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Production of xylooligosaccharides from enzymatic hydrolysis of xylan by white-rot fungi *Pleurotus*

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ABSTRACT. Hemicellulose consists of non-cellulosic polysaccharides, with xylans and mannans as their main examples. In nature, xylan can be first degraded to xylooligosaccharides and finally to xylose by certain microorganisms. White-rot fungi basidiomycetes *Pleurotus* sp. BCCB068 and *Pleurotus tailandia* were used to degrade oat-spelts xylan under submerged fermentation for a period of 40 days. The study obtained activities of endo-1,4-β-xylanase and β-xylosidase and determination of xylan products by degradation. The fungi reached significant levels of xylan degradation by *Pleurotus* sp. BCCB068 (75.1%) and *P. tailandia* (73.4%), following formations of xylooligosaccharides and sugar monomers. These *Pleurotus* strains proved to be a feasible alternative for biotechnological processes related to degradation of hemicellulose sources.

Key words: biodegradation, *Pleurotus*, xylan, hemicellulose, xylooligosaccharides.

RESUMO. Produção de xilooligossacarídeos pela hidrólise enzimática de xylana por fungos *Pleurotus*. A hemicelulose é um polissacarídeo não-celulósico, tendo como exemplos principais as xilanas e mananas. Na natureza, as xilanas podem ser degradadas por microrganismos, primeiramente a xilooligossacarídeos e finalmente a xilose. Fungos basidiomicetos *Pleurotus* sp. BCCB068 e *Pleurotus tailandia* foram utilizados para degradar xilana de aveia em fermentação submersa durante o período de 40 dias. Foram obtidas as atividades de endo-1,4-β-xilanase e β-xilosidase e a determinação dos produtos de degradação da xilana. Os fungos atingiram níveis significativos de degradação da xilana por *Pleurotus* sp. BCCB068 (75.1%) and *P. tailandia* (73.4%), seguido da formação de xilooligossacarídeos e monômeros de açúcar. Essas cepas de *Pleurotus* demonstraram ser uma alternativa viável para os processos biotecnológicos relacionados à degradação de fontes de hemicelulose.

Palavras-chave: biodegradação, *Pleurotus*, xilana, hemicelulose, xilooligossacarídeos.

Introduction

Hemicellulose is one of the most abundant polysaccharides in nature present in agricultural biomass waste, and cannot be easily converted to simple monomeric sugars due to its recalcitrant nature. Xylan, the major component of hemicellulose in plant cell walls, is the second most abundant polysaccharide after cellulose (TAN et al., 2008). Xylan polymer consists of a main chain of β-1,4- linked D-xylose residues or some substitutes including L- arabinose, D-glucose, D-galactose, D-mannose, D-glucuronic acid, 4-O-methyl glucuronic acid D-galacturonic acid residues, as well as in less proportion the L-rhamnose, L-fucose and various O-methylated neutral sugars (SUN et al., 2000). The complete degradation of this complex structure depends on different enzymes acting in synergism endo-β-1,4-xylanases hydrolyze β-1,4-bonds between D-xylose residues in the main chain producing xylooligosaccharides (XOS), and β-D-xylosidases convert xylo-oligosaccharides to xylose monomers (DEN HANN; VAN ZYL, 2003). Some other specific enzymes such as α-L-arabnofuranosidase, α-glucuronidase, as well as several esterases have also a cooperative function into the complete degradation of xylan (TENKANEN et al., 1996).

Xylan is degraded by bacteria and fungi through the production of a full complement of enzymes. The genera *Trichoderma*, *Aspergillus*, *Fusarium* and *Pichia* are considered great producers of xylanases (CHRISTAKOPOULOS et al., 1996; ADSUL et al., 2005), and basidiomycetes usually secret large amount of enzymes to degrade lignocellulosic materials: white-rot fungus *Phanerochaete chrysosporium* produces high level of α-glucuronidase

(CASTANARES et al., 1995), and *Coriolus versicolor,* a complex xylanolytic system (EL-NASSER et al., 1997). Xylanase is also produced by *Cuninghamella subvermispora* when growing on plant cell-wall polysaccharides or on wood chips (SETHURAMAN et al., 1998; SOUZA-CRUZ et al., 2004) and current investigation suggests that xylanase is a key enzyme for hemicellulose degradation. *Pleurotus ostreatus*, *Pleurotus sajor-caju* and *Trametes versicolor* (REDDY et al., 2003; VALASKOVÁ; BALDRIAN, 2006) are also producers of lignocellulolytic enzymes. Multiple endoxylanase isoenzymes are produced by microorganism and may reflect the need to produce xylanases with strict and relaxed specificities, capable of acting on different substrates (MILAGRES et al., 2005).

Xylanases are used in the management of agro-industrial wastes to degrade partially xylan to a renewable biomass, generating less environmental impact, and in the paper industry as pulp bleaching (COLLINS et al., 2002; TECHAPUN et al., 2002) to reduce the amount of chlorine in the process. Agricultural wastes can also be hydrolyzed in fermentable sugars and ethanol. Several bacteria, fungi and yeast are capable of fermenting xylose to ethanol (INGRAM et al., 1999; LEE et al., 1986). Oligosaccharides produced by the action of xylanases are used as functional food additives or alternative sweeteners with beneficial properties (PELLERIN et al., 1991; PULS et al., 1998).

The white-rot fungi basidiomycetes have been studied nowadays as xylanase sources, once its extracellular system is produced to act in a wide range of lignocellulosic materials, and because they are considered edible, these mushrooms is highly nutritional and safe (BUSWELL; CHANG, 1994) being source of important metabolites of interest to the pharmaceutical, cosmetic and food industries (JONG; DONOVICK, 1989; QINNGHE et al., 2004).

In this present study, white-rot fungi *Pleurotus* sp. BCCB068 and *Pleurotus tailandia* were evaluated regarding the degradation of the xylan matrix with production of xylanases using submerged fermentation. The ability of these fungi in degrading xylan, the principal component of hemicellulose in agro-industrial wastes, suggests further research to optimize xylanolytic enzymes, produce XOS and decrease these residues into the nature.

Material and methods

Microorganisms

The white-rot fungi *Pleurotus* sp. BCCB068 and *Pleurotus tailandia* belong to the Culture Collection of the Systematic and Microbial Physiology Laboratory (Faculty of Food Engineering – State University of Campinas, São Paulo State, Brazil) and were cultivated in PDA (Potato Dextrose Agar - Difco) at 30°C for seven days up to complete superficial growth into Petri dishes, using three portions of small disks with 6 mm in diameter, when one disk is used per 10ml of liquid medium as inoculum.

Experimental conditions

The submerged fermentation was carried out in 125 ml Erlenmeyer flasks containing 50 ml of sterile basal medium (pH 5.5) plus 1% xylan. Basal medium presented the following composition per Liter: 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.1 g urea, 0.3 g $MgSO_4.7H_2O$, 0.3 g $CaCl_2$, 5.0 mg $FeSO_4.7H_2O$, 1.56 mg $MnSO_4.H_2O$, 2.0 mg $CoCl_2$, 1.4 mg $ZnSO_4.7H_2O$. Flasks were inoculated as described above and incubated at 30°C for 40 days under aerobic static condition. Triplicate of samples were collected each 10 days of cultivation, and pH were measured. Extracellular fluids obtained by refrigerated centrifugation at 33,450 x g for 15 minutes were stored at -20 °C for enzymatic analyses and determination of xylan degradation.

Enzymatic activities

Endo-1,4-β-xylanase (EC 3.2.1.8) were assayed by measuring of reducing sugar released from substrate (MILLER, 1959). The reaction mixture containing 1 mL of culture supernatant (enzymatic extract), 1 mL of 1% oat-spelts xylan (Sigma) in 0.05 M acetate buffer pH 5.0, and 2 mL 3,5-dinitrosalicilic acid (DNS) was incubated at 50°C for 30 minutes, and the enzyme-substrate system have been shacked periodically to keep xylan in suspension. Tubes containing the reactions were read in spectrophotometer (Bausch and Lomb) at 550 nm. The values were expressed in U mL^{-1}, when one unit is μmol of xylose produced per ml of medium per minute.

β-Xylosidase activity was routinely determined by measuring the amount of p-nitrophenol released from PNPX. The assay was carried out at 50 °C in 50 mM sodium phosphate buffer (pH 6.5), with 50 μL of 10 mM PNPX (Sigma) plus an appropriately diluted enzyme solution, in a total volume of 0.5 mL. The p-nitrophenol released was measured by monitoring the increase in the absorbance at 410 nm following 10 min. of incubation. The reaction was stopped by the addition of 1.0 mL of 1.0 M sodium carbonate. One unit of b-xylosidase activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol $min.^{-1}$ mL^{-1} (IEMBO et al., 2005).

High performance liquid chromatography

The degradation of xylan was analyzed in a Shimadzu chromatographer LC-6A using an ion exchange Supelcogel C-610H column (Supelco). A volume of 20 µL was previously filtered into PVDF 0.22 µm membrane (Millipore) and injected, using 0.1% phosphoric acid in ultrapure water as mobile phase, a flow rate of 0.5 mL $min.^{-1}$ and refractive index detector RID 10A (Shimadzu), specific for carbohydrates and organic acid analyses.

Results and discussion

Results

Xylanase activity was present practically in the whole period of cultivation, and the greatest activities corresponding to 20th, 30th and 40th days were reached by *Pleurotus* sp. BCCB068: 0.64, 0.58, and 0.60 U mL^{-1}, respectively, as shown in Figure 1. *P. tailandia* presented higher activity of xylanase on 20th day (0.25 U mL^{-1}); however, very low values of this enzyme have been shown during this period (Figure 2).

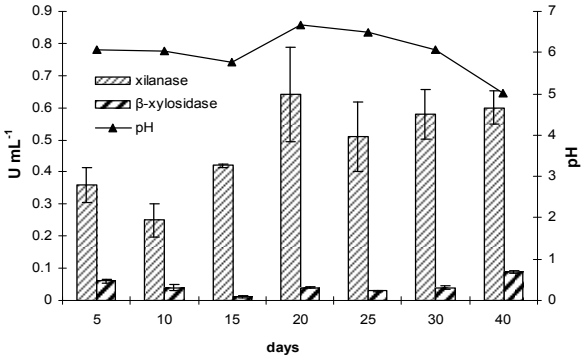

Figure 1. Activities of xylanase, β-xylosidase and pH produced by *Pleurotus* sp. BCCB068 during 40 days under submerged fermentation using xylan as carbon source. Data are the mean of triplicate and standard deviation is indicated in the error bars.

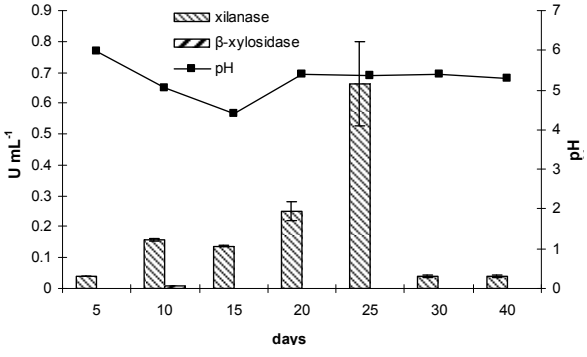

Figura 2. Activities of xylanase, β-xylosidase and pH produced by *P. tailandia* during 40 days under submerged fermentation using xylan as carbon source. Data are the mean of triplicate and standard deviation is indicated in the error bars.

In general, the fungi were capable of hydrolyzing xylan and forming some sugar monomers (xylose, arabinose, fructose, mannose, fructose, and others non-identified), and oligosaccharides as indicated in Tables 1 and 2. *Pleurotus* sp. BCCB068 shown considerable degradation of xylan on the 5th day (70.1%), followed by production of monomers and oligosaccharides. A peak of xylan hydrolysis was detected on the 20th day (75.1%), together with the best production of xylanase activity. Sugar monomers produced previously have indicated another decrease in concentration from the 15th day on due to a possible absorption of these compounds to be part of the fungal metabolism. Arabinose and xylose have been identified as products of xylan degradation by *Pleurotus* sp. BCCB068 comparing these peaks with previous analysis of carbohydrate standards (data not show). In 10 days of cultivation there was a drop of xylose peak and absence of arabinose, however, these monomers have increased on the 15th day through breakdown of XOS that were previously formed. From this period on a steady consumption of these compounds have been verified, moreover, new other compounds were formed up to 30 days of cultivation.

Table 1. Degradation of xylan, yield of xylooligosaccharides and sugar monomers by *Pleurotus* sp. BCCB068 under submerged fermentation.

Days of cultivation	% Xylan degradation	Xylooligosaccharides[a]	Sugar monomers	Non-identified intermediate metabolites[b]
5	70.1	+	Arabinose[c], Xylose[d]	+
10	55.0	+++	Xylose[d]	+++
15	42.8	++	Arabinose[c], Xylose[d]	++
20	75.1	+	ND[e]	+
25	72.7	+	ND[e]	+
30	66.9	+	ND[e]	++
40	67.2	+	ND[e]	++

[a, b]Yield expressed in intensity +/++/+++ by comparison of peak areas in the chromatograms; [c, d]sugar monomers identified by comparing retention times in the previously performed carbohydrates standard chromatogram; [e]not detected.

Table 2. Degradation of xylan, yield of xylooligosaccharides and sugar monomers by *P. tailandia* under submerged fermentation.

Days of cultivation	% Xylan degradation	Xylooligosaccharides[a]	Sugar monomers	Non-identified intermediate metabolites[b]
5	ND[g]	ND[g]	Arabinose[c], Maltose[d]	++
10	65.0	+++	ND[g]	+
15	70.2	++	Arabinose[c], Xylose[e]	++
20	73.6	+	Arabinose[c], Xylose[e], Mannose[f]	+
25	56.2	++	Mannose[f]	++
30	58.4	+	Mannose[f]	+++
40	61.2	+	Mannose[f]	+++

[a, b]Yield expressed in intensity +/++/+++ by comparison of peak areas in the chromatograms; [c, d, e, f]sugar monomers identified by comparing retention times in the previously performed carbohydrates standard chromatogram; [g]not detected.

Discussion

The highest activities of xylanase were verify by Garzillo (GARZILLO et al., 1994) following 16 days of cultivation of *P. ostreatus*; nevertheless, studies regarding the effect of growth of *P. ostreatus* 3004 and the digestibility of corncob producing cellulolytic enzymes under submerged fermentation, have verified low activities of xylanases (SERMANNI et al., 1994). Corncob contains high level of xylan (~33%) and was used as induction substrate to the production of xylanases (QINNGHE et al., 2004), as well as organic and inorganic nitrogen compounds including peptone in optimized conditions (KALL et al., 1995), promoting an increase in xylanase production by *P. ostreatus.* In our results, xylan was used as the single carbon source, and due to the absence of any co-substrate, there was low activity expressed. There was no use of such nitrogen sources, once our aim was not to optimize xylanase production, but utilize these enzymes as parameters to xylan degradation with no enrichment source. β-xylosidase enzyme was detected since the 5th day, verifying the presence of monomers through the degradation of XOS, maintaining its activity during the whole period of cultivation with some variation in values (0.01-0.09 U mL^{-1}), reaching a best activity on the 40th day, as shown Figure 1.

Analyzing pH values, *Pleurotus* sp. BCCB068 showed an oscillation from 5.03 to 6.66, and *P. tailandia*, from 4.42 to 5.99. Studies conducted by Qinnghe (QINNGHE et al., 2004) have demonstrated that *P. ostreatus* performed higher activity of xylanase in pH 5.4, confirming great mycelial growth of the same fungus in pH 5.0 (FURLAN et al., 1997). In this present work, the supernatant of *P. tailandia* revealed the highest level of xylanase activity (0.25 U mL^{-1}) in pH 5.41 with presence of xylan degradation (20th day, Figure 1). In the same period, *Pleurotus* sp. BCCB068 showed the best degradation of xylan and xylanase activity (0.64 U mL^{-1}) and pH 6.66. Burla and co-workers (BURLA et al., 1992) demonstrated that higher production of biomass to *P. ostreatus* was in pH 6.0, using basal medium supplemented by 0.2% sunflower oil and wheat straw, and a study by Bajpai (1997) has reported that from pH 5.0 though 6.5, xylanases are generally produced by fungi.

The activity of xylanase to *P. tailandia* was slightly less than described previously by *Pleurotus* sp. CCB 068, showing very low level of enzyme on the 5th day (no xylan degradation detected), and following 25, 30 and 40 days of incubation, when there was no longer an increase in the degradation rate. Nevertheless, the greatest activity of xylanase (0.25 U mL^{-1}) was observed on the 20th day, accompanied by higher levels of degradation of xylan by this fungus (Figure 2). *P. tailandia* did not show activities of β-xylosidase, except on the 10th day (0.01 UI mL^{-1}; Figure 2). Iembo (IEMBO et al., 2005) performed experiments with thermophilous fungus *Humicola grisea* in optimized conditions and found considerable value (12.98 UI mL^{-1}) when using oat-spelts xylan as carbon source. The fungus *Streptomyces* sp. CH7 growing in xylan substrate also reached great levels of β-xylosidase: 26.3 and 6.6 U mL^{-1} of protein, according to Pinphanichakarn and colleagues (PINPHANICHAKARN et al., 2004). Both studies carried out by these authors showed the stability of enzyme between pHs 6 and 9.

Regarding the results obtained by *Pleurotus tailandia*, only on the 5th day was hydrolysis of xylan not observed; however, a significant degradation of this matrix was detected on the remaining days. Higher degradations of xylan were reached from the 10th day on (Table 2). Moreover, XOS were produced early on the 10th day, as well as a gradual disappearance of these compounds up to the 25th day. In this period, a new production of oligosaccharides and sugar monomers was detected, with a drop in these productions on the 40th day, due to its consumption by the fungal metabolism. Sugar monomers were produced through the breakdown of XOS, and it is likely some metabolites produced in the xylan degradation presented the same retention time of xylan into the chromatogram, causing an overlaying of peaks, indicating a false raise of xylan peak.

According to the chromatographic profiles presented by fungi, there was a concomitant degradation of xylan and XOS, as well as assimilation of sugars and other products, suggesting the action of an enzymatic system containing xylanases and other hydrolytic and oxidative enzymes produced to degrade this complex carbon source. Studies conducted by Cotta and Zeltwanger (COTTA; ZELTWANGER, 1995) have shown the use of xylan as substrate by *B. fibrisolvens* reaching 75% of degradation with production of soluble acid-alcohols, and these products have been utilized by *S. ruminantum* to produce XOS. The degradation of oat spealt xylan and subsequently XOS by *B. fibrisolvens* generated a mixture of low-molecular-weigh intermediate metabolites containing several compounds with different levels of polymerization (e.g. xylose, arabinose and uronic acid), according to Hespell and Cotta (HESPELL; COTTA, 1995). Products from acid hydrolysis of xylan formed a variety of mixture of XOS when these products

were depending on several xylanolytic enzymes present into medium, and the degradation of XOS produced by xylanases was better metabolized than by acid hydrolysis procedure (COTTA; ZELTWANGER, 1995).

Conclusion

Despite the low production of endo-1,4-β-xylanase in this present work, a considerable degradation of xylan indicated the action of other specific and unspecific enzymes not studied in this work, responsible for xylan hydrolysis.

In conclusion, *Pleurotus* sp. BCCB068 and *P. tailandia* were capable of degrading xylan in up to 75%, producing XOS and several sugar monomers. These results have shown a promising applicability in residues with high concentration of hemicellulose, which when delignified are viable matrices for the production of oligosaccharides. Therefore, complementary studies may be done to optimize degradation processes and enzymatic production, once these fungi have shown a great potential for future biotechnological processes.

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