by substituting values of X_0, V_0, S_0, Y_{SM} and μ as determined previously from the batch operated reactor.

Samples are taken from the bioreactor at 6 hr time interval for the analysis of biomass and glucose concentration.

For biomass analysis, OD of the samples at 660 nm are measured by using UV-visible spectrophotometer.

For determination of glucose concentration in a sample a high performance liquid chromatograph (HPLC)/GOD-POD is used.

4. Task required :-

calculation of feed flow rate F .

Time profiles of biomass growth and glucose concentration in the reactor under batch and fed batch mode.

Comment on glucose utilization from the media.

Time	OD ₆₀₀	Biomass (OD ₆₀₀ /L)	Glucose concentration (g/L) × 10 ⁻²
T ₁ (0 th hour)	0.203	0.203	66.44
T ₂ (4 th hr)	0.4997	0.4997	163.56
T ₃ (8 th hr)	0.6786	0.6786	222.137
T ₄ (22 nd hour)	0.2443	0.2443	81.60
T ₅ (24 th hr)	0.1239	0.1239	40.55

Formula: $\frac{I}{\text{standard}} \times 100 \text{ mg/L}$

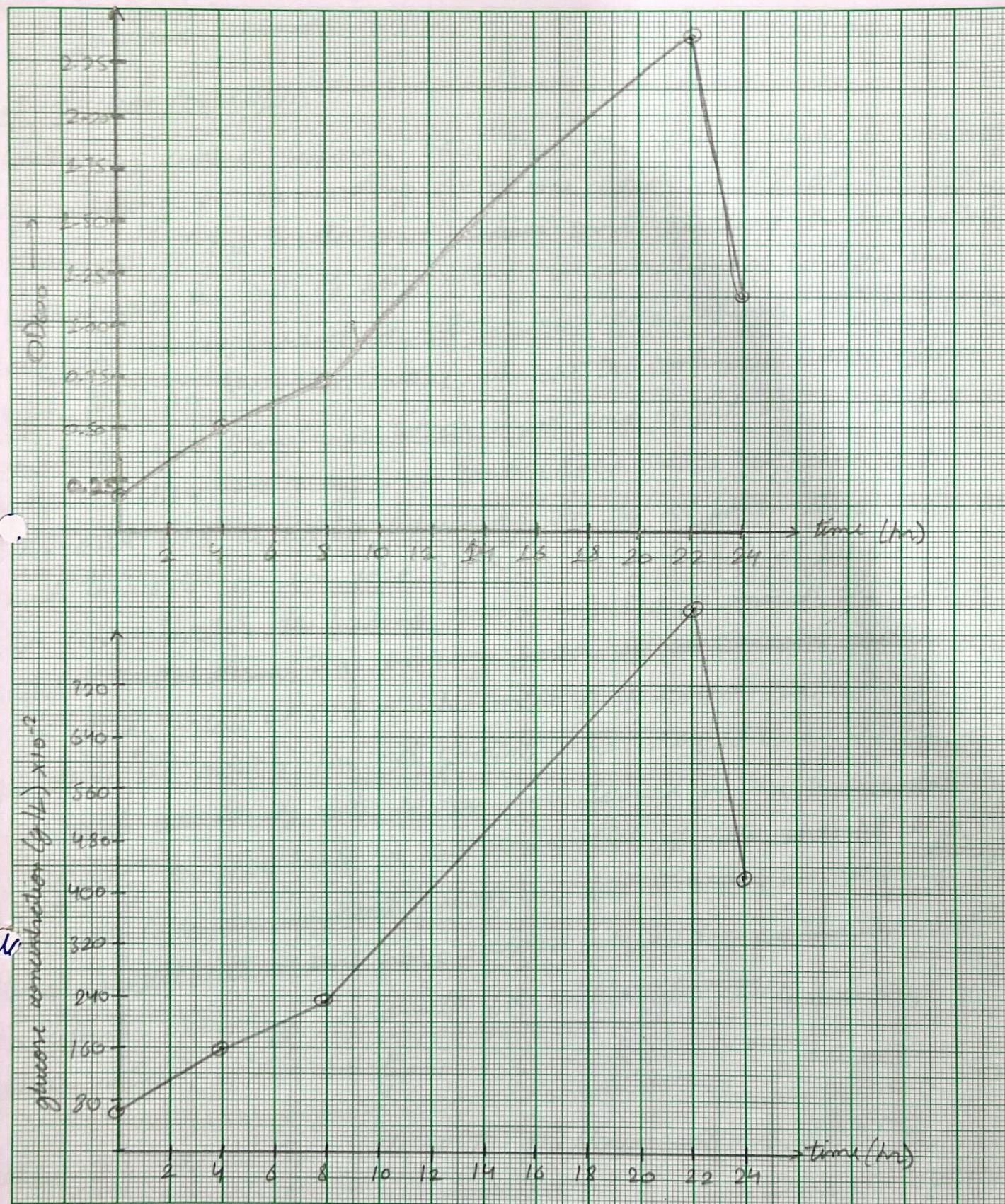
Standard: $\frac{0.3201 + 0.2909}{2} = 0.3055$

End flow rate: observation $F = 2.3 \text{ mL/min}$

The concentration of glucose decreases from ($T=22 \text{ hr}$) ($81.6 \times 10^{-2} \text{ g/L}$) to ($T=24 \text{ hr}$) ($40.55 \times 10^{-2} \text{ g/L}$). We are using fed bed batch reactor to increase the growth time especially log phase time, as this reactor increases the time window for growth.

Experiment 9

" " " " " Sugarcane juice



Experiment 9

→ Aim: Continuous production of ethanol using *Saccharomyces cerevisiae*

Introduction/Principle:

Continuous cultures perform better than the batch cultures economically by reducing or eliminating downtime, which is lost for cleaning, sterilizing of the reactor and medium and restarting the bioreactor. Furthermore, the levels of parameters (concentration of carbon and nitrogen sources, product formation, pH, redox balance etc) will change with time in the batch culture, whereas the continuous culture afford a steady state growth in which input and output are in perfect balance. There are 3 types of continuous culture, viz., chemostat, turbostat and auxostat. Depending on the set parameters, chemostat and turbostat are also known as nutristat or pH stat. In an auxostat, the budding rate is adjusted to match the rate of cellular metabolism. The primary form of continuous culture is a steady-state CFSTR or chemostat. A chemostat ensures a constant growth in the reactor. The net growth rate is equal to the dilution rate. A constant cell density rate is maintained in the turbostats by adjusting the flow rate. A tub turbostat operates well at high flow rates (near the washout points) and is useful in selecting cellular subpopulation that have adapted to a particular state. Characteristic operating parameter for continuous reactors is the dilution rate, D :

$$D = \frac{F}{V}$$

where D is the dilution rate, F is the flow rate and V is the reactor working volume.

Methodology:-

1. Preparation of medium (YPD):

The YPD medium containing 2% each of yeast extract, peptone and dextrose is used for both seed culture and production medium. The initial pH is adjusted to 5.5. 50 or 100 mL of the medium was taken in a 250 or 500 mL of Erlenmeyer flask and sterilized in autoclave. The medium is inoculated with the slant culture, *S. cerevisiae* and incubated in a rotatory shaker at 30°C and 120 rpm.

2. Preparation of inoculum :-

For seed culture preparation, the bacterium is grown in an Erlenmeyer flask (250 mL) containing YPD medium. The flask is then incubated at 30°C and 120 rpm in an orbital shaking incubator for 48 hrs.

3. Experimental procedure :-

- Start/initiate batch fermentation as per earlier method.
- A fed with fresh growth medium or glucose is started and an equal volume of culture broth is removed from the bioreactor when the culture reaches the ~~exponentiated~~ exponential growth phase or when the culture becomes substrate limited.
- Collect the samples from at regular interval of time from the reactor and exit.
- Continuous fermentation is carried out at various dilution rates.

4. Task required :-

- Operate the bioreactor at a particular dilution rate.
- Time profile of biomass growth and glucose conc. in the reactor under batch and continuous modes of operation.
- Determine the growth rate of microorganism and substrate concentration using standard protocols.
- determine ethanol conc. in the fermentation broth by using ethanol kit or GC

$$D = \frac{F}{V}, F = 2.3 \text{ mL/min} = 2.3 \times 60 \times 10^{-3} \text{ L/hr}$$

$$V = 1.2 \text{ L}$$

$$D = \frac{2.3 \times 60 \times 10^{-3}}{1.2} = 0.115 \text{ hr}^{-1}$$

Biomass reading:

<u>Batch</u>	<u>Time</u>	<u>Absorbance (OD_{600nm})</u>
T ₁	9:15 AM (Day 2)	1.2974
T ₂	10:30 AM (Day 2)	1.3076
T ₃	3:15 PM (Day 2)	0.4833
T ₄	11:30 PM (Day 1)	1.843
T ₅	11:11 AM (Day 2)	12.082
T ₆	3:30 PM (Day 2)	2.074

GOD-POD readings

- Standard absorbance = 0.3244

<u>Batch</u>	<u>Time</u>	<u>Absorbance</u>	<u>Concentration(g/L)</u>
T1	9:30AM (day 1)	0.0223	0.068742
T2	11:15AM (Day 1)	0.0131	0.040382
T3	3:15PM (Day 1)	0.0171	0.04038 0.052713
T4	11:30 PM (Day 1)	0.0162	0.049978
T5	11:11 AM (Day 2)	0.086	0.265105
T6	3:30 PM (Day 2)	0.0025	0.007707

→ Results :-

- D (dilution rate) = 0.115 hr^{-1}
- Biomass concentration increases with time and decreases after a point.
- Glucose concentration first decreases, then increase till a point and then decreases.

