18BTC107J BIOPROCESS PRINCIPLES

SEMESTER- IV

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 B.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**

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Name: Class:

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Experiment No: 1

Date:

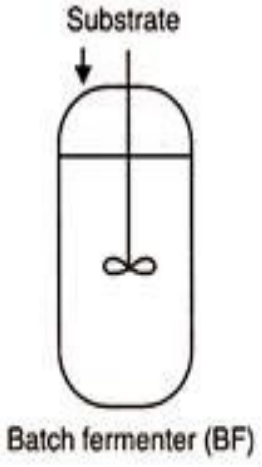
TYPES OF FERMENTATION

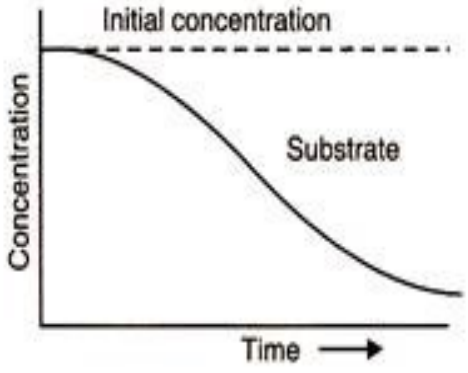
Based on the modes of operation the fermentation process are classified as batch, continuous and fed-batch fermentation

Batch fermentation

In batch fermentation, a closed system where an initial and limited amount of sterilized medium is introduced into a fermenter. The nutrient medium is inoculated with the microorganism and kept for incubation for a definite period under optimum conditions. Oxygen is provided in the form of air and pH is controlled by acid, base or any antifoaming agent, are being added during fermentation process. During fermentation a change in the composition of the culture medium (biomass and metabolites) as the microbes undergo different phases of growth and metabolism. The culture broth is harvested, separated and purified when the desired product is formed.

Batch fermentation are widely used in primary and secondary metabolites production under definite culture conditions that supports the fastest growth rate and the maximum growth could be utilized for the biomass production. In order to obtain optimum yield of primary metabolites, prolongation of exponential growth phase is required whereas it should be reduced in case of secondary metabolites.





Batch Reactor: Volume – constant

Material Balance Equation: Input = Output + Accumulation + Utilization

Substrate Balance: Amount of substrate entering/unit time = Amt. of substrate leaving/unit time + amount of substrate utilized + amount of substrate remaining in the reactor

The specific growth rate is generally found to be a function of three parameters,

- The concentration of growth limiting substrate, S (g/L)
- The maximum specific growth rate, μmax (time^{-1})
- Substrate saturation constant, K_S (g/L)

Monod Equation,

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

Where S (g/L)- concentration of the essential growth medium component, μ - specific growth rate hr^{-1}, μ_m- Maximum specific growth rate (hr^{-1}) 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 (μ_m/2).

Batch time with respect to substrate, product and cell concentration,

$$t_b = \frac{1}{\mu m} ln\ ln\ [1 + \frac{Y_{xs}}{X_0} (S_0 - S_F)]$$

$$t_b = \frac{1}{\mu m} ln\ ln\ [1 + \frac{\mu_m}{X_0 q_p} (P_F - P_0)]$$

$$t_b = \frac{1}{\mu m} ln\ ln\ [\frac{X_F}{X_0}]$$

t_b- batch time (Time);

Y_{xs}- Yield coefficient (g/g);

S_0 & S_F- Initial and final substrate concentration (g/L);

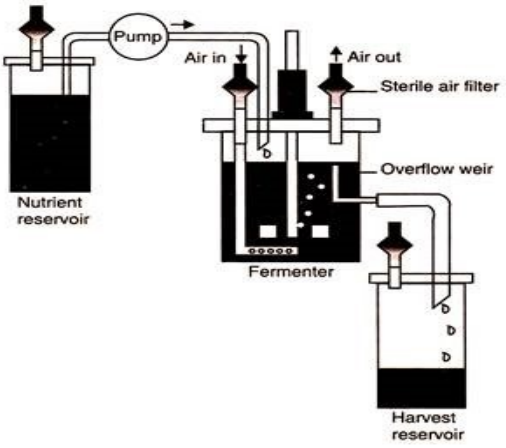
P_0 &P_F- Initial and final product concentration (g/L);

X_0 &X_F- Initial and final cell concentration (g/L);

q_p- specific product formation rate (g/g/h)

Continuous fermentation

A closed system of fermentation that operated for an indefinite period of time. Here fresh sterilized nutrient medium is added intermittently or continuously and at the same time in order to recover cells and fermented products, an equivalent amount of spend medium along with the microbes are withdrawn either intermittently or continuously. As a result, concentration and volume of the nutrient medium is optimally maintained in an automatic manner. However, continuous fermentation process lowers the operating cost as it has high productivity with less down time. In this mode of operation, medium and inoculum are initially added to the reactor and constant volume of the broth is maintained. This process has been generally used for organic solvents, starter culture, antibiotics and single cell protein production.



Continuous fermentation can be carried out in three ways as follows,

Single stage fermentation

In this process, the nutrient medium and culture are kept in continuous mode of operation by balancing the input and the output of the harvested culture and the nutrient medium in a single fermenter.

Multiple stage fermentation

Two or more fermenters are operated simultaneously where growth phase and synthetic phase of the fermentation process are being carried out in different fermenters. This process is more suitable for those fermentation in which the different phases (growth and synthetic) of the microbes are not simultaneous.

Recycle fermentation

A portion of the nutrient medium is withdrawn and added back into the culture vessel. Therefore, the

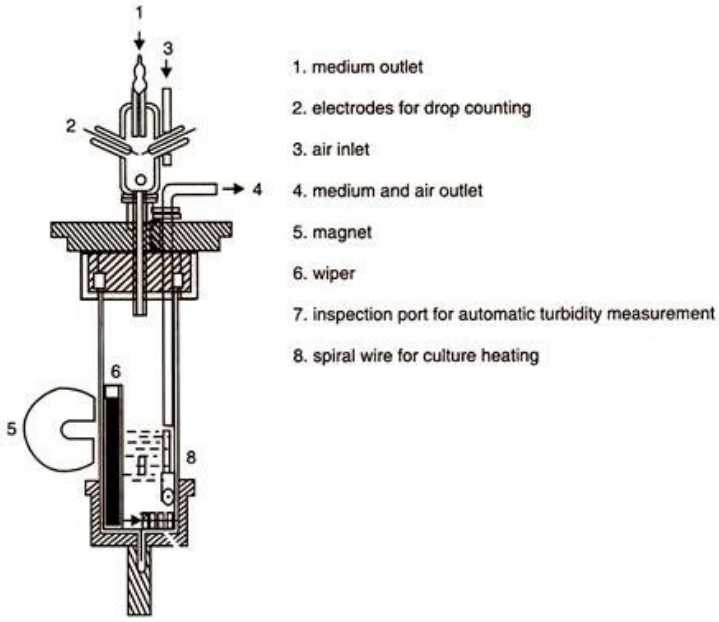
culture is recycled back into fermenter and this method is mainly carried out for hydrocarbon fermentation process. Whereas recycling of cells provides increase in the cell population that result in greater productivity of the desired product.

1) Turbidostat Method:

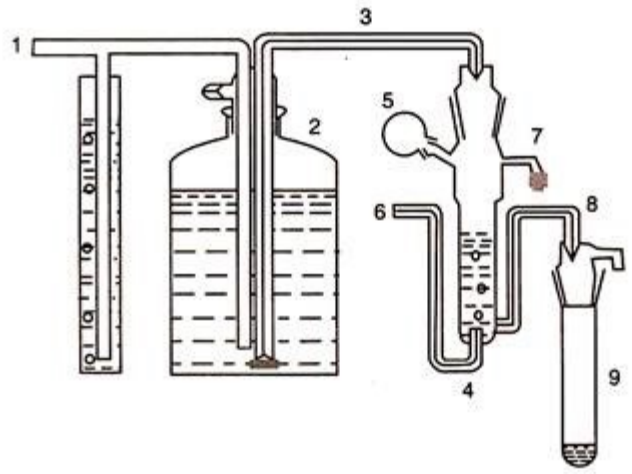
In this process, total cell count has been kept constant by measuring the turbidity of the microbial culture during the fermentation process. Hence, the nutrient feed rate and the culture withdrawal rate can be regulated. Those fermentation process which employs turbidostat, should be carried out at low cell population that utilize the less amount of substrate and reduce the wastage of larger amount of substrate that is being unused.

2) Chemostat method:

A constant value of nutrient feed rate and the withdrawal rate of harvest culture are maintained which is achieved by controlled growth rate of microbial culture by adjusting the concentration of any chemical compound used in the nutrient medium (oxygen, carbon and nitrogen source) that act as growth limiting factor. Sometimes pH, temperature and the presence of toxic byproducts generated during fermentation process also act as a growth limiting factor. This fermentation process is performed most often than the turbidostat due to the presence of less amount of unused medium and fewer mechanical problems.



Turbidostat Model



1. Air inlet
2. Mariotte's bottle
3. Capillary for medium inlet
4. Culture vessel
5. Inoculation port
6. Air inlet
7. Air outlet
8. Overflow capillary
9. Sampling tube

Chemostat

The flow of medium into the vessels is related to the volume of vessel by terms of dilution rate (D),

$D = \frac{F}{V}$ (time^{-1})

Where

F= Flow rate (m^3/time)

V= volume (m^3)

Residence time (R), reciprocal of dilution rate

$$\tau = \frac{1}{D}\ (Time)$$

The net change in the cell concentration over time period is expressed as,

$$\frac{dx}{dt} = \mu_x - D_x$$

At steady state, $\mu = D$

Therefore, $D = \frac{\mu max\ .S}{Ks+S}$

S- steady state concentration of substrate in chemostat

$$S' = \frac{K_s D}{\mu_{max} - D}$$

The concentration of the cell in the chemostat at steady state can be related to yield coefficient as

$$X' = Y_{X/S}\,(S_i - S')$$

Si- Initial substrate concentration (g/L)

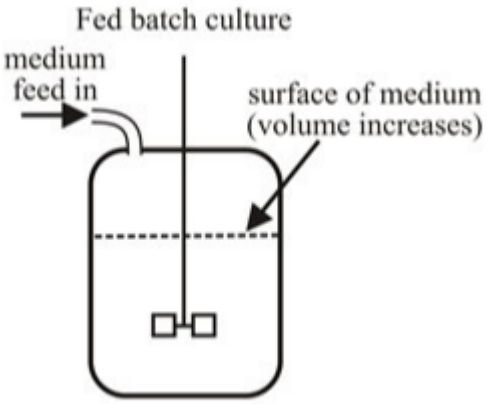
S' – steady state substrate concentration (g/L)

Y – Yield coefficient (g/g)

X'- Steady state cell concentration (g/L)

Fed batch fermentation

A modified form of batch fermentation process where substrate is periodically added in regular intervals as the fermentation process progress. Hence, an optimal concentration of the substrate is maintained. This process is essential for those fermentation processes where the secondary metabolites are being subjected to catabolic repression by the presence of high nitrogen, carbohydrates or glucose concentration in the nutrient medium. The critical elements of the medium are provided in lesser amount during the start of the fermentation process while during the production phase the substrate are being added in small amount. This process is mostly widely employed for the penicillin production. Fixed, variable and cyclic were the three types of fed batch fermentation process.



Based on the process requirements the fermentation are classified as aerobic and anaerobic fermentation

Time of the fed batch cell biomass weight in fed batch reactor at a given time t,

$$X_t = X_0 + (Y_{xs} S_i F)\, t_{fb}$$

X_t – Cell concentration at given time't' (g/L);

X_0 – Cell concentration at initial time (g/L);

Y_{xs} – Yield coefficient (g/g)

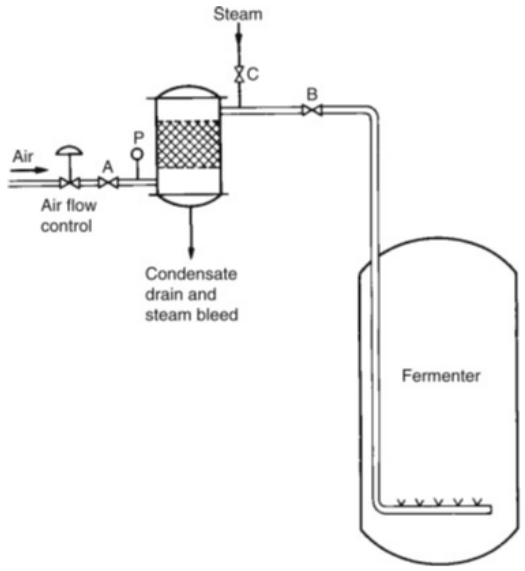
S_i - Initial substrate concentration (g/L)

F- Flow rate (m^3/ time)

t_{fb} – Time for fed-batch cycle (time)

Aerobic fermentation

Aerobic fermentation is carried out in the presence of oxygen. Most of products of human utility are generally produced by this type of fermentation.



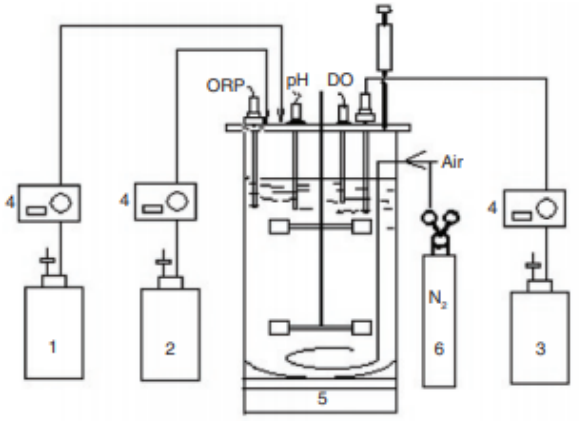
Aerobic fermenter

Anaerobic fermentation

Fermentation carried out in the absence of oxygen and the anaerobic microbes are of two types, obligate and facultative anaerobes. For example, the *Clostridium sp.,* remain active only in the absence of oxygen or cannot withstand oxygen for optimum production of the desired product. Similarly, lactic acid bacteria are facultative anaerobes that are able to remain active in the presence of small amount of oxygen. However, in case of yeast an initial aeration is required for building the high cell yield before creating an anaerobic condition.

In this fermentation process, anaerobic condition is created by pumping the inert gases like nitrogen, argon and removing the oxygen present in the head space of the fermenter or by the emergence of certain gases like hydrogen and carbon dioxide. Sometimes soon after the sterilization, the nutrient medium is inoculated at the bottom of the fermenter and also stationary/viscous medium also used for creating the

anaerobic condition.



Anerobic fermenter

1) Base solution Feed solution
2) Collection reservoir
3) Three pumps
4) Fermenter vessel with pH, DO (dissolved oxygen) and ORP (redox) probe
5) Nitrogen
6) gas tank

Fermentation can further be classified as static or surface culture and submerged fermentation

Static/ surface fermentation

In this fermentation process, solid or liquid substratum may be utilized where microorganism grow on those substrate by simultaneously withdrawing the nutrients from the substrates. Those fermentation processes are mostly desirable for the sporulation based products.

Submerged fermentation

In this method, nutrient substratum will be in the liquid state where the microorganism grows inside the substratum. Generally, spargers and impeller blades are utilized to maintain the uniform culture condition.

Solid state fermentation (SSF)

Solid state fermentation is where solid substratum (moist solid substrates) is used to define the microbial

growth in the absence or near absence of free water. Recently, SSF has shown much promising development in the several bioprocess products.

Significance of the experiment:

Critical thinking:

1) If you want to obtain the secondary metabolite from a microbial metabolism which type of fermenter operation you opt?

2) For production low value high volume product which fermenter would be more suited?

3) Which geometry of the fermenter is suitable and efficient for aerobic process? Tall and narrow? Short and shallow? Justify the answer.

Experiment No: 2&3

Date:

BIOREACTOR OPERATION

Aim: To study the design, construction and control systems of a bioreactor.

Principle:

A bioreactor is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Bioreactors are extensively used for food processing, fermentation, waste treatment, etc. On the basis of the agent used, bioreactors are grouped into the following two broad classes: (i) those based on living cells and, (ii) those employing enzymes. But in terms of process requirements, they are of the following types: (i) aerobic, (ii) anaerobic, (iii) solid state, and (iv) immobilized cell bioreactors.

All bioreactors deal with heterogeneous systems dealing with two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other. Chemical engineering principles are employed for design and operation of bioreactors. But, in general, theoretical explanation usually lags behind technical realization.

A bioreactor should provide for the following: (i) agitation (for mixing of cells and medium), (ii) aeration (aerobic fermenters; for O_2 supply), (iii) regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level, etc., (iv) sterilization and maintenance of sterility, and (v) withdrawal of cells/medium (for continuous fermenters). Modern fermenters are usually integrated with computers for efficient process monitoring, data acquisition, etc.

Basic Functions of a Fermenter:

1. It should provide a controlled environment for optimum biomass/product yields.

2. It should permit aseptic fermentation for a number of days reliably and dependably, and meet the

requirements of containment regulations. Containment involves prevention of escape of viable cells from a fermenter or downstream processing equipment into the environment.

3. It should provide adequate mixing and aeration for optimum growth and production, without damaging the microorganisms/cells. The above two points (items 2 and 3) are perhaps the most important of all.

4. The power consumption should be minimum.

5. It should provide easy and dependable temperature control.

6. Facility for sampling should be provided.

7. It should have a system for monitoring and regulating pH of the fermentation broth.

8. Evaporation losses should be as low as possible.

9. It should require a minimum of labour in maintenance, cleaning, operating and harvesting operations.

10. It should be suitable for a range of fermentation processes. But this range may often be restricted by the containment regulations.

11. It should have smooth internal surfaces, and joints should be welded wherever possible.

12. The pilot scale and production stage fermenters should have similar geometry to facilitate scale-up.

13. It should be contrasted using the cheapest materials that afford satisfactory results.

Key parts of the bioreactor:

Agitator – This facilitates the mixing of the contents of the reactor which eventually keeps the "cells" in the perfect homogenous condition for better transport of nutrients and oxygen for adequate metabolism of cell to the desired product(s).

The agitator can be top driven or bottom which could be basically magnetic / mechanically driven. The bottom driven magnetic /mechanical agitators are preferred as opposed to top driven agitators as it saves adequate space on the top of the vessel for insertion of essential probes (Temperature, pH, dissolved oxygen foam, CO_2 etc) or inlet ports for acid, alkali, foam, fresh media inlet /exit gases etc. However mechanical driven bottom impellers need high quality mechanical seals to prevent leakage of the broth.

Types of agitators:

Disc turbine

Open turbines of variable patch.

Propellers

Baffle – The purpose of the baffle in the reactor is to break the vortex formation in the vessel, which is usually highly undesirable as it changes the centre of gravity of the system and consumes additional power.

- Baffles are metal stripes roughly $1/10^{th}$ of the vessel diameter and are attached radially to the wall.
- Normally 4 baffles are used, but in vessel over 3 dm^3 diameter 6-8 baffles may be used.

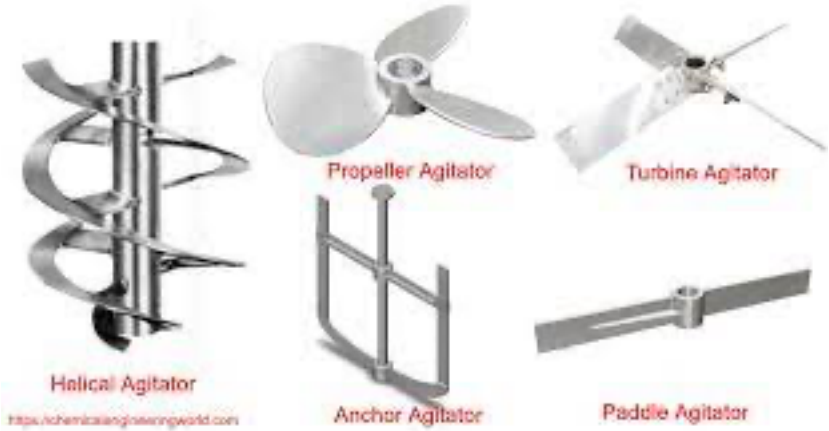
Sparger – In aerobic cultivation process thepurpose of the sparger is to supply oxygen to the growing cells. Bubbling of air through the sparger not only provide the adequate oxygen to the growing cells but also helps in the mixing of the reactor contents thereby reducing the power consumed to achieve a particular level of (mixing) homogeneity in the culture.

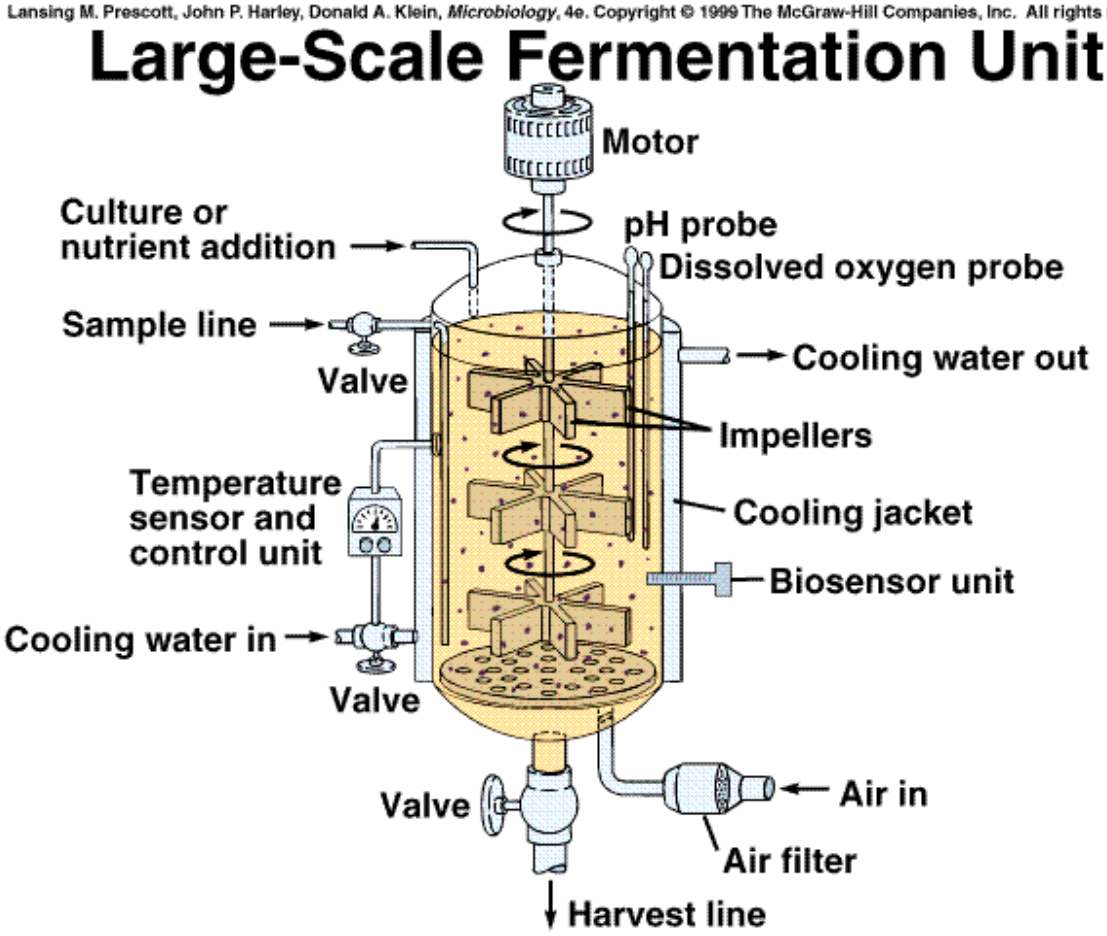
Three basic types of sparger are used:

Porous sparger.

Orifice sparger.

Nozzle sparger





Bioreactor (Bottom Driven)

Jacket – The jacket provides the annular area for circulation of constant temperature water which keeps the temperature of the bioreactor at a constant value. The desired temperature of the circulating water is maintained in a separate Chilled Water Circulator which has the provision for the maintenance of low/high temperature in a reservoir. The contact area of jacket provides adequate heat transfer area wherein desired temperature water is constantly circulated to maintain a particular temperature in the bioreactor.

Body construction:

For a small scale (1 to 30 dm^3) glass and/ or stainless steel is used because it can withstand repeated steam sterilization cycles.

Two basic types are used:

A glass vessel with a round or flat bottom and top flanged carrying plates.

A glass cylinder with stainless steel top and bottom plates. This bioreactor may be sterilized in situ. (AISI graded steel are now commonly used in bioreactor construction).

Peripheral parts:

Reagent pumps

Pumps are normally part of the instrumentation system for pH and antifoam control.

Peristaltic pumps are used and flow rate is usually fixed with a timed shot and delay feed system of control.

Medium feed pumps

and reservoir bottles

Medium feed pumps are often variable speed to give the maximum possible range of feed rates. The reservoir bottles are usually larger, but are prepared in the same as normal reagent bottles.

Rotameter

A variable area flow meter indicates the rate of gas flow into a bioreactor. A pressure regulator valve before the rotameter ensures safe operation.

Stirrer glands and bearings:

These are used for the sealing of the stirrer shaft assembly and can be operated aseptically for a long duration. Four basic types of seal assembly have been used,

The stuffing box (packed gland seal)

The simple bush seal

The mechanical seal

Magnetic drive.

Basic control systems for the operation of the bioreactor are described below:

Temperature Measurement and control – The measurement of the temperature of the bioreactor is done by a **thermocouple or Pt -100 sensor** which essentially sends the signal to the Temperature

controller. The set point is entered in the controller which then compares the set point with the measured value and depending on the error, either the heating or cooling finger of the bioreactor is activated to slowly decrease the error and essentially bring the measured temperature value close to the set point.

pH measurement and control – The measurement of pH in the bioreactor is done by the autoclavable pH probe. The measured signal is compared with the set point in the controller unit which then activates the acid or alkali to bring the measured value close to the set point. However, before the pH probe is used, it needs to be calibrated with two buffers usually in the pH range which is to be used in the bioreactor cultivation experiment. The probe is first inserted in (let us say) pH 4 buffer and the measured value is corrected by the zero knob of the controller. Thereafter the probe is put in pH 7 buffer and if needed the measured value is corrected by the asymmetry knob of the controller. The pH probe is now ready for use in the range 0-7 pH range.

Dissolved oxygen controller – The dissolved oxygen in the bioreactor broth is measured by a dissolved oxygen probe which basically generates some potential corresponding to the dissolved oxygen diffused in the probe. Before the measurement can be done by the probe it is to be calibrated for its zero and hundred percent values. The zero of the probe is set by (zero knob) the measured value of the dissolved oxygen when the broth is saturated with nitrogen purging. Similarly the hundred percent of the instrument is calibrated by the measured value of dissolved oxygen when broth is saturated with purging air in it. After calibration the instrument is ready for the measurement of the dissolved oxygen in the broth. In the event of low oxygen in the fermentation broth, more oxygen can be purged in the bioreactor &/or stirrer speed can be increased to enhance the beating of the bubbles which essentially enhances the oxygen transfer area and net availability of oxygen in the fermentation broth.

Foam control – The fermentation broth contains a number of organic compounds and the broth is vigorously agitated to keep the cells in suspension and ensure efficient nutrient transfer from the dissolved nutrients and oxygen. This invariably gives rise to lot of foam. It is essential that control of the foam is done as soon as possible.

Speed control- Speed control relies on the feedback from tachometer located with drive motor determining the power delivered by the speed controller to maintain the speed set point valve set by the user. A digital display shows the actual speed in rpm, as determined by the tachometer signals.

Table 1: Measurements of various parameters in a bioreactor

Measurements	Methods	Remarks
Agitator speed	Frequency counter tacho generator	More precise less reliable.
Agitator power	Torque sensor. Electrical power.	Difficult. Recommended.
Temperature	Resistance Thermometer Thermistor Thermocouple	Probably best Fragile Satisfactory Not recommended
Flow rate	Rota meter Orifice meter Thermal mass flow meter	Satisfactory Less accurate Set-point control
Dissolved oxygen	Galvanic probe Polarographic probe	Widely used Widely used
pH	pH electrode	Widely used
Foam	Conductivity probe	Widely used
Redox	Redox electrode	Empirical valve
Turbidity	Turbidity sensor	Complex
Liquid feed rate	Peristaltic pump Syringe pump Magnet flow meter	Widely used Limited capacity Large
Pressure	Pressure transducer	Satisfactory

Significance of the experiment:

Critical thinking:

When do you expect an uncontrolled foam formation during fermentation?

Is virus a contaminant during fermentation? If so in which type of culture you would expect its contamination?

Enlist the components of fermenter required for efficient aeration? Justify its purpose.

Experiment No: 4

Date:

ESTIMATION OF GLUCOSE BY DINITRO SALICYLIC ACID (DNS) ASSAY METHOD

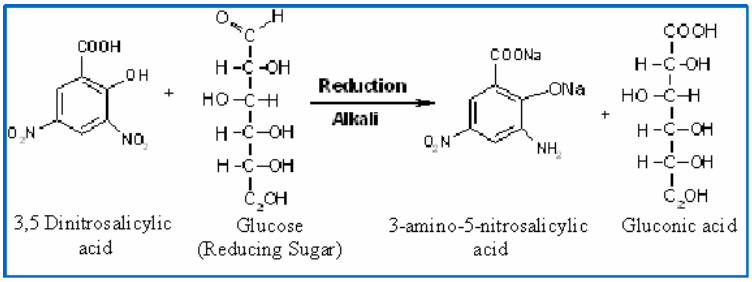
Aim :

To estimate the concentration of Glucose by DNS method

Principle:

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 (1 mg /ml of deoionized water)

Dinitrosalicylic Acid Reagent Solution

Dinitrosalicylic acid: 10 g

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Potassium sodium tartrate solution, 40%

Deionized water : 1 lit

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 1 ml with deionized water
- 1 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 standardcurve 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 1%Na-K Tartrate (ml)	O.D at 540 nm
1	Blank	-	1	1		1	
2	0.1		0.9	1		1	
3	0.2		0.8	1		1	
4	0.3		0.7	1		1	
5	0.4		0.6	1		1	
6	0.5		0.5	1		1	
7	0.6		0.4	1		1	
8	0.7		0.3	1		1	
9	0.8		0.2	1		1	
10	0.9		0.1	1		1	
11	1.0		0	1		1	

Incubate the tubes at 90ºC for 10 - 15 minutes.

Result and Interpretation:

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

Significance of the experiment:

Experiment No: 5 & 6

Date:

MEDIA OPTIMIZATION BY PLACKETT AND BURMAN

Aim

To optimize the concentration and composition of various nutrients in the media using the Plackett and Burman method

Theory

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 satisfactorily 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:

It will produce the maximum yield of product or biomass per gram of substrate used.
It will produce the maximum concentration of product or biomass.
It will permit the maximum rate of product formation.
There will be the minimum yield of undesired products.
It will be of consistent quality and be readily available throughout the year.
It will cause minimal problems during media making and sterilization.
It will cause 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 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 are 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 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 variable can be designated as a dummy variable. Alternatively, factors known to have no effect 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 show 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 the 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.

Plackett-Burman design for seven variables

Trial	Variables						
	A	B	C	D	E	F	G
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

H denotes a high level value; L denotes a low level value

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 in analyzing the data (Table 1 and 2) using Nelson's (1982) example are as follows:

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

∑ (H)

∑ (L)

Difference effect

Mean square

Mean square for error

F- Test

Thus the effect of

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

This value should be near zero for the dummy variables.

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

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

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

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

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

Factor mean square

Error mean square

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

If dummy variable is zero consider Error mean square value as one

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. Fructose	0.5	4.0
C. Glycine	0.1	1.0
D. Xylose	0.5	4.0
E. Sucrose	0.5	4.0
F. Yeast extract	0.01	1.0
G. Casamino acid	0.05	0.5
Ammonium Chloride	2.0	
KH_2PO_4	3.0	
$MgSO_4$ (IM)	1 ml	

Medium was inoculated with 1 ml of *E. coli* in each medium.

Zero time optical density reading was noted.

The culture was incubated in 37ºC in a shaker.

`

Optical density was measured at regular time intervals and recorded.

The highest optical density reading was taken as its response.

RESULT:

1. The optimized concentration of the media was found to be in _____

2. The component_____ influences the production of biomass.

TABLE 2: PLACKETT-BURMAN RESPONSE TABLE

Trial	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**Response(OD) at 540 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 OF YIELD SHOWN IN TABLE 2.

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

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

Significance of the experiment:

Critical thinking:

[1] Why you need a precise optimization of media components?

[2] In what way statistical methods help other than classical / conventional methods?

[3] microorganisms require essential macro-nutrients to grow. You have two choice (1) starch syrup (2) Molasses. Which one you will choose and why?

Experiment No: 7

Date:

BATCH HEAT STERILIZATION AND THERMAL DEATH KINETICS

Aim

To study the sterilization kinetics of the given sample of microorganism and determine the holding time at 121°C for the medium.

Introduction

A fermentation product is produced by the culture of a certain organism, or organisms, in a nutrient medium. If a foreign microorganism invades the fermentation, the following consequences may occur.

i. The medium would have to support the growth of both the production organism and the contaminant, thus resulting in a loss of productivity,

ii. If the fermentation is a continuous one then the contaminant may 'outgrow' theproduction organism and displace it from the fermentation,

iii. The foreign organism may contaminate the final product, e.g., single-cell proteinwhere the cells, separated from the broth, constitute the product,

iv. The contaminant may produce products which make subsequent extraction of thefinal product is difficult.

v. The contaminant may degrade the desired product,

vi. Contamination of a bacterial fermentation with phage could result in lysis of the culture.

Avoidance of contamination may be achieved by

i. Using pure inoculums to start the fermentation.

ii. Sterilizing the medium and the fermentor vessel, and all the materials employed in the process.

iii. Maintaining aseptic conditions during the fermentation.

Liquid medium is most commonly sterilized in batch in the vessel where it will be used. The liquid is heated to sterilization temperature by introducing steam into the coils or the jacket of the vessel; alternatively, steam is bubbled into the medium or the vessel is heated electrically. If direct steam injection is used allowance must be made for dilution of the medium by condensate which typically adds 10-20% to the liquid volume; quality of the steam just also is high to avoid contamination *of* the medium by metal ions or organics.

Depending upon the rate of heat transfer from the steam or electrical element, raising the temperature of the medium in large fermentors can take a significant period or time. Once the holding or sterilization temperature is reached the temperature is held constant for a period of time t_{hd}. Cooling water in the coils or jacket of the fermentor is then used to reduce the medium temperature to the required value.

For operation of batch sterilization also destroys nutrients in the medium. To minimize this loss, holding times at the sterilization temperature must be kept as short as possible. Cell death occurs at all time during batch sterilization, including heating up and cooling down periods. The holding time t_{hd} can be minimized by taking into account cell death during these periods

KINETICS OF CELL DEATH:

The destruction of microorganisms by steam is described as a first order chemical reaction and represented by the equation and represented by the equation,

$-dN/dt = KN$…….(1)

Where, N= Number of viable organism

t = time of sterilization treatment

K = reaction rate constant of the reaction or specific death rate

$$\int -dN/dt = \int KN$$

Upon rearragement and differentiating with time limit of t=0 and ‘t’ h,

$$\int -dN/N = \int K \, . \, dt$$

$Nt/No = e^{-Kt}$

Where,

No = Number of viable organisms present at the start of sterilization treatment,

Nt = Number of viable organisms present after the treatment period, t.

K = K_d (specific death rate constant) ($Time^{-1}$)

On taking natural logarithm of equation,

ln (Nt/No) = -Kt…….(2)

Specific Death Rate

In the first order reaction the reaction rate increases with increase in temperature due to increase in the reaction rate constant, which in the case of destruction of microorganism is the specific death rate (K).

Relation between temperature and reaction rate constant is given by Arrhenius equation:

$\int d \ln K/dt = E / RT^2$

$K = A e^{-E/RT}$ .....(3)

Where, E = Activation energy ($cal \cdot mol^{-1}$)

R = gas constant ($cal \cdot mol^{-1} \cdot K^{-1}$)

T= absolute temperature (K)

A = Arrhenius constant

The term ln(No/Nt) is called as Del factor or Nabla factor and the sterilization criterion is represented by the term Δ.

Del factor:

It is a measure of fractional reduction in viable organism count produced by certain heat and time regime.

$\Delta = \ln(No/Nt) = A*t*e^{(-E/RT)}$

$\ln(No/Nt) = A*t*e^{(-E/RT)}$

$\Delta = A*t*e^{(-E/RT)}$

$$t_{hd} = \frac{ln\frac{N0}{Nt}}{K}$$

K = K_d (specific death rate constant)

t_{hd} – hold time (time)

No – initial number of cells

Nt – number of cells at time 't'

PROCEDURE

The batch fermentor was filled with 7L distilled water.

Then the temperature was raised from 30^0C onwards. Increase in temperature per minute was noted till it reaches 121^0C.

The exhaust valve was closed at 95^0C.

At 121^0C, the outlet valve was opened to drawn out the condensed water so as to prevent from any contamination.

After 20 minutes, the cooling process was started (i.e.) temperature cooling down from 121^0C to 30^0C. Then the decrease in temperature per minute was noted.

The graph was plotted between Temperature vs. Time and the holding time was calculated.

RESULT:

The holding time for the medium at 121°C was found to be _____

TABLE 1: STERILIZATION KINETICS

S.no	Time (min)	Temperature (˚C)	Temperature (K)	k(1/min)
1				
2				

3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				

TABLE 2: HEATING PERIOD DETERMINATION

Time interval	Heating temperature (K)	Heating $Ae^{-Ed/RT}$	COOLING TEMPERATURE (K)	COOLING $Ae^{-Ed/RT}$

Significance of the experiment:

Critical thinking questions

[1] Whether excessive sterilization of nutrient media has critical effect on fermentation process?

[2] How will you sterilize the thermally unstable compounds?

[3] Calculate the time required to kill 99% of spores present in the sample at 100 °C. Provided specific death rate is 0.23 min^{-1} and it follows the first order kinetics.

[4] If pasteurization is also a kind of sterilization technique why it is not heated to 121 °C rather it is heated at ~63 for 30 °C min

Experiment No: 8-10

Date:

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.

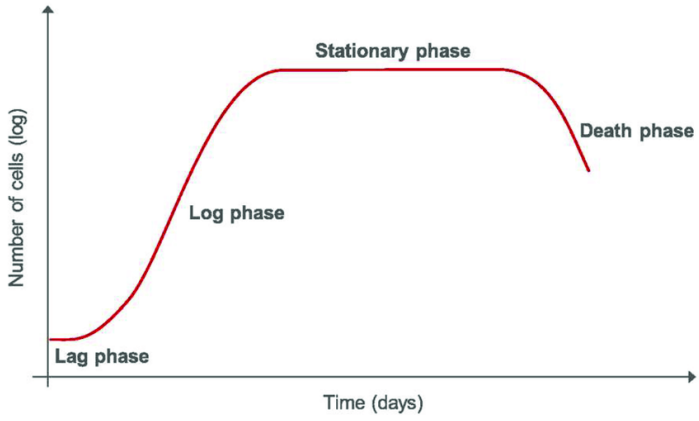
Principle

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:

Lag phase: During this period the cell adapts to the new environment by synthesizing necessary enzymes for the utilization of available substrates.

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.

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.

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

Where S (g/L)- 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 growthof 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.

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

where, X = biomass conc (g/L), t = time

On integrating eqn (1)

$X_t = X_0 . e^{\mu t}$ …..(2)

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

Where, X_o = initial biomass concentration (g/L), X_t = biomass concentration after time t (g/L)

On taking "ln",

$$X_t = ln\ ln\ X_0 + \mu t \ldots\ldots\ldots (3)$$

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

$\mu = \frac{lnln\ OD\ 1 - lnln\ OD\ 2}{t2 - t1}$ (Time^{-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,

$T_d = \frac{ln ln\, 2}{\mu}$ (Time)

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:

$Y_{x/s} = \frac{\Delta_x}{\Delta_s} = \frac{X_t - X_0}{S_0 - S_t}$ ------(5)

X_o - initial concentration of biomass (g/L) X_t = concentration of biomass at time't' (g/L), S_0 = initial substrate concentration (g/L), S_t = Residual substrate concentration at time't' (g/L).

Materials required

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

Glucose Yeast extract medium:

Glucose 3- 15 g/L

Yeast extract 5 g/L

NH_4Cl 1 g/L

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/L
$MnCl_3.4H_2O$	20 mg/L
$NiCl_2.6H_2O$	1 mg/L
$Na_2MoO_4.2H_2O$	50 mg/L

ZnSO4.7H2O 5mg/L

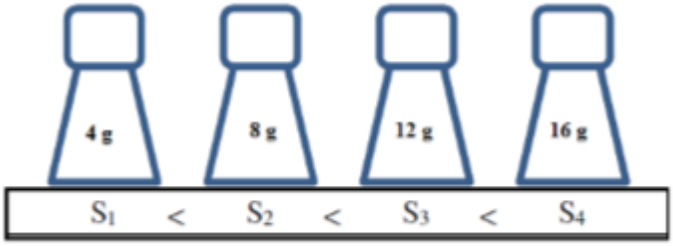
pH: 7.0

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.

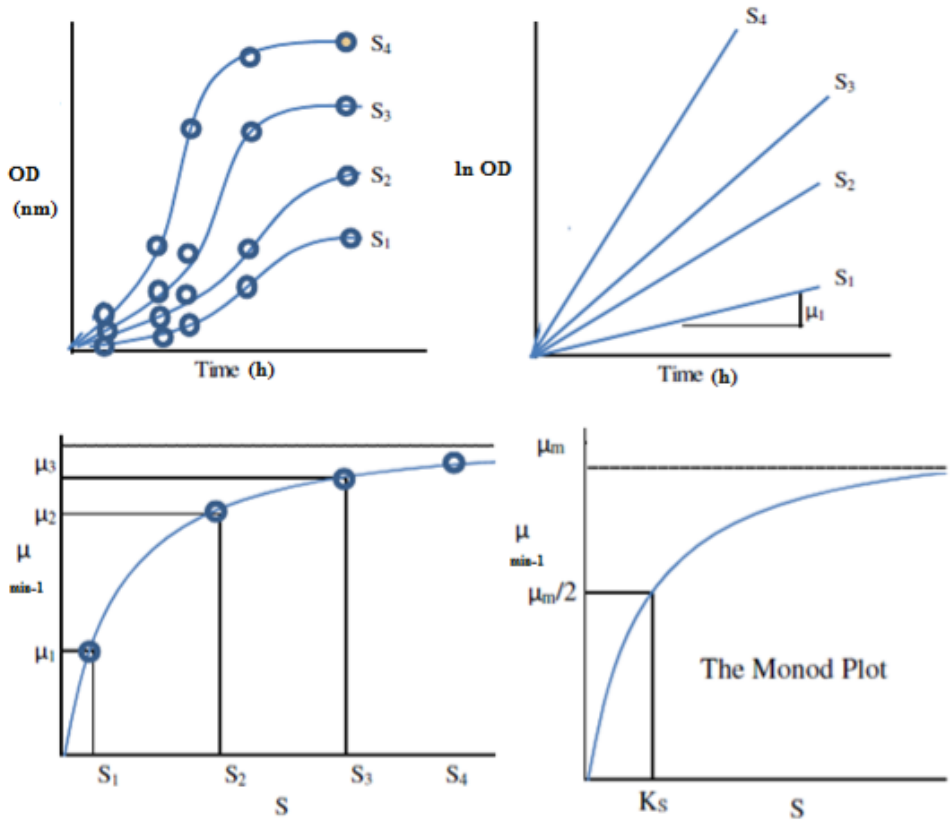
Add 1 ml 40 % Sodium Potassium tartrate solution.

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**	**15 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**	**15 g/L**	**3 g/L**	**6 g/L**	**9 g/L**	**12 g/L**	**15 g/L**

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	15 g/L	3 g/L	6 g/L	9 g/L	12 g/L	15 g/L	3 g/L	6 g/L	9 g/L	12 g/L	15 g/L

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

Specific growth rate (from calculation) μ = ……sec

Doubling time (from graph) t_d = ……sec

Doubling time (from calculation) t_d = ……sec

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

Significance of the experiment:

Critical thinking questions

How does a microbe metabolize a mixture of carbon source?

Is there any advanced techniques employed to find the population of bacterial consortia rather than using a traditional microscopy?

Assume if lactose has been provided as a carbon source for the bacteria initially, after which the culture has been transferred to another flask containing glucose. What kind of growth curve would you expect? Draw and explain.

Experiment No: 11

Date:

ENZYME IMMOBILIZATION

Aim:

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

Principle:

Immobilized enzymes have a wide range of applications, among which we can highlight food processing and industrial chemistry. The use of immobilized enzymes for chemical analysis has generated considerable interest in recent years. Any process or technique based on the use of immobilized enzymes involves the choice of the support type and the immobilization method, together with the description and later optimization of other numerous variables specific to each process. The supports used are often of a polymeric character, being networks of high molecular weight which can adsorb solvent, mainly water, but are themselves insoluble. The interactions responsible for the solvent adsorption are, among others, capillary forces, osmosis and polymer/solvent molecular interactions, resulting in a porous structure where the enzymes can lodge.

The enzymes are found in a new state, in which their properties are very much influenced by a number of factors, in particular, those related with the chemical and physical nature of the support. Immobilization can alter the distribution of the substrate, reaction products or hydrogen ions between the microenvironment and the macro environment of the enzyme, all of this as consequence of the electrostatic or hydrophilic/hydrophobic interactions between the base and the components of the surroundings. Diffusional restrictions may also appear, in particular, on the diffusion of the substrate and products between the interior of the support and the macro environment. Finally, immobilization may cause changes in the structure of the enzyme and even steric impediments, depending on how the enzyme links to the support. These effects are reflected

in the activity and kinetics of the action of the enzyme and stability, and mainly affect its interactions with the substrate and, consequently, its specific features of enzymatic action.

Sodium alginate is a natural polysaccharide obtained from kelp and seaweed, brown algae belonging to the phylum *Phaeophyta*. It is mainly composed of thousands of oxidized sugar "units" joined together to form an ionic polymer The repeating units are six-membered rings containing negatively charged $–CO_2^–$ groups. The C-1 carbon atom of one ring is connected via an oxygen atom to iouthe C-4 carbon atom of the next ring in the polymer chain (Fig.1).

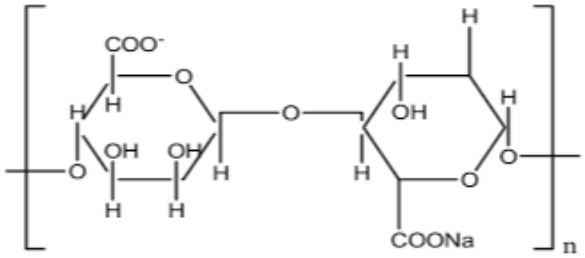
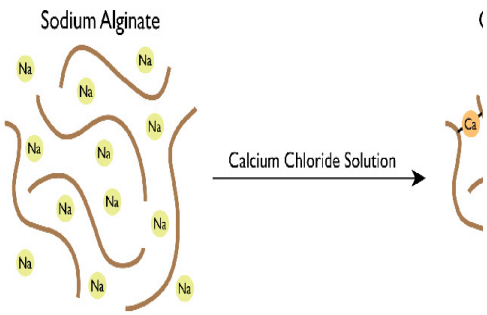


Fig.1 Sodium alginate

The presence of ionic $–CO_2^–$ side chains, as well as numerous –OH groups, make this natural polymer extremely hydrophilic or "water-loving." The resulting solution is thick, viscous, and smooth. Replacing the sodium ions in sodium alginate with calcium ions leads to cross-linking between the polymer chains and gives an insoluble gel, calcium alginate. Each Ca^{2+} ion can bind to at least two carboxylate groups in the polymer. If the two $–CO_2^–$ groups are on different (adjacent) polymer molecules, then the effect of adding divalent cations is to tie together or cross-link individual polymer molecules into a large, three-dimensional network. The cross-linked polymer swells up in contact with water to form an insoluble gel. Studies have shown that the polymer behaves like a giant chelating ligand (similar to EDTA), and that each Ca^{2+} ion is bound to four $–CO_2^–$ groups.



2 Na(Alginate) + Ca^{++} -------> $Ca(Alginate)_2$ + 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 re-dissolved by immersing the alginate.

Preparation of immobilized enzymes by entrapping by an insoluble support matrix:

Materials required:

Sodium Alginate (3%)

0.2 M $CaCl_2$

Alpha amylase enzyme (1%)

Procedure:

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 0.2 M $CaCl_2$ 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

Significance of the experiment:

Critical thinking questions

[1] Whether nanoparticles/ nano fibers can be used for immobilization of enzyme? How? Does the material feature improves the enzyme immobilization?

[2] What happens if high molecular weight ionic polymer is exposed to highly charged cations?

[3] What would happen if you add potassium or magnesium chloride solution instead of calcium chloride to interact with sodium alginate?

Experiment No: 12

Date:

ENZYME IMMOBILIZATION KINETICS

Aim:

To study the effect of immobilization of α-amylase enzyme by gel entrapment method and to find out the kinetic parameters for both free and immobilized enzyme.

Principle:

Entrapment is the physical enclosure of enzymes in a small space. Matrix entrapment and membrane entrapment, including micro-encapsulation are the two major methods of entrapment. Matrices used for enzyme entrapment are usually polymeric materials such as calcium alginate, agar, carangeenin, polyacrylamide and collagen.

Sodium alginate + Enzyme + Calcium chloride

↓

Calcium alginate beads entrapped with enzyme.

Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

Materials required:

Alpha-amylase

Calcium chloride

Sodium alginate

0.5 to 2.5% Starch solution.

Procedure:

- Dissolve 3g of sodium alginate in 100 ml distilled water to make a 3% (w/v) solution. After sodium alginate is completely dissolved, leave the solution undisturbed for 30 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float. The concentration of sodium alginate can be varied between 6-12 % (w/v) depending on the desired hardness.
- Prepare 100ml of 0.2M calcium chloride solution in a beaker.
- Mix approximately 5 mg of α-amylase enzyme with 5 ml of 3% sodium alginate solution. Add the sodium alginate and α-amylase mixture drop wise to the calcium chloride solution using peristaltic pump or syringe and needle at room temperature.
- Allow the beads in calcium chloride solution for 20 min to deplete the excess calcium.
- Use equal quantity of α-amylase enzyme solution (5 mg of alpha amylase in 5 ml of deionized water) for studying free enzyme kinetics.
- Take 50 ml of 0.5 to 2.5% starch solution in 10 conical flasks (both for free and immobilized enzyme)
- Add immobilized enzyme (prepared beads – 5 mg of alpha amylase with 5 ml of sodium alginate) and free enzyme solution (5 mg/5ml) to the respective conical flasks
- Incubate the conical flasks in rotary shaker (100 rpm, 40 °C) for 10 minutes..
- After 10 minutes, withdraw 1 ml of sample from all the conical flasks and dilute it with 2 ml of distilled water.
- Add 3 ml of DNS reagent to calculate the amount of glucose [P] (mmol/ml) released.
- Calculate the reaction rate (V) is equal to d[P]/dt.

- Draw the graphs for [S] vs V (Michaelis Menten) and 1/[S] vs 1/[V] (Lineweaver Burk plot) for both free and immobilized enzyme systems.
- Find the values of V_{max} and K_m for both free and immobilized enzyme system from Michaelis Menten and Lineweaver Burk plots.

TABULATION:

Table1. Free enzyme

Substrate concentration (mg/ml)	**OD at 540 nm**	**Conc. of glucose [P] mg/ml**	**Conc. of glucose [P] mmol/ml**	**V mmol/ml.min**	**1/V**	**1/[S]**

Table 2. Immobilized enzyme

Susbtrate concentration (mg/ml)	OD at 540 nm	Conc. of glucose [P] mg/ml	Conc. of glucose [P] mmol/ml	V mmol/ml.min	1/V	1/[S]

RESULT

Effect of immobilization of α-amylase enzyme by gel entrapment method was studied.

Maximum reaction rate (Vmax) for free enzyme = …… mmol/ml.min

Rate constant (Km) for free enzyme = …… min^{-1}

Maximum reaction rate (Vmax) for immobilized enzyme = …… mmol/ml.min

Rate constant (Km) for immobilized enzyme = …… min^{-1}

SIGNIFICANCE OF THE EXPERIMENT:

Critical thinking questions

(1) As you discussed the advantages of immobilization, list the disadvantages of the immobilization of enzymes and whole cell.

(2) Why immobilization is formed in the shape of sphere? Why not rectangle / triangle or any geometric shape?

(3) Elucidate different immobilization techniques employed commercially?

Experiment No: 13-15

Date:

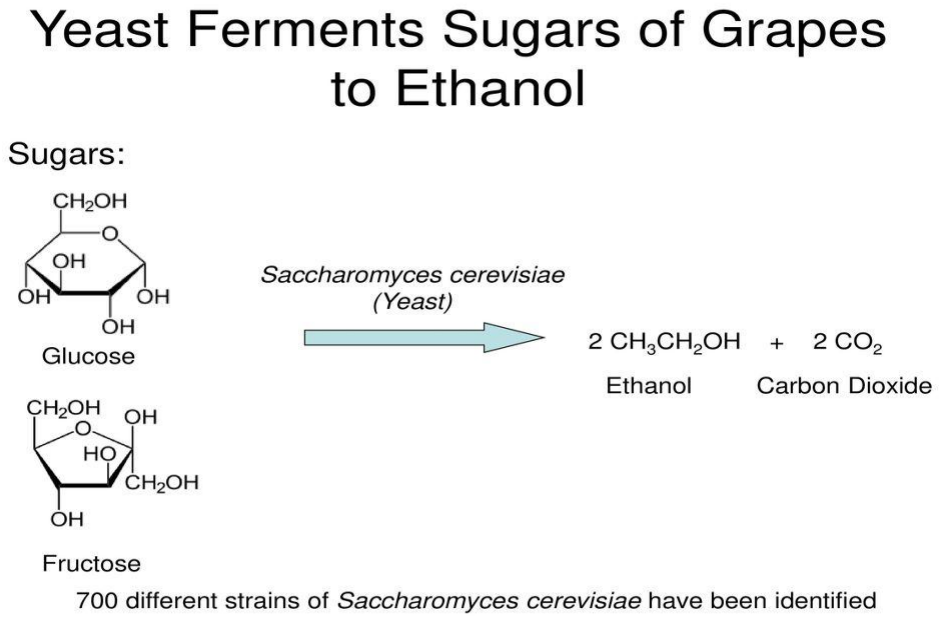
PRODUCTION OF ETHANOL BY YEAST

Aim

To produce ethanol from grape fruit juice by the yeast fermentation process

Principle

The most popular and best-known baker's yeast – *Saccharomyces cerevisiae* is used for alcohol production through anaerobic fermentation. The yeast is used for brewing beer, making bread, making wine, ethanol and distilled beverages. The yeasts appear to be more tolerant of ethanol than other strains of yeasts so that they can produce the wine that contains 20% v/v of alcohol whereas brewer's yeasts yield only 9% v/v of ethanol. Yeasts are grown on grapes for making wine anaerobically and the yield of alcohol from the fermentation depends on the amount of substrate (sugars) that is being utilized during the fermentation process.



PROCEDURE:

Add fresh and healthy black grapes in a glass beaker and squeeze them to collect the juice
Filter the collected juice
Transfer the filtered juice to a sterile Erlenmeyer flask and close tightly
Add 1.5 g of brewer's yeast and 200 g of sugar per kg of grapes
Incubate the flask at 15 – 20°C for 8-15 days

REAGENT PREPARATION:

Potassium dichromate solution ($K_2 Cr_2O_7$):

Weigh 34 g of Potassium dichromate and dissolve in 500 mL of distilled water in a one-liter standard measuring flask.

Concentric sulphuric acid (Conc. H_2SO_4 – 5 M):

Measure 325 mL of concentric sulphuric acid and slowly add to the potassium dichromate solution by keeping it in an ice bucket.

DNS Solution

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%

QUANTIFICATION OF BIOMASS:

1 mL of the fermented culture was collected at regular time intervals and centrifuged at 8000 rpm for 10 minutes
Supernatant was transferred to another eppendrof tube and air dried cell pellet weight was noted down

$Weight\ of\ the\ biomass\ (cell\ pellet) = ((Weight\ of\ the\ eppendrof\ with\ pellet) - (Weight\ of\ the\ empty\ eppendrof))$

PREPARATION OF STANDARD CURVE FOR ETHANOL ESTIMATION:

Prepare 2 % (v/v) Ethyl alcohol as a stock solution

Take 0.1, 0.2, 0.3, 0.4 and 0.5 ml of this 2 % alcohol and add it to the test tubes and makeup to 10.0 ml using distilled water

Take 0.5 mL of wine sample collected at regular time intervals and make it up to 10.0 mL using distilled water

Add 1.0 mL of potassium dichromate solution to all the test tubes

Incubate the test tubes at 90 ˚C for 15 minutes

Measure optical density at 590 nm

Plot the standard graph between the concentration of ethanol and optical density

Calculate the concentration of ethanol in the wine sample at different time intervals from the standard graph (mg/mL)

PREPARATION OF STANDARD CURVE FOR GLUOSE ESTIMATION:

Prepare glucose standards ranging from 0.1 to 1 mL by using dry clean and labelled test tubes (Refer table)

Take 0.5 mL of wine sample collected at regular time intervals

Make all the samples 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 and vortex them for few seconds

Place all the test tubes in water bath at 90º C for 10-15 minutes to develop red-brown color

Cool the test tube in the running tap water and add 1 mL of 40% potassium sodium tartrate (Rochelle salt) solution to all the test tubes and vortex them for few seconds (Note: All the tubes must be cooled to room temperature before reading since the extinction is sensitive to temperature change)

Read the Optical density of the colored solutions at 540 nm using the solution in tube 1 as a blank

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

Calculate the concentration of glucose in the wine sample at different time intervals from the standard graph (mg/mL)

Evaluation of ethanol yield and productivity

The Stoichiometric equation from reducing sugars to ethanol can be written as

$$C_6H_{12}O_6 \longrightarrow 2\,C_2H_5OH + 2\,CO_2$$

The yield of ethanol can be calculated from the below equation

Fermentation efficiency: Ratio between the theoretical and observed yield of ethanol

$$Fermentation\ efficiency = \left(\frac{Observed\ ethanol\ yield}{Theoretical\ ethanol\ yield}\right) \times 100$$

TABULATION

Table: 1 Biomass estimation

S. No	Hours	Weight of the cell biomass (g)
1	0	
2	12	
3	24	
4	36	
5	48	
6	60	
7	72	
8	84	

9	96	
10	108	

Estimation of Ethanol

Table: 2 Standard table

S. No	Conc. of ethanol (mg/mL)	Volume of sample (mL)	Volume of water (mL)	Volume of chromic acid (mL)		O.D at 590 nm
					Incubate at 90°C for 15 mins	

Table:3

S. No	Hours	Vol. of sample (mL)	Vol. of water (mL)	Vol. of chromic acid (mL)		O.D at 590 nm	Conc. of ethanol (mg/mL)
	0						
	12				Incubate at 90°C		
	24						
	48						

	60				**for 15 mins**		
	72						
	84						
	96						
	108						

Estimation of Glucose

Table: 4 Standard table

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 1%Na-K Tartrate (mL)**	**O.D at 540 nm**
1	Blank	-	3	3	**Incubate the tubes at 90ºC for 10 - 15 minutes.**	1	
2	0.1		2.9	3		1	
3	0.2		2.8	3		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	

Table: 5

S. No	Hours	Vol. of standard solution (mL)	Vol. of Distilled water (mL)	Vol. of DNS reagent (mL)		Vol. of 1% Na-K Tartrate (mL)	O.D at 540 nm	Conc. of glucose (mg/mL)
1	0			3		1		
2	12			3		1		
3	24			3		1		
4	36			3		1		
5	48			3		1		
6	60			3		1		
7	72			3		1		
8	84			3		1		
9	96			3		1		
10	108			3		1		

Incubate the tubes at 90ºC for 10 - 15 minutes.

Graph: Concentration of standard ethanol (mg/mL) vs Optical Density (@590 nm)

Concentration of ethanol (wine sample) (mg/mL) vs Optical Density (@590 nm)

Concentration of Standard Glucose (mg/mL) vs Optical Density (@540 nm)

Concentration of Glucose (wine sample) (mg/mL) vs Optical Density (@540 nm)

RESULT:

Significance of the experiment:

Critical thinking questions

[1] Does the accumulation of glucose, ethanol and CO_2 levels during fermentation become toxic to yeast?

[2] If *S.cerevisiae* is capable of utilizing glucose (C6) sugar, what will be the fate of C5 sugar if it is present in the mixture of sugar? Is there any possible to utilize all the sugars simultaneously?

[3] Do human gut microbes produce ethanol? How? Does it have impact on human health?