

## Induction of cellulase and hemicellulase activities of *Thermoascus aurantiacus* by xylan hydrolyzed products

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**Abstract** *Thermoascus aurantiacus* is able to secrete most of the hemicellulolytic and cellulolytic enzymes. To establish the xylanase inducers of *T. aurantiacus*, the mycelia were first grown on glucose up until the end of the exponential growth phase, followed by washing and re-suspension in a basal medium without a carbon source. Pre-weighed amounts of xylose (final concentration of 3.5 mg/ml), xylobiose (7 mg/ml) and hydrolyzed xylan from sugarcane bagasse (HXSB) which contained xylose, xylobiose and xylotriose (6.8 mg/ml) were evaluated as inducers of xylanase. It was observed that xylose did not suppress enzyme induction of *T. aurantiacus* when used in low concentrations, regardless of whether it was inoculated with xylobiose. Xylobiose promoted fast enzyme production stopping after 10 h, even at a low consumption rate of the carbon source; therefore xylobiose appears to be the natural inducer of xylanase. In HXSB only a negligible xylanase activity was determined. Xylose present in HXSB was consumed within the first 10 h while xylobiose was partially hydrolyzed at a slow rate. The profile of  $\alpha$ -arabinofuranosidase induction was very similar in media induced with xylobiose or HXSB, but induction with xylose showed some positive effects as well. The production profile for the xylanase was accompanied by low levels of cellulolytic activity. In comparison, growth in

HXSB resulted in different profiles of both xylanase and cellulase production, excluding the possibility of xylanase acting as endoglucanases.

**Keywords** Xylanase · Cellulase · *Thermoascus aurantiacus* · Induction · Xylobiose

### Introduction

The use of thermophilic enzymes in biotechnological processes began three decades ago (Sonnleitner and Fiechter 1983). Some of these enzymes are commercially available such as hemicellulases (Enzymatic Deinking Technology, LCC). Besides high thermostability, they have a longer useful life, of several hours at 70°C or a number of days at 60°C (Alam et al. 1994). Cellulases, xylanases and hemicellulose-debranching enzymes (Parry et al. 2002; Gomes et al. 2000; Roche et al. 1994; Khandke et al. 1989; Kalogeris et al. 2001, 1998; Maslen et al. 2007; Vardakou et al. 2003) were intensively studied. These later enzymes, also known as accessory enzymes, have many applications such as in the modification of hemicellulose, which can be used for hydrogel production (Gabrieli et al. 2000), or for addition with cellulosic pulp to improve the quality of paper (Lima et al. 2003).

Cellulases and hemicellulases are of industrial interest in supplementation of animal feed and for the manufacturing of food, drinks and bread (Maat et al. 1992; Bedford and Classen 1992). There are also further potential applications of these enzymes in the textile industry, for the bleaching of cellulose pulp and for second generation ethanol production (Kuhad and Singh 1993). In these applications the price of the enzymes must be low to make their use economical. For this reason, research has turned to study inducers and low

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cost materials as substrates for specific enzyme production (Leite et al. 2008). Components of lignocellulosic materials have emerged as a source of low cost substrates for growing several fungi with respect to enzyme production (Dobrev et al. 2007; Couri et al. 2000; Kang et al. 2004; Kovács et al. 2009; Juhász et al. 2005).

*Thermoascus aurantiacus* is a prominent microorganism for thermostable enzyme production (Leite et al. 2008; Chang and Holtzapfle 2000; Mansfield et al. 1999). Extracellular xylanase synthesis by *T. aurantiacus* is largely inducible. Compared to growth on wood xylans, fungi produce enzymes in larger amounts than on other carbon sources (Milagres et al. 2004). Since xylan is a large polymer, which cannot penetrate the mycelia, it must be anticipated that xylanase is induced by fragments of xylan. The studies on regulation of xylanases in fungi are complicated by the fact that in most species production of xylanases is accompanied by production of cellulolytic enzymes independent of the carbon source used as the growth support (Biely et al. 1980). Numerous xylanases and cellulases of fungi, including *T. aurantiacus* have been shown to possess certain substrate cross-specificity once xylanases have cellulose binding domains with little affinity to recognize xylan or cellulose (Da Silva et al. 2005). A description of catalytic properties of *T. aurantiacus* enzymes, their stability and xylanase families were recently revised (Brienzo et al. 2009a).

Endoglucanase from *T. aurantiacus* is more stable than endoglucanases produced by other fungi (Parry et al. 2002). Significant reduction in the cost of cellulases production by means of cheaper substrates and increased volumetric productivity will be important for their applications. Lignocellulosic substrates are used for production of cellulolytic enzymes under submerged cultivation or solid-state fermentation. However, fungal growth on insoluble substrates has been connected with a delayed enzyme production that lowers productivity (Kang et al. 2004; Juhász et al. 2005; Gomes et al. 2000). The search for compounds related to the structure of lignocellulosic materials responsible for cellulase and hemicellulase induction remains a challenge. In the present study xylan hydrolyzate containing small-molecular-weight carbohydrates; xylose, xylobiose and hydrolyzed xylan from sugarcane bagasse (HXSB); were used to grow *T. aurantiacus* in an attempt to find potential inducers of cellulases and hemicellulases.

## Materials and methods

### Microorganism

A strain of *T. aurantiacus* ATCC 204492, an isolate of eucalyptus chips (Auer et al. 1988), was grown on potato

dextrose agar (PDA) at 45°C for 4–5 days and stored at 4°C. Before experiments the cultures were transferred onto the same fresh medium in the incubator at the same condition.

### Cultivation

Mycelial biomass was grown on 25 ml of basal medium in 125 ml Erlenmeyer flasks. The basal medium was comprised of Vogel's salt (Vogel 1956), which was supplied with 1% of glucose for growth. The medium containing basal medium and glucose was inoculated with 4–5 day old spores at a concentration of  $1 \times 10^4$  spores/ml. The cultivation was carried out at static conditions for 72 h at 45°C.

### Induction experiments

Mycelia grown in the basal medium supplied by glucose were washed with 100 ml of cold distilled water and suspended on 25 ml of basal medium (Vogel's salt medium without glucose) and supplied with 20 mM of xylose (final concentration of 3.5 mg/ml), xylobiose (7 mg/ml) or hydrolyzed xylan from sugarcane bagasse (HXSB) (6.8 mg/ml). The mixtures were incubated in a rotary shaker at 45°C and 120 rpm. After 6, 10, 24 and 30 h samples were taken, centrifuged at 10,000xg for 15 min and the supernatant was then used to determine enzyme activity and substrate consumption. A control assay was run in parallel at the same conditions without a carbon source.

### Growth experiments

*Thermoascus aurantiacus* was cultivated on xylose, cellobiose, sugarcane bagasse xylan (XSB), birchwood xylan, oat spelt xylan, carboxymethylcellulose (CMC), avicel and hydrolyzed xylan from sugarcane bagasse (HXSB). The media (50 ml) was prepared with Vogel's salts, at a pH of 5.8 containing 1% of the applicable carbon source. The 250 ml Erlenmeyer flasks were autoclaved for 15 min at 121°C, followed by inoculation with  $2 \times 10^5$  spores/ml. The flasks were agitated for 96 h at 120 rpm and 45°C. Samples were taken every 12 h and centrifuged at 10,000xg for 15 min followed by determination of enzymatic activities. A control assay was run in parallel at the same conditions without the carbon source.

### Enzymes assays

Xylanase activity was assayed on birchwood xylan by liberation of reducing saccharides (Miller 1959). One unit (U) of xylanase is defined as the amount of enzyme required to liberate 1  $\mu$ mol of xylose from xylan during

1 min at the standard conditions. Endoglucanase assay was performed at the same conditions as xylanase using a solution of carboxymethylcellulose (0.44%) and glucose as a standard (Tanaka et al. 1981).  $\beta$ -xylosidase was performed with p-nitrophenyl- $\beta$ -xyloside (Tan et al. 1987). The assays of  $\beta$ -glucosidase and  $\alpha$ -arabinofuranosidase were performed at the same conditions using p-nitrophenyl- $\beta$ -glucoside and p-nitrophenyl-arabinofuranoside as substrates, respectively. The enzyme activity was expressed as  $\mu\text{mol}$  of p-nitrophenol formed per milliliter per minute of enzyme solution (U/ml).

### Production of HXSB

The xylan of sugarcane bagasse was extracted with hydrogen peroxide in alkaline media according to optimized condition in previous work (Brienzo et al. 2009a, b). The xylan (2%, w/v) was hydrolyzed by an enzymatic extract of *T. aurantiacus* (60 UI/g xylan), at a pH 5, a temperature of 50°C and with an agitation of 120 rpm. After reaching the reaction time of 96 h, the pH of the medium was adjusted to 6.5 and the tubes were heated in boiling water for 5 min. Upon completion, the samples were centrifuged at 10,000 $\times$ g for 15 min and the reaction products were further analyzed by HPLC to determine the degree of polymerization (DP) of the xylooligosaccharides.

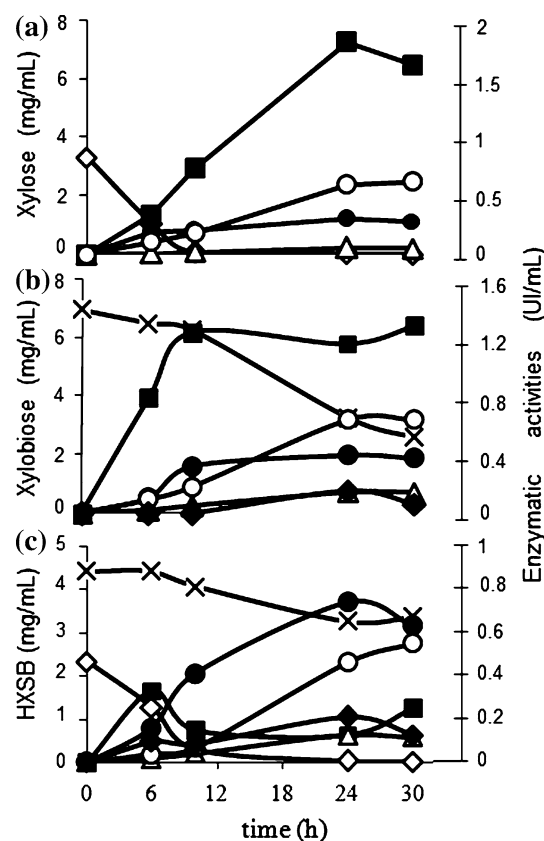
### Determination of substrate consumption

Xylose, xylooligosaccharides (XOs) and cellobiose were monitored by liquid chromatography with a refractive index detector and a BIO-RAD Aminex HPX-42A column (300  $\times$  7.8 mm). Before injection, samples were filtered through a Sep Pak C18 filter. Aliquots of filtered sample (20  $\mu\text{l}$ ) were injected onto the HPLC system. The components of the medium such as xylooligosaccharides, xylose and cellobiose were eluted using distilled/deionized water as the mobile phase. The concentration of xylooligosaccharides were quantified using standard xylose (Sigma) and xylobiose (X2), xylotriose (X3), xyloetraose (X4) and xylopentaose (X5) (Megazyme-Irlanda).

## Results and discussion

### Hemicellulases and cellulases inducers

Cellulases and hemicellulases are produced at different levels in the same induction medium by *T. aurantiacus* (Fig. 1). It was hypothesized in this work that compounds related to the hemicellulose structure can be responsible for hemicellulase and cellulase production. To check whether xylan derivatives act as inducers, the washed-mycelia



**Fig. 1** Time course of enzyme production by *T. aurantiacus* in washed glucose-grown mycelia transferred to basal medium supplied with low concentrations: xylose (open diamond), xylobiose (multiplication symbol) and hydrolyzed xylan from sugarcane bagasse (HXSB). Enzyme activities determined were: xylanase (filled square), arabinofuranosidase (open triangle), endoglucanase (closed circle),  $\beta$ -glucosidase (open circle) and  $\beta$ -xylosidase (closed triangle). Standard deviation of triplicate assays less than 5%

were incubated in a basal medium supplied with a low concentration of xylose, xylobiose or hydrolyzed xylan from sugarcane bagasse (HXSB). The control test was conducted in the absence of inducers. No enzymatic activity and no growth of mycelia growth was detected in the control, confirming that the enzymatic activities in the cultures were really induced by the saccharides.

Enzymes activities related to cellulose and hemicellulose degradation were monitored at various time intervals (Fig. 1). Xylanase was detected in all media, but showed a higher activity in media containing xylose and xylobiose compared to media containing hydrolyzed xylan from sugarcane bagasse (HXSB), which is composed of a mixture of xylose, xylobiose and xylooligosaccharides. The production of xylanase peaked at 24 h in media containing xylose, even after the exhaustion of xylose, which occurred after 10 h of incubation (Fig. 1a). Although reports concerning the inducing effect of xylose have been somewhat controversial, the common notion is that xylose at low

concentrations acts as an inducer and at higher concentrations as a repressing carbon source (Aro et al. 2005). It was demonstrated that genes encoded for the xylanolytic enzymes in fungal species are induced by xylose (Purkathofer and Steiner 1995). With respect to *T. aurantiacus*, Gomes et al. (1994) observed that readily metabolized substrates were ineffective as inducers of xylanase. Furthermore in *A. niger*, xylose induced expression of several other genes in addition to those encoding xylanases, including genes related to the side chain cleaving enzymes,  $\alpha$ -glucuronidase, acetylxylan esterase and feruloyl esterase (faeA) (Aro et al. 2005). These enzymes could be induced by the chemical or mechanical release of specific oligosaccharides from xylan, or by some other unknown sensing mechanism (Chavez and Bull 2006).

Induction of xylanase with xylobiose was initiated in a short period of time and increased up to the first 10 h, even at low consumption of the carbon source. After this time the endoxylanase activity remained constant suggesting that low amounts of xylobiose can induce endoxylanase activity (Fig. 1b).  $\beta$ -xylosidase can both cleave xylobiose into xylose and perform the reverse reaction through a transglycosylation step and make the real inducer, which would lead to enzyme production (Gomes et al. 2000). The  $\beta$ -xylosidase of *T. aurantiacus* has transxylosidase activity, as xylotriose and xylotetraose were produced in low content from xylobiose (Matsuo et al. 1998).

Only negligible xylanase activity was determined in the media supplied with HXSB, showing a peak at 6 h of cultivation. Medium supplemented with HXSB produced mainly endoglucanase and  $\beta$ -glucosidase. The xylose content of HXSB was totally consumed after 18 h, while xylobiose was partially consumed at a slower rate than in the media with only this carbon source. At the end of the experiment xylobiose was the only remaining sugar, and only 24% had been consumed (Fig. 1c). Comparatively, in the assay with xylobiose as the only carbon source its consumption was 62%. A simultaneous consumption of xylose and xylobiose was observed in the HXSB media.  $\beta$ -xylosidase produced in HXSB can cleave xylobiose into xylose, which could cause repression depending on the balance of hydrolysis and uptake, however this effect was not apparent.

The profile of endoglucanase activity was coincident with the xylanase in xylose and xylobiose media; however the enzymes produced were four times lower. On the other hand, the levels of endoglucanase and  $\beta$ -glucosidase were the same as those produced in xylobiose, suggesting a constitutive expression. Endoglucanase activity has been detected in cultures of *T. aurantiacus* where HXSB was supplied, suggesting that an induction mechanism is involved in the presence of oligosaccharides. It is possible that a compound derived from carbohydrates released from sugarcane bagasse has provoked an inductive intracellular signal.

#### Effect of carbon source on enzyme production

*Thermoascus aurantiacus* was grown on media containing various carbon sources. After 4 days of growth the activities of cellulases and hemicellulases were determined (Table 1). The highest level of xylanase (70 U/ml) was obtained in the medium containing xylan from sugarcane bagasse (XSB). In other xylan sources, the activity was smaller by about five orders of magnitude. Low basal activity of xylanase was detected on carboxymethylcellulose and avicel medium. The ability of xylanase production was observed in other microorganisms growing in the cellulosic substrate. Carboxymethyl cellulose or xylan can induce xylanases production by *P. funiculosum* (Rao et al. 1988). Raw material can also induce this microorganism. High production was obtained when sugar cane bagasse, rice straw and wheat straw were used (Fadel and Fouda 1993).

The highest xylanase activity which was detected in medium containing sugarcane bagasse xylan can be attributed to its high solubility. The chemical composition of xylan from bagasse may have influenced the xylanase production. The content of xylose (76%), lignin (5.9%), arabinose (4.6%), glucose (4.9%) and uronic acid (5.7%) was quite different from oat spelt xylan and birchwood xylan (Brienzo et al. 2009b).

The  $\alpha$ -arabinofuranosidase activity was detected in growth media provided by hemicellulose resources, revealing that the production of this enzyme is coupled with the expression of hemicellulolytic system. The  $\alpha$ -arabinofuranosidase activity

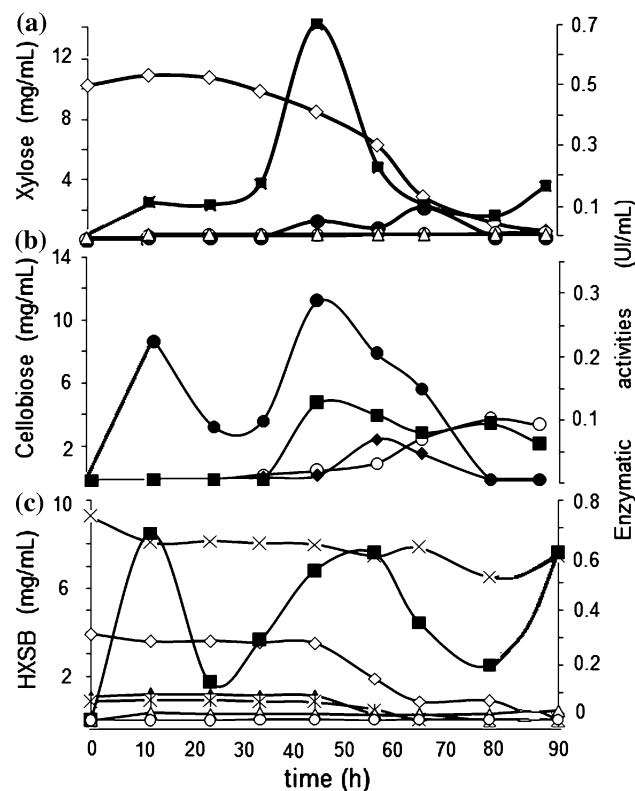
**Table 1** Enzymatic activities (U/ml) in the filtrates after 4 days growth of *T. aurantiacus* on various carbon sources

Carbon source	Xylanase	$\beta$ -xylosidase	Arabinofuranosidase	Endoglucanase	$\beta$ -glucosidase
Sugarcane bagasse xylan	76.7 $\pm$ 1.9	0	0.04 $\pm$ 0.01	0	0.23 $\pm$ 0.02
Birchwood xylan	9 $\pm$ 1.5	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	1.6 $\pm$ 0.2	0.12 $\pm$ 0.02
Oat spelt xylan	16 $\pm$ 1.3	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	2.42 $\pm$ 0.2	0
Carboxymethyl cellulose	0.5 $\pm$ 0.1	0	0	0	0.24 $\pm$ 0.02
Avicel	1.0 $\pm$ 0.1	0	0	0	0.02 $\pm$ 0.02

was performed on artificial substrate 4-NPh-  $\alpha$ -L-Araf and some  $\alpha$ -L-arabinofuranosidases, e.g. those of family 43, show a very low specific activity on this substrate.

The total of xylanase production was much lower in the presence of soluble carbohydrates (xylose, cellobiose and HXSB) than in the medium with xylan. Soluble carbohydrates are easily consumed and do not require elevated enzyme production at growth condition. The growth of *Thermoascus aurantiacus* in xylose produced mainly xylanase and endoglucanase, and lower activities of  $\beta$ -glucosidase and  $\alpha$ -arabinofuranosidase (Fig. 2a). The peak of endoxylanase production occurred at 46 h, whilst the consumption of xylose starts diminishing.

Cellobiose was totally consumed up to 90 h, although soon after 20 h of incubation a regular level of glucose was released. A peak of xylanase coincident with that of the endoglucanase appeared at approximately 45 h of fungal growth (Fig. 2b). Cellobiose induced endoglucanase activity superior to xylanase, confirming the inductive effect of cellobiose on the production of cellulolytic enzymes by *T. aurantiacus*.



**Fig. 2** Time course of enzyme production by *T. aurantiacus* growth in 1% of xylose, cellobiose, hydrolyzed xylan from sugarcane bagasse (HXSB). The substrate consumption was monitored: xylobiose (multiplication symbol), xylose (open triangle) and xylooligosaccharides (asterisk). Enzyme activities determined were: xylanase (filled square), arabinofuranosidase (open triangle), endoglucanase (closed circle),  $\beta$ -glucosidase (open circle) and  $\beta$ -xylanase (filled circle). Standard deviation of triplicate assays less than 10%

Xylanase produced in the medium supplied with hydrolyzed xylan from sugarcane was very low (Fig. 2c), similar to the levels produced by washed-mycelia, despite the use of a relatively high substrate concentration (20 mM). According to the literature, the enzyme production is proportional to the amount of the inducer within a range. At high inducer concentrations this correlation is lost (Biely et al. 1980).

Xylanase was induced by HXSB after a short lag period and two peaks of enzyme production were detected at 12 and 66 h, going along with slow time-consuming of xylobiose. The second peak was coincident with the reduction in the xylose content (Fig. 2c). The induction time in xylan was extended for 50 h; however, the synthesis of the enzyme continued for a long period.

## Conclusions

*Thermoascus aurantiacus* secreted most of the hemicellulolytic and cellulolytic enzymes when cultivated in xylooligosaccharides which acted as potent inducers for the production of xylanases.

Other stimulators were xylooligosaccharides which were in fact more effective stimulators of endoxylanase production than xylose. Almost all xylobiose remained in the medium after fungal growth as a consequence of low  $\beta$ -xylosidase activity produced by *T. aurantiacus*. Therefore, xylobiose must be the natural inducer of endoxylanase. Regardless of the inoculation mode, spores or washed-mycelia, the production of xylanase was always higher when xylose or xylobiose were added separately. Xylose does not repress enzyme induction in *T. aurantiacus* when used in low quantities.

Low activities of cellulases are produced by this fungus regardless of inducer. Despite excellent growth on easily metabolizable carbohydrates (e.g. xylose, xylobiose, cellobiose and HXSB), only constitutive levels of cellulases were produced. Cellulase activity was always determined in the presence of xylanase activity, suggesting that both induced activities are linked.

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