Experiment No. 6

Batch operation in a Bioreactor to study bacterial growth kinetics

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: A bioreactor provides better control in terms of temperature, pH, antifoam addition, DO etc. in successful fermentation processes.

When microbial cells are inoculated into a fresh culture medium under batch conditions and their increase in concentration is monitored, several distinct phases of growth can be observed. There is an initial lag phase, which is of variable duration. This is then followed by the exponential growth phase, where cell number 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 against time. Following this is a short phase of declining growth, and then the stationary phase. Here the cell numbers are highest. 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;

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

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

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

During the exponential phase in batch, we can write:

$$dS/dt = kS$$

where 'S' is the substrate concentration at time 't' and 'k' is the specific substrate utilization rate constant.

Rate of substrate utilization will be estimated by analyzing the glucose concentration through GOD (Glucose oxidase)-POD (Peroxidase) method.

Principle of GOD-POD method:

Glucose oxidase enzyme (GOD) 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). Nascent oxygen then couples with 4-aminoantipyrine and phenol to form red quinoneimine dye. The intensity of colour is directly proportional to concentration of glucose in the sample. The intensity of color is measured colorimetrically and compared with that of a standard treated similarly [1].

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{amino-4-antipyrine} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + 4H_2O$$

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

$$q_p = 1/X * [dP/dt]$$

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

Principle of SDS-PAGE:

Electrophoresis is the process of migration of charged molecules in response to an an electric field. The rate of migration depends on the net charge, size and shape of the molecule, the voltage gradient of the electric field E, and the frictional resistance of the supporting medium f, which impedes their movement. Proteins have a net charge at any pH other than their isoelectric point pI, thus when placed in an electric field, proteins will migrate towards the electrode of the opposite charge. This principle is used to separate molecules of differing charges.

Electrophoresis in acrylamide gels is referred to as Polyacrylamide gel electrophoresis. Polyacrylamide gels which were first used for electrophoresis by Raymond &Weintraub (1959) are chemically inert and particularly stable. By chemical copolymerization of acrylamide monomers with crosslinking reagent N-N'- methylene bis-acrylamide a clear transparent gel which exhibits little endosmosis is obtained.

The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of Ammonium persulfate and a catalyst N,N,N'N'-Tetramethylenediamine(TEMED).

SDS-PAGE is the most commonly used gel electrophoretic system for analyzing proteins. This method is based on the separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. SDS is an anionic detergent which binds strongly to and denatures proteins to produce linear polypeptide chains. On average one SDS molecule will be present for every two amino acids. The presence of β- mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. The detergent binds to the hydrophobic region of the denatured protein in a constant ratio of about 1.4g of SDS/gm of

protein. The protein-SDS complex carries net negative charges, hence move towards the anode and the seperation is based on the size of the proteins. Most SDS-PAGE gels are cast with a molar ratio of Bisacrylamide: Acrylamide of 1:29 which has been shown empirically to be capable of resolving polypeptides that differ in size by little as 3%.

The Polyacrylamide gel is cast as a separating gel topped by a stacking gel. The stacking gel has properties that cause the proteins in the sample to be concentrated into a narrow band at the top of the separating gel. This is achieved by utilizing differences in ionic strength and pH between the resolving buffer and the stacking gel and involves a phenomenon known as isotachophoresis. The stacking gel is of high porosity and buffered with Tris-cl buffer at pH6.8, whereas separating gel contains high percentage of acrylamide and is cast in Tris-cl buffer at pH 8.8. The upper (and lower) electrophoresis buffers contain Tris at pH 8.3 with glycine as counterion.

Stacking principle: Glycine at pH 6.8 of the stacking gel remain in neutral zwitterionic form with only a fraction 1% in the negative glycinate form. This prevents glycine to be an effective carrier of current. The Cl^- ions remain effective current carriers at pH6.8 and migrate rapidly towards the anode. The SDS-coated protein molecules and dye which have charge to mass ratio> glycine but less than that of Cl^- must now migrate to carry the electrophoresis current behind the Cl^- and ahead of the glycine. There is only a small quantity of protein-SDS complexes so they concentrate in a thin band sandwiched between the cl- ions and the glycine molecules at the interface between stacking and separating gels.

The higher pH of the separating gel favours ionization of glycine, carrying a higher charge to mass ratio than that of the proteins. Now the newly formed glycinate ions move faster than the proteins with mobility approaching that of the cl^- ions. The negatively charged protein-SDS move according to their relative mobilities and are separated by the sieving effect of the separating gel according to size. The high mobility of the tracking dye assures that it will migrate faster than the proteins.

Reagents and equipments required:

A. **Equipment:** Flasks, Spectrophotometer, Sample tubes, Micropipette, Buffers and apparatus for SDS-PAGE.

B. **Reagents**
   - Flask of inoculum culture
   - Growth media

C. Organism

- *Pseudomonas fluorescens*

D. Media composition

- **For culture maintenance:** LB agar medium, 25 g/l

- • **For Growth media in flask for** *Pseudomonas fluorescens*
- **For Growth media in flask**

Chemical name	Composition (g/l)
Glucose	100.0
Potassium Dihydrogen Phosphate	2.0
Yeast Extract	10.0

Inoculum- 4%

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 manufacturers' 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 (1 lpm).
6. Samples are taken at regular interval of time for the measurement of biomass, glucose and fusion protein concentrations.

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: 0hr, 2hrs, 4hrs, 6hrs, 8hrs, 10hrs, 12hrs and 24hrs. 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 relationship.
3. The samples are centrifuged and the supernatant is stored for glucose (SUBSTRATE) and fusion protein (PRODUCT) estimation.
4. GOD-POD method is used to determine specific substrate utilization rate of the microorganism and ethanol is estimated using HPLC.

Day2:

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 two equal volumes of 500µl in two micro centrifuge 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 50ml flasks as per the dilution chart provided (Page no. 02)

↓

Take O.D. of different dilutions at 600 nm

↓

Take the weights of dry and labeled microcentrifuge tubes up to 4 decimal points (As many no. as the dilutions)

↓

------ (a)

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

↓

Suspend the cell pellet 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 point) ------ (b)

↓

Take the difference of (a) & (b) to get the cell biomass dry weight

By plotting a calibration curve between cell biomass dry weight and optical density and estimating the slope from the calibration curve to convert OD data into Dry Cell Weight data using correlation provided in the equation (i).

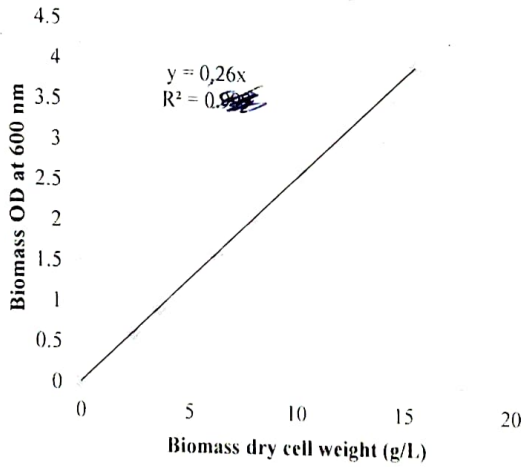

Fig.: Calibration curve for Cell Dry Weight measurement

Calculations:

1 Biomass OD @ 600 nm 0.4 Biomass dry cell weight (g/L)..... ..... (i)

PROCEDURE FOR GOD-POD METHOD

Reagents

Glucose reagent (L1): 150ml

Glucose standard (S): 5 ml (100mg/dL)

Reagent preparation

Reagents are ready to use

Procedure:

1. All the samples are diluted 10 times with dH_2O
2. 10µl of the diluted sample is pipetted out and mixed with 1000µl of Glucose reagent (L1)
3. After proper mixing, incubation is carried out at 37°C for 15 minutes (or at 25°C for 30 minutes)
4. Absorbance is checked at 546 nm.

The following are prepared in dry microcentrifuge tubes labelled as Blank (B), Standard (S) and Test (Your sample) (T):

Addition sequence	**B (ml)**	**S (ml)**	**T (ml)**
Glucose reagent (L1)	1.0	1.0	1.0
Distilled H_2O	0.01	-	-
Glucose standard (S)	-	0.01	-
Sample	-	-	0.01

Calculations:

$$\text{Total Glucose in g/L} = \frac{\text{Abs. of Test sample}}{\text{Abs. of Standard}} \times 100$$

Abs. of glucose standard = 0.1598 Equation ------- (ii)

i) Chemicals/Reagents/Buffers:

1. Stock acrylamide solution: 30g acrylamide,0.8g bisacrylamide. Make up to 100ml in distilled water and filter through whatman No1 filter and store in amber bottle at 4 c. (***CARE: Acrylamide monomer is a neurotoxin. Take care in handling acrylamide(wear gloves)and avoid breathing).***

2.Buffers:

a) Seperating gel buffer:1.875M Tris-cl,pH 8.8

b) Stacking gel buffer : 0.6M Tris-Cl,pH 6.8

3.10%w/v Ammonium persulfate. Make fresh. Store at 4 c.(***Care: Always use in Fumehood)***

4.10% w/v Sodium dodecyl sulfate (SDS)

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

6.Sample buffer

0.6M Tris-HCl, pH 6.8	5.0ml
10% SDS	0.5g
Sucrose	5.0g
β-mercaptoethanol	0.25ml
Bromophenol blue (0.5% stock)	5.0ml

Make up to 50ml with distilled water

7.Electrophoresis buffer: Tris(12g), glycine(57.6g), and SDS(2.0g).Make up to 2l with water. No pH adjustment is necessary

8.Protein Stain:0.1% Coomassie brilliant blue R250 in 50% methanol,10%glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the solution through whatmann filter paper. (***Note: Coomassie brilliant blue is harmful by inhalation or ingestion. Wear appropriate gloves & safety glasses while handling)***

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

10.Protein sample

11. Standard Protein molecular weight markers.

ii) Glasswares and others:

Conical flask
Beaker
Graduated cylinder

b) Method:

1. Clean the internal surfaces of the gel plates with methylated spirits, dry, then join the gel plates together to form the cassette, clamp it in a vertical position.
2. In an Erlenmeyer flask or disposable plastic tube, prepare the separating gel by mixing the following: ***(NOTE1)***

	For15% gels	For 10%gels
1.875M tris-HCl, pH8.8	8.0ml	8.0ml
Water	11.4ml	18.1ml
Stock acrylamide	20.0ml	13.3ml
10%SDS	0.4ml	0.4ml
Ammonium persulfate (10%)	0.2ml	0.2ml

3. Degas this solution under vacuum for about 30sec. ***(NOTE2)***
4. Add 14µl of TEMED and gently swirl the flask to ensure even mixing.
5. Using a Pasteur pipet transfer this separating gel mixture to the gel cassette carefully down one edge. Continue adding this solution until it reaches a position 1cm from the bottom of the comb that will form the loading wells.
6. To ensure that the gel sets with a smooth surface very carefully run distilled water down one edge into the cassette using a Pasteur pipet.
7. While the separating gel is setting prepare the 4% stacking gel solution. Mix the following in a 100ml Erlenmeyer flask or disposable plastic tube.
8.

0.6M Tris-Hcl, pH6.8	1.0ml
Stock acrylamide	1.35ml
Water	7.5ml
10%SDS	0.1ml
Ammonium persulfate (10%)	0.05ml

   Degas this solution under vacuum for about 30 sec
9. When the separating gel has set, pour off the overlaying water. Add 14 µl of TEMED to the stacking gel. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a

clean Teflon comb into the stacking gel solution, being careful to avoid trapping of air bubbles. Place the gel in a vertical position at room temperature and allow to set for 20min.

Preparation of samples and running the gel:

10. About 10µl of protein sample and 5µl of sample buffer are mixed by vortexing. The sample is than heated for 5min at 95-100°C to denature the proteins. The sample is than kept in ice ***(Note3)***

11. After polymerization is complete, remove the Teflon comb. Rinse out any unpolymerised acrylamide solution from the wells using electrophoresis buffer and assemble the cassette in the electrophoresis tank. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. (***Note: Do not prerun the gel before loading the samples, since this procedure will destroy the discontinuity of buffer system.)***

12. Load upto5-10µl of each of the samples (unknown and standard) in a predetermined order into the wells.

13. Connect the electrophoresis apparatus to the powerpack (the positive electrode should be connected to the bottom buffer reservoir), and pass a current of 30mA through the gel (constant current) for large format gels, or 200V (constant voltage) for minigels (Biorad).The gel is run until the bromophenol blue reaches the bottom of the resolving gel. This will take 2.5-3.0h for large format gels(16µmx16µm) and about 40min for mini gels (10µm x7µm) *(Safety care: Always turnoff &disconnect the power supply before removing the lid)*

14. Dismantle the gel apparatus, pry open the gel plates;remove the gel,discard the stacking gel,and place the separating gel in stain solution.

15. Staining should be carried out with shaking,for a minimum of 2h at room temperature. Destain the gel by soaking it in the methanol:acetic acid solution on a slowly rocking platform for 4-8 hrs.

16. After destaining, store the gels in H_2O containing 20% glycerol

17. The gel can now be used for immunoblotting to determine the protein sample

Task Required

1. Convert the X, S, P experimental data provided in the table 1 into gL-1 using Equation 1 and 2; Figures 1 and 2.

2. Plot a graph between biomass concentrations (X) vs. time and ln X vs. t.

3. Calculate the specific growth rate (µ) in each sampling interval and also in logarithmic phase. Linear part of the ln X vs. t graph, which is exponential phase of growth, will be taken for specific growth calculation for logarithmic phase.

4. Calculate the specific substrate uptake rate (qs) and specific product formation rate (qp) and tabulate all the results in table 2

5. Explain the results.

Table-2

t	*X* (gL-1)	*S* (gL-1)	P (gL-1)	Time interval	ln(X_t/X_{t-1})	μ (h-1)	q_s (g of S/g of X/time)	q_p (g of P/g of X/time)
t_0 (0th h)	Xo	S0	P0		-	-		
t_1 (2nd h)	X1	S1	P1	t1 to t0	ln(X1/X0)	ln(X1/X0)/(t_1-t_0)	(S_0 –S_1)/{(X_1-X_0)*(t_1-t_0)}	(P1 – P_0)/{(X_1-X_0)*(t_1-t_0)}
t_2 (4th h)	-	-	-	-	-	-	-	-
t_3(6th h)	-	-	-	-	-	-	-	-
t_4(8th h)	-	-	-	-	-	-	-	-
t_5(10th h)	-	-	-	-	-	-	-	-
t_6(12th h)	-	-	-	-	-	-	-	-
t_7(24th h)	-	-	-	-	-	-	-	-

References

1. Sharma, S. P., Anjankar, A. P., & Kale, A. (2017). Comparison of glucose levels using glucometer and GOD-POD Method in diabetic patients. *Int. J. Clin. Biochem. Res.*, *4*, 6-10.
2. Mukherjee, M., Sarkar, P., Goswami, G., & Das, D. (2019). Regulation of butanol biosynthesis in Clostridium acetobutylicum ATCC 824 under the influence of zinc supplementation and magnesium starvation. *Enzyme and microbial technology*, *129*, 109352.