CHAPTER 3 METHODOLOGY

The methodology used to accomplish the objectives of the thesis which consist of reagents, materials and equipment shall be explained. Moreover, the method of acid, alkaline pretreatment, chemical analysis of sugarcane bagasse, sample analysis and statistic analytical are also presented in this chapter. The flow diagram of work plan is shown in Figure 3.1

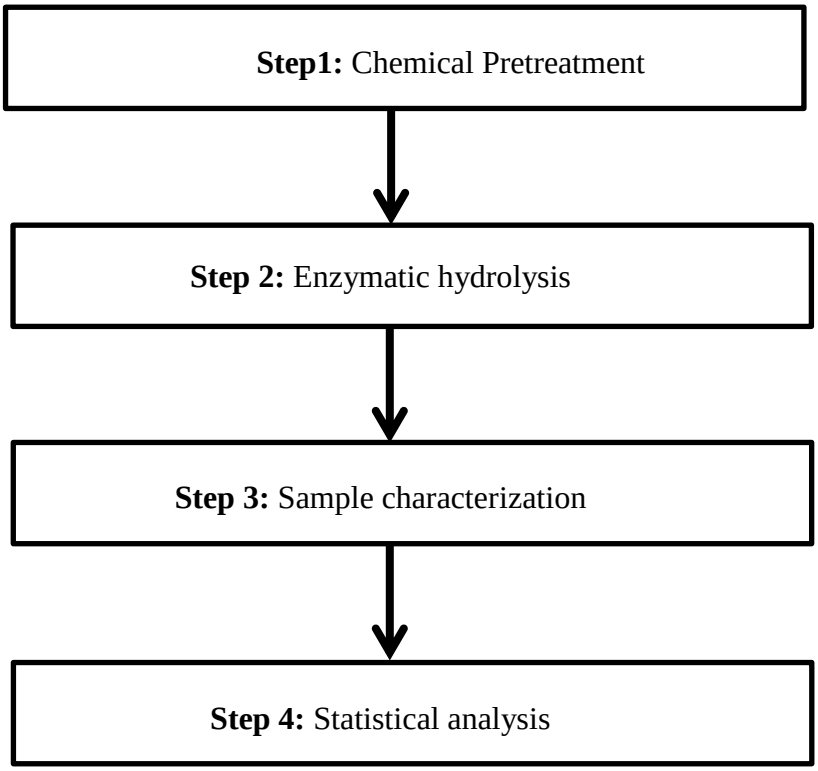

Figure 3.1 The flow diagram of work plan.

3.1 Raw materials, Equipment and Chemical Reagents

3.1.1 Reagents and Materials

1. Sugarcane bagasse
2. Sodium hydroxide (NaOH)
3. Potassium hydroxide (KOH)
4. 98% Sulfuric acid (H_2SO_4)
5. 37% Hydrochloric acid (HCL)
6. Ethanol (C_2H_5OH)
7. Acetone (CH_3OCH_3)
8. Butanol (C_4H_9OH)
9. Distilled water (DI H_2O)
10. Citric acid

11. 1 M sodium hydroxide solution
12. Cellulase enzyme
13. Cellobiase
14. 0.05 M Sodium citrate Buffer (pH = 4.8)

3.1.2 Equipment

1. Beaker
2. 1000 mL Erlenmeyer flask
3. Autoclave
4. Pipette
5. Specular
6. Dropper
7. Conventional Oven
8. Plastic Bag (Zip Lock)
9. Analytical balance
10. Volumetric flask (2000 mL and 1000 mL)
11. Nitrile Glove
12. Stirring Rod
13. Aluminum foil
14. Paraffin Tap
15. Cylinder
16. Cycling
17. Suction (250 ml, 500 ml and 1000 ml)
18. pH Paper
19. Glass Bottle
20. Sieve Tray (2.00 mm, 0.85 mm, 0.425 mm, 0.180 mm, Receiver Tray)
21. Sieve Shaker
22. Vacuum Pump
23. Buchner Funnel
24. Micropipette
25. 0.22 mm Nylon Filter
26. Hammer mill
27. Hot Plate
28. Thermometer
29. Test Tube
30. Refrigerator
31. Moisture can
32. High Performance Liquid Chromatography (HPLC)
33. UV-Visible spectrophotometer
34. Desiccator
35. Water bath

Sugarcane Bagasse used in this study was received from MITR PHOL Company, Supanburi. It was dried in the conventional oven at 80 ^{o}C for 30 min and milled to a size less than 1 mm. The milled sample was separated the particle size by used sieve tray and the screened products size larger than 425 mm but smaller than 0.850 mm was studied in this project. Moreover, the bagasse sample was determined the moisture content.

3.2.2 Chemical Pretreatment

1. Acid Pretreatment

40 g. of bagasse was transferred into 1,000 mL of 2 Erlenmeyer flasks (40 g. of bagasse in each 1,000 mL of flask) and then 1% (v/v) H_2SO_4 was added as 1:5 solid to liquid ratio. The sample was mixed together and closed with an Aluminum foil. The sample was autoclaved at 105 ^{o}C, 115 ^{o}C and 130 ^{o}C for 30, 60 and 90 minutes. The pretreatment was repeated by using H_2SO_4 concentration of 3% and 5%. The retained solid after filtration was washed with 60 ^{o}C distilled water until reached neutral pH and dried at 60 ^{o}C for 24 hours. After that, the sample was stored in sealed plastic bags at room temperature before measuring the cellulose, hemicellulose and insoluble lignin content. The 10 mL of filtrate was filtered through 0.22 mm of Nylon filter before storing in refrigerator at -30 ^{o}C. The filtrate was analyzed to determine the concentration of monosugar, oligosugar, furfural, Hydroxymethylfurfural (HMF), acetic acid and insoluble lignin. The pretreatment was repeated again by using hydrochloric acid (HCL) instead of H_2SO_4 at the same condition [3, 8].

2. Alkaline Pretreatment

40 g. of bagasse was transferred into 1,000 mL of 2 Erlenmeyer flasks (40 g. of bagasse in each 1,000 mL of flask) and then 1% (w/v) NaOH was added as 1:5 solid to liquid ratio. The sample was mixed together and closed with an Aluminum foil. The sample was autoclaved at 105 ^{o}C, 115 ^{o}C and 130 ^{o}C for 30, 60 and 90 minutes. The pretreatment was repeated by using NaOH concentration of 3% and 5%. The retained solid after filtration was washed with 60 ^{o}C distilled water until reached neutral pH and dried at 60 ^{o}C for 24 hours. After that, the sample was stored in sealed plastic bags at room temperature before measuring the cellulose, hemicellulose and insoluble lignin content. The 10 mL of filtrate was filtered through 0.22 mm of Nylon filter before storing in refrigerator at -30 ^{o}C. The filtrate was analyzed to determine the concentration of monosugar, oligosugar, furfural, Hydroxymethylfurfural (HMF), acetic acid and insoluble lignin. The pretreatment was repeated again by using potassium hydroxide (KOH) instead of NaOH at the same condition [3, 8].

3.2.3 Screening of Multivariable Parameters Affecting Lignocellulosic Material Pretreatment Using Fractional Factorial Design (FFD)

The Fractional Factorial Design (FFD) was applied to evaluate the significant factors from multivariable parameters for removing lignin and hemicellulose from

lignocellulose material, while promoting glucose and xylose and avoiding the formation of enzyme inhibitor substrates such as furfural and hydroxymethylfurfural (HMF). This experiment design was not conclude the interactive effect of variables. The three independent variables (Temperature, Time and Concentration) were divided into 2 levels; high level (+) and low level (-). Due to 2 – levels of FFD together with resolution III operation of full factorial design, the number of experiment was consisted 2^{3-1} or 4 runs for each chemical pretreatment; moreover, 2 central points were added to estimate the error of design of experiment. In conclusion, the total number of experiment was 24 runs for 4 chemical types. The data of design of experiment (DoE) by using Fractional Factorial Design (FFD) was shown in Table 3.1 – 3.8. The significant effects of each variable were determined at confidant level of 95% (p = 0.05).

Table 3.1 Coded and real values of FFD [14].

Variable	**Variable code**	**Level**		
		-1	**0**	**1**
Sodium hydroxide (NaOH) (%w/v)	A	1	3	5
Potassium hydroxide (KOH) (%v/v)	B	1	3	5
Sulfuric acid (H_2SO_4) (%v/v)	C	1	3	5
Hydrochloric acid (HCl) (%v/v)	D	1	3	5
Temperature (^oC)	E	105	115	130
Reaction time (min)	F	30	60	90

Table 3.2 Factional Factorial Design of sodium hydroxide (NaOH) pretreatment.

No.	Components		
	A	E	F
1	5	130	90
2	5	105	30
3	1	130	30
4	1	105	90
5	3	115	60
6	3	115	60

Table 3.3 Factional Factorial Design of potassium hydroxide (KOH) pretreatment.

No.	Components		
	B	E	F
1	5	130	90
2	5	105	30
3	1	130	30
4	1	105	90
5	3	115	60
6	3	115	60

Table 3.4 Factional Factorial Design of sulfuric acid (H_2SO_4) pretreatment.

No.	Components		
	C	E	F
1	5	130	90
2	5	105	30
3	1	130	30
4	1	105	90
5	3	115	60
6	3	115	60

Table 3.5 Factional Factorial Design of hydrochloric acid (HCl) pretreatment

No.	Components		
	D	E	F
1	5	130	90
2	5	105	30
3	1	130	30
4	1	105	90
5	3	115	60
6	3	115	60

3.2.4 Pretreated Hydrolysis

1. Preparation of 1 M NaOH 500 ml

$$\frac{1\ mole}{1000\ ml}\ x\ 500\ ml = 0.5\ mole$$

Mw of NaOH = 39.9971 g/gmole

NaOH = 0.5 mole x 39.9971 g/gmole = 19.9985 g.

Then, 19.9985 g. of NaOH was dissolved in 500 ml of water to obtain 1 M NaOH

2. Preparation of 50mM Citric acid 500 ml

$$\frac{0.05\ mole}{1000\ ml}\ x\ 500\ ml = 0.025\ mole$$

Mw of Citric acid = 192.12 g/gmole

Citric acid = 0.025 mole x 192.12 g/gmole = 4.803 g.

Then, 4.803 g. of citric acid was dissolved in 500 ml of water to obtain 50 mM citric acid

3. Preparation of Citrate Buffer pH 4.80

1 M NaOH was added into citric acid solution unit the pH of mixed solution reaches to 4.8. After that, the mixture was autoclaved at the temperature of 121 ^{o}C for 15 min.

4. Enzymatic Hydrolysis

0.50 g. of dried pretreated solid was transferred into the test tube and then the 10 ml of cellulase enzyme (0.0600 g of cellulase in 40 ml of 0.05 M citrate buffer at pH of 4.8) and 2 ml of cellobiase (25 µl of cellobiase in 25 ml of 0.05 M citrate buffer at pH of 4.8) were added into the tube and then the mixture was mixed together. The enzymatic hydrolysis was carried out in shacking water bath under the temperature of 50 ^{o}C for 48 hour. Before starting reaction, the mixture was sampled at the reaction time of 0 minute. After 48 hour, the sample was centrifuged to separate the solid from hydrolyzate and then 5 ml of sample was freeze before sugar analysis. The monomeric sugar was analyzed to determine the concentration of xylose and glucose by using high performance liquid chromatography (HPLC).

3.2.5 Statistical Analysis

Data was subjected to analysis of variance (ANOVA) by using The Unscramble X version 10.3 with 95% confident level (p = 0.05).