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Saccharification with *Phanerochaete chrysosporium* and *Pleurotus ostreatus* enzymatic extracts of pretreated banana waste

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Lignocellulosic biomass has a great potential as raw material for second and third generation biofuels since it is the most abundant carbohydrate on earth and the main component of agricultural waste; however, saccharification of lignocellulosic biomass is crucial for the establishment of a carbohydrate-based economy. The use of fungal enzymes is the preferred procedure for lignocellulosic saccharification. Fungi such as basidiomycetes (e.g. *Phanerochaete chrysosporium*) produce cellulolytic/hemicellulolytic and ligninolytic enzymes, which are responsible for lignocellulose degradation. In this study the saccharification of banana flour prepared from pseudostem and green non commercial-grade fruit (1:1), two of the main agro-waste of banana industry was investigated. The material was pretreated by physical and chemical processes including drying and grinding, followed by 3% HCl or 3% NaOH hydrolysis, or a sequential pretreatment with 3% HCl first and then 3% NaOH and heated at 121°C, at 15 Lb/in² for 15 min. The highest concentration of reducing sugars (RS) was obtained with acid hydrolysis (42.41 gL⁻¹). Crude cellulolytic-ligninolytic enzymatic extracts from *Pleurotus ostreatus* and *P. chrysosporium* cultured on banana waste as the only carbon source were prepared and used for the saccharification. Surprisingly, *P. chrysosporium* crude extract produced a decrease in RS (2.27 gL⁻¹). Although *P. ostreatus* cellulose activity (17,777.78 UL⁻¹) was almost half compared to *P. chrysosporium*'s (31,296.30 UL⁻¹), the former produced an increment in the release of RS (63.65 gL⁻¹). In Mexico, banana is one of the main crops and generates large agricultural waste after harvest. According to the results obtained with acid-heat pretreatment followed by saccharification with *P. ostreatus* enzymatic crude extract, banana agro-waste can be considered as a potential feedstock for RS-based bioproducts like bioethanol.

Key words: Banana waste, lignocellulosic, pretreatments, saccharification, reducing sugars.

INTRODUCTION

At present, oil is the most widely used fuel in a great majority of activities; however, it is a finite and scarce resource, with exceptionally high operation costs in addition to major environmental impacts. Lignocellulosic biomass, the most abundant carbohydrate source on

earth, is a potential candidate for obtaining energy in the form of charcoal, hydrogen, ethanol and biogas; the last three requiring hydrolysis of the lignocellulosic material (Posso, 2002; Zaldivar et al., 2001). Production of biofuels and other bioproducts from lignocellulose is expected to promote rural economies, enhance energy security and alleviate environment pollution.

The use of agricultural waste as raw material is particularly convenient because it results in a significant reduction in the cost of production (Hahn-Hägerdal et al.,

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high cost of enzymes (Hahn-Hägerdal et al., 2006). Alternatively, 2006; Quintero et al., 2006). However, for the establishment of lignocellulose-based biofuel economy, the saccharification of these materials is necessary (Sathitsuksanoh et al., 2009). Agricultural waste includes a diversity of grasses, rice and wheat husks, among others. Mexico is a major fruit producer and the waste from this activity represents a viable option for obtaining renewable energy and other bioproducts. One of the main crops in Mexican agriculture is banana. The Mexican banana industry cultivates approximately 77,301 hectares producing 2.2 million tons per year. Only the fruit is commercialized, while the pseudostem, leaves and rachis are discarded (SAGARPA, 2007); these lignocellulosic residues could represent an important source of raw material for carbohydrate-based derivatives. The principal commercial banana is *Musa acuminata* Cavendish, AAA, cv. 'Grand Naine'. The composition of banana 'Grand Naine' lignocellulosic residues can be considered as feedstock since they are relatively low in lignin and rich in carbohydrates. The pseudostem contains approximately 60 to 65% hollocellulose and 12 to 18% lignin (Cordeiro et al., 2004; Khalil et al., 2006) and rachis is 38% hollocellulose and 10.5% lignin (Oliveira et al., 2007). The packing companies also discard a considerable amount of fruit which does not meet commercialization standards (SAGARPA, 2007); fruit peel has 28% hollocellulose and 14% lignin (Monsalve et al., 2006) and fruit pulp contains 72% starch (Garcés-Molina, 2004).

To efficiently process lignocellulosic wastes, the lignin structure must first be modified, because this tridimensional phenylpropane-based polymer prevents the access to the cellulose and hemicellulose polymers, which are the source of hexoses and pentoses, the substrates for the fermentative production of ethanol or hydrogen, among others (Galbe and Zacchi, 2007; Fonseca et al., 2008). Biomass processing and conversion usually involves two sequential steps: physicochemical pretreatment/fractionation of lignocellulose and enzymatic hydrolysis of cellulose. The structural modifications of lignocellulose are achieved by physicochemical pretreatments; first a pulverization or grinding process is carried out to mechanically reduce the size of the biomass particles and increase the contact surface for subsequent processes (Ferrer et al., 2002; Monsalve et al., 2006). When the biomass requires a more aggressive treatment, steam and pressure are applied (Sreenath et al., 2001; Sharma et al., 2007). In the case of chemical pretreatments, strong acids and bases such as H₂SO₄ or HCl (Chen et al., 2007; Ferrer et al., 2002; Hernández-Salas et al., 2009) and NaOH (Monsalve et al., 2006; Mussatto et al., 2006) are used at different concentrations and temperatures. The use of microorganisms or enzymes which facilitate the release of sugars can also be employed. Currently, cellulosic-based products like ethanol are expensive because of the

enzymes obtained from lignocellulolytic microorganisms are used for saccharification (Patel et al., 2007; Sharma et al., 2007).

The white rot Basidiomycete *Phanerochaete chrysosporium* is able to completely degrade cellulose, hemicellulose and lignin (Kersten and Cullen, 2007). This fungus has been used for saccharification of lignocellulosic agro-waste of paddy straw, wheat straw, sugarcane bagasse (Havannavar and Geeta, 2007), cotton stalks (Kerem et al., 1992) and coffee pulp (Parani and Eyini, 2010) among others; therefore, it can be an option for saccharification of other agricultural residues as banana wastes. In addition, the edible mushroom *Pleurotus ostreatus* has been successfully cultured on banana agrowastes (leaf, pseudostem) to produce ligninolytic and cellulolytic enzymes such as laccase (Lac), lignin peroxidase (LiP) and cellulases (Reddy et al., 2003). This fungus was proved to efficiently degrade banana pseudostem biomass (Ghosh et al., 2004). In addition, *Pleurotus* species have been shown to degrade lignocellulosic agro-industrial wastes (Havannavar and Geeta, 2007; Parani and Eyini, 2010) and they have also been used to improve digestibility of lignocellulosic biomass (Adamovi et al., 1998; Alborés et al., 2006; Mukherjee and Nandi, 2004).

Lignocellulosic materials are largely heterogeneous, making it necessary to determine for each case the type of pretreatment and biological saccharification to achieve the greatest release of RS (Angenent, 2007; Hahn-Hägerdal et al., 2006; Masera et al., 2005). The objective of this work was to evaluate the release of RS from banana waste by different sequences of physicochemical pretreatments and subsequent saccharification with crude enzymatic extracts obtained from the lignocellulolytic fungi *P. ostreatus* and *P. chrysosporium*.

MATERIALS AND METHODS

Biological material

The fungi *P. ostreatus* ATCC 38540 and *P. chrysosporium* CDBB-H-686 (from the microbial collection of CINVESTAV, Mexico) were used to obtain the enzymatic extracts for the saccharification.

Substrate and pretreatment

Green non commercial-grade banana fruit and pseudostems (*Musa acuminata* Cavendish, AAA, cv. 'Grand Naine') were collected in a plantation and packing company located in Teapa, Tabasco State, Mexico (located in the south of the state between the parallels 17°32' north latitude and 92°57' west longitude). The substrates were mechanically cut to a size of approximately 2 cm, dried in a Felisa® stove to constant weight (Mussatto et al., 2006) and subsequently reduced in size using a PAGANI® grinder to particles of about 2 mm in diameter (Baig et al., 2004; Chen et al., 2007). Banana pseudostem and fruit flours were mixed (1:1). Three chemical pretreatments were evaluated, each one by triplicate. Ratio of non-wood lignocellulosic solid to liquid solutions was 6 g:

25 ml. In the first pretreatment, 3% HCl was used for 15 min; in the second pretreatment, 3% NaOH was applied for 90 min (Mussatto et al., 2006); for the third pretreatment, 3% HCl was added for 15 min then neutralized and 3% NaOH was added for 90 min (Chen et al., 2007; Zhao et al., 2008). After each pretreatment, heat was applied at 121 °C at 15 L^b for 15 min and the reducing sugar were quantified by the DNS method (Miller, 1959).

Enzymatic extracts

The mycelia of *P. ostreatus* and *P. chrysosporium* were first grown in PDB for fast growth. After 2 weeks, mycelia were filtered in aseptic conditions, washed with sterile water and 3 g of each mycelium was transferred to 100 ml of sterile 0.1 N acetate buffer (pH of 4.8) containing 6 g of lignocellulosic banana flour as only carbon source. Fungi were cultured at room temperature and agitation was done at 100 rpm.

To establish the day for recovering the enzymatic extracts, the cellulase activity was measured every day for 17 days. Two independent experiments, with three replicates each were conducted.

Enzyme assays

Cellulase activity was assayed in 0.1 M Na citrate pH 4.8, using 1% (w/v) CMC as substrate and addition of the fungal culture supernatant. Mixtures were incubated for 30 min at 50 °C (Kim et al., 1996). The release of RS was measured as described earlier. One activity unit was defined as the amount of enzyme that released 1 μmol of RS (expressed as glucose) in one minute.

Lac activity was determined according to Li et al. (1999), following the rate of oxidation of 5 mM ABTS in 0.1 M Na acetate at pH 5.0. Oxidation was measured at 420 nm ($\epsilon_{420} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$). LiP was measured in 100 mM Na succinate, at pH 3.0, with 4.0 mM veratryl and 0.1 mM H₂O₂ following veratraldehyde production at 310 nm ($\epsilon_{310} = 9300 \text{ M}^{-1} \text{ cm}^{-1}$) (Tien and Kirk, 1988). MnP was measured in 100 mM Na succinate, at pH 4.0, 25 mM Na lactate, 20 mM MnSO₄, 7 mM phenol red and 0.1 mM H₂O₂. Phenol red oxidation rate was measured at 431 nm ($\epsilon_{431} = 18,300 \text{ M}^{-1} \text{ cm}^{-1}$) (Archibald, 1992). One activity unit of laccase (Lac), lignin peroxidase (LiP) or manganese peroxidase (MnP) was defined as the amount of enzyme that released 1 μmol of product per minute. These enzymes were measured every two days for 17 days. Enzyme activities were reported as UL⁻¹.

Enzymatic saccharification

The cell free crude enzymatic extracts from 10 days fungal cultures were recovered by centrifugation at 3000 rpm during 20 min. To determine the efficiency for saccharification of banana lignocellulosic residue of each fungal culture, the whole enzymatic crude extract (100 ml each) was independently added to pretreated flours (6 g each). The flasks were incubated in an orbital shaker at 100 rpm and 35 °C for 96 h. Aliquots were sampled every 24 h to evaluate the RS. pH was monitored during this process. Two independent experiments, with three replicates each were conducted.

Statistical analysis

All experiments were carried out in triplicate and the results expressed as means ± standard deviation. To analyze the experi-

mental data, Statgraphics plus v. 4.1 (Statistical Graphics Corp. Warrenton, VA) software was used.

Scanning electron microscopy (SEM)

For SEM analysis, samples were vacuum infiltrated with 2.5% glutaraldehyde in 0.2 M sodium phosphate, at pH 7.3, fixed for 48 h at room temperature and rinsed several times in buffer solution. The samples were dehydrated using gradient solutions from 30 to 85% (v/v) ethanol, followed by absolute alcohol and carbon dioxide. Samples were mounted on stubs and coated with gold in a sputter coater (Samdri-795, Tousimis Research Corporation, Rockville, Maryland, USA). The images were captured with a JEOL JSM-6360 LV scanning electron microscope.

RESULTS

Pretreatments

Three different pretreatments were evaluated on the flour mixture prepared from the banana pseudostem and green non commercial-grade banana fruit. Sequential addition of 3% (v/v) HCl (incubated for 15 min) followed by 3% (v/v) NaOH (for 90 min) produced 0.45 gL⁻¹ of RS which was slightly lower than the result obtained with the reference (0.52 gL⁻¹), where deionized water was added. Alkali or acid alone produced 0.81 and 0.87 gL⁻¹ of RS, respectively, being the higher values obtained with these treatments.

The mixtures of flours were submitted to the same treatments followed by humid heat at 121 °C for 15 min with a double purpose; as physical lignocellulose pretreatment and also to prevent microbial contamination during saccharification. RS were measured after each treatment and showed that sequential HCl-NaOH-heat steps produced 1.13 gL⁻¹ and NaOH-heat pretreatment released 1.37 gL⁻¹. RS measured in the reference (water) was 1.62 gL⁻¹. The highest saccharification was observed with the HCl-heat pretreatment, which yielded 42.41 gL⁻¹ RS.

Scanning electron microscopy (SEM) was conducted to evaluate microstructural changes in the banana waste after pretreatments. NaOH-heat and sequential HCl-NaOH-heat produced a porous flat surface area, apparently eliminating the rough external surface. More efficient biomass structure modification was achieved by HCl-heat pretreatment, exposing internal structures which were seen as fibers. The electronic scanning images are shown in Figure 1.

Enzymatic activities

Cellulase, laccase, manganese peroxidase and lignin peroxidase were monitored in *P. chrysosporium* and *P. ostreatus* submerged agitated cultures. The culture medium was supplemented with banana lignocellulosic waste as carbon source. Because

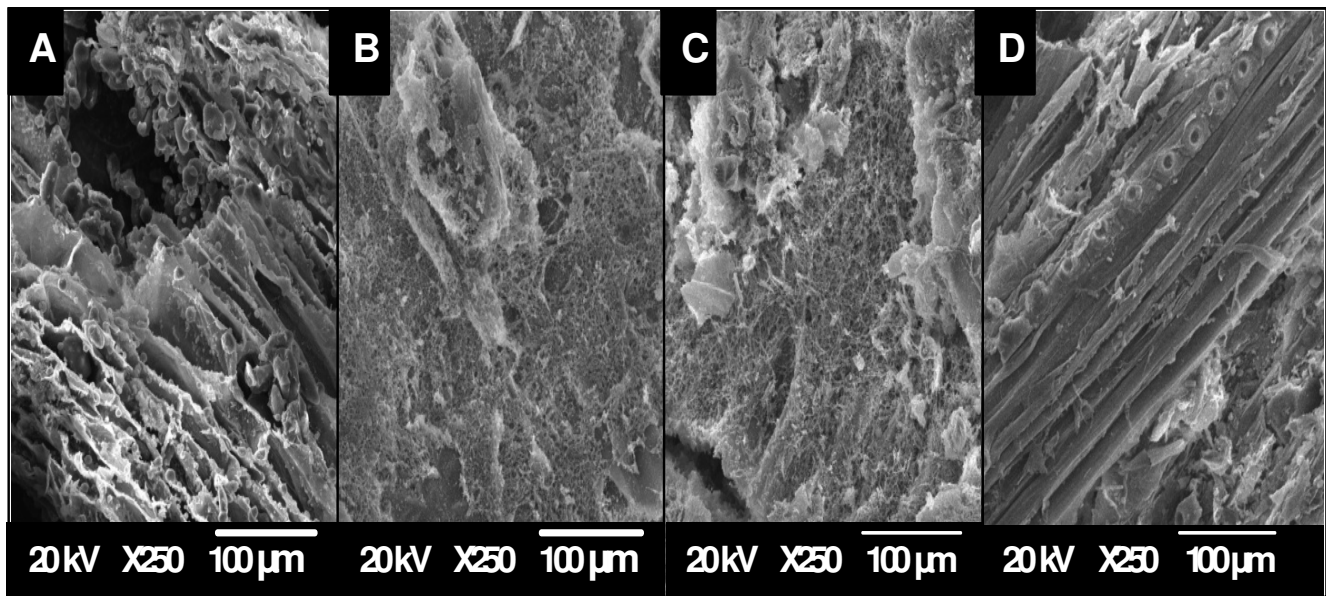


Figure 1. SEM images of banana lignocellulosic material (mixture of pseudostem and non-commercial grade banana fruit) before (A) and after (B-D) pretreatments; (B) HCl-NaOH-heat; (C), NaOH-heat; (D) HCl-heat pretreatment.

it was considered as important for further discussion, the results of the two independent experiments monitoring the cellulase activity are presented in Figures 2A and B. The first peak of cellulase activity was observed in *P. chrysosporium* culture during the first four days with a maximum value of $23,148 \text{ UL}^{-1}$. At the 5th day, no activity was detected. A second peak of cellulase activity was also detected in this fungal culture, from the 8th to the 12th day, with a maximum activity of $30,000 \text{ UL}^{-1}$ at day 9 and 10 (Figures 2A and B). After the 13th day, the *P. chrysosporium* culture had no cellulase activity.

In the case of *P. ostreatus*, a single broad peak of cellulase activity was observed, from the 9th to the 13th day. Maximum activity was maintained during those days, at approximately $20,000 \text{ UL}^{-1}$ (Figures 2A and B). The analysis of variance (ANOVA) showed no significant difference ($n = 3$, $p = 0.05$) in the cellulase activity at days 9 and 10 for *P. chrysosporium* extracts and days 9 to 13 for *P. ostreatus*.

Activities of ligninases were measured in these crude extracts at every second day. In *P. chrysosporium*, MnP activity started from the first day of culture (Figure 3) and increased after the fifth day, reaching a maximum value of 258.48 UL^{-1} on the 7th day. The other fungus also produced MnP from the fifth day, displaying two peaks with maximum values of 183.88 and 444.68 UL^{-1} on days 7 and 13 (Figure 3). LiP activity appeared on the seventh day and reached a maximum of $6,509.64 \text{ UL}^{-1}$ on the 9th day in *P. chrysosporium*. No significant LiP activity was measured in *P. ostreatus* crude extracts (Figure 4). Lac activity was higher in *P. ostreatus* culture (Figure 5) and

was detected in this fungal culture from 5th to 9th day, with a maximum value of 730.16 UL^{-1} on the 7th day. In the case of *P. chrysosporium* crude extract, it showed two peaks of Lac activity that were lower than that in *P. ostreatus*. Maximum Lac activity in *P. chrysosporium* was detected also at seventh day, reaching a value of 341.27 UL^{-1} . The second maximum Lac activity value was measured on the 15th day, with a value of 146.83 UL^{-1} .

Enzymatic saccharification of banana waste

For the saccharification of lignocellulosic biomass, both kind of enzyme families (ligninases to open structure and cellulases to hydrolyze the carbohydrate) are important. In this case, since banana biomass was physically and chemically pretreated, cellulases were the most important enzymes for saccharification. The crude enzymatic extracts (supernatants) from the 10th day of fungal cultures were used, since the cellulase activity was at the maximum in both cultures at that day. For saccharification of pretreated banana biomass, the whole enzymatic crude extracts (100 ml) were used in each case.

Fungal enzymatic saccharification after NaOH-heat and sequential HCl-NaOH-heat pretreatments was poor. Initial RS (0.81 gL^{-1}) increased to 2.28 gL^{-1} with *P. ostreatus* and 1.57 gL^{-1} with *P. chrysosporium* crude enzymatic extracts in the case of NaOH-heat pretreatment. For sequential HCl-NaOH heat pretreated material, maximum RS values were 5.58 and 3.72 gL^{-1} with *P. ostreatus* and *P. chrysosporium* enzymes, respectively

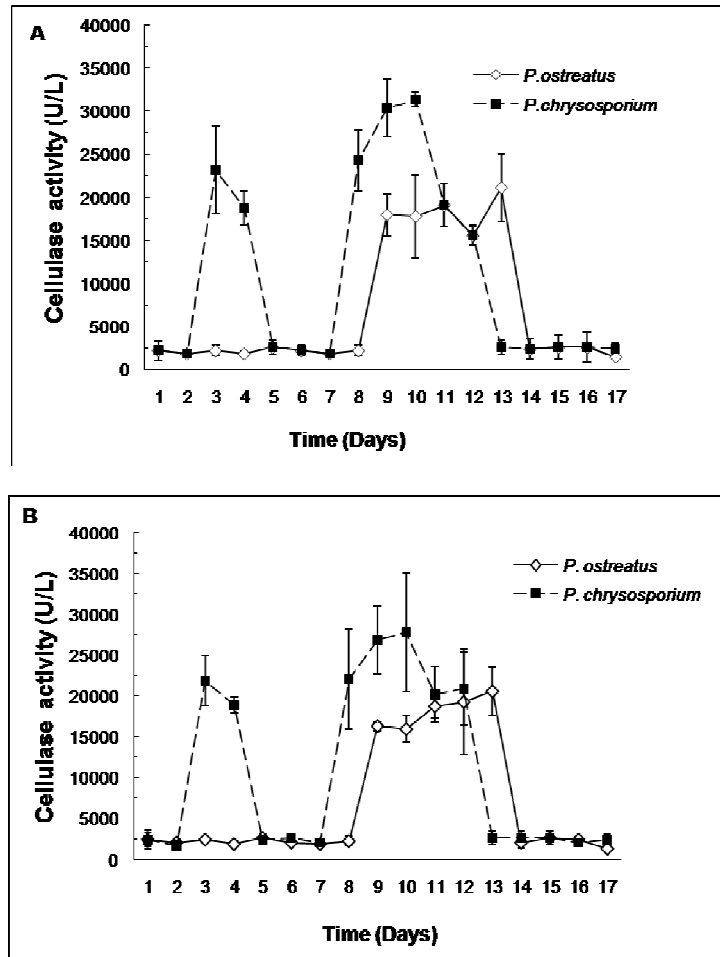


Figure 2. Time course for cellulase activity of *P. ostreatus* and *P. chrysosporium* in liquid medium. Panel A and B correspond to independent experiments. Data represent the means of triplicate and error bars indicate SD.

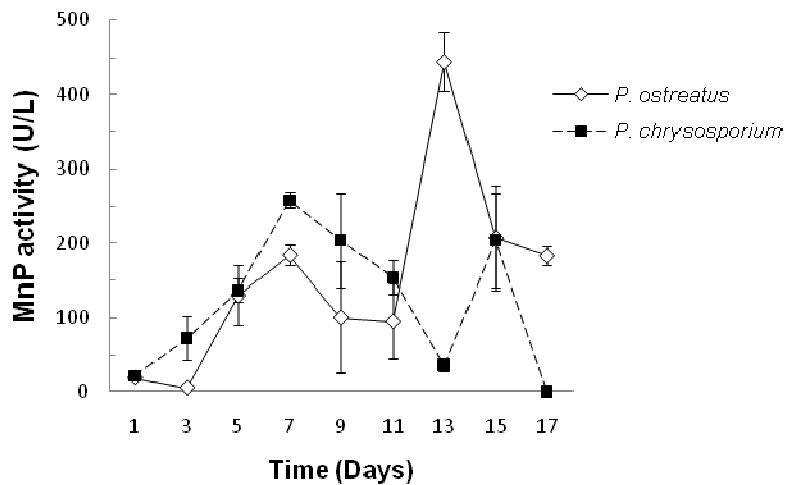


Figure 3. MnP activity production in crude enzymatic extracts of *P. ostreatus* and *P. chrysosporium* cultures. Activity was determined following oxidation of phenol red. Data represent the means of triplicate and error bars indicate SD.

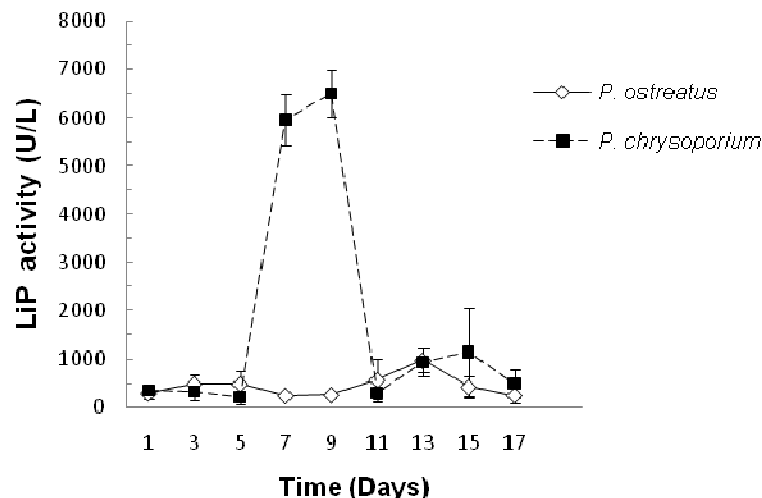


Figure 4. Lip activity determined by the VA assay in *P. ostreatus* and *P. chrysosporium* cultures. Data represent the means of triplicate and error bars indicate SD.

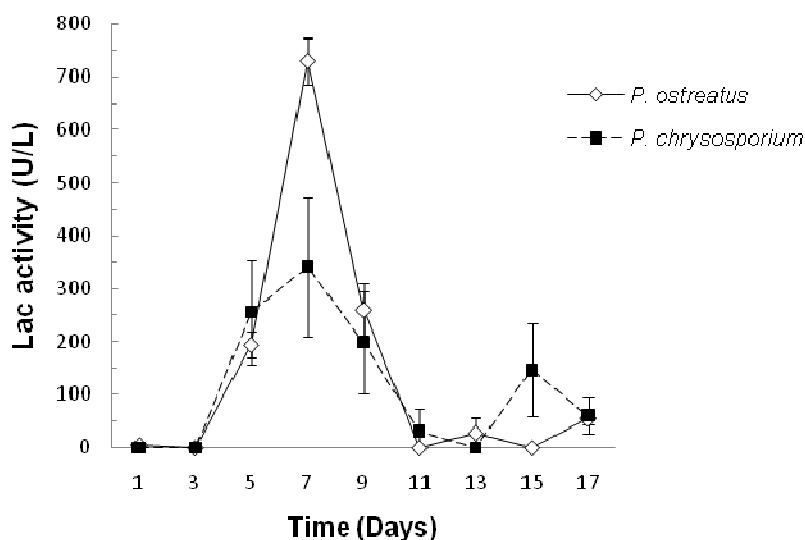


Figure 5. Lac activity profiles of *P. ostreatus* and *P. chrysosporium* cultures. Activity was measured by the ABTS assay. Data represent the means of triplicate and error bars indicate SD.

(data not shown). These results were negligible.

With HCl-heat pretreated banana lignocellulosic flour, *P. chrysosporium* enzymatic extract produced no evident release of RS, on the contrary, after 24 h after addition of this enzymatic preparation, the RS decreased from 42.41 to 31.56 gL⁻¹. On the second day, the amount of RS was 37.26 gL⁻¹. However, on the third day, a dramatic decrease in RS to 2.27 gL⁻¹ was measured (Figure 6A). This behavior was reproducible; the same profile in the measured RS was observed in a second independent experiment (Figure 6B).

The best result of RS was obtained when *P. ostreatus* enzymatic extract was added to the HCl-heat pretreated biomass. In this case, reducing sugar was 63.65 gL⁻¹ at the third day of saccharification. At the fourth day, there was a little decrease to 57.46 gL⁻¹ (Figure 6A). Figure 6B shows similar results in an independent experiment.

Table 1 corresponds to the cellulase and ligninases activities measured in *P. ostreatus* and *P. chrysosporium* enzymatic crude extracts harvested on the 10th day. *P. chrysosporium* extract contained almost double activity of the cellulase and more than five times the activity of MnP,

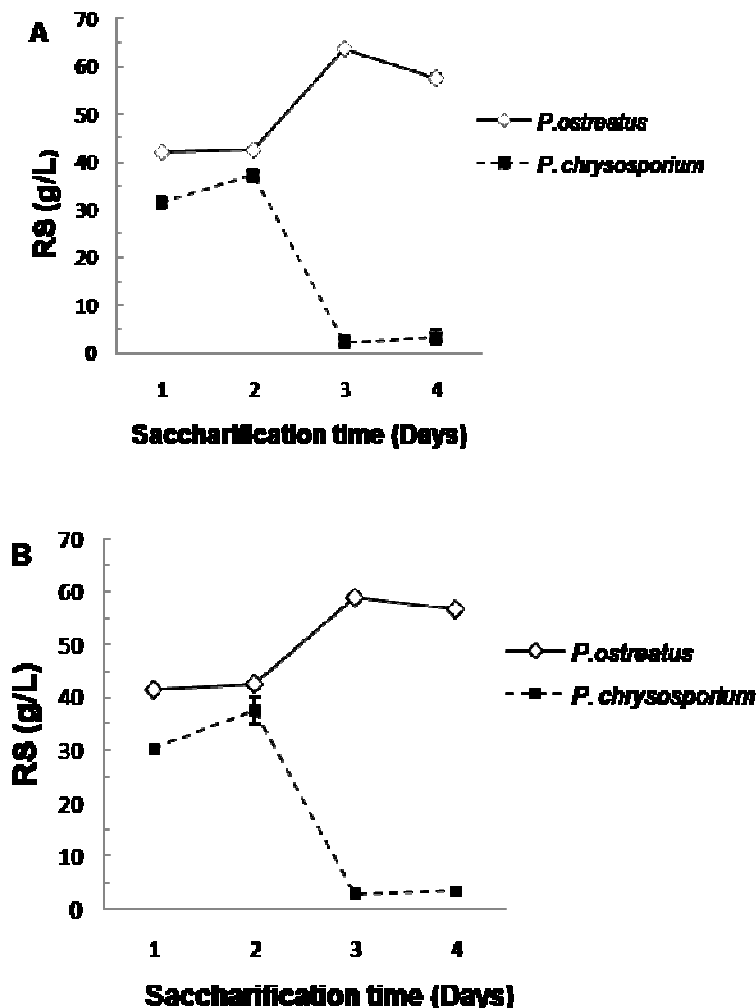


Figure 6. Enzymatic saccharification of HCl-heat pretreated banana lignocellulosic biomass with *P. ostreatus* and *P. chrysosporium* cell free crude extracts. Panel A and B correspond to independent experiments. Data represent the means of triplicate and error bars indicate SD.

when compared with *P. ostreatus* extract. On the contrary, Lac activity was twice in the *P. ostreatus* extract. The more remarkable difference in these activities was in LiP. *P. chrysosporium* had 38 times the activity measured in *P. ostreatus* extract.

DISCUSSION

Lignocelluloses are often a major waste from forestry, agriculture and municipalities. However, the suitability of lignocelluloses to be used as feedstock in many industries depend on the success in saccharification. Enzymatic hydrolysis of unpretreated lignocelluloses is usually not effective because of high stability of the materials. Baig et al. (2004) saccharified unpretreated banana pseudostem-leaf mixed flour; they used the

enzymatic extract of *Trichoderma lignorum* cultured on unpretreated lignocellulosic material and obtained a maximum of 1.34 gL^{-1} reducing sugar. Similarly, Patel et al. (2007) saccharified independently bagasse and rice husks, using individual and mixed fungi and it was observed that, the combination of *Pleurotus sajor-caju* and *Aspergillus awamori* released 0.34 gL^{-1} of RS with rice husk and with bagasse and *Phanerochaete chrysosporium* released 0.90 gL^{-1} RS. These data show the incapacity of the enzymes to penetrate and reach the cellulose when the lignin has not been opened or eliminated.

Better results were obtained when raw materials were pretreated, making them accessible to microbial and enzymatic attack. Zhu et al. (2006) hydrolyzed rice husk residue, including a microwaves/acid/alkali pretreatment and subsequently saccharified it with commercial cellu-

Table 1. Cellulase and ligninase activities measured in *P. ostreatus* and *P. chrysosporum* crude enzymatic extracts harvested on the 10th day of culture. Fungi were grown in liquid medium containing banana lignocellulosic biomass as carbon source. Cell free enzymatic crude extract was used for the saccharification of the pretreated biomass.

Fungi	Cellulase activity (U/L)	MnP activity (U/L)	LiP activity (U/L)	Lac activity (U/L)
<i>P. ostreatus</i>	17,777.78 ± 4746.67	57.59 ± 33.39	95.58 ± 62.57	193.12 ± 38.95
<i>P. chrysosporum</i>	31,296.30 ± 809.54	322.95 ± 47.52	3,611.54 ± 993.31	88.62 ± 47.34

Data represent the means of triplicate ± standard deviation.

lases; the released RS yield was 35.6 gL⁻¹. Similar results were reported by Sreenath et al. (2001) with microwaves pretreatment on alfalfa fibers before adding commercial cellulases (final RS yield of 40 gL⁻¹). Chen et al. (2007) reported high saccharification of maize stalks with a combination of cellulases from *Trichoderma reesei* ZU-02 and *Aspergillus niger* ZU-07 after pretreatment to the waste with H₂SO₄ (1:6) for 3 h; saccharification released 89.5 gL⁻¹ RS.

In this work, two fungal enzymatic extracts were employed to saccharify banana agrowastes (mixture of pseudostem and non-commercial grade banana fruit). The material was dried and ground until fine flour. Size of particles, solutions/concentration and time used for pretreatments, as well as conditions used for saccharification (pH, temperature, incubation time) were chosen from procedures described in the literature (Baig et al., 2004; Sharma et al., 2007; Taherzadeh and Karimi, 2008), following criteria of convenience and economy. For example, particles of 2 mm were not big but it was neither the smallest size used in the literature, which means more expensive process cost (Baig et al., 2004; Monsalve et al., 2006). This size was used in some reports (Chen et al., 2007), as milling improves surface area and therefore, accessibilities of chemical and enzymes to the cellulose. pH 4.8 was used according to Talebnia et al., (2008) and Vaithanomsat et al., (2009). Baig et al. (2004) studied the influence of different factors in saccharification of banana leaves and pseudostems and found similar results at temperatures between 30 and 45°C; 35°C, an intermedium temperature was used in this study.

After drying and grinding the material, three chemical pretreatments were applied. NaOH-heat and sequential HCl-NaOH heat produced no significant release of RS. Alkaline pretreatments removed lignin and a part of the hemicelluloses, but usually require long time and high concentration of the base (Taherzadeh and Karimi, 2008). Lignocelluloses are largely heterogeneous and pretreatments which are good for some materials are not good for others (Hernández-Salas et al., 2009). The success of a particular pretreatment depends on which specific lignocellulosic biomass is used. However, acids are more used for chemical pretreatment (Taherzadeh and Karimi, 2007; Talebnia et al., 2008). Acids are not effective in dissolving lignin, but they can disrupt it and

increase cellulose susceptibility to enzymatic hydrolysis. Acids can be used either as a pretreatment of raw biowastes before enzymatic hydrolysis or as the method of hydrolyzing them to fermentable sugars (Taherzadeh and Karimi, 2008). In this study, the best result was obtained with acid-heat pretreatment. Heat improves disruption of the lignocellulosic structure because it increases kinetic energy and facilitates mass transfer inside the lignocellulose; in addition, pressure also improves penetration of solutions into the biomass. This explains the fact that, pretreatment with HCl-heat released 42.41 gL RS while HCl with no heat produced 0.87 gL⁻¹. Release of sugars is a combination of acid and heat-pressure effect on the biomass since the material subjected to 121°C and 15 Lb/in² in water (reference condition) released only 0.52 gL⁻¹ RS (not shown). Sequential HCl-NaOH pretreatment, with or without heat, produced also a poor release of RS, (less than 2 gL⁻¹), because acid was neutralized by the base before heating. SEM results confirmed that, the acid-heat pretreatment was the most effective in opening up the biomass structure (Figure 1), which was congruent with the better recovery of RS.

Pretreatment of banana agrowastes have been previously reported. Monsalve et al. (2006) worked on banana peels and obtained a RS yield of 20 gL⁻¹ after sequential NaOH-H₂SO₄ pretreatment and predicted that the yield of RS will increase to 32 gL⁻¹ when fruit endosperm was included. In this study, a higher release of RS was achieved even though 50% of pseudostem was included in the flour. Pseudostem and non-commercial grade banana fruit are among the most abundant wastes in the Mexican banana industry; a reason why it would be more economic to work with the mixture at industrial scale, saving on waste separation cost. For comparison, the released RS in both raw materials were measured independently. The released RS from fruit after HCl-heat was 62.53 gL⁻¹ meanwhile, for pseudostem 23.10 gL⁻¹ were obtained (data not shown).

Sharma et al. (2007) analyzed the saccharification of a mixture of two agroindustrial residues from India, kinnow waste and banana peels. After grinding, the substrates were mixed in a proportion of 4:6 (kinnow waste: banana peels) and the mixture was hydrolyzed by steam explosion, yielding 63 gL⁻¹ RS. However, this technique requires a high energy input. Other limitations include the

destruction of a part of xylanases, the incomplete disruption of the lignin-carbohydrate matrix and the generation of toxic compounds (furfurals, hydroxymethyl furfurals) which can inhibit the activity of fermentative microorganisms. In addition, it requires special facilities not commonly available, particularly in developing countries. In this work, heat by sterilization conditions in autoclave (121 °C at 15 Lb/in²) was used, which was commonly employed in any laboratory and in many industries.

Several works have reported the use of commercial enzymes for saccharification (Lynd et al., 2005; Sreenath et al., 2001; Zhu et al., 2006); however, because of high enzyme cost, more economic alternatives are being investigated, such as the domestic production of enzymes using lignocellulolytic microorganisms (Chen et al., 2007; Leathers, 2004; Sharma et al., 2007). For saccharification, the domestic preparation of lignocellulolytic enzymes with two fungal cultures was produced. *P. ostreatus* and *P. chrysosporium* were cultured in liquid artificial medium (acetate buffer supplemented with banana lignocellulosic flour). In both cases, the crude enzymatic extracts were harvested at the 10th day of culture, since cellulase activity was maximum with both fungi. The cellulase activity was not adjusted but the whole volume of the extracts was added. This means that, cellulase activity was 17,777.78 UL⁻¹ for *P. ostreatus* and 31,296.30 UL⁻¹ for *P. chrysosporium*. Even when cellulase activity in *P. chrysosporium*'s extract was twice than that of *P. ostreatus*'s (Figure 2A), the latter produced better saccharification (63.65 gL⁻¹ RS at third day) while *P. chrysosporium* produced a dramatic depletion of RS (2.27 gL⁻¹). This was a surprising result since *P. chrysosporium* is one of the best known fungus that produces large amounts of lignocellulolytic enzymes.

This fungus is able to completely degrade cellulose, hemicellulose and lignin (Kersten and Cullen, 2007) and has been extensively used for the saccharification of lignocellulosic agrowastes (Havannavar and Geeta, 2007; Kerem et al., 1992; Parani and Eyini, 2010). Although, contradictory to what was expected initially, this result is largely reproducible (Figure 6B) and it was not due to microbial contamination during saccharification as the complete process was conducted under aseptic conditions. In addition, when RS depletion was observed an aliquot was plated (4th day); neither bacterial nor fungal colonies were found on LB or PDA media after two weeks (data not shown). Data for cellulase activity in *P. ostreatus* and *P. chrysosporium* crude enzymatic extracts were also reproducible, being half in *P. ostreatus* (Figure 2B).

This unusual result has biotechnological importance and it is worth to be discussed since the strategy to induce lignocellulolytic enzymes using lignocelluloses is currently proposed to avoid the use of expensive substrates. The sudden decrease of RS strongly suggested an enzymatic-mediated transformation. After analysis of the other enzymatic activities, a probable

explanation to this result can be proposed. *P. chrysosporium* extract had higher activities of peroxidases than *P. ostreatus* (Figures 3 and 4). MnP activity was more than five times (322.95 UL⁻¹) and LiP activity was 38 times more (3,611.54 UL⁻¹) in *P. chrysosporium* than that of *P. ostreatus*. These enzymes require the presence of H₂O₂, which in ligninolytic fungi, is produced by various pathways with pyranose oxidases (from D-glucose, D-xylose), glyoxal oxidase (from glyoxal, methyl-glyoxal), aryl alcohol dehydrogenase (from benzaldehyde and benzoic acid) and FAD-dependent aryl alcohol oxidase (from benzyl alcohol, 4-methoxybenzyl alcohol, veratryl, cinnamyl and coniferyl alcohols) (Izumi et al., 1990; Kersten and Kirk, 1987; Kersten and Cullen, 2007; Shah and Nerud, 2002; Koker et al., 2004); some of these enzymes consume sugars directly or indirectly. In *P. chrysosporium* cultures, the glucose oxidase is the main system for H₂O₂ production, for it consumes glucose (Kelley and Reddy, 1986). *P. chrysosporium* secretes glucose oxidase in liquid culture (Daniel et al., 1992) when the fungus is grown using lignocelluloses as carbon source (Daniel et al., 1994). Therefore, based on this information, reduction in the RS by the addition of *P. chrysosporium* enzymatic extract (Figures 6A and B) may be explained in part by the presence of pyranose oxidases, which are some of the principal sources of H₂O₂ in multiple fungi including *P. chrysosporium* (Daniel et al., 1994). However, it is also intriguing that the RS depletion occurred at the third day. Why was it delayed? Which mechanism explains that the decrease did not occur from the beginning? The delay could be explained by the presence of an inactive enzyme at the beginning of the culture, which was further activated *in vitro* by the production of an activator (the system, even when it was not *in vivo* but *in vitro*, was very complex, containing many compounds from the pretreated biomass and many different enzymes, not only discussed here, but produced the fungi). Probably more plausible, is to expect the presence of a metabolite (released from lignocelluloses or produced during the pretreatment) which at the beginning of saccharification inhibited the pyranose oxidases present in the fungal enzymatic extract. Removal of this inhibitor (by *P. chrysosporium* peroxidases, which are largely able to degrade xenobiotics) triggered the activity of pyranose oxidases, resulting in the dramatic decrease of RS in the third day. Glucose oxidase is not the only explanation. Currently, it is believed that aryl-alcohol oxidase and glyoxal oxidase are the main enzymes responsible for the production of extracellular H₂O₂ (Shah and Nerud, 2002). Glyoxal and methyl-glyoxal can be produced by oxidation of ethylene glycol, acetaldehyde and also from glucose. Chemical or biological conversion of RS in oxoaldehydes (or other metabolites) can activate *in vitro* the target enzymes triggering the catalytic (enzymatic-mediated) degradation of RS into oxoaldehydes (for H₂O₂ production by glyoxal oxidase), explaining the depletion

of RS on the third day. Although, both proposals are largely speculative, a high demand of H_2O_2 is expected for sustaining the *P. chrysosporium* LiP and MnP activities for ligninolysis, since this white-rot basidiomycete is precisely one of the most powerful ligninolytic fungus known so far. "The continuous production of H_2O_2 requires a large drain of the reducing equivalents, which is not a problem for the white-rot fungi, since there is excessive carbon in nature" (Shah and Nerud, 2002).

P. chrysosporium has been used in solid state saccharification and fermentation; meanwhile in this study the sac-charification was conducted in "liquid state", using the enzymatic extract produced in the submerged culture. In solid state saccharification, these enzymes act sequentially (time regulated). First, peroxidases and pyranose oxidases act coordinately to open part of the lignin and expose the cellulose and hemicellulose. When these polymers are accessible, cellulases and hemicellulases act to hydrolyze them into simple sugars, which are, at least in part, absorbed by the fungus as nutrient. This probably explains why the results in this study ("no saccharification of the pretreated material but depletion of RS") are different from those of other reports. Saccharification of HCl-heat pretreated material with *P. ostreatus* produced an increment of 50% (63.65 gL^{-1}) above the RS achieved after pretreatment (42.41 gL^{-1}). Reddy et al. (2003) reported the use of banana leaf and pseudo-stem agrowaste to produce lignocellulolytic enzymes from *P. ostreatus* and *P. sajor-caju* in solid culture. Enzyme activities could not be compared because they expressed unit values in other terms, but the profiles were similar. They measured cellulase and high activity of Lac in *P. ostreatus* and LiP was not detected. On the contrary, *P. sajor-caju* was able to produce LiP on these materials.

The banana residues are suitable for the cultivation of highly nutritious edible mushroom as the oyster *P. ostreatus* (Ghosh et al., 2004; Reddy et al., 2003) and *Volvariella volvacea* (Belewu and Belewu, 2005). These fungi are easily digestible and they have no cholesterol. In addition, they are rich in statins (which control cholesterol in blood) and glutamic acid (which helps the immune and nervous systems). Therefore, they can be able to improve human public health being an alternative application for the lignocelluloses.

Dried-grinded-HCl-heat pretreatment released 42.41 gL^{-1} RS. So, these materials are attractive because acid can be used either as a pretreatment for enzymatic hydrolysis or as a cheap method of hydrolysis to release the fermentable sugars. However, the 50% increment in RS with *P. ostreatus* extract was not negligible specially when scaling up of the process. Both procedures are appropriate, depending on the goal. Since an acceptable saccharification of the banana residual biomass was achieved, this is suitable to be used in Mexico as biocompost, biofertilizer, for cattle feeding and biofuel

production (bioethanol, biohydrogen and methane).

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Abbreviations

RS, Reducing sugars; **DNS**, 3,5-dinitrosalicylic acid; **PDB**, potato dextrose broth; **HCl**, hydrochloric acid; **NaOH**, sodium hydroxide; **CMC**, carboxymethylcellulose; **H_2O_2** , hydrogen peroxide; **H_2SO_4** , sulfuric acid; **Lac**, laccase; **ABTS**, 2,2'-azinobis-(3-ethyl benzothiazoline)-6-sulfonate; **LiP**, lignin peroxidase; **MnP**, manganese peroxidase; **ϵ** , molar extinction coefficient.

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