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Characterization of the Solid-State and Liquid Fermentation for the Production of Laccases of *Pleurotus ostreatus*

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Additional information is available at the end of the chapter

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Abstract

In this chapter, the activity and isoenzymes number of laccases of *Pleurotus ostreatus* grown in solid-state and liquid fermentations are reported. An atypical behavior of this fungus with relation on enzyme production was observed, since the major laccase activity levels were observed in liquid fermentation, whereas the solid-state fermentation has been recognized as better system for enzyme production.

Keywords: laccases, *Pleurotus ostreatus*, solid-state fermentation, submerged fermentation, ligninolytic enzymes

1. Introduction

Laccases are enzymes oxygen oxidoreductases produced by plants, insects, bacteria, and fungi. The most studied laccases are fungal origin, mainly of white rot fungi using different culture systems, mainly in solid-state fermentation (SSF) and liquid fermentation (SmF). In general, it has been suggested that the solid-state fermentation is better for the production of metabolites and enzymes compared with SmF [1]; however, in recent studies has been observed that the basidiomycete *Pleurotus ostreatus* grown in SmF reported higher laccases values compared to those when the fungus grown in solid-state fermentation. *P. ostreatus* strain ATCC 32783 has been studied for the production of intracellular laccases of peripheral and central vegetative mycelium [2], have also been evaluated the solid-state fermentation and SmF systems for laccases

production, observing different levels of activity and number of isoenzymes depending on the culture system [3, 4]. When compared some strains of *P. ostreatus* with ATCC 32783, the latter being better than others [5]. In other study, the effect of pH of the culture medium on the laccases activity of *P. ostreatus* developed in SmF was evaluated, where the activity was seven times higher when the pH of the medium did not change [6]. It has been reported the description of a novel gene encoding a laccase of *P. ostreatus* ATCC 32783 called lacP83, which is preferentially expressed in liquid culture conditions [7]. Recently, the effect of initial pH of development from 3.5 to 8.5 on the laccase activity was evaluated; the pattern of production and the expression profile of five laccase genes of *P. ostreatus* ATCC 32783 grown in SmF, showing that the pH of 8.5 favors biomass production but not enzymatic activity and shows repression of gene expression, however, the pH 4.5 showed higher laccase enzyme activity, reaching up to 78,500 U/L [8].

2. *Pleurotus ostreatus*

2.1. *Pleurotus ostreatus*: phases of growth and composition

The genus *Pleurotus* (Jacq.: Fr.) Kummer (Pleurotaceae, higher Basidiomycetes) comprises a group of edible ligninolytic mushrooms, which have two phases of growth: one is called vegetative or mycelial and is seen as strands of hyphae, which colonize the substrate and the other, the reproductive or fruit body, is represented by the mushroom itself that in basidiomycetes, is called the basidiome. *P. ostreatus* is characterized by a white spore print, with an eccentric stipe and a fan or oyster-shaped pileus or cap (5–25 cm). The Latin *Pleurotus* means “beside the ear” and *ostreatus* means “oyster shaped.” This fungus is commonly named “oyster mushroom” for the resemblance of its fruiting body a white shell [9]. In this case, the spores are located in a special structure called basidium. In the *P. ostreatus* growth, after spore germination (or inoculation of *in vitro*-grown mycelia), the substrate is invaded by microscopic filaments called hyphae. Hyphae continually grow and branch to form a network of hyphae. Mycelial growth is generally coupled with increased enzyme production and respiration. Hyphae absorb digestive products, penetrating the substrate until its complete hyphal invasion. The vegetative growth is in direct contact with the support (substrate), providing the nutritive materials required for mushroom growth (**Figure 1**) [10].

The growth of all parts of a fungus occurs at the hyphal tips. The mycelial structure grows by synthesizing new wall at the hyphal apex, as they increase in length, additional sites for wall synthesis are formed in the subapical region, originating lateral branches that synthesize wall again confined to the hyphal tip [11]. The formation of a new branch requires the production of a new apex from the existing mature hyphal cell. It has been reported that some enzymes such as proteinases could create weakened zones in the cell wall, which could be pushed out by cytoplasmic flow to initiate branching. So in the mycelial growth, in addition to activation of the cell wall synthesis, enzymes such as chitin synthetase and proteinases, which might weaken the lateral cell walls, are also important. During growth of vegetative hyphae, a large amount of nutrient absorbed from the substrate is stored. Polysaccharide may be stored in the form of glucans in secondary wall layers and/or as glycogen granules in the cytoplasm in the cell. The cells in a hypha are separated by a cross-wall called septum. Septa placement has been

reported to depend on the position of nuclear division. The septum is formed by chitin deposition on a preformed ring of actin microfilament. Dolipore septa divide hyphae into compartments or cells where movement of cytoplasmic material between them is carefully regulated. It gives rigidity to the hyphae and it can help avoid further injury if damage occurs at the hyphal tip [12]. In a developed colony, the hyphae tip growth or peripheral growth zone forms continuity between hyphae because of the presence of dolipore septa. The growing tips have a constant forward advance, so the mycelium that is left behind seems no longer involved in the growth. At the beginning of the fructification, the characteristic invasive growth of the vegetative mycelium in the substrate is modified. The fruit body initial is formed by increase in mycelial mass, with the formation of additional hyphal branching between the hyphae. The fruit body formation begins with the aggregation of hyphae to form a “knot” that will develop into a primordium and then a mature fruit body with differentiated stem and cap. It has been reported that the most important quantitative change in the cell wall during fruiting is the almost total loss of water-soluble glucan, instead, chitin has been reported important in fruit body development, which is essential for elongation of the stem hyphal walls. The precursor of chitin is N-acetylglucosamine and it is incorporated in the elongation of the hyphae of fruit bodies during expansion. Glycogen is accumulated in the base of fruit bodies at very earlier stages of growth and then disappears from the base as it is accumulated in the cap. In fruit body development, carbohydrates from the culture medium are temporarily store in R-glucan (alkali insoluble glucan) in the wall of mycelia and fruit body primordium hyphae, which is utilized for pileus development in the growing fruit bodies. *Pleurotus* species are cosmopolitan reported mainly as subtropical mushrooms. The optimal temperatures for growth of the mycelium are around 25–28°C and the range of pH is about 6.0–7.0. For fruit body formation, optimal temperature, relative humidity, CO₂, and light are 10–21°C, 85–90%, <1000 ppm, and 1000–1500 lx, respectively [13].

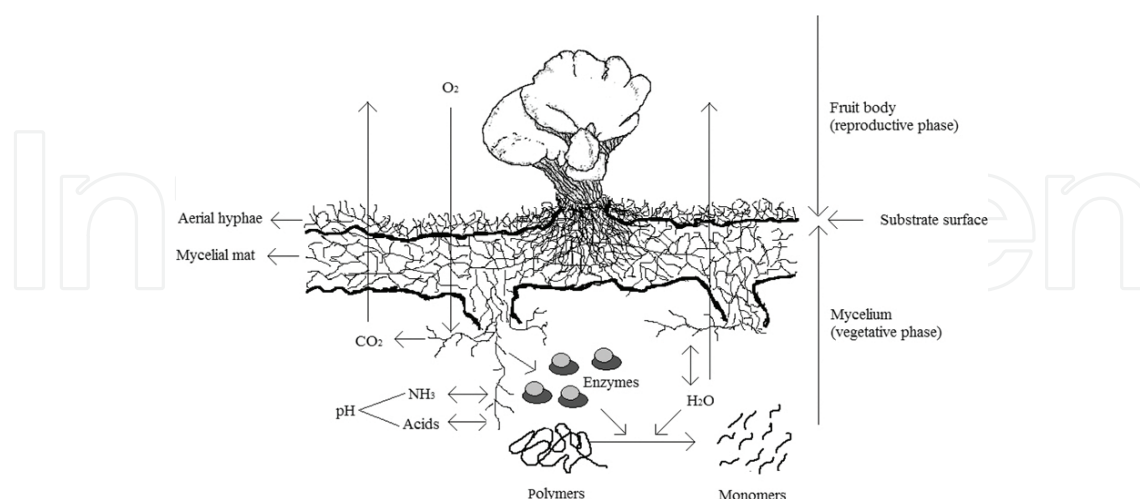


Figure 1. Schematic representation of the growth of *Pleurotus ostreatus* [10].

P. ostreatus can be considered as functional food with nutritional and health benefits in addition to nutritional value [14]. This mushroom contains vitamins as well as an abundance of essential

amino acids. It also has proteins, lipids, ash, glycosides, tocopherols, phenolic compounds, flavonoids, carotenoids, folates, organic acids, etc. [15, 16]. In general, mushrooms contain 90% water and 10% dry matter, and their nutritional value can be compared to those of eggs, milk, and meat [17]. The total energetic value of cultivated species of *P. ostreatus* is 151 J in 100 g of fresh mushrooms [18].

P. ostreatus is the second most cultivated edible mushroom worldwide after *Agaricus bisporus* [13]. Technological improvements have made possible this mushroom cultivation worldwide. It has ability to degrade several lignocellulosic substrates due to its ability to secrete a wide range of hydrolyzing and oxidizing enzymes [19] and can be produced on natural materials from agriculture, woodland, animal husbandry, and manufacturing industries [13].

2.2. Ligninolytic enzymes of *Pleurotus* spp.

From an ecophysiological point of view, white-rot basidiomycetes are microorganisms able to degrade lignin efficiently. However, the degree of lignin degradation with respect to other wood components depends on the environmental conditions as well as the fungal species involved. *Pleurotus* species cause white rot of wood and other lignocellulosic materials, due to their oxidative and extracellular ligninolytic system. The fungal degradation occurs exocellularly, either in association with the outer cell envelope layer or extracellularly, because of the insolubility of lignin, cellulose, and hemicellulose. Three ligninolytic enzyme families have been reported as the enzymatic complex from *Pleurotus* species; manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2) but lack lignin peroxidase. Recently, was reported that in *Pleurotus ostreatus*, the role that generally played the lignin peroxidase, has been assumed by versatile peroxidase. [20]. Studies on the enzymes secreted by *P. ostreatus* have shown that the concerted action of laccase and aryl-alcohol oxidase produce significant reduction in the molecular mass of soluble lignosulphonates [21].

Additional peroxidases, such as dye decolorizing peroxidases have also been detected in *P. ostreatus* [22, 23]. Lignin biodegradation is an oxidative process, as a consequence *Pleurotus* enzymes can be involved in such processes. The manganese peroxidase gene family (mnps) of *P. ostreatus* is composed of five Mn^{2+} -dependent peroxidases (mnp3, 6, 7, 8, and 9) and four versatile peroxidases (mnp1, 2, 4, and 5), all having related gene and protein structure [24]. Mn^{2+} -dependent peroxidases (MnP) catalyze the H_2O_2 -dependent oxidation of lignin and its derivatives [25]. Mn is an obligatory cosubstrate for these enzymes, as it is required to complete the catalytic cycle. In fact, the oxidation of lignin and other phenols by MnP is dependent on free Mn^{2+} ions. This peroxidase does not oxidize nonphenolic lignin structures. It lacks sufficient oxidative potential to cleave the major nonphenolic units of lignin. MnP contains Mn^{2+} -binding catalytic site that is formed by three acidic residues (two Glu and one Asp) and generates Mn^{3+} , which acts as a diffusible oxidizer on phenolic or nonphenolic lignin units through lipid peroxidation reactions [26, 27]. In many fungi, MnP thought to play a crucial role in the primary attack of lignin because it generates a diffusible and strong oxidant (Mn^{3+}). Organic acids such as oxalate and malonate are secreted by white-rot fungi, stimulating the MnP reaction throughout the stabilizing of Mn^{3+} [28, 29]. Versatile peroxidases feature Mn-binding residues as well as conserved Trp involved in the electron transfer that enables

oxidation of nonphenolic compounds. Versatile peroxidases possess two catalytic sites, one for the direct oxidation of low- and high-redox potential compounds, and the other for oxidation of Mn in a preferred manner [23, 30–33]. This dual activity mode of action enables versatile peroxidases to modify a wide range of substrates. It has been suggested a role for versatile peroxidases of *P. ostreatus* in the transformation of azo dyes [23, 30, 34] and carbamazepine [35].

Laccases are blue copper oxidases that catalyze the one-electron oxidation of *ortho*- and *para*-diphenols, aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical. Their oxidation of the phenolic units in lignin generates phenoxy radicals. Laccases also catalyze the demethoxylation of several lignin model compounds [36–38], such oxidation activity is accompanied by the reduction of molecular oxygen to water. In laccase, histidine and aspartic residues are involved in binding the phenolic compounds, and histidine residue itself is involved in the binding of nonphenolic substrates [39]. Laccase activity in fungal cultures can be increased by the addition of different aromatic compounds to the media, producing different forms of laccase due to the supplementation of aromatic compounds [40, 41]. The ligninolytic system of *P. ostreatus* makes this organism useful in several practical applications of cell-free or purified forms of peroxidases in bioremediation and biotransformation of persistent organic pollutant.

2.3. Laccases

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are enzymes classified as multicopper oxidases. These glycoproteins have the redox ability of copper ions to catalyze the oxidation of a wide range of aromatic substrates where water is obtained as by-product from the reduction of molecular oxygen [42, 43]. Laccases were first time reported in the Japanese lacquer tree (*Rhus vernicifera*) [44]. Laccases has been observed in plants, insects, bacteria, but the most studied are from the fungi classified as of rot-white, where are considered as ligninolytic enzymes because lignin sources are the best substrate for the growth of these fungi. These enzymes occur mainly in basidiomycetes, deuteromycetes, and ascomycetes, but their production in lower fungi has never been observed [45]. There exists a wide diversity of laccases including isoenzymes produced by fungi that have very different physicochemical properties. Numbers of isoenzymes depend on the fungal species [2, 7, 46–48]. In general, laccases show molecular weight between 40 and 100 kDa with 10–50% of their total weight of glycosylation and with isoelectric point (pI) around pH 4.0. It has been reported that the glycosylation in fungal laccase plays a role in secretion, copper retention, susceptibility to proteolytic degradation, and thermal stability [49, 50]. The growth conditions of fungi and their physiological states are responsible for the expression of different laccase isoenzymes, which are coded by gene families and differentially regulated [4, 7, 41, 51, 52]. In *P. ostreatus*, 12 possible genes encoding laccases have been reported and only described and characterized 7 isoenzymes laccase: lacc2 [47], lacc4 [53], lacc6 [54], lacc9 [55], lacc10 [56, 57], lacc12 [58] and lacP83 [7]. The characteristics of some purified enzymes from *P. ostreatus* using 2,6-dimethoxyphenol (DMP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), syringaldazine (SYR) and guaiacol (GUA) as substrate are reported in Table 1[59–65].

Fungal species	Optimum pH of activity using different substrates				pI	MW (kDa)	Optimum temperature (°C)	Reference
	DMP	ABTS	SYR	GUA				
<i>P. ostreatus</i>			5.8		3.6	67	50	[59]
<i>POXA1b</i>	4.5	3.0	6.0		6.9	62		[54]
<i>P. ostreatus</i>								
<i>POXA1w</i>	3.0–5.0	3.0	6.0		6.7	61	45–65	[60]
<i>P. ostreatus</i>								
<i>POXA2</i>	6.5	3.0	6.0	6.0	4.0	67	25–35	[61]
<i>P. ostreatus</i>								
<i>POXA3a</i>	5.5	3.6		6.2	4.1	83–85	35	[47]
<i>P. ostreatus</i>								
<i>POXA3b</i>	5.5	3.6		6.2	4.3	83–85	35	[47]
<i>P. ostreatus</i>								
<i>POXC</i>	3.0–5.0	3.0	6.0	6.0	2.9	59	50–60	[56–61]
<i>P. ostreatus</i>								
<i>Lcc2</i>		4.0–5.5	6.2–6.5	6.0–8.0		46	50	[62]
<i>P. pulmonarius</i>								
<i>P. florida</i>					4.1	77		[63]
<i>P. sajor-caju</i> IV		2.1			3.6	55		[64]
<i>P. eryngii</i> I		4.5			4.1	65	55	[65]
<i>P. eryngii</i> II		4.5			4.2	61	55	[65]

Table 1. Characteristics of laccases from *Pleurotus* spp.

Laccases have a high capacity and nonspecific oxidation which allow their use in many biotechnology applications, such as detoxification of wastewater produced in pulp bleaching process [66] and from industrial plants [67], treatment of elimination of phenolic compounds in beer and processed fruit juices [68], in effluent discoloration and modification of textile fibers [69], as biosensors [70], as drug testing (to distinguish morphine from codeine) [71]. Another important application is in environmental remediation; laccases have shown ability of degrading hazardous compounds that have carcinogenic and/or mutagenic effects, including polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), pentachlorophenols (PCP), toluene, benzene, xylene (BTEX), ethylbenzene, and trinitrotoluene (TNT) [72].

2.4. Production of laccases of *Pleurotus* in solid-state and submerged fermentation

SSF has been defined as the bioprocess carried out in the absence or near absence of free water by the use a solid matrix with high water adsorption; the solid matrix could be biodegradable

or inert, but in both cases must possess enough moisture to support growth and metabolism of the microorganism. Biodegradable solid matrix acts as support and source of nutrients and in the inert matrix, it is only the support and the culture liquid medium must be added [73, 74]. SSF has been a very efficient process for the production of enzymes by filamentous fungi [75, 76], possibly, they reproduce the natural living conditions [77].

SSF is the best culture system to study the morphological and metabolic differences between aerial hyphae and those that penetrate in the solid matrix [78]. It has been reported that SSF is better system than SmF for production of fungal enzymes, because it provides higher volumetric productivities, is less sensible to catabolite repression and yields enzymes with a higher stability at temperature and/or pH [1]. The fermentation could be carried out in less time and the productions of undesirable proteases that degrade enzymes of interest are minimized [78, 79]. Several studies in this field have determined the physiological differences during the growth of microbial cells in the two types of processes. The use of an adequate support for performing SSF is essential, since the success of the fermentation depends on it [80]. Castanera et al. [81] reported that laccase gene transcription is upregulated in an induced SmF but downregulated in the SSF, when were determined the laccases expression profiles in different fungal strains under SmF and SSF using wheat straw extract as inducer.

In a study was found that *P. ostreatus* grown in SSF on wheat bran and vinasse produced twice laccase activity (20 U/ml and three isoforms) than those reported in SmF (10 U/ml and two isoforms) [82]. It has been reported the intracellular activity and isoenzymes number of laccase of 10 strains of *Pleurotus* spp. grown on agar without addition of inducers. Differences in the *in vitro* activities using different substrates (2,6-dimethoxyphenol, *p*-anisidine, or *o*-tolidine) were observed between all the strains; zymogram patterns were similar for strains within same species, independently of any of the three substrate used [2]. Similar results were found in the extracellular extracts obtained of the same strains grown as above was mentioned [7]. In other study was used the same composition of the culture medium to grow *P. ostreatus* (ATCC 32783) in both SmF and SSF using polyurethane foam (PUF) as inert support. Atypical behavior was observed, since in SmF the fungus reported a laccase activity of 13,000 U/L with a biomass production of 5.6 g/L and four laccase isoforms, while SSF had a much lower laccase activity (2430 U/L), with biomass production of 4.5 g/L and three laccase isoforms. These results show that *P. ostreatus* performs much better in SmF than in SSF [4].

P. ostreatus (ATCC 32783) was grown at different initial pH of the culture medium in SSF using PUF. In general, the fungus showed high values of specific growth rate at all pH tested, the higher were at pH 3.5 and 8.5 (0.078 and 0.082 h⁻¹, respectively), whereas at pHs of 4.5, 6.5, and 7.5 were 0.047, 0.034, and 0.047 h⁻¹. Furthermore, the maximum biomass values were low, about 3.7 g/L in all cases. The maximum values of laccase activity were approximately 40,000 U/L observed in fermentation development at pH 4.5 and 6.5. The largest number of isoenzymes was observed in fermentations carried out at pH 7.5 and 8.5 [83].

Pleurotus pulmonarius (Fr) Quélet was cultivated on SSF using corn cob as substrate to produce laccase. The addition of 25 mM CuSO₄ increased from 270 to 1420 U/L the laccase production [84]. In other study, *Pleurotus pulmonarius* was grown on SSF using several natural supports, obtaining high laccase activities in wheat bran (2860 ± 250 U/L), pineapple peel (2450 ± 230 U/

L), and orange bagasse (2100 ± 270 U/L) [85]. Recently, the growth of *Pleurotus eryngii* on SSF using different agricultural wastes was reported and its laccase activity was evaluated in mycelium, primordium, and fruiting body. Laccase activities were comparably low in mycelial and primordium. The highest laccase activity was obtained in fruiting body developed on both wheat straw and cotton stalk. The laccase activities of 125.65 and 205.83 U/L of fruiting body were observed on wheat straw +5% of rice bran and on cotton stalk +5 % of rice bran, respectively [86]. Maximum activity of laccase during vegetative phase of growth of *P. ostreatus* can be directly correlated with degradation of lignin in this stage [87].

Different strains of *Lentinula edodes* and *Pleurotus* species were compared for the first time for their ability to produce lignocellulolytic enzymes in SmF and SSF using various plant raw materials. Two strains of *Lentinula edodes* (IBB 123 and IBB 363) appeared to be better producers of laccase than oyster mushrooms. In SSF, *Lentinula edodes* IBB 123 reached laccase activity of 57 U/flask on day 7 of fermentation. *Pleurotus tuber-regium* IBB 624 showed 20 U/flask of laccase activity after 10 days of fermentation, other fungi of this genus produced only 7–16 U/flask of laccase during 7 or 10 days of SSF [88].

P. ostreatus and *Pleurotus sajor-caju* were grown in SSF and their ability to produce laccase and carboxymethylcellulase (CMCase) on different agricultural wastes was studied. *Pleurotus* was inoculated on viticulture wastes, wheat straw, paddy straw, sesame straw, sawdust, and the mixtures of these wastes with wheat bran. Different mycelial growth times were related with different patterns of enzyme activities. During the incubation period, *P. ostreatus* showed its highest values of laccase activity at 10th day and decreased gradually until the first harvest. The highest laccase activity was observed on mixture of wheat straw:bran (2:1) (5.48 U/mg), followed by on paddy straw:bran (2:1) (4.36 U/mg) and on viticulture wastes:bran (2:1) (3.51 U/mg) at 10th day of mycelial growth. The lowest laccase activity was obtained on viticulture wastes (0.30 U/mg) [89]. It has been reported that laccase activity could be regulated, increasing the activity in morphogenesis during the mycelial growth and then the enzyme level decreases rapidly [90]. The laccase production of an indigenous strain of *P. ostreatus* (HP-1) was studied on SSF. Culture parameters, including type and concentration of substrate, moisture content, inoculum size, temperature, pH, surfactant presence, and nitrogen source, were optimized by conventional one factor at a time methodology. Maximum laccase activity of 3952 U/g of dry substrate was obtained with wheat straw as substrate, incubation temperature 28°C, five agar plugs as inoculum, pH 5.0, 60% moisture content, surfactant concentration 0.015 g/L, and combination of L-asparagine and NH_4NO_3 at 10 mM concentration each as nitrogen source. Laccase activity was increased with the use of various aromatic inducers and CuSO_4 . Highest laccase activity of 14189 U/g of dry substrate was obtained using 0.28 mM CuSO_4 under optimized conditions [91]. *P. ostreatus* was grown in SSF conditions for production of laccase, manganese peroxidase, and lignin peroxidase. Highest enzymes levels (laccase 455.11, manganese peroxidase 210.77, and lignin peroxidase 54.50 U/ml) were observed at 7 days in a medium containing 5 g wheat straw (66% w/w moisture), 4 ml inoculum at pH 4.5 and 30°C, using 1% (v/v) glycerol as carbon source, 0.2% (w/w) urea as nitrogen source, 1% (w/v) 2,2-azinobis 3-ethylbenzthiazoline 6 sulphonate as laccase inducer and 1% (w/v) MnSO_4 for

manganese peroxidase, 1% (w/v) CuSO_4 as metal ion for laccase, and Mn^{2+} for manganese peroxidase [92].

Different concentrations of apple pomace were evaluated on laccase production by *P. ostreatus*. During the first four days of fermentation, there was not laccase production. The maximum laccase activity (114.64 U/ml), was observed at 9 days of fermentation in the medium with 2.5% (w/v) apple pomace. This activity was approximately 2.8 times (30.24 U/ml) and 0.9 times (60.49 U/ml) higher than that of *P. ostreatus* with 5% (w/v) and without apple pomace, respectively. These results suggest that *P. ostreatus* might use the nutrient content of apple pomace (rich in carbohydrates, dietary fiber, and minerals) without laccase activity in the initial stages of cultivation (approximately 4 days) [93].

Recently, optimization of the laccases production of *P. ostreatus* grown on sugarcane bagasse in SSF was worked. Water activity, pH, temperature, and concentrations of CuSO_4 , $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , asparagine, and yeast extract were variables used in the optimization. The concentrations of CuSO_4 and $(\text{NH}_4)_2\text{SO}_4$ had a significant influence on the production of laccase, but the use of yeast extract and the addition of ferulic acid as inducer provided increases of laccase activity of 5.7 times and 2.0 times, respectively. The highest laccase activity of 151.6 U/g was produced at the 5th day of SSF [94].

Cocultivation of *P. ostreatus* MTCC 1804 and mutant *Penicillium oxalicum* SAUE-3.510 was studied for the production of xylanase-laccase mixture under SSF condition. Growth compatibility between both fungi was analyzed by growing them on potato dextrose agar plate, obtaining 58% and 33% higher levels of xylanase and laccase production, respectively. A mixture of sugarcane bagasse and black gram husk (3:1) was the best solid substrate and support for fungal colonization and enzyme production during co cultivation. Maximum activity values of xylanase (8205.31 IU/g) and laccase (375.53 IU/g) during SSF were observed using 4 g of solid support with 80% of moisture content. The coculture system was efficient in the production of xylanases and laccases that may be employed in agroindustrial waste degradation [95].

P. ostreatus (ATCC 32783) was grown under SmF conditions and found the production profile of laccases as well as the isoenzymes patterns through zymograms. Four laccase isoenzymes were produced, one throughout the fermentation time and three only during growth stationary phase. The maximum laccase activity (12200 U/L) was observed in the growth stationary phase. Kinetic parameters of a purified isoenzyme (enzyme produced throughout the fermentation), such as the apparent molecular weight of 43.7 kDa, K_m 90 μM , V_{\max} 1.18 $\Delta\text{Abs}/\text{min}$, and pI of 2.3, were obtained [3].

On the other hand, the growth and activity of laccases from five different strains of *P. ostreatus* developed under SmF conditions with and without copper added to the culture medium was studied. It was observed that the concentration of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.25 g/L) did not affect the growth of the strain ATCC 32783, however, other strains showed lower growth rates and less biomass, the ATCC 201216 strain almost was inhibited. ATCC 32783 strain showed the highest values of laccase activity in the presence of copper reaching up to 37490 U/L, whereas in the culture without copper was obtained 1086 U/L; ATCC 201216 strain in the presence and absence

of copper produced 1400 and 1000 U/L, respectively. These results suggest that not all strains have the same answer to the presence of Cu in the culture medium, and the sensitivity to Cu be could use to select strains with high laccase production for commercial exploitation [5].

The activity and isoenzymes number of laccase from *P. ostreatus* ATCC 32783 grown in SmF conditions using a buffered and nonbuffered media were studied. For both culture media, the initial pH was 3.5. Laccase activity was around 100–500 U/L during the 100–400 h (approximately) of fermentation in both media. Buffered culture medium showed minimal pH changes, while the pH in nonbuffered medium changed drastically, reached a value of 6.5 after 240 h of fermentation. The highest laccase activity (3200 U/L) at 500 h of fermentation was obtained in the buffered medium and in nonbuffered culture medium was only of 450 U/L. One laccase isoenzyme was observed during the entire fermentation process in both media, but in the nonbuffered medium, an additional isoenzyme was produced when the pH reached a value of 6.5. These results suggest that some laccase isoenzymes are regulated by pH signals and also observed that the fungus produces metabolites to regulate the pH of the medium [6].

A gene called lacP83 that encode a laccase isoenzyme of *P. ostreatus* ATCC 32783 grown in SmF was described. Using the PCR inverse strategy, a 2887 bp sequence was obtained from a genomic library of *P. ostreatus*. The coding sequence was of 1527 bp long with 17 exons and the protein encoded had 509 amino acids, shows a putative signal peptide and conserved Cu binding domains. In the promoter region (466 bp upstream of ATG), putative binding transcription factors such as metal response element, xenobiotic response element, a stress response element, and a defense response element were found. The gene and protein sequences of lacP83 had 85–94% and 90–96%, respectively, of similarity with laccases of *Pleurotus* previously reported. This laccase showed differences in its promoter sequence and apparent molecular weight [7].

Recently, the effect of pH on the expression of five genes of laccases (lacc1, lacc4, lacc6, lacc9 and lacc10) and isoenzymes profiles produced by *P. ostreatus* ATCC 32783 developed under SmF conditions was evaluated. The initial pH of the culture media was adjusted at 3.5, 4.5, 6.5, and 8.5. In this research, it was observed that pH is a very important factor for growth, development and production of enzymes, and metabolites of this fungus. The specific growth rate increased with the increase of initial pH of the culture medium, higher biomass values were obtained at pH 6.5 and 8.5; highest laccases activity was obtained at initial pH of culture media of 4.5 and 6.5 and determined at the same values of pH reaching up to 77,500 U/L. The isoenzyme patterns were different depending on the initial pH of the culture medium, to acidic pH was observed up to three isozymes (29, 47, and 65 kDa), at pH near neutrality were observed four isoenzymes (29, 38, 47, and 65 kDa), and alkaline pH three isozymes (29, 47, and 65 kDa) were observed. Since the expression of four genes of laccases (lacc1, lacc4, lacc6, and lacc10) and four isoenzymes was observed, it was suggested that lacc6, lacc10, lacc4, and Lacc1 correspond to isoenzymes of 65, 47, 38, and 29 kDa, respectively. The authors suggest that the pH has a very important role as a transcriptional factor that determines the expression profile and pattern of production of laccase enzymes under conditions SmF [8].

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