# CHAPTER III

# MATERIALS AND METHODS

Lactic acid fermentation in this study is carried out in a shake flask using *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741 as lactic acid producer.

## 3.1 Chemicals

1. Meat extract: Merck, Germany.
2. Yeast extract: Merck, Germany.
3. Peptone from casein: Merck, Germany.
4. D(–)glucose andydrous: Merck, Germany.
5. Tween-80: Merck, Germany.
6. di-Potassium hydrogen phosphate, $K_2HPO_4$: Ajax Finechem, Australia.
7. Magnesium sulphate, $MgSO_4$: Ajax Finechem, Australia.
8. Manganese sulphate, $MnSO_4$: Merck, Germany.
9. Calcium carbonate, $CaCO_3$: Ajax Finechem, Australia.
10. Chitosan: Seafresh Chitosan (LAB), Thailand.
11. Loofa sponge: Loofa sponge bathing scrub factory, Thailand
12. Sugarcane bagasse: Local market, Thailand.
13. Tamarind fruit fibre: Local market, Thailand.
14. Coconut fibre: Bedding stuff factory, Thailand.

## 3.2 Equipments

1. Refrigerated incubator shaker: Innova 4330, USA
2. UV-vis spectrophotometer: UV-2450, Shimadzu, Japan.
3. Glucose/Lactate analyzer: Biochemistry 2700, Yellow Spring Instrument, USA.
4. Scanning Electron Microscopy: JSM-5410LV, JSM, Japan.
5. Zetasizer Nano: ZS 90, Malvern, UK.
6. Viscometer: DV – II +, Brookfield, USA.

## 3.3 Methods

### 3.3.1 Stock cell suspension

*L. salivarius* is kindly provided by Thailand Institute of Scientific and Technological Research (TISTR). Stock culture is stored at -20°C in the mixture of modified MRS broth and glycerol at 1:1 ratio. Each starter culture is obtained by two successive propagations at 37°C in preculture medium. The preculture medium composes of meat extract 10 g $L^{-1}$, yeast extract 5 g $L^{-1}$, peptone from casein 10 g $L^{-1}$, glucose 20 g $L^{-1}$, tween-80 1 g $L^{-1}$, $K_2HPO_4$ 2 g $L^{-1}$, $MgSO_4$ 0.2 g $L^{-1}$, and $MnSO_4$ 0.2 g $L^{-1}$. The medium is sterilized in autoclave for 15 minutes at 121°C before use. The cells are concentrated by decantation or centrifugation to obtain starter culture. All procedures are done in aseptic condition.

### 3.3.2 Carrier preparation

In the beginning, loofa sponge derived from the factory is washed with tap water to remove dirt and impurities and oven dry. Then it is cut into square shape with the approximate dimension 2 x 2 cm [48, 55]. Sugarcane bagasse, waste from the juice shop, is bathed in tap water in order to remove remained sugar. Tamarind fruit fibre, waste from the tamarind concentrated juice factory, is thoroughly cleaned off the tamarind fruit pulp. The last one, coconut fibre, is soaked in tap water to eliminate dirt and small particles. To ensure good cleaning before used as immobilized support carrier, all materials are boiled in deionized water for 30 minutes, left in deionized water for 24 hours, and finally dried in a hot air oven at 70°C [31, 36, 48, 55]. All fibres obtained from this step are named "untreated fibre".

Pre-treatment with hydrogen peroxide is done by immerse the fibres in various concentration of hydrogen peroxide solution (0.25%, 0.5%, 1.0%, and 2.5%) at 60°C for several time interval (20, 40, and 60 minutes) in water bath [27]. After that the obtained fibres are washed several times with deionized water and dried at 70°C. Derived material is classified as "$H_2O_2$-treated fibre".

Surface modification by chitosan is applied to the $H_2O_2$-treated fibre. A 1% w/v of chitosan solution in 2% v/v acetic acid is prepared as modified reagent. The

fibres are incubated in chitosan solution for 30 minutes [23] and dried at 50°C in a hot air oven. Obtained material from this process is called "$H_2O_2$-chitosan-treated fibre".

### 3.3.3 Adsorption of cell to matrices

The cell suspension containing 16-h-old cells ($OD_{600}$ after 100 fold diluted = 0.30-0.35) is inoculated to the fibres in the medium and incubated in rotary shaker at 50 rpm and 37°C for 24 hours. The immobilized biocatalyst is separated from the fermentation broth and washed with sterile normal saline twice to remove free and loosely bound microorganisms.

### 3.3.4 Lactic acid fermentation

The cells of *L. salivarius* immobilized on the treated fibres are inoculated in a medium containing glucose 50 g $L^{-1}$, others are the same as in preculture medium. The fermentation is propagated at the same condition with the immobilized step. Sampling is done regularly. In repeated batch mode, immobilized biocatalysts are transferred aseptically to fresh medium and new run is carried out in the same configuration as the main run.

### 3.3.5 Sample analysis

Cell mass concentration is determined by measuring the optical density of the cell solution at 600 nm. Depending on the $OD_{600}$, the fermentation broth was transferred to pre-weighed aluminum cup and dried at 95°C for 24 hours. After that the calibration curve of $OD_{600}$ versus cell dry weights will be obtained by correlating the cell dry weight with $OD_{600}$.

Glucose and lactic acid concentration in fermentation broth are measured by YSI 2700 biochemistry, a biosensor [56 – 58].

During the course of fermentation, samples of carriers are collected to canning electron microscopy (SEM). The carriers are sputter-coated by gold and examined using JSM 5410LV (JEOL, Japan) scanning electron microscope.