CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and equipment

3.1.1 *Chemicals*

Chemicals	Companies	Grade
KH_2PO_4	Carlo Erba	Laboratory
K_2HPO_4	Carlo Erba	Laboratory
$CaCl_2.2H_2O$	Ajax Finechem	Laboratory
Glucose	Ajax Finechem	Laboratory
$MgSO_4.7H_2O$	Ajax Finechem	Laboratory
NaOH	Ajax Finechem	Laboratory
Agar powder	Ajax Finechem	Laboratory
Sodium potassium tartrate	Ajax Finechem	Laboratory
Yeast extracts	Himedia	Laboratory
HCl	Merck	Laboratory
Trichloro acetic acid	Merck	Laboratory
Nutrient broth	Scharlau	Laboratory
Skim milk	Scharlau	Laboratory
Polyethylene Glycol (PEG) 1000	Acros Organic	Laboratory
PEG 4000	Fluka	Laboratory
PEG 6000	Fluka	Laboratory
PEG 10,000	Sigma	Laboratory
3,5- Dinitrosalicylic acid	Fluka	Laboratory
Azocasein	Fluka	Laboratory
Tris(hydroxymethyl) aminomethane hydrochloride	Fluka	Laboratory
Comassie brilliant blue G250	Fluka	Laboratory

3.1.2 *Equipment*

Equipments	Model	Companies
Centrifuge	Kubota 5100	Kubota cooperation , Japan
Fermentors (1L)	Biostat Q	Renown technical Co. Ltd, Japan
Spectrophotometer	4001/4	Spectronic instrument, USA
Incubator	National TB-179	Masushita electric work, Japan
Shaker	Supperline	Masushita electric work, Japan
Light microscope	Olympus BH-2	Olympus, Japan
Water-bath	Laudra A-100	Laudra GMB & Co, Germany
Whirly mixer	Vortex genie 2	Scientific industries, INC, USA
Pump LDC	LDC 4100	LDC,USA
Integrator	LDC monitor IV	LDC,USA
Refractive index detector	C-P1A	Shimadzu, Japan
Electron microscope	JSM 5410 LV	JSM ,Japan
Dryer	Samdri 780	Samdri ,Japan
Sputter	SCD O40	Banzors, Japan

3.2 Methods

3.2.1 Microorganism, medium and culture conditions:

3.2.1.1 Microorganism:

Strain *Bacillus subtilis* TISTR 25 was supplied by Associate Prof. Napa Siwarungson, Faculty of Science, Chulalongkorn University and was maintained on nutrient agar plates at 4°C (no longer than 2 months).

3.2.1.2 Medium:

One litre medium contained 1 g of KH_2PO_4; 0.5 g of $MgSO_4.7H_2O$; 0.01 g of $CaCl_2.2H_2O$; 20 g of yeast extracts; and 5 g of glucose in distilled water.

3.2.1.3 Skim milk agar plate:

Before cultivation, the cells were activated in skim milk agar plate. Skim milk agar was made by mixing 20 g of skim milk and 20g of agar powder in 1000 ml of distillated water. After sterilization at 121 ° C for 10 minutes, it was poured into plates and cooled. The colony from nutrient agar plate was streaked and moved to skim milk agar plate aseptically. The petridishes were left overnight at ambient temperature (30°C± 2°C). By checking the clear zone around the colony we would know the activation of bacterial that was the larger area the higher activation.

3.2.1.4 Starter:

Cells from skim milk plate were aseptically transferred into 500 ml flask containing 250 ml of medium. The flask was shaken for 17 hours at ambient temperature (30°C± 2°C) at 250 rpm before inoculation.

3.2.1.5 Shake flash experiments:

25 ml of starter was aseptically transferred into sterile 225ml culture solution in 500 ml flaskṡ. These culture contained flasks were shaked at 250 rpm and at ambient temperature 30°C± 2°C. The samples were taken out every 12 hours in laminar flow chamber.

3.2.1.6 Conventional alkaline protease fermentation in fermentor:

Medium for conventional alkaline protease was prepared as explained in 3.2.1.2. Fermentation was done at 37°C± 0.5°C, which was determined to be optimal temperature for *Bacillus subtilis* TISTR 25 growth. 50 ml of starter was inoculated aseptically into steriled 450 ml medium with agitator speed 250 rpm, air feed was kept constant at 1 v/v and pH was automatic controlled at pH 7 constantly.

3.2.1.7 Alkaline protease fermentation in ATPs in fermentor:

To avoid precipitation or any by-reactions (if any), PEG and potassium phosphate in powder and wax form were steriled separately from medium and glucose. After sterilization, medium prepared in 3.2.1.2 was fed into fermentor containing sterile PEG and potassium phosphate. The two-phase system was then obtained. Medium should be sterile and then fed to fermenter when fermenter temperature is about 80°C, this will help easily create the two-phase system. Fermentation conditions were 250 rpm of agitation, air feed was

constant at 500 ml/minute and pH was automatically controlled at pH 7 constantly. The samples were taken out every 12 hours.

3.2.2 Analytical methods:

3.2.2.1 Phase diagram construction:

A series of ATPs were made with varied system compositions at pH 7 in screwed cap test tubes of 50 ml at 30°C± 2°C. These tubes were kept overnight before analysis by HPLC to define amount of PEG and potassium phosphate in each phase.

3.2.2.2 Partition coefficient experiments:

ATPs was prepared for different compositions at pH 7 and at 30°C± 2°C with total volume was 20 ml, 0.1 ml of alkaline protease (about 45 unit) was added into each sample tubes. Each tubes were whirly mixed 3 times, kept overnight and were tested for alkaline protease activity in both phases.

3.2.2.3 Phase volume ratio:

Five ml of sample solution was taken from the fermentor for phase volume ratio measurement using a volumetric cylinder.

After defining phase volume ratio, samples were centrifuged at 3,500 rpm for 15 minutes. For conventional fermentation, the clear solution was kept for further analysis. For ATPs fermentation, the top phase sample was taken out by micropipette with a small amount leaved at the interface. The bottom phase was taken out carefully through the interface. All samples were analyzed for enzyme activity, glucose, and protein concentration.

3.2.2.4 Alkaline protease activity assay:

- *Reagents preparation*

+ *Tris-HCl (Tris-hydroxymethyl) aminomethane hydrochloride) 0.1 molar*: Dissolve 12.1 g of Tris-HCl in 800 ml distilled water. The solution was set to a pH of 10.5 by using concentrated NaOH and HCl solutions and then distilled water was added to make total volume of 1000 ml. The solution was then kept at 4° C.

+ *Azocasein solution 0.2%w/v*: 2 g of Azocasein was dissolved in 1000 ml aqueous solution which contained 4 ml of absolute ethanol and 2 ml of absolute toluene. This solution was kept away from light by using opaque bottle and stored at 4 ° C.

+ *TCA (trichloroacetic acid) 10% w/v*: Dissolve 100 g of TCA in 1000 ml of distilled water. Keep the solution away from light by using an opaque bottle and stored at 4 °C.

- *Analysis*:

A 0.2ml of sample solution was added to 1.8 ml of Tris-HCl, and the solution was then mixed with 2ml of Azocasein solution to initiate the reaction. The incubation time was 20 minutes at 45°C and the reaction was stopped by adding 0.4 ml of TCA as a protein-precipitating agent (Srinivas et al, 1996) before plunging the test tubes into an ice-cold bath. The enzyme blank sample was prepared by replacing the fermented broth with the initial fermentation medium. After centrifugation at 3,500 rpm for 15 min, the supernatant was tested by spectrophotometer at a 440 nm wavelength. The enzyme activity (unit/ ml) was calculated by multiplying the absorbance by 10, which was the slope of the standard curve.

One unit of enzyme is defined as the quantity of enzyme which liberates 1 μg of tyrosine per minute under assay conditions.

3.2.2.3 Total protein assay

- *Reagents preparation*

+ *HCl 0.6 N*: Dilute 50 ml of 37 %v/v HCl with 950 ml of distilled water. This solution is kept in a screw-capped bottle at 4 °C.

+ *CBB (Comassie brilliant blue G250) solution 0.06 % w/v:* 0.06 g of CBB was dissolved in 100 ml of 0.6N HCl. Then, this solution was filtered with filter paper "Whatman" no. 1. This solution is not stable at all, so need to be prepared every time before use.

- *Analysis*:

0.1 ml of sample solution was mixed with 2.4 ml of distilled water and 1 ml of CCB solution. The mixture was tested by spectrophotometer at the wave length of 620 nm. Amount of protein (mg/l) was calculated by dividing absorbance with 0.0014, which was the slope of the standard curve (figure 3.1).

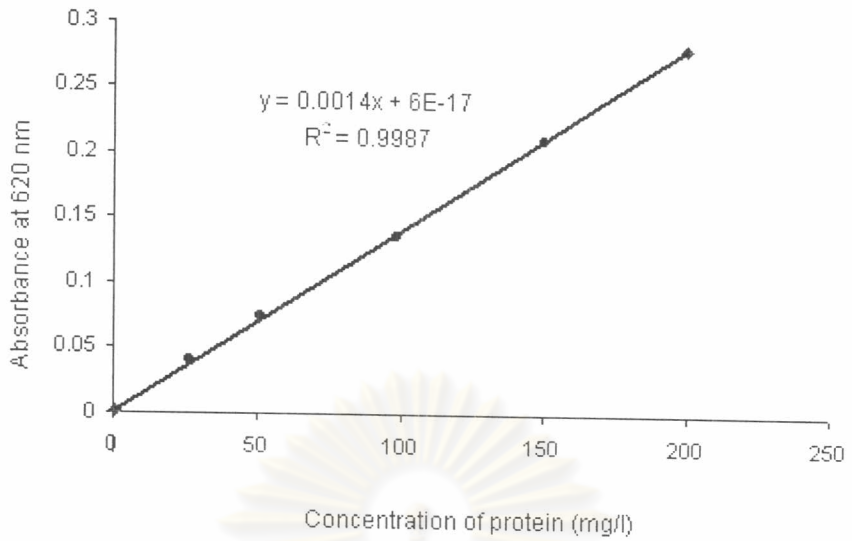

Figure 3.1: Standard curve for protein assay

3.2.2.4 Glucose assay

- *Reagents preparation*

+ *DNS reagent*: 16 g of NaOH was dissolved in 200 ml of distilled water to make on 8 %wt NaOH solution. 10 g of 3,5- dinitrosalicylic acid was then added in the prepared solution. 500 ml of distillated water was then added before 300 g of sodium potassium tartrate was dissolved. The solution then was made up to 1000 ml by distilled water and at last kept in opaque bottle for 2-3 days before using.

- *Analysis*:

0.1 ml of sample solution was added to 1 ml of DNS reagent. The mixture was whirly mixed and then incubated at 100 °C for 5 minutes. The reaction is stopped by plunging the test tubes into cold bath (mixture of ice and water). Then 10 ml of distilled water was added and whirly mixed before testing at 520 nm of wave length by spectrophotometer. The standard curve was made by variation of concentration of glucose (not higher than 5 g/l).

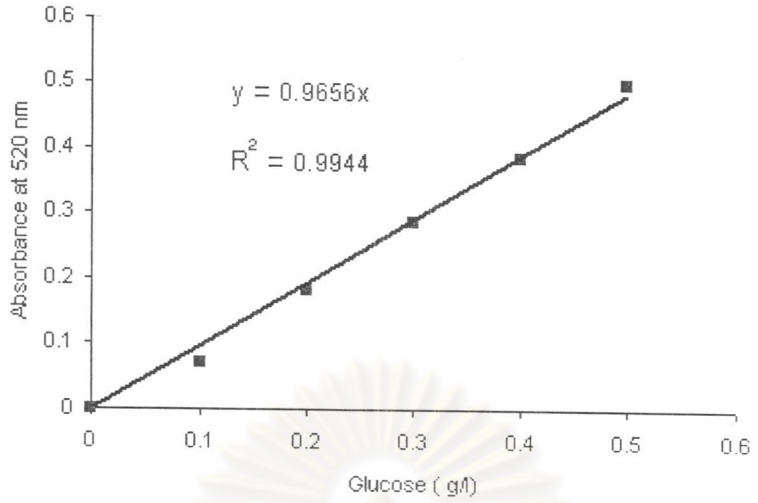

Figure 3.2: Standard curve for glucose analysis.

3.2.2.5 HPLC (High performance liquid chromatography)

The conditions to define amount of PEG X and phosphate was as the following:

- *Conditions for PEG X (1000, 4000, 6000, and 10000) determination:*

The column was Merck Lichrocart C_8, with 125mm x 4mm in dimension. The mobile phase was solution of 50% v/v up to 80% v/v of methanol in water, with flow rate was 1.0 ml/min using refractive index detector at 25 °C and 1 microlitre of injection volume.

- *Conditions for phosphate determination:*

The column was Dupont Zorbax-SAX, 250 mm x 4.6 mm in dimension. The mobile phase was 1.3 ml/ min of aqueous phosphoric acid pH 2.38 using refractive index detector at 25 °C and 1 microlitre of injection volume.

3.2.2.5 Electron microscopy:

Scanning electron microscopy was used to visualize the shape of cells. Cells were centrifuged at 10,000 rpm for 5 minutes, suspended in 0.1 M phosphate buffer pH 7.2 and then was filtered with membrane of 0.4 micrometre pore size. Cells deposited on membrane surface were fixed by 2.5 %wt gluteradehyde in 0.1 M phosphate buffer pH 7.2 and left overnight. After that it was washed by

phosphate buffer pH 7.2, and followed by distilled water washing, and dehydrated with gradually EtOH treatment series as the following: 1 time for 10 minutes for each solution (30%, 50%, 70%, and 90% v/v) and 3 times with absolute EtOH for 5 minutes each. Then cells were dried at critical point, mounted on a stub and coated with pure gold by sputter. Finally, cells were observed under 10,000 times magnification.