CHAPTER IV
RESULTS AND DISCUSSION

4.1 Biomass Characterizations

4.1.1 Chemical Composition of Corncobs

Corncobs were measured the quantities of cellulose, hemicellulose, and lignin. The results are shown in Table 4.1 as the percentage of dry weight unit.

Table 4.1 Chemical composition of corncobs

Chemical components	Dry solid (%)
Cellulose	39.31
Hemicellulose	34.46
Lignin	10.47
Others	15.76

Table 4.1 presents the composition of corncobs, which consists of 39.31% cellulose, 34.46% hemicelluloses, and 10.47% lignin, respectively. Commonly lignocellulosic biomass such as corn, cassava, sugarcane etc. is mainly comprised of cellulose (38–50%), hemicelluloses (23–32%), and lignin (15–30%) % wt. of biomass material on dry basis along with smaller amounts of extractive and ash. Cellulose is a main structural component in plant cell walls, which its structure is a homopolymer consists of repeating β–D–glucose units. This linear structure is connected by β–1,4 glycosidic bonds. The cellulose in a lignocellulosic biomass can be divided into two parts: a crystalline structure and amorphous structure. Crystalline cellulose is a major proportion of cellulose while a small proportion is amorphous cellulose. Cellulose is a highly crystalline material which mainly affects to resist enzymatic hydrolysis accessibility. Hemicellulose represents as the connection between cellulose and lignin, which results in the network of cellulose-hemicellulose-lignin more rigidity. Hemicellulose is a heteropolymers of carbohydrate which consists of five-carbon sugars (e.g. xylose and arabinose) and

six-carbon sugars (e.g. mannose, glucose, and galactose). Hemicellulose has a lower molecular weight than cellulose and the structure is random, amorphous, and branched that causes hemicellulose can hydrolyze easily. In addition, lignin made up of three types of phenolic acids (p–coumaryl, coniferyl, and sinapyl alcohol) and they linked in a three dimensional structures affected lignin particularly difficult to hydrolyzed (Kumar *et al.*, 2009).

4.2 A Comparison between Dilute Sulfuric Acid and Phosphoric Acid at an Optimal Pretreatment Condition

4.2.1 Monomeric Sugar and Furfural Yield

The purposes of the pretreatment process are to remove lignin and hemicellulose, disrupt the cellulose crystalline structure, and increase the porosity of the materials. Dilute acid pretreatment helps to break down the crystalline cellulose and hemicellulose polymers from lignocellulosic biomass to form individual sugar molecules, which can be fermented into biofuels (Wyman 1994). It would be recognized that the structure of hemicellulose is branches with short lateral chains, helps them be simple to hydrolyze than cellulose. Furthermore, pretreatment process could increase surface area and porosity of lignocellulosic structure in order to increase enzymatic digestibility of cellulose in enzymatic hydrolysis process; therefore, the enzyme can be easy to hydrolyze polysaccharide to monosaccharide. Corncobs were pretreated with dilute acid under these following conditions: 2% w/w at 120 °C for 5 min and 15:1 liquid-to-solid ratio (LSR) in case of sulfuric acid (Tangmanasakul, 2011), while phosphoric acid is 2% w/w at 140 °C for 10 min and 10:1 liquid-to-solid ratio (LSR) (Satimanont, 2012). However, liquid-to-solid ratio (LSR) in sulfuric acid (H_2SO_4) optimal condition has not been investigated in the previous work. Therefore, comparison of sulfuric condition between 10:1 liquid-to-solid ratio (LSR) and 15:1 under the same condition will be investgated. When this process was completed, the monomeric sugar yield was measured from the product in liquid phase, prehydrolysate.

Table 4.2 and Figure 4.1 illustrate monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatments

by using 2% (w/w) with different pretreatment times, temperatures, and LSRs and it was found that the monomeric sugar yield was increased with decreased pretreatment LSR. The major component in prehydrolysate from pretreatment process was xylose. Owing to the structure of hemicellulose which is a random, amorphous, and branches, therefore, xylose presented as the part that can be readily to hydrolyze than glucose. In term of monomeric sugar (glucose, xylose, and arabinose) yield for sulfuric acid condition, the trend clearly presents that it increased with pretreatment LSR from 15:1 to 10:1. These results were supported by previous works, which reported that the appropriate ratio is 10:1 which acid and corncobs are being well-mixed with the highest monomeric sugar yield (Jeevan *et al*, 2011, Satimanont, 2012). When LSR was too high, it caused loss volume and reduced total monomeric sugar. Sufficient liquid is required to dissolve hemicelluloses, lignin, and their degradation products; too small LSR decreases the solubility because less liquid is present, and deposition of dissolved hemicellolses, leading to a decrease in total monomeric sugar (Jeevan *et al*, 2011). However, furfural formation at a 10:1 LSR was higher than at a 15:1 LSR because of high amount of xylose yield which presented in prehydrolysate can be converted to higher furfural (Satimanont, 2012). When comparing with phosphoric acid operating condition, monomeric sugar contents are lower than sulfuric condition with 10:1 LSR according to higher temperature and longer time from 120 °C to 140 °C and 5 min to 10 min, respectively. Satimanont, 2012 reported that increasing pretreatment temperatures and times can drive xylose degradation into furfural. Under severity pretreatment conditions (> 120 °C), the xylose yield decreased owing to xylose degradation into furfural which can obstacle the micro-organism growing in fermentation process (Ezeji *et al.*, 2007). The result of total monomeric sugar obtained at a 10:1 LSR in sulfuric acid condition is greater than that in phosphoric acid condition with lower temperature and time. The highest total monomeric sugar (35.72 g/l) was obtained in pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR. However, the glucose, xylose, and arabinose concentrations were not much different from reducing yield of phosphoric acid pretreatment. While, the highest furfural content of 0.37 g/l can be found in pretreated corncobs with 2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR.

Table 4.2 Total sugar yield of corncobs in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment using 2% (w/w) with different pretreatment times, temperatures and LSRs

Conditions	Glucose (g/l)	Xylose (g/l)	Arabinose (g/l)	Total sugar (g/l)	Furfural (g/l)
2% H_2SO_4 at 120 °C for 5 min and 15:1 LSR	1.74	17.89	3.47	23.10	0.11
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR	2.79	27.60	5.33	35.72	0.17
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR	2.08	26.86	5.15	34.09	0.37

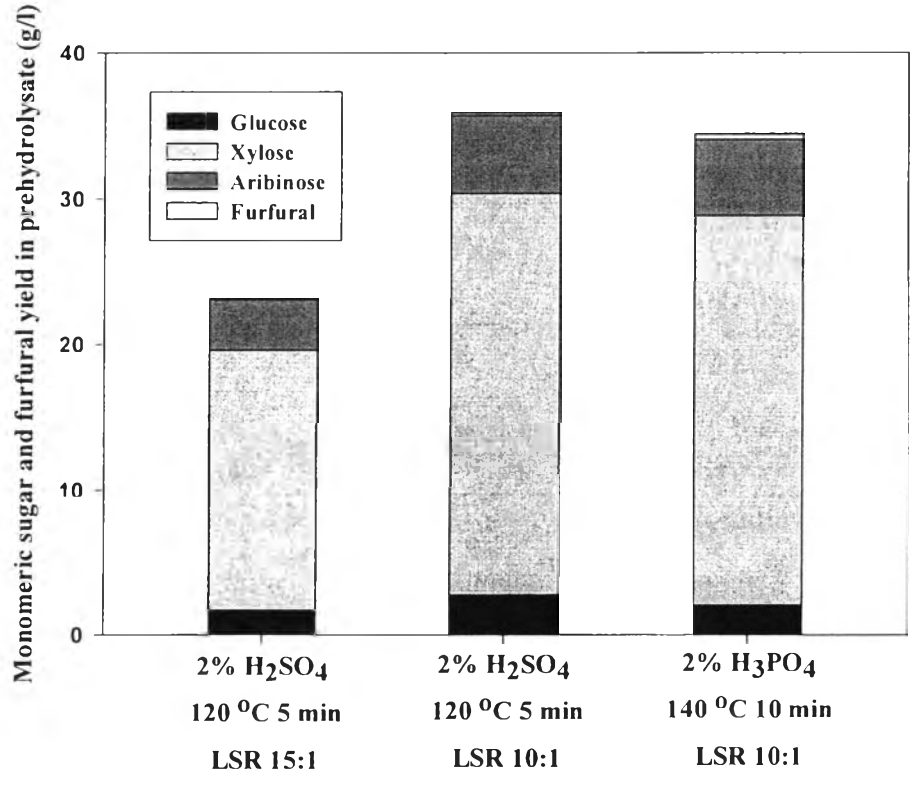

Figure 4.1 Monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment by using 2% (w/w) with different pretreatment times, temperatures and LSRs.

4.2.2 Surface Area Analysis

Surface area, pore volume, and pore diameters of corncobs were measured and the results are shown in Table 4.3.

Table 4.3 BET surface area, total pore volume, and average pore diameter of samples

Sample	Surface area (m^2/g)	Total pore volume (cm^3/g)	Average pore diameter (nm)
Untreated corncobs	3.83	$6.20x10^{-3}$	64.7
Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 15:1	4.41	$1.36x10^{-2}$	123.4
Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 10:1	4.58	$1.20x10^{-2}$	104.8
Pretreated corncobs with 2% H_3PO_4 at 140 °C for 10 min and LSR 10:1	3.90	$1.32x10^{-2}$	116.1

Accessible cellulose surface area is one of the main factors which have influenced to the ease of enzymatic hydrolysis of pretreated lignocellulosic biomass. Table 4.3 shows the physical properties of dilute acid pretreated corncob compared with untreated corncob. It was found that the surface area and total pore volume of pretreated corncobs were higher than the untreated corncobs after dilute sulfuric and phosphoric acid pretreatments. In case of sulfuric acid pretreatment conditions at 2% H_2SO_4 at 120 °C for 5 min, it indicated that surface area of the substrates pretreated at a 15:1 LSR was lower than the substrates pretreated with 10:1 LSR. The highest surface area of 4.58 m^2/g and total pore volume of $1.20x10^{-2}$ cm^3/g were obtained under the pretreatment condition at 120 °C, 5 min pretreatment times, 2% (w/w) H_2SO_4 with 10:1 LSR. On the other hand, surface area of pretreated corncobs with 2% (w/w) H_3PO_4 at 140 °C for 10 min with 10:1 LSR was not much different from untreated corncobs. For average pore diameter, all pretreated samples have higher diameter than unpretreated sample, which is benefit to contact of the enzyme (Mercier and Pinnavaia, 1997).

4.2.3 X-ray Diffraction Analysis

The degree of cellulose crystallinity is a major factor affecting enzymatic hydrolysis of the substrate (Yoshida *et al.*, 2008). Crystallinity is believed as an important feature affecting enzymatic saccharification of cellulose. CrI refers to the fraction of crystalline material in the sample and CrI value depends on the compositions in lignocellulosic materials. Crystallinity refers as a measure of ordered orientation of cellulose crystallites and it would be increased when removing of amorphous component (hemicellulose and lignin) as well as disruption of hydrogen bonding within and in between cellulose chains, which occur in a high energy treatment process such as steaming (Kumar *et al.*, 2009). Biomass crystallinity as expressed by crystallinity index (CrI) was determined according to:

$$CrI = 100 \times [(I_{002} - I_{amorphous})/I_{002}]$$

in which, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about $2\theta = 22°$ and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about $2\theta = 18°$ (Segal *et al.*, 1959). The minimum intensity in the region of the recommended locations for the intensity of the amorphous fraction (18° for cellulose Iβ and 16° cellulose II) (Azubuike *et al.* 2012). Crystallinity increased with changing in pretreatment temperature, time, acid type, and LSR, as compared with the untreated corncob and the results are illustrated by XRD patterns in Figure 4.2. Table 4.4 presents crystallinity index of pretreated corncob with dilute sulfuric and phosphoric acid under various pretreatment conditions and untreated corncob. The higher intensity peak indicates that more crystalline part of pretreated corncobs. Pretreated corncobs with 2% H_2SO_4 at 120 °C for 5 min and LSR 10:1 gives the highest crystallinity index, 51.11. In the meantime, the CrI index of pretreated corncobs with 2% H_3PO_4 at 140 °C for 10 min and LSR 10:1 is not much different from the previous one. Kim *et al.*, (2003) reported that the crystallinity index increased with pretreatment reflecting removal of the amorphous portion of biomass. Besides, Yoshida *et al.*, (2008) discovered that the crystallinity will be increased after pretreatment process because the removal of lignin which is considered to be amorphous covering cellulose in lignocellulosic biomass.

Table 4.4 Crystallinity index (%) of untreated and treated corncobs

Sample	Crystallinity index
Untreated corncobs	32.26
Pretreated corncobs with 2% H_2SO_4 at 120°C for 5 min and LSR 15:1	47.50
Pretreated corncobs with 2% H_2SO_4 at 120°C for 5 min and LSR 10:1	51.11
Pretreated corncobs with 2% H_3PO_4 at 140°C for 10 min and LSR 10:1	48.94

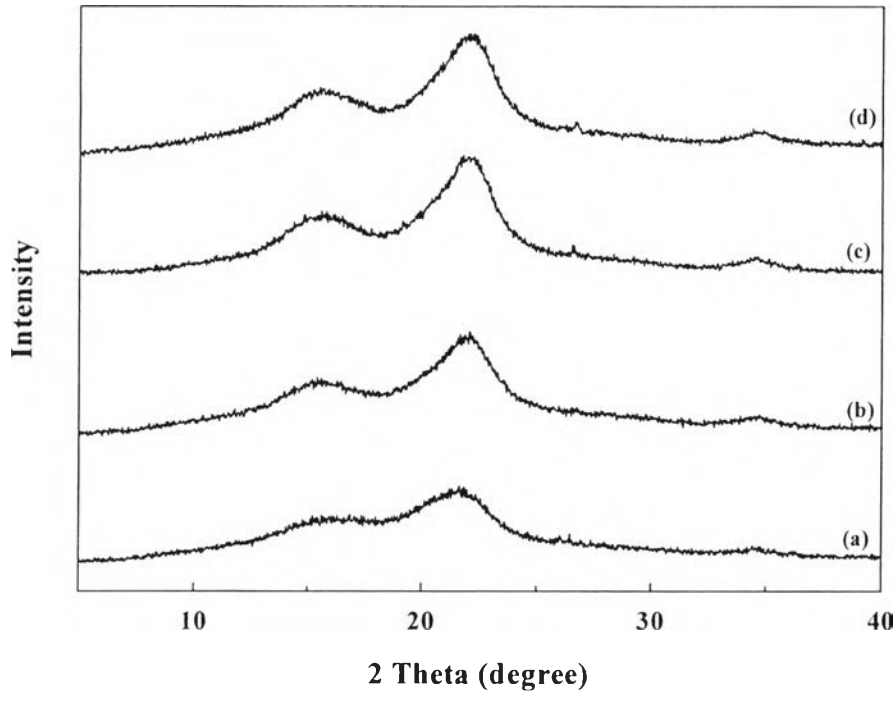

Figure 4.2 X-ray diffraction patterns of the corncob. Symbols; (a) fresh corncob, (b) pretreated corncob at 120 °C, 5 min of pretreatment time by using 2% (w/w) H_2SO_4 at a 15:1 LSR, (c) pretreated corncob at 140 °C, 10 min pretreatment time by using 2% (w/w) H_3PO_4 at a 10:1 LSR, (d) pretreated corncob at 120 °C, 5 min pretreatment time by using 2% (w/w) H_2SO_4 at a 10:1 LSR.

4.2.4 Scanning Electron Microscope (SEM)

The pretreatment process can disorganize the crystalline cellulose in dilute acid pretreated corncob to an amorphous form. The damaged structure of dilute acid pretreated corncob has high surface area which can increase enzymatic accessibility, as shown in Figure 4.3. Figure 4.3 presents the structure of dilute acid pretreated corncob after pretreatment process at condition of 2% (w/w) H_2SO_4 and H_3PO_4, under different pretreatment temperatures, times, and LSRs by using scanning electron microscope. Figure 4.3 (a–d) shows the SEM images of fresh corncob, pretreated samples with sulfuric acid with different LSRs, and pretreated samples with phosphoric acid, respectively. The SEM pictures show that fresh corncob is non-porous, bulging, smooth and uniform surfaces. In contrast, SEM images of the dilute acid pretreated corncob shows significant collapse and destruction structures. For dilute sulfuric pretreatment with 15:1 LSR, Figure 4.3 (b), many of bulges have oval characteristic which become hole and the average shape size is 61 μm wide and 156 μm long while, at a 10:1 LSR. Figure 4.3 (c) presents more porous and deeply cracks which could increase the surface area of the samples than that of 15:1 LSR and has average diameter 150 μm. In addition, pretreated corncobs with phosphoric acid cause circle shape crack with 132 μm of average diameter. The dilute acid pretreated corncob had a rougher surface and more porous than fresh corncob. This kind of cracks was essential for enzymatic hydrolysis of cellulose because they can increase surface area and porosity of lignocellulose (Gabhane *et al.*, 2011).

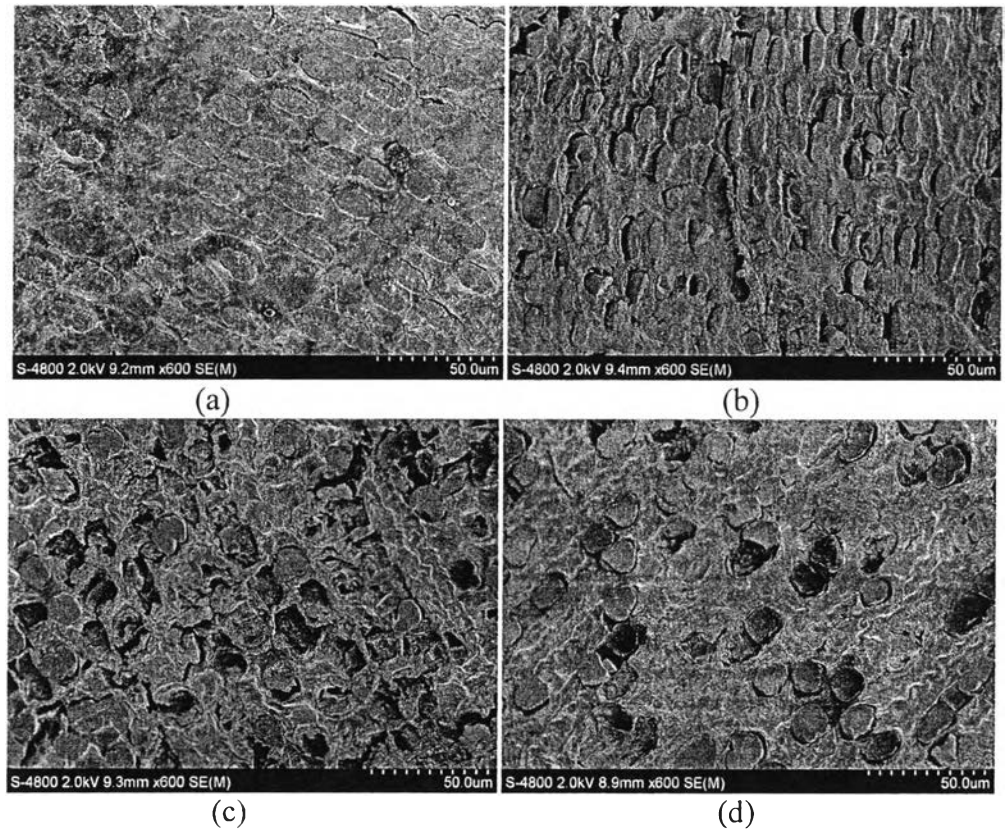

Figure 4.3 Scanning electron microscope images of samples:

(a) untreated,

(b) after pretreated with 2% (w/w) H_2SO_4 with 15: 1 LSR at 120°C for 5 min,

(c) after pretreated with 2% (w/w) H_2SO_4 with 10: 1 LSR at 120°C for 5 min, and

(d) after pretreated with 2% (w/w) H_3PO_4 with 15: 1 LSR at 140°C for 10 min.

4.3 Effect of Overliming Step in Prehydrolysate

4.3.1 Monomeric Sugar and Furfural Yield

During pretreatment of lignocellulosic substrates, various toxic compounds are formed that can inhibit microorganism in fermentation step. Furfural and 5-hydroxymethyl furfural (HMF) are two furan derivatives which are formed by the further hydrolysis of the sugars (pentoses and hexoses). Furfural is mostly found after pretreatment and other inhibitors such as 5-hydroxymethy furfural or 5-HMF and acetic acid are found secondly. These furans are available in relatively high concentration in the hydrolyzates and known as serious inhibitors to many other

microorganisms (Purwadi *et al.*, 2004). To enhance the efficiency of the fermentation, many detoxification techniques are performed; for example, alkaline detoxification, activated charcoal etc. Lime detoxification or overliming is widely known as one of inhibitor removal technique which gives high efficiency and most economical choice (Larsson *et al.*, 1999). $Ca(OH)_2$ adjustment of pH has been reported the result in better fermentability than NaOH adjustment due to the precipitation of toxic compounds (Palmqvist and Hahn-Hägerdal, 2000).

Table 4.5 and Figure 4.4 show total monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment by using 2% (w/w) with different pretreatment times, temperatures, and LSRs with and without overliming step.

Table 4.5 Total sugar yield of corncobs in prehydrolysate after dilute sulfuric and phosphoric acid pretreatment using 2% (w/w) and different pretreatment times, temperatures and LSR with and without overliming step

Conditions	Total monomeric sugar (g/l)	Furfural (g/l)
2% H_2SO_4 at 120°C for 5 min and 15:1 LSR	23.10	0.11
2% H_2SO_4 at 120°C for 5 min and 15:1 LSR (with overliming step)	21.81	0.10
2% H_2SO_4 at 120°C for 5 min and 10:1 LSR	35.72	0.17
2% H_2SO_4 at 120°C for 5 min and 10:1 LSR (with overliming step)	32.53	0.13
2% H_3PO_4 at 140°C for 10 min and 10:1 LSR	34.09	0.37
2% H_3PO_4 at 140°C for 10 min and 10:1 LSR (with overliming step)	34.82	0.33

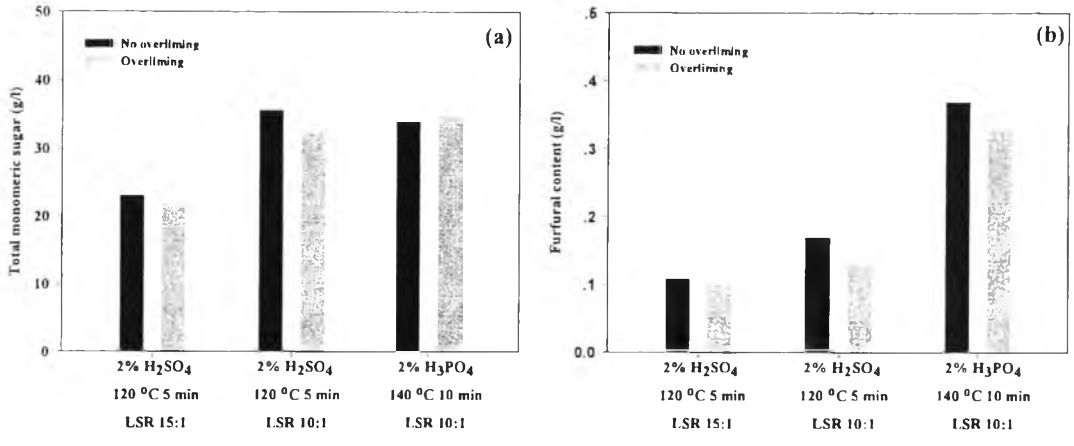

Figure 4.4 Comparison of monomeric sugar and furfural yield of corncob in prehydrolysate after dilute sulfuric and phosphoric acid pretreatments by using 2% (w/w) with different pretreatment times, temperatures and LSRs with and without overliming step.

From Figure 4.4 (a), after overliming step the concentration of total monomeric sugar of sulfuric pretreated were reduced slightly (5–9%). Larsson *et al.*, 1999 have indicated that small decreases in concentrations of fermentable sugar were observed owing to dilution with either acid or $Ca(OH)_2$ when pH was adjusted to 10. Moreover, heating step in the overliming procedures leading to some sugar decompose at high temperature. Pentose sugars are less stable than hexose sugars and the observed decline resulted primarily from a reduction in xylose, the most abundant sugar (Martinez *et al.*, 2000). However, degradation of fermentable sugars in lime detoxification has the lowest percentage compared to other methods (Larsson *et al.*, 1999). For furfural content as well known as a volatile compound can cause lower membrane permeability (Palmqvist *et al.*, 1999). Figure 4.4 (b) presents furfural content obtained from with and without overliming step and the treatment of hydrolysate with $Ca(OH)_2$ resulted in a decline in furfural in every condition. For sulfuric acid condition with 15:1 LSR has 0.01 g/l of furfural reduction. While, sulfuric acid and phosphoric acid conditions with 10:1 LSR can reduce 0.04 g/l of furfural content. Some furfural has evaporated in heating step of overliming. In addition, $Ca(OH)_2$ over-liming involved precipitation of toxic components and

instability of a few inhibitors at high pH (Chandel *et al.*, 2011). Agblevor *et al.*, 2004 used Carbon-13 Nuclear Magnetic Resonance Spectroscopy (^{13}C-NMR) to elucidate the functional groups involved in the overliming reaction has descript that overliming process can remove aliphatic and aromatic acids or esters, and other aromatic and aliphatic compounds functional group. The result in inhibitors removal leads to improve the ABE fermentability.

4.3.2 Acetone-Butanol-Ethanol Production

From the result of mononeric sugar in Table 4.6, high monomeric sugar content which was prehydrolysate from pretreated corncobs with 2% H_2SO_4 10:1 LSR at 120 °C for 5 min and 2% H_3PO_4 10:1 LSR at 140 °C for 10 min also with and without overliming step were investigated for ABE production. The *C. beijerinckii* culture was growing in glucose-based P2 medium (control) and liquid part of prehydrolysate from the previous condition which previously mentioned above. According to Table 4.6, pretreatment with overliming step shows an increase in ABE yield while the pretreatment without overliming step has low ABE yield. The highest ABE yield, 23% belongs to liquid prehydrolysate fermentation of pretreated corncobs with 2% H_2SO_4 10:1 LSR at 120 °C for 5 min followed by overliming step. On the other hand, ABE yield from liquid prehydrolysate fermentation obtained from pretreated corncobs with 2% H_3PO_4, 10:1 LSR at 140 °C for 10 min followed by overliming step has found high secondly, 17%. However, furfural content shows slightly decrease in overliming step and the ABE yields were increasing significantly. This can imply that furfural is not the major inhibitor affecting to ABE fermentation (Ezeji *et al.*, 2007).

Table 4.6 Acetone-Butanol-Ethanol production between dilute sulfuric acid and phosphoric acid at an optimal pretreatment condition of corncobs followed by with and without overliming step

Samples	Total ABE (g/l)	ABE yield (%)	ABE productivity
Control	8.59	23	0.12
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR	0.05	3	0.01
2% H_2SO_4 at 120 °C for 5 min and 10:1 LSR with overliming step	5.21	23	0.07
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR	0.06	4	0.01
2% H_3PO_4 at 140 °C for 10 min and 10:1 LSR with overliming step	3.15	17	0.02

4.4 Optimization of Acetone-Butanol-Ethanol Production Employing Response Surface Methodology (RSM)

4.4.1 Development of a Model for ABE Production

Factors affecting the statistical analysis were pH, temperature, and reaction time. The ranges were approximately 4.8–6.6 for pH, 37–50 °C for temperature, and 24–72 h for reaction time. Table 4.7 presents the RSM experimental design matrix with three factors at three levels and experimental results.

Table 4.7 Three level central composite design (CCD) design and response of dependent variable (ABE production)

Run	Coded values			Real values			Total ABE production (g/l)
	pH	Temperature	Reaction time	pH	Temperature (°C)	Reaction time (h)	
1	1	1	1	6.6	50	72	0.58
2	1	1	-1	6.6	50	24	0.13
3	1	-1	1	6.6	37	72	11.50
4	1	-1	-1	6.6	37	24	8.79
5	-1	1	1	4.8	50	72	0.18
6	-1	1	-1	4.8	50	24	0.14
7	-1	-1	1	4.8	37	72	11.64
8	-1	-1	-1	4.8	37	24	2.74
9	1	0	0	6.6	43.5	48	0.16
10	-1	0	0	4.8	43.5	48	0.15
11	0	1	0	5.7	50	48	0.21
12	0	-1	0	5.7	37	48	8.80
13	0	0	1	5.7	43.5	72	0.17
14	0	0	-1	5.7	43.5	24	0.14
15	0	0	0	5.7	43.5	48	0.17
16	0	0	0	5.7	43.5	48	0.21
17	0	0	0	5.7	43.5	48	0.17
18	0	0	0	5.7	43.5	48	0.21
19	0	0	0	5.7	43.5	48	0.16
20	0	0	0	5.7	43.5	48	0.19

The ANOVA was carried out to determine the significance of the model equation and the model term. Table 4.8 shows statistical analysis of the coefficient. The following polynomial equation was obtained using multiple regression analysis.

$$Y = 0.16 + 0.62X_1 - 4.19X_2 + 1.2X_3 - 0.68X_1X_2 - 0.71X_1X_3 - 1.38X_2X_3 - 0.02X_1^2 + 4.28X_2^2 - 0.02X_3^2 \quad \ldots(1)$$

where the code variables were: Y = Total ABE (g/l); X_1 = pH; X_2 = temperature (°C), and X_3 = reaction time (h).

Table 4.8 Statistics for the regression of the optimization model

Coefficient	Value	Standard error	*t*-value	*P*-value
a_0	0.16	0.3396	0.475	0.6450
a_1	0.62	0.3124	1.995	0.0741
a_2	-4.19	0.3124	-13.402	<0.0001
a_3	1.20	0.3124	3.851	0.0032
a_{12}	-0.68	0.3492	-1.958	0.0787
a_{13}	-0.71	0.3492	-2.037	0.0690
a_{23}	-1.38	0.3492	-3.948	0.0027
a_{11}	-0.02	0.5956	-0.039	0.9697
a_{22}	4.28	0.5956	7.189	<0.0001
a_{33}	-0.02	0.5956	-0.039	0.9697

Table 4.9 ANOVA for the regression

Source of variation	Degree of freedom	Sum of Square	Mean Square	*F*-static	Prob>*F*
Model	5	9.754	1.951	6361.604	<0.0001
Residual	5	0.002			
Total	10	9.756	0.976		
R^2	0.97				

Table 4.8 shows *t*-value and the corresponding *P*-value along with the parameter estimate. The *P*-values indicate the significance of the regression coefficient, which the significance will be higher with smaller *P*-values. Therefore,

the linear effect of temperature (a_2) and reaction time (a_3), the quadratic effect of temperature (a_{22}) and the interaction between temperature and reaction time (a_{23}) are the most influential factors ($P < 0.05$). On the contrary, the linear effect of pH (a_1) as well as the quadratic effect of pH and reaction time (a_{11} and a_{33}), and the interactive effect of pH with temperature and reaction time (a_{12} and a_{13}) on ABE production were all slight as indicated by the large P-values. In order of significance of three variables on ABE production, the most importance is temperature followed by reaction time and pH, which can be remarked from the *P*-values, respectively.

The quality of the regression was evaluated by Fisher's statistical test for analysis of variance (ANOVA) as shown in Table 4.9. The regression statistics showed that the model represented an accurate representation of the experimental data, as the computed $F_{\text{statistic}}$ (6361.604) is much larger than $F_{0.05,5,5}$ (5.05). The coefficient of determination or R^2 value of 0.97 is a measure of the amount of variation around the mean, explained by the model indicated that only 3% of all variation for response could not be explained by the model and expresses enough fit. Generally, a regression model with R^2 higher that 0.9 is considered to have a very high correlation (Haaland, 1989). Moreover, it is suggested that R^2 value should be at least 0.8 for a good fit of a model (Joglekar and may, 1987).

4.4.2 Effect of Parameters on ABE Production

Figure 4.5 presents the three-dimension response surfaces and two-dimensional contour plots with ABE production as the response. The response models are mapped against two experimental factors while the other factor is maintained constant at its central value. Figure 4.5 shows that the variation of temperature influenced the ABE production greatly across the test range, while the effect of pH has small effect. ABE production increased to a peak with decreased in temperature and pH. The effect of pH and reaction time is shown in Figure 4.6; it can indicate that total ABE production was high with increased pH and reaction time. Figure 4.7 demonstrates the variation of temperature and reaction time which can be indicated that the variation of temperature is very important than reaction time. Figure 4.7 (b) illustrates two-dimensional contour plot with changing of time, and ABE production time presents almost constant with raised temperature. The results

of the studied parameters effect show that temperature has the most significant of variable followed by pH and reaction time, respectively. This trend is reasonable and corresponding with *P*-value in the previously mentioned.

The temperature of the medium has been recognized for a long time to be very important for optimum solvent production. The previous results showed that the most preferable temperature of microorganism, *Clostridium species*, in ABE fermentation step is 35–37 °C (Mcwell and Kristiansen, 1985, Voget *et al.*, 1985) as the highest yield of produced ABE. The results presented that the sensitivity of microorganism to temperature higher than 37 °C, leading to lower ABE production significantly. On the other hand, effect of pH has found secondly significant. Maddox *et al*, 2000 has described that if the pH decreases below 4.5 before sufficient acids are produced, "acid crash" which is solventogenesis may not take place. Whereas the pH of ABE fermentation initiated at the value near 6.8 and after that the pH was decreased to lower value (5.0–5.5), which encourage solventogenesis and resulted in a proper butanol production (Qureshi *et al.*, 2008). ABE fermentation is composed of two main phases such as acidogenesis and solventogenesis. The batch fermentation can be completed within 2–6 days depending on the condition and the type of substrate employed. The final total concentration of solvents was produced in batch fermentation with ranges from 12 to 20 g/l, which was separated from the fermentation broth by distillation (Lee *et al.*, 2008).

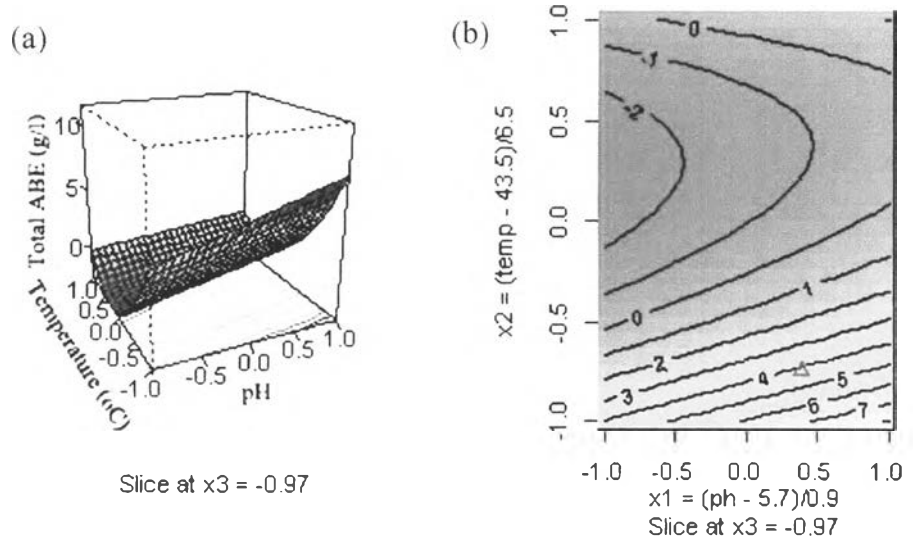

Figure 4.5 Response surface and contour plot for ABE production : effects of temperature and pH.

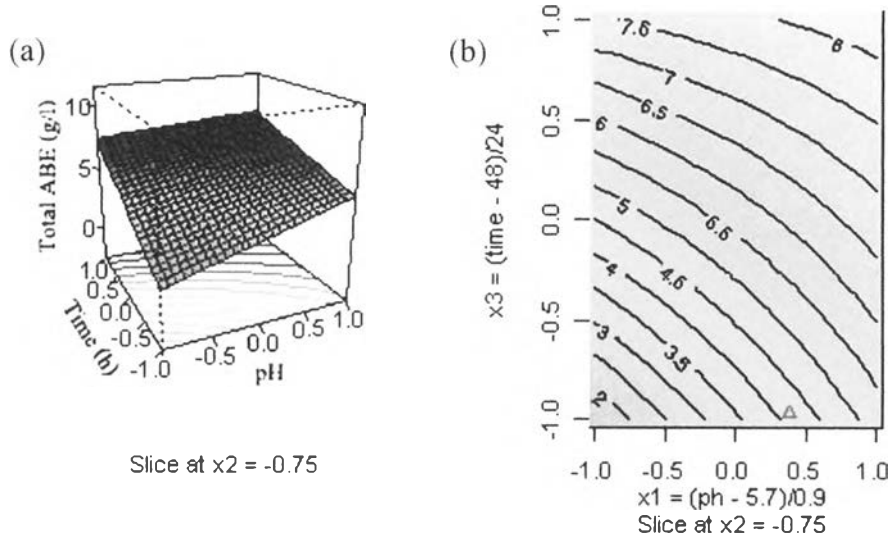

Figure 4.6 Response surface and contour plot for ABE production : effects of pH and reaction time.

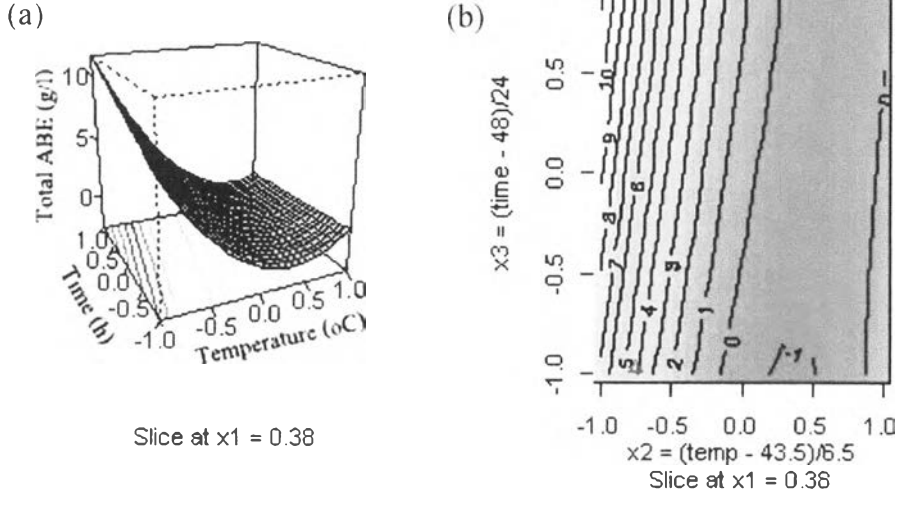

Figure 4.7 Response surface and contour plot for ABE production : effects of temperature and reaction time.

4.4.3 Optimization of ABE Production and Confirmation Experiment

The optimum conditions for maximum ABE concentration, calculated by setting the partial derivatives to zero of the following equation:

$$Y = 0.16 + 0.62X_1 - 4.19X_2 + 1.2X_3 - 0.68X_1X_2 - 0.71X_1X_3 - 1.38X_2X_3 - 0.02X_1^2 + 4.28X_2^2 - 0.02X_3^2$$

The results showed the optimum condition at pH 6.2, 47.3 °C temperature, and 54.8 h reaction time. However, the predictable result of total ABE concentration was -0.707 g/l which is the unpractical value and shows minimum response of the model. This problem had occurred by the assumption that condition between hydrolysis condition and fermentation condition might enhance efficiency of simultaneous saccharification and fermentation (SSF). However, microorganism growth decreased when temperature higher than 37 °C and presents saddle surface response leading to unable to serve an optimal value. Voget *et al*, 1985 and Mcwell and Kristiansen, 1985 have descripted that microorganism species of *clostridium* to produce butanol has high effective at temperature range of 35–37 °C. Referring to the previous work (Boonsombuti *et al.*, unplublished data), using 60 g/l of glucose as a substrate *C. beijerinckii* TISTR1461 can produce 4.84, 7.98, 0.26, and 13.07 g/L of acetone, butanol, ethanol, and total ABE, respectively, and the highest utilized glucose concentration of 46 g/L was gained. New optimal condition experiment will be selected base on partial derivative of Eq.(1) of 13.07 g/l (the assumption of maximum total ABE production from *C. beijerinckii* TISTR1461) using 0.1 for step, size from the center in lower temperature path as shown in Table 4.10. Experiment number 10, the distance of 1.8, was suitable and corresponded with the assumption condition that was predicted the total ABE resulting of 13.578 g/l.

In order to confirm the validity of the optimization strategy, confirmation experiments were conducted with two replicates under selected previously mentioned condition. Total ABE concentration of 11.82 g/l was obtained in condition of pH 6.30, 35.7 °C and 61.2 h. Qureshi *et al.*, 2008 has recommended that temperature of 35 °C and a pH 5.0–6.5 could enhance the rate of hydrolysis for simultaneous saccharification and fermentation (SSF).

Table 4.10 Experiment design and results for RSM

No.	Dist	Coded values			Real values			Predicted total ABE (g/l)
		X1	X2	X3	pH	Temp	Time	
1.	0.0	0.551	0.579	0.284	6.254	40.848	58.368	-0.707
2.	1.0	0.616	-0.408	0.432	6.254	40.848	58.368	3.698
3.	1.1	0.622	-0.507	0.447	6.260	40.205	58.728	4.626
4.	1.2	0.628	-0.606	0.462	6.265	39.561	59.088	5.643
5.	1.3	0.635	-0.704	0.477	6.272	38.924	59.448	6.738
6.	1.4	0.641	-0.803	0.492	6.277	38.281	59.808	7.932
7.	1.5	0.648	-0.902	0.506	6.283	37.637	60.144	9.213
8.	1.6	0.654	-1.000	0.521	6.289	37.000	60.504	10.570
9.	1.7	0.661	-1.099	0.536	6.295	36.357	60.864	12.030
10.	1.8	0.667	-1.198	0.551	6.300	35.713	61.224	13.578
11.	1.9	0.673	-1.296	0.566	6.306	35.076	61.584	15.197
12.	2.0	0.680	-1.395	0.581	6.312	34.433	61.944	16.922

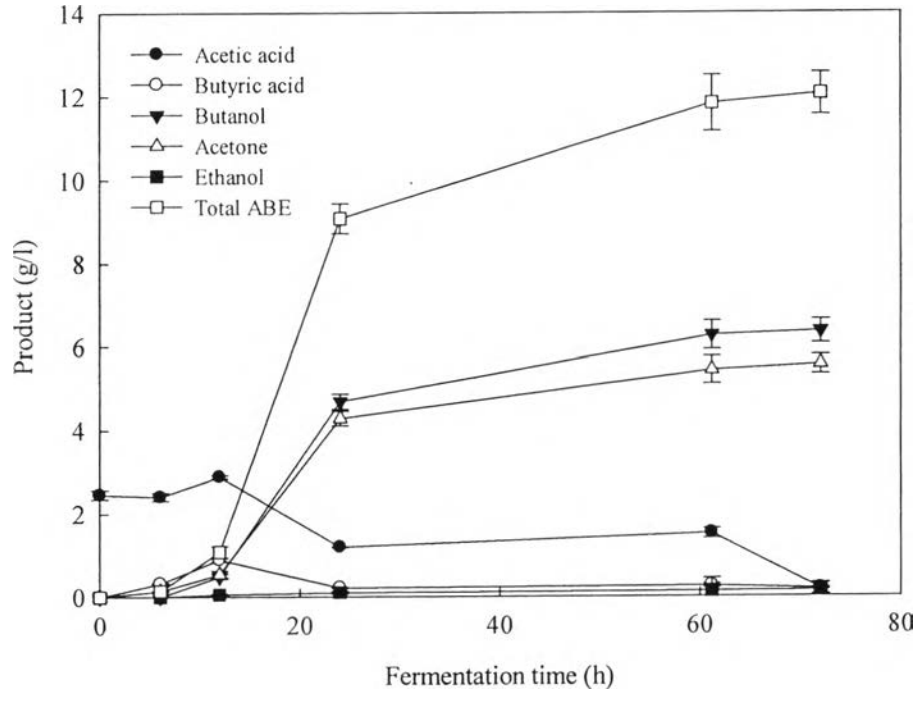

Figure 4.8 Simultaneous saccharification and fermentation (SSF) of corncobs using *C. beijerinckii* TISTR1461 at the condition of pH 6.30, 35.7 °C, and 61.2 h with 20 g/l glucose as sugar supplementation.

The fermentation profiles of the experiment are shown in Figure 4.8. During 24 h of process period, ABE production was fast, but it considerably slowed down during the next 61.2 h. However, it was noticed that the SSF process continued during this period, though at a very slow rate. The initial amounts of sugars present in the system were 21.95 g/l of glucose and 0.12 g/l of cellobiose. During fermentation, monomeric sugars of 6.87 g/l glucose, 0.77 g/l xylose, and 0.09 g/l arabinose, respectively. At the end of fermentation (61.2 h), 0.72 g/l glucose, 0.56 g/l xylose, 6.26 g/l butanol, 5.43 g/l acetone, and 0.13 g/l ethanol were measured. As mentioned previously, it clearly presented that the limitation of resulting in maximum of produced total ABE (11.82 g/l) was the deficiency of sugar at the end of fermentation (Qureshi *et al.*, 2008). Furthermore, one of the most critical problems in ABE fermentation is butanol toxicity. Jones and Woods, (1986) has reported the minimum concentration of butanol which no observable decrease in growth occurred was 4–4.8 g/l. While, 7–13 g/l of butanol to culture medium resulted in a 50% inhibition of growth and the addition of acetone and ethanol up to 40 g/L reduced growth by 50%.

In order to confirm whether fermentation was inhibited by sediment present in the hydrolysate. Figure 4.9 shows chromatogram of Gas Chromatography Mass Spectroscopy (GC-MS) analysis comparing of control experiment (20 g/l of glucose as a substrate) in Figure 4.9(a) and SSF condition of pH 6.30, 35.7 °C, and 61.2 h in Figure 4.9(b). The retention time of 1.91 min, 4.17 min, 6.94 min, 8.20 min, and 11.96 min refer to acetone, butanol, acetic acid, butyric acid and glycerin, respectively. The results of chemical compounds in both of experiment were mainly found acetone, butanol, acetic acid, butyric acid. While SSF resulting in glycerin compound which degraded from membrane phospholipids in corncob cells plant and has no inhibit effect to the culture of *C. beijerinckii*.

The productivity and yield were also compared in control experiment, with sugar and non-sugar supplementation of SSF batch as shown in Figure 4.10. When SSF batch was supplemented with 20 g/l glucose, the productivity of 0.22 g/l·h and a yield of 0.43 were recorded. While non-sugar supplementation batch resulted in a productivity of 0.09 g/l·h and a yield of 0.62. In the control experiment

using glucose as a substrate, a productivity of 0.14 g/l·h and a yield 0.38 were obtained.

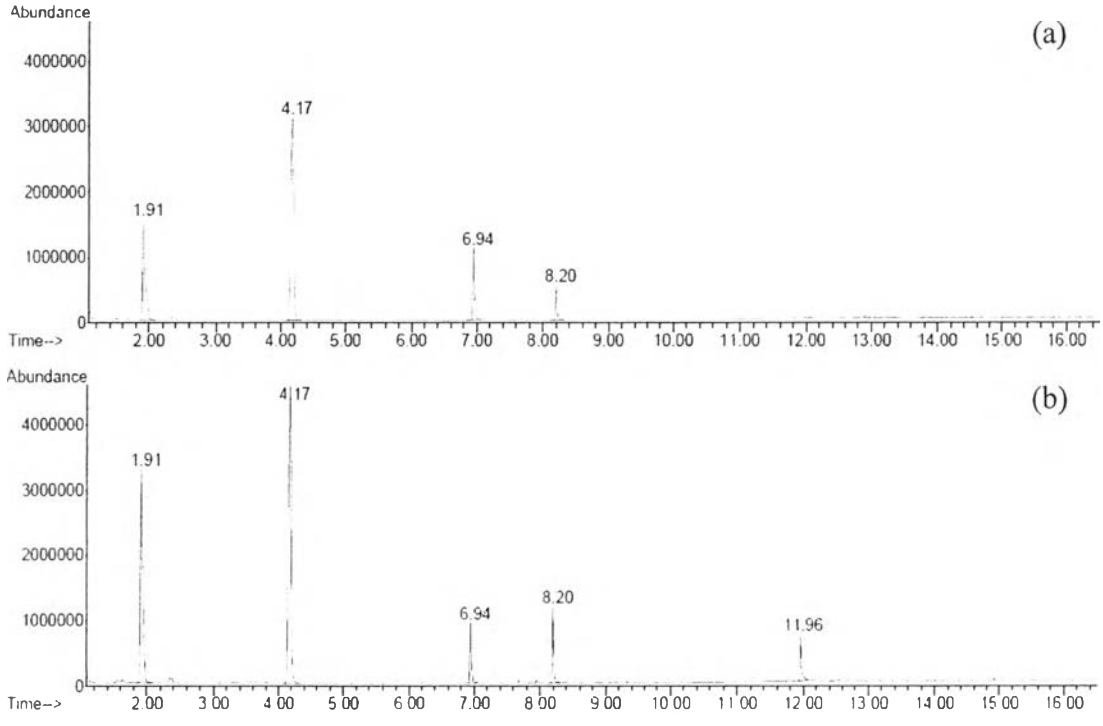

Figure 4.9 Chromatogram for Gas Chromatography Mass Spectroscopy (GC-MS) analysis. (a) control experiment (20 g/l of glucose as a substrate) ;(b)condition of pH 6.30, 35.7 °C, and 61.2 h with 20 g/l glucose as sugar supplementation.

The outcome in the lowest productivity of SSF non-sugar supplementation batch due to lack of sugar which negatively affected to ABE production (Qureshi *et al.*, 2008). In addition, the total ABE production in sugar supplementation batch was greater than control experiment by 159%, and SSF with sugar supplementation improved total ABE concentration from 4.81 g/l to 11.82 g/l comparing to without sugar supplementation batch. Qureshi *et al.*, 2008 suggested that at the beginning of fermentation, the culture was deficient in sugar because it used sugar faster than released by enzymatic hydrolysis. Moreover, the appropriate sugar concentration without inhibit enzyme was lower than 60 g/l.

Table 4.11 Acetone-Butanol-Ethanol production at the condition of pH 6.30, 35.7 °C and 61.2 h

Samples	Total ABE (g/l)	ABE yield	ABE productivity
Control (20 g/l of glucose as a substrate)	7.43	0.38	0.14
SSF with sugar supplementation	11.82	0.43	0.22
SSF with non-sugar supplementation	4.81	0.62	0.09

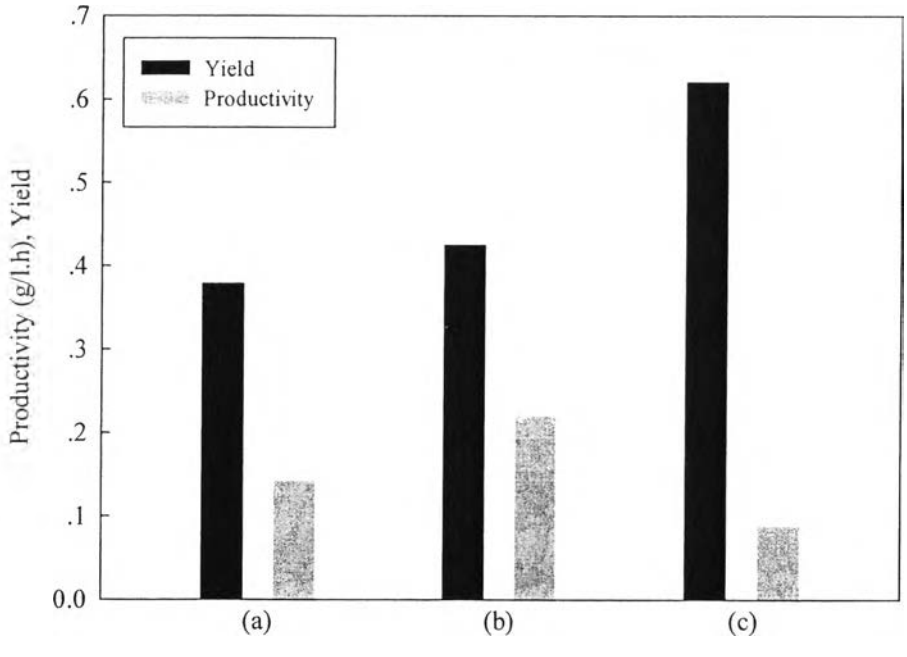

Figure 4.10 Productivity and yield of ABE production at the condition of pH 6.30, 35.7 °C, and 61.2 h in (a) control experimental (20 g/l of glucose as a substrate); (b) SSF with sugar supplementation (20 g/l glucose); (c) SSF non-sugar supplementation.