OPEN ACCESS
Freely available on line

Universitas Scientiarum

Univ. Sci. 2015, Vol. 20 (1): 117-127
doi: 10.11144/Javeriana.SC20-1.eple

ORIGINAL ARTICLE

Production of lignocellulolytic enzymes from floriculture residues using *Pleurotus ostreatus*

Balkys Quevedo-Hidalgo[1]✉, Paulo Cesar Narváez-Rincón[3], Aura Marina Pedroza-Rodríguez[2], Mario Enrique Velásquez-Lozano[4]

Abstract

Floriculture is a vital agro-industrial sector in the Colombian economy; the export of flowers positively impacts employment and the balance of trade. However, this industry could negatively impact the environment if its waste products are not handled properly. These flower residues, rich in lignin, hemicellulose and cellulose, could be a cost-effective raw material to produce enzymes. Here, we evaluate the production of lignocellulolytic enzymes by degradation of *Chrysanthemum* and *Rosa* residues using *Pleurotus ostreatus*, and manganese sulfate and copper sulfate as inductors. From the two residues, we obtained laccase, manganese peroxidase, endoglucanase, exoglucanase, and β-glucosidase. The use of inductors, favored all enzyme activities except for β-glucosidase. The enzymes that displayed the highest activity were laccase (4,693.4 U/L and 2,640 U/L from the residues of *Chrysanthemum* and *Rosa*, respectively) and β-glucosidase (9,513 U/L and 6,811.9 U/L). The enzyme that showed the lowest activity was endoglucanase (11.5 U/L and 15.4 U/L). Under the conditions evaluated, the best substrate for enzyme production is *Chrysanthemum* wastes; the extracts obtained had higher enzymatic activity than the extracts from *Rosa* residues.

Keywords: floriculture residues; *Chrysanthemum*; *Rosa*; lignocellulolytic enzymes; *Pleurotus ostreatus*

Edited by Alberto Acosta ✉

1. Grupo de Biotecnología Ambiental e Industrial (GBAI), Laboratorio de Biotecnología Aplicada, Departamento de Microbiología, Pontificia Universidad Javeriana. Bogotá, Colombia.

2. Grupo de Biotecnología Ambiental e Industrial (GBAI), Laboratorio de Microbiología ambiental, Departamento de Microbiología, Pontificia Universidad Javeriana.

3. Grupo de Procesos Químicos y Bioquímicos, Laboratorio de Ingeniería Química, Departamento de Ingeniería Química y Ambiental, Universidad Nacional de Colombia. Bogotá, Colombia.

4 Grupo de Procesos Químicos y Bioquímicos, Laboratorio de Ingeniería Química, Departamento de Ingeniería Química y Ambiental, Universidad Nacional de Colombia. Bogotá, Colombia.

Received: 01-06-2014 **Accepted:** 01-08-2014
Published on line: 08-09-2014

Citation: Quevedo-Hidalgo B, Narváez-Rincón PC, Pedroza-Rodríguez AM, Velásquez-Lozano ME (2015) Production of lignocellulolytic enzymes from floriculture residues using *Pleurotus ostreatus*. *Universitas Scientiarum* 20(1): 117-127 doi: 10.11144/Javeriana.SC20-1.eple

Funding: Pontificia Universidad Javeriana; Universidad Nacional de Colombia.

Electronic supplementary material: N/A

Introduction

Forestry and agricultural activities such as the production of paper, lumber and many other agro industries generate large amounts of waste. These residues are considered an environmental contamination issue. A high percentage of these residues are indiscriminately burned, generating greenhouse gases, and their outdoor decomposition generates leaching into water bodies and promotes the proliferation of pests (Asocolflores 2002).

Lignocellulose is the major structural component of woody and non-woody plants and represents an important source of renewable organic matter. Lignocellulose contains lignin, hemicellulose and cellulose, compounds with chemical properties

that increase the biotechnological potential of the lignocellulosic substrate (Malherbe & Cloete 2002). To exploit the potential of lignocellulose as feedstock to produce high added-value products, a physical, chemical or biological pretreatment process is always necessary to modify its three-dimensional structure. Some biological pretreatments have been studied, among these; degradation using enzymes produced by fungi (Stajić et al. 2006, Membrillo et al. 2008, Bettin et al. 2011, Větrovský et al. 2013), including the lignocellulosic degradation using *P. ostreatus* (Isikhuemhen & Mikiashvilli 2009, Quevedo-Hidalgo 2012, Thakur et al. 2013).

P. ostreatus is a white rot basidiomycete that grows on a variety of agricultural lignocellulosic residues and produces a great number of extracellular enzymes. For example, Baldrian et al. (2005) reported the production of cellulolytic and hemicellulolytic enzymes endo-1,4-β-glucanase, exo-1,4-β-glucanase, 1,4-β-glucosidase, endo-1,4-β-xylanase, 1,4-β-xylosidase, endo-1,4-β-mannanase and 1,4-β-mannosidase, and ligninolytic enzymes such as Mn-peroxidase and laccase, by growing *P. ostreatus* on wheat straw in the presence and absence of Cu, Mn, Pb, and Zn. Elisashvili et al. (2006) studied the production of cellulase, xylanase, laccase and manganese peroxidase in submerged fermentation of mandarin peels and tree leaves. Thakur et al. (2013) reported the production of ligninolytic enzymes using *P. ostreatus* from biologically and chemically pretreated wheat straw and banana stem substrates.

A typical fungal cellulolytic system comprises multiple enzymes. Cellulases are usually mixtures of several enzymes, mainly endoglucanase, exoglucanase and β-glucosidase (Sun & Cheng 2002). Endoglucanases internally hydrolyze cellulose chains, exoglucanases externally hydrolyze cellobiose units from the non-reducing or reducing ends of cellulose microfibers and 1,4-β-glucosidase hydrolyzes the bonds linking glucose molecules (Baldrian & Valášková 2008). By catalyzing the hydrolysis of alkyl- and aryl- β-glucosides, diglucosides, and oligosaccharides, 1,4-β-glucosidase plays a considerable role on the decomposition of lignocellulosic substrates; it hydrolyzes cellobiose into glucose, thereby preventing end-product inhibition of exoglucanases (Huang, 2011).

Cellulases and other enzymes with similar or complementary enzymatic pathways are used in the food, beer, wine, animal feed, textile, laundry and paper industries, as well as in agriculture research (Bhat 2000). Laccase and manganese peroxidase are enzymes that have potential industrial applications because these enzymes remove oxidized phenolic compounds and a variety of aromatic compounds. The conversion of toxic compounds and textile dyes present in wastewater and the bleaching and removal of lignin from wood fibers and non-wood are two examples of industrial applications of these enzymes (Lundell et al. 2010).

Floriculture is one of the main activities of the agricultural sector in Colombia, with a 13 % share of the world trade of flowers, ranking second after the Netherlands, which has a 42 % market share. According to Asocolflores (2010), more than 50 types of flowers are produced over an area of 7,266 hectares in Colombia. The main types of flowers are *Rosa* (33 %), carnations (12.7 %), mini carnations (6.7 %), *Chrysanthemum* and pompons (8 %) (Asocolflores 2010). The floriculture sector generates approximately 1.5 m^3/ha/day or between 0.8 and 1 t/ha/week of vegetable residues, depending on the type of flower and the production cycle. Approximately 9,591 t/month of residues are generated by *Rosa* production while 2,325 t/month are generated by *Chrysanthemum* cultures. Residues result from the cutting, post-harvesting and planting startup steps.

Considering the volume of floriculture residues generated in Colombia and the fact that enzyme production from floriculture wastes has not been reported previously, in this article, we evaluate enzyme production from the degradation of lignocellulose residues of *Rosa* and *Chrysanthemum* using *P. ostreatus*. The production of laccase, manganese peroxidase, endoglucanase, exoglucanase and β-glucosidase was evaluated after a submerged fermentation process using flower residues and *P. ostreatus*. Enzyme extracts containing all the enzymes mentioned previously were obtained from the two assessed residues. The process using the *Chrysanthemum* residues produced the extracts with the highest enzymatic activities.

Materials and methods

Cultivos del Norte (Tocancipá, Colombia) supplied the *Chrysanthemum* and *Rosa* residues (stems and leaves). The residues were analyzed for lignin, cellulose and hemicellulose contents using the neutral detergent fiber method (NDF) (Van Soest et al. 1991). Phosphorus, calcium, potassium, zinc, magnesium, copper and manganese content was assessed using flame atomic absorption spectrophotometry (Thermo Scientific ICE3000) following the procedure described in ASTM D1971-11 Practice B for digestion and ASTM D4691-11 for measuring the elements. Nitrogen content was determined by the procedure described in ASTM D5373-08 (**Table 1**). Before degradation, the residues were dried at 104 °C for 24 h and milled to a particle size smaller than 1 mm. All reagents used in this study were of analytical grade. *P. ostreatus* (HPB/P3) were obtained from the Biotechnology Laboratory of the Pontificia Universidad Javeriana (Bogotá, Colombia). Stock cultures of this fungus were maintained on wheat bran agar at 4 °C.

Enzyme production from *Chrysanthemum* and *Rosa*

Two assays were performed for each residue, one with and one without inductors addition (in total four cultures, each one run by triplicate) to evaluate enzyme production from *Chrysanthemum* and *Rosa*.

Fungal inoculum were grown on a static culture at 30 ± 2 °C in 250 mL Erlenmeyer flasks containing 50 mL of the following (per liter): 50 g of floriculture wastes, 2 g of yeast extract, 5 g of peptone, 0.075 g of $MnSO_4.H_2O$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_20$, and 175 g of wheat bran. This medium was inoculated with ten fungal disks (5 mm diameter) taken from the edge of the mycelium grown on bran extract agar in petri dishes (7-day old). After seven days, mycelial biomass was obtained and was inoculated (5 % of dry mycelium based on substrate volume) to the degradation stage.

The degradation of *Chrysanthemum* and *Rosa* residues was performed in a 1.5 L bioreactor (TECFERM, Procesos Biotecnológicos y Medioambientales, Bogotá, Colombia) with an agitation speed of 240 ± 5 rpm and aeration rate of 2 vvm for 24 h, with *P. ostreatus* in submerged fermentation under the optimum conditions as determined by Quevedo-Hidalgo et al. (2012) for reducing sugars. The optimum conditions for *Chrysanthemum* and *Rosa* residues were 6.3 % (w/v) concentration and pH 5.6, and 11.8 % (w/v) concentration and pH 5.6, respectively (Quevedo-Hidalgo et al. 2012). For the assays without inductors, Cu^{+2} and Mn^{+2} concentrations were the same as those originally present in each residue. For the assays with inductors, copper sulfate and manganese sulfate were added simultaneously; each concentration was 7.5 mM.

The culture was separated from the fungal biomass and insoluble components of the culture medium by centrifugation (at 22,000 g for 15 min) at 4 °C, followed by filtration using a Millipore membrane (0.45 μm). The extract was stored at -20 °C and it was used to determine the presence of the following enzymes: laccase, manganese peroxidase (MnP), endoglucanase, exoglucanase, and β-glucosidase.

Finally, because *Chrysanthemum* and *Rosa* residues contain Zn^{+2} and Cu^{+2}, we added Zn^{+2} or Cu^{+2} to final concentrations of 20, 40 and 80 ppm to assess laccase

Table 1. Characterization of the *Chrysanthemum* and *Rosa* residues.

Residue	Cellulose	Hemicellulose	Lignin	N	Ca	K	Mg	Cu	Fe	Mn	Zn
	Content (weight %)							Content (mg/kg)			
Chrysanthemum	49.6	7.5	17.5	2	0.63	1.78	0.26	24.6	258	70.7	58.8
Rosa	38.4	8.8	9.5	1.94	0.78	0.88	0.19	8.99	256	86.3	82.3

activity in the extracts. Each assay was performed by triplicate. Laccase activities with added Zn^{+2} or Cu^{+2} were compared to the activities obtained without the addition of these metals.

Evaluation of the solid fraction obtained from the degradation process of *Chrysanthemum* and *Rosa* residues

Because the solid fraction of the residues can contain adsorbed enzymes, they were washed 5 times by centrifugation with distilled water (at 1,500 g for 10 min at 4 °C). The remaining solids were re-suspended with 50 mM sodium citrate buffer pH 5.0 and stirred at 150 rpm for two hours at 4 °C. The supernatant was separated by centrifugation at 10,800 g for 10 min at 4 °C, then filtered through a 0.45 µm Millipore membrane and used to measure the enzymatic activities. The enzymatic activity of the solid fraction was related with the extract to determine the percentage of adsorption. The process was evaluated with and without the addition of these inductors to establish the effects of manganese and copper sulfate on the enzymatic activity.

Enzymatic assays

All assays were performed in triplicate using an Evolution 60 UV/Visible Thermo spectrophotometer. Laccase activity (EC 1.10.3.2) was determined with the substrate ABTS (2.2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (Sigma, St. Louis, MO, USA). The assay mixture contained 100 µL of 0.5 mM ABTS, 100 µL of 60 mM sodium acetate buffer (pH 4.5), and 800 µL of the culture filtrate; the reaction was carried out during 3 min. Oxidation of ABTS was monitored by measuring the absorbance increase at 436 nm (ξ_{436}= 29,300 M^{-1} cm^{-1}). One unit of laccase activity was defined as 1 µmol $ABTS^{+}$ formed per minute (Bourbonnais et al. 1997, Tinoco et al. 2001).

The manganese peroxidase (MnP, E.C. 1.11.1.13) activity was determined by the oxidation of 2.6-dimethoxyphenol (2.6-DMP) (Sigma, St. Louis, MO, USA) at 468 nm (ξ_{468}= 49,600 M^{-1} cm^{-1}), and the reaction mixture contained 450 µL of culture supernatant, 500 µL of 10 mM 2.6-DMP in 100 mM sodium acetate buffer (pH 5.0), 50 µL of 0.4 mM $MnSO_4$, and 30 µL of 22 mM H_2O_2; this reaction was carried out during 3 min (Santoyo et al. 2008). Given that both MnP and laccase can oxidize 2.6-DMP, the laccase activity was subtracted using data from control experiments performed in the absence of H_2O_2 and manganese. One unit of MnP activity was defined as 1 µmol of 2.6-DMP oxidized per minute.

The activity of endoglucanase (EC 3.2.1.4) was measured with carboxymethylcellulose (CMC) (low viscosity, Sigma, St. Louis, MO, USA). The reaction mixture contained 1 mL of 2 % (w/v) substrate in 50 mM sodium citrate buffer (pH 5.0), and 1 mL sample (Ghose 1987). The reaction mixture was incubated at 40 °C for 60 min; the reaction was stopped by boiling the sample for 5 minutes. The sample was then cooled for 5 minutes and centrifuged at 2,500 g for 15 min. The amount of reducing sugars released was determined using the Somogy-Nelson method (Somogy 1952). A standard curve was obtained by using glucose. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars as glucose per min.

The activity of exoglucanase (EC 3.2.1.91) was determined using *p*-nitrophenyl-β-D-cellobioside (PNPC, Sigma, St. Louis, MO, USA). The reaction mixture contained 160 µL of 5 mM PNPC in a 50 mM sodium acetate buffer (pH 5.0) and a 40 µL sample. Reaction mixtures were incubated at 40 °C for 60 min. The reaction was stopped by adding 100 µL of 0.5 M sodium carbonate, and the absorbance was read at 400 nm. Enzyme activity was calculated by using a standard curve of *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per min (Valášková & Baldrian 2006).

The activity of β-glucosidase (EC 3.2.1.21) was determined using *p*-nitrophenyl-β-D-glucoside as the substrate (PNPG, Sigma, St. Louis, MO, USA) with the same method described for exoglucanase (Valášková & Baldrian 2006).

Statistical analysis

To establish significant differences between *Chrysanthemum* and *Rosa* residues as substrates and with and without inductors, we performed an ANOVA with Tukey test using a confidence interval

of 95 % (α = 0.05). Data were tested for normality (Shapiro-Wilk) and subjected to the Levene test for homogeneity in the group variances. In all the cases, differences of $p < 0.05$ were regarded as statistically significant. SAS 9.0 for Windows was used.

Results

Enzyme production from *Chrysanthemum* and *Rosa*

According to the ANOVA, statistically significant differences were observed for the enzymatic activity of laccase, exoglucanase and β-glucosidase ($p<0.0001$), and for endoglucanase ($p=0.0007$), between the different cultures completed.

The enzymatic activities of *Chrysanthemum* residues were higher than those of *Rosa* residues, except for endoglucanase (**Figure 1**). Regarding the enzymes involved in the degradation of lignin, laccase and MnP, the maximum enzyme activity for both residues was obtained by adding inductors. The behavior of endoglucanase and β-glucosidase was different and the maximum enzyme activity for both residues was obtained without inductors (Figure 1). The addition of inductors did not increase exoglucananse enzymatic activity.

Enzyme activities for the degradation of *Chrysanthemum* residues were 4,693.4 U/L and 673.8 U/L for laccase and for MnP, respectively (**Table 2**). Laccase activity was 4.8 times higher when the inductors Cu^{+2} and Mn^{+2} were added, while the MnP activity was 62 % higher. On the other hand, the behavior for endoglucanase and β-glucosidase was different, and the presence of inductors decreased their activity. The addition of inductors reduced the

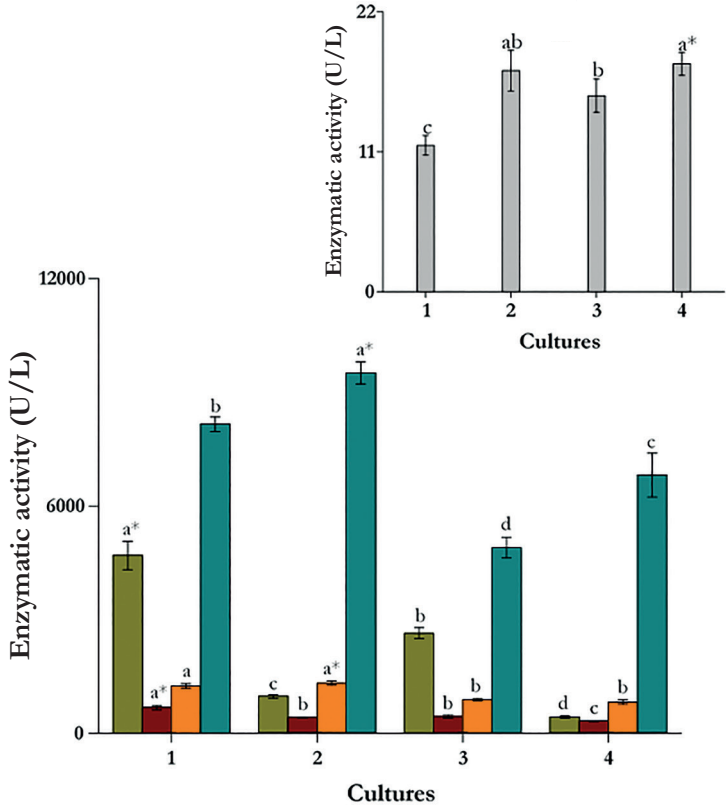

Fig. 1. Enzymatic activity as a function of the residue used as substrate and the presence of inductors. Culture 1: *Chrysanthemum* residues with inductors. Culture 2: *Chrysanthemum* residues without inductors. Culture 3: *Rosa* residues with inductors. Culture 4: Rosa residues without inductors. Laccase MnP Endoglucanase β-glucosidase Exoglucanase . Letters represent Tukey homogeneous subsets. a* corresponds to the culture with the best activity for each enzyme, followed in order by a, b, c and d.

Table 2. Enzyme activity in the liquid extract after the degradation of the *Chrysanthemum* residue using *P. ostreatus*.

Enzyme	With inductors	Without inductors
Laccase (U/L)	4,693.4±374.9	974.1 ±40.3
MnP (U/L)	673.8 ±58.4	415.7 ±9.9
Endo-1,4-β-glucanase (U/L)	11.5 ±0.8	17.4 ±1.6
Exoglucanase (U/L)	1,248.7 ±57.5	1,325 ±55.6
1,4- β-glucosidase (U/L)	8,158.3±189.7	9,513 ±291.7

Table 3. Enzyme activities in the liquid extract after the degradation of the *Rosa* residue using *P. ostreatus*.

Enzyme	With inductors	Without inductors
Laccase (U/L)	2,640±142.3	429 ±29
MnP (U/L)	439.2 ±35	322 ±12
Endo-1,4-β-glucanase (U/L)	15.4 ±1.3	17.9 ±0.9
Exoglucanase (U/L)	884.3 ±28	825.6 ±56
1,4- β-glucosidase (U/L)	4,900.3 ±275.5	6,811.9 ±577.2

enzymatic activity about 34 % and 14 %, respectively. When inductors were added, exoglucanase activity decreased to less than 6 %.

Regarding the enzymes involved in the degradation of lignin from *Rosa* residues, the maximum enzyme activity was 2,640 U/L for laccase and 439.2 U/L for MnP when inductors were used (**Table 3**). When Cu^{+2} and Mn^{+2} were added, the activities of these enzymes increased six times and 36 %, respectively. As described for *Chrysanthemum* residues, the addition of inductors reduced the enzymatic activity of endoglucanase and β-glucosidase. Their activities were reduced about 14 % and 28 %, respectively. Unlike the behavior observed with *Chrysanthemum* residues, exoglucanase activity augmented 7 % when inductors were added.

Cu^{+2} addition increased laccase activity in all cases (**Table 4**), whereas the addition of Zn^{+2} decreased it in both *Chrysanthemum* and *Rosa* extracts (Table 4). The maximum reduction in activity was found in *Rosa* extract.

Evaluation of the solid fraction obtained from the degradation process of *Chrysanthemum* and *Rosa* residues

The presence of enzymes in the solid fraction was assessed to check whether they were adsorbed in the

Table 4. Enzyme activities in the liquid extract after the degradation of the *Rosa* residue using *P. ostreatus*.

Increase in laccase activity (%)			Reduction in laccase activity (%)		
Final Cu^{+2} concentration in the enzymatic reaction	*Chrysanthemum*	*Rosa*	Final Zn^{+2} concentration in the enzymatic reaction	*Chrysanthemum*	*Rosa*
20 ppm	10	2	20 ppm	0.2	12
40 ppm	13	7	40 ppm	8	21
80 ppm	19	41	80 ppm	21	30

Table 5. Enzyme activities in the solid fraction after the degradation of the *Chrysanthemum* and *Rosa* residues using *P. ostreatus*. Data are the average of three measurements ±SD.

Enzyme	*Chrysanthemum*		*Rosa*	
	Activity (U/L)	Adsorption (%)	Activity (U/L)	Adsorption (%)
Laccase	203.2±6.6	4	281.2±9.6	9.6
MnP	28.8±2.5	4	118.1±6.2	21.2
Endo-1,4-β-glucanase	5.5±0.5	32.4	6.5±0.5	29.6
Exoglucanase	20.80±2.1	1.6	814.4±14.1	48
1,4- β-glucosidase	438.13±18.8	5	210±15	4.1

residue. For both residues, in all cases, the enzyme activity was higher in the liquid fraction than in the solid (**Table 5**). The percentage of adsorbed laccase enzymes was low for both residues. Approximately 30 % of the endoglucanase was adsorbed for both residues.

Discussion

Enzyme production from *Chrysanthemum* and *Rosa*

The laccase activity obtained in this study was higher than the activity reported by other researchers using *P. ostreatus* and measuring the enzymatic activity with ABTS. For example Isikhuemhen & Mikiashvilli (2009) obtained 164.6 U/L for laccase from wheat straw mixed with solid waste obtained from the anaerobic digestion of litter from the commercial production of broiler chickens. Another example is the study developed by Větrovský et al. (2013); these authors reported 16.7 mU/ml when laccase was produced from a synthetic CLN medium with cellulose as the source of carbon and high C/N ratio typical of wood. The highest laccase activity obtained by these authors was 206.8 mU/ml using *Fomes fomentarius*.

As previously mentioned, the addition of inductors increased the laccase activity 4.8 times with *Chrysanthemum* and 6 times with *Rosa*. Other authors have reported the effect of the inductors, copper and manganese, in improving laccase activity depending on the substrate, organism, crop, and the concentrations of these metals (Giardina et al. 2000, Baldrian et al. 2005, Stajić et al. 2006, Lu & Ding 2010). For example, Baldrian et al. (2005) reported that average laccase activity increases 79 % with Cu addition in cultures of *P. ostreatus* on wheat straw. Stajić et al. (2006) reported peaks of laccase activity, grown on dry mandarin peels, which depended on the concentration of Cu^{+2}, the time of cultivation and the *P. ostreatus* strain.

According to the characterization of the waste (Table 1), the copper contents in the residues of *Rosa* and *Chrysanthemum* were 8.99 ppm and 24.6 ppm, respectively. This difference explains why laccase activity was higher when *Chrysanthemum* residues were used as a substrate. Copper induces laccase activity, as reported in cultures of *Trametes versicolor* (Collins 1996), *P. ostreatus* (Palmieri 2000), *Trametes pubescens* (Galhaup & Haltrich 2001), and *Botryosphaeria* sp. (Dekker & Barbosa 2001), and *P. ostreatus* (Tinoco et al. 2011). However, some studies have showed that high copper concentrations can be inhibitory (Murugesan et al. 2009).

The lignin content is another factor that may influence the difference found in the laccase activity of these residues. The lignin content was 17.5 % for *Chrysanthemum* residues and 14.8 % for *Rosa* residues (Table 1). Tinoco et al. (2011) observed that the addition of lignin to the culture medium had a strong positive influence on the production of laccase by *P. ostreatus*. When copper was added simultaneously, the activity of that enzyme was higher than the sum of the values determined using each inductor separately; this shows a synergistic effect on the production of the enzyme (Tinoco et al. 2011). The lignin and copper contents in *Chrysanthemum* residues are higher than those in *Rosa* residues (Table 1), explaining why laccase activities were higher in the former residue.

The MnP activity increased 62 % and 36 % with the addition of inductors for *Chrysanthemum* and *Rosa* residues, respectively. The addition of Mn^{+2} can have positive or negative effects on the MnP activity; as a result, this metal must be evaluated for each process. For example, Giardina et al. (2000) produced MnP using *P. ostreatus* in a solid culture of poplar sawdust; they reported an increase in the MnP activity as a consequence of the addition of Mn^{+2} to the culture. On the other hand, Baldrian et al. (2005), reported that the MnP activity was negatively affected by the presence of Mn^{+2}, Cu^{+2} and Pb^{+2} and that the MnP activity was lower in the presence of Mn^{+2} than in the absence of this metal when *P. ostreatus* was cultured on lignocellulose.

Concerning the evaluation of the enzymes involved in the degradation of cellulose, the results show that the enzyme complex necessary to hydrolyze cellulose was present in the obtained extract. According to the results of this work, the activity of β-glucosidase stresses the production from *Chrysanthemum* and *Rosa* residues, with or without inductors, because the activities are higher than the values reported by other studies. For instance, Větrovský et al. (2013) obtained an activity of 3.88 U/ml using a synthetic medium while the value obtained in our work was 9,513 U/L using *Chrysanthemum* residue without inductors. The activities of endo- and exo-glucanases were different with and without inductors, so we can affirm that copper and manganese have effect on the activity of these enzymes, although this is less important than the effect of these inductors on laccase and MnP. The activity of β-glucosidase was 14 % lower when the process was performed with the addition of copper and manganese. That reduction can be explained because copper is an inhibitor as reported by Lin et al. (2010). These authors found that the presence of Cu^{+2} (0.1 mM) inhibited the activity of β-glucosidase, and this activity was not increased by the addition of Mn^{+2} (0.1 mM).

Enzymes that exhibited activity after the degradation of the *Chrysanthemum* and *Rosa* residues have several industrial uses. For example, laccase is used in industrial processes such as bleaching of stains and the degradation of polycyclic aromatic hydrocarbons; β-glucosidase also plays an important role in the degradation of lignocellulosic substrates. The addition of β-glucosidase speeds up sugar production, which is the end product of the degradation of lignocellulosic substrates. This enzyme catalyzes cellobiose hydrolysis, avoiding inhibition of endoglucanase and exoglucanase synthesis (Kumar et al. 2008). Therefore, establishing the production conditions to obtain β-glucosidase with high activity (9,513 U/L) and productivity (396.4 U/L/h) is an essential contribution to the study of the hydrolysis of cellulose.

Because the effects of copper and zinc on laccase activity can be either positive or negative, we conducted experiments adding these metals to laccase-catalyzed reactions. Results showed that laccase activity increased with the addition of Cu^{+2} and diminished with the addition of Zn^{+2}. The effect of zinc can be explained by the results of Murugesan et al. (2009). Their results indicated that laccase activity increased when Ca^{+2}, Cu^{+2}, Co^{+2} and Zn^{+2} ions were added to the culture medium at low concentrations (up to 1 mM), and decreased if the concentrations of Cu^{+2} and Zn^{+2} were increased to 5 mM. It is then possible that the initial concentration of Zn^{+2} in the residues evaluated in this work might be higher than the limit inhibitory concentration and that the addition of zinc reduced laccase activity. Although the inhibitory limit concentration was not determined in this work, the initial concentration of Zn^{+2} was higher in *Rosa* than in *Chrysanthemum* (Table 1). The previous explains why the reduction in laccase activity is higher in the former residue with the same final Zn^{+2} concentration in the enzymatic reaction.

Evaluation of the solid fraction obtained from the degradation process of *Chrysanthemum* and *Rosa* residues

The adsorption percentages for exoglucanase and MnP depend largely on the type of waste. In general, more enzymes were adsorbed on *Rosa* than on *Chrysanthemum* residues (Table 5); other researchers observed similar behavior. For example, Valášková & Baldrian (2006) reported the presence of laccase, MnP, exoglucanase, and β-glucosidase in the solid fraction of a liquid culture of *P. ostreatus* in a synthetic medium; the percentages of adsorption of these enzymes were 38.8 %, 5.4 %, 87 % and 65 %, respectively. As in this study, the percentage of adsorption of exoglucanase was the highest. This behavior can be explained by the irreversible adsorption of cellulases by the residue, specifically by cellulose, and the addition of surfactants is recommended. Surfactants change the surface tension between the liquid and solid phases and minimize the binding of cellulases to cellulose (Sun & Cheng 2002).

Conclusion

In this study, ligninases and cellulases were produced from *Chrysanthemum* and *Rosa* residues using *P. ostreautus* in a submerged culture. Under the conditions evaluated here, the best substrate was the *Chrysanthemum* residue; the obtained extract had higher enzymatic activity than extracts from the *Rosa* residue. The highest enzymatic activities for both residues were obtained for laccase and β-glucosidase and the lowest for endoglucanase. The activity of β-glucosidase was higher without the presence of inductors, whereas laccase activity was enhanced by the presence of inductors.

Acknowledgements

The authors acknowledge the financial support from Pontificia Universidad Javeriana and the División de Investigación de la Sede Bogotá (DIB) at Universidad Nacional de Colombia. We also thank Cultivos del Norte for providing the *Chrysanthemum* and *Rosa* residues.

Conflict of interest

Potential conflict of interest none reported.

References

Asocolflores (2010) Global Reporting Initiative (GRI). www.asocolflores.org. Retrieved January 2014.

Asocolflores, Ministerio de Medio Ambiente y Sociedad de Agricultores de Colombia. (2002). Guía ambiental para la floricultura. www.minambiente.gov.co/documentos/floricultor.pdf. Retrieved august 2011

ASTM Standard (2008) ASTM D5373-08 . American Standard Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Laboratory Samples of Coal. *ASTM International, West Conshohocken*. www.astm.org

ASTM standard (2011a) ASTM D4691-11. Standard practice for measuring elements in water by flame atomic absorption spectrophotometry. *ASTM International, West Conshohocken*. www.astm.org

ASTM Standard (2011b) ASTM D1971-11. Standard practices for digestion of water samples for determination of metals by flame atomic absorption, graphite furnace atomic absorption, plasma emission spectroscopy, or plasma mass spectrometry. *ASTM International, West Conshohocken*. www.astm.org

Baldrian P, Valášková V (2008) Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiology Reviews* 32:501-521 doi: 10.1111/j.1574-6976.2008.00106.x

Baldrian P, Vendula V, Merhautová, Gabriel J (2005) Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. *Research in Microbiology* 156:670-676 doi: 10.1016/j.resmic.2005.03.007

Bettin F, Osorio da Rosa L, Montanari Q et al. (2011) Growth kinetics, production, and characterization of extracellular laccases from *Pleurotus sajor-caju* PS-2001. *Process Biochemistry* 46:758–764 doi: 10.1016/j.procbio.2010.12.002

Bhat MK (2000) Cellulases and related enzymes in biotechnology. *Biotechnology Advances* 18:355-383.

Bourbonnais R, Paice G, Freiermuth B, Bodie E, Borneman S (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Applied and Environmental Microbiology* 63:4627-4632.

Collins PK, Field J, Dobson A (1996) Oxidation of anthracene and benzo pyrene by laccases from *Trametes versicolor*. *Applied and Environmental Microbiology* 62:4563-4567.

Dekker RFH, Barbosa AM (2001) The effects of aeration and veratryl alcohol on the production of two laccases by the ascomycete Botryosphaeria sp. *Enzyme and Microbial Technology* 28:81-88.

Elisashvili V, Penninckx M, Kachlishvili E, Asatiani M, Kvesitadze G. (2006) Use of Pleurotus dryinus for lignocellulolytic enzymes production in submerged fermentation of mandarin peels and tree leaves. 38:998-1004 doi: 10.1016/j.enzmictec.2005.08.033

Galhaup CC, Haltrich DD (2001) Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology* 56:225-32 doi: 10.1007/s002530100636

Ghose T, K (1987) Measurement of cellulase activities. *Pure and Applied Chemistry* 59:257-268.

Giardina P, Palmieri G, Fontanella B, Rivieccio V, Sannia G (2000) Manganese Peroxidase Isoenzymes Produced by Pleurotus ostreatus Grown on Wood Sawdust. *Archives of Biochemistry and Biophysics* 376:171-179 doi: 10.1006/abbi.1999.1691

Huang R, Su R, Qi W, He Z (2011) Bioconversion of Lignocellulose into Bioethanol: Process Intensification and Mechanism Research. *Bioenergy Research* 4:225-245 doi: 10.1007/s12155-011-9125-7

Isikhuemhen OS, Mikiashvilli NA (2009) Lignocellulolytic enzyme activity, substrate utilization, and mushroom yield by Pleurotus ostreatus cultivated on substrate containing anaerobic digester solids. *Journal of Industrial Microbiology & Biotechnology* 36:1353-62 doi: 10.1007/s10295-009-0620-1

Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of Industrial Microbiology & Biotechnology* 35:377-91 doi: 10.1007/s10295-008-0327-8

Lin Y, Chen G, Ling M, Liang Z (2010) A method of purification, identification and characterization of β-glucosidase from Trichoderma koningii AS3.2774. *Journal of Microbiological Methods* 83:74-81 doi: 10.1016/j.mimet.2010.07.019

Lu X, Ding S (2010) Effect of Cu^{2+}, Mn^{2+} and aromatic compounds on the production of laccase isoforms by Coprinus comatus. *Mycoscience* 51:68-74 doi: 10.1007/s10267-009-0002-6

Lundell TK, Mäkelä MR, Hildén K (2010) Lignin-modifying enzymes in filamentous basidiomycetes: ecological, functional and phylogenetic review. *Journal of Basic Microbiology* 50:5-20 doi: 10.1002/jobm.200900338

Malherbe S, Cloete T (2002) Lignocellulose biodegradation: Fundamentals and applications. *Reviews in Environmental Science and Biotechnology* 105-114

Membrillo I, Sánchez C, Meneses M, Favela E, Loera O (2008) Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. *Bioresource Technology* 99:7842-7847 doi: 10.1016/j.biortech.2008.01.083

Murugesan K, Kim Y-M, Jeon J-R, Chang Y-S (2009) Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*. *Journal of Hazardous Materials* 168:523-529 doi: 10.1016/j.jhazmat.2009.02.075

Palmieri GG, P; Bianco, C; Fontanella, B. (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology* 66:920-924 doi: 10.1128/AEM.66.3.920-924.2000

Quevedo-Hidalgo B, Narváez-Rincón P, Pedroza-Rodríguez A, Velásquez-Lozano M (2012) Degradation of *Chrysanthemum* (Dendranthema grandiflora) wastes by *Pleurotus ostreatus* for the production of reducing sugars. *Biotechnology and Bioprocess Engineering* 17:1103-1112 doi: 10.1007/s12257-012-0227-7

Santoyo F, González AE, Terrón MC, Ramírez L, Pisabarro AG (2008) Quantitative linkage mapping of lignin-degrading enzymatic activities in *Pleurotus ostreatus*. *Enzyme and Microbial Technology* 43:137-143 doi: 10.1016/j.enzmictec.2007.11.007

Somogy N (1952) Notes on sugar determination. *The Journal of Biological Chemistry* 195:19-23.

Stajić M, Persky L, Friesem D, et al. (2006) Effect of different carbon and nitrogen sources on laccase and peroxidases production by selected *Pleurotus* species. *Enzyme and Microbial Technology* 38:65-73 doi: 10.1016/j.enzmictec.2005.03.026

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology* 83:1-11

Thakur S, Shrivastava B, Ingale S, Kuhad R, Gupte A (2013) Degradation and selective ligninolysis of wheat straw and banana stem for an efficient bioethanol production using fungal and chemical pretreatment. *3 Biotech* 3:365-372 doi: 10.1007/s13205-012-0102-4

Tinoco, Pickard, Vázquez D (2001) Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains. *Letters in Applied Microbiology* 32:331-335

Tinoco R, Acevedo A, Galindo E, Serrano-Carreño L (2011) Increasing *Pleurotus ostreatus* laccase production by culture medium optimization and copper/lignin synergistic induction. *Journal of Industrial Microbiology & Biotechnology* 38:531-40 doi: 10.1007/s10295-010-0797-3

Valášková V, Baldrian P (2006) Estimation of bound and free fractions of lignocellulose-degrading enzymes of wood-rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. *Research in Microbiology* 157:119-124 doi: 10.1016/j.resmic.2005.06.004

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition.

Symposium: Carbohydrate Methodology, Metabolism, and Nutritional Implications in Dairy Cattle. *Journal of Dairy Science* 74:3583-3597

Větrovský T, Baldrian P, Gabriel J (2013) Extracellular enzymes of the white-rot fungus *Fomes fomentarius* and purification of 1,4-[beta]-glucosidase. *Applied Biochemistry and Biotechnology* 169:100-9 doi: 10.1007/s12010-012-9952-9

Producción de enzimas lignocelulolíticas a partir de residuos de floricultura empleando *Pleurotus ostreatus*

Resumen. En Colombia la floricultura es un sector agro-industrial importante, con impactos positivos en el empleo y la balanza comercial. Sin embargo, tiene impacto negativo en el medio ambiente porque genera alto volumen de residuos. Estos residuos, ricos en lignina, hemicelulosa y celulosa, podrían ser una materia prima de bajo costo para la producción de enzimas. En este trabajo se estudió en la producción de enzimas lignocelulíticas por la degradación con *Pleurotus ostreatus* de residuos de *Chrysanthemum* y *Rosa*, usando como inductores sulfato de manganeso y de cobre. A partir de ambos residuos se obtuvieron lacasa, manganeso peroxidasa, endoglucanasa, exoglucanasa y β-glucosidasa. Los inductores favorecieron todas las actividades enzimáticas, excepto para β-glucosidasa. Las enzimas que tuvieron mayores actividades fueron lacasa (4,693.4 U/L y 2,640 U/L a partir del residuo de *Chrysanthemum* y *Rosa*, respectivamente) y β-glucosidasa (9,513 U/L y 6,811.9 U/L). La enzima que tuvo menor actividad fue endoglucanasa (11.5 U/L y 15.4 U/L). Bajo las condiciones evaluadas, el mejor residuo para producción de enzimas fue *Chrysanthemum*, porque los extractos tuvieron mayor actividad enzimática que los producidos a partir de *Rosa*.

Palabras clave: residuos de floricultura; *Chrysanthemum*; *Rosa*; enzimas lignocelulolíticas; *Pleurotus ostreatus*

Produção de enzimas lignoceluliticas a partir de resíduos de floricultura utilizando *Pleurotus ostreatus*

Resumo. Na Colômbia, a floricultura é um importante setor da indústria agrícola, com impactos positivos sobre o sector laboral na balança comercial. Além disto, tem impacto negativo sobre o meio ambiente, pois gera grandes volumes de resíduos. Estes resíduos, com altos conteúdos em lignina, hemicelulose e celulose, podem ser uma matéria-prima de baixo custo para a produção de enzimas. Neste trabalho foi estudada a produção da enzimas lignoceluliticas pela degradação com *Pleurotus ostreatus* de resíduos de *Chrysanthemum* e *Rosa*, utilizando como indutores sulfatos de cobre e manganês. A partir destes resíduos foram obtidos lacase, manganês peroxidase, endoglucanase, exoglucanase e β-glicosidase. O uso de indutores favoreceu as atividades enzimáticas, exceto β-glicosidase. As enzimas que apresentaram atividades mais elevadas foram lacase (4,693.4 U/L e 2,640 U/L a partir do *Chrysanthemum* e *Rosa*, respetivamente) e β-glicosidase (9,513 U/L e 6,81.9 U/L). A enzima que apresentou menor atividade foi a endoglucanase (11.5 U/L e 15.4 U/L). Nas condições testadas, o melhor resíduo para a produção da enzima foi *Chrysanthemum*, porque os extratos tinham uma atividade enzimática mais elevada que aqueles produzidos a partir de *Rosa*.

Palavras-chave: resíduos de floricultura; *Chrysanthemum*; *Rosa*; enzimas lignoceluliticas; *Pleurotus ostreatus*