CHAPTER III

MATERIAL AND METHODS

Ethanol production in this study is carried out in shake flasks for batch culture and packed bed reactor for continuous culture. Palm sugar and sugar cane molasses are used as carbon and energy source for the producing yeast. A flocculating yeast strain, *Saccharomyces cerevisiae* M30 is immobilized in alginate-Al_2O_3 and its ethanol productivity is determined.

Most of materials and methods used in this work are of common practice in cell cultivation, cell immobilization, and fermentation technologies. Methods for reinforced carrier's preparation are constructed based on simple sensible aseptic produces that can be readily applied on bench scale fermentation experiments. Except molasses other chemicals are of analytical grade.

3.1. Microorganism

Yeast *Saccharomyces cerevisiae* M30 strain is kindly provided by Prof. Dr. Savithree Limthong, from Department of Microbiology, Kasetsart University, Bangkok.

3.2 Stock cell suspension and immobilization

Yeast *S. cerevisiae* M30 strain were stored in PDA agar slants at 4 °C. Each starter culture was obtained by transferring cells from an agar slant into 500 ml Erlenmeyer flask containing 100 ml sterilized cultivation medium and assigned immobilized material. The cultivation medium composed of 5% w/v sugar from palm sugar, 0.05% w/v $(NH_4)_2SO_4$, 0.01% w/v KH_2PO_4, 0.0035% w/v $MgSO_4.7H_2O$ was added as the nutrient and Al_2O_3 powder was added as immobilized material. The initial pH of the medium was adjusted at 5.0. The cultivation medium was sterilized in

autoclave for 15 minutes at 121°C before used. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20-24 hours in order to obtain high cell density. The late exponential phase cells were harvested by decantation to obtain stock cell suspension. All procedures were carried out in aseptic condition.

3.3 Immobilization cell

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto the surface of Al_2O_3 powder. The second was entrapment of Al_2O_3-cells in Ca-alginate matrix.

Sterilized Al_2O_3 powder was immersed in pre-culture medium and incubated with cell for 20-24 hours to induce natural cells adhesion. Al_2O_3 -cell mixture was added with and equal volume (1:1 v/v) of Na-alginate solution to form an alginate-Al_2O_3 -cell mixture. The mixture was used to construct Al_2O_3 doped alginate gel (AEC) carriers. The formation was initiated by adding the alginate-Al_2O_3-cell mixture drop wisely into sterile 0.12M $CaCl_2$ solution by using Gilson Pipetman auto pipette. Alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapping Al_2O_3 –cell. AEC carriers were left to harden in $CaCl_2$ solution for 30 minutes and then rinsed 3 times with sterile 0.9% w/v NaCl solution to remove excess Ca^{2+} and untrapped cells.

3.4 Fermentation

3.4.1 Batch Fermentation

Sugar cane molasses was used as carbon source for the fermentation medium. The medium contained initial sugar concentration of 220 g/l, 0.05% w/v $(NH_4)_2SO_4$ and the initial pH was adjusted at 5.0. The prepared medium was sterilized at 121°C for 15 min. Experiments were initiated by transferring prepared cell suspension or immobilized cells into 500 ml Erlenmeyer flasks containing 250 ml of the fermentation medium in order to

promote anaerobic condition which was favorable ethanol fermentation. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 72 hours. The considerable variables in batch system were including:

1. The effect of Al_2O_3 on immobilization of *S. cerevisiae* M30 was studied for the activity comparison of EC (Entrapment in alginate culture), AEC (Adsorption Al_2O_3-Entrapment alginate culture) and SC (Suspended cells culture), EC carrier was prepared by using 1.5% (w/v) of Na-alginate and AEC carrier was prepared by using 1.5% (w/v) of Na-alginate and 5 % (w/v of alginate) of Al_2O_3 with bead diameter of 4 mm and 6 mm.
2. The effect of bead diameter was studied for various bead diameter of 2 mm, 4 mm and 6 mm that were prepared by using different size of auto pipette tip (1 ml, 5 ml and 10 ml, respectively, as shown Figure 3.1) with 1.5% (w/v) of Na-alginate and 5% (w/v of alginate) of Al_2O_3.

Figure 3.1 Auto pipette tip 1 ml, 5 ml and 10 ml

3. The effect of Na-alginate concentration on fermentation was conducted at various concentrations ranging from 1.5%, 2%, 2.5% and 3% w/v with 5% (w/v of alginate) of Al_2O_3 and the optimum bead diameter.

4. The effect of Al_2O_3 concentration on fermentation was carried out at various concentrations ranging from 3.3%, 5.0%, 6.7% and 8.3% (w/v of alginate) with the optimum bead diameter and Na-alginate concentration.
5. The effect of AEC carrier expansion, the square shape (20 x 20 x 4 mm^3) immobilized cell carrier was prepared by pour the Na-alginate-Al_2O_3-cell solution (with the optimum AEC carrier preparation) to the tray and then pour $CaCl_2$ solution for formed cross-link of alginate gel. Each piece was cut by scissor while it soaks in $CaCl_2$ solution.

3.4.2 Continuous Fermentation

The suitable condition from batch system was applied for the continuous fermentation in a packed bed column with working volume around 0.6 L. The sterile molasses solution with the initial sugar concentration of 220 g/l, 0.05% w/v $(NH_4)_2SO_4$ and the initial pH at 5.0 was used as a fermentative medium. Before the bed was packed in the column, the immobilized cells in AEC (alginate-Al_2O_3-cell) carrier was cultivated with fermentative medium in Innova 4330 refrigerated incubator shaker at 150 rpm, 33°C for 24 hours to increase the immobilized cells inside the carrier. The bioreactor was sterilized by 70% (v/v) ethyl alcohol and then packed with AEC carrier. Temperature of the system is controlled at 32 ± 1 °C by the circulating water (28 °C) through the reactor jacket. CO_2 was released through a vent at the top of the column. The fermentative medium was fed to the bottom of the fermentor continuously by means of a peristaltic pump through sterile silicon tubing. Effluent liquid overflowed from an outlet port at the top of the bioreactor, maintaining a constant level of fermentation broth in the column. The carriers were trapped inside the bioreactor covered with a metal mesh filter. For improved mass transfer, the bed was divided into 9 stages by the tray packed with porous plastic (PVA) material (Figure 3.2) to prevent from liquid maldistribution. A magnetic stirring bar was placed in the bottom of the reactor for the feed mixing. The recycle process was performed at the end of each dilution rate and every 3 days of the stability test (Figure 3.3). The fermented broth was circulated by a peristaltic pump from the outlet

tube at the 5th sampling port into the inlet tube at the 1st sampling port and flowed upward through the packed column back to the outlet tube. The circulation was performed for 10 cycles with the overall flow rate of 2×68 ml/min. Schematic of the packed bed bioreactor and the recycle process were shown in Figure 3.2 and 3.3.

The studied variables in continuous system were including:

1. The dilution rates which was varied from 0.09, 0.16, 0.22 to 0.28 h^{-1}. The dilution rate was changed after the steady state of the system (at least 5 times of the retention times).
2. The stability test, was performed after the last dilution rate test (D = 0.28 h^{-1}). The stability test was carried out at D = 0.09 h^{-1} for the total operation time of 30 days.

Sampling is done regularly with volume of 5 ml for every 8 hour. The samples are frozen before analysis of sugar, ethanol, and cell concentration in order to enable all samples to be analyzed at the same time.

3.5 Analytical methods

3.5.1 Sugar concentration

Sugar concentration is determined using a modified 3,5-dinitrosalicylic acid (DNS) reagent method through a corresponding standard curve. Briefly, 0.1 ml of sample is with 37% w/v HCl. After the hydrolysis is stopped, the sample is neutralized using 30% w/v NaOH. Centrifugation is performed and the supernatant is reacted with DNS reagent before the color intensity is measured by spectrophotometer at 520 nm.

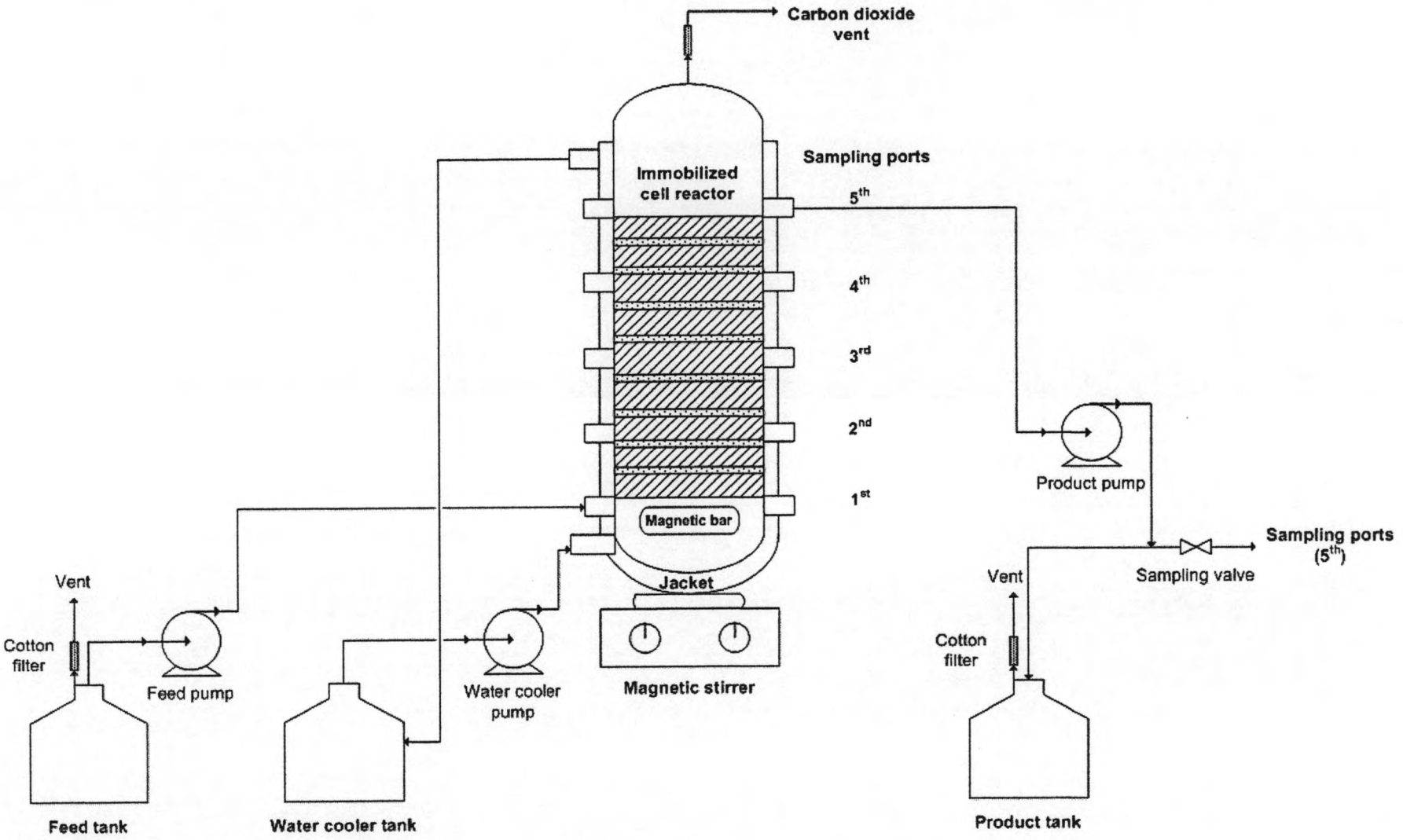

Figure 3.2 Schematic diagram of immobilized cell packed bed reactor.

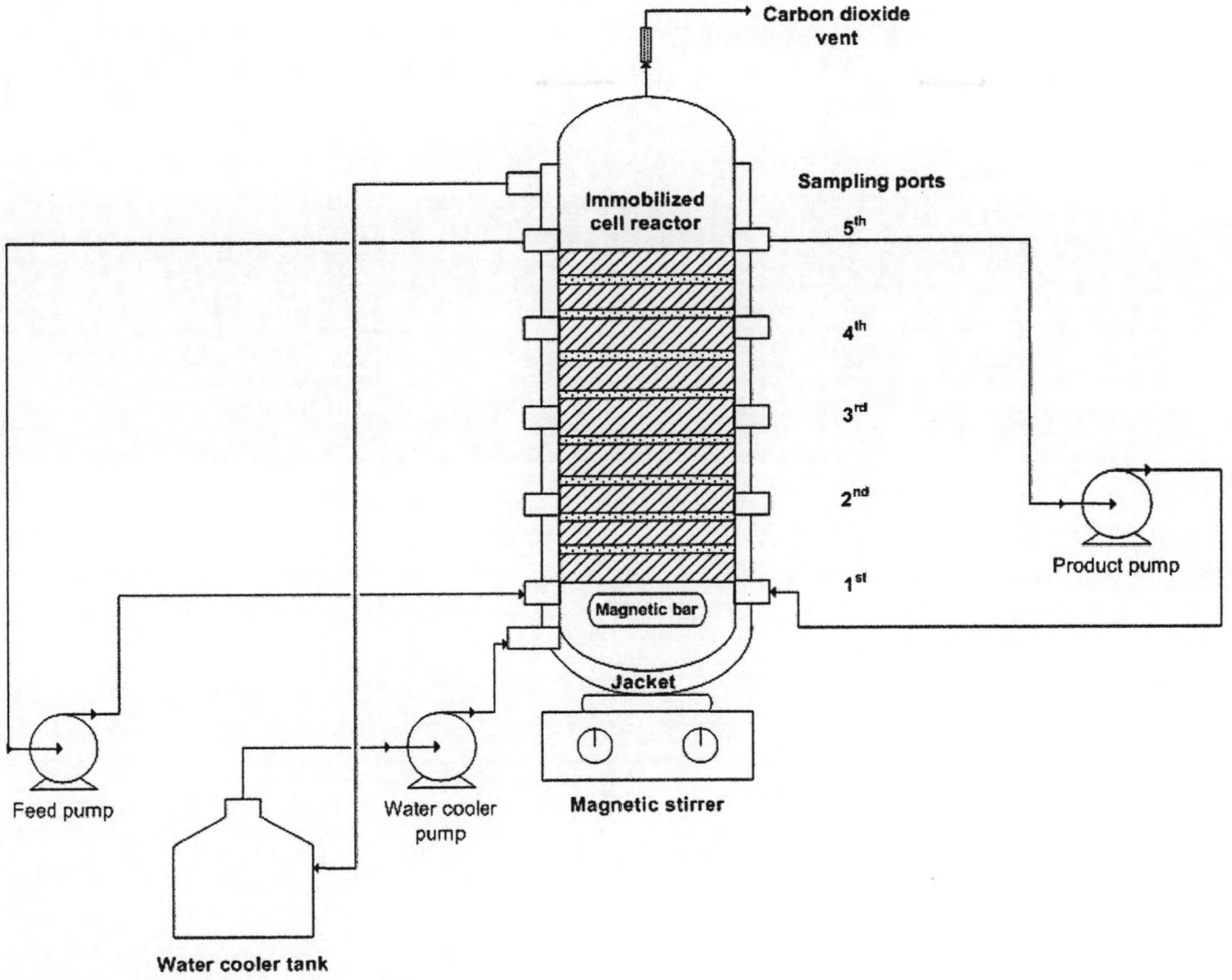

Figure 3.3 Schematic diagram of recycle process.

3.5.2 Ethanol concentration

Ethanol assay is conducted by gas chromatography using a Shimadzu Model GC $7A_G$ equipped with Flame Ionization Detector (FID). A column with length of 2 m, outer diameter of 3.3 mm, and packed with Porapak Q 80-100 mesh is used in collaboration with N_2 as carrier gas. Flow rate of N_2 is 50 ml/min. The oven and detector temperature are 190°C and 240°C respectively. The samples are injected with volume of 1μL and injection temperature of 240°C.

3.5.3 Cell concentration

Free and immobilized cell concentrations are measured by spectrophotometer at 600 nm. The cell pellet is washed with 0.1 N HCl and resuspended in water. A known mass of the carriers is dissolved in sodium citrate 0.5 M. After the alumina powder is removed, the suspension is treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. The cell dry weight was obtained using a calibration curve. The cell dry weight was proportional to cell turbidity and absorbance at 600 nm. Drying of all cells obtained from the 24 h culture broth and then performed in oven at 100°C for 2 hours.

3.5.4 Scanning Electron Microscopy (SEM)

Samples of immobilized cells in alginate- Al_2O_3 beads were frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken on a JEOL JSM-5410LV (JEOL, Tokyo) scanning electron microscope.

3.5.5 Mechanical properties measurement

The EC and AEC carrier in form of rectangular was tested for mechanical properties in term of tensile strength and percent elongation at brake by Universal Testing Machine-H 10 KM (Hounsfield). The test condition follows ASTM D882. The sample

was cut into strip-shaper specimens 10 mm in width and 10 cm in length. At least five specimens were used for each carrier tested.

3.5.6 BET Surface analysis

Pore size, pore volume, and surface area of the carriers were measured by a Brunauer-Emmett-Teller (BET) surface area analyzer (Model ASAP 2000). For sample preparation, the wet sample was soaked in ethanol aqueous solution of 30, 50, 70, 95% (v/v) for 10 minutes in each solution, respectively and 100% (v/v) for 10 minutes, 3 times. After that the sample was dried by using supercritical drying method. Then it was placed in the sample cell, which was heated up to 70 °C and held at this temperature for 12 hours. Afterward, the samples were cooled down to room temperature in desiccator and ready to measure the surface area. There were three steps to measure the surface area: adsorption step, desorption step and calibration step.

3.5.7 Calculation of fermentation parameters

Fermentation efficiency for bioreactor system was expressed as follows:

- Immobilization yield (Y_I, %)

$$Y_I = \frac{X_I}{X_T} \times 100$$

- Yield of sugar consumption (Y_S, %)

$$Y_S = \frac{S_0 - S_F}{S_0} \times 100$$

- Yield of ethanol production ($Y_{P/S}$, g ethanol/g sugar)

$$Y_{P/S} = \frac{P_F - P_0}{S_0 - S_F} \times 100$$

- Ethanol productivity (Q_P, g/l h)

$$Q_P = \frac{P_F}{fermentation\ time} = P_F \times D$$

X_I ; immobilized cell concentration (g/l)

X_E ; free cell concentration (g/l)

X_T ; total cell concentration (g/l)

S_0 ; initial sugar concentration (g/l)

S_F ; final sugar concentration (g/l)

P_0 ; initial ethanol concentration (g/l)

P_F ; final ethanol concentration (g/l)

D ; dilution rate (h^{-1})