CHAPTER IV
RESULTS AND DISCUSSION

4.1 Composition of Rice Straw

The fiber concentration of rice straw was examined by using the Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Digestible Lignin (ADL) procedures (van Soest *et al.*, 1991). The composition determination was determined by the following equations:

$$NDF = Cellulose + Hemicellulose + Lignin \quad (4.1)$$

$$ADF = Cellulose + Lignin \quad (4.2)$$

$$ADL = Lignin \quad (4.3)$$

A native rice straw in this research came from surin province which consisted of the main component of cellulose (49.56%) and hemicellulose (21.22%) as shown in Table 4.1. From these two main components, it could be used to examine the theoretical maximum of total sugar concentration from the equation as shown below:

$$\text{Theoretical maximum of total sugar concentration} = (1.11 \times \text{cellulose}) + (1.136 \times \text{hemicellulose}) \quad (4.4)$$

Where 1.111 was the factor that converted cellulose to equivalent glucose and 1.136 was the factor that converted hemicellulose to equivalent xylose (Cheng *et al.*, 2011).

From this equation, 79.17% of rice straw has been converted to sugar. When using 30 g/L of substrate solution which was calculated by the ratio of rice straw (g) and volume of sodium citrate buffer (L), the maximum total sugar concentration was 23.75 g/L.

Table 4.1 Composition of untreated rice straw

Composition	Percentage
Cellulose	49.56
Hemicellulose	21.22
Acid detergent lignin	3.27
Extractive	7.53
Others (acid soluble lignin, protein and ash)	18.42

4.2 Optimization of the Total Sugar Concentration using Response Surface Methodology (RSM)

The chemical pretreatment of rice straw which used aqueous-[EMIM][Ac] combined with microwave technology gave the fermentable sugar, as shown in Table 4.2. The results showed that glucose concentration was derived in the range of 11.81-17.83 g/L while-xylose concentrations was obtained beginning from 2.91 to 4.06 g/L. From all pretreatment conditions, the hydrolysate from pretreated rice straw at 50% [EMIM][Ac], at 160 °C for 55 min gave the highest glucose and total sugar concentration. In this condition, glucose, xylose and total sugar concentration were 17.83, 3.30 and 21.13g/L, respectively. However, when using response surface methodology (RSM) with a full three-level factorial designs to examine the optimal condition, the three-dimensional response surface in Figure 4.1 and two-dimensional contour plots for the concentration of total sugar in Figure 4.2 could not show the clear optimal condition which was outside the experiment region. However, this model could predict the optimal condition from the trend of reducing sugar concentration and provided the polynomial equation from the following:

$$Y_1 = 19.7646 + 2.3958x_1 + 1.1165x_2 - 1.1001x_1^2 - 1.6953x_2^2 + 0.63\,x_1x_2 \qquad (4.5)$$

Where Y_1 was the total sugar concentration (g/L), x_1 was the pretreatment temperature (°C) and x_2 was the pretreatment time (min).

From this equation, when apply the partial derivatives to zero with respect to the corresponding variables, the maximum total sugar concentration of 21.58 g/L was derived when rice straw was pretreated at 50% [EMIM][Ac], at 162 °C for 48 min which was 90.86% of theoretical yield (sugar concentration / maximum total sugar concentration).

Table 4.2 Experimental design and results of the central composite design of Aqueous-[EMIM][Ac]/Microwave pretreatment

Run	Temp (°C)	Time (min)	Glucose (g/L)	Xylose (g/L)	Total sugar (g/L)
1	140	25	12.18	2.91	15.09 ± 1.15
2	140	40	11.47	3.57	15.04 ± 0.19
3	140	55	11.82	3.46	15.28 ± 1.18
4	150	25	11.81	3.33	15.14 ± 0.68
5	150	40	16.07	3.72	19.80 ± 2.85
6	150	55	14.89	4.06	18.95 ± 1.18
7	160	25	17.12	3.57	20.69 ± 3.21
8	160	40	16.54	3.70	20.24 ± 0.35
9	160	55	17.83	3.30	21.13 ± 0.32

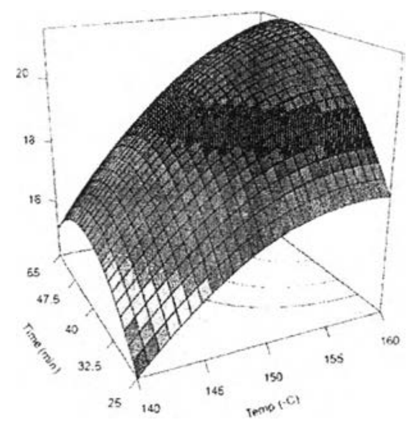

Figure 4.1 Response surface for total sugar concentration: effects of temperature and time.

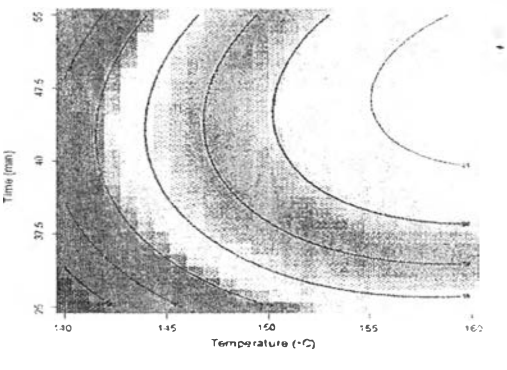

Figure 4.2 Contour plots for total sugar concentration: effects of temperature and time.

4.3 Confirmation of Total Sugar Concentration from Response Surface Methodology (RSM)

Table 4.3 Comparison of theoretical and actual total sugar concentration

Methods	Concentration (g/L)
Total sugar concentration from RSM	21.58
Actual total sugar concentration	19.59
% Error	9.24

From using response surface methodology (RSM) to determine total sugar concentration, the optimal condition of rice straw pretreatment at 162 °C for 48 min provided 21.58 g/L of total sugar concentration. However, from Table 4.3, 19.59 g/L can be produced in the actual pretreatment at this condition that was a 9.24% error of this model to examine the total sugar concentration.

4.4 Comparison of Chemical Pretreatment Method of Rice Straw

In this study, the optimal condition of ionic liquid pretreatment of rice straw has been compared to other chemical pretreatment methods which were alkali and acid pretreatments. For alkali pretreatment, rice straw was pretreated by 0.5% (w/w) concentration of NaOH at 140 °C for 15 min which was reported as the optimal condition of alkali pretreatment (Cheng *et al.*, 2011) while pretreatment condition of 2% (w/w) concentration of HNO_3 at 100 °C for 7 min gave the maximum fermentable sugar for acid pretreatment of rice straw (Chittibabu *et al.*, 2012).

4.4.1 Total Sugar Concentration

After pretreatment, pretreated rice straw by different methods were hydrolyzed by cellulase enzyme at 50 °C for 72 h and the total sugar concentrations were measured by HPLC. From Table 4.4, the results showed that the condition of 0.5% NaOH, at 140 °C for 15 min presented the highest sugar concentration of 22.32 g/L and the following was condition of 50% [EMIM][Ac], at 162 °C for 48 min which gave the 19.59 g/L of total sugar concentration, being higher amount of sugar than 2% HNO_3 pretreatment and untreated rice straw which were 5.47 g/L and 4.84 g/L, respectively.

Table 4.4 Comparison of total sugar concentration by different chemical pretreatment methods

Methods	Total sugar concentration (g/L)
Untreated	4.84 ± 0.10
50% [EMIM][Ac], at162 °C for 48 min	19.59 ± 1.07
0.5% NaOH, at 140 °C for 15 min	22.32 ± 1.31
2% HNO_3, at 100 °C for 7 min	5.47 ± 0.69

4.4.2 Crystallinity Index by X–Ray Diffraction

The different total sugar concentration of three chemical pretreated rice straw can be indicated that structure changes of each pretreated samples would occur. Therefore, the analysis of untreated and pretreated rice straw was required. Crystallinity index of biomass which can be provided by X–Ray Diffraction Analyzer (XRD) was used to describe the relative amount of crystalline material in cellulose and to interpret changes in cellulose structure (Park *et al.*, 2010). The crystallinity index can be examined by following equation:

$$CrI = \frac{I_{002} - I_{amorphous}}{I_{002}} \times 100\% \tag{4.3}$$

where, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about $2\theta = 22.5°$ and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about $2\theta = 18.6°$.

The XRD profiles of untreated and pretreated rice straw are shown in Figure 4.3 and the crystallinity index of samples obtained from XRD patterns are shown in Table 4.5.

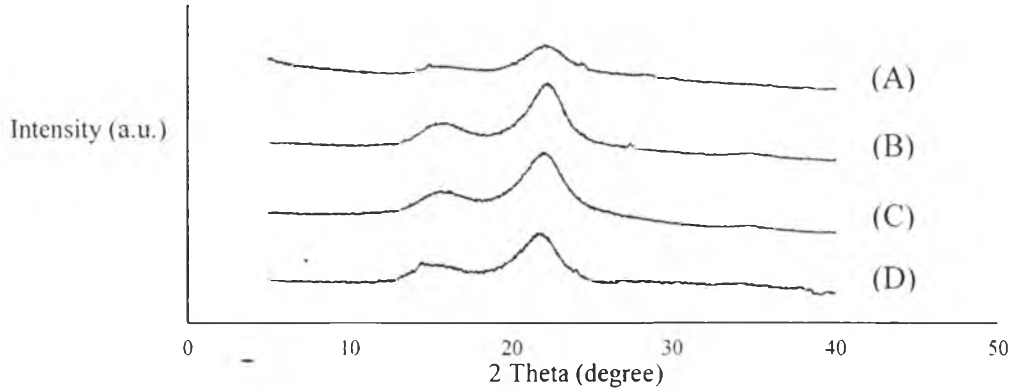

Figure 4.3 XRD patterns of untreated rice straw (A), pretreated rice straw by 0.5%NaOH (B), 2%HNO_3 (C) and 50% [EMIM][Ac] (D).

From the profile, the native cellulose was obtained two main peaks at 15° and 22.5° corresponding to 101 and 002 planes of crystalline portion of cellulose while amorphous portion was shown at 18.6° and the crystallinity index was found to be 44.03% which was less compared to other pretreated samples. After pretreatment, the intensity of crystalline peak increased significantly at 2 theta=22.5° and slightly increased at 2 theta=15°, leading to an increse of crystallinity of cellulose. The crystallinity of NaOH and HNO_3 were observed to be 56.54% and 50.99%, respectively. The increased crystallinity in alkali and acid pretreated rice straw showed the similar results to Rahnama *et al.*, 2013 who found a rise of crystallintiy from 50.81% in untreated rice straw to 62.41% in NaOH pretreated rice straw and Hsu *et al.*, 2010 presented the crystallinity of rice straw after H_2SO_4 pretreatment increased from 57% in untreated sample to the range from 58% to 65% depending on the increased of pretreatment temperature from 160 °C to 180 °C. However, the contrast results of aqueous-[EMIM][Ac] pretreated rice straw was shown, compared to the other studies that found the decrease of crystallinity after using ionic liquid pretreatment. Weerachanchai *et al.*, 2012 showed the decrease of crystallinity of rice straw after increasing the pretreatment temperature but the increase of crystallinity was observed when pretreatment temperature was 180 °C. In this temperature, the low sugar conversion was found due to the decomposition of rice straw to biochar. Nevertheless, the pretreatment of rice straw at 162 °C 48 min gave only high crystallinty but not presented the low sugar conversion, it can be indicated that aqueous-[EMIM][Ac] pretreated rice straw has not decompose to biochar. The increase of crystalline portion of rice straw after pretreatment could be possible due to the removal of amorphous structure surrounding the cellulose which are lignin and hemicellulose leading to an exposure of the crystalline portion of cellulose. A dominant of cellulose in pretreated rice straw can imporove the enzyme accessibility leading to an enhancement of hydrolysis rate and sugar conversion (Bak *et al.*, 2009).

Table 4.5 Crystallinity index of rice straw before and after pretreatment

Rice straw samples	Crystallinity index
Untreated	44.03
50% (w/w) [EMIM][Ac], at162 °C for 48 min	46.69
0.5% (w/w) NaOH, at 140 °C for 15 min	56.54
2% (w/w) HNO_3, at 100 °C for 7 min	50.99

4.4.3 Crystallinity Index and Functional Group by Fourier Transform Infrared Spectroscopy

The FTIR profiles of untreated and pretreated rice straw are shown in Figure 4.4 and the crystallinity index obtained from FTIR were shown in Table 4.6. The total crystallinity index (TCI) was calculated by the absorbance ratio of 1373 cm^{-1} and 2900 cm^{-1} (Nelson and O'Connor, 1964). The results showed that the crystallinity index of pretreated rice straw presented the higher value than untreated sample supporting the results of XRD. Moreover, untreated rice straw showed the sharp peak at the absorbance band located at1516 and 1639 cm^{-1} but presented the smaller band and broad peak after using three chemical pretreatments. These two absorption bands corresponded to aromatic C-O stretching mode for lignin (Stewart *et al.*,1995) and doublet phenolics of remained lignin (Sene *et al.*,1994), respectively. The decrease of these bands after pretreatment provided the removal of lignin in the composition of rice straw. Whereas the absorbance band at 1732 cm^{-1} was assigned to alkyl ester of the acetyl group in hemicellulose (Hsu *et al.*,2010). When rice straw was pretreated, reduced the absorbance at this band was observed. It can be indicated that hemicellulose was extracted especially in HNO_3 pretreated rice straw. Consequently, the structural changes of cellulose was found after three chemical pretreatments which could be easier to hydrolyze by cellulase enzyme.

Table 4.6 Total crystallinity index of rice straw before and after pretreatment

Rice straw samples	Crystallinity index
Untreated	1.05
50% (w/w) [EMIM][Ac], at 162 °C for 48 min	1.10
0.5% (w/w) NaOH, at 140 °C for 15 min	1.45
2% (w/w) HNO_3, at 100 °C for 7 min	1.36

Total crystallinity index (TCI) = $A_{1373cm^{-1}}/A_{2900cm^{-1}}$

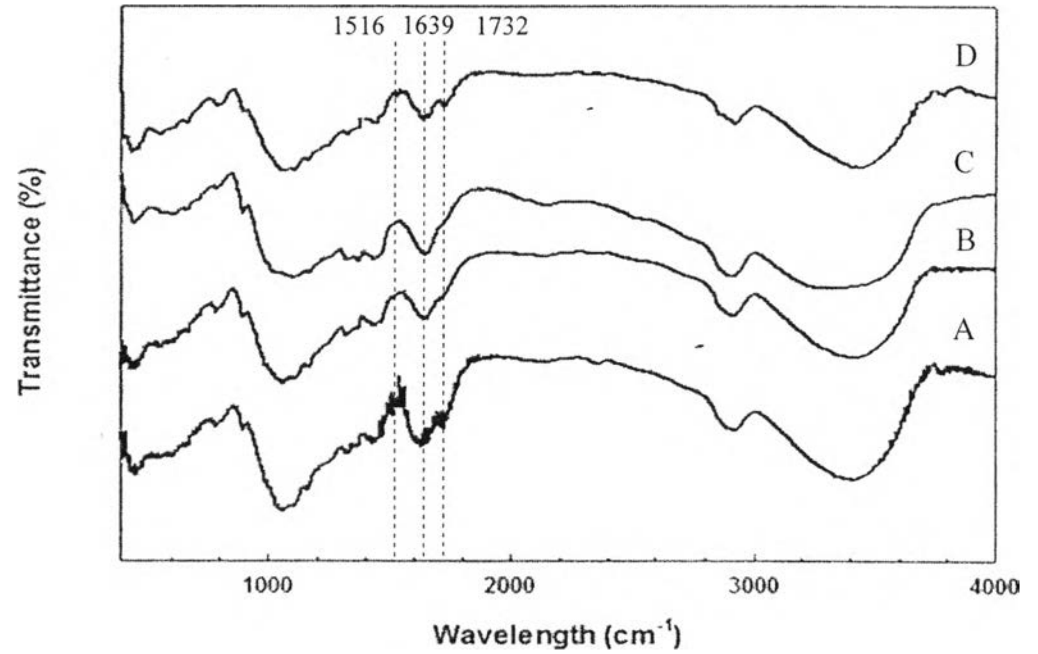

Figure 4.4 FTIR spectra of untreated rice straw (A), pretreated rice straw by 50% [EMIM][Ac] (B), 0.5%NaOH (C) and 2%HNO_3 (D).

4.4.4 Thermal Analysis

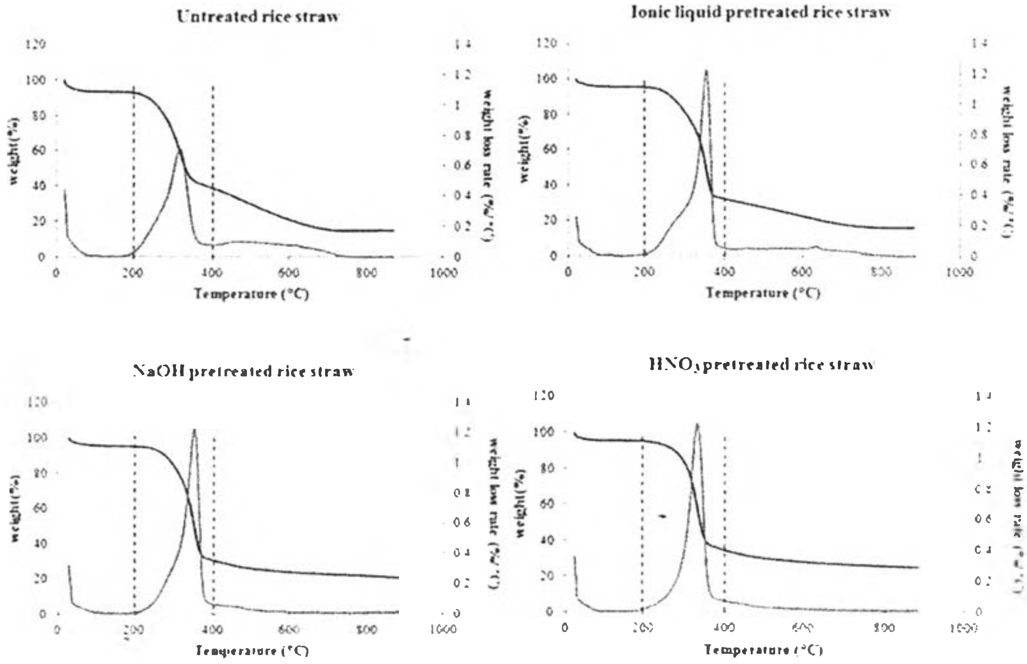

Figure 4.5 The TGA curves of untreated and three chemicals pretreated rice straw.

The TGA was used to found weight losses (TG) and its first derivative (DTA) of lignocellulosic structure which was relevant to the composition of cellulose, hemicelluloses, and lignin fractions in biomass (Chen and Kuo, 2010; Zabaniotou *et al.*, 2008). The TG curves of biomass presented the evaporation of moisture and light volatile at the temperature below 200 °C (Abdullah *et al.*, 2010). After that, the temperature between 200 °C and 300 °C showed the decomposition of hemicellulose and continuously decomposed to 400 °C and the degradation of cellulose by breaking of glycosidic linkages of the glucose chain was observed at 300-400 °C whereas lignin decomposed at the wide range of temperature starting at 200 °C (Benítez-Guerrero *et al.*, 2014). However, the small amount of lignin compared to cellulose and hemicellulose component could not show the clearly peak but there was reported that the shoulder or tail that appeared above 400 °C under inert atmosphere was associated with lignin (Di Blasi, 2008; Tihay and Gillard, 2010). From Figure 4.5 which provided the TG and DTA results of untreated and three chemicals pretreated rice straw found that the temperature from 200 °C to 400 °C showed the overlapping peak which mainly consisted of cellulose and hemicellulose in untreated, ionic liquid, and NaOH pretreated samples while HNO_3 pretreated rice straw could not appear the shoulder of hemicellulose peak that could be pointed out that HNO_3 has removed more hemicellulose. However, the DTA peak of pretreated rice straw showed the higher peak of cellulose degradation than untreated rice straw indicated the cellulose has been more exposed after using pretreatment which can be confirmed by the drop dramatically of weight loss in TG curves. Moreover, the cellulose peak shifted to higher temperature of pretreated samples referred to the increment of thermal stability which related to more exposure of cellulose and the enhancement of crystallinity in XRD and FTIR. And at temperature higher than 400 °C of untreated rice straw showed higher shoulder than the pretreated samples which can be concluded that the lignin content was decreased when pretreatment processes were used.

4.4.5 Surface Morphology

The images of scanning electron microscope (SEM) of the untreated and the pretreated rice straw with different methods are shown in Figure 4.6. From Figure 4.6 (A), the untreated rice straw presented the smooth and rigid surface area. After pretreatment processes, morphology of rice straw has been changed as shown in Figure 4.6(B)- 4.6(D). Pretreated sample showed the destroyed structure which can generate pores and rough surface area. From the SEM analysis, it can support crystallinity index results from XRD and FTIR that pretreatment can help to remove lignin and hemicellulose which are the barrier of cellulose hydrolysis. Similarly, Hou *et al.*, 2013 found that the increase of surface area and pore volume of pretreated rice straw by ionic liquid around 3-23 fold and 2-11 fold, respectively, as compared to the untreated sample. It was reported that the increment of the delignification degree could be observed, which may enhance the initial sugars release rates and polysaccharides digestibility.

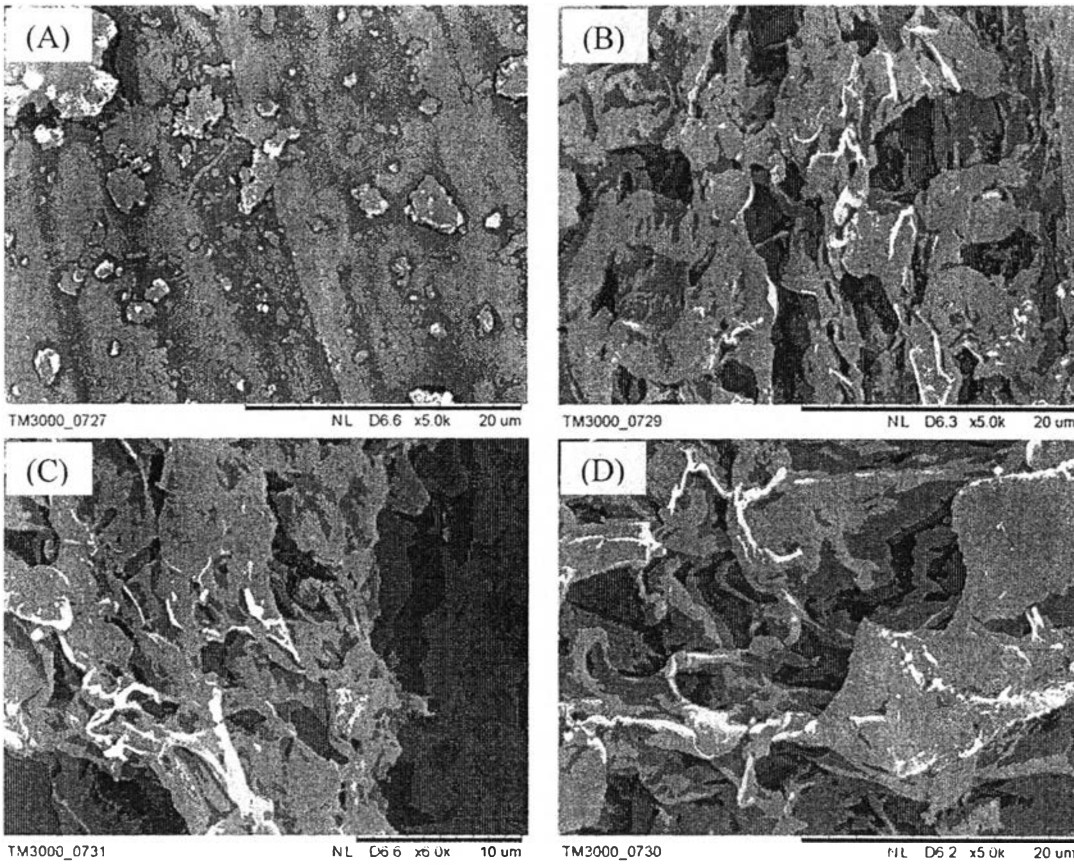

Figure 4.6 SEM images of untreated rice straw (A), pretreated rice straw by 50% [EMIM][Ac] (B), 2%HNO_3 (C) and 0.5%NaOH (D).

4.4.6 Surface Area and Pore Diameter

From the SEM results which showed the different structure of pretreated rice straw have been confirmed by surface area and pore diameter as shown in Table 4.7. BET analyzer provided the low surface area and pore diameter of untreated sample approximately 5.57 m^2/g and 63.77 Å, respectively, when pretreatment processes were applied, the higher value of surface area and pore size were found. The highest average pore diameter can be provided by 50% of ionic liquid pretreatment whereas HNO_3 gave the most increment of surface area. From the sugar concentration results of pretreated samples, it can be indicated that increasing of surface area and pore diameter can enhance the sugar yield but an increasing of pore diameter played an important roll than surface area. It was due to the removal of more hemicellulose in HNO_3 pretreatment and lignin in ionic liquid and NaOH pretreatment. The extraction of hemicellulose and lignin could increase the porosity of rice straw; therefore, the enzymes could easily penetrate to attack cellulose leading to the high sugar production.

Table 4.7 Surface area and pore diameter of rice straw before and after pretreatment

Rice straw samples	Surface area (m^2/g)	Pore diameter (Å)
Untreated	5.57	63.77
50% (w/w) [EMIM][Ac], at162 °C for 48 min	8.87 (+59.25%)	132.80 (+108.25%)
0.5% (w/w) NaOH, at 140 °C for 15 min	11.30 (+102.87%)	85.48 (+34.04%)
2% (w/w) HNO_3, at 100 °C for 7 min	12.06 (+116.52%)	79.53 (+24.71%)

4.5 Comparison of Acetone-Butanol-Ethanol (ABE) Concentration of Pretreated Rice Straw

The liquid fraction from enzymatic hydrolysis was used to produce Acetone-Butanol-Ethanol (ABE) in fermentation step by using *Clostridium beijerinckii* TISTR1461 at 37 °C for 72 h. The results from gas chromatography (GC) presented the ABE concentrations at each pretreatments of rice straw in Figure 4.7. The highest ABE concentration was found in liquid solution from NaOH pretreated rice straw. While total sugar derived by HNO_3 pretreated rice straw cannot produce ABE at the same fermentation condition. Moreover, when ABE yield and %productivity were observed in Table 4.8, the results showed that the maximum yield of 0.47 was found in total sugar from ionic liquid pretreated rice straw which consumed the amount of total sugar only 4.83 g/L but can produce ABE concentration of 2.27 g/L. However, when examined the ABE production per hour, the results showed that NaOH pretreated rice straw can provide the highest productivity of 8.82% whereas sugar from ionic liquid pretreated rice straw can gave only 3.15% which was less than control solution (glucose solution) and untreated rice straw. Additionally, the sugar concentration from HNO_3 pretreated samples was small higher than untreated rice straw but cannot present the amount of ABE. It could be possible that sugar derived by HNO_3 pretreated rice straw may have some inhibitors that can be toxicity to microorganisms leading to low concentration of ABE. Similarly, the study of Tutt *et al.*, 2012 found that cellulose can be dissolved in HNO_3 better than H_2SO_4 and HNO_3 also eliminated more hemicellulose but compounds formed during HNO_3 pretreatment were more difficult to remove with washing that can cause a negative impact on fermentation.

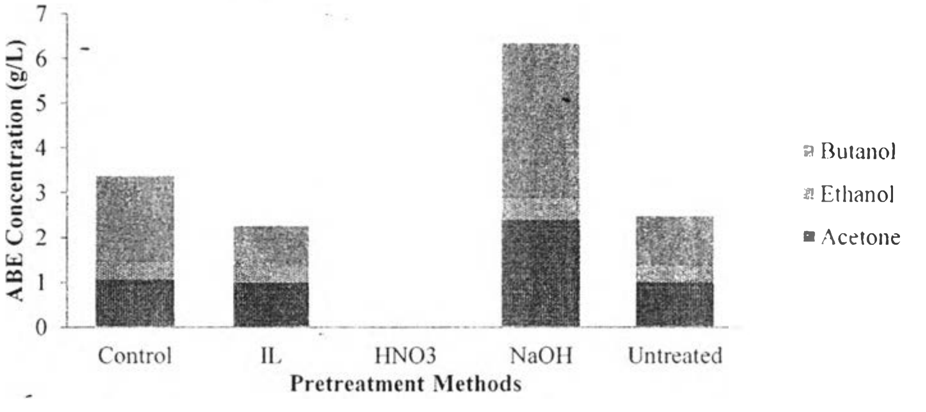

Figure 4.7 ABE concentration of chemical pretreated rice straw in fermentation technique.

Table 4.8 The ABE yield and productivity of pretreated rice straw at 72 h

Method	Control	IL	HNO_3	NaOH	Untreated
ABE Conc. (g/L)	3.38	2.27	0	6.35	2.48
ABE Yield	0.21	0.47	0	0.29	0.42
%Productivity (g/L h)	4.70	3.15	0	8.82	3.44
Initial Sugar (g/L)	25.13	22.54	6.94	24.23	6.43
Utilized Sugar (g/L)	16.31	4.83	0	21.90	5.92
Remaining Sugar (g/L)	8.82	17.71	6.94	2.33	0.51

Moreover, the results from gas chromatography-mass spectrometry (GC-MS) shown in Appendix C could be used to detect other components from the different molecular weight in solution after fermentation step. In liquid fraction from untreated rice straw gave the similar component to GC results which contained the acetone, butanol and ethanol. Additionally, the high amount of acetic and butyric acids was found in this solution. From the path way of ABE fermentation, acetic and butyric acids were produced in exponential phase (acidogenesis). After that, the path way has shifted to the solventogenic phase to produce acetone, butanol and ethanol (Chang, 2010). When the high amount of acids was observed, it can be indicated that the conversion to ABE products were less. Whereas solution from pretreated rice straw consisted of only acetic acid and the highest amount of acetic acid was presented in HNO_3 pretreated rice straw which related to the disappearance of ABE products. Furthermore, other components were found in solution from pretreated samples which were 1-hydroxy-2-propanone and isosorbide. For 1-hydroxy-2-propanone can be called as hydroxyacetone which was the one product of cellulose decomposition in fast pyrolysis (Shen *et al.,* 2013). While isosorbide can be taken place by converting cellulose through simultaneous hydrolysis and hydrogenation reactions to produce sorbitol and be further dehydrated to isosorbide (Li *et al.,* 2013). From observation of 1-hydroxy-2-propanone and isosorbide, it might be possible that three chemicals used in pretreatment and thermal condition brought into the cellulose degradation. Moreover, 2-metylpropanal or isobutyraldehyde was presented solution from NaOH pretreated rice straw. Isobutyraldehyde was an intermediate product in isobutanol production pathway which was different pathway to produce butanol as shown in Fig. 4.8. It might be hypothesized that isobutanol production could be produced by using *Clostridium beijerinckii* in solution from NaOH pretreated rice straw but it had to develop and concern more other factors that related to the isobutanol prodution.

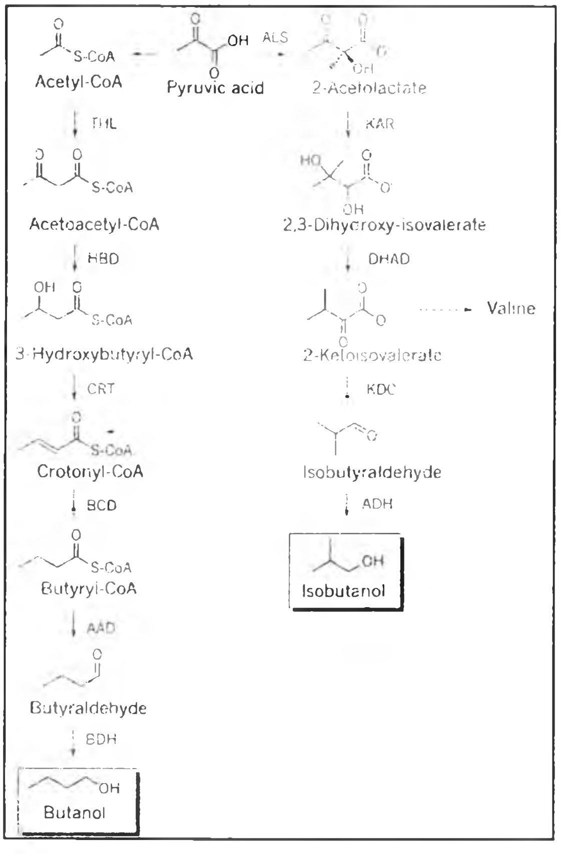

Figure 4.8 Comparison of butanol and isobutanol pathway (Peralta-Yahya *et al.*, 2012)