CHAPTER 8

ENZYMATIC HYDROLYSIS AND PRELIMINARY OF BIO-ETHANOL PRODUCTION

8.1 Introduction

There are two main processes involved in the lignocellulosic biomass conversion, hydrolysis of cellulose in the biomass to produce fermentable sugars and fermentation of the sugar to bio-ethanol. Cellulose and hemicelluloses are hydrolyzed to soluble monomeric sugars (hexoses and pentoses) by using cellulases and hemicellulases, respectively. The major sugars in biomass hydrolysis are glucose and xylose with significantly less amounts of arabinose, galactose and mannose (Gray *et al*., 2006). When cellulose and hemicelluloses are hydrolyzed, a mixture of monosaccharide was fermented. Fermentation involves microorganisms which consume sugar as a food source.

The optimization values of some important factors of sugar fermented microorganism such as initial pH, concentration of inoculums and incubation times are important for ethanol production. The optimum value could be developed by using an effective experimental design. Response surface methodology employing Central Composite Design, commonly called a CCD, is a collection of mathematical and statistical techniques for building empirical models. There are many application of CCD to search the optimizing conditions or factors for the production of important products such as enzymes and chemical products (Khurana *et al*., 2007).

The objective of this study was to apply CCD method to evaluate the optimal value of some important factors for ethanol production from agricultural residues by *Saccharomyces cerevisiae* TISTR 5088 and could be attained of ethanol production.

8.2 Materials and methods

8.2.1 Lignocellulose substrates preparation

Three agricultural residues (sugarcane bagasse: coffee husk: rice husk, 57:6:37 % w/w) were pretreated by biological pretreatment (presented in Chapter 7) and used as solid substrate for ethanol production.

8.2.2 Microorganisms and culture medium

In this study, *T. aurantiacus* SL16W (Kongbuntad *et al*., 2006) was used for fungal hydrolysis and produced cellulase and xylanase from agricultural residues. The fermented yeast, *Saccharomyces cerevisiae* TISTR 5088 from Thailand Institute of Scientific and Technology Research, Thailand, was used for ethanol fermentation. Yeasts were maintained in MY media (Yu *et al*, 2009) and one loop from yeast slant was used to inoculate 50 ml of the pre-culture media (MY media without glucose) in 250 ml Erlenmeyer flasks and cultivated on a rotary shaker (140 rpm) at room temperature for 24 hr and used as fermented yeast inoculums.

8.2.3 Microbial growth analysis

Optical density of yeast cultures was measured spectophotometrically at 600 nm (Patle and Lal, 2007). Yeast concentration was verified by colony counts on MY agar plate to determine the colony forming units (CFU) per milliliter.

8.2.4 Experimental design for ethanol fermentation

To determine the factors effecting on ethanol production by *S. cerevisiae* TISTR 5088 was done using central composite design (CCD). Three important factors were initial pH, inoculums concentration, and incubation time. The different levels of their coded and actual values for CCD following octagon design, the design points (α) for 3 factors as ±1.68 are shown in Table 8.1. All trials were performed in triplicate, and the mean was determined. The data were analyzed for significant difference between treatments using analysis of variance (ANOVA). To establish if differences between individual trails were significant ($p< 0.05$) and multiple linear regression analysis was performed using software Design-expert 6.0.2. The maximum ethanol production was taken as the dependent variable of response (Y). The observed values were analyzed and fitted to the second-order model equation is as follows;

$$Y= \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (8.1)$$

Where, Y is the predicted variable response (ethanol yield) and β is the regression coefficients given by the model and x_i and x_j are the independent factors of the experiment (Akay, 2007). If the curve shape of the response surface plot is elliptical or circular then it is presumed that the correlation between the variables is most significant.

Table 8.1 Codes and actual levels of the independent variables for design experiment

Variables	Code levels				
	-1.68	-1	0	1	1.68
pH	3.32	4.0	5.0	6.0	6.68
Inoculums (%)	0.32	1	2	3	3.68
Time (hr)	3.18	10	20	30	36.8

8.2.5 Fungal hydrolysis

Pretreated substrates were hydrolyzed by fungus *T aurantiacus* SL16W under solid state cultivation, 45^{o}C for 10 days. Available sugars were extracted by 10 mM sodium acetate buffer pH 5.0 and centrifugation 10,000 rpm, 4^{o}C for 10 min. Clearly supernatant was concentrated to final buffer concentration of 100 mM by evaporation technique and the amount sugar from hydrolysis was estimated in terms of release of reducing sugars by DNS method (Miller, 1959).

8.2.6 Ethanol production

Reducing sugar produced from fungal hydrolysis was used for ethanol fermentation by *S. cerevisiae* TISTR 5088 under batch fermentation. One ml of soluble sugar was added to fermentation flask (125 ml) and sterilized by autoclave 110 ^{o}C for 10 min. Two percentages of inoculums (about 1×10^{6} CFU/ml) was added to fermentation flask. The test flasks were fermented on a rotary shaker, mild speed (100 rpm) at room temperature for 120 hr and sampling every 24 hr. Ethanol production during fermentation was measured by gas chromatography.

8.2.7 Ethanol measurement

Ethanol produced during fermentation was measured by gas chromatography; GC (Konik HRGC 4000 B) by using 1% of butanol as internal standard (Appendix F).

8.3 Results and discussion

8.3.1 Fungal hydrolysis

Three lignocelluloses (sugarcane bagasse, coffee husk and rice husk in the ratio of 57: 6: 37 %w/w) were mixed and pretreated by white rot fungus, *T. polyzona* WR710-1 (presented in Chapter 7). The pretreated lignocelluloses were used as solid substrates for reducing sugar production (fungal hydrolysis) by *T. aurantiacus* SL16W under solid state cultivation. The reducing sugar was determined. The highest yield of reducing sugar around 0.15 mg/ml was detected after 10 days and decreased up to 29.09% at day 12 of incubation (Fig. 9.1). Pretreated substrates showed more reducing sugar production compared with control (unpretreated) substrates, 78.72% increasing rate (Fig. 9.2). One of the reasons of increasing hydrolyzed product because of the pretreatment increased the available surface area (pore volume) for the enzymatic attack (Alvira *et al*., 2010). *Thermoascus aurantiacus* has been proposed as good microorganism for bioconversion of lignocellulosic biomass to sugars and offer the great potential to be used in industrial scales (Dashtban *et al*., 2009). There are many publications about fungal hydrolyzed from *T. aurantiacus* (Gomes *et al*., 2000).

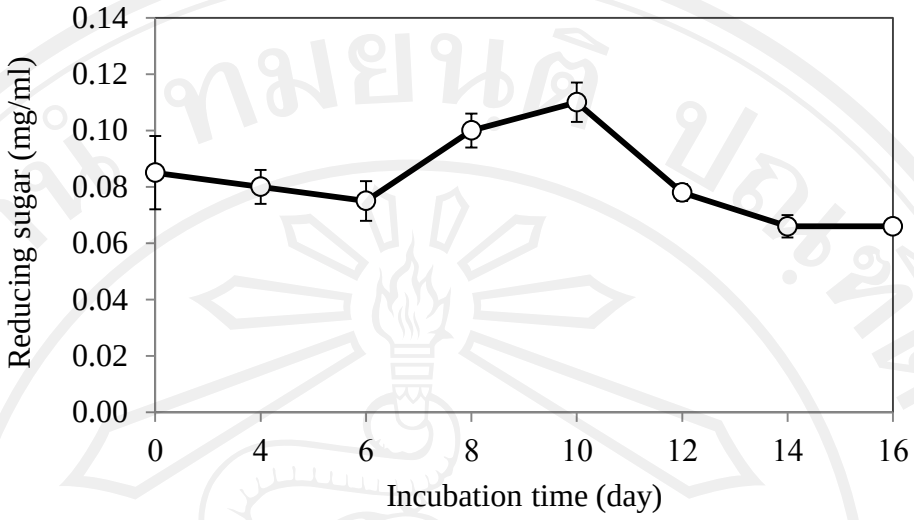

Fig. 8.1 Time-course of reducing sugar production from mixed agricultural residues by *Thermoascus aurantiacus* SL16W under solid state fermentation. Error bars mean standard deviation of three replications.

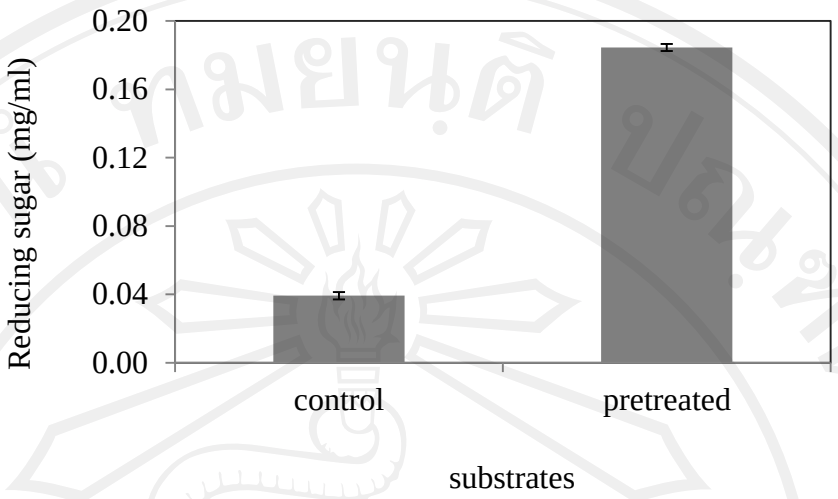

Fig. 8.2 Reducing sugar production by fungal hydrolysis (*Thermoascus aurantiacus* SL16W) from biological pretreated agricultural residues compared with substrate control (untreated), incubated at 45^{o}C, static for 10 day.

8.3.2 The response surface methodology experiment results of ethanol fermentation

Some important factors of ethanol fermentation are the initial pH, the inoculums (yeast) concentration, and the incubation time. The suitable levels for these parameters were also determined using CCD. From the experimental design matrix, the 15 trials were performed using different combination of the variables and ethanol production by *S. cerevisiae* TISTR 5088 were measured (Table 8.2).

Table 8.2 Matrix corresponding to CCD experimental designs together with the ethanol production (observed experimental data)

Trials	pH	Inoculums (%)	Incubation time (hr)	Ethanol yield* (g/L)
1	4.0	1.0	48.0	0.000±0.00 ^{e}
2	6.0	1.0	48.0	0.012±0.01 ^{e}
3	4.0	3.0	48.0	0.000±0.00 ^{e}
4	6.0	3.0	48.0	0.000±0.00 ^{e}
5	4.0	1.0	96.0	0.000±0.00 ^{e}
6	6.0	1.0	96.0	0.036±0.01^{de}
7	4.0	3.0	96.0	0.010±0.00 ^{e}
8	6.0	3.0	96.0	0.110±0.02^{cd}
9	3.3	2.0	72.0	0.000±0.00 ^{e}
10	6.7	2.0	72.0	0.065±0.01^{de}
11	5.0	0.3	72.0	0.181±0.01^{bc}
12	5.0	3.7	72.0	0.172±0.00^{bc}
13	5.0	2.0	31.6	0.194±0.01^{bc}
14	5.0	2.0	112.4	0.256±0.06^{ab}
15	5.0	2.0	72.0	0.284±0.08 ^{a}

* Results are mean of ±SD of three determination. Values with the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

The highest ethanol production (0.28±0.08 g/L) was observed in the 15th trial of the experimental setup when initial sugar about 1.0 mg/ml. The results of the ANOVA for ethanol production were shown in Table 8.3. The quadratic regression

showed the model was significant, the value of F-test (the ratio of mean square due to regression to mean squares to real error) less than 0.05 indicated the significance of the model terms.

Table 8.3 ANOVA for variable factors for ethanol production fitted to the response surface quadratic model

Source	Mean Square	F value	Prob > F	
Model	0.023	4.289	0.0032	significant
X_1	0.010	1.783	0.1968	
X_2	0.000	0.084	0.7752	
X_3	0.009	1.659	0.2124	
X_1^2	0.173	31.90	0.0001	
X_2^2	0.057	10.50	0.0041	
X_3^2	0.032	5.943	0.0242	
$X_1 X_2$	0.001	0.126	0.7263	
$X_1 X_3$	0.004	0.711	0.4092	
$X_2 X_3$	0.002	0.424	0.5221	
Residual	0.005			
Lack of Fit	0.017	11.464	0.0001	significant
Pure Error	0.002			

X_1= pH, X_2= concentration of inoculums and X_3= incubation time, $R^2 = 0.890$

On the basis of quadratic polynomial equation of mixture design model in Eq. 8.1, the effect of independent factors; rice husk, coffee husk and sugarcane bagasse on the specific activity of xylanase were analyzed in Eq. 8.2;

$$Y = -3.034 - 0.120X_1^2 - 0.069X_2^2 - 0.0005X_3^2 \quad (8.2)$$

Equation 8.2 showed the quadratic model for three components where Y was the ethanol production (g/L), X_1, X_2, X_3 were pH, concentration of inoculums and incubation time, respectively which non significant values were not shown in the equation.

The 3D response surface and the contour plots are generally the graphical representations of the regression equation were shown in Fig 8.3, 8.4 and 8.5, from which the values of ethanol productivity for the different levels of the variables can be predicted. The 3D contour plots in Fig.8.3 showed that there is a significant mutual interaction between pH and concentration of inoculums, which fixed the levels of incubation time at 20 hr. The contour plots in Fig. 8.4 showed that the interaction between pH and incubation time which fixed the concentration of inoculums at 1.5%, and Fig. 8.5 was the interaction between incubation time and the concentration of inoculums which fixed levels of pH at 4.8 From the model equation (Eq. 8.2), the result indicated that optimal values of the test variables were pH (X_1 = 5.04), the concentration of inoculums (X_2 = 1.97 %) and incubation time (X_3 = 22.27 hr) with the ethanol production (corresponding Y = 0.31 g/L).

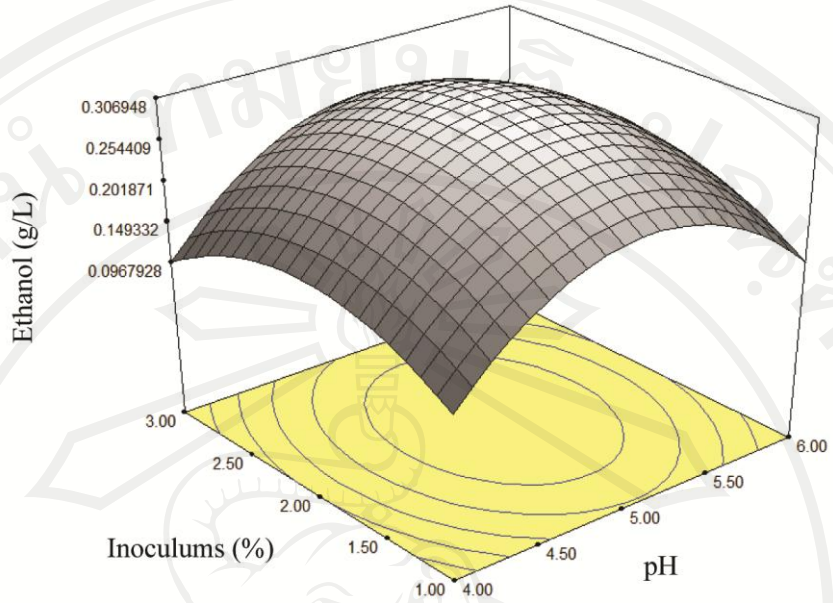

Fig. 8.3 Response surface plots of ethanol production (g/L) and the effect of inoculums and pH which incubation time was fixed at 20 hr.

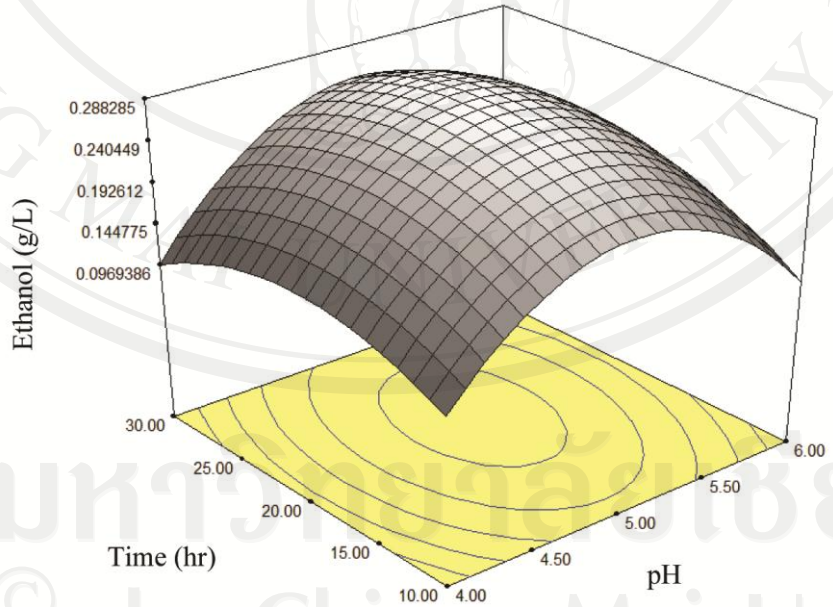

Fig. 8.4 Response surface plots of ethanol production (g/L) and the effect of incubation time and pH which concentration of inoculums was fixed at 1.5 %.

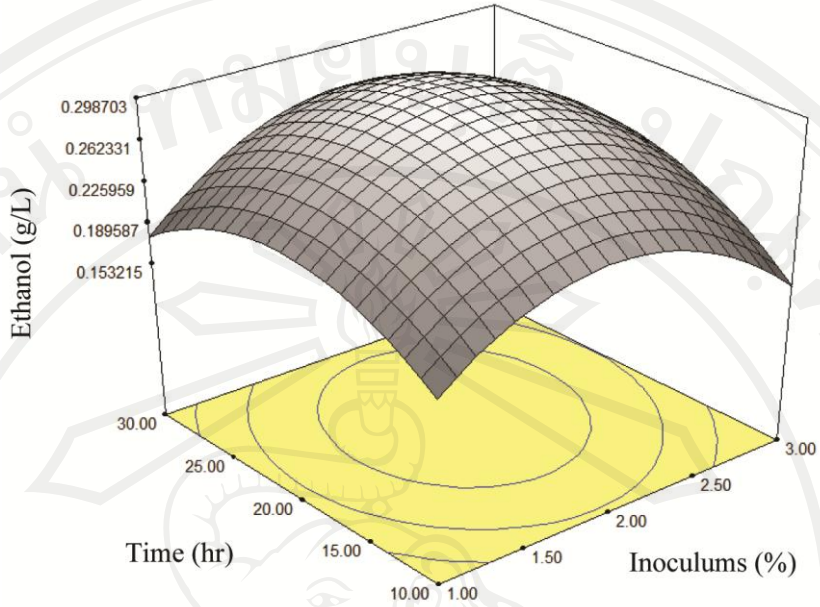

Fig. 8.5 Response surface plots of ethanol production (g/L) and the effect of incubation time and concentration of inoculums which pH was fixed at 4.8.

The response surface showed that the interaction of differential variables tested of ethanol fermentation and useful to built the model for selection of the optimal value in each variable that related to ethanol production. Many publications were studied on the application of response surface methodology for ethanol production (Sasikumar and Viruthagiri, 2008; Lin *et al*., 2010; Pradeep *et al*., 2012).

8.3.3 Ethanol production

After fungal hydrolysis, the reducing sugars was concentrated and determined by DNS method. Ethanol fermentation by *S. cerevisiae* TISTR 5088 under optimal condition from CCD experiment and the initial reducing sugar was concentrated to about 20 mg/ml. The result was shown in Fig. 8.6, the highest ethanol production (4.6 g/L) was presented at 24 hr of incubation and yeast cell showed slightly grew from 2.6×10^6 to 9.1×10^6 CFU/ml at the first period of incubation, while reducing sugar

remained 50% from initial yield (19.3 mg/ml). Ethanol yield reduced when incubated up to 48 hr, may be because ethanol was evaporated. Based on a theoretical yield of 0.51 g ethanol/g sugars, the ethanol yield from this experiment was calculated to be 0.24 g ethanol/g sugar or 47.06 % of the theoretical yield.

This study is a preliminary study for ethanol production from agricultural residues. From the results showed that the production of ethanol was low, may be because the other important factors were not determined, such as optimal condition, temperature and bioreactor. Thus, more experiment should be study in future to improve yield of ethanol.

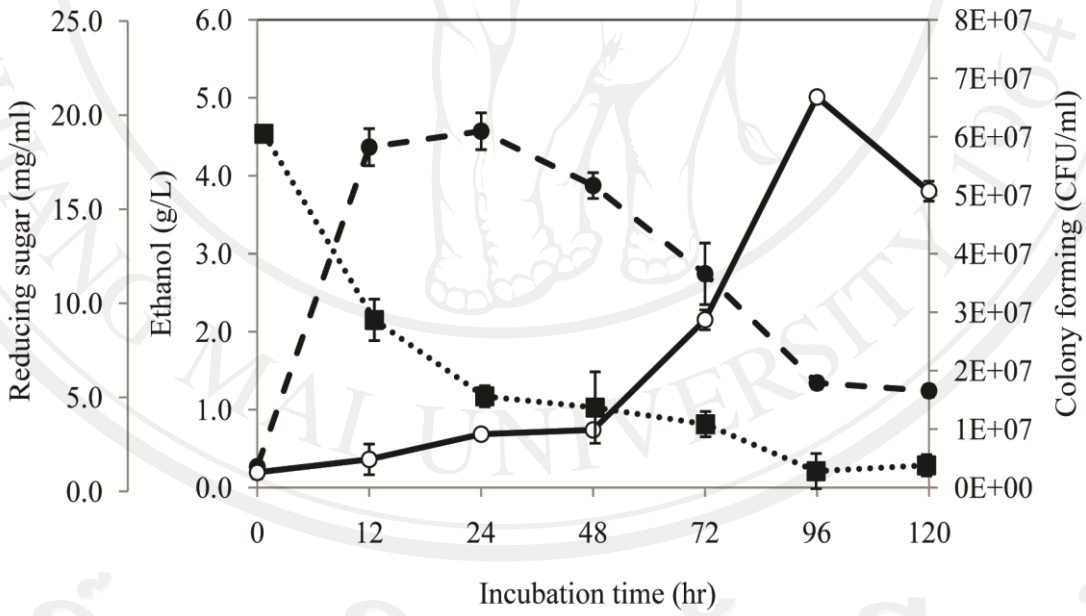

Fig 8.6 Reducing sugar (■), *S. cerevisiae* TISTR 5088 growth (O) and ethanol production (●) measurement from separated saccharification and fermentation. Error bars mean standard deviation of three replications.