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ORIGINAL RESEARCH PAPER

Identification of thermostable β-xylosidase activities produced by *Aspergillus brasiliensis* and *Aspergillus niger*

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Abstract Twenty *Aspergillus* strains were evaluated for production of extracellular cellulolytic and xylanolytic activities. *Aspergillus brasiliensis*, *A. niger* and *A. japonicus* produced the highest xylanase activities with the *A. brasiliensis* and *A. niger* strains producing thermostable β-xylosidases. The β-xylosidase activities of the *A. brasiliensis* and *A. niger* strains had similar temperature and pH optima at 75°C and pH 5 and retained 62% and 99%, respectively, of these activities over 1 h at 60°C. At 75°C, these values were 38 and 44%, respectively. Whereas *A. niger* is a well known enzyme producer, this is the first report of xylanase and thermostable β-xylosidase production from the newly identified, non-ochratoxin-producing species *A. brasiliensis.*

Keywords *Aspergillus brasiliensis* · *Aspergillus niger* · β-Xylosidase · Thermostability

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Introduction

Much attention has been given to xylan-degrading enzymes, including their potential industrial use in the animal feed, bread-making, and the paper and pulp industries (Bajpai 1999; Polizeli et al. 2005). More recently, particular attention has been given to the enzymes involved in enzymatic degradation of arabinoxylan in relation to ''second generation'' biofuel processes (Sørensen et al. 2005). Xylans consist of a linear backbone of β-1,4–linked D-xylopyranosyl units, which may carry various substitutions. The enzymatic degradation of xylans in turn requires action of different enzyme activities. β-Xylosidases (EC 3.2.1.37) attack the non-reducing ends of short xylooligosaccharides to liberate xylose and catalyse the cleavage of xylobiose (Rasmussen et al. 2006; Sørensen et al. 2003). β-Xylosidase activity is rate limiting in arabinoxylan hydrolysis (Poutanen and Puls 1988) and addition of pure β-xylosidase, purified from *Trichoderma reesei*, to a commercial, hemicellulolytic enzyme blend, ''Ultraflo L'' from *Humicola insolens* (Novozymes A/S, Bagsværd, Denmark), significantly boosts xylose liberation during enzymatic wheat arabinoxylan degradation (Sørensen et al. 2005).

Filamentous fungi are widely used as enzyme producers and are generally considered as more potent xylanase producers than bacteria and yeast (Haltrich et al. 1996; Polizeli et al. 2005). Strains of

the genus *Aspergillus* have received particular attention due to their potential thermotolerance and production of thermostable enzymes (Castro et al. 1997). Thermostable enzymes are of interest because elevation of the reaction temperature (up to a certain limit) generally increases the reaction rate and reduces the risk of microbial contamination (Collins et al. 2005). Members of the *Aspergillus* section *Nigri* are particularly efficient producers of several types of extracellular enzymes (Samson et al. 2004; Serra et al. 2006). This group of fungi currently includes 17 species (Arbarca et al. 2004). This work was undertaken to identify novel xylan degrading enzyme activities in *Aspergillus* spp. with particular attention on identifying fungal strains producing thermally robust β-xylosidase activity.

Materials and methods

Chemicals

Wheat bran was from Cerealia Mills A/S [as per Sept. 2006 ''Lantmännen Mills''] (Vejle, Denmark). Soluble wheat arabinoxylan was obtained from Megazyme (Bray, Ireland).

Microorganisms

The 20 aspergilli strains were all obtained from the IBT fungal culture collection at BioCentrum-DTU, Technical University of Denmark (Lyngby, Denmark). Table 1 shows the *Aspergillus* strains tested.

Growth media

All fungi were initally cultivated on two different solid media to check growth and purity: Czapek-Dox yeast autolysate (CYA) agar and malt extract/agar (MEA) (Frisvad and Thrane, 2002). Afterwards, a basic liquid growth medium (GM) containing oat spelt xylan was used for the xylanase activity experiments, while a GM containing wheat bran was employed to screen for cellulolytic enzyme activities. The GM medium had the following basic composition: 3 g $NaNO_3$ l^{-1}, 0.5 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 1.3 g $K_2HPO_4 \cdot 3H_2O$ l^{-1}, 0.5 g KCl l^{-1}, 10 mg $FeSO_4 \cdot 7H_2O$ l^{-1}, 0.05 mg $CuSO_4 \cdot 5H_2O$ l^{-1} and 0.1 mg $ZnSO_4 \cdot 5H_2O$ l^{-1}. For the cultivations for xylanase activity screenings, the oat spelt xylan (Sigma) was added to 30 gl^{-1}. For the cellulolytic activity screenings the final wheat bran level in the GM was 600 gl^{-1}. Prior to inoculation, the medium was autoclaved at 121°C for 15 min. After five days of incubation in the different growth media, the cultures were filtered through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England).

Table 1 Species from the genus *Aspergillus* evaluated in this work. Where known, the CBS number is also given

	IBT no.	CBS no.
A. aculeatus	3244	172.66
A. aculeatus	3679	114.80
A. aculeatus	21050	–
A. brasiliensis	21946	101740
A. carbonarius	4353	127.49
A. carbonarius	4977	115.49
A. carbonarius	21702	–
A. foetidus	4602	564.65
A. foetidus	16906	121.28
A. japonicus	4559	113.48
A. japonicus	13519	–
A. heteromorphus	13961	117.55
A. homomorphus	21893	101899
A. lacticoffeatus	22029	101885
A. lacticoffeatus	22032	101886
A. niger	3250	554.65
A. niger	16911	113.33
A. sp.	12227	–
A. sp.	14352	312.89
A. tubingensis	4981	126.52

Determination of xylanolytic and cellulolytic activities

Xylanolytic and cellulolytic activities were measured on filtered culture broths using Azo-wheat arabinoxylan and Azo-CM-Cellulose according to the procedures described by Megazyme (Bray, Ireland). Absorbances were measured at 590 nm, and the results were based on triplicate activity determinations on the filtered broth samples from each growth medium.

Determination of protein content

Protein levels were assessed directly on the filtered broths by the Lowry method using BSA as the standard.

Action on wheat arabinoxylan

Aliquots of soluble wheat arabinoxylan (1 mg) were dissolved in 1 ml deionised water. Each substrate sample was then incubated with 0.1 g enzyme protein·kg^{-1} dry wt wheat arabinoxylan in parallel evaluations for: (a) 2 h at 40°C; (b) 20 min at optimal temperature, i.e. 60°C for *A. japonicus*, and 75°C for *A. niger* and *A. brasiliensis*. After each incubation the samples were immediately heated at 100°C for 10 min to halt the enzyme reaction. The samples were then centrifuged at 20,000 *g* for ten minutes and the level of xylose liberated was determined by high performance anion exchange chromatography (Sørensen et al. 2003)

Determination of β-D-xylosidase activity

β-Xylosidase enzyme activity was measured by the *p*-nitrophenol method using 2.5 mM *p*-nitrophenol-β-D-xylopyranoside (Sigma) in 50 mM citrate buffer at pH 5 (Poutanen and Puls 1988). After incubation at 40°C for 20 min, the reaction was terminated by addition of 1 M Na_2CO_3 to a final concentration of 0.33 mM and the *p*-nitrophenol release from the substrate was measured at 400 nm. One katal of β-xylosidase activity per ml broth was calculated as equivalent to degradation of 1 mol β–1,4-xylosidic linkages s^{-1} as calculated from a standard curve of *p*-nitro-phenol. The results shown are averages of triplicate measurements made on each broth sample.

Statistics

The initial cellulolytic and xylanolytic activity data were analyzed by principal component analysis using The Unscrambler version 9.6 (Camo Software AS, Oslo, Norway). Significance of the β-xylosidase assay results was established at $P \leq 0.05$. Differences in activities were determined by one-way analysis of variance with 95% confidence intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

Results and discussion

Cellulolytic and xylanolytic activities of *Aspergillus* strains

Of the 20 strains tested in the screening work, *A. brasiliensis* (IBT–21946, CBS 101740, IMI 381727) isolated from Brazilian soil, *A. japonicus* (IBT–13519) isolated from soil in Burkina Faso, and *A. niger* (IBT-3250, CBS 554.65) isolated from tannin in Connecticut, USA, were found to have highest xylan-degrading activity and were selected for further study of their production of β-xylosidase activity.

pH optima

For all three of the selected *A. niger*, *A. japonicus* and *A. brasiliensis* strains, the highest β-xylosidase activities were at pH 5 (Fig. 1). For the *A. niger* strain, the β-xylosidase activities at pH 3 and 4 were equal to that at pH 5. For the *A. niger* and *A. brasiliensis* strains the lowest activities were at pH 6 whereas the β-xylosidase activity produced by *A. japonicus* at pH 6 was almost as high as that at pH 5. The *A. japonicus* activity was generally lower than that of the other two strains, however (Fig. 1).

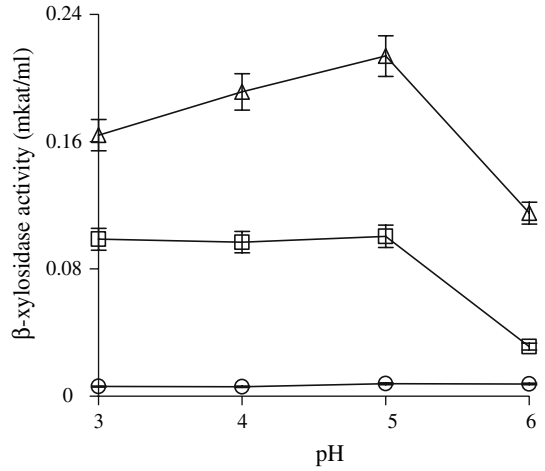

Fig. 1 pH optima of the β-xylosidase enzymes at dilution factor 2. β-xylosidase enzymes activity per ml broth was measured at pH 3 to 6 and at 40°C. *A. niger* (□), *A. japonicus* (○), *A. brasiliensis* (Δ)

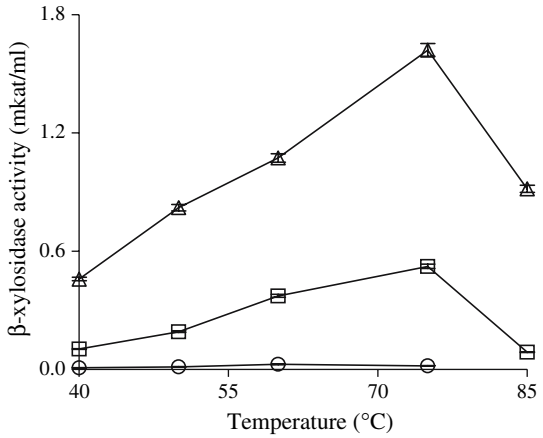

Fig. 2 Temperature optima: β-xylosidase activity per ml broth was measured after incubation for 1 h at pH 5 at 40, 50, 60, 75 and 85°C. *A. niger* (□), *A. japonicus* (○), *A. brasiliensis* (Δ)

Temperature optimum

For the β-xylosidase activities produced by the *A. niger* and *A. brasiliensis* strains the optimal temperature was 75°C while the optimal temperature for the *A. japonicus* strain was at 60°C (Fig. 2). In general, these data for temperature and pH optima agree well with what has been reported previously by others (Table 2), but the temperature optima of the *A. niger* and *A. brasiliensis* strains studied here were clearly in the high end of the available data (Table 2). Previously, β-xylosidase activity produced by an *A. niger* strain was found to have temperature optimum above 75°C (Uchida et al. 1992), whereas, to the best of our knowledge, this is the first report of a high temperature optimum β-xylosidase activity production by *A. brasiliensis* and *A. japonicus*.

Thermal stability

Among the three strains, the thermal stability of the β-xylosidase activity produced by *A. japonicus* dropped rapidly above 40°C retaining 61% of the original activity after 1 h at 50°C, and only 16% at 60°C (Fig. 3). In contrast, 99% of the original β-xylosidase activity was retained after 1 h incubation of the *A. niger* culture broth at 60°C; after 1 h at 75°C the activity had dropped to 44% of the original activity (Fig. 3). The β-xylosidase activity produced by *A. brasiliensis* retained 62% of the activity after 1 h at 60°C, and 38% of the activity after 1 h at 75°C (Fig. 3). A β-xylosidase enzyme produced by *T. reesei* maintained only 20% of its activity at 70°C (Poutanen and Puls 1988), while a β-xylosidase produced by *A. phoenicis* maintained approximately 25% of the original enzyme activity after 1 h of incubation at 70°C while no activity was found after 1 h at 75°C (Rizzatti et al. 2001). Kiss and Kiss (2000) have reported that the β-xylosidase activity produced by *A. carbonarius* is completely lost after 30 min of incubation at 70°C, whereas Christov et al. (1999) found *A. oryzae* to produce β-xylosidase activity having a

Table 2 Properties of β-xylosidases produced by *Aspergillus* spp. Assay time is mentioned when known

Strain	Optima		Assay time (min)	Reference
	pH	*T* (°C)		
A. awamori (= *A. niger*)	6.5	70	(not reported)	Kormelink et al. (1993)
A. carbonarius	4.0	60	30	Kiss and Kiss (2000)
A. fumigatus	4.5	75	20	Kitpreechavanich et al. (1986)
A. nidulans	5.0	50	30	Kumar and Ramon (1996)
A. niger	5.0	>75	10	Uchida et al. (1992)
A. niger	3.8–4.0	70	(not reported)	Rodionova et al. (1983)
A. oryzae	4.0	60	10	Kitamoto et al. (1999)
A. phoenicis (= *A. niger*)	4.0–4.5	75	(not reported)	Rizzatti et al. (2001)
Trichoderma reesei	4.0	60	10	Poutanen and Puls (1988)
T. reesei and Talaromyces emersonii	3.0–3.5	60	10	Rasmussen et al. (2006)
A. niger (IBT-3250)	5.0	75	20	This work
A. japonicus (IBT-13519)	5.0	60	20	This work
A. brasiliensis (IBT-21946)	5.0	75	20	This work

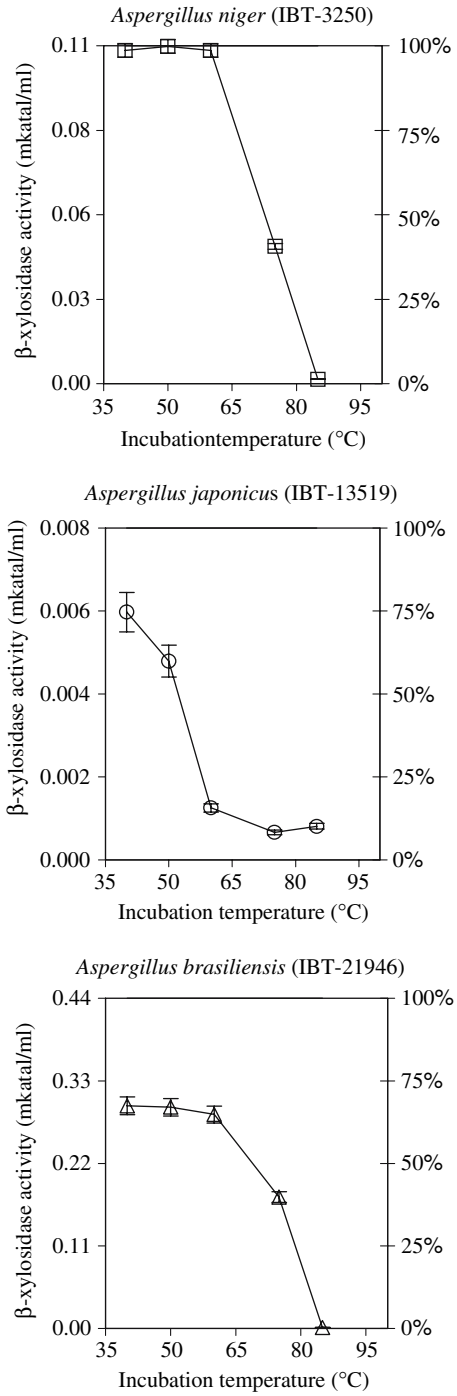

Fig. 3 Temperature stability: β-xylosidase activity per ml broth was measured after incubation for 1 h at pH 5 and at each particular temperature

half life of 4 h at 60°C. The stability of the β-xylosidase activities produced by the *A. niger* IBT-3250 and *A. brasiliensis* IBT-21946 strains were thus better than the stability of previously studied β-xylosidases produced by strains of the genus *Aspergillus* or by *Trichoderma reesei*. *A. brasiliensis* is moreover a new specie in section *Nigri* which has the advantage of not being able to produce the mycotoxin ochratoxin A (Samson et al. 2004).

Table 3 Specific activity measured over 10 min at pH 5 and at optimal temperature; the optimal temperature given in parenthesis

Strain	IBT no.	Specific activity (mkat/mg protein)
A. niger	3250	0.93 (75°C)
A. japonicus	13519	0.04 (60°C)
A. brasiliensis	21946	1.54 (75°C)

Specific β-xylosidase activity

Among the three strains, *A. brasiliensis* had the highest specific β-xylosidase activity (Table 3). The underlying results indicated an almost similar protein production by *A. niger* and *A. japonicus* (0.56–0.62 mg protein/l), whereas the *A. brasiliensis* produced 85% and 70% more protein (1.05 mg/l) than *A. niger* and *A. japonicus*, respectively. When comparing the calculated specific β-xylosidase activities, *A. brasiliensis* moreover exhibited 66% higher specific activity than *A. niger* and 368% higher specific β-xylosidase activity than *A. japonicus*.

Table 4 β-xylosidase activity [a] on polymeric wheat arabinoxylan

IBT	Species	Activity [b] 2 h at 40°C	Activity [c] 20 min at optimal temperature
3250	*A. niger*	1.07	6.84
13519	*A. japonicus*	0.08	0.52
21946	*A. brasiliensis*	11.08	8.18

[a] Data are averages of two replicate determinations for each assay run. Average standard coefficient of variation on all measurements: <8%

[b] mg xylose released from wheat arabinoxylan per mg enzyme protein per minute at pH 5 and 40°C

[c] mg xylose released from wheat arabinoxylan per mg enzyme protein per minute at pH 5 and 60°C for *A. japonicus* and at pH 5 and 75°C for *A. niger* and *A. brasiliensis*

Action on wheat arabinoxylan

The data obtained on the genuine wheat arabinoxylan substrate (Table 4) verified the potency the *A. brasiliensis* β-xylosidase activity and confirmed the thermal activity of the *A. niger* and *A. brasiliensis* β-xylosidase enzymes on a polymeric xylan substrate.

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