CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Palm pressed fiber

Palm pressed fiber (PPF) was kindly provided by Thai-Taro & Oil, Co., Ltd., Suratthanee Province, Thailand.

1.2 Microorganisms and fermentation condition

1.2.1 *Zymomonas mobilis* TISTR405

Z. mobilis TISTR405 was purchased from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. For short-term storage, stock cultures were stored on Zymomonas medium agar at 4°C and subcultured every 3-4 weeks. For long-term storage, the cultures were suspended in sterile 40% (v/v) glycerol and stored in 1 ml volumes at -70°C. When required, individual aliquots were thawed rapidly and regenerated at 30°C in liquid Zymomonas medium (Davis *et al.*, 2006).

Seed culture of *Z. mobilis* TISTR405 was prepared by liquid Zymomonas medium (pH 5.5) and incubated at 30°C. Fermentation was started with 10% (v/v) inoculum at an absorbance of 0.5 at 660 nm which indicated cells in the exponential growth phase (Davis *et al.*, 2006).

1.2.2 *Candida shehatae* TISTR5843

C. shehatae TISTR5843 purchased from TISTR, Thailand, was maintained on yeast-malt (YM) agar slant. Stock cultures were stored at 4°C and subcultured every 3-4 months (Chandel *et al.*, 2007; Lebeau *et al.*, 2007).

The inoculum of *C. shehatae* TISTR5843 was prepared by harvesting the cells grown for 24 h at 30°C in the culture medium containing 15.0 g/l glucose and a cell suspension was prepared in sterile water containing 0.85% NaCl. The cell number was maintained by adjusting the OD at 600 nm in the range of 0.6–0.8 (corresponding to dry weight of cell mass 0.8–0.95 g/l) (Appendix E). Each 250 ml

Erlenmeyer flask containing 100 ml working volume was inoculated with 10% (v/v) of starter and incubated at 30°C on a rotary shaker (150 rpm) (Chandel *et al.*, 2007).

1.2.3 *Saccharomyces cerevisiae* TISTR5017

S. cerevisiae TISTR5017 obtained from Microbiological Laboratory, Faculty of Agro-Industry, Prince of Songkla University, Thailand, was stocked on YM agar (Lebeau *et al.*, 2007), kept at 4°C and subcultured every 2 weeks.

The inoculum of *S. cerevisiae* TISTR5017 was prepared as described above for *C. shehatae* TISTR5843 (Chandel *et al.*, 2007; Lebeau *et al.*, 2007).

1.3 Media

Yeast malt (YM) agar contained (g/l): malt extract 5.0, glucose 10.0, yeast extract 3.0, peptone 5.0, agar 20, and pH 6.4 (Chandel *et al.*, 2007; Lebeau *et al.*, 2007).

Zymomonas medium contained (g/l): glucose 20, yeast extract 10 bacteriological peptone 10 and agar 15 (Davis *et al.*, 2006).

1.4 Enzyme

The liquid cellulase, Accellerase 1000, from *Trichoderma reesei* was kindly provided by Danisco US Inc. (Genencor Division). Carboxymethyl cellulose (CMC) (Sigma-Aldrich) was used as a substrate for determination of cellulase (CMCase) activity. Cellulose-PPF, obtained from delignification of PPF, was a substrate for glucose production.

1.5 Chemicals and reagents

All chemicals and reagents employed were of analytical or reagent grade and commercial grade. Their lists and sources were shown in Appendix A

1.6 Instruments

Most of the instruments used in this work were in Faculty of Agro-Industry, and some equipments were supported by Scientific Equipment Center (SEC), Prince of Songkla University, Thailand. All lists and sources of instrument are given in Table 8.

Table 8. List of some instruments.

Instrument name	Model	Source
Autoclave	SX-700	Tomy, USA
Balance	BP 221S	Sartorius, Germany
Bio-safety cabinet	25 MANOMETER	DWYER Instruments Inc., USA
Centrifuge	UNIVERSAL 32R	Hettich Zentrifugen
Fermentor	MDL 300	B.E. Marubishi, Japan
Gas chromatography (GC)	GC Model 6850	Hewlett-Packard, USA
High performance liquid chromatography (HPLC)	HPLC Model 1100	Hewlett-Packard, USA
Hot air oven	ULM 500	Memmert, USA
Micro centrifuge	Centrifuge A 14	Jouan, France
pH meter	Delta 320	TOLEDO, China
Rotary Evaporator	SB 651	Rikakai Co.LTD., Tokyo
Scanning electron microscope	Quanta400	
Shaker	SK3-PO SSeriker II	Welsource CO.,LTD
Spectrophotometer	Anthos Zenyth 200rt	Anthos labtec instruments, Austria
Water bath	MP	Judabo, Perkin-elmer (Thailand) Co., LTD

2. Analytical methods

2.1 Sugars analysis

2.1.1 Qualitative method

The sugar compositions of palm pressed fiber (PPF)'s hemicellulose were determined by Thin Layer Chromatography (TLC). TLC Silica gel 60 F_{254} aluminum sheet (Merck) employing isopropyl alcohol, ethyl acetate and water (3:3:1) were used. N-(1-napthyl)-ethylenediamine (0.3 g in 5 ml of conc. H_2SO_4 and 100 ml of methanol) was a spray dye. Standard sugars were arabinose, rhamnose, xylose,

fructose, galactose, glucose and mannose (50 mg/ml of each sugar) (Bandaiphet, 2007). The hemicellulose extracted from PPF were digested by 5N TFA at 120°C for 90 min (Marzialetti *et al.*, 2008). Hydrolysates and standard sugars were spotted on TLC, sprayed with N-(1-napthyl)-ethylenediamine, and then dried at 103°C for 20 min. Colors and R_f values of standard sugars were considered to estimate the sugar type and constituents of these hydrolysates.

2.1.2 Quantitative method

2.1.2.1 High performance liquid chromatography (HPLC)

The sugars were determined by HPLC (Hewlett-Packard, Agilent 1100, USA) with Zorbax NH_2 column (4.6×250 mm, 5-Micron, Agilent, USA) combined with RI detector. Mobile phase was acetonitrile and water in the ratio of 75:25 v/v, operating temperature was controlled at 25°C and flow rate was set at 0.7 ml/min. Standard sugars were run in the same condition (Rahman *et al.*, 2006).

2.1.2.2 Somogyi-Nelson method

Residual reducing sugar was determined by Somogyi-Nelson method as described in Appendix B (Somogyi, 1952; Nelson, 1944).

2.2 Enzyme assay

Cellulase enzyme solution and CMC (1%) were prepared in sodium acetate buffer (0.05 M, pH 4.8). 0.5 ml of 1% CMC was incubated with 0.5 ml of enzyme solution at 50°C for 30 min. The reaction was terminated by boiling for 10 min (Sharma *et al.*, 2001). The reducing sugar produced as a result of CMC hydrolysis was determined by the Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944). One enzyme unit (U) liberates 1 μmol of reducing sugar per minute at 50°C, pH 4.8 and for 30 min. The saccharification values were calculated by using the formula (Eq. 1) given by Sharma et al. (2004) and Chen et al. (2008):

$$Saccharification\ value\ (\%) = \frac{\text{Re}ducing\ sugar\ (g) \times 0.9}{Polysaccharides\ in\ substrate\ (g)} \times 100 \quad \ldots\ldots\ldots (1)$$

2.3 Ethanol, furfural and acetate determination

Ethanol, furfural (or furaldehyde) and acetate were analyzed by GC-FID (HP 6850, Hewlett Packard). The Stabilwax®-DA column and operating condition were modified from Suwansaard *et al.*, 2009. Briefly, flow rate of helium was 1.2 ml/min. The temperatures of injection port and detection port were 230°C and 250°C, respectively. Injection volume was 1 µl. The initial temperature of oven was 70°C for 1 min followed with a ramp of 20°C/min to final temperature of 180°C and then hold for 2 min.

2.4 Protein estimation

The protein was determined by Folin's method using bovine serum albumin as a standard protein (Appendix C) (Chapla *et al.*, 2010).

2.5 Electron microscopic scanning

The samples were soaked in 3.5% glutaraldehyde for 6 h, and dried by treatment with 50, 70, 90, 95, and 100% ethanol, followed by overnight retention of samples in a desiccator for the removal of moisture. The samples were then detected by a scanning electron microscope (SEM) (Quanta400, FEI) (Yu *et al.*, 2007).

3. Methods

This research aimed to produce value added products from palm press fiber (PPF) such as hemicelluloses, furfural, xylose, cellulose, glucose, and ethanol. All experiments were carried out as outline in Fig. 11.

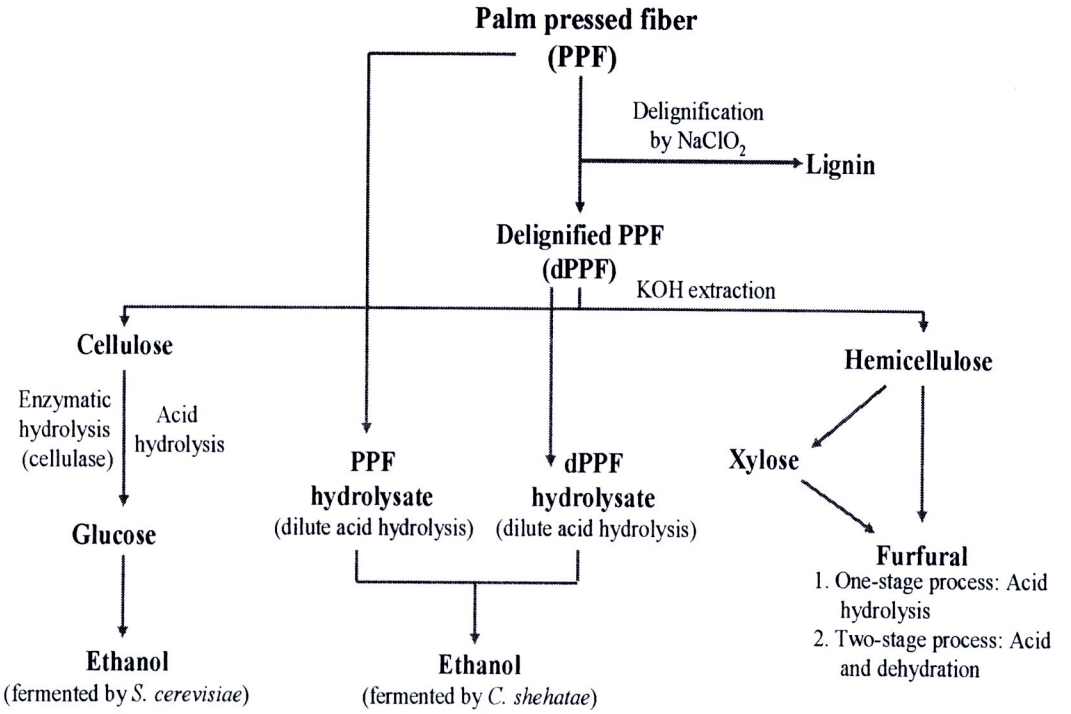

Figure 11. Flow chart of this research work.

3.1 PPF preparation and its composition

The PPF was sun-dried and ground to a particle size of <0.5 mm by sieve cut off (35 mesh analytical sieve, Fritsch, Germany). After oven-dried at 103°C overnight, the PPF powder was kept in a plastic bag and stored at room temperature (30°C). Prior to pretreatment, composition of PPF was analyzed, according to the standard methods (A.O.A.C., 1990).

3.2 Delignification process

PPF to sodium chlorite ($NaClO_2$) in the ratio of 10:1 (w/w) was dissolved in 0.01% acetic acid solution, at 70°C for 1 h and repeated 3-4 times (Collings *et al.*, 1978). The delignified PPF (dPPF) was then separated by centrifugation (5,720 x *g* for 20 min) and twice washed with warm water (< 60°C in order to prevent autohydrolysis of C5 sugars), then centrifuged again, and finally added acetone to remove water. After incubation at 45°C overnight, the amount of the

dPPF was calculated using the equation (2). The composition of PPF and dPPF were analyzed according to the standard methods (A.O.A.C., 1990).

$$Delignified\ PPF\ (\%) = \frac{Weight\ of\ Delignified\ PPF\ (g)}{Weight\ of\ PPF\ (g)} \times 100 \quad \text{.....(2)}$$

3.3 Extraction and optimization of hemicellulose by alkaline hydrolysis

3.3.1 Procedure of hemicellulose extraction by alkaline hydrolysis

The dPPF was mixed with potassium hydroxide (KOH) solution and kept in ice waterbath with shaking to obtain the highest efficiency of extraction (Aquino *et al.*, 2002). After centrifugation (7,244 x *g* for 30 min), the supernatant was neutralized with 5% (v/v) acetic acid and then hemicellulose was precipitated by adding 95% ethanol in the ratio of 1:1, v/v (Prasertsan and Oi, 2001). The hemicellulose after centrifugation (7,244 x *g* for 10 min) was then freeze dried. The hemicellulose yield was calculated using Eq. (3) (A.O.A.C., 1990) and the experimental protocol is illustrated in Fig. 12.

$$Hemicellulose\ (\%) = \frac{Weight\ of\ hemicellulose\ (g)}{Weight\ of\ PPF\ (g)} \times 100 \quad \text{.....(3)}$$

3.3.2 Hemicellulose component

The sugar composition of hemicellulose was determined after hydrolysis with 5% (v/v) sulfuric acid at 120°C for 30 min (Rahman *et al.*, 2006), the supernatant was neutralized (2 N NaOH) and filtered through 0.2 μm syringe filter, then analyzed by HPLC as described in Section 2.1.2.1.

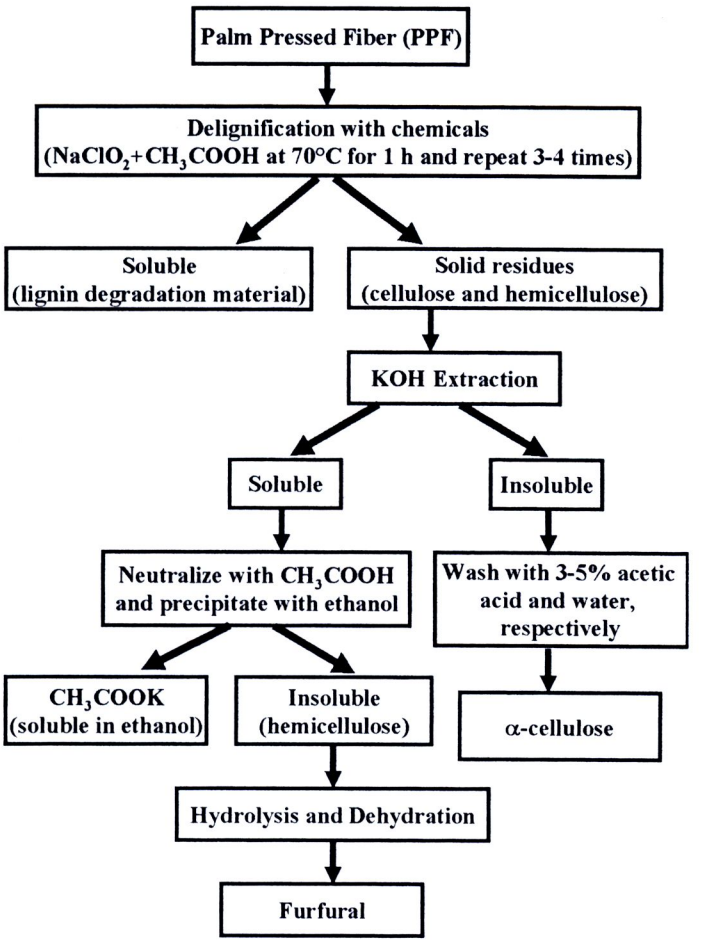

Figure 12. Experimental protocol used to determine the yields of hemicellulose and furfural.

3.3.3 Optimization of hemicellulose extraction by using RSM

Three parameters affecting KOH hydrolysis; KOH concentration (10-50% w/v), ratio of delignified PPF to KOH (1:20-1:50 (w/v)) and reaction time (20-60 min) were studied. In order to describe these parameters (as the independent variables) on hemicellulose production (as the dependent variable), 20 batch experiments were conducted by central composite designs (CCD). The concentration ranges were 10-50% w/v KOH (central value = 30% w/v), the PPF to KOH ratio of 1:20-1:50 (w/v) (central value = 1:35), and reaction time of 20-60 min (central value = 40 min). 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). The variables are coded according to Eq. (4) whereas a

quadratic model (Eq. (5) (Box *et al.*, 1978; Sangkharak and Prasertsan, 2007; O-Thong *et al.*, 2008) was used to evaluate the optimization of environmental parameters and stepwise regression analysis (Rahman *et al.*, 2007).

$$\overline{Xi} = \frac{Xi - Xi^*}{\Delta Xi}, \quad i = 1, 2, 3,..., k \quad ..... \quad (4),$$

where $\overline{Xi}$ is the code value of an independent variable, Xi is a real value of an independent variable, Xi^* is the real value of an independent variable at the center point of the experiment, and ΔXi is the step change value.

$$Y_1 = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad ....(5),$$

where Y_1 is the expected response value predicted from RSM of hemicellulos ; β_i, β_j and β_{ij} are the parameters estimated from regression results. The response variable (Y_1) was fitted using a predictive polynomial quadratic equation (5) in order to correlate the response variable to the independent variables. Regression analyze of hemicellulose was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA) (trial version). The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the F-test. The confirmation of this experiment was conducted by the Design Expert v. 7.

3.4 Production and optimization of furfural from extracted hemicellulose

3.4.1 Production of furfural by one-stage process

The extracted hemicellulose was then used for production of furfural using one-stage process. This experiment was carried out in COD test tube and heated by COD heating box. Amount of substrate (hemicellulose extracted from dPPF) used in this research was 0.5 g. The reaction temperature was tested at 120 and 150°C, the sulfuric acid concentration at 5 and 10% (v/v), the 3% (v/v) sulfuric acid to hemicellulose ratio (liquid to solid ratio, LSR) was 1:8 and 1:10 (w/v), and the reaction time of 30-120 min was studied. Furfural was analyzed by gas chromatography (GC-FID) as described in Section 2.3.

3.4.2 Production of furfural by two-stage process

3.4.2.1 Optimization of hydrolysis process by RSM

PPF was delignified by using sodium chlorite and acetic acid (Collings *et al.*, 1978) and hemicellulose was extracted (as described above). Hemicellulose was then used for production of furfural using two-stage process. Each stage of furfural production was optimized independently. This experiment was carried out in flask and heated by hot air oven. Amount of substrate (hemicellulose extracted from dPPF) used in this research was 10 g. In the first stage process or xylose production process, 30 batch experiments were also conducted by the central composite design (CCD). The range of reaction temperature (X_1) was 100-150°C (central value = 125°C), the sulfuric acid concentration (X_2) was ranged from 1-10% v/v (central value = 5.50% v/v), sulfuric acid to hemicellulose ratio (liquid to solid ratio, L/S ratio) (X_3) was ranged from 8- 10 (v/w) (central value = 1:9 v/w), and the reaction time (X_4) of 30-120 min (central value = 75 min) was studied. The variables are coded according to Eq. (4) whereas a quadratic model (Eq. (5)) was used to evaluate the optimization of environmental parameters and stepwise regression analysis (Rahman *et al.*, 2007). Data analysis was calculated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA) (trial version) as described in Section 3.3.3.

3.4.2.2 Optimization of dehydration process by RSM

For the second stage or furfural production process, sulfuric acid concentration was fixed at the same acid concentration obtained from hydrolysis process. In this study, 13 batch experiments were conducted by CCD. Reaction temperature was ranged from 120-160°C (central value = 140°C) and reaction time at 30-150 min (central value = 90 min) was tested. Furfural was analyzed in the liquid phase by gas chromatography (GC-FID) (modified from Suwansaard *et al.*, 2009). The variables are also coded according to Eq. (4) similarly with a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis. Regression analyze of furfural was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA).

3.5 Hemicellulosic hydrolysate production

3.5.1 Hemicellulosic hydrolysate production

Dilute sulfuric acid was varied from 0%, 5% and 10% (v/v) and carried out in 100 ml Duran bottoms. The delignified PPF (dPPF) was mixed with dilute acid in the ratio of 1:10 (w/w) (Herrera *et al.*, 2003; Téllez-Luis *et al.*, 2002; Yáñez *et al.*, 2004; Rahman *et al.*, 2006). Operating temperatures of hydrolysis were varied between 75 and 148°C. 5 ml of samples were taken at various time intervals in the range of 30-180 min. After reaction, solids were separated from aqueous solution by filtration and used as a substrate for glucose production by enzymatic hydrolysis. The filtrate was analyzed for xylose, glucose, furfural and acetic acid.

3.5.2 Kinetics models

The acid hydrolysis models for cellulose hydrolysis involves polymer glucan of cellulose is degraded to glucose and then subsequently converted to decomposition products (Eq. 6) (Rahman *et al.*, 2006).

$$glucan \longrightarrow glucose \longrightarrow decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots (6)$$

Hemicellulose can be hydrolyzed by acid and proposed by different studies, which can be divided into two different kinetic mechanisms (Rahman *et al.*, 2006). The first mechanism, xylan is first converted to xylooligosaccharide, which is future converted to xylose by acid hydrolysis and then xylose is subsequently decomposed to furfural. Another mechanism, xylan is converted to xylose without include the intermediate formation of xylooligosaccharide and finally xylose is decomposed to furfural (Eq. 7). The final results of both mechanisms are same.

$$xylan \longrightarrow xylose \longrightarrow decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (7)$$

Therefore, it can be generalized as Eq. 8:

$$polymer \xrightarrow{k_1} monomer \xrightarrow{k_2} decomposition\ products \quad \ldots\ldots\ldots\ldots\ldots (8)$$

where k_1 is the rate of monomer production (min^{-1}) and k_2 is the rate of monomer decomposed (min^{-1}). Based on this reaction, model and solving differential equations, monomer concentration *(M)* as a function of time *(t)* can be represented by equation 9:

$$M = \left[\frac{k_1 P_0}{(k_2 - k_1)}\right]\left(e^{-k_1 t} - e^{-k_2 t}\right) + M_0 e^{-k_2 t} \qquad (9)$$

where P and M represents concentration of polymer and monomer, respectively. The subscript 0 represents at time $t = 0$. P_0 was fixed to 35.77 g/l (see in Chapter 3 section 3.7 and Eq. 27). Assuming M_0 to be nearly equal to 0, Eq. 9 can be modified to Eq. 10:

$$M = \left[\frac{k_1 P_0}{(k_2 - k_1)}\right]\left(e^{-k_1 t} - e^{-k_2 t}\right) \qquad (10)$$

The kinetic model for cellulose hydrolysis involves pseudo-homogeneous irreversible first order reactions represented by equation (11):

$$glucan \xrightarrow{k_3} glucose \xrightarrow{k_4} decomposition\ products \qquad (11)$$

where k_3 is the rate of glucose production (min^{-1}) and k_4 is the rate of glucose decomposed (min^{-1}). Actually, the operating conditions in this investigation did not want decomposition products. Thus the Eq. (11) can be modified as Eq. (12):

$$glucan \xrightarrow{k_3} glucose \qquad (12)$$

According to solving differential equations, concentration of glucose *(G)* as a function of time can be represented as Eq. (13):

$$G = G_0(1 - e^{-k_3 t}) \qquad (13)$$

where G_0 is the potential glucose concentration calculated by regression analysis. In addition, the kinetic model of furfural generation is from xylose degradation as Eq. (14). Thus the model of furfural concentration *(F)* as a function of time can be represented as Eq. (15):

$$xylose \xrightarrow{k_4} furfural \qquad (14)$$

$$F = F_0(1 - e^{-k_4 t}) \qquad (15)$$

where F_0 is the potential furfural concentration calculated by regression analysis and k_4 is also the furfural production rate (min^{-1}).

Acetic acid is a decomposition product obtained by acetyl group's degradation from hemicelluloses. The model for acetic acid generation can be represented as Eq. (16):

$$acetyl\ groups \xrightarrow{k_5} acetic\ acid \qquad (16)$$

Acetic acid concentration (A) in the hydrolysates as a function of time can be represented as Eq. (17):

$$A = A_0(1 - e^{-k_5 t}) \qquad (17)$$

where A_0 is the potential acetic acid concentration calculated by regression analysis and k_5 is the acetic acid production rate (min^{-1}).

Eqs. (10), (13), (15) and (17) were applied to sulfuric acid hydrolysis of delignified PPF. Non-linear regression analyses were performed to obtain the kinetic parameters and constants. The results were statistically evaluated by using the statistical one-tailed *F*-test to the variances. Coefficient of determination (R^2) and *F*-test probability were obtained to establish the significance of the models (Rahman *et al.*, 2006; Téllez-Luis *et al.*, 2002).

For predicting all products, the modified model was developed by relating kinetic parameters with sulfuric acid concentration by empirical equation (Eq. 18) (Rahman *et al.*, 2006).

$$k_m = k_0 C_a^n \qquad (18)$$

where m is in the range of 1 to 5; k_0 and n are the regression parameters; C_a is sulfuric acid concentration expressed in % v/v.

3.6 Cellulosic hydrolysate production

3.6.1 Cellulosic hydrolysate production by using enzymatic hydrolysis

The solids, cellulose obtained from hemicellulose extraction, were hydrolyzed with the cellulase (Accellerase 1000, activity 2500 U/ml). Cellulosic hydrolysate was determined total reducing sugars by the Somogyi-Nelson method using glucose as standard (Somogyi, 1952; Nelson, 1944). The cellulosic hydrolysate was then used as a source for bio-ethanol production. Parameters affecting cellulose hydrolysis, which were pH, temperature, substrate concentration, cellulose dosage and incubation time were optimized.

3.6.1.1 Effect of pH on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M) with different pH ranging from 3.6 to 6.0. Substrate or cellulose (2 g/l) was added in 25 ml of total volume. The incubation condition was at 50°C for 24 h. The reducing sugar was determined by the Somogyi-Nelson method.

3.6.1.2 Effect of temperature on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). Substrate concentration of 2 g/l was added in 25 ml of total volume and then incubated for 24 h at various temperatures ranging from 35°C to 70°C. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.3 Effect of substrate concentration on cellulase activity

Cellulase (0.1 ml/25ml or 500 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). The various substrate concentrations

ranging from 0.4 g/l to 20 g/l was added into 25 ml of enzyme solution, and then incubated for 24 h at the optimal temperature. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.4 Effect of of cellulase dosage

Cellulase varied from 50 µl/25 ml to 1000 µl/25 ml (or 416 U/g substrate to 8,333 U/g substrate) was prepared in sodium acetate buffer (0.05 M with the optimal pH). The optimal substrate concentration was added into 25 ml of enzyme solution, and then incubated for 24 h at the optimal temperature. The reducing sugar produced was determined by the Somogyi-Nelson method.

3.6.1.5 Effect of incubation time and saccharification

The optimal cellulase concentration was prepared in sodium acetate buffer (0.05 M with the optimal pH). The optimal substrate concentration was added into 25 ml of enzyme solution and then incubated at the optimal temperature. The sample was taken from 5.0 min to 4320 min (72 h). The reducing sugar produced was determined by the Somogyi-Nelson method.

The saccharification values were calculated by using Eq. 19 (Chen *et al.*, 2008). All experiments were done in duplicate.

$$Saccharification\ value\ (\%) = \frac{\text{Re}ducing\ sugar\ (g) \times 0.9}{Polysaccharides\ in\ substrate\ (g)} \times 100 \quad \ldots\ldots\ldots \quad (19)$$

3.6.2 Cellulosic hydrolysate production by using concentrated sulfuric acid hydrolysis

Cellulose of PPF, obtained from PPF delignification process, was mixed with concentrated sulfuric acid (72%) at various solid-liquid ratios (1:10-1:20 g/ml). The reaction was carried out at room temperature (30°C) for 90 min. After that, cellulose of PPF hydrolysates was then diluted with distilled water in the range of 1-5% and then boiled. Reaction time was controlled in the range of 60-180 min. After boiling, the cellulose of PPF hydrolysates were cooled immediately on ices and adjusted to the final volume of 720 ml. Reducing sugars, xylose, furfural and acetic acid were determined.

3.7 Ethanol production by *C. shehatae* TISTR5843 in synthetic medium

3.7.1 Optimization of ethanol production in synthetic medium

C. shehatae TISTR5843 was firstly cultured in YM medium and incubated at 30°C for 24 h on a shaker with shaking speed of 180 rpm. Starter was prepared by adjusting to obtain OD_{600} of 0.5. After that, 10% (v/v) (20 ml) of starter culture was transferred to the 180 ml working volume of modified YM broth (Lebeau *et al.*, 2007) consisting of glucose or xylose 15 g/l; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; yeast extract 3.0 g/l. pH was adjusted to 5.0 by using 1 N NaOH. The routine cultivation was incubated at 30°C on a rotary shaker (180 rpm). 4.0 ml of samples were taken every 6 h to determine the ethanol yield, residual glucose, pH, acetic acid, furfural and dry cell weight (DCW).

3.7.1.1 Effect of glucose concentration

C. shehatae TISTR5843 was cultured in the modified YM medium containing various glucose concentrations of 4, 7, 12, 24, 45 and 75 g/l and cultured under the same condition.

3.7.1.2 Effect of xylose concentration

C. shehatae TISTR5843 was cultured in the modified YM medium containing various xylose concentrations of 4, 8, 20, 40, 60 and 90 g/l and cultured under the same condition.

3.7.1.3 Effect of glucose to xylose ratio

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of glucose to xylose ratio of 10:0, 8:2, 6:4, 5:5, 4:6, 2:8 and 0:10 (w/w). and incubated as described in Section 3.3.7.1.1.

3.7.1.4 Effect of temperature

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio obtained from Section 3.3.7.1.3. To study the effect of temperature, the cells were incubated at room temperature (30°C) and 35°C.

3.7.1.5 Effect of initial pH

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio with various initial pH of 3.0, 4.0,

4.5, 5.0 and 6.0. The cells were cultured at the optimum temperature obtained from Section 3.3.7.1.4.

3.7.1.6 Effect of shaking speed

C. shehatae TISTR5843 was cultured in the modified YM medium consisting of the optimum glucose to xylose ratio with the optimum pH from Section 3.3.7.1.5. The cells were cultured at the optimum temperature. The shaking speed was various at 60, 120, 180 and 240 rpm.

3.8 Ethanol production in cellulosic hydrolysate

3.8.1 Selection of ethanolic producing yeasts and bacteria

Starter culture of *C. shehatae* TISTR5843 and *S. cerevisiae* TISTR5017 were prepared by cultivating in YM medium and incubated at 30°C for 24 h with the optimum shaking obtained from Section 3.7.1.6 (Lebeau *et al.*, 2007). After that, 10% (v/v) of starter (OD_{600}=0.5) was transferred to the cellulosic hydrolysate with 200 ml working volume. The cellulosic hydrolysate containing 7.8 g/l glucose was supplemented with; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; yeast extract 3.0 g/l and pH 5.0. The yeast cells were cultured at 30°C for 72 h with the optimum shaking obtained from Section 3.7.1.6. During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration and residual sugars.

Seed culture of *Z. mobilis* TISTR405 was prepared by cultivating in the medium (pH 5.0) containing the following components: 100 g/l glucose, 10 g/l yeast extract, 1 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$ and 0.5 g/l $MgSO_4.7H_2O$ under stationary incubation at 30°C (Ruanglek *et al.*, 2006; Bandaru *et al.*, 2006). Fermentation was started with 10% (v/v) inoculum after 12 h cultivation and at an absorbance of approximately 0.5 at 660 nm which indicated cells in the exponential growth phase (Davis *et al.*, 2006). After that, 10% (v/v) of starter (OD_{660}=0.5) was transferred to the 200 ml working volume of cellulosic hydrolysate (7.8 g/l glucose) supplemented with; 10 g/l yeast extract and 10 g/l bacteriological peptone and cultivated at 30°C for 72 h on a shaker (180 rpm). During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration and residual sugars.

The strain either yeast or bacteria giving the highest ethanol concentration, ethanol yield and ethanol productivity was selected to further investigate.

3.8.2 Optimization of ethanol production from cellulosic hydrolysate by the selected stain

The selected strain was cultivated in the cellulosic hydrolysate (200 ml working volume) supplemented with nutrients as described in section 3.8.1. The optimization was performed by RSM. The initial pH (X_4) was tested in the range of 4.0 to 6.0, and cultured at 30 °C for 72 h. Shaking speed (X_5) was varied in the range of 120 to 240 rpm and inoculum size with cell concentration (X_6) of 0.725 to 1.20 g/l corresponded to OD_{600} of 0.5-1.0. During fermentation, samples (2 ml) were taken every 6 h until 72 h cultivation to determine the ethanol concentration, residual sugars, residual acetate, pH and dry cell weight.

20 batch experiments were also conducted by central composite designs (CCD). The variables were also coded according to Eq. (4), and a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis, as described above. The expected response values predicted from RSM consist of ethanol concentration (g/l), ethanol yield (g ethanol/g sugar) and ethanol productivity (g/l/h). Regression analysis of ethanol production was illustrated by Design Expert v. 7 (Stat-Ease. Inc., MN, USA). The control condition contained the initial pH of 5.0, shaking speed of 180 rpm, initial cell concentration of 0.725 g/l without any nutrient supplementation.

3.9 Effect of inhibitory compounds presented in PPF hydrolysate on ethanol production by *C. shehatae* TISTR5843

Acetate, furfural and vanillin (derivative of lignin) are the main inhibitors present in lignocellulosic hydrolysate treated by dilute acid (Olsson and Hahn-Hägerdal, 1996; Delgenes *et al.*, 1996). Acetate concentration was ranged from 0-10 g/l, furfural concentration was ranged from 0-2 g/l and vanillin concentration was ranged from 0-2 g/l (Delgenes *et al.*, 1996). The fermentations were controlled at the optimum initial pH obtained from Section 3.7.1.5, the optimum shaking obtained from Section 3.7.1.6, and cell concentration of 0.725 g/l (OD_{600}=0.5). During

fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Sreenath *et al.*, 2001), residual acetic acid (Suwansaard *et al.*, 2009), furfural (Mansilla *et al.*, 1998), furfuryl alcohol, pH and dry cell weight (DCW). The control treatment was 180 ml PPF hydrolysate without any inhibitor supplementation.

3.10 Ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

3.10.1 Effect of nitrogen source, nitrogen concentration and C/N ratio on ethanol production from hydrolysate of PPF by *C. shehatae* TISTR5843

Starter culture of *C. shehatae* TISTR5843 was prepared by cultured in the YM broth and incubated at 30°C for 24 h with shaking at 180 rpm (Lebeau *et al.*, 2007). After that, 10% (v/v) (20 ml) of initial cell concentration of 0.725 g/l was transferred to the PPF hydrolysate with 180 ml working volume. The PPF hydrolysate containing 10 g/l xylose was supplemented with; KH_2PO_4 10 g/l; $(NH_4)_2SO_4$ 5.0 g/l; malt extract 3.0 g/l; and pH 5.0 (obtained from section 3.7) otherwise indicated. The yeast cells were cultured at 30°C for 72 h with shaking speed of 180 rpm (obtained from Section 3.7).

Three parameters consisted of nitrogen source (NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone), nitrogen concentration of 0-10 g/l and C/N ratio varied from 2.8/1-9.3/1 (w/w) were studied with supplementation of acetate (4.25 g/l) and furfural (0.67 g/l).

3.10.1.1 Effect of nitrogen source

NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3PO_4$, urea, yeast extract, peptone and tryptone were tested as a nitrogen source to obtain the maximum ethanol production by *C. shehatae* TISTR5843. 3 g/l (based on nitrogen content) of all nitrogen sources were added into the PPF hydrolysate medium. During fermentation, samples (2 ml) were taken at 24 h and 48 h to determine the ethanol concentration (Zhu *et al.*, 2006).

3.10.1.2 Effect of nitrogen concentration

The selected nitrogen source which was peptone was studied the concentration in the ranged of 0-10 g/l in the PPF hydrolysate. During fermentation,

samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al.*, 2006).

3.10.1.3 Effect of C/N ratio

C/N ratio was studied in the ranged from 2.8/1-9.3/1. During fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al.*, 2006).

3.10.2 Effect of dilution of PPF hydrolysate on ethanol production by *C. shehatae* TISTR5843

PPF hydrolysates were diluted with distill water in the range of no dilution, 1/2 dilution, 1/3 dilution and 1/5 dilution (Suwansaard, 2010) with trace elements supplementation as described section 3.10.1. During fermentation, samples (2 ml) were taken every 12 h until 96 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Sreenath *et al.*, 2001), pH and dry cell weight (DCW).

3.10.3 Optimization of factors affecting on cells growth and ethanol production by *C. shehatae* TISTR5843 in PPF hydrolysate

Due to the complex composition of PPF hydrolysate, three parameters consisted of initial pH in the range of 4.0 to 6.0, shaking speed of 60 to 180 rpm and inoculum size or cells concentration varied from 0.9 to 1.5 g/l were studied by RSM. During fermentation, samples (2 ml) were taken every 6 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al.*, 2009) and furfural (modified from Suwansaard *et al.*, 2009). Ethanol yield was calculated from ethanol concentration divided by xylose consumption (Kim *et al.*, 2008).

In order to describe the effects of initial pH, shaking speed and initial cells concentration (as the independent variables) on ethanol production (as the dependent variables), 20 batch experiments were conducted by central composite designs (CCD) (Box *et al.*, 1978; Sangkharak and Prasertsan, 2007; O-Thong *et al.*, 2008). The variables are coded according to Eq. (4), and a quadratic model (Eq. (5)) was also used to evaluate the optimization of environmental parameters and stepwise regression analysis, as described above. The expected response values predicted from RSM consist of ethanol concentration (g/l), ethanol yield (g ethanol/g sugar) and ethanol productivity (g/l/h). The response variable was fitted using a predictive

polynomial quadratic equation (5) in order to correlate the response variable to the independent variables (O-Thong *et al.*, 2008). Regression analysis of ethanol production was illustrated by Design Expert v. (Stat-Ease. Inc., MN, USA). The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface plots. The quality of the fit of quadratic model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the F-test (O-Thong *et al.*, 2008).

3.11 Ethanol production from PPF hydrolysate in 3 L reactor by *C. shehatae* TISTR5843

3.11.1 Batch fermentation

To produce large quantity of ethanol and compare the predict yield, scaling-up from Erlenmeyer flask (250 ml) to reactor (3 L) with different geometry was studied under controlled agitation condition. The optimal condition of ethanol production achieved from Erlenmeyer flask (section 3.10) was used as a control condition. During fermentation, samples (2 ml) were taken every 12 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al.*, 2009), furfural (modified from Suwansaard *et al.*, 2009) and dry cell weight (DCW).

3.11.2 Fed-batch fermentation

This work was carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The initial working volume was 450 ml fresh medium combined with 10% starter culture. Fresh medium of the second and the third cycles were approximately 500 and 1000 ml at 48 and 96 h cultivation time, respectively. During fermentation, samples (2 ml) were taken every 12 h until 144 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al.*, 2009), furfural (modified from Suwansaard *et al.*, 2009) and dry cell weight (DCW).

3.11.3 Semi-continuous fermentation

This work was also carried out in 3 L fermentors. The experiments were consisted of 3 cycles of fresh medium. The initial working volume was 1,820 ml fresh medium combined with 10% starter culture (2,000 ml total volume). Fresh

medium of the second and the third cycles were approximately 1000 ml at 48 and 96 h cultivation time, respectively. During fermentation, samples (2 ml) were taken every 12 h until 144 h to determine the ethanol concentration (Zhu *et al.*, 2006), residual xylose (Somogyi, 1952; Nelson, 1944), residual acetic acid (Suwansaard *et al.*, 2009), furfural (modified from Suwansaard *et al.*, 2009) and dry cell weight (DCW).

3.12 Bioethanol production by immobilized *C. shehatae* TISTR5843 using palm pressed fiber as a support

PPF was delignified (dPPF) by method described in section 3.2. The sizes of natural supports were < 5.0 mm for small PPF particles (sPPF) and small delignified PPF particles (sDPPF), and 5-20 mm for large PPF particles (lPPF).

Starter culture of immobilized *C. shehatae* TISTR5843 on PPF and DPPF was prepared by adding 100 g/l supports into a liquid yeast cell (0.725 g/l) medium containing 25 g/l glucose, 10 g/l KH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 3 g/l yeast extract and 3 g/l malt extract (initial pH 5) with working volume of 200 ml in the Erlenmeyer flasks. The routine experiments were incubated on an orbital shaker (150 rpm) for 18 h at room temperature (30°C). The 10% (v/v) of immobilized starter culture was introduced to a fresh medium with 200 ml working volume. Samples were taken to determine dried cell weight (DCW) (Fujii *et al.*, 1999), ethanol production by GC-flame ionized detector using a column and the operational condition as described in section 2.3, and residual glucose by HPLC as described in Section 2.1.2.1. The immobilized system was detected by a scanning electron microscope as described in section 2.5.

Repeated batches fermentation (4 cycles) was carried out as described in Kopsahelis et al. 2007. Briefly, the fermented liquids were decanted after the end of each fermentation batch, and then fresh medium was added for the next fermentation batch. Samples of the fermented liquids were collected and analyzed for ethanol, residual sugar and volatile-by products.