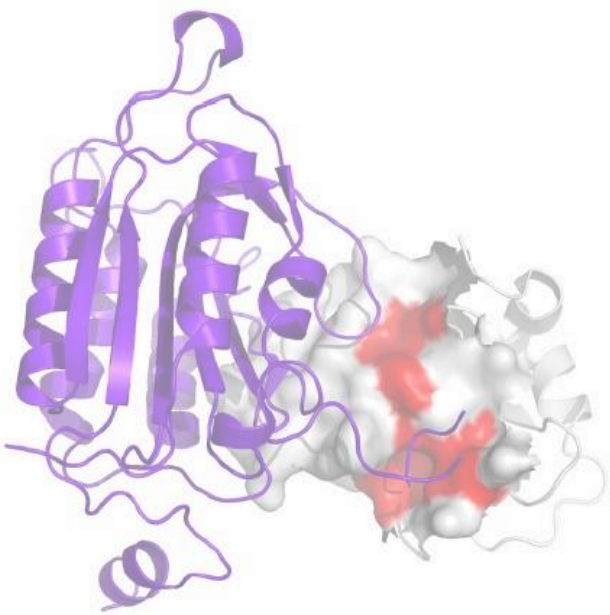

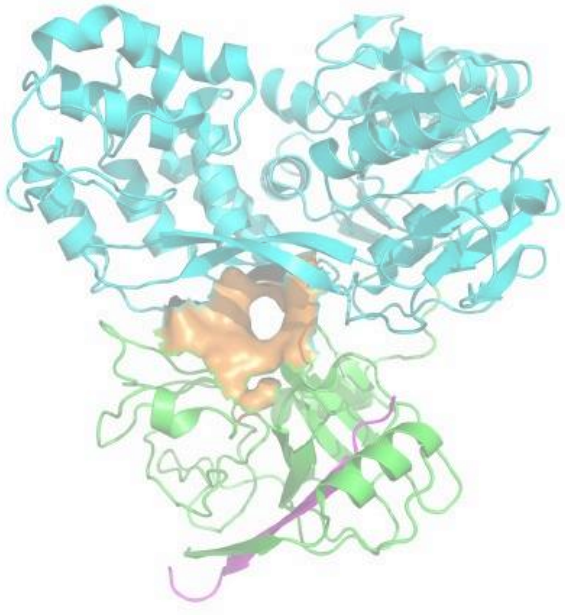

ENZYME SCIENCE AND ENGINEERING

BBL433 Lab Manual

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GENERAL LAB RULES

- All the students should reach the UG lab before or at 2:30 pm sharp
- Lab coats are compulsory for all the students
- Students would perform all the experiments in pair
- All the protocols would be uploaded to the teams before the respective experiment. Kindly refer to it before and during the experiments
- Students are requested to handover the pipettes and other lab materials issued to them before leaving the lab
- If a student faces difficulty in performing the protocol, they must ask their TAs to help
- All the students must record their results and do the analysis before their next lab
- Eating and drinking are not allowed in the lab
- Students should work sincerely during lab hours and not indulge in talking in groups

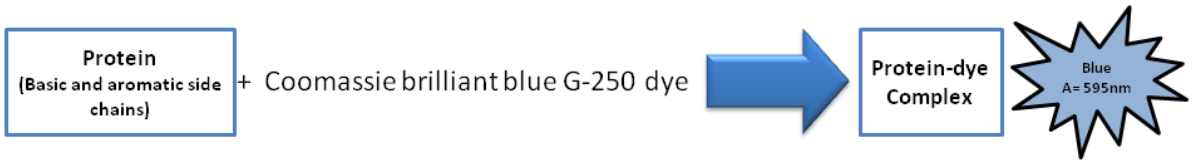
LIST OF EXPERIMENTS

1. Protein quantification by using the Bradford method
2. Assay of β-Glucosidase
3. Assay of Alcohol Dehydrogenase (ADH)
4. Isolation of invertase enzyme from *Saccharomyces cerevisiae* and calculation of enzyme activity
5. Kinetic Analysis of enzyme-catalyzed reaction (Determination of Km and Vm)
6. Kinetic Characterization of the enzyme and in presence of an inhibitor
7. Enzyme entrapment in calcium alginate beads
8. Kinetic characterization of enzyme immobilized in calcium alginate beads
9. Study conversion rate in CSTR enzyme reactors
10. Study conversion rate in PFR enzyme reactors

1. Protein Quantification by using the Bradford method

BACKGROUND

The Bradford protein assay is used to measure the concentration of total protein in the sample. The binding of protein molecules to the Coomassie dye under acidic conditions results in the color change from brown to blue which can be quantified. The method primarily measures the presence of the basic amino acids- lysine, arginine, and histidine which form the protein-dye complex. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.



MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Weighing Balance
3. Cuvettes
4. Pipettes and Tips
5. Eppendorfs
6. Test tubes/ Falcons

REAGENTS

1. Bradford reagent
2. Bovine Serum Albumin (BSA) (2mg)
3. Standard solutions of protein
3. Distilled Water

PROTOCOL

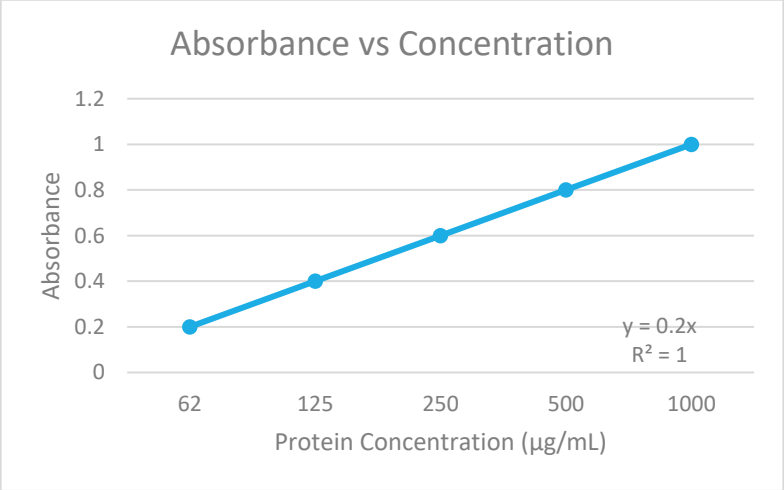
1. Dilute the stock solution of protein (2 mg/mL) in the range of 50 to 1000 µg/mL using distilled water to prepare the standards. Prepare each stock with a final volume of 100µL.

Note: You can either use the standards of concentration- 62.5, 125, 250, 500, 1000 µg/mL or prepare of your own choice.

2. After preparing the dilutions and blank (water and Bradford reagent) sample, add 1mL of Bradford reagent to each tube along with the unknown samples. Incubate for 10 minutes at room temperature away from light.
3. Set the spectrophotometer to 595nm. Zero the instrument using a blank sample. Measure the absorbance of the standards and unknown samples.
4. Also, measure the absorbance of BSA stock solution (2mg/mL) in the spectral range of 260nm to 400nm and prepare an absorbance curve.

CALCULATIONS

- Prepare a standard curve of absorbance versus protein concentration.
- Determine the concentrations of unknown samples from the curve equation.



POINTS TO REMEMBER

1. The dilutions should be carefully made from the stock solution
2. Perform the experiments away from light or in dark
3. Make sure the reagents are not contaminated. Cuvettes should be clean.
4. The incubation time should not be less than 5 minutes or more than 30 minutes.

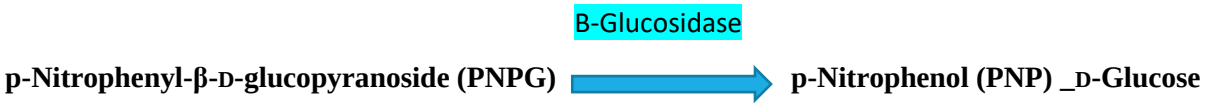
NOTES:

1. The Bradford assay is rapid, sensitive, and has high reproducibility. It is widely used to determine protein concentration due to its ease of use, formation of stable protein-dye complex, and occurrence of minimal interferences.
2. The limitation of the Bradford assay is the incompatibility with low concentrations of detergents, which are routinely used to solubilize membrane proteins. However, the detergent substances can be removed by gel filtration, dialysis, or precipitation of the proteins with calcium phosphate.

2. Assay of β- Glucosidase

BACKGROUND

β-Glucosidase is an enzyme that acts upon the β 1→4 bonds linking two glucose or glucose-substituted molecules. It catalyzes the hydrolysis of terminal non-reducing residues in β-D- glucosides with the release of glucose. β – glucosidase activity can be measured by monitoring the formation of a chromogenic product p-nitrophenol, which is formed by hydrolysis of p-nitrophenol – β- D – glucopyranoside (PNPG). The amount of PNP formed can be measured by determining the absorbance at 405nm. The amount of PNP produced is proportional to the amount of β- glucosidase at the time of the reaction. The reaction is stopped by adding Na_2CO3 which shifts the pH of the reaction mixture to alkaline (pH = 11.0). At this pH most of the PNP is converted to the yellow-colored anionic form and β- glucosidase is inactivated.



MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Water Bath
3. Vortex Mixer
4. Cuvettes
5. Pipettes and Tips
6. Eppendorfs
7. Test tubes/ Falcons

REAGENTS

1. Citrate Buffer (50mM) (pH: 4.8)
2. PNPG solution (1mM) in Citrate buffer
3. PNP solution (1mM) in Citrate buffer
4. Sodium Carbonate solution (1N) in distilled water
5. Distilled water

PROTOCOL

I. For standard Curve-

a. Serially dilute the PNP solution in the range of 10µM to 100µM with citrate buffer to the final volume of 1mL. You can use- 20, 40, 60, 80, 100 µM dilutions or prepare of your choice. (Note: Use Citrate buffer to dilute the PNP; not water)

b. Add 0.5mL of 1N Na_2CO_3 to each tube and measure the OD at 405nm against the reagent blank (Blank= 1 mL Citrate buffer + 0.5 mL 1N Na_2CO_3)

II. For Enzyme Assay-

a. To 100µL of the enzyme, add 0.9 mL of PNPG solution, pre-equilibrated at 50^oC

b. Incubate the solution in a water bath for exactly 10 minutes

c. Add 0.5 mL of 1N Na_2CO_3 to each tube to stop the reaction

d. Measure the absorbance at 405nm [Blank= 0.1mL of Citrate buffer + 0.9mL PNPG + 0.5 mL of 1N Na_2CO_3]

CALCULATIONS

IU/mL= [µmoles of PNP released * dilution of enzyme]/ [Incubation time in minutes * volume of enzyme in mL]

POINTS TO REMEMBER

1. The dilutions should be carefully made from the stock solution in citrate buffer
2. Make sure the reagents are not contaminated. Cuvettes should be clean.
3. Use the pre-incubated PNPG at 50^oC
4. The incubation time should be exactly 10 minutes

3. Assay of Alcohol Dehydrogenase (ADH)

BACKGROUND

Alcohol dehydrogenase (ADH) refers to a family of enzymes that catalyze the reversible oxidation of primary or secondary alcohols to aldehydes or ketones respectively. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes. These enzymes use either NAD^+ or $NADP^+$ as their coenzyme which is reduced during dehydrogenation. A typical example is alcohol dehydrogenase (ADH) which catalyzes the following reaction:

$$R - CH_2 - OH + NAD^+ \rightarrow R - CHO + NADH + H^+$$

Reduced NAD^+(NADH) exhibits strong UV absorption at 340 nm whilst the oxidized form has virtually no absorption at this wavelength. Therefore, if one starts with a mixture of ethanol, NAD^+ and enzyme in buffer, the reaction proceeds until equilibrium is established. The reaction may be followed by measuring the increase in absorbance of the solution at 340 nm as NADH is formed.

MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Cuvettes
3. Pipettes and Tips
4. Micro-centrifuge Tubes (MCTs)

REAGENTS

1. 0.05 M Phosphate Buffer (pH = 8.8)
2. 25mM NAD^+
3. 2M Ethanol

PROTOCOL

1. Prepare reaction systems in MCTs as per follows:
    a. Test: *0.6 ml Phosphate buffer + 0.2 ml NAD^+ + 0.1 ml Enzyme (Final Reaction Volume = 0.9 ml)*
    b. Blank: *0.75 ml Phosphate buffer + 0.2 ml NAD^+ + 0.1 ml Enzyme (Final Reaction Volume = 1.050 ml)*

2. Mix well and transfer 1 ml of Blank solution to the cuvette and calibrate the blank in UV spectrophotometer at 340nm.
3. Now, start the reaction by adding 0.15 ml of alcohol to the Test and mix well. Immediately transfer the 1 ml from it to the cuvette and measure absorbance at 340 nm.
    a. Keep the cuvette inside the UV spectrophotometer and take readings at every 30 seconds for 2-3 minutes.
4. Using the readings of change in absorbance per unit time (dA/dt) calculate the unit activity of the enzyme.

CALCULATIONS

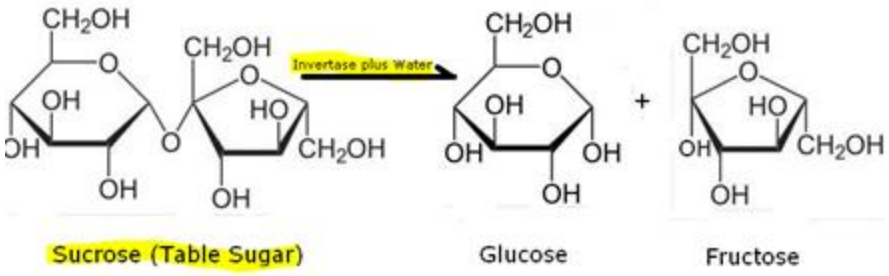
According to the Lambert-Beer law, absorbance is proportional to formed NADH concentration (Molar Extinction Coefficient for NADH at 340nm = 6.220 L/mM/cm).

1. Calculate the moles of NADH produced per minute using the beer-lambert's law.
2. Enzyme activity is calculated by the following formula: **[Absorbance* Dilution factor]/6.22**
3. Now calculate the unit activity of the enzyme based on the following definition:
    a. One unit will convert 1.0 µmole of ethanol to acetaldehyde per minute at pH 8.8 at 25^{o}C. Therefore, one unit will also convert 1.0 µmole of NAD^{+} to NADH per minute.

4. Isolation of invertase enzyme from *Saccharomyces cerevisiae* and calculation of enzyme activity

BACKGROUND

Invertase, also called beta-fructofuranosidase cleaving the terminal non-reducing beta-fructofuranoside residues, is a glycoprotein with an optimum pH of 4.5 and stability at 50 °C. It catalyzes the hydrolysis of the disaccharide sucrose into glucose and fructose. The resulting mixture of fructose and glucose is referred to as "inverted sugar" because of the inversion of its optical property from the positive rotation to the negative rotation. It is widely distributed in the biosphere, especially in plants and microorganisms. Saccharomyces cerevisiae commonly called baker's yeast is the chief strain used for the production and purification of the enzyme. Invertase in nature exists in different isoforms. In yeasts, it is present either as extracellular Invertase or intracellular Invertase. In plants, there are three isoforms each differing in biochemical properties and subcellular locations. Invertase in plants is essential not only for metabolism but also help in osmoregulation, development and defense system. In humans, the enzyme acts as an immune booster, as an anti-oxidant, an antiseptic and helpful for bone cancer or stomach cancer patients in some cases.



MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Water Bath
3. Cuvettes
4. Pipettes and Tips
5. Eppendorfs
6. Test tubes/ Falcons

REAGENTS

1. 0.1M sodium bicarbonate solution

2. 100% ethanol
3. 5mM Tris-Cl, pH 7.4
4. 20mM sucrose solution (freshly prepared)
5. Alkaline DNS
6. 50mM acetate, pH 4.8, 3ml per group

PROTOCOL

1. Centrifuge 1.5ml yeast extract solution at 7500rpm for 2min at 4°C
2. Add Ice-cold ethanol to the supernatant to get a final concentration of 29%
3. Invert-mix the sample, and place on ice for 2 min followed by centrifugation at 10000rpm for 10min at 4°C
4. Transfer the supernatant to another tube; remove the ethanol completely and resuspend the pellet in 600µL5mM Tris-Cl, pH 7.4
5. Add Ice-cold ethanol to the supernatant to get a final concentration of 40% (be precise on the volume, calculate carefully)
6. Invert-mix the sample, place on the ice for 2min, and centrifuge at 10000rpm for 10min at 4°C
7. Activity testing is to be done on the five suspensions (SAMPLES) obtained after centrifugation.

Activity testing:

a. 5µLof sample + 50µLfreshly diluted 20mM sucrose solution

b. Incubate the mixture at room temperature for 5min

c. Add 200µLalkaline DNS to mixture

d. Incubate the mixture at 90°C for 5min

e. Add 200µL 50mM acetate, pH 4.8 to the mixture

f. Blank- 5µL Tris-Cl (instead of sample) + sucrose+ DNS+ acetate

Dilute the samples 1:10 with distilled water

g. Record all the absorbance reading taken at 540nm and calculate the Enzyme activity using standard curve.

Standard curve:

The stock solution of glucose is 20mM. Prepare five dilutions in the range- 0mM to 20mM [0, 4, 8, 12, 16, 20 mM].

a. 50 µL of glucose + 200 µL of DNS

b. Incubate the mixture at 90°C for 5min

c. Add 200µL 50mM acetate, pH 4.8 to the mixture

d. Blank- 5µL water (instead of sample) + DNS+ acetate [Treat the blank same as your samples]

Dilute the samples 1:10 with distilled water

e. Record all the absorbance reading taken at 540nm and prepare the standard curve.

5. Kinetic Analysis of enzyme-catalyzed reaction (Determination of Km and Vm)

BACKGROUND

For an enzyme showing the typical Michaelis-Menten kinetics:

$$V_0 = \frac{V_{max}[S]}{[S] + K_m} \quad \Rightarrow \quad \frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

The kinetic parameters, K_m and V_{max} can be determined by plotting a graph of between 1/V vs 1/[S]. This plot is called the Lineweaver-Burk plot. The intercept on the Y-axis gives $1/V_{max}$ and the slope equals K_m/V_m. In view of the difficulty in obtaining reliable estimates of K_m and V_{max}, Eisenthal and Cornish-Bowden suggested a new approach. At a constant [S] and V, a hypothetical plot of V_{max} vs K_m is linear. When $K_m = 0$, $V_{max} = v$ and at $V_{max} = 0$, $K_m = -[S]$. Each data point of the reaction, i.e., V_i and S_i will yield a straight line and the intersection of these lines will give a reliable estimate of the true K_m and V_{max}.

MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Water Bath
3. Cuvettes
4. Pipettes and Tips
5. Eppendorfs
6. Test tubes/ Falcons

REAGENTS:

1. 1M sucrose solution (freshly prepared)
2. Alkaline DNS
3. 50mM sodium acetate buffer, pH 4.8
4. 100IU/ml invertase enzyme
5. Distilled water

PROTOCOL:

Prepare five different concentrations of sucrose (0 to 300mM) and follow the below mentioned steps for each dilution (Stock Concentration= 1M)

a. 5µLof enzyme + 50µLfreshly diluted sucrose solution

b. Incubate the mixture at room temperature for 5min

c. Add 200µL alkaline DNS to mixture

d. Incubate the mixture at 90°C for 5min

e. Add 200µL 50mM acetate, pH 4.8 to the mixture

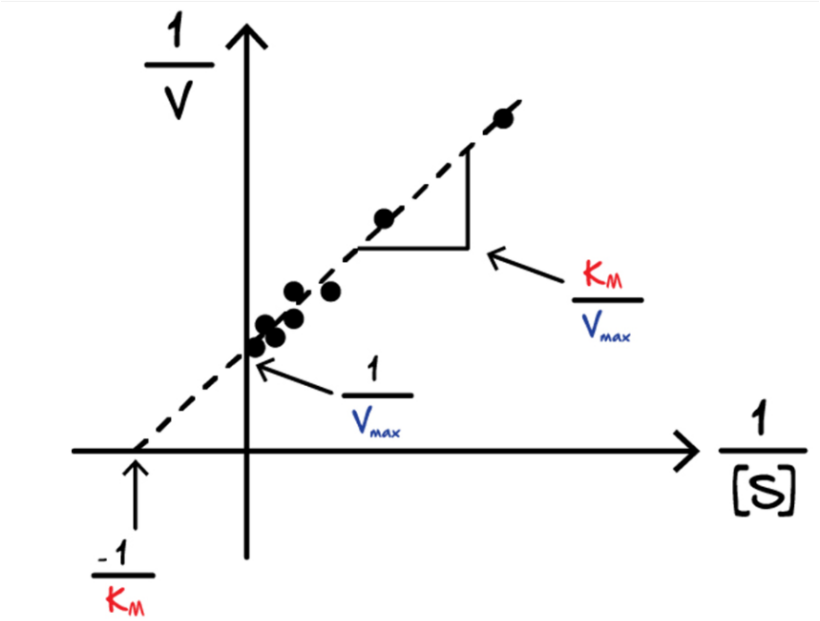
f. Blank- 5µL Tris-Cl (instead of sample) + sucrose+ DNS+ acetate

Dilute the samples 1:100 with distilled water

g. Record all the absorbance reading taken at 540nm and calculate the Enzyme activity using standard curve.

RESULTS:

Prepare a lineweaver- burk plot as shown-



6. Kinetic Characterization of the enzyme and study of substrate inhibition

BACKGROUND

For an enzyme showing the typical Michaelis-Menten kinetics:

$$V_0 = \frac{V_{max}[S]}{[S] + K_m} \quad \Rightarrow \quad \frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

The kinetic parameters, K_m and V_{max} can be determined by plotting a graph of between 1/V vs 1/[S]. This plot is called the Lineweaver-Burk plot. The intercept on the Y-axis gives $1/V_{max}$ and the slope equals K_m/V_m. In view of the difficulty in obtaining reliable estimates of K_m and V_{max}, Eisenthal and Cornish-Bowden suggested a new approach. At a constant [S] and V, a hypothetical plot of V_{max} vs K_m is linear. When $K_m = 0$, $V_{max} = v$ and at $V_{max} = 0$, $K_m = -[S]$. Each data point of the reaction, i.e., V_i and S_i will yield a straight line and the intersection of these lines will give a reliable estimate of the true K_m and V_{max}. The three main mechanisms for the inhibition of the enzyme-catalyzed reactions can be kinetically expressed as:

1. Competitive inhibition:

$1/V = 1/V_{max} + Km/Vm\ (1+1/Ki).1/S$

Vm is unaffected but the apparent Km is increased by a factor (1+1/Ki)

2. Non-competitive inhibition:

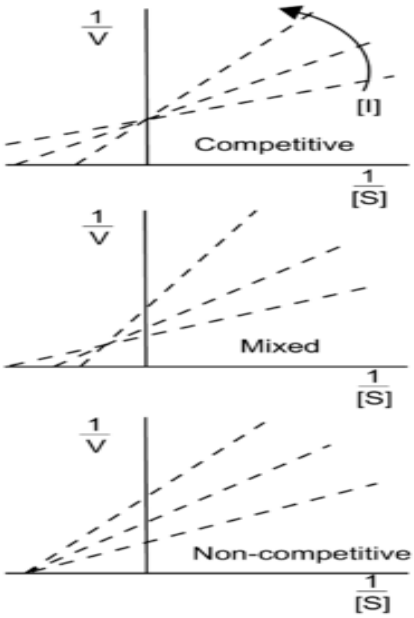
$1/V = 1/Vm\ (1+1/Ki) + Km/Vm(1+1/Ki).1/S$

Km remains unaffected but Vm is decreased by a factor 1/(1+1/Ki)

3. Mixed inhibition:

$1/V = 1/Vm(1+1/Ki) + Km/Vm.1/S$

Here both Km and Vm are affected by inhibitor.



MATERIALS AND EQUIPMENT

1. Spectrophotometer
2. Water Bath
3. Cuvettes
4. Pipettes and Tips
5. Eppendorfs
6. Test tubes/ Falcons

REAGENTS:

1. 1M sucrose solution (freshly prepared)
2. Alkaline DNS
3. 50mM sodium acetate buffer, pH 4.8
4. 100IU/ml invertase enzyme
5. $CuSO_4$ (two concentrations)
6. Distilled water

PROTOCOL

a. 5µL of sample + 50µL freshly diluted sucrose solution + 545µL distilled water/$CuSO_4$ conc. 1/ $CuSO_4$ conc. 2

b. Incubate the mixture at 30°C for 5min

c. Add 200µL alkaline DNS to mixture

d. Incubate the mixture at 90°C for 5min

e. Add 200µL 50mM sodium acetate buffer, pH 4.8 to the mixtures

f. Blank - 50µL distilled water (instead of sucrose)

Dilute the samples 1:10 with distilled water

g. Record all the absorbance readings taken at 540nm and plot the Lineweaver-Burk plot for all three cases.

RESULTS

1. **Without Inhibitor**

Tube	**Absorbance**	**Sucrose concentration**	**µmoles of sucrose hydrolysed**	**µmoles of sucrose hydrolysed/ min (v_{max})**	**1/[S]**	**$1/V_{max}$**
Blank						
1						
2						
3						
4						
5						

2. **With Inhibitor (1mM)**
3. **With Inhibitor (10mM)**

7. Enzyme entrapment in calcium alginate beads and kinetic characterization of enzyme immobilized in calcium alginate beads

Background

Immobilization of enzyme by gel entrapment involves the entrapment of the biocatalyst within a polymeric network. The biocatalyst after mixing with the aqueous polymeric solution is forced through a fine orifice (syringe) into a salt solution that insolubilizes the mixture through ion exchange. The shape and size of beads can be controlled by choosing the orifice diameter and the distance of the nozzle from the liquid surface. Gel entrapment usually does not result in any adverse modification of the enzyme conformation and can provide high yield of immobilization.

Materials and Equipments

1. Spectrophotometer
2. Water Bath
3. Cuvettes
4. Pipettes and Tips
5. Eppendorfs
6. Test tubes/ Falcons
7. Syringes

Reagents:

1. 30 mL Acetate buffer (0.05 M, pH 5)
2. 15 mL Sodium alginate 2% w/v in acetate buffer
3. 15 mL invertase enzyme extracted from yeast
4. 1 mL 1 M sucrose in acetate buffer
5. 100 mL 4% w/v $CaCl_2$ in acetate buffer
6. Alkaline DNS
7. 100µL invertase enzyme
8. Distilled water

PROCEDURE:

1. The enzyme was mixed to the sodium alginate solution (equal volumes) to bring the final concentration of sodium alginate to 1% w/v.
2. This slurry was added drop-wise using a pump/syringe into chilled calcium chloride solution under gentle continuous stirring at 5-10 rpm.
3. The calcium alginate beads were cured in calcium chloride solution for 30 minutes and washed thoroughly with the sodium acetate buffer.
4. The gel beads were suspended in acetate buffer.
5. The diameter was measured and also weight of beads formed by pipette and syringe using graph paper and weighing balance.

Kinetic characterization of the calcium alginate beads-

Prepare 3 different concentrations of sucrose ([S]=K_m, [S]<K_m and [S]>K_m) and follow the below mentioned steps for each dilution (Stock concentration of sucrose= 1M)

a. 5µLof free/immobilized enzyme + 50µLfreshly diluted sucrose solution

b. Incubate the mixture at 30 °C for 5min

c. Add 200µL alkaline DNS to mixture

d. Incubate the mixture at 90°C for 5min

e. Add 200µL 50mM acetate, pH 4.8 to the mixture

f. Blank (no sucrose) + DNS+ acetate

Dilute the samples appropriately with distilled water.

Record all the absorbance reading taken at 540nm and calculate the enzyme activity using standard curve.

8. Immobilized beads in PBR

BACKGROUND:

Immobilization of enzyme by gel entrapment involves the entrapment of the biocatalyst within a polymeric network. The biocatalyst after mixing with the aqueous polymeric solution is forced through a fine orifice (syringe) into a salt solution that insolubilizes the mixture through ion exchange. The shape and size of beads can be controlled by choosing the orifice diameter and the distance of the nozzle from the liquid surface. Gel entrapment usually does not result in any adverse modification of the enzyme conformation and can provide high yield of immobilization.

REAGENTS AND MATERIALS REQUIRED:

1. Invertase-entrapped calcium alginate beads
2. 0.1M sucrose solution
3. Alkaline DNS
4. 50mM sodium acetate buffer, pH 4.8
5. Spectrophotometer
6. Water Bath
7. Glass Wool
8. PBR set up

PROCEDURE:

1. Fill ⅓ height of glass vessel with glass wool.
2. Add prepared beads (40ml)
3. Fill the vessel with glass wool.
4. Adjust flow rate to 2mL/min.
5. Connect input and output lines and pass substrate through the packed bed.
6. Collect 50 µL sample every 0, 10, 20, 30, 40, 50, 60 mins.
7. Perform kinetic characterization of the calcium alginate beads –
    g. Add 200µL alkaline DNS to 50µL sample obtained from the reactor.

    ↓

    h. Incubate the mixture at 90^{o}C for 5min

    ↓

    i. Add 200µL 50mM acetate, pH 4.8 to the mixture

    ↓

    j. Blank (without enzyme) + sucrose + DNS+ acetate

    ↓ Dilute the samples appropriately with distilled water.

Record all the absorbance reading taken at 540nm and calculate the enzyme activity using standard curve.

9. Immobilized beads in CSTR

BACKGROUND:

Immobilization of enzyme by gel entrapment involves the entrapment of the biocatalyst within a polymeric network. The biocatalyst after mixing with the aqueous polymeric solution is forced through a fine orifice (syringe) into a salt solution that insolubilizes the mixture through ion exchange. The shape and size of beads can be controlled by choosing the orifice diameter and the distance of the nozzle from the liquid surface. Gel entrapment usually does not result in any adverse modification of the enzyme conformation and can provide high yield of immobilization.

REAGENTS AND MATERIALS REQUIRED:

1. Invertase-entrapped calcium alginate beads
2. 0.1M sucrose solution
3. Alkaline DNS
4. 50mM sodium acetate buffer, pH 4.8
5. Spectrophotometer
6. Water Bath
7. Glass Wool
8. CSTR set up

PROCEDURE:

1. The input of the vessel is covered with glass wool.
2. Add 200 ml of sucrose solution to the CSTR reactor.
3. Add 40ml of immobilized invertase calcium alginate beads.
4. Adjust the flow rate to 2 ml/ min.
5. Add the magnetic bead into the solution and stir the solution.
6. Collect 50 µL sample at every 0, 10, 20, 30, 40, 50, 60 mins.
7. Perform kinetic characterization of the calcium alginate beads –
   a. Add 200µL alkaline DNS to 50µL sample obtained from the reactor.

   ↓

   b. Incubate the mixture at 90^{o}C for 5min

   ↓

   c. Add 200µL 50mM acetate, pH 4.8 to the mixture

   ↓

   d. Blank (without enzyme) + sucrose + DNS+ acetate

   ↓ Dilute the samples appropriately with distilled water.

Record all the absorbance reading taken at 540nm and calculate the enzyme activity using standard curve.

10. Immobilized beads in batch reactor

BACKGROUND:

Immobilization of enzyme by gel entrapment involves the entrapment of the biocatalyst within a polymeric network. The biocatalyst after mixing with the aqueous polymeric solution is forced through a fine orifice (syringe) into a salt solution that insolubilizes the mixture through ion exchange. The shape and size of beads can be controlled by choosing the orifice diameter and the distance of the nozzle from the liquid surface. Gel entrapment usually does not result in any adverse modification of the enzyme conformation and can provide high yield of immobilization.

REAGENTS AND MATERIALS REQUIRED:

1. Invertase-entrapped calcium alginate beads
2. 0.1M sucrose solution
3. Alkaline DNS
4. 50mM sodium acetate buffer, pH 4.8
5. Spectrophotometer
6. Water Bath
7. Batch reactor set up

PROCEDURE:

1. Take 200 ml of sucrose.
2. Add 40 ml beads to the batch reactor.
3. Collect sample every 0, 10, 20, 30, 40, 50, 60 mins.
4. Perform kinetic characterization of the calcium alginate beads –
   a. Add 200µL alkaline DNS to 50µL sample obtained from the reactor.

   b. Incubate the mixture at 90^{o}C for 5min

   c. Add 200µL 50mM acetate, pH 4.8 to the mixture

   d. Blank (without enzyme) + sucrose + DNS+ acetate

Dilute the samples appropriately with distilled water.

Record all the absorbance reading taken at 540nm and calculate the enzyme activity using standard curve.