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ORIGINAL PAPER

Carbon source directs the differential expression of β-glucosidases in *Stachybotrys microspora*

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Abstract *Stachybotrys microspora* is a filamentous fungus characterized by the secretion of multiple β-glucosidases. The production of these enzymes was studied under culture with variable carbon sources. The highest activity was produced on glucose (0.66 U ml^{-1}) whereas galactose, lactose, cellobiose, Avicel cellulose, carboxymethylcellulose (CMC), wheat bran and gruel allowed intermediate production levels ranging between 0.08 and 0.48 U ml^{-1}. The zymogram analysis showed that complex sugars such as Avicel cellulose and CMC induced the expression of several β-glucosidases whereas all tested simple sugars (mono and disaccharides) induced the expression of one single β-glucosidase, each time different. The most efficient β-glucosidase named bglG was produced on glucose which continues to be, at the same time, its strong inhibitor. The bglG N-terminal sequence confirmed that it is a novel β-glucosidase. According to its large molecular weight, this enzyme was assumed to belong to family 3 of β-glucosidases. RT-PCR analysis showed that family 3 expressions were induced on glucose while those of family 1 were repressed. Finally, with the use of different combinations of glucose and various carbon sources at different ratio, we showed that such sources direct the differential expression of β-glucosidases in *S. microspora* since our strain co-produced the β-glucosidases corresponding to each carbon source.

Keywords *Stachybotrys microspora* · Differential expression · Production · β-glucosidase · Glucose

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Introduction

Cellulose represents the most abundant carbohydrate substance in nature. It is a bio-polymer of glucose units related by β1, 4 glucosidic linkages (Suto and Tomita 2001). Cellulose degradation requires a multi-enzymatic system composed of three enzymes which are respectively: the endoglucanases (EC3.2.1.4) that randomly attack cellulose in amorphous zones releasing oligomers, the cellobiohydrolases (EC3.2.1.91) that liberate cellobiose from reducing and non reducing ends and finally the β-glucosidases (EC3.2.1.21) that hydrolyse cellobiose into glucose. β-Glucosidases (BGL) are present in all living organisms (Bacteria, Archaea, and Eukarya) (Esen 1993) and perform a wide range of functions. In bacteria and fungi, β-glucosidases play a key role during cellulose degradation by the hydrolysis of cellobiose and cellooligosaccharides into glucose units (Esen 1993; Shewale 1982; Sternberg et al. 1977). In the process of saccharification of lignocellulosic biomass, cellooligosaccharides and cellobiose are often produced by the limited hydrolysis of cellulosic materials; something that leads to inefficient ethanol fermentation, whereas their hydrolytic product, glucose, is the best substrate for ethanol production (Nakata et al. 2006) by yeast (Takeshi et al. 2008; Tokuhiro et al. 2008; Yu et al. 2008).

β-Glucosidases have potential roles in various fields such as the food, pharmacology and cosmetic industries and also in the valorisation of some products, due to the properties of this enzyme to convert and to synthesize biomolecules of high added value (Esen 1993). Indeed, β-glucosidase can either degrade or synthesize small carbohydrate polymers, depending on particular experimental conditions (Pérez et al. 2008).

The cellulolytic systems are generally inducible and repressible. The inducer of cellulase production is mainly

cellulose (Suto and Tomita 2001) and its derivatives (CMC, HEC) and other oligosaccharides, disaccharides (sophorose, gentiobiose, laminaribiose, lactose) (Suto and Tomita 2001; Sehnem et al. 2006) and even monosaccharides like xylose (Suto and Tomita 2001; Smaali et al. 2004). In contrast, cellulases, as many other hydrolases, are repressed by glucose (Suto and Tomita 2001) and other simple sugars.

We have shown that a novel fungal strain belonging to *Stachybotrys microspora,* isolated by our group is a filamentous fungus with a β-glucosidase rich system. When grown on CMC or on Avicel cellulose, it produces several different β-glucosidase activities (Amouri and Gargouri 2006). Two of these have been already purified and biochemically characterized (Amouri and Gargouri 2006; Saibi et al. 2007). These two purified enzymes, named M9 and F2 respectively, present different biochemical and kinetic properties but are endowed with the same capacity of hydrolysing cellobiose into glucose units. Hence they are considered as cellobiases. A third extracellular β-glucosidase, named bglG, was purified from *Stachybotrys microspora* and used as a tool to show the chaperone effect of a wheat protein called Dehydrin (DHN-5). Indeed, we showed for the first time that dehydrin protected this beta glucosidase against denaturing temperatures (Brini et al. 2010).

In the present work, we report on the regulation of β-glucosidase expression by carbon sources. Several monosaccharides, disaccharides, polysaccharides, alcoholic sugar and by-products were tested as carbon sources in liquid culture of the fungus. Zymogram analysis allowed us to discriminate between the different β-glucosidases secreted by our strain and that of the profile which reflects the substrate used.

Materials and methods

Biological material

The biological strain used in this work is a filamentous fungus that was identified as *Stachybotrys microspora* by the Centraalbureau voor Schimmelcultures-the Netherlands (Amouri and Gargouri 2006).

This fungal strain was grown on PDA (potato dextrose agar) medium at 30°C for 4 days, in order to collect the spores which are harvested in 0.1% Tween 80 solution and used as inoculums. Liquid pre-cultures were carried out in modified Mandels medium (Mandels and Reese 1957) containing per litre 2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 1 g yeast extract, 0.69 g urea, 0.3 g $CaCl_2{\cdot}2\,H_2O$, 0.3 g $MgSO_4{\cdot}7H_2O$, 1 ml Tween 80 and 1 ml trace elements solution composed of $MnSO_4$ 1.6 g l^{-1}, $ZnSO_4$ 2 g l^{-1}, $CuSO_4$ 0.5 g l^{-1}, $CoSO_4$ 0.5 g l^{-1} supplemented with 1% glucose. After 3 days, the mycelium was used to inoculate cultures in fresh medium of the same composition which contain each selected carbon source.

Batch-culture studies

The ability of this strain to utilize glucose, galactose, xylose, lactose, cellobiose, sucrose, glycerol, Avicel cellulose, CMC, wheat bran, gruel and sugar cane bagasse was examined in modified Mandels medium. Carbon sources were dispensed at 1% in 100 ml medium into 500 ml baffle Erlenmeyer flasks. Mandels medium without carbon source (but still containing 0.1% yeast extract) was used as a negative control. Time course of β-glucosidase production in shake-flask batch cultures was carried out at 30°C under shaking condition (150 rev min^{-1}). Samples were withdrawn at different times during 10 days, centrifuged at 4,580*g* for 20 min and supernatants were assayed for β-glucosidase activity and analysed by zymogram. Each culture condition was carried out in triplicate.

Enzyme assay

β-Glucosidase activity was assayed by monitoring the quantity of the *p*-nitrophenol liberated from the hydrolysis of the *p*-nitrophenyl-ß-D-glucoside (*p*-NP-Glc) at 5 mM concentration under the following experimental assay conditions (pH 5.0, 50°C and 100 mM Sodium acetate buffer). The assay reaction was performed in a volume of 3 ml. One unit of β-glucosidase activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute in the reaction mixture. Every enzymatic value resulted from the average of three repetitions. We should note that enzyme activity was normalised by adding denatured enzyme to the auto zero in order to eliminate any potential interference during spectrophotometer measurements.

Determination of protein concentration

Protein concentration was determined using the method of Bradford with bovine serum albumin as a standard. We determined the protein concentration in order to normalize the amount of protein from different culture preparation used during zymogram analysis.

Enzyme chromatography

The extracellular proteins of fed-batch culture of A19 strain were filtered and centrifuged; the supernatant, which was concentrated with ammonium sulfate precipitation at 80% of saturation, was applied to a MonoQ anion exchange column equilibrated with buffer A (20 mM Tris–HCl, pH 8). The proteins were eluted out with a linear gradient (500 ml) of NaCl from 0 to 1.0 M in buffer A. The active

fractions were pooled, concentrated, dialysed against 20 mM Tris–HCl, pH 8.0, and analysed for β-glucosidase activities.

Protein electrophoresis and zymogram analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Favre 1973) was carried out on 10% separating gel and 5% stacking gel. β-Glucosidase activity was also revealed by zymogram analysis. For this purpose, proteins were mixed with the same loading buffer as in SDS-PAGE, but were not heated before migration on SDS-gel (Laemmli and Favre 1973). After electrophoresis, the gel was incubated for 2 h in buffer (Tris–HCl 20 mM, pH 8.0) to get rid of SDS, allowing the renaturation of proteins. After 15 min of equilibration in 0.1 M sodium acetate buffer pH 5.0, the gel was superposed against an overlay of 1% agar containing 25 μg ml^{-1} of MU-Glc. After a suitable period of incubation, the system was observed under UV light, with excitation at 366 nm and emission at 445 nm. The location of the activity is indicated by the fluorescence emitted by methylumbelliferol released via enzyme action.

Determination of the aminoterminal sequence

The purified enzyme was subjected to N-terminal sequence analysis using an automated protein sequencer (Procise 492 cLc, Applied Biosystem).

RNA isolation and expression levels

Total RNA was extracted from *Stachybotrys microspora* mycelium, grown on glucose culture medium, using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA concentration was determined and 5 μg of total RNA was used for reverse transcription. Synthesis of first strand cDNA was carried out with oligo-dT primer by First Strand cDNA Synthesis Kit (Fermentas, Lithuania) using the M-MuLV reverse transcriptase enzyme (Fermentas, Lithuania), as recommended by the manufacturer. cDNA was subsequently amplified using oligonucleotide primers specific for family 1 and family 3 β-glucosidases and β-actin according to the following temperature profile: 94°C (5 min), followed by 40 cycles of 94°C (30 s), 50°C (30 s), and 72°C (2 min). The reaction was accomplished with an additional extension for 7 min at 72°C. The primer sequences derived from a compilation of fungal β-glucosidases that allowed us to define consensus sequences; for which specific primers were designed, and thus enabled us to isolate four different genes, to clone them and to sequence them (Abdeljalil and Gargouri, in preparation). The consensus sequences were LDHLQRA for the family 1; FMCSYNQ and QSEAKLV for family 3. The corresponding primers were the following: "family 1": 5′-CTGGATCACCTTCAACGAGC-3′ (sense) and Oligo(dT)18 Primer (antisense), "family 3": 5′-TTC ATGTGCTCGTACAACCAG-3′ (sense) and 5′-CAGTCCGA AGCCAAACTCGTA-3′ (antisense) Concerning the β-actin, primers were 5′-AGCGTGGTATCCTCACGCTC-3′ (sense) and 5′-CTTCATGATGGAGTTGAACG-3′. The PCR products were electrophoresed through 1% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Inhibition of β-glucosidase by glucose

Glucose was added to the enzyme activity assay at various final sugar concentrations not exceeding 100 g l^{-1}(at pH 5.0 and 50°C). This assay was carried out as described before and the residual activity was determined.

Hydrolysis of cellobiose into glucose

About 2.5 g l^{-1} of cellobiose solution, prepared in 0.1 M sodium acetate buffer pH 5, was added to enzyme sample, at final volume of 2 ml and incubated at 50°C. Aliquots (20 μl) were taken during various time intervals. The amount of released glucose was determined by the use of GOD kit. The δ-gluconolactone, specific inhibitor of β-glucosidases, was added at a final concentration of 1 mM in order to check if the enzyme is really a β-glucosidase. The identity of released glucose was confirmed by HPLC analysis combined to sugar column (Aminex HPX-87-C).

Results

Differential expression of β-glucosidases from *S. Microspora*

Various carbon sources were tested namely glucose, xylose, galactose, cellobiose, lactose, sucrose, glycerol, sorbitol, CMC, Avicel cellulose, wheat bran, gruel (a by-product of the wheat manufactories) and sugarcane bagasse. Mandels medium without any carbon source was used as control.

All substrates induced β-glucosidase production at different levels (Table 1, Fig. 1a) except glycerol which could be therefore considered as non-inducing or even repressing carbon source (Table 1, Fig. 1d). Glycerol gave the same result as the medium starved for carbon source (Table 1, Fig. 1d). Unexpectedly, the highest activity was observed with glucose (0.66 U ml^{-1}) which is generally regarded as a universal repressor of the majority of hydrolases, followed closely by gruel (0.48 U ml^{-1}) and lactose (0.24 U ml^{-1}). It is interesting to note that gruel gave the highest titre among the by-products, a result that could be explained by its

Table 1 Production level of β-glucosidase activity by *S. microspora* grown on different carbon sources

Carbon source	β-Glucosidase activity (U ml^{-1})
Simple sugars	
Glucose	0.66 ± 0.04
Galactose	0.12 ± 0.03
Xylose	0.08 ± 0.01
Disaccharides	
Lactose	0.24 ± 0.02
Sucrose	0.14 ± 0.01
Cellobiose	0.12 ± 0.005
Complex sugars	
Avicel Cellulose	0.09 ± 0.01
CMC	0.10 ± 0.01
By-products	
Gruel	0.48 ± 0.03
Wheat bran	0.08 ± 0.01
Sugar cane bagasse	0.14 ± 0.01
Sugar-alcohols	
Sorbitol	0.08 ± 0.01
Glycerol	0.00 ± 0.00
Biological control	
Carbon starved	0.02 ± 0.01

The different carbon sources were used at the concentration of 1% in the culture medium. Each value was determined in triplicate, standard deviations (SD) were therefore given

CMC carboxymethylcellulose

richness in starch, in comparison with wheat bran and bagasse. This starch would be converted by amylases into small oligosaccharides and glucose which can therefore induce the β-glucosidase system (Table 1). Figure 1b, which shows the biomass production in different culture conditions, proves that *Stachybotrys* strain grows similarly in the presence of the various carbon sources, so there was no preference for one source over another.

The zymogram analysis showed a differential expression pattern of β-glucosidases depending on the carbon source (Fig. 1c, d). Indeed, one single and major β-glucosidase was detected: the same one, named bglG, on glucose and on cellobiose; another one was induced by lactose while Avicel cellulose and CMC produced roughly all β-glucosidases with the bglG as the lowest one (Figs. 1c, 2d).

The monoQ anion exchange separation of enzymes, produced on cellulose, led to the identification of many different β-glucosidases (see the fractions F1, F2 and F3 on Fig. 2a, b) whereas, on glucose, only the fraction G was identified and corresponded to the largest one seen on the zymogram of Fig. 2c, d.

This "glucose induced" β-glucosidase, bglG, was purified and subjected to NH_2-terminal sequencing. The determined sequence NH2-YYMFVMPEE did not show any homology with the available known sequences.

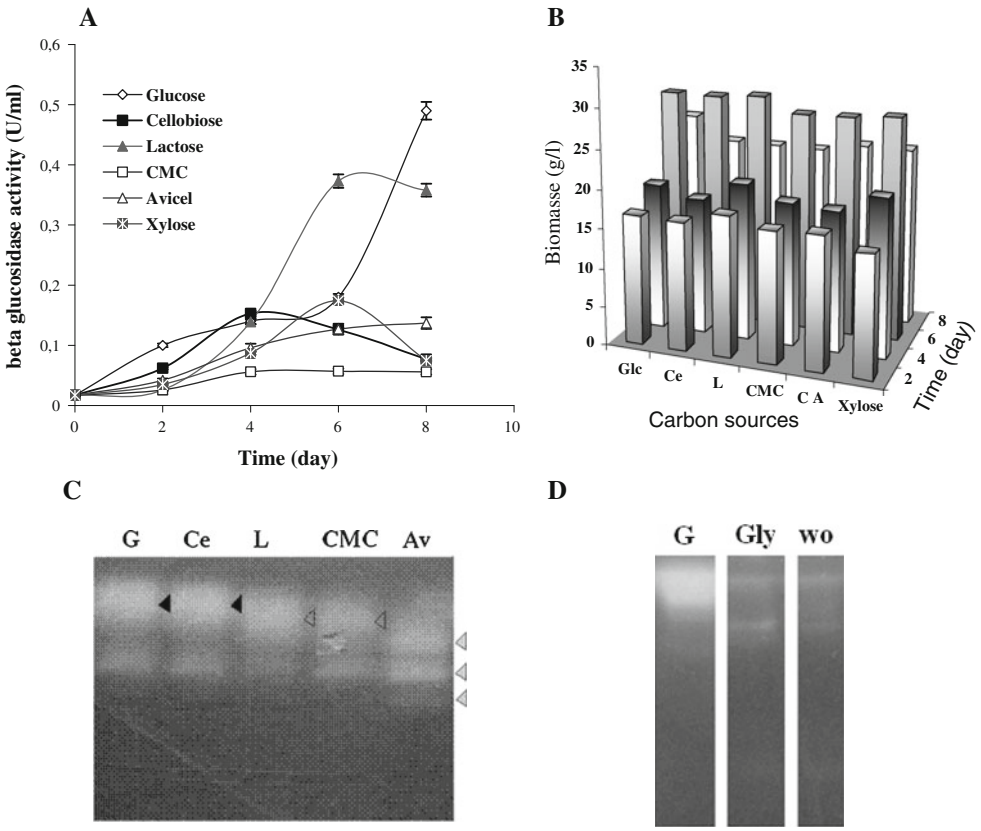

Fig. 1 Effect of various carbon sources on the β-glucosidases production by *S. microspora*. **a** Time course of β-glucosidase activity using various carbon sources. Glucose, cellobiose, lactose, CMC, Avicel cellulose, and xylose were used individually, at the concentration of 1% in the culture medium. Samples were withdrawn every two days during 8 days. **b** Biomass evolution of the same cultures in **a**. **c**, **d** Zymogram showing the differential expression of different β-glucosidase of *S. microspora* at 5 days of growth on various carbon sources at 1% (w/v); with *G* glucose, *Ce* cellobiose, *L* Lactose, *CMC* carboxymethylcellulose. *Av* Avicel cellulose, *Gly* glycerol) and *wo* no carbon source added

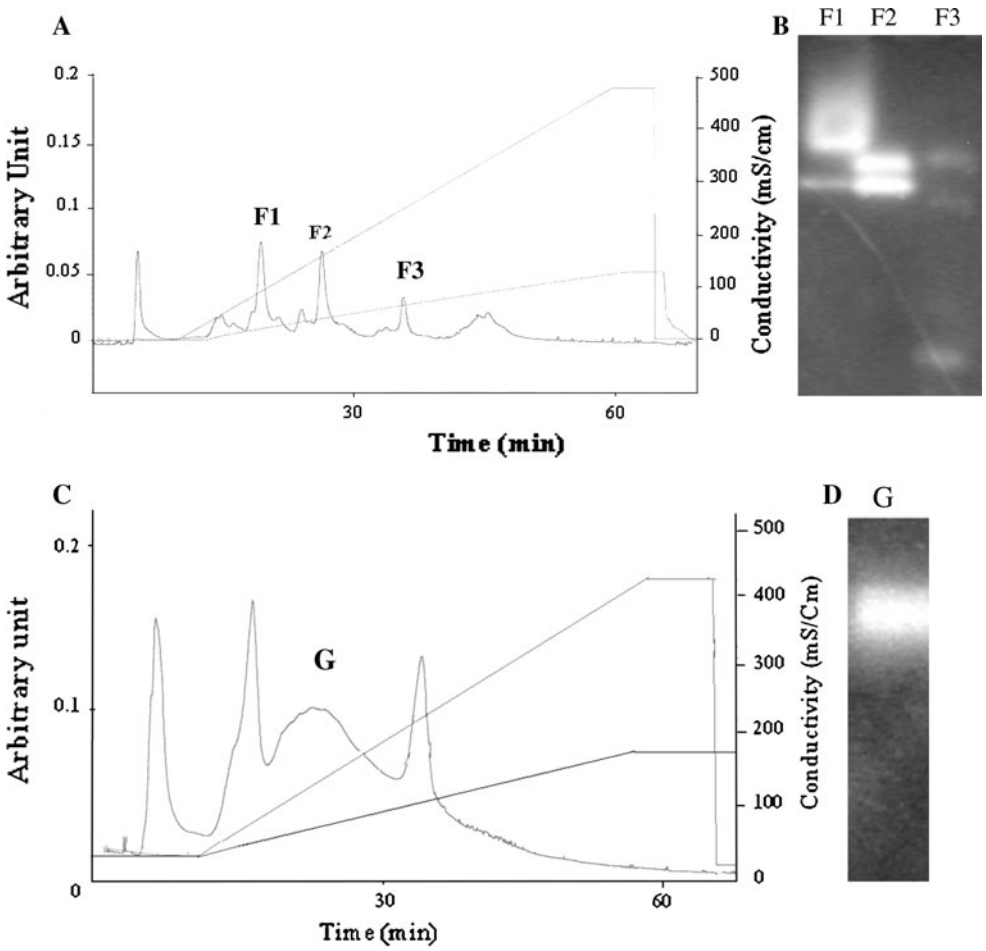

Fig. 2 Separation of β-glucosidase through chromatography and electrophoresis. **a**, **c** Chromatographic profiles (from monoQ anionic exchange FPLC column, equilibrated in Tris–HCl 20 mM pH8 and eluted with a 0–1 M NaCl gradient) of supernatant of *S. microspora,* cultivated for 5 days on Avicel cellulose and glucose respectively. **b**, **d** Zymograms of the indicated fractions resulting from chromatographic analyses

Dual effect of glucose on β-glucosidase production and activity in *S. microspora*

Although glucose is considered as a repressor of the majority of cellulolytic activities in most known microorganisms, it appears to behave here, at 1% concentration, as a strong inducer of β-glucosidase. Therefore, we studied the effect of glucose concentration in the culture medium on β-glucosidase production. Time course of β-glucosidase production was studied during 10 days on 5 glucose concentrations in Mandels medium (0.5, 1, 2, 3 and 4%). Figure 3a shows that 1% glucose was the best inducing concentration; whereas at higher glucose concentrations, a decrease of β-glucosidase production was observed. This result was enhanced by zymogram analysis (Fig. 3b) that shows the gradual decrease of the major β-glucosidase activity with the increase of glucose in the culture medium.

All these results impelled us to look for the effect of glucose on the enzyme activity. Therefore, β-glucosidase activity was determined using various glucose concentrations ranging from 0 to 100 g l^{-1}. Figure 3c shows clearly that glucose, at a concentration higher than 1%, acts as a strong inhibitor of bglG.

RT-PCR results corroborates the zymogram analysis

According to the molecular weight of the "glucose induced" enzyme as determined on SDS-PAGE, we assumed that such activity could be encoded by a gene belonging to family 3 β-glucosidase, since such genes are generally much longer than those encoding family 1 β-glucosidases. To follow the same path, RT-PCR experiments (Fig. 4) were performed on RNA extracted from *S. microspora* grown on 1% glucose used as sole carbon source. Specific primers were designed to isolate different β-glucosidases genes by PCR, RT-PCR and Nested-PCR (data not shown). The analysis of RT-PCR revealed a high level of expression of family 3 β-glucosidases. On the contrary, no amplification was detected for family 1 β-glucosidase genes which seem to be repressed in the same culture condition. Hence, no amplification product

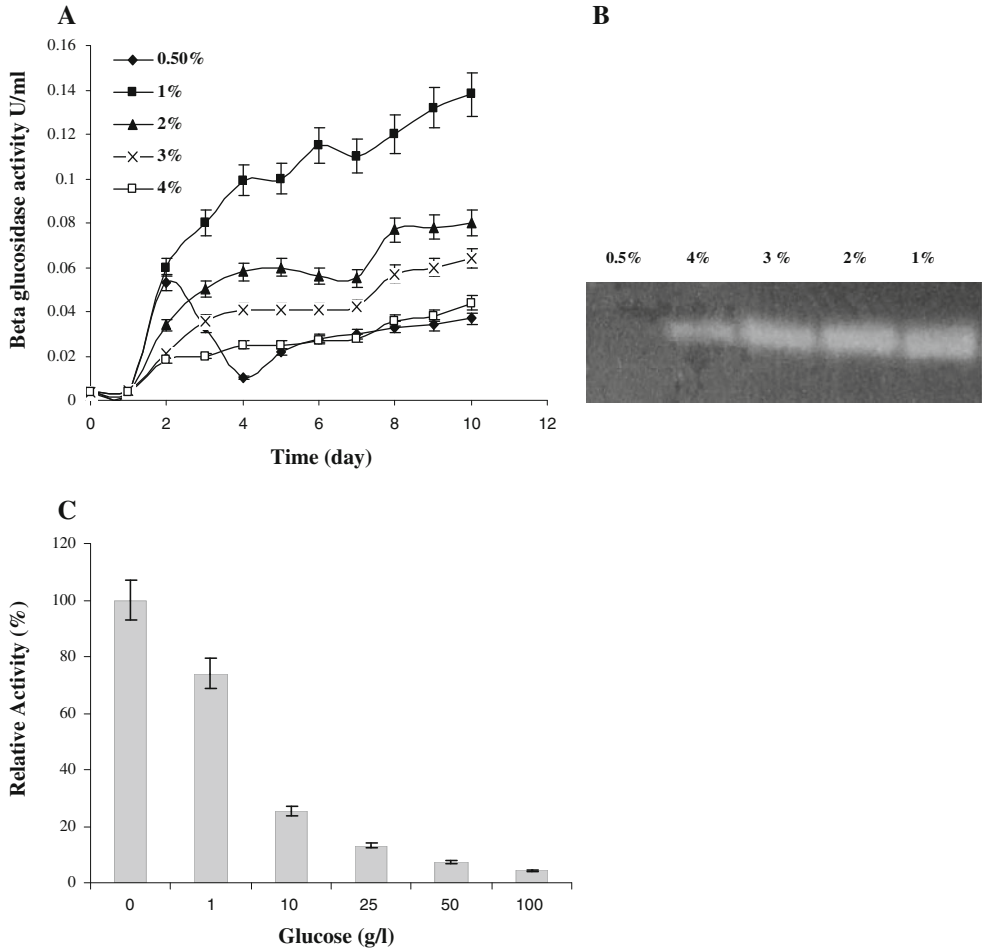

Fig. 3 Effect of glucose on the bglG production and activity. **a** Time course production of β-glucosidase activity using glucose as a sole carbon source at different concentrations: 0.5, 1, 2, 3 and 4% (w/v). **b** Corresponding Zymogram analysis. **c** Effect of glucose concentration on the purified bglG activity

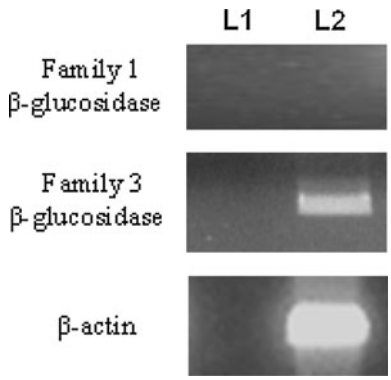

Fig. 4 RT–PCR analysis of family 1 and family 3 β-glucosidase mRNAs. *Lane1* Control reaction, performed using non-reverse transcribed RNA as a template in order to check for genomic DNA contamination; *Lane 2* PCR amplification of cDNA using specific primers of family 1 and family 3 β-glucosidases genes. The actin transcript was used as an internal control. RT-PCR products were analysed by electrophoresis on 1% agarose gel

was detected in negative control reactions. These results corroborate the biochemical studies that suggested a differential expression of β-glucosidases.

The production of *S.microspora* β-glucosidases is substrate specific

Zymogram analysis showed clearly that on glucose, a large β-glucosidase (the largest one with the coronet electrophoretic mobility) was seen on the top of the gel, named bglG. This enzyme exhibited a strong activity, since a few minutes of overlay application were sufficient to reveal the fluorescence under UV (Figs. 1c, 2d). On lactose, another one named bglL (Fig. 1c) was revealed.

In order to study the expression and regulation of bglG and bglL, the production of each enzyme was assessed on a culture liquid medium in the presence of both carbon sources at proportion of 1/3, 1/1 and 3/1 of glucose and lactose respectively (1/3, 1/1 and 3/1 ratios corresponding to 0.25/0.75 g, 0.5/0.5 g and 0.75/0.25 g of glucose and lactose successively). Figure 5 shows the co-production of two β-glucosidases when glucose and lactose were used simultaneously in the medium culture, but the expression level was ratio dependent. Indeed, at the ratio of 1/3 (glucose/lactose) the bglL seems to be strongly produced. At the 3/1 ratio, the reciprocal state was obtained and at the 1/1 ratio, we observed a strong co-production of both

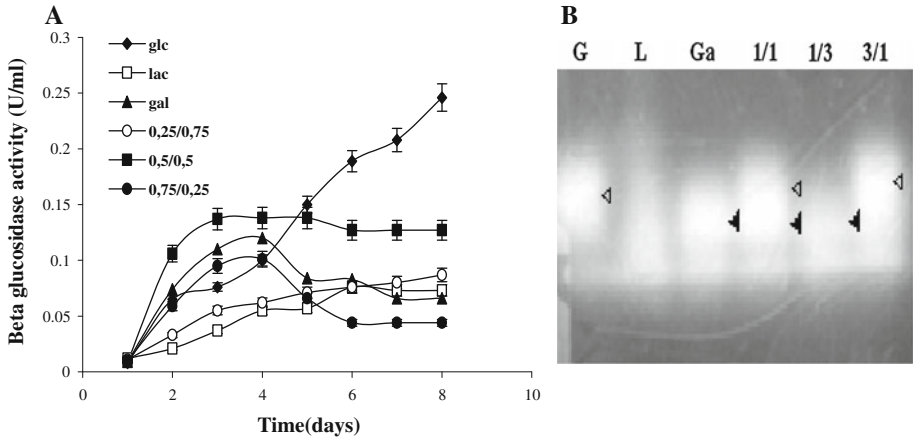

Fig. 5 The carbon source determines the nature of the β-glucosidase produced. **a** Time course of β-glucosidase production and co-production from 1% of glucose, lactose, galactose or glucose/lactose cultures; the various ratios of glucose/lactose are indicated. 1/3, 1/1 and 3/1 are equivalent to 0.25/0.75 g, 0.5/0.5 g and 0.75/0.25 g of glucose lactose combinations. **b** Corresponding zymogram with *G* glucose, *L* lactose, *Ga* galactose, 1/1: glucose/lactose: 1/1, 1/3: glucose/lactose: 1/3 and 3/1: glucose/lactose: 3/1

enzymes (bglG and bglL). Figure 5a suggests also that the β-glucosidase activity appeared much earlier and more efficiently in the presence of galactose (at 1%) than that of lactose. Figure 5b shows that this activity rather corresponds to bglL, already noted on lactose (Fig. 5b). Samples analysed in Fig. 5 are taken at the fifth day of culture. The days before, only a single β-glucosidase was observed in the presence of galactose, which migrates on SDS-PAGE at the same level. These results suggest that it could correspond to the unique bglL.

A similar result was obtained in the presence of glucose and CMC (data not shown). Indeed, in the presence of CMC, our strain expressed all of the β-glucosidases except bglG. The addition of glucose to CMC stimulated the expression of all β-glucosidases including the bglG enzyme.

BglL is a β-glucosidase with cellobiase capacity and not a β-galactosidase

When grown on lactose as unique carbon source, *Stachybotrys microspora* secreted a major pNPGase activity, named bglL (Fig. 1a). In order to verify that bglL is a β-glucosidase and not a β-galactosidase or another monosaccharidase, we tested its capacity to hydrolyse cellobiose into glucose units. Figure 6a shows such behaviour in the extracellular enzymes produced by A19 on lactose, enabling us to conclude that bglL is a true cellobiase with a glucose recovery of 25% after 26 h of reaction. The conversion of cellobiose into glucose was followed by HPLC analysis (using simple sugar column Aminex-87-C): Fig. 6b, c show the chromatogram at time zero and after 26 h of the conversion reaction. Moreover, when 1 mM of δ-gluconolactone, a potent inhibitor of β-glucosidases, was added at the middle of the reaction, it abolished strongly and immediately the conversion of cellobiose into glucose. All these findings prove that bglL is a β-glucosidase and not a β-galactosidase or any other monosaccharidase.

Discussion

Fungal strains producing multiple β-glucosidases have rarely been reported. *Stachybotrys microspora* escaped this rule and allowed us to ask several questions about the causes of such diversity. The current work was thus undertaken to study the regulation of β-glucosidases production in *S. microspora* and to show that they i.e. β-glucosidases are differentially induced using various carbon sources. A major result concerning glucose, generally admitted as a universal repressor of the majority of hydrolases, which is shown here as the best inducer of the major and strong β-glucosidase activity, named bglG. We have also shown that the carbohydrate polymers (CMC and Avicel cellulose) induced the expression of a high number of β-glucosidases while simple sugars (mono and disaccharides such as glucose, galactose, cellobiose and lactose) induced the expression of a single β-glucosidase, each time different. For instance, both lactose and galactose induced the same bglL enzyme.

Moreover, when we used cellobiose as sole carbon source, *Stachybotrys microspora* produced the same bglG enzyme with an optimum at the fourth day, after which the production decreased, whereas on glucose, we never observed such a drop. This result is similar to the one described by Makoto et al. (2004), where oxidative enzymes seem to be important for cellulose degradation, such as the cellobiose dehydrogenase (CDH) which has been isolated from cellulose-degrading culture of *Phanerochaete*

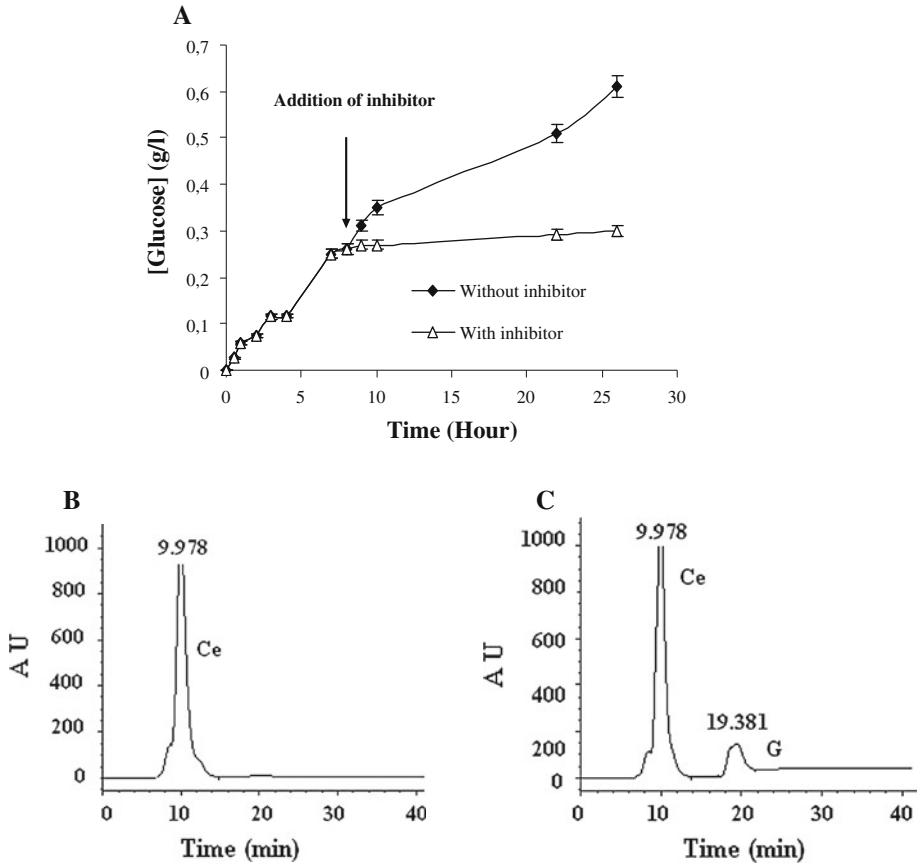

Fig. 6 Hydrolysis of cellobiose into glucose by extracellular proteins of A19 when grown on lactose (producing exclusively bglL as β-glucosidase). **a** The conversion kinetics of cellobiose into glucose by bglL. The addition of δ-gluconolactone (1 mM), specific inhibitor of the β-glucosidases, in the time course of the reaction, annihilated the hydrolysis capacity of bglL. **b**, **c** HPLC chromatography profiles at time T = 0 h and T = 26 h of hydrolysis reaction, with *Ce* cellobiose and *G* glucose

chrysosporium (Ayers et al. 1978; Bao et al. 1993). Since this fungus secretes one extracellular β-glucosidase and one CDH, it has been suggested that cellobiose is firstly oxidized by CDH to cellobionolactone which was further hydrolysed to glucose and δ-gluconolactone by extracellular bgl (Eriksson 1978), recalling that the δ-gluconolactone is a strong inhibitor of β-glucosidase. Similarly, we proved the presence of CDH during the growth on cellobiose but not on glucose-based medium (data not shown). This finding could explain the drop in the enzyme production when cellobiose was used.

Using lactose and galactose, our fungal strain produced one major β-glucosidase bglL different from bglG. We can hypothesize that this enzyme is not really induced by lactose but rather by its hydrolysis product, which is galactose. Concordant with this hypothesis, the bglL activity appeared much earlier and more efficiently in presence of galactose than that of lactose (Fig. 5a, b). Probably, the lag would give enough time to the fungus to cleave lactose into glucose and galactose which would separately and respectively induce the bglG and bglL synthesis. This raised the following question: why, when lactose was cleaved into glucose and galactose, did we detect by zymogram only the bglL and not the bglG? It is plausible to guess that glucose, resulting from lactose hydrolysis, was present at a concentration lower that the optimal inducing concentration of 1%. Moreover, the glucose would be more efficiently converted into biomass than galactose. Thus, we can consider galactose as the effective inducer of the same enzyme, bglL, observed on lactose.

According to the results given from Fig. 6 analysis, we show that bglL is a β-glucosidase and not a β-galactosidase or another monosaccharidase. Hence, *Stachybotrys microspora* secretes several β-glucosidases, four of which have been characterized (Amouri and Gargouri 2006; Saibi et al. 2007; Saibi and Gargouri 2010, submitted for publication) and bglL (listed in this work).

The multiplicity of β-glucosidases is rarely reported among fungi, as they have usually one or two genes. Mammals have five β-glucosidases, yet their purification and biochemical characterization showed that they are different and are not deriving from translational maturation or alternative splicing, excepted two acidic β-glucosidases that derived from alternative splicing from a single gene (Premkumar et al. 2005).

To go further in our investigations on the multiplicity of β-glucosidases in *S. microspora*, we can claim that this multiplicity results from the existence of several corresponding genes, but it could also result from proteolysis or aggregates or different glycosylated forms of the same

enzyme or it can be multiple products of a single gene. We cannot exclude such a hypothesis, but at the same time we think that some results are concordant with a certain multiplicity of β-glucosidases in our strain:

(a) The purification of three β-glucosidases which are different at their biochemical properties (Amouri and Gargouri 2006; Saibi et al. 2007 and this work,) as well as their NH_2-terminal sequence since the first one (M9) "YDGENVRIGGRGSFVPGISFHVPTGVNLAY" (Amouri and Gargouri 2006) was clearly different from that of bglG, "YYMFVMPEE", determined in this work.
(b) In the same context, we have isolated until now four different β-glucosidase genes (AbdelJalil and Gargouri, in preparation). So, at least four different enzymes do really exist and do not simply derive from proteolysis, aggregation or different glycosylation.
(c) Generally, proteolysis occurs at the end of culture, due to the release of endogenous proteases. In our case, we observed the same variability of β-glucosidases at the first days as well as the last days of the culture (data not shown).

Moreover, several combinations of two different carbon sources at various ratios (glucose/lactose and Glucose/CMC) in the cultures showed the co-production of each enzyme separately and suggested that these different enzymes are expressed disjointly.

Taken concurrently, these results suggest that *S. microspora* produces at least three classes of β-glucosidases which are differently regulated: the bglG induced exclusively by glucose or cellobiose, the bglL induced by galactose or lactose and the remaining β-glucosidases which are mainly induced by polysaccharides such as CMC or Avicel cellulose.

According to its large molecular weight, the BglG enzyme was assumed to belong to family 3 of β-glucosidases. Since we have not yet isolated the corresponding gene, we planned to follow its transcription levels by an indirect procedure. Therefore, we have designed primers corresponding to family 3 consensus parts and performed the RT-PCR analysis, shown in Fig. 4. Such analysis showed that family 3 expressions were induced on glucose while those of family 1 were repressed.

According to all these results, we consider that the carbon source directs the differential expression of β-glucosidases in *S. microspora*. Carbon sources affect the transcription of different β-glucosidases via its direct or indirect interaction with various transcription factors. It is already known, in *Trichoderma* for instance, that cellulase genes are regulated by either activators such as ACEII or repressors such as ACEI, CRE1 and others (Aro et al. 2003). However, the molecular mechanisms by which the fungus senses the composition of the extracellular milieu and modulates the expression of these enzymes are unknown.

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