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Recombinant Enzymes - From Basic Science to Commercialization

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Chapter 3
Common Laboratory Procedure

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Abstract This chapter presents all common laboratory procedures or protocols that researchers normally perform during studies of recombinant enzyme production. The principle and theory behind each experiment is presented to help the reader understand each step that is conducted in an experiment so the reader will be able to select and modify the procedures according to the type of enzyme they are working with. Examples of procedures and the results obtained during recombinant bromelain production are used to develop readers' understanding.

Keywords Casein · Colorimetric protein assay · Protein assay · Turbidity

3.1 Introduction

There are many experiments involved with recombinant enzyme research. However, when dealing with the production of recombinant enzymes, there are a few experiments that are typically repeatedly performed throughout the pre-commercialization stage. Among the popular analyses that are commonly conducted are bacterial growth analysis, total protein content, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme activity assays. The listed assays are important because they will help researchers to calculate the productivity of recombinant enzyme fermentation.

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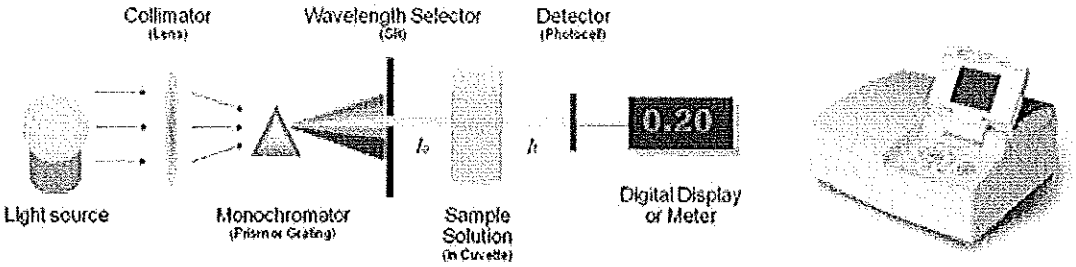


Fig. 3.1 Schematic diagrams on bacterial growth observation by turbidity. (From chemwiki.ucdavis.edu)

3.2 Estimating Bacterial Growth by Turbidity Measurement

Turbidity measurement is used for this type of research because it provides fast results. As bacteria multiply in a liquid medium, the medium becomes turbid. Therefore, the more turbid the medium becomes, the greater the number of bacteria that are present. The instrument used in this protocol is a spectrophotometer.

3.2.1 Principle

According to Tortora et al. [1], a beam of light is transmitted through a collimator (a lens), a monochromator (to select the suitable wavelength) and finally a bacterial suspension to a light-sensitive detector (Fig. 3.1). Once the number of bacteria increases, less light will reach the detector, and the instrument will indicate the absorbance or optical density (OD).

3.2.2 Objective of Experiment

This experiment aims to quantitate the amount of bacteria in the culture and plot a graph of OD600 versus time of fermentation.

3.2.3 Materials and Methods

Consumable item

Disposable pipette tips (200 µL and 1 mL)
Cuvette (15 mL)

Equipment

No	Equipment	Usage
1	Pipettes and dispenser (200 µL)	To add any solution into cuvette
2	Spectrophotometer (A600 nm)	To read absorbance at 600 nm

Chemicals and Reagents

No	Chemicals	Manufacturer
1	Bacteria culture	NA
2	Luria broth	Sigma-Aldrich, St. Louis, USA

Methodology

1. Add 1 ml of sterile Luria broth into a cuvette and read the OD600. Set this as a blank for other samples.
2. Take 1 ml of sample for every hour and add into a cuvette and read the OD600.
3. Plot a graph of OD600 against time.

3.2.4 Results and Discussion

Table 3.1 shows the raw data collected during our observation of the absorbance reading of a bacterial culture. The raw data gathered in Table 3.1 is then processed and translated into a graph. The graph (Fig. 3.2) shows that the bacterial growth is at the lag phase from the inoculation time until 4 h, and then, the culture entered the log phase until 11 h of fermentation. The bacterial growth reached a maximum level (OD600 = 6.128) before the culture entered its death phase.

3.3 Total Protein Assay

3.3.1 Principle

The BRADFORD protein assay was first reported by Bradford [2] in 1976. It is a colorimetric protein assay that is based on the binding between the hydrophobic pockets of the protein sample at the non-polar region of the dye through van der Waals forces.

Table 3.1 Raw data presenting absorbance of bacteria culture at OD600 during 12 h of fermentation

Hour	OD600			Average	Std. Deviation
	R1	R2	R3		
1	0.037	0.04	0.031	0.036	0.003
2	0.048	0.044	0.042	0.044	0.002
3	0.097	0.091	0.093	0.093	0.002
4	0.0286	0.32	0.331	0.226	0.140
5	1.272	1.099	1.29	1.220	0.086
6	1.71	2.476	2.058	2.081	0.313
7	2.606	3.08	2.818	2.834	0.193
8	3.614	3.806	3.808	3.742	0.090
9	4.594	5.221	4.933	4.916	0.256
10	5.364	5.31	6.32	5.664	0.463
11	6.812	6.741	5.102	6.218	0.789
12	5.151	6.649	4.908	5.569	0.769

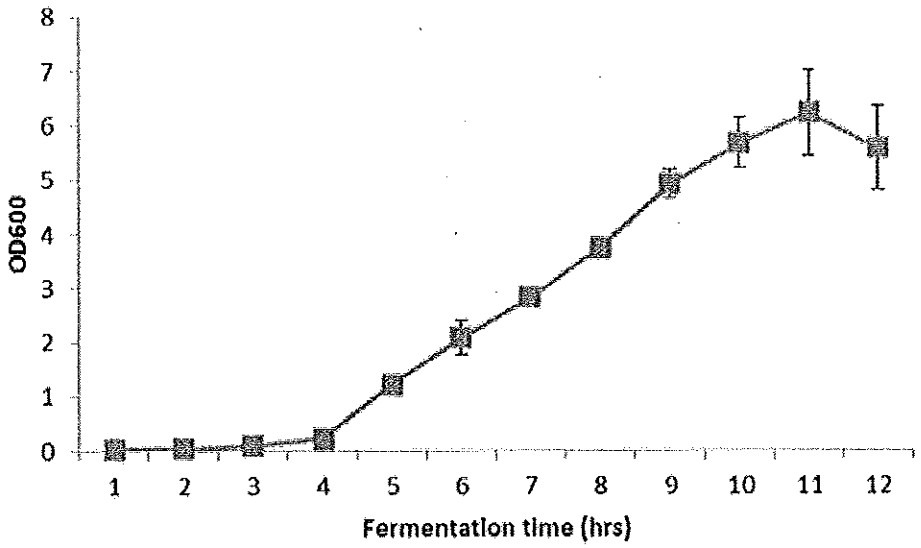


Fig. 3.2 Processed data of bacteria culture absorbance versus the *fermentation time*

The binding stabilizes the blue form of the Coomassie dye. Therefore, the amount of the complex present in solution is a measure of the protein concentration and is estimated by the absorbance reading. The absorbance spectrum for the dye is at 595 nm.

3.3.2 Objective of Experiment

This experiment aims to measure the amount of total protein produced by bacterial fermentation.

3.3.3 Materials and Method

Consumable items

Disposable pipette tips (200 µL and 1 mL)
Cuvette
Microcentrifuge tube (2 mL)

Equipment

No	Equipment	Usage
1	Pipettes and dispenser (20 & 200 µL)	To add any solution into tube
2	Spectrophotometer (A595 nm)	To read absorbance at 595 nm

Chemical and reagents

No	Chemical	Manufacturer
1	Bradford reagent	Bio Basic, Canada
2	Bovine Serum Albumin (BSA)	Merck, Germany

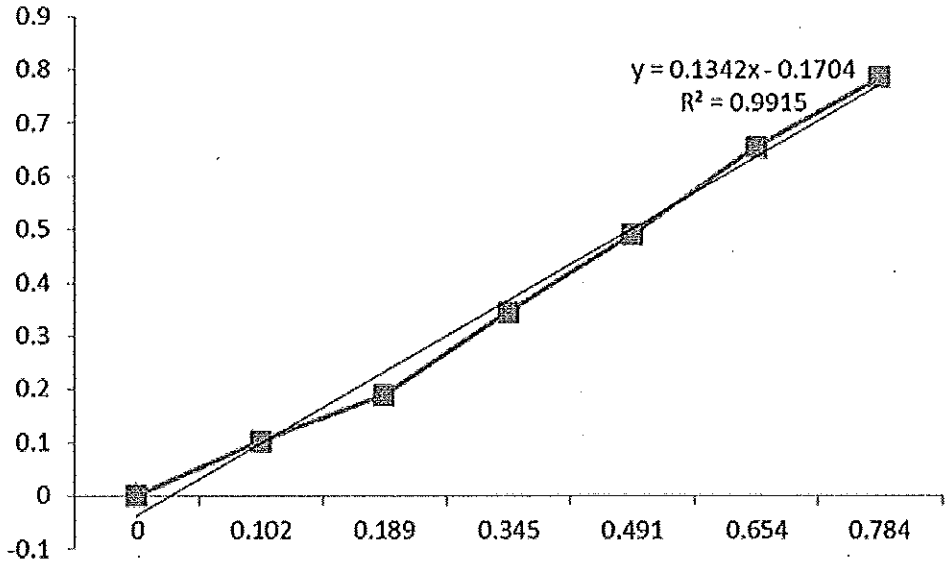


Fig. 3.3 Standard curve develop and use to calculated total protein content

Methodology

1. Prepare 1 mg/mL BSA as a stock.
2. Dilute this BSA to five different concentrations from 0–100 μg/mL in a 2 mL final volume.
3. Add 800 μL of diluted BSA into a microcentrifuge tube and then add 200 μL of Bradford reagent into the same tube.
4. Gently mix the solution by inverting few times and then leave at room temperature for 15 min.
5. Measure the absorbance at 595 nm using a spectrophotometer.
6. Use sample buffer as a blank.
7. Record the absorbance reading, and plot a graph of the concentration of BSA versus absorbance.
8. Repeat step 3, but this time replace BSA with the protein sample.
9. Calculate the amount of total protein using the calibration curve drawn based on the BSA standard (Fig. 3.3).

3.3.4 Results (Table 3.2)

To calculate the total protein, use the below equation, which is deduced from the equation on the graph:

$$x = \frac{y + 0.1704}{0.1342}$$

If the absorbance for a protein sample is 0.456, the total protein is

$$x = \frac{0.456 + 0.1704}{0.1342}$$

$$x = 4.66\ \mu g$$

Table 3.2 Raw data obtained for total protein content using BSA as standard

Protein content (μg)	A595		
	1	2	Average
0	0	0	0
10	0.098	0.105	0.102
20	0.203	0.174	0.189
40	0.351	0.338	0.345
60	0.491	0.490	0.491
80	0.638	0.669	0.654
100	0.783	0.784	0.784

3.4 Enzyme Activity Assays

In principle, an enzyme activity assay is used to determine the quality of the process in each unit operation during pre-commercialization of a recombinant enzyme. For example, the fermentation process is not considered worthwhile if it produces high biomass, but the expressed protein is not in an active form or the spray-drying process finally decreases the enzyme activity more than 30%. Enzyme activity assays essentially involve allowing the reaction of an enzyme on a selected substrate in a suitable reaction buffer at a certain temperature and for a certain incubation period. The enzyme will catalyze a specific substrate [3], and thus different enzymes will require different substrates for the enzyme activity assay to be performed. Table 3.3 below shows examples of common enzyme assays based on the enzyme tested. The enzyme activity assay procedure in this chapter is based on bromelain, a protease. Therefore, the substrate chosen will be specific for a protease, such as Na-CBZ-L-lysine p-nitrophenyl ester (LNPE) (synthetic substrate) and casein (natural substrate) (Table 3.3).

Table 3.3 Common substrates and brief methods for enzyme activity measurement using the spectrophotometric approach

Enzyme	Substrate	Temperature (°C)	Incubation time (min)	pH	Wavelength (nm)
Bromelain (protease)	Na-CBZ-L-Lysine p-Nitrophenyl Ester (LNPE)	25	5	4.6	340
Protease	Casein	37	10	7.5	660
Lipase	pNP-palmitate	40	5	8.0	410
Cellulase	Glucose (HK)	37	120	5	340
Xylanase	RBB-Xylan	40	10	6	590
α-Amylase	Soluble starch	20	3	6.9	540
Maltase	Maltose	25	30	6.0	340
Pepsin	Hemoglobin	37	10	2	280

Na-CBZ-L-Lysine p-Nitrophenyl Ester + Bromelain> p-Nitrophenol + Na-CBZ-L-Lysine

Fig. 3.4 Chemical reaction of LNPE with bromelain

3.4.1 Enzyme Activity Assay Using LNPE as Substrate

3.4.1.1 Principle

This procedure follows Sigma's continuous spectrophotometric rate determination protocol (Sigma-Aldrich, Germany). In this assay, Na-CBZ-L-Lysine p-Nitrophenyl Ester (LNPE) acts as a substrate in the bromelain (protease) reaction. When bromelain digests LNPE, the p-nitrophenol and Nα-CBZ-$_L$-Lysine are released from the reaction (Fig. 3.4). p-Nitrophenol has a color that is quantifiable and measured as an absorbance value using a spectrophotometer at 340 nm absorbance. The greater the amount of p-Nitrophenol that is released from LNPE, the more color generated, and the stronger the bromelain activity is. The absorbance values per minute generated by the blank will be subtracted from the absorbance values per minute of the bromelain or sample activity. One unit activity of bromelain represents one unit of enzyme releasing 1.0 μmole of p-nitrophenol from Nα-CBZ-$_L$-lysine p-nitrophenol ester per minute at pH 4.6 at 25 °C.

3.4.1.2 Objective of Experiment

The objective of this experiment is to measure bromelain enzyme activity.

3.4.1.3 Materials and methods

Consumable item

Disposable pipette tips (200 μL and 1 mL)
Falcon tube (15 mL)

Equipment

No	Equipment	Usage
1	Pipettes and dispenser (200 μL)	To add any solution into tube
2	Dispenser (2.6 and 2.7 mL)	To add any solution into e tube
3	Spectrophotometer (A340 nm)	To read absorbance at 340 nm
4	pH meter	To read pH

Chemicals and reagents

No	Chemicals	Manufacturer
1	Sodium acetate, trihydrate	Sigma-Aldrich, St. Louis, USA
2	Potassium chloride	Sigma-Aldrich, St. Louis, USA
3	L-Cysteine, hydrochloride, monohydrate	Sigma-Aldrich, St. Louis, USA
4	LNPE	Sigma-Aldrich, St. Louis, USA
5	Acetonitrile	Sigma-Aldrich, St. Louis, USA
6	1 M HCl	Sigma-Aldrich, St. Louis, USA
7	Commercial bromelain (3–7 units/mg)	Sigma-Aldrich, St. Louis, USA

Methodology

Reagent preparation

1. 100 mL of Reagent A (30 mM Sodium Acetate Buffer with 100 mM Potassium Chloride and 1.0 mM L-Cysteine), pH 4.6, at 25 °C.
   a. Add 40.8 mg of sodium acetate trihydrate into 50 mL of deionized dH_2O.
   b. Add 17.6 mg of L-cysteine hydrochloride monohydrate into the solution.
   c. Add 745.5 mg of potassium chloride into the solution.
   d. Adjust pH 4.6 with 1 M HCl at 25 °C.
   e. Bring volume to 100 mL with deionized dH_2O.
2. 1 mL Reagent B (50 mM Nα-CBZ-$_L$-Lysine p-Nitrophenyl Ester (LNPE)), *Freshly Prepare*
   a. Add 21.9 mg of LNPE into 0.2 mL of acetonitrile.
   b. Bring volume to 1.0 mL with deionized dH_2O.

Bromelain Enzyme Solution

1. Add 100 mg of commercial bromelain (3–7 units/mg) into 1666.7 mL of Reagent A to obtain 0.2–0.4 units/mL bromelain solution.

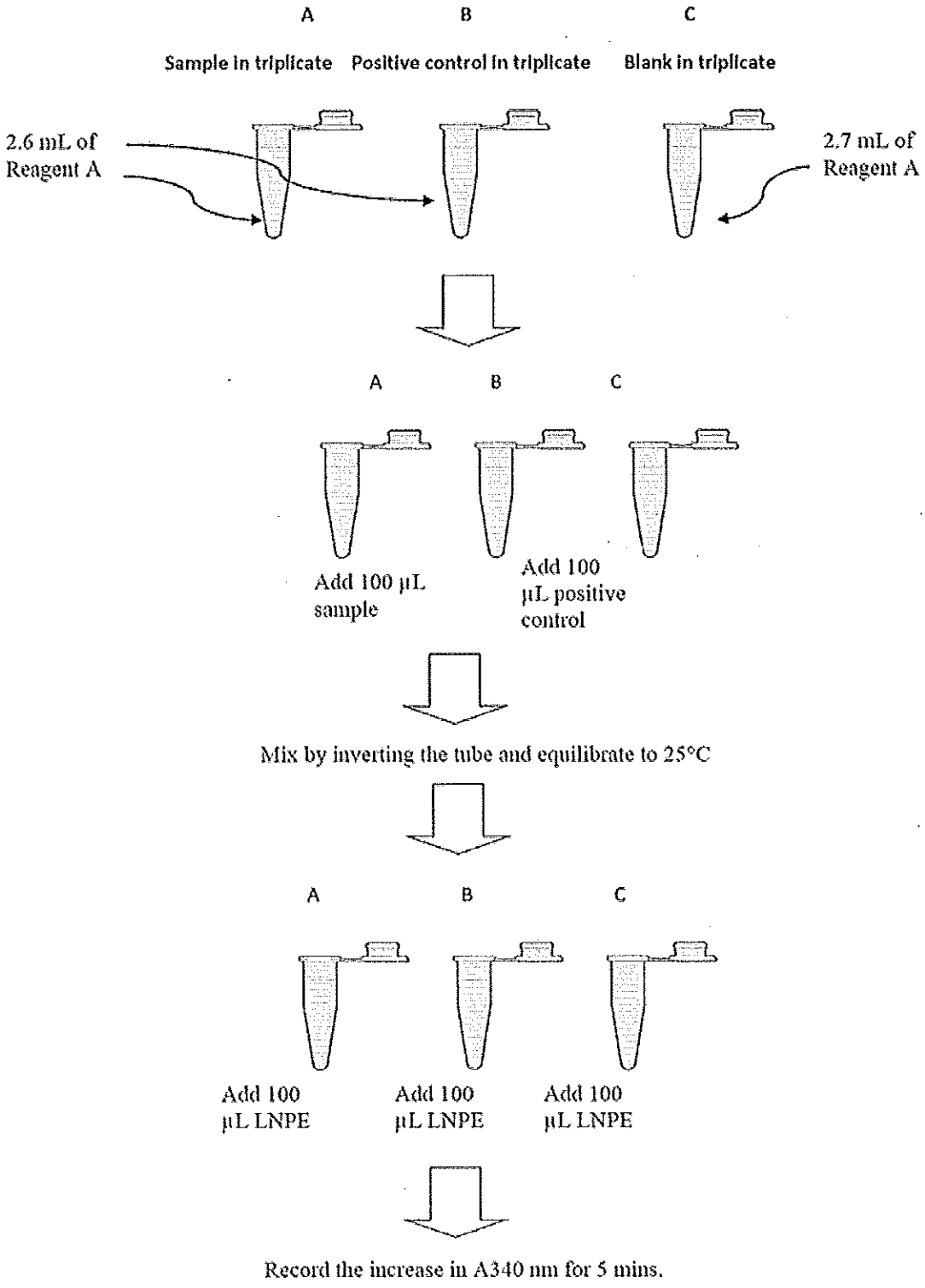
Important notes

Allow sample solution to incubate on ice for approximately 2 h.

The LNPE solution should be used as soon as possible as it will hydrolyze while resting.

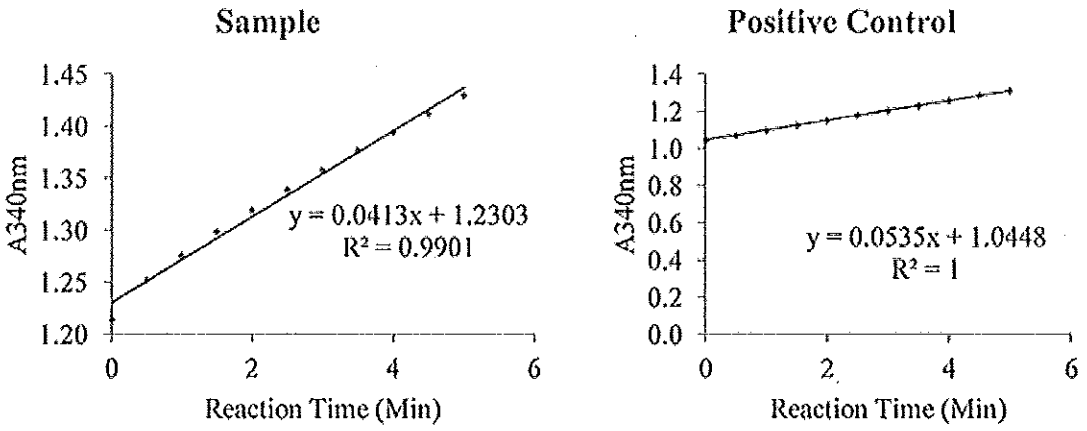
Flow of experiment

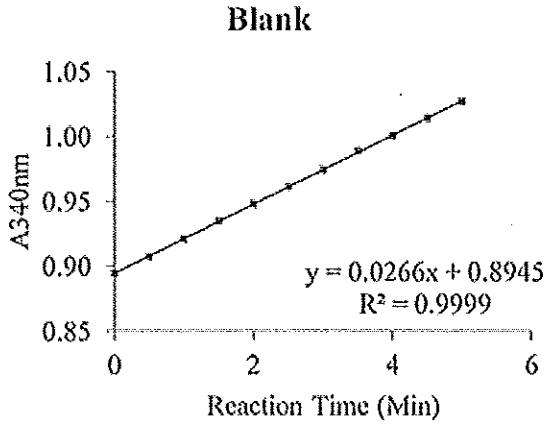
1. Sample, blank and positive control



3.4.1.4 Results and Discussions

Examples of readings obtained after 5 min follow:





Calculation of Enzyme Activity

$$\frac{\text{Units}}{\text{ml}}\text{enzyme} = \frac{\left(\frac{\Delta 340\text{nm}}{\text{min}}_{\text{sample}} - \frac{\Delta 340\text{nm}}{\text{min}}_{\text{blank}}\right)(2.8)(\text{dilution factor})}{(6.32\ \text{milimolar extinction coefficient of p-nitrophenol at 340nm})(0.1\ \text{ml of sample used})}$$

Example of actual case

Blank (Table 3.4)

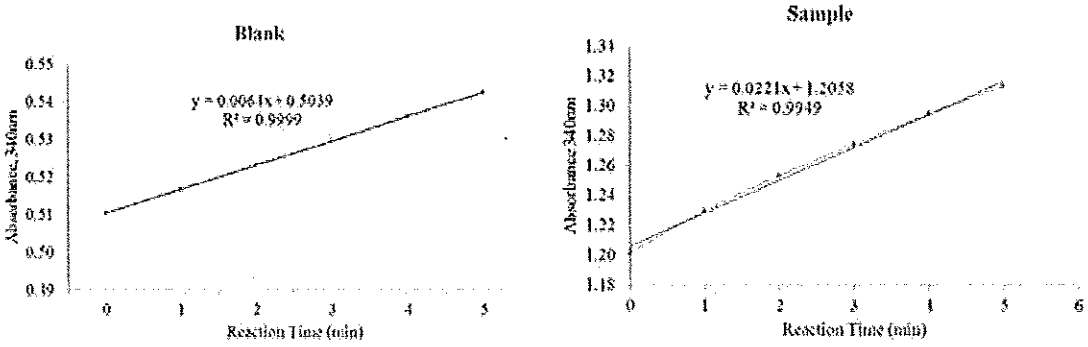


Table 3.4 Raw data obtained for enzyme activity of blank

Reaction Time (min)	Absorbance at 340 nm				
	1	2	3	Average	Std Dev
0	0.1076	0.9355	1.0387	0.6939	0.5104
1	0.1099	0.9492	1.0518	0.7036	0.5167
2	0.1130	0.9637	1.0658	0.7142	0.5231
3	0.1161	0.9785	1.0795	0.7247	0.5295
4	0.1194	0.9924	1.0955	0.7358	0.5363
5	0.1226	1.0072	1.1088	0.7462	0.5424

Table 3.5 Raw data obtain for enzyme activity of samples

Reaction Time (min)	Absorbance at 340 nm				
	1	2	3	Average	Std Dev
0	1.192	1.217	1.197	1.202	0.013
1	1.222	1.243	1.223	1.229	0.012
2	1.247	1.267	1.245	1.253	0.012
3	1.268	1.290	1.265	1.274	0.014
4	1.288	1.309	1.286	1.294	0.013
5	1.308	1.328	1.303	1.313	0.013

Sample (Table 3.5)

Calculations

$$\Rightarrow \frac{\text{Units}}{\text{ml}}\text{enzyme}$$

$$= \frac{\left(\frac{\Delta 340\text{nm}}{\text{min}}_{\text{sample}} - \frac{\Delta 340\text{nm}}{\text{min}}_{\text{blank}}\right)(2.8)(\text{dilution factor})}{(6.32\ \text{milimolar extinction coefficient of p-nitrophenol at 340nm})(0.1\ \text{ml of sample used})}$$

$$= \frac{\left(0.0221_{\text{sample}} - 0.0064_{\text{blank}}\right)(2.8)(1\ \text{dilution factor})}{(6.32\ \text{milimolar extinction coefficient of p-nitrophenol at 340nm})(0.1\ \text{ml of sample used})}$$

$$= 0.0696$$

$$\Rightarrow \sigma^2(Z) = \sigma^2(A) + \sigma^2(B)$$

$$\sigma\left(0.0221_{sample} - 0.0064_{blank}\right) = \sqrt{0.0007447^2 + 0.00009343^2} = 0.00075$$

$\therefore$ net accumulative error.

In summary, the bromelain activity should be reported as

$$\Rightarrow 0.0696 \pm 0.00075 \frac{U}{ml}$$

3.4.1.5 Conclusion

The enzyme activity of the recombinant bromelain is 0.0696±0.0075 U/ml.

3.4.2 Enzyme Activity Assay Using Casein as Substrate

3.4.2.1 Principle

This procedure follows Sigma's non-specific protease activity (Sigma-Aldrich, Germany). In this assay, casein acts as a substrate for the reaction. When protease digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin and Ciocalteu's Phenol, or Folin's reagent, primarily reacts with free tyrosine to produce a blue-colored chromophore, which is quantifiable and measured via an absorbance value on a spectrophotometer at 660 nm absorbance. A greater level of tyrosine released from casein indicates the generation of more chromophores and stronger protease activity. The absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. Using the standard curve, the activity of protease samples can be determined in terms of units, which is the amount in micromoles of tyrosine equivalents released from casein per minute.

3.4.2.2 Objective of Experiment

To measure recombinant bromelain enzyme activity

3.4.2.3 Materials and methods

Consumable item

Disposable pipette tips (200 µL and 1 mL)
Microcentrifuge tube (1.5 mL)
Microcentrifuge tube (2.5 mL)
0.4 µm Millipore membrane filter

Equipment

No	Equipment	Usage
1	Pipette (200 and 1000 µL)	To add solution into the microcentrifuge tubes
2	Centrifuge	To separate supernatant from precipitate
3	Spectrophotometer (A660 nm)	To read absorbance at 660 nm
4	Water bath	To regulate the temperature of anything subjected to heat to obtain the desired temperature

Chemicals and reagents

No	Chemicals	Manufacturer
1	Phosphate dibasic, trihydrate	Sigma-Aldrich, St. Louis, USA
2	Casein	Sigma-Aldrich, St. Louis, USA
3	Sodium hydroxide	Sigma-Aldrich, St. Louis, USA
4	Hydrochloric acid	Sigma-Aldrich, St. Louis, USA
5	Trichloroacetic acid	Sigma-Aldrich, St. Louis, USA
6	Folin's Phenol reagent	Sigma-Aldrich, St. Louis, USA
7	Anhydrous sodium carbonate	Sigma-Aldrich, St. Louis, USA
8	Sodium acetate	Sigma-Aldrich, St. Louis, USA
9	Calcium acetate	Sigma-Aldrich, St. Louis, USA
11	L-tyrosine	Sigma-Aldrich, St. Louis, USA
12	Commercial bromelain	Sigma-Aldrich, St. Louis, USA

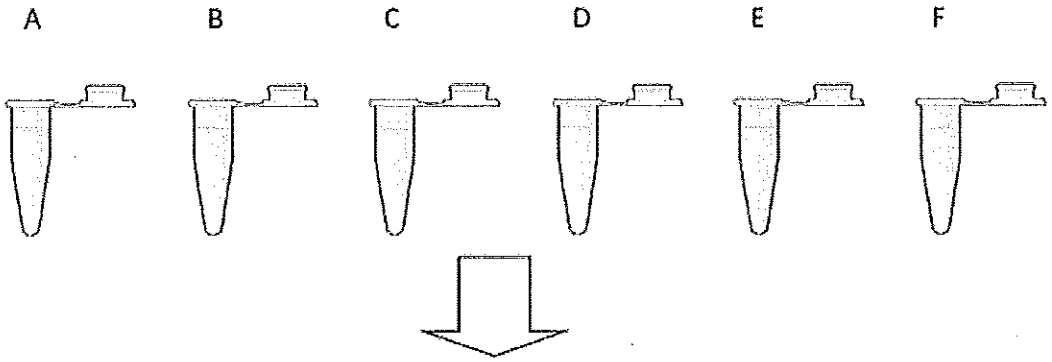
3.4.2.4 Methodology

Reagent preparation

1. 50 mM Potassium phosphate buffer, pH 7.5 at 37 °C.
   a. Add 1140 mg of potassium phosphate dibasic trihydrate into 90 mL of purified dH_2O.
   b. Incubate at 37 °C and adjust pH to 7.5 using 1 N NaOH or 1 N HCl.
   c. Bring volume to 100 mL with purified dH_2O.
2. 0.65 % (w/v) casein solution.
   a. Add 65 mg of casein into 9 mL of 50 mM potassium phosphate buffer.
   b. Gradually increase the solution temperature with gentle stirring to 80–85 °C for approximately 10 min until a homogenous dispersion is achieved (*IT IS VERY IMPORTANT NOT TO BOIL THE SOLUTION*).
   c. Adjust pH to 7.5 using 1 N NaOH or 1 N HCl.
   d. Bring volume to 10 mL with 50 mM potassium phosphate buffer.
3. 110 mM trichloroacetic acid solution
   a. Dilute 6.1 N trichloroacetic acid stock with dH_2O by adding 1 mL of 6.1 N trichloroacetic acid stock into 55 mL of dH_2O (*TRICHLOROACETIC ACID IS A STRONG ACID AND SHOULD BE HANDLED WITH CARE*).

4. 0.5 mM Folin and Ciocalteu's or Folin's Phenol Reagent
   a. The solution will react with tyrosine to generate a measurable color change that will is directly related to the activity of proteases.
      Folin's Phenol reagent is an acid and should be handled with care.
5. 500 mM sodium carbonate solution
   a. Add 530 mg of anhydrous sodium carbonate to 10 mL of purified water.
6. 10 mM sodium acetate buffer with 5 mM calcium acetate, pH 7.5, at 37°C (enzyme diluents solution).
   a. This solution is conveniently prepared in a 20X stock solution. To prepare in 1 liter, add 27.22 g of sodium acetate trihydrate and 15.82 g of calcium acetate hydrate to 700 ml of distilled water. Let the mixture dissolve with stirring or mild heating. Once completely dissolved, equilibrate the solution first at 37°C and then adjust the pH to 7.5. Bring the solution volume to 1 l. For working solution, simply dilute to 1X concentration.
      This solution is to dissolve solid commercial bromelain or to dilute enzyme solution.
      Let the solution be ice chilled before use.
7. Commercial bromelain solution (as a positive control)
   a. Immediately before use, dissolve commercial bromelain to 0.1–0.2 unit/ml in enzyme diluent solution.
   b. Commercial bromelain solution serves as a positive control for the quality control assay and as validation for the calculation that we have to perform to determine enzyme activity.
8. 1.1 mM L-tyrosine standard stock solution
   a. Add 0.2 mg of L-tyrosine into 1 mL of purified water in a tube.
   b. Heat the tube gently in a water bath until it dissolves (*DO NOT BOIL THIS SOLUTION*)
   c. Allow it to cool to room temperature.
   d. This solution will be diluted further to generate the standard curve.

Flow of experiment for Standard Curve



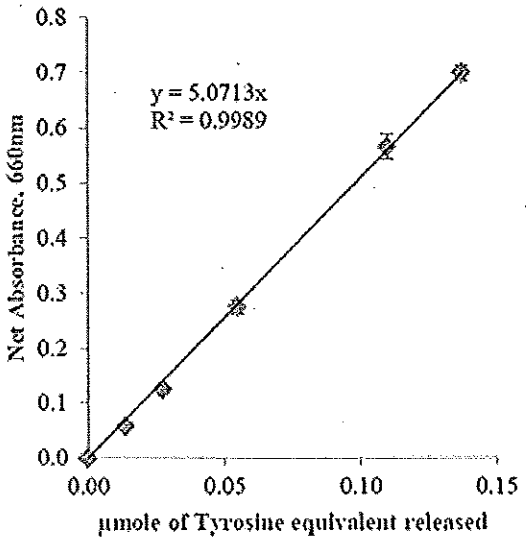
Add 1.25 mL 500 mM Na_2CO_3

Add 250 µL 0.5 M Folin & Ciocalteu's Reagent

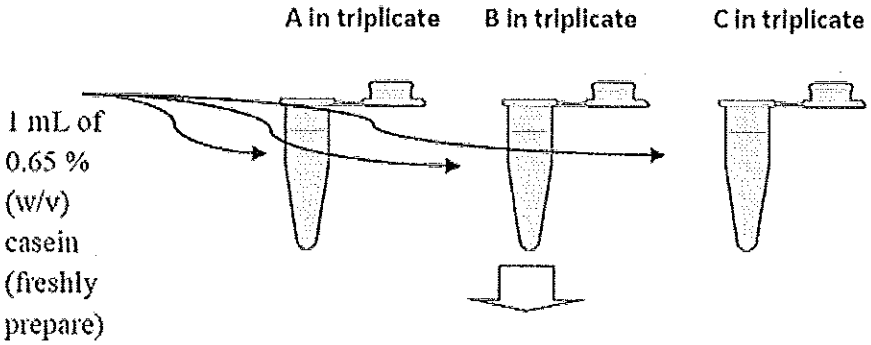
Vortex briefly and incubate at 37°C for 30 min

Collect supernatant using milipore membrane filter and syringe and read absorbance at 660 nm

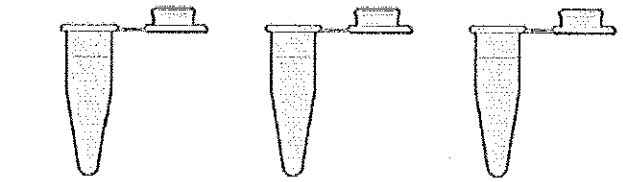
Construct an XY-scattered graph and generate the linear equation using MS Excel as shown below



1. Sample, blank and positive control (Table 3.6)



Equilibrate to 37°C, 5 min



Add 200 µL sample | Add 200 µL dH_2O | Add 200 µL positive control

Swirl and incubate at 37°C, 10 min

Using 0.45 µm milipore membrane filter and syringe, discharge precipitate and transfer 500 µL supernatant to new tube

Add 1.25 mL 500 mM Na_2CO_3

Add 250 µL 0.5 M Folin & Ciocalteu's Reagent

Mix solution by gently inverting the tube several time, then centrifuge 5 min at 14,000 rpm

Collect supernatant using milipore membrane filter and syringe and read absorbance at 660 nm.

Table 3.6 Example of data obtain to generate standard curve

Tube	A in triplicate	B in triplicate	C in triplicate	D in triplicate	E in triplicate	F in triplicate
Concentration (μmole)	Blank	0.014	0.028	0.055	0.110	0.140
L-tyrosine (μL)	0	0.013	0.025	0.050	0.100	0.125
dH_20 *(μL)*	0.500	0.487	0.475	0.450	0.400	0.375

Table 3.7 Raw data for enzyme activity from sample

Run	Test sample replicates reading	Average σ	Blank replicates reading	Average σ
1	0.3180 0.3117 0.2845	0.3047±0.0178	0.0224 0.0211 0.0247	0.0227±0.0018

Calculation of enzyme activity

1. ΔA660 nm=Average ΔA660 nm (sample)—Average ΔA660 nm (blank).
2. Determine the μmole tyrosine equivalent released using the standard curve linear equation.

$$\text{Activity } \frac{\text{unit}}{\text{ml}} = \frac{(\mu\text{mole tyrosine equivalent released})(2.2\text{ ml total volume})}{(0.2\text{ ml enzyme solution})(0.5\text{ ml colorimetric volume})(10\text{ min})}$$

3. For determination of solid protease I enzyme diluent, divide the activity of enzyme in units/mL by the concentration of solid used in this assay originally in mg/mL.
4. Therefore, the activity of protease is

$$\frac{\text{units}}{\text{mg}} = \frac{\dfrac{\text{Units}}{\text{mL}}\text{enzyme}}{\text{mg}\dfrac{\text{solid}}{\text{mL}}\text{enzyme}}.$$

3.4.2.5 Results and Discussions (Table 3.7)

$$\textit{Net average absorbance}, 660\,nm = 0.3047 - 0.0227 = 0.2820$$

$$\Rightarrow \sigma^2(Z) = \sigma^2(A) + \sigma^2(B)$$

$$\sigma(0.3047 - 0.0227) = \sqrt{0.0178^2 + 0.0018^2} = \mathbf{0.0179}$$

$\therefore$ First net accumulative error.

From the bromelain assay's standard curve, using a spreadsheet, the following linear equation is obtained:

$$y = 5.071x$$

The estimated errors for both the slope and y-intercept are as highlighted below in the table.

Best-fit values	
Slope	5.071±0.04975
Y-intercept when X=0.0	0.000±0.00000
Goodness of Fit	
r^2	0.9995

From the linear equation

$$0.2820 = 5.071x$$

$$\therefore = \frac{0.2820}{5.071} = 0.0556\,(\mu mole\ tyrosine\ equivalent\ released)$$

$$\Rightarrow \left(\frac{\sigma(Z)}{Z}\right)^2 = \left(\frac{\sigma(A)}{A}\right)^2 + \left(\frac{\sigma(B)}{B}\right)^2$$

$$\left(\frac{\sigma\left(\frac{0.2820}{5.071}\right)}{0.0556}\right)^2 = \left(\frac{\sigma(0.2820)}{0.2820}\right)^2 + \left(\frac{\sigma(5.071)}{5.071}\right)^2$$

$$\sigma\left(\frac{0.2820}{5.071}\right) = 0.0556 \times \sqrt{\left(\frac{\sigma(0.2820)}{0.2820}\right)^2 + \left(\frac{\sigma(5.071)}{5.071}\right)^2}$$

$$= 0.0556 \times \sqrt{\left(\frac{\mathbf{0.0179}}{0.2820}\right)^2 + \left(\frac{0.0498}{5.071}\right)^2}$$

$$= 0.0036 \quad \therefore \text{Second net accumulative error.}$$

Thus, from here, bromelain activity can be determined using the following:

$$\Rightarrow Bromelain\ activity\left(\frac{U}{ml}\right) = \frac{(\mu mole\ tyrosine\ equivalent\ released)(2.2\ ml)}{(10\ min)(0.2\ ml)(0.5\ ml)}$$

$$= (\mu mole\ tyrosine\ equivalent\ released)(2.2)$$

$$= 0.0556(2.2)$$

$$= 0.1223\ {}^{U}/_{ml}$$

In summary, the bromelain activity should be reported as

$$\Rightarrow \mathbf{0.1223 \pm 0.0036}\,\frac{U}{ml}$$

3.4.2.6 Conclusion

The enzyme activity of the recombinant bromelain is $0.1223 \pm 0.0036 U / ml$.

3.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.5.1 Principle

SDS-PAGE is a technique to separate proteins using electrophoresis. SDS is an anionic detergent that is able to destroy most of the complex structure of proteins and is strongly attracted toward an anode, whereas the polyacrylamide gel is used as a support medium and restrains larger protein molecules from migrating as fast as smaller protein molecules. Because the charge-to-mass ratio is nearly the same among the SDS-denatured polypeptides, the separation of protein is dependent exactly on the differences in the relative molecular mass of the polypeptides.

3.5.2 Objective of Experiment

This experiment aims to separate proteins according to their molecular weight and is normally used by researchers to obtain a qualitative measurement of the final product during recombinant enzyme production.

3.5.3 Materials and Methods

Consumable items

Disposable pipette tips (20, 200 and 1000 µL)
Microcentrifuge tubes
Gloves

Equipment

No	Equipment	Usage
1	Pipettes and dispenser (20, 200 and 1000 µL	To add any solution into microcentrifuge tubes and gel's wells
2	SDS-PAGE apparatus or electrophoresis set	To run the protein sample
3	Water bath	To boil the protein sample
4	Rocking platform	For use during gel staining
5	Gel imager	To document the gel picture

Chemicals and Reagents

No	Chemicals	Manufacturer
1	Tris	Sigma-Aldrich, St. Louis, USA/Merck, Germany
2	HCl	Sigma-Aldrich, St. Louis, USA/Merck, Germany
3	Acrylamide	Sigma-Aldrich, St. Louis, USA/Merck, Germany
4	Bis-acrylamide	Sigma-Aldrich, St. Louis, USA/Merck, Germany
5	Ammonium persulphate	Sigma-Aldrich, St. Louis, USA/Merck, Germany
6	Glycine	Sigma-Aldrich, St. Louis, USA/Merck, Germany
7	SDS	Sigma-Aldrich, St. Louis, USA/Merck, Germany
8	Coomassie Brilliant Blue	Sigma-Aldrich, St. Louis, USA/Merck, Germany
9	Isopropanol	Sigma-Aldrich, St. Louis, USA/Merck, Germany
10	Acetic acid	Sigma-Aldrich, St. Louis, USA/Merck, Germany
11	TEMED	Sigma-Aldrich, St. Louis, USA/Merck, Germany

Methodology

1. Set up the SDS-PAGE apparatus according to manufacturer's instructions (each manufacturer has a different apparatus set up).
2. Prepare the 15% separating gel as shown in the table below

Components	15% Separating Gel (mL)
30% Acrylamide, 0.8% Bis-acrylamide	10
1.875 M Tris-HCl Buffer (pH 8.8)	4
10% SDS	0.2
Ammonium persulphate	0.1
TEMED	0.0187
Distilled water	5.7
Total	20

3. Transfer the gel mixture into the gel cassette by using a Pasteur pipette. Allow solidification for 15–20 min.
4. Prepare the 4% stacking gel as shown in the table below.

Components	4% Stacking gel (mL)
30% Acrylamide, 0.8% Bis-acrylamide	1.35
0.6 M Tris-HCl Buffer (pH 6.8)	1
10% SDS	0.1
Ammonium Persulfate	0.05
TEMED	0.015
Distilled water	7.5
Total	*10*

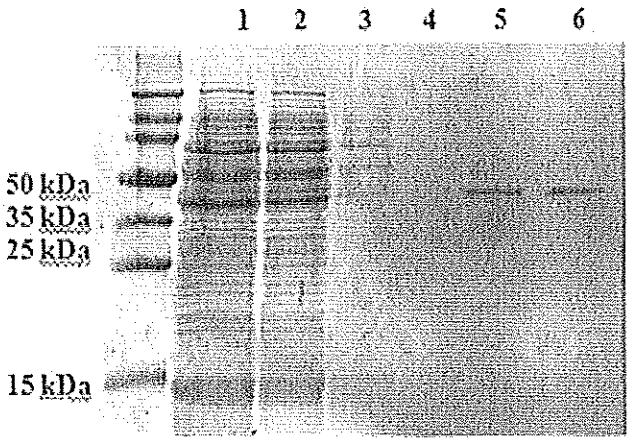


Fig. 3.5 The SDS page results for protein purification under native conditions. The first lane (M) is the protein marker (10–150 kDa) followed by a protein sample from different purification steps. Lanes *1* and *2* contain crude recombinant bromelain. Lanes *3* and *4* contain samples from different washing steps, and lanes *5* and *6* contain eluted recombinant bromelain

5. Load the gel mixture on the top of the separating gel, insert a 0.75 mm comb between the two glass plates and allow to solidify for 20 min.
6. Once solidified, fill in the top reservoir and bottom tank with the electrophoresis buffer.
7. Mix the protein sample with 5 X sample buffer at a ratio 4:1, and denature the sample by heating to 90–100 °C for 5 min.
8. Load the denatured sample using a syringe needle just above the bottom of the well.
9. Use a constant current of 30 mA to run the electrophoresis until the blue marker reaches the bottom of the gel.
10. Stain the gel with Coomassie blue for 40–60 min. Finally, destain the gel and document the picture using the gel documentation system.

3.5.4 Results

Figure 3.5 shows the protein bands in different lanes from different samples after fermentation in a bioreactor and purification using affinity chromatography.

References

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3. Karp G (2002) Cell and molecular biology: concepts and experiments, 3rd edn. Wiley, New York