Optimization of Endoglucanase Production by *Trichoderma harzianum* KUC1716 and Enzymatic Hydrolysis of Lignocellulosic Biomass

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Trichoderma species are widely used for the commercial production of cellulolytic enzymes. In the present investigation, medium components were optimized using a central composite design and response surface methodology to produce endoglucanase (EG) from *Trichoderma harzianum* KUC1716. 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 EG (1.97 U/mL) production was obtained with 1.85% cellulose, 0.48% soy peptone, and 0.10% thiamine HCl. EG production in the optimized medium was 2.6 fold higher than in the unoptimized medium. In addition, the crude enzyme preparation from *T. harzianum* KUC1716 supplemented with β-glucosidase from *Schizophyllum commune* KUC9397 was used to hydrolyze various types of lignocellulosic materials and showed significant saccharification yields on all lignocellulosic materials, surpassing that of a commercial enzyme cocktail. It was verified that the crude enzyme preparation derived *from T. harzianum* KUC1716 could replace the commercial enzymes. This highlights the potential of the crude enzymes for use in biomass conversion systems.

Keywords: Endoglucanase; Optimization; Response surface methodology; Saccharification; Trichoderma harzianum

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INTRODUCTION

Lignocellulosic materials are abundant, renewable, and sustainable resources worldwide, thus are important sources for various types of industrial processed sugars that can be fermented to ethanol and other organic chemicals (Cardona and Sánchez 2007). Recently, due to the constant fluctuation of oil prices, the significance of biofuel production from lignocellulosic biomass as an alternative energy source has been intensified, and efficient conversion of lignocelluloses to biofuel is gaining interest (Bak *et al*. 2009).

Cellulose, hemicellulose, and lignin are the three main components of lignocellulosic biomass. Via pretreatment and enzymatic saccharification, cellulose and hemicellulose can be degraded to fermentable sugars, which can be further fermented to ethanol or other products. Among the components, cellulose is the main structural component of lignocellulose, and it is a long chain of glucose molecules. The conversion of cellulose to glucose involves the concerted action of three classes of enzymes: endo-β-

1,4-glucanases (EG, EC 3.2.1.4), exo-cellobiohydrolases (CBH, EC 3.2.1.91), and β-glucosidases (BGL, EC 3.2.1.21). EGs randomly hydrolyze internal glycosidic bonds in cellulose to yield reducing or non-reducing new chain ends for CBHs, which subsequently hydrolyze the newly generated cellulose chain ends. By the synergistic action of these enzymes, cellobiose units are released and act as a direct inhibitor of those cellulolytic enzymes. Finally, BGLs hydrolyze cellobiose to glucose and are therefore required to complete the degradation of cellulose.

Trichoderma harzianum is frequently reported as a control agent against fungal pathogens (Arantes and Saddler 2010; Howell 2003). However, recent studies have also revealed the potential of this fungus for cellulase production and industrial applications (Ahmed *et al.* 2009; Castro *et al.* 2010). Likewise, *T. harzianum* has also become a promising system for xylanase production under appropriate conditions (Franco and Ferreira 2004).

In the present study, the medium components, carbon, nitrogen, and vitamin sources, affecting EG production in *Trichoderma harzianum* KUC1716, were optimized by a central composite design (CCD) using response surface methodology (RSM). A fungal enzyme cocktail from *T. harzianum* KUC1716 and *Schizophyllum commune* KUC9397 was utilized to hydrolyze various types of lignocellulosic materials, and its performance was compared to that of a commercial enzyme cocktail (Cellulast 1.5L and Novozyme 188) by evaluating saccharification yields.

EXPERIMENTAL

Microorganism and Identification

An efficient EG-producing microorganism, *T. harzianum* KUC1716, was provided by the Korea University Culture Collection (KUC, Seoul, Korea). 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 part of translation elongation factor (TEF) gene was amplified from isolated genomic DNA with primers EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and TEF rev (5'-GCCATCCTTGGAGATACCAGC-3') as described by Carbone and Kohn (1999) and by Samuels *et al.* (2002). Sequencing of the amplified TEF gene was performed, and the sequence was deposited in GenBank (accession number KR820004).

Inoculum and EG Preparation

T. harzianum KUC1716 was grown on a malt extract agar plate for a week at room temperature. After the cultivation, a spore suspension was obtained by adding 3 mL distilled water to the plate, scraping, and collecting. A total of 10^6 spores were inoculated into a modified basal medium based on Mandels' medium for EG production (Juhász *et al.* 2005). The inoculum 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 for a week, the cultures were centrifuged, and the supernatant was used to measure cellulase activity. EG activity was measured using carboxymethyl cellulose (CMC) according to the method of Ghose (1987). One unit per mL of enzyme activity was defined as the amount of enzyme required to release 1 μmol of glucose equivalents from CMC per milliliter of culture medium per minute. All experiments were performed in triplicate.

Effect of Medium Components on EG Production

The effects 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 EG production were studied. In addition, carbon source concentrations were varied between 1, 2, and 3% (w/v), nitrogen source concentrations were varied between 0.1, 0.25, 0.5, and 0.75% (w/v), and vitamin concentrations were varied between 0.01, 0.05, 0.1, 0.25, and 0.5%. To determine the optimal medium composition for EG production, the different carbon sources, nitrogen sources, and vitamins were examined sequentially, and the concentration of the best sources were optimized through the CCD.

CCD and Response Surface Analysis

A 20-run CCD using RSM was used to optimize medium components for EG production from *T. harzianum* KUC1716. Table 1 shows the ranges and levels of the independent variables. The CCD consisted of a 2^3 full factorial design at a distance of 1.68179 from the origin with 6 central points (Table 2). Based on preliminary experiments, three independent variables that affect EG activity, cellulose (X_1, %), soy peptone (X_2, %), and thiamine HCl (X_3, %), were chosen, and the range for each factor was studied. EG activity (U/mL) served 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 the independent variables.

Table 1. Component Ranges and Levels of the Independent Variables

Independent variable	Factor	Ranges and levels				
	xi	-1.68	-1	0	1	1.68
Cellulose (%, w/v)	x1	0.32	1	2	3	3.68
Soy peptone (%, w/v)	x2	0.08	0.25	0.5	0.75	0.92
Thiamine HCl (%, w/v)	x3	0.016	0.05	0.1	0.15	0.184

Enzymatic Hydrolysis

Saccharification was performed in 20 mL bottles in 50 mM sodium acetate buffer (pH 5.0). Four lignocellulosic material samples pre-treated by soaking in aqueous ammonia (SAA) at 30 °C and 70 °C were provided from Kyonggi University, and their chemical composition is presented in Table 4. Substrate concentrations were 1.5% dry mass (w/w). The working volume of enzymatic hydrolysis was eventually 10 mL in the 20 mL bottle. Reactions were performed at 50 °C under shaking at 200 rpm for 60 h according to the National Renewable Energy Laboratory method (Selig *et al.* 2008). To hydrolyze the biomass, the fungal enzyme cocktail was composed of enzymes from *T. harzianum* KUC1716 and *Schizophyllum commune* KUC9397. In a previous study, enzyme

preparation obtained by *T. harzianum* KUC1716 showed low β-glucosidase activity. Consequently, enzyme preparation produced by *S. commune* KUC9397, absent of filter paper unit but high β-glucosidase activity, was supplemented to improve the hydrolysis yield. For an enzyme cocktail control, Celluclast 1.5 L (Novozymes, Franklinton, NC) and Novozyme 188 (Novozymes A/S, Bagsvaerd, Denmark) were used. The protein content and enzyme activities of the enzyme preparations are summarized in Table 5. After saccharification, enzymatic hydrolysates were boiled for 5 min to inactivate the enzymes and centrifuged at 12,000 rpm for 5 min. Supernatants were filtered with 0.2 μm syringe filters (No. 729022, Macherey-Nagel, Germany). The total reducing sugars were measured by the DNS method (Qi *et al.* 2004). The saccharification rate (%) was calculated as the ratio of sugar content to initial cellulose and hemicellulose content in the dry substrate. All saccharification experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Effects of Different Nutrient Sources

In the present study, the significance of different carbon, nitrogen, and vitamin sources was investigated to improve the composition of the medium for endoglucanase production using *T. harzianum* KUC1716. Among the carbon sources, cellulose and Avicel had the strongest inducing effect on the formation of endoglucanase activity, whereas cellobiose and glucose resulted in low levels of endoglucanase activity across their concentration ranges (data not shown). In general, high enzyme activity is detected when organisms are grown on complex substrates as opposed to when they are grown on easily metabolized substrates (Haltrich *et al.* 1994; Sun and Chen 2008).

Most nitrogen sources in this study had a strong inducing effect on the production of endoglucanase by *T. harzianum* KUC1716. Enzyme production was greatly affected by soy peptone across its concentration range (data not shown). Although it is common to add peptone to improve cellulase yields (Krishna *et al.* 2000; Jun *et al.* 2009), soy peptone is also known as an effective nitrogen source for *Trichoderma* sp. (Seyis and Aksoz 2005; Rodriguez-Gomez and Hobley 2013).

Little attention has been paid to the role of vitamin source in cellulase production by *Trichoderma* sp. In a previous study, we reported the effect of thiamine-HCl on cellulase production by *Schizophyllum commune*, which improved cellulase yield significantly (Lee *et al.* 2014). As expected, thiamine-HCl showed effective enhancement of endoglucanase production in *T. harzianum* KUC1716.

The combination of 2% cellulose, 0.5% soy peptone, and 0.1% thiamine HCl was the best for the production of endoglucanase, and interaction effects among the ingredients on endoglucanase production were further assessed.

Statistical Optimization of Endoglucanase Production by RSM

The interaction effects of varying the concentrations of three independent culture medium components (cellulose, soy peptone, and thiamine HCl) on EG production were 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 EG production as a function of the independent variables tested. The final response equation that represents a suitable model for EG production is given below,

$$Y = 1.15 - 0.15X_1 - 0.09X_2 - 0.07X_3 + 0.085X_1X_2 + 0.068X_1X_2 + 0.01X_2X_3 - 0.17X_1^2 - 0.16X_2^2 - 0.11X_3^2 \quad (2)$$

where Y represents EG 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 2. The CCD of RSM and the Mean Experimental Responses of Endoglucanase Production from *T. harzianum* KUC1716

Run	Coded value			Actual value			Activity (U/mL)
	x_1	x_2	x_3	x_1	x_2	x_3	
1	0	0	0	2	0.5	0.1	1.02
2	-1	1	1	1	0.75	0.15	0.60
3	0	-1.68	0	2	0.08	0.1	0.75
4	0	0	0	2	0.5	0.1	0.98
5	0	0	-1.68	2	0.5	0.016	0.87
6	1	-1	1	3	0.25	0.15	0.56
7	0	0	1.68	2	0.5	0.18	0.76
8	1	-1	-1	3	0.25	0.05	0.58
9	-1.68	0	0	0.32	0.5	0.1	0.76
10	0	0	0	2	0.5	0.1	1.37
11	0	1.68	0	2	0.92	0.1	0.58
12	-1	1	-1	1	0.75	0.05	0.86
13	1	1	-1	3	0.75	0.05	0.54
14	0	0	0	2	0.5	0.1	1.07
15	1	1	1	3	0.75	0.15	0.48
16	-1	-1	1	1	0.25	0.15	0.94
17	0	0	0	2	0.5	0.1	1.24
18	0	0	0	2	0.5	0.1	1.22
19	-1	-1	-1	1	0.25	0.05	1.31
20	1.68	0	0	3.68	0.5	0.1	0.48

The model's coefficient of determination (R^2) implies that 87.6% of the sample variation for EG production could be explained by the three independent variables (Table 3). A lower coefficient of variation (CV) indicates higher experimental reliability. The CV of these data is 16.32%, indicating high experiment reliability. The computed F-value (7.87) implies that the model is significant. The P-value (0.0017) was also very low, indicating the model's significance. In addition, the model's lack of fit (0.6405) suggests that the obtained experimental data fit well with the model. In this case, five model terms (X_1, X_2, X_1^2, X_2^2, and X_3^2) were found to be significant for EG production. Cellulose concentration (X_1) had an extremely significant effect ($P < 0.0029$) on EG 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 zero, 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 = -0.15, X_2 = -0.09, and X_3 = -0.07. Therefore, the optimal concentrations of cellulose, soy peptone, and thiamine HCl are 1.85, 0.48, and 0.10% (w/v), respectively. With this medium composition, the maximum predicted EG production is 1.77 U/mL. The experimentally measured enzyme activity (1.97 U/mL) matches the predicted value and represents a 2.6-fold enhancement in EG activity.

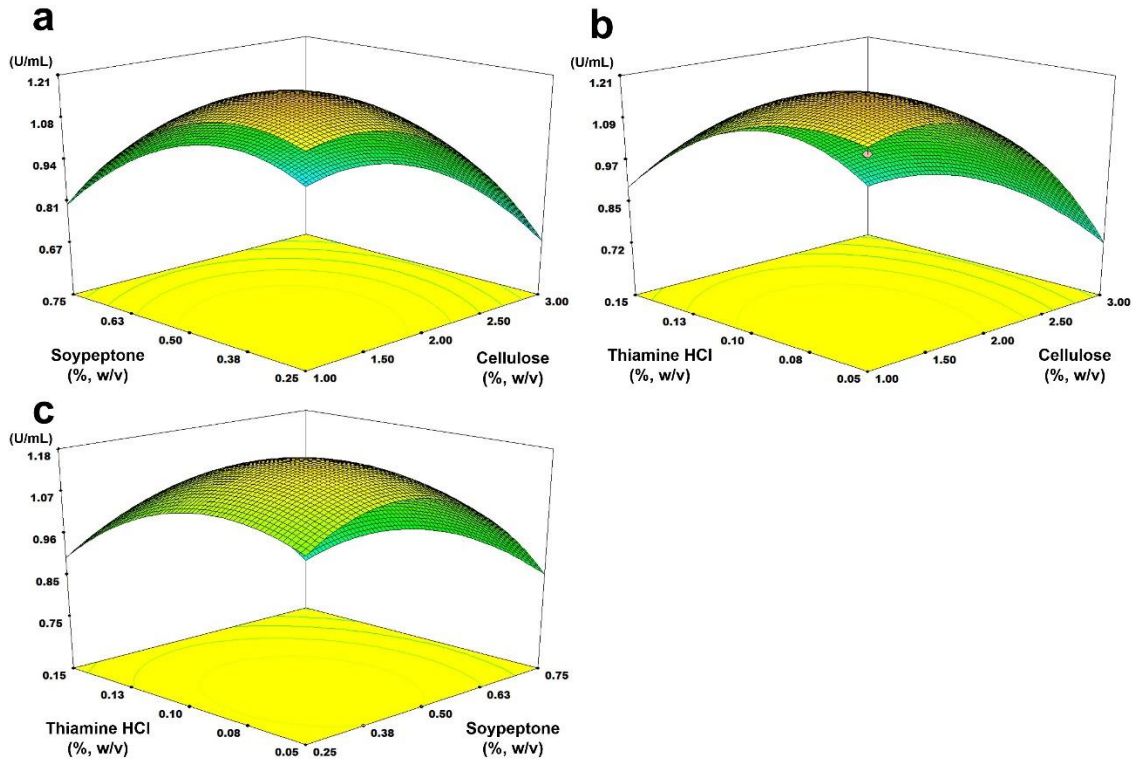

Fig. 1. Statistical optimization of enzyme production using RSM. a. Three dimensional response surface plot for the interaction between cellulose and soy peptone. b. Three dimensional response surface plot for the interaction between cellulose and thiamine HCl. c. Three dimensional response surface plot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

Enzymatic Hydrolysis of Lignocellulosic Materials

Enzymatic hydrolysis of four types of agricultural residue was performed by the enzyme cocktails from *T. harzianum* KUC1716 and *Schizophyllum commune* KUC9397. *T. harzianum* KUC1716 produced large amounts of endoglucanase and exoglucanase but relatively low β-glucosidase activity. To date, no natural microorganism that produces an ideal enzyme preparation for biomass hydrolysis has been discovered (Maeda *et al.* 2011). Therefore, β-glucosidase from *S. commune* KUC9397 producing high activity of the enzyme was supplemented to hydrolyze lignocellulosic materials. The enzymatic saccharification yields of SAA-pretreated lignocellulosic materials are shown in Fig. 2. Overall, pretreatments performed at higher temperature were effective for hydrolyzing lignocellulosic materials. The saccharification yields were significantly ($p<0.05$) higher with higher temperature at pretreatment, perhaps because higher temperature allowed for

more efficient pretreatment and better structural preparation of the substrate for enzymatic saccharification. Correlations were also obtained between lignocellulose composition and saccharification yield. The cellulose content had a positive correlation with saccharification yield obtained from the fungal enzyme cocktail ($F=26.62$, $p<0.01$, and $R^2=0.72$) and the commercial enzyme cocktail ($F=86.53$, $p<0.01$, and $R^2=0.89$). In addition, the lignin content showed a negative correlation with the yield obtained from the fungal enzyme cocktail ($F=7.93$, $p<0.05$, and $R^2=0.44$) and the commercial enzyme cocktail ($F=9.16$, $p<0.05$, and $R^2=0.47$). It is apparent that the lower lignin ratio improved enzyme accessibility to the polysaccharide chain and resulted in improved saccharification efficiency.

Table 3. ANOVA for the Selected Quadratic Model

Source	Sum of squares	df.	Mean square	F-value	p-value (Prob > F)
Model	1.3757	9	0.1529	7.87	<0.0017
X_1	0.2985	1	0.2985	15.37	<0.0029
X_2	0.1037	1	0.1037	5.34	<0.0435
X_3	0.0590	1	0.0590	3.04	<0.112
$X_1 X_2$	0.0577	1	0.0577	2.97	<0.1153
$X_1 X_3$	0.0366	1	0.0366	1.89	<0.1997
$X_2 X_3$	0.0008	1	0.0008	0.04	<0.8429
X_1^2	0.4406	1	0.4406	22.69	<0.0008
X_2^2	0.3680	1	0.3680	18.95	<0.0014
X_3^2	0.1608	1	0.1608	8.28	<0.0164
Residual	0.1942	10	0.0194		
Lack of Fit	0.0808	5	0.0162	0.71	<0.6405
Pure Error	0.1134	5	0.0227		
Cor. Total	1.5699	19			
$R^2=0.8763$; Adj. $R^2=0.7650$; CV=16.32 *df.* Degree of freedom					

In terms of enzyme efficiency, it was apparent that the fungal enzyme cocktail was more efficient at hydrolyzing lignocellulosic materials than the commercial enzyme cocktails. Although this finding could not be explained by the enzyme activities included in the enzyme cocktails, it is likely that accessory enzymes enhance the overall hydrolysis by solubilizing lignocellulose, which hinders access to the cellulose and hemicellulose.

Table 4. Chemical Composition of Pretreated Lignocellulosic Materials

Biomass	Pretreatment temp.	Total components (%)			
		Cellulose	Hemicelluloses	Lignin (Klason)	Other
Barley straw	Raw material	37.88	37.39	14.68	10.05
	Treated at 30°C	49.75	29.81	14.12	6.32
	Treated at 70°C	56.52	28.83	10.43	4.22
Empty Fruit Bunches	Raw material	31.86	26.3	24.4	17.44
	Treated at 30°C	36.77	29.81	22.02	11.4
	Treated at 70°C	37.44	29.08	19.22	14.26
Miscanthus	Raw material	37.30	29.16	18.63	14.91
	Treated at 30°C	43.88	30.64	15.89	9.59
	Treated at 70°C	54.71	30.15	9.7	5.44
Rice straw	Raw material	40.64	23.28	10.14	25.94
	Treated at 30°C	49.59	26.31	8.55	15.55
	Treated at 70°C	57.12	25.82	8.99	8.07

Table 5. Activities of the Enzymes Used for Enzymatic Hydrolysis

Enzyme preparation	Protein content (mg/g biomass)	FPA (FPU/g biomass)	EG (U/g biomass)	CBH (U/g biomass)	BGL (U/g biomass)
Fungal enzyme cocktail	29.3	15.0	153.6	11.3	15.0
Commercial enzyme cocktail	25.8	15.0	166.6	14.8	15.0

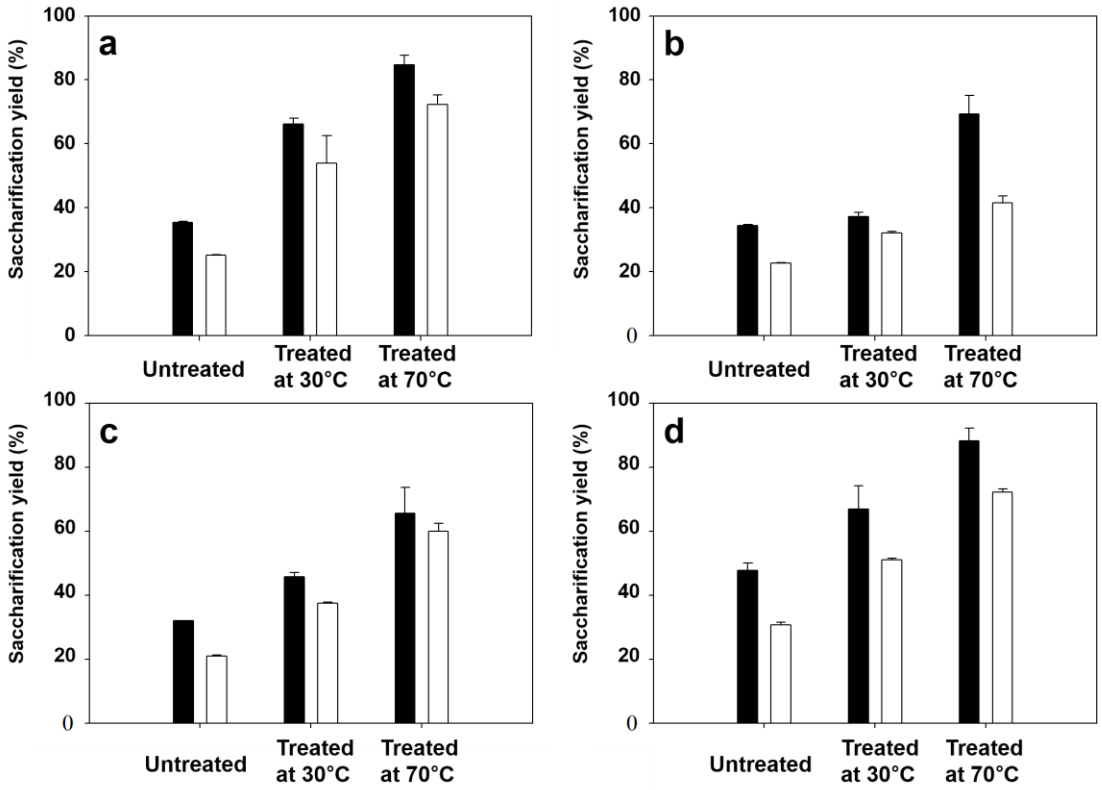

Fig. 2. Saccharification yields of lignocellulosic materials over 60 hours. The error bars represent the standard error of triplicate experiments. a. Hydrolysis yield of barley straw. b. Hydrolysis yield of EFB. c. Hydrolysis yield of miscanthus. d. Hydrolysis yield of rice straw. (■, fungal enzyme cocktail; □, commercial enzyme cocktail)

Accessory enzymes such as pectinase might have considerable potential to increase the overall performance of cellulase enzyme mixtures and achieve effective hydrolysis of pretreated lignocellulosic substrates (Hu *et al.* 2011). It was verified that the crude enzyme preparation derived from *T. harzianum* KUC1716 and *S. commune* KUC9397 could replace the commercial enzymes. This highlights the potential of such crude enzymes for use in cellulosic biomass conversion systems.

CONCLUSIONS

1. Medium optimization enhanced endoglucanase production 2.6 fold.
2. Crude fungal enzyme from *T. harzianum* KUC1716 showed hydrolysis potential equal to that of commercial enzymes.
3. This study demonstrated an efficient process to produce endoglucanase by *T. harzianum* KUC1716 and its potential for biomass hydrolysis for bioethanol applications.

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