CHAPTER 3

RESULTS AND DISCUSSIONS

Many valuable products could be produced from palm press fiber (PPF) using either by chemical or biological methods. PPF was firstly removed lignin to obtain delignified PPF (dPPF). Hemicellulose was then extracted from dPPF and used as a biomaterial for furfural production via one and two stage process; and xylose production by dilute acid hydrolysis. Cellulose is another product after KOH extraction of dPPF. It is used as a source for glucose, which is a substrate for ethanol production. The PPF is not only used directly as a substrate for ethanol production by *Candida shehatae* TISTR5843 but also used as a carrier in cells immobilization.

3.1 PPF composition

PPF consists of cellulose, hemicellulose and lignin which is a complex structure acting as a protective physical barrier (Taniguchi *et al.*, 2005) and can not be removed by steam pretreatment (Öhgren *et al.*, 2007). In this study, PPF was delignified by alkali (KOH) method and its composition was compared to that of PPF (Table 9). The cellulose, hemicellulose and lignin contents of the PPF in this study (32.06%, 25.83% and 17.28%, respectively) were within the range of the content of cellulose (24-40%), hemicelluloses (14-26%) and lignin (12-27%) of the PPF in other studies (Aziz *et al.*, 2002; Kelly-Yong *et al.*, 2007; Gutiérrez *et al.*, 2009).

3.2 Delignification of PPF

In this study, delignification process by sodium chlorite and acetic acid could remove more than half (57.7%) of total lignin content (from 17.28% to 7.31%) (Table 9). This was lower than those reported by using the same method (60%) (Ahlgren and Goring, 1971) and sodium hydroxide (NaOH) at 120°C (77.2%) (Koba and Ishizaki, 1990). NaOH pretreatment was not used in this study as it required higher reaction temperature and hemicellulose content decreased (~13% of the initial content) (Koba and Ishizaki, 1990). In this study there was no loss of the

hemicellulose during the delignification process. In addition, delignification by using NaOH and microwave-assisted NaOH pretreatment resulted in 81% and 86% lignin removal, respectively, with the high loss of hemicellulose (76-84%) compared to the initial content (Zhu *et al.*, 2006). This means that the substrate for value added products was decreased simultaneously. In this study, lignin was removed due to the attack of ClO_2 (generated during delignification process) directly to the aromatic ring of lignin to decompose quickly and completely to form chlorous acid such as fumaric acid, oxalic acid and monochloroacetic acid (Collings *et al.*, 1978).

Table 9. Compositions of palm pressed fiber (PPF) and delignified PPF (dPPF).

Parameters	Composition (%)		
	PPF[a]	dPPF[b]	dPPF[c]
Crude fiber	81.49 ± 1.86	76.82	86.31 ± 2.02
Cellulose	32.06 ± 0.64	37.70	42.36 ± 1.07
Hemicellulose	25.83 ± 1.12	34.67	38.96 ± 0.67
Lignin	17.28 ± 0.18	7.31	8.21 ± 0.37
Protein	17.10 ± 0.20	12.04	13.53 ± 0.59
Lipid	12.89 ± 1.28	2.68	3.01 ± 0.10
Moisture	5.04 ± 0.48	5.70	6.41 ± 0.29
Ash	8.30 ± 0.02	7.58	8.52 ± 0.38

[a] based on 1 g PPF
[b] based on 1 g PPF:1 g PPF converted to 0.89 g dPPF
$dPPF^b = dPPF^c \times 0.89$
[c] based on 1 g dPPF

The benefits of lignin removal are the increase of hemicelluloses (from 25.83% to 34.67%) and cellulose (from 32.06 to 37.70%) as well as reduce lignin content (from 17.28% to 7.31%) which is the inhibitor in downstream process for ethanol production (Delgenes *et al.*, 1996; Limtong *et al.*, 2000; Taniguchi *et al.*, 2005; Karimi *et al.*, 2006). Nevertheless, this sodium chlorite delignification process caused the reduction of the minor components of PPF such as protein from 17.10 to 12.04% and lipid from 12.89 to 2.68%. Loss of the protein content was similar to that

using acid chlorite delignification of Alfalfa silage (from 17.8% to 13.8%) (Ely *et al.*, 1956). Therefore, the native hemicellulosic polysaccharide-protein and polysaccharide-protein-polyphenol complexes might be partially modified by this deligification method (Hedley, 2001).

3.3 Optimization of hemicellulose extraction by alkaline hydrolysis

3.3.1 Experimental design by using Response Surface Methodology (RSM)

Experimental ranges and levels of independent process variables; KOH concentration (X_1; 10-50% w/v), the dPPF to KOH ratio (X_2; 1:20-1:50 w/v) and reaction time (X_3; 20-60 min) as well as dependent process variables (responses); hemicellulose (Y_1) concentration, are given in Table 10. The hemicelluloses in the range of 20.98-42.93% and percentage of extraction in the range of 53.85-110.19% were generated. However, 110.19% extraction was resulted from the mixture of hemicellulose and cellulose. High level of hemicellulose (33.96-42.93%) and percentage of extraction (87.17-110.19%) were obtained by using the moderate (30% w/v) and high (50% w/v) KOH concentrations (33.96-38.88% hemicellulose and 87.17-99.79% extraction in trials 6-15, and 38.72-42.93% hemicellulose and 99.38-110.19% extraction in trials 16-20, respectively). While KOH concentration had a profound effect on the hemicellulose production, the reaction time had much less effect. For example, at 30% KOH and the dPPF to KOH ratio of 1:35 w/v (trials 8 and 9); the hemicellulose yield and percentage of extraction increased only 4.84% (from 35.97% to 37.71%) and 4.83% (from 92.33% to 96.79%), respectively, with 3-folds increase of reaction time (from 20 min to 60 min). To evaluate the results, the data in Table 10 were subjected to regression analysis, using the following quadratic equation (20):

$$Y_1 = 20.22 + 1.24X_1 - 460.56X_2 - 0.10X_3 - 0.015X_1^2 + 6545.454X_2^2 + 0.002X_3^2 + 2.456X_1X_2 + 0.001X_1X_3 + 0.819X_2X_3 \quad \text{..... ..... (20)}$$

where X_1, X_2 and X_3 are the actual values of KOH concentration, the dPPF to KOH ratio and reaction time, respectively (Table 10). The models illustrated the high determination coefficients (R^2=*0.97*) (Table 11) explaining 97% of variability in the

responses of hemicellulose. The high adjusted determination coefficients (adjusted R^2=*0.93*) indicated high significance of the model (O-Thong *et al.*, 2008). In addition, the ANOVA quadratic regression demonstrated that the model was significant, as evidenced from a very low probability (*P<0.0001*) while the lack of fit of the model was not significant (*P=0.7578*). Low variation coefficient value (C.V.=5.23%) indicated a high precision and reliability of the experiments (O-Thong *et al.*, 2008). The significance of each coefficient was determined by probability values (Table 11). Linear term of X_1 and quadratic term of X_1^2 were significant (*P<0.05*), demonstrated that maximizing for hemicellulose production required an optimum value of KOH concentration. To find the optimum values, estimation of hemicellulose yield over the three independent variables (X_1, X_2 and X_3) in terms of response surfaces were conducted. For hemicelluloses production (Fig. 13A-13C), results indicated that the KOH concentration (Fig. 13A and 13B) had a significant effect while the dPPF to KOH ratio (w/v) (Fig. 13A and 13C) and reaction time (Fig. 13B and 13C) gave no significant difference (*P>0.05*) on hemicellulose production. The maximum hemicellulose yield of 42.93% giving the maximum percentage of extraction of 110.19% were obtained by operating at 50% KOH concentration with the dPPF to KOH ratio of 1:50 (w/v) for 60 min reaction time. It could be implied that some cellulose were also extracted under this condition. The reaction time had no effect on hemicellulose extraction which agreed to the results of the extracted hemicelluloses (6.04-6.51%) from palm cake using 20 min to 8 h reaction time at 80°C (Prasertsan and Oi, 2001).

The advantages of alkali extraction are that there are no any by-products (furan derivatives and acetic acid), low cost, and high yield (Carrillo *et al.*, 2005). Mechanism of alkaline extraction is a saponification of intermolecular ester bonds cross-linking hemicellulose and other components, for example, hemicellulose linked by lignin and hemicelluloses linked by itself (Sun and Cheng, 2002). In the process of hemicellulose extraction by using potassium hydroxide (KOH), it can be reacted with acetic acid to form potassium acetate (CH_3COOK). In the step of hemicellulose precipitation by ethanol, CH_3COOK is soluble in ethanol and the pellet of hemicelluloses can be separated easily.

Table 10. Central composite experimental design matrix defining potassium hydroxide (KOH) concentration (%, w/v) (X_1), the PPF to KOH ratio (w/v) (X_2), and reaction time (min) (X_3) and results on hemicelllulose concentration.

Trials	Parameters			Response (Y_1)	% extraction
	X_1	X_2	X_3	Hemicellulose (%)	
1	10	1:50	20	24.74 ± 0.36	63.850
2	10	1:20	20	21.72 ± 3.28	55.75
3	10	1:50	60	24.48 ± 1.90	62.83
4	10	1:20	60	24.08 ± 1.21	61.81
5	10	1:35	40	20.98 ± 0.40	53.85
6	30	1:50	40	36.66 ± 1.37	94.10
7	30	1:20	40	38.88 ± 2.91	99.79
8	30	1:35	20	35.97 ± 0.67	92.33
9	30	1:35	60	37.71 ± 0.47	96.79
10	30	1:35	40	37.51 ± 0.61	96.28
11	30	1:35	40	35.97 ± 0.46	92.32
12	30	1:35	40	37.04 ± 3.04	95.07
13	30	1:35	40	36.88 ± 4.03	94.66
14	30	1:35	40	36.68 ± 0.81	94.15
15	30	1:35	40	33.96 ± 1.49	87.17
16	50	1:50	20	38.72 ± 0.71	99.38
17	50	1:20	20	40.94 ± 1.78	105.08
18	50	1:50	60	42.93 ± 1.78	110.19
19	50	1:20	60	41.22 ± 1.22	105.80
20	50	1:35	40	39.12 ± 2.24	100.41

Table 11. Model coefficient and analysis of variance estimated by ANOVA for hemicellulose production from dPPF.

Parameter	Hemicellulose production	
	Coefficient estimate	Probability (*P*)
Intercept	36.86	-
X_1	8.69	<0.0001*
X_2	-0.27	0.6478
X_3	0.83	0.1754
X_1X_2	0.49	0.4596
X_1X_3	0.30	0.6500
X_2X_3	-0.16	0.8028
X_1^2	-6.07	0.0002*
X_2^2	0.65	0.5613
X_3^2	0.72	0.5210
Model	-	< 0.0001
Lack of fit	-	0.7578
R^2	0.97	-
Adjusted R^2	0.93	-
C.V.	5.23	-

*Significant level at 95%

C.V. = Coefficient of variation

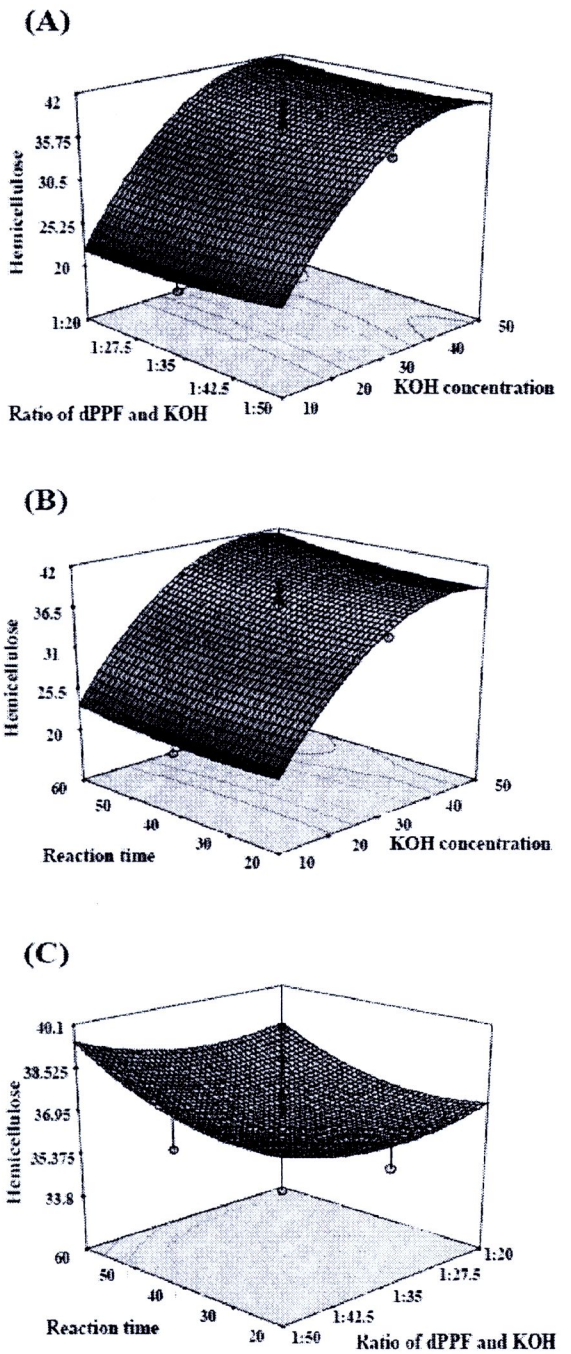

Figure 13. Three-dimensional graphs of the quadratic model for hemicellulose yield (%) (A-C) within the central composite design (CCD). Experiments A fixed reaction time at centre point of 40 minutes; Experiments B fixed the PPF: KOH ratio at centre point of 1:35 (w/v); and Experiments C fixed KOH concentration at centre point of 30 % (w/v).

3.3.2 Confirmation experiments and adequacy of the model of hemicellulose production

The optimal condition, close to the original content, calculated by RSM contained 28.88% (w/v) KOH concentration, the dPPF to KOH ratio of 1:20 (w/v) and reaction time of 20 min. To confirm the validity of the statistical experimental strategies of hemicellulose extraction, three replicates of batch experiments were performed under the optimal condition compared to the control and the central parameters (Table 12). Results from confirmation experiments indicated that the experimental values of hemicellulose yield (38.67±1.21%) was close to its predicted values (36.78%) with 99.25% extraction and low deviation of 5.14%. There was no significant difference of both hemicellulose yield between the experimental values and the predicted value *($P<0.05$)*. After optimization, hemicellulose extracted from dPPF increased 1.60 fold, compared with the control condition. Furthermore, the efficiency of hemicellulose production was 99.25% (Table 12). The results suggested that the model could be used as a tool for hemicellulose production.

Table 12. The confirmation experiments for hemicellulose contents after extraction at the optimal condition.

Trials	Conditions	X_1	X_2	X_3	Hemicellulose (%)		% extraction
					Predicted	Measured	
-	Optimal[a]	28.88	1 : 20	20	36.78[b]	38.67 ± 1.21[b] (5.14%[c])	99.25
10	Central	30	1 : 35	40	39.56[d]	37.51 ± 0.61	96.28
-	Selected	24	1 : 50	30	35.94[d]	24.11 ± 3.13	61.88

X_1: KOH concentration (%, w/v), X_2: the PPF to KOH ratio (w/v) and X_3: reaction time (min).

[a]: based on hemicellulose extraction, [b]: not significant at level $P<0.05$

[c] Deviation (%) = [(Measured value – predicted value) x 100]/predicted value.

[d] calculated by Eq. 20

3.4 Composition of the extracted hemicellulose

The hemicellulose was digested to monomeric sugars by 5% sulfuric acid at 120°C for 30 min and analyzed by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).

3.4.1 Characterizations of sugars in hemicellulose hydrolysate using TLC (qualitative method)

The types of monomeric sugar in PPF could be separated based on their polarities by thin layer chromatography (TLC, normal phase silica gel 60 F254 (Merck)). Mobile phase was isopropyl alcohol, ethyl acetate and water in the ratio of 3:3:1, and N-(1-napthyl)-ethylenediamine as sprayed dye. The results of experiments are shown in Fig. 14.

The retention factor (R_f) of unknown samples (No. 8-11) were calculated and compared to the various standard sugars (Fig. 14). The R_f values of standard sugars consisting of arabinose, rhamnose, xylose, fructose, galactose, glucose and mannose were 0.53, 0.71, 0.64, 0.52, 0.46, 0.53 and 0.57, respectively, meanwhile the unknown samples were observed to consist of two bands with the R_f values of 0.54 and 0.65. It was illustrated that the first band of hemicellulose hydrolysate might be arabinose, fructose and glucose because of their R_f value compared to standard sugar. The second band (0.65-0.66) was xylose (0.64). The carbohydrates in the fiber was consisted of 56.4% glucose, 36.0% xylose, 5.9% arabinose, and 1.7% mannose (Koba and Ishizaki, 1990). Since the result of the first lane was unclear, thus HPLC was used to identify these components.

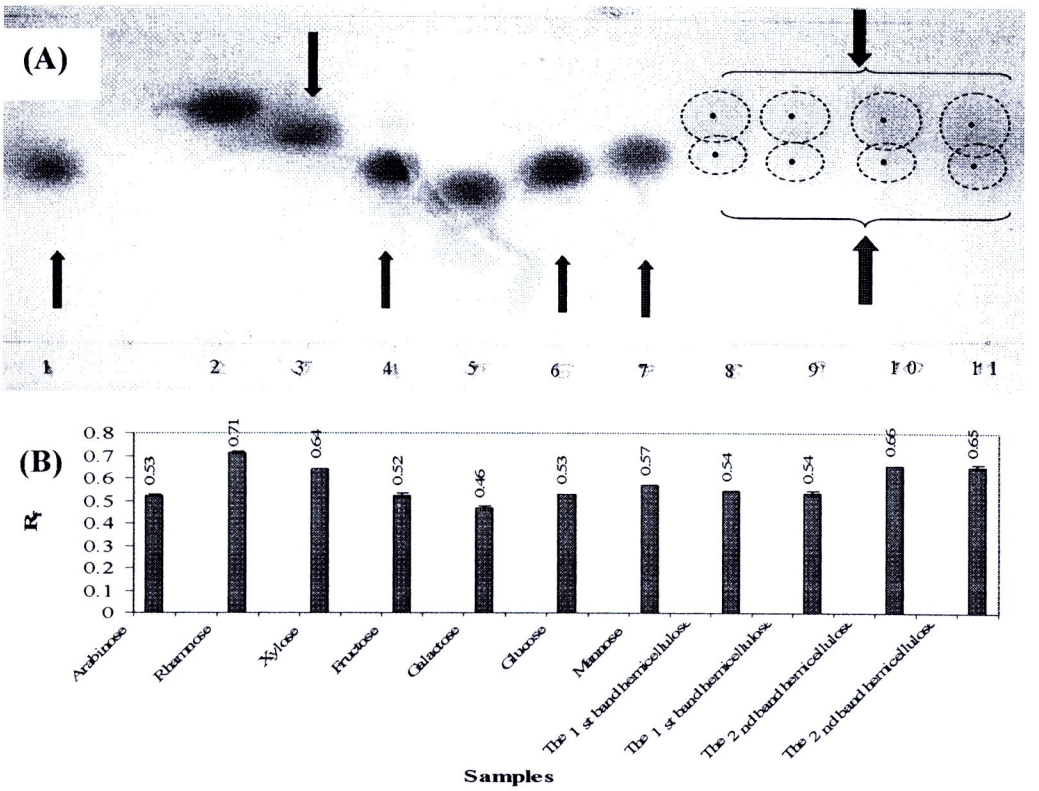

Figure 14. (A); TLC chromatogram of digested PPF by 2 N TFA at 120°C for 90 min; 1: standard arabinose, 2: standard rhamnose, 3: standard xylose, 4: standard fructose, 5: standard galactose, 6: standard glucose, 7: standard mannose, 8 and 9: PPF, 10 and 11: extracted hemicellulose from PPF. (B); the retention factor (R_f) values of various standard sugars and hydrolysate samples of PPF.

3.4.2 Characterizations of sugars in hemicellulose hydrolysate using HPLC (quantitative method)

The composition of hemicellulose was determined by HPLC. Hemicellulose was digested to monomeric sugars by sulfuric acid and xylose (80%) was found to be the main sugar in the extracted hemicelluloses of PPF and glucose (15%) was the second compound without of any other sugars. In general, the inhibitory compounds generated during hydrolysis were acetic acid and furfural whereby acetic acid was from the hydrolysis of the acetyl groups bound to the hemicellulosic monomers (Herrera *et al.*, 2003; Rahman *et al.*, 2006), while furfural,

is a derivative product from xylose hydrolysis (Herrera *et al.*, 2003; Rahman *et al.*, 2006). While 3.16% of acetic acid was detected, there was no detection of furfural. Therefore, the hydrolytic condition in this study may be suitable for xylose production as it gave low concentration of both acetic acid and furfural.

3.5 Furfural production from the extracted hemicellulose

3.5.1 Furfural production using one-stage process

The operational conditions, assayed and experimental results are illustrated in Table 13. Furfural was produced in the range of 0.06-0.86 g/l. The maximum furfural production (0.86 g/l) was achieved using high temperature (150°C), low LSR (8 ml/g), low sulfuric acid concentration (5% v/v) for 90 min reaction time. In this study, it was found that the requirement for giving high furfural production (0.80-0.86 g/l) was achieved under high reaction temperature in all experiments together with more than 60 min reaction time. It is similar to the suggestion of Parajó and Santos (1995) that the requirement of furfural production could be under high temperature (>110°C), high acid concentration (> 0.2%) and low L/S ratio (< 10/1 g/g). On the other hand, the acid hydrolysis had the disadvantages that pentosan removal was lower and reaction period was longer (90 min) (Punsuvon *et al.*, 2008). Comparison of furfural yields generated from different lignocellulosic materials is given in Table 14. The yield expressed as grams furfural/g initial dry substrate. Moderate temperature (100°C-134°C) produced furfural in the range of 3.34-13.36 wt% (Abad *et al.*, 1997; Mansilla *et al.*, 1998; Vázquez *et al.*, 2007). When compared the furfural yield of this study (3.44 wt%) to other one stage processes (3.34 wt%), the amount of furfural yields was the same as that obtained at 5% H_2SO_4, 150°C for 90 min (Mansilla *et al.*, 1998). Using the moderate temperature combined with pretreated substrate gave the advantages of lower equipment, simple and easy. However, using moderate temperature gave lower furfural yield than the high temperature condition (Table 14). The highest furfural yield of 70% can be obtained at high temperature (240°C) with short time condition (Montané *et al.*, 2002), but many disadvantages on cost of equipment and high energy usage. To achieve higher furfural yields with lower cost, two-stage process (hydrolysis followed by dehydration process) (Dias *et al.*, 2005) will be employed for further investigation.

Table 13. Operational conditions and experimental data on the furfural production from the hemicellulose extracted from dPPF.

Exper.	T (°C)	LSR (ml/g)	H_2SO_4 (% v/v)	Time (min)	XC (g/l)	FC (g/l)
1	120	10	5	0	0	0
				30	0.59	0.06
				60	1.00	0.11
				90	2.40	0.22
				120	5.92	0.31
2	120	10	10	0	0	0
				30	3.44	0.13
				60	6.18	0.45
				90	6.09	0.58
				120	6.17	0.11
3	120	8	5	0	0	0
				30	0.75	0.06
				60	1.46	0.17
				90	2.53	0.29
				120	6.34	0.41
4	120	8	10	0	0	0
				30	5.26	0.16
				60	5.81	0.52
				90	8.56	0.68
				120	8.53	0.71
5	150	10	5	0	0	0
				30	2.17	0.06
				60	3.30	0.61
				90	4.56	0.81
				120	4.20	0.85
6	150	10	10	0	0	0
				30	4.22	0.34
				60	3.70	0.84
				90	2.48	0.80
				120	0.96	0.55
7	150	8	5	0	0	0
				30	1.71	0.15
				60	3.70	0.78
				90	4.60	0.86
				120	6.01	0.82
8	150	8	10	0	0	0
				30	4.92	0.61
				60	7.35	0.81
				90	3.31	0.61
				120	2.15	0.42

LSR = liquid/solid ratio (ml/g), XC = xylose content in hemicellulose (g/l),
FC = furfural concentration (g/l)

Table 14. Comparison of furfural yield produced from various lignocellulosic materials using one-stage process.

Type of raw material	Condition	Furfural yield (%)[a]	References
Sorghum straw	6% phosphoric acid at 134°C for 300 min	13.36%	Vázquez *et al.*, 2007
Eucalyptus globulus wood	0.4 g conc. HCl/100 g at 130°C for 45 min	4.48 g/l (3.8%)	Abad *et al.*, 1997
Rice hull	10.5% H_2SO_4	5.55%	Gladkova,
Rice hull	One stage: 20% (w/w) H_2SO_4 at 125°C, 1.5 atm for 30 min	3.34%	Mansilla *et al.*, 1998
Corn cubs	ND	10%	Jaeggle,
Bagasse	ND	8-9%	1975 cited
Cotton husks	ND	8-9%	by Mansilla
Hard wood	ND	6-8%	*et al.*, 1998
Beech bark	ND	5-6%	
Rice husk	ND	6%	
Sunflower hull	ND	8-9%	
Hemicellulose of dPPF	One stage: 5% H_2SO_4 at 150°C for 90 min, liquid/solid ratio of 8 ml/g	3.44%[b] (0.86 g/l)	This study

ND = no detail

* % (grams furfural/g initial dry substrate)

** Transformation unit of g/l to %, calculated by = $\left(\frac{0.86 \times A}{1000}\right)\left(\frac{100}{0.2}\right)$,

where A = adjusted volume after hydrolysis (10 ml)

0.2 = initial weight of extracted hemicellulose (g)

3.5.2 Furfural production using two-stage process

3.5.2.1 Optimization of hydrolysis process by RSM

Response surface methodology is an efficient tool to establish the relationship of the interesting variables (at least two variables) with the obtained responses. The data analysis was developed by fitting the experimental data in a smooth curve, which is plotted by calculation of specific predicted response (Khanna and Srivastava, 2005). Therefore, response surface analysis establishes a relationship between variables and responses more professionally than the traditional design (Launen *et al.*, 1999). The effective variables in the hydrolysis stage were optimized and the pentose sugars in the extracted hemicellulose of PPF would be used as substrate for furfural production. Results of the thirty experiments (Table 15)

indicated that xylose was generated in the range of 0.43-12.58 g/l. High xylose concentrations (10.21-12.58 g/l) were achieved at the reaction temperature of 100-125 °C, the acid concentration of 5.5-10 % v/v, L/S ratio of 8-10 ml/g and reaction time of 30-75 min (trial 5, 7, 10, 11, 13, 15 and 16-21). Higher values of reaction temperature (150 °C) and reaction time (120 min) tremendously reduced the xylose yield to 0.43-1.87 g/l (average 0.99 g/l) (trials 23 and 28-30), which was nearly 92 % lower yield compared to the high xylose concentration (average 12.36 g/l). This was due to the degradation of xylose to furfural (Rahman *et al.*, 2006; Rahman *et al.*, 2007; Punsuvon *et al.*, 2008). The maximum xylose production (12.58 g/l) was achieved under 125 °C, 5.5 % sulfuric acid, L/S ratio of 9 ml/g for 30 min (trial 16). In addition, small amount of by-products were also formed; 0.16 to 1.18 g/l furfural, 1.21-8.22 g/l acetate and 0.02-5.88 g/l glucose. To evaluate the influence of these variables on xylose yield (g/l), the design matrix of experimental conditions with the corresponding xylose yield values (Table 15) were subjected to regression analysis, generating the following quadratic equation (21):

$$\begin{aligned} Xylose\ (g/l) = & -130.88 + 2.27X_4 + 2.26X_5 + 1.03X_6 - 0.09X_7 - 0.01X_4X_5 \\ & - 0.01X_4X_6 - 0.0005X_4X_7 + 0.003X_5X_6 - 0.004X_5X_7 + 0.016X_6X_7 \\ & - 0.009X_4^2 - 0.078X_5^2 - 0.038X_6^2 + 0.00008X_7^2 \quad \text{.....(21)} \end{aligned}$$

where X_4, X_5, X_6 and X_7 are the actual values of reaction temperature, sulfuric acid concentration, L/S ratio and reaction time, respectively. The model presented a high value of regression coefficient (R^2 = 0.90) explaining 90% of variability in the response. The value of the adjusted determination coefficient (adjusted R^2 = 0.81) is quite high, indicating a high significance of the model (Tanyildizi *et al.*, 2005; O-Thong *et al.*, 2008). The ANOVA quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher's F-test with a very low probability ($P<0.0001$). Moreover, a lower of variation coefficient value (CV=15.15%) indicated a high precision and reliability of the experiments (O-Thong *et al.*, 2008). The optimum conditions for maximizing xylose production yield, calculated by setting the partial derivatives of Eq. 21 to zero with respect to the corresponding variables, were a reaction temperature of 120°C, a sulfuric acid

concentration of 5.7% (v/v), an L/S ratio of 8.5 ml/g, and a reaction time of 31 min. The maximum response value for xylose production yield was estimated as 13.01 g/l. A dimensional and contour plot was based on Eq. 20 with varying the four variables within the experimental range (Fig. 15). The main goal of response surface analysis is to unravel the optimum combination of variables in order to maximize the response.

The response surface of xylose production indicated that xylose yield increased with increasing reaction temperature (up to 120 °C) (Fig. 15(a) and 15(b)) and sulfuric acid concentration (up to 5.5 %, v/v) (Fig. 15(a), 15(d) and 15(e)). This is in agreement with the suggestion of other researchers that acid concentration is an important parameter for release of sugars (Rahman *et al.*, 2006; Rahman *et al.*, 2007). The maximum xylose yield was achieved after 30 min of reaction (Fig. 15(c), 15(e) and 15(f)) and decreased with prolonged degradation to furfural (Rahman *et al.*, 2006; Rahman *et al.*, 2007) especially at longer reaction time (Table 15 trials 16, 15 and 14 for 30, 75 and 120 min, respectively). The hydrolysis process for release of sugars should therefore employ higher acid concentration with lower reaction time to minimize the formation of furfural in the resulting hydrolysate (Rahman *et al.*, 2006).

However, reaction temperature (X_4) and reaction time (X_7) had an individual significant influence on xylose production. The significance of each coefficient was calculated by probability values which are listed in Table 16. The variables with a significant effect on xylose production were the reaction temperature (X_4) and time elapsed (X_7) ($P<0.05$). It also showed that linear terms of X_4 and X_7, interaction term of X_4X_5, and quadratic term of X_4^2 are significant ($P<0.05$), demonstrating that the xylose production required a suitable reaction temperature, reaction time and sulfuric acid concentration for the highest xylose production.

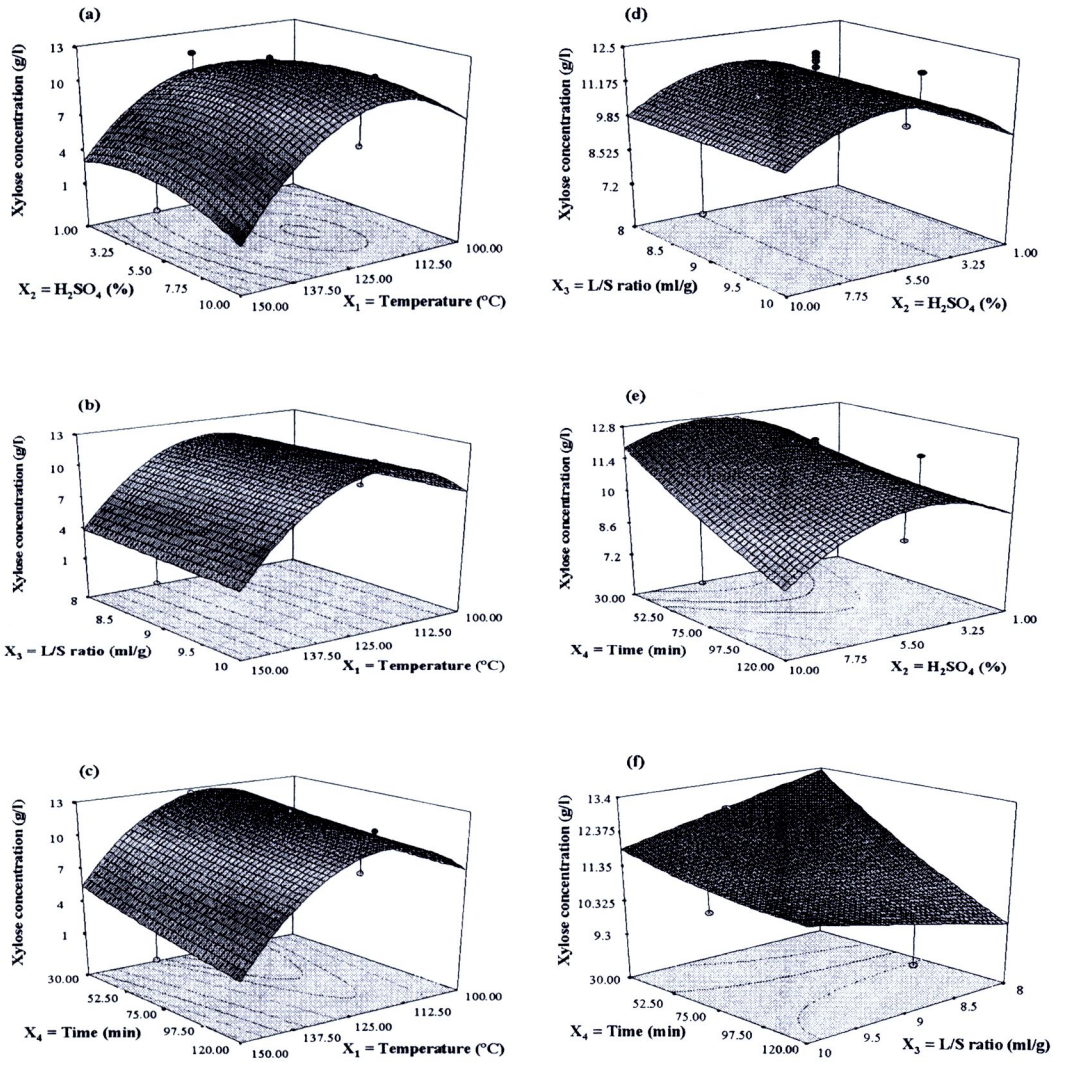

Figure 15. Three-dimensional graphs of the quadratic model for xylose yield (g/l) (a-f) within the central composite design (CCD): (a) fixed L/S ratio and reaction time at centre point of 9 ml/g and 75 min; (b) fixed H_2SO_4 and reaction time at centre point of 5.5% and 75 min; (c) fixed H_2SO_4 and L/S ratio at centre point of 5.5 % and 9 ml/g; (d) fixed reaction temperature and time at centre point of 120°C and 75 min; (e) fixed reaction temperature and L/S ratio at centre point of 120°C and 9 ml/g; (f) fixed reaction temperature and H_2SO_4 at centre point of 120°C and 5.5%.

Table 15. Central composite experimental design matrix defining reaction temperature (°C) (X_4), sulfuric acid concentration (% v/v) (X_5), L/S ratio (ml/g) (X_6), and reaction time (min) (X_7) and results on productions of xylose (Y_2), glucose (Y_3), furfural (Y_4) and acetate (Y_5) from the hemicellulose extracted from dPPF.

Trial	Variables				Responses			
	X_4	X_5	X_6	X_7	Xylose (g/l)	Glucose (g/l)	Furfural (g/l)	Acetate (g/l)
1	100	1	8	30	4.11	0.05	0.42	1.32
2	100	10	10	120	6.21	3.76	0.98	5.48
3	100	1	10	30	3.67	0.02	0.38	1.21
4	100	1	10	120	8.34	0.40	0.44	1.75
5	100	10	10	30	10.21	1.89	0.96	5.02
6	100	1	8	120	5.89	0.67	0.52	1.88
7	100	10	8	30	10.97	2.08	1.07	5.32
8	100	5.5	9	75	9.48	1.67	0.82	4.89
9	100	10	8	120	5.12	3.89	1.05	5.72
10	125	1	9	75	11.18	0.68	0.48	2.32
11	125	5.5	9	75	12.49	3.27	0.92	7.21
12	125	10	9	75	7.21	3.92	0.99	7.97
13	125	5.5	9	75	12.19	3.34	0.87	7.42
14	125	5.5	9	120	9.31	3.67	1.11	7.52
15	125	5.5	9	75	10.87	3.29	0.94	7.61
16	125	5.5	9	30	12.58	2.12	0.88	7.25
17	125	5.5	9	75	12.17	3.44	0.96	7.47
18	125	5.5	10	75	10.75	3.21	0.82	7.32
19	125	5.5	9	75	12.35	3.50	0.90	7.56
20	125	5.5	9	75	11.97	3.35	0.99	7.39
21	125	5.5	8	75	10.75	3.62	1.02	7.69
22	150	5.5	9	75	1.27	4.17	1.18	8.07
23	150	1	8	120	0.58	1.34	0.58	6.12
24	150	10	10	30	2.46	3.68	0.82	7.96
25	150	10	8	30	4.47	3.81	0.97	8.12
26	150	1	8	30	6.98	0.56	0.50	2.22
27	150	1	10	30	3.47	0.41	0.40	1.95
28	150	1	10	120	1.87	1.29	0.56	2.92
29	150	10	8	120	0.43	5.88	0.23	8.22
30	150	10	10	120	1.09	5.52	0.16	8.01

Table 16. Model coefficient and analysis of variance estimated by ANOVA for xylose production.

Parameter	Xylose production	
	Coefficient estimate	Probability (*P*)
Intercept	11.48	-
X_4	-2.35	<0.0001*
X_5	0.06	0.8923
X_6	-0.01	0.9770
X_7	-1.06	0.0279*
X_4X_5	-1.00	0.0475*
X_4X_6	-0.31	0.5166
X_4X_7	-0.56	0.2416
X_5X_6	0.01	0.9798
X_5X_7	-0.79	0.1060
X_6X_7	0.70	0.1501
X_4^2	-5.41	0.0003*
X_5^2	-1.59	0.1854
X_6^2	-0.04	0.9739
X_7^2	0.16	0.8932
Model	-	< 0.0001
R^2	0.90	-
Adjusted R^2	0.81	-
C.V.	15.15	-

*Significant level at 95%

R^2 = Regression coefficient

C.V. = Coefficient of variation

For better understanding of xylose production, three main by-products (furfural, acetate and glucose) from acid hydrolysis of xylose (Rahman *et al.*, 2006; Herrera *et al.*, 2003; Garrote *et al.*, 2001) were illustrated in the response surface plots (Fig. 16). Furfural concentration increased with increasing sulfuric acid concentration from 0 to 5.5 %, (v/v) (Fig. 16(a), 16(d) and 16(g)) and decreasing L/S ratio from 10 to 8 ml/g (Fig. 16(d)). In addition, reaction temperature and reaction time had profound effect on furfural formation (Fig. 16(a) and 16(g), respectively), which increased with either increasing reaction temperature and shorter reaction time, or decreasing reaction temperature with longer reaction time. Acetate is generated from degradation of acetyl groups of hemicellulose (Rahman *et al.*, 2006; Herrera *et al.*, 2003; Garrote *et al.*, 2001). Acetate concentration increased with increasing sulfuric acid concentration (in the range of 0-10 %, v/v) (Fig. 16(b), 16(e) and 16(h)) and increasing reaction temperature (100-137 °C) (Fig. 16(b)). Meanwhile, both L/S ratio (Fig. 16(e)) and reaction time (Fig. 16(h)) had no influence on acetate production. This demonstrated that 30 min reaction time was optimum for production of xylose from hemicellulose as no acetate was detected at prolonged reaction time. Glucose was also generated from hemicellulose hydrolysis and its concentration increased with the increase of sulfuric acid concentration (0-10 %, v/v) (Fig. 16(c), 16(f) and 16(i)), reaction temperature (100-150 °C) (Fig. 16(c)), and reaction time (30-120 min) (Fig. 16(i)). Glucose could not been produced so much in the diluted acid hydrolysis because of a little glucose content in hemicellulose (Rahman *et al.*, 2006).

3.5.2.2 Confirmation experiments and adequacy of the model of hydrolysis process

Three replicates of batch experiments were performed under the optimal condition calculated by RSM (Table 17). Results from confirmation experiments indicated that the experimental value of xylose yield (12.32 ± 2.42 g/l) was no significant difference *($P<0.05$)* from its predicted value (13.01 g/l). After optimization, xylose production from dPPF hemicellulose increased 5.4 and 1.4 folds, compared with the control and the central conditions, respectively.

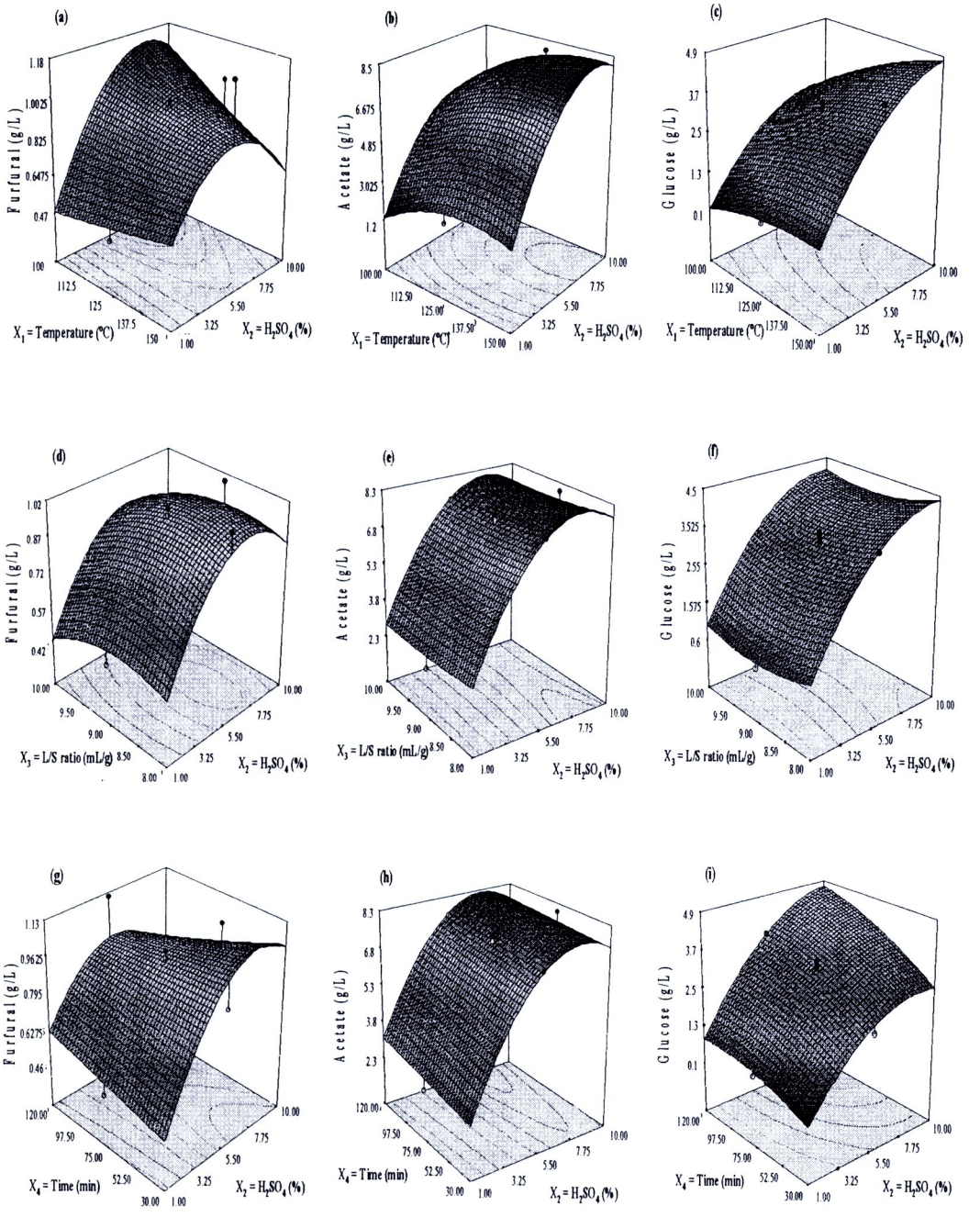

Figure 16. Three-dimensional graphs showing the effect of reaction temperature, H_2SO_4 concentration, L/S ratio and reaction time on furfural (a, d, g), acetate (b, e, h) and glucose (c, f, i) productions.

Table 17. The confirmation experiments for xylose contents after hydrolysis using the optimal condition.

Trials	Conditions	X_4	X_5	X_6	X_7	Xylose (g/l)	
						Predicted	Measured
-	Optimal[a]	120	5.70	8.5	31	13.01[b]	12.32 ± 2.42 [b] (5.30[c]%)
11, 13, 15, 17, 19, and 20	Central	125	5.50	9	75	8.70[d]	12.01 ± 0.58
-	Selected	100	1	10	15	4.21[d]	3.21 ± 1.23

X_4: reaction temperature (°C), X_5: H_2SO_4 concentration (%, v/v), X_6: L/S ratio (ml/g) and X_7: reaction time (min).

[a]: based on xylose production

[b]: not significant at level $P<0.05$

[c] Deviation (%) = [(Measured value – predicted value) x 100]/predicted value.

[d] calculated by Eq. 21

3.5.2.3 Optimization of dehydration process by RSM

Many plant materials contain the polysaccharide hemicellulose, a polymer of sugars containing five carbon atoms each. When heated with sulfuric acid, hemicellulose undergoes hydrolysis to yield these sugars, principally xylose. Under the same conditions of heat and acid, xylose and other five carbon sugars undergo dehydration, losing three water molecules to become furfural (Dias *et al.*, 2005). In this study, the optimum hydrolysis process was used to give the substrate for dehydration process. Two variables (reaction temperature and time) were optimized as it was suggested that reaction time needs to be reduced and temperature should be increased to enhance furfural production (Carrasco *et al.*, 1991; Vedernikov *et al.*, 1993). The results of all thirteen experiments were summarized (Table 18) with the furfural production in the range of 0-8.52 g/l. The maximum furfural production (8.52 g/l) was achieved under reaction temperature of 140°C for 90 min reaction time.

Table 18. Central composite experimental design matrix of dehydration process defining reaction temperature (°C) (X_8) and reaction time (min) (X_9) and results on production of furfural in one stage process.

Trials	Variables		Response
	X_8	X_9	Furfural (g/l)
1	120	150	4.92
2	120	30	2.34
3	120	90	6.42
4	140	90	8.30
5	140	150	3.83
6	140	90	8.20
7	140	30	5.24
8	140	90	8.43
9	140	90	8.40
10	140	90	8.52
11	160	30	3.00
12	160	150	0
13	160	90	1.42

Results indicated that high furfural production (4.92-8.52 g/l) could be achieved and higher temperature with shorter time (140°C/90 min) gave higher furfural yield than lower temperature with longer time (120°C/150 min). To evaluate the influence of both variables on furfural yield, the design matrix of experimental conditions with the corresponding furfural yield values (Table 18) were subjected to regression analysis, generating the equation (22):

$$Furfural(g/l) = -160.68 + 2.30X_8 + 0.29X_9 - 0.001X_8X_9 - 0.008X_8^2 - 0.001X_9^2 \quad \text{.....(22)}$$

where X_8 and X_9 are the actual values of reaction temperature and reaction time, respectively (Table 18). The model presented a high value of regression coefficient

(R^2 = 0.93). The value of the adjusted determination coefficient (adjusted R^2 = 0.88) is quite high, indicating a high significance of the model (Tanyildizi *et al.*, 2005; O-Thong *et al.*, 2008). The ANOVA quadratic regression model demonstrated that the model was highly significant, as evidenced from the Fisher's *F-test* with a very low probability ($P=0.0006$). Moreover, a smaller coefficient of variation (C.V. = 9.53 %) indicated a high precision and reliability of the experiments (O-Thong *et al.*, 2008). The optimum conditions for maximizing furfural production were the reaction temperature of 135.2 °C and a reaction time of 90.3 min. The maximum response value for furfural production yield was estimated as 8.21 g/l.

A dimensional and contour plot based on Eq. 22 with varying the two variables within the experimental range was illustrated in Fig. 17. The response surface of furfural production indicated that furfural yield increased with increasing reaction temperature in the range of 120-135 °C. The optimum reaction time was 90 min. However, reaction temperature (X_8) and reaction time (X_9) had an individual significant influence on furfural production. The significance of each coefficient was calculated by probability values which are listed in Table 19. It is clear that the variable with a significant effect on furfural production was the term of reaction temperature (X_8) ($P<0.05$). Linear term of X_8, interaction term of X_8X_9, and quadratic terms of X_8^2 and X_9^2 are significant ($P<0.05$), demonstrating that the furfural production required a suitable reaction temperature and reaction time for the highest furfural production.

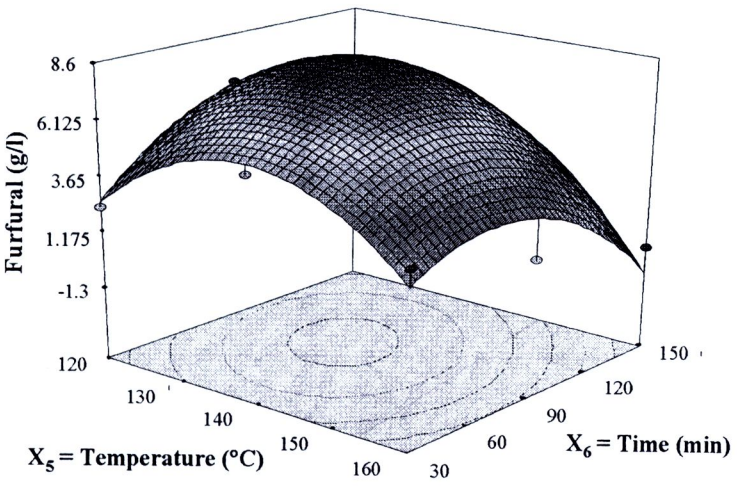

Figure 17. Graph of the quadratic model for furfural production (g/l) within the central composite design (CCD) of reaction temperature and reaction time.

Table 19. Model coefficient of furfural production estimated by ANOVA.

Parameter	Furfural production	
	Coefficient estimate	Probability (*P*)
Intercept	8.03	-
X_8	-1.54	0.0082*
X_9	-0.31	0.4946
X_8X_9	-1.39	0.0311*
X_8^2	-3.25	0.0012*
X_9^2	-2.64	0.0039*
Model	-	0.0006
R^2	0.93	-
Adjusted R^2	0.88	-
C.V.	9.53	-

*Significant level at 95%

R^2 = Regression coefficient

C.V. = Coefficient of variation

3.5.2.4 Confirmation experiments and adequacy of the model of furfural production

Results of confirmation experiments (Table 20) and commercial pure xylose experiments (Fig. 18) were done in three replicates under the optimal conditions calculated by RSM. It was indicated that the experimental values of furfural yield (8.67 g/l) was very similar to its predicted value (8.21 g/l) with no significantly difference *($P<0.05$)*. However, furfural yield from xylose in the hydrolysate (8.67 ± 0.62 g/l) was 41 % higher than that from commercial pure xylose (5.11±0.29 g/l) under the similar conditions. As shown in Fig. 18, commercial pure xylose was easily converted to furfural (8.75 ± 0.17 g/l) within 30 min and slightly decreased thereafter because of its degradation to form formic acid and levulinic acid (Jing and Lü, 2007). After optimization, furfural production increased 5.4 and 1.4 folds, compared to the control and the central conditions, respectively. Comparison of furfural yields generated from various lignocellulosic materials is given in Table 21. Results indicated that using the moderate temperature (100°C-135°C) produced furfural in the range of 10.3-17.3 % (Punsuvan *et al.*, 2008; Mansilla *et al.*, 1998). The furfural yield from two-stage process was 5.04 folds higher than that from one-stage process (from 3.44 wt% (0.86 g/l) to 17.34 wt% (8.67 g/l)). After optimizations of hydrolysis and dehydration processes, the furfural yield of 17.34 % was obtained at reaction temperature of <200 °C which is economic prospects of operation and equipment costs reduction. Besides the optimization process, a high furfural yield could be obtained from substrate pretreatment (delignification) and optimization processes (hydrolysis and dehydration steps).

Table 20. The confirmation experiments for furfural yield after hydrolysis and dehydration processes using the optimal condition predicted by RSM.

Trials	Conditions	X_8	X_9	Furfural (g/l)		
				Predicted	Measured	Deviation[c] (%)
-	Optimal[a]	135.24	90.34	8.21[b]	8.67±0.62 [b]	5.60
4, 6, 8, 9, and 10	Medium	140	90	9.92[d]	8.37 ± 0.12	15.62
-	Selected 1	120	30	2.32[d]	1.81 ± 0.23	21.98

X_8: reaction temperature (°C) and X_9: reaction time (min).
[a]: based on furfural production
[b]: not significant at level $P<0.05$
[c] [(Measured value – predicted value) x 100]/predicted value.
[d] calculated by Eq. 22

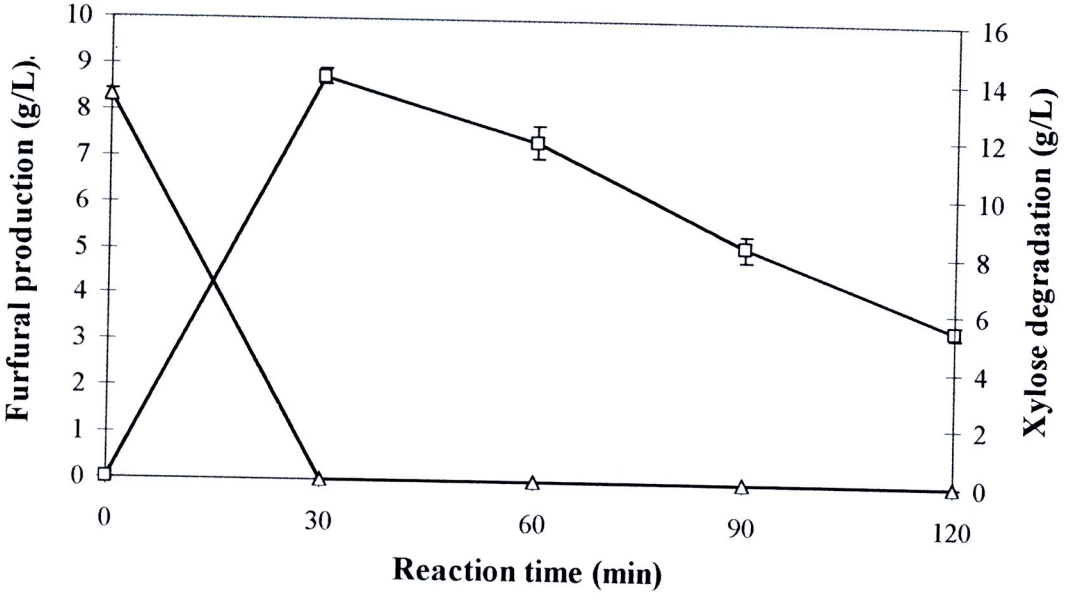

Figure 18. Time course of furfural production (□) and pure xylose degradation (Δ) at 135.2 °C for 90.3 min.

Table 21. Comparison of furfural yield produced from various lignocellulosic materials using two-stage process.

Type of raw material	Conditions	Furfural yield (%)[a]	References
Rice hull	Two stage: (i) Hydrolysis: 3 % H_2SO_4 for refluxing 30 min, (ii) Dehydration: 15 % H_2SO_4 and taking 250 mL of steam distillation	~10.5 %	Mansilla *et al.* (1998)
Hemicellulose (xylose) of Bagasse	Two stage: (i) Hydrolysis: steam explosion and concentrated 6 folds, (ii) Dehydration: 3 % H_2SO_4 at 121 °C for 1 h	~10.3 %	Punsuvon *et al.*(2008)
Hemicellulose of dPPF	Two stage: (i) Hydrolysis: 5.7 % H_2SO_4, L/S ratio 9 mL/g, at 120 °C for 31 min, (ii) Dehydration: 5.7 % H_2SO_4 at 135 °C for 90 min	17.34 %[b] (8.67 g/L)	This study
	- One stage: 5% H_2SO_4, L/S ratio of 8 mL/g, at 150 °C for 90 min	3.44 % (0.86 g/L)	This study

[a] % (grams furfural/g initial dry substrate)

[b] Transformation unit of g/l to %, calculated by = $\left(\frac{8.67 \times A}{1000}\right)\left(\frac{100}{0.5}\right)$

where A = adjusted volume after hydrolysis (10 ml)

0.5 = initial weight of extracted hemicellulose (g) for two-stage process of furfural production

3.6 Cellulosic hydrolysate production

3.6.1 Cellulosic hydrolysate production by enzymatic hydrolysis

3.6.1.1 Effect of pH on cellulase activity

Cellulose of PPF was used as a substrate for glucose production by enzymatic hydrolysis. To determine the effect of pH on cellulase on this material, the pH was varied from 3.6 to 6.0. The reaction temperature was controlled at 50°C for 24 h. The results are shown in Fig. 19. The optimal pH giving the highest yield of

reducing sugar (2.1 g/l) was 4.8. However, the optimal pH of saccharification of rice straw was 5.0 by cellulase (*Trichoderma reesei*) (Kaur *et al.*, 1998).

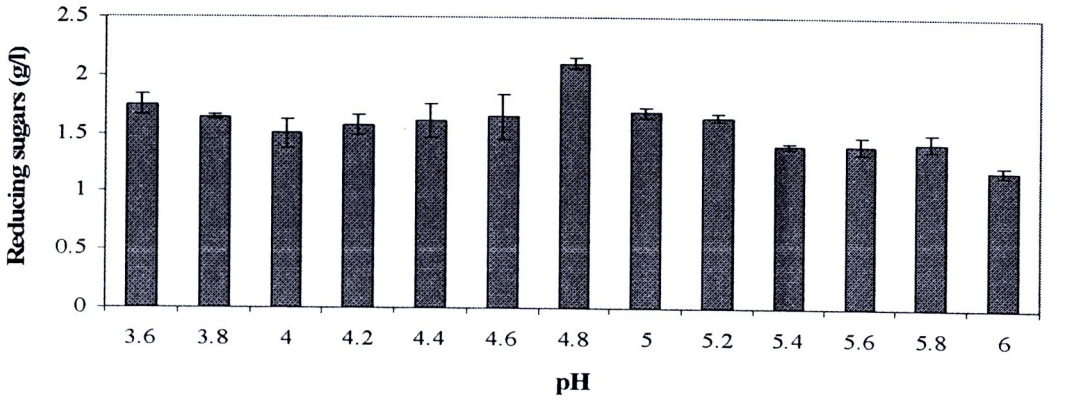

Figure 19. Effect of pH of cellulase on reducing sugar production from cellulose of PPF at substrate concentration of 2 g/l with cellulase dosage of 500 U/g substrate under 50°C for 24 h.

3.6.1.2 Effect of temperature on cellulase activity

Reaction temperature of enzymatic hydrolysis was varied from 35°C to 70°C. The pH of reaction was controlled at 4.8 (section 3.6.1.1). The results of effect of reaction temperature are shown in Fig. 20. The optimal reaction temperature was 50°C giving the highest reducing sugar of 1.59 g/l. This optimal reaction temperature was similar to the result of Kaur *et al.* (1998). The cellulase activity would decrease in the temperature condition more than 50°C due to denature of the enzyme.

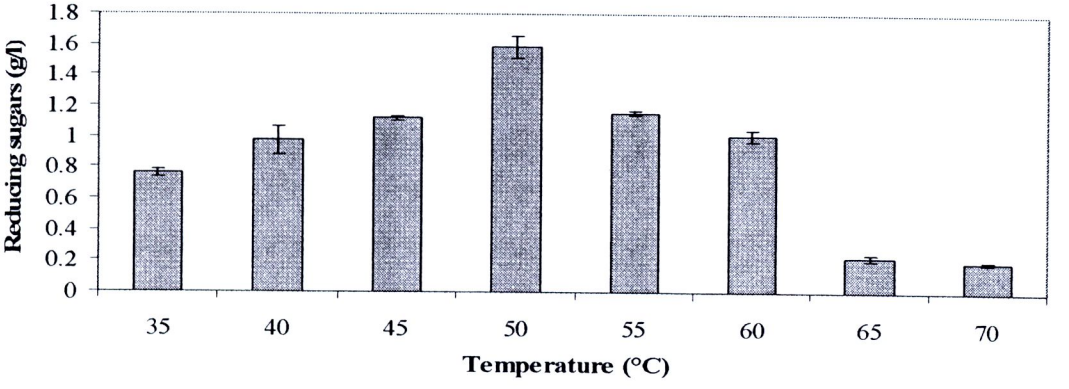

Figure 20. Effect of reaction temperature on reducing sugar production from cellulose of PPF at substrate concentration of 2 g/l with cellulase dosage of 500 U/g substrate under pH 4.8 for 24 h.

3.6.1.3 Effect of substrate concentration on cellulase activity

This study was conducted by varying the concentration of cellulose from 0.4 g/l to 20 g/l. The reaction was controlled at pH 4.8 and 50°C (section 3.6.1.1 and 3.6.1.2), respectively for 24 h. These experimental results are shown in Fig. 21. The optimal substrate concentration of cellulose was 12 g/l (833 U/g substrate) giving the maximum reducing sugar yield of 6.1 g/l. When the substrate concentration was higher than 12 g/l, reducing sugars concentration did not increase because cellulase activity was inhibited by product inhibition.

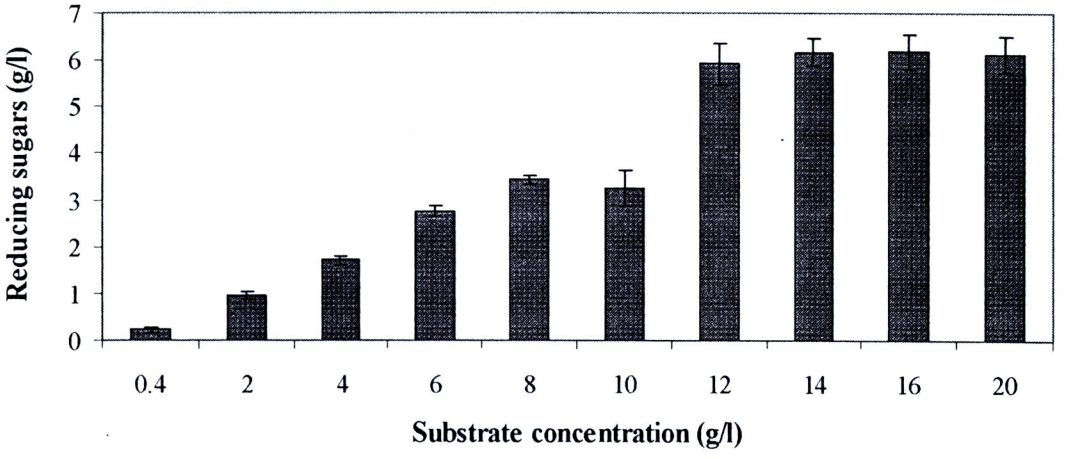

Figure 21. Effect of substrate concentration (cellulose) on reducing sugar production by enzymatic hydrolysis at cellulase dosage of 500 U/g substrate under 50°C, pH 4.8 for 24 h.

3.6.1.4 Effect of cellulase dosage

To determine the effect of cellulase dosage on reducing sugar production, the cellulase concentration was ranged from 416 U/g substrate to 8,333 U/g substrate. The reaction was controlled at pH 4.8, 50°C, and cellulase concentration of 12 g/l obtained from section 3.6.1.1, 3.6.1.2 and 3.6.1.3, respectively, for 24 h. The optimum cellulase concentration was 4,166 U/g substrate (Fig. 22) giving the highest reducing sugar yield of 7.4 g/l. The decrease cellulase loadings from 4,166 to 416 U/g substrate significantly decreased reducing sugars yields from 7 to 1 g/l which is similar with the results of Sathitsuksanoh *et al.* (2010). Meanwhile, percent of enzymatic hydrolysis of steam-exploded corn stover increased from 65 to

80% with increasing cellulase dosage from 10 to 25 IU/g glucan (Fang *et al.*, 2010). Moreover, incubation time was an important factor affecting on release of reducing sugars. Therefore, incubation time would be studied in the next work.

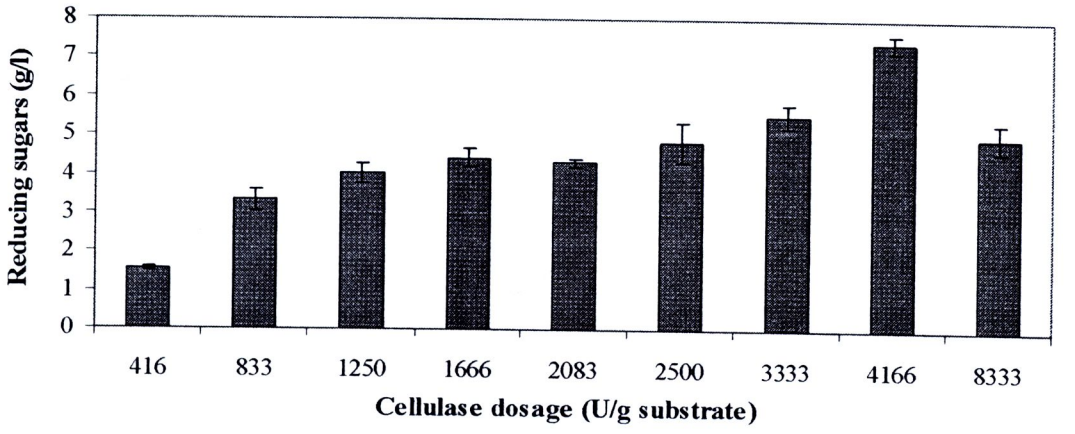

Figure 22. Effect of cellulase dosage on reducing sugar production from cellulose of PPF at substrate concentration of 12 g/l under 50°C, pH 4.8 for 24 h.

3.6.1.5 Effect of incubation time and saccharification value

To study the incubation time of reducing sugar production from cellulose by enzymatic hydrolysis, the hydrolysates were taken from 5 min to 4,320 min. The reaction was controlled at pH 4.8, 50°C, cellulose concentration of 12 g/l, and cellulase dosage of 4,166 U/g substrate obtained from section 3.6.1.1, 3.6.1.2, 3.6.1.3, and 3.6.1.4, respectively. The optimal incubation time for cellulose hydrolysis was 900 min (15 h) giving the highest reducing sugar yield of 7.9 g/l. In addition, the saccharification of this material was 60% (Fig. 23). Glucan digestibility of delignified PPF was much greater for 15 h, whereas 12-24 h and 48 h were the suitable glucan digestibility of Bamboo (Sathitsuksanoh *et al.*, 2010) and steam-exploded corn stover (Fang *et al.*, 2010), respectively. These results illustrated that cellobiose and glucose may affect the hydrolysis rate after 15-24 h incubation time (Yang *et al.*, 2010). The fast initial rate at the beginning of hydrolysis was due to preferential hydrolysis of the amorphous region and then the rate decreased as the enzyme encountered the more recalcitrant crystalline region (Laureano-Perez *et al.*, 2005; Yang *et al.*, 2010). It was observed, with the crystallinity index measured by X-ray assay, that the crystalline

cellulose had been shown to be more recalcitrant than amorphous portions because the crystallinity index had increased after enzymatic hydrolysis meaning the amorphous portion was more readily hydrolyzed than the crystalline portion (Cao and Tan, 2005; Zhu *et al.*, 2008; Yang *et al.*, 2010). The optimal values of these parameters and the highest reducing sugar yield are summarized in Table 22.

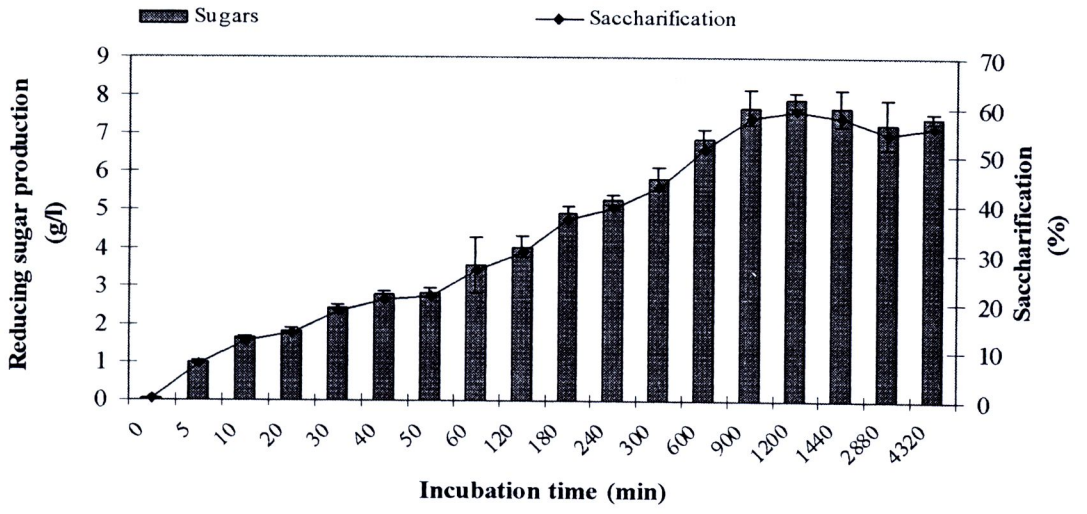

Figure 23. Effect of incubation time on reducing sugar production and saccharification values from cellulose by using cellulase hydrolysis at substrate concentration of 12 g/l with cellulase dosage of 4,166 U/g substrate under 50°C, pH 4.8 for 24 h.

Table 22. Summary of the optimal value of each parameter for the highest reducing sugar yield production.

Parameters	Optimal point
1. pH	4.8
2. Temperature (°C)	50
3. Substrate concentration (g/l)	12
4. Enzyme dosage (U/g substrate)	4,166
5. Incubation time (min)	900
6. Saccharification (%)	60
7. Reducing sugar (g/l)	7.9

3.6.2 Cellulosic hydrolysate production by concentrated sulfuric acid hydrolysis

3.6.2.1 Optimization for reducing sugars production by concentrated sulfuric acid using RSM

To understand the nature of the α-cellulose hydrolysis reaction, these experiments were conducted based on the effect of the solid (cellulose)-liquid (sulfuric acid) ratio (X_{10}), the dilute H_2SO_4 concentration in the hydrolysis (X_{11}) and reaction time (X_{12}) combined with statistical design. Levels of solid-liquid ratio, sulfuric acid concentration and reaction time were not only important factors for glucose production by chemical process, also imperative response-dependent variables for by-products formation (Table 23). Fischer's *F*-test demonstrated that the model applied was significant ($P<0.05$). The significant terms were calculated using *t*-test and the responses under different combinations were analyzed by analysis of variance (ANOVA). Only significant terms were selected to receive the maximum value of coefficient of determination (R^2) (Box *et al.*, 1978) as shown in Eqs. (23)-(27). In this study, the values of R^2 varied from 0.83-0.93, which suggested that the model gave quite good fit. Equations obtained for reducing sugars (Eq. 23) and xylose production (Eq. 24) by sulfuric acid hydrolysis were:

$$\text{Reducing sugars, } Y_7\ (g/l) = -14.65 + 0.45x_{11} - 0.03x_{11}^2 \quad \text{.....(23)}$$

$$\text{Xylose, } Y_8\ (g/l) = -(8.19\times10^{-4}) - (4.33\times10^{-6})x_{11}^2 \quad \text{.....(24)}$$

In the pretreatment step, sulfuric acid (72% v/v) hydrolysis at room temperature for 90 min and then diluted to various acid concentrations at 120°C produced reducing sugars (0.25-0.55 g/l) and xylose (<0.0002 g/l). The lowest value of reducing sugars (0.25 g/l) was obtained from 2 conditions consisting of; (i) 1:15 (w/v) solid-liquid ratio, 1.0% (v/v) sulfuric acid concentration for 180 minutes (treatment number 4), and (ii) 1:10 (w/v) solid-liquid ratio, 1.0% (v/v) sulfuric acid concentration for 120 minutes (treatment number 8). Therefore, 1.0% (v/v) sulfuric acid concentration is not suitable for reducing sugars production due to the complex structure of cellulose (Herrera *et al.*, 2003).

Table 23. Results of the experimental design for response surface analysis for producing reducing sugars and by-products formation.

Run	Real value			Product response		By-products response		
	X_{10}	X_{11}	X_{12}	Reducing sugars, Y_7 (g/l)	Xylose, Y_8 (g/l)	Furfural, Y_9 (g/l)	Acetic acid, Y_{10} (g/l)	5-HMF, Y_{11} (g/l)
1	1:20	3	60	0.42	0.000127	1.05	1.32	0.22
2	1:20	1	120	0.27	0.000126	0.99	0.00	0.00
3	1:15	3	120	0.50	0.000135	0.00	1.47	0.43
4	1:15	1	180	0.25	0.000113	0.20	1.62	0.03
5	1:15	3	120	0.50	0.000146	0.00	1.49	0.35
6	1:15	3	120	0.48	0.000146	0.00	1.49	0.40
7	1:15	3	120	0.49	0.000146	0.00	1.49	0.41
8	1:10	1	120	0.25	0.000131	1.03	0.40	0.04
9	1:10	5	120	0.46	0.000131	2.05	1.33	0.53
10	1:20	5	120	0.42	0.000112	1.93	0.68	0.31
11	1:20	3	180	0.50	0.000139	1.02	1.66	0.45
12	1:15	5	60	0.55	0.000123	1.22	1.24	0.40
13	1:15	3	120	0.49	0.000149	0.00	0.95	0.38
14	1:15	5	180	0.30	0.000101	3.74	1.84	0.52
15	1:10	3	180	0.47	0.000129	1.03	1.40	0.47
16	1:10	3	60	0.34	0.000138	1.03	0.80	0.15
17	1:15	1	60	0.26	0.000135	0.00	0.00	0.00

The lowest value of xylose, however, is normal because these substrates have been used for xylose production prior to this study (Section 3.7). In treatment number 12, the hydrolysis conditions required the highest level of sulfuric acid concentration (5%, v/v), the moderate level of solid-liquid ratio (1:15, w/v), and the lowest level of reaction time (60 min) to give the highest concentration of reducing sugars (0.55 g/l). Equations representing by-products formation from sulfuric acid hydrolysis were equations 25-27:

$$Furfural,\ Y_9\ (g/l) = 331.47 - 1.25x_{11} + 0.02x_{10}^2 + 0.22x_{11}^2 \quad \text{.....(25)}$$

$$Acetic\ acid,\ Y_{10}\ (g/l) = -178.53 + 1.84x_{11} + 0.02x_{12} - 0.11x_{11}^2 \quad \text{.....(26)}$$

$$5-HMF,\ Y_{11}\ (g/l) = -25.95 + 0.79x_{11} + 0.01x_{12} - 0.03x_{11}^2 \quad \text{.....(27)}$$

where x_{10}, x_{11} and x_{12} represent the actual values of solid-liquid ratio, sulfuric acid concentration and reaction time, respectively.

The sulfuric acid hydrolysis of cellulose of PPF also gave the formation of by-products (furfural, acetic acid and 5-HMF) (Table 23). Furfural and acetic acid are by-products from xylose degradation (Dias *et al.*, 2005); however, 5-HMF is a by-product from degradation of glucose (Larsson *et al.*, 1999; Karimi *et al.*, 2006). Sulfuric acid (72% v/v) hydrolysis produced furfural (0-3.74 g/l), acetic acid (0-1.84 g/l) and 5-HMF (0-0.53 g/l) in the same range of various factors. The less or no 5-HMF formation was observed from treatment number 2, 4, 8 and 17, the lowest of sulfuric acid concentration (1.0%, v/v) was used in these treatments. Therefore, the factor of sulfuric acid concentration is an important factor affecting on 5-HMF formation. The generation of furfural and acetic acid was very low due to the low xylose content in the substrate.

The effects of solid-liquid ratio, sulfuric acid concentration and reaction time on needed product and by-products productions were analyzed by three dimension (3D) graph obtained from RSM. Plus (+) and minus (-) symbol represented the positive and negative effects on the response as shown in Eqs. (23)-(27). However, interaction of the factors had a pronounced effect on reducing sugars

optimization indicating the importance of these factors for increasing of reducing sugars yield and decreasing of by-products yields.

Interaction of solid-liquid ratio, sulfuric acid concentration and reaction time on reducing sugars production using cellulose as a substrate revealed the response surface of reducing sugars production (Fig. 24A-24C) in which one variable kept at the optimal level and the other two variables varied within the experimental ranges. These results show the interaction between three parameters influencing significantly on reducing sugars production. The optimal conditions for reducing sugars production as mainly glucose from cellulose were 1:15 (w/v) of solid-liquid ratio, 5.0% (v/v) of sulfuric acid concentration and 60 minutes reaction time giving the maximum reducing sugars concentration of 0.55 g/l (Fig. 24A-24C). Reducing sugars produced by sulfuric acid hydrolysis increased with the increase of sulfuric acid concentration up to a value of 4% (v/v) (Fig. 24A and 24C). Similar with the effect of solid-liquid ratio and reaction time, the reducing sugars decreased when solid-liquid ratio and reaction time increase rather than 1:15 (w/v) and 90 minutes, respectively (Fig. 24B and 24C). However, the reducing sugars obtained from this study were very low. It might be low reaction time (90 min) and low reaction temperature (room temperature, 28-30°C) for decrystallization of cellulose. Xiang et al. (2003) reported that the α-cellulose form treated by concentrated sulfuric acid of 65% at high temperature (more than 200°C) can be changed from fibrous form to gelatinous form within 4 hours. The results was successfully hydrolyzed α-cellulose around 95% after carrying out at 120°C, 4% H_2SO_4 for 90 min.

At the optimal level of reducing sugars production (0.55 g/l), furfural (1.22 g/l), acetic acid (1.24 g/l) and 5-HMF (0.40 g/l) were also produced by reducing sugars degradation processes. The results (Fig. 25A-25I) demonstrated that furfural, acetic acid and 5-HMF increased when sulfuric acid concentration and reaction time increased while solid-liquid ratio had quite no effect on all by-products formation.

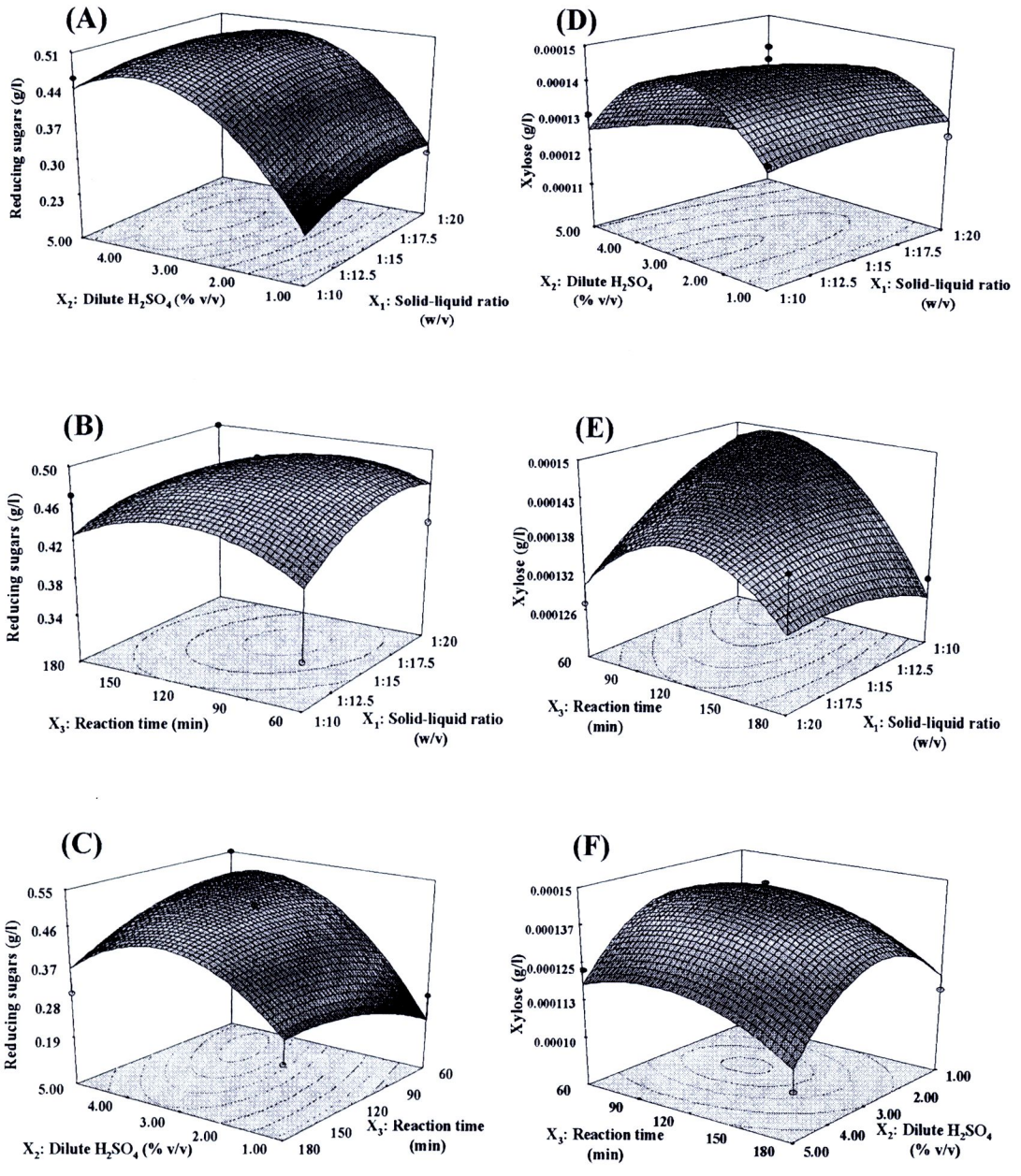

Figure 24. Three-dimensional graphs of the quadratic model for reducing sugars yield (g/l) (A-C) and xylose yield (g/l) (D-F) by using Box-Behnken design: (A and D); fixed reaction time at centre point of 120 minutes, (B and E); fixed the dilute sulfuric acid at centre point of 3% (v/v), and (C and F); fixed the solid-liquid ratio at centre point of 1:15 (w/v).

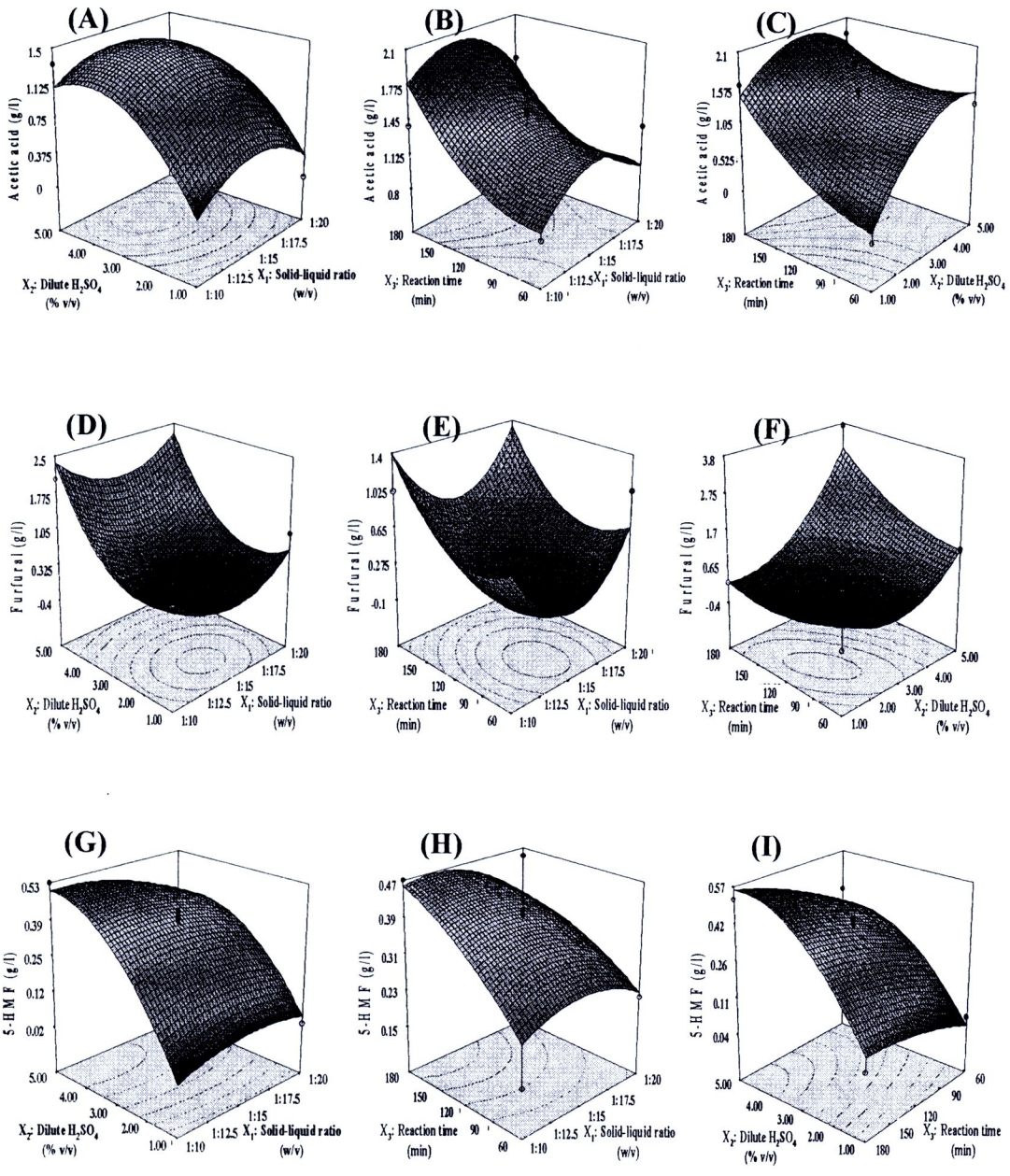

Figure 25. Three-dimensional graphs of the quadratic model for Acetic acid (g/l) (A-C) furfural (g/l) (D-F) and 5-HMF (g/l) (G-I) by using Box-Behnken design: (A, D and G); fixed reaction time at centre point of 120 minutes, (B, E and H); fixed the dilute sulfuric acid at centre point of 3% (v/v), and (C, F and I); fixed the solid-liquid ratio at centre point of 1:15 (w/v).

3.6.2.2 Model validation and confirmation

Verification experiments performed at the predicted conditions was selected to confirm the model. The experiment was conducted in triplication. The results (Table 24) demonstrated that the experiment values were similar to the predicted values and gave the percentage of deviation from predicted value at 3.68-13.33%, indicating the validity and adequacy of the predicted model.

Table 24. Comparison of predicted and experimental values of five responses (Y_7-Y_{11}) at the optimal levels predicted from model using response surface method (RSM).

Conditions	Response	Predicted value[a]	Experimental value[b]	Deviation[c] (%)
X_{10}=1:16 (w/v)	Y_7= Reducing sugars	0.52	0.54 ± 0.04	3.85
X_{11}=4.0%(v/v)	(g/l)			
X_{12}= 86 (min)	Y_8= Xylose (g/l)	0.000136	0.000131 ± 0.00005	3.68
	Y_9= Furfural (g/l)	0.45	0.51 ± 0.06	13.33
	Y_{10}=Acetic acid (g/l)	1.36	1.42 ± 0.18	4.41
	Y_{11}= 5-HMF (g/l)	0.39	0.35 ± 0.09	10.26

Parameters: X_{10} = solid-liquid ratio (w/v), X_{11} = sulfuric acid concentration (%, v/v), X_{12} = reaction time (min).

[a] Predicted value obtained from RSM model.

[b] Observed value determined from experiments.

[c] [(Observed value – predicted value) x 100]/predicted value.

3.6.2.3 Comparison of reducing sugar produced between enzymatic hydrolysis and concentrated sulfuric acid

In order to select the better method for producing reducing sugar from cellulose of PPF, concentrated sulfuric acid hydrolysis and enzymatic hydrolysis were studied. Enzymatic hydrolysis method is a better method than concentrated sulfuric acid hydrolysis method for reducing sugar production (Table 25). Therefore,

enzymatic hydrolysis method was selected to produce reducing sugar as a substrate for producing ethanol in the further investigation. However, the optimal conditions of concentrated sulfuric acid hydrolysis (solid-liquid ratio (X_{10}) = 1.16 w/v, sulfuric acid concentration (X_{11}) 4.0 %, v/v, reaction time (X_{12}) 86 min) were unclear because very low reaction time and temperature were given in these studies. Moreover, the inhibitor compounds in both hydrolysates of enzymatic method and concentrated sulfuric acid should be studied.

Table 25. Comparison of reducing sugar and inhibitors between concentrated sulfuric acid (72% v/v) and enzymatic hydrolysis.

Parameters	Enzymatic hydrolysis*	Concentrated sulfuric acid hydrolysis**
1. Reducing sugar (g/l)	7.9	0.54
2. Furfural (g/l)	0	0.51
3. Acetate (g/l)	6.8	1.42
4. 5-HMF (g/l)	0	0.35

* pH 4.8, temperature 50°C, substrate concentration 12 g/l, enzyme dosage 80 U/g substrate, and 900 min incubation time

**Solid-liquid ratio (X_{10}) = 1.16 w/v, sulfuric acid concentration (X_{11}) 4.0 %, v/v, reaction time (X_{12}) 86 min

3.7 Production of delignified PPF (dPPF) hydrolysate

3.7.1 Delignified PPF (dPPF) hydrolysate

The component of the main fractions of dPPF was; cellulose 42.36 ± 1.07%, hemicellulose 38.96 ± 0.67%, and lignin 8.21 ± 0.37% (Table 9). The hemicellulose fraction of this material was mainly xylan 80.8% (w/w). If we assume that xylan is completely converted to xylose without formation of any decomposition products, then P_0 can be represented by equivalent amount of xylose by Eq. (28) and (29):

$$P_0 \; of \; dPPF = \left(\frac{X_{P0} \times 150 \times 10}{132 \times LSR} \right) = 35.77 \; g \; xylose / l \qquad (28)$$

$$P_0 \; of \; PPF = \left(\frac{X_{P0} \times 150 \times 10}{132 \times LSR} \right) = 29.35 \; g \; xylose / l \qquad (29)$$

where X_{P0} is the initial xylan polymer presented in the PPF or dPPF on dry basis (25.83 g xylan/100g PPF or 31.48 g xylan/100g dPPF; calculated from (38.96x80.8)/100), 150/132 is the stoichiometric factor (Rahman *et al.*, 2006) and LSR is liquid solid ratio (10 g liquid/g dPPF).

The maximum release of xylose from hemicellulose was 30.67 g/l under dilute sulfuric acid (5% v/v), giving 85.74% of potential concentration of xylose. Reaction temperature and reaction time of xylose production were important parameters affecting on release of xylose (Fig. 26a). In the autohydrolysis at 75-148°C, the maximum of xylose production (9.10 g/l) required higher reaction temperature (120-148°C) and reaction time (180 min). Moreover, under condition of 10% sulfuric acid gave the maximum xylose released of 23.54 g/l. It was observed that with increase in acid concentration, concentration of xylose in the dPPF hydrolysate was decreased (Fig. 26b and 26c). Experimental results illustrated that probably there are some decomposition reaction leading to dehydration of xylose to furfural (Rahman *et al.*, 2006).

Glucose was also released during acid hydrolysis but the concentration was low (0.20-6.05 g/l) (Fig. 27). The maximum of glucose released in dPPF hydrolysate was 6.05 g/l under 10% sulfuric acid concentrations at 148°C for 180 min (Fig. 27c). On the other hand, with 0 and 5% sulfuric acid concentration, the maximum glucose released was 1.51 and 5.33 g/l, respectively. Normally, the release of glucose could be either from hemicellulose or cellulose chain (Téllez-Luis *et al.*, 2002; Rahman *et al.*, 2006). Thus, the glucose released in this study is from both hemicellulose and cellulose; however, it comes basically from hemicelluloses by using dilute acid (Téllez-Luis *et al.*, 2002).

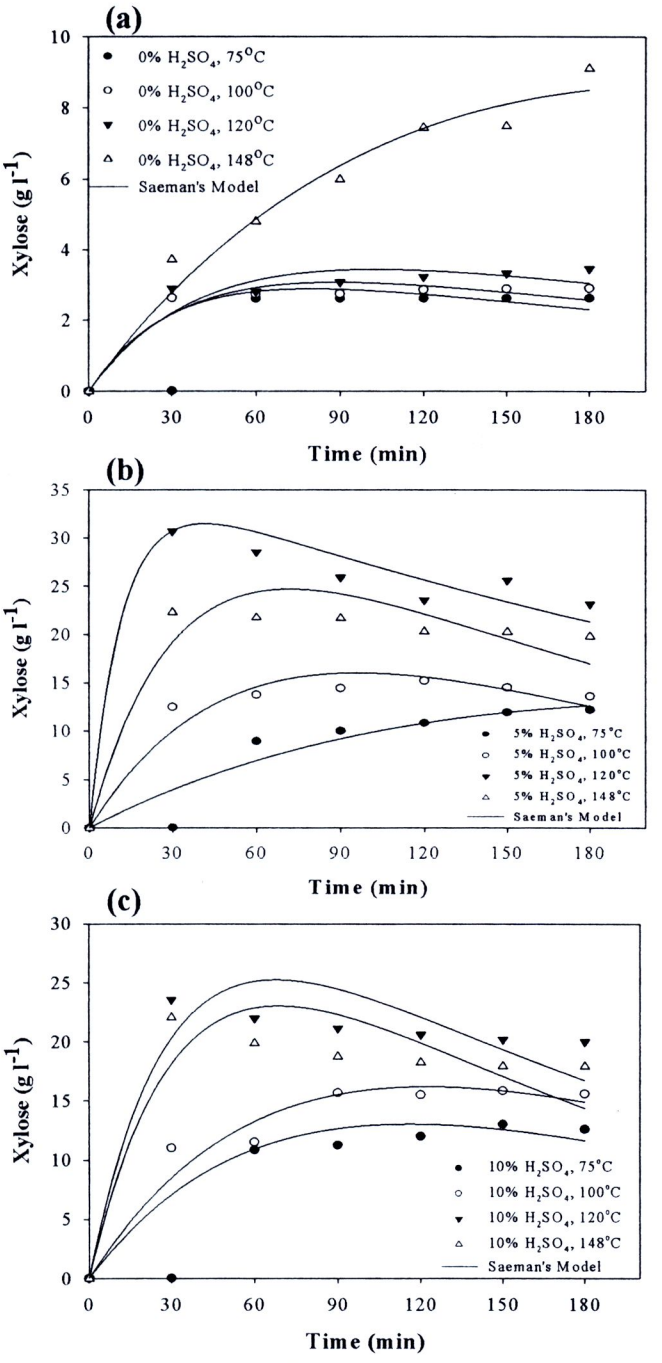

Figure 26. Experimental (dot) and predicted (line) concentrations of xylose released from dPPF at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

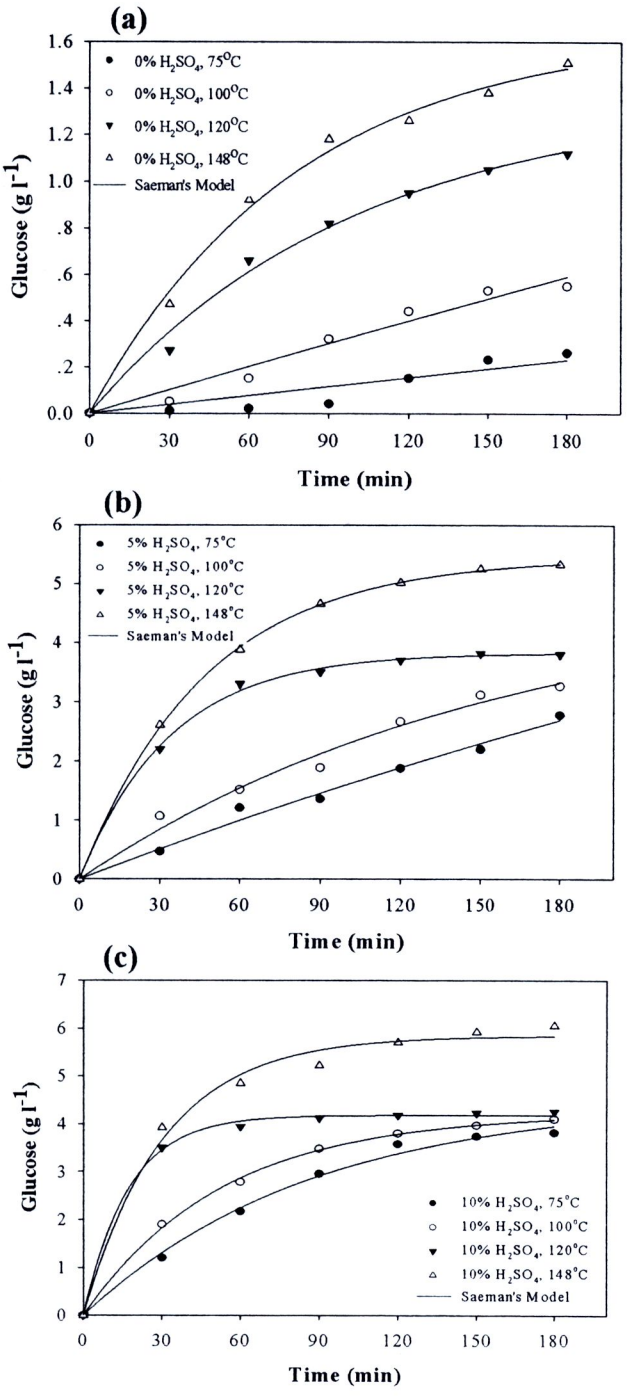

Figure 27. Experimental (dot) and predicted (line) concentrations of glucose released from dPPF at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

The furfural formation as a decomposition product from xylose in the dPPF hydrolysate is shown in Fig. 28. It was demonstrated that when sulfuric acid concentration and reaction temperature were increased from 0 to 5% (Fig. 28a and 28b) and 75 to 148°C (Fig. 28a), furfural concentration was increased in the hydrolysate. The highest concentration of furfural (Fig. 28b) was 1.16 g/l when sulfuric acid concentration and reaction time were 5% and 60 min, respectively.

During acid hydrolysis, acetic acid is generated from acetyl groups of hemicellulose (Rahman *et al.*, 2006). The maximum and minimum generations of acetic acid in the dPPF hydrolysate were 8.02 and 0.65 g/l when sulfuric acid concentrations were 10 and 0%, respectively, and reaction time of 90 and 30 min, respectively (Fig. 29).

3.7.1.1 Kinetic model of xylose production

Kinetic and statistical parameters obtained from dPPF hydrolysis at various reaction temperatures (75-148°C) with various sulfuric acid concentrations (0-10%) is shown in Table 26. Experimental and predicted data for xylose production with various acid concentrations, reaction temperatures and reaction times are shown in Fig. 26. It was demonstrated that with both sulfuric acid concentrations (5% and 10%, v/v) and ≥100°C of reaction temperature, xylose production rate (k_1) was higher than the decomposition rate (k_2) which were 0.0102-0.0821 min^{-1} and 0.0044-0.0510 min^{-1}, respectively. When the reaction temperature was 75°C, xylose production rate (k_1) and the decomposition rate (k_2) under 0%, 5% and 10% sulfuric acid were the same values of 0.0033-0.0031 min^{-1}, 0.0042 min^{-1} and 0.085-0.0087 min^{-1}, respectively. Therefore, the 75°C of reaction temperature had no effected on xylose production. The determination of coefficient R^2 showed a good agreement between experimental and predicted data for all regressions. It was also demonstrated that with increase in acid concentration, the values of k_1 and k_2 were also increased. It can be implied that the optimum reaction time to obtain maximum release of xylose and minimum release of furfural and acetic acid in the dPPF hydrolysate is essential.

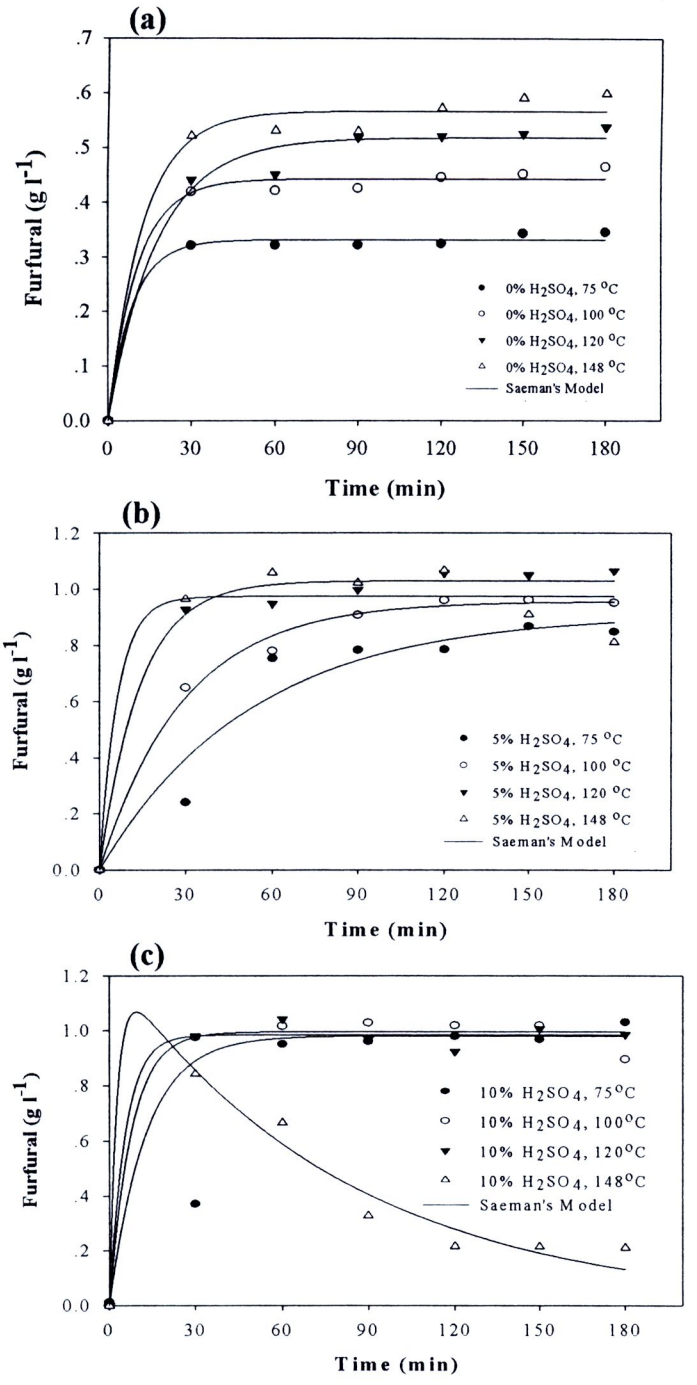

Figure 28. Experimental (dot) and predicted (line) concentrations of furfural generated in dPPF hydrolysate at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

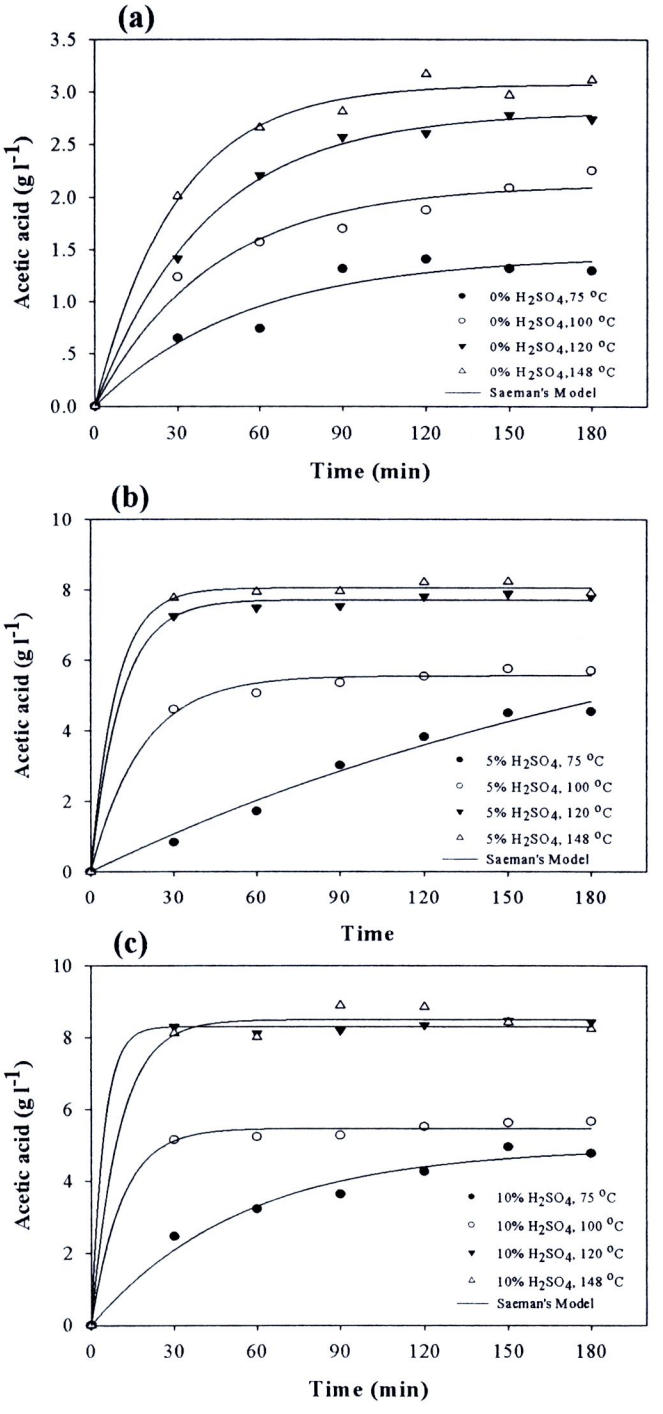

Figure 29. Experimental (dot) and predicted (line) concentrations of acetic acid generated in dPPF hydrolysate at: (a) autohydrolysis (0% sulfuric acid) at various reaction temperature of 75-148°C, (b) 5% sulfuric acid hydrolysis, and (c) 10% sulfuric acid hydrolysis with the same reaction temperature.

Table 26. Kinetics and statistical parameters of xylose, glucose, furfural and acetic acid released during dilute sulfuric acid hydrolysis of delignified palm pressed fiber (dPPF) at various reaction temperatures.

	0% H_2SO_4				5% H_2SO_4				10%H_2SO_4			
	75°C	100°C	120°C	148°C	75°C	100°C	120°C	148°C	75°C	100°C	120°C	148°C
Xylose												
k_1 (min^{-1})	0.0033	0.0031	0.0030	0.0030	0.0042	0.0128	0.0821	0.0293	0.0085	0.0102	0.0922	0.0277
k_2 (min^{-1})	0.0031	0.0276	0.0228	0.0063	0.0042	0.0044	0.0310	0.0052	0.0087	0.0065	0.0510	0.0064
R^2	0.96	0.96	0.95	0.98	0.94	0.97	0.98	0.95	0.96	0.97	0.94	0.92
Glucose												
k_3 (min^{-1})	0.0051	0.0004	0.0100	0.0128	0.0019	0.0064	0.0297	0.0212	0.0114	0.0189	0.0582	0.0328
G_0	1.32	1.70	1.35	1.65	4.45	4.87	3.83	5.45	4.54	4.23	4.18	5.84
R^2	0.91	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Furfural												
k_4 (min^{-1})	0.1141	0.0954	0.0286	0.0794	0.0189	0.0341	0.0717	0.1612	0.0794	0.1312	0.1806	0.3706
F_0	0.33	0.44	0.52	0.57	0.91	0.96	1.03	0.97	0.98	0.99	0.98	0.01
R^2	0.99	0.99	0.99	0.99	0.97	0.99	0.99	0.97	0.99	0.99	0.99	0.98
Acetic acid												
k_5 (min^{-1})	0.0180	0.0234	0.0247	0.0342	0.0041	0.0546	0.0918	0.1108	0.0185	0.0919	0.2213	0.0998
A_0	1.44	2.12	2.81	3.07	9.18	5.56	7.72	8.05	4.94	5.47	8.31	8.50
R^2	0.97	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

k_1 is the rate of xylose production (min^{-1}), k_2 is the rate of xylose decomposed (min^{-1}), k_3 is the rate of glucose production (min^{-1}), k_4 is the furfural production rate (min^{-1}), and k_5 is the acetic acid production rate (min^{-1})
G_0 is the potential glucose concentration, F_0 is the potential furfural concentration, and A_0 is the potential acetic acid concentration

Table 27. Generalized models for kinetic parameters prediction of delignified palm pressed fiber (dPPF) hydrolysis with dilute sulfuric acid at 120°C.

Products	Models	R^2
Xylose	$k_1 = 0.063C_a^{0.1660}$ (30)	0.99
	$k_2 = 0.0092C_a^{0.7370}$ (31)	0.99
Glucose	$k_3 = 0.0065C_a^{0.9511}$ (32)	0.99
Furfural	$k_4 = 0.0173C_a^{1.0011}$ (33)	0.95
Acetic acid	$k_5 = 0.0214C_a^{1.0017}$ (34)	0.98

The generalized model for prediction of xylose production rate is calculated by Eq. (30) and represented by empirical Eq. (18) where k_1 is represented with acid concentration (C_a). Similarly, xylose degradation rate (k_2) is represented by empirical Eq. (31). The determination coefficients R^2 for both parameters were in good agreement which is shown in Table 27. Combination of Eq. (30) and (31) with the model of xylose production and degradation, it is possible to predict xylose concentration at any time and acid concentration within the time period (0-180 min) and acid concentration (0-10%). The generalized model predicted that maximum xylose concentration of more than 25 g/l could be achieved by treated dPPF with 5% sulfuric acid at reaction temperature of 120°C for 30 min. The dependence of xylose concentration with various acid concentrations and internal range of time at fixed reaction temperature of 120°C are shown by response surface in Fig. 30(a). Therefore, reaction time should be 30 min to obtain the maximum release of xylose with the minimum decomposition products in the hydrolysate.

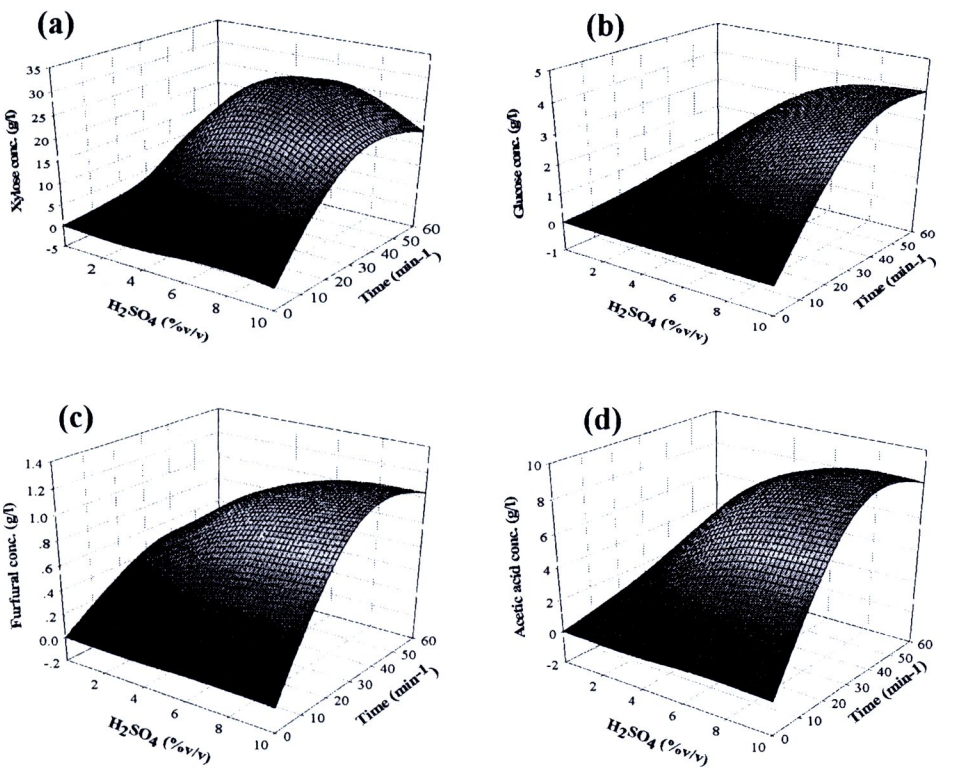

Figure 30. Effect of sulfuric acid concentration and reaction time on generalized model for prediction of; (a) xylose production, (b) glucose production, (c) furfural formation and (d) acetic acid release.

3.7.1.2 Kinetic model of glucose production

Glucose is a secondary product in many hydrolysis of biomasses, i.e. oil palm empty fruit bunch (OPEFB) (Rahman *et al.*, 2006), sorghum straw (Téllez-Luis *et al.*, 2002) and dPPF as well. It is difficult to determine how much glucose released either from cellulose or hemicellulose (Téllez-Luis *et al.*, 2002). In the model of glucose production (Eq. 13), G_0 and kinetic parameter k_3 were obtained by regression. The determination coefficient (R^2) was very high agreement with experimental and predicted data. The G_0 (potential glucose concentration) were in the range of 1.32-5.84 g/l, corresponding to 3.14-13.90% of total cellulose in dPPF (see Appendix F). The values of k_3 were obtained in the range of 0.0004-0.0582 min^{-1} (Table 26). The values of k_3 increased with the increase in both acid concentration and reaction temperature. Therefore, the model for prediction of glucose production was developed correlated with acid concentration, reaction temperature and kinetic

parameter k_3. The value of k_3 was lower than that of corresponding value of k_1 (xylose production) because the structure of hemicellulose is amorphous which is easier hydrolysis than crystalline of cellulose (Rahman *et al.*, 2006).

The hydrolysis of cellulose is dependent on degree of crystallinity (Alvira *et al.*, 2010) and swelling state of cellulose (Rahman *et al.*, 2006). Experimental and predicted data of glucose production in the resulting dPPF hydrolysate is given in Fig. 27. Therefore, the combination of Eq. (32) and glucose production model was feasible to predict release of glucose within the experimental ranges. The highest glucose production predicted by the models was 5.84 g/l at 10%v/v (0.1 M) sulfuric acid concentration, 148°C for 180 min. The k_3 is presented by empirical Eq. (32). The determinant coefficient R^2 was well fitted which was given in Table 27. The response surface plot of the generalized model for glucose production with increase in acid concentration and reaction time is shown in Fig. 35(b). From the response surface plot, there was no decomposition reaction occurred during the hydrolysis process because the degradation of glucose to HMF requires high temperature (180-230°C) and pressure (1.5-2.0 MPa) (Karimi *et al.*, 2006). To obtain maximum xylose and glucose concentration in this hydrolysate, it is necessary to keep the reaction time at 30 min where glucose concentration is very low.

3.7.1.3 Kinetic model of furfural formation

Furfural is the principle degradation product of xylose in the acid hydrolysis of dPPF. Kinetic and statistical parameters for furfural are given in Table 26. The values of F_0 (potential furfural concentration) and kinetic parameter k_4 were within the range of 0.01-1.03 g/l and 0.0286-0.3706 min^{-1}, respectively. The determinant coefficients R^2 were well fitted with furfural formation model. The experimental and predicted data is shown in Fig. 28. The k_4 was increased with the increase in both sulfuric acid concentration and reaction temperature. A generalized model of furfural production was modified to correlate k_4 with acid concentration and reaction temperature for prediction of furfural concentration at any acid concentration, reaction temperature and time within the operating range (Rahman *et al.*, 2006).

The empirical Eq. (33) represents the generalized model which is shown in Table 27. The response surface graph of generalized model for furfural

formation is given in Fig. 30c. From this response surface graph, furfural production increased with increase in acid concentration and reaction time. Furfural is well known as an inhibitory compound to fermentation process and thus its concentration must be minimized to facilitate optimum use of dPPF hydrolysate for ethanol (Sun and Cheng, 2002) and xyliyol productions (Rahman *et al.*, 2006). Therefore, resulted from response surface graphs of xylose (Fig. 30a) and furfural (Fig. 30c) production, the hydrolysis process for the highest xylose production and the lowest furfural formation should be conducted with higher acid concentration and lower reaction time. In this study, the generalized model of furfural formation can facilitate to predict concentration of furfural. Hence, the selection of acid concentration and reaction time can be carried out in order to obtain the maximum xylose concentration in the dPPF hydrolysate while keeping concentration of furfural at minimum level.

3.7.1.4 Kinetic model of acetic acid production

Acetic acid is principle product released from acetyl group degradations of hemicellulose in acid hydrolysis of dPPF and other lignocellulosic materials (Rahman *et al.*, 2006; Herrera *et al.*, 2003; Téllez-Luis *et al.*, 2002; Garrote *et al.*, 2001). Kinetic and statistical parameters for acetic acid are given in Table 26. The values of A_0 (potential acetic acid concentration) and kinetic parameter k_5 were within the range of 1.44-9.18 g/l and 0.0041-0.2213 min^{-1}, respectively. The determinant coefficients R^2 were well fitted with acetic acid production model. The experimental and predicted data is shown in Fig. 29. The value of kinetic parameter k_5 was increased with increase in acid concentration and reaction temperature. A generalized model for prediction of acetic acid production was conducted to correlate k_5. This is obtained by Eq. (34) as shown in Table 27. The value of regression parameter n for acetic acid production k_5 was higher than that of regression parameter n for xylose production k_1 in all experiments (Table 26). These phenomenon shows that the effect of acid on acetyl removal from hemicellulose was higher compared to that of effect of acid on xylose generation. In another word, xylose was easily produced from dPPF observed by the degradation of acetyl group of hemicellulose. However, the value of regression parameter n for acetic acid production k_5 obtained in some studies was lower than that of regression parameter n for xylose production k_1

(Rahman *et al.*, 2006). There was a big different thing between this work and Rahman *et al.* (2006) work which is raw material pretreatment, delignification process. Due to the lignin removal from the surface of PPF, the effect of acid on acetyl groups degradation was increased which was the reason for obtaining the higher value of k_5.

The response surface plot of generalized model for acetic acid released in the dPPF hydrolysis is shown in Fig. 30d. It was observed that acetic acid concentration was increased with increase in sulfuric acid concentration and reaction time which is similar to the experiments of Rahman *et al.* (2006) and Téllez-Luis *et al.* (2002). Therefore, to maximize xylose concentration in the resulting of dPPF hydrolysate should be conducted the experiments at high sulfuric acid concentration and low reaction time for keeping the concentration of acetic acid at minimum level.

3.7.1.5 Comparison of xylose production rate with other lignocellulosic materials under acid hydrolysis

Xylose production rate (k_1) would be higher resulted from not only acid concentration, but also type of acid. Table 28 clearly shows that a higher k_1 value was obtained from 6% sulfuric acid (0.0798 min^{-1}) than 6% hydrochloric acid (0.0333 min^{-1}). Moreover, sulfuric acid gave lower xylose decomposition (k_2) and acetic acid generation (k_5). However, a higher furfural formation (k_4) would be obtained from sulfuric acid than hydrochloric acid.

Pretreatment process, delignification, might increase furfural and acetic acid production. When compared the k_5 value between dPPF (0.0918 min^{-1}) and OPEFB (0.0189 min^{-1}), a higher k_5 value was obtained from dPPF because of lower content of lignin resulted to increase acid hydrolysis on hemicellulose (Table 28).

Table 28. Comparison of xylose production rate (k_1), xylose decomposition rate (k_2), glucose production rate (k_3), furfural formation rate (k_4), and acetic acid generation rate (k_5) on various lignocellulosic materials by acid hydrolysis.

Sample	Conditions	k_1 (min^{-1})	k_2 (min^{-1})	k_3 (min^{-1})	k_4 (min^{-1})	k_5 (min^{-1})	References
Sorghum straw	6% HCl, 122°C for 70 min	0.0333	0.0047	0.0204	0.0047	0.1992	Herrera *et al.*, 2003
Sorghum straw	6% H_2SO_4, 100°C for 60 min	0.0798	0.0001	0.0626	0.0129	0.0971	Téllez-Luis *et al.*, 2002
OPEFB*	6% H_2SO_4, 120°C for 15 min	0.1695	0.0057	0.0518	0.0118	0.0189	Rahman *et al.*, 2006
dPPF	5% H_2SO_4, 120°C for 30 min	0.0821	0.0310	0.0297	0.0717	0.0918	This study

* Oil palm empty fruit bunch

3.7.2 Comparison of dPPF hydrolysate and PPF hydrolysate

In order to reduce a cost of raw material pretreatment, xylose yields from both dPPF and PPF were compared. If the xylose concentrations obtained from above materials were not significantly difference, PPF would be selected and used as a substrate for producing xylose. However, amounts of by-products (acetic acid and furfural) have to be considered as well. The experiments were designed by conventional method. Reaction times were controlled at 0, 30 and 60 min. Sulfuric acid was diluted in the range of 1-6% (v/v). The ratio of solid and liquid (PPF or dPPF and diluted sulfuric acid) and reaction temperature were fixed at 1:10 (w/v) and 120°C, respectively. Results are shown in Fig. 31.

For xylose production, the optimal condition for xylose production from both PPF and dPPF was 2% H_2SO_4 and 30 min reaction time at 120°C giving the highest xylose concentration of 27.23 g/l and 28.7 g/l, respectively (Fig. 31a) with the percentage of xylose extraction of 92.78% and 80.23%, respectively. The concentration of sulfuric acid had significantly effected on xylose production ($P \leq 0.05$), but there are no significantly differences between sample (PPF and dPPF), and reaction time (30 min and 60 min) ($P > 0.05$) as shown in Fig. 31a. However, the optimal condition gave the glucose concentration of 2-4 g/l (Fig. 31b).

Analysis of acetic acid, the selected condition of PPF and dPPF hydrolysis gave acetic acid concentrations of 5.99 g/l and 10.56 g/l, respectively, (Table 29 and Fig. 32a). For furfural analysis, the optimal condition of PPF and dPPF hydrolysis also gave furfural concentrations of 0.42 g/l and 0.55 g/l, respectively.

Therefore, PPF was more suitable material than dPPF (Table 29) due to (i): giving the same xylose yield (Fig. 31a); (ii): lower of acetic acid and furfural (Fig. 32a and 32b); (iii): reducing the cost in delignification process and also decreasing environmental emission from the wastes of delignification process (ClO_2 gas and wastewater) (Collings *et al.*, 1978).

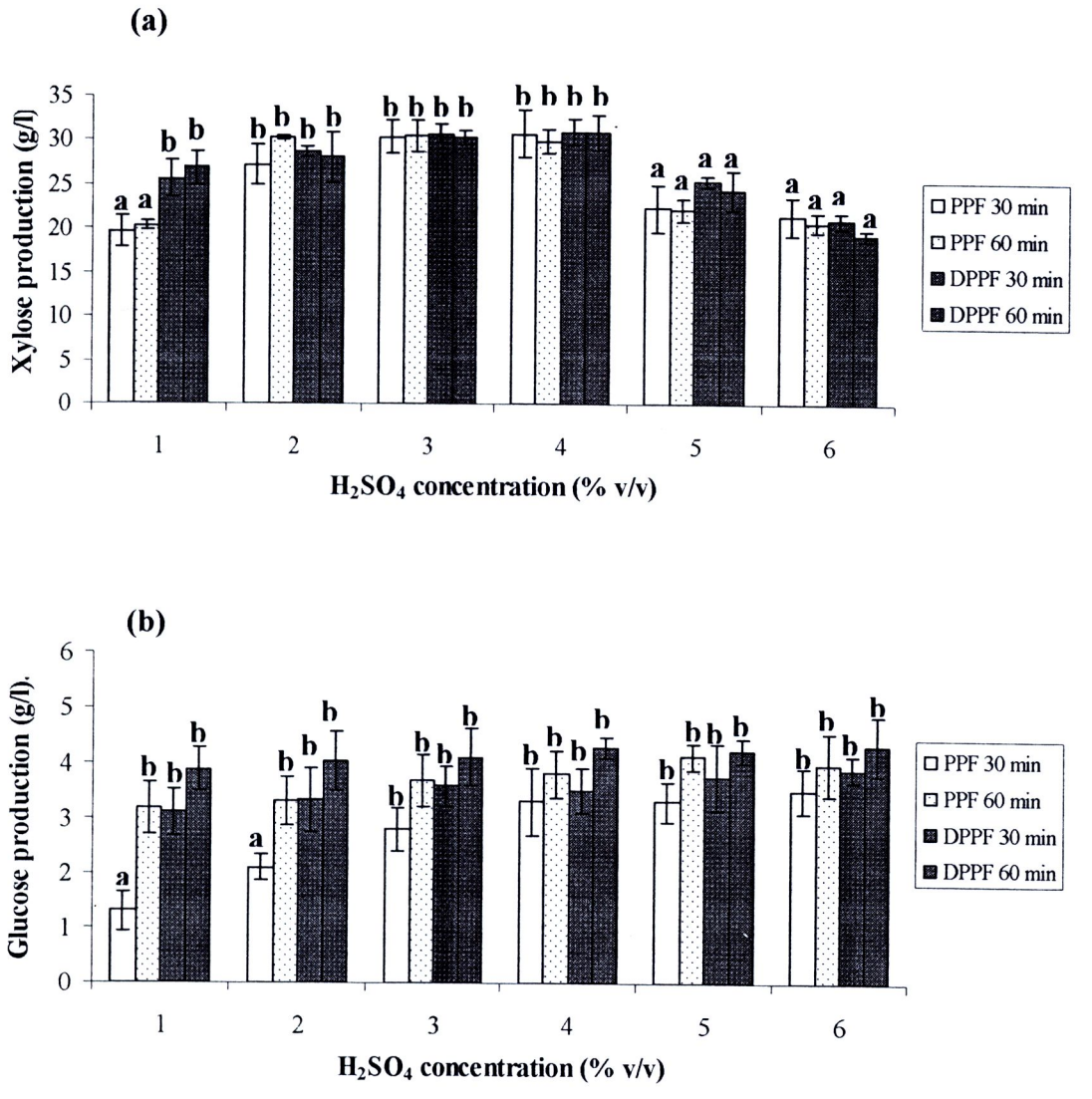

Figure 31. Comparative studies of xylose (a), and glucose productions (b) from PPF and dPPF using various diluted sulfuric acid at 30 and 60 min.

a, b are significantly differences of sugars concentration at $\alpha = 0.05$

Table 29. Comparison of reducing sugars and by-products formation during various acid hydrolysis conditions from lignocellulosic materials.

Raw materials	Conditions	SLR* (g:g)	Products yields				References
			Xylose (g/l)	Glucose (g/l)	Furfural (g/l)	Acetic acid (g/l)	
1. Oil palm empty fruit bunch	H_2SO_4 4%, 115 °C, 60 min	1:8	30.81	2.2	0.8	2.0	Rahman, *et al.*, 2007
2. Oil palm empty fruit bunch	H_2SO_4 6%, 120 °C, 15 min	1:8	29.4	2.34	0.87	1.25	Rahman, *et al.*, 2006
3. Sorghum straw	HCl 6%, 122 °C, 70 min	1:10	16.2	3.8	2.0	1.9	Herrera, *et al.*, 2003
4. Sorghum straw	H_2SO_4 6%, 100 °C, 60 min	1:10	18.27	6.78	0.7	1.35	Téllez-Luis, *et al.*, 2002
5. Cardboard	H_2SO_4 3%, 130 °C, 180 min	1:10	10.7	9.2	nd	nd	Yáñez, *et al.*, 2004
6. DPPF	H_2SO_4 5%, 120 °C, 30 min	1:10	26.67	2.2	0.65	7.25	This study
7. DPPF	H_2SO_4 2%, 120 °C, 30 min	1:10	28.70	3.5	0.55	10.56	This study
7. PPF	H_2SO_4 2%, 120 °C, 30 min	1:10	27.23	2.3	0.42	5.99	This study

* SLR: Solid-Liquid ratio

The xylose yields obtained from PPF (27.23 g/l) and dPPF (26.67-28.70 g/l) were similar with oil palm empty fruit bunch (OPEFB) (29.40-30.81 g/l) (Table 29). However, there were differences when compared to sorghum straw and cardboard because of the different composition of each material.

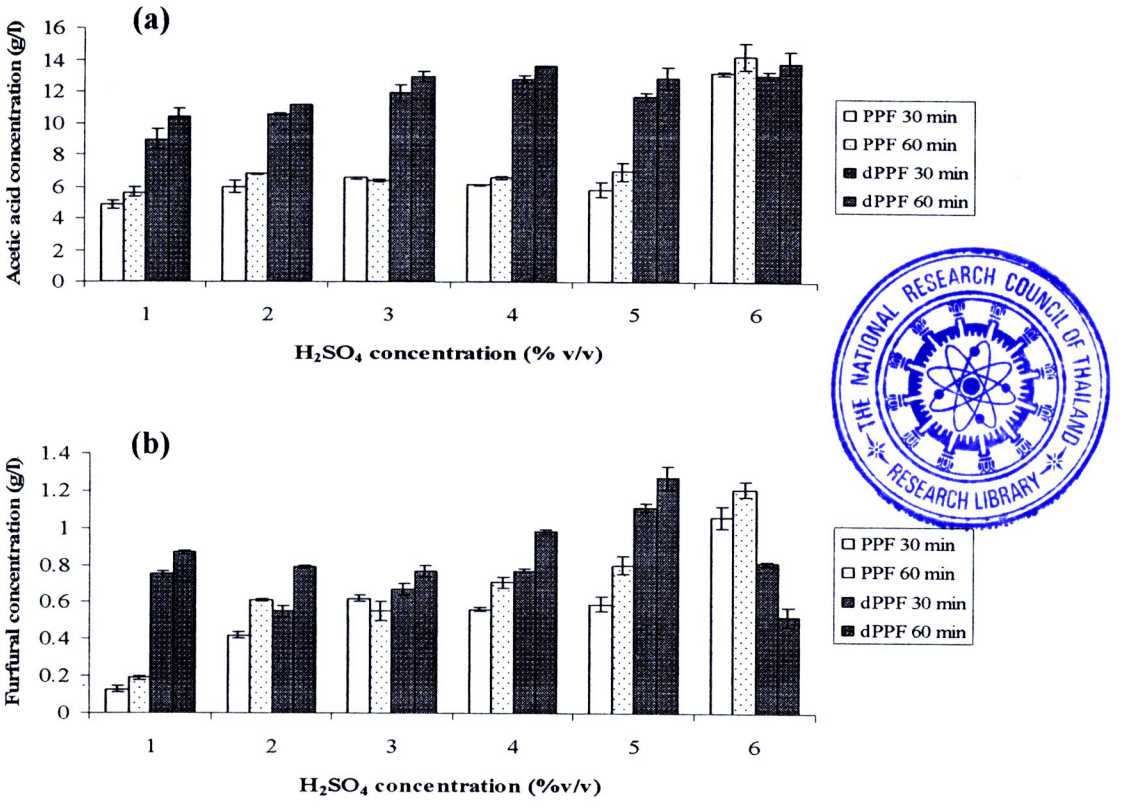

Figure 32. Comparative studies of acetic acid (a) and furfural productions (b) from PPF and dPPF using various diluted sulfuric acid.

3.8 Ethanol production in synthetic glucose or/and xylose medium by *Candida shehatae* TISTR5843

3.8.1 Effect of glucose concentration

Candida shehatae TISTR5843 is effective ethanolic producing yeast that is able to use directly glucose, xylose or glucose and xylose as substrates to produce ethanol. *Candida* species are known as sugar-tolerant yeasts (Petrovska *et al.*, 2000). In order to prevent substrate inhibition, the substrate concentrations were studied and ranged of 4, 7, 12, 24, 45 and 75 g/l, which usually found in cellulosic hydrolysates, and cultured for 96 hours at 30°C on the rotary shaker (180 rpm). The

considerate parameter for ethanol production is ethanol yield, which is a measurement of how much substrate is converted into ethanol. It is well known that 0.51 g ethanol is produced from 1 g glucose. However, the carbon flow in cells is also used for biomass production. Therefore, the theoretical ethanol yield is approximately 0.46-0.48 g ethanol/g glucose (Kopsahelis *et al.*, 2007).

Glucose concentrations at 4, 7, 12, 24 and 45 g/l were consumed closely to zero by *C. shehatae* TISTR5843 within 12, 18, 24, 30 and 48 h (Fig. 33b), respectively, giving ethanol yields of 0.31, 0.37, 0.46, 0.45 and 0.37 g ethanol/g glucose and productivities of 0.054, 0.106, 0.222, 0.343 and 0.314 g ethanol/l/h, respectively. However, the glucose concentration of 75 g/l, glucose has been consumed to 22.19 g/l at 48 h cultivation giving ethanol yield and productivity of 0.24 g ethanol/g glucose and 0.290 g ethanol/l/h, respectively. It is demonstrated that the glucose concentrations of 12-24 g/l were suitable for ethanol production due to giving the highest ethanol yield (0.45-0.46 g/g) and productivity (0.343 g ethanol/l/h). However, the ethanol yield and productivity at the glucose concentration of 75 g/l were lower than those of glucose concentration of 45 g/l (Fig. 33a) because of substrate inhibition at the initial fermentation observing from the quirt longer lag phase and product inhibition at the cultivation of 48 h observing from the residual glucose of 22.19 g/l (Fig. 33a).

Acetate concentration of 5, 10 and 15 g/l decreased cell growth and ethanol production of *C. shehatae* ATCC22984 in the range of 5-20% and 20-40%, respectively (Delgenes *et al.*, 1996). However, acetate concentration of 1.0 g/l presented at the initial cultivation might be from some of glucose degradation (Qian *et al.*, 2005). During fermentation, the pH of cultured broth decreased from 5.0 to 3.5-4.5 because of many acid by-products production as well as carbon dioxide (CO_2) generation during yeast cell growth. The pH affects on alcohol dehydrogenase (ADH) activity (Nie *et al.*, 2007). The optimal pH of ADH in this study was 4.5 at 12-18 h (Fig. 33a) (starting point of ethanol production) and Fig. 34b (pH changing to 4.5), which gave the maximum ethanol production (Fig. 33a). This result is similar to those results of Nie et al (2007), who reported the optimal pH of ADH by *Candida parapsilosis* was 4.5. However, the pH was dropped to 3-4 and changed back to closely pH 4.5 after 18 h cultivation time in the glucose concentrations of 4, 7, 12 and

23 g/l (Fig. 34b). After cultivation, the pH of broth at glucose concentrations of 45 and 75 g/l was 3.2 and 2.8, respectively. Cell growths of all glucose concentration experiments were in the stationary phase within 42-48 h (Fig. 34a) with the maximum DCW of 3.46-20.21 g/l.

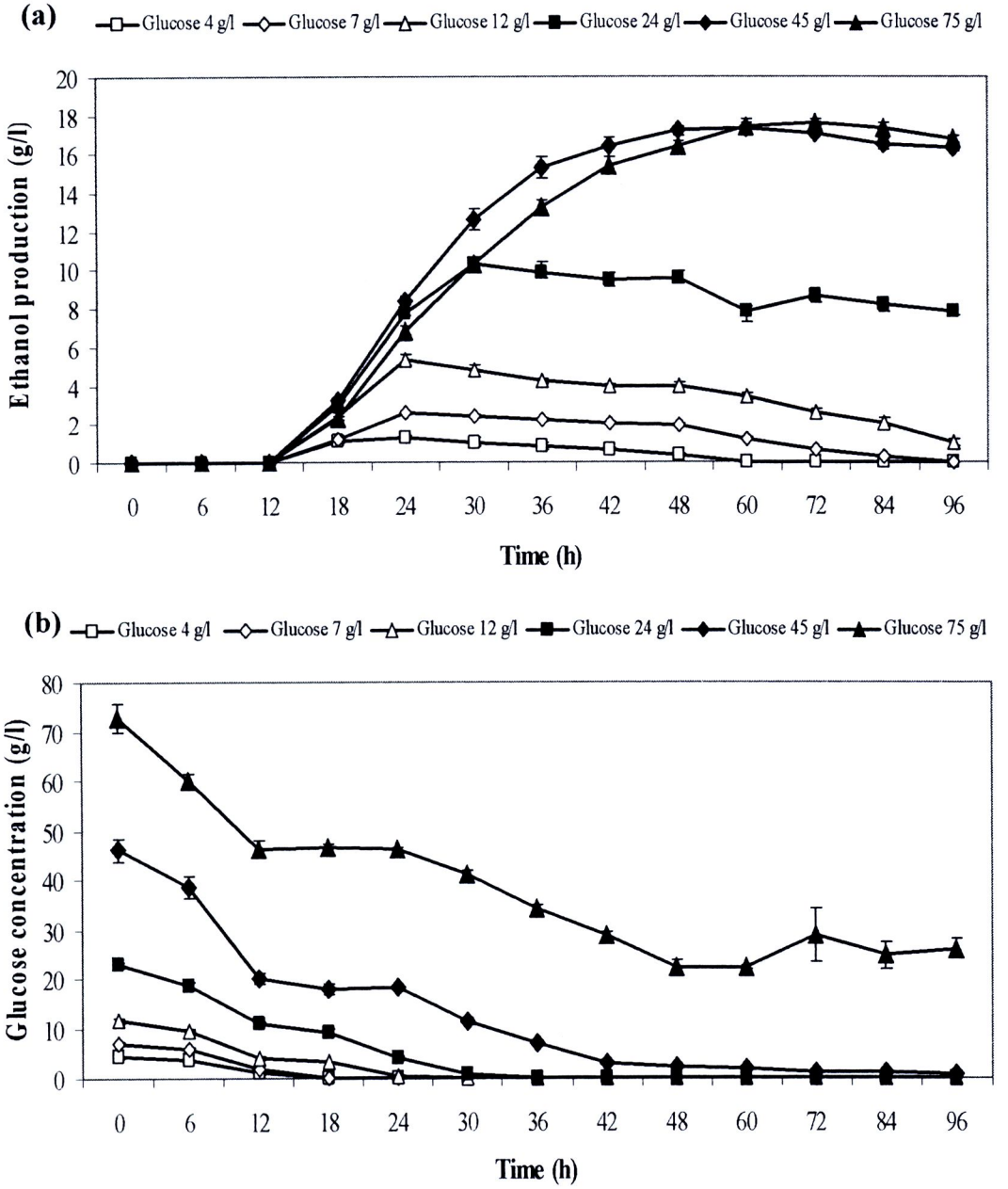

Figure 33. Time course of ethanol production (a) and glucose consumption (b) by *Candida shehatae* TISTR5843 in various glucose concentrations at 180 rpm, room temperature (28-30°C).

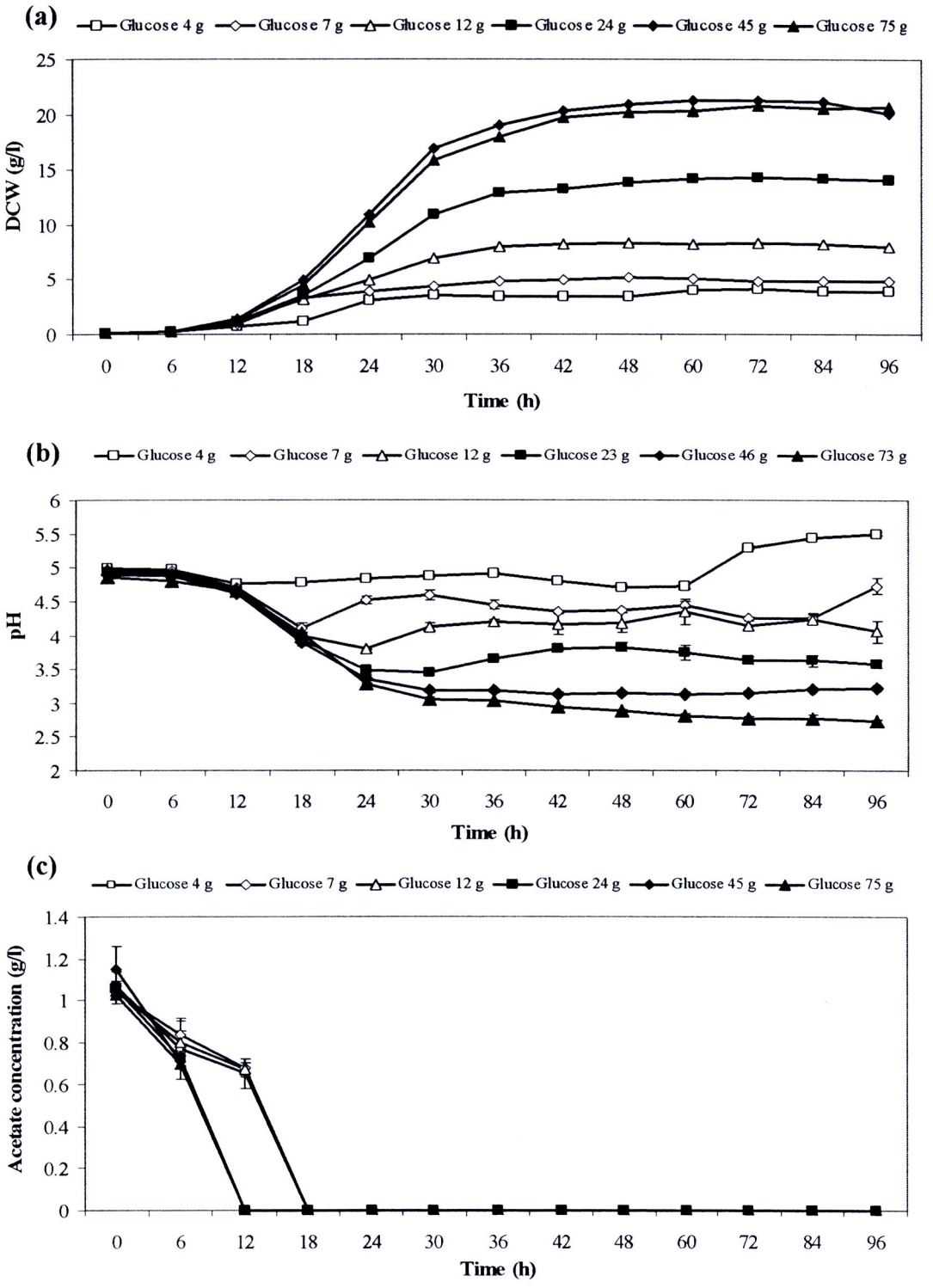

Figure 34. Time course of DCW (a), pH changes (b) and acetate residuals (c) during ethanol production by *Candida shehatae* TISTR5843 in various glucose concentrations at 180 rpm, room temperature (28-30°C).

3.8.2 Effect of xylose concentration

Effect of xylose concentrations on ethanol production was studied at 4, 8, 20, 40, 60 and 90 g/l and cultured with shaking of 180 rpm at room temperature (28-30°C) for 96 h without any furfural and acetate supplementation. Xylose concentrations of 4, 8, 20 and 40 g/l were consumed closely to zero by *C. shehatae* TISTR5843 within 18, 24, 36 and 60 h (Fig. 35b), respectively, giving ethanol yields and productivities of 0.33, 0.45, 0.42 and 0.29 g ethanol/g xylose and 0.058, 0.122, 0.194 and 0.186 g ethanol/l/h, respectively. It is demonstrated that, the xylose concentrations of 8 g/l gave the highest ethanol yield while the xylose concentration of 20 g/l gave the highest productivity. The xylose concentrations of 60 and 90 g/l could be also used to produce ethanol (13-14 g/l) as shown in Fig. 35a. However, they gave lower ethanol yield (0.22 and 0.16 g ethanol/g xylose, respectively) and lower productivity (0.138 and 0.146 g ethanol/l/h, respectively) than glucose concentration of 20 g/l because of (i) substrate inhibition at the initial fermentation, (ii) product inhibition at the cultivation time of 72 h observing from the residual glucose of 10 and 40 g/l (Fig. 35b), and (iii) by-products inhibition (Fig. 36a and 36b).

Furfural presented in this study could be produced from xylose degradation under autoclave (120°C for 10 min). Unfortunately, acetate also presented. Acetate was consumed within 18 h cultivation because it is less toxic than furfural (Fig. 36b). Furfural concentration less than 0.5 and 1.0 g/l was transformed completely to another form by using alcohol dehydrogenase-coupled with $NADH+H^+$ for furfuryl alcohol formation, and by using aldehyde dehydrogenase (AIDH)-coupled with NAD^+ for furoic acid formation (Modig *et al.*, 2002; Zhao *et al.*, 2005), within 18 and 84 h cultivation, respectively. While furfural concentration of 2.3 g/l decreased to 1.0 g/l after 84 h cultivation (Fig. 36a). This transformation is called "detoxification" (Keating *et al.*, 2006). The equation of transformation of furfural to furoic acid and furfuryl alcohol was shown in equation (35) and (36), respectively (Modig *et al.*, 2002);

$$Furfural + NAD^+ \xrightarrow{AIDH} furoic\ acid + NADH + H^+ \quad (35)$$

$$Furfural + NADH + H^+ \xrightarrow{ADH} furfuryl\ alcohol + NAD^+ \quad (36)$$

Moreover, the present of furfural inhibits the cell growth and ethanol production. Furfural concentrations of 0.5, 1.0 and 2.0 g/l could decrease the cell growth and ethanol production of *C. shehatae* ATCC22984 in the range of 20-90% (Delgenes *et al.*, 1996). Furthermore, the transformation of furfural to the less toxic compounds is based on amount of air presented during fermentation. Horváth *et al* (2003) reported that under respiratory metabolism of *Saccharomyces cerevisiae* CBS8066 furfural is converted completely to furoic acid whereas furfural is converted to furfuryl alcohol (analyzed in section 3.10) under anaerobic condition.

For the pH change during fermentation, the pH dropped to 3-4 and changed to closely pH 4.5 after 24 h cultivation in the xylose concentrations of 4 and 8 g/l (Fig. 37b) whereas the pH of the xylose concentrations of 20-90 g/l dropped to 2.75-3.5 and no changing to pH 4.5 (Fig. 37b). These phenomena might be from many acid by-products production as well as carbon dioxide (CO_2) generation during yeast cell growth. For determination of DCW during cultivation, cell growth of among xylose concentrations of 4, 8 and 20 g/l was in the stationary phase within 42 h (Fig. 37a), while the stationary phase of cell growth of 40, 60 and 90 g/l xylose was 48 h cultivation, respectively (Fig. 37a).

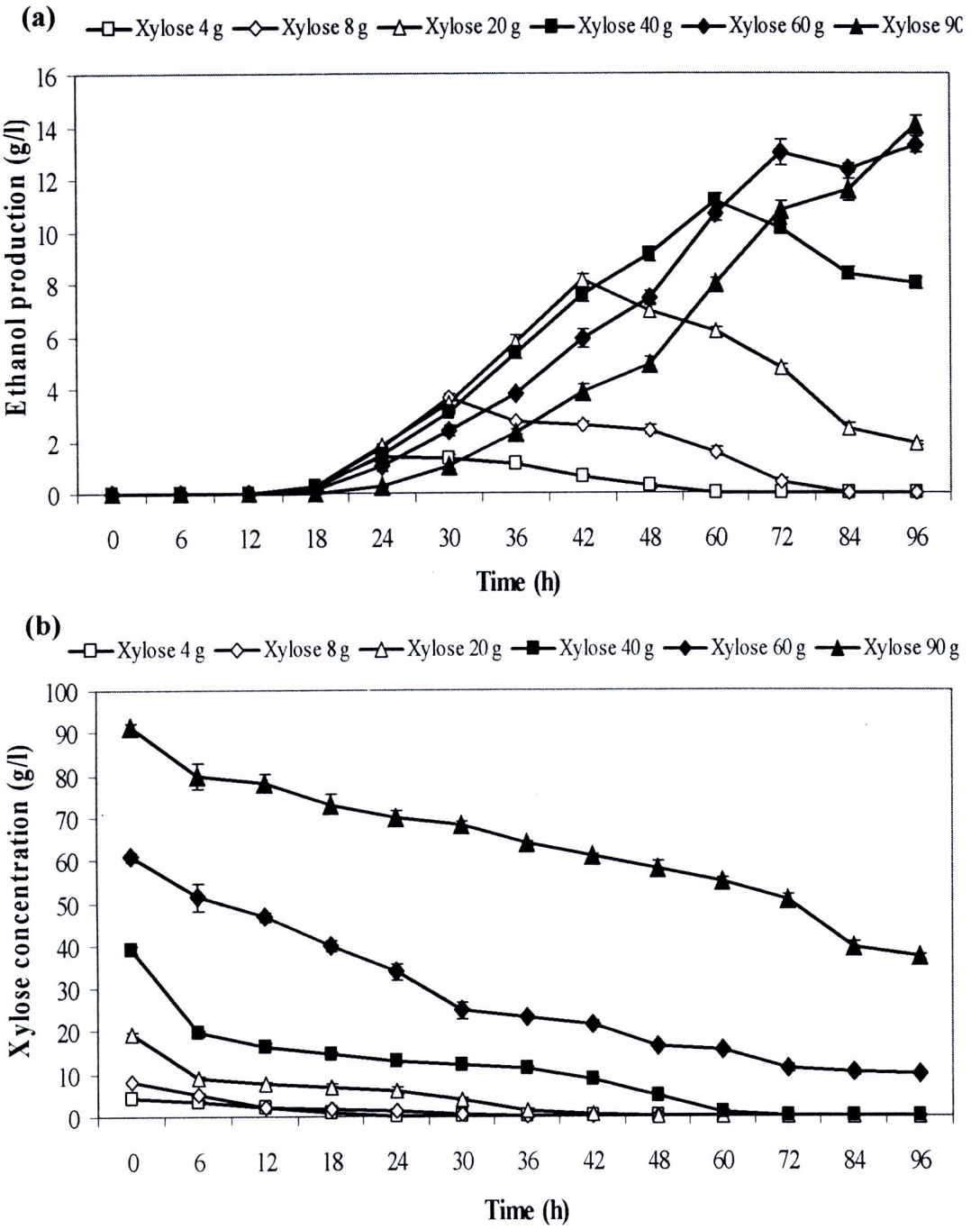

Figure 35. Time course of ethanol production (a) and xylose consumption (b) by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

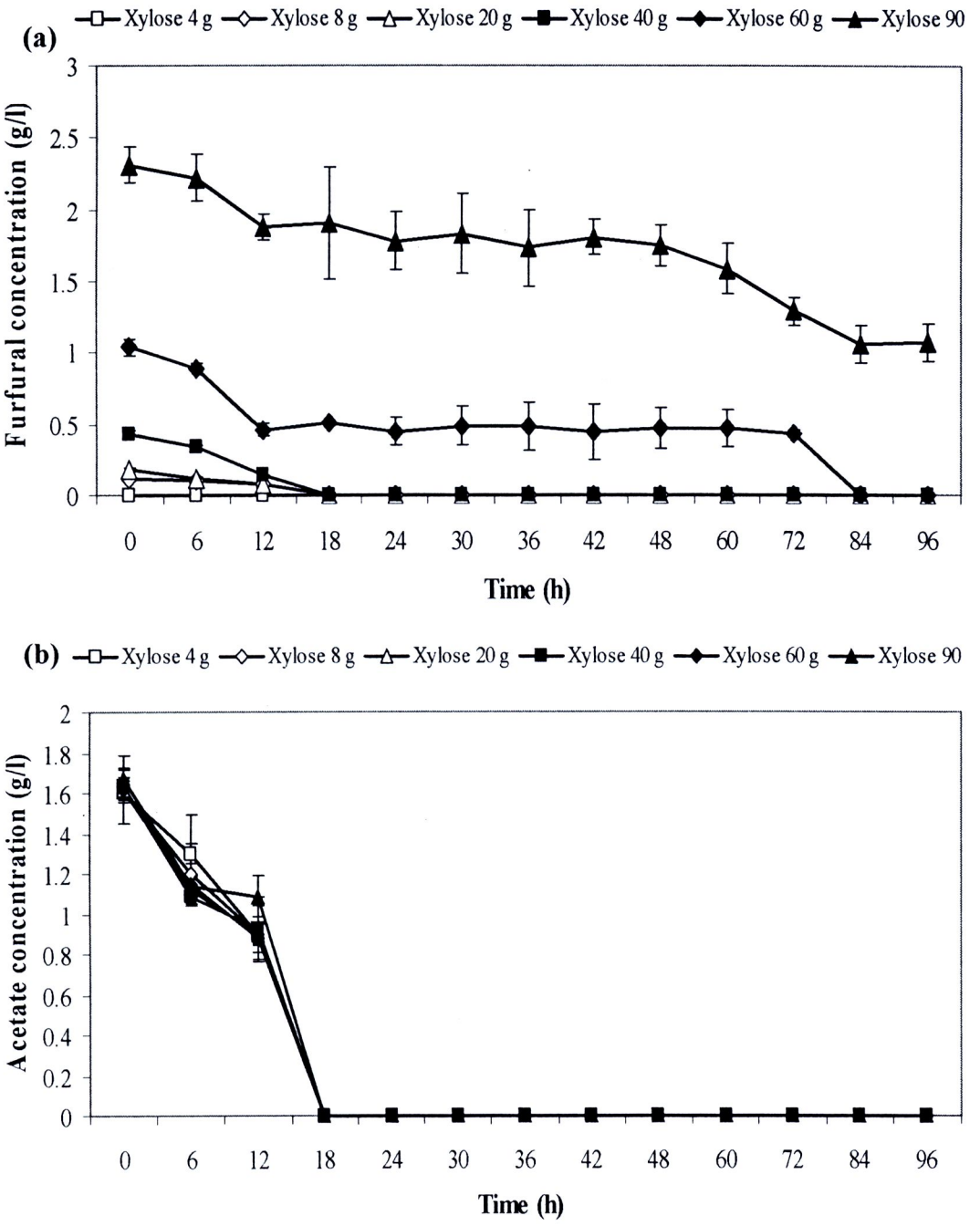

Figure 36. Time course of furfural residuals (a) and acetic acid residuals (b) in ethanol production by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

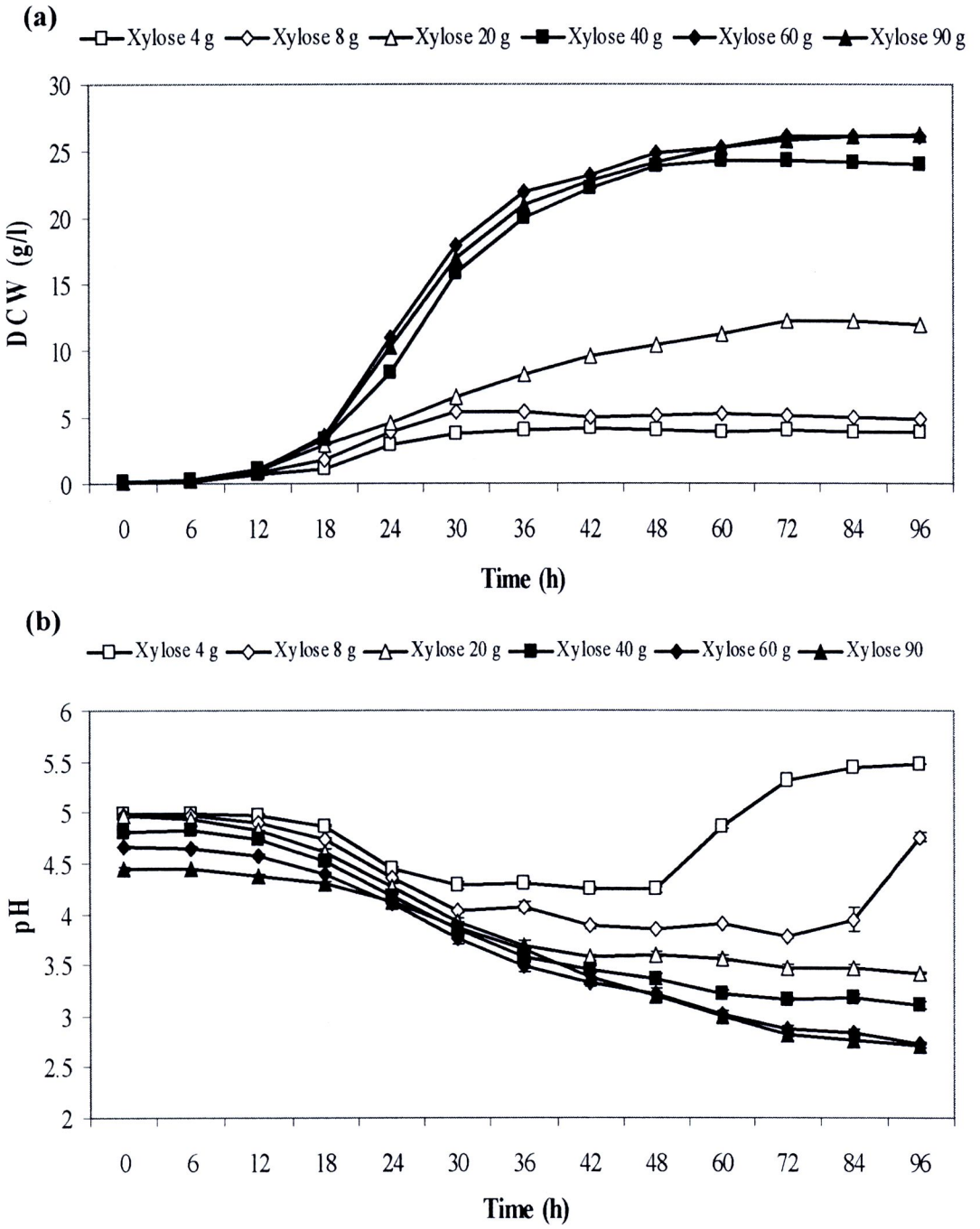

Figure 37. Time course of DCW (a) and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in various xylose concentrations at 180 rpm, room temperature (28-30°C).

3.8.3 Effect of glucose to xylose ratio

C. shehatae TISTR5843 is ethanolic producing yeast that is able to use directly glucose and xylose as substrates to produce ethanol (Delgenes *et al.*, 1996). Mixture of both monomerric sugars is an effective process to generate ethanol. The optimal glucose to xylose ratio, therefore, is a necessary parameter and need to be optimized. In these experiments, various glucose to xylose ratios were ranged from 10:0, 8:2, 6:4, 5:5, 4:6, 2:8 to 0:10 (w/w) (10 g/l total sugars) and cultured for 72 h at 30°C with shaking speed of 180 rpm. Results demonstrated that both sugars at all glucose to xylose ratios were consumed close to zero within 30 h (Fig. 38b), giving ethanol yields of 0.45, 0.43, 0.44, 0.44, 0.43, 0.43 and 0.42 g ethanol/g sugar while the productivities were 0.175, 0.178, 0.178, 0.174, 0.139, 0.111 and 0.109 g ethanol/l/h, respectively. It is illustrated that, the glucose to xylose ratios of 10:0, 8:2, 6:4, 5:5 and 4:6 g/l were the optimum ratios of glucose to xylose because of giving the highest ethanol yields (0.43-0.45 g ethanol/g sugar) and the highest ethanol productivities (0.139-0.178 g/l/h).

The ethanol yields and ethanol productivities decreased when the furfural generated after sterilization increased (Fig. 39a). However, furfural concentrations could be transformed to lower toxic compound within 24 h (Fig. 39a).The highest furfural generation was obtained from the glucose to xylose ratio of 0:10 (w/w). The acetate was consumed by *C. shehatae* TISTR5843 within 30 h cultivation time (Fig. 39b).

The pH of all glucose to xylose ratios dropped to 3.9-4.1 and then changed closely to pH 4.5 after 42 h cultivation. The ethanol production was maximum when pH changed to 4.5 due to its optimum pH for alcohol dehydrogenase (ADH) (Banerjee *et al.*, 1981) (Fig. 40b). However, ethanol concentration of all experiments decreased after 42 h cultivation time because of alcohol dehydrogenaseII (ADH2) (Banerjee *et al.*, 1981). ADH2 is an enzyme that is able to use ethanol as a substrate to covert back to acetyldehyde and then converted to acetate for producing energy and reducing power in the cell under limitation of substrate and higher ethanol concentration in broth (Banerjee *et al.*, 1981). For DCW determination, cell growth of all glucose to xylose ratios would reach stationary phase within 42 h (Fig. 40a).

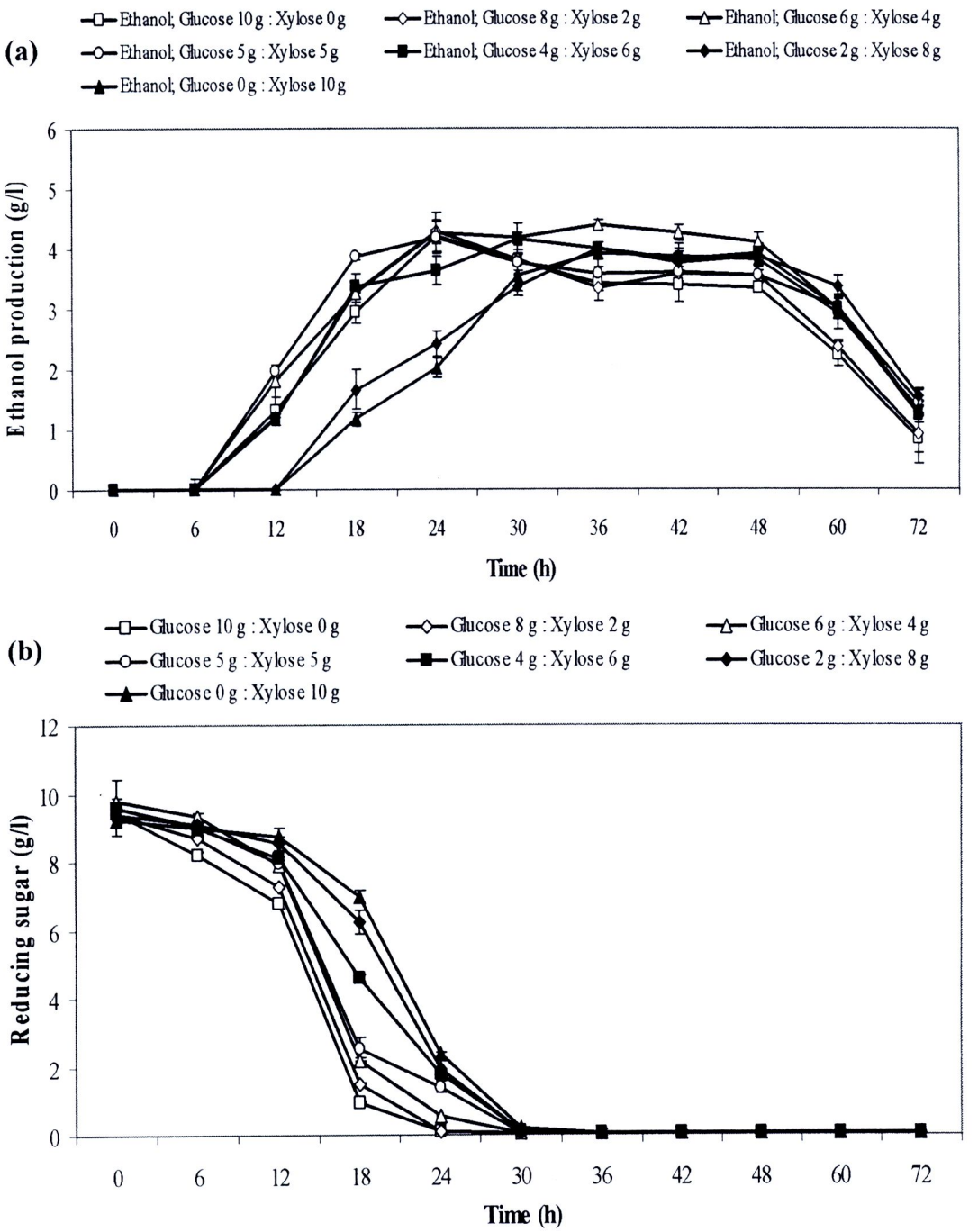

Figure 38. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

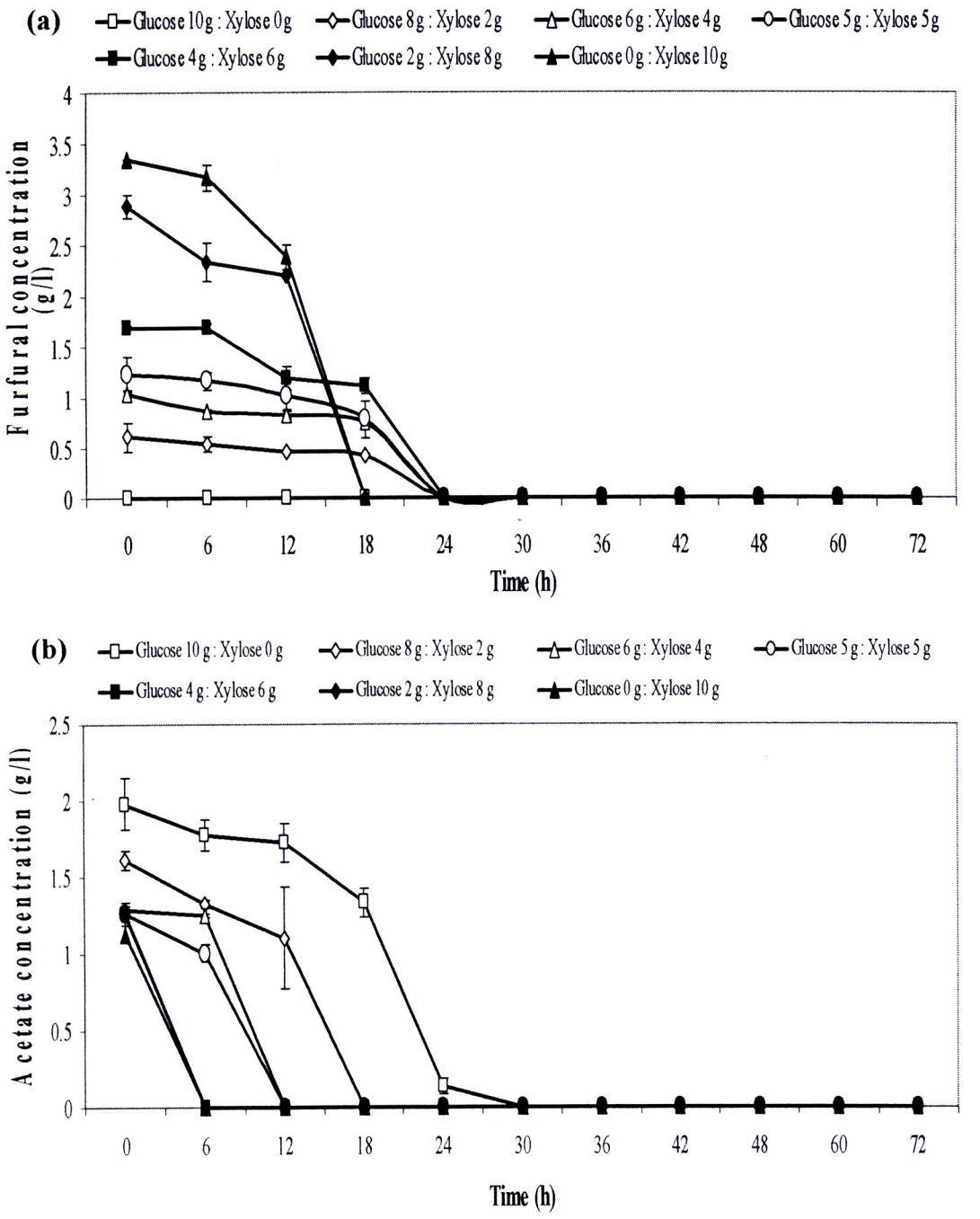

Figure 39. Time course of furfural (a) and acetic acid (b) concentration during ethanol production by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

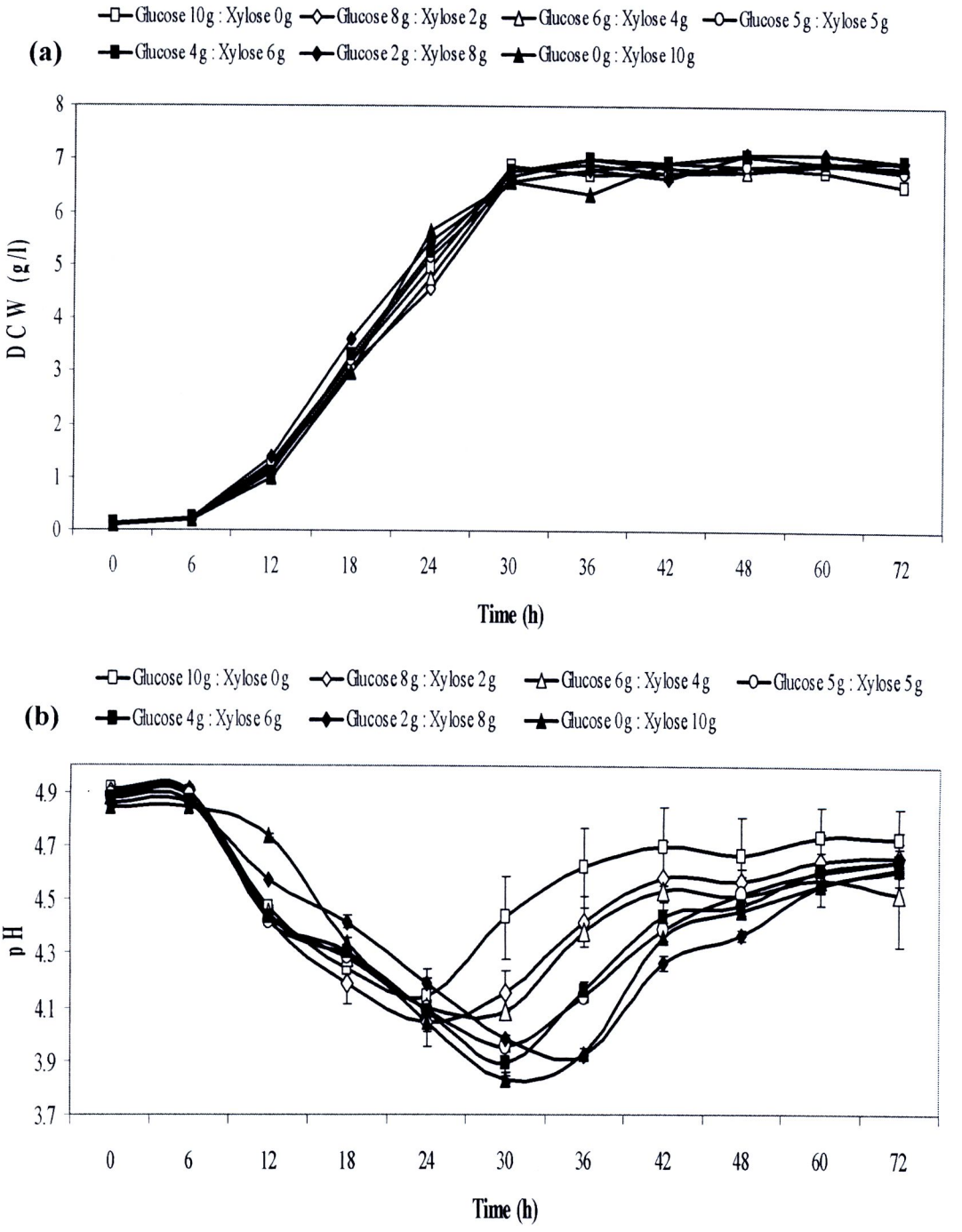

Figure 40. Time course of DCW (a) and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in various glucose to xylose ratios at 180 rpm, room temperature (28-30°C).

3.8.4 Effect of initial pH

pH of the medium plays important role in cell growth and ethanol production. The several of initial pH of medium were ranged of 3.0-6.0. Fermentation was carried out in synthetic medium containing glucose to xylose ratios of 2:8 (w/w) which is the same sugars ratio presented in PPF hydrolysate (Section 3.10). The incubation conditions were 180 rpm at room temperature (30°C) for 72 h.

At initial pH of 3.0, 4.0, 4.5, 5.0 and 6.0, the ethanol yields and ethanol productivities were 0.35, 0.36, 0.44, 0.45 and 0.34 g ethanol/g sugar and 0.052, 0.089, 0.134, 0.136 and 0.081 g/l/h, respectively. Results indicated that initial pH of 4.5-5.0 was optimum for ethanol production and cells growth because it gave the highest ethanol concentration (3.22-3.27 g/l), the highest ethanol yield (0.44-0.45 g ethanol/g sugar), the highest ethanol productivity (0.134-0.136 g/l/h) as shown in Fig. 41a, and as well as the highest DCW (7.01-7.11 g/l) as shown in Fig. 42b. The fermentation of *C. shehatae* FPLY-049 in wood hydrolysate, the maximum ethanol production was found at pH 6.0 with completely fermented within 48 h and had low acetate content (Sreenath and Jeffries, 2000). All sugars in culture medium were consumed almost completely at 48 h cultivation (Fig. 41b).

The initial pH of 4.5 gave the highest and the fastest of cells growth observed from increase of DCW (Fig. 42b). The initial pH of 4.0, 4.5 and 5.0 slightly decreased within 24 h and then slightly increased to nearly pH 4.5 (Fig. 42c). There was no acetate formation whereas 0.16-0.17 g/l furfural was detected and it could be converted to the less toxic compounds such as furfuryl alcohol and furoic acid (described in section 3.8.2) within 30-36 h (Fig. 42a).

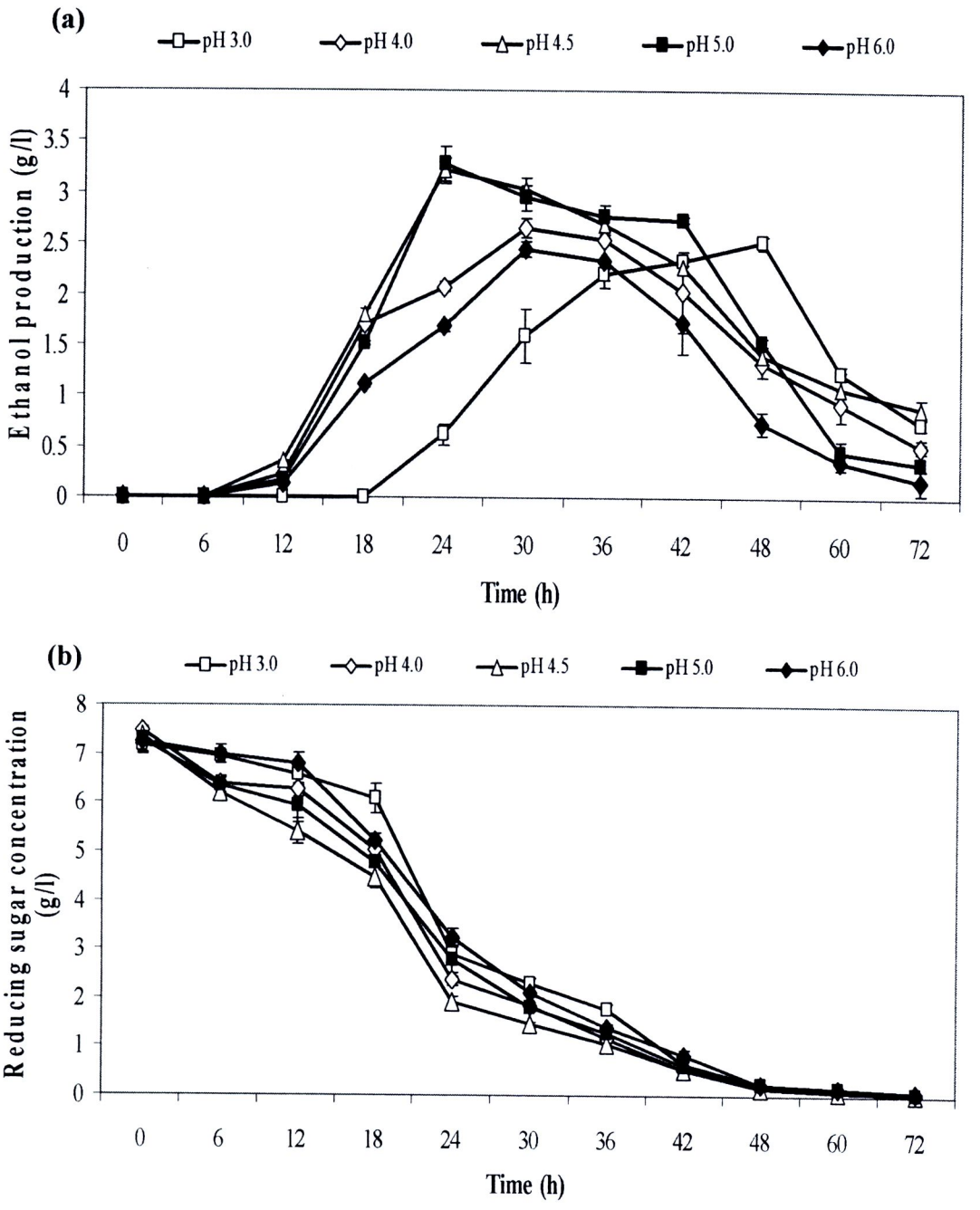

Figure 41. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various initial pH (3.0-6.0) under 2:8 (w/w) of glucose to xylose ratios at 180 rpm, room temperature (30°C).

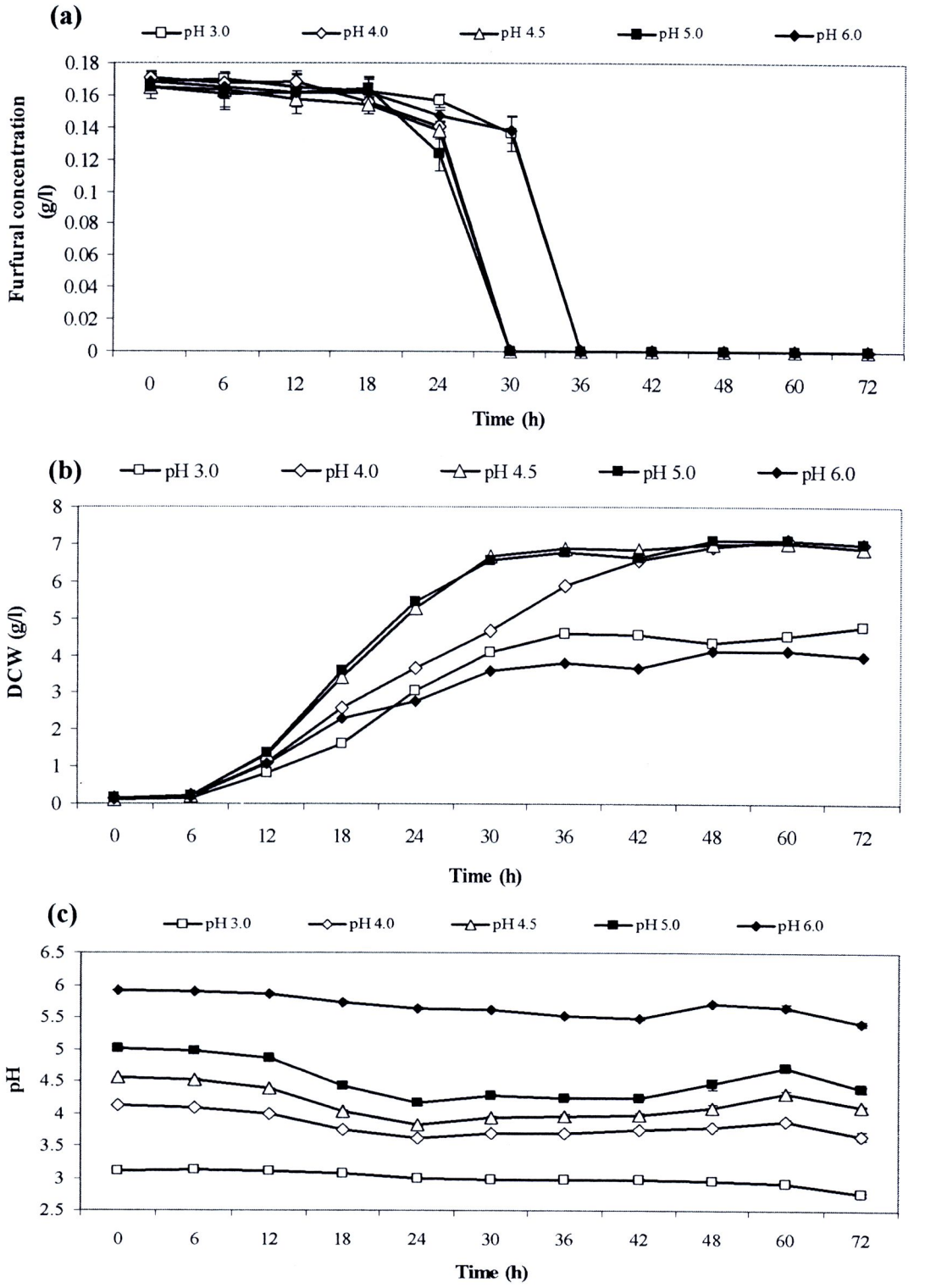

Figure 42. Time course of furfural concentrations (a), DCW (b) and pH changes (c) during ethanol production by *Candida shehatae* TISTR5843 in various initial pH (3.0-6.0) under 2:8 (w/w) of glucose to xylose ratios at 180 rpm, room temperature (30°C).

3.8.5 Effect of temperature

Temperature for cultivating *C. shehatae* TISTR5843 is an important factor concerning metabolism of cells and enzymes activities. At 35°C incubation, no cell growth (Fig. 43b) was detected with reveal to less sugar consumption (Fig. 44b). At room temperature (30±2°C), 3 g/l ethanol was produced at 30 h cultivation (Fig. 43a) giving ethanol yield of 0.42 g ethanol/g substrate and ethanol productivity of 0.103 g/l/h.

Acetate was consumed by *C. shehatae* TISTR5843 within 18 h cultivation (Fig. 44a) and furfural was transformed to lower toxic compound within 24 h (Fig. 44a) at room temperature. No consumptions of acetate and no transformation of furfural were also detected at 35°C (Fig. 44a).

The pH was changed between 4.0-4.5 at the room temperature (Fig. 44b) whereas no pH change was appeared under 35°C due to no growth. In additionally, temperature had no effect on any enzyme level over the range of 20-30°C (Alexander *et al.*, 1988). However, when the temperature is higher than 30°C, enzyme activity reduced. The stationary phase of cells growth at room temperature was 42 h (Fig. 44b).

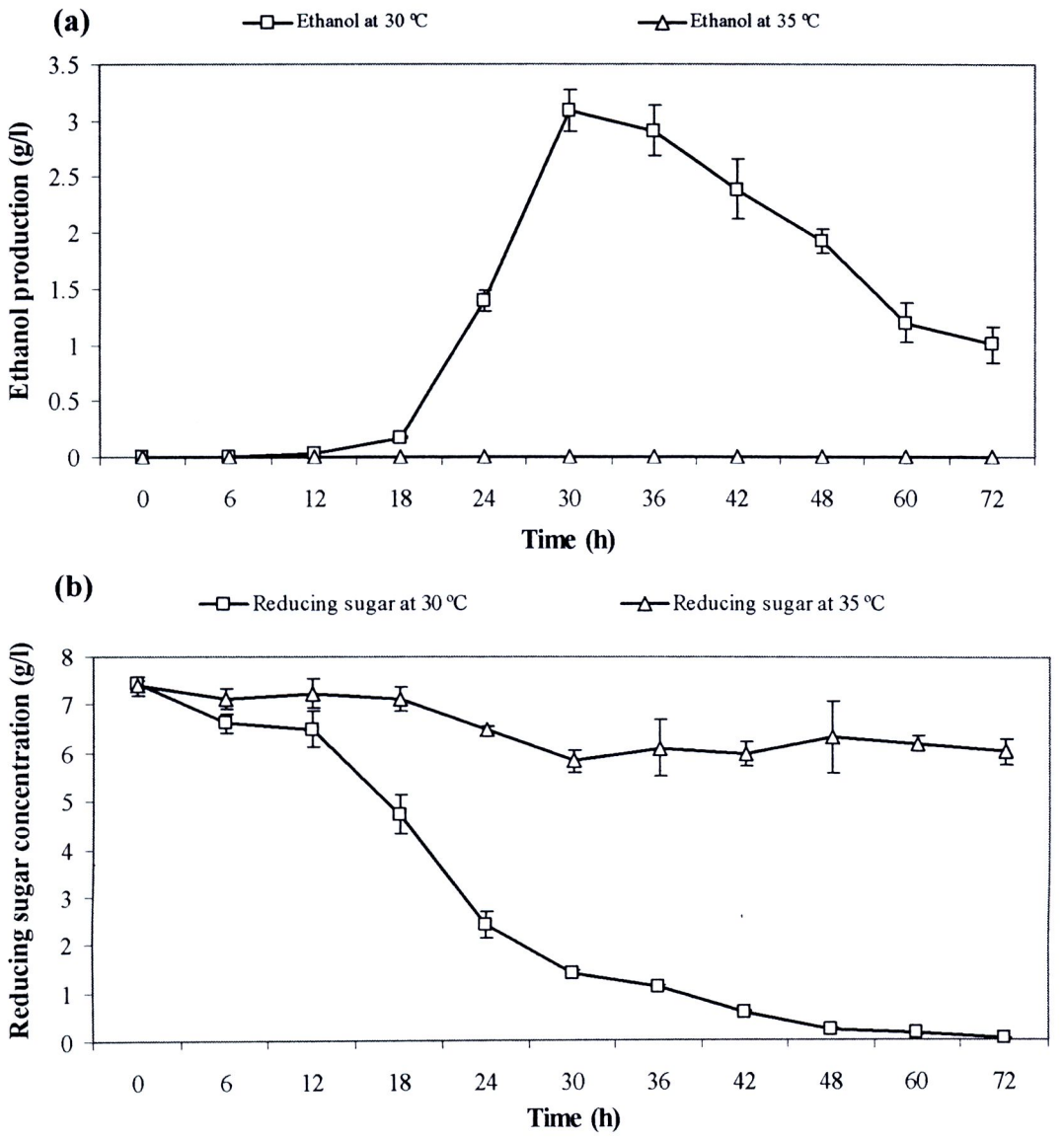

Figure 43. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in 2:8 (w/w) of C6 to C5 ratios at 180 rpm, pH 5.0 under 30°C and 35°C.

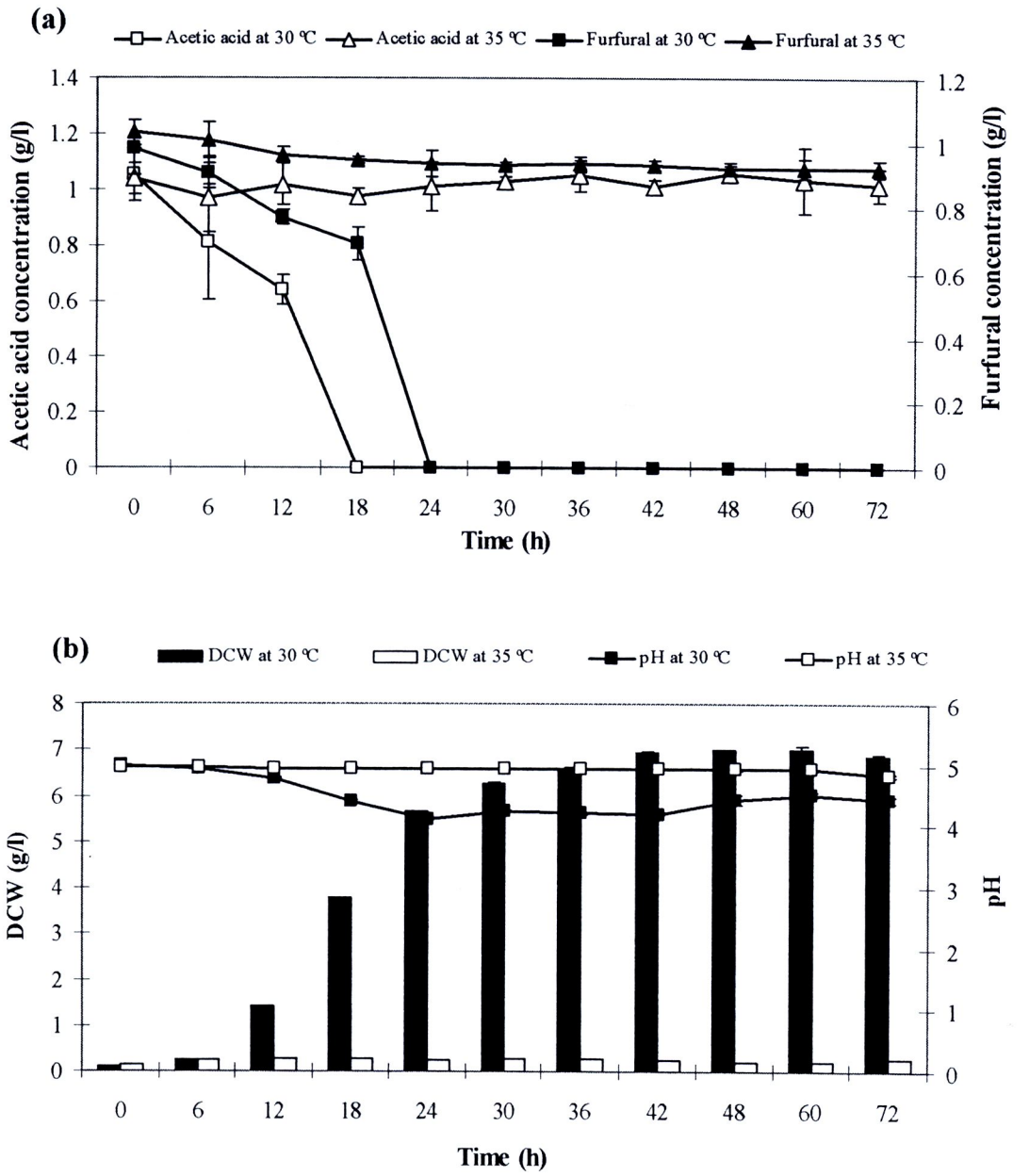

Figure 44. Time course of acetic acid and furfural concentrations (a) DCW and pH changes (b) during ethanol production by *Candida shehatae* TISTR5843 in 2:8 (w/w) of glucose to xylose ratios at 180 rpm, pH 5.0 under room temperature (30°C) and 35 °C.

3.8.6 Effect of shaking speed

Effect of shaking speed at 60, 120, 180 and 240 round per minute (rpm) was studied. Cultivation was conducted in the synthetic medium containing 2:8 (w/w) of glucose to xylose ratios with the initial pH of 5 at room temperature (30°C) for 72 h.

Ethanol yields and ethanol productivities of this factor were 0.35, 0.39, 0.43 and 0.40 g ethanol/g sugar, and 0.051, 0.091, 0.106 and 0.071 g/l/h, respectively. The results clearly indicated that at the optimum shaking speed was 180 rpm, giving the highest ethanol concentration of 3.17 g/l, the highest ethanol yield and productivity, as well as the fastest ethanol production (within 30 h cultivation) (Fig. 45a). Sugar consumption under various shaking speed of 120, 180 and 240 rpm were closely to zero within 48 h whereas the sugar consumption under shaking speed of 60 rpm slightly decreased during cultivation because *C. shehatae* TISTR5843 required more oxygen for cell growth (Fig. 45b) (Delgenes *et al.*, 1996).

Furfural concentration of 0.16 g/l presented in the culture medium was produced by xylose degradation after sterilization at 110°C for 15 min. Furfural was converted to the less toxic compounds within 30 h under shaking speed of 120, 180 and 240 rpm while furfural was transformed at 60 h cultivation at shaking speed of 60 rpm (Fig. 46a). For pH investigation, pH slightly decreased within 30-36 h and then slightly increased to pH 3.9-4.2 thereafter in all shaking speed (Fig. 46c). However, at the shaking speed of 240 rpm gave the highest cells growth (Fig. 46b) and the lowest ethanol production (Fig. 45a) because of high oxygen resulted in high metabolism of cells growth (Jeffries and Alexander, 2000). These results are similar with the experiments of Prior *et al.* (1988). When the oxygen supply to an aerobic condition of *C. shehatae* was reduced to oxygen-limited and anoxic conditions, the accumulation of ethanol and the specific activity of alcohol dehydrogenase increased upto 4-folds (Prior *et al.*, 1988). Additionally, Jeffries and Alexander (2000) suggested that at low level of aeration must be maintained to obtain good xylose fermentation and growth. However, too much oxygen supply is detrimental because xylose-fermenting yeast appears to both produce and consume ethanol at the same time.

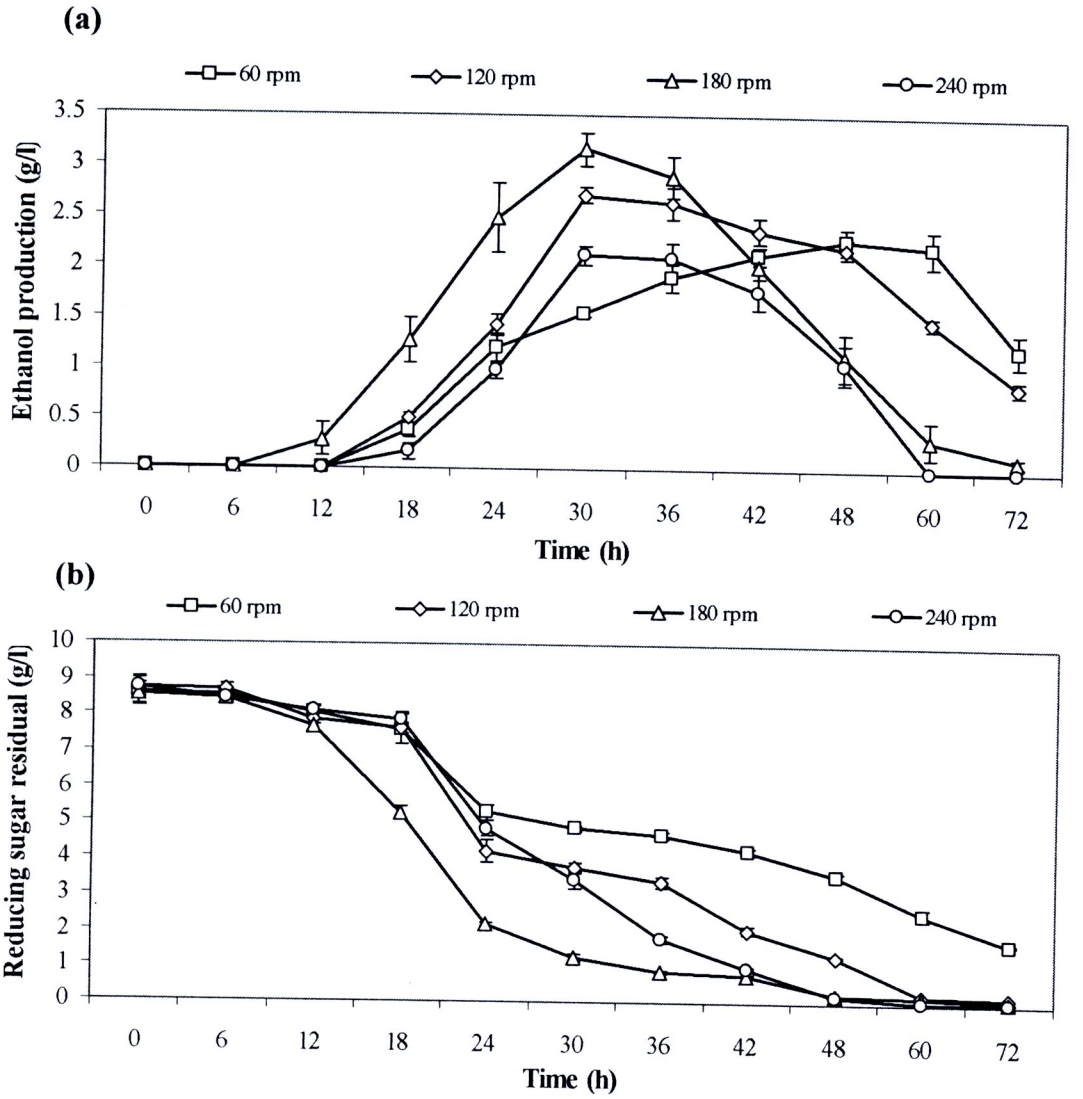

Figure 45. Time course of ethanol production (a) and reducing sugar consumption (b) by *Candida shehatae* TISTR5843 in various shaking speed in synthetic medium containing 2:8 (w/w) of glucose to xylose ratios, pH 5 at room temperature (30°C).

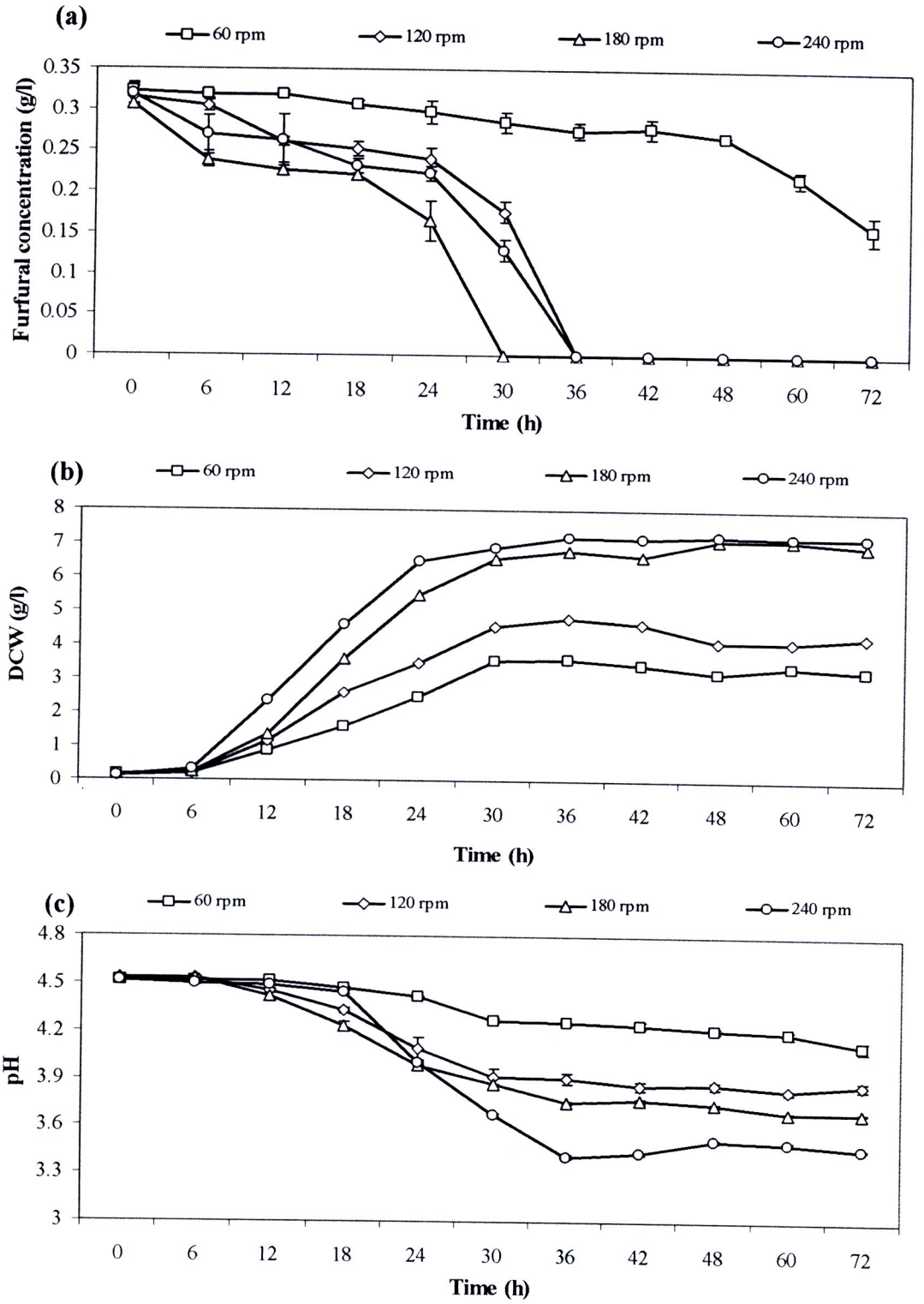

Figure 46. Time course of furfural concentrations (a), DCW (b) and pH changes (c) during ethanol production by *Candida shehatae* TISTR5843 in various shaking speed in synthetic medium containing 2:8 (w/w) of glucose to xylose ratios, pH 5 at room temperature (30°C).

The ethanol yields, ethanol productivities and ethanol concentrations at the optimum condition are summarized in Table 30. When cultured the yeast cell in the synthetic medium containing glucose as a sole carbon source, the optimum concentration was 24 g/l. However, when cultured the yeast cell in the synthetic medium containing xylose as a sole carbon source, the optimum concentration was 20 g/l. The co-substrate, glucose and xylose were studied. The optimum glucose to xylose ratio in the medium was 2:8 w/w with initial pH of 5. Incubation conditions were achieved at room temperature (30°C) with the shaking speed of 180 rpm. The highest ethanol yields, ethanol productivities and ethanol concentrations at optimum condition were 0.39-0.43 g ethanol/ g sugar, 0.103-0.330 g/l/h and 0.37-1.25%, respectively.

Table 30. Comparisons of ethanol yields, ethanol productivities and ethanol percentages by *Candida shehatae* TISTR5843 in synthetic medium at the optimum parameters affecting on ethanol production.

Factors		Ethanol yield (g ethanol/g sugar)	Ethanol productivity (g/l/h)	Ethanol concentration (g/l)
1. Glucose concentration	4 g/l	0.31	0.054	1.29
	7 g/l	0.37	0.106	2.55
	12 g/l	0.46	0.221	5.32
	24 g/l	0.45	0.343	10.29
	45 g/l	0.37	0.359	17.22
	75 g/l	0.24	0.290	17.40
2. Xylose concentration	4 g/l	0.33	0.058	1.39
	8 g/l	0.45	0.122	3.67
	20 g/l	0.42	0.194	8.14
	40 g/l	0.29	0.186	11.15
	60 g/l	0.22	0.138	13.29
	90 g/l	0.16	0.146	14.00
3. Glucose to xylose ratio	10:0 (w/w)	0.45	0.175	4.20
	8:2 (w/w)	0.43	0.178	4.28
	6:4 (w/w)	0.44	0.178	4.26
	5:5 (w/w)	0.44	0.174	4.18
	4:6 (w/w)	0.43	0.139	4.17
	2:8 (w/w)	0.43	0.111	3.99
	0:10 (w/w)	0.42	0.109	3.91
4. Temperature	30 °C	0.42	0.103	3.09
	35 °C	0	0	0
5. Initial pH	3.0	0.35	0.052	2.51
	4.0	0.36	0.089	2.66
	4.5	0.44	0.134	3.22
	5.0	0.45	0.136	3.27
	6.0	0.34	0.081	2.44
6. Shaking speed	60 rpm	0.35	0.051	2.27
	120 rpm	0.39	0.091	2.72
	180 rpm	0.43	0.106	3.17
	240 rpm	0.40	0.071	2.12

3.9 Ethanol production from enzymatic cellulosic hydrolysate

3.9.1 Selection of ethanolic producing yeasts and bacteria

In order to obtain the maximum ethanol production from cellulosic hydrolysate (almost glucose 7.9 g/l) obtained from enzymatic hydrolysis (Section 3.6), *C. shehatae* TISTR5843, *S. cerevisiae* TISTR5017, and *Z. mobilis* TISTR405 were studied for ethanol production from cellulosic hydrolysate at room temperature (30 ± 2°C) with shaking speed of 180 rpm for 72 h (Abbi *et al.*, 1996). The maximum ethanol production were 2.25 ± 0.06 g/l, 2.82 ± 0.11 g/l and 2.46 ± 0.04 g/l achieved at 60, 24, and 30 h, respectively (Table 31). The results clearly indicated that *S. cerevisiae* TISTR5017 was the best ethanolic producing strain from using cellulosic hydrolysate containing glucose, giving not only the highest ethanol concentration but also ethanol yield (0.34 g/g sugar) and ethanol productivity (0.118 g/l/h).

Table 31. Comparison of ethanol production in cellulosic hydrolysate (glucose 7.9 g/l) by *C. shehatae* TISTR5843, *S. cerevisiae* TISTR5017, and *Z. mobilis* TISTR405.

Strains	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
C. shehatae TISTR5843	2.25 ± 0.06	0.27	0.047
S. cerevisiae TISTR5017	2.82 ± 0.11	0.34	0.118
Z. mobilis TISTR405	2.46 ± 0.04	0.30	0.068

3.9.2 Optimization of ethanol production in cellulosic hydrolysate by selected strain

RSM was used as a statistical tool to find the optimum condition for maximizing ethanol production in cellulosic hydrolysate by the selected strain, *S. cerevisiae* TISTR5017. Three major parameters, initial pH (X_{13}), shaking speed (X_{14}) and initial cell concentration (X_{15}), were studied. The responses of RSM model, ethanol concentration (Y_{12}), ethanol yields (Y_{13}), and ethanol productivity (Y_{14}), were

shown in Table 32. At the initial pH of 4.0 (run 1-5), no ethanol production was detected because of no cells growth. Low ethanol concentration (2.00-2.49 g/l), ethanol yield (0.24-0.30 g ethanol/g sugar) and ethanol productivity (0.083-0.104 g/l/h) were obtained at the condition of initial pH of 5.00 and 6.00 and shaking speed of 240 rpm (run 14, 19 and 20). High ethanol concentration (3.14-3.94 g/l), ethanol yield (0.38-0.48 g ethanol/g sugar) and ethanol productivity (0.131-0.164 g/l/h) were achieved at the initial pH of 5.00 and 6.00 and shaking speed of 120 and 180 rpm (run 6-13 and 15-18). To evaluate the results, the data in Table 32 were subjected to regression analysis, using the following quadratic equations 37-39:

$$Y_{12} = -55.59 + 20.21X_{13} + 0.06X_{14} + 1.30X_{15} - 0.0045X_{13}X_{14} - 0.22X_{13}X_{15} - 0.0005X_{14}X_{15} - 1.77X_{13}^2 - 0.0001X_{14}^2 - 0.12X_{15}^2 \quad (37)$$

$$Y_{13} = -6.86 + 2.49X_{13} + 0.007X_{14} + 0.16X_{15} - 0.0006X_{13}X_{14} - 0.028X_{13}X_{15} - 0.00004X_{14}X_{15} - 0.22X_{13}^2 - 0.00001X_{14}^2 - 0.016X_{15}^2 \quad (38)$$

$$Y_{14} = -2.33 + 0.85X_{13} + 0.002X_{14} + 0.05X_{15} - 0.0002X_{13}X_{14} - 0.009X_{13}X_{15} - 0.00002X_{14}X_{15} - 0.075X_{13}^2 - 0.000005X_{14}^2 - 0.004X_{15}^2 \quad (39)$$

These models presented the high determination coefficients (R^2 = 0.97, 0.97 and 0.97, respectively) (Table 33) explaining 0.97% of variability in the responses of ethanol concentration, ethanol yield and ethanol productivity. The adjusted determination coefficients (adjusted R^2 = 0.95) indicated the high significance of these models. The ANOVA quadratic regression demonstrated that among models were significant, as evidenced from high *F*-values ($F=41.54$, 41.44 and 42.33, respectively) with a very low probability ($P<0.0001$). Low variation coefficient value (C.V. = 14.01%, 14.07% and 13.89%, respectively) indicated a high precision and reliability of the experiments (O-Thong *et al.*, 2008). The significance of each coefficient was determined by probability values. The variables with a significant effect on ethanol production were the initial pH (X_{13}) and shaking speed (X_{14}) ($P<0.05$). Linear terms of X_{13} and X_{14} and quadratic terms of X_{13}^2 and $X_{13}X_{14}$ were significant ($P<0.05$), demonstrating that maximizing ethanol production required a suitable value of initial pH and shaking speed.

Estimation of ethanol concentration, ethanol yield and ethanol productivity over X_{13}, X_{14} and X_{15} in terms of response surfaces are shown in Fig. 47. The effect of initial pH and shaking speed on ethanol production, fixed the initial cells concentration at 0.7 g/l, are shown in Fig. 47(a), 47(d) and 47(g). The maximum ethanol concentration (3.94 g/l), ethanol yield (0.48 g ethanol/g sugar) and ethanol productivity (0.164 g/l/h) were achieved at initial pH of 5.0 and shaking speed of 120 rpm. The results of ethanol production indicated that both initial pH and shaking speed had significant effect on ethanol production. The pH affects on cell membrane permeability and the solubility of some components of the medium: thus, a modification in the pH might also cause some micronutrient to precipitate and so become impossible to be assimilated (Sánchez *et al.*, 1997). The dissolved oxygen tension (DOT) is also particularly critical in attaining maximal ethanol production with xylose-fermenting yeasts. *C. shehatae* require aeration for maximal ethanol production (Jeffries and Jin, 2000). In addition, rotary of the shaker should be effective enough to provide gentle mixing and surface aeration during the first period of the growth phase (Phisalaphong *et al.*, 2006). However, much more oxygen supplied into fermentation system would be caused of reduction of ethanol production because the pathway of *C. shehatae* produced cell mass more than ethanol (Jeffries and Jin, 2000).

The response surface plots of the initial pH and initial cells concentration interaction were shown in Fig. 47(b), 47(e) and 47(h) when shaking speed was fixed at 180 rpm. The initial pH had a significant effect on ethanol production while the initial cells concentration had no effect. The response surface plots of shaking speed and initial cells concentration interaction are shown in Fig. 47(c), 47(f) and 47(i) when initial pH was fixed at 5.0. Shaking speed had a significant effect on ethanol production while the initial cells concentration had no effect.

Table 32. Central composite experimental design matrix defining initial pH (X_{13}), shaking speed (rpm) (X_{14}), and initial cells concentration (g/l) (X_{15}) and results on ethanol production in cellulosic hydrolysate after cultivation of *S. cerevisiae* TISTR5017 for 72 h at room temperature (30°).

Run	Parameter			Ethanol concentration (Y_{12}) (g/l)	Ethanol yield (Y_{13}) (g /g sugar)	Ethanol productivity (Y_{14}) (g/l/h)
	X_{13}	X_{14}	X_{15}			
1	4	120	0.40	0	0	0
2	4	120	1.00	0	0	0
3	4	180	0.70	0	0	0
4	4	240	1.00	0	0	0
5	4	240	0.40	0	0	0
6	5	180	0.40	3.18	0.39	0.133
7	5	180	1.00	3.54	0.43	0.148
8	5	180	0.70	3.66	0.45	0.153
9	5	180	0.70	3.66	0.45	0.153
10	5	180	0.70	3.66	0.45	0.153
11	5	180	0.70	3.66	0.45	0.153
12	5	180	0.70	3.66	0.45	0.153
13	5	180	0.70	3.66	0.45	0.153
14	5	240	0.70	2.05	0.25	0.086
15	5	120	0.70	3.94	0.48	0.164
16	6	120	0.40	3.52	0.43	0.147
17	6	120	1.00	3.14	0.38	0.131
18	6	180	0.70	3.23	0.39	0.134
19	6	240	0.40	2.49	0.30	0.104
20	6	240	1.00	2.00	0.24	0.083

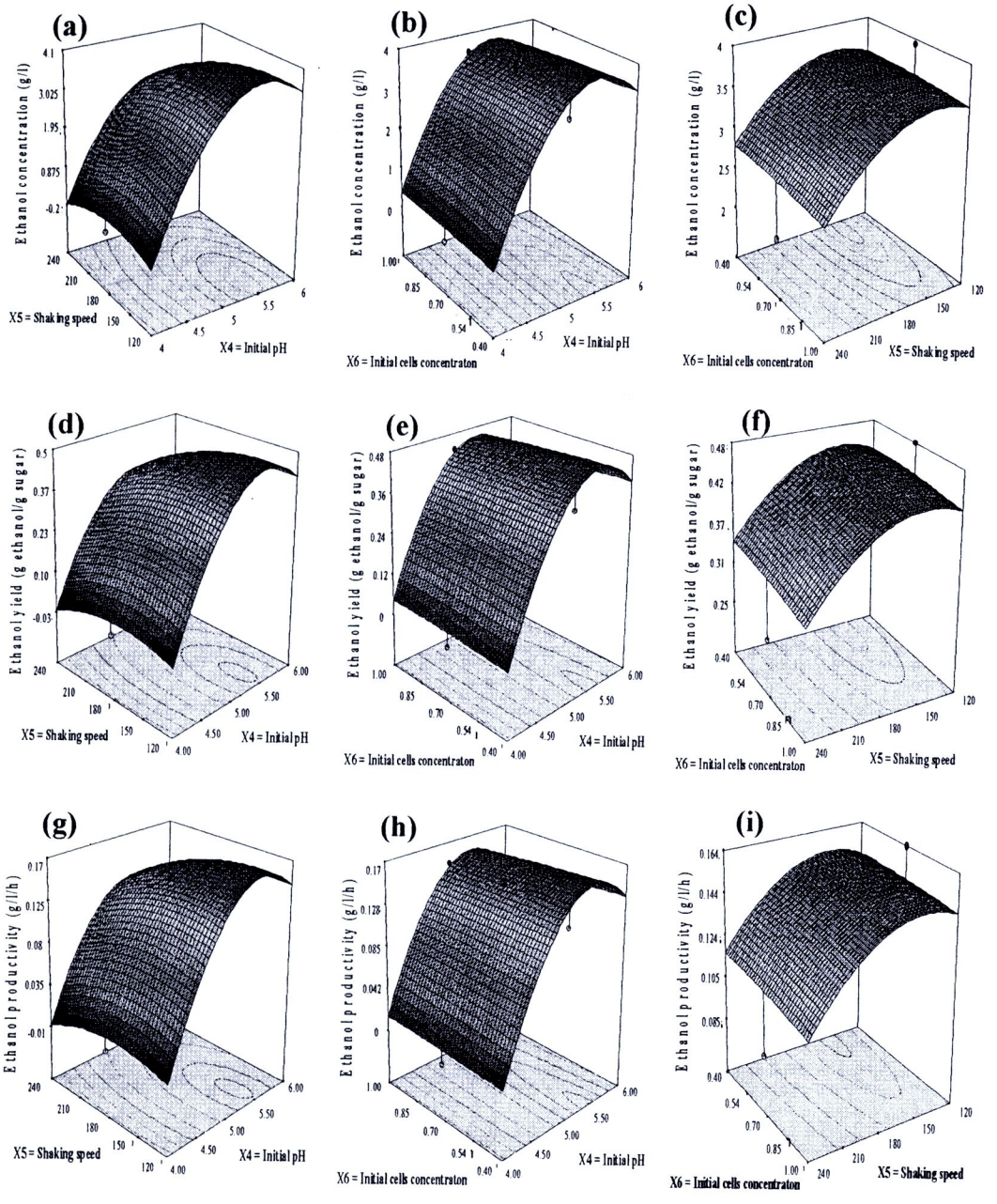

Figure 47. Three-dimensional graphs of the quadratic model of ethanol production in a cellulosic hydrolysate medium by *S. cerevisiae* TISTR5017. for ethanol concentration (a-c), ethanol yield (d-f) and ethanol productivity (g-i) within the central composite design (CCD): a, d and g; fixed initial cells concentration at centre point of 0.70 g/l, b, e and h; fixed shaking speed at centre point of 180 rpm, c, f and i; fixed initial pH at centre point of 5.0.

Table 33. Analysis of variance (ANOVA) for ethanol production in a cellulosic hydrolysate.

Responses (Y)	Source	Sum of square	Degree of freedom	Mean square	F-value	P-value
Ethanol concentration (Y_{12})	Model	44.15	9	4.91	41.54	< 0.0001
	R^2	0.97				
	Adjusted R^2	0.95				
	C.V.	14.01				
Ethanol yield (Y_{13})	Model	0.66	9	0.074	41.44	< 0.0001
	R^2	0.97				
	Adjusted R^2	0.95				
	C.V.	14.07				
Ethanol productivity (Y_{14})	Model	0.077	9	0.009	42.33	<0.0001
	R^2	0.97				
	Adjusted R^2	0.95				
	C.V.	13.89				

3.9.3 Confirmation experiments and adequacy of the models of ethanol production in cellulosic hydrolysate

To confirm the validity of the statistical experimental strategies of ethanol production from cellulosic hydrolysate, three replicates of batch experiments were performed under the optimal condition conducted by RSM with the initial pH of 5.40, shaking speed of 137 rpm and initial cells concentration of 0.72 g/l. Results of confirmation experiments (Table 34) indicated that the experimental values of ethanol production (3.98 ± 0.42 g/l, 0.48 ± 0.02 g ethanol/g sugar, and 0.167 ± 0.04 g/l/h) were similar to the predicted values (4.06 g/l, 0.49 g ethanol/g sugar, and 0.170 g/l/h, respectively). There was no significant difference between the experimental values and the predicted values *($P<0.05$)*. The ethanol production using the initial condition (control) (Table 34) and the optimum condition (Table 34) was 2.82 and 3.98 g/l, respectively. After optimization, ethanol production from this hydrolysate medium increased 1.41-fold (from 2.82 to 3.98 g/l).

Table 34. The confirmation experiments for ethanol production in cellulosic hydrolysate by *S. cerevisiae* TISTR5017 cultivated under the optimal condition.

Substrates	Trials	Condition	Initial pH	Shaking speed (rpm)	Initial cells concentration (g/l)	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
Cellulosic hydrolysate	-	Optimal[a]	5.40	137	0.72	3.98 ± 0.42	0.48 ± 0.02	0.167 ± 0.04
	9	Medium	5.00	180	0.70	3.75	0.46	0.156
	-	Control or initial	5.00	180	0.4	2.82	0.34	0.118

[a] Based on ethanol production.

3.10 Ethanol production from PPF hydrolysate

3.10.1 Comparison of ethanol production between synthetic xylose medium and PPF hydrolysate by *C. shehatae* TISTR5843

Prior to produce ethanol from PPF hydrolysate, comparison of ethanol production from both synthetic xylose medium (28 g/l xylose) without supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) and PPF hydrolysate (28 g/l xylose approximately) by *C. shehatae* TISTR5843 was investigated. *C. shehatae* TISTR5843 was selected due to its ability to consume xylose (Delgenes *et al.*, 1996). Meanwhile, *S. cerevisiae* TISTR5517, selected and used to produce ethanol from cellulosic hydrolysate, could not use xylose as a substrate to produce ethanol. The optimum extrinsic parameters of ethanol production in synthetic xylose medium by *C. shehatae* TISTR5843 were set at initial pH of 5.0, cell concentration of 0.725 g/l (OD_{600}=0.5), shaking speed of 180 rpm, and incubated at room temperature (30°C) (Section 3.8: Table 30).

For synthetic xylose medium, ethanol production was started at 18 h (0.29 g/l) cultivation and increased rapidly until 48 h (8.14 g/l). In PPF hydrolysate medium, ethanol production was also begun at 18 h cultivation (0.17 g/l) and slightly increased until 96 h (3.30 g/l) (Fig. 48) because of generation of inhibitory compounds from dilute acid hydrolysis (Delgenes *et al.*, 1996; Rahman *et al.*, 2006) i.e. 4.25 g/l acetate and 0.67 g/l furfural were generated. The ethanol yields and ethanol productivities of both synthetic xylose and PPF hydrolysate medium were 0.43 and 0.16 g ethanol/g xylose, and 0.194 and 0.034 g/l/h, respectively. The results showed that the inhibitory played an important role in ethanol production by *C. shehatae* TISTR5843. Therefore, xylanase usage might be a good method to produce xylose without any inhibitors generation. However, xylose production by using xylanase have to study in case of the cost of enzyme was expensive. Therefore, the further studied of this research was an investigation of the effect of inhibitory compounds in PPF hydrolysate for the production of ethanol by *C. shehatae* TISTR5843. Consequently, the effect of nitrogen source and its concentration and carbon to nitrogen (C/N) ratio (concerning to cells growth) were studied in synthetic xylose medium.

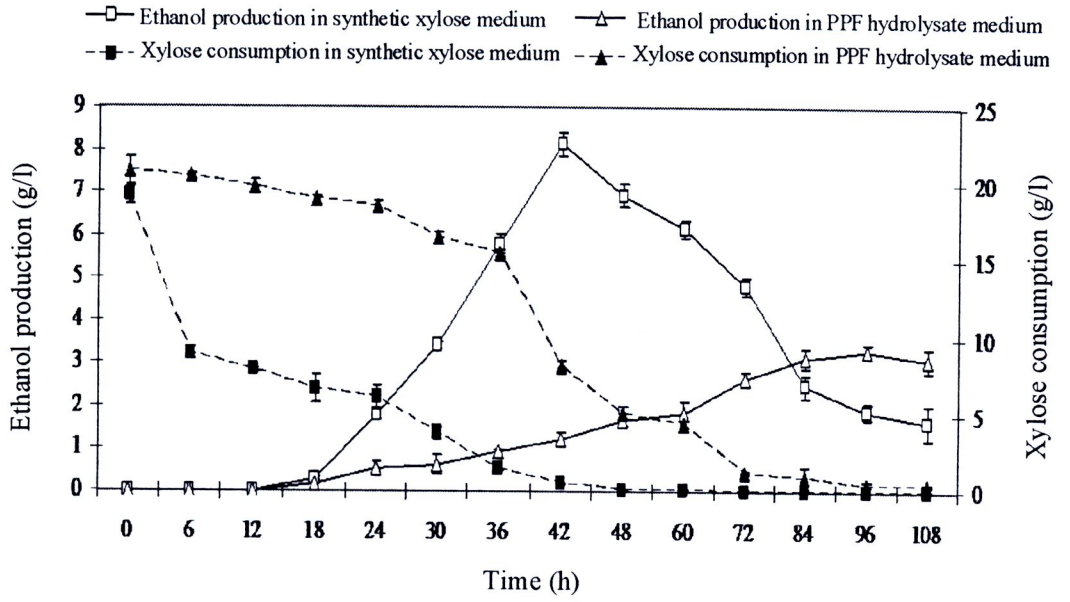

Figure 48. Time course comparison of ethanol production in synthetic xylose and PPF hydrolysate mediums by *Candida shehatae* TISTR5843 under 20 g/l xylose, initial pH of 5.0, shaking speed of 180 rpm at room temperature (30°C).

3.10.2 Effect of nitrogen source, nitrogen concentration and C/N ratio on ethanol production

3.10.2.1 Effect of nitrogen source

NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone were used as nitrogen sources. Each of nitrogen sources was supplemented into the xylose medium in amount of 3 g/l based on nitrogen content in each compound. The synthetic xylose medium was 28 g/l xylose with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l), which is the similar xylose concentration and inhibitors concentration in PPF hydrolysate. The optimum extrinsic parameters of ethanol production in synthetic xylose medium by *C. shehatae* TISTR5843 were set at initial pH of 5.0, cell concentration of 0.725 g/l (OD_{600}=0.5), shaking speed of 180 rpm, and incubated at room temperature (30°C) (Section 3.8:

Table 30). In this case, the cultures were incubated for 24 h and 48 h. As seen in Fig. 49 peptone was the optimum nitrogen source giving the maximum ethanol production of 3.05 g/l. The reduction of ethanol production (8 to 3 g/l) resulted from furfural and acetate added into the synthetic xylose medium.

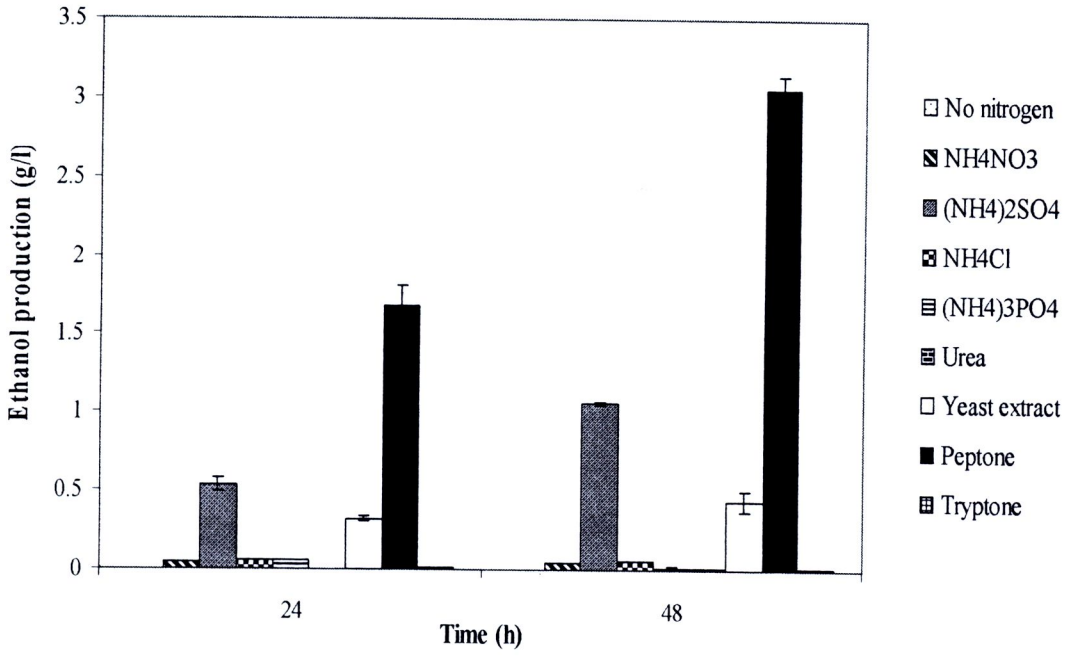

Figure 49. Effect of nitrogen sources on ethanol production by *Candida shehatae* TISTR5843 with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) at optimum pH of 5.0 and initial cell concentration of 0.725 g/l incubated at 30°C on a rotary shaker (180 rpm) for 24 and 48 h.

3.10.2.2 Effect of peptone concentration

The highest ethanol production (4.75 g/l) was obtained at 3 g/l peptone with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l) corresponded to a C/N ratio of 9.3 (Fig. 50). Substantial reduction in ethanol production was observed at peptone concentration higher than 5 g/l. Ethanol production decreased with the increase of nitrogen content because of presented much more nitrogen (Abd-Aziz *et al.*, 2001). In this study, therefore, peptone at 3 g/l was the optimal nitrogen concentration for producing ethanol by *C. shehatae* TISTR5843.

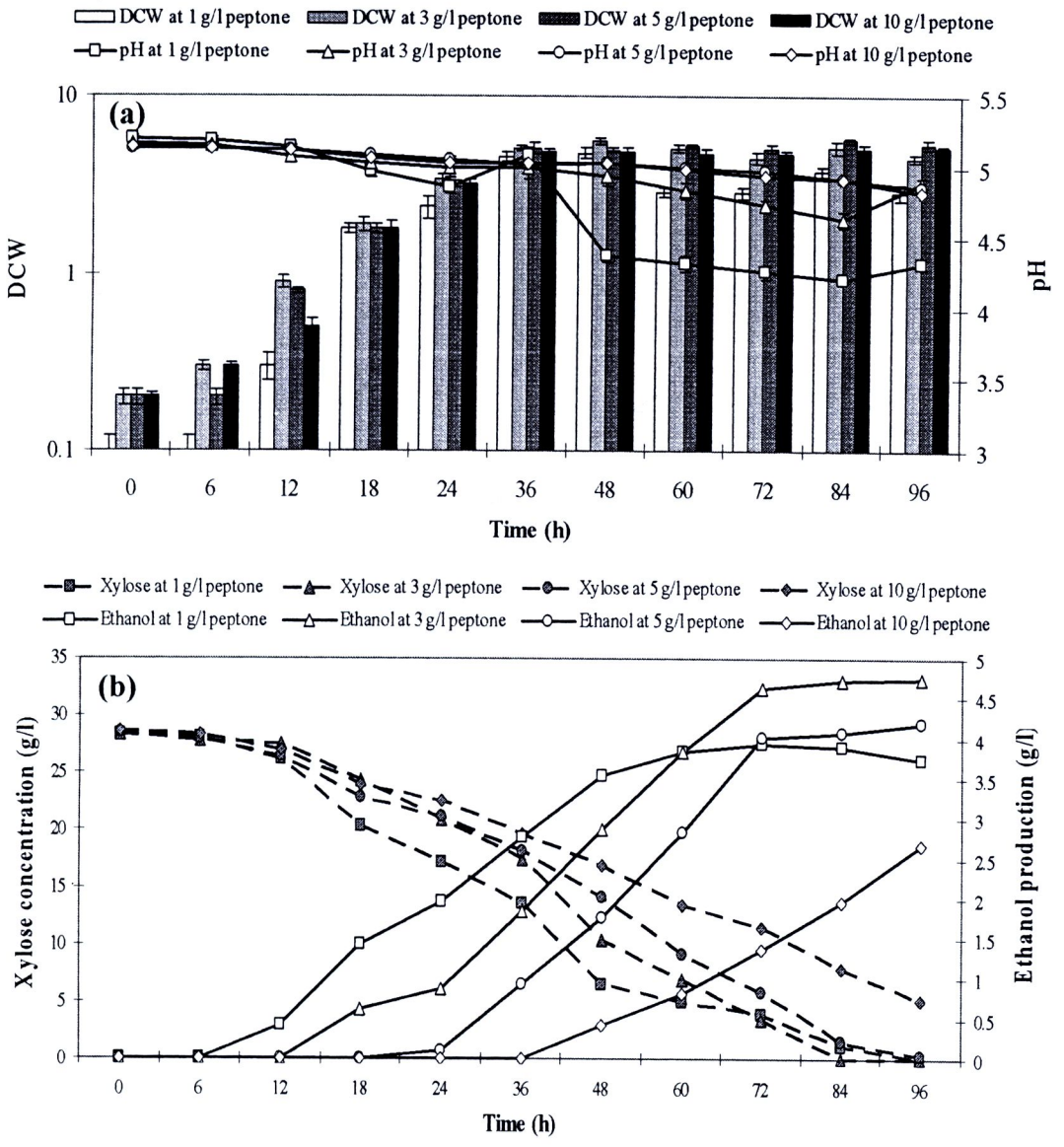

Figure 50. Time course of cell growth and pH change (a), and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 under various peptone concentrations of 1-10 g/l in synthetic xylose medium with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l), pH5 with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

To set the composition in synthetic medium as close as in PPF hydrolysate, the 28 g/l xylose medium was supplemented with 4.25 g/l acetate and 0.67 g/l furfural (as the same concentration in PPF hydrolysate). The performance of direct fermentation of xylose to ethanol by *C. shehatae* TISTR5843 at different C/N ratios is shown in Table 35 and described in the previous section. On the other hand, *C. shehatae* TISTR5843 grew faster and reached a maximum cell concentration (5.6 g/l) and produced a maximum ethanol production (4.75 g/l) at the C/N ratio of 9.3.

Table 35. The performance of ethanol production from xylose medium with supplementation of 4.25 g/l acetate and 0.67 g/l furfural at different C/N ratios by *Candida shehatae* TISTR5843.

Fermentation performance	C/N ratio			
	2.8	5.6	9.3	28
Maximum cell concentration (g/l)	5.2	5.3	5.6	4.8
Maximum ethanol concentration (g/l)	2.68	4.20	4.75	3.93
Ethanol yield (g of ethanol per g of xylose)	0.12	0.17	0.19	0.16
Cell yield (g of cell per g of xylose)	0.221	0.279	0.311	0.218
Fermentation time (h)	48	48	48	48
Overall productivity (g/h)	0.056	0.087	0.099	0.081

28 g/l xylose was used in all fermentations

3.10.3 Effect of inhibitory compounds in PPF hydrolysate on ethanol production

Normally, after diluted acid hydrolysis of biomass, several inhibitor compounds were generated such as acetate, furfural, vanillin, cinnamaldehyde, ρ-hydroxybenzaldehyde and syringaldehyde (Olsson and Hahn-Hägerdal, 1996; Delgenes *et al.*, 1996). In this study, acetate, vanillin and furfural were studied because they are the major inhibitor compounds in PPF hydrolysate.

Compared to the control culture (without any inhibitor supplementations), all supplemented cultures of *C. shehatae* TISTR5843 showed a

decrease in cells growth and ethanol production (Table 36). Vanillin, derivative of lignin, showed the highest inhibitory effect (Fig. 51). Both growth and ethanol production processes were almost totally inhibited at an initial vanillin concentration of 1.0 g/l (Table 36). The high toxicity of vanillin on activities by xylose-fermenting microorganisms was reported by Tran and Chambers (1985), Nishikawa *et al* (1988) and Delgenes *et al* (1996). The ethanol produced by *P. stipitis* CBS 5776 in the xylose fermentation with 0.09 g/l of vanillin was 2.2 times lower of that in the control (Tran and Chambers, 1985). *K. pneumoniae* ATCC 8724 grown in the presence of 0.5 g/l of vanillin showed that growth and 2,3-butanediol production from xylose were lower 80 and 56.1% , respectively compared to the control (Nishikawa *et al.*, 1988). An initial vanillin concentration of 1.0 g/l, both growth and ethanol production processes by *C. shehatae* ATCC22984, *P. stipitis* NRRL Y 7124 and *S. cerevisiae* CBS 1200 were almost totally inhibited (Delgenes *et al.*, 1996).

In contrast to vanillin, allowing for concentration effects, acetate was the less toxic compound for *C. shehatae* TISTR5843. At the highest acetate concentration of 10 g/l, growth and ethanol production decreased 26.78% and 43.01%, respectively, compared to the control culture (Table 36 and Fig. 52). The sensitivity of the fermentation activities toward acetate appeared to be strain dependent as illustrated by Tran and Chambers (1985), Watson *et al* (1984) and Delgenes *et al* (1996). With an acetate concentration of 11.9 g/l, the ethanol production by *P. stipitis* CBS 5776 decreased 24% (Tran and Chambers, 1985), whereas 13 g/l of acetic acid almost completely inhibited ethanol production from xylose by *P. tunnophilus* (Watson *et al.*, 1984). At an initial acetate concentration of 15 g/l, ethanol production by *C. shehatae* and *P. stipitis* decreased 64% and 25%, respectively (Delgenes *et al.*, 1996). The undissociated acetic acid permeates the cell membrane and then dissociates in the cytoplasm where the pH is almost neutral. The cell uses energy to pump out surplus H^+ ions in order to maintain its intracellular pH. This eventually leads to cell death at high acetic acid concentrations (Palmqvist *et al.*, 1996; Nigam, 2001).

Table 36. Growth and ethanol production by *C. shehatae* TISTR5843 in synthetic xylose medium in the presence of inhibitory compounds.

Compounds	Concentration (g/l)	DCW (g/l)	Ethanol concentration (g/l)	DCW (% of control)	Ethanol (% of control)
Control	Acetate = 1.6 Furfural = 0.2	12.96	12.88 (0.46)	100	100
Acetate	2.5	4.92	8.41 (0.30)	37.96	65.30
	5	4.25	7.58 (0.27)	32.79	58.85
	10	3.47	5.54 (0.20)	26.78	43.01
Furfural or furaldehyde	0.5	4.97	7.51 (0.27)	39.35	58.31
	1	1.32	5.94 (0.21)	11.19	46.12
	2	0	0	0	0
Vanillin	0.5	4.62	3.95 (0.14)	35.65	30.67
	1	0.24	0.04 (0)	1.85	0.31
	2	0	0	0	0
Acetate and furfural	4.25 and 0.67[a]	4.18	4.56 (0.16)	32.25	35.40

[a]The same amount of inhibitor concentration presented in the PPF hydrolysate
Control: amount of malt extract was 3 g/l

For furfural effect showed that the furfural concentration of 1 g/l gave cell growth and ethanol production of 11.19% and 46.12 %, respectively, of the control whereas the completely inhibited effect of cell growth and ethanol production was obtained from the initial furfural concentration of 2 g/l (Table 36). In general, furfural is inhibitory to yeast metabolism at a level of 1.0 g/l and greater (Nigam, 2001). Furfural affects the growth and metabolism of microorganisms. It delays the onset of fermentation while it is assimilated and degraded (Palmqvist *et al.*, 1996). However, a suitable amount of furfural present in lignocellulosic hydrolysate can be beneficial for ethanolic fermentation of xylose because furfural uses NADH to generate NAD^+, which is used for transformation of xylitol to xylulose by xylitol dehydrogenase (XDR) in xylose phosphate pathway (Wahlbom and Hahn-Hägerdal, 2002). Furfural can be converted to furfuryl alcohol by *C. shehatae* TISTR5843

(Martín *et al.*, 2007) with the maximal conversion rate of 0.025 g/l/h (Fig. 53c and 54), which is 3-folds lower than that of *S. cerevisiae* (Martín *et al.*, 2007). However, the limited substrate concentration and the present of furfuryl alcohol decreased the growth rate as well as the specific rate of ethanol production (Fig. 53c and 54) because furfuryl alcohol inhibited alcohol dehydrogenase (ADH) and xylose reductase, which are enzymes for furfural reduction (Nigam, 2001). Consisting of individual (furfural, acetic acid and others), combination and all together in medium had the different inhibitory effected on fermentability. Nigam (2001) reported that the effect of all together > combination > individual are proved.

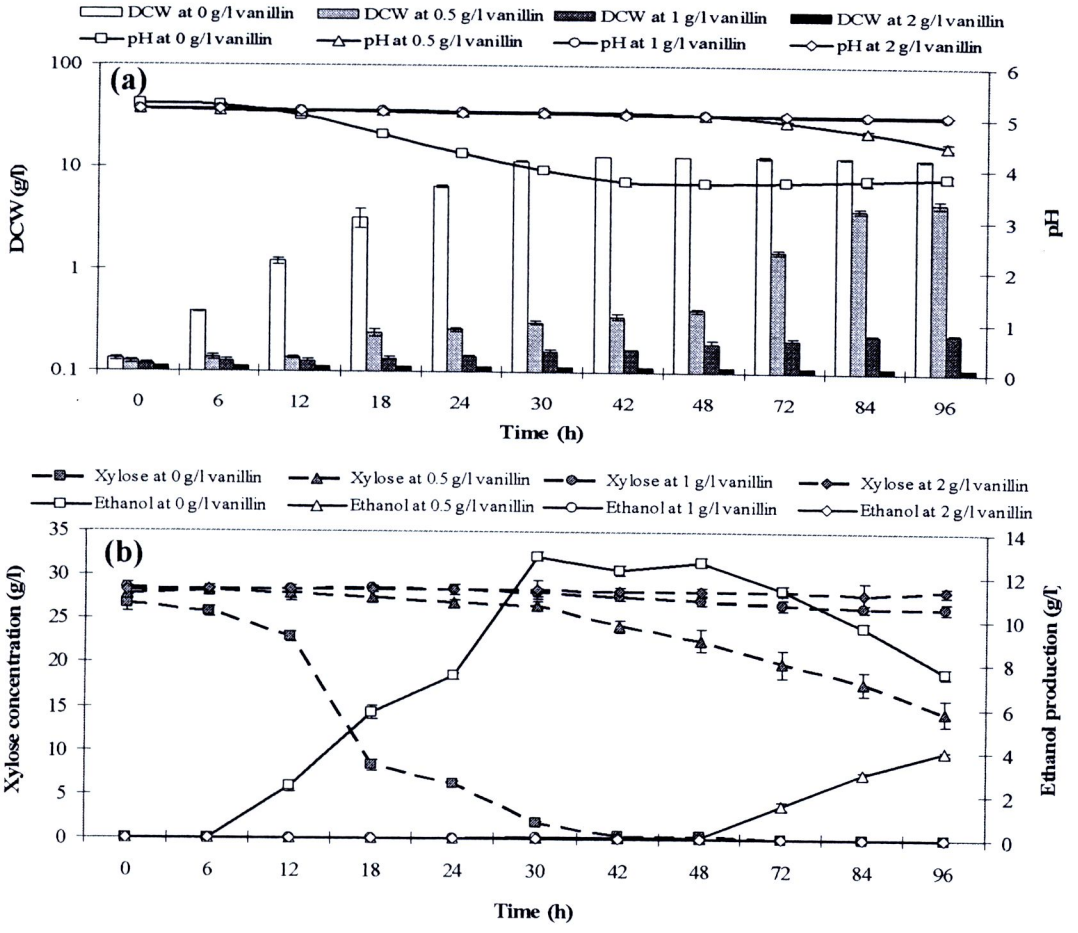

Figure 51. Time course of growth and pH change (a), and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 under various vanillin supplementations of 0-2 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

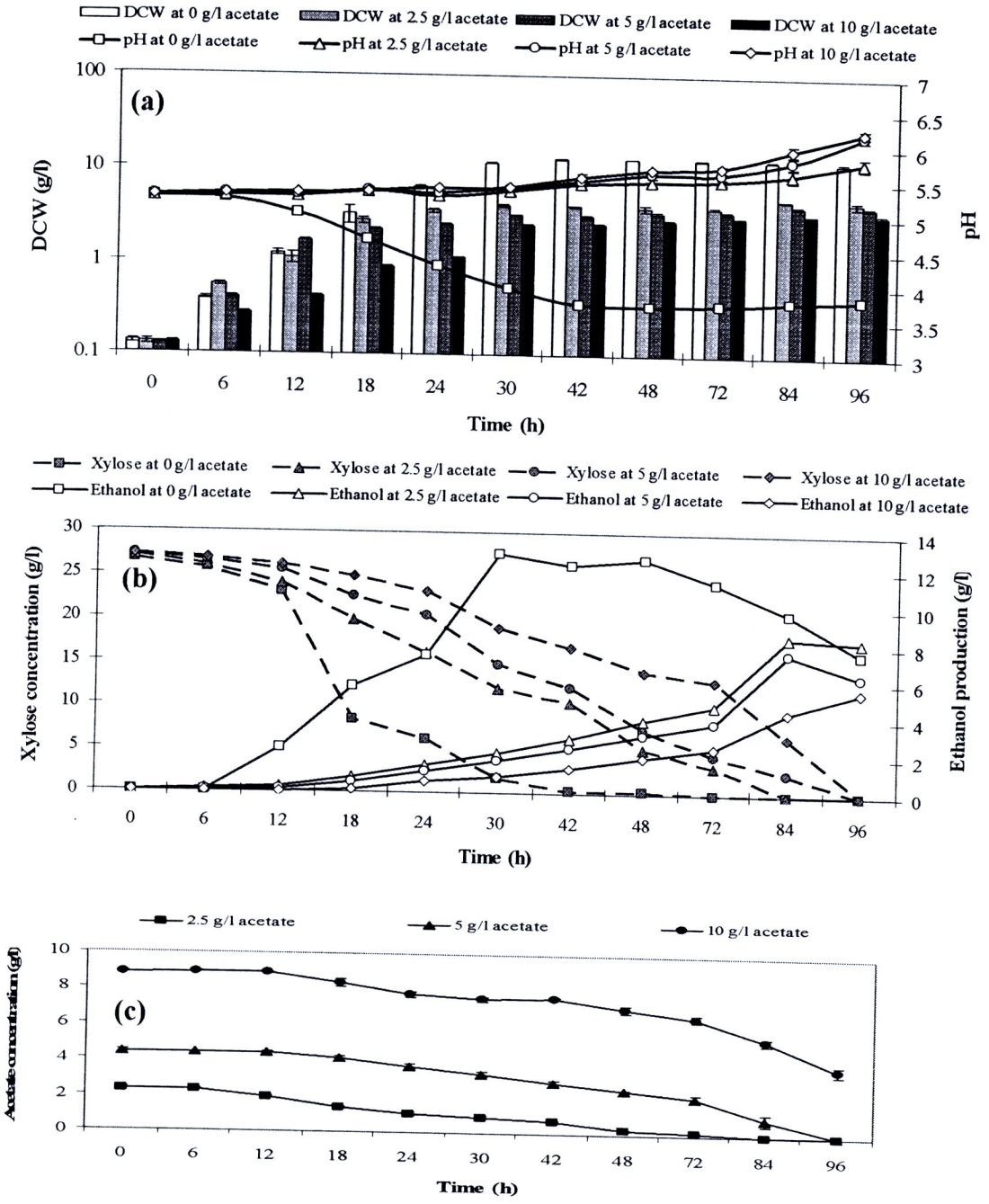

Figure 52. Time course of growth and pH change (a), xylose consumption and ethanol production (b), and acetate reduction (c) by *Candida shehatae* TISTR5843 under various acetate supplementations of 0-10 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm) in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l.

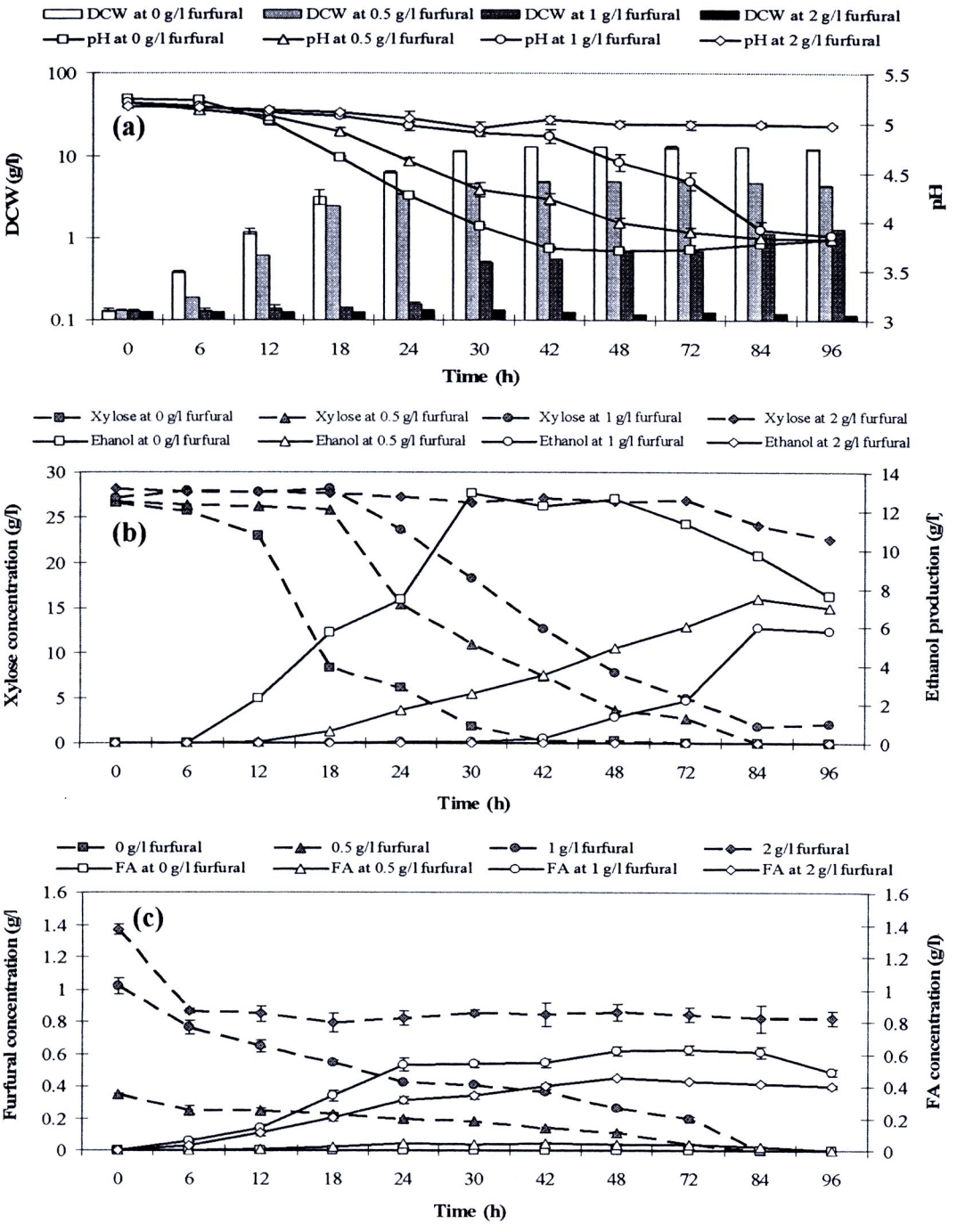

Figure 53. Time course of growth and pH change (a), xylose consumption and ethanol production (b), and furfural reduction with furfuryl alcohol (FA) generation (c) by *Candida shehatae* TISTR5843 under various furfural supplementations of 0-2 g/l in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

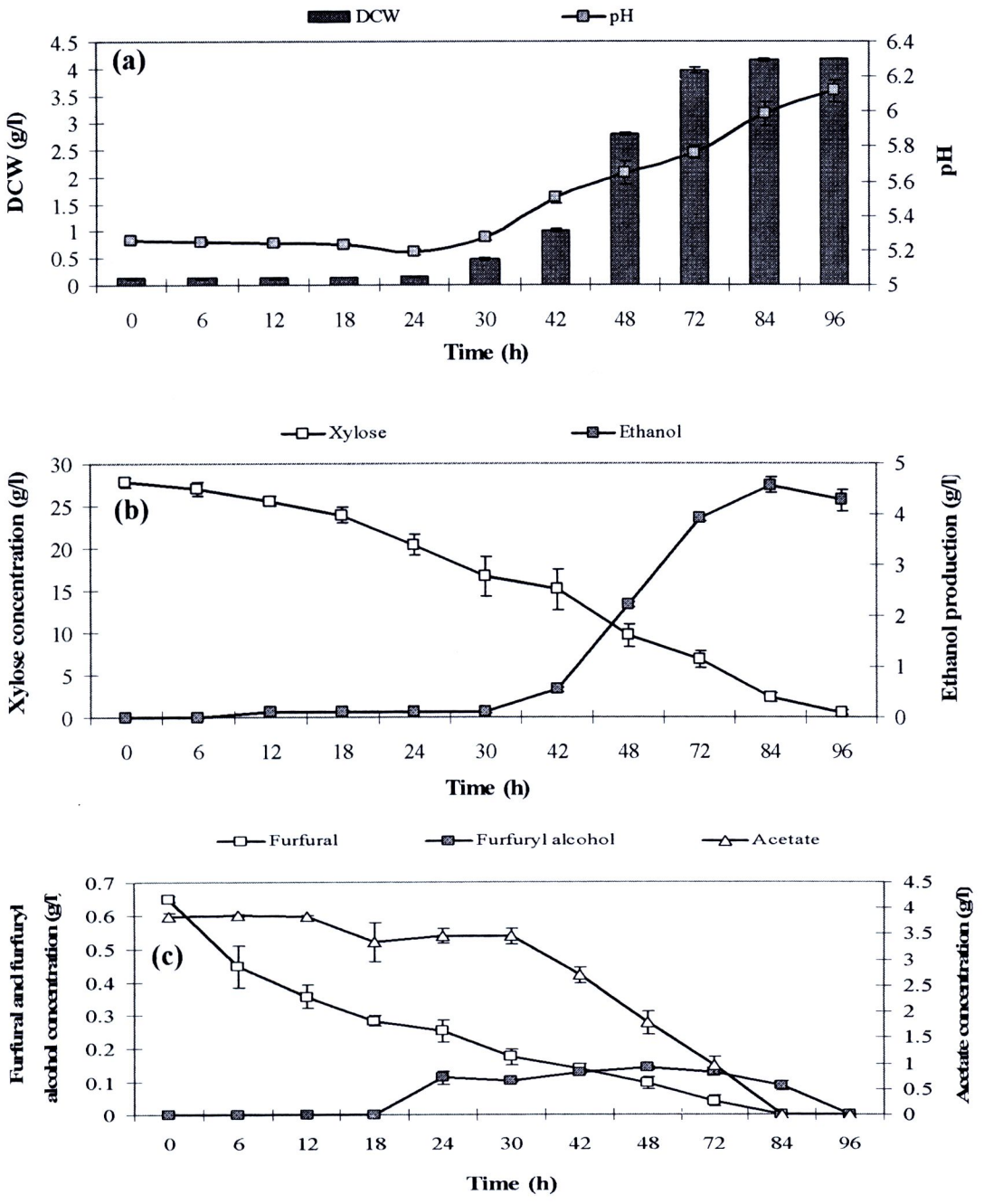

Figure 54. Time course of growth and pH change (a), xylose consumption and ethanol production (b), furfural and acetate reduction with furfuryl alcohol (FA) generation (c) by *Candida shehatae* TISTR5843 under mixture of 4.25 g/l acetate and 0.67 g/l furfural in synthetic xylose medium (pH5) with the initial cell concentration of 0.725 g/l at 30°C on a rotary shaker (180 rpm).

3.10.4 Effect of dilution factor of PPF hydrolysate on ethanol production

A large amount of inhibitory compounds in PPF hydrolysate resulted in the inhibition of cell growth and ethanol production, using diluted PPF hydrolysate may be a suitable method in order to reduce inhibition in the fermentation process. Inhibitors can be abated prior to fermentation through this process (Martín *et al.*, 2007; Nichols *et al.*, 2010). Detoxification improves the fermentability of hydrolysates, it is for economical reasons desirable to limit the requirements for detoxification to a minimum when compared to chemical and physical detoxifications (Martín *et al.*, 2007). In this study, no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution were studied.

Cells growth at 1/5 dilution of PPF hydrolysate was found to be the fastest of growth rate within 36 h because of the lowest content of inhibitory compounds presented in hydrolysate (Fig. 55a and 55b). However, cells growth of no dilution, 1/2 dilution and 1/3 dilution of PPF hydrolysate increased rapidly after all inhibitors were consumed and transformed to less toxic compound at 60 h cultivation time (Fig. 55a and 56).

Xylose concentration of no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution of PPF hydrolysate were consumed closely to zero by *C. shehatae* TISTR5843 within 84, 36, 36 and 24 h (Fig. 55b), respectively, giving maximum of ethanol production of 5.21, 4.51, 3.02 and 1.56 g/l, respectively (Fig. 55b). Ethanol yields and productivities of these dilutions were 0.19, 0.32, 0.30, 0.27 g/g, and 0.062, 0.125, 0.084, 0.065 g/l/h, respectively. Thus, the 1/2 dilution was a suitable dilution for production of ethanol because of giving the maximum ethanol yield and ethanol productivity. The 1/2 dilution was used in the further experiment of optimization of ethanol production in PPF hydrolysate.

Acetate was consumed by *C. shehatae* TISTR5843 within 48-84 h cultivation time (Fig. 56a) for 1/2-1/5 dilutions, while acetate consumption of no dilution was consumed from 6.00 to 0.92 g/l (84.67 % reduction). Furfural concentrations could be transformed totally to furfuryl alcohol within 60 h (Fig. 56b).

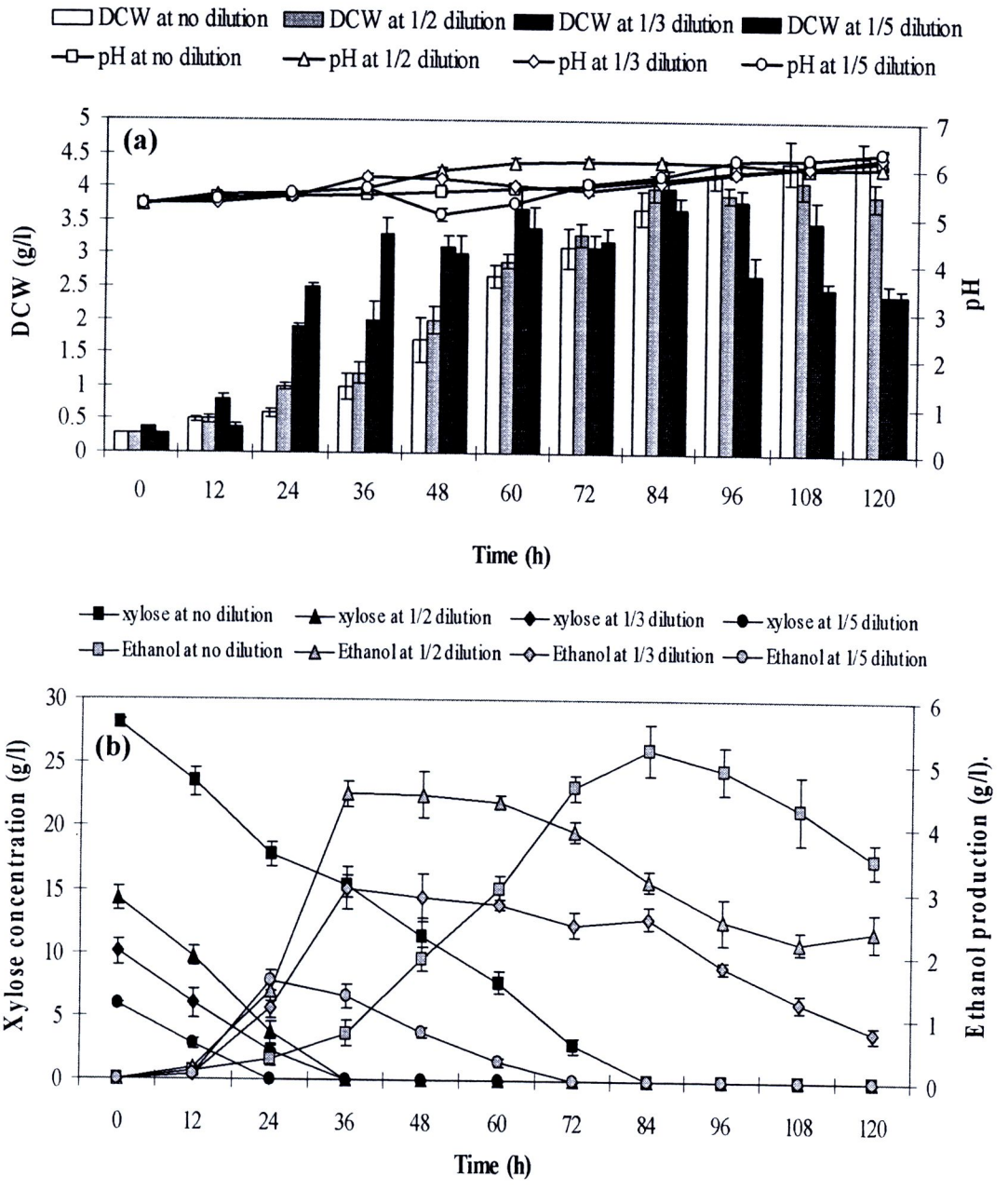

Figure 55. Time course of growth and pH change (a) and xylose consumption and ethanol production (b) by *Candida shehatae* TISTR5843 in various PPF hydrolysate dilutions (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

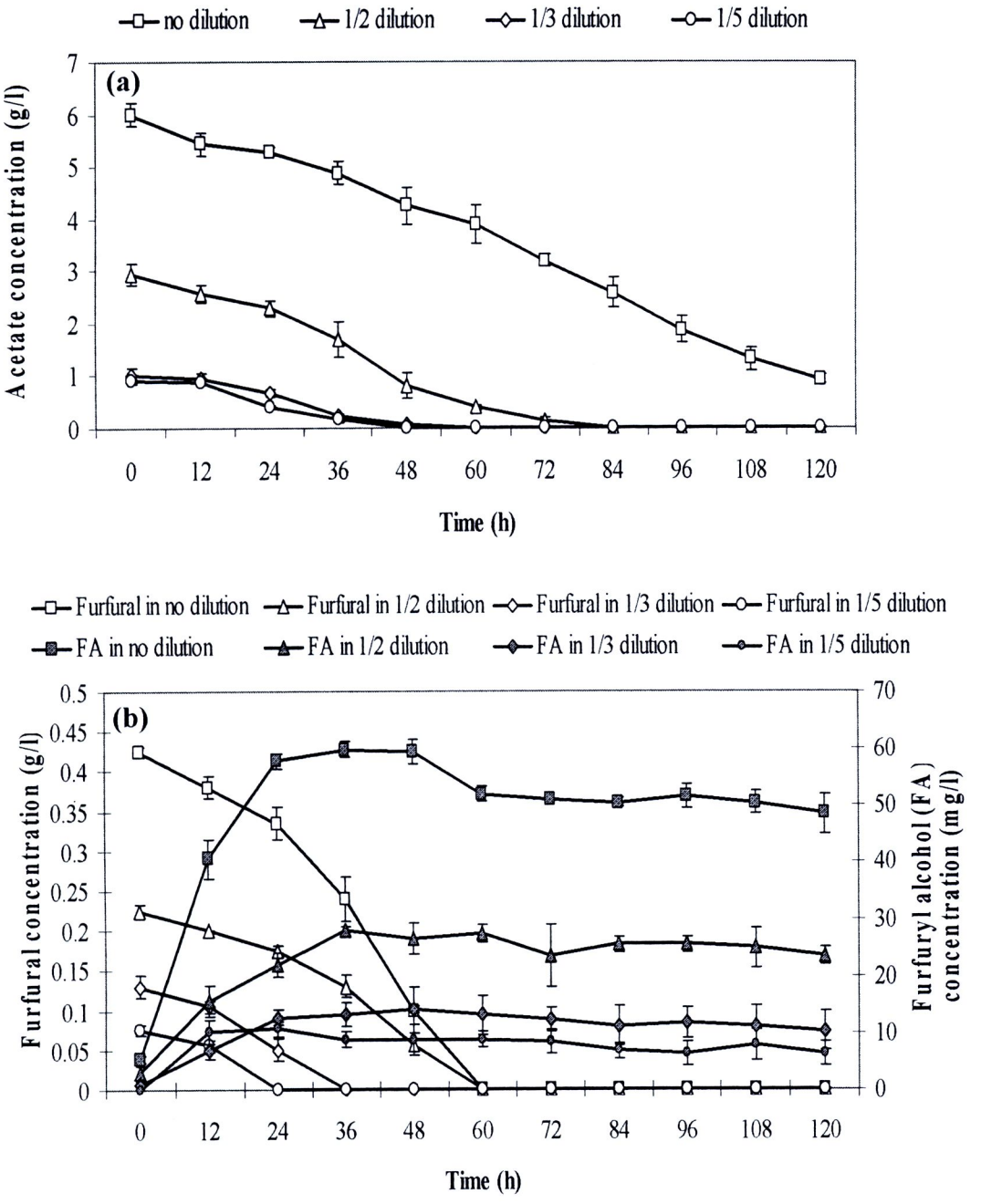

Figure 56. Time course of acetate consumption (a) and furfural reduction with furfuryl alcohol (FA) generation (b) by *Candida shehatae* TISTR5843 in various PPF hydrolysate dilutions (pH5) with the initial cell concentration of 0.725 g/l. The incubation condition was at 30°C on a rotary shaker (180 rpm).

3.10.5 Optimization of ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

3.10.5.1 Effect of initial pH, cells inoculum and shaking speed on ethanol production from PPF hydrolysate using RSM

Effect of initial pH, cells inoculum (OD_{600}) and shaking speed had been done in synthetic xylose medium. However, these parameters have to be optimized again due to the different ingredients in both synthetic xylose medium and PPF hydrolysate medium. Initial pH was varied in the range of 4-6, shaking speed was set in the range of 60-180 rpm and cells inoculum was controlled in the range of 0.9-1.5 g/l (Table 37). These experiments were operated at dilution of PPF hydrolysate medium of 1/2 dilution giving the initial xylose concentration of 13 g/l. The RSM was used a technique to find the optimal condition for maximizing ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate.

The ethanol concentration ranged from 0 to 5.15 g/l, the ethanol yield ranged from 0 to 0.40 g ethanol/g sugar and ethanol productivity ranged from 0 to 0.143 g/l/h were obtained in this study (Table 37). Low ethanol concentration (0-2.18 g/l), ethanol yield (0-0.17 g ethanol/g sugar) and ethanol productivity (0-0.061 g/l/h) were obtained using the initial pH of 4.00 and 6.00 (Table 37 at run 1-5 and run 16-17) and shaking speed of 60 rpm (run 16-17). Moderate ethanol concentration (2.93-4.65 g/l), ethanol yield (0.24-0.36 g ethanol/g sugar) and ethanol productivity (0.081-0.129 g/l/h) were obtained using the initial pH of 5.00 and 6.00 (run 6, 15 and run 18-20) and shaking speed of 60, 120 and 180 rpm (run 6, 15 and run 18-20). High ethanol concentration (5.04-5.15 g/l), ethanol yield (0.39-0.40 g ethanol/g sugar) and ethanol productivity (0.140-0.143 g/l/h) were achieved by using the moderate initial pH of 5.00 (run 7-14) and the moderate shaking speed of 120 rpm (run 7-14). However, no significance ($P<0.05$) of ethanol concentration (5.04-5.08 g/l), ethanol yield (0.39 g ethanol/g sugar) and ethanol productivity (0.140-0.141 g/l/h) were obtained using the initial pH of 5.00, shaking speed of 120 rpm and the initial cell concentration of 0.9 and 1.5 g/l (run 8 and 7). Therefore, there was no effect of the initial cell concentration in the range of 0.9-1.5 g/l on ethanol production from hemicellulosic hydrolysate by *C. shehatae* TISTR5843.

Table 37. Central composite experimental design matrix defining initial pH (X_{16}), shaking speed (rpm) (X_{17}), and initial cells concentration (g/l) (X_{18}) and results on ethanol production from hemicellulosic hydrolysate after cultivation of *Candida shehatae* TISTR5843 under 1/2 dilution factor of PPF hydrolysate medium (13 g/l initial xylose content) for 72 h at room temperature (30°).

Run	Parameter			Response (Y_{15}) Ethanol concentration (g/l)	Response (Y_{16}) Ethanol yield (g ethanol/g sugar)	Response (Y_{17}) Ethanol productivity (g/l/h)
	X_{16}	X_{17}	X_{18}			
1	4	120	1.2	0	0	0
2	4	60	0.9	0	0	0
3	4	180	0.9	0	0	0
4	4	180	1.5	0	0	0
5	4	60	1.5	0	0	0
6	5	180	1.2	4.65	0.36	0.129
7	5	120	1.5	5.08	0.39	0.141
8	5	120	0.9	5.04	0.39	0.140
9	5	120	1.2	5.13	0.39	0.142
10	5	120	1.2	5.13	0.39	0.142
11	5	120	1.2	5.15	0.40	0.143
12	5	120	1.2	5.10	0.39	0.142
13	5	120	1.2	5.15	0.40	0.143
14	5	120	1.2	5.14	0.40	0.143
15	5	60	1.2	3.89	0.31	0.108
16	6	60	0.9	2.10	0.16	0.058
17	6	60	1.5	2.18	0.17	0.061
18	6	180	0.9	2.93	0.24	0.081
19	6	120	1.2	4.42	0.35	0.123
20	6	180	1.5	3.00	0.24	0.083

To evaluate the results, the data in Table 37 were subjected to regression analysis, using the following quadratic equations (40)-(42):

$$Y_{15} = -77.97 + 30.20X_{16} + 0.04X_{17} + 2.02X_{18} + 0.003X_{16}X_{17} + 0.08X_{16}X_{18} - 0.0001X_{17}X_{18} - 2.92X_{16}^2 - 0.0002X_{17}^2 - 1.16X_{18}^2 \quad (40)$$

$$Y_{16} = -5.98 + 2.31X_{16} + 0.003X_{17} + 0.23X_{18} + 0.0003X_{16}X_{17} + 0.01X_{16}X_{18} - 0.22X_{16}^2 - 0.00002X_{17}^2 - 0.14X_{18}^2 \quad (41)$$

$$Y_{17} = -2.16 + 0.84X_{16} + 0.001X_{17} + 0.056X_{18} + 0.0001X_{16}X_{17} + 0.0025X_{16}X_{18} - 0.000008X_{17}X_{18} - 0.08X_{16}^2 - 0.00001X_{17}^2 - 0.03X_{18}^2 \quad (42)$$

The models presented the high determination coefficients (R^2 = 0.98, 0.98 and 0.98, respectively) (Table 38) explaining 98% of variability in all responses of ethanol concentration, ethanol yield and ethanol productivity. The adjusted determination coefficients (adjusted R^2 = 0.97, 0.97 and 0.97, respectively) indicated the high significance of these models (O-Thong *et al.*, 2008). In addition, the ANOVA quadratic regression demonstrated that among models were significant, as evidenced from high F-values (F=65.77, 60.90 and 64.72, respectively) with a very low probability ($P<0.0001$). Low variation coefficient value (C.V.=11.86%, 12.27% and 11.96%, respectively) indicated a high precision and reliability of the experiments (Table 38) (O-Thong *et al.*, 2008). The significance of each coefficient was determined by probability values. The variables with a significant effect on ethanol production were the initial pH (X_{16}) and shaking speed (X_{17}) ($P<0.05$). Linear term of X_{16} and quadratic terms of X_{16}^2 and X_{17}^2 were significant ($P<0.05$), demonstrating that maximizing ethanol production required a suitable value of initial pH and shaking speed.

Estimation of ethanol concentration (Y_{15}), ethanol yield (Y_{16}) and ethanol productivity (Y_{17}) over X_{16}, X_{17} and X_{18} in terms of response surfaces are shown in Fig. 57. The effect of initial pH and shaking speed was studied on ethanol production when initial cells concentration was fixed at 1.2 g/l (Fig. 57a, 57d and

57g). The maximum ethanol concentration (5.15 g/l), ethanol yield (0.40 g ethanol/g sugar) and ethanol productivity (0.143 g/l/h) were achieved at initial pH of 5.20 and shaking speed of 120 rpm whereas the minimum value (0-3 g/l, 0-0.24 g ethanol/g sugar and 0-0.081 g/l/h, respectively) was obtained at initial pH of 4.0 and 6.0, and at shaking speed of 60 and 180 rpm. These results indicated that both initial pH and shaking speed had significant effect on ethanol production as they affected directly on fermentation concerning on alcohol dehydrogenase (ADH) activity (Prior *et al.*, 1988). The optimal initial pH of 5.20 gave the highest ADH activities (Nie *et al.*, 2007). For the effect of shaking speed (optimum at 120 rpm), oxygen-limitation condition was reported to increase the specific activity of ADH up to 4-folds with the occurrence of ethanol accumulation in *C. shehatae* (Prior *et al.*, 1988). Excess oxygen is detrimental because xylose-fermenting yeasts appear to produce as well as consume ethanol at the same time (Alexander *et al.*, 1988).

The response surface plots of initial pH and initial cells concentration interaction were given in Fig. 57b, 57e and 57h when shaking speed was fixed at 120 rpm. These figures indicated that only the initial pH had a significant effect on ethanol production while the initial cells concentration had no effect. The effect of initial pH on ethanol production has been studied in *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The optimum initial pH of both strains was 4.18-6.39 (Wang *et al.*, 2008; Yu *et al.*, 2009) and 4.93 (Bandaru *et al.*, 2006), respectively, giving the highest ethanol production. The response surface plots of shaking speed and initial cells concentration interaction were given in Fig. 57c, 57f and 57i when the initial pH was fixed at 5.0. These figures illustrate that the shaking speed had a significant effect on ethanol production while the initial cells concentration had no effect. These results are similar with the report of Sreekumar et al (1999). The inoculum concentration (6 and 10 log CFU) had no effect on ethanol production by *Zymomonas mobilis* (6.57 and 5.71% w/v). However, the effect of inhibition is reduced when higher cells inocula were used (Palmqvist *et al.*, 1996). The substances give rise to a lag phase proportional to their concentration. The ethanol productivity decreased, but the maximum ethanol yield was constant (Palmqvist *et al.*, 1996).

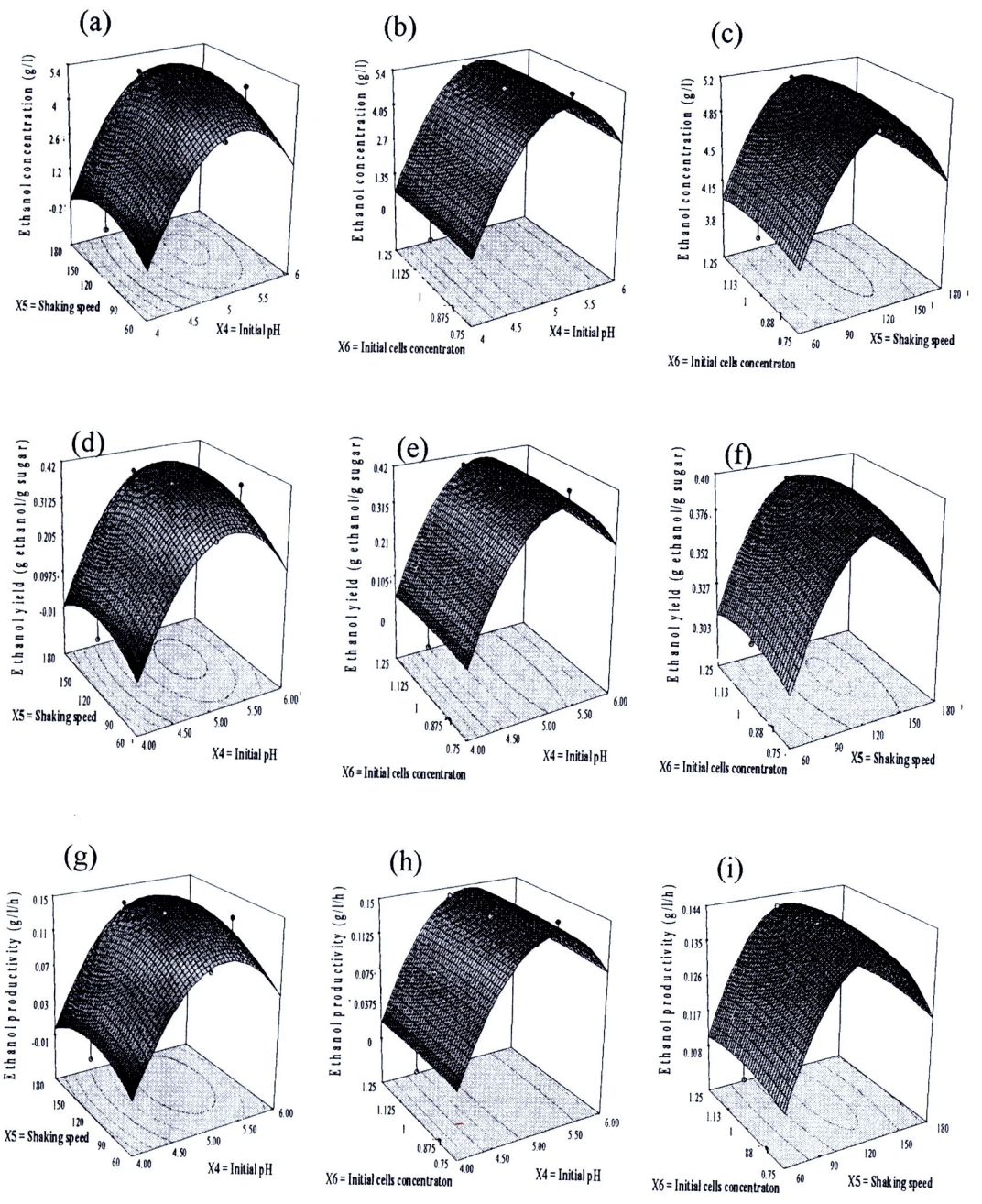

Figure 57. Three-dimensional graphs of the quadratic model of ethanol production in PPF hydrolysate by *Candida shehatae* TISTR5843: ethanol concentration (a-c), ethanol yield (d-f) and ethanol productivity (g-i) within the central composite design (CCD): a, d and g; fixed initial cells concentration at centre point of 1.2 g/l, b, e and h; fixed shaking speed at centre point of 120 rpm, c, f and i; fixed initial pH at centre point of 5.0.

Table 38. Analysis of variance (ANOVA) for ethanol production in PPF hydrolysate medium.

Responses (Y)	Source	Sum of square	Degree of freedom	Mean square	F-value	P-value
Ethanol concentration (Y_{15})	Model	85.53	9	9.50	65.77	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	11.86				
Ethanol yield (Y_{16})	Model	0.51	9	0.057	60.90	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	12.27				
Ethanol productivity (Y_{17})	Model	0.066	9	0.001	64.72	< 0.0001
	R^2	0.98				
	Adjusted R^2	0.97				
	C.V.	11.96				

3.10.5.2 Confirmation experiments and adequacy of the models of ethanol production from PPF hydrolysate

To confirm the validity of the statistical experimental strategies of ethanol production from PPF hydrolysate, three replicates of batch experiments were performed under the optimal condition calculated by RSM, containing initial pH of 5.25, shaking speed of 135 rpm and initial cells concentration of 1.08 g/l (OD_{600}=0.9). The results from confirmation experiments indicate that the experimental values of ethanol production (5.25 ± 0.72 g/l, 0.40 ± 0.02 g ethanol/g sugar, and 0.146 ± 0.011 g/l/h) were agreed with the predicted values (5.32 g/l, 0.41 g ethanol/g sugar, and 0.148 g/l/h). There was no significant difference between the experimental values and the predicted values *(P>0.05)*. The ethanol production using the initial condition (control) (Table 39) and the adjusted condition (Table 39, trial 10) were 4.12 and 5.25 g/l, respectively. After optimizing the condition of ethanol production from this hydrolysate medium, ethanol concentration, ethanol yield and ethanol productivity increased 1.27, 1.21 and 2.56 folds, respectively.

3.10.6 Effect of sterilized and non-sterilized PPF hydrolysate mediums on ethanol production by *C. shehatae* TISTR5843

Due to inhibitors contained in PPF hydrolysate medium, sterilization and non-sterilization of this medium for ethanol production have to compare in order to prevent inhibitors generation and saving of energy. This study has been controlled under optimum condition of initial pH of 5.25, shaking speed of 135 rpm, cells inoculums of 1.08 g/l (OD_{600}=0.9) (Section 3.10.5) and room temperature for 108 h. Xylose concentration of sterilized and non-sterilized PPF hydrolysate medium were consumed closely to zero by *C. shehatae* TISTR5843 within 36 h (Fig. 58a) giving maximum of ethanol production of 4.71 and 4.47 g/l, respectively (Fig. 58a). Ethanol yields and productivities of both mediums were 0.38, 0.37 g/g, and 0.131, 0.124 g/l/h, respectively. After statistical determination, there were not significant differences in both ethanol yield and ethanol productivity between sterilized and non-sterilized PPF hydrolysate medium *(P>0.1)*. Thus, non-sterilized PPF hydrolysate medium was used for production of ethanol in the scale up experiment. The pH change, acetate and furfural reduction of both mediums had the same trend (Fig. 58b and 58c).

Table 39. The confirmation experiments for ethanol production in PPF hydrolysate medium (13 g/l xylose) by *C. shehatae* TISTR5843 cultivated under the optimal condition.

Substrates	Trials	Condition	Initial pH	Shaking speed (rpm)	Initial cells concentration (g/l)	Ethanol concentration (g/l)	Ethanol yield (g/g sugar)	Ethanol productivity (g/l/h)
PPF	-	Optimal[a]	5.25	135	1.08	5.25 ± 0.72	0.40 ± 0.02	0.146 ± 0.011
hydrolysate	13	Central	5.00	120	1.20	5.13 (36 h)	0.39	0.142
medium	-	Selected	5.00	180	0.5	4.12 (72 h)	0.33	0.057

[a] Based on ethanol production.

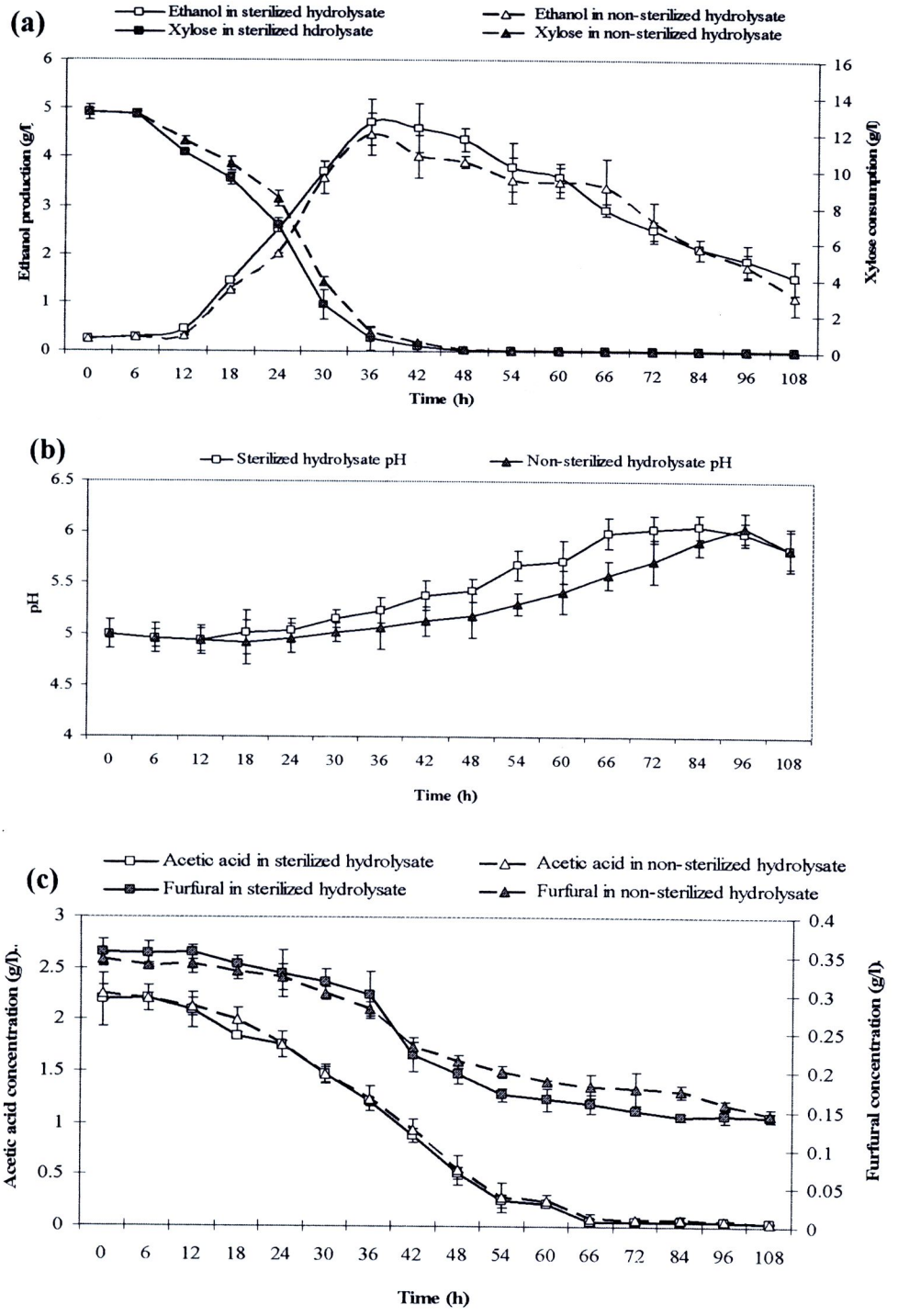

Figure 58. Time course of xylose consumption and ethanol production (a), pH change (b), and acetate and furfural reduction (c) by *Candida shehatae* TISTR5843 under sterilized and non-sterilized PPF hydrolysate medium (pH5.25) with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm).

3.10.7 Ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate medium in 3 L reactor

3.10.7.1 Comparison of ethanol production from Erlenmeyer flask and 3-L reactor using batch process

Prior to study the scale up of ethanol production in PPF hydrolysate medium by *C. shehatae* TISTR5843, comparison on ethanol production in Erlenmeyer flask (250 ml) and reactor (3 liters) have to been done in order to obtain some available data, i.e. cultivation for maximum ethanol production, cultivation for inhibitors reduction, and comparison of ethanol production between flask and reactor due to the difference of container, which is perhaps affected. In this experiment, PPF hydrolysate medium was prepared and operated under non-sterilized with 1/2 dilution with initial pH of 5.25, and agitation speed of 135 rpm. The initial cells inoculum was 1.08 g/l. The routine experiment was carried out at room temperature (30°C) on an agitation speed (135 rpm) for 120 h.

Xylose concentration of non-sterilized PPF hydrolysate medium in both flask and reactor were consumed closely to zero by *C. shehatae* TISTR5843 within 48 h giving maximum of ethanol production of 4.47 and 4.07 g/l, respectively (Fig. 59a). Ethanol yields and productivities of both systems were 0.40, 0.39 g/g, and 0.093, 0.084 g/l/h, respectively. However, the ethanol productivity was decreased from 0.124 (see section 3.10.6) to 0.084 g/l/h due to expansion of cultivation for maximum ethanol production from 36 h (see section 3.10.6) to 48 h. A longer lag phase might be from the inhibitory compounds in hydrolysate. The furfural content in this study was approximately 360 mg/l in both flask and reactor (Fig. 59d) which was higher than that furfural content in the section 3.10.6 (330-340 mg/l) resulting in longer cultivation time. Meanwhile the acetic acid content (~2200 mg/l) was constant (Fig. 59c). Moreover, there was no significant difference between flask and reactor in both pH change and DCW (P>0.05) (Fig. 59b).

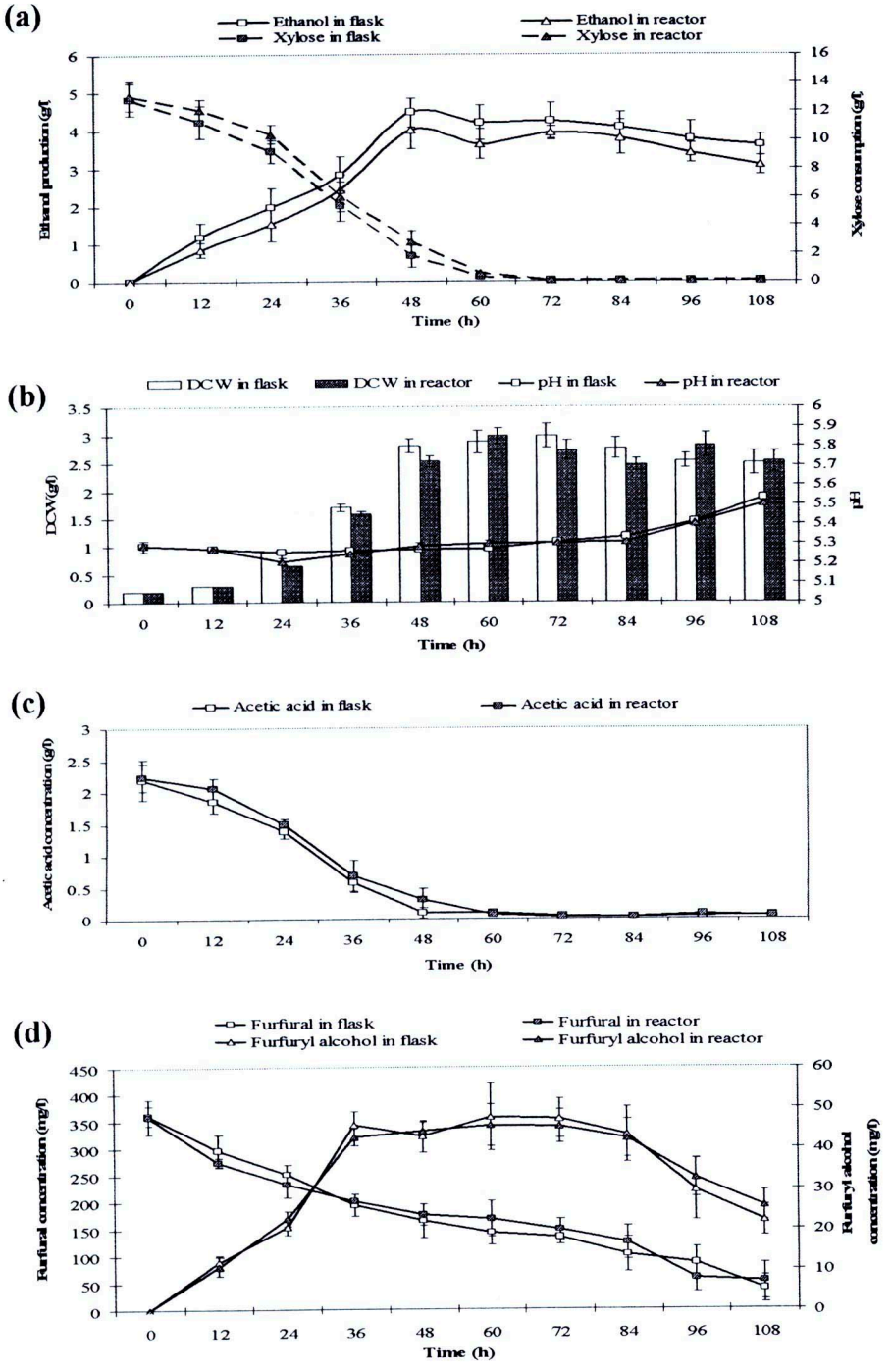

Figure 59. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), acetic acid reduction (c), furfural reduction and furfuryl alcohol production (d) by *Candida shehatae* TISTR5843 under non-sterilized with 1/2 dilution of PPF hydrolysate medium (pH 5.25) in both flask and reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm).

3.10.7.2 Ethanol production in PPF hydrolysate medium under fed-batch fermentation

To increase the ethanol production from PPF hydrolysate medium by *C. shehatae* TISTR5843, a fed-batch process was studied. Fed-batch culture is a batch culture feeding continuously or sequentially with substrate without the removal of fermentation broth. It is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins, enzymes and other compounds (Roukas, 1996). Fed-batch culture compared to the conventional batch culture has several advantages including very low concentration of residual sugars, higher dissolved oxygen in the medium (from added fresh medium), decreased fermentation time, higher productivity and reduced toxic effects of the medium components which are present at high concentrations (Roukas, 1996) as well as eliminating substrate inhibition (Ozmichi and Kargi, 2007). This work was carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The fresh medium added into the reactor in the second and next cycle has been no diluted in order to control the system was 1/2 dilution. The data from the batch process (section 3.10.7.1) were used for operating the fed-batch system including maximum ethanol production time (48 h, Fig. 59a), xylose residue and inhibitory compounds residues. The experimental kinetic values of ethanol production, cells growth and inhibitors reduction in the bioreactor during 6 days (3 cycles) of fed-batch fermentation of PPF hydrolysate medium (13 g/l of xylose) are presented in Table 40. The ethanol concentration in broth from bioreactor gave the maximal value of 3.92 g/l in the first cycle and slightly decreased in the further cycles (from 3.92 to 3.61 g/l and finally to 2.99 g/l at the last cycle (Table 40)). The cause of this phenomenon is from inhibitory compounds (furfural and acetate) presented in PPF hydrolysate medium (Fig. 60c and 60d) (Delgenes *et al.*, 1996). Fig. 60c and 60d indicated that *C. shehatae* TISTR5843 was not able to completely reduce furfural and acetate within 48 h (the first cycle) resulted in the accumulation of these toxic compounds to the next cycles (Fig. 61). Therefore, substrate uptake rate and ethanol production were decreased in the subsequent cycles (Fig. 61).

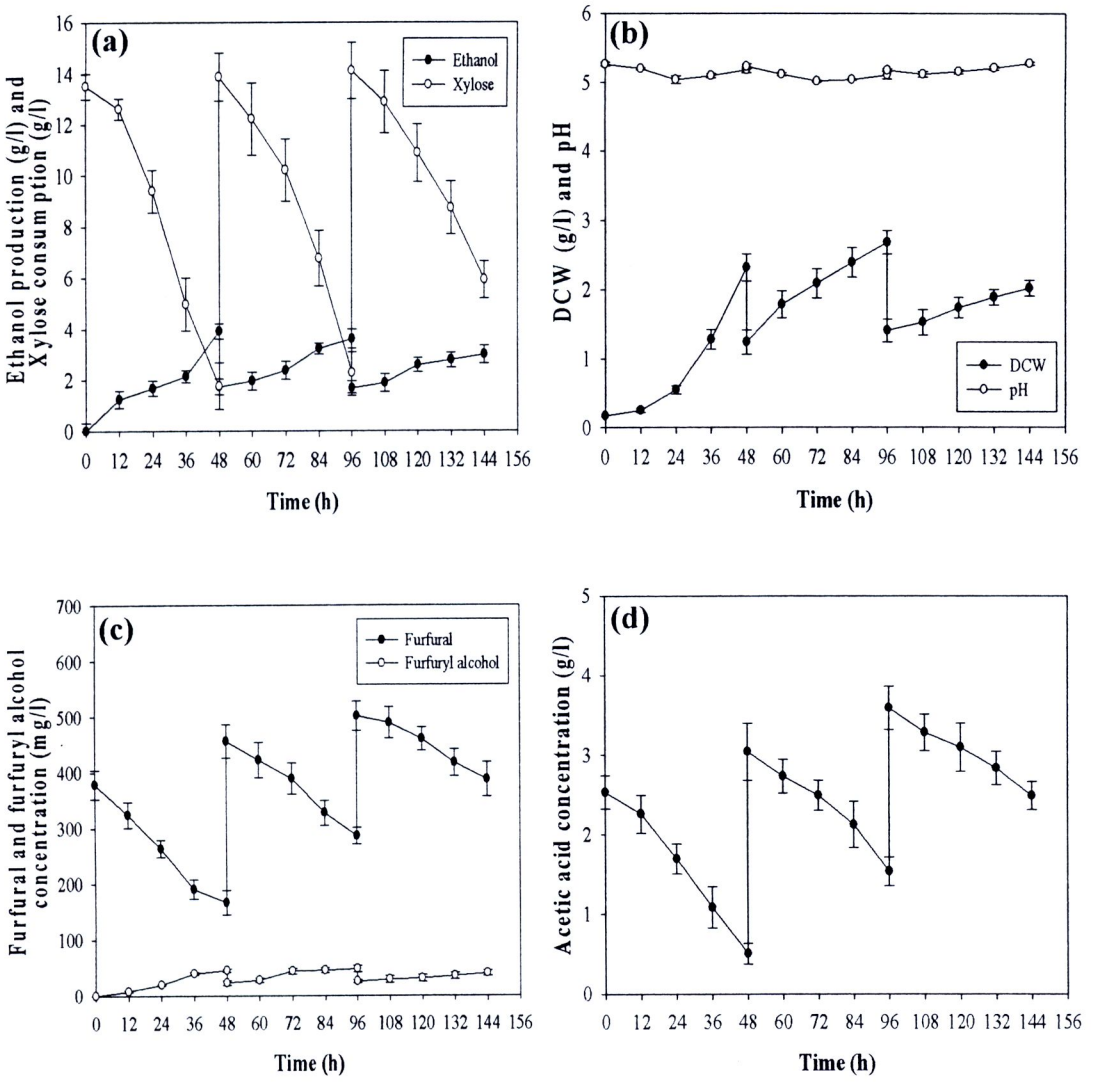

Figure 60. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), furfural reduction and furfuryl alcohol generation (c), acetic acid reduction (d) by *Candida shehatae* TISTR5843 under non-sterilized fed-batch process with 1/2 dilution (pH5.25) in 3 liters reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm) for 144 h.

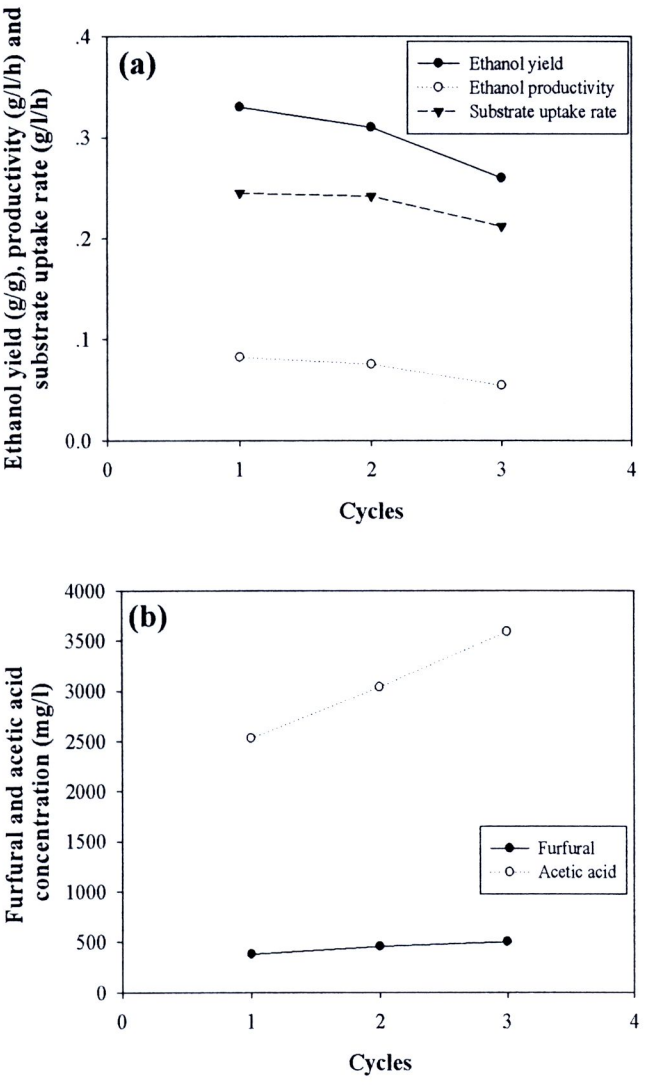

Figure 61. Reduction of ethanol production and substrate uptake rate (a) and accumulation of furfural and acetate (b) in all cycles of fed-batch fermentation by *Candida shehatae* TISTR5843 in 3 liters non-sterilized PPF hydrolysate.

Table 40. Fermentative kinetics of fed-batch and semi-continuous processes in PPF hydrolysate medium by *Candida shehatae* TISTR5843.

	Fed-Batch			Semi-continuous		
	1st	2nd	3rd	1st	2nd	3rd
Maximum ethanol (*P*, g/l)	3.92	3.61	2.99	4.02	3.71	2.79
Ethanol yield (*Yp/s*, g/g)	0.33	0.31	0.26	0.36	0.34	0.20
Substrate uptake rate (*Qs*, g/l.h)	0.245	0.242	0.212	0.232	0.229	0.181
Ethanol productivity (*Qp*, g/l.h)	0.082	0.075	0.054	0.084	0.077	0.058
Conversion of substrate to ethanol (%)	86.98	83.57	72.24	85.06	79.82	63.88

3.10.7.3 Ethanol production in PPF hydrolysate medium under semi-continuous fermentation

When a portion of the fermentation broth is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next batch culture, the system is operated as a repeated fed-batch culture or semi-continuous culture. In addition to increased productivity, semi-continuous culture has the advantages which are (i) it doesn't require new inocula for each consecutive fed-batch and (ii) the contamination of the medium is lower than in the continuous culture. Thus semi-continuous culture is considered one of the most useful systems for economical ethanol production (Roukas, 1996). This work was also carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium.

The experimental kinetic values of ethanol production in the bioreactor during 6 days (3 cycles) of semi-continuous fermentation of PPF hydrolysate medium (13 g/l of xylose) are presented in Table 40. The ethanol concentration in broth drained from bioreactor gave the maximal value of 4.02 g/l in the first cycle and slightly decreased in the further cycles (from 4.02 to 3.71 g/l and finally to 2.79 g/l at the last cycle (Table 40)). The causes of this phenomenon were described in the previous section 3.10.7.2 and shown in Fig. 62 and 63.

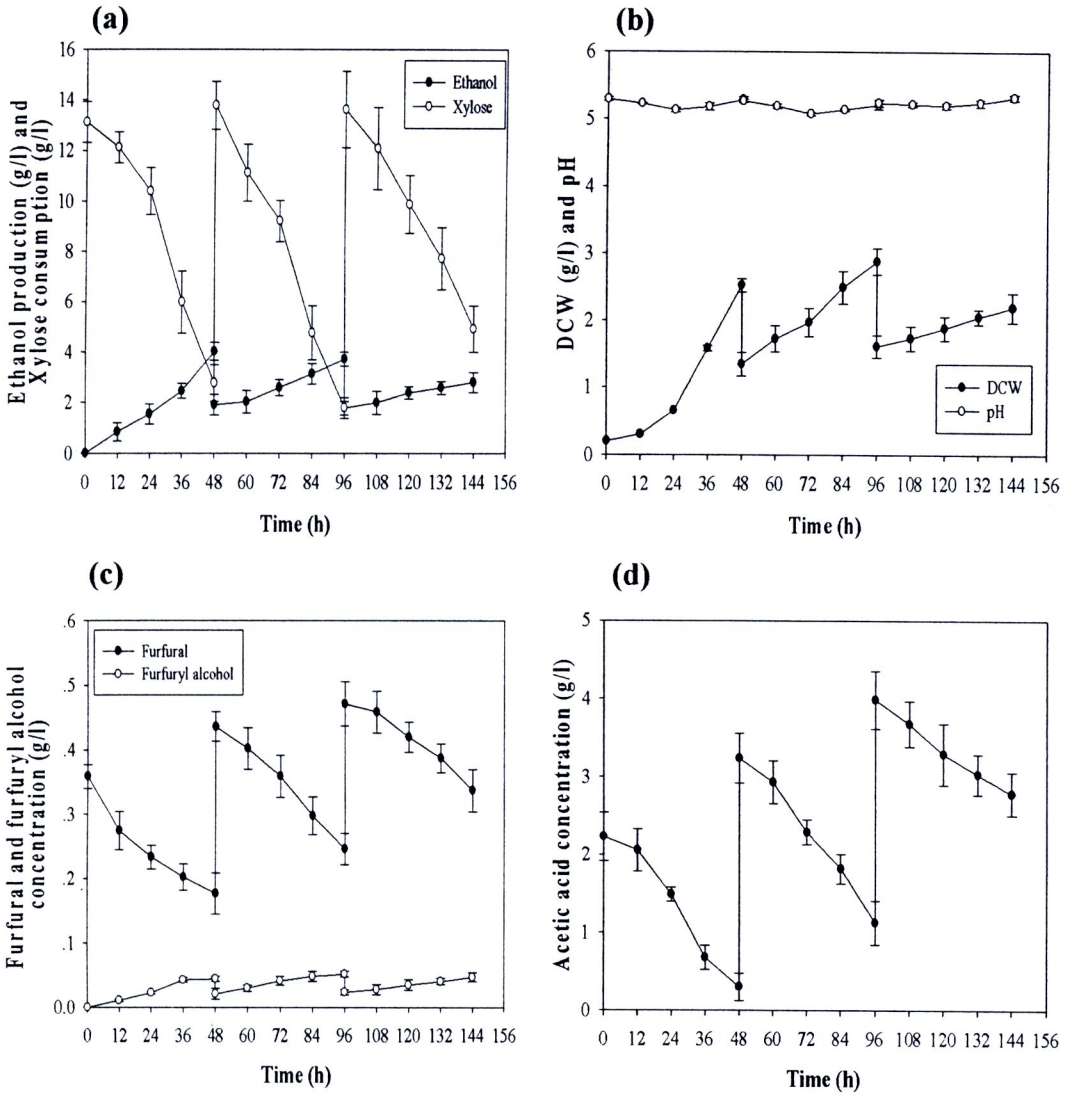

Figure 62. Time course of xylose consumption and ethanol production (a), pH change and DCW (b), furfural reduction and furfuryl alcohol generation (c), acetic acid reduction (d) by *Candida shehatae* TISTR5843 under non-sterilized semi-continuous process with 1/2 dilution of PPF hydrolysate medium (pH5.25) in 3 liters reactor with the initial cell concentration of 1.08 g/l. The incubation condition was at 30°C on an agitation speed (135 rpm) for 144 h.

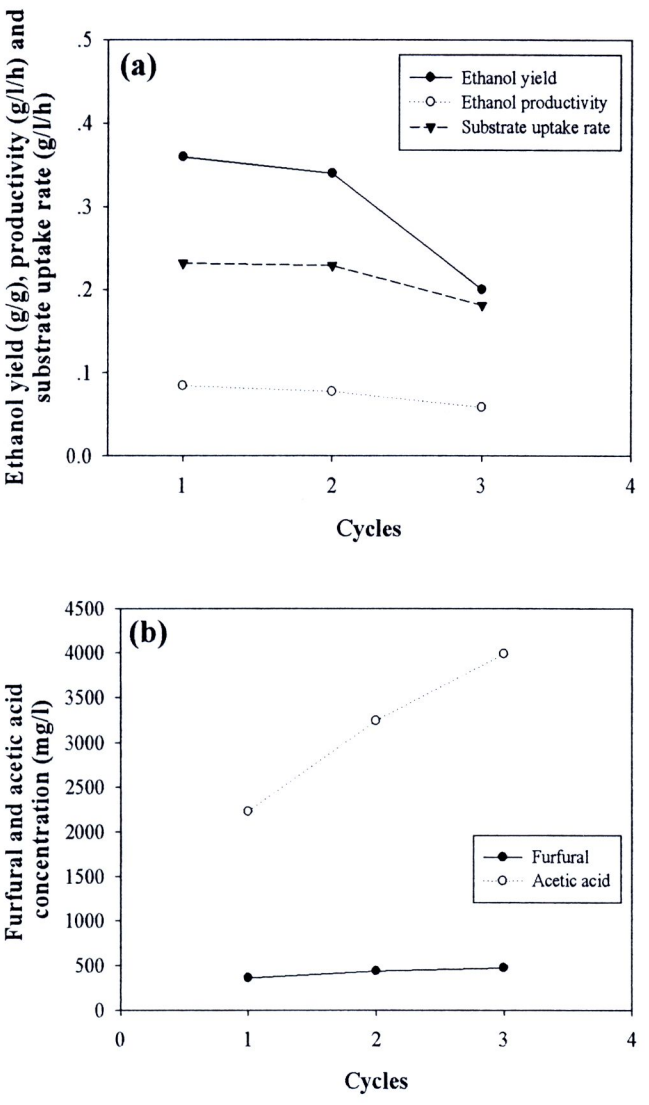

Figure 63. Reduction of ethanol production and substrate uptake rate (a) and accumulation of furfural and acetate (b) in all cycles of semi-continuous fermentation by *Candida shehatae* TISTR5843 in a 3 liters non-sterilized PPF hydrolysate.

3.11 Potential of ethanol production by immobilized yeast cells on PPF as a natural support

3.11.1 Immobilization of yeast cells on PPF and delignified PPF (dPPF)

A limitation of the operational stability, which is a cell desorption in immobilization system has been an issue (Yu *et al.*, 2007). Scanning electron microscopic technique was used as a tool to study the yeast cells adsorption in sPPF, lPPF and sDPPF (Fig. 64). The lPPF is fibrous and non porosity form (Fig. 64a). After milling process, the surfaces area and porosity increased in sPPF as well as in sDPPF (Fig. 64b and 64c). The yeast cells were observed on the outer surface of lPPF only (Fig. 64d); however, a population of yeast cells was observed on all surfaces of sPPF and sDPPF (Fig. 64e and 64f). The porous structure of sPPF and sDPPF increased mass transportation which resulted in high biomass concentration in the range of 3.71- 4.20 g DCW/l (Fig. 64d-64f).

3.11.2 Kinetic analysis of immobilization for ethanol production in the batch fermentation

The kinetic values were investigated with respect to the use of PPF as a natural support for ethanol production by *C. shehatae* TISTR5843 (Table 41). The maximum ethanol production (P_{max}) was observed at 36 h cultivation by immobilized yeast cells on lPPF, sPPF and sDPPF with the values of 10.7, 11.3 and 11.5 g l^{-1}, respectively (Fig. 65 and Table 41). The P_{max} values of immobilized cells on sPPF and sDPPF increased 4.2 and 6.2%, respectively; however, this value of immobilized cells on lPPF was not significantly different from the value of thoes free cells ($P<0.05$). The results are similar to the study of Behera et al (2010) which reported the ethanol productions by yeast cells entrapped in Ca-alginate was higher than free cells. The substrate uptake rate (Qs) of immobilized cells on sPPF and sDPPF were higher than that of free cells with the values of 5.25 and 5.46%, respectively. Similarly, the ethanol yield (Yp/s) obtained from immobilized cells was higher than that of free cells with the values of 4.55 and 6.82%, respectively. Correspondingly, the ethanol productivity (Qp) and sugar consumption (%) of immobilized cells were slightly more than to that of free cells. All cell concentrations were rapidly increased within 30 h cultivation and slightly increased thereafter due to the limitation of carbon source

(Fig. 65c). Generally, the performance of ethanol production by immobilized cells is much better than free cells because the immobilization system protect cell from ethanol inhibition (Abbi *et al.*, 1996).

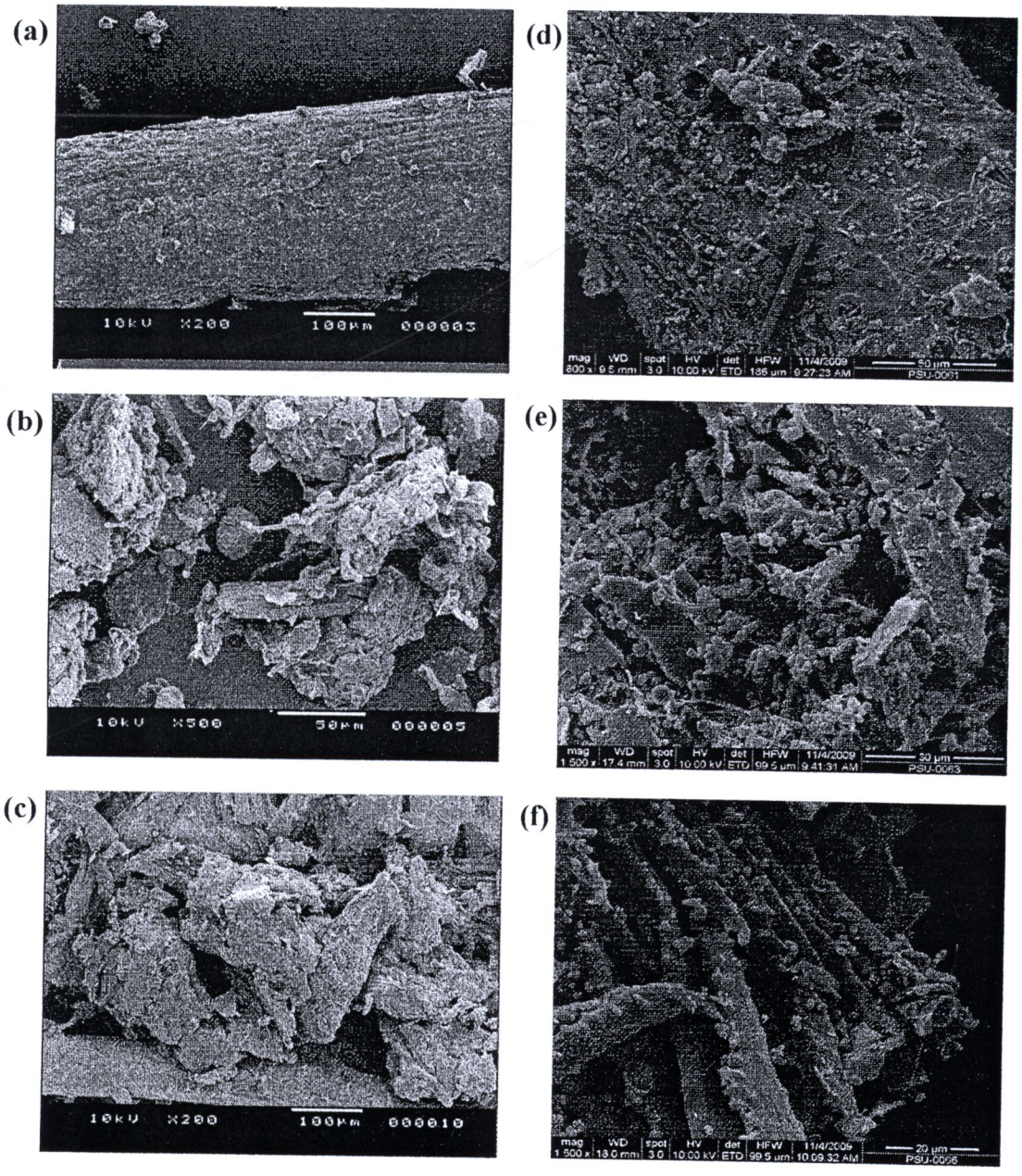

Figure 64. Scanning electron micrograph of (a) lPPF, (b) sPPF and (c) sDPPF; and immobilized cells adhere to the surface of (d) lPPF, (e) sPPF and (f) sDPPF.

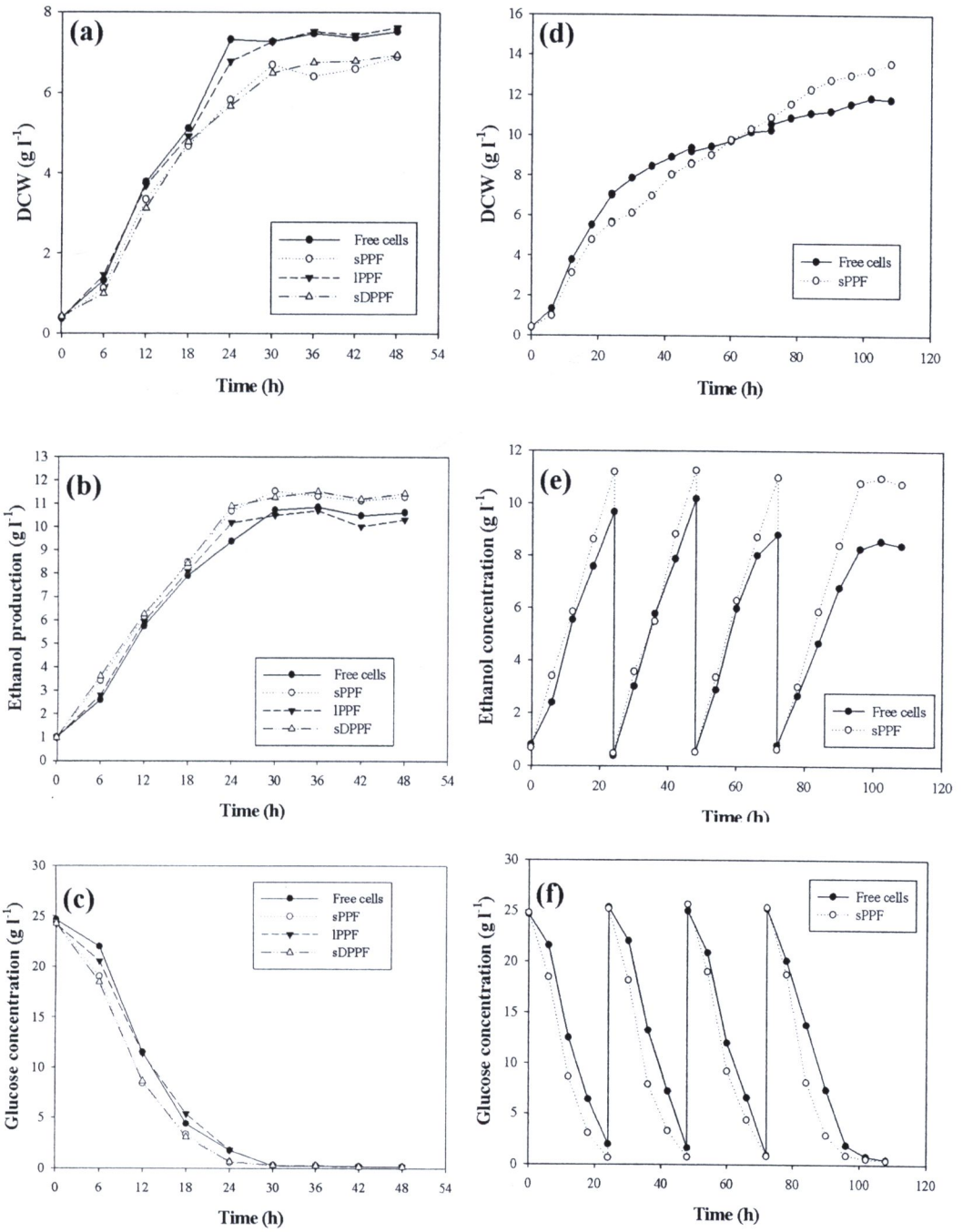

Figure 65. Kinetics of ethanol production by free and immobilized cells of *Candida shehatae* TISTR5843 in batch fermentation, in panel (a) cell growth, (b) ethanol production and (c) glucose consumption; and in repeated batches fermentation, in panel (d) cell growth, (e) ethanol production and (f) glucose consumption at room temperature (30°C) with shaking speed 150 rpm.

The ethanol yields by free and immobilized cells obtained from this study consist with theoretical value (Table 41). The sPPF was chosen as a support in further study of repeated batches fermentation because the ethanol yield was not significantly different compared to ethanol production by immobilized on sDPPF ($p<0.05$). Also, sDPPF is concerned in the sector of environment and economic because PPF need to be treated by chemical (delignification).

Table 41. Growth and fermentation kinetics of free and immobilized cells by *Candida shehatae* TISTR5843 adhered on various supports in batch fermentation.

	Free cells	Immobilized cells on		
		lPPF	sPPF	sDPPF
Maximum ethanol production (P_{max}, g ethanol/l)	10.8[a]	10.7[a]	11.3[b]	11.5[b]
Maximum cells concentration (X_{max}, g DCW/l)	7.52	7.62	6.89	6.93
Specific growth rate (μ, h^{-1})	0.02	0.02	0.02	0.02
Cell yield (*Yx/s*, g DCW/g glucose)	0.31	0.31	0.28	0.29
Ethanol yield (*Yp/s*, g ethanol/g glucose)[c]	0.44[a]	0.44[a]	0.46[b]	0.47[b]
Substrate uptake rate (*Qs*, g glucose/l/h)	0.95	0.93	1.00	1.00
Ethanol productivity (*Qp*, g ethanol /l/h)	0.30	0.30	0.31	0.32
Glucose consumption (%)[d]	98.2	98.0	99.1	99.0

All cases were performed in triplicate; values varied less that 10%.

[a] and [b] are significant difference at $p<0.05$.

[c] $$Ethanol\ yield\ (g\ ethanol\ g\ glucose^{-1}) = \frac{Ethanol\ production\ (g\ l^{-1})}{Glucose\ consumption\ (g\ l^{-1})}$$

[d] $$Glucose\ consumption\ (\%) = \frac{Glucose\ consumption\ (g\ l^{-1})}{Glucose\ content\ in\ medium\ (g\ l^{-1})} \times 100$$

3.11.3 Kinetic analysis of immobilization for ethanol production in the repeated batch fermentation

To increase ethanol productivity and retain the ethanol yield by recycling the cells, the repeated batches fermentation was studied. The free and immobilized cells were recycled into a fresh medium every 24 h. The cultivation conditions were pH 5.0 at room temperature (30°C) with an agitation speed of 150 rpm. A comparison of ethanol production by free and immobilized cells in four repeated batch experiments was shown in Fig. 65. The ethanol concentrations, ethanol yields and ethanol productivities of free and immobilized cells increased in repeated batches fermentation in the range of 10.78-30.12%, 9.52-22.22% and 11.90-32.35%, respectively (Table 42).The ethanol yield of immobilized cells decreased 9.52 % after the second cycle and 6.38% at the forth cycle (Table 42). It has been reported that ethanol production by immobilized cells on spent grain and delignified spent grain in four repeated batches process decreased 22.81% and 15.08%, respectively, (Kopsahelis *et al.*, 2007). Ethanol production by immobilized *C. shehatae* NCL-3501 was constant for 3 cycles and decreased thereafter (Abbi *et al.*, 1996).

Both ethanol productivity and ethanol yield of immobilized cells were greater than free cells. There is an evidence supported that the significantly higher percentage of saturated fatty acids in immobilized cells leads to the grater ethanol tolerance, greater yeast cells survival and greater ethanol productivity compared to free cells (Krisch and Szajáni, 1997). In addition, immobilized cells can retain enzyme activities for a long time because of the physiological changes in cells which affect the cell compositions such as proteins, lipids, RNA, DNA, and inorganic substances (Yu *et al.*, 2007).

In conclusion, the pretreatment of PPF, milling and delignification increase surface area of natural support, which enhances cell adsorption and ethanol production. The immobilized yeast demonstrates the operational stability without decrease its activity. Therefore, PPF has a potential as a natural supporting material in the immobilization system.

Table 42. Fermentative kinetics of free and immobilized cells by *Candida shehatae* TISTR5843 adhered on sPPF in four repeated batches fermentation.

	Free cells				Immobilized cells on sPPF			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Maximum ethanol production (P_{max}, g ethanol l^{-1})	9.7	10.2	8.8	8.3	11.2	11.3	11.0	10.8
Ethanol yield (*Yp/s*, g ethanol g glucose^{-1})	0.42	0.42	0.38	0.36	0.46	0.47	0.44	0.44
Substrate uptake rate (*Qs*, g glucose $l^{-1}h^{-1}$)	0.94	0.99	1.00	0.97	1.00	1.02	1.03	1.02
Ethanol productivity (*Qp*, g ethanol $l^{-1}h^{-1}$)	0.40	0.42	0.37	0.34	0.47	0.47	0.46	0.45
Glucose consumption (%)	92.0	93.6	96.4	92.5	97.4	97.1	96.8	96.5

All cases were performed in triplicate; values varied less that 10%.