Current Trends in Biotechnology and Pharmacy
Vol. 11 (4) 414-423 October 2017, ISSN 0973-8916 (Print), 2230-7303 (Online)

Screening, Production and Partial Characterization of Xylanases from Woodchips Fungi with Potential Application in Bioethanol Production

Ntsoaki Leticia Mosina[1], Suresh Babu Naidu Krishna[2*], Lucretia Ramnath[1] and Roshini Govinden[1]

[1]Department of Microbiology, School of Life Sciences, Westville Campus, University of KwaZulu-Natal, Durban-4000, South Africa. Email - Govindenr@ukzn.ac.za; Tel: +27 31 260 8281

[2]Research & Post Graduate Support, Durban University of Technology, Durban-4000, South Africa. Email - Sureshk@dut.ac.za; Tel: +27 31 373 3093

*For Correspondence - sureshk@dut.ac.za

Abstract

The objective of this present study was to isolate, identify and screen for potential fungal isolates from local wood chips with respect to xylanase production. The fungal strains were isolated from degrading wood chips. All the fungal strains were screened for their ability to produce xylanase by the plate screening method using Congo red as indicator. Two isolates were further selected among them and screened for the production of the enzymes in liquid medium and identified by 18S rRNA. *Phialophora alba*, had the highest xylanase activity of 24.43 U/ml with temperature optima at 50°C and 90°C respectively. Enzymatic hydrolysis of pre-treated sugarcane bagasse using crude enzyme resulted in the production of 0.36 g/ml reducing sugars after 48 hours. This is the first report of a thermophilic xylanase from *Phialophora alba*. These results suggest that the application of this xylanase in bioethanol production may be very promising.

Keywords: Xylanase, *Phialophora alba*, Screening, Cultural conditions, Bioethanol production

Introduction

Xylanases (EC 3.2.1.8) catalyse the hydrolysis of internal β (1, 4) glyosidic linkages connecting xylopyranosyl units in xylan. This polysaccharide is the most abundant among the hemicellulosic materials and contributes to cohesion and integrity of plant cell walls, at the interface between lignin and cellulose (1, 2). Diverse forms of these enzymes exist, showing varying folds, mechanisms of action, substrate specificities, hydrolytic activities (yields, rates and products) and physicochemical characteristics (3).

Interest in xylanolytic enzymes has intensified in the past two or three decades due to their potential industrial application in the food, feed, and pharmaceutical industries and for sustainable production of fuels and chemicals (4). Also, they can be applied in some processes in which cellulolytic activity must be absent, to preserve vegetal fibres, in the pulp and paper industries (1), and in the processing of flax (5), hemp and jute in the textile industries (6). For commercial purposes, many xylanases have been highly expressed in heterologous systems, such as *Escherichia coli*, *Bacillus* spp. and *Pichia pastoris* (7, 8). The most widely used xylanases are from the fungal genera *Trichoderma*, *Aspergillus* and *Penicillium*, and these enzymes are generally highly active over a temperature range of 40–60° C (9). At these temperatures, complete saccharification of biomass polysaccharides requires a long reaction time with high contamination risks (10). Therefore, high-temperature active xylanases are needed to enhance the mass transfer and reduce the substrate viscosity (11).

When a new efficient xylanase-producing microorganism is isolated, it is essential to purify and characterize the enzymes to know the action towards substrates of each component of a xylanolytic complex, its regulation and biochemical properties in order to develop more competitive processes. In the current study, several fungal strains were isolated from Eucalyptus spp. woodchips and tested for their ability to produce xylanases. Among 46 strains isolated (Table 3.1), a strain producing a thermostable and thermoactive xylanase was identified using PCR amplification and sequencing to be *Phialophora alba*.

Materials and Methods

Isolation and initial screening of microbial strains: A primary screening was conducted among microbial strains for xylanase production from wood chips collected from Sappi Saiccor, Umkomaas, KwaZulu-Natal, South Africa. Microorganisms were screened for xylanase activity on nutrient agar plates supplemented with 1% birchwood xylan (Sigma-Aldrich, Switzerland) and 1% low sulfonate lignin (Sigma-Aldrich, Switzerland), respectively. The pure cultures were incubated at 50°C to select for thermophilic microorganisms. Zones of clearing which were indicative of enzyme activity were visualized upon staining with 0.1% Congo Red and destaining with 1 M NaCl. The diameters of the zones of hydrolysis were subsequently measured. Fungal isolates were selected based on differences in macroscopic characteristics the size of the zone of hydrolysis and subjected to further analysis. These strains were maintained on potato-dextrose agar plates with subculturing every three months.

Production of xylan-degrading enzyme: Enzymes were produced by cultivation of the fungal isolates using 50 ml medium in 250 ml shake flasks on an orbital shaker at 200 rpm using modified mineral media (pH 5) for the cultivation of fungi as described by Gomes et al. (12). One litre of media contained 5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2$. $2H_2O$, 5 g NH_4NO_3, 1% Birchwood xylan and 1 ml trace-element solution [g.l-1: $MnCl_2.4H_2O$ (0.03), $ZnSO_4.7H_2O$ (1.4), H_3BO_3 (0.3), $CoCl_2.6H_2O$ (0.2), $CuCl_2.2H_2O$ (0.01), $NiCl_2.6H_2O$ (0.02), $NaMoO_4.H_2O$ (0.0277).$6H_2O$, $CoCl_2.6H_2O$]. Samples were withdrawn periodically under aseptic conditions and assayed for xylanase activities. One millilitre of culture medium was removed from each flask and centrifuged at 13 000 rpm for five minutes. Time course for optimal enzyme production was determined for six isolates using the cell-free supernatant. The amount of reducing sugars liberated was determined using the DNS (dinitrosalicylic acid) assay. All experiments were done in triplicate.

Enzyme assays: Xylanase activity was assayed spectrophotometrically at 540 nm by the dinitrosalicylic acid (DNS) method as described previously (13) using birchwood xylan (1%) as substrate. Xylanase activity was expressed as nkat.ml-1 where one unit of enzyme activity (nkat) was defined as the amount of enzyme one nmol of xylose liberated per second, under the assay conditions.

Total protein concentration was determined using the Bradford assay (14) using bovine serum albumin (BSA) as the standard. Specific activity was determined by dividing xylanase activity (nkat.ml-1) by protein concentration (mg.ml-1) and expressed as U, where one U is equivalent to one $nkat.mg^{-1}$.

Temperature and pH stability: The optimum temperature of crude xylanase was determined by incubating the culture filtrates in a water bath at various temperatures (from 40 to 90°C) for 15 min before the reaction was stopped. The optimum pH of crude xylanase was determined at optimum temperature by carrying out the enzyme assay at different pH values (pH 4-9) using the following buffers: using the DNS assay as described by Bailey et al. (13) 50 mM citrate phosphate (pH 4.0, 5.0 6.0, 7.0, 8.0 and 9.0).

Native PAGE gel electrophoresis: A 10% Native PAGE gel supplemented with 1% Beechwood xylan (250 µl) was done as previously described by Ninawe et al. (15). Briefly, a substrate gel was

prepared containing 1% Birchwood xylan. The PAGE gel was placed onto the substrate gel and incubated for 1 hour at 50°C. After incubation, the protein gel was stained and destained. The activity gel was stained for 2 hours with 0.1% Congo Red and destained with 1 M NaCl. The gel was fixed by washing with 0.5% acetic acid. The crude enzyme was loaded, separated by electrophoresis and subsequently stained with Congo red for 1 hour and destained with 1M NaCl

Molecular identification of Fungal isolates: Genomic DNA extraction: The fungal isolates were cultivated in malt extract broth (MEB) (Sigma-Aldrich, Switzerland) and incubated at 30°C for 3 days. After incubation, cultures were centrifuged at 10 000 × g for 3 minutes and the pellets were re-suspended in 200 µl of sterile distilled water. Both fungal and bacterial DNA extraction was performed using a ZR soil microbe DNA kit (Zymo Research, USA). DNA samples were stored at -20°C.

PCR amplification of the 18S ribosomal ITS region of the 18S rRNA gene: The primer pair ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-CCTCCGCTTATTGATATGCTAAG-3') were used to amplify the ITS1-5.8S-ITS2 region of the fungal isolates F1 and F10. The universal bacterial primers 63F (5'-CAGGCCTAACA CATGCAAGTC-3') and 1387R (5'-GGGCGG(A/T)GTGTACAAGGC-3') were used for the amplification of a segment of the bacterial genomic DNA. In the PCR reaction mixture 10 mM buffer (5 µl), 25 mM MgCl2 (2 µl), 2.5 µM each of the forward and reverse primers (2.5 µl), 250 U Super-ThermTaq polymerase (Fermentas, South Africa)) (0.5 µl), 10 mM dNTPs (5 µl) were added. Five microlitres of template DNA was added to the reaction mixture and brought up to volume with sterile double distilled water. Amplification of a 50 µl PCR mix was performed using the following thermal cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 31 cycles of denaturation (95°C) for 1 minute; annealing (55°C) for 1 minute and extension (72°C) for 1 and a half minutes. A final extension step was conducted at 72°C for 5 minutes.

PCR products were visualized by electrophoretic analysis on a 1% agarose gel. Once the presence of the PCR amplicon was confirmed, they were sent for sequencing to Inqaba Biotech (Pretoria, South Africa). BLAST searches of the amplified regions were conducted to identify the isolates.

Enzymatic hydrolysis of pre-treated sugarcane bagasse: Enzymatic hydrolysis was performed using 1% (w/w) substrate in 50 mM citrate buffer (pH 5) with a final reaction volume of 50 ml as described by Adsul et al. (16). Briefly, the sugar cane bagasse (SCB) was dried in an oven overnight at 60°C. The dried samples were milled to particles of 0.5-1 cm in size and passed through 0.5 mm and 1 cm screens. The milled SCB (20 g) was mixed with 450 ml of distilled water and served as no pretreatment control. Another experiment was set up in which the milled SCB was pre-treated with alkali by adding 20 g of the milled substrate to an Erlenmeyer flask containing 450 ml of 2N NaOH. The flask was incubated at 30°C for 20 hours followed by several washing steps with distilled water to neutralize the pH of the preparation. Thermal and pressure pretreatment were conducted simultaneously by autoclaving (121°C, 0.103 MPa) an Erlenmeyer flask containing 20 g of substrate and 450 ml of distilled water for 90 minutes. All preparations (untreated, thermal/pressure and alkali) were filtered through 0.45 µm filters to remove the liquid phase. The remaining SCB was used in subsequent hydrolysis studies. Enzymatic hydrolysis was performed using 1% (w/w) substrate in 50 mM citrate buffer (pH 5) with a final reaction volume of 50 ml. The thermally inactivated crude enzyme and partially pure xylanase served as a control for each pretreatment process and the untreated SCB. Hydrolysis of the pretreated SCB was carried in duplicate for each type of treatment. All flasks were incubated at 50°C with shaking at 150 rpm. Samples (1 ml) were withdrawn after 0, 24 and 48 hours. All samples were filtered using 0.45 µm filters and the supernatant assayed for residual sugars using the DNS assay. All

experiments and analyses were carried out in triplicate.

Results and Discussion

Enzyme production: Amongst 46 microorganisms isolated from *Eucalyptus* spp. woodchips, 46% were positive for xylanase activity. Furthermore, mixed population of bacterial and fungal species were isolated from *Eucalyptus* spp. woodchips. Selection of isolates was based on differences in morphology, macroscopic characteristics and the size of the zone of hydrolysis. Only the fungal isolates were selected for further characterization. (Table 1). Fungal isolates F1, F2, F6 and F10 displayed relatively large zones of clearing (Table 1) in comparison to the bacterial isolates (data not shown). No ligninase activity was observed for the fungal isolates.

Among the selected microorganisms, fungal isolates F2 and F10 produced optimal quantities of xylanase after 5 days while F1 and F10 required a cultivation period of 7 days in order for optimal amounts of xylanases to be achieved. F1 and F10 demonstrated highest enzyme activity amongst the fungal isolates with specific activities of 12.25 U and 24.43 U, respectively. This is in agreement with enzyme titres obtained in studies conducted by Gomes et al. (12) and Palaniswamy et al. (17). Based on the desirable activities displayed by selected isolates, further characterization in terms and pH and temperature optimum, stability, molecular identification and molecular weight was determined

Enzyme Characterization

Xylanase production and optimization of fermentation parameters: Preliminary studies were conducted in 0.1 M sodium citrate buffer, pH 5 containing birchwood xylan as the primary carbon source for production of xylanase at under shaking conditions (200 rpm). For xylanase activity, the DNS method (18) is used under standard conditions. The fungal isolates F1, F2, F6 and F10 produced high levels of xylanase. Highest levels of production were observed for F1 and F10 with specific activities of 12.58 U and 24.23 U, respectively after 7 days of cultivation (Fig. 1). Enzyme activity in relation to protein concentration was low for the remaining fungal isolates in comparison to F1 and F10.

The pH optima determined for the two fungal

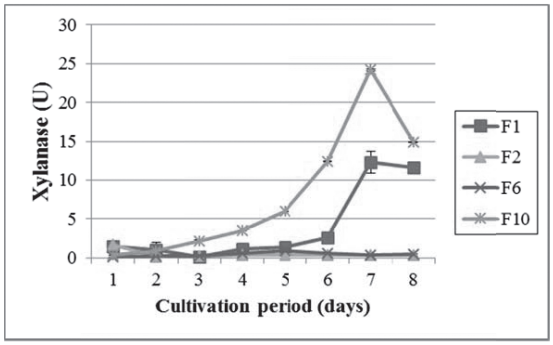
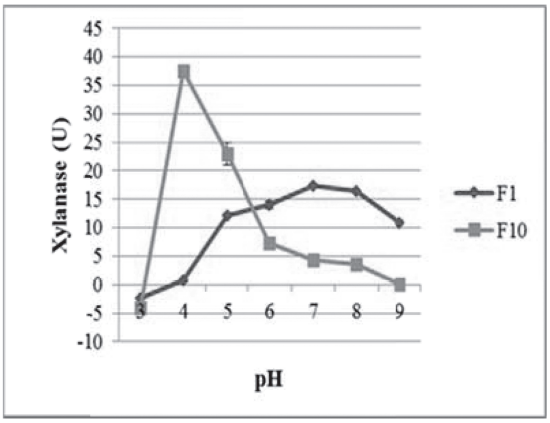


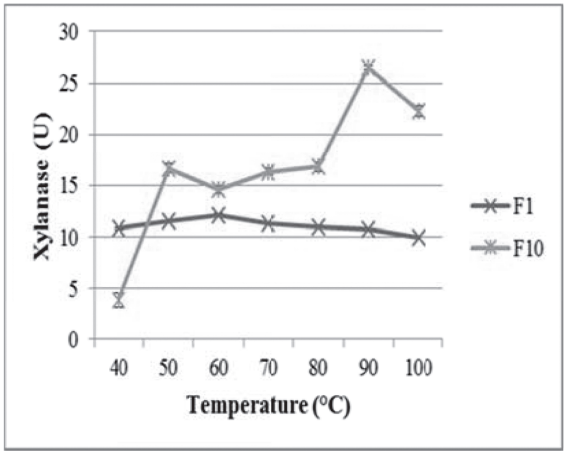
Fig 1. Time course for optimal xylanase production for fungal isolates F1, F2, F6 and F10 isolated from woodchips.

(F1 and F10) xylanases, F1 xylanases displayed a neutral optimum pH with a corresponding specific activity of 17.33 U. However, F10 xylanases displayed an acidic pH optimum of pH 4 and a corresponding specific activity of 37.51 U (Fig. 2a & b). F1 displayed a broad optimum temperature within the range of 40-100ºC with a slight increase at 60ºC (specific activity of 12.15 U). However, F10 displayed two distinct peaks



2a. pH optima of F1 and F10 xylanases.

within its temperature profile at 50ºC and 90ºC with corresponding production levels of 16.68 U and 24.5 U.



2b. Temperature optima of F1 and F10 xylanases
Fig. 2. pH and temperature optima studies of fungal isolates F1 and F10.

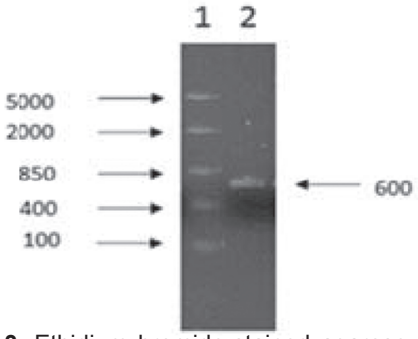


Fig. 3. Ethidium bromide-stained agarose get containing PCR products 18S rRNA amplicon of F10 fungal DNA lane I 100 bp Fast Ruler middle range DNA ladder, lane 2 18S PCR amplicon

Molecular identification of fungal isolate: Substantial xylanase activity of 37.51U was detected in the culture supernatant of F10 strain when birchwood xylan (1%) is used as substrate. The crude enzyme was precipitated using 20% ammonium sulphate to homogeneity and native PAGE and is performed. Native PAGE analysis

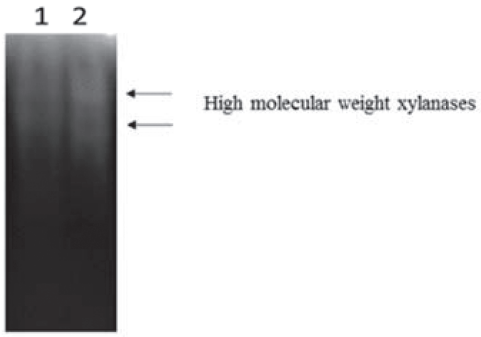


Fig. 4. 10% Native PAGE gel supplemented with 1% beechweed xylan showing the isozymes present in the crude enzyme.
lane 1 : crude enzyme, *lane* 2:10+ concentrated crude enzyme. Each *lane* was loaded with 15 ug of crude enzyme

(Fig. 4) indicated the presence of more than three high molecular weight xylanase isozymes.

Bioethanol production using crude enzyme: The enzymatic hydrolysis of SCB was established using the crude enzyme and partially pure xylanase preparations from *P. alba*. The milled SCB was subjected to two different types of pretreatment conditions prior to enzymatic saccharification (alkali and temperature/pressure). The reducing sugars liberated after pretreatment with alkali and high temperature and pressure was difficult to determine as the high NaOH concentrations in the alklali-treated SCB interfered with the DNS assay. Sugar concentrations below 0.4 g/ml were present after 24 and 48 hours (Fig 5) in the untreated control in the absence of enzyme. The addition of crude enzyme preparation to the control produced tenfold higher reducing sugar levels after 48 hours.

(N: untreated+buffer; NP: untreated+partially pure xylanase; NC: untreated+crude enzyme; T: thermal treatment+buffer; TP: thermal treatment+partially pure xylanase; TC: thermal treatment+crude enzyme; A: alkaline treatment+buffer; AP: alkaline treatment+partially pure xylanase; AC: alkaline treatment+crude enzyme). (All data points with bars are means ± standard deviation (n=3)).

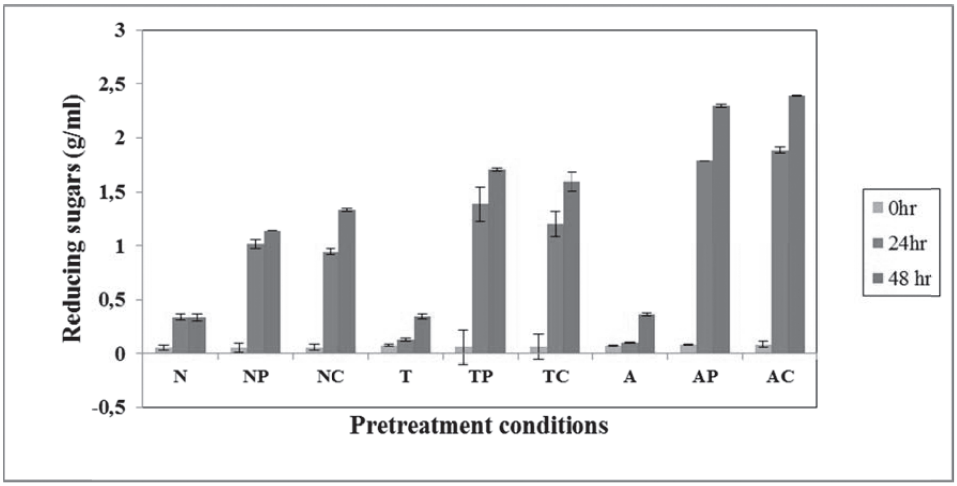


Fig 5. Enzyme hydrolysis of sugarcane bagasse using partially pure xylanase and crude enzyme (15 IU/g) from *P. alba*

Discussion

Woodchips are known to contain a plethora of microorganisms with diverse characteristics and enzymatic capabilities. Bacterial and fungal isolates have been isolated from such environmental samples (19). The enzymatic capabilities of these microorganisms have also been exploited for various applications in industrial biotechnology. In the current study, of the 32 microorganisms isolated from *Eucalyptus* spp. woodchips, 46% were positive for xylanase activity. Of the xylanase producers, 8 isolates were selected for further enzyme characterization. Selection of isolates was based on differences in morphology, macroscopic characteristics and the size of the zone of hydrolysis. The time required for optimal enzyme production was determined. Among the selected fungal isolates, F2 and F10 produced optimal quantities of xylanase after 5 days and F1 and F10 demonstrated highest enzyme activity with specific activities of 12.25 U and 24.43 U, respectively. Similar enzyme titres were obtained in studies conducted by Gomes et al. (12) and Palaniswamy et al.(17). Based on the desirable activities displayed by the above-mentioned isolates, further characterization in terms and pH and temperature optimum, stability and determination of protein size was conducted.

All isolates displayed a pH optima of 7 except for F10 which exhibited an acidic pH optimum of 4. Most xylanases have pH optima ranging from 4.5-7; however, reports of a xylanase produced by *Talaromyces emersonii* with an acidic pH optimum have been made (20). Good stability at their optimum pH was observed for the crude xylanases which indicates that their application in the paper and pulp industry is promising. Biopulping requires thermostable, alkaline xylanase (21) while bioleaching requires thermostable, acidic xylanases (22).

High xylanolytic activity was observed for crude fungal xylanases isolated from wood chips. F1 remained stable for 105 minutes at optimum pH with greater than 99% of activity being retained. Desired stability was exhibited by the crude xylanases at pH 5 as 98% of activity was retained for 90 minutes. A similar trend was observed for F10. Approximately 98% of xylanases activity was retained at pH 4 and 96% at pH 5 for 75 and 60 minutes. The xylanase activity displayed by these isolates is consistent with

what has been reported in literature. However, in most instances such activities have been observed after optimization of media composition by different carbon sources such as wheat straw, maize straw and oat hay as inducers of enzyme activity in order to minimize cost by manipulating media components. (23).

F10 displayed temperature optima at two peaks at 50ºC and 90ºC respectively (Fig 2b). This suggests that more than one type of xylanase may be present each with its own optimum temperature and specific activity which agrees with reports that multiple xylanases may be produced by a single microorganism (2). As a result of genetic redundancy fungi like *Aspergillus niger* and *Trichoderma viride* are known to produce fifteen and thirteen xylanases, respectively (24). The complexity of xylan requires more than one type of enzyme to ensure penetration into the cell. Furthermore, desirable activity was observed at 100ºC for both fungal isolates indicative of the production of thermoactive enzymes necessary for application in bioprocessing. Fungal xylanases are typically more stable at elevated temperatures than bacterial xylanases. There have been reports of fungal xylanases which retained 100% of activity between 65ºC and 80ºC for 30 minutes after optimization of growth media (25). Thermostability can be attributed to the presence of a thermostabilising domain, expressed either as discrete entities or as their natural fusions with the catalytic module have capacity to bind various carbohydrates and potentiate hydrolytic activity (26). Among the fungal isolates in current study, differences in thermal stability were observed as F1 was more stable than F10 xylanase. Such differences in stability could be attributed to the presence of co-factors and salts in the media which tend to elicit varying effects on proteins in general.

The thermophilic fungus in the present study was identified as *P. alba* by 18S rRNA amplicon of F10 fungal DNA. Further, edited sequence were used to determine the identity of the fungus against a database of known microorganisms. The fungus was thus, identified as *P. alba* (HM 116755) with a 99% homology.

Initial electrophoretic analysis yielded no visible bands. To enhance the visibility of the bands, the crude extracts were concentrated 10× and bands were only visualized for F10 although clearing in the native substrate PAGE gels indicate fairly high specific activity for several isozymes. Therefore, in the near future, the crude extracts need to be further purified to enhance visibility of bands for electrophoretic analysis. Once bands have been visualized, zymogram analysis may be carried out to determine molecular weight of xylanases.

In the present study the effect of two pretreatment strategies (viz., high temperature, pressure and alkali pretreatment) of SCB in conjunction with enzymatic hydrolysis was determined. The effect of pretreatment alone on SCB could not be established as the high NaOH concentration interfered with the DNS assay and precluded determination of sugars released. In the current study, the additive effect of enzyme treatment (partially pure xylanase and crude enzyme preparation) with pretreatment strategies was assessed (Fig 5). Milling was responsible for some degradation of the SCB as low baseline levels of reducing sugars were detected in all samples at 0 hours. Whilst, both enzyme preparations had a moderate effect on the milled SCB as less than 0.35 mg/ml reducing sugars were produced after 48 hours. According to Corrales et al. (27), the pretreatment of lignocellulosic residues including SCB is essential in order to achieve efficient hydrolysis to monomeric sugars. Untreated SCB represents the fully intact polymer complex of lignocelluloses.

Under thermal pretreatment conditions, the crude enzyme produced the maximum amounts of reducing sugars after 48 hours with sugar concentrations of 1.2 g/ml and 1.6 g/ml detected at 24 and 48 hours, respectively. Thus the reaction time determines the amount of reducing sugars liberated. This is in agreement as reported by Saores et al. [28], where the amount of reducing

Table 1. Microorganisms isolated from woodchips (Sappi Saiccor) and their respective cellulase, ligninase, and xylanase activity

Pure Isolates	Species	Phyla Affiliation	Best Match database (Gene Bank Accession No.)	Similarity (%)	Xylanase
F1	*Paecilomyces* sp.	Ascomycota	AB217858.1	99	+++
F2	*Aspergillus fumigatus*	Ascomycota	GU992275.1	100	++
F3	*Phanerochaete chrysosporium*	Basidiomycota	AF475147.1	100	-
F4	*Paecilomyces formosus*	Ascomycota	GU968673.1	99	++
F5	*Paecilomyces formosus*	Ascomycota	GU968664.1	99	-
F6	*Geosmithia arqillacea*	Ascomycota	GU165722.1	98	++
F7	*Penicillium verruculosum*	Ascomycota	HM469420.1	99	+
F8	*Acremonium implicatum*	Ascomycota	FN706553.1	98	+
F9	*Aspergillus fumigatus*	Ascomycota	GU566217.1	100	++
F10	*Phialophora alba*	Ascomycota	HM116755.1	99	++
F12	*Curvularia* sp.	Ascomycota	HQ631061.1	100	+

- : absence of zones; + : 1-18 mm; ++ : 19-29 mm; +++: 30-40 mm

sugars from steam pretreated SCB were liberated over a 72 hour period.

Alkaline pretreatment coupled with enzyme hydrolysis proved to be the most effective SCB preparation resulting in the highest production of reducing sugars. A similar study by Hernandez-Salas et al. (29) using a cocktail of enzymes showed a higher concentration of reducing sugars with alkali treated SCB compared to either acid hydrolysed or steam pretreated bagasse. Approximately 11-20% reducing sugars were generated over a 4 hour period at 55°C.

The results obtained in the current study are consistent with reports that fungi produce xylanases with higher activity than bacteria (30, 31).The fungal xylanases have auxiliary application in biopulping and bioethanol production.

Conclusions

The current study presented evidence for the presence of thermostable and thermoactive xylanolytic enzymes produced from woodchip-inhabiting microorganisms. The desirable activity, pH and thermostability of F1 and F10 are indicative for their potential application in a range of bioprocesses. Potential applications include bioethanol production, biobleaching and biopulping which are evidenced by the high activity observed at pH 5, pH 4 and pH 9. As robust biocatalysts, F1 and F10 xylanases show potential to withstand the elevated temperatures and extremes in pH during industrial processing. Furthermore, xylanase activity observed at 90ºC indicates that these enzymes can be used in the degradation of hemicellulose-rich sugarcane bagasse and agricultural wastes for biofuel production following a thermal pre-treatment step. Therefore, the cooling step which is usually implemented after thermal pre-treatment may be eliminated as the enzymes function at the same temperature.

P. alba isolated in this study would be more useful for enzyme production for industrial applications. Further purification to homogeneity, analysis of the characteristics of xylanase produced by *P. alba* will be needed in order to apply the results to industry.

Conflict of interest

The authors declare no conflict of interest.

References

1. Kumar, V., Marín-Navarro, J. and Shukla, P. (2016). Thermostable microbial xylanases for pulp and paper industries: trends, applications and further perspectives. World Journal of Microbiology and Biotechnology, 32(2): p. 34.
2. Collins, T., Gerday, C. and Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiology Reviews, 29(1): p. 3-23.
3. Shallom, D. and Shoham, Y. (2003). Microbial hemicellulases. Current Opinion in Microbiology, 6(3): p. 219-228.
4. Silva, L.A.O., Terrasan, C.R.F. and Carmona, E.C. (2015). Purification and characterization of xylanases from *Trichoderma inhamatum.* Electronic Journal of Biotechnology, 18(4): p. 307-313.
5. Fillat, A., Roncero, M.B. and Vidal, T. (2011). Assessing the use of xylanase and laccases in biobleaching stages of a TCF sequence for flax pulp. Journal of Chemical Technology & Biotechnology, 86(12): p. 1501-1507.
6. Bai, W., Wang, Q. and Ma, Y. (2014). Progress in the thermophilic and alkalophilic xylanases]. Sheng Wu Gong Cheng Xue Bao, 30(6): p. 828-37.
7. Du, Y., et al. (2013). Characterization of three novel thermophilic xylanases from *Humicola insolens* Y1 with application potentials in the brewing industry. Bioresource Technology, 130: p. 161-167.
8. Jhamb, K. and Sahoo, D.K. (2012). Production of soluble recombinant proteins in *Escherichia coli:* effects of process conditions and chaperone co-expression on cell growth and production of xylanase. Bioresource technology, 123: p. 135-143.
9. Ahmed, S., Riaz, S. and Jamil, A. (2009). Molecular cloning of fungal xylanases: an overview. Applied Microbiology and Biotechnology, 84(1): p. 19-35.
10. Berka, R.M., et al. (2011). Comparative genomic analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. Nature Biotechnology, 29(10): p. 922-927.
11. Margaritis, A., Merchant, R.F. and Yaguchi, M. (1986). Thermostable cellulases from thermophilic microorganisms. Critical Reviews in Biotechnology, 4(3): p. 327-367.
12. Gomes, I., et al. (2000). Simultaneous production of high activities of thermostable endoglucanase and β-glucosidase by the wild thermophilic fungus *Thermoascu-saurantiacus.* Applied Microbiology and Biotechnology, 53(4): p. 461-468.
13. Bailey, M.J., Biely, P. and Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology, 23(3): p. 257-270.
14. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2): p. 248-254.
15. Ninawe, S., Kapoor, M. and Kuhad, R.C. (2008). Purification and characterization of extracellular xylanase from *Streptomyces cyaneus* SN32. Bioresource Technology, 99(5): p. 1252-1258.
16. Adsul,M., et al.(2005). Enzymatic hydrolysis of delignified bagasse polysaccharides. Carbohydrate Polymers, 62(1): p. 6-10.
17. Palaniswamy, M., Pradeep, B.V., Ramaswamy, S. And Angayarkanni, J. (2008). Isolation, identification and screening of potential xylanolytic enzyme from litter degrading fungi. African Journal of Biotechnology, 7(12): 1978-1982.

18. Miller, G.L. (1959). Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Analytical Chemistry, 31(3): p. 426-428.

19. Straatsma, G., et al. (1994). Ecology of thermophilic fungi in mushroom compost, with emphasis on *Scytalidium thermophilum* and growth stimulation of *Agaricus bisporus* mycelium. Applied and environmental microbiology, 60(2): p. 454-458.

20. Maheshwari, R., Bharadwaj, G. and Bhat, M.K. (2000). Thermophilic fungi: their physiology and enzymes. Microbiology and Molecular Biology Reviews, 64(3): p. 461-488.

21. Anuradha, P., et al. (2007). Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields. Current Science, 92(9): p. 1283-1286.

22. Yang, V., et al. (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp. isolated from kraft pulp. Journal of Industrial Microbiology & Biotechnology, 15(5): p. 434-441.

23. Goyal, M., et al. (2008). Xylanase production with xylan rich lignocellulosic wastes by a local soil isolate of *Trichoderma viride*. Brazilian Journal of Microbiology, 39(3): p. 535-541.

24. Wong, K., Tan, L. and Saddler, J.N. (1988). Multiplicity of beta-1, 4-xylanase in microorganisms: functions and applications. Microbiological Reviews, 52(3): p. 305.

25. Singh, S., Madlala, A.M. and Prior, B.A. (2003). *Thermomyces lanuginosus:* properties of strains and their hemicellulases. FEMS Microbiology Reviews, 27(1): p. 3-16.

26. Charnock, S.J., et al. (2000). The X6 "Thermostabilizing" domains of xylanases are carbohydrate-binding modules: structure and biochemistry of the *Clostridium thermocellum* X6b Domain. Biochemistry, 39(17): p. 5013-5021.

27. Corrales, R.C.N.R., et al. (2012). Structural evaluation of sugar cane bagasse steam pretreated in the presence of CO_2 and SO_2. Biotechnology for Biofuels, 5(1): p. 36.

28. Soares, I., et al. (2011). Effects of washing, milling and loading enzymes on the enzymatic hydrolysis of a steam pretreated sugarcane bagasse. Industrial Crops and Products, 33(3): p. 670-675.

29. Hernández-Salas, J., et al. (2009). Comparative hydrolysis and fermentation of sugarcane and agave bagasse. Bioresource Technology, 100(3): p. 1238-1245.

30. Sunna, A. and Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. Critical Reviews in Biotechnology, 17(1): p. 39-67.

31. Ratanachomsri, U., et al. (2006). Thermostable xylanase from *Marasmius* sp.: purification and characterization. Journal of Biochemistry and Molecular Biology, 39(1): p. 105.