CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial Strains

Saccharomyces cerevisiae M30 and *Kluyveromyces marxianus* DMKU 3-1042 strain were kindly provided by Prof. Savitree Limtong, Dr.Eng. (Department of Microbiology, Kasetsart University, Bangkok). Stock cultures were stored in PDA agar slants at 4 °C.

3.1.2 Chemicals

The details of chemicals used in this experiment are shown in Table 3.1

Table 3.1 The chemicals used in this experiment

Chemical	Supplier
- Sucrose	Ajax Finechem
- Sodium hydroxides (NaOH)	Merck
- Hydrochloric acid (HCl)	J.T. Baker
- Ammonium sulfate $(NH_4)_2SO_4$	Ajax Finechem
- Magnesium sulfate $(MgSO_4.7H_2O)$	APS
- 3,5-dinitrosalicylic acid (DNS)	Fluka
- Potassium dihydrogen ortho-phosphate (KH_2PO_4)	Ajax Finechem

- Potato dextrose agar (PDA)	Himedia
- Absolute ethanol	Merck
- Na-K tartrate	Carlo Erba

3.1.3 Equipments

1 Scanning electron microscopy, SEM (JOEL JSM-5410LV, Japan).

2 UV-visible spectrophotometer, UV-Vis (UV 2450, Shimadzu, Japan).

3 Gas chromatography (Shimadzu Model GC $7A_G$, Japan).

4 Autoclave (Model Tomy Autoclave SS-325, Ner ima-ku, Tokyo, Japan).

5 Refrigerated incubator shaker,(Innova 4330, New Brunswick Scientific, USA).

6 Peristaltic pump (WATSON MARLOW 505U, England).

7 YSI (Model 2700 SELECT Biochemistry Analyzer, Yellow Springs, Ohio 45387 USA).

3.2 Methods for fermentation.

3.2.1 Methods for stock cell suspension preparation

Transferring cells from an agar slant into 500 ml Erlenmeyer flask containing 100 ml sterilized cultivation medium. The cultivation medium was composed of 10% w/v sugar from palm sugar, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ at pH 5. The medium was sterilized in autoclave for 15 minutes at 121°C. Cell cultivation was carried out in the Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 200 rpm, 33, 37, 40 and 45 °C for 24 hours.

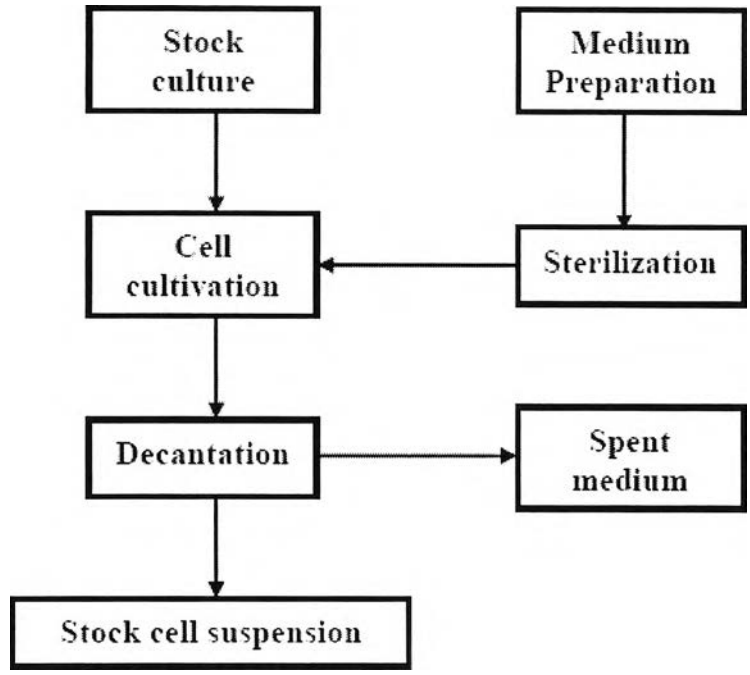

Figure 3.1 Diagram for methods of stock cell suspension preparation

3.2.2 Methods for immobilization and coimmobilization.

3.2.2.1 Thin shell silk cocoon.

Thin shell silk cocoons of 2.5 g and 250 ml of culture medium in 500 ml flask were autoclaved for 15 minutes at 121°C. Stock cell suspension of 10 ml was added to the medium, which was called monoculture. For coimmobilization, equal volume of 5 ml of each *K. marxianus* and *S. cerevisiae* stock cell suspension were mixed together. The mixed cell suspension was then added to the medium, which was called mixed culture. After that the sterilized thin shell silk cocoons was added into the monoculture and mixed culture. The immobilized cells were obtained after the incubation of the suspension mixture for 24 hr. The method are shown in **Figure 3.2**

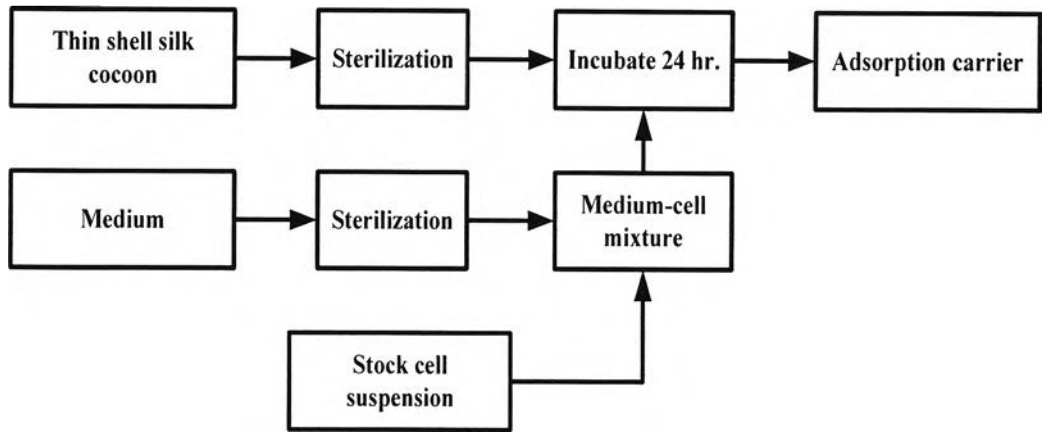

Figure 3.2 Preparation of thin shell silk carrier

3.2.2.2 Alginate-loofa.

Sodium alginate of 30 g/l was made by dissolving Na-alginate powder in NaCl 9 g/l solution. It was autoclaved for 15 minutes at 121°C and kept overnight at 4°C to facilitate deaeration. Stock cell suspension was added to the alginate solution to form alginate-cell mixture with volumetric ratio of 1:10. The mixture was used to construct for entrapment alginate-loofa cube (EALC). Cubic loofa sponge (20 x 20 x 2 mm^3) 2.5 g for batch fermentation and 18 g for continuous fermentation were dipped into alginate-cell mixture before transferred to 14.7 g/l $CaCl_2$ solution to form EALC. EALC carriers were left to harden in $CaCl_2$ solution under mild stirring for 15 minutes. The carrier was then rinsed 3 times with NaCl 9 g/l. The method are shown in **Figure 3.3**

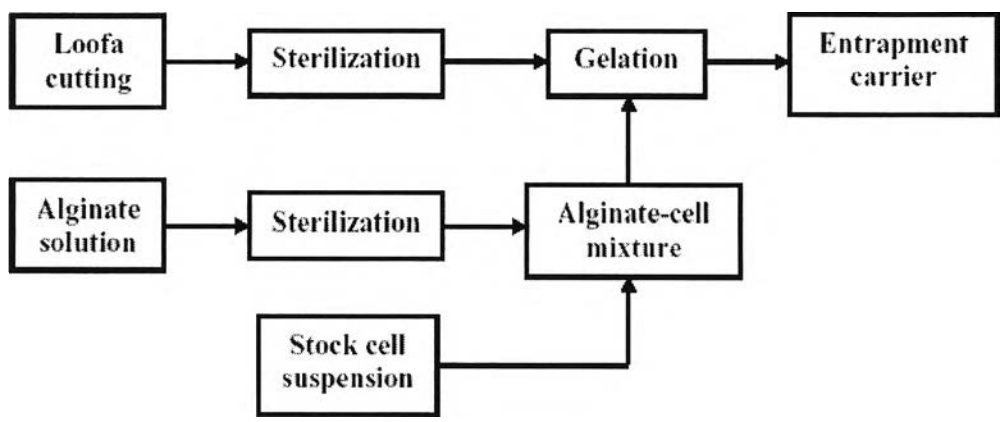

Figure 3.3 Preparation of Alginate-Loofa carrier

3.2.3 Methods for ethanol fermentation.

3.2.3.1 Batch Fermentation

Sugar cane juice and molasses were used as carbon source for the fermentation medium. The medium contained initial sugar concentration of 220 g/L, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ 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 the 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 200 rpm. The fermentation was performed at the temperature varied from 33°C to 45°C for 72 hours. 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. The considerable variables in batch system were including:

1. The materials for immobilized, are thin shell silk cocoon and alginate-loofa.
2. The substrates used as carbon source are sugar cane juice and molasses.
3. The effect of temperature on fermentation was examined in the temperatures range of 33°C - 45°C.
4. The effect of mono and mixed cell cultures was studied by using suspension cells or immobilized cells in monocultures of *K. marxianus* DMKU 3-1042 or mixed cultures of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30.

3.2.3.2 Continuous Fermentation

The suitable condition from the batch system was applied for the continuous fermentation in a packed bed column with working volume of 0.7 L. The sterile sugar cane juice and molasses solutions with the initial sugar concentration of 220 g/l, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ with the

adjusted pH at 5.0 was used as a fermentative medium. Sampling was aseptically took with volume of 2 ml for every 8 hours. 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.

3.2.4 **Analytical methods**

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 hydrolyzed with 0.5 ml of 37% w/v HCl. After the hydrolysis is stopped, the sample is neutralized using 0.5 ml of 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. The residual glucose concentration was also determined by the glucose analyzer (YSI, Model 2700 SELECT Biochemistry Analyzer, Yellow Springs, Ohio 45387 USA).

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 temperatures are 190°C and 240°C respectively. The samples are injected with volume of 1μL and injection temperature of 240°C.

Cell concentration was determined by cell dry weight method. Yeast cells were harvested by centrifugation for 15 min at 20,000 rpm. The pellets were washed with HCl 0.1 N; after that it was washed twice with distilled water and weighed after 24 h of drying at 100°C. At the beginning and the end of fermentation, samples of carrier were collected for Scanning Electron Microscopy (SEM).

3.2.5 **Calculation of fermentation parameters**

Fermentation efficiency for bioreactor system was expressed as follows:

1 Immobilization yield (Y_I, %)

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

2 Yield of sugar consumption (Y_S, %)

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

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

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

4 Ethanol productivity (Q_P, g/l h)

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

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

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)