CHAPTER III

MATERIALS AND METHODS

Lactic acid production in this study is carried out in a batch fermentor using lactic acid bacteria, *Lactobacillus salivarius subsp. salivarius* ATCC 11741.Cassava starch hydrolysate and brewer's yeast autolysate are used as carbon and nitrogen source for the producing lactic acid.

3.1 Chemicals

- Meat extract (Merck, Germany)
- Yeast extract (Merck, Germany)
- Peptone from casein (Merck, Germany)
- D-glucose anhydrous ($C_6H_{12}O_6$ = 180.16, Ajax Finechem, Australia)
- Tween 80 (Merck, Germany)
- di-Potassium hydrogen phosphate (K_2HPO_4 = 174.18, Carlo Erba reagents, Italy)
- Magnesium sulphate ($MgSO_4.7H_2O$ = 246.47, Ajax Finechem, Australia)
- Manganese sulphate ($MnSO_4.H_2O$ = 169.01, Ajax Finechem, Australia)
- 3,5 Dinitrosalicylic acid (DNS , Fluka, China)
- Potassium sodium tartrate (KNaC4H4O6.4H2O = 282.22, Ajax Finechem, Australia)
- n-Hexane (95%, Fisher Scientific, United Kingdom)
- Isooctane (Carlo Erba reagents, Milan, Italy)
- Hydrochloric acid (HCl 36.5-38%, J.T. Baker, NJ, USA)
- Sodium hydroxide (NaOH = 40, Ajax Finechem, Australia)

3.2 Equipments

- Fermentor CSTR, 4 in series (Biostat Q, B. Braun Biotech International, Germany)
- Water bath shaker (XY-80, Japan)
- UV-Vis spectrophotometer (UV -2450, Shimudzu, Japan)

- High performance liquid chromatography (HPLC, prevail C18, 5 micron)
- Autoclave (SS-325, Japan)
- Refrigerated incubator shaker (Innova 4330, USA)
- Laminar air flow hood (ISSCO larminar flow model VS-124)
- Magnetic stirrer / Hot plate (RCT Basic, Ika labortechnik, Germany)
- Centrifuge (Kubota 7820 and 5100, Japan)
- autopipette (Pipetman P100, P1000, and P5000, USA)
- Desiccator (SR Lab, Thailand)
- Stirrer vertical (RW 20 DZM, Ika labortechnik, Germany)
- Distillation Unit Nitrogen analyzer (BUCHI 339)

3.3 Methods

3.3.1 Lactic acid fermentation

3.3.1.1 Microorganism

Lactobacillus salivarius subsp. salivarius ATCC 11741 (from Thailand Institute of Scientific and Technological Research, TISTR, Thailand) was used in this study. Stock cultures were maintained at -80 ^{0}C in MRS broth (Difco, Detroit, MI, USA) contains glycerol ratio 1:1. *Lactobacillus salivarius* was reactivated by two successive propagations at 37 ^{0}C for 24 h in preculture medium (Figure 3-1). The preculture medium contains the following (g/l): 10 meat extract; 5 yeast extract; 10 peptone from casein; 20 glucose; 1 tween-80; 2 K_2HPO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.2 $MnSO_4 \cdot H_2O$.

3.3.1.2 Fermentation medium

Fermentation medium consisted of brewer's yeast autolysate (29-114 ml/l) or yeast extract (5 g/l,) (The brewer's yeast autolysate, BYA, was reconstituted at 29 ml/l, corresponding to yeast extract concentration of 5 g/l); glucose (from cassava starch hydrolysate, CSH, 70-100 g/l) or D-glucose (20-100 g/l); meat extract (10 g/l); peptone (10 g/l); Tween-80 (1 g/l); K_2HPO_4 (2 g/l); $MgSO_4 \cdot 7H_2O$ (0.2 g/l); $MnSO_4 \cdot H_2O$ (0.2 g/l).

3.3.1.3 Fermentor conditions

Batch culture was carried out in 1-L fermentor (Biostat Q, B. Braun Biotech International, Germany) (Figure 3-2). Temperature was controlled at 37 ^{0}C, stirrer speed was 100 rpm, and no aeration was used. pH was varied from 5.0 to 6.0 by the automatic addition of 4 M NaOH and 4 M HCl.

Figure 3-1 Inoculum flask of *L.salivarius subsp. salivarius*

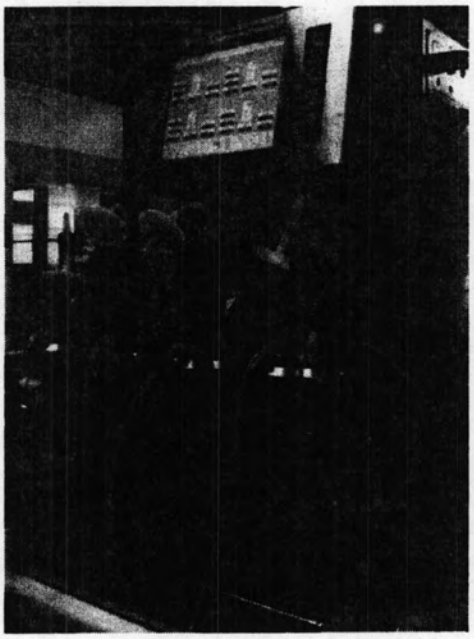

Figure 3-2 Fermentor CSTR, 4 in series

3.3.1.4 Biomass analysis

Depending upon the OD_{600}, the fermentation broth was transferred to pre-weighed, dry aluminum cups and dried for 24 h at 95 ^{0}C. Dry cell weights (DCW) were correlated with optical density measurements at 600 nm to obtain a calibration of OD_{600} vs. DCW. OD_{600} readings were subsequently used to determine DCW.

3.3.1.5 Glucose analysis by DNS reagent method (Miller, 1959) [46]

1. Reagents

- 3-5 Dinitrosalicylic acid 5 g
- Sodium hydroxide 8 g
- Potassium sodium tartrate 150 g
- Add distilled water to 500 ml

2. Procedures

1. Add 0.5 ml of DNS reagent to 0.5 ml of glucose sample in lightly capped test tube. (To avoid the loss of liquid due to evaporation)

2. Heat the mixture at 90 ^{0}C for 5-15 minutes to develop the red-brown color.

3. After cooling to room temperature in a cold water bath, add distilled water 5 ml and record the absorbance with a spectrophotometer at 540 nm.

3.3.1.6 Lactic acid analysis

Lactic acid concentration in fermentation broth were measured by HPLC using a refractive index detector (RI detector) detection with a prevail C18 250 mm × 4.6 mm. Elution was with 20 mM H_3PO_4 at 1 ml/min.

3.3.2 Brewer's yeast autolysis [47].

3.3.2.1 Preparation of brewer's yeast

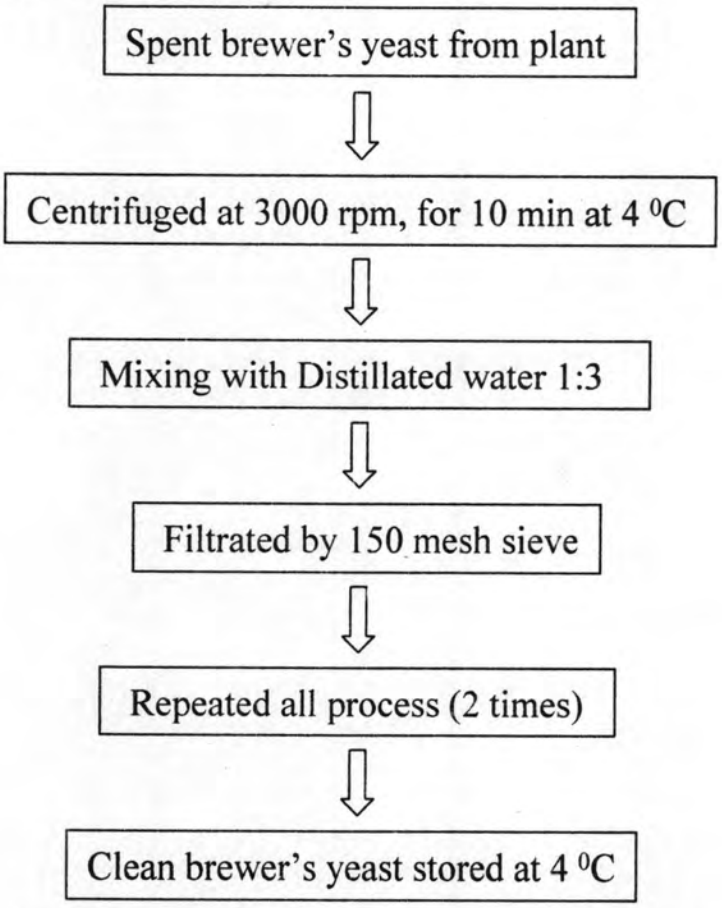

Figure 3-3 preparation of clean brewer's yeast cream

3.3.2.2 Brewer's yeast autolysis

Autolysis was started after adjusted pH of brewer's yeast cream to 6.0. Temperature was controlled at 50 ^{0}C, stirrer speed was 100 rpm, pH 6.0, 13 h.

3.3.2.3 Debittering procedure [16]

The brewer's yeast autolysate was extracted with n-hexane to remove the bitter substance. The sample to solvent ratio was 1:5 by volume. The BYA and n-hexane were mixed by using a motor stirrer in a plastic container for 10 min. Thereafter the mixture was centrifuged at 10,000 rpm for 10 min and each phase was separated in a separating funnel to recover the aqueous phase as the product.

3.3.2.4 Bitterness analysis (European Brewery Convention, EBC, 1987)

1. Add 1 ml of HCl 6 N and 10 ml of isooctane to 10 ml of sample.
2. Centrifuge at 3000 rpm, for 20 min, at 4 ^{0}C.

3. Record the absorbance of the isooctane layer at 275 nm against a reference of pure isooctane, by spectrophotometer.

For the calculation of bitterness units

$$\text{Bitterness (EBU)} = OD_{275} \times 50$$

1 EBU = 1 mg iso alpha-acids / 1 l solution

3.3.2.5 Total nitrogen analysis by Kjeldahl method [48]

The kjeldahl method may be broken down into three main steps:

Digestion – the decomposition of nitrogen in organic samples utilizing a concentrated acid solution. This is accomplished by boiling a homogeneous sample in concentrated sulfuric acid. The end result is an ammonium sulfate solution.

Distillation – adding excess base to the acid digestion mixture to convert NH_4^+ to NH_3, followed by boiling and condensation of the NH_3 gas in a receiving solution.

Titration – to quantify the amount of ammonia in the receiving solution

$$\%\ \text{Total nitrogen} = \frac{1.4007 \times N_{H2SO4} \times V_{H2SO4}}{V_{sample}}$$

N_{H2SO4} = Normality of standard acid (sulfuric acid)

V_{H2SO4} = Volume of sulfuric acid for titration

V_{sample} = Volume of sample used