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

Isabel Membrillo [a], Carmen Sánchez [b], Marcos Meneses [d], Ernesto Favela [c], Octavio Loera [c,*]

[a] *División de Química y Bioquímica, Tecnológico de Estudios Superiores Ecatepec, Ecatepec 55210, Mexico*
[b] *Universidad Autónoma de Tlaxcala, CICB, Laboratorio de Biotecnología, C.P. 90000 Tlaxcala, Mexico*
[c] *Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, 09340 Mexico D.F., Mexico*
[d] *Programa en Ganadería, Colegio de Postgraduados, Km. 36.5, Carr. Mexico-Texcoco, Montecillo 56230, Mexico*

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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

* Corresponding author.
E-mail address: loera@xanum.uam.mx (O. Loera).

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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