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Characterization of hemicellulases from thermophilic fungi

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Abstract The thermophilic fungi *Thermomyces lanuginosus*, *Malbranchea cinnamomea*, *Myceliophthora fergusii* and the thermotolerant *Aspergillus terreus* were cultivated on various carbon sources, and hemicellulolytic and cellulolytic enzyme profiles were evaluated. All fungi could grow on locust bean galactomannan (LBG), Solka floc, wheat bran and pectin, except *T. lanuginosus*, which failed to utilize LBG for growth. Different levels of cellulase and hemicellulase activities were produced by these fungal strains. Depending on the carbon source, variable ratios of thermostable hydrolytic enzymes were obtained, which may be useful in various applications. All strains were found to secrete xylanolytic and mannanolytic enzymes. Generally, LBG was the most efficient carbon source to induce mannanase activities, although *T. lanuginosus* was able to produce mannanase only on wheat bran as a carbon source. Xylanolytic activities were usually highest on wheat bran medium, but in contrast to other investigated fungi, xylanase production by *M. fergusii* was enhanced on pectin medium. Preliminary thermostability screening indicated that among the investigated species, thermotolerant glycosidases can be found. Some of the accessory activities, including the α-arabinosidase activity, were surprisingly high. The capability of the produced enzymes to improve the hydrolysis of lignocellulosic pretreated substrate was evaluated and revealed potential for these enzymes.

Keywords Mannanase · Xylanase · Cellulase · Thermophilic fungi · Hydrolysis

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Introduction

Although hydrolysis of plant cell wall polysaccharides has been a subject of study for decades (see reviews by Béguin and Aubert 1994; Aro et al. 2005), efficient enzymatic hydrolysis for the conversion of lignocellulose still is a matter of intensive research. Lignocellulose consists of the three major structural polymers, cellulose, hemicelluloses and lignin. The carbohydrates consist of the backbone of β-1,4-linked pyranosyl units of the monomeric sugars; glucose in cellulose, xylose in xylans and mannose in mannans or mannose and glucose in glucomannans. Hydrolysis of these carbohydrates proceeds through breakdown of the β-1,4-glycosidic linkages in the polysaccharide backbones by cellulases, xylanases, mannanases and

glucanases. The heteropolymeric mannans and xylans also require side group cleaving enzymes, including α-galactosidase, α-glucuronidase and α-arabinosidase. In addition, esterases are needed for the removal of acetyl groups from xylans in hardwood and annual plants, as well as from acetylated galactoglucomannan from soft woods. Finally β-glucosidase, β-xylosidase and β-mannosidase are needed to hydrolyze small oligomers into monomers. A complete hydrolysis of native polymers requires the concerted action of all these enzymes. During fractionation and pretreatments, however, the composition of lignocellulosic raw materials is profoundly modified and consequently, the optimal enzyme mixtures will depend on the structure of the substrate. For more specific modifications of lignocellulosic carbohydrates tailored enzyme compositions can be used (Viikari et al. 2002; Singh et al. 2003; Collins et al. 2005; Polizeli et al. 2005).

There is an increasing need to develop enzyme preparations with enhanced kinetic efficiency and stability at high temperatures, as well as enzymes with complementary activity profiles. Especially elevated thermal stability may be regarded as one of the main targets to improve hydrolysis technologies for biomass conversion. Fungal hydrolytic enzymes are induced variably by different substrates and significant differences have been observed with respect to their quantity, properties and action pattern. Recent evaluation of genomic and proteomic data from several fungi have indicated that these organisms have an extremely large source for hydrolytic polysaccharide-degrading enzymes (Carbohydrate Active Enzymes database, Cantarel et al. 2011). Production of many of these enzymes is subject to induction and repression based on the carbon sources used in the media.

Thermophilic fungi in tropical areas appear usually in lignocellulosic environments, such as decaying forest and agricultural residues or composts (Maheshwari et al. 2000). Thus, they are able to produce a variety of plant cell wall depolymerizing cellulases and hemicellulases. Many modestly thermophilic or even mesophilic fungi are known to produce thermostable enzymes. Xylanases of thermophilic moulds have been widely investigated (Ghatora et al. 2006). One of the most extensively studied thermophilic fungi is *Thermomyces lanuginosus* which is known to be a prolific producer of thermostable xylanases (Singh et al. 2003). Other thermostable hemicellulases, such as mannanases, have been studied only to a more limited extent. A thermostable mannanase secreted by the thermotolerant mesophilic mould *Aspergillus fumigatus* (originally identified as *T. lanuginosus* by Puchart et al. 1999) has been characterized (Puchart et al. 2004). *Thermomonospora fusca* KW3 is described as a mannan degrader with an ability to act on a wide variety of mannan substrates (Hilge et al. 1998). Even less well known are thermostable accessory enzymes needed for complete hemicellulose hydrolysis or cellulases, especially endoglucanases with minor hydrolytic activity. Production of various less studied thermostable hemicellulases is of current interest in several biotechnological areas, including the total hydrolysis of various lignocellulosic raw materials to platform sugars. Catalytically more effective, thermostable enzymes are actively searched for improving biomass conversion processes (Viikari et al. 2007).

In this work, production of lignocelluloses degrading enzymes by selected four thermophilic fungal strains on different complex substrates was studied. A miniaturized assay system described by Xiao et al. (2005) was tested and adopted for the preliminary evaluation of thermostability of the enzymes produced. Characteristics of hydrolytic activities in the selected thermotolerant fungi at elevated temperatures were examined and compared with a commercial hydrolase mixture.

Materials and methods

Microorganisms and enzymes

The thermophilic fungi *Myceliophthora fergusii* MTCC 9293, *Malbranchea cinnamomea* (previously *Malbranchea sulfurea)* MTCC 9294, *T. lanuginosus* MTCC 9331 and thermotolerant *Aspergillus terreus* FBCC 1369 were isolated from Sagar, Madhya Pradesh, India. All the strains were isolated during a survey of thermophilic fungi from litter and decaying woods in 2003. During screening of thermophilic fungi these strains were found to produce endo-β-xylanase on medium containing wheat bran as the substrate and were selected from among fifty thermophilic fungi isolated from decaying litter and wood (Kango 2003; Kango et al. 2003).

In the present study it was envisaged to evaluate these strains belonging to different genera for production of thermostable hemicellulases. The identity of *A. terreus* was confirmed by the analysis of the genomic ITS-region using standard methodology (Heinonsalo et al. 2001). Stock cultures were maintained on yeast extract-phosphate-soluble starch agar (YpSs) slants (Cooney and Emerson 1964) at +4°C. Commercial enzymes (Celluclast 1.5L and Novozym 188, Novozymes A/S, Bagsværd, Denmark) were used as the reference cellulase and supplementary β-glucosidase preparations, respectively. The thermostable monocomponent enzymes were a kind gift of ROAL Ltd (Rajamäki, Finland). Thermostable mixture contained: CBHI (*Acremonium thermophilum* Cel7A), CBHII (*Chaetomium thermophilum* Cel6A), EGII (*Thermoascus aurantiacus* Cel5A) and a xylanase (*T. aurantiacus* Xyn10A). The hydrolysis experiments with the thermostable mixture were supplemented with 2 mg β-glucosidase (*A. thermophilum* Cel3A) per g dry matter of the substrate (Szijártó et al. 2011).

Fungal cultivations

To determine the temperature regime for the growth, fungi were grown at two temperatures viz. 45 and 18°C on Emerson's YpSs agar medium. The liquid cultures were grown on a rotary shaker at 110 rpm in 250 ml Erlenmeyer flasks containing 50 ml of basal medium with the following composition (g l^{-1}): Yeast extract 14.0, $(NH_4)_2SO_4$ 2.1; $MgSO_4 \cdot 7H_2O$ 3.0, $CaCl_2 \cdot 2H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.5 and KH_2PO_4 10.0. Final pH was adjusted to 5.0. As the carbon source, the cultures contained either powdered cellulose Solka floc (1% w/v), wheat bran (2% w/v; from local store), locust bean galactomannan (0.5% w/v, Sigma) or citrus pectin (1% w/v, Sigma). The flasks were inoculated with two mycelial discs of 7 days old cultures of *M. cinnamomea*, *M. fergusii*, or *T. lanuginosus*, pregrown on YpSs agar plates and grown for 96 h at 45°C. *A. terreus* was inoculated as above, but cultivated for 96 h at 37°C using a rotary shaker at 140 rpm. Cultures of *M. cinnamomea*, *T. lanuginosus* and *A. terreus* were filtered through Miracloth (Calbiochem) and clear supernatants were used for enzyme assays. Because of turbidity of the culture medium, *M. fergusii* cultures were filtered prior to enzyme assays through glass fibre filters (Whatman GF-C).

Enzyme activity assays

The total cellulase activity (FPU) was estimated by measuring reducing sugars liberated from Whatman filter paper strips (60 mm × 10 mm) (IUPAC 1987; Wood and Bhat 1988). Endoglucanase, xylanase and mannanase activities were measured using hydroxyethyl cellulose (1% w/v, Fluka) (Bailey and Nevalainen 1981), birchwood xylan (1% w/v, Roth) (Bailey et al. 1992) and locust bean gum (0.5% w/v) (Stålbrand et al. 1993), as substrates, respectively, dissolved in 50 mM Na-citrate buffer, pH 5.0. Enzyme samples were incubated at 50°C for 10 min. The reaction was stopped by adding the dinitrosalicylic acid (DNS) reagent and reducing sugars were measured at 540 nm against the blank (Miller 1959). Activities of β-glucosidase, β-xylosidase, β-mannosidase, α-arabinofuranosidase and α-galactosidase were estimated using 1 mM *p*-nitrophenyl-β-D-glucopyranoside, 5 mM *p*-nitrophenyl-β-D-xylopyranoside, 2 mM *p*-nitrophenyl-β-D- mannopyranoside, 2 mM *p*-nitrophenyl-α-L-arabinopyranoside or 2 mM *p*-nitrophenyl-α-D-galactopyranoside as substrate (all from Sigma), respectively, according to the method described by Bailey and Nevalainen (1981). The absorbance was measured at 400 nm using *p*-nitrophenol (Merck) as a standard. All activities were expressed as nkat ml^{-1}. For evaluation of general esterase activity, 10 mM *p*-nitrophenyl acetate (Sigma) was used. The 50 mM stock substrate was dissolved in DMSO, and diluted with 50 mM Na-citrate buffer, pH 5.0. Enzyme samples were incubated for 10 min, and reaction was terminated by boiling the samples for 10 min. Liberated *p*-nitrophenol was measured as described above. The stability of the substrate in water solutions was followed and the degradation remained below 15% of the applied amount during the reaction time. All assays were conducted in duplicates and the results represent average values.

The preliminary screening of endoglucanase, xylanase and mannanase activities was assayed by modifying the methods of Bailey and Nevalainen (1981), Poutanen et al. (1987) and Xiao et al. (2005). Activities were assayed as follows: 40 µl of the substrate in 50 mM Na-citrate buffer, pH 5.0, tempered to 50°C in a PCR-apparatus (iCycler, PerkinElmer), was incubated with 20 µl of the enzyme sample. Enzyme samples were added with a multichannel pipette. The plate was sealed by aluminium foil to

avoid evaporation. The reaction was stopped by adding 60 µl of DNS reagent, after which the temperature in the system was increased to 95°C for 5 min. Samples were transferred from the PCR-plates onto microplates that were readable in a multiscan reader (Thermo Labsystems Multiscan EX, MTX Labsystems Inc., USA). Assays for β-glucosidase, β-xylosidase, β-mannosidase and α-galactosidase followed methods described (Bailey and Nevalainen 1981; Poutanen et al. 1987), with modifications: 180 µl of preheated substrate at 50°C in 50 mM Na-citrate buffer, pH 5.0, was incubated with 20 µl of the enzyme solution in the PCR-apparatus. The reaction was stopped by adding 100 µl of 1 M Na_2CO_3, after which the samples were removed into a microtiter plate and the absorbance was measured at 405 nm *p*-nitrophenol was used a standard.

Analysis of the thermostability was assayed by incubating the samples in the PCR-apparatus at 65°C for 1 h with the foil coverage, after which the samples were assayed as described above. Activities before and after thermal treatment were calculated, and residual activity at 50°C was regarded as a measure of thermal stability. Optimum temperatures were determined by varying the temperature used at intervals of 10° in the standard assay in the range at 30–80°C. Incubation time was 10 min.

Hydrolysis experiments

Hydrolysis experiments were carried out using hydrothermally pretreated wheat straw (*Triticum aestivum* L.) obtained from Inbicon A/S (Fredericia, Denmark), pretreated according to Larsen et al. (2008). The feedstock was first pre-soaked in 3 g/l acetic acid at 80°C for 10 min and then steamed at 195°C for 12 min in a continuous reactor operated at 50 kg/h feed rate and a straw to water ratio of 1:5. The pretreated fibers were washed and pressed, and stored frozen (−20°C) in aliquots until use. The cellulose (glucan) content of the solids was 58.9% of DM, comprising 94 % of all carbohydrates. The major hemicellulose sugar was xylose (3.6% of DM). Hydrolysis studies were carried out in 10 ml Falcon tubes with 5 ml working volume at 2% (w/w) solids (DM) in 0.05 M sodium-citrate buffer (pH 5.0). To avoid loss of sugars due to possible microbial contamination the reaction mixture also contained 0.02% NaN_3.

The proteins from the culture filtrates of 7 days old cultivations of *M. cinnamomea* and *M. fergusii* on wheat bran medium were concentrated about 20–40-fold by precipitation by adding three volumes (v/v) of cold (4°C) ethanol. The precipitates were dissolved in 25 mM Na-citrate buffer, pH 5.0, and were further concentrated by Amicon no. 8400 stirred ultrafiltration apparatus using PM10 membranes (M_r cut-off point 10,000) at 4°C. The enzyme dosage was set on the protein basis at 12 mg per g DM of substrate. Duplicate tubes were prepared for each test point and the whole reaction mixtures were taken as samples. Enzymatic hydrolyses were stopped by boiling the tubes for 10 min. Appropriate references were prepared and run together with the experimental series. The amount of total sugars formed after acid and enzymatic hydrolysis was determined as reducing sugars. All analyses were carried out in triplicates, with excellent reproducibility. The standard deviation of the measurements was consistently below 5%. Monomeric sugars from selected samples were analyzed by high performance anion exchange chromatography with pulsed amperometric detection system as described in Pakarinen et al. (2011).

Results and discussion

Fungal temperature regime and growth on different carbon sources

Growth tests on Emerson's YpSs agar medium in different temperatures clearly indicated that three of the species were thermophilic, whereas *A. terreus* showed lower temperature regime compared to the other investigated species (Table 1). *A. terreus* therefore was designated thermotolerant, and the other species thermophilic (Cooney and Emerson 1964).

The three thermophilic fungal species, *M. cinnamomea*, *M. fergusii*, and *Thermomyces lanuginosus* grew well in shake flasks at 45°C, whereas *A. terreus* required a slightly lower temperature to enable efficient growth. *M. fergusii*, *M. cinnamomea* and *A. terreus* were able to utilize all the tested carbon sources. Although the inducible character of the secretion of hydrolytic enzymes was clearly observed with all the investigated species, significant variation was also noted especially with respect to regulation of production of various hydrolases on a particular carbon source. A complex carbon source, such as wheat bran, was found to act as a potential carbon

Table 1 Sources and growth characteristics of fungi in moderate and high temperatures

Strain	Habitat of isolation	Growth	
		18°C	45°C
Thermomyces lanuginosus MTCC 9331	Wheat straw	−	+++
Myceliophthora fergusii MTCC 9293	Compost	−	++++
Malbranchea cinnamomea MTCC 9294	Soil	−	+++
Aspergillus terreus FBCC 1369	Storage seeds	+	++

− No growth, + very poor, ++ poor, +++ good, ++++ excellent

source and inducer for these fungi. Wheat bran is an efficient inducer of hemicellulases containing a high amount of soluble carbohydrates, required for the initiation of growth, hemicelluloses for induction, as well as organic nitrogen. Especially production of many hemicellulases was induced, whereas cellulolytic activity was less related to a special carbon source used in this study (Tables 2, 3, 4). The observed pattern was not surprising, and is typical among cellulolytic fungi. *T. lanuginosus* MTCC 9331 was the only organism which failed to grow on medium containing locust bean gum. Previously, the limited ability of *T. lanuginosus* strains to grow on media with only mannan (LBG) as a carbon source and to produce mannanolytic enzymes has been reported (Puchart et al. 1999). In the following, the secretion of individual enzymes is described in more detail.

Enzymes hydrolysing galactomannan

The consortium of different mannanolytic enzymes could be beneficial for a complete hydrolysis of glucomannan containing substrates. Synergistic action of mannanases along with other enzymes; α-galactosidase, acetyl glucomannan esterases, β-mannosidase and β-glucosidase, is required for complete hydrolysis of acetylated galacto(gluco)mannans (Tenkanen 1998; Clarke et al. 2000; Dhawan and Kaur 2007). Supplementation of *Aspergillus awamori* culture filtrate containing β-mannanase and β-mannosidase with commercial β-glucosidase and α-galactosidase has been shown to enhance the hydrolysis of galactomannan (LBG) (Kurakake and Komaki 2001). The *A. terreus* strain clearly produced most significant levels of mannanase under the culture conditions used (Table 2). Mannanase activity was strongly induced by LBG in this fungus. Similarly, the mannanase activity in *M. cinnamomea* was induced by this carbon source whereas in *M. fergusii*, also cellulose appeared to induce a notable mannanase activity. Similar elevated expression of mannanase activity induced by cellulose has been observed e.g. in *Sclerotium rolfsii* (Sachslehner et al. 1998). The activity levels obtained with *M. fergusii* were, however, relatively low when compared to *A. terreus* on locust bean gum.

The *T. lanuginosus* secreted low mannanase activity when cultivated on wheat bran medium (Table 2). Wheat bran has previously been observed to be a good carbon source for mannan-degrading enzyme production in *Penicillium simplicissimum*, although efficient induction required supplementation of LBG (Luonteri et al. 1998). Obviously in *T. lanuginosus*, complex carbon sources, such as wheat bran, were most favourable for the induction of mannan hydrolysing activities, and more efficient than the polymeric galactomannan, LBG. Puchart et al. (1999) screened several *T. lanuginosus* strains and found that mannanase production was restricted to only three strains of the 17 tested. A mannanase-positive isolate was, however, later identified as a strain of *A. fumigatus* (Puchart et al. 2004), in which species also α-galactosidase activity was characterized (Puchart et al. 2000).

Interestingly, *T. lanuginosus* produced a high α-galactosidase activity on wheat bran medium, but no activity on LBG. On the other hand, *A. terreus* produced measurable α-galactosidase activities on all media, but highest on the LBG medium. Obviously, the levels of α-galactosidase activity were associated with the high mannanase activity (Table 2). Locust bean gum has an average degree of substitution of galactose: mannose of 1:4.

The β-mannosidase activities produced by the tested strains on various media were quite low. No activity could be detected by the *M. fergusii* and *A. terreus* strains on any media. The highest activity was produced by *M. cinnamomea*. The apparent β-mannosidase activities measured against the artificial *p*-nitrophenyl substrate have been low also in other fungal species, such as *A. awamori* (Rätto and Poutanen 1988; Kurakake and Komaki 2001), *Rhizopus niveus* (Fujmoto et al. 1997), *Aspergillus niger*

Table 2 Mannanolytic activities produced by the tested strains

Fungus	Medium			
	Wheat bran	Locust bean gum	Solka floc	Pectin
	Mannanase activity (nkat ml^{-1})			
M. fergusii	6.8	29	30	BDL
M. cinnamomea	BDL	45	BDL	BDL
T. lanuginosus	6.3	BDL	BDL	BDL
A. terreus	19	130	7.3	7.3
	α-Galactosidase activity (nkat ml^{-1})			
M. fergusii	0.5	0.2	0.2	0,2
M. cinnamomea	BDL	0.2	BDL	BDL
T. lanuginosus	35	BDL	5.7	8.5
A. terreus	8.5	45	0.5	0.5
	β-Mannosidase activity (nkat ml^{-1})			
M. fergusii	BDL	BDL	BDL	BDL
M. cinnamomea	0.2	0.9	0.2	0.1
T. lanuginosus	0.2	BDL	0.1	BDL
A. terreus	BDL	BDL	BDL	BDL

Activities in various media were assayed after 4 days of cultivation

BDL Below detection level

Table 3 Endoglucanase and β-glucosidase activity produced by the tested strains in various media

Fungus	Medium			
	Wheat bran	Locust bean gum	Solka floc	Pectin
	Endoglucanase activity (nkat ml^{-1})			
M. fergusii	14	BDL	17	9
M. cinnamomea	9	14	11	10
T. lanuginosus	BDL	BDL	BDL	16
A. terreus	17	9	9	BDL
	β-Glucosidase activity (nkat ml^{-1})			
M. fergusii	5.3	4.2	8.3	3.6
M. cinnamomea	0.5	0.1	0.2	0.2
T. lanuginosus	5.1	ND	3.6	5.8
A. terreus	22	22	2.3	2.2

BDL Below detection level

(Itoh and Kamiyama 1995) and *Trichoderma reesei* (Rätto and Poutanen 1988) (Table 2).

The β-1,4-linkages between glucose and mannose units in glucomannans are also hydrolyzed by endoglucanases (Stålbrand et al. 1993). Unspecific endoglucanases, such as the EGI from *T. reesei*, also hydrolyze β-1,4-linkages in xylans. Therefore, the endoglucanase activities were also followed in the strains studied. The filter paper activities were low or absent in all strains (results not shown). Interestingly, endoglucanase activity indicated variation in the production patterns (Table 3). No endoglucanase activity was detected in *T. lanuginosus* cultivations on wheat bran, solka floc or locust bean gum, which is in accordance with previous studies (Singh et al. 2003). The endoglucanase production in *T. lanuginosus* showed somewhat unexpected production pattern, as EG was produced on pectin as the carbon source. The other strains produced varying levels of endoglucanases on various media. Previously, a strain of *Myceliophthora* has been shown to produce a variety of hydrolytic enzymes, including endoglucanases and β-glucosidase on rice straw (Badhan et al. 2007).

Along with β-mannosidase, also β-glucosidase is needed for a complete hydrolysis of small oligomers consisting of glucose and mannose. Relatively low level of β-glucosidase activity was observed in the culture filtrates of test organisms on different carbon sources (Table 3); only *A. terreus* grown on locust bean gum and wheat bran, produced elevated levels of β-glucosidase. In contrast, much lower activity was measured on Solka floc and pectin containing media.

Enzymes hydrolyzing xylans

A direct correlation between the enzymatic xylan removal and efficiency of enzymatic hydrolysis of cellulose has been observed (Selig et al. 2008). For a complete hydrolysis of xylans from hardwood or annual plants, in addition to endoxylanases, potentially also acetylesterases, α-arabinosidases and ferulic acid esterases, as well as β-xylosidases could be needed. All tested strains were xylanolytic. *T. lanuginosus* was a superior producer of xylanases; whereas activities produced by other tested fungi were clearly lower (Table 4). Several *T. lanuginosus* strains have been shown to produce high level of xylanase also on other lignocellulosic substrates, such as barley husk and corn cobs in liquid media (Gomes et al. 1993; Hoq and Deckwer 1995; Chadha et al. 1999, Singh et al. 2003). Wheat bran was a good carbon source for xylanase production for three of the tested species; only *M. fergusii* did not efficiently produce xylanase

Table 4 Xylanolytic enzymes produced by the tested strains in various media

Fungus	Medium			
	Wheat bran	Locust bean gum	Solka floc	Pectin
	Xylanase activity (nkat ml^{-1})			
M. fergusii	27	BDL	54	72
M. cinnamomea	124	BDL	30	BDL
T. lanuginosus	3,420	BDL	324	BDL
A. terreus	171	BDL	33	0.2
	α-Arabinosidase activity (nkat ml^{-1})			
M. fergusii	0.35	ND	ND	ND
M. cinnamomea	0.30	ND	ND	ND
T. lanuginosus	0.32	ND	ND	ND
A. terreus	1.1	ND	ND	ND
	Acetyl esterase activity (Units ml^{-1})			
M. fergusii	11.7	ND	ND	ND
M. cinnamomea	11.4	ND	ND	ND
	β-Xylosidase activity (nkat ml^{-1})			
M. fergusii	0.2	ND	ND	ND
M. cinnamomea	0.4	BDL	BDL	BDL
T. lanuginosus	0.2	BDL	BDL	0.1
A. terreus	0.7	BDL	0.2	0.1

BDL Below detection level

on wheat bran, but higher titers were obtained on Solka floc cellulose and, unexpectedly, on the pectin medium. As could be expected, none of the strains produced xylanase on locust bean galactomannan. Xylanase production by *A. terreus* has been previously investigated on oat spelt xylan and the production levels were in the same range as was obtained in this study (Marques et al. 2003; Hrmová et al. 1989).

The activities of the accessory enzyme α-arabinosidase remained at low levels (Table 4). On wheat bran medium *A. terreus* produced the highest α-arabinosidase activities, followed by *T. lanuginosus*, *M. cinnamomea* and *M. fergusii*. Esterase activity is required for the liberation of acetic acid from acetylated polymeric and oligomeric xylans. Xylans may also contain esterified aromatic substituents, including feruoyl and *p*-coumaroyl side groups. In this work, the general esterase activity was estimated from *M. cinnamomea* and *M. fergusii* by using *p*-nitrophenyl acetate as substrate. Both *M. cinnamomea and M. fergusii* produced esterases on wheat bran. The measured β-xylosidase activities were low in all tested strains. Again, wheat bran was the most favourable substrate for the production of β-xylosidase (Table 4).

Thermal stability of the enzymes

Albeit the fungi showed good or moderate thermotolerance for growth (Table 1), no clear trend was observed on the thermal stability of the hydrolytic enzyme activities, as tested by the mini-scale screening assays. However, several hydrolases with good thermal stability, i.e. expressing activity without notable loss of activity after incubation at 65°C for 1 h, were found indicating that these species offer potential sources for further characterization of thermostable hydrolases. In each tested species, at least one enzyme showed excellent thermal stability (Table 5).

The mannanolytic enzyme system in the studied species appeared to have slightly lower temperature tolerance compared to the xylanolytic enzyme system, based on mini-scale screening tests and the temperature optima analyses (Tables 5, 6). Only about 15–25% of the original mannanase activity in various fungal species was retained after the incubation. Part of the α-galactosidase activity in samples from all three species was lost after 1 h treatment at 65°C (Table 5). The temperature optima of the α-galactosidase in *M. fergusii* and *M. cinnamomea* was 50°C, about the same as in the commercial *T. reesei* preparation Celluclast, used as reference enzyme. Recently, Shankar et al. (2009) reported the production and properties of α-galactosidase from *A. terreus*, which showed, in contrast to the results in this work, very good thermal stability. The thermostable α-galactosidase from *A. fumigatus* was shown to remain stable for up to 6 h at 60°C (Puchart et al. 2000, 2004).

Good thermal stabilities of xylanases in *M. cinnamomea* and *M. fergusii* were observed, as well. Also the strain of *T. lanuginosus* investigated here possessed xylanase with good thermal stability. The temperature optimum and thermal stability of *T. lanuginosus* xylanases is known to be good, although also considerable variation has been reported to occur between the strains (Cesar and Mrša 1995; Singh et al. 2000). The thermostability characteristics of the endoxylanases of *M. fergusii* and *M. cinnamomea* were further investigated. The endoxylanases of *M. fergusii* and *M. cinnamomea* possessed temperature optimum at 70°C, which exceeds the temperature

optimum of *T. reesei* xylanase, present in the Celluclast enzyme mixture, by 10°C (Fig. 1a). As with xylanase activity, the optimum temperature for *M. fergusii* and *M. cinnamomea* β-xylosidase exceeded that of Celluclast, and was highest at 70°C. At this temperature Celluclast had lost about 25% of its maximal activity (Fig. 1b).

The temperature optima of the α-arabinosidases of *M. fergusii* and *M. cinnamomea* indicated very high stability of the enzymes, retaining high activity even at over 80°C (Table 6). Luonteri et al. (1995) have previously characterized three α-arabinosidases from a strain of *A. terreus*. It was found that *A. terreus* arabinosidases lost their thermal stability in temperatures over 60°C, although no optimal temperature for activity was reported. Thermostable α-arabinosidases, with optimum activity at 70°C, have previously been characterized in *Thermoascus aurantiacus* (Roche et al. 1994).

The general esterase activity in *M. fergusii* and *M. cinnamomea* appeared to show activity within a wide temperature regime, as the enzymes acted well at 30–80°C without losing more than 10% of the activity. This stability pattern exceeds the *T. reesei* activity in Celluclast, which showed maximal esterase activity at 60°C (Table 6). Above this temperature, the activity in Celluclast was rapidly lost at higher temperatures, without any remaining activity at 70°C.

The temperature optima of *M. fergusii* and *M. cinnamomea* endoglucanases were determined to be high, with maximal activity in *M. fergusii* obtained at 70°C and in *M. cinnamomea* even above 80°C (Fig. 1c). The measured temperature optimum of the *M. fergusii* β-glucosidase was at the same level as *T. reesei*, around 70°C, whereas in *M. cinnamomea* the maximum activity was obtained at 60°C (Fig. 1d). However, 1 h incubation at 65°C retained the activity well, and thus the β-glucosidase stability appeared to be good in both species (Table 5).

Hydrolysis experiments

The properties of the thermostable enzymes from *M. cinnamomea* and *M. fergusii* were further preliminarily evaluated for their hydrolytic capabilities using hydrothermally pretreated wheat straw as the lignocellulose substrate. Concentrated enzyme solutions of culture filtrates from the wheat bran medium were selected, as a broad range of cellulolytic and hemicellulolytic enzymes in reasonable amounts were produced on this medium by the fungi studied. The basic enzymes used were the commercial *T.reesei* preparation, Celluclast, as well as a thermostable enzyme preparation composed of the major partially purified cellulolytic enzymes. Because of the synergistic behaviour of cellulases, it was expected that the use of thermostable enzyme preparations from *M. fergusii* and *M. cinnamomea* would enhance the hydrolytic potential of the Celluclast or the thermostable cellulase mixture. The enzymes were dosed on the protein basis in different combinations the level of comparison 12 mg/g substrate. Thus, the activities of the preparations varied with respect to the enzyme activity ratios; i.e. cellulases and individual hemicellulases. The thermostable hemicellulases were also evaluated alone at a relatively high dosage (9 mg/g substrate). The enzyme mixtures were tested at 45°C, suitable for the performance of Celluclast, and at 55°C, suitable for the thermostable cellulase mixture (Szijártó et al. 2011), representing also the maximum operating temperature of the *T. reesei* enzymes.

When tested alone, the thermostable enzyme preparations of *M. fergusii* and *M. cinnamomea* showed fairly poor ability to hydrolyze cellulose in the lignocellulosic substrate, as could be expected (Fig. 2). The hydrolysis yield by the *M. fergusii* preparation at 55°C was clearly increased as compared to hydrolysis at 45°C, especially due to the cellulolytic activity in the thermostable fungal preparation. Elevated cellulose

Table 5 Thermal stabilities of various fungal hydrolases: activities remaining after 1 h treatment at 65°C

Fungus	Enzyme activity, % of the original					
	Endoglucanase	Xylanase	Mannanase	β-Glucosidase	β-Xylosidase	α-Galactosidase
M. fergusii	60	61	17	50	74	16
M. cinnamomea	18	43	22	81	95	9
T. lanuginosus	21	46	26	52	10	16
A. terreus	47	21	15	16	90	14

Table 6 Temperature optima of the α-galactosidase, α-arabinosidase and acetyl esterase activities of *M. fergusii*, *M. cinnamomea* and the commercial Celluclast enzyme mixture

Fungus	Temperature optima (°C)		
	α-Galactosidase	α-Arabinosidase	Acetyl esterase
M. fergusii	50	>80	80
M. cinnamomea	50	>80	80
Celluclast	50	80	60

hydrolysis correlated well with the excellent thermostability values determined for *M. fergusii* endoglucanase, in particular. Also xylose release was improved, particularly with the *M. fergusii* enzyme treatment, correlating with the good thermostability values recorded for the xylanolytic enzymes (Table 5). At increased temperature, the ability of the *M. cinnamomea* preparation to hydrolyze cellulose was slightly improved whereas no significant change in xylan hydrolysis could be observed (Fig. 2).

Improvement of the hydrolysis of the pretreated wheat straw by the commercial cellulase preparations was further studied by addition of the thermostable fungal hemicellulase preparations (Fig. 3a, b). The *T. reesei* based commercial preparation (9 mg/g substrate) was supplemented by the thermostable enzymes at 45°C (dosage of 3 mg/g substrate). The hydrolysis yield at 45°C was improved especially by the *M. cinnamomea* preparation, and the yield was equal to the result obtained with the higher dosage of the commercial enzyme used, 12 mg/g substrate (Fig. 3a). The basis of comparison even on protein basis is, however, difficult due to the uncharacterized composition of the thermostable hemicellulases. As expected, the yields by the thermostable hemicellulases alone were modest.

It is well recognized that in prolonged hydrolysis, the enzymes of *T. reesei* lose some of the activity already at 55°C. Therefore, the potential of the thermostable enzymes were evaluated in combination with a mixture of partially purified thermostable cellulases, composed to mimic the ratios of the major cellulases in efficient hydrolase preparations. At elevated temperature, the *M. fergusii* preparation was clearly more efficient, reaching almost the same level of hydrolysis as the higher dosage (12 mg/g substrate) of the thermostable cellulase preparation (Fig. 3b). The elevated hydrolytic effect was expectedly due to the hemicellulase and endoglucanase activities.

The analysis of monosaccharides indicated the composition of the raw material, i.e. a fairly low content of xylan in the solids. Interestingly, the thermostable fungal preparations were able to release also minor hemicellulose originating sugars; arabinose, mannose and galactose (Table 7). These components were not identified after the harsh analytical acid hydrolysis. The hydrolysis of mannose with a high dosage of *M. fergusii* enzymes was doubled when

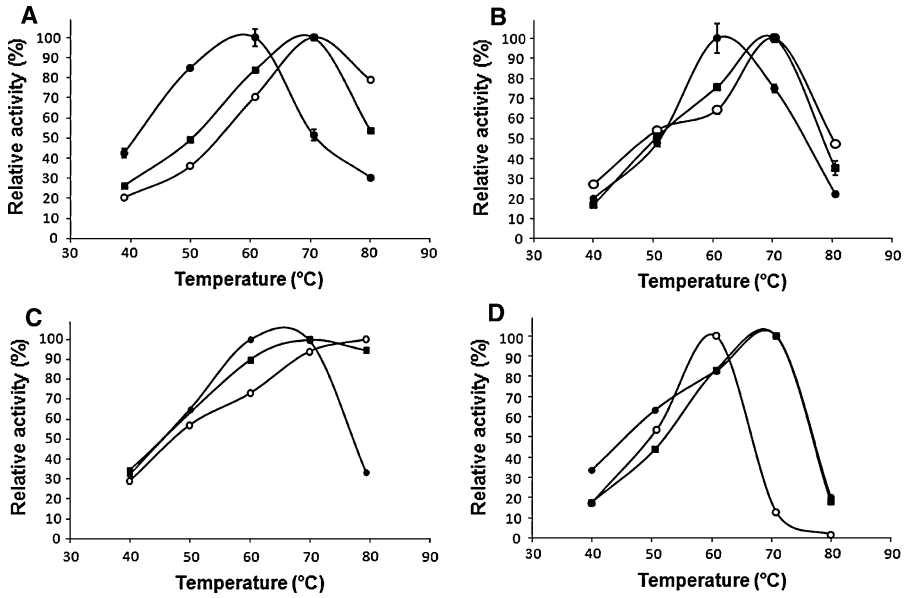

Fig. 1 The temperature optima of the four hydrolytic enzymes from *M. fergusii* (*fiilled square*), *M. cinnamomea* (*empty circle*) and the commercial Celluclast enzyme mixture (*filled circle*). **a** xylanase, **b** β-xylosidase, **c** endoglucanase, **d** β-glucosidase

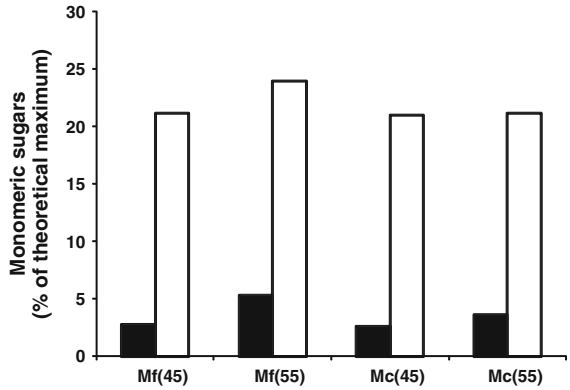

Fig. 2 The amount of glucose (*filled square*) and xylose (*empty square*) (expressed as % of theoretical) liberated in the hydrolysis of pretreated wheat straw after 72 h by enzymes of *M. fergusii* (Mf) and *M. cinnamomea* (Mc) in the two temperatures shown in *brackets*

the temperature was increased from 45 to 55°C. Celluclast, the thermostable enzyme mixture, or *M. cinnamomea* enzyme liberated no or only trace amounts of galactose, arabinose or mannose. It is interesting to note that the enzymes of *M. fergusii* grown on wheat bran, although containing relatively low mannanolytic activities (Table 2), was able to release mannose from the lignocellulosic substrate.

It is well known that in lignocellulosic substrates the microfibrils and fibre surfaces are interlinked and even coated by xylans (in hardwood, softwood and annual plants) and galactoglucomannans (mainly in softwood). Even low amounts of hemicelluloses hinder the efficient hydrolysis. Thus, hemicellulolytic enzymes have been shown to have a boosting effect in the hydrolysis of lignocellulosic substrates (Öhgren et al. 2007; Selig et al. 2008; Gao et al. 2011). The results show that an optimal mixture of hemicellulases and cellulases is needed for improving the hydrolysis. In the present work, the comparison of the results may have been unfair for the unpurified thermostable hemicellullolytic enzymes, as they may have contained also a higher fraction of enzymes, not advancing lignocelluloses hydrolysis. Further experiments would, however, be needed to evaluate the potential of these enzymes.

Conclusions

All tested thermophilic strains produced considerable amounts of hemicellulolytic enzymes. However, considerable variation occurred in the enzyme activity profiles on various carbon sources, indicating the inducible nature of hemicellulase production. The overall pattern of mannanolytic activities in *A. terreus* was interesting as it produced significant amounts of β-mannanase, α-galactosidase and β-glucosidase on LBG medium. Only *M. cinnamomea* produced notable β–mannosidase activity on LBG. In the present study, high xylanase activity was observed in *T. lanuginosus* cultivated on wheat bran and good mannanase yields in *A. terreus* cultivated on LBG. Screening of the four test strains for cellulases showed that only low level of

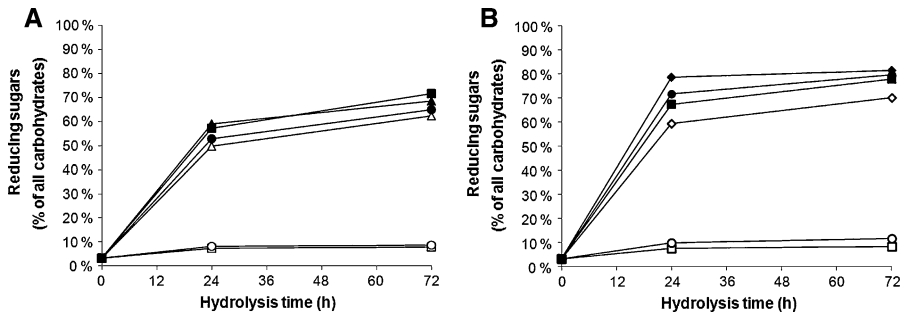

Fig. 3 Hydrolysis (as % conversion of theoretical) of pretreated wheat straw at 45°C (**a**) and 55°C (**b**) by various enzymes, dosed as mg protein per gram of substrate. **a** Celluclast 12 mg (*filled triangle*), Celluclast 9 mg (*empty triangle*), Celluclast 9 mg, supplemented by *M. fergusii* enzymes 3 mg (*filled circle*), *M. fergusii* 9 mg (*empty circle*).Celluclast 9 mg, supplemented by *M. cinnamomea* enzymes 3 mg (*filled square*), *M. cinnamomea* 9 mg (*empty square*). **b** Thermostable cellulases (T) 12 mg (*filled diamond*), T 9 mg (*empty diamond*), T 9 mg supplemented by *M. fergusii* enzymes 3 mg (*filled circle*), *M. fergusii* 9 mg (*empty circle*). T 9 mg supplemented by 3 mg *M. cinnamomea* (*filled square*), *M. cinnamomea* 9 mg (*empty square*)

Table 7 Carbohydrate composition of hydrolysates of hydrothermally pretreated wheat straw after 72 h hydrolysis at 45°C or 55°C, using different combinations of Celluclast (C), thermoenzyme mixture (T), *M. fergusii* (Mf) or *M. cinnamomea* (Mc) enzymes

Enzyme	Dosage (mg g^{-1} DW)	Glucose (%)	Xylose (%)	Arabinose (%)	Mannose (%)	Galactose (%)	Total amount (%)
Hydrolysis at 45°C							
C	9	50.5	3.0	0.0	0.0	0.0	53.5
C + Mf	9 + 3	62.0	3.7	0.0	0.0	0.0	65.7
Mf	9	3.2	1.3	0.1	0.1	0.6	5.4
C + Mc	9 + 3	69.4	4.1	0.0	0.0	0.0	73.4
Mc	9	3.1	1.3	0.1	0.1	0.1	4.7
C	12	59.5	3.4	0.0	0.0	0.0	63.0
Hydrolysis at 55°C							
T	9	65.8	4.0	0.0	0.0	0.0	69.8
T + Mf	9 + 3	67.6	4.2	0.0	0.0	0.1	72.0
Mf	9	5.8	1.4	0.1	0.2	1.3	8.6
T + Mc	9 + 3	67.0	4.1	0.0	0.0	0.0	71.1
Mc	9	3.9	1.2	0.1	0.0	0.0	5.3
T	12	72.3	4.4	0.0	0.0	0.0	76.7

Enzymes were dosed based on protein (mg g^{-1} substrate DW). The sugars were analyzed by HPLC and the values are expressed as % of total carbohydrates in the substrate

total cellulase activity (FPase) was produced on the carbon sources. Notable levels of general esterases were observed in *M. fergusii* or *M. cinnamomea* on wheat bran medium. Further analysis of esterase activity with special substrates to identify acetyl xylan esterase and feruloyl esterase activity would clarify the esterase characteristics in these fungi.

The good thermostability of the enzymes, an important characteristic of hydrolases in several application areas, was found with several hemicellulases as well as with endoglucanases. The co-operativity of these enzymes is important in achieving complete hydrolysis of various hemicelluloses; xylan and (galacto)glucomannan containing substrates. The performance of the produced hemicellulases was preliminary tested as complementary enzymes in the hydrolysis of hydrothermally pretreated wheat straw. The hemicellulolytic enzymes were shown to have a beneficial effect in the hydrolysis of lignocellulosic substrates. Thermostable xylanases, mannanases and side group cleaving activities have several other applications in various areas, as well.

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