Chiang Mai J. Sci. 2012; 39(2): 270-280
http://it.science.cmu.ac.th/
Contributed Paper

Optimization of Solid S tion for Reducing Sugar Production from Residues of Sweet Sorghum by *Trichoderma harzianum*

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Received: 28 July 2011
Accepted: 27 December 2011

ABSTRACT

Sweet sorghum is an attractive feedstock for ethanol production. The juice extracted from the fresh stem contains sucrose, glucose, and fructose and can be readily fermented to alcohol. The solid fraction residue, the so-called bagasse, is a lignocellulose which can further be processed to ethanol. In this work, optimal conditions for reducing sugar production from sweet sorghum bagasse under Solid State Fermentation (SSF) by *Trichoderma harzianum* was investigated. Response Surface Methodology (RSM) using Box-Behnken Design (BBD) for optimization of culture conditions was performed; these parameters were initial moisture content, inoculum size and incubation time. The reducing sugar production from sweet sorghum bagasse by means of SSF was achieved after sequential of acid and alkaline pretreatments. The experimental data showed that the highest value of reducing sugar from cellulose pulp were produced at initial moisture content of 77.5%, inoculum size as 10.5% and 56 hours of incubated time. Under these conditions, reducing sugar of 10.34% (g/g dry materials) was obtained.

Keywords: Sweet sorghum bagasse; Box-Behnken Design; *Trichoderma harzianum.*

1. INTRODUCTION

Sweet sorghum is recognized as an alternative potential feedstock for ethanol production, since it is a high biomass and sugar yielding crop. It contains soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) [1]. The juice extracted from the fresh stem can be easily converted to ethanol. The remaining solid residue is called bagasse, which is a byproduct representing about 30% of the plant fresh weight. Bagasse, an important residue from sweet sorghum processing, can become an important biomass source for saccharification and

fermentation for bioethanol production.

Sweet sorghum bagasse defined as the lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. Cellulose is a linear polymer that comprises glucose subunits linked by β-1,4 glycosidic bonds. These long chains are connected together by hydrogen bonds and Van der Waals forces. Cellulose is usually presented as a crystalline form while amorphous cellulose represents as small amount of non-organized cellulose chains forms. Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is composed of xylose, mannose, galactose, glucose, arabinose and glucuronic acids, and are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds. Lignin is responsible for forming a physical seal that is an impenetrable barrier in the plant cell wall [2].

In order to obtain sugar, it is necessary to degrade the polymers to monomer, which can be done by either physical, or chemical, or biological methods. Chemically pretreatment with enzymatic hydrolysis of lignocellulosic material has received attention due to its potential as an environmentally friendly process, which may provide an efficient and specific process technique [3]. For the degradation of lignocellulosic materials, a broad range of cellulolytic enzymes are necessary. Cellulose can be hydrolysed by action of cellulase such as β-glucosidases, cellobiohydrolases and endoglucanases [3]. Enzyme hydrolysis of hemicellulose is much more complex. Complete breakdown of this heterogeneous biopolymer requires the action of several hydrolytic enzymes in the group of hemicellulases such as xylanase, arabinase, etc [4].

Numerous bacterial and filamentous fungi can produce cellulolytic enzymes. One of the most extensively studied cellulolytic microorganisms is *Trichoderma spp.* which is also industrially used for enzyme production. In the natural habitat, filamentous fungi can be growing on the solid lignocellulosic particles. Accordingly, the growth of fungi can facilitate the production of enzyme. The optimum approach for enzyme production is based on Solid State Fermentation (SSF), which involves the growth of microorganisms on moist solid substrates in the absence of free water [5]. The use of SSF has many advantages, such as no need for complex and sophisticated machinery, easy product recovery, low energy demand, high volumetric productivity and often a high yield of products [6]. Moreover, the SSF can use inexpensive and widely available agricultural residues as substrates [7].

In this present work, sweet sorghum bagasse in different forms, namely untreated bagasse, cellulignin, and cellulose pulp, are analyzed for the percentages of chemical compositions. These three substrates are used for comparison of reducing sugar production by *Trichoderma harzianum*. In this experiment, moisture content, inoculum size and incubation time were the chosen as three factors to be optimized followed the Box-Behnken Design (BBD).

2. MATERIALS AND METHODS

2.1 Substrate preparation and pretreatment

Sweet sorghum (*Sorghum bicolor (L.)* Moench) bagasse from the cultivar of Suwan-4 was obtained from Suwan farm, Kasetsart University. This material was thoroughly washed and dried at 65°C until constant weight was obtained. The air-dried bagasse was then milled and subsequently sieved to a size particle of 2-4 mm. 1% H_2SO_4 solution was added to the untreated

material at ratio of 1:20, then, autoclaved at 121°C for 20 min for an efficient hemicellulose removal from sweet sorghum bagasse. After reaction the solid residue (cellulignin) was separated, washed with water until the pH was neutral, and dried at 65°C.

The cellulignin was, then, treated with 4%(w/v) NaOH solution at a solid:liquid with ratio of 1:20 and consequently autoclaved at 121°C for 20 min, conditions previously optimized for an efficient lignin removal from sweet sorghum bagasse. At the end of the reaction, the residual solid material (cellulose pulp) was separated, washed with water to remove the residual alkaline, and dried at 65°C.

2.2 Microorganisms

The fungal strain *Trichoderma harzianum*, was obtained from Uniseeds Co., LTD. The inoculum of *T. harzianum* was in powder form and contained 10^8 spores per gram.

2.3 Optimization of reducing sugar production under SSF

Reducing sugar production under SSF was conducted in 250 ml flask covered with cotton. Each flask containing 5 g dried sweet sorghum bagasse, which used as the carbon source. Standard Mandel medium in 50 mM sodium citrate buffer (pH 4.8) that contained of 0.3 g/l urea, 1.4 g/l $(NH4)_2SO_4$, 2.0 g/l $KH_2P\ O_4$, 0.4 g/l $CaCl_2{\cdot}2H_2O$, 0.3 g/l $MgSO_4{\cdot}4H_2O$, 0.75 g/l peptone, 0.25 g/l yeast extract, 5 mg/l $FeSO_4{\cdot}7H_2O$, 1.6 mg/l $MnSO_4{\cdot}4H_2O$, 1.4 mg/l $ZnSO_4{\cdot}7H_2O$ and 20 mg/l $CoCl_2{\cdot}6H_2O$, was added to substrate for adjusting initial moisture content before autoclaving at 121°C for 20 min. Each flask was inoculated with fungal spore of *T. harzianum* and incubated at 25°C.

Response surface methodology (RSM) was applied for optimization of the reducing sugars production. Cellulose pulp was selected as substrate for the whole experiments. Three parameters namely size of inoculum (X_1), moisture content (X_2), and incubated time (X_3) with the ranges of minimum (-1), maximum (+1) and central point (0) were assigned as design of experiment of reducing sugar production.

The levels of parameters for experimental design are shown in Table 1. The total of 15 experimental runs with three variables was designed according to a Box-Behnken Design (BBD) using the statistical software MINITAB release 14. The behavior of the system was explained by the following quadratic model equation.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (1)$$

Where Y is the predicted response, β_0 intercept β_1, β_2 and β_3 linear coefficient, β_{11}, β_{22} and β_{33} square coefficient, and β_{12}, β_{13} and β_{23} interaction coefficients.

Table 1. Experimental range and coded levels of factors for reducing sugars production.

Factors	Symbol	Range and levels		
		-1	0	1
Inoculum size (%w/w)	X_1	5	10	15
Moisture content (%w/w)	X_2	70	80	90
Incubated time (hours)	X_3	36	48	60

3. ANALYTICAL METHODS

The composition of materials (untreated bagasse, cellulignin and cellulose pulp) were then analyzed by the methods of Goering and Vansoest (1970) [8] to evaluate cellulose, hemicellulose, lignin and ash content in the biomass.

After suitable periods of time, reducing sugar was extracted from the fermented medium by adding 100 ml distilled water to each flask. The flasks were then shaken at 200 rpm for 2 hr at 60°C. The suspended materials and fungal biomass were separated using filter paper.

The total reducing sugars in the filtrate were determined by the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959) [9]. The composition of reducing sugars (glucose, xylose and arabinose) in hydrolysates was determined by HPLC with a refractive index (RI) detector and Rezex RPM Monosaccharide 00H-0135-K0 (300x7.8 mm) column. The samples were filtered through syringe filters (Regenerated cellulose: 0.45 μm) and thus injected in chromatograph under the conditions: column temperature 75°C, distilled water as mobile phase at a flow rate of 0.8 ml/min and injection volume of 10 μl. The concentration of these compounds was obtained from the calibration curves of the standard solution.

4. RESULT AND DISCUSSIONS

4.1 Chemical Composition of Substrates

The enzymatic hydrolysis of cellulose was reported to be affected by many factors including porosity (accessible surface area) of materials, cellulose fiber crystallinity, hemicelluloses and lignin content [10]. Cellulose, hemicelluloses, lignin and ashes content of sweet sorghum bagasse before and after pretreatment are shown in Table 2.

Sweet sorghum bagasse was initially pretreated with dilute sulfuric acid at high temperature to solubilize the hemicelluloses fraction. While acid hydrolysis occurred, the acid also hydrolyzed the polysaccharides, especially hemicelluloses that was easier to be hydrolyzed than cellulose [11]. The structural complexity of cellulose was more than that of hemicelluloses, then cellulose hydrolysis could only be occurred with strong acids. An acid pretreatment by 1% H_2SO_4 effectively reduced the content (% dry weight) of cellulose and hemicellulose by 8.48% and 28.02%, respectively (data not shown). Therefore, the cellulose and lignin fractions remained almost the same in solid phase [12]. Subsequently, the solid residue (cellulignin) reacted with sodium hydroxide to solubilize the lignin.. This was typical of alkaline pretreatment, which generally had a stronger effect on

Table 2. Chemical compositions of sweet sorghum bagasse in the original form (untreated bagasse), after the acid pretreatment (cellulignin) and after sequential acid and alkaline pretreatments (cellulose pulp).

Composition (%dry weight)	Untreated bagasse	Cellulignin	Cellulose pulp
Cellulose	54.64	73.27	95.62
Hemicellulose	28.15	0.21	0.13
Lignin	16.07	25.35	3.67
Ash	1.14	1.17	0.58

lignin than cellulose and hemicelluloses [13]. Lignin could be well-solubilized in bases. However, upon an alkaline pretreatment with 4% NaOH at high temperature, this situation still reacted with the bonds of hemicelluloses and cellulose. Pretreated biomass was swollen, which led to the minimization in degree of polymerization, decrease in crystallinity, disruption of the lignin structure, and separation of structural linkages between lignin and carbohydrates [14]. As a result shown in Table 2, the purity of cellulose fraction was increase from 54.64% (from untreated bagasse) to 95% (from cellulose pulb).

4.2 Effect of Pretreatment on Reducing Sugar Production

In this respect, different forms of sweet sorghum bagasse (untreated bagasse, cellulignin and cellulose pulp) were tested for the production of reducing sugar by *T. harzianum* under SSF. An inoculum (10% w/w) was added to a medium of 70% (w/w) moisture content and incubated at 25°C for 96 hours. As shown in Figure 1, the highest reducing sugar of 8.95% (w/w), was obtained with cellulose pulp as the substrate for 2 days of incubation time, followed by untreated bagasse and cellulignin of 4.79 and 4.14 % (w/w), respectively. The hemicellulose and lignin removal were relatively resistant to microbial digestion. On the contrary, the treatment with *T. harzianum* did cause extensive changes in structure and accessibility of cellulose that had more digestible surface area and more swelling [15]. Because of the highest percentages of obtained reducing sugar, cellulose pulp was then selected as substrate for the optimization of reducing sugar production.

4.3 Optimization of Reducing Sugar Production under SSF

In the present study, BBD is used to find the optimal conditions of reducing sugar production from cellulose pulp derived from sweet sorghum bagasse under SSF by *T. harzianum*. The performing of 15 experiments of three variables namely inoculum size, moisture content and

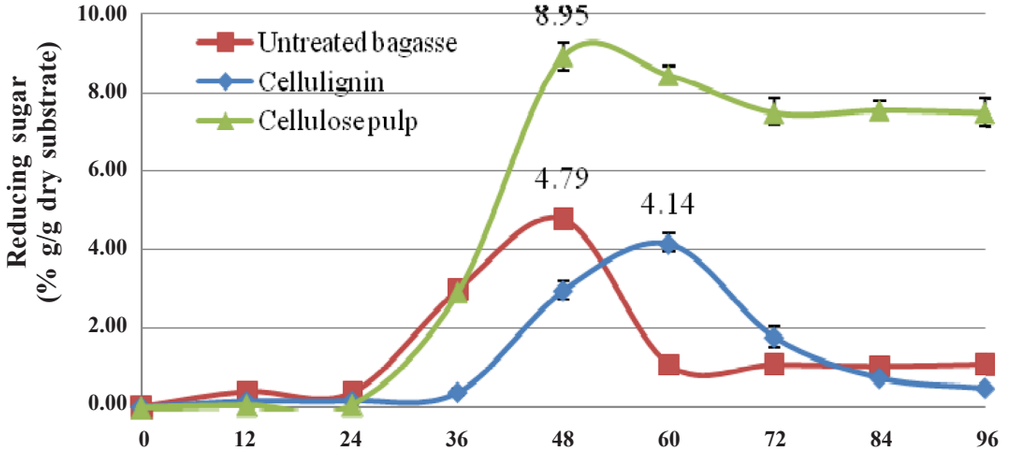

Figure 1. Comparison of reducing sugar production by *T. harzianum* under SSF using different form of sweet sorghum bagasse (untreated bagasse, cellulignin and cellulose pulp) as the substrate (10% inoculum, 70% moisture content).

incubated time, were chosen as shown in Table 3. Details of three different ranges in each parameter were explained in Table 1. The maximum reducing sugar was 9.97% (g/g dry substrate) at the mid values of the parameters condition, which was shown as experimental run number 15 in Table 3.

The statistical software MINITAB release 14 was used in design of experiments, to determine the coefficients of linear, quadratic and interaction terms, as well as to build the quadratic model and 3D response surface plots.

The BBD was used to study the linear, quadratic and interaction effects of various parameters on reducing sugar production. The experimental results were analyzed by regression analysis consisting of the effect linear, quadratic and interaction which gave the following regression equation with reducing sugar production as a function of inoculum size (X_1), moisture content (X_2), and incubated time (X_3):

$$Y = -211.251+2.555X_1+4.086X_2+1.828X_3-0.096X_1^2-0.026X_2^2-0.015X_3^2+0.004X_1X_2-0.017X_1X_3+0.000X_2X_3 \quad (2)$$

Reducing sugar (Y) at specific combination of three variables could be predicted by substituting the corresponding values of each variable in Eq. (2). The predicted values from Eq. (2) of reducing sugar of each experimental runs were shown in the final column of Table 3.

Table 3. Reducing sugar from cellulose pulp in experiments obtained by BBD.

Run number	Level of experimental factors			Reducing sugar % (g/g dry substrate)	
	Inoculum (X_1)	Moisture (X_2)	Time (X_3)	Experimental	Predicted
1	-1	-1	0	6.05 ± 0.11	5.29
2	1	-1	0	6.21 ± 0.21	6.42
3	-1	1	0	2.87 ± 0.15	2.66
4	1	1	0	3.83 ± 0.20	4.59
5	-1	0	-1	0.10 ± 0.05	0.69
6	1	0	-1	4.64 ± 0.48	4.24
7	-1	0	1	7.92 ± 0.45	8.31
8	1	0	1	8.42 ± 0.56	7.83
9	0	-1	-1	3.13 ± 0.13	3.31
10	0	1	-1	1.50 ± 0.05	1.13
11	0	-1	1	8.59 ± 0.35	8.96
12	0	1	1	6.86 ± 0.32	6.68
13	0	0	0	9.80 ± 0.28	9.78
14	0	0	0	9.56 ± 0.46	9.78
15	0	0	0	9.97 ± 0.41	9.78

The probability value (p-value) was a tool for evaluating the significance and contribution of each parameter and the statistical polynomial model equation. The smaller p-value was an indicator of high significance of corresponding coefficient [16]. The regression coefficient in the response surface model for the linear, quadratic and interaction effects of the variables were shown along with p-value in Table 4. The p-value suggested that the coefficient for the linear effect of inoculum size ($p < 0.05$), moisture content ($p < 0.01$) and incubated time ($p < 0.01$) were statistically significant for reducing sugar production.

Analysis of variance (ANOVA) fitted for the model (data not shown). The p-value of the regression model ($p < 0.001$) implied that the model was significant. In addition, the coefficient of variation ($R^2 = 0.98$) indicated a high correlation between the observed and predicted values from model Eq. (2). Therefore, this equation could be used for predicting the amount of reducing sugar production under conditions varied with only three variables in the experimental range.

Table 4. Estimated regression coefficient and corresponding p-value for reducing sugar production.

Term	Coefficient	p-value
Constant	-211.251	0.001**
Inoculum size (X_1)	2.555	0.017*
Moisture content (X_2)	4.086	0.001**
Time (X_3)	1.828	0.004**
Inoculum × Inoculum	-0.096	0.001**
Moisture × Moisture	-0.026	0.001**
Time × Time	-0.015	0.003**
Inoculum × Moisture	0.004	0.608
Inoculum × Time	-0.017	0.040*
Moisture × Time	0.000	0.948

* $p < 0.05$, ** $p < 0.01$

4.4 Evaluation of the Interactions of Each Parameter

The two-dimensional (2D) contour plots and three-dimensional (3D) response surface of the interactions were presented in Figure 2A-C. These observations also identified the optimal conditions with the maximum response for the levels of the factors in the design of experiments. The maximum response was referred by the surface confined in smallest ellipse in the contour plot. The perfect interaction between the independent variables could be shown when elliptical contours were obtained [16].

Moisture content had influence on reducing sugar production. The optimal initial moisture content of medium in reducing sugar production was observed at 77.5% (w/w). The optimal moisture

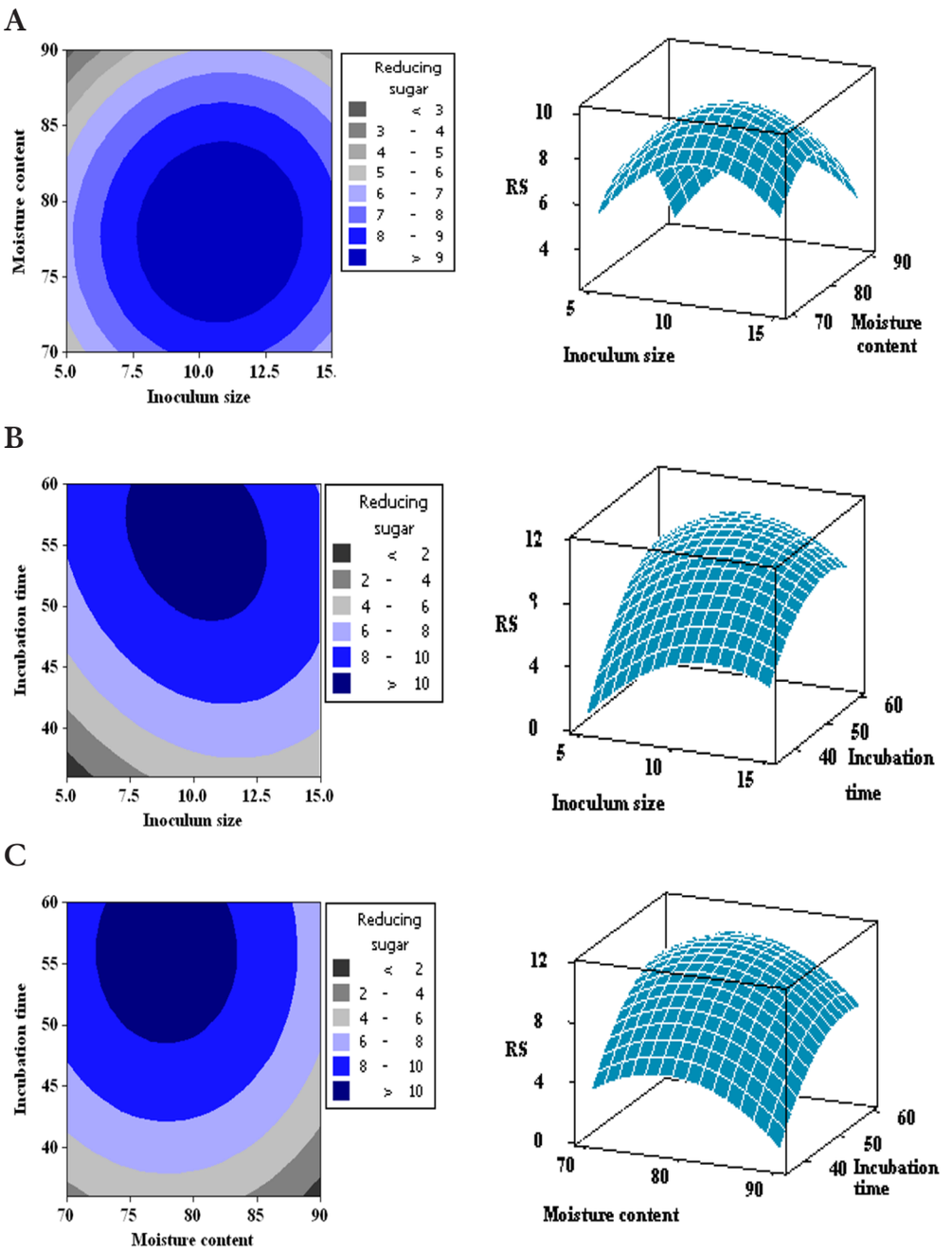

Figure 2. Contour plot and response surface.
A. Effects of inoculum size and initial moisture content at a constant incubated time.
B. Effects of inoculum size and incubated time at a constant initial moisture content.
C. Effects of initial moisture content and incubated time at a constant inoculum size.

content had been approximated to the previous report on scaling up of cellulase production by *T. harzianum* on a mixture of sugar cane bagasse and wheat bran in SSF system [17]. Fungi were well-known to favor a moist environment which positively affected their growth. The appropriate moisture of substrate was one of the critical factors influencing the SSF. It was observed that the moisture enabled better utilization of the substrate by microorganisms and the efficiency of mass transfer in the solid phase depended on the substrate characteristics. However, further too much increase in moisture content negatively influenced the volume of production, resulting as water. It reduced surface area of the biomass, and made the water film thicker, which reduced the accessibility of the air to the biomass. Since the transfer of oxygen affected the growth and metabolism, the substrate should contain suitable amount of water to enhance mass transfer. Therefore, the water content of solid substrates was one of the key factors in SSF [18].

Optimum inoculum density was important consideration for SSF process. An increase in inoculum size would ensure a rapid proliferation and biomass synthesis. However, over crowding number of spores in the inoculum led to the competition for the nutrients resulted in the decreased metabolic activity of the organism. With optimum inoculum size for the reducing sugar production, there was a balance between proliferating biomass and availability of nutrients that supported production of reducing sugar [19]. In current study, the optimal inoculum size of *T. harzianum* was 10.5% (w/w) $\sim 5x10^7$ spores/g dry substrate.

Application of RSM with BBD predicted that the maximum reducing sugar production must occur at decoded values of condition parameters as 10.5% (w/w) inoculum size, 77.5% (w/w) moisture content, and 56 hours of incubated time. The reducing sugar should reach 10.80% (g/g dry substrate). A repeat fermentation for reducing sugar production by *T. harzianum* under optimal conditions was carried out following the validation of optimized parameters. After performing the fermentation under optimal conditions, the obtained reducing sugar was 10.34% (g/g dry substrate). Since the difference between predicted and actual result was only about 4.35%, it should be regarded as acceptable.

4.5 Analysis of Reducing Sugar Composition

The compositions of reducing sugar existing in the enzymatic hydrolysates of cellulose pulp under the optimal conditions by *T. harzianum*, were analyzed by HPLC. Standard curves of cellobiose, glucose, xylose, arabinose and mannose concentration were described first, all showing good linear relativity. The retention time of cellobiose, glucose, xylose, arabinose and mannose concentration are 8.73, 10.20, 10.90, 12.54 and 12.82 min, respectively. Based on these standard curves, the concentration of reducing sugar in the enzymatic hydrolysates of cellulose pulp could be calculated by the peak area The percentage of dry weight (% w/w) was summarized as in table 5, which was 1.11% of cellobiose, 7.57% of glucose, 0.91% of xylose and 0.29% of arabinose and mannose. Glucose and cellobiose obtained from the degradation of cellulose, which was the major element of cellulose pulp as shown in Table 2. Cellobiose was a disaccharide, derived from the condensation of two glucose molecules linked in a

Table 5. Compositions of reducing sugar existing in the enzymatic hydrolysates of cellulose pulp.

Type of Reducing Sugar	Quantity (%w/w)
Cellobiose	1.11
Glucose	8.03
Xylose	0.91
Arabinose and Mannose	0.29

β(1→4) bond. The result showed that glucose was the main component of reducing sugar in the enzymatic hydrolysates. Glucose was the most desired reducing sugar to acquire, since it could be easily fermented to ethanol by various kinds of yeast.

5. CONCLUSIONS

The results obtained in this study had shown that filamentous fungus *T. harzianum* was able to produce cellulolytic enzyme for reducing sugar production from sweet sorghum bagasse as lignocellulose biomass. The optimal conditions obtained through a statistical BBD were successfully determined to maximize the reducing sugar production. The result from the second order polynomial model developed indicated that the optimal conditions for reducing sugar production from cellulose pulp under SSF by *T. harzianum* were the initial moisture content of 77.5%, inoculum size of 10.5%(w/w) and 56 hours of incubated time which give the maximum reducing sugar of 10.34% (g/g dry substrate). The reducing sugar obtained contains glucose as the major component.

ACKNOWLEDGEMENTS

This study was supported by the Graduate School Kasetsart University Research and Development Institute (KURDI), National Center of Excellence for Petroleum, Petrochemicals and Advanced Materials, S&T Postgraduate Education and Research Development Office (PERDO), Center for advanced Studies in Industrial Techechnology (NRU), Department of Chemical Engineering, Faculty of Engineering, Kasetsart University.

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