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Research Article

Enhancement of β-Glucosidase Activity from a Brown Rot Fungus *Fomitopsis pinicola* KCTC 6208 by Medium Optimization

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Abstract β-Glucosidase, which hydrolyzes cellobiose into two glucoses, plays an important role in the process of saccharification of the lignocellulosic biomass. In this study, we optimized the activity of β-glucosidase of brown-rot fungus *Fomitopsis pinicola* KCTC 6208 using the response surface methodology (RSM) with various concentrations of glucose, yeast extract and ascorbic acid, which are the most significant nutrients for activity of β-glucosidase. The highest activity of β-glucosidase was achieved 3.02% of glucose, 4.35% of yeast extract, and 7.41% ascorbic acid where ascorbic acid was most effective. The maximum activity of β-glucosidase predicted by the RSM was 15.34 U/mg, which was similar to the experimental value 14.90 U/mg at the 16th day of incubation. This optimized activity of β-glucosidase was 23.6 times higher than the preliminary activity value, 0.63 U/mg, and was also much higher than previous values reported in other fungi strains. Therefore, a simplified medium supplemented with a cheap vitamin source, such as ascorbic acid, could be a cost effective mean of increasing β-glucosidase activity.

Keywords Ascorbic acid, β-glucosidase, *Fomitopsis pinicola* KCTC 6208, Medium optimization, Response surface methodology

The dependence on fossil fuel as an energy source has been depleting fossil fuels and increased the environmental pollution with global warming. Thus, environmental friendly technologies for the production of renewable energy using various biomasses have been the focus of extensive development. Corn and sugar cane have been mainly used for bioenergy resources but it has a conflict between "food vs. energy." To overcome this difficulty, energy resources should be changed to a non-food based type of biomass such as cellulosic biomass.

Cellulosic biomass is commonly composed of cellulose, hemicellulose and lignin and the most abundant organic compounds in the biosphere [1, 2]. Its potential use as a bioenergy resource has been long discussed and many approaches have been attempted to produce biofuels such as ethanol, butanol, etc. Among them, the most important step is to prepare fermentable sugars from the cellulosic biomass. Cellulose, which is a major cell-wall constituent in higher plants, is a linear polysaccharide consisting of β-1,4 linked glucose residues. Enzymatic hydrolysis for conversion of cellulose to the fermentable monomeric sugar, glucose, involves the synergistic activity of three types of cellulases: endo-1,4-β-D-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [3].

In particular, β-glucosidase plays an important role in the process of cellulose saccharification by hydrolyzing cellobiose to two glucoses. The renewable fermentable sugar from cellulose could be the solution not only for the valuable substrates required for production of biofuels and biochemicals but also in many potential areas such as pharmaceutical, cosmetic, textile, food and detergent industries [3, 4]. Recently, pretreatment of cellulosic biomass by environmentally friendly manufacturing processes, such as enzymatic hydrolysis, has aroused the interest of many researchers [5, 6]. However, such processes are costly because the commercial enzyme price occupies 40% of the total process cost [7]. Therefore, studies on improving enzyme activity may be a

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solution to reducing the cost of saccharification. To increase enzyme activity, the optimization of various culture conditions has been studied by controlling pH, temperature, incubation period, carbon, and nitrogen sources. In a previous study, the Deswal group investigated the culture conditions for enhanced cellulase production from a newly isolated brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation [8] and reported that ascorbic acid was an essential nutrient for enhancing activity of β-glucosidase from *Fomitopsis* sp. This group performed optimization of solid state fermentation using cellulases of *Fomitopsis* sp. But this study was only identified the effect of each single factor (nitrogen, carbon source and amino acid) on production of cellullase. They did not measure the combinatorial effect of each nutrient source. Considering that previous study, we also studied the effect of ascorbic acid for increasing β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208. To identify the factors involved in increasing of β-glucosidase activity, we used the response surface methodology (RSM) to enhance the activity. This method has several outstanding advantages in optimization of effective factors [9]. It could save time in obtaining optimized conditions as well as in identifying the relation affecting factors at optimized result.

In this study, we investigate the optimization of a highly efficient β-glucosidase activity in the brown-rot fungus *F. pinicola* KCTC 6208.

MATERIALS AND METHODS

Microorganism and cultivation. The microorganism used in this study, *F. pinicola* KCTC 6208, was obtained from the Korean Collection for Type Culture (KCTC). The *F. pinicola* KCTC 6208 was cultured on potato dextrose agar (Difco, Detroit, MI, USA) plates and incubated for 7 days at 27°C. For inoculum preparation, four mycelial discs (0.5 cm diameter each) were inoculated in each Erlenmeyer flask (500 mL) containing 150 mL of potato dextrose broth (Difco) and incubated at 27°C under shaking cultivation conditions for 5 days. The mycelia was homogenized with pestle and mortar under sterile conditions and used as primary inoculum for further experiments.

Optimization of carbon and nitrogen sources for β-glucosidase activity. Various carbon sources (2.5%), including cellulose, carboxymethylcellulose (CMC), glucose, sucrose, and maltose, were examined in the media containing 2.5% yeast extract to find the optimal carbon source for maximum β-glucosidase activity.

Experimental design and analysis by the RSM. The RSM was used to enhance a mathematical analysis between three major independent nutrient factors involved in enhancing activity of β-glucosidase in *F. pinicola*. The effect of the manipulatable variables on enzyme activity were investigated using a Box-Behnken design [10]. In this study, activity of β-glucosidase was analyzed with the following medium conditions: glucose (X_1) of 2~4%, yeast extract (X_2) of 0.5~6.5%, and ascorbic acid (X_3) of 1.4~11.4%. The results of the variable effect on activity of β-glucosidase were analyzed by quadratic polynomial equations. The general form of the predictive polynomial quadratic equation is as follows (Eq. 1) [11]. Where y is the response, is a constant, is a linear coefficient, is a quadratic coefficient and is an interactive coefficient.

$$\begin{aligned}\beta\text{-Glucosidase activity (U/mL)} = {} & -30.4866 + 3.3178\ X_1 \\ & + 17.7322\ X_2 + 3.1901\ X_3 - 0.1492\ X_1 X_2 - 0.0285\ X_1 X_3 \\ & + 0.0965\ X_2 X_3 - 0.3054\ X_1^2 - 2.7088\ X_2^2 \\ & - 0.1872\ X_3^2\ (p = 0.041,\ F = 10.12,\ r^2 = 0.9681) \qquad (1)\end{aligned}$$

Where X_1 is the glucose concentration (%, w/v), X_2 is the yeast extract concentration (%, w/v), and X_3 is the ascorbic acid concentration (%, w/v).

Enzyme assay and protein determination. β-Glucosidase activity was assayed in 250 μL of a 100 mM sodium acetate buffer (pH 4.5) containing 1 mM 4-nitrophenyl β-D-glucopyranoside (*p*NPG) as a substrate. at 50°C for 30 min, the reaction was stopped by adding 25 μL of 2 M Na_2CO_3 and the amount of *p*-nitrophenol released was monitored at 415 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μM of *p*NP per min at 50°C and pH 4.5. The Bradford method was used for determination of protein concentration in the enzyme solution [12], using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Effect of carbon sources on β-glucosidase activity. The effect of carbon sources on the induction of β-glucosidase activity in *F. pinicola* KCTC 6208 was investigated. Carbon sources tested for induction of β-glucosidase were glucose, sucrose, cellulose, CMC, and maltose at a concentration of 2.5% (w/v). As shown in Fig. 1A, glucose was found to be the best carbon source. Maltose was the second followed by sucrose, cellulose, and CMC (Fig. 1A). This result is similar to that of *Trichosporon asahii* [13]. However, other groups reported that CMC was the best carbon source for the induction of β-glucosidase [14-17].

Effect of nitrogen sources on β-glucosidase activity. To optimize the nitrogen sources, various nitrogen including peptone, soy peptone, tryptone, yeast extract, and urea source were tested in a same concentration (2.5% w/v). Fig. 1B showed that maximum activity of β-glucosidase at yeast extract as a nitrogen source. The soy peptone showed second highest for β-glucosidase activity followed by tryptone, peptone, and urea. In a previous study, *Trichoderma* sp. showed higher β-glucosidase activity when it was grown on yeast extract as a nitrogen source [18].

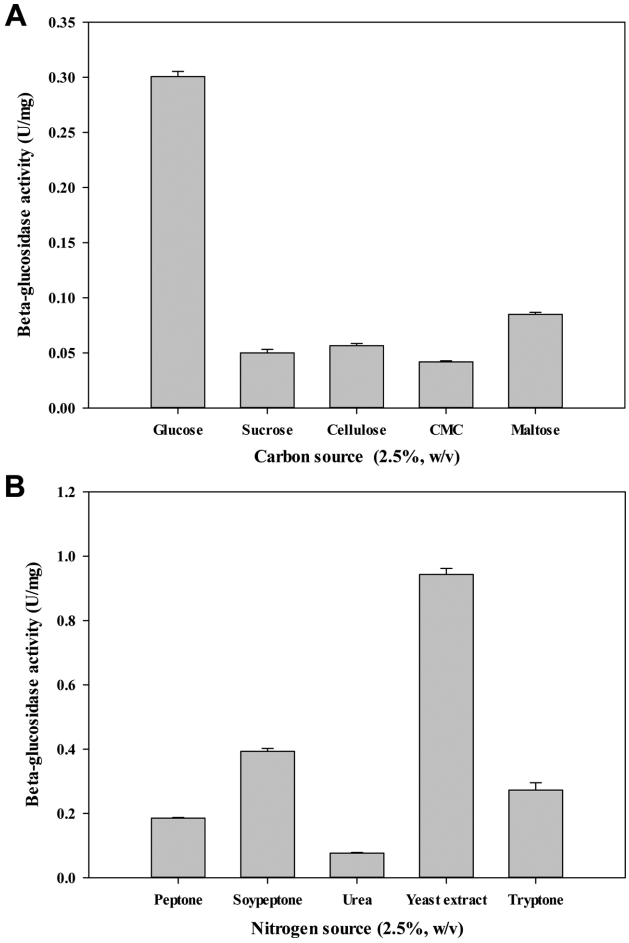

Fig. 1. Effects of various sources on β-glucosidase activity. A, Effect of carbon sources: the concentration of each carbon source was 2.5% with 2.5% yeast extract as a nitrogen source; B, Effect of nitrogen source: the concentration of each nitrogen source was also 2.5% with 2.5% glucose as a carbon source. The enzymatic reaction was performed at 27°C for 30 min in a 100 mM sodium acetate buffer (pH 4.5). The value represents the mean of three replicate determinations; the error bar indicates the standard deviation.

Optimization of β-glucosidase enhancing activity by RSM. RSM was employed to investigate the effect of nutrients and the combinatorial synergy effects of selected components by identifying the optimal mixing ratio and the optimal concentration of each nutrient. Glucose and yeast extract, which were determined to be the best carbon and nitrogen sources, with ascorbic acid were subjected to a central composition design (CCD). As the optimization result, 6.31 U/mg of β-glucosidase activity was obtained under the conditions of 3.0% of glucose, 4.5% of yeast extract, and 0.8% of ascorbic acid. Interestingly, 0.63 U/mg of β-glucosidase activity was obtained with the same component medium without ascorbic acid. Addition of ascorbic acid resulted in 10.0-fold increase in β-glucosidase activity, meaning that ascorbic acid is a significant factor in increasing the activity of β-glucosidase in *F. pinicola* KCTC 6208 (Table 1). To evaluate the influence of the manipulated variables on highly efficient β-glucosidase activity, the experimental conditions were designed by the RSM. Thirteen experiments were performed under the various conditions. The relation between the predicted value and actual value by the CCD is shown in Table 2. These two values are very similar at each experiment condition. The regression square is larger than 0.97, which means these modeling results were a very suitable approach on enzyme activity (Fig. 2). Generally, a value of $p > F$ under 0.05 is acceptable as a significant model. In this study, the *p*-values from some of the variations did not satisfy the condition of less than 0.05. However, all the variations were used for the quadratic equation until a high r^2 value (> 0.96) was maintained [19]. This indicator (> 0.96) showed that all variations in the actual values could be supported by the RSM. Only 4% of the results were unexpected data. The quadratic models derived from the analysis of variance (ANOVA) results are expressed as Eq. (1). β-Glucosidase activity was found to be related to specific constant gravity as a function of each medium concentration by the RSM. More details of the ANOVA are described in Table 3. The *F*-value of 10.12 implied that this model is significant, and the *p*-value was 0.0413, which means that there was only a 4.5% chance that there were predicted values larger than actual values. The *p*-value was an indicator that checked the significance of each variable's coefficient. Based on the *p*-value, Table 3 shows the priority of impact on β-glucosidase activity.

The optimal concentrations of glucose, yeast extract, and ascorbic acid for the induction or increasing activity of β-glucosidase were 3.02%, 4.35%, and 7.41%, respectively (Fig. 3). The maximum β-glucosidase activity predicted by

Table 1. Effects of ascorbic acid on β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208

Components (%)	β-Glucosidase activity	
	Unit (U/mL)	Unit (U/mg)
Glucose 2.0, yeast extract 4.5, ascorbic acid 0.0	0.93 ± 0.12	0.89 ± 0.17
Glucose 2.0, yeast extract 4.5, ascorbic acid 0.8	6.15 ± 0.22	5.87 ± 0.15
Glucose 2.5, yeast extract 3.5, ascorbic acid 0.0	1.89 ± 0.01	1.14 ± 0.09
Glucose 2.5, yeast extract 3.5, ascorbic acid 0.8	5.75 ± 0.25	5.26 ± 0.12
Glucose 3.0, yeast extract 4.5, ascorbic acid 0.0	0.89 ± 0.01	0.63 ± 0.06
Glucose 3.0, yeast extract 4.5, ascorbic acid 0.8	6.99 ± 0.02	6.31 ± 0.13

Value represents the mean of three replicate determinations ± standard deviation.

Table 2. Central composite design (CCD) and estimated response values for optimization of β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208

Run	Glucose (%, w/v)		Yeast extract (%, w/v)		Ascorbic acid (%, w/v)		β-glucosidase activity			
	X_1	Code	X_2	Code	X_3	Code	Predicted (U/mL)	Predicted (U/mg)	Actual (U/mL)	Actual (U/mg)
1	3	0	0.5	−1	1.4	−1	4.72	3.37	4.93 ± 0.19	3.16 ± 0.15
2	2	−1	3.5	0	1.4	−1	6.23	4.66	5.92 ± 0.14	4.08 ± 0.17
3	4	1	6.5	1	6.4	0	11.64	10.98	11.54 ± 0.10	10.20 ± 0.05
4	2	−1	6.5	1	6.4	0	10.64	11.20	11.45 ± 0.14	11.73 ± 0.04
5	3	0	6.5	1	11.4	1	10.93	10.73	10.73 ± 0.21	10.93 ± 0.10
6	4	1	3.5	0	11.4	1	10.58	9.40	10.89 ± 0.15	9.98 ± 0.12
7	2	−1	0.5	−1	6.4	0	8.47	7.01	8.57 ± 0.15	7.79 ± 0.05
8	4	1	0.5	−1	6.4	0	8.78	8.58	7.97 ± 0.08	8.05 ± 0.07
9	3	0	3.5	0	6.4	0	14.22	15.34	14.22 ± 0.12	14.90 ± 0.04
10	3	0	0.5	−1	11.4	1	7.81	8.28	8.31 ± 0.16	8.23 ± 0.19
11	2	−1	3.5	0	11.4	1	10.59	9.69	9.99 ± 0.15	8.95 ± 0.11
12	4	1	3.5	0	1.4	−1	7.56	6.30	8.16 ± 0.15	7.04 ± 0.08
13	3	0	6.5	1	1.4	−1	6.64	7.52	6.14 ± 0.14	7.57 ± 0.13

Value represents the mean of three replicate determinations ± standard deviation.

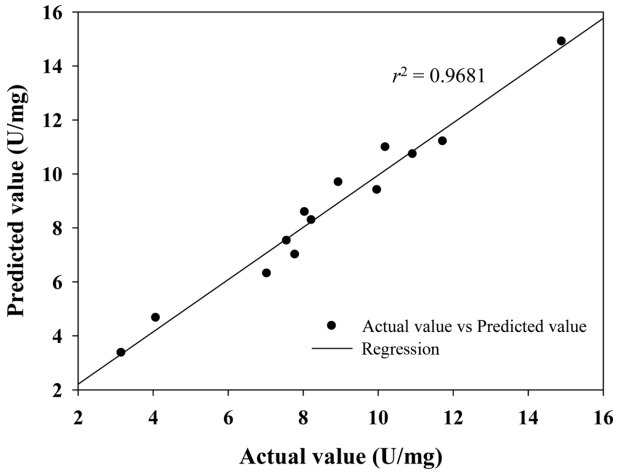

Fig. 2. Predicted and experimental values of β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208.

Table 3. Parameter estimation and analysis of variance (ANOVA) of the design for β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208

Source of variation	df	Sum of squares	Mean squares	*F*-value	Probability > *F*
Model	9	110.43	12.27	10.12	0.0413
X_1	1	21.78	21.78	17.97	0.0240
X_2	1	0.92	0.92	0.76	0.4467
X_3	1	32.97	32.97	27.20	0.0137
X_1X_2	1	0.80	0.80	0.66	0.4758
X_1X_3	1	0.73	0.73	0.60	0.4940
X_2X_3	1	0.93	0.93	0.77	0.4453
X_1^2	1	17.27	17.27	14.25	0.0326
X_2^2	1	16.77	16.77	13.84	0.0338
X_3^2	1	50.04	50.04	41.28	0.0076
Error	3	3.64	1.21	-	-
Total	12	114.07	-	-	-

X_1, yeast exctract; X_2, glucose; X_3, ascorbic acid.

the RSM was 15.34 U/mg, which was in perfect agreement with experimental value, 14.90 U/mg. This result shows an increase of 23.6 times that of the first optimization value, 0.89 U/mL. In this study, the obtained β-glucosidase activity value was the best of all the reported other strains. Barbosa *et al.* [20] reported optimized culture conditions of β-glucosidase activity from *Debaryomyces pseudopolymorphus*. Optimized medium conditions for β-glucosidase production was obtained as 1.25% of cellobiose, 0.05% of tween 80, and 0.4% of NH_4NO_3 over 72 hr. The result showed a 10-fold increase in the β-glucosidase activity (0.02~0.20 U/mL) compared to the first optimization [20]. In another study, the Maeda *et al.* [21] reported that evaluated nitrogen sources, such as urea and yeast extract, were of greater importance for β-glucosidase production in *Penicillium funiculosum*. They obtained in a 6.7-fold increase in β-glucosidase activity (1.375 U/mL) over the first optimization [21]. According to the obtained conditions using RSM, *F. pinicola* KCTC 6208 incubation for high β-glucosidase activity was conducted. As a result, a optimal incubation time for high β-glucosidase activity in *F. pinicola* KCTC 6208 was obtained at after the 16th day (14.90 U/mg) (Fig. 4).

In conclusion, glucose, yeast extract, and ascorbic acid were identified as significant nutrient factors that enhance the activity of β-glucosidase from *F. pinicola* KCTC 6208. The identified optimal concentrations of glucose, yeast extract, and ascorbic acid for a high activity of β-glucosidase were 3.02%, 4.35%, and 7.41%, respectively. Among the nutrients, ascorbic acid was found to be the most significant nutrient affecting the activity of β-glucosidase. The predicted maximum β-glucosidase activity by the RSM was 15.34 U/mg, which was in perfect agreement with the experimental value of 14.90 U/mg at the 16th day during incubation, which

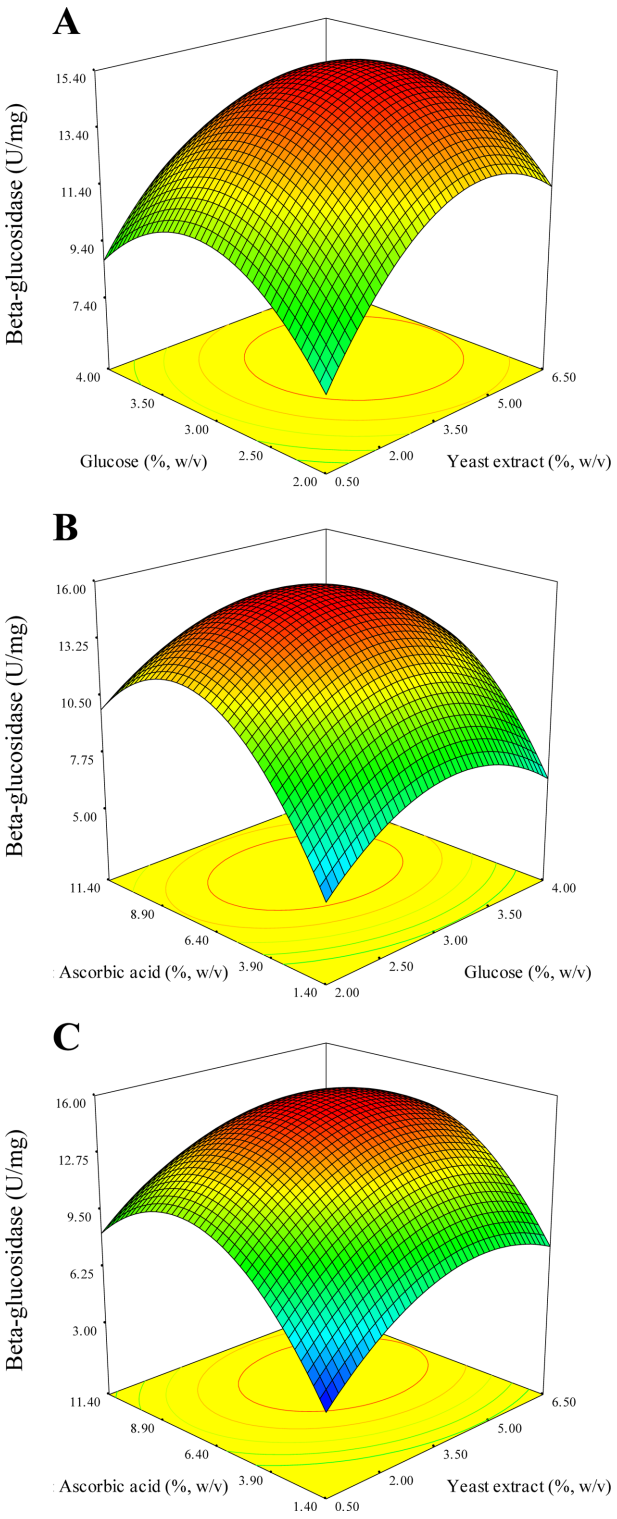

Fig. 3. 3D response surface display showing the relative effect of two variables on β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208. A, Interaction between glucose and yeast extract; B, Glucose and ascorbic acid; C, Yeast extract and ascorbic acid.

also showed a 23.6-fold increase over the first optimization value of 0.63 U/mg. Thus, a simplified medium supplemented

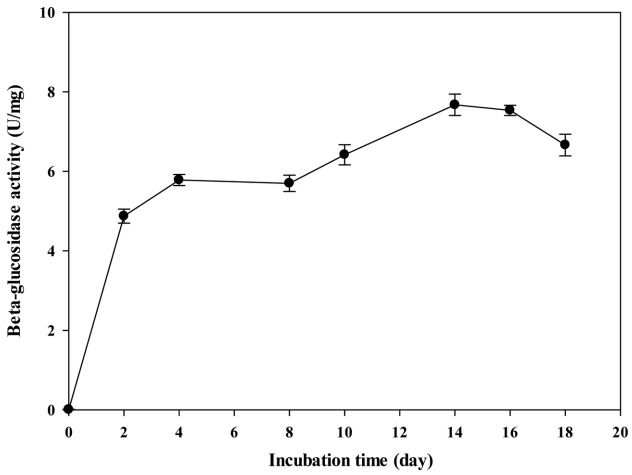

Fig. 4. Effect of incubation time on β-glucosidase activity in *Fomitopsis pinicola* KCTC 6208. The assay reaction performed at 27°C in a 100 mM sodium acetate buffer (pH 4.5). The value represents the mean of three replicated determinations; the error bar indicates the standard deviation.

with a simple vitamin source, such as an ascorbic acid, was proved to be a cost effective method for increasing activity or production of β-glucosidase because the cost of cellulase is a major determinant in the biofuel and biochemical industry using lignocellulosic biomass. Therefore, development of optimum conditions for enhancing activity of β-glucosidase will reduce the cost required for enzyme production, and media optimization will be also a very important process to save the cost required for the bioenergy industry.

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