Chapter III

Materials and Methods

Most of the materials and methods used in this work are of common practice in cell cultivation, cell immobilization, and fermentation technologies. A flocculating yeast strain, *Saccharomyces cereviceae* M30 was chosen based on its high ethanol productivity. Methods for reinforced carriers' preparation were constructed based on simple sensible aseptic procedures that can be readily applied on bench scale fermentation experiments. Complete details of the methods used in this study are provided in Appendix A. Except molasses, palm sugar, and chitosan all chemicals were of analytical grade.

General flow diagram of experimental works carried out in this study is provided by Figure 3.1. In the inoculum development stage, highly concentrated cell suspension was obtained by decantation. This cell suspension was entitled as stock cell suspension. The stock cell suspension was then used for carrier preparation. With exception of loofa sponge carrier, all carriers were prepared by gelation. The carriers were employed for batch ethanol fermentation in shake flasks culture at 150 rpm, 33°C. Samples of the fermentation were analyzed for sugar, cell, and ethanol concentration. Electron micrographs of the carriers were obtained by scanning electron microscopy (SEM). Fermentation parameters were reported in the form of yield factors.

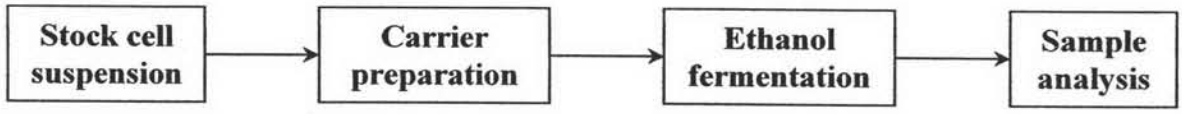

Figure 3.1 General flow diagram of experimental work

3.1 Stock cell suspension

Stock cell suspension preparation steps are outlined by Figure 3.2. *S. cereviceae* M30 strain was kindly provided by Assoc. Prof. Savitree Limtong, Dr.Eng. from Department of Microbiology, Kasetsart University, Bangkok. Stock culture was stored in PDA agar slant. Each starter culture was obtained by transferring cells from an agar slant into 500 mL Erlenmeyer flask containing 150 mL sterilized cultivation medium. The cultivation medium was composed of 5% w/v sugar from palm sugar, 0.05% w/v

$(NH_4)_2SO_4$, 0.01% w/v KH_2PO_4, and 0.0035% w/v $MgSO_4.7H_2O$ at pH 5. The medium was sterilized in autoclave for 20 minutes at 121°C before use. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours before harvesting. The cells were concentrated by decantation to obtain stock cell suspension. All procedures were done in aseptic condition.

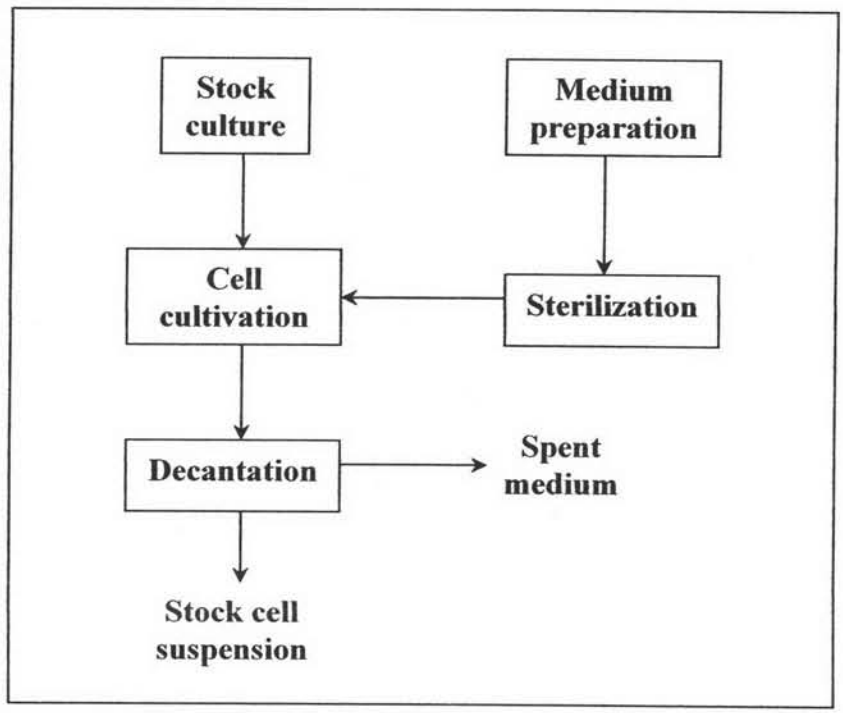

Figure 3.2 Methods of stock cell suspension preparation

3.2 Carrier preparation

For preliminary examination of cell adhesion onto loofa sponge, various shapes of originated from different parts of loofa was employed in batch fermentation. Alginate gel was used for both cell adsorption and entrapment purposes. Cell immobilization for chitosan was done only by adsorption because of the harsh condition encountered in chitosan gelation which was harmful to cell activity.

3.2.1 Loofa sponge carrier preparation

Dried loofa sponge was cut into 5 different shapes, which were:

1. Axial sponge. An entire loofa sponge with diameter of 3 cm was cut axially to obtain a ring structure with height of 0.5 cm.

2. Core sponge. Core sponge with diameter of 1.5 cm and 0.5 cm height was prepared from the central part of the sponge.
3. Cubic sponge. Peripheral part of the sponge was cut into cubic of 2 x 2 cm.
4. Rolled cylinder. Rectangle with dimension 2 x 3 cm obtained from the peripheral part was rolled to form a cylinder of 2 cm in height and 0.5 cm in diameter.
5. Sponge bead. Spherical sponge was formed from rolled peripheral part cubic with dimension of 1 x 1 cm.

After cutting, the sponges were sterilized with autoclave for 20 min at 121°C. Formation of the sponge to the desired shape was carried out aseptically after sterilization.

3.2.2 Alginate-loofa carrier preparation

Preparation steps of entrapment alginate-loofa carriers are shown in Figure 3.3. Sodium alginate 3% w/v was made by dissolving Na-alginate powder in NaCl 0.9% w/v solution. It was autoclaved for 5 minutes at 121°C and kept overnight at 4°C to facilitate deaeration. Stock cell suspension was added to the alginate solution to form an alginate-cell mixture with volumetric ratio of 1:10. The mixture was used to construct 4 types of gel carriers: entrapment alginate bead (EAB), entrapment alginate-loofa bead (EALB), entrapment alginate-loofa cube (EALC), and entrapment alginate-loofa cylinder (EALY).

EAB (∅=2 mm) formation was initiated by adding the alginate-cell mixture drop wisely into $CaCl_2$ 1.47% w/v using a syringe with internal diameter of needle of 1.2 mm. Small pieces of peripheral sponge of loofa (4 x 4 mm) were rolled by hand to form spherical shape and dipped into alginate-cell mixture before transferred to $CaCl_2$ 1.47% w/v to form EALB carrier (∅=3 mm). EALC with block dimension of 8 x 8 x 1 mm was formed in the same way as EALB by using cubic sponge (8 x 8 mm) instead of rolled spherical sponge. In case of EALY formation, rolled 2 x 1 cm sponge was used. All gel carriers were left to harden in $CaCl_2$ solution under mild stirring for 15 minutes. The carriers were then rinsed 3 times with NaCl 0.9% w/v.

Figure 3.4 outlines preparation steps of adsorption based carriers. In adsorption alginate loofa carrier preparation, alginate solution was used as the precursor solution. After rinsing, the carriers were sterilized in autoclave for 5 min at 121°C. They were then immersed in stock cell suspension for 1 hour to induce natural cells adhesion. All aforementioned procedures were done in aseptic condition to minimize contamination.

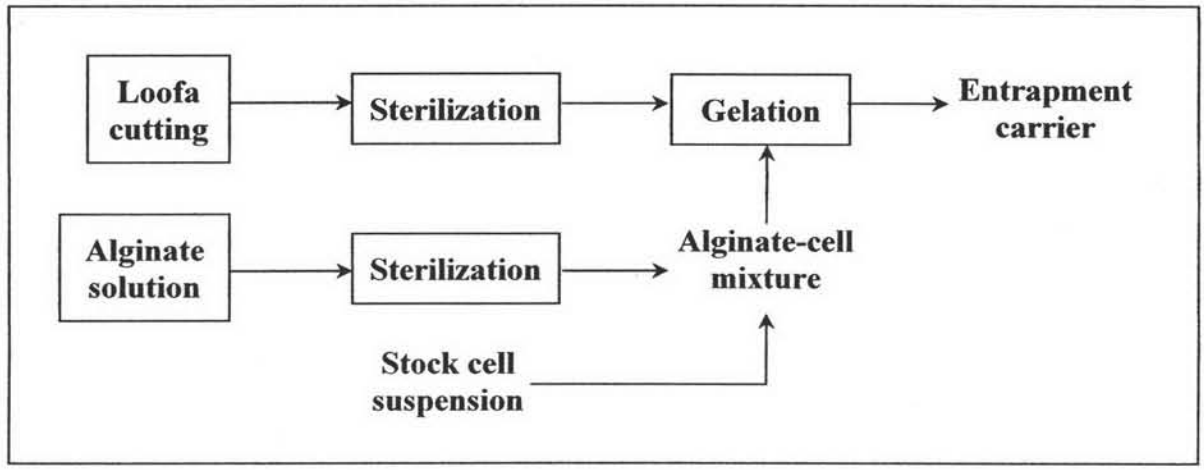

Figure 3.3 Preparation of entrapment based carrier

3.2.3 Chitosan-loofa carrier preparation

As chitosan-loofa carrier is classified as adsorption based carrier, its preparation methods is also well represented by Figure 3.4. Chitosan flakes were slowly added to 1M acetic acid to form 2% w/v monomer solution of chitosan. Chitosan-loofa carriers were formed similarly as adsorption alginate-loofa carriers by exchanging $CaCl_2$ with NaOH 1M as gelating agent. The carriers were left to harden for 24 hours before rinsed with deionized water. After sterilization for 5 min at 121°C, the carriers were immersed in stock cell suspension for 1 hour.

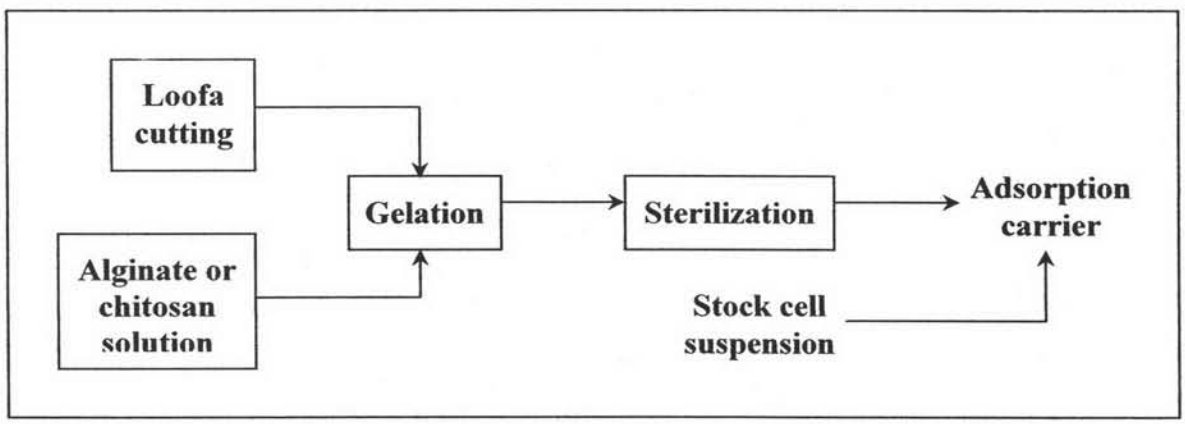

Figure 3.4 Preparation of adsorption based carrier

3.3 Ethanol fermentation

In early experiments (until fermentation 3), palm sugar was chosen as sugar source because of its clear color characteristic which was more favorable for analytical purpose.

In fermentation 4 and 5, molasses was utilized instead of palm sugar as it represented the most widely used raw material for fermentative ethanol production.

In palm sugar based medium, the composition of fermentation medium was similar with cell cultivation except that the sugar concentration was increased to optimum level of 200-220 g/L [43]. In case of molasses based medium, 0.05% w/v ammonium sulfate was added as the nutrient. The volume of medium was adjusted to 250 mL in 500 mL Erlenmeyer flask in order to promote anaerobic condition which was favorable ethanol fermentation by yeast. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C.

Suspended cell (SC) culture was prepared by inoculating stock cell suspension into fermentation medium in 500 mL Erlenmeyer flask. Immobilized cell cultures were inoculated aseptically with a known mass of their respective carriers. Sampling was done regularly. The samples were frozen before analysis of sugar, ethanol, and cell concentration in order to enable all samples to be analyzed at the same time. In repeated batch mode, decanted cells for SC or filtered carriers in case of immobilized cell systems were transferred aseptically to fresh medium and new run was carried in the same configuration as the main run.

3.4 Sample analysis

Sugar concentration was determined by a modified 3,5-dinitrosalicylic acid (DNS) reagent method through a corresponding standard curve. Briefly, sample was hydrolyzed with HCl 37% in boiling water bath for 10 minutes. After hydrolysis, the sample was neutralized using NaOH 30% w/v. Centrifugation was performed and the supernatant was reacted with DNS reagent before the color intensity was measured by spectrophotometer at 520 nm.

Ethanol assay was 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 was used in collaboration with N_2 as carrier gas. Flow rate of N_2 was 50 ml/min. The oven and detector temperature were 190°C and 240°C respectively. The samples were injected with volume of 1μL and injection temperature of 240°C.

Free and immobilized cell concentrations were measured as cell dry weight. For free cell determination, liquid samples from fermentation were centrifuged (2000 rpm, 15

minutes). The cell pellet was washed with HCl 0.1 N and resuspended in water. The cell concentration was measured by spectrophotometer at 600 nm. In case of immobilized cell carriers, a known mass of the carriers was dissolved in sodium citrate 0.05 M. After the sponge was removed, the suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Drying of all cells was performed in oven at 100°C for 2 hours.

During the course of fermentation, samples of carrier were collected for scanning electron microscopy (SEM). The carriers were sputter-coated by gold and examined using JSM 6700-F (JEOL, Japan) scanning electron microscope.

Immobilization yield (Y_I, %) was defined as the ratio of immobilized cell concentration (X_I, g/L) to total cell concentration (X_T, g/L) at the end of each batch. X_T was calculated as summation of free cell concentration (X_E, g/L) and X_I. The yield of sugar consumption (Y_S, %) was considered as the ratio of sugar consumption ($S_0 - S_F$, g/L) to starting sugar level (S_0, g/L). S_F was the value of final sugar concentration (at the end of batch). The ethanol yield factor ($Y_{P/S}$, g ethanol/g sugar) was the ratio of ethanol production of each batch ($P_F - P_0$, g/L) to sugar consumption ($S_0 - S_F$). P_F and P_0 was ethanol concentration at the end and beginning of ethanol fermentation batch respectively.