CHAPTER IV

EXPERIMENTS

This chapter will be focused on materials and methods of batch fermentation and continuous fermentation coupling with microfiltration. The characteristics of microfiltration are also done by fermentation broth.

4.1 Apparatus

- Air compressor model 0.2 OP-5S of Hitachi., Ltd., Japan.
- Autoclave model HL 24ADY of Hirayama Manufacturing Corporation, Japan.
- Centrifuge model Kubota 5100 of KUBOTA Corporation, Japan.
- Centrifuge model Kubota 7820 of KUBOTA Corporation, Japan.
- Ceramic membrane model 1M-1 of Toshiba Ceramic Corporation., Ltd., Japan.
- Digital pH / Temperature meter model 671 of JENCO Electronics., Ltd., Taiwan.
- Dissolved oxygen meter model OM-14-L1 of Horiba, Japan.
- Fermenter model MINI-JAR-FERMENTER KMJ of Mituwa, Japan.
- Glucose analyzer model YSI 27 of Yellow Springs Instrument Corporation, USA.
- Incubator Shaker of Gesells Chaft Fur Labortechnik, Germany.
- Laminar Flow Culture Boot model VS-124 of ISSCO, USA.
- Level transmitter of Technic Pool, Thailand.
- Peristaltic pump model Dual pump of New Brunswick Scientific CO., INC., USA.
- pH meter and controller of Mituwa, Japan.

- pH transmitter model INGOLD 2500 of Mettler Toledo AG, Switzerland.
- Rotary pump model SUS-316 of Nakakin Corporation., Ltd., Japan.
- Spectrophotometer model Spectronic 20D of Milton Roy Company, USA.
- Spectrophotometer model 220A of Hitachi, Japan.
- Water bath model Julabo of Labortechnik, GMBH, Germany.

4.2 Chemical reagents

- Buffer concentration kit (YSI 2357) of Yellow Springs Instrument, USA.
- Calcium chloride dihydrate ($CaCl_2 .2H_2O$) of MERCK, Germany.
- Casein of BDH, England.
- Hydrochloric acid (HCl) of BDH, England.
- Magnesium sulfate heptahydrate ($MgSO_4 . 7H_2O$) of FLUKA, Switzerland.
- Potassium dihydrogen phosphate (KH_2PO_4) of MERCK, Germany.
- Sodium bicarbonate ($NaHCO_3$) of MERCK, Germany.
- Sodium carbonate anhydrous (Na_2CO_3) of MERCK, Germany.
- Sodium hydroxide (NaOH) of EKA NOVEL, Sweden.
- Sulfuric acid (H_2SO_4) of J.T.Baker, USA.
- tri-Chloroacetic acid (TCA) of MERCK, Germany.
- TAKATHERM (α-amylase) of Cinnamon, Thailand.
- OPTIDEX (glucoamylase) of Cinnamon, Thailand.
- SAG 30 (Antifoam) of OSi Specialities, Thailand.
- Soybean waste and extruded sunflower seed waste from oil processing of Bangkok Feed Meal Co.,Ltd. , Thailand.

4.3 Strain

The bacteria employed in this study was Bacillus subtilis TISTR 25 obtained from Thailand Institute of Scientific and Technological Research in a lyophilized (freeze-dried) form.

4.4 Medium

The complex medium comprised of the following substances. The unit was shown in gram per liter. [28]

KH_2PO_4	1	g
$MgSO_4 .7H_2O$	0.5	g
$CaCl_2 .2H_2O$	0.1	g
Starch hydrolysate	2.5	g (equivalent to glucose)
Protein hydrolysate	20	g

4.4.1 Starch hydrolysation

Starch hydrolysate is a D-glucose riched compound which was termed after the termination of starch hydrolysation process [29]. Catalysed by enzymes, the yield of hydrolysation is approximately 95 % conversion (Appendix B). As shown in Figure 4-1, the starch would be completely hydrolysed by employing α-amylase (TAKA-THERM, commercial name) and glucoamylase (OPTIDEX, commercial name), subsequently. Before being catalysed by α-amylase, starch must dispersed in solution and rendered susceptible to breakdown. Industrially, starch solubilization could be accomplished by heat treatment (gelatinization). Once gelatinized, starch is broken down by α-amylase catalysis to a soluble dextrin hydrolysate and this procedure is called liquefaction.

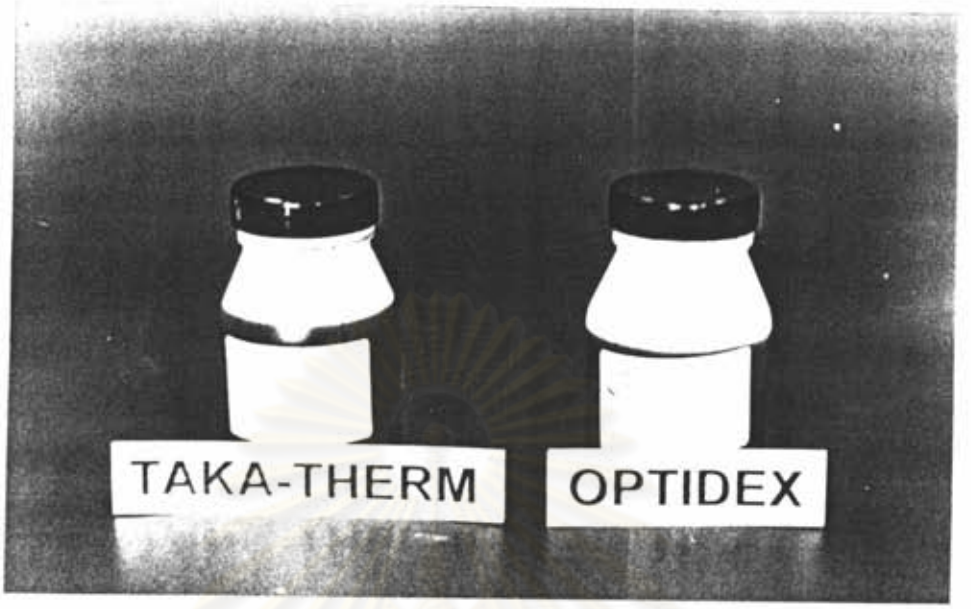

Figure 4-1 Enzymes for starch hydrolysation

Left: TAKA-THERM (α-amylase) Right : OPTIDEX (glucoamylase)

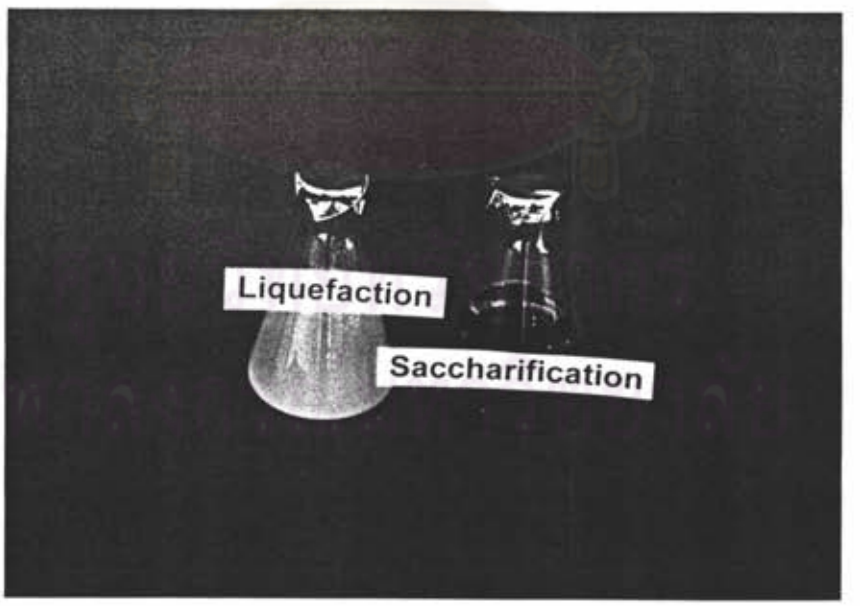

Figure 4-2 Hydrolyzed starch

Left : in liquefaction process Right : in saccharification process

Then a subsequent process called saccharification is achieved by glucoamylase catalysis that will hydrolyze dextrin hydrolysate to glucose units. Some more details about their procedure will be described below.

4.4.1.1 Liquefaction

Starch powder was dispersed in an aqueous solution to a 30% weight ratio and was then adjusted to the pH of 6.0 using 1N NaOH. The α-amylase solution was later added to the prepared solution to a ratio of 0.25 ml α-amylase solution per kilogram of starch. The liquefaction process lasted 90 minutes at 90 °C with continuous stirring. Prolonged hydrolysis by α-amylase resulted in the formation of small quantities of glucose and maltose. Dextrin slurry (Figure 4-2,left) was obtained after the complete liquefaction.

4.4.1.2 Saccharification

The pH of dextrin slurry from liquefaction process was adjusted to the pH of 4.2 with 1N of HCl and glucoamylase was added to the ratio of 0.5 ml glucoamylase per kilogram of starch. The operating temperature was controlled at 60 °C for 2 days. Prolonged reaction would not significantly affect the final glucose concentration. High glucose content mixture or starch hydrolysate (Figure 4-2,right) was obtained after the complete saccharification.

4.4.2 Protein hydrolysation

The most effective medium formula for the highest protease production by Bacillus subtilis TISTR 25 in shaken flask fermentation was employed in this experiment. The medium was obtained from the acid hydrolysation of soybean residual and extruded sunflower seed residual from oil production. After complete hydrolysation, abundant amino acids were achieved. The details of hydrolysation were given as follows :

Six grams of each soybean waste and extruded sunflower residual were mixed together (Figure 4-3, A and B). Then 40 ml (1N.) of H_2SO_4 was added to the

mixtures. The mixtures were autoclaved them at 121°C and 15 psi for 40 minutes. After being cooled to room temperature, the mixtures (suspension) were then adjusted neutral pH using 10N NaOH. The 80 ml of water was then added to the hydrolyzed mixtures, which were later centrifuged at 5000 rpm 20 °C for 20 minutes. The obtained clear mixtures were kept at -20 °C for storage. Protein hydrolysate (Figure 4-3, C) was obtained after the hydrolysation was completed.

Figure 4-3 Materials and end product of protein hydrolysation

(A) Soybean waste (B) Extruded sunflower seed waste

(C) Hydrolyzed protein or protein hydrolysate

4.5 Strain storage

Freeze-dried culture was revived by making a deep mark around the ampule about one inch from the tip of it, wiped with a piece of gauze moisten with 70% alcohol. The ampule was wrapped around with the gauze and was broken at the score area, lightly flamed at the open end. Using a sterile Pasteur pipette to aseptically add 0.3 to 0.4 ml of the appropriate broth to the freeze-dried material. A few drops of this suspension were also transferred to an agar slant or plate which they were streaked on it. The tube and plate were incubated at the appropriate temperature.

4.5.1 Short-term storage

Aseptically, some suspension from revived freeze-dried culture was transferred using a sterile loop and streaked on an nutrient agar slant. The slant was incubated at 37 °C for 24 hours, and was kept at 4 °C. Employing this method, the culture could be kept for about 2 - 3 months

4.5.2 Long-term storage

Covered with 50% glycerol, the culture which was first transferred to nutrient broth could be stored for a year at temperature of -20 °C. Glycerol prevents microorganisms from ice crystal formed by water composition in the broth.

4.6 Experimental procedures

4.6.1 Batch fermentation preparation

Each two culture agar slant tubes (Figure 4-4) were filled with 5 ml normal saline solution and were then shaken thoroughly, the slant-washed normal saline solution was aseptically transferred to two seed flasks (Figure 4-5). Each of 500 ml seed flasks was prepared by filling 150 ml of dissolved medium composition adjusted to the pH of 7.0 by adding 1.0 N NaOH. The prepared medium were then

sterilized at 121 °C , 15 lb_f/in^2 for 20 minutes. Note that every substance involving fermentation process must be sterilized before entering to the process. The two seed flasks were incubated at 37 °C for 18 hours. A KMJ 3 liter glass fermenter used in batch process was also prepared in the same fashion. The pH of the experimental broth was controlled by a pH controller equipped with acid and base pump as demonstrated in Figure 4-6 and Figure 4-7. Oxygen transfer in the medium was proportional to both agitation speed and aeration rate besides operating temperature and medium compositions which both of them were fixed through the process. The temperature of the experimental broth was controlled at 37 °C by heating element.

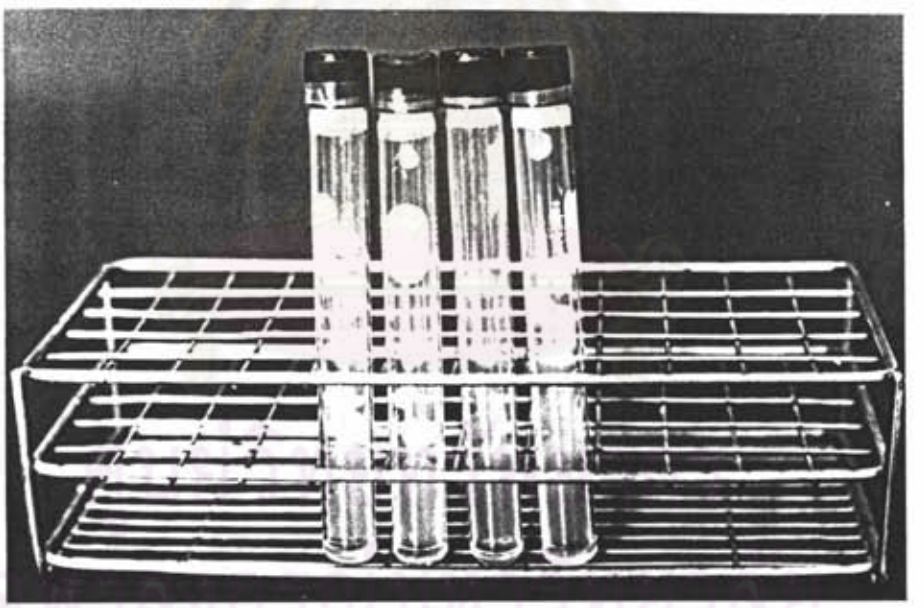

Figure 4-4 Culture slants of Bacillus subtilis TISTR 25

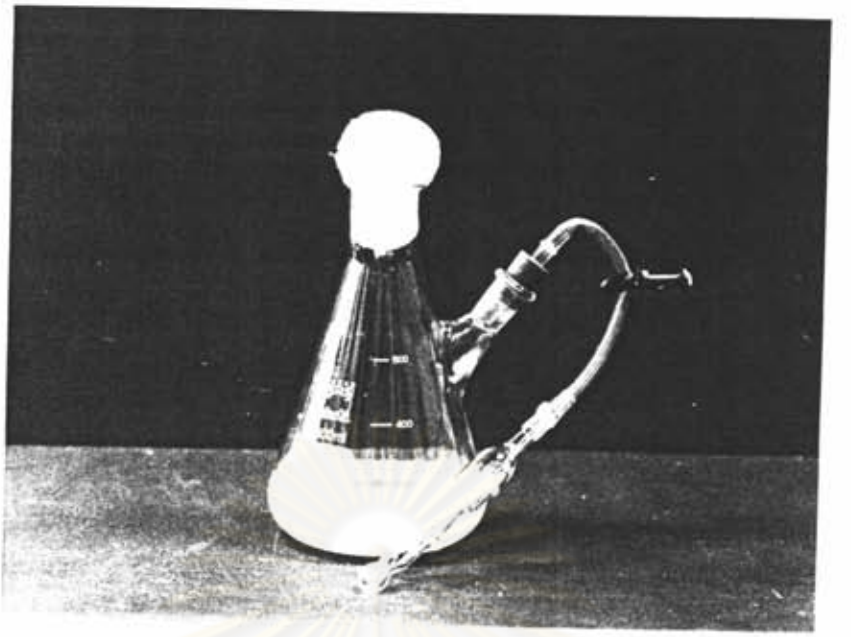

Figure 4-5 A culture seed flask of Bacillus subtilis TISTR 25

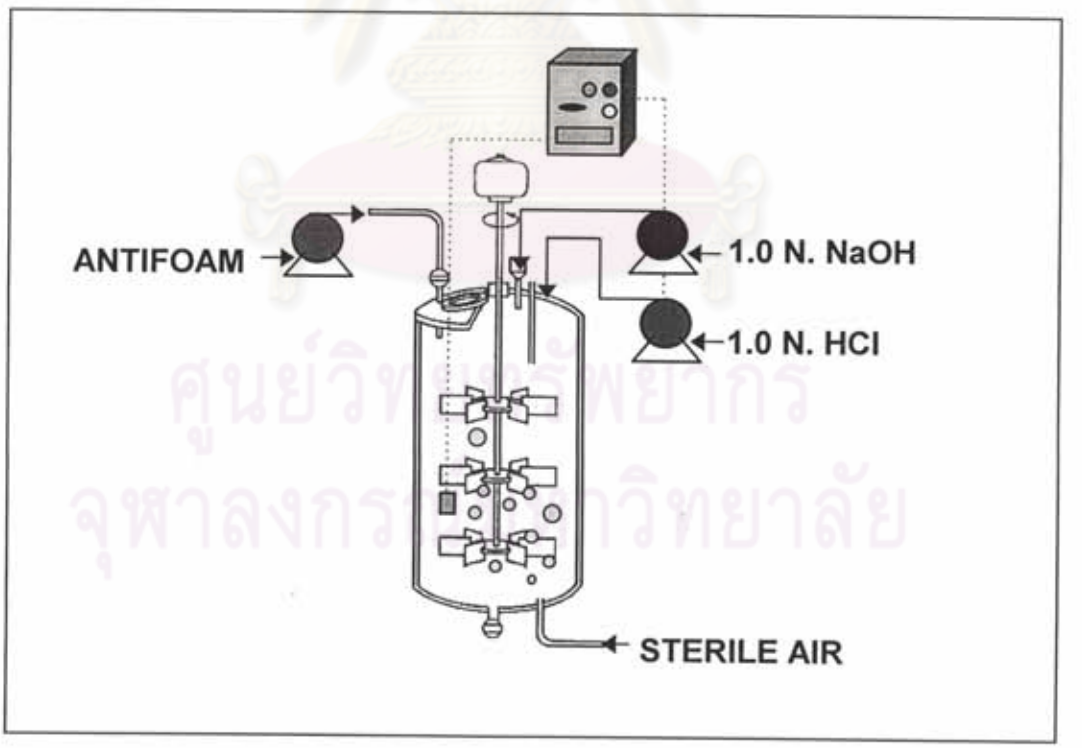

Figure 4-6 Schematic diagram of the batch system applied for the protease production

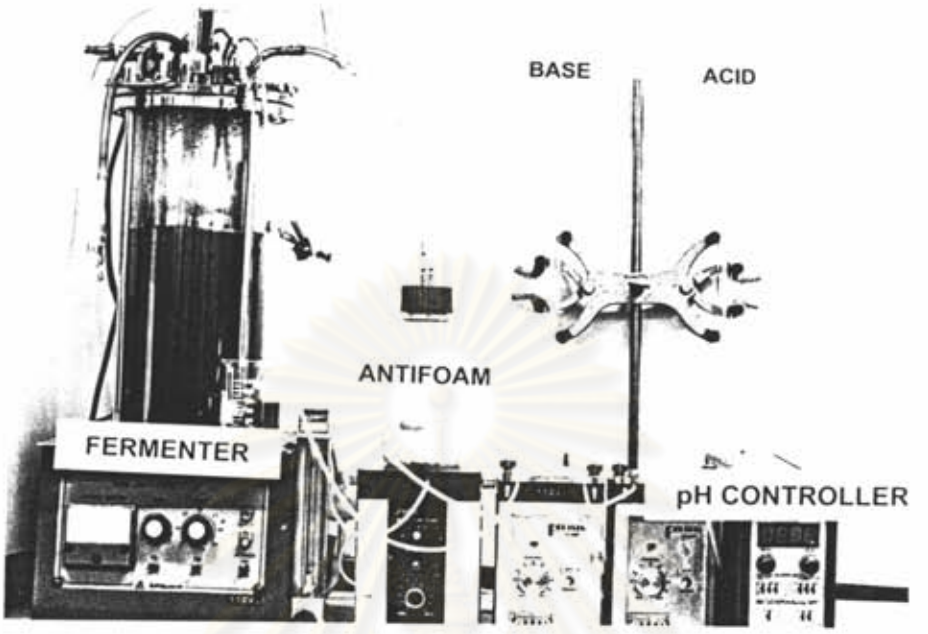

Figure 4-7 Photograph of batch fermentation process

A batch process was initiated by innoculating of the two culture seed flasks into the sterilized fermenter which contained 2,700 ml of sterilized medium and then the aeration of 1.0 vvm (volume of air per volume of medium per minute) and the agitation speed of 350 rpm (revolution per minute) were applied to achieve a saturated dissolved oxygen condition in the medium (Appendix B). An antifoam was filled into the fermenter to reduce foam. In the early log phase, 10 ml of sample was withdrawn every 2 hours to analyze cell, glucose and protease concentrations until the process reached the end of the 10^{th} hour, after which sampling time was switched to every-4-hour collection. The process would be terminated at the 46^{th} hour of fermentation.

4.6.1.1 The study of controlled pH effect on growth and protease production in batch fermentation

As much as previous basic data was concerned, the shaken flask fermentation could not provide controlled pH systems, hence the batch fermentations with controlled pH were set to clarify the optimum pH for growth and protease production under 37 °C and saturated dissolved oxygen condition. The controlled pH values were varied from 6.0 to 8.0 with a step of 0.5.

4.6.1.2 The study of starch hydrolysate concentration effect on growth and protease production in batch fermentation

The aims of this experiment were to elucidate the optimum initial glucose concentration which was suitable for cell growth and protease production and to obtain maximum specific growth rate by varying initial starch hydrolysate concentration (equivalent to the glucose concentration of 1.0, 1.8, 3.2, 4.5 and 7.5 g/l, respectively).

4.6.2 The study of microfiltration characteristics

The objective of this study was to obtain the suitable operating condition of microfiltration unit which was applied in cell recycling system. As much as the microfiltration theory is concerned, there are five parameters that affect the filtration process. Temperature and pH were held constant at their optimal point throughout the fermentation process, so these two parameters could be neglected in this study. Pressure, recirculation velocity or recirculation flow rate, and cell concentration were optimized for the microfiltration unit.

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4.6.2.1 Cleaning and regenerating the filter

A 1M-1 alumina ceramic filter used in microfiltration process (Figure 4-8) consisted of 19 carbon tubes with inside coated microfiltering membrane as illustrated in Figure 4-9. Each tube in multichannel ceramic supporter is 4 mm. in internal diameter, 85 cm. long. With 0.2030 m^2 of filtration area, and 0.2 μm in pore size, this multichannel ceramic filter could be employed to filter microbial cell.

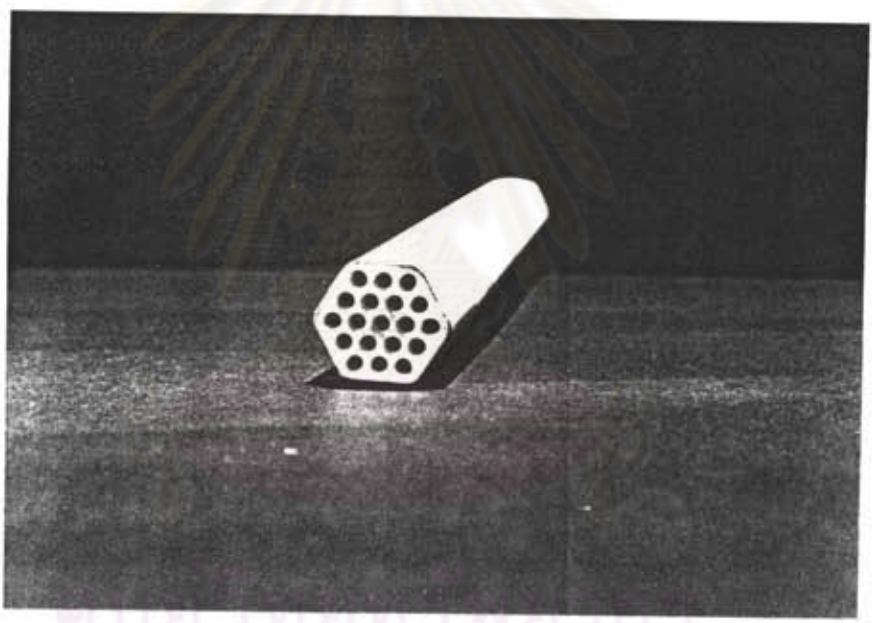

Figure 4-8 Ceramic filter (type 1M - 1)

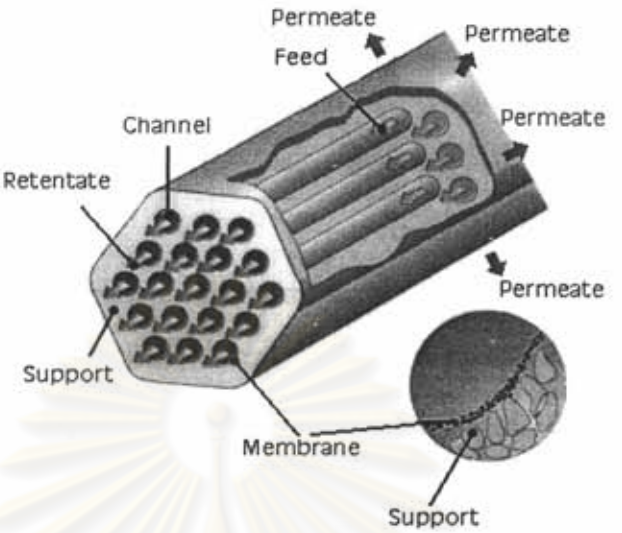

Figure 4-9 Cross-flow filtration in a multichannel element (19 channels)

After each operation, the ceramic membrane was cleaned by rinsing and back flushing with distilled water so that some particles or cells were removed; however, the residuals were removed by immersion of ceramic filter in a 1.5 % NaOH solution for 12 - 24 hours (HCl can also be used) which would break microbial cells by osmotic pressure. The 30 minute sterilization of the membrane was then carried out in order to eliminate the residual protein and cell debris out of the membrane surface. The regenerated ceramic filter should have the same characteristic , same permeate flux at the same condition, as those before the operation otherwise rinsing and back flushing should be done on that filter again.

4.6.2.2 Experimental set up

The schematic of microfiltration system is shown in Figure 4.10. Distilled water was employed for filtration at 0.0^{+} kg_f/cm^2 applied pressure and 0.4 m^3/hr recirculation flow rate in order to get reference water flux. The obtained pure water flux is used to check the cleanness of the regenerated filter by comparing to the pure water flux of the regenerated filter to it. If it is less than the reference value, it will be cleansed again. Fermentation broth was pumped from a process tank or fermenter

(No.4) and flowed pass a flow meter (No.7) and a ceramic filter (No.9), then returned to the process tank. The permeate flux was a criteria reflecting the filtration performance and the permeate flux was calculated from permeate flow rate. Hence the permeate volume was measured at a certain time period until it reached the same constant value as that of the distilled water experiment. The parameters (cell concentration, pressure and recirculation flow rate) used for each experiments were illustrated in Table 4-1.

Table 4-1 The operating condition of microfiltration system for the study of the effects of pressure, recirculation flow rate, and cell concentration on permeate flux

Run	Types of fluid	Pressure (kg_f/cm^2)	Recirculation flow rate (m^3/hr)
I	Distilled water	0.0^+	0.4, 0.5, 0.6
II	Distilled water	0.2^+, 0.4^+, 0.6^+, 0.8^+	0.4
III	1.81 g/l	0.0^+	0.4, 0.5, 0.6
IV	1.81 g/l	0.2^+, 0.4^+, 0.6^+, 0.8^+	0.4
V	4.24 g/l	0.0^+	0.4, 0.5, 0.6
VI	4.24 g/l	0.2^+, 0.4^+, 0.6^+, 0.8^+	0.4
VII	8.70 g/l	0.0^+	0.4, 0.5, 0.6
VIII	8.70 g/l	0.2^+, 0.4^+, 0.6^+, 0.8^+	0.4
IX	14.42 g/l	0.0^+	0.4, 0.5, 0.6
X	14.42 g/l	0.2^+, 0.4^+, 0.6^+, 0.8^+	0.4

Note : X g/l in column "Types of fluid" refers to cell concentration in fermentation broth

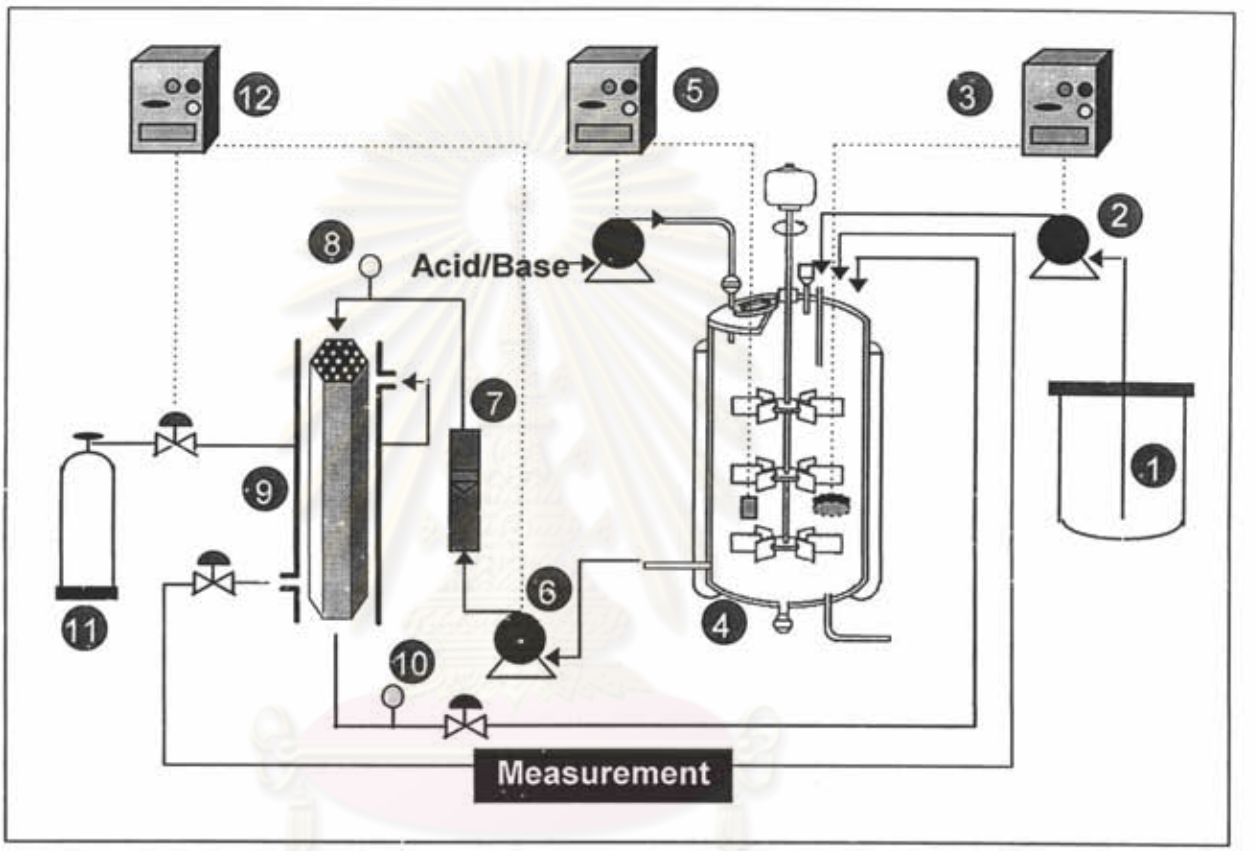

Figure 4-10 Schematic diagram of microfiltration system applied for the protease production (modified from Apichart, 1996)

1. Feed tank
2. Feed pump
3. Level controller
4. Fermenter
5. pH controller
6. Recirculation pump
7. Flow meter
8. Inlet pressure gauge
9. Microfiltration module
10. Outlet pressure gauge
11. N_2 storage tank
12. Control block

4.6.3 The experimental setup of the continuous fermentation coupling with microfiltration

The reason why the continuous fermentation with microfiltration was selected in this experiment was due to the fact that many continuous fermentation with microfiltration processes can increase their product productivity by employing the use of cell recycling technique as mentioned in the literature review section.

An Eylla - M100 2 liter fermenter was equipped with the microfiltration unit, a 1M-1 ceramic membrane module in a stainless housing. All connections were sterilized by applying 100 °C steam for 30 minutes. As schematic diagram of the cell recycling system illustrated in Figure 4-11, an operation was started with a batch startup and then the medium would be pumped to the fermenter by a peristaltic pump (No.2). The cultivation process occurred batchwise until glucose ran out or cell concentration reached a constant value under the same operating conditions as those of a previous batch fermentation (37 °C, pH 7.0, aeration rate of 1.0 vvm and agitation speed of 350 rpm). The continuous operation was started with introducing fresh medium into the fermenter and the fermentation broth was circulated through microfiltration unit by a 0.75 kW sanitary rotary pump (No.6) with the 0.4 m^3/hr recirculation flow rate and 0.0^+ kg_f/cm^2 pressure. The permeate flow rate was controlled in order to adjust dilution rate instead of feed pump manipulation. The volume of broth in fermenter was controlled at 1.0 liter by a level controller which controlled the medium feed pump, the peristaltic one. Acid and base were also equipped with the fermenter.

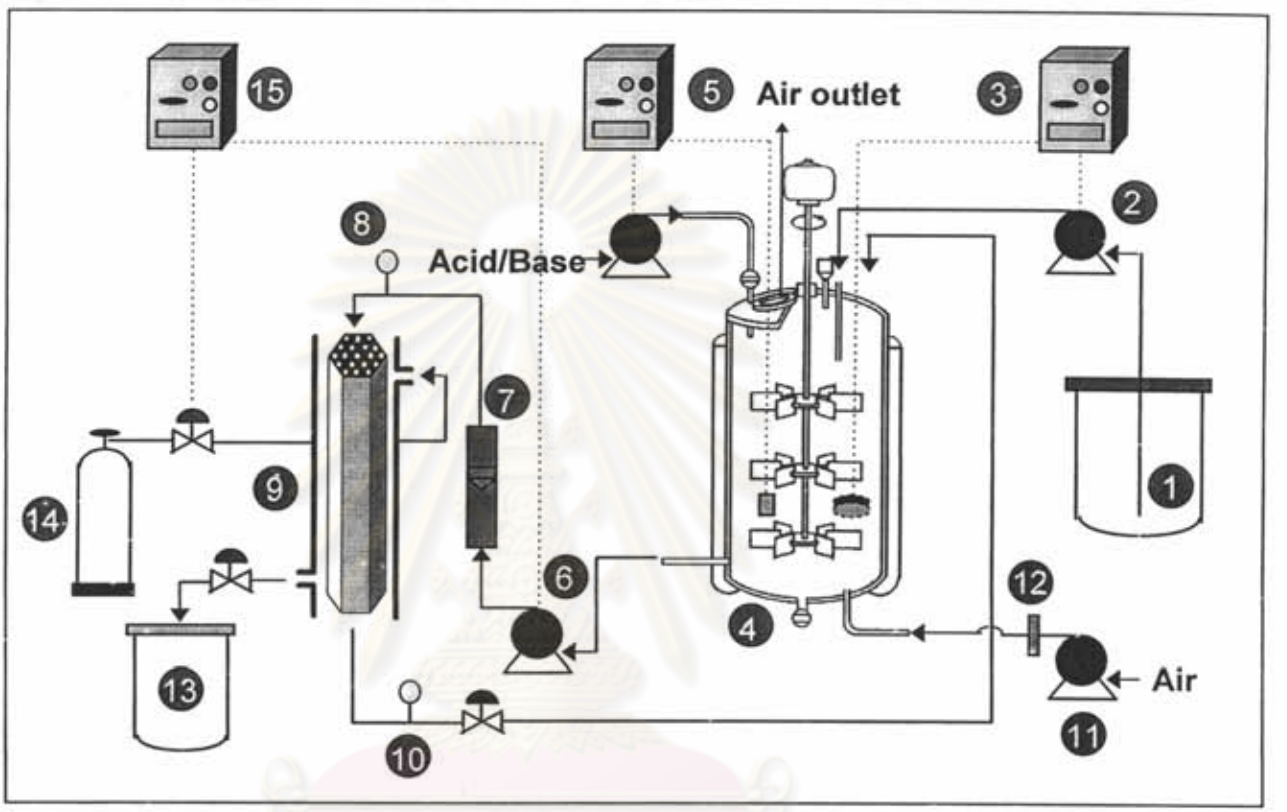

Figure 4-11 Schematic diagram of the cell recycling system applied for the protease production (modified from Apichart, 1996)

1. Feed tank
2. Feed pump
3. Level controller
4. Fermenter
5. pH controller
6. Recirculation pump
7. Flow meter
8. Inlet pressure gauge
9. Microfiltration module
10. Outlet pressure gauge
11. Air compressure
12. Air filter
13. Permeate storage tank
14. N_2 storage tank
15. Control block

4.6.3.1 Study of the effect of dilution rate on growth and protease production in continuous with microfiltration fermentation

The aim of this experiment was to elucidate the optimum dilution rate which was suitable and practical for steady state protease production by varying the permeate flow rate which directly affected the dilution rate of 0.11, 0.2, and 0.3 hr^{-1}, respectively

4.6.3.2 Study of the stability of protease production at constant suitable dilution rate which was obtained from item 4.6.3.1

The purpose of this experiment was to clarify the stability of protease production by prolonging cultivation time at the dilution rate of 0.3 hr^{-1} including the influence of twice cell bleeding.

4.7 Analysis

4.7.1 Determination of cell concentration

Cell concentration was determined by two methods

4.7.1.1 Optical density method

Cell concentration could be measured using the spectrophotometry technique by measuring the optical density. The fermentation samples were properly diluted in order to be in linear range of absorbance. Spectrophotometer type spectronic 20D was used at the fixed wavelength of 420 nm. to determine cell concentration. A 5 ml fermentation sample was centrifuged at 3500 rpm for 10 minutes after which the supernatant was drained, and fresh water was added to the tube to make a proper dilution. The diluted culture in cuvette was inserted into a spectrophotometer set at 420 nm and the optical density was measured.

4.7.1.2 Dry weight method

A 5 ml of sample was dried in the hot air oven at 90 °C for a day or until its weight were constant.

4.7.2 Determination of glucose concentration

Glucose was determined by a glucose analyzer type YSI 27. The 25 µl clarified sample was injected to glucose analyzer by the Syringepet (25 µl micropipette) then, the sample was oxidized to hydrogen peroxide by oxidase enzyme which was immobilized within a commercial membrane. The hydrogen peroxide was then measured by electrochemical oxidation at a platinum anode. The glucose analyzer was calibrated by injection of 200 mg/dl standard glucose, hence the sample had to be diluted with distilled water to obtain proper dilution for an accurate measurement.

4.7.3 Determination of protease

In 1956 an international Enzyme Commission was set up to bring more order into the existing nomenclature and measurement of activity, and their report was adopted in 1961 by the International Union of Biochemistry. The Commission defined that "one unit activity of enzyme is that amount which will catalyse the transformation of one micromole of substrate per minute under defined conditions" [1]. Protease concentration could be expressed in term of unit activity and its unit activity is defined as **"one unit activity of protease is that amount which will catalyse the transformation of one micromole of tyrosine from casein per minute under defined conditions".** Details of determination of protease is described as follows :

Firstly, the fermentation sample was centrifuged at 3400 rpm for 10 minutes. Then the supernatant was withdrawn and kept for further experiments. In another tube, 1 ml of 0.5% casein in buffer solution (pH 10.5) was added together with 0.9 ml of the same buffer solution. The prepared substrate solution was incubated

in a constant 45 °C water bath. Next, the 0.1 ml. of sample supernatant was pipetted to a prepared substrate tube under the controlled temperature. Mixed well, and incubate in the water bath at 45 °C. After 20 minutes, the tube was cooled down by dipping it in a coolbath and the reaction was stopped by an immediate addition of 2 ml. of 10% TCA. The solution was recentrifuged at 3400 rpm for 15 minutes. The absorbance of the supernatant was measured at the wavelength of 280 nm which could then be related to protease unit activity using the tyrosine standard curve. Note that the defined operating conditions were pH 10.5 (carbonate buffer solution), and 45 °C