Optimization of Saccharification Conditions of Acid-pretreated Sweet Sorghum Straw Using Response Surface Methodology

Sukanya Phuengjayaem[1], Aphisit Poonsrisawat[1], Amorn Petsom[2] & Siriluk Teeradakorn[3]

[1] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

[2] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

[3] The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok, Thailand

Correspondence: Siriluk Teeradakorn, The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10330, Thailand. Tel: 66-2218-8073. E-mail: tsiriluk@chula.ac.th

Abstract

This study focused on the cellulase production from *C. versicolor* TD17, white rot fungi. The maximum cellulase activity of 0.897 U/ml was obtained after 5 days of cultivation using 20 g/l cellobiose as a carbon source and 2 g/l ammonium sulfate supplemented with 0.3 g/l urea as nitrogen sources. Enzymatic saccharification of acid-pretreated sweet sorghum straw (SSS) using in house cellulase was optimized using Response Surface Methodology (RSM), variable five-code-level, four-factor; 1.0-7.0% w/v acid-pretreated SSS, 15-35 FPU/g dry substrate of cellulase enzyme, pH 3 to 7 and temperatures 30 to 70 °C. The optimal conditions were 1% w/v acid-pretreated SSS, 25 FPU/g dry substrate of cellulase, pH 5, 50 °C and 72 h cultivation. A maximal glucose yield of 0.440 g/g dry substrate was obtained.

Keywords: saccharification, acid pretreatment, sweet sorghum straw, response surface methodology

1. Introduction

The industrial revolution has generated an increasing need for energy. Petroleum was in great demand and its use has been spread, therefore, rapid price rising of petroleum-based fuels have recently increased. As a consequence, ethanol production as an alternative to petroleum-based fuels can be produced from biomass, a plentiful renewable resource. To increase the productivity and cost effectiveness of ethanol production, lignocellulose is one of potential choices due to sufficient abundance and generating very low net greenhouse emissions (Ikeda et al., 2007). It reduces carbon monoxide emission by blending bioethanol into gasoline. Apart from sugarcane (in Brazil), corn grain (in USA), tapioca starch and sugarcane molasses (in Thailand), other agricultural raw materials rich in fermentable carbohydrates, including sorghum, have been of particular interest for biological transformation into ethanol for use as fuel or fuel additive (Laopaiboon et al., 2009).

Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a tropical grass that can be cultivated in nearly all temperatures and in a wide range of tropical climates. Sweet sorghum is a drought-resistant agricultural crop that can remain dormant during dry periods because it is tolerant of water-logging, salinity and alkalinity. Sweet sorghum is also reported to remain dormant under more favorable environmental and nutritional conditions (Laopaiboon et al., 2009). Sweet sorghum is an inexpensive and abundant renewable cellulose resource that can be synthetically used in additives as a raw material for ethanol production and has byproducts with high additional value. This grain's stalk has high polysaccharide content comprised of cellulose and hemicellulose. Cellulose is a major fraction of lignocellulosic biomass that can be hydrolyzed to glucose by cellulase enzymes. The natural structure of this biomass makes it difficult for microorganisms to utilize these components to produce ethanol.

The main processes of lignocellulose biomass to ethanol conversion consist of pretreatment, enzymatic hydrolysis (saccharification) and ethanol fermentation. Saccharification is a critical step for sugar production. The pretreatment breaks the lignin seal and alters substrate composition, which is essential for lignin removal, hemicellulose pre-hydrolysis, cellulose crystallinity reduction, and increasing lignocellulosic material porosity. Pretreatment significantly improves sequential enzyme attack for maximal sugar productivity. Pretreatment methods such as H_2SO_4 or HCl treatment give high hemicellulose sugar recovery in the liquid fraction with most

of the cellulose remaining in the solid residue for sequential enzymatic saccharification (Qi et al., 2009). Enzymatic saccharification is the second step in ethanol production from cellulosic material. The main hydrolysis product of cellulose is glucose, whereas hemicellulose yields pentoses and hexoses. Cellulases are key enzymes for bioconversion of cellulosic biomass to useful products. Enzyme saccharification activity is important to produce reducing sugars from cellulosic biomass, especially glucose. This ability is influenced by enzyme component composition (Ikeda et al., 2007). A complete cellulase system consists of three extracellular enzymes that are required for complete cellulose breakdown to simple sugars. The cellulase system contains Endoglucanase (EG), Exoglucanases and β-glucosidases (BGL). Cellulase production is the most important step in achieving economical ethanol production from renewable cellulosic material. Cellulolytic enzyme components are different in each microorganism species. Many microorganisms that produce various cellulolytic enzymes have been studied for several decades; e.g., fungi, bacteria and actinomycetes. Most commercial cellulase production research has focused on fungi, such as *Trichoderma reesei*, *Aspergillus niger* and *Penicillium brasilianum*. Of these fungal genera, the well-studied *Trichoderma reesei* fungus has been famous for producing commercial cellulolytic enzymes with relatively high enzymatic activity. However, *Trichoderma* enzymes do not effectively hydrolyze cellulose biomass alone (Sun & Cheng, 2002) because low β-glucosidase activity is produced relative to the total cellulase activity, which is inhibited by glucose. Because inhibiting the product of BGL controls saccharification, enzymatic hydrolysis efficiency cannot be improved much by increasing enzyme loading, and high enzyme amounts are added for saccharification (Sukumaran et al., 2009). White-rot fungus is an interesting use for lignocellulose degradation because of its ability to degrade all lignocellulosic material components completely. A statistical approach was applicable to improve enzymatic saccharification process performance and develop more economical cellulolytic enzyme production (Levin et al., 2008). Enzymatic saccharification efficiency depends on several process parameters, such as enzyme loading, substrate concentration, temperature and pH, which often interact with one another. Traditional methods for optimizing a multifactorial system include dealing with "one-factor-at-a-time," which involves changing one independent variable while fixing other variables to investigate an individual factor's influence on process performance. This "one-factor-at-a-time" method ignores interactions among different factors, and has been criticized for having little chance of finding optimal conditions. This single-dimensional search is laborious, time-consuming, expensive and incapable of reaching a true optimum because it does not estimate interactions among experimental variables. A statistical method was recently used as an alternative and more efficient approach. Response surface methodology (RSM) was applied to identify optimal conditions for reducing sugar production from enzyme saccharification of pretreated sweet sorghum straw by analyzing the effect of multiple variables on overall process speed and efficiently with minimal experiments while ensuring a high degree of statistical significance in the results (Qi et al., 2009; Levin et al., 2008; Jeya et al., 2010).

In this study, enzymatic saccharification of acid-pretreated sweet sorghum straw was investigated using cellulolytic enzyme from *Coriolus versicolor* TD17 compared with commercial cellulase from *Trichoderma reesei*. Moreover, optimal saccharification conditions were studied using the RSM method.

2. Method

2.1 Fungal Strain and Culture Conditions

Coriolus versicolor TD17 was kindly offered from Emeritus Professor Yataka Kitamoto, Japan. The fungal strain was maintained in potato dextrose agar at 4 °C. Small pieces (20-40 mm^2) of mycelium without agar were cultured on a fresh, sterile potato dextrose agar (PDA) slant for 10 days at 30 °C before being aseptically transferred to a 250 ml Erlenmeyer flask containing 50 ml potato dextrose broth (PDB). Initial media pH was adjusted to 5.0 with either 1N NaOH or HCl. The media was then autoclaved for 20 min at 121 °C. Cultivation conditions were 30 °C with 150 rpm shaking for 48 h. Next, 10% v/v submerged culture was used as the inoculum for cellulase production.

2.2 Cellulase Production From Coriolus versicolor TD17

Basal media composition for cellulase production was based on Mandel's method (Mandels, 1975). The culture media contained 20 g/l carboxymethylcellulose (CMC), 3.0 g/l ammonium sulfate, 1.0 g/l peptone, 0.3 g/l urea, 2.0 g/l potassium di-hydrogen phosphate, 0.5 g/l magnesium sulfate, 1.0 g/l tween-80 and 2.0 ml/l trace metal solution. The trace metal solution contained 2.5 g/l ferrous sulfate, 0.8 g/l manganese sulfate, 0.7 g/l zinc sulfate and 1.0 g/l cobalt chloride, pH 4.0. Media was sterilized by autoclaving at 121 °C for 15 min. The crude filtrate from the cultivation culture was used as crude cellulase in enzymatic saccharification studies.

2.3 Effect of Carbon Sources on Cellulase Production

Different carbon sources were studied using basal media supplemented with either 20 g/l of

carboxymethylcellulose, cellobiose, avicel, or α-cellulose as the sole carbon source for the optimization experiment.

2.4 Effect of Nitrogen Sources on Cellulase Production

Different nitrogen sources were studied using basal media supplemented with either 4 g/l ammonium sulfate, ammonium nitrate, peptone, yeast extract, urea or control nitrogen sources (ammonium sulfate, peptone and urea) for the optimization experiment.

2.5 Effect of Combined Nitrogen Sources on Cellulase Production

The influence of each nitrogen source (ammonium sulfate, peptone and urea) was investigated using basal media in which a single nitrogen source was taken from the production media and compared with only ammonium sulfate as a nitrogen source and control media that contained three nitrogen sources as shown in Table 1.

Table 1. Various nitrogen sources in the culture media

Abbreviation	Nitrogen sources		
	$(NH_4)_2SO_4$	Peptone	Urea
NPU(control)	+	+	+
N	+	-	-
NP	+	+	-
NU	+	-	+

2.6 Sweet Sorghum Straw Pretreatment

Sweet sorghum straw (SSS) was obtained from The Suphanburi Field Crops Research Center in Thailand. The SSS consisted of 44.51% cellulose, 38.62% hemicellulose, 6.18% lignin and 10.69% ash. Chopped SSS was dried in an oven at 70 °C to a constant weight. In total, 30 grams chopped sweet sorghum straw was suspended in 300 ml of 3% sulfuric acid solution at 120 °C, for 10 minutes. After pretreatment, the hydrolyzate was neutralized with 40% NaOH, centrifuged and filtered through 0.45 µm filters before analyzing total reducing sugars with the DNS method and monomeric sugars (glucose, xylose, galactose, arabinose and mannose) by HPLC. The solid residue was collected by filtration and washed extensively with distilled water until a neutral pH was obtained. Acid-pretreated SSS was dried in an oven at 70 °C to a constant weight and used as a substrate for the saccharification experiment.

2.7 Acid-Pretreated Sweet Sorghum Straw Saccharification

A typical hydrolysis mixture consisted of 0.1 g acid pretreated SSS, 20 FPU/g dry substrate of cellulase from Celluclast 1.5, Novozyme or cellulase from *Coriolus versicolor* TD17 and 2.0 ml sodium phosphate buffer (pH 6.0). Microbial contamination was prevented by adding 0.01 mg/ml sodium azide. The mixture was incubated at 50°C in a rotary shaker at 150 rpm for 7 days. Samples were obtained from the reaction mixture at different time intervals. The samples were cooled, centrifuged for 10 min at 10,000 rpm, and the supernatant was used to analyze total reducing sugars by the DNS method and monomeric sugars by HPLC.

2.8 Enzyme Assays

Cellulase activity was assayed using a method that was described by Mandels and Weber (Mandels and Weber, 1969). The activity was estimated using 2% w/v carboxymethylcellulose in citrate buffer (50 mM, pH 4.8) as a substrate. The reaction mixture contained 1 ml citrate buffer, 0.5 ml substrate solution and 0.5 ml diluted enzyme solution, and the reaction mixture was incubated at 50 °C for 30 min. The liberated reducing sugars were estimated using the DNS method. One unit of enzyme activity was defined as the amount of enzyme that was required to yield one micromole reducing sugar and was expressed as glucose per min under the assay conditions.

2.9 Monomeric Sugar Analyses

High-performance liquid chromatography (HPLC) was used to determine monomeric sugar concentrations (xylose, glucose, galactose, arabinose and mannose) using an Aminex HPX-87P column (Bio-Rad, Richmond, USA) with a refractive index detector. The analysis was performed at 85 °C using Milli-Q water as the eluent with a flow rate of 0.6 ml/min. Sample peak areas were identified and quantified by comparing with the retention times of known analytical standards (glucose, xylose, galactose, arabinose and mannose).

2.10 Statistical Analyses

All of the experiments were performed in triplicate and the related data were expressed as average values. Enzymatic saccharification experimental data were analyzed using SPSS software with one-way analysis of variance (ANOVA) followed by Tukey's multiple range method test to compare means. Differences in means were judged to be significant when p values for the null hypothesis were 0.05 or less.

2.11 Response Surface Methodology (RSM)

A factorial, central composite design (CCD) for four factors with replicates at the center point and star points were used in this investigation. Saccharification condition variables were 1–7% substrate concentration, 15–35 FPU/g substrate cellulase concentration, temperature30-70 °C and pH 3-7. Each variable at five coded levels (-α, -1, 0, +1, +α) was assessed using statistical analysis and RSM as demonstrated in Table 2. The actual variable levels for the CCD experiments were selected based on using the initial levels as the center points. A total of thirty experimental trials, including sixteen for factorial design, eight for axial points (two for each variable) and six for replication of the central points were performed. The Design-Expert 8.0 statistical software package (Stat-Ease, Inc., Minneapolis, USA) was used for experimental data regression analysis and to plot the response surface.

Table 2. Variables and their levels for central composite experimental design

Variables	Units	Symbol	Code levels				
			-2	-1	0	1	2
Substrate	% w/v	A	1%	2.5%	4%	5.5%	7%
Cellulase	FPU/g dry substrate	B	15	20	25	30	35
Temperature	°C	C	30	40	50	60	70
pH	-	D	3	4	5	6	7

3. Results

3.1 Effect of Carbon Sources on Cellulase Production

The effect of carbon sources carboxymethylcellulose, cellobiose, avicel and α-cellulose on cellulase production was investigated at different cultivation times.

The experimental results demonstrated in Figure 1, indicated that cellobiose was the best carbon source followed by carboxymethylcellulose whereas avicel and α-cellulose had no significant effect on cellulase production. The highest cellulase activity (0.829 U/ml after 9 days of cultivation) was obtained when cellobiose was used as the sole carbon source. Carboxymethylcellulose, avicel and α-cellulose cellulase activities were 0.446 U/ml, 0.276 U/ml and 0.239 U/ml after 9 days cultivation time, respectively. In the presence of either avicel or α-cellulose, cell dry weight was significantly increased, in contrast with cellulase activity. Including avicel and α-cellulose in the media supported high *C. versicolor* TD17 cell growth but resulted in minimal cellulase production. The obvious high *C. versicolor* TD17 cell growth in the presence of avicel or α-cellulose as a carbon source was four times higher compared with using carboxymethylcellulose and cellobiose as carbon sources. This might be an error from the cell growth measurement method. These values included residual avicel or α-cellulose because we used cell dry weight to represent cell growth.

The cellulase activity time course study using cellobiose as a carbon source revealed a significant increase in enzyme production with cultivation time. Cellulase activity was 0.621 U/ml and 0.829 U/ml after 2 and 9 days of cultivation time, respectively, which was because of rapid cellulose hydrolysis in the media. Further increases in fermentation (after 9 days) resulted in decreased cellulase activity.
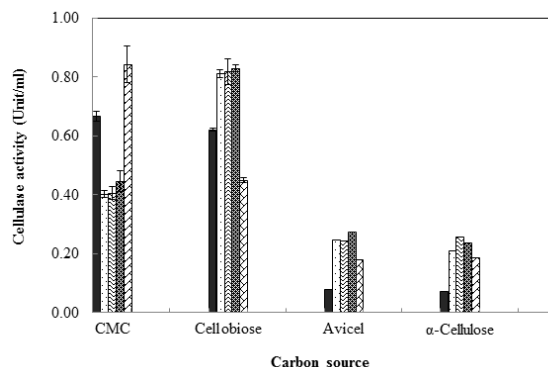
Figure 1. Effect of carbon sources on cellulase production from *Coriolus versicolor* TD17

Cells were cultivated for 11 days in 250 ml erlemeyer flasks with an initial various carbon source of 20 g/l. Symbol key: (■) 2 days; (□) 5 days; (▨) 7 days; (▨) 9 days; (▨) 11 days. The error bars in the figure indicated the standard deviation (SD) among three parallel replicates.

The analyses of variance for all of the data using SPSS software (data not shown) indicated that the means of various carbon sources used for cellulase production from *C. versicolor* TD17 were statistically different with a 95% confidence interval with Tukey's test result at $\alpha = 0.05$. A maximal cellulase activity of 0.7064 U/ml was obtained with 20 g/l cellobiose followed by carboxymethylcellulose, avicel and α-cellulose, respectively. This result may be attributable to cellulase enzyme induction because cellulose is a universal inducer of cellulase synthesis (Paul & Varma, 1993). Considering the time course of cellulase production, the highest cellulase amount was obtained at the 9th day of cultivation. Increasing cultivation time from 2-9 days exhibited a significant increase cellulase production, whereas increasing cultivation time from 5-9 days had no significant on cellulase production, but cellulase production was significantly decreased when cultivation time was extended longer than 9 days. This observation was in close agreement with the results of SzabÓ et al., who demonstrated optimal cellulase production to be 129 mg cellulase/g dry substrate from *Phanerochaete chrysosporium* when using 20 g/l cellobiose as a carbon source (SzabÓ et al., 1996).

3.2 Effect of Nitrogen Sources on Cellulase Production

Cultivation with various nitrogen sources such as ammonium sulfate, ammonium nitrate, peptone, yeast extract and urea were substituted for control nitrogen sources (ammonium sulfate, peptone and urea) in the basal media. The experimental results demonstrated in Figure 2, indicated that ammonium nitrate was the best nitrogen source followed by ammonium sulfate, control media, urea, peptone and yeast extract.
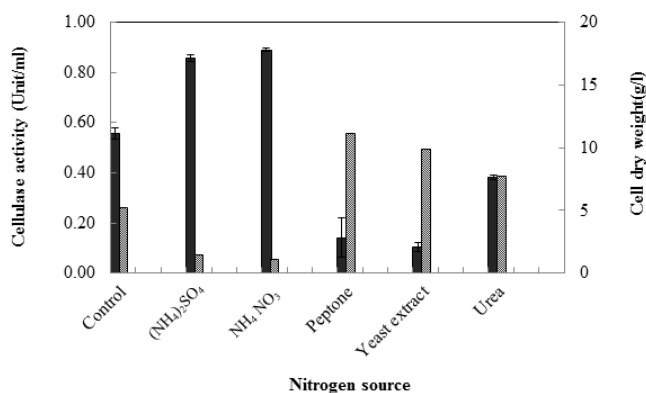

Figure 2. Effect of nitrogen sources on cellulase production from *Coriolus versicolor* TD17

Cells were cultivated for 5 days in 250 ml erlemeyer flasks with an initial various nitrogen source of 4 g/l. Symbol key: (■) cellulase activity (U/ml); (▨) cell dry weight (g/l). The error bars in the figure indicated the standard deviation (SD) among three parallel replicates. *Control (ammonium sulfate, peptone and urea).

The analyses of variance for all of the data using SPSS software (data not shown) demonstrated that various nitrogen sources that are used as a substrate for cellulase production from *C. versicolor* TD17 were statistically different with a 95% confidence interval followed by Tukey's test at α = 0.05. Maximum cellulase activity of 0.888 U/ml was obtained with 4 g/l ammonium nitrate as the sole nitrogen source followed by ammonium sulfate, control, urea, peptone and yeast extract, respectively. However, with ammonium nitrate or ammonium sulfate as substrate, there were no significant effects on cellulase production. Considering the cost of the nitrogen source, using ammonium sulfate will improve the economic cost of cellulase production from *C. versicolor* TD17. Adding an organic nitrogen source, such as peptone or yeast extract resulted in increased cell growth but gave a small amount of enzyme production. More literature data indicated that inorganic nitrogen sources should be used for economic aspects. From our results, ammonium sulfate itself could act as a suitable nitrogen source for cellulase production. Furthermore, the optimal ammonium sulfate concentration was investigated. These results demonstrated that 2 g/l ammonium sulfate yielded maximum cellulase activity of 0.606 U/ml 5 days after cultivation (data not shown). Several adjustments in combination with nitrogen sources were also investigated for greater cellulase production (Liu & Yang, 2007).

3.3 Effect of Combined Nitrogen Sources on Cellulase Production

The influence of each nitrogen source on cellulase production and cell growth from *C.versicolor* TD17 was investigated using media in which only a single nitrogen source was taken from the production media compared with using only ammonium sulfate as a nitrogen source and control media that consisted of three nitrogen sources. Experimental results were shown in Figure 3, which indicated that the culture media that consisted of only ammonium sulfate as a nitrogen source exhibited the maximum cellulase activity of 0.579 U/ml higher than the control media. Culture using media without urea demonstrated lower cellulase activity than the control media, whereas the dry cell weight was similar to the control. Cultivation using the media without peptone demonstrated high cell growth but produced less cellulase than the control media. Therefore, urea and peptone were necessary for cell growth. These results indicated that supplementing with more than one nitrogen source affected cell growth.
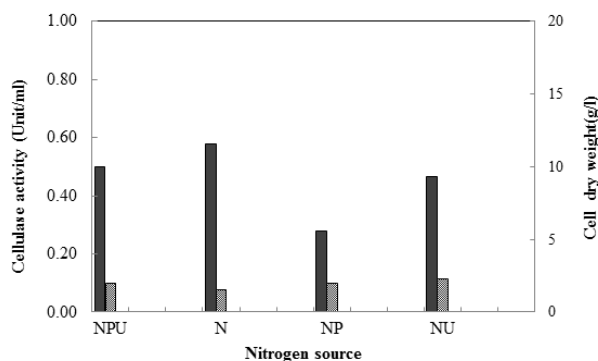

Figure 3. Effect of combined nitrogen sources on cell growth and cellulase production from *Coriolus versicolor* TD17

Cells were cultivated for 5 days in 250 ml erlemeyer flasks with combined nitrogen sources. Symbol key: (■) cellulase activity (U/ml); (▧) cell dry weight (g/l). *Control (NPU = ammonium sulfate, peptone and urea).

The analyses of variance was performed for all of the data using SPSS software (data not shown) and demonstrated that combined nitrogen sources when used as substrates for cellulase production from *C.versicolor* TD17 were statistically different with a 95% confidence interval and Tukey's test was α = 0.05. Maximum cellulase activity (0.579 U/ml) was obtained when 2 g/l ammonium sulfate was used as a sole nitrogen source followed by a control nitrogen source (NPU), ammonium sulfate with urea (NU) and ammonium sulfate with peptone (NP). There were no significant differences in cellulase production when using control media or ammonium sulfate with urea (NU) serving as a nitrogen source. The statistical analysis results clearly demonstrated that supplementation with 2 g/l ammonium sulfate was optimal for cellulase production but neither urea nor peptone had any effect on cellulose production. However, we used 2 g/l ammonium sulfate supplement with 0.3 g/l urea, which had a synergistic effect on cell growth. These results were in accordance with those that were reported by Ikeda (Ikeda et al., 2007). Various cultivation media and cellulase production conditions from

different microorganism species sources were also summarized in Table 3.

Table 3. Comparison of cellulase production by different microorganism species and substrates

Microorganism Species	Carbon Sources	Nitrogen Sources	pH	Temp. (^{o}C)	Cultivation Time (Days)	Cellulase Activity (U/ml)	References
Trichoderma reesei Rut C-30	25 g/l cellulose, 25 g/l sugar beet pulp	0.3 g/l urea, 1.4 g/l $(NH_4)_2SO_4$, 0.25 g/l yeast extract	5.0	28	4	0.450	(Olsson et al., 2003)
Aspergillus Niger	1.0% cellulose	3 g/l urea	5.0	28	7	0.824	(Narasimha et al., 2006)
Streptomyces Sp. (Strain J2)	0.2% glucose	0.2% NH_4Cl	7.0	30	3	0.432	(Jaradat et al., 2008)
Aspergillus Niger	9.6% sawdust	0.3 g/l urea, 1.4 g/l $(NH_4)_2SO_4$, 1.0 g/l peptone	4.0-4.5	28	4	0.181	(Acharya et al., 2008)
Bacillus alcalophilus S39	1% CMC	0.7% yeast extract	7.0	30	3	2.070	(Taleb et al., 2009)
Coprinellus disseminates	20 g/l glucose	1 g/l yeast extract	6.4	37	7	0.925	(Singh et al., 2009)
Agaricus arvensis	20 g/l rice straw	5 g/l tryptone	-	-	12	2.600	(Jeya et al., 2010)
Coriolus versicolor TD17	20 g/l cellobiose	2 g/l $(NH_4)_2SO_4$, 0.3 g/l urea	5	30	5	0.897	This work

3.4 Optimization of Cellulase Saccharification Conditions From C. versicolor TD17 *Using Response Surface Methodology*

Experiments were performed using saccharification conditions that were obtained by central composite design of four independent variables: substrate concentration, cellulase concentration produced by *C. versicolor* TD17, temperature and pH. Experimental values were expressed as the total reducing sugars and glucose concentration obtained after 72 h saccharification. Thirty experiments were performed in triplicate corresponding to each combination and were summarized in Table 4.

Table 4. Central composite design (CCD) four factor variables at five code levels by cellulase enzyme from *Coriolus versicolor* TD17

Sample No.	Substrate (%w/v)	Enzyme (FPU/g DS)	Temp. (°C)	pH	Actual glucose (g/g DS)	Predicted glucose (g/g DS)
1	5.5	20	40	6	0.058	0.023
2	4.0	25	50	5	0.103	0.100
3	2.5	30	60	4	0.069	0.115
4	5.5	30	60	6	0.017	0.023
5	4.0	25	50	5	0.089	0.100
6	5.5	30	40	4	0.139	0.107
7	2.5	20	40	6	0.150	0.148
8	4.0	25	50	5	0.113	0.100
9	5.5	30	60	4	0.059	0.040
10	2.5	20	60	4	0.099	0.120
11	2.5	20	40	4	0.233	0.239
12	5.5	20	60	6	0.025	0.002
13	2.5	20	60	6	0.004	0.047
14	5.5	20	40	4	0.100	0.076
15	5.5	20	60	4	0.036	0.037
16	2.5	30	60	6	0.058	0.060
17	2.5	30	40	6	0.178	0.189
18	5.5	30	40	6	0.114	0.072
19	2.5	30	40	4	0.260	0.262
20	4.0	25	50	5	0.119	0.100
21	4.0	25	50	5	0.115	0.100
22	4.0	15	50	5	0.100	0.064
23	4.0	25	70	5	0.042	0.040
24	4.0	25	50	7	0.000	0.026
25	4.0	25	50	3	0.122	0.082
26	1.0	25	50	5	0.440	0.332
27	4.0	25	30	5	0.114	0.128
28	4.0	35	50	5	0.136	0.108
29	7.0	25	50	5	0.087	0.132
30	4.0	25	50	5	0.132	0.100

Applying the RSM yielded the regression equation, which had an empirical relationship between the glucose concentration and the test variables in coded units. The statistical significance of the model equation (Equation 1) was assessed using the *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model was shown in Table 5.

Table 5. Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of Squares	Mean Square	F-value	p-value Prob > F
Model	0.183	1.31×10^{-2}	6.75	0.0005
A-Substrate (%w/v)	6.09×10^{-2}	6.09×10^{-2}	31.36	< 0.0001
B-Enzyme (FPU/g DS)	2.79×10^{-3}	2.79×10^{-3}	1.44	0.2501
C-temperature($^{\circ}$C)	4.26×10^{-2}	4.26×10^{-2}	21.94	0.0004
D-pH	1.69×10^{-2}	1.69×10^{-2}	8.72	0.0105
AB	5.84×10^{-5}	5.84×10^{-5}	0.03	0.8647
AC	6.33×10^{-3}	6.33×10^{-3}	3.26	0.0925
AD	1.44×10^{-3}	1.44×10^{-3}	0.74	0.4037
BC	7.73×10^{-4}	7.73×10^{-4}	0.40	0.5381
BD	3.23×10^{-4}	3.23×10^{-4}	0.17	0.6894
CD	3.21×10^{-4}	3.21×10^{-4}	0.17	0.6903
A^2	2.97×10^{-2}	2.97×10^{-2}	15.29	0.0016
B^2	3.47×10^{-4}	3.47×10^{-4}	0.18	0.6788
C^2	5.03×10^{-3}	5.03×10^{-3}	2.59	0.1298
D^2	8.58×10^{-3}	8.58×10^{-3}	4.42	0.0541

Form the model, F value was 12.83 and probability values (P-values) > F values or $\alpha < 0.0001$, which implied that the model was significant ($P < 0.05$). This model with a very low probability value demonstrated high significance for the regression model. These data indicated that the model was statistically significant with a 95% confidence interval, a coefficient of determination (R^2) of 0.8709, the coefficient of the varation (CV) of 39.915 %, lack of fit of 0.0090 (significance) and pure error of 0.00069. The model is stronger and the predicted response is better as the R^2 value becomes closer to 1.0. However, Ferreira et al. reported that R^2 value > 0.75 indicate the model's tendency (Ferreira et al., 2009). Therefore, the model was applicable for predicting saccharification conditions in this study. Reaction variable interaction was presented using ANOVA. These data demonstrated that individual variables on saccharification conditions were significantly ($P < 0.05$) affected by substrate concentration, enzyme concentration, temperature and pH. The effect of substrate concentration was significant ($P < 0.0001$), while the effect of enzyme concentration was not significant ($P = 0.2501$). Temperature and pH were the most significant variables because of very low probability values ($P = 0.0004$ and 0.0105)

The RSM application yielded the following overall second-degree quadratic polynomial equation for saccharification, which is given by the following equation:

$$Y=0.1165-0.0504A+0.0108B-0.0421C-0.0267D+1.9109\times10^{-3}AB+0.0199AC+9.4834\times10^{-3}AD-6.9507\times10^{-3}BC$$
$$+4.4947\times10^{-3}BD+4.4810\times10^{-3}CD+0.0329A^2-3.557\times10^{-3}B^2-0.0135C^2-0.0177D^2 \qquad (1)$$

Where Y denotes the glucose concentration (g glucose/g dry substrate), A is the substrate concentration (%w/v), B is the enzyme concentration (FPU/g dry substrate), C is the temperature (°C), and D is the pH. Model accuracy was assessed by plotting between the actual and the predicted glucose concentrations. The normality assumption was satisfactory because normal residuals fell along a straight line as shown in Figure 4.
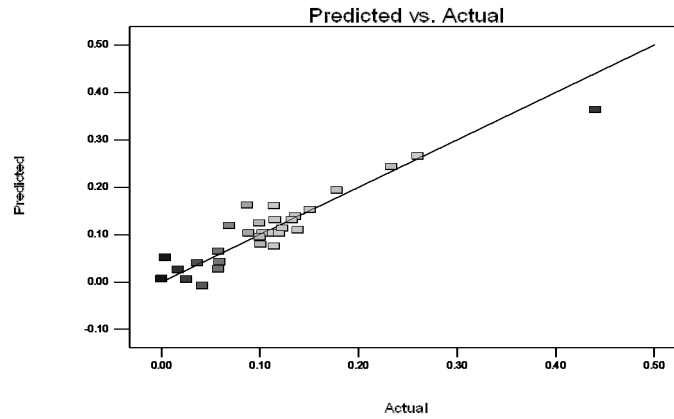
Figure 4. Correlation between the actual and the predicted glucose concentration that was liberated from acid-pretreated sweet sorghum straw using cellulase enzyme from *Coriolus versicolor* TD17

The response was plotted on the Z-axis to obtain 3D response surface plots (g glucose/g dry substrate) against any two variables while keeping the other variable constant at its '0' level.
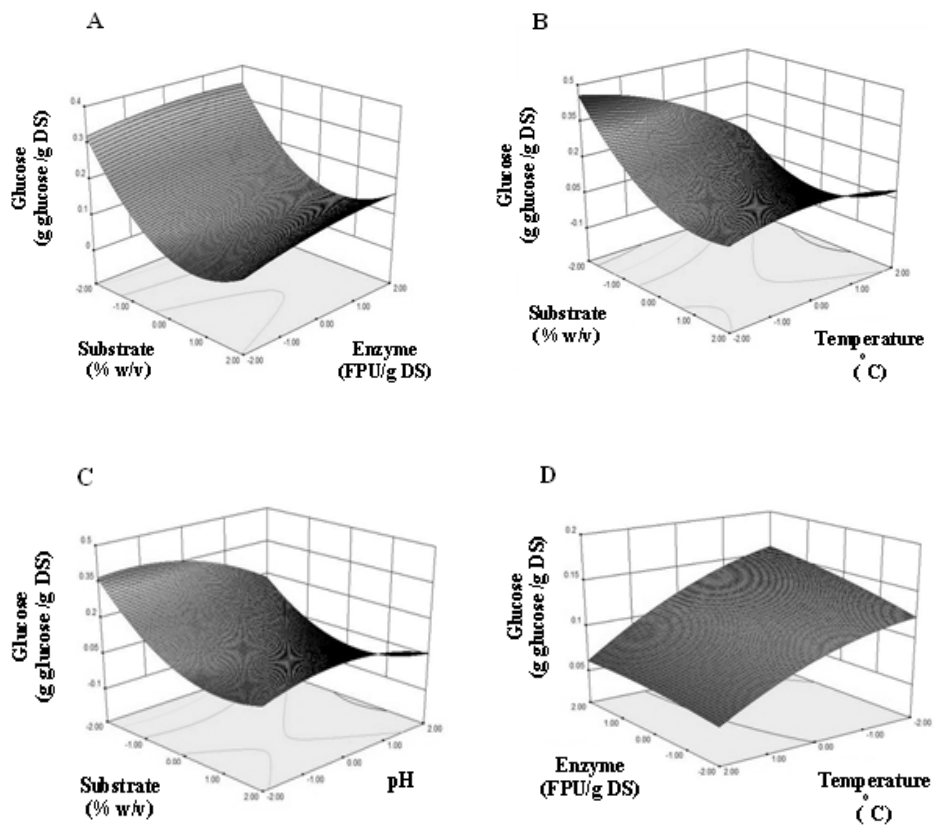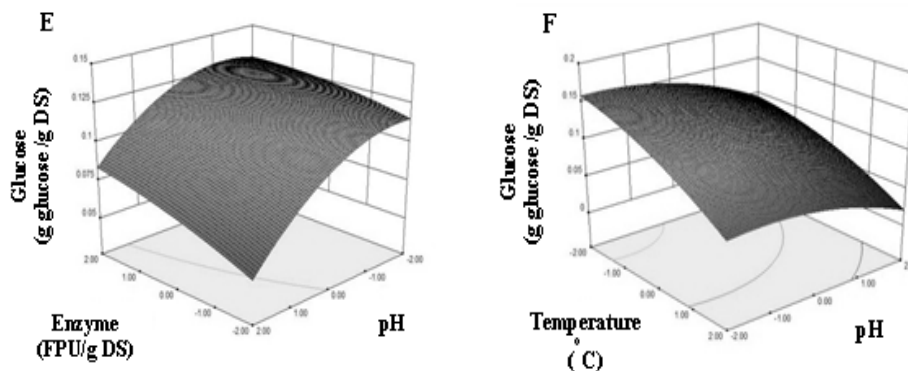
Figure 5. Response surface plots demonstrating interactions among variables in converting the acid pretreated SSS to glucose

(A) interaction between substrate and enzyme concentrations; (B) interaction between substrate concentration and temperature; (C) interaction between substrate concentration and pH; (D) interaction between enzyme concentration and temperature; (E) interaction between enzyme concentration and pH; (F) interaction between temperature and pH. Perturbation graph demonstrating the effect of each variable on saccharification, while keeping other variables at respective '0' levels. A – substrate concentration (% w/v); B – enzyme concentration (FPU/g dry substrate); C – temperature (°C); D – pH. Axis X and Y denoted vaiables (substrate, enzyme, temperature and pH); axis Z denoted saccharification yield (glucose).

Isoresponse contour and surface plots for optimal saccharification condition were demonstrated in Figure 5. From Figure 5A, substrate concentration in the range 1-7% w/v had a negative effect, which resulted in significantly decreased glucose concentrations. Increasing the enzyme concentration from 15 to 35 FPU/g dry substrate did not further increase glucose concentration. The optimal substrate concentration was approximately 1.0-2.5% w/v with temperature between 30 and 40 °C and pH between 3 and 5 (Figure 5B and Figure 5C). The optimal glucose yield was obtained at low temperatures (30 °C) but gradually decreased at temperatures over 50 °C (Figure 5D). Increasing enzyme concentrations (15-35 FPU/g dry substrate) produced no significant differences in glucose yield. The optimal enzyme concentration was approximately 15-35 FPU/g substrate with a pH between 3 and 5 (Figure 5E. The optimal temperature and pH were between 30 and 50 °C and 3 and 5 (Figure 5F), respectively. These data demonstrated that individual variables on saccharification conditions were significantly ($P < 0.05$) affected by substrate concentration, enzyme concentration, temperature and pH. The effect of substrate concentration was significant ($P < 0.0001$), while the effect of enzyme concentration was not significant ($P = 0.2501$). Temperature and pH were the most significant variables because of very low probability values ($P = 0.0004$ and 0.0105).

In summary, response surface methodology was successfully applied to optimize enzymatic saccharification conditions of the acid-pretreated SSS by applying cellulase from a white rot fungus, *Coriolus versicolor* TD17. Optimal saccharification conditions were obtained using 1% of the acid pretreated SSS, 25 FPU/g dry substrate in house cellulase, pH 5 and a temperature of 50 °C, which provided a maximum yield of 0.440 g glucose/g dry substrate (Run No.26 in Table 4) at 72 h incubation time. Optimal saccharification conditions of several commercial cellulase and substrate concentrations were in the range 15–35 FPU/g dry substrate and 1.0-5.0% w/v, respectively (Cara et al., 2008; Jeya et al., 2010).

Table 6. Summary of the combined yield of monosugars that were liberated from sweet sorghum straw acid pretreatment (stage 1) and enzymatic saccharification of the pretreated sweet sorghum straw using cellulase from *Coriolus versicolor* TD17 (stage 2)

Conditions	Yield $_{avg}$ (g monosugar /g dry substrate)			
	Glucose	Xyl	Gal, Man, Ara	Total sugars
Stage 1	0.234±0.079	0.208±0.073	0.235±0.164	0.676±0.230
Stage 2	0.440±0.001	0	0	0.440±0.001
Stage1+Stage2	0.674±0.080	0.208±0.073	0.235±0.164	1.116±0.231

Overall SSS hydrolysis including acid pretreatment and enzymatic saccharification was shown in Table 6, maximum glucose yield was 0.674 g/g dry substrate, the maximum xylose yield was 0.208 g/g dry substrate and other monosugars (galactose, mannose and arabinose) were 0.235 g/g dry substrate. Optimal conditions for lignocellulosic material hydrolysis by cellulolytic enzymes gave the indicated values for "maximum fermentative sugar concentration" that are summarized in Table 7.

Table 7. Summary of the optimal conditions for cellulase activity to saccharify lignocellulosic materials

Source of cellulase	Enzyme concentration (FPU/g DS)	Substrate Concentration (g/g DS)	Temperature (ºC)	pH	Reducing sugars (g/g-DS)	References
Trametes hirsute	30 FPU/g-substrate	2.25% rice straw	25-35	5	0.685 g RS/g DS	(Jeya et al., 2010)
Agaricus arvensis	65 FPU/g-substrate	10% poplar	37	5	0.293 g RS/g DS	(Jeya et al., 2010)
Novozyme NS50013 (Cellulase complex)	60 FPU/g substrate	0.40 % pretreated rock-rose	50	4.86	0.313 g RS/g DS	(Ferreira et al., 2009)
	60 FPU/g substrate	0.27% pretreated broom	50	4.5	0.448 g glu./g DS	(Ferreira et al., 2009)
Commercial cellulase from *Aspergillus niger*	0.16(v/v)	50% food waste	46.3	5.2	0.117 g RS/g DS	(Kim et al., 2008)
Commercial cellulase from Novozymes	15 FPU/g substrate	5% olive tree biomass	50	4.8	0.363 g glu./s DS	(Cara et al., 2008)
Coriolus versicolor TD17	25 FPU/g dry substrate	1 % w/v sweet sorghum straw	50	5	0.440 g glu /g dry substrate	This work

4. Conclusions

Optimal cellulase production from *C.versicolor* TD17 was obtained using 20 g/l cellobiose, 2 g/l ammonium sulfate and supplemented with 0.3 g/l urea. Maximum activity (0.897 U/ml) was obtained after 5th day of cultivation. From RSM study, optimal saccharification conditions of cellulase from *C.versicolor* TD17 were 1%

w/v acid-pretreated SSS, 25 FPU/g dry substrate, 50 °C and pH 5. Similarly, optimal saccharification conditions by commercial cellulase from *T. reesei* were 2.5% w/v acid-pretreated SSS, 15 FPU/g dry substrate, 40 °C and pH 4. The maximum yield of 0.440 and 0.558 g glucose/g dry substrate (data not shown) were obtained after 72 h, respectively. SSS has a potential as an alternative renewable resource for liberating fermentative sugars.

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