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Expression of a Glucose-tolerant β-glucosidase from *Humicola grisea* var. *thermoidea* in *Saccharomyces cerevisiae*

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Abstract A β-glucosidase gene (*bgl*4) from *Humicola grisea* var *thermoidea* was successfully expressed in *Saccharomyces cerevisiae*. The recombinant protein ($BGL4^{Sc}$) was initially detected associated with yeast cells and later in the culture medium. $BGL4^{Sc}$ showed optimal pH and temperature of 6.0 and 40 °C, respectively, and an apparent molecular mass of 57 kDa. The enzyme showed activity against cellobiose and synthetic substrates, and was inhibited more than 80% by Fe^{2+}, Cu^{2+}, Zn^{2+}, and Al^{3+}. Using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate, $BGL4^{Sc}$ presented a V_{max} of 6.72 μmol min^{-1} mg total $protein^{-1}$ and a K_m of 0.16 mM under optimal conditions. Most important, $BGL4^{Sc}$ is resistant to inhibition by glucose and the calculated K_i value for this sugar is 70 mM. This feature prompts $BLG4^{Sc}$ as an ideal enzyme to be used in the saccharification process of lignocellulosic materials for ethanol production.

Keywords β-Glucosidase · *Humicola grisea* var. *thermoidea* · *Saccharomyces cerevisiae* · Glucose tolerance · Biomass conversion

Introduction

Agricultural wastes such as sugar cane bagasse are a rich source of lignocellulosic material which can be used as a substrate to produce ethanol. However, the conversion of lignocellulosic material to fermentable sugars is a complex process. For example, the conversion of cellulose to glucose involves the concerted action of three classes of enzymes: endo-β-1,4-glucanases (EC 3.2.1.4), exo-cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (β-D-glucosidic glucohydrolases, EC 3.2.1.21) [1]. The limiting step in the enzymatic saccharification of cellulosic material is the conversion of short-chain oligosaccharides and cellobiose (resulted from the synergistic action of endoglucanases

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and cellobiohydrolases) to glucose, a reaction catalyzed by β-glucosidases [2]. It is well established that cellobiose inhibits the activities of most cellobiohydrolases and endoglucanases [3–6]. β-Glucosidases reduce cellobiose inhibition by hydrolyzing this disaccharide to glucose, thus allowing the cellulolytic enzymes to function more efficiently [6–8]. Most of the known microbial β-glucosidases are inhibited by glucose [5, 9–11], therefore, the use of a glucose-insensitive enzyme is of great interest for the development of an industrial process involving enzymatic conversion of lignocellulosic biomass to fermentable sugars [6, 12].

The fungus *Humicola grisea* var. *thermoidea* is known to produce several thermostable cellulases [13–16] and has been considered for saccharification of sugar cane bagasse [17]. *H. grisea* is known to be a good source of β-glucosidases presenting six different characterized activities (BGL1–6) [18–20]. Some important features of the *H. grisea* β-glucosidase system, such as high specific activity, optimal pH, and temperature values, have been exploited to complement cellulolytic extracts. For example, BGL4 has been used to supplement the *Trichoderma reesei* β-glucosidase activity in a cellulose saccharification process [21]. β-Glucosidase may also be expressed in heterologous systems in order to confer new cellulose-degrading capabilities to the host strain. Some β-glucosidase genes have been expressed in *S. cerevisiae* aiming at their characterization and application [22–24]. *S. cerevisiae* has also been used to co-express fungal exo/endocellulases and β-glucosidases to drive the production of ethanol from lignocellulosic materials [25, 26]. As a first step to develop a *S. cerevisiae* strain capable of producing ethanol from cellobiose, we report the heterologous expression and characterization of BGL4, a β-glucosidase from *H. grisea* var *thermoidea*.

Materials and Methods

Strain, Media, and Culture Conditions

Saccharomyces cerevisiae MFL is an auxotrophic mutant for leucine obtained by *LEU2* gene disruption of the industrial strain FTPT472 which was isolated from an industrial fermentor at Fundação Tropical de Pesquisas Tecnológicas André Tosello (Campinas, Brazil). *S. cerevisiae* MFL was cultured in buffered minimal medium containing 0.67% yeast nitrogen base without aminoacids, 2% glucose, 0.4% aspartic acid, and 0.4% glutamic acid (pH 5.0), supplemented or not with 50 μg ml^{-1} leucine. Cultures were incubated at 30 °C on a rotatory shaker at 200 rpm.

DNA Manipulations

Standard protocols were followed for DNA manipulations [27]. *H. grisea* total RNA was obtained by the TRIzol® method (Invitrogen, Carlsbad, CA, USA) from mycelium grown on ball-milled straw (BMS). Transformation of *Escherichia coli* and *S. cerevisiae* followed standard protocols [28, 29]. Double-stranded DNA sequencing was performed with the MegaBACE® Dye Terminator kit (GE Healthcare, Piscataway, NJ, USA). Computer sequence analysis was carried out using the Phrap and Phred programs [30].

Cloning of *bgl*4 Gene

Total RNA extracted from a culture of *H. grisea* grown on BMS was used as template in a reverse-transcription reaction using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad,

CA, USA) and a specific primer, 3βglu (5′-GGATCCTTACTCCTTGCGAATCAAGC TATC-3′), that was designed based on the *bgl*4 gene sequence data available at Genbank (accession number AB003109). The coding region of the cDNA was then amplified by PCR with primers 3βglu and 5βglu (5′-GGATCCATCATGTCTCTTCCTCCGGA-3′), both containing a *Bam*HI restriction site in their 5′ends. The amplicon was purified with the QIAquick® PCR Purification kit (Qiagen, Valencia, CA, USA) and cloned into pGEM-T® Easy (Promega, Madison, WI, USA). The ligation mixture was used to transform competent *E. coli* DH5α cells which were selected on LB agar plates supplemented with ampicillin (100 μg ml^{-1}) and X-gal (40 μg ml^{-1}).

Heterologous Expression of *bgl*4

A recombinant plasmid containing *bgl*4 was digested with *Bam*HI and the full-length cDNA was ligated into dephosphorylated *Bam*HI-digested YEp351PGK [31]. The resulting plasmid, pYBG4, was used to transform *S. cerevisiae* MFL. Transformants expressing β-glucosidase activity were selected on glucose minimal medium agar plates overlayed with 4-methylumbelliferyl-β-D-glucoside (MUG; Sigma, St. Louis, USA) agarose solution (1 mM MUG, 50 mM sodium acetate pH 6.0, 0.75% agarose [*w*/*v*]) and positive clones were visualized under UV light. A recombinant clone expressing BGL4 was grown in minimal medium for 12 h and cells were harvested and treated with glass beads (three sets of 45-s agitation in the presence of 10% glass beads, intercalated by three incubations of 15 s on ice) to release cell-associated proteins.

Enzymatic Assays

β-Glucosidase activity was routinely assayed in a 1 ml reaction mixture containing 5 mM *p*-nitophenyl-β-D-glucopyranoside (pNPG; Sigma, St. Louis, USA), 50 mM acetate buffer (pH 6.0), and an appropriate dilution of enzyme preparation. After 10 min incubation at 40 °C, the reaction was stopped by adding 1 ml of 1 M Na_2CO_3, and *p*-nitrophenol (pNP) release was monitored at A_{405nm}. One unit of β-glucosidase activity corresponded to the amount of enzyme necessary to release 1 μmol of pNP min^{-1} under these conditions. Activities on other synthetic substrates and in the presence of cations were determined under the same conditions. Activity on cellobiose was assayed using 1% cellobiose (Sigma, St. Louis, USA) in 50 mM acetate buffer (pH 6.0) and an appropriate dilution of enzyme preparation. After 60 min of incubation at 40 °C, the release of glucose resulting from the hydrolysis of cellobiose was determined by the glucose oxidase method (Glucox 500 kit, Doles Reagentes e Equipamentos para Laboratórios Ltda, Brazil). Optimal temperature and pH were determined under standard conditions by changing the assay temperature or pH buffers. K_m and V_{max} were calculated by the Lineweaver and Burk double-reciprocal plot method [32]. Inhibition of pNPG hydrolysis by glucose was tested by adding the inhibitor at different concentrations to the standard reaction mixture and then performing the assay under optimal conditions (10 min, 40 °C, pH 6.0).

Protein Analytical Methods

Protein concentration was determined at A_{595nm} by using the Bio Rad protein assay (Hercules, CA, USA). Molecular mass was determined using a calibration curve derived from a mixture of marker proteins (Sigma, St. Louis, USA): myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), egg

albumin (45 kDa), and carbonic anhydrase (29 kDa). Polyacrylamide gels were silver-stained using the *PlusOne Silver Staining Kit Protein* (GE Healthcare, Piscataway, NJ, USA). A zymogram was performed to assess β-glucosidase activity after gel incubation at room temperature for 2 h in 50 mM acetate buffer (pH 6.0) containing 1 mM MUG. The hydrolyzed substrate was visualized under UV light.

Results

Primers 3βglu and 5βglu, designed for the isolation of the *bgl*4 cDNA, were used in a reverse-transcription reaction followed by PCR using total RNA from BMS-grown *H. grisea* as template. This reaction resulted in a 1,431-bp amplicon that was cloned into pGEM-T easy followed by DNA sequencing. The plasmid bearing the *bgl*4 cDNA was *Bam*HI-digested and the~1.4 kb fragment was ligated into *Bgl*II-digested YEp351PGK for constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* promoter and terminator sequences. After confirmation of insert orientation, the resulting construct was designated pYBG4 (Fig. 1).

Plasmid pYBG4 was used to transform *S. cerevisiae* MFL and transformants were screened for β-glucosidase activity using MUG as substrate. Positive clones were selected by their ability to form fluorescent halos around colonies on minimal medium agar plates overlayed with MUG. Bright fluorescence due to the formation of methylumbelliferone was clearly observed around β-glucosidase-producing colonies, but not in the colony-bearing YEp351PGK (negative control; Fig. 2).

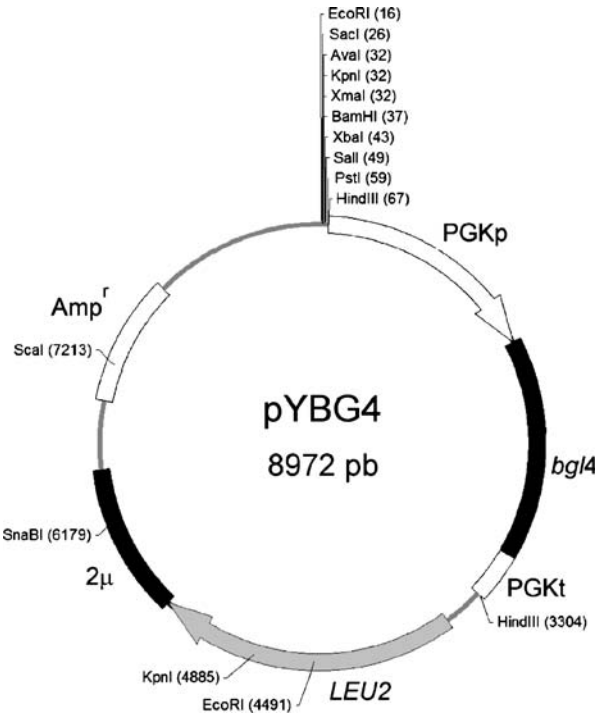

Fig. 1 Physical map of plasmid pYBG4

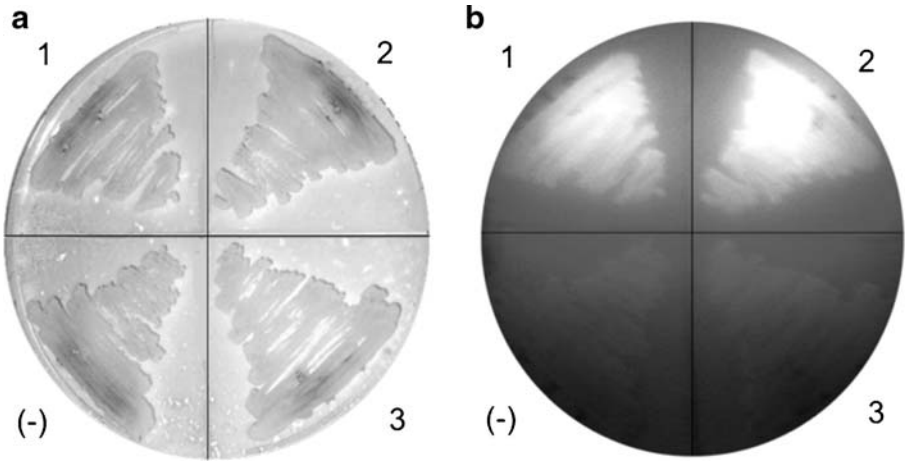

Fig. 2 Expression of *bgl*4 gene in yeast. *S. cerevisiae* MFL strain was transformed with pYBG4 and YEp351PGK plasmids. **a** A negative control *(−)* and three independent transformants (*1–3*) were streaked on glucose minimal medium agar plates and incubated at 30 °C for 2 days. **b** Detection of β-glucosidase activity by UV-stimulated fluorescence of MUG cleavage products

After growing *S. cerevisiae* cells in liquid culture, β-glucosidase activity was determined by the use of the pNPG substrate. No activity was detected in the reference yeast strain carrying YEp351PGK. Positive clones showed β-glucosidase activity associated with cells at exponential phase whereas no activity was detected in the culture medium (Fig. 3). At late exponential phase, cell-associated activity decreased. From that time on enzyme activity increased in the supernatant until stationary phase when no activity was detected in association with cells.

Recombinant BGL4 produced in *S. cerevisiae* ($BGL4^{Sc}$) was analyzed by SDS-PAGE which revealed the presence of a 57 kDa band which was absent in the control strain (Fig. 4a). Zymogram analysis showed that only the 57-kDa protein was active on the fluorescent substrate, indicating that it corresponded to $BGL4^{Sc}$ (Fig. 4b). The recombinant enzyme was more active on pH values in the range of 5.0 to 7.0 with the highest activity at pH 6.0. The enzyme retained about 100% of the relative activity after incubation for 30 h in buffers with pH values from 7.0 to 10.0 at 4 °C. The optimal temperature observed at pH 6.0 was 40 °C. $BGL4^{Sc}$ retained more than 80% of the original activity after 1 h incubation

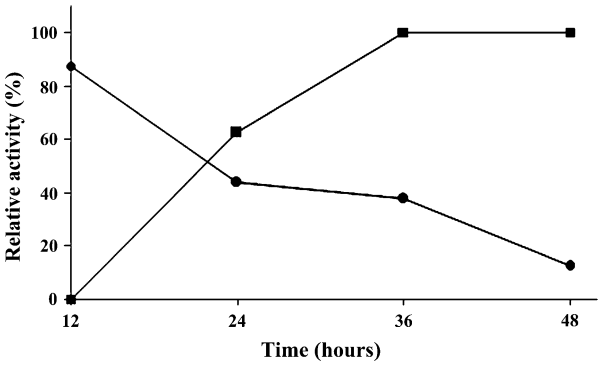

Fig. 3 β-Glucosidase activity during growth of recombinant *S. cerevisiae* expressing BGL4. (*filled square*) Supernatant; (*filled circle*) Periplasm

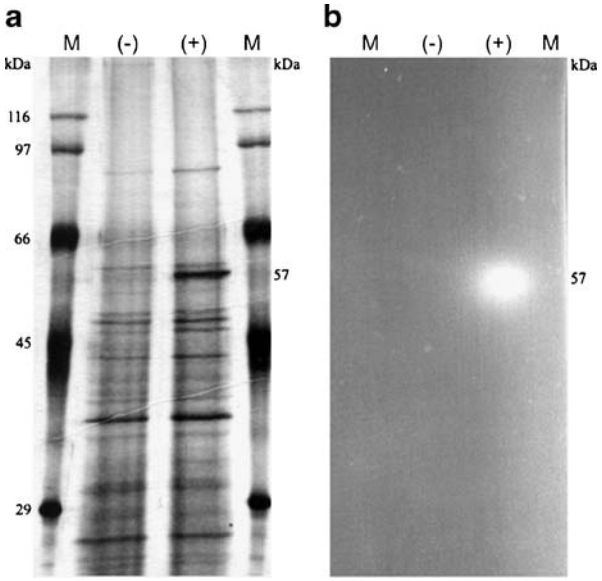

Fig. 4 Eletrophoretic analysis and zymogram of periplasmic extracts. **a** Silver-stained 12% SDS-PAGE gel. **b** Zymogram using MUG as substrate and visualized under UV light. *M* molecular weight marker, *(−)* negative control, *(+)* cell expressing BGL4^{Sc}

at 40 °C. The kinetic parameters for hydrolysis of pNPG were calculated by the Lineweaver–Burk reciprocal plot. BGL4^{Sc} hydrolyzed pNPG with a V_{max} and apparent K_m values of 6.72 μmol pNP min^{-1}mg total protein^{-1} and 0.16 mM, respectively (Table 1). According to the Hill plot, glucose caused a competitive inhibition with a K_i value of 70 mM (Fig. 5). The substrate specificity of BGL4^{Sc} was analyzed by hydrolysis tests using chromogenic/fluorescent substrates and cellobiose. The highest activities were observed for hydrolysis of pNPG and cellobiose. The enzyme was also able to hydrolyze *p*-nitrophenyl-β-D-cellobioside (pNPC), *o*-nitrophenyl-β-D-galactopyranoside (oNPGal), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 4-methylumbellipheryl-β-D-glucopyranoside (MUG), 4-methylumbellipheryl-β-D-cellobioside (MUC) and 4-methylumbellipheryl-β-D-lactoside (MUL; Table 2). Little or no activity was detected with *p*-nitrophenyl-β-D-xylanopyranoside (pNPX). When the effect of different metallic ions were tested BGL4^{Sc} exhibited more than 80% inhibition by 5 mM Fe^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+}, and ~50% by Co^{2+}. Glucose at the concentration of 5 mM was not sufficient to cause any inhibition of BGL4^{Sc} activity while the *H. grisea* total β-glucosidase activity was inhibited by about 50% under the same conditions.

Table 1 Properties of BGL4 produced by different organisms.

Organism	Location of activity	T (°C)	pH	K_m (pNPG) (mM)	V_{max}[a]	Reference
S. cerevisiae	Periplasmic space	40	6.0	0.16	6.72	This work
H. grisea	Extracellular	55	6.0	0.34	8.70	[20]
A. oryzae	–	55	6.0	0.32	25	[21]

K_m for pNPG

[a] V_{max} was measured as μmol product min^{-1} mg protein^{-1}

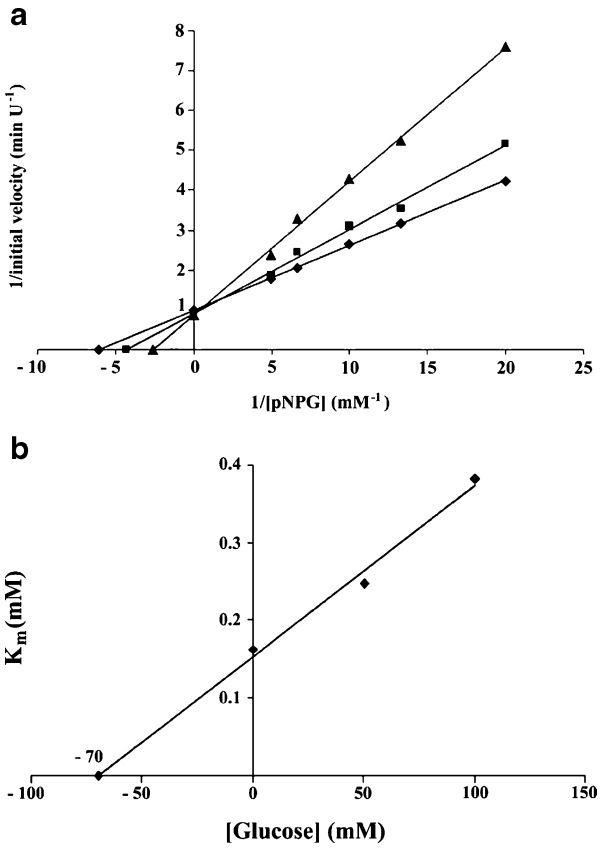

Fig. 5 **a** Lineweaver–Burk plot of pNPG hydrolysis by recombinant β-glucosidase in the presence of glucose. Each curve corresponds to a different concentration of added glucose, namely (*filled diamond*) 0 mM, (*filled square*) 50 mM, and (*filled upright triangle*) 100 mM. **b** Hill plot, *X*-intercepts translate into a K_i of ~70 mM

Discussion

H. grisea produces at least six β-glucosidases with different substrate specificities [20] and about 80% of the total β-glucosidase activity is lost in the presence of glucose; however, the remaining activity is tolerant to high levels of this sugar [13]. This work describes the cloning and expression in *S. cerevisiae* of one of these β-glucosidase genes, namely *bgl*4. The *bgl*4 coding sequence was expressed in *S. cerevisiae* and the recombinant enzyme was successfully detected. At early phases of the culture, recombinant β-glucosidase activity was shown to be associated with the yeast cells and no β-glucosidase activity was detected in the medium culture. However, the cell pellet was capable of hydrolyzing pNPG, probably due to the ability of this synthetic substrate to diffuse through the cell wall. No pNPG hydrolysis was observed in the cell pellet of the control strain. These data suggest that at the beginning of cell growth, the enzyme could be located in the periplasmic space. As the culture ages, the enzyme begins to diffuse to the culture medium until all the activity is detected in the supernatant. This behavior was also described for other fungal cellulases expressed in *S. cerevisiae* [22, 33, 34]. The recombinant β-glucosidase from *S. cerevisiae*

Table 2 Hydrolysis of different substrates by $BGL4^{Sc}$.

Substrate	Activity (μmol min^{-1} mg^{-1})
p-nitrophenyl-β-D-glucopyranoside (pNPG)	1.10
p-nitrophenyl-β-D-cellobioside (pNPC)	0.49
p-nitrophenyl-β-D-xylanopyranoside (pNPX)	0.02
o-nitrophenyl-β-D-galactopyranoside (oNPGal)	0.26
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	+
4-methylumbellipheryl-β-D-glucopyranoside (MUG)	+
4-methylumbellipheryl-β-D-cellobioside (MUC)	+
4-methylumbellipheryl-β-D-lactoside (MUL)	+
Cellobiose	4.00

+ Activity not quantified

showed an optimal temperature of about 40 °C, which is different from the observed optimal temperature for the native enzyme, 50 °C [20]. This difference may reflect different patterns of protein glycosylation between yeast and filamentous fungi. However, the optimal temperature shift to 40 °C might be advantageous for the development of a simultaneous saccharification and fermentation (SSF) process since yeast alcoholic fermentation temperature occurs between 30 and 37 °C. $BGL4^{Sc}$ presented lower K_m and V_{max} than the values observed for the native enzyme (Table 1). These alterations of kinetic parameters could be explained by the fact that we have used a non-purified source of this enzyme. Otherwise, $BGL4^{Sc}$ maintained the same substrate specificity and high affinity for cellobiose as the native enzyme.

$BGL4^{Sc}$ was shown to be highly tolerant to glucose inhibition, with a K_i value of ~70 mM. Effective inhibition by glucose is frequently observed on β-glucosidases, whose K_i values range from 0.35 to 100 mM when assayed with pNPG as substrate [35]. Recently, it has been reported the identification of glucose-tolerant β-glucosidades from compositing soil fungi, which were active in the presence of 300 mM glucose [12]. The observation that $BGL4^{Sc}$ is not inhibited by glucose is of great biotechnological interest. In the development of a process for the production of ethanol from cellulose, the use of an enzyme with this feature could relieve the inhibition of endoglucanase and cellobiohydrolase caused by the accumulation of cellobiose during the bioconversion process. We envision that the construction of an industrial *S. cerevisiae* strain expressing glucose-tolerant β-glucosidase such as *bgl*4 will be of great importance in the development of the consolidated bioprocessing of lignocellulosic materials.

Conclusions

The *bgl*4 gene from *H. grisea* var *thermoidea* coding for β-glucosidase was successfully expressed in *S. cerevisiae* and the observation that the heterologous enzyme was resistant to glucose inhibition shows its advantageous use in the saccharification process of lignocellulosic materials, thus paving the way to ethanol production from biomass.

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