British Biotechnology Journal
4(1): 30-39, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Screening of Filamentous Fungi for Xylanases and Cellulases Not Inhibited by Xylose and Glucose

L. F. C. Ribeiro[1], L. F. Ribeiro[1], J. A. Jorge[2] and M. L. T. M. Polizeli[2*]

[1]Immunology and Biochemistry Department of Faculdade de Medicina de Ribeirão Preto – USP, Ribeirão Preto, SP, Brazil.
[2]Biology Department of Faculdade de Filosofia Ciências e Letras de Ribeirão Preto – USP, Ribeirão Preto, SP, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Author LFCR participated in all operations of this manuscript. This work is part of her Doctor thesis. Author LFR carried out the Lichenase and laccase assays. He performed the analysis of data concerning these enzymes. Authors JAJ and MLTMP designed the study and wrote the protocol performed. Author MLTMP revised the manuscript and she is final responsible for all information presented. All authors read and approved the final manuscript.

Original Research Article

Received 21st July 2013
Accepted 5th October 2013
Published 26th October 2013

ABSTRACT

Aims: Screening different filamentous fungi for thermostable xylanases and cellulases that would not be inhibited by xylose and glucose, respectively.
Methodology: Samples of fungi collected in the Atlantic forest region, Minas Gerais, Brazil, and some fungi from our Culture Collection were used in this screening. All fungi were grown in liquid media containing 1% sugar cane bagasse (SCB). After that, an aliquot of the crude broth was incubated at different temperatures (from 4 to 60 °C) in carboxymethyl cellulose (CMC) or xylan-media plates, for 12 hours. After this period, the plates were stained with Congo Red. Fungi that presented the best results (larger halos) were tested for the effect of adding xylose and glucose in the xylanase and cellulases activities, respectively. Crude extracts obtained from fungi grown in SCB were used for laccase and lichenase assay.
Results: The screening on agar plates with CMC/xylan presented halos of different sizes. From all tested fungi, the best cellulase producer was *Malbranchea pulchella,* which also

**Corresponding author: Email: polizeli@ffclrp.usp.br;*

presented the most thermostable xylanase. *Penicillium griseofulvum* presented bigger halos at all temperatures tested, but the xylanase lost almost 14% of its stability in higher temperatures. The effect of xylose and glucose on the enzymatic activities recorded dose-dependent. It was observed that 20% activation of the enzymes produced by *M. pulchella* with 30 mM glucose or 20 mM xylose to cellulase and xylanase, respectively. It was observed a loss of less than 20% for *P. griseofulvum* xylolytic activity using 50 mM xylose. Lichenase was detected in some fungi prospected but laccase was not detected.
Conclusion: *Malbranchea pulchella* was a good producer of xylanase and cellulase tolerant to xylose and glucose, respectively. Other studies must be performed with this fungus so that it can be used in the future for biotechnological purposes.

Keywords: Malbranchea pulchella; xylanase; cellulases; xylose inhibition; glucose inhibition.

1. INTRODUCTION

Microbial enzymes have been commercially exploited and successfully used on industrial scale to catalyze several chemical processes [1]. In this context, filamentous fungi that are good enzyme producers are particularly interesting due to their easy cultivation and high production of extracellular enzymes with large industrial potential [2]. These enzymes may be applied in detergent, drinks and food, textile, animal feed, barking, pulp and paper, chemical and biomedical product industries [3,4].

Plant cell wall is a complex structure that surrounds and protects the cell. Its major structural components are cellulose, hemicelluloses and lignin. The degradation of this complex structure requires a complete enzymatic system that includes cellulases, xylanases ligninases and laccase [5]. Cellulolytic enzymes, which hydrolyze cellulose releasing glucose, can be divided into three categories: endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase or exoglucanase (exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1, 4-β-D-glucosidase, BG, EC 3.2.1.21) [6,7]. Lichenase, a cellulolytic enzyme, endo-1,3-1,4-β-glucanase, hydrolyzes the internal 1,4-β-glucosyl linkages when the glucosyl residue is linked at the O-3 position [8]. Xylanases degrade plant fibers made of xylan hemicellulose releasing xylose monomers or oligomers [3]. They comprise an enzymatic complex composed by endo-β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8), β-D-xylosidase (1,4-β-xylan xylanohydrolase, EC 3.2.1.37), and acting together with debranching enzymes (esterases) [2]. Laccase (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing oxidase that catalyzes the reduction of molecular oxygen to water, bypassing a stage of hydrogen peroxide production [9].

Cellulases and xylanases have great potential for industrial application, like bioconversion of lignocelluloses into fermentable sugar that may be used by yeasts to produce ethanol [10,11]. Laccase is an interesting enzyme in this context because it may fragment the lignin, releasing cellulose and hemicelluloses, easing the action of cellulases and xylanases [12].

Brazil has a large diversity of microorganisms due to the fact it comprises a vast physical territory where there are large areas of forests and vegetal diversity. There is a possibility of the existence of some fungi that are excellent producers of cellulases, xylanases and laccases that remain unknown. The aim of this work was to select filamentous fungi from nature in the region of Uberlândia-MG and from the mycoteca of our laboratory that are good

producers of xylanases, cellulases and laccases that are thermostable and not inhibited by their products.

2. MATERIALS AND METHODS

2.1 Microorganism

Filamentous fungi were isolated from decomposing trees in Atlantic forest region, Minas Gerais, Brazil. Fungal identification was performed at Departamento de Micologia da Universidade Federal de Pernambuco, Brazil. Stock cultures were maintained at 4°C in oat extract agar media and constant replications were performed at regular intervals

2.2 Screening on Agar Medium

Filamentous fungi were cultivated for 72 hours at optimum temperature of each fungus in modified SR [13] liquid medium. Ten milligrams yeast extract and 10 mg of peptone were added for 100 mL medium. The medium was modified to make a not so rich medium, so the fungus could grow using mainly the sugarcane bagasse as the carbon source and not yeast extract. Media were filtrated and used as enzymatic extract. A volume of 10 µL of extracts was placed inside a cavity of 7 mm diameter made in an agar medium. This medium contained the same salt solution used for SR medium added with 0.5% (w/v) xylan Birchwood (SIGMA) or 0.5% (w/v) carboxymethylcellulose sodium salt (CMC) with low viscosity (SIGMA). The cavity was filled on its base with agar 1% (w/v). These plates (90 mm x 15 mm) containing about 0.5 cm of agar layer depth were incubated at 30, 40 and 50 °C for 15 hours and stained with Congo Red [14].

2.3 Xylose and Glucose Effect on Xylanase and Cellulose Activities

Enzymatic assays were done by adding xylose or glucose in different concentrations (0 to 70 mM) in the reaction mixture. The substrate for xylanase was 0.2 % Remazol Brilliant Blue R-D-Xylan and for cellulose was 0.2 % Remazol Brilliant Blue Carboxymethylcellulose [15]. Both reactions were carried out with 100 mM sodium acetate buffer pH 5.0 and incubated at 50°C for 5 and 30 minutes, respectively. The reaction was stopped with the addition of 2 volumes of ethanol absolute. After centrifugation at 4000 x g for 5 minutes, the absorbance of the supernatant was read at 595 nm.

2.4 Quantitative Assay for Xylanase Activity

The amount of xylanase produced was measured by using 1% xylan Birchwood, SIGMA, as substrate [16]. Xylanase activity was assayed in 200 µL of a reaction mixture containing 50 µL of crude broth, 100 µL of 1% xylan from Birchwood (prepared in 0.1 M sodium acetate buffer, pH 5.0) and 50 µL of 0.1 M sodium acetate buffer, pH 5.0. The mixture was incubated at 50°C for 5 min. The reaction was stopped by the addition of 200 µL of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 5 min [17]. The absorbance at 540 nm was read and the amount of reducing sugars released was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme that releases 1 µmol of xylose in 1 min under the assay conditions.

2.5 Quantitative Assay for Cellulase Activity

Cellulase (CMCase) activity was determined by mixing 100 µL of 1% (w/v) Carboxymethyl cellulose sodium salt, medium viscosity, SIGMA with 0.7 degree of substitution, (prepared in 0.1 M sodium acetate buffer pH 5.0) with 150 µL of crude broth and 50 µL 0.1M sodium acetate buffer. The reaction mixture was incubated at 50°C for 30 min. The reaction was stopped by the addition of 300 µL of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 5 min. The absorbance at 540 nm was read and the amount of reducing sugars released was quantified using glucose as standard. One unit of enzyme activity is defined as the amount of enzyme that releases 1 µmol of glucose in 1 min under the assay conditions.

2.6 Quantitative Assay for Lichenase Activity

β-1,3–1,4-Glucanase activity was assayed by the determination of reducing sugars released from lichenan substrate (MP Biomedical – Solon, USA) using the 3,5-dinitrosalicylic acid (DNS) method [17]. The assay mixture (0.5% (w/v) lichenan, 50 mM MES - 2-(N-morpholino) ethanesulfonic buffer, pH 6.0) was incubated with the crude broth for 10 min and the reaction was stopped by the addition of DNS reagent. The absorbance at 540 nm was read and the amount of reducing sugars released was quantified using glucose as standard. One unit of enzyme activity is defined as the amount of enzyme that releases 1 µmol of glucose in 1 min under the assay conditions.

2.7 Quantitative Assay for Laccase Activity

Laccase activity was determined by the rate of oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which was monitored at 420 nm ($\varepsilon = 36000\ M^{-1}cm^{-1}$) [18]. The assay mixture contained crude broth, 1 mM ABTS and 50 mM acetate buffer, pH 4.5.

2.8 Soluble Protein Assay

Protein content of the culture supernatant was determined according to the method described by Bradford [19] using bovine serum albumin (BSA) as standard.

2.9 Analysis of Reaction Products by TLC – Thin Layer Chromatography

Xylan and CMC degradation products were qualitatively determined by thin-layer chromatography (TLC) on precoated TLC sheets (silica gel; DC-Alufolien Kieselgel 60, Merck) revealed with n-butanol-ethanol-water (5:3:2, vol/vol/vol). The products were visualized by spraying the layers with a 1:1 (vol/vol) mixture of 0.2% methanolic orcinol and 20% sulfuric acid [20].

3. RESULTS AND DISCUSSION

The screening on agar plates with CMC or xylan media presented many halos with different sizes (Table 1). This experiment was done to select the fungus that produced enzymes with the best activities and higher thermostability. From all the tested fungi, the best cellulolytic enzyme producers were *Malbranchea pulchella* and *Penicillium griseofulvum.* Among those, *M. pulchella* presented the higher stability at 50°C (Fig. 1 and Table 1), which can be

indirectly measured by the size of the halo. Since this fungus is classified as a thermophilic mold [21], this result was expected. The best producer of xylolytic enzymes was *Aspergillus clavatus*, that presented bigger halos at all the temperatures tested, but it lost almost 14% of its stability in higher temperatures (50°C).

Table 1. Diameter of halos produced by the hydrolysis of CMC or xylan from Birchwood

Specie	Halo diameter in CMC (cm)				Halo diameter in xylan (cm)			
	4°C	30°C	40°C	50°C	4°C	30°C	40°C	50°C
M. pulchella	0 ±0	1.30 ±0.1	1.63±0.1	1.82 ±0.1	0 ±0	1.80 ±0.1	1.94 ±0.0	2.22 ±0.1
T. longibrachiatum	0 ±0	1.22 ±0.1	1.41±0.0	1.01 ±0.0	0 ±0	1.82 ±0.0	2.05 ±0.1	2.03 ±0.1
A. clavatus	0 ±0	0 ±0	1.13±0.1	1.34 ±0.1	1.21±0.0	2.89 ±0.1	2.61 ±0.0	2.51 ±0.0
A. terreus albino	0 ±0	1.30 ±0.0	1.50±0.0	1.61 ±0.0	0 ±0	1.47 ±0.0	1.73 ±0.0	1.72 ±0.0
F. oxysporum	0 ±0	1.2 ±0.1	1.42±0.1	1.40 ±0.1	0 ±0	1.01 ±0.1	1.28 ±0.1	0 ±0
P. griseofulvum	1.0±0.0	1.61 ±0.1	1.78±0.1	1.61 ±0.1	0 ±0	1.62 ±0.0	1.83 ±0.0	1.60 ±0.0
Aspergillus sp	0 ±0	1.22 ±0.1	1.39±0.0	1.0 ±0.1	0 ±0	1.31 ±0.0	1.51 ±0.1	1.52 ±0.1

The experiment was conducted in triplicate.

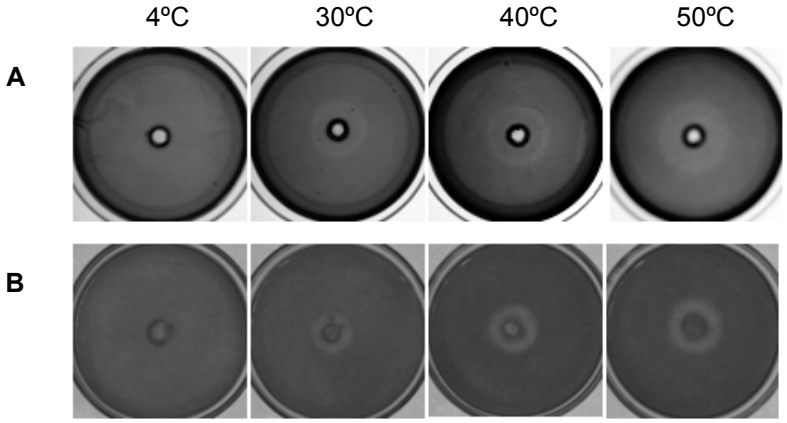

Fig. 1. *M. pulchella* halos in xylan (A) and CMC (B) after the incubation at different temperatures. Plates were incubated at the temperatures for 15 hours

The halo detection generated by the enzymatic hydrolysis is an easy and valuable way to do this screening. There is still a resistance in using methods of screening for filamentous fungi. Some authors use this technique by inoculating the fungus directly on the plate and measuring the generated halo [22,23], but this assay is not always straight forward because depending on the fungus, it may sporulate too much and the spores may spread over the plate as previously seen in our results (not shown). If this happens, more than a unique colony will grow on the plate, making it confusing to interpret the results.

Another advantage of our screening method is that it was possible to qualitatively analyze the thermostability by incubating the plates at different temperatures. It saves time and work because for the usual screening method it is necessary to incubate a certain amount of crude broth at different temperatures and only after that the enzymatic activity is measured [24]. When the value of cellulase activity is low, it will be even more difficult to observe differences concerning the thermostability.

3.1 Glucose and Xylose Can Enhance Glycosyl Hydrolase Activities

Another parameter that was interesting to investigate with *M. pulchella* and *P. griseofulvum* was the effect of monosaccharides, the end products of the action of the xylolytic and cellulolytic complexes. So, those activities were measured with different concentrations of xylose and glucose, respectively. As *M. pulchella* was stable at the highest temperature tested (50 °C, Table 1) and *P. griseofulvum* presented good results for xylanase and cellulose activities (Table 1), they were selected to test the effect of monosaccharides amendment to the reaction mixture. *A. clavatus* was discarded despite having the highest activity in xylan but it showed low activity in CMC (Table 1).

The results obtained showed that it seems to exist an activation of the enzymes produced by *M. pulchella* (Fig. 2, A). This activation was dose dependent, but on the other hand the inhibition of these enzymes by their end products (Figs. 2, A and B) was not observed. For the xylolytic activity of *P. griseofulvum* and for both enzymatic activities (xylolytic and cellulolytic) of *M. pulchella*, it was observed a loss of less than 20% from 20 mM and 40 mM of the monosaccharides concentration, respectively (Figs. 2, A and B) and from this on their activities remained constant. However, *P. griseofulvum* cellulases were inhibited by glucose. Xiros et al. [25] observed that for *Fusarium oxysporum* the presence of xylose in concentrations varying from 1 g/L up to 10 g/L did not inhibited xylanase activity, as it has been seen for *P. griseofulvum* in this work (Fig. 2 B).

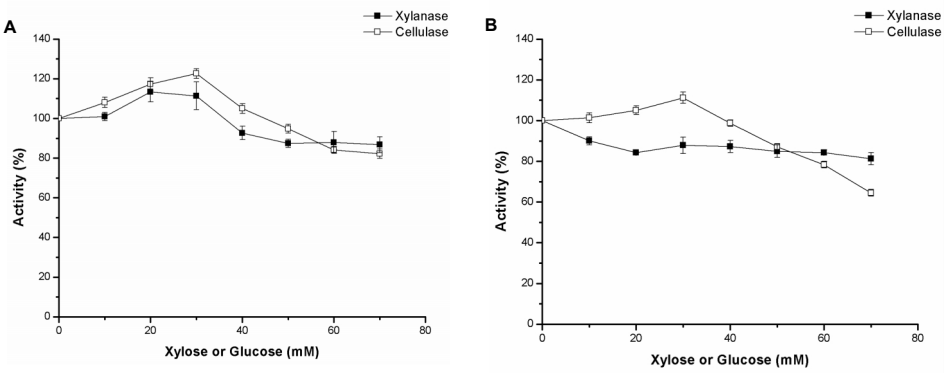

Fig. 2. Xylose or glucose effect on xylolytic and cellulolytic activities, respectively. This assay was carried out with Remazol Brilliante Blue Xylan or CMC. A – *Malbranchea pulchella*; B – *Penicillium griseofulvum*

3.2 Elucidating the End Products of Xylanases and Cellulases

In order to investigate the hydrolysis products released by the enzymes and to better understand the effect of monosaccharides on xylolytic and cellulolytic activities, an enzyme assay using xylan or CMC as substrates was done. After a period of up to 12 hours of incubation at 50°C, the crude broths were examined by TLC (Fig. 3). The results revealed that for *M. pulchella,* xylose was not released even after 12 hours of incubation. However, glucose was detected with incubation of 12 hours, but not before this time. This may be the reason that there were not strong negative effects of those sugars over xylanases and cellulases activities (Fig. 2). Those enzymes are usually inhibited by their direct products [26], but it was not observed in this work. In order to *Penicillium griseofulvum*, it was not

possible to observe the presence of both monosaccharides even after 12 hours of incubation (Fig. 3). Also, the effect of these sugars over the enzymatic activity did not present a high inhibition (Fig. 2 B), moreover cellulolytic activity showed a discrete activation with 30 mM glucose (Fig. 2 B).

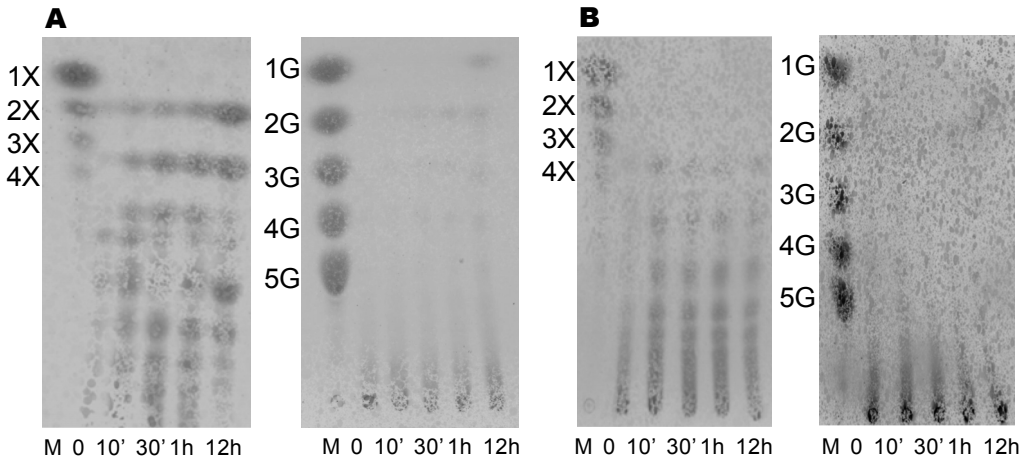

Fig. 3. TLC of xylolytic and cellulolytic enzymes, respectively. A – *Malbranchea pulchella*; B - *Penicillium griseofulvum*. 1 to 4 X represent the monomer xylose and the oligosaccharides containing 2 to 4 residues of xylose. The same for 1 to 5 G, except that it is for glucose. M – pattern of the mono and some oligosaccharides. 0 to 12h – time of incubation of the crude broth with the substrate (xylan or CMC)

3.3 Investigation of Lichenase and Laccase Activities

All seven fungi (Table 1) were grown in liquid medium containing sugar cane bagasse as carbon source because it is a complex source that could allow the screening of xylanases, cellulases and maybe ligninases. Therefore, screening for lichenase and laccase was also carried out (Table 2). Results of the xylanase, cellulase, lichenase and laccase activities are shown in Table 2. *A. clavatus* presented the highest lichenase activity (an endoglucanase that hydrolyzes specifically β1-4 linkages followed by β1-3 bonds), followed by *A. terreus* albino, *F. oxysporum* and *P. griseofulvum,* that presented lichenase activity higher 3 U/mL. These results are very useful because there is a scarcity of data in the literature regarding filamentous fungi that secrete lichenases. Laccase was not detected in any fungi, although it is mainly produced by filamentous fungi [27]. This enzyme was assayed in an attempt to make a relation between hemicellulosic activities with the degradation of lignin. In this way, the aim of this experiment was to check if a fungus that possesses laccase activity would be a better producer for xylolytic and cellulolytic enzymes. Unfortunately it was not possible to do this kind of comparison since laccase activity was not detected.

As it can be seen in the Table 2, all the fungi analyzed presented high values of xylanase activity when grown in sugarcane bagasse as the carbon source (Table 2). Souza et al. [28] found much reduced xylanase activity (smaller than 1 U/mL) when they grew *A. niveus* in sugarcane bagasse as carbon source [28], however, Mahamud and Gomes recorded it over 9 U/mL for *Trichoderma* sp grown with 2% sugarcane bagasse [29]. In order to *A. niger* it

was about 3 U/mL, smaller than the values obtained in this work in which the xylanase activity varied from 6.81 to 19.93 U/mL (Table 2).

The results for CMCase activity were very poor in comparison to the earlier reports in literature. The activity obtained in this work were lower than 0.2 U/mL (Table 2), while the literature shows activities varying from close to 0.25 U/mL [28] for *A. niger* until less than 0.3 U/mL for *Trichoderma viride* when the fungi were grown in sugarcane bagasse [30]. So it seems that the fungi tested are not good producers of cellulolytic enzymes when grown in sugarcane bagasse.

Table 2. Screening of lignocellulolytic enzymes for fungi grown in liquid medium containing sugar cane bagasse as carbon source

Sample	Specie	Enzyme Activity (U/mL)			
		Cellulase	Xylanase	Lichenase	Laccase
M. pulchella	*Malbranchea pulchella*	0.049 ±0.008	9.2 ±0.3	1.2 ±0.09	ND
LF212	*Trichoderma longibrachiatum*	ND	11.8 ±0.8	2.0 ±0.2	ND
Acla	*Aspergillus clavatus*	ND	19.93 ±1.28	5.45 ±0.41	ND
Ateralb	*Aspergillus terreus albino*	0.162 ±0.025	10.18 ±0.71	3.47 ±0.23	ND
FusRosa	*Fusarium oxysporum*	0.070 ±0.011	11.78 ±0.58	3.64 ±0.27	ND
V4	*Penicillium griseofulvum*	0.032 ±0.009	6.81 ±0.16	4.44 ±0.27	ND
LF32	*Aspergillus* sp.	0.041 ±0.005	9.57 ±0.57	2.15 ±0.11	ND

ND - Not Detected.

4. CONCLUSION

It is important to find good producers of lignocellulolytic enzymes, because of its wide application. These enzymes may be used in paper and pulp industries, bioethanol production, animal feed industry, etc. From all tested fungi, *Malbranchea pulchella* exhibited the best set of results. It presented pronounced halos in CMC and in xylan, as well as high thermostability and it was not inhibited by glucose or xylose. Actually, it presented a little activation in low concentrations of monosaccharides, both for cellulase and xylanase activities, but did not present inhibition by both sugars. This result is interesting because *Malbranchea pulchella* can be used in biorefineries aiming the production of second generation ethanol.

ACKNOWLEDGEMENTS

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho de Desenvolvimento Científico e Tecnológico (CNPq) and the National System for Research on Biodiversity (Sisbiota-Brazil, CNPq 563260/2010-6/FAPESP nº 2010/52322-3). J.A.J. and M.L.T.M.P. are Research Fellows of CNPq. L.F.C.R. and L.F.R. are recipients of FAPESP Fellowships. We thank Ricardo Alarcon and Mauricio de Oliveira for technical assistance and Mariana Cereia for English support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sohail M, Naseeb S, Serwani SK, Sultana S, Aftab S, Shahzad S, Ahmad A, Khan SA. Distribution of hydrolytic enzymes among native fungi: *Aspergillus* the pre-dominant genus of hydrolase producer. Pak. J. Bot. 2009;41(5):2567-2582.
2. Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS. Xylanases from fungi: properties and industrial applications. Appl. Microbiol. Biotechnol. 2005;67:577-591.
3. Guimarães LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo F, Aquino ACMM, Junior AB, Polizeli MLTM. Screening of filamentous fungi for production of enzymes of biotechnological interest. Braz. J. Microbiol. 2006; 37:474-480.
4. Whiteley CG, Lee DJ. Enzyme technology and biological remediation. Enz. Microb. Technol. 2006;38:291-316.
5. Caffall KH, Pattathil S, Phillips SE, Hahn MG, Mohnen D. *Arabidopsis thaliana* T-DNA mutants implicate GAUT genes in the biosynthesis of pectin and xylan in cell walls and seed testa. Mol. Plant. 2009;2,1000–1014.
6. Li YH, Ding M, Wang J, Xu GJ, Zhao F. A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulose degrading bacterium, *Bacillus* sp. AC-1. Appl. Microbiol. Biotechnol. 2006;70:430-436.
7. Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. Bioresour. Technol. 2008;99:7623-7629.
8. Pang Z, Kang YN, Ban M, Oda M, Kobayashi R, Ohnishi M, Mikami, B. Crystallization and preliminary crystallographic analysis of endo-1,3-β-glucanase from *Arthrobacter* sp. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2005;61:68-70.
9. Morozova OV, Shumakovich GP, Gorbacheva MA, Shleev SV, Yaropolov AI. “Blue” laccases. Biochemistry. 2007;72,1136-1150.
10. Viikari L, Kantelinen A, Sundqvist J, Linko M. Xylanases in bleaching: from an idea to the industry. FEMS Microbiol Rev. 2001; 13: 335-350.
11. Gomes J, Purkarthiofer HM, Kapplomiller J, Sinnner M, Steiner W. Production of high level of cellulase–free xylanase by the thermophilic fungus, *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. Appl. Microbiol. Biotechnol. 1993;39:700-707.
12. Couto SR, Herrera JLT. Industrial and biotechnological applications of laccases: A review. Biotechnol. Adv. 2006;24:500-513.
13. Rizzatti ACS, Sandrim VC, Jorge JA, Terenzi HF, Polizeli MLTM. Influence of temperature on the properties of the xilanolytic enzymes of the thermotolerant fungus *Aspergillus phoenicis*. J. Ind. Microbiol. Biotechnol. 2004;31:88-93.
14. Teather RM, Wood PJ. Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 1982;4:777-780.
15. Biely P, Mislovicová D, Toman R. Soluble chromogenic substrates for the assay of endo-1,4-beta-xylanases and endo-1,4-beta-glucanases. Anal. Biochem. 1985;144(1):142-146.

16. Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 1992;23:257-270.
17. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 1959;31(3):426-428.
18. Arnold FH, Georgiou G. Directed enzyme evolution, screening and selection methods. Methods Mol. Biol. 2003;230:3-26.
19. Bradford MM. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 1976;72:248-254.
20. Fontana JD, Gebara M, Blumel M, Schneider H, Mackenzie CR, Johnson KG. α–4–O–methyl–D–glucuronidase component of xylanolytic complexes. Methods Enzymol. 1988;160,560-571.
21. Banerjee S, Archana A, Satyanarayana T. Xylose metabolism in the thermophilic mould *Malbranchea pulchella* var. *sulfurea* TMD-8. Curr. Microbiol. 1994,29:349-352.
22. Sridevi B, Charya MAS. Isolation, identification and screening of potential cellulose-free xylanase producing fungi. Afr. J.Biotechnol. 2011;10:4624-4630.
23. Abdel-Sater MA, El-Said AHM. Xylan-decomposing fungi and xylanolytic activity in agricultural and industrial wastes. Internat. Biodeter..Biodegrad. 2001;44:15-21.
24. Alves-Prado HF, Pavezzi FC, Leite RS, de Oliveira VM, Sette LD, Dasilva R. Screening and production study of microbial xylanase producers from Brazilian Cerrado. Appl Biochem Biotechnol. 2010;161:333-346.
25. Xiros C, Katapodis P, Chirstakopoulos P. Factors affecting cellulose and hemicelluloses hydrolysis of alkali treated brewers spent gain by *Fusarium oxysporum* enzyme extract. Bior. Technol. 2011;102:1688-1696.
26. Andrić P, Meyer AS, Jensen PA, Dam-Johansen K. Effect and modeling of glucose inhibition and in situ glucose removal during enzymatic hydrolysis of pretreated wheat straw. Appl. Biochem. Biotechnol. 2010;160:280-297.
27. Xu F, In Flickinger MC, Brew SW. Encyclopedia of bioprocessing technology: fermentation, biocatalysis and bioseparations. Wiley, New York. 1999;1545-1554.
28. Souza WR, Gouveia PF, Savoldi M, Malavazi I, Bernardes LAS, Goldman LH, de Vries RP, Oliveira JVC, Goldman GH. Transcriptome analysis of *Aspergillus niger* grown on sugarcane bagasse. Biotechnol. Biofuels. 2011;4:40.
29. Mahamud MR, Gomes DJ. Enzymatic saccharification of sugar cane bagasse by the crude enzyme from indigenous fungi. J. Sci. Res. 2011;4(1),227-238.
30. Ahmed FM, Rahman SR, Gomes DJ. Saccharification of sugarcane bagasse by enzymatic treatment for bioethanol production. Malaysian J. Microbiol. 2012;8(2):97-103.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=282&id=11&aid=2380