CHAPTER II

LITERATURE REVIEW

This chapter will be focused on literature review of protease production, and the application of microfiltration to continuous fermentation.

2.1 Protease production

Atalo and Gashe [4] studied the protease production by thermophilic Bacillus sp. (P-001A) isolated from alkaline hot spring. In an optimized medium (0.1% K_2HPO_4, 0.01% $CaCl_2$, 0.01 % $MgSO_4.7H_2O$, 0.5% glucose, 0.1% yeast extract and 0.1% peptone), the maximum protease activity against casein was 65 unit/ml. The main purpose of production was to obtain protease for the degradation of fibrous proteins, such as horn, leather, nail, and hair.

One unit of protease activity was expressed as 1 microgram of amino acid released per 1 ml of culture supernatant per minute under 55 °C and pH 9.5.

Rujiwatra et al [5] reported that the protease was produced from hot spring thermophiles in shaken flask fermentation composing of 0.1% yeast extract, 0.1% tryptone, and 20% base mixture solution as medium. With the condition of 200 rpm and 65 °C, high protease activity was produced by strain T-20 and TLS 33 with the maximum of 22 and 17 unit/ml at the 24 th hour .

One unit activity was defined as the amount of enzyme digesting substrate which liberated 1 microgram of tyrosine (measured at 275 nm) in one minute at pH 7.2 (Tris-HCl buffer) and 65 °C.

Kladwang, Phutrakul, and Kanasawud [6] also studied the production of protease from Thermus S2 isolated from hot spring. Fermentation was taken place in shaken flask system which 0.1% yeast extract, 0.1% tryptone in base mixture were mixed as medium to achieve the maximum activity of 0.9 unit/ml at the 25 th hour.

One unit activity was defined as the amount of enzyme which produced inprecipitate product from azocasein with 1.0 absorbance value at 440 nm, pH 7.0 (Tris-HCl buffer) and 65 ^{o}C for one hour.

Nehete et al [7] screened a high yielding stable variant of Bacillus licheniformis by employing repeated heat (80^{o}C, 30 minutes) treatment containing 5% glucose in cultivated medium. From the fourth heat-treated culture, 1 ml suspension was aseptically plated on agar medium to select surviving spores for the next treatment. After the 7 th treatment, alkaline protease activity had increased from 85 to 196 unit/ml (2.31 fold).

One unit of protease activity was defined as the amount of enzyme digesting substrate which liberated 1mg of tyrosine in 15 minutes at pH 9.5 and 50^{o}C.

Kitada and Horikoshi [8] studied the alkaline protease production from methyl acetate by alkalophilic Bacillus sp. No. 8-1 which was isolated from soil. They stated that not only ethyl acetate but also ethyl, propyl acetate, isopropyl acetate were used as carbon sources in alkaline medium composition of 5% methyl acetate, 0.5% yeast extract, 0.1% poly peptone, 0.3% urea, 0.3% K_2HPO_4, 0.02% $MgSO_4.7H_2O$ and 1.0% $NaHCO_3$. After shaken flask fermentation at 120 rpm, 30^{o}C, pH 9.0, for 72 hr, alkaline protease was harvested at the highest yield of 600 unit/ml. The culture pH also had a great influence on cell growth and enzyme production.

One unit activity was determined by the casein 275 nm method [9] at pH 11.0 (Na_2CO_3-NaOH Buffer) 40^{o}C for 10 minutes.

Hussein and Abdel-Gwaa [10] reported the protease activity of Aspergillus flavus grown on hydrocarbons and oxygenated hydrocarbon. Their idea was focused on fungal production of exocellular enzymes from their inexpensive carbon sources.

The results indicated that ethanol promoted the maximum cell growth whereas methanol, led to a sharp decline in cell production, promoted the maximum protease production . Unit activity was not stated in this paper but quoted in previous work [10].

Helmo, Winther-Nielsen, and Emborg [12] stated that the retention of Baciilus firmus in a chemostat by a new carrier material, Luxopore, led to increased productivity of protease. Luxopore is a porous mineral product of irregular shape. When these particles are put into a fermenter, aeration and stirring make them float. Fermenters with Luxopore loading of 200 and 500 g/l were run as chemostats parallel to a control chemostat without it. The Luxopore particles contained more than 50% of the cell in the chemostats (50 mg dry cell weight/g), which had a higher cell and protease activity in the culture fluid (0.5 % glucose, 0.3% yeast extract 0.3% beef extract 0.01% sodium sesquicarbonate) than the control chemostat. The overall protease productivity was up to four times higher than that of the control.

One unit activity was defined at pH 7.6 and 37 °C by azocasein method in Anson units (AU) based on a comparison with an Alcalase standard of 1.5 AU/g.

Lee and Chang [13] studied alkaline protease production by Bacillus lincheniformis in an aqueous two-phase system composed of 5% (w/w) polyethylene glycol 6000 (PEG 6000) and 5% (w/w) dextran T500. The top phase was continuous and rich in PEG while the bottom phase was dispersed and rich in dextran. The cell were retained in the bottom phase and in the interface. The two-phase system produced less enzyme in total amount than the control in the early phase, but after 50 hr, the enzyme produced in the control system decreased while the aqueous phase system continued its production and finally the total enzyme activity reached 1.3 times of the control culture. In order to improve the productivity of protease, repeated batch cultivations were successfully carried out four times by optimizing the top phase composition of freshly added media, which resulted in 13.8, 35.9, 27.8 and 34.7 unit ml /hr of protease based on the amounts of replaced top phases, respectively.

One unit activity was defined as the amount of enzyme digesting substrate which liberated 1 microgram tyrosine in one minute at 37 °C.

Tan and Shen [14] studied the partitions of alkaline protease from Bacillus lincheniformis in low cost PEG/ Reppal PES 200 system and EHEC/ Reppal PES 200 system. Parameters which influence the partition such as pH, concentrations of polymers, salts and charged PEG were studied. The extraction results revealed that partition of alkaline protease was mainly pH dependent. When the appropriate conditions were chosen, alkaline protease from Bacillus licheniformis could be purified 3.4 times with recovery of 88% in PEG/ Reppal PES 200 system and a purification factor of 2.4 times with 84% recovery in EHEC/ Reppal PES 200 system was obtained. This process could be easier scaled up without separating the cells from the fermentation broth.

Activity of alkaline protease was determined by Tomarelli's method [15]. Azoalbumin was used as substrate and optical density was measured at 440 nm. Activity could be calculated according to a standard curve of alkaline protease obtained from SIGMA company (9.3 unit/mg)

Basically, there are two commercial protease production processes. In the tray process, extensively used in Japan, mold is introduced into a semi solid medium containing about 50 % water. The medium is either in trays or in large roller drums. The trays are incubated at constant temperature and humidity, until the maximum level of protease is obtained. After that the supernatant extraction with water or salt solution is employed to extract the crude enzyme which is then precipitated by addition of inorganic salts or organic solvent. In submerged fermentation, the batch reactor coupled with various units as shown in Figure 2-1 is set. After finishing sterilization process, the fermenter is introduced by sterile medium together with 1-10 % inoculum of actively-grown bacteria. pH, antifoam, oxygen and temperature are controlled until the maximum level of protease is obtained. After that, insoluble material is removed by continuous centrifugation. The clarified supernatant is firstly

concentrated by vacuum evaporator and is secondly precipitated and filtered by continuous filter. Finally, the solid enzyme is dried and packed.

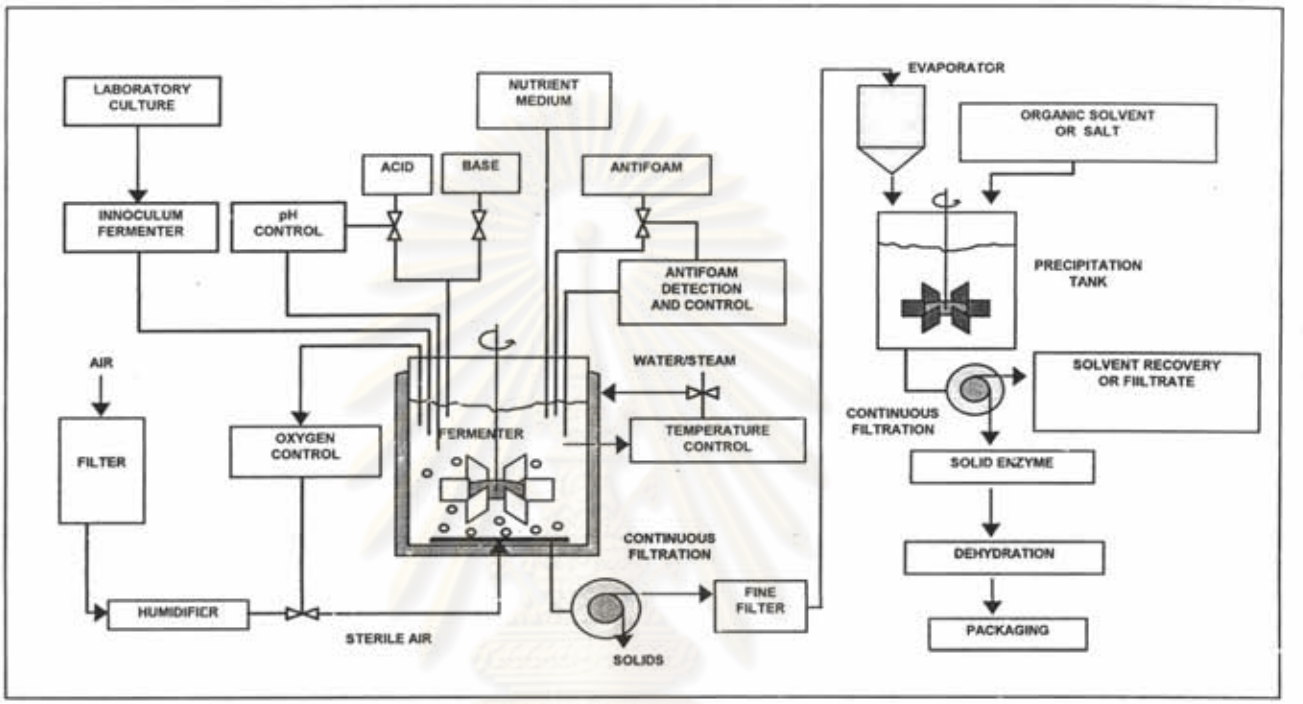

Figure 2-1 Flow diagram for commercial production of microbial protease

2.2 The application of microfiltration to continuous fermentation.

The application of microfiltration to fermentation processes has been studied to improve product productivity in fermentation. Continuous fermentation, using cross-flow microfiltration to recycle cell back to the fermenter, has been reported to increase the cell per unit volume, facilitate an increase in productivity. In cross-flow microfiltration, fermentation broth flows tangentially across the membrane surface with cell-free liquid permeating through the membrane. Accumulated cells are swept away from the membrane surface employing a high recirculation flow rate. Concentrated cells and a portion of the cell-free liquid stream are returned to the fermenter.

Minier et al. [16] improved productivity in acetone-butanol fermentation by coupling continuous fermentation with microfiltration. The membrane was a carbon tube with a microfiltering ceramic coat inside. With total recycle of cell, a solvent weight concentration of 125 g/l was obtained, which enhanced a solvent productivity of 4.5 g/l at dilution rate of 0.33 hr^{-1} .

Schlote and Gottschalk [17] improved a productivity of acetone-butanol fermentation by using cellulosetriacetate membrane to separate and recycle cells in a continuous fermentation of Clostridium acetobutylicum ATCC 824 under phosphate limitation ($0.74x10^{-3}$ molar) at dilution rate of 0.40 hr^{-1}. A solvent productivity of 4.1 g/l- hr was maintained for three months.

Pisalaphoge [18] studied the application of microfiltration for improving productivity in continuous acetone-butanol fermentation. A multitubular ceramic microfilter was used to separate and recycle cells of Clostridium acetobutylicum ATCC 824. With total recycle of cells, a dry weight concentration of 80 g/l and a solvent productivity of 6.06 g/l-hr were obtained at dilution rate of 0.55 hr^{-1} on a complex medium containing 42.4 g/l glucose.