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APPENDIX

1. Lists of Chemicals

Name of Chemicals	Product company
Agar	Difco
Ammonium nitrate	SK trading
Beef extract	Criterion
Boric acid	QRec
Casein	Himedia
Casein peptone	Himedia
Coomassie brilliant blue G	Labchem
Dipotassium Hydrogenphosphate	Univar
Folin & Ciocatteu's phenol reagent	Merck
Glucose	SK trading
Hydrochloric acid	LAB SCAN
L-tyrosine	Univar
Magnesiumsulphate heptahydrate	Ajax Finechem
Malt extract	Himedia
Ortho phosphoric acid	Univar
Peptone	Criterion
Potassium dihydrogen phosphate	Univar
Skimmilk	Himedia
Sodium carbonate	Merck
Sodium chloride	LAB SCAN
Sodium hydroxide	Univar
Sulfuric acid	QRec
Trichloroacetic acid	Merck
Yeast extract	Himedia

2. Lists of Equipments

Equipment	**Product company**	**Model**
Analytical balance	Ohaus	Adventurer
Autoclave	Iwaki	ACV-3167
Hot air oven	Memmert	
Incubator shaker	Kuhner	Lab-Therm
Kjeldahl	FOSS	Kjeltec^{TM} 8100
Laminar air flow	Nitech	NH3A
Microcentrifuge	Hettich	MIKRO20
Microscope	Olympus	Cx31
pH meter	Eutech	Cyberscan
Spectrophotometer	Spectronic	20 GENESYS
Refrigenerated centrifuge	Hettich	MIKRO 22R
Water bath	Memmert	WB10

3. Stock Solution, Reagents and Media

3.1 Stock Solutions

3.1.1 0.05 M potassium phosphate buffer, pH 7.5

Combine 83.4 ml of 1 M K_2HPO_4, 16.6 ml of 1 M KH_2PO_4, dilute the combined 1 M stock solution to 2 litters and confirm that the pH= 7.5 ± 0.1 (if the pH needs to be adjusted, use phosphoric acid or KOH). Store at room temperature. The shelf life of this solution is greater than one year.

3.1.2 0.65% (w/v) Casein Solution

0.65% w/v casein solution, prepared by mixing casein in the 50 mM potassium phosphate buffer. Heat gently (do not boil) to 80-90 °C for 10 minutes with stirring. Adjust the pH to 7.5 at 37 °C, if necessary, with either 1 M NaOH or 1 M HCl.

3.1.3 110 mM Trichloroacetic Acid Reagent (TCA)

Dilute 9 ml of Trichloroacetic Acid, 6.1 N, approximately 100% (w/v), to 500 ml with deionized water.

3.1.4 Folin & Ciocalteu's Phenol Reagent (F-C)

Dilute 10 ml of Folin & Ciocalteu's Phenol Reagent to 40 ml with deionized water.

3.1.5 500 mM Sodium Carbonate Solution (Na_2CO_3)

Prepare 500 ml in deionized water using sodium carbonate anhydrous. A 50 mM sodium carbonate solution, prepared using 53 mg/ml of anhydrous sodium carbonate in furified water.

3.1.6 1.1 mM L-Tyrosine Standard

Prepare 100 ml in deionized water using L-Tyrosine 0.2 mg/ml. Heat gently (do not boil) until tyrosine dissolves and cool to room temperature.

3.1.7 Bradford Reagent

- Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
- (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. The Bio-Rad concentrate is expensive, but the lots of dye used have apparently been screened for maximum effectiveness.

3.2 Medium Formulation

3.2.1 Enrichment medium (Wang *et al.*, 2006)

0.2 g Chitin

0.1% K_2HPO_4

0.05% NH_4NO_3

1. Dissolve 0.2 g of Chitin, 0.1 g K_2HPO_4 and 0.05g NH_4NO_3 in 100 ml water
2. Autoclave 20 min
3. Cool to room temperature

3.2.2 Minimal synthetic medium (Wang *et al.*, 2002)

2% crab shell powder (CSP)

0.1% K_2HPO_4

0.05% $MgSO_4.7H_2O$

2.0% agar

1. For 1 liter, dissolve 20 g crab shell powder, 1g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 20 g agar pH7.0
2. Autoclave for 20 min at 15 lb/sq in. Let cool to ~ 55 °C and add desired antibiotics at this point
3. Store at room temperature or at 4 °C

3.2.3 Protease production medium (Wang *et al.*, 2006)

2% crab shell powder CSP

0.1% K_2HPO_4

0.05% $MgSO_4.7H_2O$

1. Dissolve 2 g crab shell powder, 0.1 g K_2HPO_4 and 0.05 g $MgSO_4.7H_2O$ final volume adjusted to 100 ml
2. Adjust the pH of the solution to 7.0 with NaOH

3. Autoclave for 20 min at 15 lb./sq. in. Let cool to ~ 55 °C and add desired antibiotics at this point

3.2.4 LB medium

10 g tryptone

5 g yeast extract

10 g NaCl

20 g agar

1. Adjust pH to 7.0 and autoclave to sterilize
2. Suspend the solids in ~800 ml of distilled or deionized water
3. Add further distilled or deionized water, in a measuring cylinder to ensure accuracy, to make a total of 1 litre
4. Autoclave at 121 °C

4. Proximate Analysis

4.1 Protease Assay and Standard Curves

1. Suitable 4 vials that will hold about 15 ml were used. One vial will be used as a blank, and three others will be used to assay activity.

1.1 Add 5 ml of 0.65% casein solution. Let them equilibrate in a water bath at 37°C for about 5 min.

1.2 Add varying volumes of enzyme solution to three of the test sample vials, but not the blank. Mix by swirling and incubate for 37°C for exactly 10 min.

2. After this 10 min incubation, add the 5 ml of the TCA reagent to each tube to stop the reaction. Then, add an appropriate volume of enzyme solution to each tube, even the blank. Incubate the solutions at 37°C for 30 min.

Set up tyrosine standard dilutions. Use 6 dram vials (dram vials can be substituted with polypropylene tubes) that can easily hold 8 ml to the six vials, add the 1.1 mM tyrosine standard stock solutions with the following volumes in mls: 0.05, 0.10, 0.20, 0.40, 0.50. Don't add any tyrosine standard to the blank. Once the tyrosine standard solution has been added, add an appropriate volume of purified water to each of the standards to bring the volume to 2 mls.

3. After the 30 min incubation, filter each of the test solutions and the blank using a 0.45 μm polyethersulfone syringe filter.

3.1 Add the filtration 2 mls of the test samples and blank filtrate to 4 dram vials. The same type of vial in which the standards were prepared can be used.

3.2 To all of the vials containing the standards and standard blank, add 5mls of sodium carbonate. For best results, add 1 ml of Folin's reagent immediately afterwards.

3.3 Add sodium carbonate to regulate any pH drop created by the addition of the Folin's reagent.

3.4 Add sodium carbonate to the test samples and test blank. These solutions become cloudy after the addition of sodium carbonate. Add the Folin's reagent, which will react primarily with free tyrosine.

3.5 Mix the dram vials by swirling and incubate at 37°C for 30 min.

Measuring Absorbance and Calculating Enzyme Activity

1. The absorbance of the samples is measured by a spectrophotometer using a wavelength of 660 nm. Once all of the data has been collected, the standard curve can be created.

Table 6.1 Volume of tyrosine standard (ml) on μmoles of tyrosine

Volume of standard tyrosine (ml)	Tyrosine (μmoles)
0.05	0.055
0.10	0.111
0.20	0.221
0.40	0.442
0.50	0.553

2. After data points have been entered. Find the change in absorbance in the test samples by calculating the difference between the test sample absorbance and the

absorbance of the test blank. To get the activity of enzyme in units per ml, perform the following calculation:

$$\text{Units/ml Enzyme} = \frac{(\mu\text{mole tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (2)}$$

11= Total volume (in ml) of assay

10= Time of assay (in min) as per the unit definition

1= Volume of enzyme (in ml) of enzyme used

2= Volume (in ml) used in colorimetric determination

Take the number of micromoles tyrosine equivalents released obtained from the slope equation and multiply it by the total volume of the assay in ml. Divide this value by three other quantities: the time of the assay, which we ran for 10 min, the volume of enzyme used in the assay, which was varied (let's use 1ml), the volume of milliliters used in colorimetric detection, which may differ based on your cuvette. We used 2 ml.

3. Micromoles of tyrosine divided by time in minute yields measurement of protease activity called "units". We can cancel out the units for volume measurement in the numerator and denominator, leaving a measurement of enzyme activity in terms of units/ml. In order to determine the activity in a solid protease sample diluted in enzyme diluent, we divide our activity in units/ml by the concentration of solid used in this assay originally in mg/ml., leaving us with activity in terms of units/mg.

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

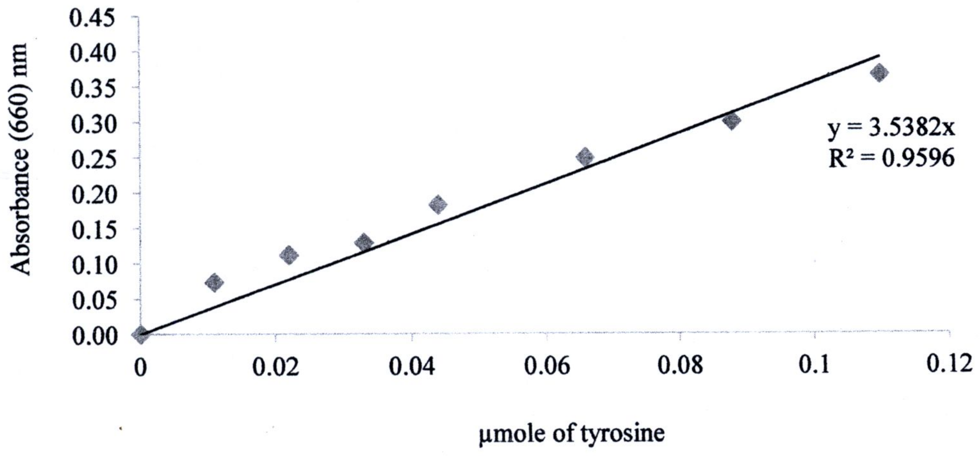

Figure 6.1 Standard curve of μmoles tyrosine and absorbance of sample

4.2 Protein Determination by Bradford Method (AOAC, 1990)

1. Warm up the spectrophotometer before use.
2. Dilute unknowns if necessary to obtain between 5 and 100 μg protein in at least one assay tube containing 100 μl sample
3. If desirred, add an equal volume of 1 M NaOH to each sample and vortex (see Comments below). Add NaOH to standards as well if this option is used.
4. Prepare standards containing a range of 5 to 100 micrograms protein (bovine serum albumin (BSA) or gamma globulin are recommended) in 100 μl volume. Add 5 ml dye reagent and incubate 5 min.
5. Measure the absorbance at 595 nm.

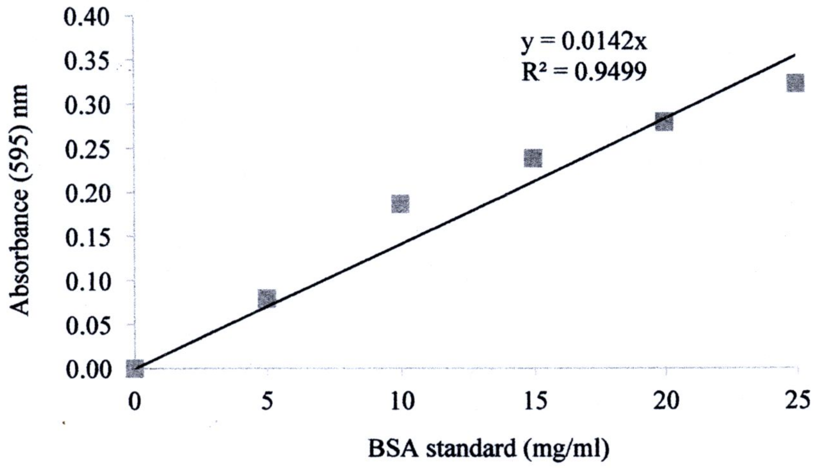

Figure 6.2 Standard curve of bovine serum albumin (mg/ml) and absorbance of sample

4.3 Nitrogen Determination by Kjeldahl

1. 1.5 g of dried sample was transferred into digestion tube
2. Added 2 tablets of catalyst and 20 ml of sulfuric acid
3. Digestion in Kjeldahl digester (Tecator Kjeltec System, Germany) at 420°C for 45 min.
4. Transferred the tube into Kjeldahl distillation and distilled for 5 min.
5. The sample was titrated with 0.184 N of sulfuric acid.

$$\text{\% total Nitrogen (N)} = \frac{(A-B) \times C \times 0.014 \times 100}{D}$$

$$\text{\% Crude Protein} = \text{\%N} \times 6.25$$

A = ml of 0.1 N standard sulfuric acid titrated with sample

B = ml of 0.1 N standard sulfuric acid titrated with blank

C = Concentration of sulfuric acid

D = Sample weight (g)

5. DNA sequence of isolate ECM04

AACACGTGGGTAACCTGCCTGTAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTT
CGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT
CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAA
TTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAG
GGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG
TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGC
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
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ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCG
AAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGT
GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC

CURRICULUM VITAE

Name	Miss Chonlachat Jaihao
Date of birth	1 November 1982
Academia background	
High school	Suratthani School, 2000
Bachelor Degree	Bachelor of Science (Biotechnology), Maejo University, 2004