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EXPERIMENTAL ARTICLES

Expression and Characterization of GH3 β-Glucosidase from *Aspergillus niger* NL-1 with High Specific Activity, Glucose Inhibition and Solvent Tolerance[1]

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Abstract—A β-glucosidase gene *bglI* from *Aspergillus niger* NL-1 was cloned and expressed in *Pichia pastoris*. The *bglI* gene consists of a 2583 bp open reading frame encoding 861 amino acids; the enzyme was classified into glycoside hydrolases 3. To improve the expression level of recombinant BGL in *P. pastoris*, fermentation conditions were optimized by the single-factor experiments. The optimal fermentation conditions were obtained: initial pH 5.0, methanol concentration 0.5% added into the culture every 24 h, and initial cell density (OD_{600}) of 10 for induction. The activity of BGL was increased from 4 U/mL to 45 U/mL in optimal conditions. The BGL was purified by ultrafiltration and $(NH_4)_2SO_4$ precipitation showing a single band on SDS-PAGE. The optimal activity was at pH 4.0 and 60°C. The recombinant enzyme was stable over a pH range of 3.0–7.0 and retained more than 85% activity after incubation at 60°C for 30 min. The kinetic experiments revealed K_m and V_{max} for *p*-nitrophenyl-β-D-glucoside of 0.64 mM and 370 U/mg, for cellobiose 8.59 mM and 1480 U/mg. The activity of BGL was not or only a little affected by many metal ions and EDTA and was enhanced by methanol or *n*-butyl alcohol. The BGL had a K_i of 48 mM for glucose and retained 76% activity in the presence of 50 mM glucose. The favorable properties of BGL offer the potential for industrial application.

Keywords: β-glucosidase, *Aspergillus niger*, expression, characterization

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β-Glucosidase is an important component of the cellulase enzyme system. Apart from being involved in cellulose degradation, it also plays an important role in hydrolyzing cellulose to fermentable glucose by relieving the inhibition of exoglucanase and endoglucanase from cellobiose [1–2]. It is an efficient way to reduce the accumulation of cellobiose in hydrolysates and increase the hydrolysis yield by additional β-glucosidase in the process of hydrolyzing cellulose by cellulase from *Trichoderma* [3–5]. Furthermore, β-glucosidase is used as a flavor enzyme to enhance the flavor of wine, tea, and fruit juices; to decompose lactose in dairy industry and catalyze glucose into functional oligosaccharides by transfer reaction and condensation reaction [6–10]. Therefore, the topic of producing high-activity and low-cost β-glucosidase has become heated in these fields.

Recently, the search for β-glucosidase with specific properties has increased significantly. A few bacterial and fungal β-glucosidases have been reported. Fungal β-glucosidases have the advantages of tolerance to organic solvent and relatively high specific activity. *Aspergillus* species are generally considered to be a good producer with high yield of β-glucosidase [11]. Generally *Aspergillus* species were found to secrete some distinct β-glucosidases when they were grown in induction culture. The major forms had a molecular mass of 130–100 kDa and were highly inhibited by glucose, belonging to family 3 of the glycoside hydrolases (GH3). Some of the minor forms with a molecular mass of 40–50 kDa exhibited tolerance to glucose. However, glucose-tolerant β-glucosidases from *Aspergillus* species have considerably lower specific activity for cellobiose than for *p*-nitrophenyl-β-D-glucopyranoside [12–14]. The β-glucosidases with lower specific activity for cellobiose will inhibit degradation of cellulose-containing biomass.

A. niger NL-1 was isolated by our laboratory for cellulose degradation. We previously found the native β-glucosidases from *A. niger* NL-1 had tolerance to organic solvents and glucose and had a molecular mass of 114 kDa. However, the activity of β-glucosidase from *A. niger* NL-1 obtained by optimization of the culture medium and condition does not meet modern-day demands of industry; moreover, the cost of the enzyme is high. For production of the recombinant protein, genetic engineering is the first choice because

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Table 1. Nucleotide sequences of the primers used

Primer	Nucleotide sequence
bgl-1	CCCGAATTCATGAGGTTCACTTTGATCG
bgl-2	CCCAAGCTTTTAGTGAACAGTAGGCAG
bgl-3	CCCGAATTCGCTGATGAATTGGCCTACTCCC
bgl-4	CCCCCGCGGTTAGTGAACAGTAGGCAGAGACGCC

it is easy, fast, and cheap [15, 16]. In the past decade, the yeast *P. pastoris* has become a common system for the expression of heterologous proteins. Several factors have made this yeast a preferred tool in protein expression: the strong and highly regulated alcohol oxidase promoter, very stable integration events in host chromosomal DNA, efficient techniques for high-cell density cultivation and commercial availability of strains and vectors.

In this paper, the β-glucosidase gene *bglI* was cloned from *A. niger* NL-1, the *bgl1* gene encoding β-glucosidase without the signal peptide was expressed in *P. pastori* GS115, the fermentation conditions were optimized, and the recombinant enzyme was purified and characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. *A. niger* NL-1 was reserved by our laboratory. *E. coli* TOP10F, *P. pastoris* GS115 (his^-mut^+), GS115(his^+ mut^S), Ablumin, GS115 (his^+ mut^+) and β-gal expression vector pPICZαA were bought from Invitrogen (Carlsbad, United States). *E. coli* JM109 and clone vector pMD18-T were from TaKaRa Biotechnology (Dalian, China). *E. coli* was cultured in Luria-Baertani (LB) medium supplemented with 100 μg/mL ampicillin. The medium for *P. pastoris* as BMGY, BMMY, YPD, YPDS, MD and MH were made up according to Invitrogen operation manual. *A. niger* NL-1 was grown at 30°C in YPD medium.

DNA manipulation. DNA was manipulated according to the standard procedures [17]. Qiagen plasmid kit and PCR purification kit (Qiagen, United States) were employed for the purification of plasmids and PCR products. DNA restriction and modification enzymes were purchased from TaKaRa (Dalian, China). DNA transformation was performed by electroporation using GenePulser (Bio-Rad, United States).

Plasmid contructions. Total RNA was isolated from 5-day-old mycelia as described by Sambrook et al. [17]. The cDNA was synthesized by using SuperScript III Reverse Transcriptase kit (Invitrogen) under the manufacturer's instructions. For amplification of the *bglI*, a pair of primers (bgl-1 and bgl-2, Table 1) was designed on the nucleotide sequence of the *A. niger bgl* cDNA (ABH01182). The PCR product was cloned into pMD18-T to yield the recombinant plasmid pMD18-BGLI.

The signal peptide was analyzed by Signal P 3.0 serve (http://www.cbs.dtu.dk/services/SignalP). Without the coding sequence for its predicted signal peptide, the *bglI* gene was amplified from pMD18-BGLI by PCR using primers bgl-3 and bgl-4 (Table 1), the PCR products were digested with *Eco*RI and *Sac*II and inserted into pPICZαA at *Eco*RI and *Sac*II sites, yielding the plasmid pPICZα-BGLI.

Expression of the recombinant *β*-glucosidase. The plasmid pPICZα-BGLI was linearized with *Pme*I and transformed into *P. pastoris* GS115. The multicopy recombinant *P. pastoris* were screened and identified on YPD plate containing Zeocin at 1000 μg/mL at 30°C for 3–4 days. The positive clones were incubated in 10 mL BMGY medium for 48 h at 30°C, 200 rpm. When the OD_{600} reached between 2~6, the cultures were harvested and resuspended in 30 mL BMMY to express β-glucosidase by adding methanol (final concentration 0.5% (vol/vol)) at OD_{600} about 1.0, and incubated further at 30°C, 250 rpm for about 120 h.

Optimization BGL expression. Culture conditions for β-glucosidase production were optimized by the single-factor experiments. The expression at different initial pH (ranging from 3.5 to 7.0) was set. Induction was continued with the addition of methanol to achieve concentrations ranging from 0.25% to 2.0% (vol/vol) at every 24 h to sustain the expression after incubation for 48 h. Different addition periods of methanol (a final concentration of 1.0%) ranging from 12 to 48 h were also tested after incubation for 48 h. The effect of initial cell density (OD_{600} 1, OD_{600} 5, OD_{600} 10, OD_{600} 15) on expression of α-glucosidase was studied.

Purification of recombinant laccase. The supernatant (200 mL) was harvested from 120 h culture of recombinant *P. pastoris* by centrifugation at 6000 *g* for 10 min, and the sample was purified by ultrafiltration (50000 ultrafiltration membrane, model number: CXA-50, Ya Dong Shanghai nuclear resin Ltd, China). The ultrafiltrate was precipitated by adding ammonium sulfate to 80%. The protein was dissolved in 25 mM citrate-phosphate buffer (pH 4.0) and dialyzed against the same buffer.

Characterization of the recombinant β-glucosidase. Quantitative assays were performed using *p*–nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate [18].

The reaction mixture comprised 1 mM pNPG, 50 mM citrate-phosphate phosphate buffer, and appropriately diluted enzyme solution in a total volume of 200 μL; the reaction was performed at 60°C for 10 min at pH 4.0. The *p*-nitrophenol release was monitored as A_{405}. One unit of the β-glucosidase activity was defined as the amount of the enzyme that produced 1.0 μmol of *p*-nitrophenol per minute under the standard assay conditions. Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

The optimal pH of the recombinant β-glucosidase was determined at 60°C for 10 min in citrate-phosphate phosphate buffer at a pH range from 3.0 to 7.0. The pH stability of the enzyme was determined by examining the residual activities under standard conditions after preincubation of the enzyme at 40°C for 30 min in the citrate-phosphate phosphate buffer of various pH. The activity of the enzyme without preincubation was defined as 100%.

To determine the optimal reaction temperature for the recombinant β-glucosidase, the enzyme activity was measured at temperatures ranging from 20 to 70°C. Thermal stability of the enzyme was determined by assaying the residual enzyme activity under the standard condition after the purified enzyme was preincubated for 30 min at various temperatures in the absence of the substrate.

The effects of metal ions on the activity of the purified enzyme were determined by adding 1 mM to the reaction mixture. The effect of EDTA on the enzyme was assayed at 10 mM. The effects of organic solvents on the enzyme were determined by adding 10, 15, 20, 30, and 40% organic solvents (ethanol, methanol, *n*-butyl alcohol, ethyl acetate, acetonitrile, and acetone) to the reaction mixture. Activity was expressed as a percentage of the activity obtained in the absence of the metal ions, chemical agents, or organic solvents.

Kinetic constant of BGL was determined by measuring the initial rates at various *p*-nitrophenyl-β-D-glucopyranoside concentrations or various cellobiose concentrations under standard reaction conditions. K_i were assayed with the substrate pNPG by adding different concentrations of glucose in range of 50 to 200 mM.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in gels containing 10% acrylamide and 0.1% SDS using a discontinuous Tris-glycine buffer system according to the method of Laemmli [19]. Proteins were stained with Coomassie Brillant Blue R-250.

Analysis of cellulose degradation. Avicel (Sigma, United States) was treated with the recombinant β-glucosidase and cellulase (from *Aspergillus niger* NL-1, data not shown), and the degradation was analyzed by the Glucose Kit (Shengsuo, Shanghai, China). The reaction mixture (1 mL) contained 25 mg cellulose, 2 U cellulase, and appropriately diluted recombinant β-glucosidase in citrate-phosphate phosphate buffer (pH 4.8) at 50°C for 6 h.

RESULTS AND DISCUSSIONS

Cloning and expression of *bglI* gene. Cellulose-containing biomass is the most abundant renewable resource on earth, whose natural degradation represents an important part of the carbon cycle within the biosphere [20]. Complete degradation of cellulose requires the synergistic action of endo-β-glucanase (EC 3.2.14), exo-β-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [21]. β-Glucosidases are commonly used in various industries, including food, agriculture, winemaking, as well as in research development. In this work, a 2583-bp DNA fragment of *bglI* gene was amplified and sequenced from cDNA library of *A. niger* NL-1, and ligated to pMD18-T to generate the plasmid pMD18-BGL. The *bglI* encoded a protein (BGL) of 861 amino acids. The sequence of the BGL belonged to family 3 of the glucoside hydrolases. It showed 98.8% homology with the gene of *A. niger* (ABH01182), which was revealed by whole-genome sequencing but has not been biochemically characterized. The *bglI* gene without the putative signal peptide was amplified from pMD18-BGL and ligated to pPICZαA at *Eco*RI and *Sac*II sites to generate the plasmid pPICZα-BGLI. Fifty independent recombinant strains of *P. pastoris* GS115 harboring pPICZα-BGLI with higher zeocin (1000 μg/mL) resistance were established. The recombinant strains were subjected to fermentation in shaking flasks and the production varied from 2 to 4 U/mL.

Expression of the recombinant β-glucosidase in *P. pastoris* GS115. For investigation of induction conditions such as pH value, time for induction, concentration of methanol, and the interval time for adding methanol, the best recombinants of Mut^+ GS115/pPICZα-BGLI were chosen and induced by different conditions to express the BGL.

P. pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *P. pastoris*. Methanol concentration in a *P. pastoris* process is therefore extremely important, since high levels of methanol may be toxic to the cells and low levels of methanol may not be enough to initiate transcription. In this study, the optimal methanol concentration was 0.5% when methanol was added every 24 h. When the methanol concentration was above or below 0.5%, the activity was low (Figs. 1a, 1b). The pH value has an influence on the metabolism of *P. pastoris* in general. The cultures were prepared with initial pH values of 3.5, 4.0, 5.0, 6.0, and 7.0. The result showed β-glucosidase activity increased as the pH values increased at a pH between 3.5 and 5.0, but the activity slightly decreased when the pH reached 5.0 (Fig. 1c). Since early expression of *bglI* can affect growth of

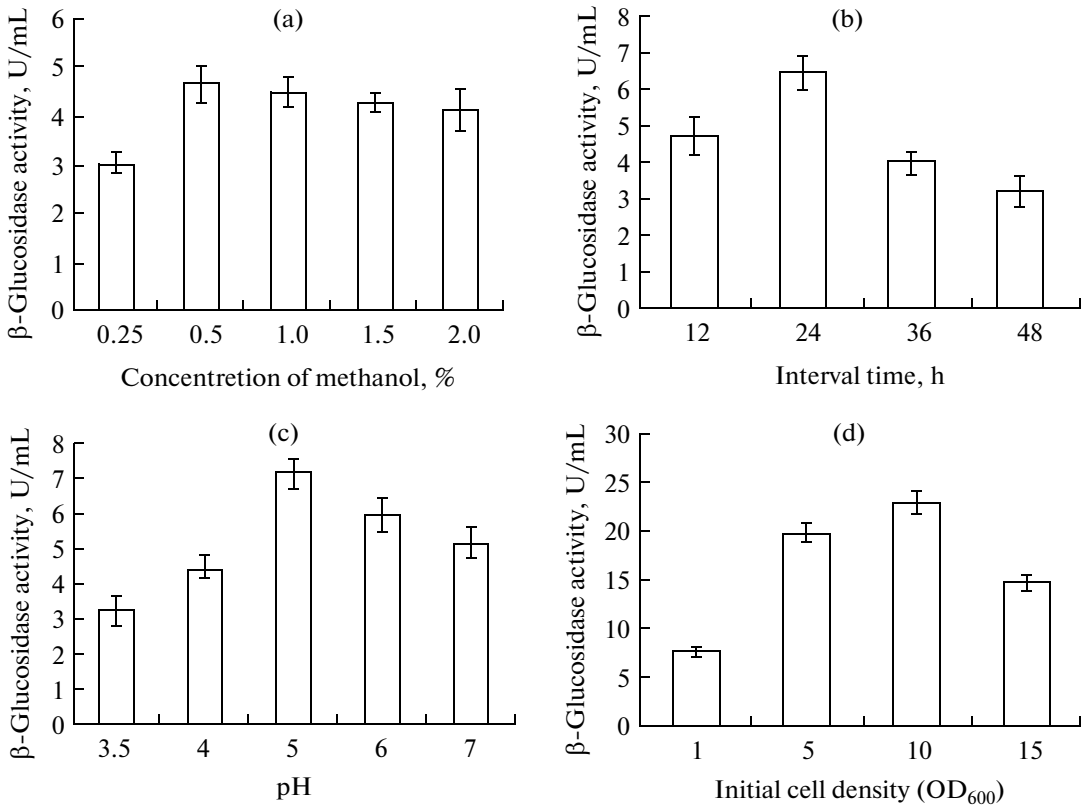

Fig. 1. The induction conditions of *P. pastoris*/pPICZα-BGLI. (a) Effect of concentration of methanol on the expression of the recombinant β-glucosidase. (b) Effect of the interval time for adding methanol on the expression of the recombinant β-glucosidase. (c) Effect of initial pH on the expression of the recombinant β-glucosidase. (d) Effect of initial cell density on the expression of the recombinant β-glucosidase.

P. pastoris and too late expression of *bglI* may affect the production of BGL, the initial cell density for induction is also important. The results showed that the optimal initial OD_{600} was 10 (Fig. 1d). By optimizing the conditions, the β-glucosidase expression level of the shake flask (23 U/mL) was about 5.5 times of extracellular β-glucosidase from *A. niger* NL-1 (data not shown).

Larger scale production was carried out in a 5 L fermenter using the optimal medium. A 300 mL inoculum in BMGY medium was used to inoculate the 5 L fermenter, which was run using the optimal medium and conditions. The β-glucosidase expression level was 1.9 times higher than that of the shake flask culture with the maximum activity of 45 U/L at the 120 h hours, which shortened the enzyme production cycle for industrial production (Fig. 2).

Purification of the recombinant β-glucosidase. The supernatant was harvested from 120 h culture of recombinant *P. pastoris* by centrifugation, and the recombinant β-glucosidase was purified by a simple precipitation. The results showed a 1.7-fold purification, which correlated with the final enzyme yield of 73%. The purification steps and relevant details such as laccase activity, specific activity and fold purification are outlined in Table 2. The recombinant β-glucosidase subunits showed a molecular mass of 121 kDa on 10% SDS-PAGE without undesired bands (Fig. 3). The molecular mass of the enzyme was greater than the theoretic molecular weight of 93.2 kDa because of

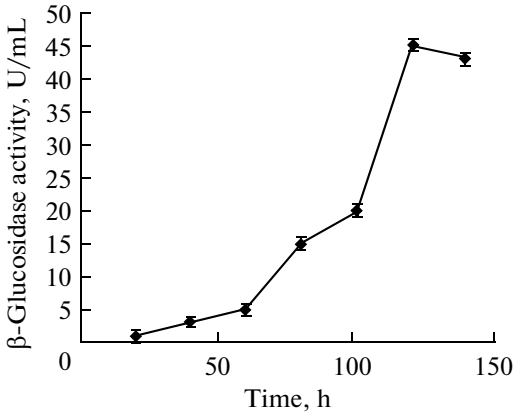

Fig. 2. Enzyme production curves of the recombinant β-glucosidase from the *P. pastoris*/pPICZα-BGLI.

Table 2. Purification of the recombinant β-glucosidase

Purification step	Total volume, mL	Total activity	Total protein, mg	Specific activity, U/mg	Yield, %	Purification (fold)
			U			
Culture filtrate	200	8600	55	156	100	1
Ultrafiltration (50 kDa)	10	6708	26	256	78	1.6
Ammonium sulfate precipitation	5	6278	23	270	73	1.7

Table 3. Characteristics of the recombinant β-glucosidases from *A. niger* NL-1 and other microorganisms

Strain	K_m, mM		V_{max}, U/mg		K_{cat}/K_m, mM^{-1} for cellobiose
	pNPG[a]	Cellobiose	pNPG	Cellobiose	
A. niger NL-1	0.68	8.59	370	1480	374
Thermoanaerobacteriwn thermosaccharolyticum DSM571 [27]	0.63	7.9	64	120	13.3
Debaryomyces vanrijiae [28]	0.77	57.9	668	84.3	2.43
A. oryzae [19]	0.55	7	1.066	353	36.1
A. niger [13]	21.7	ND	124.4	ND	ND
A. tubingensis [12]	6.2	ND	28.4	0.32	ND

Note: [a] pNPG: *p*-nitrophenyl-β-D-glucopyranoside; [b] ND: not determined.

the glycosylation of the enzyme in *P. pastoris*. By analysis of the amino acid sequence of the β-glucosidase from *A. niger* NL-1, 15 glycosylation sites were found. These may normally be relatively highly glycosylated.

Characterization of the recombinant β-glucosidase. When the activity of the recombinant β-glucosidase was assayed at 60°C, the enzyme exhibited optimal pH at 4.0 (Fig. 4b). Meanwhile, considerable levels of activity were detected at temperatures from 45 to 70°C, although the optimal temperature was at 60°C (Fig. 4a). Thermostability assays indicated that its residual activity was more than 85% after being incubated at 60°C for 30 min. The enzyme was stable from pH 3.0 to pH 7.0.

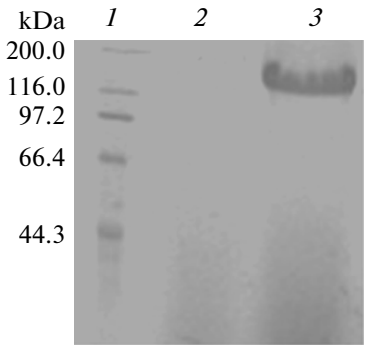

Fig. 3. SDS-PAGE analysis of the recombinant β-glucosidase. Lane *1*: protein marker, lane *2*: supernatant of *P. pastoris*/pPICZαA as negative control, lane *3*: the purified recombinant β-glucosidase (2.5 μg).

The dependence of the rate of the enzymatic reaction on the substrates concentration followed the Michaelis-Menten kinetics, with K_m and V_{max} values of 0.64 mM and 370 U/mg for *p*-nitrophenyl-β-D-glucopyranoside, and for cellobiose 8.59 mM and 1480 U/mg under optimal conditions. Although several β-glucosidases from a few fungi and bacteria show high glucose tolerant with K_i values of more than 200 mM, the V_{max} values of these enzymes for cellobiose was much lower than that of BGL. The K_{cat}/K_m value of BGL for cellobiose 347 mM^{-1} s^{-1} was higher than those of β-glucosidases in Table 3. The high specific activity for cellobiose of the BGL demonstrated a great potential of the gene in biomass degradation.

The effects of metal ions and some chemicals on the enzyme activity are shown in Table 4. In various assays, the effects of Ba^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, K^+, Fe^{2+}, Zn^{2+}, Cu^{2+}, or EDTA on the enzyme activity was

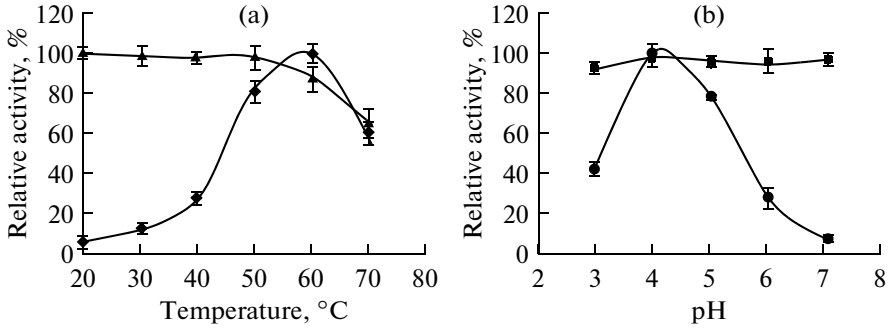

Fig. 4. Effects of pH and temperature on the activity and stability of the recombinant β-glucosidase. (a) Effect of temperature on activity (filled diamonds) and stability of the β-glucosidase (filled triangles). (b) Effect of pH on activity (filled circulars) and stability of the β-glucosidase (filled squares). The initial activity was defined as 100%.

not significant, but the enzyme activity was significantly inhibited by Ag^+. Six organic solvents were selected to test their effect on the activity of recombinant β-glucosidase, namely methanol, ethanol, *n*-butyl alcohol, ethyl acetate, acetonitrile, and acetone. The activity of the enzyme was unaffected by the concentration of ethanol below 20%, although ethanol had negative effect when the concentration of ethanol exceeded 30%. The enzyme activity was significantly enhanced by methanol or *n*-butyl alcohol, when their concentrations were not exceeding 30%. The enzyme activity was increased by 150% after addition of 30% (vol/vol) methanol into reaction mixtures (Table 5). The effect of ethyl acetate on the enzyme activity was not so significant, while the enzyme activity was significantly inhibited by acetonitrile or acetone.

Most β-glucosidases belonging to family 3 of the glycoside hydrolases (GH3) from *Aspergillus* species were subject to competitive inhibition of glucose to produce glucose, the K_i is generally 1–20 mM [10, 11]. The recombinant GH3 β-glucosidase had an unusually high tolerance to glucose; 76% of its activity was retained in the presence of 50 mM, and it had a K_i of 48 mM for glucose.

Application in producing glucose from avicel. Production of glucose from avicel by cellulase and the recombinant β-glucosidase was determined. Without addition of the recombinant β-glucosidase, the yield of glucose was only 49.3%. The yield of glucose was 63.4%, 70.5%, and 78.6% by adding the recombinant β-glucosidase 0.5, 0.75, and 1.0 U/mL, respectively (Fig. 5). With the recombinant β-glucosidase increasing, the rate of the cellulose degradation was enhanced. The result suggested that the recombinant β-glucosidase played an important role in hydrolyzing avicel by relieving the inhibition of endoglucanase from cellobiose.

In summary, the β-glucosidase gene (*bglI*) from *A. niger* NL-1 was expressed and characterized in *P. pastoris*. The expression level of *bglI* was increased from 4 U/mL to 45 U/mL in a 5 L fermentor under optimal conditions. The optimal activity was at pH 4.0 and 60°C over a 10 min assay. The recombinant enzyme was stable over a pH range of 3.0–7.0 and retained more than 85% activity after incubation at 60°C for 30 min. The kinetic expriments K_m and V_{max} for *p*-nitrophenyl-β-D-glucoside was 0.64 mM and 370 U/mg, for cellobiose 8.59 mM and 1480 U/mg. The K_{cat}/K_m value of BGL for cellobiose 347 $mM^{-1}\ s^{-1}$. The BGL had a K_i of 48 mM for glucose and retained 76% activity in the presence of 50 mM glucose. As compared on the enzyme properties, the recombinant

Table 4. Effects of chemical agents and metal cations on the recombinant β-glucosidase[a]

Chemical agents and metal cations	Residual activity, %
Control	100
Ba^{2+}	96
Mg^{2+}	95
Mn^{2+}	106
Ca^{2+}	98
K^+	97
Fe^{2+}	102
Zn^{2+}	100
Cu^{2+}	97
Ag^+	57
EDTA	98

Note: [a] Values shown are the mean of duplicate experiments, and the variation about the mean was below 5%.

Table 5. Effects of organic solvent on the recombinant β-glucosidase[a]

Final concentration of organic solvent, %	Residual activity, %					
	Ethanol	Methanol	*n*-Butyl alcohol	Ethyl acetate	Acetonitrile	Acetone
0	100	100	100	100	100	100
10	107	151	135	99	86	97
20	92	149	129	103	56	74
30	67	132	121	101	29	58
40	52	99	119	105	19	39

Note: [a] Values shown are the mean of duplicate experiments, and the variation about the mean was below 5%.

BGL was relatively high tolerant to glucose and organic solvent, more efficient in hydrolysis of cellibiose than β-glucosidases from other microorganisms. Thus, this study provides a useful β-glucosidase, which may be used to improve the enzymatic conversion of cellulosic to glucose through synergetic action.

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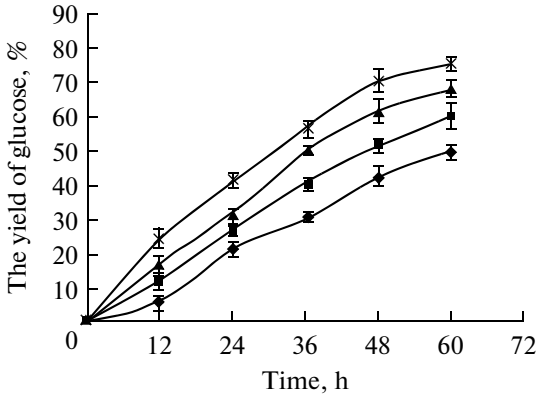

Fig. 5. Analysis of avicel hydrolysed by cellulase or by cellulase and the recombinant β-glucosidase. The reaction mixture (1 mL) contained 25 mg cellulose, 2 U cellulose, and 0 U (filled diamonds), 0.5 U (filled squares), 0.75 U (filled triangles), or 1 U (letter x) β-glucosidase in citrate-phosphate phosphate buffer (pH 4.8) at 50°C for 60 h.

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