Laccase production by *Pleurotus djamor* in agar media and during cultivation on wheat straw

Producción de lacasas en cepas de *Pleurotus djamor* creciendo en medios con agar y durante su cultivo en paja de trigo

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Resumen

Se cultivaron setenta y una cepas de *Pleurotus djamor* en agar con extracto de malta (AEM) o agar con extracto de malta y levadura (AEML) suplementada con derivados solubles de lignina (DSL) para determinar su crecimiento micelial y su capacidad de producción de lacasas *in vitro*. En promedio, los valores de producción de lacasas y diámetros miceliales fueron más altos en los cultivos de AEML-DSL que en AEM. Las cuatro cepas que presentaron los valores más altos de producción de la enzima fueron seleccionados para cuantificar la actividad durante su ciclo completo de cultivo, utilizando paja de trigo como sustrato. La actividad de la enzima fue detectada a partir del segundo día de incubación. Se observaron dos picos de actividad enzimática, el primero a los 4 días de incubación (169.8-579.8 µMol g^{-1} min^{-1}) y el segundo durante la formación y desarrollo de las fructificaciones, alcanzando valores desde 164.4 hasta 469.9 µMol g^{-1} min^{-1}. Los resultados sugieren que el sistema enzimático de lacasas en *P. djamor* está asociado a la etapa reproductiva de las cepas. Además, la alta producción de lacasas cuantificada en estas cepas, evidencia que *P. djamor* podría ser una especie de interés para su uso en otros procesos biotecnológicos.

Palabras clave: cultivo de hongos, enzimas, biomasa, crecimiento micelial

Abstract

Laccase production *in vitro* and mycelial growth were measured for seventy one *Pleurotus djamor* strains cultivated on malt extract agar (MEA) or yeast malt extract agar (YMEA) supplemented with water soluble lignin derivatives (WSLD). *In vitro* laccase production and mycelial diameters averages were higher in the YMEA-WSLD cultures than MEA cultures. The four strains with the highest enzyme production *in vitro* were selected to measure their laccase activity pattern during the culture cycle, using wheat straw as substrate. Laccase activity was detected on second day of incubation. The first peak of activity (169.8-579.8 µMol g^{-1} min^{-1}) was reached on day 4, and the second peak of activity was observed during the initiation and development of fruiting bodies, with values from 164.4 to 469.9 µMol g^{-1} min^{-1} measured. These results suggest that a laccase system in *P. djamor* is associated with the reproductive stage. Furthermore, high laccase production observed in these strains evidences that *P. djamor* may be an interesting species for use in some biotechnological processes.

Keywords: mushroom cultivation, enzymes, biomass, mycelial growth

Introduction

Laccases (EC 1.10.3.2) are copper-containing polyphenol oxidases implicated in lignin degradation by a one-electron oxidation mechanism (Baldrian, 2006). Degradation of lignin is carried out by a group of basidiomycetes categorized as white rot fungi (Mayer and Staples, 2002), although some brown rot fungi produce laccases (Lee *et al*., 2004; Baldrian, 2006).These enzymes have the ability to oxidize high redox potential sub-

strates in the presence of synthetic mediators (Strong and Claus, 2012), which allows the degradation of xenobiotic compounds (Rodríguez *et al.,* 2004; Martínez *et al.,* 2005; Piscitelli *et al.,* 2011). Extracelullar laccases are constitutively produced in small amount, however their production can be stimulated by inducing substances, mainly aromatic or phenolic compounds related to lignin or derivatives (Gianfreda *et al.,* 1999). Laccases and their production, have been studied in commercially edible fungi with the proposal of the understanding of phyisiological mechanisms regulation enzyme synthesis in lignocelullulose bioconversion could be use for improving the technological process of mushroom production (Songulashvili *et al.,* 2007). Some species of genus *Pleurotus* are mushrooms widely cultivated in the world. *Pleurotus ostreatus* (Jacq.) P. Kumm., *P. pulmonarius* (Fr.) Quél., *P. eryngii* (DC) Quél. and *P. citrinopileatus* Singer have been recognized to produce laccases isoenzymes with applications relevant to various biotechnological processes, e.g.: biopulping and biobleaching, decolourization of dyes, degradation of xenobiotics and effluent treatment (Hublik and Schinner, 2000; Robinson *et al.,* 2000; Gitatti Marques de Souza *et al.,* 2004; Hou *et al.,* 2004; Rodríguez *et al.,* 2004; Camarero *et al.,* 2005; Stajic *et al.,* 2006; Knezevi *et al.,* 2013).

Pleurotus genus comprises other potential species that are not commercially cultivated but having putative important medicinal and nutrimental properties. *Pleurotus djamor* (Rumph. ex Fr.) Boedijn (= *P. flabellatus* Sacc.*)* is a widely distributed tropical and subtropical species growing upon a great variety of lignocellulosic materials (Guzmán, 2000). This species can be grown at environmental temperature up to 30°C, having the capacity to develop fruiting bodies after 1 to 2 weeks of spawn propagation; therefore it is recommended for cultivation in tropical regions (Sánchez Vázquez *et al.,* 1997). For the development of this culture it is interesting to explore and valorize the variability inside the biological resources of this species for the understanding of physiological mechanism regulation enzyme synthesis that allow improving the yield of fruiting body production and the lignocelluloses bioconversion. Lignocellulolytic activities in *P. djamor* cultures using rice and barley straw and coffee pulp as substrates have been reported previously (Upadhyay and Fritsche, 1997; Mata *et al.,* 2005; Savoie

et al., 2007), but there is very little information available about their role in growth and fruiting body formation. Therefore, the paper describe the laccase enzyme production by wild *P. djamor* strains under wheat straw during their crop cycle, and also reports on the effect of nitrogen sources on enzyme production *in vitro*. Results are discussed with respect to the role that this enzyme plays during the development of the fructifications, as well as the ligninolytic ability of this edible mushroom.

Materials and Methods

Strains and culture conditions

Seventy one strains of *Pleurotus djamor* were used in this study. Fifty four strains provided from wild specimens growing on logs and other organic materials in decomposition, and they were collected in different geographical regions of Argentina (2), Colombia (1), Cuba (3), Guatemala (1), Japan (2), Mexico (29) and Panama (16); and the rest, 17 strains, were obtained from genetic crosses in the laboratory. All materials are deposited in the Strain Collection of the Instituto de Ecología in Xalapa, Mexico (World Federation for Culture Collections No. 789).

Pleurotus djamor strains were reseeded in two culture media: malt extract (2%) agar (1.5 %) (MEA, Bioxon); and yeast (2%), plus malt extract agar (YMEA, Bioxon). The latter media was dissolved in a water soluble lignin derivatives suspension (WSLD) at phenols concentration of 2 mM/L. WSLD was prepared from 3 g indulin AT (Sigma) that had been dissolved in 800 mL of water and brought to a boil for 5 min (Mata *et al.,* 1997). Both the MEA and YMEA-WSLD media were sterilized for 15 min at 121°C, and then 20 mL were placed in Petri dishes such that 5 replicates per strains per media were prepared. Cultures were incubated for 7 days at 27 °C.

Enzyme assays

In vitro laccase activity was studied in samples incubated for 7 days at 27°C by cutting out small discs of the solid culture medium (0.7 cm in diameter) that contained fungal mycelium from the peripheral growth zone (Salmones and Durán Barradas, 2001). Enzyme quantification was carried out in test tubes containing 1.5 mL of 0.2% ABTS [2,2´-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] solution in sodium acetate buffer

0.1 M, pH 5. One mycelium disc was placed in each tube. Samples of each condition were prepared by quintuplicate, and incubated for 15 min at 30°C, after time the reaction was stopped by immersing the tube in ice. The absorbance of the recovered solution was recorded at 436 nm (Spectronic Genesys 5, Macedar NY) (Mata *et al.*, 1997). One unit of laccase activity was expressed as 1 µmol of ABTS oxidized min^{-1} mycelium disc^{-1} and was calculated by using $\varepsilon = 29300$ M^{-1} cm^{-1}.

Mycelial growth *in vitro*
Estimates of mycelial growth were made based upon the measuring by two mycelial diameters, in Petri dishes, one taken perpendicularly from the other after 7 days of incubation.

Enzyme activity during culture cycle
The four strains with the highest enzyme production values *in vitro* (IE-116, IE-145, IE-267, IE-11and IE-633), were selected to investigate variation in enzyme production during the complete culture cycle (Velázquez Cedeño *et al.*, 2002). Moistened wheat straw samples (50 g dry weight) were deposited in polypropylene bags and sterilized at 121 °C for 1 h. Cooled substrate was homogeneously mixed with spawn prepared from sorghum seeds, ratio 5% on wet weight, according to Guzmán *et al.* (2013) and incubated in darkness at 25ºC (± 2ºC). Eighteen samples were prepared for each strain. During the running spawn period, two samples of each strain were collected at 0, 2, 4, 6 and 8 days of incubation for the determination of enzyme. On day 9, the bags were removed to facilitate fruiting. Sampling also took place during the first appearance of primordia (P), beginning of first harvest (H), and 5 days after finishing first (PH1) and second (PH2) harvests. The primordia were detected between 9 to 12 days of incubation, requiring 4 to 5 days more for the obtention of the first harvest. Crude enzyme extract was obtained from a suspension of 1 g of substrate (fresh weight) from each bag and 10 mL of sterile distilled water, rotated end-over-end for 30 min at 45 rpm, filtered through an inert material, and centrifuged twice at 10,000 g for 15 min each time. Enzyme quantification was carried out in test tubes containing 1.5 mL of 0.2% ABTS in sodium acetate buffer 0.1 M pH 5, and 25 µL crude enzyme extract. Laccase activities were measured in the filtrates after 30 min of incubation at 30°C (as describe above). Enzyme activity was expressed as U g^{-1} of cultivation substrate defined as the amount of enzyme producing 1µmol of ABTS oxidized min^{-1}g^{-1} (dry weight, obtained by differences between dry and wet weight of substrate extracted). Five measures were realized from each sample.

Estimation of mycelia metabolic activity
Fungal biomass production was associated with mycelial metabolic activity of the strains during the incubation period, and was determined using the method of fluorescein diacetate (FDA) hydrolysis (Mata *et al.*, 2002; Salmones *et al.*, 2005). This method consists by placing 0.7 g of fresh substrate in five sterile tubes; in three tubes, 10 mL of FDA (Sigma) (10 mL l^{-1}in 60 mM phosphate buffer pH 7.6) were added, and in the two remaining tubes (controls) 10 mL of phosphate buffer were added. The samples were incubated for 30 min at 30°C, after that the reaction was stopped by the addition of 10 mL of acetone. The solution was filtered (Whatman No. 1) and the absorbency of the remaining filtrate was read to 490 nm. One unit of metabolic activity was defined as 1 µMol of FDA hydrolyzed min^{-1}g^{-1} (dry weight). Material corresponded to 0, 2, 4, 6 and 8 days of incubation were analyzed.

Statistical analyses
Means and standard deviations were calculated and significance difference were estimated using Tukey's test (α=0.05). Regression analysis was completed to obtain a coefficient of determination (R^2) between the laccase activities and mycelial growth. *Statistical analysis was performed using Statistica® program.*

RESULTS AND DISCUSSION
Enzyme activity was detected in MEA cultures with an average of 2.07±1.11 for all strains and with range values from 0.22±0.19 (IE-670) to 4.93±0.43 µmol disc^{-1} min^{-1} (IE-237). Values were significantly higher in the YMEA-WSLD cultures, since only 36 strains presented enzyme activity average smaller to 5 µmol disc^{-1} min^{-1}, while 33 strains obtained means of laccase activity between 5 to 15 units, and 2 strains were upper 15 µmol disc^{-1} min^{-1} of enzymatic activity (IE-145 and IE-633). The strains cultivated in YMEA-WSLD reaching an average production value of 5.56±3.5 and the enzyme activities fluctuated between

1.05±0.11 (IE-645) until 17.29±2.93 µmol disc^{-1} min^{-1} (IE-633). This phenomenon of selective laccase inducibility had been observed earlier in other mushroom strains, as *Pleurotus* and *Lentinula* strains (Mata *et al*., 1997; Arora and Gill, 2000). In addition these results show that *P. djamor* has a great capacity for producing and inducing this enzyme, when compared to commercial strains of *P. ostreatus* and *P. pulmonarius* under similar culture conditions in which average activities of 0.5 and 1.9 µmol disc^{-1} min^{-1}, respectively (Salmones and Mata, 2005).

According to the results of enzyme activity, the YMEA-WSLD cultures had an average higher mycelial diameters (75.9±12.64 mm) than the MEA cultures (63.43±20.4 mm). After 7 days of incubation, 55 strains cultivated on MEA and 70 strains cultivated on YMEA-WSLD had covered the middle of the Petri box surface (45 mm). Correlations between the enzyme production and mycelial diameters were determined for each media culture (Figures 1 and 2). The only positive correlation between laccase activities and mycelial growth corresponding to YMEA-WSLD cultures (R^2= 0.072, =0.05). Our work suggests that as result of adding lignin derivatives to the medium, including various phenolic compounds, *P. djamor* strains presented a greater capacity to produce extracellular enzymes, and consequently the levels of digestion of lignin could be increased. In

this case, the fungus may take advantage of its competitors, a physiological characteristic searched frequently in the programs of strains selection for commercial purposes.

Based on the data presented above, the four strains with the highest values of enzyme production in YMEA-WSLD cultures were selected for the next stage (Figure 2, Table 1). When the strains were cultivated on sterilized straw, the laccase activity was detected on the second day of incubation (Figure 3). For the strains IE-116, IE-145 and IE-633, a first peak of activity (169.8-579.8 µMol g^{-1} min^{-1}) was observed on day 2, but a subsequent reduction of activity occurred during the final stage of colonization (8 days of incubation) (Figure 3). A second peak of activity was observed during the development of fruiting bodies (9 to 12 days of incubation) and 5 days after finishing first harvest, with values from 164.4 to 469.9 µMol g^{-1} min^{-1}. Activity gradually decreased following the next harvesting of fruiting bodies. In the strain IE-267, laccase activity was lower during the first week of incubation, having its maximal activity during the fructification stage. The pattern of laccase production by *P. dajmor* strains is different from that observed with other *Pleurotus* species (Geetha and Sivaprakasam, 1998; Isikhuemhen and Nerud, 1999; Velázquez-Cedeño *et al*., 2002; Elisashvili *et al*., 2003; Savoie *et al*., 2007), as well as for some of the other
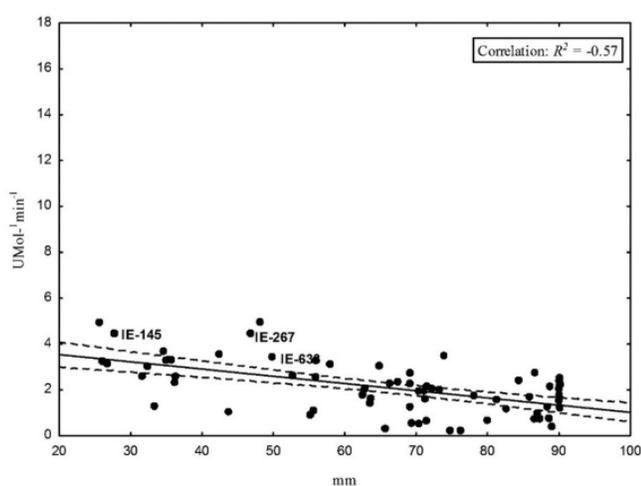
Figure 1. Relationship (R^2 = -.57) between mycelia diameter (mm) of *P. djamor* strains growing on MEA medium at seven days of incubation and enzyme activity expressed as µmol of ABTS oxidized min^{-1} mycelium disc^{-1} (€= 29300 M^{-1} cm^{-1}).
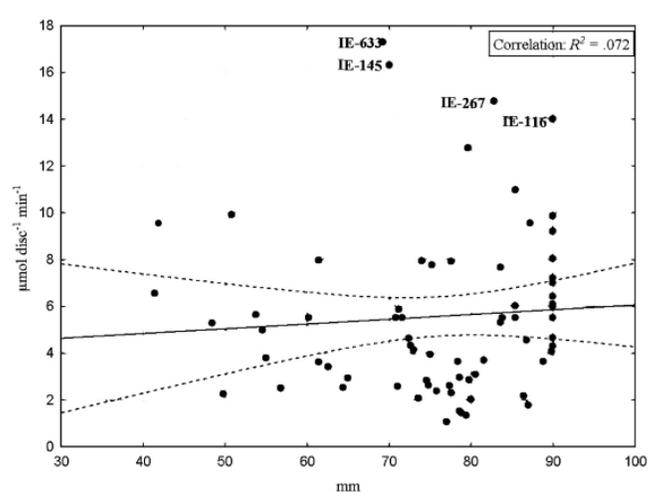
Figure 2. Relationship (R^2 =0.72) between mycelia diameter (mm) of *P. djamor* strains growing on YMEA-WSLD medium at seven days of incubation and enzyme activity expressed as µmol of ABTS oxidized min^{-1} mycelium disc^{-1} (€= 29300 M^{-1} cm^{-1}).
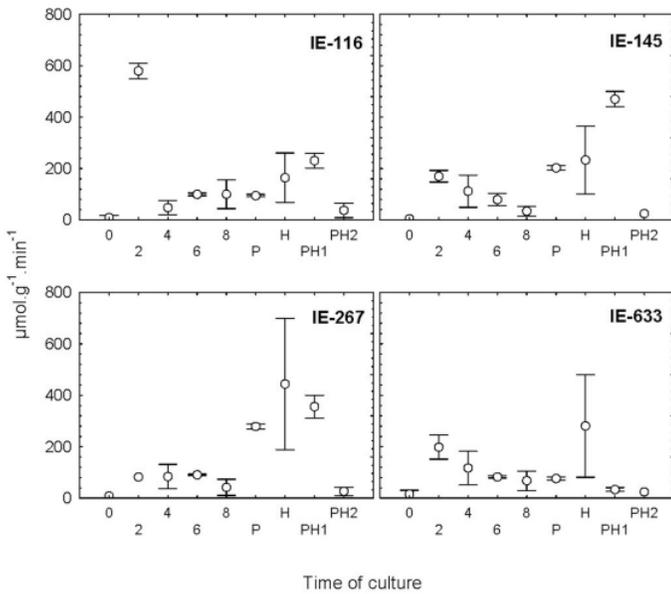
Figure 3. Laccase activities measured in extracts from wheat straw inoculated with *P. djamor* strains at different times of the culture cycle. 0, 2, 4, 6 and 8 days of incubation, primordia appearance (P), first harvest (H), 5 days after finishing first (PH1) and second (PH2) harvests.

Table 1. The highest averages of laccase activity (7 days of incubation) presented by *Pleurotus djamor* strains in the two different growth conditions evaluated

Strain	Origin	LAC activity (U g⁻¹)	Mycelial diameter (mm)
		MEA[1]	
IE-237	MEX	4.93	34.5
IE-235	MEX	4.91	42.2
IE-267[3]	MEX	4.5	73.8
IE-145	CUB	4.47	49.8
IE-121	MEX	3.69	35.0
IE-218	MEX	3.59	55.8
IE-197	MEX	3.54	25.7
IE-633	JAP	3.45	88.2
		YMEA-WSLD	
IE-633	JAP	17.29	86.6
IE-145	CUB	16.32	77.5
IE-116	MEX	14.75	82.8
IE-267	MEX	14.0	90.0
IE-156	MEX	12.76	79.6
IE-134	MEX	10.96	85.4
IE-219	MEX	9.92	50.8
IE-111	MEX	9.54	87.2

[1] MEA= malt extract agar, YMEA-WSLD= yeast, malt extract agar plus water soluble lignin derivatives suspension.
[2] MEX=Mexico, CUB=Cuba, GUAT=Guatemala, JAP= Japan.
[3] Data in bold corresponding to the strains selected for the next stage of experimentation

white rot basidiomycetes, where enzymatic activity was only detected during the early stages of substrate colonization (Savoie, 1998). Similarly to *P. djamor* cultures, Chen *et al.* (2003) reported a temporal correlation between laccase production and sporophore formation in *Volvariella volvacea* compost cultures.

Physiological functions involving in the laccase activity in *P. djamor* deserve further attention. In preliminary reports, we proposed that the occurrence of phenolics compounds present on coffee pulp could induce this enzyme activity during the colonization of the substrate (Salmones and Mata, 2002; Salmones *et al.*, 2005). Nevertheless, the ability of this species to produce high laccase activity during the reproductive stage had not been suggested in previous works, since generally this enzyme has been associated to biodegradation and detoxification of the substrates that occurred during the initial stages of colonization. The earlier fructification of *P. djamor* compared with other commercial *Pleurotus* species have led to suggestions that this tropical mushroom is poorly equipped to degrade the lignin component of lignocellulosic materials commonly used

for mushroom cultivation, but in previous paper we reported that the level of laccase production in *P. ostreatus* and *P. pulmonarius* strains, cultivated under similar conditions, could be less to *P. djamor* (Savoie *et al.*, 2007). Therefore, an increase in laccase activity during the reproductive stage of the mushrooms could be associated to play an important role in the morphogenesis of fruiting bodies. Although we have now proposed that this species produces laccases with different functions during the vegetative mycelial growth and fruiting phases.

Metabolic activities of the four *P. djamor* strains on wheat straw are shown in Figure 4. These activities were recorded for all strains starting at the second day of incubation. Statistically significant increases in metabolic activity were observed for all strains between 0 to 2 days of incubation, and between 2 to 4
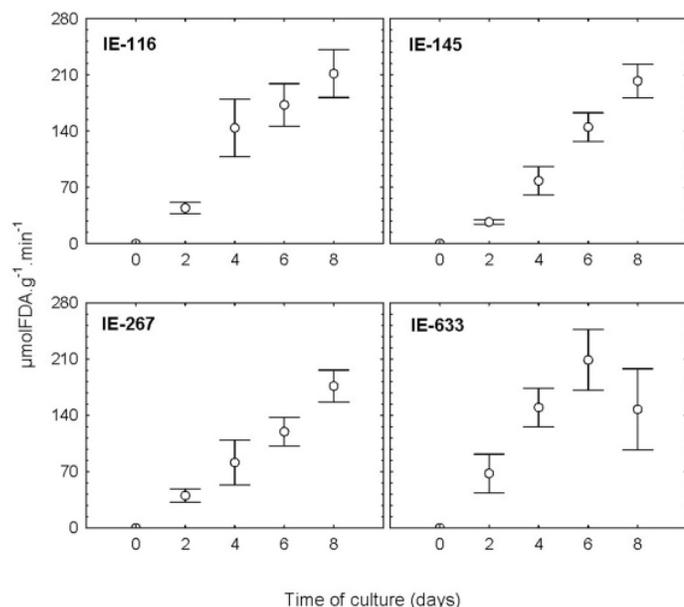
Figure 4. Metabolic activities recorded for *P. djamor* strains during the first 8 days of incubation.

days. The mycelia continued increasing metabolic activity between 4 to 6 days of incubation, however, only the strain IE-145 showed significant differences. Maximum metabolic activities were noted at 8 days of incubation for IE-116 (211.5±29.7), IE-145 (202.3±21) and IE-267 (176.5±20), and 6 days of incubation for IE-633 (209±37.9). IE-116 and IE-633 showed the greatest metabolic level among four strains tested during the incubation period. No significant correlations were found between enzyme activity and metabolic activity for all strains during the first week of incubation; although we have supposed that the primary fungal growth would be associated with enzymes generally considered to play key roles in lignin degradation.

Previous studies on metabolic activities in *Pleurotus* spp. had been focused on to propose a standardized method for estimating the capacity to produce biomass (Salmones *et al*., 2005). Although it had not been possible to establish a correlation between values for metabolic activity and biological efficiency, it had been suggested that a high capacity of biomass production depends on the ability of the strain to utilize nutrients available in the substrate, some of which are important elements to the primordium morphogenesis begins.

In conclusion, our results shown that *P. djamor* strains presented some peaks of activity in the complete cycle of culture;

this suggests that a laccase isoforms could be associated with changes in cultivation substrate composition. The role of laccases during the early fruiting could continue to be detoxification of phenolic compound, but may also play a physiological role in the development of fruiting bodies. Additionally, in previous studies we observed that under similar culture conditions, this species can present higher laccase activity than other *Pleurotus* species, as *P. ostreatus* and *P. pulmonarius*. It suggests that *P. djamor* may be an interesting species for use in different biotechnological processes.

Further studies should be conducted to understand the molecular basis of enzymatic catalysis and the regulatory mechanisms controlling the production of laccases of this species, and to study the level of variability in laccase activities as a source of improvement and identification of specific enzymes of the interest in sustainable production systems. It will be necessary to identify the molecular characteristics and parameters that improve the activity and the stability of this enzyme in order to determine the potential application of this fungus in other alternative environmental technologies.

Acknowledgments

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