Chapter 3
METERIALS AND METHODS

3.1 Microorganism

Saccharomyces cerevisiae M30, the flocculating yeast, kindly provided by the laboratory of Assoc. Prof. Savithree Limthong (department of microbioology, Kasetsart University, Bangkok, Thailand) is used for the ethanol fermentation experiments.

3.2 Apparatus

- Shaking incubator model D3006 Burgwedel of Gesellschaft für Labortechnik mbH, German
- Centrifuge model Kubota 5100 of Kubota Corporation, Japan
- Spectrophotometer model Spectronic 20 Genesys of Spectronic Instrument, USA
- Laminar flow model VS-124 of ISSCO, USA
- Autoclave model HL24ADY of Hirayama Manufacturing Corporation, Tokyo, Japan
- pH meter of Mettler Tolledo, Switzerland
- Hot air oven model ULM 500 of Menmert, Germany
- Gas chromatography

3.3 Chemicals

- Hydrochloric acids [HCl], Merck, Germany
- Sodium hydroxide [NaOH], Carlo Erba, Italy
- Ammonium sulfate [$(NH_4)_2SO_4$], Carlo Erba, Italy
- Potassium sodium tartrate [$KNaC_4H_4O_6 \cdot 4H_2O$], Carlo Erba, Italy
- 3,5-dinitrosalicylic acids [$(C_9H_9N_2^+)(C_7H_3N_2O_7^-)$], Fluka chemical, Switzerland
- Ethyl alcohol anhydrous for HPLC 99.8% (v/v), Italmar Co., Ltd., France
- Potato dextrose agar (PDA), Becton, Dickinson and Company, France

- Molasses
- HPLC water
- Reverse osmosis water

3.4 Experimental Methods

3.4.1 Inoculums

Stock cultures are stored in PDA agar slant. Precultures are prepared by transferring a stock culture to 100 mL of prepared medium in 250 mL Erlenmeyer flask and incubated at 33 °C for 20 hours before transferred to main culture. The medium for the inoculum contained 0.05% ammonium sulfate and 5% inverse sugar from molass mash and are adjusted pH to 5.0. The prepared medium is sterilized at 121°C for 20 minutes.

3.4.2 Shake flask fermentation

Shake flask fermentation is conducted in duplicate using molasses as nutrient at assigned concentration. The medium are added with 0.05%w/v ammonium sulfate adjusted at pH 5 and sterilized by autoclaving at 121°C for 20 minutes. Erlenmeyer flasks 500 mL containing 250 mL of the prepared fermentation medium are inoculated with 5% of cell suspensions. For the study of the effect of initial sugar concentration, experiments are carried out in the presence of 3%, 5%, 8%, 11%, 13%, 15%, 17%, 20%, 22%, and 25%w/v and incubate at 33°C for 72 hours. For the study of the effect of operating temperature, media are prepared with the optimal initial reducing sugar concentration determined from the earlier experiment and incubate at temperature ranging from 30 to 42°C. All fermentation flasks are shaken at 150 rpm. The fermentation is monitored for 3 days by removing 4 mL samples every 4, 6, and 8 hours on the first, the second and the third day of the fermentation respectively for cell, sugar and ethanol analyses.

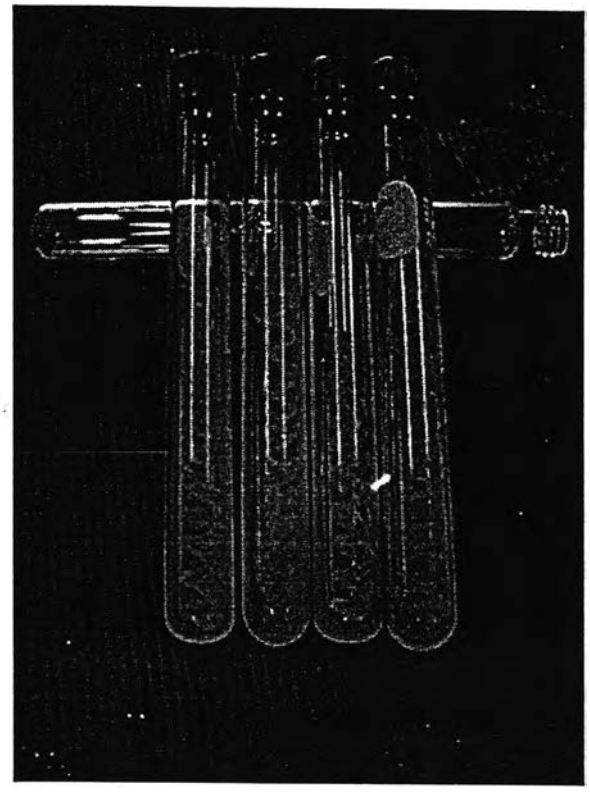

Figure 3.1 *Saccharomyces cerevisiae* M30 on Potato dextrose agar slant.

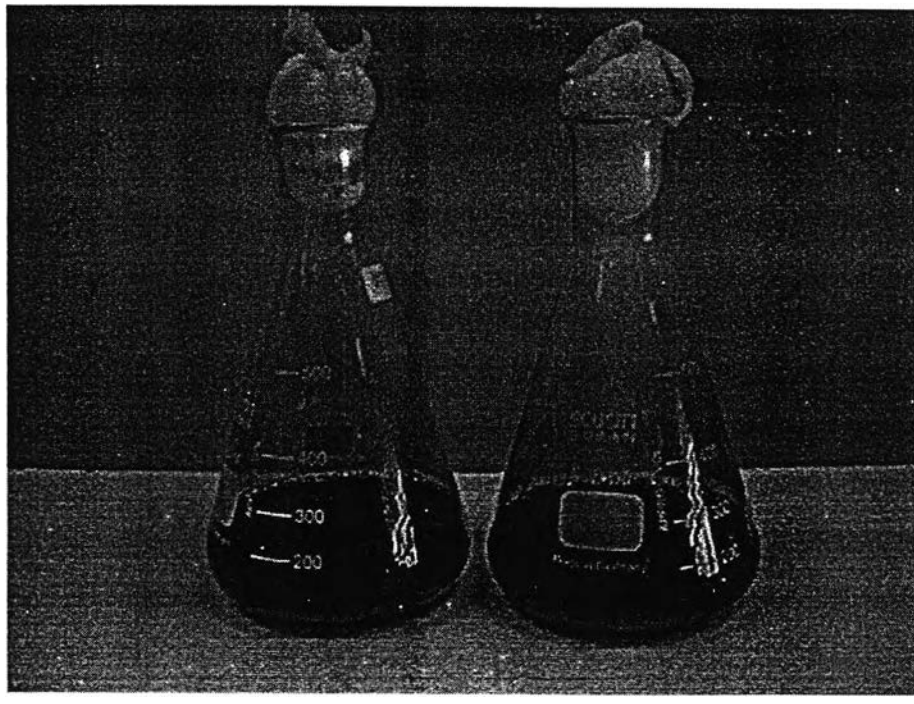

Figure 3.2 *Saccharomyces cerevisiae* M30 in 500 mL Erlenmeyer flasks containing 250 mL of the prepared fermentation media

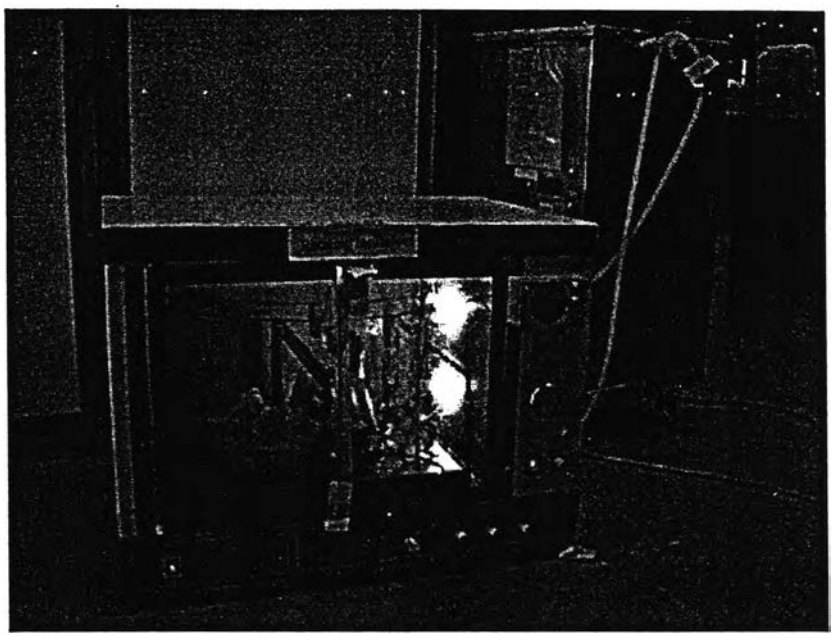

Figure 3.3 Shaking incubator with water cooling

3.5 Analytical methods

3.5.1 Cell concentration

Cell concentration is determined by cell dry weight determination. For this method, a 3-mL sample of the fermentation broth is centrifuged at 3,000 rpm for 10 minutes. The cell pellet is resuspended in 0.1 normal HCl and washed twice with distilled water and then dried for 48 hr, at 60°C and weighed.

3.5.2 Ethanol concentration

Concentrations of ethanol are determined by a gas chromatography system using a Shimadzu Model GC $7A_G$ equipped with a flame ionization detector. A column 2 m x 0.125 packed with Porapak Q 80-100 mesh is used with N_2 as carrier gas. The injector temperature is 280°C, and the detector temperature is 300°C.

3.5.3 Reducing sugar concentration

To measure the amount of sugar in sample, a 0.2 mL of sample solution is hydrolyzed in 33% HCl at 100°C for 10 minutes, neutralized with

20% NaOH solution and determined for reducing sugar content by using modified dinitrosalicylic acid method (See appendix).

3.6 Mathematical methods

An unstructured kinetic model for the cell activities in the anaerobic fermentation of molasses to produce ethanol is developed under four main factors, substrate limitation, substrate inhibition, product inhibition, and operating temperature by controlling pH and shear force effect. The microbial reactions usually show saturation at high substrate concentrations, meaning that the reaction rate approaches a maximum value. On the other hand, the reaction rate equals zero if no substrate is available. Therefore, if there is no interfered influence on cell growth, cell growth can be estimated based on Monod equation which represents substrate limitation kinetics:

$$\left\{\begin{matrix}\text{rate of biomass}\\ \text{concentration change}\end{matrix}\right\} = \left\{\begin{matrix}\text{rate of biomass concentration}\\ \text{change caused by exponential growth}\end{matrix}\right\}$$

$$\frac{dC_X}{dt} = \mu C_X = \frac{\mu_m C_S}{K_S + C_S} C_X \qquad \text{------- (3.1)}$$

However, the inhibition by substrates and products is normally observed in fermentation processes, especially at high concentration of substrates or products [Tyagi and Ghose, 1980]. Therefore, the Monod kinetic is modified in both substrate and product terms and counted with death rate and cell maintenance for biomass equation [Sainz, *et. al.*, 2003]. Reducing sugar from molasses is assigned as the sole limiting substrate of the fermentation. In this study, the effects of initial substrate concentrations, C_{S0}, on the kinetic parameters are investigated. The mathematical model of the microbiological ethanol synthesis can be written as follows:

$$\left\{\begin{matrix}\text{rate of biomass}\\ \text{concentration change}\end{matrix}\right\} = \left\{\begin{matrix}\text{rate of biomass concentration}\\ \text{change caused by exponential growth}\end{matrix}\right\} - \left\{\begin{matrix}\text{rate of biomass}\\ \text{concentration death}\end{matrix}\right\} - \left\{\begin{matrix}\text{rate of biomass}\\ \text{concentration maintainance}\end{matrix}\right\}$$

$$\mu C_X = \frac{dC_X}{dt}$$

$$= \frac{\mu_m(C_{S0})C_S}{K_S(C_{S0}) + C_S + \frac{C_S^{\,2}}{K_{SS}(C_{S0})}}\left(1 - \frac{C_P}{P_m(C_{S0})}\right)C_X - K_d(C_{S0})C_X - K_{CM}(C_{S0})C_X$$

------- (3.2)

$$\left\{\begin{matrix}\text{rate of product} \\ \text{concentration change}\end{matrix}\right\} = \left\{\begin{matrix}\text{rate of product} \\ \text{synthesis}\end{matrix}\right\}$$

$$\nu C_X = \frac{dC_P}{dt}$$

$$= \frac{\nu_m(C_{S0})C_S}{K_{SP}(C_{S0}) + C_S + \frac{C_S^{\,2}}{K_{SSP}(C_{S0})}}\left(1 - \frac{C_P}{P_{mP}(C_{S0})}\right)C_X$$

------- (3.3)

The kinetic parameters, μ_m, K_S, K_{SS}, P_m, K_d, ν_m, K_{SP}, K_{SSP} and K_{CM} are allowed to vary as a function of C_{S0}. The relationship of these parameters can be obtained by fitting experimental data over a range of C_{S0} values.

The substrate consumption rate could be stated as

$$\left\{\begin{matrix}\text{rate of substrate} \\ \text{concentration change}\end{matrix}\right\} = \left\{\begin{matrix}\text{rate by which the substrate} \\ \text{is consumed by mico - organisms}\end{matrix}\right\} + \left\{\begin{matrix}\text{rate by which the substrate} \\ \text{is converted to product}\end{matrix}\right\}$$

$$\frac{dC_S}{dt} = -\frac{1}{Y_{X/S}}\frac{dC_X}{dt} - \frac{1}{Y_{P/S}(C_{S0})}\frac{dC_P}{dt} \qquad \text{------- (3.4)}$$

Here, the yield coefficients, $Y_{X/S}$ and $Y_{P/S}$, are the relation between cell-substrate and product-substrate respectively. The yield of cell ($Y_{X/S}$) refers to the proportion of cell mass production to substrate utilization. Literature reported that yield of cell was around 0.5 g/g for *Saccharomyces cerevisiae* [Edwin, 1990]. Similarly, the yield of production ($Y_{P/S}$) refers to the proportion of product accumulation to substrate utilization.

Finally, the operating temperature relationship is applied in the mathematical model. Superposition of activation and deactivation temperature effects [Esner, *et al.*, 1980] is combined on key parameters as follow:

$$\mu_m, K_S, P_{mx}, \nu_m, P_{mp}, Y_{X/S}, \text{and } Y_{P/S} = f(C_{S0}, T) \qquad \text{------- (3.5)}$$

$$\frac{\text{Parameter}}{\text{Parameter at reference temperature}} = \gamma_i = \frac{a_i e^{-\frac{b_i}{T}}}{1 + c_i e^{-\frac{d_i}{T}}} \qquad \text{------- (3.6)}$$

where γ is the ratio of specie i parameter between its temperature and reference temperature while a, b, c, and d are the coefficients of specie i.

The example of ratio is shown below.

$$\frac{\mu_m}{\mu_m^o} = \gamma_{\mu_m} = \frac{a_{\mu_m} e^{-\frac{b_{\mu_m}}{T}}}{1 + c_{\mu_m} e^{-\frac{d_{\mu_m}}{T}}} \qquad \text{------- (3.7)}$$

The initial parameter values are primarily estimated from experiment data by using initial rate method before running the program iterations. The best-fit values of the kinetic parameter are estimated by least square method to minimize the sum of square error between the predicted and experimental data. From the developed mathematical model, after applying numerical method, the solution is solved using software package MATLAB v.6.1. All these steps can illustrate in Figure 3.4.

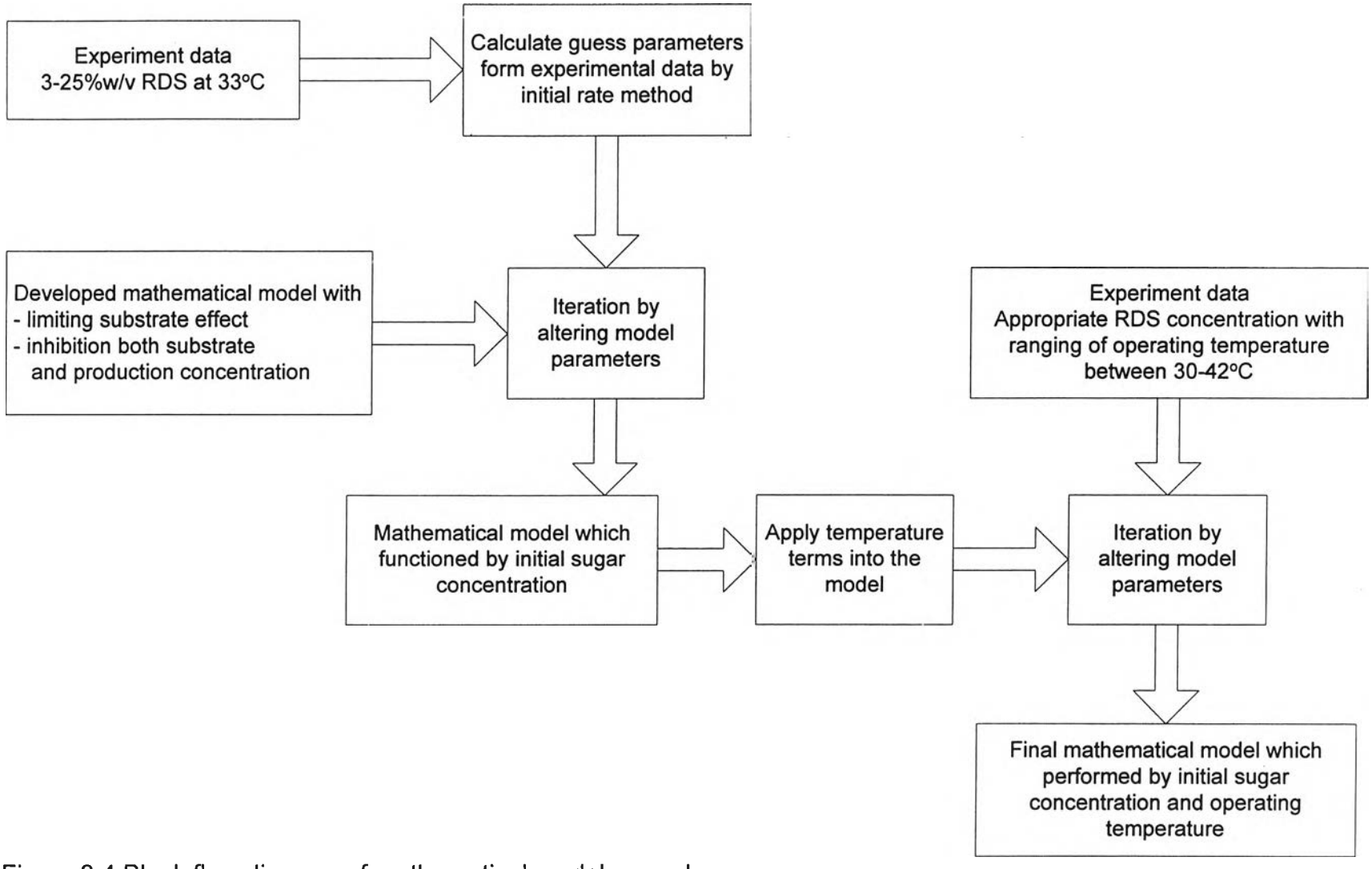

Figure 3.4 Block flow diagram of mathematical model procedure