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Screening for xylanase and β-xylosidase production from wood-inhabiting *Penicillium* strains for potential use in biotechnological applications

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Abstract

Experiments were performed to find potential sources for enzyme production for the pulp and paper industry and for biological ethanol production by screening the cellulase, xylanase and β-xylosidase activities of 36 species of *Penicillium* isolated from various wood materials in Korea. Rice straw powder (RiceP), birchwood xylan (BirchX), and beechwood xylan (BeechX) were supplied as individual carbon sources for the *Penicillium* species. All *Penicillium* species tested in this study showed little cellulase activity, but some species exhibited remarkably high xylanase and β-xylosidase activities, as determined by a filter paper assay. *P. oxalicum* showed the greatest xylanase activity on RiceP (158.70 U ml^{-1}). On the other hand, *P. brevicompactum* produced the highest active β-xylosidase on BirchX (6.25 U ml^{-1}).

Keywords: biobleaching; *Penicillium brevicompactum*; *Penicillium kloeckeri*; *Penicillium oxalicum*; xylanase; β-xylosidase.

Introduction

Xylan is the second most abundant renewable polysaccharide in nature. It is a major component of hemicelluloses in plant cell walls and is composed of heteropolymers of xylose and arabinose. Xylan deposition plays an essential role in pulping chemistry (Fengel and Wegener 1989; Jiang et al. 2006; Westbye et al. 2006; Mittal et al. 2009; dos Santos Muguet et al. 2011). The cooperative action of several enzymes, particularly xylanase and β-xylosidase, are required to completely break down branched xylan (Biely 1985; Abdel-Sater and El-Said 2001).

Because of their biological potential for various industrial uses, xylanolytic enzymes from microorganisms have attracted great attention in the past several decades (Biely 1985; Beg et al. 2001). There are two potential applications of xylanolytic enzymes. Conversion of xylan to D-xylose in paper-pulp industrial waste and agricultural waste can be achieved by xylanolytic enzymes through conjugation with cellulolytic enzymes. The role of xylanases as a coenzyme in the saccharification of cellulolytic materials has also attracted attention (Krogh et al. 2004). In the absence of cellulolytic enzymes, xylanolytic enzymes can be used for removal of xylan from dissolving pulp (Rahkamo et al. 1998; Bajpai 1999; Ibarra et al. 2009). The mechanisms of xylanase in pulp bleaching have been investigated by deJong et al. (1997). Biological bleaching of pulp is a promising industrial application of xylanase, thus the study of cellulose-free xylanases is often in focus in biobleaching processes (Beg et al. 2001; Xu et al. 2010a,b). Xylanases are also useful for studying the chemical composition of wood and fractionation of wood components (Furuno et al. 2006; Lawoko et al. 2006; Westbye et al. 2008; Nakamura et al. 2010).

β-xylosidase is able to decompose short xylooligosaccharides to monomeric xylose and exoglycosidase plays an important role in decreasing the endoxylanases-inhibiting effects of the end products. In comparison to xylanase, there are relatively few reports on β-xylosidase (Sunna and Antranikian 1997; Maheshwari et al. 2000; Lama et al. 2004), despite its importance as a part of xylan-hydrolyzing enzyme cocktails used in the recycled paper industry (Marques et al. 2003) and despite its important role in the processing of wood pulp (Tsujibo et al. 2001).

Many studies have reported on *Penicillium* species that produce highly active xylanases (Brown et al. 1976; Chaabouni et al. 1994), which were also identified in previous studies as good β-xylosidase producers (Seeta et al. 1989; Terrasan et al. 2010). Recently, Jang et al. (2011) investigated the diversity of wood-inhabiting *Penicillium* species in Korea and identified a total of 37 *Penicillium* species, including six unknown ones, from a collection of 137 isolates. In this study, a screen for cellulase- and xylanase-producing *Penicillium* species will be performed aiming at the identification of potentially novel enzyme producers and to provide preliminary data on these species for future biotechnological applications.

Materials and methods

Microbe and culture condition

Thirty-six *Penicillium* species previously classified by Jang et al. (2011) were obtained from the Korea University Culture Collection (KUC Collection). One isolate from each species was inoculated on malt extract agar media (MEA) containing 2% (w/v) malt extracts

(Bacto, Sparks, MD, USA) and 1.5% agar powder (Showa, Tokyo, Japan).

Enzyme preparation Strains were transferred to another fresh MEA plate and grown for 14 days to produce spore development. Resulting spores were suspended in 0.02% (v/v) Tween 80 and collected in e-tubes. Erlenmeyer flasks (100 ml) containing 40 ml of medium (see below) were prepared and inoculated with 400 μl of the spore suspension (10^6 spores ml^{-1}).

The medium contained 0.8% (w/v) peptone (Bacto), 0.2% yeast extract (Bacto), 0.5% KH_2PO_4, 0.5% K_2HPO_4, 0.3% $MgSO_4 \cdot 7\ H_2O$ and a carbon source of 2% of either prepared rice straw powder (RiceP), birchwood xylan (BirchX) or beechwood xylan (BeechX).

To produce RiceP, air-dried rice straw collected in Osong, Chungchungbukdo, Korea was chopped and washed with tap water. The washed rice straw was oven-dried at 60°C for 48 h, then milled with a cutting mill and sieved (160 μm) to prepare RiceP. BirchX (product no. X-0502) and BeechX (product no. X-4252) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Cultures were grown aerobically at 25°C while rotating at 150 rpm in the dark for 5 days. The cultures were prepared in duplicate or triplicate and the mean values are presented. Five days after inoculation, the entire liquid culture medium, including fungal mycelium, was transferred to 50 ml conical tubes and centrifuged at 4000 rpm for 30 min at 4°C. Media supernatants were filtered through Whatman No. 1 filter paper and the filtrates used as the crude enzyme solution.

Determination of protein concentration

The concentration of extracellular protein was determined by a 96-well plate assay based on the Bradford method (Bradford 1976).

Enzyme assay

Filter paper activity Enzyme activities were determined based on estimation of released reducing sugars using dinitrosalicylic (DNS) acid (Miller 1959) with the 60-μl filter paper assay (FPA) method (Xiao et al. 2004) in 96-well micro plates. The assay mixtures were prepared in a total volume of 60 μl consisting of 30 μl of suitably diluted crude enzymes and 30 μl of 50 mM sodium acetate buffer (pH 5.0) in a 0.2 ml PCR tube; the substrate was a 7 mm diameter Whatman No.1 filter paper disk. After 60 min of incubation at 50°C, 120 μl of DNS was added into each reaction. The reaction was then boiled for 5 min and cooled. They were transferred in a 96-well micro plate and absorbance at 540 nm was measured. Assay mixture with buffer instead of enzyme was used as absorbance blank and non-reacted mixture, added DNA reagents just before boiling, was used as enzyme blank for each sample. A filter paper unit (FPU) was defined as 1 μM of glucose equivalent released per min under the assay conditions.

Xylanase activity DNS-based detection of xylanase activities was performed according to Bailey et al. (1992) with D-xylose as a standard. The total reaction volume was reduced to 200 μl for the 96-well micro plate, a modification of the FPA assay mentioned above, to improve the screening process. A mixture of 20 μl bulk enzyme solution and 80 μl of substrate (2% birchwood xylan) in a 0.2 ml PCR tube was incubated for 5 min at 50°C. Then, 60 μl of the reaction solution was transferred to another 0.2 ml PCR tube and 120 μl of DNS reagent was added. The mixture was incubated at 95°C for 5 min and cooled. The absorbance was measured at 540 nm. One unit of xylanase activity was defined as the amount of enzyme that released 1 μM of xylose as reducing sugar equivalents per min.

β-xylosidase activity β-xylosidase was determined by measuring the concentration of *p*-nitrophenyl (*p*NP) released by *p*-nitrophenyl xylosidase (*p*NPX) at 50°C. The reaction mixture, containing 0.5 ml of diluted enzyme and 0.5 ml of 1 mM *p*NPX prepared in 0.5 mM sodium acetate buffer (pH 0.5), was incubated for 30 min at 50°C. The reaction was quenched by adding 0.2 ml of 1 M Na_2CO_3 and the absorbance at 410 nm was measured. One unit of β-xylosidase activity was defined as the amount of enzyme that released 1 μM of *p*NP min^{-1}.

Results and discussion

Filter paper assay

The results of the filter paper assay are shown in Figure 1a. The strains investigated in this study had low cellulase activities, ranging from 0 to 0.21 FPU ml^{-1}. The highest activity was found in *P. diversum* on BirchX (0.21 U ml^{-1}) and BeechX (0.18 U ml^{-1}) followed by *Penicillium* sp. 4 on RiceP (0.16 U ml^{-1}).

Based on the filter paper method, culture media with a supplied carbon source of xylan (BirchX, BeechX) generally show a higher degrading ability than RiceP, except for *P. citrinum*, *P. crustosum*, *P. expansum*, *P. ochrochloron*, *P. oxalicum*, *P. pinophilum*, *P. simplicissinum* and *Penicillium* sp. 4. In a previous study, crude enzyme extracted from media supplied with a cellulose rather than xylan carbon source had a relatively high FPU (Krogh et al. 2004). Cellulose as the sole carbon source induces cellulolytic enzymes, but RiceP does not induce cellulolytic enzymes only, because it contains diverse natural carbon sources, enzyme-inhibiting compounds and metallic ashes. Moreover, the additional components would hinder fungal growth and cellulase activity. In studies of plant extracts, inhibition of fungal growth (Cowan 1999; Bafi-Yeboa et al. 2005) and inhibition of cellulase activity by the extracts (Bell et al. 1962) were reported.

Taking into account the low average cellulase activities, the *Penicillium* species tested in this study cannot be regarded as good cellulase producers for biological ethanol production, but these species could be useful to reduce the loss of pulp yield in the process of biobleaching pulp and other processes in the paper industry.

Xylanase activity

The greatest xylanase activity was found in *P. oxalicum* on RiceP (158.7 U ml^{-1}) (Figure 1b). *P. pinophilum*, *Penicillium* sp. 6 and *P. kloeckeri* also exhibit relatively high activities on BirchX (104.7 U ml^{-1}), BirchX (96.8 U ml^{-1}) and BeechX (93.1 U ml^{-1}), respectively.

P. oxalicum has often been studied as a biological control agent (BCA) (DeCal et al. 1997) and toxic metabolite producer (Steyn 1970). There are only a few reports of xylanase production by *P. oxalicum*; Li et al. (2007) and Dwivedi et al. (2009) investigated the xylanase activity of various

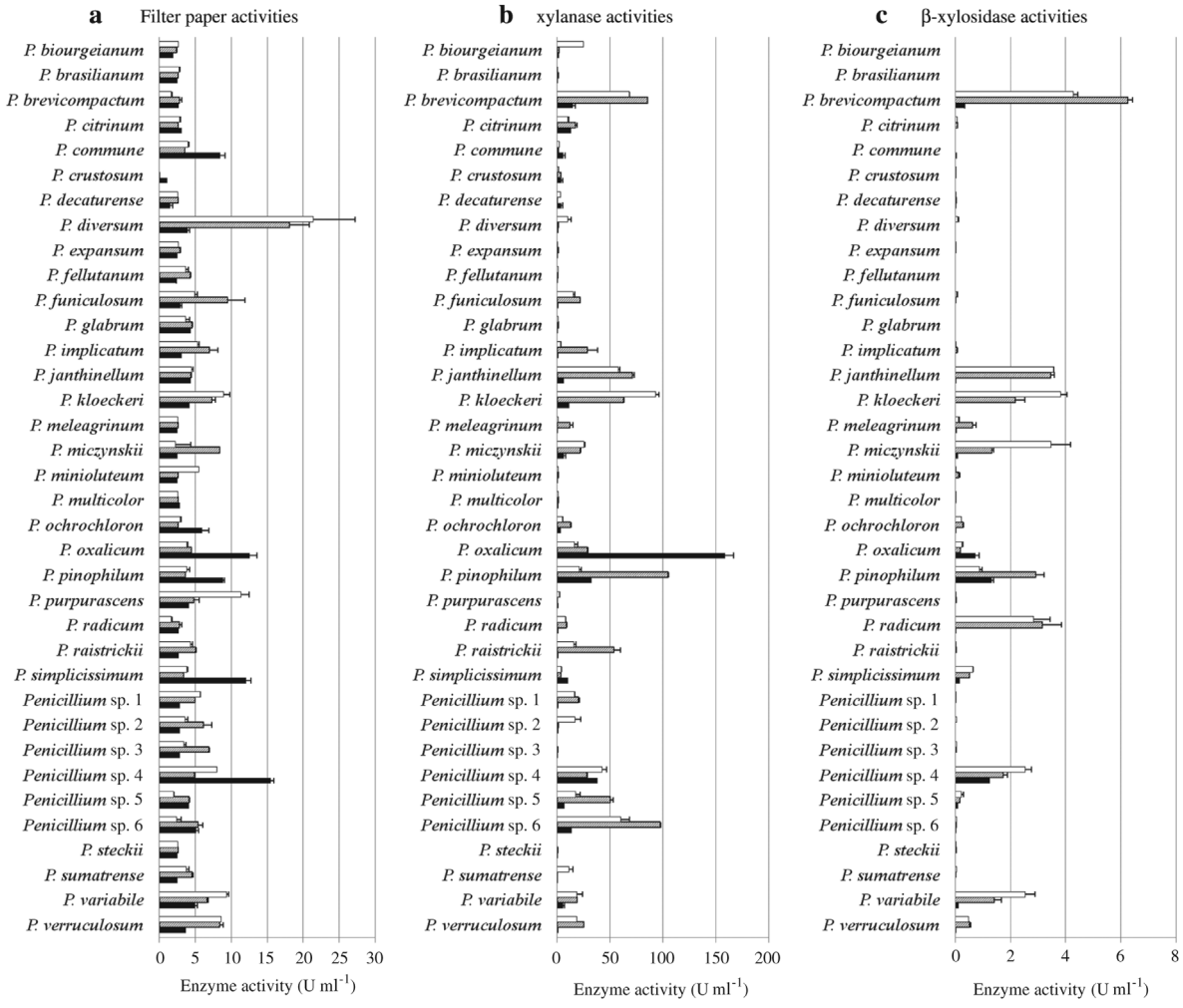

Figure 1 (a) Filter paper activities, (b) xylanase activities and (c) β-xylosidase activities of the *Penicillium* species collected from woods in Korea. (□, BeechX; ▨, BirchX; and ■, RiceP). RiceP: rice straw powder; BirchX: Birch wood xylan; BeechX: beech wood xylan.

strains of *P. oxalicum* under various culture conditions to optimize their xylanolytic enzymes (Table 1). When compared to these results, *P. oxalicum* KUC3083 have almost identical activity on RiceP to *P. oxalicum* SAU_E-3.510, a genetically modified variant of *P. oxalicum* SA-8 (Dwivedi et al. 2009). Considering the amount of extracellular proteins in *P. oxalicum*, the production of a specific xylanase by *P. oxalicum* on RiceP and BirchX is remarkable. In the case of BirchX as the carbon source, the specific xylanase activity of *P. oxalicum* is similar to that of the control. The overall xylanase activity of *P. pinophilum* on BirchX is the highest, but its specific enzyme activity is not significantly higher than that of *P. oxalicum* (Table 2). All fungi tested in this study show higher activity on BirchX and/or BeechX than

Table 1 Comparison of xylanase production by *Penicillium oxalicum* with production of other strains and under various growth conditions.

Strain	Substrate (concentration)	Culture condition in shake flask	Activity (U ml^{-1})	References
P. oxalicum ZH-30	Xylan (10 mg l^{-1})	30°C, 72 h	5.3	Li et al. (2007)
	Wheat bran (10 mg l^{-1})	30°C, 72 h	13.3	
P. oxalicum SA-8	Oat spelt xylan (5.0 g l^{-1})	30°C, 144 h	242	Dwivedi et al. (2009)
P. oxalicum SAU_E-3.510	Oat spelt xylan (5.0 g l^{-1})	30°C, 144 h	453	
P. oxalicum KUC3083	Rice straw powder (2.0 g l^{-1})	25°C, 96 h	158.7	This study
	Birchwood xylan (2.0 g l^{-1})	25°C, 96 h	28.3	
	Beechwood xylan (2.0 g l^{-1})	25°C, 96 h	16.0	

Table 2 Specific enzyme activities of the five *Penicillium* species on the three carbon sources.

	Enzyme activity (U ml^{-1}) on			Specific activity[a] (U mg^{-1}) on		
	RiceP	BirchX	BeechX	RiceP	BirchX	BeechX
P. brevicompactum[b]	0.33±0.02	6.25±0.18	4.28±0.15	6.44±0.02	56.93±0.50	57.56±16.18
P. janthinellum[b]	0.03±0.00	3.47±0.13	3.56±0.02	0.24±0.00	35.96±0.21	42.01±4.95
P. kloeckeri[b]	0.01±0.00	2.17±0.35	3.82±0.22	0.11±0.00	18.69±3.37	90.41±14.33
P. oxalicum[c]	158.70±8.05	28.34±0.51	15.99±3.51	7116.23±721.42	1096.52±299.93	3115.38±741.53
P. pinophilum[c]	32.22±0.05	104.67±0.77	20.83±1.80	302.23±6.51	752.94±71.72	234.06±4.51

RiceP, rice straw powder; BirchX, Birch wood xylan; BeechX, beech wood xylan. [a]Specific activity was expressed as units of enzyme activity per milligram of total extracellular protein in crude enzyme solution. [b]β-xylosidase activities. [c]xylanase activities.

RiceP, except *P. oxalicum* which is most active on RiceP. This result is likely due to fungal preferences for specific substrates.

β-xylosidase activity

In the genus *Penicillium*, β-xylosidase activity varies widely between species (Figure 1c). Many species have almost no β-xylosidase activity, but some strains (*P. brevicompactum*, *P. janthinellum*, *P. kloeckeri*, *P. oxalicum*, *P. pinophilum*, *P. radicum* and *P. variable*) demonstrated significant activities. *P. brevicompactum* reveals the highest activity (BirchX; 6.25 U mg^{-1}, BeechX; 4.28 U ml^{-1}) followed by *P. janthinellum* (BeechX; 3.56 U ml^{-1}, BirchX; 3.47 U ml^{-1}) and *P. kloeckeri* (BeechX; 3.82 U ml^{-1}, BirchX; 2.17 U ml^{-1}).

Interestingly, the highest specific β-xylosidase activity was observed on *P. kloeckeri* on BeechX and not *P. brevicompactum* on BirchX (Table 2). Obviously, the individual enzyme systems of the fungi are very different. Although crude enzymes of *P. kloeckeri* have relatively low β-xylosidase activity compared with *P. brevicompactum*, the specific enzymes that hydrolyze 1, 4- β-D-xylan to D-xylose produced by *P. kloeckeri* might be more efficient than those produced by *P. brevicompactum*. In this case, increasing the secretion of the target enzyme through mutagenesis is possible, as suggested by Brown et al. (1987).

The β-xylosidase activity of *P. brevicompactum* and *P. kloeckeri* was not yet reported, but in the present study these species are more active with this regard than the other *Penicillium* species. Accordingly, they are good candidates as β-xylosidase producers for industrial applications. But the fact, that the specific β-xylosidase activity of *P. kloeckeri* is significant only on BeechX indicates that further research is needed concerning the details of the β-xylosidase producing system of *P. kloeckeri*.

Conclusions

Concluded from the screening results for cellulase and hemicellulase activities, the crude enzymes produced by *P. oxalicum* had low total cellulase activity, but its xylanase activities were the highest among the *Penicillium* species tested. Enzymes obtained from *P. brevicompactum* and *P. kloeckeri* also showed remarkable β-xylosidase activity. Based on this study, these species can be recommended as potential enzyme producers for biotechnological applications.

The activity of each *Penicillium* species was not optimal, because all the growth and testing conditions were kept constant to ensure identical conditions for a better comparability. In other words, the performance of the promising species (*P. oxalicum*, *P. brevicompactum*, and *P. kloeckeri*) still can be improved under individually optimized growing conditions.

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