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Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains

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Abstract

Two strains of *Pleurotus ostreatus* (IE-8 and CP-50) were grown on defined medium added with wheat straw extract (WSE). Mycelia from these cultures were used as an inoculum for solid fermentation using sugar cane bagasse (C:N = 142). This substrate was used separately either as a mixture of heterogeneous particle sizes (average size 2.9 mm) or as batches with two different particle sizes (0.92 mm and 1.68 mm). Protein enrichment and production of lignocellulolytic enzymes on each particle size was compared. The effect of ammonium sulphate (AS) addition was also analyzed (modified C:N = 20), this compound favored higher levels of protein content. Strain CP-50 showed the highest increase of protein content (48% on particle size of 1.68 mm) when compared to media with no additional N source. However, strain IE-8 produced the highest levels of all enzymes: xylanases (5.79 IU/g dry wt on heterogeneous particles) and cellulases (0.18 IU/g dry wt on smallest particles), both without the addition of AS. The highest laccase activity (0.040 IU/g dry wt) was obtained on particles of 1.68 mm in the presence of AS. Since effect of particle size and addition AS was different for each strain, these criteria should be considered for diverse biotechnological applications.

Keywords: Particle size; Enzymatic activity; Fungal growth; Classified substrate; *Pleurotus ostreatus*

1. Introduction

Over the last three years in Mexico, sugar cane production was nearly 46 million ton annually (SAGARPA, 2006). Sugar cane production releases bagasse as a by-product, which is a lignocellulosic material providing an abundant and renewable energy source. Polysaccharides in this material can be used for bioethanol production, enzyme production, and fertilizing compounds for plant growth after a suitable composting process (Pandey, 2003; Viniegra-González et al., 2003). Although its digestibility is very poor due to the presence of lignin, sugar cane bagasse represents a potential source of energy as a supplement in animal feeding (Campos et al., 2004).

The white rot fungus *Pleurotus ostreatus* produces a wide range of extracellular lignocellulolytic enzymes, also named fibrolytic enzymes, including: xylanases, cellulases, and laccases (Sun et al., 2004). All these enzymes contribute in the degradation of cell wall content in sugar cane bagasse. However, there are some reports describing that lignocellulolytic enzymes production by *P. ostreatus* depends strongly on the strain, substrate composition and conditions of cultivation (Stajić et al., 2006).

Since the last decade, many studies about the application of solid-substrate fermentation (SSF) were focused on adding an extra value to agro industrial residues; several processes have been developed in order to enhance protein content in starchy fruits residues, enzyme production, and

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metabolite synthesis (Pandey, 2003). In general, in those processes nutrient sources are simultaneously physical supports for microbial growth. Therefore substrate degradation is attributed to cell bound enzymes or extracellular enzymes (Nandakumar et al., 1994). Enzyme actions on the substrate depend upon the physical properties of the materials including the crystalline or amorphous nature, accessible area, surface area, porosity and mainly particle size (Pandey, 2003; Viniegra-González et al., 2003). The effect of particle size on growth and product formation in SSF has been studied by different authors (Reddy et al., 2003; Zadrazil and Puniya, 1995), but no reports are available concerning specific induction of lignocellulolytic enzymes as a response to particle geometry in sugar cane bagasse. The aim of the present work was to obtain a vigorous inoculum and then evaluate both the effect of an inorganic N source and different particle sizes of sugar cane bagasse, on the production of protein and lignocellulolytic enzymes on solid substrate fermentation using two *P. ostreatus* strains.

2. Methods

2.1. Microorganisms and mycelial growth

Two strains of *P. ostreatus* were used: IE-8 and CP-50. Both strains belong to the mycological culture collection of the Colegio de Posgraduados in Texcoco, Mexico. Strains IE-8 and CP-50 were grown on plates containing 20 ml of malt extract agar (MEA) enriched with different N sources (urea or ammonium sulphate) in order to obtain a C:N ratio of 20 g:g (according to the commercial medium composition of MEA by DIBICO®). In another series of experiments, MEA was also prepared entirely dissolved in wheat straw extract (WSE) which was obtained as follows: 100 g of chopped wheat straw were suspended in 1 L of distilled water and maintained at 85 °C during 1 h. The liquid was then filtered by a cotton cloth. Due to evaporation, volume was adjusted to 1 L with distilled water. All culture media were autoclaved at 15 psi for 15 min. Three plates per medium for every strain were inoculated with a 6 mm mycelium-agar plug, obtained from simple MEA plates, and maintained at 29.5 ± 0.5 °C for 5 d. Radial growth rate (mm/h) from triplicates was determined by measuring colony diameter every day (Trinci, 1974). Means from each medium were subjected to a statistical analysis using the NCSS 2001® software.

2.2. Substrate characterization

Dry sugar cane bagasse was subjected to a sieving procedure employing mesh-size sieves of: 4, 6, 8, 12, 16, 20, 24, 35, 48, 50, and 60 (Mc Cabe et al., 2002). After this, bagasse particles were classified according to three diameter sizes and were used as substrates for SSF. The smallest particles (0.92 mm) were collected from fractions between meshes 16 and 20 (−16, +20), intermediate particles (1.68 mm) were collected from fractions (−8, +12), finally, heterogeneous bagasse (0.25–5.5 mm, average 2.9 mm diameter) was also used as a substrate. The geometrical ratio (fiber length: diameter or L/D) was determined for each particle size as an average value from 100 fibers per fraction. These L/D values corresponded to 12.3 ± 0.44, 8.7 ± 1.1, and 18 ± 0.55 for particles sizes of 0.92, 1.68, and 2.9 mm, respectively. For all three selected fractions, chemical composition was determined according to the methodology proposed by Bassi (2005) and Van Soest et al. (1991).

2.3. Solid substrate fermentation

Sugar cane bagasse was rehydrated with hot water (90 °C) for 30 min; then excess moisture was drained and final moisture content was determined to be 80%. Sugar cane bagasse was analyzed according to Bassi (2005) and Van Soest et al. (1991) and C:N ratio was determined to be 142:1 (protein 1.7%, cellulose 53.7%, hemicellulose 19.2%, lignin 18.2%, ashes 3.1%, and moisture 4.2%). This C:N value was obtained considering that N content in protein is 16% (Nelson and Cox, 2000), in this case 0.28% of bagasse corresponded to nitrogen and 40% of carbohydrates was considered as carbon. When ammonium sulphate (AS) addition was required in experimental trials, C:N ratio was adjusted to 20.

Five grams of this substrate were placed in 250 ml flasks and then were autoclaved at 121 °C for 15 min. Each flask was inoculated with four mycelium plugs (6 mm diameter each) obtained from MEA–WSE medium as described above. Biomass from mycelium plugs was carefully covered with substrate using a sterile spatula. Cultures were kept at 29.5 ± 0.5 °C during 8 d under static conditions.

After the incubation period, the content of each flask was suspended in 50 ml of sodium citrate buffer (50 mM, pH 5.0) for 30 min within an ice bath. Solids were separated by filtering through a gauze cloth, and filtrate was then centrifuged (4 °C, 10 000 rpm, 30 min). Supernatant was used for measurements of enzyme activity and soluble protein. Solids were dried at 70 °C 24 h, then ground in a blender to measure insoluble protein attached to substrate particles. Triplicates were used for each experimental treatment and statistical analyses were realized using the NCSS 2001® software.

2.4. Analytical procedures

Protein was determined according to Bradford (1976), Bovine Serum Albumin (40 mg/L) was used as standard; xylanase activity was estimated by DNS method (Miller et al., 1960) using a 0.5% solution of Birchwood xylan as substrate, previously dissolved in a sodium citrate buffer (50 mM, pH 5.3) according to Loera and Córdova (2003); laccase activity was determined registering oxidation of 2,2-azo-bis-(ethylbenzothiazoline-6-sulfonic acid) (ABTS) in an acetate buffer (0.5 mM, pH 5) at 420 nm

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every 20 s during 2 min (Wolfenden and Willson, 1982); carboxymethyl cellulase (CMCase) activity was estimated by DNS method ($\lambda = 575$ nm) using a solution of 1% carboxymethyl cellulose as a substrate, dissolved in a sodium citrate buffer (50 mM, pH 4.8) according to Miller et al (1960); filter paper activity (FPA) in the extracted filtrates was measured also at pH 4.8 employing a piece of filter paper (1 cm × 6 cm, No. 41) according to the procedures by and Decker et al. (2003). All activities were calculated from triplicates and statistical analyses were realized using the NCSS 2001® software. Values were expressed in international units (IU), where one unit (IU) of enzyme activity is defined as the amount of enzyme required to release 1 μmol of product per minute under the given assay conditions. Activities were referred to initial substrate dry weight (IU/g dry wt).

3. Results and discussion

3.1. Mycelial growth and inoculum preparation for SSF

P. ostreatus exhibited different growth patterns according to the type of inoculum used for SSF (Sainos et al., 2006). These authors also reported a correlation between productivity parameters and some enzymatic profiles in a variety of inocula using *P. ostreatus*. Consequently, inoculum preparation has to be considered as an important initial aspect for SSF cultures.

In order to obtain a rapid growing mycelium to be used as an inoculum, three different N sources were analyzed (Table 1). In the case of strain IE-8, the radial growth rate was 0.323 mm/h using WSE, only 5.0% higher than that value for AS (0.308 mm/h) and 38% higher than rates determined in urea containing medium (0.234 mm/h). For strain CP-50 the same pattern was observed. However, Novotný et al. (2001) observed that mycelia growth rates of *P. ostreatus* reached up to 1.25 mm/h (using enriched soil plus glucose and malt extract) and 2.5 mm/h (with glucose plus ammonium tartrate) indicating that some strains preferred more complex N sources. Our results did not show significant differences in radial growth for strains IE-8 and CP-50 using either AS or WSE (Table 1). Wheat straw extract was used as an N source for inoculum preparation for SSF studies, since this extract can easily be incorporated in media for petri dishes. However, AS was added directly on sugar cane bagasse in order to modified the C:N ratio as mentioned below.

Table 1
Radial growth rate (mm/h) for two strains of *P. ostreatus* on different nitrogen sources

Nitrogen source	Strain	
	IE-8	CP-50
Urea	0.234 ± 0.011^{a}	0.167 ± 0.008^{a}
Ammonium sulphate	0.308 ± 0.014^{b}	0.275 ± 0.017^{b}
Wheat straw extract	0.323 ± 0.008^{b}	0.294 ± 0.016^{b}

Means per column with different superscript letters are significantly different after a Duncan's test comparison for every nitrogen source ($p \leqslant 0.05$).

3.2. Analysis of protein and lignocellulolytic enzymes using different particle sizes

Sugar cane bagasse was classified after sieving and composition for all fractions was the same: Lignin 19% ± 1.9, hemicellulose 20% ± 1.8, cellulose 57% ± 3.3, and ashes 3.2% ± 0.7. Then fractions of sieved sugar cane bagasse alone (C:N = 142) or supplemented with AS (C:N = 20) were used for both strains as supports for SSF. After 8 d of incubation on every condition, both total protein and enzymes production were determined.

3.2.1. Total protein production

Protein enrichment after growth of *P. ostreatus* on sugar cane bagasse with different particle size is summarized in Table 2. In general, media supplemented with AS (C:N = 20) favored higher levels of protein content when compared to media with no additional N source (C:N = 142). Strain CP-50 showed the highest increase of protein content (48%) on particle size of 1.68 mm. However, for this strain after growing on substrate particles of 0.92 mm there was not significant difference in protein content after the addition of AS.

3.2.2. Enzymes production

Enzyme production was determined for different activities: xylanases, laccases, and cellulases, since these enzymatic families are involved in the breakdown of cell wall components. Xylanase activity produced by *P. ostreatus* growing on sugar cane bagasse with different particle size is shown in Fig. 1. Strain IE-8 was more susceptible to the presence of AS, since xylanase activity on 2.9 mm particles was 145% higher when AS was not added (5.79 IU/g dry wt) in comparison to that value obtained in presence of AS (2.36 IU/g dry wt). Similarly, using the smallest particle evaluated in this work (0.92 mm diameter) without AS,

Table 2
Protein enrichment by two strains of *P. ostreatus* after 8 d of growth on sugar cane bagasse added with ammonium sulphate (C:N = 20) and compared with the same substrate with no additional N source (C:N = 142)

Strain	C:N ratio	Average particle diameter (mm)		
		0.92	1.68	2.9
IE-8	20	0.630 ± 0.09^{a}	N.D.	0.430 ± 0.04^{a}
	142	0.526 ± 0.02^{a}	0.522 ± 0.02^{a}	0.342 ± 0.01^{b}
CP-50	20	0.566 ± 0.02^{a}	0.742 ± 0.03^{b}	0.758 ± 0.03^{c}
	142	0.563 ± 0.02^{a}	0.500 ± 0.02^{a}	0.668 ± 0.03^{d}

Data represent a mean for triplicates and are expressed as mg of total protein per g dry weight of fermented substrate. Initial total protein was 0.3 ± 0.028 mg/g dry wt.
ND = no determined. Means in a column with different superscript letters are significantly different after a Duncan's test comparison ($p \leqslant 0.05$).

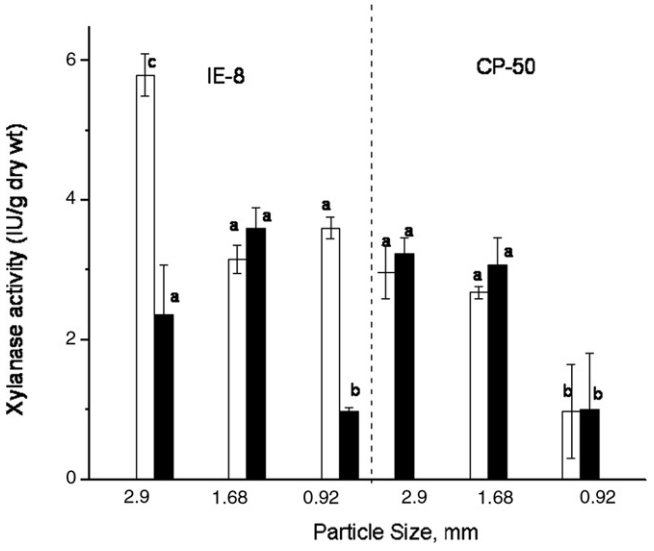

Fig. 1. Xylanases production by two strains of *P. ostreatus* grown on sugar cane bagasse after 8 d. Black bars correspond to ammonium sulphate addition. Means per column with different superscript letters are significantly different after a Duncan's test comparison ($p \leqslant 0.05$).

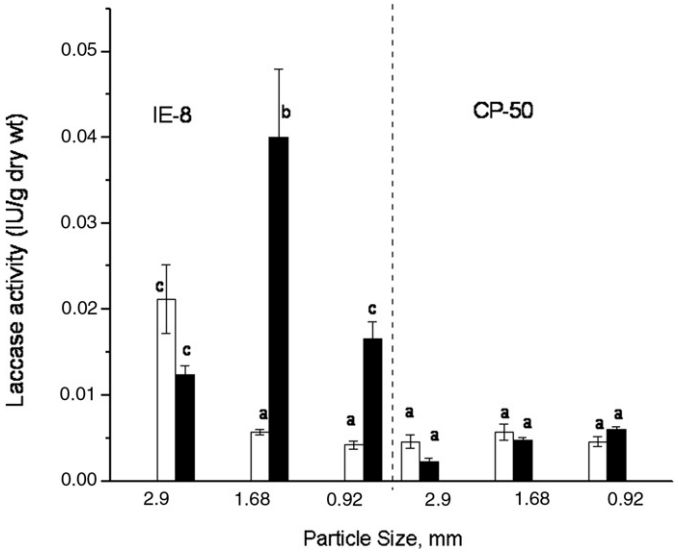

Fig. 2. Laccase production by two strains of *P. ostreatus* grown on sugar cane bagasse after 8 d. Black bars correspond to ammonium sulphate addition. Means per column with different superscript letters are significantly different after a Duncan's test comparison ($p \leqslant 0.05$).

xylanase activity (3.6 IU/g dry wt) was almost four times higher than in sugar cane bagasse supplemented with this inorganic N source (0.98 IU/g dry wt). However, when enzyme activity was analyzed from extracts obtained on middle sized particles (1.68 mm diameter) no significant effect on xylanase production was observed. For strain CP-50 the presence of AS did not have a significant effect for all particle sizes (Fig. 1). Our production levels were substantially higher than those values reported by Reddy et al. (2003), who observed xylanases titre between 0.64 and 1.6 IU/g dry wt for *P. ostreatus* and between 2.4 and 3.4 IU/g dry wt for *Pleurotus sajor-caju* after 20 d of culture on banana wastes. More recently, Seyis and Aksoz (2005) reported xylanase levels as specific activity, in terms of units per mg of protein, by *Trichoderma harzianum* grown on agro industrial wastes in the presence of different N sources, where maximal production levels reached 26.5 U/mg after one week of culture, whereas our *P. ostreatus* strain IE-8 reached 16.5 U/mg as the highest value considering protein content shown in Table 2.

Laccase production by both strains is shown in Fig. 2. This figure shows that neither AS or particle sizes had an effect on laccase production by strain CP-50. However, presence of AS in medium with every particle size exhibited a strong effect on laccase titre by strain IE-8, with the highest variation for middle sized particles (1.68 mm), since activity levels were 0.04 IU/g dry wt and 0.006 IU/g dry wt, with or without ammonium sulphate, respectively. Similarly, for small particles (0.92 mm) the same trend was observed. However, on heterogeneous particles (2.9 mm average diameter), laccase activity production without AS addition was almost double (0.021 IU/g dry wt) compared to enzymatic extracts obtained in the presence of this N source (0.012 IU/g dry wt). Variation in laccase profiles attributed to intrinsic differences of *Pleurotus* strains have been reported (Tellez-Tellez et al., 2005).

For other *P. ostreatus* strains, which have been cultivated under SSF, phenol oxidases production was significantly higher. Krishna Prasad et al. (2005) showed that levels of laccase activity up to 750 IU/g dry wt can be achieved after optimizing culture conditions (glucose, wheat bran, urea, and yeast extract) and using inducers (xylidine and N sources). Reddy et al. (2003), who worked with *P. ostreatus* found titres of 15.8 and 27.4 IU/g dry wt after 10 and 20 d, respectively. Although it has been suggested that N limitation is necessary for laccase production, Stajić et al. (2006) reported titres of laccase activity up to 32.1 IU/g dry wt after 10 d of cultivation for *P. ostreatus* under SSF on grapevine sawdust added with NH_4NO_3 (C:N = 40). Nandakumar et al. (1994) showed that the type of substrate available in wheat bran (starch or cellulose) causes sequential production of enzymes. They propose a mathematical model which gives the progression of substrate degradation (size reduction) during fermentation in terms of particle dimensions; our results agree with this point, since the present study suggests that exposure to different materials of sugar cane bagasse in particles with different size and geometry, affects laccase levels for both strains. Interestingly, a report by Tellez-Tellez et al. (2005) showed that for strains of *Pleurotus* belonging to the same species, differences in activity can be explained by the expression level of the same isoforms, rather than differential expression of laccase genes. These results were achieved comparing diverse strains under the same culture condition.

In the case of cellulases, both maximal CMCase (0.18 IU/g dry wt) and FPA activity (0.013 IU/g dry wt) were obtained for strain IE-8 in small particles (0.92 mm

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diameter) with no AS added (Figs. 3 and 4). Other studies (Reddy et al., 2003) reported that CMCase activity for *P. ostreatus* grown on banana waste reached 0.81 IU/g dry wt and almost nothing of FPA after 20 d. In the present study, both size particle and N source addition had an effect on CMCase and FPase synthesis. Strain IE-8 produced CMCase activity on 1.68 mm particles only with AS, whereas a strong increase was observed in FPA on small particles with no AS addition. However, the strongest effect of AS addition for strain CP-50 was detected in extracts obtained from large particles (Fig. 4). Blandino et al. (2002) have showed that both particle size and chemical composition of blends containing wheat grains and milled wheat affect the rate of microbial growth and therefore, the patterns observed by polysaccharides production in *Aspergillus awamori* after 7 d of cultivation at 30 °C. In relation to those observations, our results showed that enzymatic induction occurred to a different extend depending on particle sizes and the presence of AS, since sugar cane bagasse initially presented the same composition for every experiment.

Our results show that the geometrical ratio and size of sugar cane bagasse fibers strongly influence the profile of enzymatic activities. Sugar cane vascular beams exhibit a secondary wall, where a major fraction of rigid and cellulosic materials are located internally. Thus, in particles with grater *L*/*D* ratio, lignin is more abundant on the substrate surface; therefore the induction of laccases could be favored as shown for strain IE-8. It might be convenient to carry out some studies, based on cultures with periodic sampling, aimed at identifying variations intrinsically related to every strain. As mentioned above, variation in enzyme profiles attributed to strain differences have been reported (Tellez-Tellez et al., 2005; Arana et al., 2004; Reddy et al., 2003). In terms of enzyme synthesis response after AS addition, strain CP-50 was less sensitive than strain IE-8, which reveals that the regulatory effect of N depends also on differences inherent to every strain and it can not be assumed as a general feature. Finally, effect of particle size and addition AS was different for each strain: CP-50 showed the highest increase of protein content (48% on particle size of 1.68 mm) when compared to media with no additional N source. However, strain IE-8 produced the highest levels of all enzymes; in consequence these criteria should be considered when designing specific biotechnological applications.

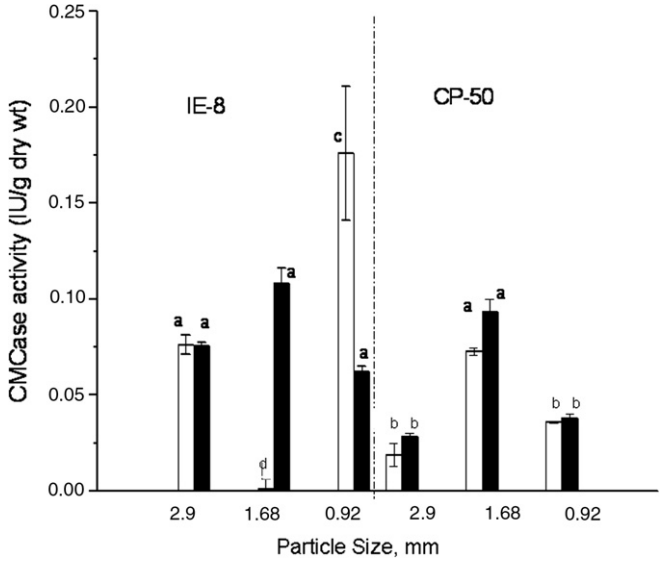

Fig. 3. CMCase activity production by two strains of *P. ostreatus* grown on sugar cane bagasse after 8 d. Black bars correspond to ammonium sulphate addition. Means per column with different superscript letters are significantly different after a Duncan's test comparison ($p \leqslant 0.05$).

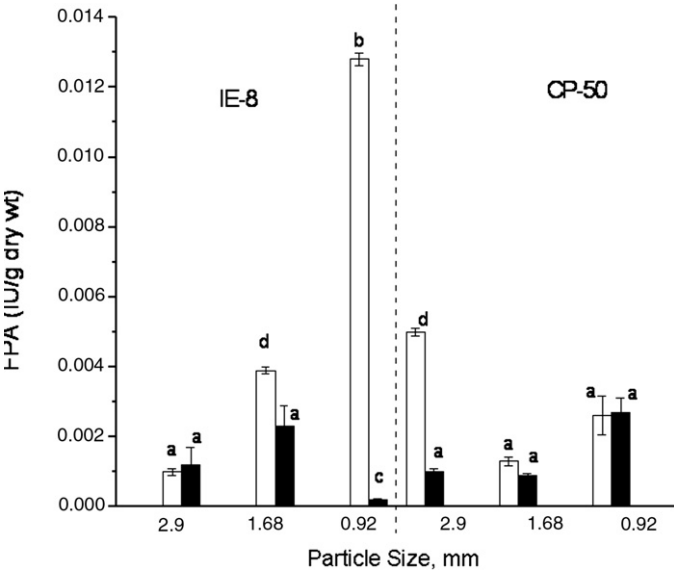

Fig. 4. FPAase production by two strains of *P. ostreatus* grown on sugar cane bagasse after 8 d. Black bars correspond to ammonium sulphate addition. Means per column with different superscript letters are significantly different after a Duncan's test comparison ($p \leqslant 0.05$).

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References

Arana, A., Téllez, A., Loera, O., Terrones, C., González, A., 2004. Comparative analysis of laccase-isozymes patterns of several related *Polyporaceae* species under different culture conditions. Journal of Basic Microbiology 44, 79–87.

Bassi, T., 2005. <http://www.mejorpasto.com.ar/UNLZ/2004/TX4.htm> (accessed 22.10.2005).

Blandino, A., Iqbalsyah, T., Pandiella, S.S., Cantero, D., Webb, C., 2002. Polygalacturonase production by *Aspergillus awamori* on wheat in solid-state fermentation. Applied Microbiology and Biotechnology 58, 164–169.

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, 248–254.

Campos, F.P., Sampaio, A.A.M., Bose, M.L.V., Vieira, P.F., Sarmento, P., 2004. Evaluation of *in vitro* gas production of roughages and their mixtures using the curves subtraction method. Animal Feed Science and Technology 116, 161–172.

Decker, S.R., Adney, W.S., Jennings, E., Vinzant, T.B., Himmel, M.E., 2003. Automated filter paper assay for determination of cellulase activity. Applied Biochemistry and Biotechnology 107, 689–704.

Krishna Prasad, K., Venkata Mohan, S., Sreenivas Rao, R., Ranjan Pati, B.P.N., Sarma, P.N., 2005. Laccase production by *Pleurotus ostreatus* 1804: optimization of submerged culture conditions by Taguchi DOE methodology. Biochemical Engineering Journal 24, 17–26.

Loera, O., Córdova, J., 2003. Improvement of xylanase production by a parasexual cross between *Aspergillus niger* strains. Brazilian Archives of Biological Technology 46, 177–181.

Mc Cabe, W.L., Smith, J.C., Harriot, P., 2002. Unit Operations in Chemical Engineering, sixth ed. McGraw-Hill, pp. 1199.

Miller, G.L., Blum, R., Glannon, W.E., Burton, A.L., 1960. Measurement of carboxymethylcellulase activity. Analytical Biochemistry 2, 127–132.

Nandakumar, M.P., Thakur, M.S., Raghavarao, K.S.M.S., Ghildyal, N.P., 1994. Mechanism of solid particle degradation by *Aspergillus niger* in solid substrate fermentation. Process Biochemistry 29, 545–551.

Nelson, D.L., Cox, M.M., 2000. Lehninger, Principios de Bioquímica, third ed. Barcelona, España, Omega, pp. 1264.

Novotný, C., Rawal, B., Bhatt, M., Patel, M., Sasek, V., Molitoris, H.P., 2001. Capacity of *Irpex lacteus* and *Pleurotus ostreatus* for decolorization of chemically different dyes. Journal of Biotechnology 89, 113–122.

Pandey, A., 2003. Solid-state fermentation. Biochemical Engineering Journal 13, 81–84.

Reddy, G.V., Ravindra Babu, P., Komaraiah, P., Roy, K.R.R.M., Kothari, I.L., 2003. Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor.caju*). Process Biochemistry 38, 1457–1462.

SAGARPA, 2006. Servicio de información y estadística agroalimentaria y pesquera. <http://www.siap.sagarpa.gob.mx> (accessed 21.02.2006).

Sainos, E., Díaz-Godínez, G., Loera, O., Montiel-González, A.M., Sánchez, C., 2006. Growth of *Pleurotus ostreatus* on wheat straw and wheat-grain-based media: biochemical aspects and preparation of mushroom inoculum. Applied Microbiology Biotechnology 72, 812–815.

Seyis, I., Aksoz, N., 2005. Xylanase production from *Trichoderma harzianum* 1073 D3 with alternative carbon and nitrogen sources. Food Technology and Biotechnology 43, 37–40.

Stajić, M., Persky, L., Friesem, D., Hadar, Y., Wasser, S.P., Nevo, E., Vukojević, J., 2006. Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. Enzyme and Microbial Technology 38 (1–2), 65–73.

Sun, X., Zhang, R., Zhang, Y., 2004. Production of lignocellulolytic enzymes by *Trametes gallica* and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. Journal of Basic Microbiology 44, 220–231.

Tellez-Tellez, M., Sanchez, C., Loera, O., Diaz-Godinez, G., 2005. Differential patterns of constitutive intracellular laccases of the vegetative phase for *Pleurotus* species. Biotechnology Letters 25, 1391–1394.

Trinci, A.P.J., 1974. A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. Journal of General Microbiology 81, 225–236.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nostarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Viniegra-González, G., Favela-Torres, E., Aguilar, C.N., Romero-Gómez, S., Díaz-Godínez, G., Augur, C., 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. Biotechnology Engineering Journal 13, 157–167.

Wolfenden, B.S., Willson, R.L., 1982. Radical cations as reference chromogens in studies of one-electron transfer reactions: pulse radiolysis studies of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate). Journal Chemical Society, Perkin Trans, II, 805–812.

Zadrazil, F., Puniya, A.K., 1995. Studies on the effect of particle size on solid-state fermentation of sugarcane bagasse into animal feed using white-rot fungi. Bioresource Technology 54, 85–87.

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