CHAPTER III
EXPERIMENTAL

This chapter contains information of microorganism, chemicals, instruments, raw materials, the preparation and determination of main components with TAPPI T203 which compared with DTG/TGA data, spectrophotometric method and GC-FID analysis, respectively.

3.1 Microorganism

Saccharomyces cerevisiae was employed to produce cellulosic ethanol in this research. This microorganism was obtained from Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand. In addition, it was popular used for ethanol production with the advantage of a simple ethanol production. It could grow in aerobic conditions and used in baking and brewing industrials. Therefore, it was one of the bioethanol producing organisms used in industrial processes. However, it could not convert all of main components in non pretreated lignocellulosic biomass to be cellulosic ethanol.

3.2 Chemicals and Instruments

3.2.1 Chemicals

All chemical reagents were at least of analytical reagent grade. Ammonium sulphate ($(NH_4)_2SO_4$), potassium dichromate ($K_2Cr_2O_7$), sodium chlorite ($NaClO_2$), sodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were purchased from Ajax Finechem (Australia). 3,5-Dinitrosalicylic acid ($C_7H_4N_2O_7$) was obtained from Fluka (China). Hexane (C_6H_{14}), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), methanol (CH_3OH), phenol (C_6H_5OH), potassium sulphate (K_2SO_4), sodium sulphate (Na_2SO_4) and sulphuric acid (H_2SO_4) were purchased from Carlo Erba (Italy). Magnesium sulphate ($MgSO_4$) and potassium dihydrogenphosphate (KH_2PO_4) were obtained from Fluka (Switzerland). Ethanol (C_2H_5OH), glacial acetic acid (CH_3COOH), potassium sodium tatrate tetrahydrate

($C_4H_4KNaO_6.4H_2O$) and 2-propanol (C_3H_7OH) were purchased from QRec™ (New Zealand). Agar powder, malt extract powder and yeast extract powder were obtained from Himedia (India). D(+) Glucose anhydrous ($C_6H_{12}O_6$) was purchased from Calbiochem (Germany). Peptone was purchased from Oxoid (U.K.). Sodium sulphite (Na_2SO_3) was obtained from BDH Limited Pooled (England). The water was purified by Milli-Q system, Millipore (USA).

3.2.2 Instruments

Electrical autoclave; Model No.1941x, Wisconsin Aluminum Foundry (U.S.A.). Fourier Transform Infrared (FTIR) Spectrometer; Spectrum One; Perkin Elmer (Germany). Gas Chromatograph equipped a Flame Ionization Detector (GC-FID); Model 9A, Shimazu (Japan), packed column, carbowax 20 M, 10 wt% on chromosorb WAW 80/100 mesh, inner diameter 2.00 mm and length of column 1.00 m. Spectrophotometer; Model AGILENT 8453 UV-Visible Spectroscopy System (Germany). Differential/thermal gravimetric analyzer (DTG/TGA); Pyris diamond, Perkin Elmer (Germany).

3.3 Raw materials

The fresh durian and pineapple peel were collected from the Food Service Center in Khon Kaen University (KKU), Khon Kaen Province, Thailand, as shown in Fig. 3.1 (A) and (B). The durian and pineapple peel were washed thoroughly with distilled water. They were minced and dried at 80°C in a hot-air oven for 24 hrs. After that, they were crushed into powder by a fruit blender (Philippe, Holland) and sieved to get the particles with a size of 45-697 μm. These samples were characterized by using a FTIR with KBr pellet method.

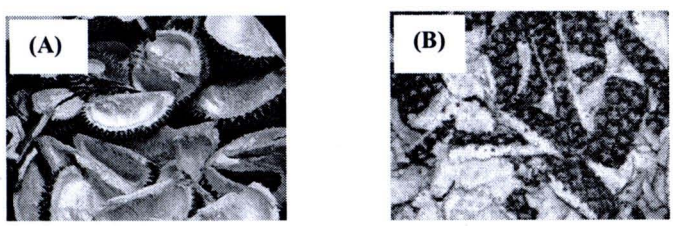

Figure 3.1 Fresh durian (A) and fresh pineapple peel (B)

3.4 Procedures

3.4.1 Determination of the main components by a DTG/TGA

Each of raw durian and pineapple peel powder were performed in the temperature range of 30-830°C at 10°C/min. Flame gas used was nitrogen/oxygen gas at flow rate of 60 mL/min (Nishiyama et al., 2002).

3.4.2 Removal and determination of the main components in durian and pineapple peel via removable processes

This method was performed using processes of Technical Association of Pulp and Paper Industrial T203 test method (TAPPI, 1994-1995).

3.4.2.1 Removal of ester compounds sample (Sample 1)

Each of raw durian and pineapple peel powder was extracted with the solvent, a mixure of hexane: methanol, (2:1) v/v to remove ester compounds by shaking at 180 rpm for 30 min and dried in a fume hood for solvent disposal. This step, the fat and oil was removed from raw durian and pineapple peel.

3.4.2.2 Removal of lignin sample (Sample 2)

Dried powder of sample 1 was soaked of the sample 1 in 150 mL DI water, 1.5 g sodium chlorite and 10 drops of 18 M glacial acetic acid in a water bath at 70°C for 1 hr (Boerjan et al., 2003). Then, they were washed thoroughly with distilled water, dried in an oven at 80°C for 1 hr until get constant weight.

3.4.2.3 Removal of hemicelluloses sample (Sample 3)

The powder of sample 2 was soaked in 0.25 M sodium hydroxide for a day and then were boiled at 70°C for 1 hr following the TAPPI T203 test method (TAPPI, 1994-1995). After that, they were washed thoroughly with distilled water, dried in an oven at 80°C for 1 hr and weighed. The dried cellulose powder was obtained. Finally the three samples were characterized by using a FTIR spectrometer with KBr pellet method. In addition, stepwise of the TAPPI T203 test method was illustrated in Fig. 3.2.

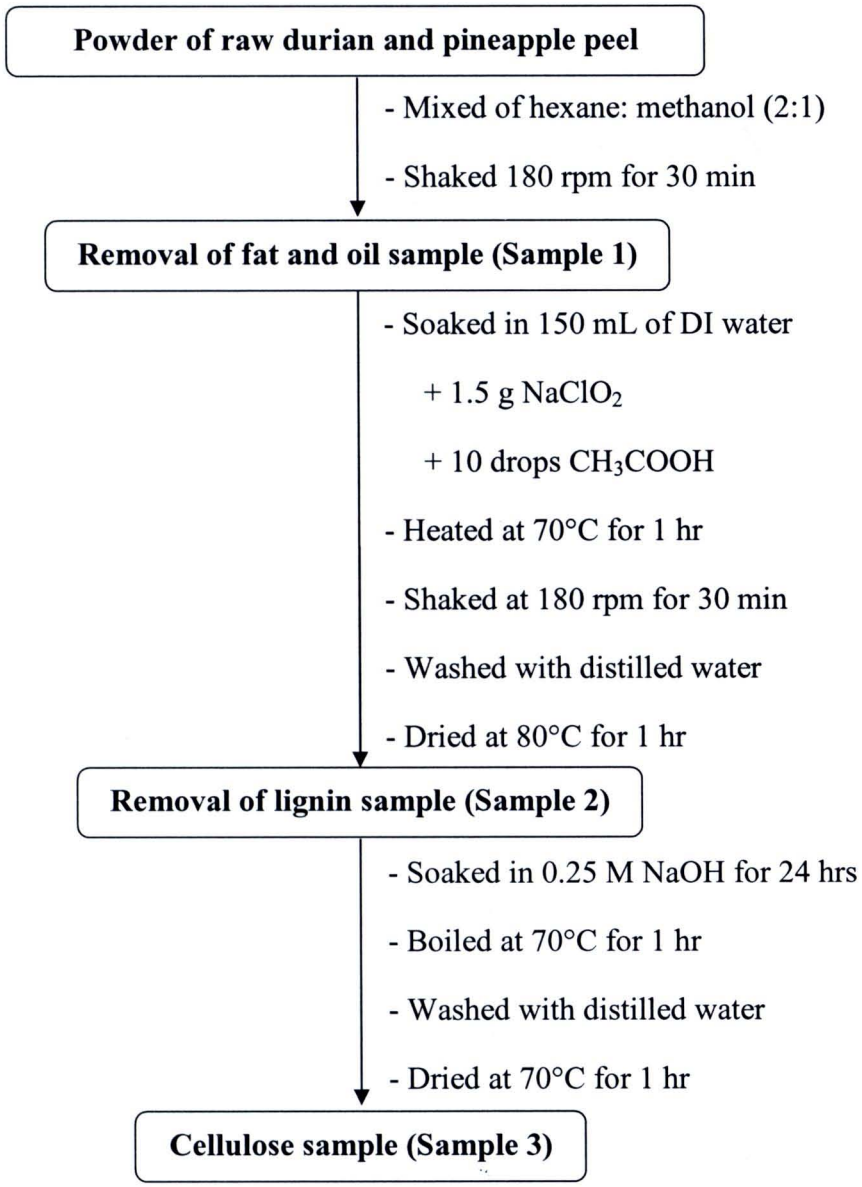

Figure 3.2 The stepwise of TAPPI T203 test method

3.4.3 Dilute acid hydrolysis for total reducing sugars

3.4.3.1 The dilute acid hydrolysis in an electrical autoclave

Each of three samples were hydrolyzed with 0.2 M sulfuric acid using electrical autoclaving at 120°C, 15 psi for 30 min. The working conditions for dilute acid hydrolysis were modified from the experiment of Xu et al. (2003). Then, the obtained hydrolyzed solution was a filtered through filter paper.

3.4.3.2 The diluted acid hydrolysis on a hot plate

Each of three samples were hydrolyzed with 0.2 M sulfuric acid using hot plate at 120°C for 30 min. The working conditions for diluted acid hydrolysis were modified from the experiment of Xu et al. (2003) and applied for hydrolysis on a hot plate. Then, the obtained hydrolyzed solution was filtered through a filter paper.

3.4.4 Dinitrosalicylic acid method for determination of total reducing sugars

The standard glucose and total reducing sugars were determined according to dinitrosalicylic acid method as described by Miller (1959).

3.4.4.1 Preparation of standard glucose

The 100.0 mg L^{-1} (Stock solution) was prepared as follow the 10 mg of D(+) Glucose anhydrous ($C_6H_{12}O_6$) was completely soluted in 100 mL of DI water and stored at 4°C. This stock solution was prepared every six months. Working solutions were prepared by appropriate dilution of the stock solutions in DI water.

3.4.4.2 Method validation

The method validation was investigated using dinitrosalicylic acid method. The parameters were: linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy.

- Linearity

Linearity was investigated in triplicate analysis of standard glucose ranged from 1.0 to 20.0 mg L^{-1}. All standard glucose was daily prepared by dilution of the stock solutions containing 100.0 mg L^{-1} of each standard, as shown in Fig. D1 and D2, respectively.

- LOD and LOQ

Limit of detection (LOD) and limit of quantitation (LOQ) were obtained with blank solutions containing all reagents, except glucose. In this study, LOD and LOQ were calculated as glucose concentration giving a signal equal to 3SD and 10SD of blank (n=5), respectively.

- Accuracy and precision

Accuracy was studied in term of recovery by spiking the sample with known concentrations of each analyst (1.0 and 5.0 mg L^{-1}) and was analyzed in five replicates. While precision was studied in term of relative standard deviation of recovery.

3.4.4.3 Real sample analysis

The total reducing sugars were determined at wavelength 570 nm by a UV-VIS spectrophotometer. These methods could as expressed in Fig. 3.3.

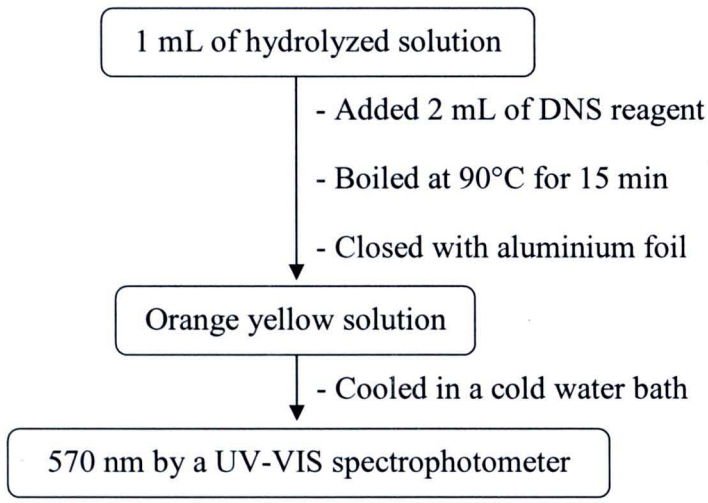

Figure 3.3 The stepwise of dinitrosalicylic acid method

3.4.5 Batch fermentation for bioethanol production

3.4.5.1 Yeast growth and nutrition

Saccharomyces cerevisiae, obtained from Department of Microbiology, Faculty of Science, Khon Kaen University, was maintained at 4°C on slants of nutrient agar, as shown in Fig. 3.4 (A). Always, before the fermentation, *S. cerevisiae* was activated on a Petri dish of nutrient agar (0.3 g of Malt extract, 0.3 g of Yeast extract, 0.3 g of Peptone, 1.0 g of Glucose and 1.5 g of Agar in 100 mL of DI water). It was incubated at room temperature for 24 hrs. Then, it was streaked into nutrient broth (all ingredients like a Petri dish of nutrient agar, except agar powder) and stirred for all time at room temperature for 48 hrs, as shown in Fig. 3.4 (B).

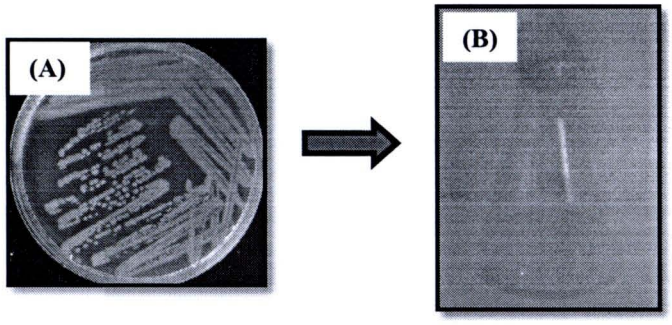

Figure 3.4 *S. cerevisiae* on medium plate (A) and in the growth broth (B)

3.4.5.2 Batch fermentation for cellulosic ethanol production

For the batch fermentation process, the hydrolyzed solution of durian and pineapple peel were adjusted pH at 7 using 2 M sodium hydroxide and then the solution was filtered through a 0.2 mm filter membrane. These solutions were added into the synthetic medium according to Brown et al. (1981) (1.0 g/L yeast extract, 1.0 g/L $MgSO_4$, 2.0 g/L $(NH_4)_2SO_4$ and 0.5 g/L KH_2PO_4). After that they were sterilized using an electrical autoclave at 120°C, 15 psi for 30 min. Then, *S. cerevisiae* broth was measured OD_{600} equal to 0.1 for determination of the yeast's colony. They were then loaded into the culture with a working volume of 50 mL medium. Finally, the batch fermentation was carried out by a rotary shaker with speed 100 rpm at 30°C for 18 hrs. These methods can be concluded in Fig. 3.5.

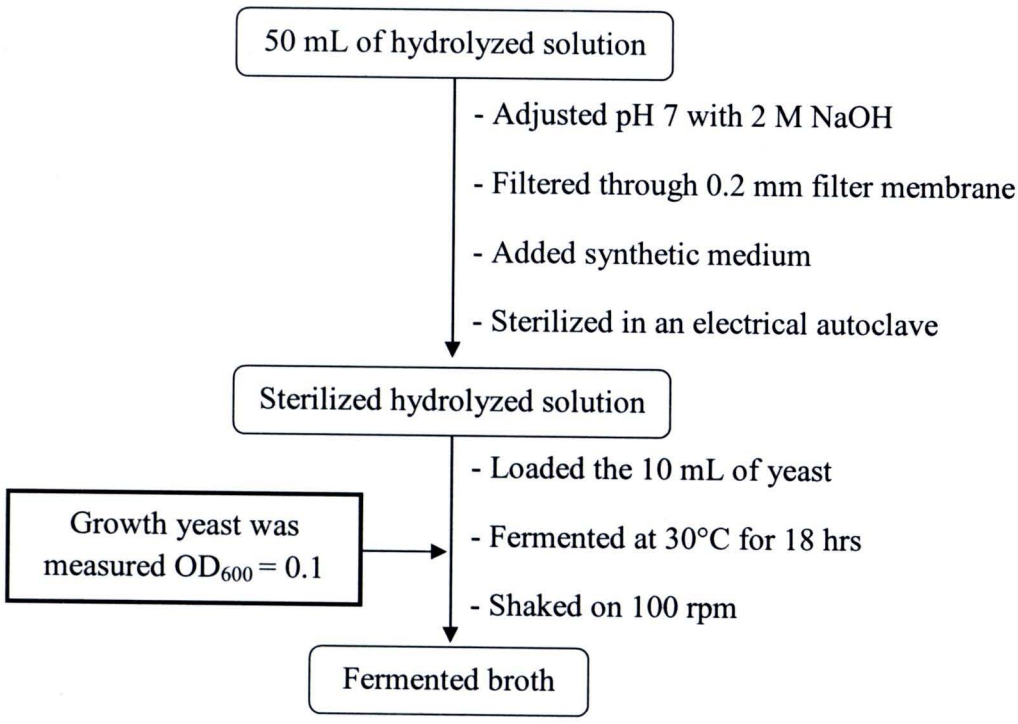

Figure 3.5 The stepwise of the batch fermentation

3.4.6 The cellulosic ethanol analyzed by a GC-FID

The standard ethanol and the cellulosic ethanol in fermented broth were monitored by a GC-FID.

3.4.6.1 Preparation of standard ethanol and internal standard

The 15.0 M of standard ethanol (Stock solution) and 15.0 M of 2-propanol (Internal standard) as follow 15 mL of each ethanol and 2-propanol were completely soluted in 100 mL of DI water and stored at 4°C. This standard ethanol and 2-propanol solution were prepared every six months. Working solutions of standard ethanol were prepared by appropriate dilution of the stock solutions in DI water.

3.4.6.2 The optimum conditions for GC-FID

An injector was operated at 250°C. The flame ionization detector was kept at 200°C. Nitrogen gas was used as carrier gas at flow rate of 30 mL/min. The temperature was programmed at 120°C for 1.4 min, from 120°C to

240°C at 30°C/min, then hold 5 min at 240°C. The internal standard used was 2-propanol (Caylak and Vardar, 1998), as shown in Fig. 3.6.

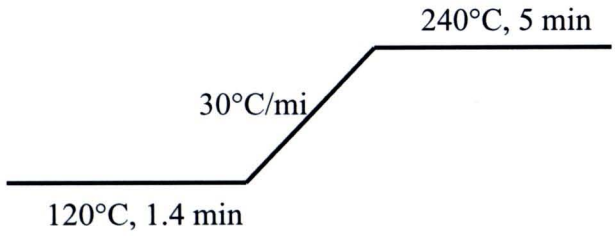

Figure 3.6 The optimum conditions for GC-FID analysis

3.4.6.3 The study on retention time (t_R) of standard ethanol and 2-propanol

1 μL of each the standard ethanol and 2-propanol were injected into an injector part of GC-FID under optimum conditions in section 3.4.6.2.

3.4.6.4 Method validation

The parameters were: linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy.

- Linearity

Linearity was investigated in triplicate analysis of standard ethanol ranged from 1.0 to 15.0 M. All standard ethanol was daily prepared by dilution of the stock standard solutions containing 15.0 M of each standard.

- LOD and LOQ

LOD and LOQ were obtained with blank solutions containing all the reagents, except ethanol. In this study, LOD and LOQ were calculated as ethanol concentration giving a signal equal to 3SD and 10SD of blank (n=5), respectively.

- Accuracy and precision

Accuracy was studied in term of recovery by spiking the sample with known concentrations of each analyst (1.0, 5.0 and 9.0 M) and was analyzed in five replicates. While precision was studied in term of relative standard deviation of recovery.

3.4.6.5 Real sample analysis

The obtained fermented broth was centrifuged at 1500 rpm for 10 min and filtered through a 0.45 μm millipore filter membrane. Then, 1μL of these samples were injected into an injector of a GC-FID. They were monitored under optimum conditions in section 3.4.6.2. These methods could conclude in Fig. 3.7.

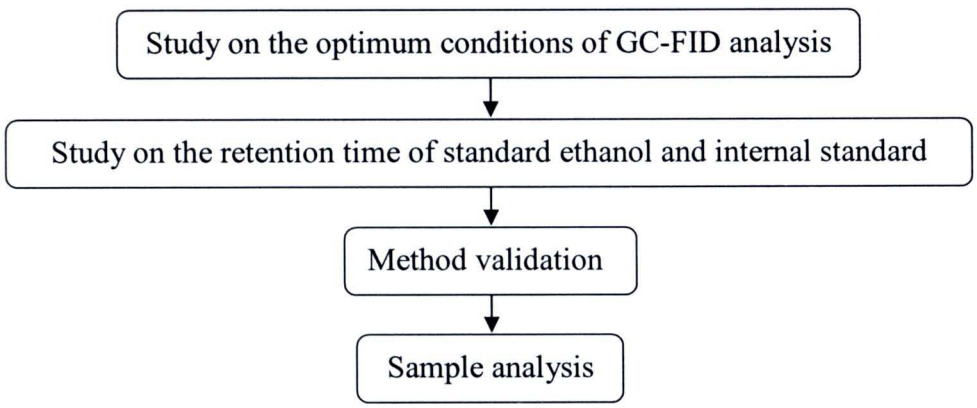

Figure 3.7 The stepwise of determination of cellulosic ethanol by a GC-FID