

Characterization of β -Glucosidase Produced by the White Rot Fungus *Flammulina velutipes*

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β -Glucosidase production by the white rot fungus *Flammulina velutipes* CFK 3111 was evaluated using different carbon and nitrogen sources under submerged fermentation. Maximal extracellular enzyme production was 1.6 U/ml, corresponding to a culture grown in sucrose 40 g/l and asparagine 10 g/l. High production yield was also obtained with glucose 10 g/l and asparagine 4 g/l medium (0.5 U/ml). Parameters affecting the enzyme activity were studied using *p*-nitrophenyl- β -D-glucopyranoside as the substrate. Optimal activity was found at 50°C and pHs 5.0 to 6.0. Under these conditions, β -glucosidase retained 25% of its initial activity after 12 h of incubation and exhibited a half-life of 5 h. The addition of MgCl₂, urea, and ethanol enhanced the β -glucosidase activity up to 47%, whereas FeCl₂, CuSO₄, Cd(NO₃)₂, and cetyltrimethylammonium bromide inflicted a strong inhibitory effect. Glucose and cellobiose also showed an inhibitory effect on the β -glucosidase activity in a concentration-dependent manner. The enzyme had an estimated molecular mass of 75 kDa. To the best of our knowledge, *F. velutipes* CFK 3111 β -glucosidase production is amongst the highest reported to date, in a basidiomycetous fungus.

Keywords: β -Glucosidase, *Flammulina velutipes*, submerged fermentation

Introduction

β -Glucosidases (β -D-glucoside glucohydrolase, E.C. 3.2.1.21), under physiological conditions, catalyze the hydrolysis of β -1,4-glycosidic bonds from the nonreducing termini presented in alkyl- and aryl- β -D-glycosides, as well as different oligosaccharides (containing 2–6 monosaccharides). These enzymes are widespread in nature, occurring in all domains of living organisms, Archaea, Eubacteria, and Eukaryotes, in which they play varied functions [39]. They represent an important group of enzymes because of their potential uses in various biotechnological processes, including biomass degradation [44], the production of fuel ethanol from cellulosic agricultural residues [22], release of aromatic compounds in the flavor industry [13], and synthesis of useful β -glucosides [24].

Cellulose is the most abundant polymer on Earth, and its complete degradation is accomplished by a cellulolytic complex, which involves the synergistic action of three

enzymes: 1,4- β -D-endoglucanase (E.C. 3.2.1.4), 1,4- β -D-cellobiohydrolase (CBH, E.C. 3.2.1.91), and β -glucosidase. It is generally accepted that the endoglucanases and the CBHs act cooperatively and synergistically in depolymerizing cellulose to cellobiose and oligosaccharides, which are then converted by β -glucosidase to glucose [3]. The deficiency in β -glucosidase activity causes the accumulation of the disaccharide cellobiose, leading to the repression of enzyme biosynthesis and end-product inhibition of the upstream enzymes, which result in a limited hydrolysis yield [44]. Therefore, commercially available cellulolytic preparations are often supplemented with β -glucosidase to boost the overall activity, such as that prepared from *Trichoderma reesei* cellulases [8, 41]. β -Glucosidase is the rate-limiting factor in the conversion of cellulose to glucose for the subsequent production of fuel ethanol. Product inhibition and thermal inactivation of β -glucosidase constitute two major barriers to the development of enzymatic hydrolysis of cellulose as a commercial process. There is an

increasing demand for the identification and production of β -glucosidases, especially those insensitive to product inhibition and with high stability [31].

The white rot fungus *Flammulina velutipes* is a xylophagous fungus belonging to the *Physalacriaceae* family. Recently, *F. velutipes* strain Fv-1 demonstrated its potential for the conversion of lignocellulosic biomass to ethanol by consolidated bioprocessing, a methodology that combines enzyme production, enzymatic saccharification, and ethanol fermentation in one step, thus reducing the total cost of bioethanol production [22]. The objective of this research was to evaluate the β -glucosidase production by *F. velutipes* CFK 3111 and to determine the influence of some parameters on the activity and stability of this enzyme.

Materials and Methods

Organism and Culture Conditions

F. velutipes CFK 3111 (*Physalacriaceae*, *Agaricales*, *Basidiomycota*) was obtained from the culture collection of Laboratorio de Micología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. The strain was maintained in malt-extract agar (MEA; malt extract 1.2%, glucose 1%, and agar 2%) at 4°C. Growth media consisted of basal medium MgSO₄·7H₂O 0.5 g, KH₂PO₄ 0.5 g, K₂HPO₄ 0.6 g, CuSO₄·5H₂O 0.4 g, MnCl₂·4H₂O 0.09 mg, H₃BO₃ 0.07 mg, NaMoO₄·2H₂O 0.02 mg, FeCl₃ 1 mg, ZnCl₂·H₂O 2.5 mg, biotin 0.005 mg, thiamine hydrochloride 0.1 mg, and distilled water up to 1 L. Culture media were supplemented with the indicated carbon source (1 or 4% (w/v)) and nitrogen source (0.4 or 1% (w/v)). Erlenmeyer flasks (125 ml) containing 25 ml of growth media were inoculated with 5 mm diam plugs cut out from the margin of a 7-day-old colony growing on MEA and incubated statically at 28°C. The final pH of all media was

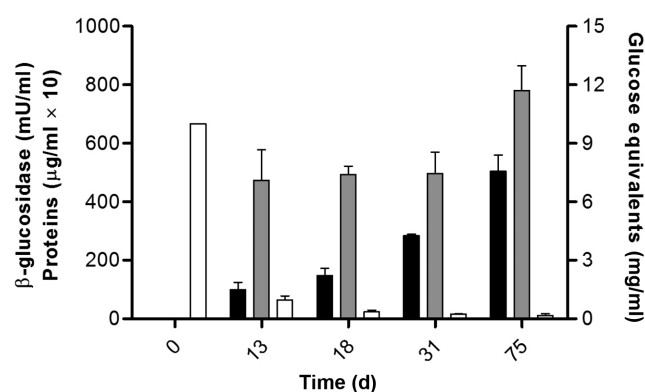


Fig. 1. Time course of β -glucosidase production (black bars), extracellular protein (grey bars), and glucose consumption (white bars) during growth in GA medium. Error bars denote standard deviation.

adjusted to 6.5. Erlenmeyer flasks were sealed with Parafilm-covered cotton plugs and by a loose cap of aluminum foil in order to avoid excessive water evaporation during extended incubation periods. Cultures were harvested at proper intervals (depicted in Fig. 1), 0.5 ml aliquots of the supernatant were collected aseptically, and at day 75, the last sampling day, whole cultures were filtered through a filter paper using a Büchner funnel, dried overnight at 70°C, and weighed. Growth was estimated by measuring the biomass production. When crystalline cellulose was used as a carbon source, the fungal biomass was estimated by *N*-acetyl glucosamine quantification [29]. Culture supernatant samples were used for β -glucosidase activity, soluble proteins, and consumption of reducing sugars determinations. Unless otherwise stated, all chemicals used were of reagent grade and purchased from Sigma (St. Louis, MO, USA).

Enzyme Assay

The β -glucosidase activity was assayed by using *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG; Sigma) as the substrate. First, 0.45 ml of *p*NPG (0.02% in 50 mM sodium acetate buffer; pH 4.8) was mixed with 0.05 ml of appropriately diluted enzyme solution. The reaction mixture was incubated at 50°C for 30 min and stopped by the addition of 1 ml of 0.1 M Clark and Lubs buffer (50 ml of 0.1 M H₃BO₃ in 0.1 M KCl, plus 40.8 ml of 0.1 M NaOH and double-distilled water up to 100 ml; pH 9.8) [10]. The amount of *p*-nitrophenol released was determined spectrophotometrically by measuring the absorbance of the solution at 430 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the assay conditions. Specific activity (U sp) was expressed as U per milligram of dry biomass weight [6].

Analytical Determinations

Glucose was measured by the Somogyi-Nelson [26, 35] method. The extracellular proteins were measured in the culture supernatants using the Bradford method [5] with the BioRad protein assay reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA) as the standard protein.

Effects of Temperature and pH on β -Glucosidase Activity and Stability

For testing β -glucosidase activity and stability, supernatants from cultures with GA (glucose 10 g/l and asparagine 4 g/l) were assayed. The effect of pH on β -glucosidase activity was determined in the range 2.6–8.0 by incubating the crude enzyme at 50°C for 30 min in *p*NPG dissolved in 50 mM citrate phosphate buffer (pHs 2.6–7.0) or 50 mM phosphate buffer (pHs 6.0–8.0). The optimum temperature for β -glucosidase activity was examined after incubating the crude enzyme for 30 min at various temperatures from 30°C to 70°C with *p*NPG in 50 mM sodium acetate buffer (pH 4.8) as substrate. In the experiments testing the effect of pH on the enzyme stability, crude enzyme sample were preincubated for up to 15 h at 50°C and 60°C at the pH range 2.6 to 8.0 in the buffers

described above. Samples were withdrawn at proper intervals (depicted in Fig. 3) for enzyme activity determination at standard assay conditions. Residual enzyme activity was calculated by comparison with non-preincubated supernatants [1].

Effects of Various Additives on β -Glucosidase Stability

Crude enzyme sample used in these experiments were desalted against distilled water at 1:1,000 ratio (v/v) using dialysis tubing of molecular weight cut-off 12,000 Da (Sigma-Aldrich, St. Louis, MO, USA), and then concentrated against sucrose for 4 h. The stability of β -glucosidase against urea, maltose, and fructose, selected salts (ZnSO_4 , FeCl_2 , KCl , MgCl_2 , MnCl_2 , $\text{Cd}(\text{NO}_3)_2$, $\text{Cr}(\text{NO}_3)_2$, SO_4Cu), chelators (ethylenediaminetetraacetic acid (EDTA) and Azida), and surfactants (sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB)), was tested at 1 and 10 mM. For this, the culture fluid was preincubated with the individual additives at 30°C. Samples were collected after 30 min for enzyme activity determination at standard assay conditions. Control with a preincubation in the absence of any additive was recorded as 100% activity. In addition, the effect of organic solvents on β -glucosidase stability was assayed. Culture filtrate was incubated with methanol (50% (v/v)), acetic acid (50% (v/v)), or ethanol (25, 50, and 75% (v/v)) at 30°C. Samples were collected at selected times and residual activities were determined.

Effects of Glucose and Cellobiose on β -Glucosidase Activity

The effects of glucose or cellobiose on β -glucosidase activity were conducted using the standard reaction mixture with the addition of the individual sugars at final concentration 50–200 mM. Activity in the control with no sugar added was recorded as 100% [14].

Polyacrylamide Gel Electrophoresis

Enzyme extract was analyzed by non-denaturing polyacrylamide gel electrophoresis (PAGE), using 9% acrylamide. Proteins separated in the gel were stained with Coomassie Brilliant Blue R-250, and their molecular mass estimated with standard markers: myosin (208 kDa), phosphorylase B (114 kDa), BSA (81.2 kDa), ovalbumin (47.9 kDa), carbonic anhydrase (31.5 kDa), trypsin inhibitor (24.8 kDa), and lysozyme (16.6 kDa). The β -glucosidase activity was detected in Native-PAGE in which the lanes of the gel were loaded with 10 mU activity. The bands were visualized by incubating the gel at 50°C with 0.02% pNPG in 50 mM sodium acetate buffer (pH 4.8) for 2 h. The gel was washed with distilled water, and 0.1 M Clark and Lubs buffer (pH 9.8) was added and incubated until a yellow band appeared; the gel was photographed immediately.

Statistical Analysis

The data presented are the average of the results of three replicates. Analysis of variance was tested by the software Statgraphics ver. 5.1. The significant differences between treatments were compared by Fisher's LSD multiple range test at 5% level of probability.

Results

Effects of the Carbon and Nitrogen Sources on β -Glucosidase Production

The effects of the carbon and nitrogen sources on biomass and enzyme activity are shown in Table 1. Although all of the liquid media evaluated were able to support mycelial growth of *F. velutipes*, the biomass production was greatly affected by the culture medium used, and β -glucosidase production showed to be independent of growth. Among the nitrogen sources tested, asparagine was the nutrient that enhanced the highest production of β -glucosidase, where cultures supplemented with this amino acid yielded maximal volumetric and specific activities in all the combinations assayed. Negligible to very low activities were observed when ammonium sulfate was used as the nitrogen source, and intermediate activities were found with glutamic acid. Higher asparagine concentrations in the culture media yielded more biomass (data not shown) but comparable specific activities. Regarding the carbon source used, the highest enzyme activities were detected for glucose, cellobiose, and sucrose, whereas very low yields were achieved with fructose, crystalline cellulose, or lactose. Similar biomass production was observed in cultures grown either with cellobiose or glucose, but slightly higher enzyme activity was achieved with glucose. Overall, of the media assayed, the highest enzyme activity was observed at day 47 in cultures with sucrose 40 g/l and asparagine 10 g/l (1.6 U/ml), and high values were also achieved with GA (0.5 U/ml). As glucose is commonly used as a carbon source, data on fungal growth and production of different enzymes are vast, and thus GA medium was selected for further analysis to facilitate full comparisons with those data reported in the literature.

The kinetics of β -glucosidase production and enzyme activity in GA medium are depicted in Fig. 1. Extracellular proteins and β -glucosidase activity increased steadily up to day 18, when glucose was almost exhausted from the medium (96% of the glucose was consumed by the fungus). Afterwards, enzyme activity increased but at a slower rate until the last sampling day.

Effects of Temperature and pH on β -Glucosidase Activity and Stability

The effect of temperature on enzyme activity is shown in Fig. 2A. β -Glucosidase showed maximal activity at 50°C, where at this temperature activity was more than 2-fold higher than that detected at 30°C. The effect of pH was tested in the range 2.6–8.0 (Fig. 2B), where β -glucosidase

Table 1. Effects of carbon and nitrogen sources on β -glucosidase production (mU/ml).

Carbon source (g/l)	Nitrogen source											
	Glutamic acid				L-Asparagine				Ammonium sulfate			
	4 g/l		10 g/l		4 g/l		10 g/l		4 g/l		10 g/l	
	mU/ml	U sp	mU/ml	U sp	mU/ml	U sp	mU/ml	U sp	mU/ml	U sp	mU/ml	U sp
Glucose	10	262.9 $\pm 14.6^{(31)}$	ND				496.8 $\pm 55.1^{(75)}$	5.8 $\pm 0.1^{(75)}$			3.9 $\pm 0.6^{(75)}$	0.1 $\pm 0.1^{(75)}$
	40			378.4 $\pm 130.4^{(31)}$	ND				563.3 $\pm 147.0^{(47)}$	3.3 $\pm 0.3^{(47)}$		4.8 $\pm 0.6^{(75)}$ 0.2 $\pm 0.1^{(75)}$
Fructose	10	76.8 $\pm 2.9^{(31)}$	ND				288.4 $\pm 77.5^{(47)}$	5.0 $\pm 0.3^{(47)}$			13.9 $\pm 1.3^{(75)}$	0.3 $\pm 0.1^{(75)}$
	40			341.2 $\pm 48.0^{(47)}$	2.1 $\pm 0.1^{(47)}$				1051.4 $\pm 4.3^{(47)}$	8.0 $\pm 0.1^{(47)}$		46.1 $\pm 9.7^{(75)}$ 1.1 $\pm 0.2^{(75)}$
Sucrose	10	73.7 $\pm 17.5^{(47)}$	1.0 $\pm 0.2^{(47)}$				932.6 $\pm 180.9^{(75)}$	12.1 $\pm 0.2^{(75)}$			3.9 $\pm 1.1^{(75)}$	0.1 $\pm 0.1^{(75)}$
	40			634.7 $\pm 26.4^{(47)}$	3.9 $\pm 0.1^{(47)}$				1609.5 $\pm 332.2^{(47)}$	10.5 $\pm 0.2^{(47)}$		4.5 $\pm 0.6^{(75)}$ 0.1 $\pm 0.1^{(75)}$
Crystalline cellulose	10	122.4 $\pm 5.3^{(47)}$	ND				271.3 $\pm 54.7^{(75)}$	ND			26.1 $\pm 2.1^{(18)}$	ND
	40			220.8 $\pm 72.4^{(31)}$	ND		218.4 $\pm 4.9^{(47)}$	ND			707.3 $\pm 286.7^{(75)}$	ND
Cellobiose	10					494.0 $\pm 101.2^{(47)}$	10.1 $\pm 0.2^{(47)}$			681.9 $\pm 115.9^{(47)}$	11.6 $\pm 0.2^{(47)}$	
Lactose	10					217.9 $\pm 21.5^{(47)}$	5.0 $\pm 0.1^{(47)}$					
	40									271.4 $\pm 70.1^{(47)}$	2.6 $\pm 0.3^{(47)}$	

The values shown correspond to the peak of enzyme production. The numbers above the columns indicate the day these maxima were achieved. Specific activity (U sp) was calculated per milligram of dry biomass. The values are the mean of three replications \pm SD. ND, Not determined.

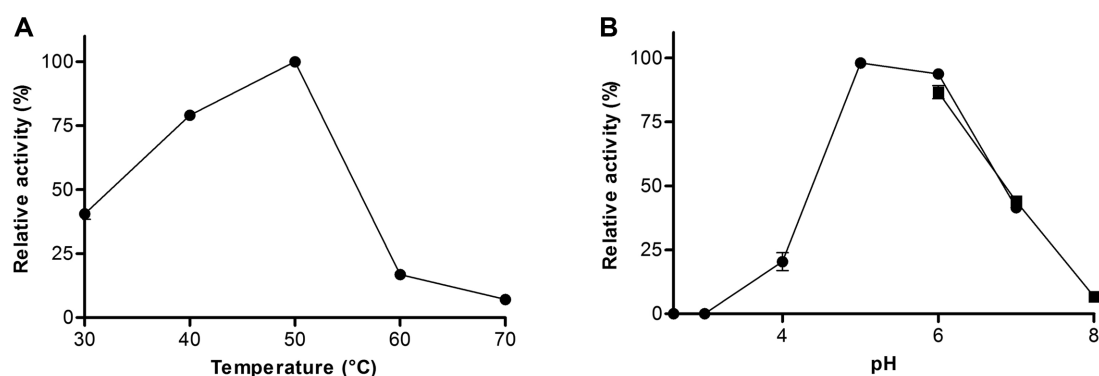


Fig. 2. Effects of (A) temperature and (B) pH with citrate phosphate buffer (●) and phosphate buffer (■) on β -glucosidase activity. Error bars are the standard deviations. When not shown, the error bars fall within the symbols.

activity was substantially affected, and the enzyme exhibited highest activity at pH 5, although it maintained up to 95% and 44% of its activity at pH 6.0 and 7.0, respectively. No

differences owing to buffer composition were detected. Stabilization studies were performed at different pHs ranging from 2.6 to 8.0 at 50°C and 60°C. The residual

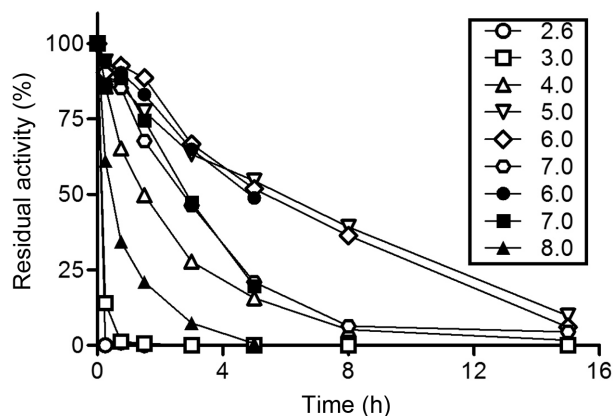


Fig. 3. pH stability assays, carried out by incubating the enzyme at 50°C at different pHs from 2.6 to 7.0 with citrate phosphate buffer (white symbols) and from 6.0 to 8.0 with phosphate buffer (black symbols).

activity was quantified at proper intervals. At 50°C, β-glucosidase was highly stable at pHs 5.0 and 6.0, exhibited a half-life of 5 h, and kept 25% of its activity after 12 h of incubation (Fig. 3). The stability as function of pH value was also measured at 60°C. At this temperature, the highest

Table 2. Effects of additives on β-glucosidase stability.

Reagent	Residual activity (%) ^b	
	1 mM	10 mM
None ^a	100 ± 3.5	100 ± 1.5
ZnSO ₄	100.2 ± 5.2	84.8 ± 7.4
FeCl ₂	106.2 ± 2.8	14.8 ± 1.2
KCl	107.3 ± 2.3	101.6 ± 2.0
SDS	121.2 ± 0.5	80.8 ± 0.3
MgCl ₂	147.2 ± 0.9	99 ± 0.4
MnCl ₂	111.9 ± 0.3	95.7 ± 0.4
Cd(NO ₃) ₂	78.5 ± 0.3	99.3 ± 0.2
Cr(NO ₃) ₂	114.9 ± 4.1	82.6 ± 3.0
SO ₄ Cu	103.5 ± 0.6	67.6 ± 0.5
Urea	125.5 ± 1.2	99.7 ± 0.8
Azida	ND	104.8 ± 0.1
EDTA	105.2 ± 5.4	107.7 ± 2.8
CTAB	88.0 ± 6.7	17.6 ± 3.0
Maltose	105.4 ± 4.4	103.8 ± 1.3
Fructose	101.8 ± 1.5	98.9 ± 1.7

The values are the mean of three replications ± SD.

^aThe activity assayed in the absence of additives was considered as 100%.

^bResidual activity was evaluated after preincubation for 30 min at 30°C with the individual additives.

ND, Not determined.

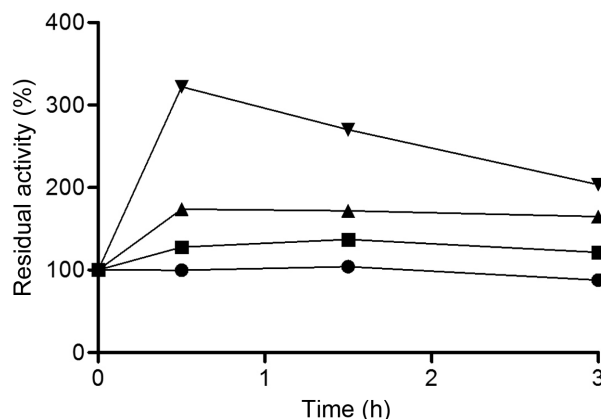


Fig. 4. Effect of ethanol on β-glucosidase stability.

Culture filtrates were incubated with ethanol 0% (●), 25% (■), 50% (▲), and 75% (▼) at 30°C. Samples were collected at selected times and residual activities were determined.

half-life of the enzyme was 9 min at pH 6.0 (data not shown).

Effects of Various Additives on β-Glucosidase Stability

The effects of various reagents on β-glucosidase stability were investigated (Table 2). Significant inhibition was observed with Cd²⁺ and CTAB at 1 mM concentration. When the concentration was increased to 10 mM, potent inhibition (up to 80%) was observed with CTAB, and some inactivation was found with SDS and the metal ions Zn²⁺, Cr²⁺, and Cu²⁺. Fe²⁺ almost abolished the β-glucosidase activity. In contrast, Mg²⁺, Mn²⁺, SDS, and urea at 1 mM enhanced the β-glucosidase activity, especially Mg²⁺, which exhibited a potent activation effect with 47.2% of activity increase. No significant effects were found with the sugars maltose and fructose, or with Azida and EDTA.

The effects of various organic solvents on β-glucosidase stability are depicted in Table 3 and Fig. 4. Ethanol 50% (v/v) caused an increase of 23% in β-glucosidase activity, whereas a minimum increase of 5% was observed with methanol.

Table 3. Effects of organic solvents on β-glucosidase stability.

Solvent (50% (v/v))	Residual activity (%) ^b
None ^a	100 ± 0.5
Methanol	105 ± 0.2
Ethanol	123 ± 0.1
Acetic acid	0 ± 0.1

The values are the mean of three replications ± SD.

^aThe activity assayed in the absence of additives was considered as 100%.

^bResidual activity was evaluated after preincubation for 40 min at 30°C with the individual organic solvents.

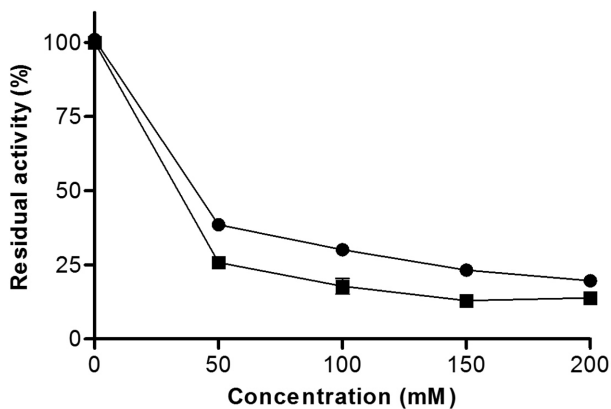


Fig. 5. Effects of glucose and cellobiose on β -glucosidase activity.

The relative activity was determined by measuring β -glucosidase at 50°C in 50 mM sodium acetate buffer (pH 4.8), in the presence of glucose (●) or cellobiose (■).

Contrariwise, total inhibition was caused by acetic acid. Ethanol activation was concentration-dependent (Fig. 4), and when used at 75% concentration, the enzyme activity increased more than 3-fold after incubation for 30 min, but from this time onward the activity declined.

Effects of Glucose and Cellobiose on β -Glucosidase Activity

The carbohydrates glucose and cellobiose inhibited the β -glucosidase activity (Fig. 5). Between them, cellobiose caused the more marked inhibitory effect. When glucose and cellobiose were used at 50 mM, β -glucosidase retained 38.5% and 25.8% of its initial activity, respectively, whereas at higher concentrations (up to 200 mM), the enzyme still retained around 20% of its activity.

Polyacrylamide Gel Electrophoresis

Native gel of the crude extract revealed the presence of a single activity band that corresponded with one β -glucosidase with an approximately molecular mass of 75 kDa (Fig. 6). Such band had a coincident Rf with a more intense colored protein band revealed in the SDS-PAGE after Coomassie Blue staining.

Discussion

Effects of the Carbon and Nitrogen Sources on β -Glucosidase Production

F. velutipes produced high quantities of extracellular β -glucosidase when grown in defined liquid medium with different carbon and nitrogen sources. Cultures with

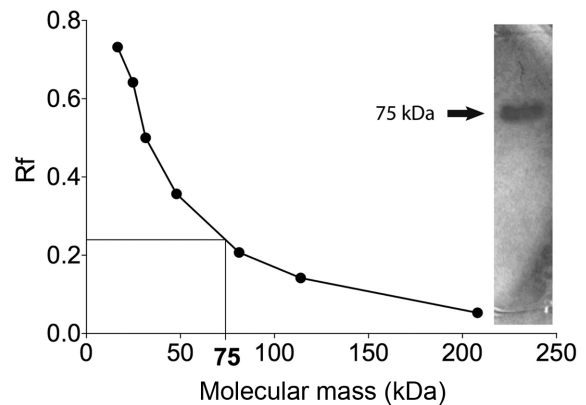


Fig. 6. Apparent molecular mass of β -glucosidase from *F. velutipes*.

Activity was revealed after incubating the native gel in *p*-nitrophenyl- β -D-glucopyranoside 0.02% at 50°C.

asparagine and sucrose produced the highest volumetric activity yields. High titers were also obtained with asparagine and fructose, cellobiose, or glucose. Enzyme activities were comparable with those yielded by several imperfect fungi species such as *Aspergillus*, *Penicillium*, and *Trichoderma*, which are considered the main β -glucosidase producers [17, 37]. Although there is growing interest in the search for new fungal β -glucosidases, the vast majority of studies were performed with imperfect fungi, and there are scant reports that deal with basidiomycetous fungi, not only for biotechnological purposes but also for physiological research. Among them, Vitrovský *et al.* [38] evaluated the production of lignocellulose-degrading enzymes in several white rot fungi and obtained up to 0.026 U/ml, with *Fomes fomentarius* being the major producer. Morais *et al.* [25] studied β -glucosidase production by *Pleurotus ostreatus* grown in different culture media containing agro-industrial wastes, where the highest activity detected was 0.385 U/ml with straw-pepper-potato extract [37]. In *Volvariella volvacea*, β -glucosidase activity in supernatants reached 0.13 U/ml [7], and in *Trametes trogii* was 0.25 U/ml [21]. Recently, similar titers of β -glucosidase activity (1.5 U/ml) were produced by another strain of *F. velutipes* [22] in a medium with yeast extract, peptone, and glucose as nitrogen and carbon sources. To the best of our knowledge, *F. velutipes* CFK 3111 β -glucosidase production is amongst the highest reported to date, in a basidiomycetous fungus.

Differences in enzyme activity as a response to different sources could be explained as being due to a promotion of fungal growth and subsequent major enzyme production. It has been demonstrated in *Telephora terrestris* that secretion

of laccase enzyme is biomass-dependent [18]. Thus, because of the physiological effect that nutrients had on growth, and to avoid interference of possible biomass-dependent activities, it was necessary to calculate the activities per milligram of mycelium. When considering *F. velutipes* specific activities, cultures with sucrose and asparagine attained maximal titers (10.5–12.1 mU/mg of dry biomass). Similarly, cellobiose, the natural substrate of β -glucosidase, was as efficient as sucrose in enzyme induction.

Partial Crude Enzyme Characterization

Effects of temperature and pH on β -glucosidase activity and stability. Physicochemical parameters were determined for *F. velutipes* crude enzyme towards *p*NPG substrate. Optimal activity conditions were observed at 40°C–50°C and pHs 5.0–6.0, a range similar to several fungal β -glucosidases, such as those from *Stachybotrys microspora* [33], *Humicola insolens* [36], and *Daldinia eschscholzii* [19]. β -Glucosidase retained 25% of its initial activity after 12 h of incubation at 50°C, at pHs 5.0 and 6.0, and exhibited a half-life of 5 h. In comparison, β -glucosidase from *Ferrodobacterium islandicum* [15] retained its activity for over 5 h at 50°C and showed a half-life of 15 min at 100°C. The *Scytalidium thermophilum* enzyme was thermostable up to 60 min when incubated at 50°C, at pH 6.5, and exhibited a half-life of 20 min when incubated at 55°C [45]. *Pyrococcus furiosus* β -glucosidase maintained 80% to 90% of its activity after 30 min of incubation at 100°C, with half-lives of 85 h at 100°C and 13 h at 110°C [20]. *Microbispora bispora* produced a β -glucosidase that was more active at 60°C, retaining 70% of its activity after 48 h of incubation [40].

Effects of Various Additives on β -Glucosidase Stability

Up to 92% inactivation was observed with the metal cations Fe^{2+} , Cu^{2+} , and Cd^{2+} , which may suggest that thiol groups are involved in the catalytic reaction at the active site. A similar effect was observed when studying the β -glucosidase from *T. reesei*, where the enzyme showed drastic inhibition by these cations; thus, it was speculated that the thiol-group Cys 168 may play a key role in inhibition mechanisms by cations [16]. The surfactant CTAB was another destabilizing compound that also caused noticeable loss of enzyme activity. This molecule commonly used detergent could interfere with hydrophobic sites, causing denaturation and consequently enzyme inactivation. On the other hand, enzyme activity was stimulated by Mg^{2+} and Mn^{2+} . A previous report showed that β -glucosidase activity from *T. reesei* was enhanced by up to 226% in the presence of MnCl_2 [16]. The enzyme from *F. velutipes* was

not so strongly enhanced, but this cation provoked the highest enzyme stimulation among compounds assayed in this study. Strong activation by Mg^{2+} was observed in the β -glucosidases of phylogenetically distant species such as *Neocallimastix patriciarum* [9], *D. eschscholzii* [19], and *Clostridium cellulovorans* [16], which suggest that Mn^{2+} and Mg^{2+} ions may assist the enzyme reaction in a highly conserved catalytic site. Ethanol also exerted activity enhancement, especially in short time intervals, with more remarkable effects the more concentrated the solution was. At ethanol 75% (v/v), enzyme activity increased more than 3-fold. It has been proposed that alcohol activation of some β -glucosidases may be due to their glycosyltransferase activities [4, 28]. In an environment containing high amounts of alcohols and relatively low amounts of water, many β -glucosidases can preferentially use the alcohols as acceptors for the glycosyl moiety during catalysis of *p*NPG, resulting in elevated reaction rates. There is vast literature where this effect is reported. For example, the β -glucosidase activity of *Aureobasidium pullulans* was enhanced 15% by ethanol 7.5% (v/v) [31]. Ethanol and methanol at 5% (v/v) increased *Stereum hirsutum* β -glucosidase activity about 30% [27], and in *Aspergillus oryzae* and *A. niger*, activity was stimulated 30% with ethanol 15% (v/v) [30, 42].

Effects of Glucose and Cellobiose on β -Glucosidase Activity

A decrease in enzyme activity was observed in the presence of glucose and cellobiose, possibly as β -glucosidase is sensitive to product and substrate inhibition, respectively. This effect was described in many other microorganisms [32, 34].

Molecular Mass of *F. velutipes* β -Glucosidase

The molecular mass of β -glucosidase was estimated as 75 kDa. This value falls within the wide range of molecular masses reported for basidiomycetous fungi, varying between 50 and 256 kDa [7, 23].

Concluding Remarks

During recent years, studies involving β -glucosidases are becoming more frequent given the key role of this enzyme in ethanol production for biofuels. Most commercial cellulolytic enzymes are obtained from *T. reesei*, such as the case of Celluclast 1.5L (Novozymes A/S), which has potent endoglucanase and CBH activities, but has to be supplemented with extra β -glucosidase activity from another source in order to improve cellulose hydrolysis; for example, Novozym 188 (Novozymes A/S) that is prepared from *A. niger*. Recent

reports have shown different strategies to reach more efficient processes leading to cellulose hydrolysis, such as immobilization [2, 11, 43], stability enhancement [12], and manipulation of culture parameters [46]. The production and characterization of novel β -glucosidases, such as the one described in this work, are part of the recent search for enzymes with robustness and high specificity towards cellobiose. Besides contributing with more efficient biotechnological tools in cellulose exploitation, ongoing investigations will expand our knowledge on the physiology of fungal β -glucosidase regulation.

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