Optimization of Medium Components for β-glucosidase Production in *Schizophyllum commune* KUC9397 and Enzymatic Hydrolysis of Lignocellulosic Biomass

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Medium components for β-glucosidase (BGL) production in *Schizophyllum commune* KUC9397 were optimized using a central composite design and response surface methodology. From the various medium components tested, cellulose, soy peptone, and thiamine HCl were selected as the optimal carbon, nitrogen, and vitamin sources, respectively. The highest BGL production was obtained with 2.96% cellulose, 2.30% soy peptone, and 0.11% thiamine HCl. BGL production in the optimized medium was increased 7.2-fold compared to production in an unoptimized medium. Crude enzyme preparation from *S. commune* KUC9397 was used to saccharify pretreated lignocellulosic biomass. The crude enzyme preparations showed statistically equal saccharification rates as Cellobiase, a commercial BGL. This finding indicates that crude enzymes produced by *S. commune* KUC9397 have good potential for application in cellulosic biomass conversion systems in place of Cellobiase.

Keywords: β-glucosidase; Optimization; Saccharification; Schizophyllum commune

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INTRODUCTION

Lignocellulosic biomass, which is composed of cellulose, hemicelluloses, and lignin, is an important feedstock for biofuels (de Almeida *et al*. 2014). Cellulose is the main constituent of wood tissue and the most abundant carbohydrate polymer. Cellulose can be hydrolyzed into monomeric sugars and finally converted into valuable biochemicals and bioenergy. Cellulose hydrolysis is affected by the synergistic action of various cellulases, including endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). Although BGL does not actually hydrolyze cellulose, the enzyme plays an important role in cellulose hydrolysis: BGL efficiently eliminates end-product inhibition by cellobiose (Jeya *et al*. 2010), increasing the rate of cellulose hydrolysis. Singh and Bishnoi (2012) reported an increase in sugar yield with increased BGL loading. However, enzymatic hydrolysis of cellulosic biomass requires high enzyme loadings, and cellulase production is considered to be the most expensive step of enzymatic hydrolysis. Significantly reduced cellulase cost is therefore critical to the development of an economical process for bioethanol production from lignocellulosic biomass (Singhania *et al*. 2007). Several researchers have made an effort to reduce enzyme production costs or to enhance enzyme activity. Cheaper biomass substrates, cheaper saccharification techniques, and microbial

strains with high enzyme productivities can be used to reduce costs (Mekala *et al*. 2008). Additionally, media composition accounts for a considerable portion of cellulase production costs (Han *et al*. 2009), and many studies have employed statistically designed experiments and response surface methodology (RSM) to optimize medium composition (Haltrich *et al*. 1994; Singhania *et al*. 2007; Han *et al*. 2009; Valte *et al*. 2010; Nagraj *et al*. 2014) .

In the present study, the medium components that affect BGL production in *Schizophyllum commune* KUC9397 were optimized with a central composite design (CCD) using RSM. Crude enzymes produced in the optimized medium were utilized for saccharification of pretreated lignocellulosic biomass. Saccharification efficiency was evaluated in comparison with Cellobiase, a commercial enzyme.

EXPERIMENTAL

Microorganism and Identification

An efficient BGL-producing microorganism, *S. commune* KUC9397, was selected from 159 basidiomycetes provided by the Korea University Culture Collection (KUC, Seoul, Korea) after screening for BGL activity. The microorganism was maintained on potato dextrose agar (Difco, USA). Genomic DNA for identification of the fungus was extracted according to the procedure described by Huh *et al.* (2011). The internal transcribed spacer (ITS) region was amplified from isolated genomic DNA using the ITS universal primer set ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') / ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White *et al.* (1990) and by Gardes and Bruns (1993). Sequencing of the amplified ITS rDNA region was performed, and the sequence was deposited in GenBank (accession number KJ535691).

Inoculum Preparation and BGL Production

Five agar plugs with mycelium were inoculated into 100 mL of potato dextrose broth (Difco, USA) and pre-cultured at 27 °C and 150 rpm. After 4 days of incubation, *S. commune* KUC9397 cells were washed with two volumes of sterile distilled water. The cells were then homogenized and inoculated into a modified basal medium based on Mandels' medium for BGL production (Juhász *et al*. 2005). A 1% inoculum from the pre-culture was added to 250 mL flasks containing 100 mL of basal medium with various carbon, nitrogen, or vitamin concentrations. After incubation at 27 °C and 150 rpm, the cultures were centrifuged, and cellulase activity in the supernatant was measured. All experiments were performed in triplicate.

Effect of Medium Components on BGL Production

The effect of carbon sources (*e.g*., avicel, cellobiose, cellulose, and glucose), nitrogen sources (*e.g*., casein, corn steep liquor, soy peptone, tryptone, and yeast extract), and vitamin source (*e.g*., thiamine HCl) on BGL production was studied. In addition, carbon source concentrations were varied between 1, 2, 3, and 4% (*w/v*), nitrogen source concentrations were varied between 0.5, 1, 2, and 3% (*w/v*), and vitamin concentrations were varied between 0.01, 0.05, 0.1, and 0.2%. To determine the optimal medium composition for BGL production, the different carbon sources, nitrogen sources, and vitamins were examined sequentially.

CCD and Response Surface Analysis

A 20-run CCD using RSM was used to optimize medium components for BGL production from *S. commune* KUC9397. Table 1 shows the ranges and levels of the independent variables. The CCD consisted of a 2^3 full factorial design at a distance 1.68179 from the origin with 6 central points (Table 2). Based on preliminary experiments, the three independent variables that affect BGL activity, cellulose (X_1, %), soy peptone (X_2, %), and thiamine HCl (X_3, %), were chosen and the range for each factor was studied. BGL activity (U/ml) was determined as the dependent output variable.

A second-order polynomial equation was fit to the experimental data to predict the optimum point. For three factors, the model equation is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (1)$$

where Y is the predicted response, b_0 is the intercept term, b_1, b_2, and b_3 are linear coefficients, b_{11}, b_{22}, and b_{33} are squared coefficients, b_{12}, b_{23}, and b_{13} are interaction terms, and X_1, X_2, and X_3 are independent variables.

Enzymatic Saccharification of Pretreated Lignocellulosic Biomass

BGL produced in optimized medium was used for saccharification of pretreated lignocellulosic biomass, and the crude enzyme's saccharification ability was compared with that of a commercial enzyme. Four types of biomass were provided by Prof. J. S. Kim, Kyonggi University and were pretreated by soaking in aqueous ammonia, and their chemical compositions are presented in Table 3. Enzymatic hydrolysis was carried out on 1.5% substrates in 10 mL of 0.05 M sodium acetate buffer (pH 4.8) at 50 °C on a shaking incubator at 200 rpm for 72 h according to the National Renewable Energy Laboratory method (Selig *et al*. 2008). To evaluate the saccharification efficiency of the crude enzyme preparation produced in this study, two different enzyme combinations were created with Celluclast® 1.5L (Sigma-Aldrich, USA), Cellobiase (Sigma-Aldrich, USA), and the crude enzyme preparation. For each combination, Celluclast® 1.5 L was added to a filter paper units (FPU) loading level of 30 FPU, and Cellobiase and the crude enzyme preparation were added to a cellobiase units (CBU) loading level of 30 CBU. The protein content and enzyme activities of these enzymes are summarized in Table 4. After saccharification, glucose and xylose production were measured by high performance liquid chromatography (HPLC). The saccharification rate (%) was calculated as the ratio of glucose and xylose content to initial cellulose and hemicellulose content in the dry substrate. All saccharification experiments were carried out in triplicate.

HPLC Analysis of Enzymatic Hydrolysates

Enzymatic hydrolysates were boiled for 5 min to inactivate the enzymes and centrifuged at 12,000 rpm for 5 min. Samples were filtered with 0.2 μm syringe filters (No. 729022, Macherey-Nagel, Germany), diluted 5-fold with deionized water, and measured by HPLC (Agilent 1100 series) with a refractive index detector (RID-10Am Shimadzu, Japan) and Aminex HPX-87H column (300 X 7.8 min, Bio-Rad Laboratories, USA). Operation conditions included an oven temperature of 50 °C and a flow rate of 0.8 mL/min with dilute sulfuric acid (0.005 N).

RESULTS AND DISCUSSION

Microorganism Identification

The microorganism was identified by phylogenetic analysis based on ITS sequencing data. The sequencing data were compared to the GenBank database using a BLAST search. The data most closely matched (99%) *Schizophyllum commune* (JQ676210). Therefore, the screened fungus was named as *S. commune* KUC9397 (accession no. KJ535691).

Table 1. Experimental Ranges and Levels of the Independent Variables

Independent variable	Factor	Ranges and levels				
	x_i	-1.68	-1	0	1	1.68
Cellulose (%, *w/v*)	x_1	1.32	2	3	4	4.68
Soy peptone (%, *w/v*)	x_2	0.32	1	2	3	3.68
Thiamine HCl (%, *w/v*)	x_3	0.016	0.05	0.1	0.15	0.184

Effect of Carbon Source, Nitrogen Source, and Vitamin

The effects of various carbon sources, nitrogen sources, and vitamins on BGL yield were investigated using a basal medium. BGL production was influenced by the carbon, nitrogen, and vitamins supplied. It appeared as though 3% cellulose, 2% soy peptone, and 0.1% thiamine HCl was the best combination of ingredients for BGL production, and these three sources were further investigated to determine their interaction effect on BGL production. Glucose and cellobiose resulted in little BGL production over the concentration range investigated, while substrates such as avicel and cellulose induced high enzyme production (data not shown). Cellulose (3%) resulted in the highest BGL activities. It has been reported that complex substrates have a marked positive effect on cellulase biosynthesis, whereas cellulase biosynthesis is repressed in the presence of readily metabolizable carbon sources such as glucose or cellobiose (Niranjane *et al*. 2007).

BGL production was strongly influenced by the nitrogen source. All nitrogen sources tested promoted BGL production, but organic nitrogen sources were found to be especially favorable for cell growth and cellulase production. This result is in agreement with many reports in the literature (Haltrich *et al*. 1994; Deswal *et al*. 2011; Gautam *et al*. 2011). Peptones have been found to support maximum cellulase production by *Trichoderma reesei* (Enari and Markkanen 1977), and they are known to be suitable nitrogen sources, especially soy peptone (Baig 2005). In this study, 2% soy peptone induced maximal BGL production. Soy peptone is an inexpensive nitrogen source, so its use may decrease the cost of fermentations for industrial enzyme production in addition to increasing enzyme yields.

Many researchers have reported that the presence of vitamins influences the rate of biosynthesis of many metabolites and increases enzyme activities (Báez-Saldaña *et al*. 2004; Chen *et al*. 2004; Deswal *et al*. 2011; Lee *et al*. 2001). Additionally, vitamins are easy to deliver during liquid fermentation (Joo *et al*. 2009). To examine the effect of vitamins on BGL production and to select a suitable vitamin concentration, thiamine HCl ranging from 0.01 to 0.2% (*w/v*) was added to medium containing 3% cellulose and 2% soy peptone. As the concentration of thiamine was increased from 0.01 to 0.1%, BGL activity increased to a maximal activity of 42.3 U/mL at 0.1% thiamine. This activity was

2.1-fold higher than that attained without thiamine. When the thiamine concentration was further increased to 0.2%, however, BGL activity significantly decreased.

Table 2. The CCD of RSM and the Mean Experimental Responses of BGL Production from *S. commune* KUC9397

Run No.	Coded value			Actual value			Activity (U/ml)
	x_1	x_2	x_3	x_1	x_2	x_3	
1	-1	-1	-1	2	1	0.05	17.16
2	1	-1	-1	4	1	0.05	23.98
3	-1	1	-1	2	3	0.05	31.80
4	1	1	-1	4	3	0.05	24.22
5	-1	-1	1	2	1	0.15	15.08
6	1	-1	1	4	1	0.15	27.05
7	-1	1	1	2	3	0.15	41.24
8	1	1	1	4	3	0.15	24.26
9	-1.68	0	0	1.32	2	0.1	17.67
10	1.68	0	0	4.68	2	0.1	31.66
11	0	-1.68	0	3	0.32	0.1	03.42
12	0	1.68	0	3	3.68	0.1	22.86
13	0	0	-1.68	3	2	0.016	36.36
14	0	0	1.68	3	2	0.184	34.23
15	0	0	0	3	2	0.1	40.49
16	0	0	0	3	2	0.1	44.49
17	0	0	0	3	2	0.1	41.62
18	0	0	0	3	2	0.1	39.55
19	0	0	0	3	2	0.1	42.67
20	0	0	0	3	2	0.1	40.12

Optimization of Culture Medium Components by Applying RSM

The interaction effect of varying the concentrations of three independent culture medium components (cellulose, soy peptone, and thiamine HCl) on BGL production was investigated. The results of the CCD experiments are presented with mean experimental responses in Table 2. The regression equations obtained after an analysis of variance (ANOVA) give the level of BGL production as a function of the independent variables tested. The final response equation that represents a suitable model for BGL production is given below,

$$Y = 41.42 + 1.30X_1 + 5.19X_2 + 0.50X_3 - 5.42X_1X_2 - 0.53X_1X_3 + 1.06X_2X_3 - 5.47X_1^2 - 9.54X_2^2 - 1.71X_3^2 \quad (2)$$

where Y represents BGL production, and X_1, X_2, and X_3 are the concentrations of cellulose (%, *w/v*), soy peptone (%, *w/v*), and thiamine HCl (%, *w/v*), respectively.

Table 3. Chemical Compositions of Pretreated Biomass

Pretreated biomass	Total components (%)			
	Cellulose	Hemicelluloses	Lignin (Klason)	Others
Barley straw	56.52	28.83	10.43	4.22
Empty fruit bunch	37.44	29.08	19.22	14.26
Miscanthus	54.71	30.15	9.70	5.44
Rice straw	57.12	25.82	8.99	8.07

Table 4. Activities of the Enzymes Used for Enzymatic Hydrolysis

	Protein content (mg/g biomass)	FPU (FPU/g biomass)	EG (U/g biomass)	CBH (U/g biomass)	BGL (CBU/g biomass)	XYL (U/g biomass)	BXL (U/g biomass)
Combination 1	8.27	30.23	54.21	5.44	29.59	589.75	9.61
Combination 2	6.81	29.94	53.99	3.42	29.93	758.56	9.47

The model's coefficient of determination (R^2) was 0.936 (Table 5), indicating a high correlation between the experimentally observed and predicted values. A lower coefficient of variation (CV) indicates higher experimental reliability (Singh and Bishnoi 2012). The CV of these data was 13.11%, indicating high experiment reliability. The computed F-value (16.18) implies that the model was significant. The P-value was also very low ($P < 0.0001$), indicating the model's significance. In addition, the model's lack of fit (0.0194) suggests that the obtained experimental data were fit well by the model. In this case, four model terms (X_2, X_1X_2, X_{12}, and X_{22}) were found to be significant for BGL production. Soy peptone concentration (X_2) had an extremely significant effect ($P < 0.0006$) on BGL production and was much more influential than the other variables explored in this study.

Response surface curves demonstrate the effect of two independent variables while a third variable remains fixed at a zero level, and curves for this study are depicted in Fig. 1. The model predicts the optimal values of the most significant three variables to be X_1 = -0.04, X_2 = 0.30, and X_3 = 0.24. Therefore, the optimal concentrations of cellulose, soy peptone, and thiamine HCl are 2.96, 2.3, and 0.113% (*w/v*), respectively. With this medium composition, the maximum predicted BGL production is 42.22 U/mL. The experimentally measured enzyme activity (43.51 U/mL) matches the predicted value and represents a 7.2-fold enhancement to BGL activity. Additionally, this optimized medium induced a significant amount of BGL production, contrary to previous reports of BGL production with other *S. commune* strains: Desrochers *et al.* (1981) and Steiner *et al.* (1987) reported maximum BGL activity of 22.2 U/ml and 34.9 U/ml, respectively, under optimized conditions at 30 °C.

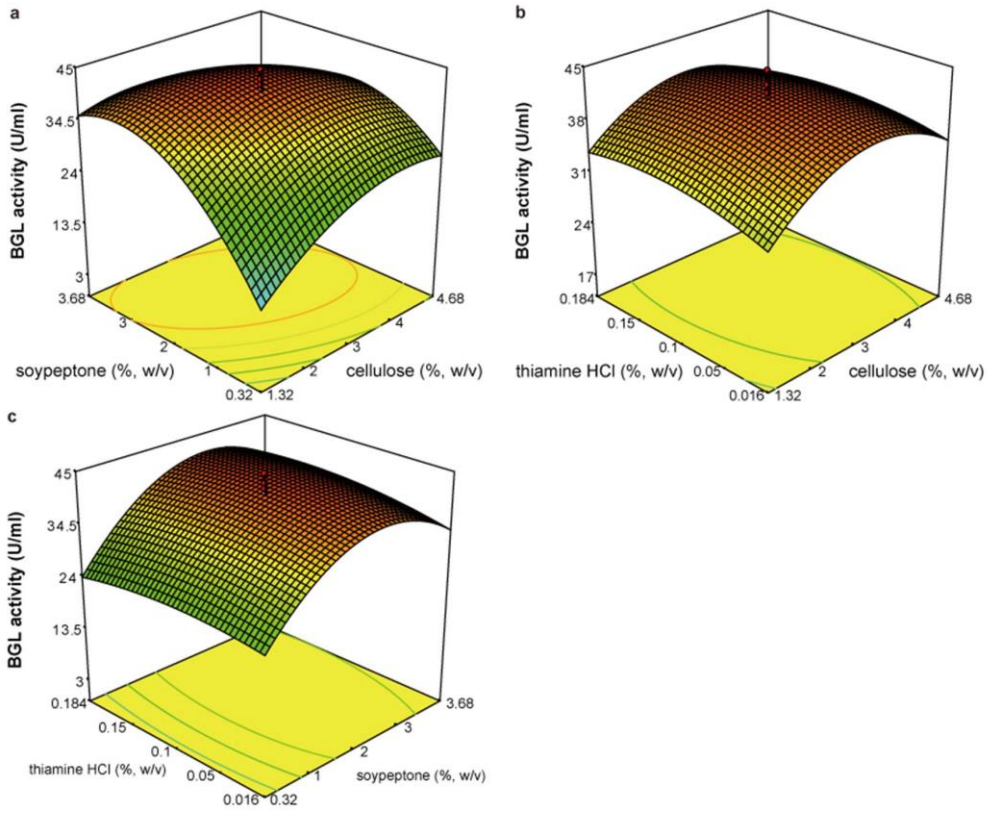

Fig. 1. Statistical optimization of enzyme production using RSM. a. Three dimensional response surface plot for the interaction between cellulose and soy peptone. b. Three dimensional response surface plot for the interaction between cellulose and thiamine HCl. c. Three dimensional response surface plot for the interaction between soy peptone and thiamine HCl.

An Evaluation for Saccharification Efficiency of *S. commune* KUC9397

To evaluate the saccharification efficiency of *S. commune* KUC9397, crude enzymes produced under the optimized conditions were mixed with Celluclast® 1.5L (Combination 2), and the sugar hydrolysis profile for this combination was compared with the profiles for commercial enzymes (Combination 1). Alkali-pretreated biomass was hydrolyzed for 72 h with an enzyme dose of 30 FPU/g and 30 CBU/g of substrate.

As shown in Fig. 2(c), the total saccharification rates of enzyme combinations 1 and 2 were 34.0 to 69.7% and 32.7 to 60.9%, respectively. On the other hand, there were no significant differences between the total saccharification rates of combinations 1 and 2 for all of the biomass substrates tested except barley straw (Fig. 2). The total protein content of combination 2 was lower than combination 1: the EG and CBH doses were slightly lower in combination 2. Despite the low enzyme dosage, the saccharification rates of combination 1 and 2 were not statistically significant, which is an encouraging result. The low protein content of combination 2 implies that the enzymes produced by *S. commune* KUC9397 have high specific activities. To further increase specific activities, the production of nonessential enzymes should be reduced, and the relative ratio of critical enzymes to nonessential enzymes should be enhanced. This results in reduced enzyme cost (Banerjee *et al*. 2010). Improving specific activities is one method to reduce enzyme cost. EG, CBH, and BGL are the most important components for cellulose hydrolysis and act synergistically to degrade crystalline cellulose to glucose. BGL converts the oligosaccharides produced by EG and CBH into glucose (Kawai *et al*. 2013). Low BGL

activity leads to cellobiose accumulation and reduced biomass conversion efficiency (Nakazawa *et al*. 2012). BGL derived from *S. commune* KUC9397 therefore played a crucial role in glucose production from cellulose.

Table 5. ANOVA for the Selected Quadratic Model

Source	Sum of squares	*df*	Mean square	*F* value	*P* > *F*
Model	2,253.41	09	0,250.38	16.18	< 0.0001
X_1	, 023.09	01	0,023.09	01.49	< 0.2498
X_2	0 368.54	01	0,368.54	23.82	< 0.0006
X_3	0,003.47	01	0,003.47	00.22	< 0.6458
X_1^2	0,431.10	01	0,431.10	27.86	< 0.0004
X_2^2	1,312.71	01	1,312.71	84.85	< 0.0001
X_3^2	0,042.19	01	0,042.19	02.73	< 0.1297
X_1X_2	0,234.90	01	0,234.90	15.18	< 0.0030
X_1X_3	0,002.26	01	0,002.26	00.15	< 0.7104
X_2X_3	0,009.01	01	0,009.01	00.58	< 0.4630
Residuals	0,154.71	10	0,015.47		
Lack of fit	0,137.66	05	0,027.53	08.07	< 0.0194
Pure error	0,017.05	05	0,003.41		
Cor total	2,408.12	19			
R^2 = 0.9358; Adj R^2 = 0.8779; CV = 13.11 *df*: degree of freedom					

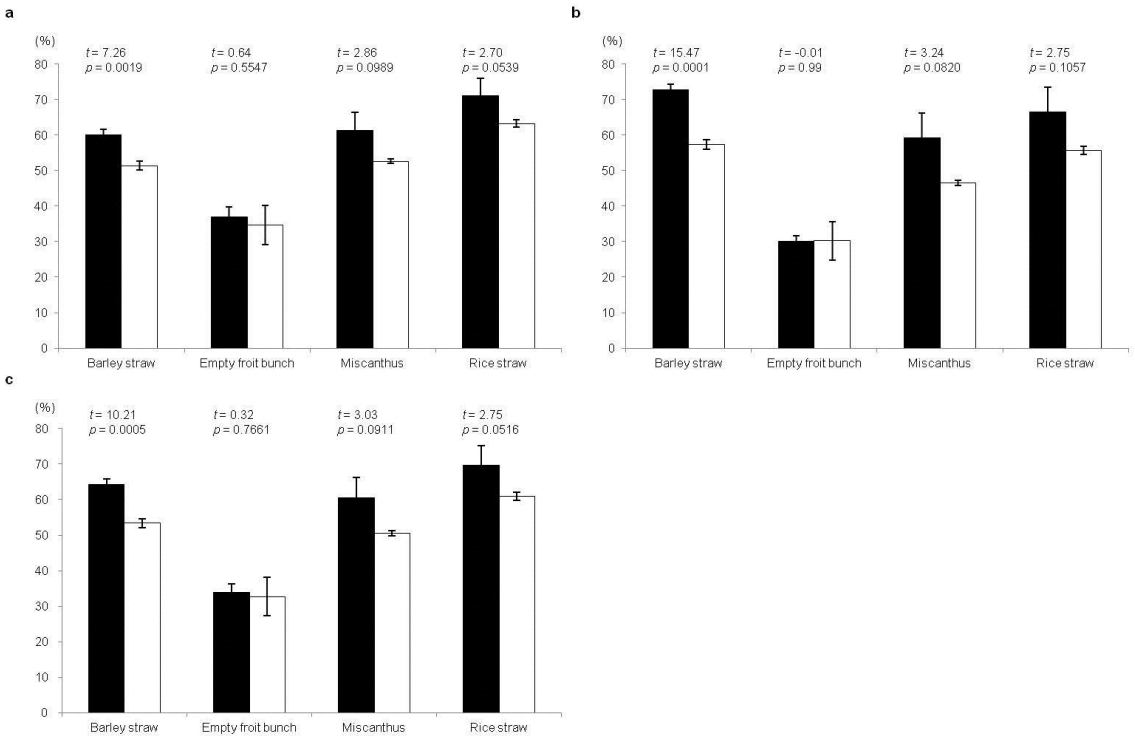

Fig. 2. The % yield of sugars released during enzymatic hydrolysis over 72 hours as measured by HPLC. The error bars represent the standard error of triplicate experiments. a. The yields of released glucose. b. The yields of released xylose. c. The total saccharification rates. (■, combination 1; □, combination 2)

Hemicellulases, such as endo-β-1, 4-xylanase (XYL), randomly cleave internal xylan bonds, and β-xylosidase (BXL) hydrolyzes xylo-oligosaccharides to xylose. Although enzyme combination 2 had a fairly high XYL activity, xylan conversion efficiency was not increased compared to that of combination 1. Thus, cellulases were responsible for the total saccharification rate rather than hemicellulases, and our crude enzyme preparation could be used in place of commercial enzymes such as Cellobiase. BGL derived from *S. commune* KUC9397 has the potential for applications in industrial fermentation systems.

CONCLUSIONS

1. An efficient BGL-producing fungus was screened and identified as *S. commune* KUC9397 based on genotypic characteristics.
2. BGL production from this fungus was improved 7.2-fold by optimizing the medium composition using a statistical approach.
3. Crude enzymes derived from *S. commune* KUC9397 were shown to be potential replacements for Cellobiase, a commercial BGL.
4. This study highlights the potential of our crude enzyme for cellulosic biomass conversion systems.

ACKNOWLEDGMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2A10011390) and by the Technology Development Program (309016-05) for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry, and Fisheries, Republic of Korea.

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Article submitted: April 14, 2014; Peer review completed: May 16, 2014; Revised version received and accepted: May 30, 2014; Published: June 4, 2014.