British Biotechnology Journal
2(3): 115-132, 2012

SCIENCEDOMAIN *international*
www.sciencedomain.org

Optimization of Cultural and Nutritional Parameters for the Production of Laccase by *Pleurotus ostreatus* ARC280

Maysa A. Elsayed[1], Mohamed M. Hassan[1], Ali M. Elshafei[1*], Bakry M. Haroun[2] and Abdelmageed M. Othman[1]

[1]Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt.
[2]Department of Botany and Microbiology, Faculty of Sci., Al-Azhar University, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. All authors contributed, read and approved the final manuscript.

Research Article

Received 13th March 2012
Accepted 20th May 2012
Online Ready 25th June 2012

ABSTRACT

Aims: To optimize laccase production by submerged fermentation using an edible mushroom *Pleurotus ostreatus* ARC280.
Study Design: Laccase activity was assayed by monitoring the product formation rate of enzymatic oxidation of syringaldazine spectrophotometrically at 525 nm.
Place and Duration of Study: Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre, Dokki, Cairo, Egypt, between May 2009 and October 2010.
Methodology: *Pleurotus ostreatus* ARC280 was maintained on potato dextrose agar medium. The liquid medium used for the laccase production by the fungal culture during its growth in submerged fermentation was selected from eight liquid media for inducing laccase production. Parameters such as incubation period, temperature, pH of the production medium, carbon and nitrogen sources and other nutritional parameters were studied using syringaldazine as a model substrate for laccase activity determination.
Results: In the present work, Eight media with different components were screened. The enzyme formed by *Pl. ostreatus* ARC280 was localized mainly in the extra-cellular fraction. Laccase formation reaches its maximum value with specific activity of about 140 U/mg protein at the twenty-sixth day of incubation, pH 5.0 and 28ºC. Among the various wastes used, corn stover induces the highest laccase production with specific activity of

**Corresponding author: Email: alielshafei@yahoo.com;*

75.48 U/mg protein. Soluble starch at 1.5% (w/v) and ammonium sulfate was found to be the best carbon and nitrogen sources for laccase formation, respectively. The optimal concentrations of Tween-80 and $CuSO_4$. $5H_2O$, were found to be 0.1% (v/v) and 100µM and cause enzyme induction by about 44% and 19% than control, respectively.
Conclusion: Laccase production by *Pl. ostreatus* ARC280 has been shown to depend markedly on the composition of the culture medium, carbon, nitrogen content and inducer compounds and governed by parameters such as pH of the production medium and other nutrition parameters.

Keywords: Laccase; Pleurotus ostreatus; production; induction; optimization.

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), which belong to the group of polyphenoloxidases are multi-copper-containing enzymes which reduce molecular oxygen to water and simultaneously perform one-electron oxidation of various substrates such as diphenols, methoxy-substituted monophenols and aromatic and aliphatic amines (Munusamy et al., 2008a; Kudanga et al., 2011; Elshafei et al., 2012). Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups. These enzymes were known to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines including diphenols and polyphenols to diamines, aromatic amines, benzenethiols and substituted phenols (Kiiskinen et al., 2002; El-Shora et al., 2008; Sivakumar et al., 2010). Laccases were first detected in the sap of the Japanese laquer tree *Rhus vernicifera* and subsequently in many other plants (Yoshida, 1883; Desai and Nityanand, 2011), insects (Kramer et al., 2001), Archae and bacteria (Claus, 2003). However, most laccases are found and studied in lignin-degrading basidiomycetes (Thurston, 1994; Baldrian, 2006; Prabu et al., 2006). Among the basidiomycetes, white-rot fungi have received special attention because of their ability to mineralize lignin by secreting oxidative enzymes, such as peroxidases and laccases (Khammuang and Sarnthima, 2009; Halaburgi et al., 2011). Investigators reported that fungal laccases existed as extracellular as well as intracellular enzymes as one or more isoenzymes (Bertrand et al., 2002; Baldrian, 2006).

Mushrooms are saprophytic fungi belonging to the class of the Basidiomycetes. They grow in moist places with decomposing organic matter and are very important in nutrient cycling (Subramanian, 1995). Although there are more than 2000 species of edible mushrooms nowadays, only the champignon (*Agaricus bisporus*), the giant mushrooms (*Pleurotus ostreatus and Pleurotus edodes*) are among the most cultivated ones (Bononi et al., 1999). The *Pleurotus* genus gathers several species, such as *Pl. ostreatus*, *Pl. pulmonaris*, *Pl. sajor caju*, *Pl. cornucopiae* and *Pl. ostreatoroseus*. *Pleurotus* is spread all around the world in its natural habitat, mainly in forest environments (Bononi et al., 1999) and produce lignocellulosic enzymes, mainly laccase (LAC) and Mn-peroxidase (MnP), which convert lignocellulosic residues into food (Bernardi et al., 2008).

Applications of laccase in biotechnology include: textile dye or stain bleaching (Fu and Viaraghavan, 2001; Kirby et al., 2000; Pointing and Vrijmoed, 2000), paper-pulp bleaching (Annunziatini et al., 2005; Grönqvist, et al., 2003), synthetic dye decolorization (Baldrian,

2004; Nagai et al., 2002), bioremediation (Jaouani et al., 2005; Mohidem and Mat, 2009), biosensors (Timur et al., 2004; Khammuang and Sarnthima, 2008), chemical synthesis (Karamyshev et al., 2003), immunoassays (Jordaan and Leukes, 2003) and the detoxification of contaminated soil and water (Filazzola et al., 1999; Munusamy et al., 2008b).

1.1 Main Objective

A good strategy to increase the productivity of the laccase fermentation process is the optimization of the fermentation medium and then enhancing laccase activity by using inducers. Therefore, the objective of this study was to optimize laccase production by the edible white-rot fungus, *Pleurotus ostreatus* ARC280 by studying the physiological conditions required for the production of the enzyme under study, which also be beneficial to the development of laccase fermentation industry.

2. MATERIALS AND METHODS

2.1 Microorganism

Pleurotus ostreatus ARC280 was obtained from Agriculture Research Center (ARC), Egypt.

2.2 Chemicals and Media

The enzyme substrate (syringaldazine) was supplied by Sigma, USA. The other chemicals used in this study were of analytical grade and higher purity. For maintenance and propagation of the fungal culture, potato-dextrose agar (PDA) medium was used. The liquid medium used for the laccase production by the fungal culture during its growth in submerged fermentation is composed as follows, (g/l): glucose, 10.5; yeast extract, 5; $(NH_4)_2SO_4$, 2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.02; $CaHPO_4$, 0.3; $ZnSO_4$, 0.2; $MnSO_4$, 0.2 and $CuSO_4.5H_2O$, 0.25 (Tlecuitl-Beristain et al. 2008). The medium was adjusted to an initial pH value of 5.0, and then sterilized by autoclaving at 1.5 atmosphere and 121ºC for 20 min.

2.3 Preparation of Cell Free Filtrates

At the end of the incubation period, the cultures were filtrated using Whatman No.1 filter paper. The culture filtrate was used directly for enzyme activity determination.

2.4 Preparation of Cell Free Extracts

Pl. ostreatus ARC280 mats were filtered and homogenized in citrate phosphate buffer (100 mM; pH 5.0) and cold washed sand in a cold mortar (4ºC). The crude homogenate was then centrifuged at 5000 rpm for 10 min. The upper layer containing cell free extracts was separated by decantation and used as the crude endocellular enzyme preparation (Verdin et al., 2004; Elshafei et al., 2012).

2.5 Enzyme Assay

Laccase activity was assayed by monitoring the product formation rate of enzymatic oxidation of syringaldazine spectrophotometrically at 525nm (ε_{525} = 65,000 M^{-1} cm^{-1})

(Leonowicz and Grzywnowicz, 1981; Minussi et al., 2007). The assay mixture (2.0 ml) contained syringaldazine, 0.1µmole; citrate-phosphate buffer (pH 5.0), 90 µmoles and appropriate volume of diluted enzyme. One unit laccase activity was defined as the change in the absorbance of 0.001 per sec (Prabu and Udayasoorian, 2005; Aríca et al., 2009). Protein content was estimated by the modified procedure of Lowry et al. (1951). Citrate-phosphate buffer was prepared according to the method presented by Gomori (1955). Moreover, the final pH was accurately adjusted by using PHM 62 Standard pH meter. All the data were statistically evaluated according to the method described by Kenney and Keeping (1962). All experiments were performed in triplicate. The means and standard deviation (Mean ± S.D.) were calculated for each experiment.

2.6 Influence of Media Composition

Eight liquid media were tested for inducing laccase production. These media compositions were described in these references: Medium 1, Chawachart, et al., 2004; Medium 2, Chefetz et al., 1998; Medium 3, Minussi et al., 2007; Medium 4, Da Re et al., 2008; Medium 5, Dhaliwal et al., 1992; Medium 6, Difco Manual, 1672 and Bora, 2003; Medium 7, Tlecuitl-Beristain et al., 2008 and Medium 8, Khlifi et al., 2010. All media were adjusted to an initial pH value of 5.0, then sterilized by autoclaving at 1.5 atmosphere and 121ºC for 20 min. *Pleurotus ostreatus* ARC280 was grown on the eight different media under both static and shake culture conditions (150 rpm) using New Brunswick scientific Co. Inc. Edison N. J. USA shaker at 28 ± 2ºC for 26 days of incubation and the cell-free filtrate (CFF) was then used as an enzyme source. The activity of the formed laccase was assayed using syringaldazine as a substrate.

3. RESULTS AND DISCUSSION

3.1 Effect of Different Media Composition

Eight liquid media were tested for inducing laccase production with compositions described previously in materials and methods section. Results obtained in Table 1 indicate that, *Pl. ostreatus* ARC280 laccase was mainly formed under static condition. Results also showed that in the diverse range of culture media used for the cultivation of *Pl. ostreatus* ARC280, better enzyme production was obtained maximally in the medium No7 under both static and shake conditions with specific activities of 150.47 and 57.52 U/mg proteins, respectively. Laccase production by fungi has been shown to depend markedly on the composition of the culture medium, carbon, nitrogen content and inducer compounds (Ravankar and Lele, 2006; Adejoye and Fasidi, 2010). Bora (2003) stated that, in some media, the addition of some inducers may induce the laccase production whilst in other media they did not.

3.2 Localization of Laccase Produced by *Pl. ostreatus* ARC280

Pl. ostreatus ARC280 was cultured and incubated under static condition on the medium No7. After incubation, the levels of laccase activity in both cell-free filtrate (CFF) and cell-free extract (CFE) were compared using syringaldazine as a substrate**.** Results in Table 2 indicate that, the enzyme formed by *Pl. ostreatus* ARC280 expressed as specific activities, was detected mainly in the extra-cellular fraction (CFF), and the relative specific activities of the corresponding endo-cellular enzyme values represent only about 48% and 67% of the corresponding relative activities for exo-cellular enzyme level with static and shake conditions respectively which mean that the enzyme is mainly excreted extracellularly. In the

following experiments, the filtrated fermented medium (cell-free filtrate, CFF), was employed as a laccase source under static culture condition.

Table 1. Effect of different media on the formation of laccase produced by *Pl. ostreatus* ARC280

Medium number	Aeration condition	Final pH	Specific activity (U/mg protein)
1	static	4.8	1.96 ± 0.21
	shake	4.5	51.11 ± 5.16
2	static	4.8	3.92 ± 0.72
	shake	4.5	57.33 ± 4.67
3	static	5.0	1.47 ± 0.79
	shake	4.5	0.68 ± 0.03
4	static	4.5	0.18 ± 0.10
	shake	4.5	0.36 ± 0.01
5	static	4.5	28.28 ± 3.73
	shake	4.5	40.50 ± 1.14
6	static	4.8	3.96 ± 0.21
	shake	4.5	33.92 ± 2.70
7	static	5.0	150.47 ± 1.54
	shake	4.5	57.52 ± 1.05
8	static	5.0	25.80 ± 0.75
	shake	5.5	3.81 ± 0.17

Table 2. Localization of laccase produced by *Pl. ostreatus* ARC280

Enzyme source	Static		Shake	
	Specific activity (U/mg protein)	Relative specific activity (%)	Specific activity (U/mg protein)	Relative specific activity (%)
Exo-cellular	150.47 ± 1.54	100.00	57.52 ± 1.05	100.00
Endo-cellular	71.72 ± 3.28	47.66	38.62 ± 0.30	67.14

3.3 Incubation Period

In this experiment, *Pl. ostreatus* ARC280 was grown on media No7 under static culture condition for thirty-one days of incubation. Samples of the fermented medium were collected periodically for the determination of laccase activity. Results cited in Fig. 1 indicate that, the highest specific activity was obtained at the twenty-sixth day of growth of *Pl. ostreatus* ARC280 where laccase formation reaches its maximum value with specific activity of 140.03 U/mg protein. A decrease in enzyme level was noticed by increasing the incubation period above this value.

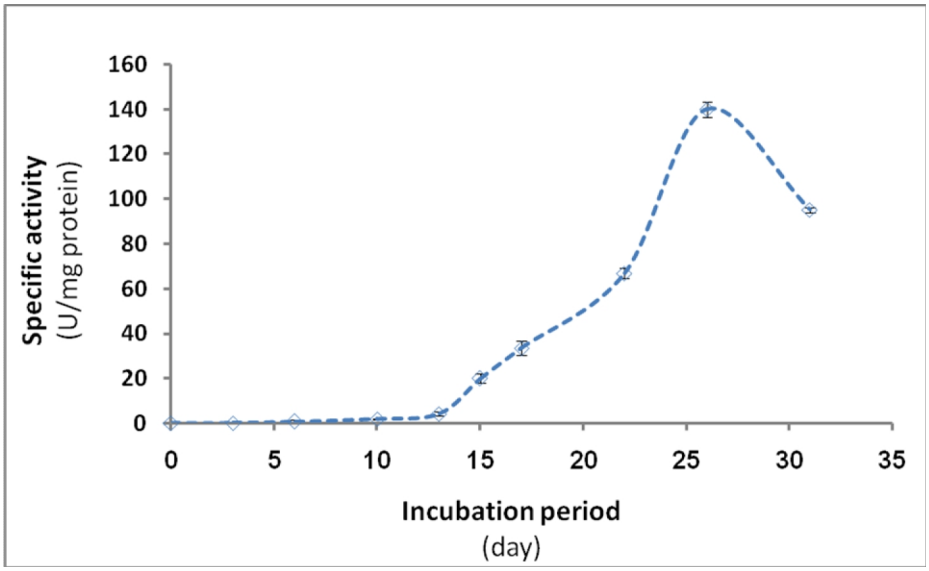

Fig. 1. Effect of incubation period on laccase formation by *P. ostreatus* ARC280

Many investigators reported different incubation periods for optimum production of laccase. The ligninolytic enzyme system of white rot fungi, although may be present in the primary phase of growth, usually is triggered in response to N or C depletion, attaining its maximum in the idiophase when the mycelial dry weight is decreasing (Kaal et al.,1995). Das et al. (2011) stated that *Pl. ostreatus* (MTCC, 1802) was able to produce highest quantity of laccase in the 25th day of culture, whereas the other species (*Pl. florida* (ITCC, 3308), *Pl. flabellatus* (MTCC, 1799), *Pl. sajorcaju* (MTCC, 1806) and *P. pulmonarius* (MTCC, 1805) could even effectively exhibit their optimum laccase activities in the 26th day of culture in agreement with the results obtained. On the other hand, Elisashvili et al. (2008) and Sivakumar et al. (2010) reported that maximum laccase production was obtained at the 7th and 10th day of incubation in case of *Lentinus edodes* and *Ganoderma sp*, respectively. Oppositely, Cavallazzi et al. (2005) found maximum laccase activity with *Lentinula edodes* after 30 days of incubation, whilst Chawachart et al. (2004) reported that laccase activity still increased after 36 days of cultivation of *Coriolus versicolor* strain RC3 when grown on rice bran solid media.

3.4 Initial pH Values

A series of pH values ranging from 4.0 to 7.0 were studied in this experiment. Results obtained are shown in Fig. 2. From which it is clear that maximal formation of *P. ostreatus* ARC280 laccase took place at pH 5.0 and laccase formation occurred at a narrow range of pH values, whereas considerably low levels of enzyme were obtained at pH values below and above this value. This may be attributed to the fact that change in pH value may alter the three-dimensional structure of the enzymes (Shulter and Kargi, 2000). Haltrich et al., (1996) stated that most of fungal cultures prefer a slightly acidic pH in the medium for growth and enzyme biosynthesis, in agreement with the results obtained. In addition, Patel et al. (2009), Adejoye and Fasidi (2010) and Sivakumar et al. (2010) reported that *Pleurotus ostreatus* HP-1, *Schizophyllum commune* and *Ganoderma sp.* gave the optimum laccase production at pH 5.0, 5.5 and 6.0, respectively.

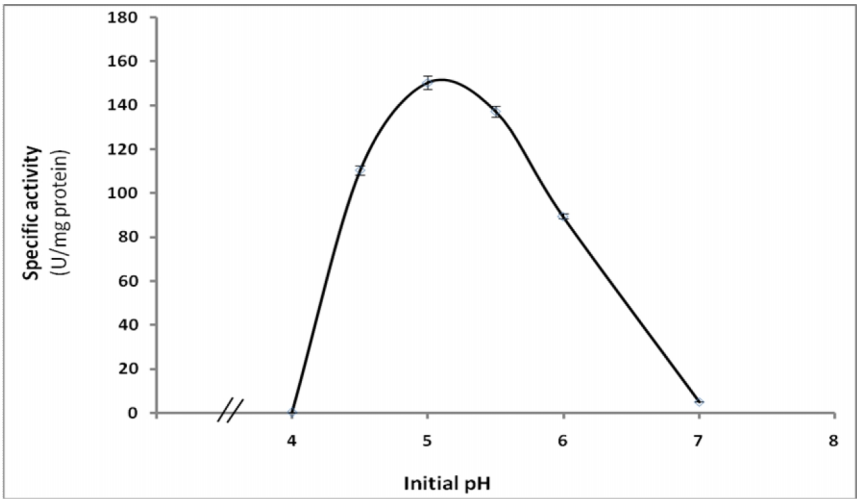

Fig. 2. Influence of different initial pH values on the formation of *Pl. ostreatus* ARC280 laccase

3.5 Incubation Temperature

In this experiment, *Pl. ostreatus* ARC280 was grown at various degrees of temperatures ranging from 25 to 35ºC. Results cited in Fig. 3 demonstrate that *Pl. ostreatus* ARC280 is able to grow and produce laccase within the range of incubation temperatures studied. The optimal temperature for fungal growth and laccase formation was found to be 28ºC with specific activity of 146.94 U/mg protein. Results also indicated that, by increasing the incubation temperature above 28ºC, a gradual decrease in laccase formation occurred until 32ºC and a null growth was obtained at 35ºC, and subsequently no enzyme activity could be detected at that degree of temperature.

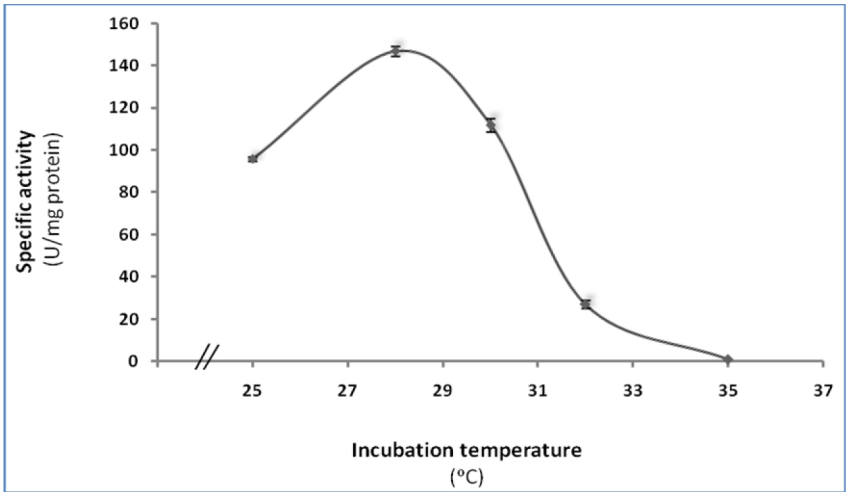

Fig. 3. Influence of incubation temperature on the formation of *Pl. ostreatus* ARC280 laccase

This can be interpreted by the alteration of cell membrane composition and stimulation of protein catabolism. Lonsane et al., (1985) and Krishna, (1999) stated that, the optimum cultivation temperature depends on the growth kinetics of the microorganism employed rather than on the enzyme produced. Many authors also reported that 28ºC was the best temperature for laccase production by *Schizophyllum commune* edible mushroom (Adejoye and Fasidi, 2010) and *Pleurotus ostreatus* HP-1 (Patel et al., 2009). On the other hand, Zadrazil et al., (1999) reported that temperatures higher than 30ºC caused reduction in ligninolytic enzymes activity.

3.6 Inoculums' Size

Inoculum plays a significant role in enzyme production. A lower level of inoculum may not be sufficient to initiate growth, whereas a higher level may cause competitive inhibition (Sabu et al., 2005). Sharma et al. (1996) reported that inoculum size controls and shortens the initial lag phase, as smaller inoculum size increased the lag phase. In this experiment, different inoculum sizes were tested ranging from 2 to 7 agar plugs (14 mm in diameter) were cut from actively growing fungal mycelium and inoculated in the production medium. Results obtained indicated that laccase production was progressively increased up to six agar plugs. Inoculum size of seven discs had an adverse effect on laccase formation Fig. 4, which can interpreted by the fast depletion of nutrients, resulting in a decrease in metabolic activity (Patel et al., 2009). In agreement with our results, Patel et al., (2009) reported that maximum laccase production was obtained with inoculum size of five agar discs.

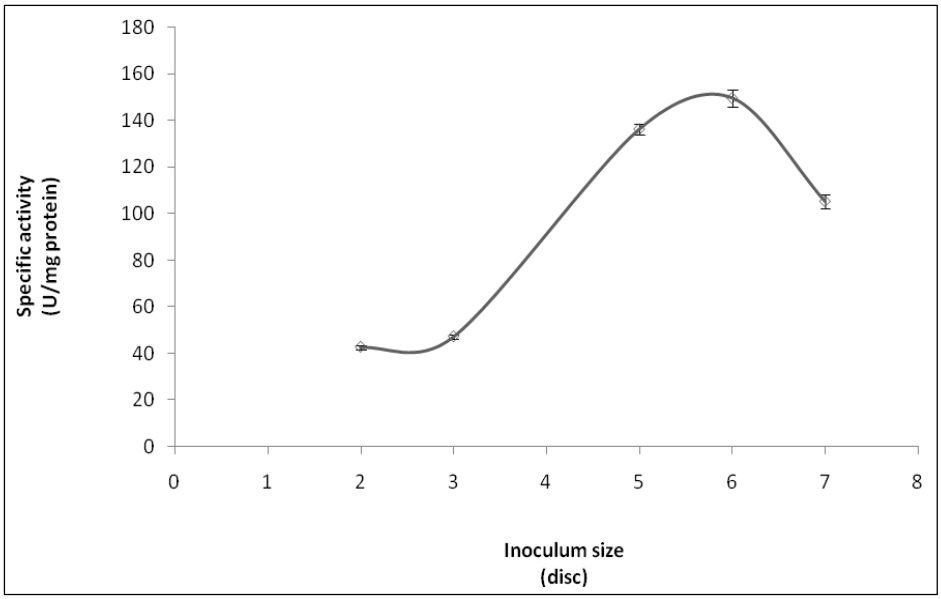

Fig. 4. Influence of inoculums' size on the formation of *Pl. ostreatus* ARC280 laccase

3.7 Effect of the Addition of Lignocellulosic Wastes

Different lignocellulosic wastes (wheat bran, wheat straw, sawdust, rice bran, rice straw, corn stalk, corn stover and sugar cane bagasse) were washed, dried, cut and grinded to equal sizes then used instead of glucose in the media for laccase production after

adjustment of media initial pH values at pH 5.0. Results cited in Table 3 showed that the maximum activity was recorded with glucose (as control) with specific activity of 152.15 U/mg protein.

Patel et al., 2009 stated that, wheat straw was found to be the best substrate. Whereas, higher laccase activities were found in *Pl. ostreatus* HAI 493 and *Pl. pulmonarius* HAI 572 under conditions of SSF of grapevine sawdust (Stajić et al., 2006). On the other hand, rice bran as a carbon source was found to be the most efficient substrate for laccase production by *Coriolus versicolor* strain RC3 compared to glucose, wheat bran and rice straw meal (Chawachart et al., 2004).

Table 3. Influence of lignocellulosic wastes on the formation of *Pl. ostreatus* ARC280 laccase

Lignocellulosic waste	Visual growth	Activity (U/ml)	Specific activity (U/mg protein)
Glucose (Control)	++++	541.67	152.15 ± 2.39
Wheat bran	++++	27.33	5.24 ± 0.29
wheat straw	+++	26.00	7.81 ± 0.38
Saw dust	++	176.67	56.81 ± 0.85
Rice bran	++++	11.33	3.00 ± 0.01
Rice straw	+++	92.67	26.86 ± 0.72
Corn stalk	++++	88.67	23.46 ± 1.04
Corn stover	++++	285.33	75.48 ± 3.27
Sugar cane bagasse	++++	29.33	8.81 ± 0.63

3.8 Effect of Carbon Source

The effect of conventional carbon sources on adaptation of the fungus for the production of laccase is of importance. For studying the effect of different carbon sources on the formation of laccase, monosaccharides (galactose and fructose), disaccharides (maltose and sucrose) and polysaccharides (carboxymethyl cellulose (CMC) and soluble starch) were used instead of glucose (control). Each carbon source was added at a concentration of 10.05 g/l to the growing medium described by Tlecuitl-Beristain et al., 2008 (medium 7), as the main carbon source. The obtained results showed that, soluble starch was found to be the best inducer for laccase formation. Alternatively, galactose, fructose, maltose and CMC significantly repressed laccase formation by *Pl. ostreatus* ARC280 (Fig. 5). In agreement with our results, among the carbon sources, starch supported the maximum laccase production from *Ganoderma sp*. (Sivakumar et al*.,* 2010). On the other hand, maximum laccase production by *Pl. ostreatus* HP-1 was obtained with 1% (w/v) glucose containing medium (Patel et al., 2009). Mansur et al. (1997) showed that the use of fructose instead of glucose resulted in a 100-fold increase in the specific laccase activity of basidiomycetes. Earlier it was suggested that easily assimilable components such as glucose, allow for constitutive laccase production but repress its induction in several fungi (Bollag and Leonowicz, 1984). An alternative to avoid this time delay in laccase production is to use a carbon source that is not very easily assimilable (D'Souza-Ticlo et al., 2009). Undoubtedly, laccase production is dependent on the microbial taxa employed (Osma et al., 2007).

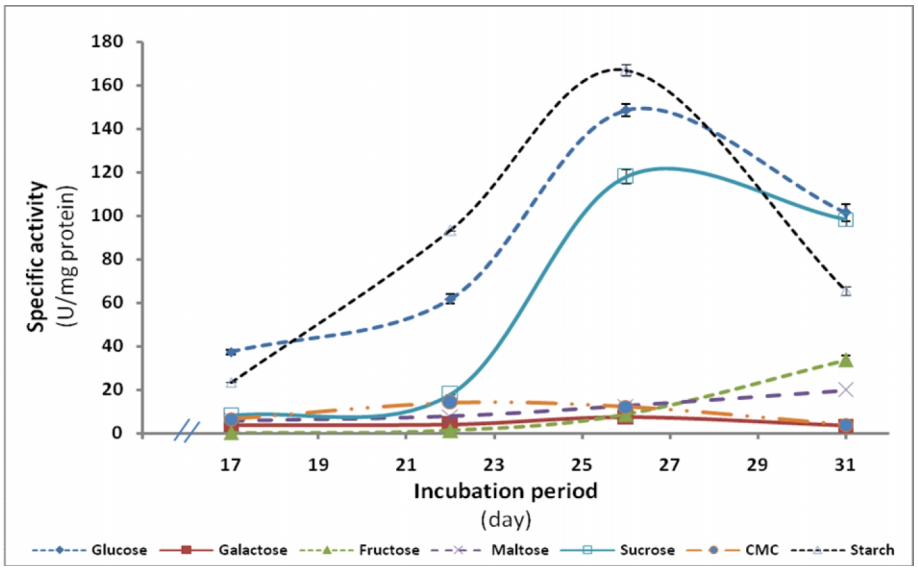

Fig. 5. Influence of carbon source on the formation of *Pl. ostreatus* ARC280 laccase

3.9 Influence of Soluble Starch Concentration

From the results of the preceding experiment, it was evident that soluble starch was the best tested carbon source for laccase formation by *Pl. ostreatus* ARC280. Therefore, it was necessary to test the effect of soluble starch concentration on enzyme formation. Soluble starch was added to the culture medium instead of glucose at six different concentrations ranging from 2.5 to 25 g/l. It is clear from Fig. 6, that the enzyme specific activity was increased with increasing soluble starch concentration up to 15 g/l, which is the best concentration of soluble starch for laccase formation. Increasing soluble starch concentration above this value resulted to a decrease in enzyme level.

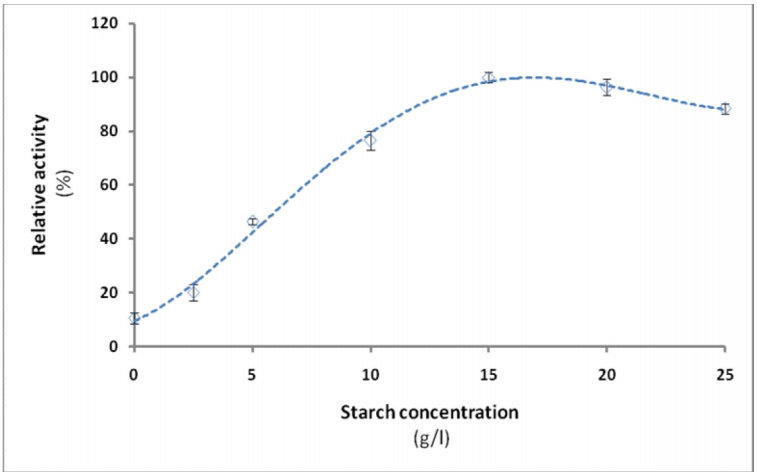

Fig. 6. Influence of soluble starch concentration on the formation of *Pl. ostreatus* ARC280 laccase

3.10 Effect of Different Nitrogen Sources

Various nitrogen compounds (sodium nitrate, ammonium chloride, urea, L(+)asparagine, L(+)glutamine, L-alanine, DL–alanine, L-arginine and L-tryptophan) were added separately to the culture medium in amounts equivalent to the amount of nitrogen in ammonium sulfate (control) in the medium described by Tlecuitl-Beristain et al., 2008 (medium 7) for *Pl. ostreatus* ARC280. The highest level of enzyme formation expressed in terms of specific activity was obtained with ammonium sulfate followed by L-arginine. The rest of nitrogen sources gave also considerable amounts of laccase except urea which gave the lowest enzyme activity. Different proportions of laccase were obtained in all media containing different nitrogen sources (Table 4).

Table 4. Influence of different nitrogen sources on the formation of *Pl. ostreatus* ARC280 laccase

Nitrogen source	Final pH	Protein (mg/ml)	Activity (U/ml)	Specific activity (U/mg protein)
Ammonium sulfate (Control)	5.0	2.63	431.33	164.00 ± 5.24
Sodium Nitrate	5.0	3.07	216.42	70.50 ± 2.25
Ammonium chloride	5.0	2.82	229.83	81.50 ± 2.09
Urea	5.5	3.75	10.67	2.85 ± 0.00
L(+) asparagine	5.5	3.76	99.30	26.41 ± 0.96
L(+) glutamine	6.0	3.57	243.30	68.15 ± 3.08
L- alanine	5.0	3.44	213.33	62.01 ± 4.05
DL– alanine	5.5	3.13	174.00	55.59 ± 0.65
L- arginine	5.0	3.38	358.33	106.01 ± 6.47
L- tryptophan	6.0	10.35	135.83	13.12 ± 3.61

In agreement with this result, Gogna et al. (1992) stated that, the most widely used nitrogen sources for fungal ligninolytic enzyme production are ammonium salts. Stajić et al. (2006) showed 396 U/l activity of laccase by *Pl. ostreatus* HAI 493 and 100 U/l by *Pl. eryngii* with ammonium sulfate as nitrogen source. On the other hand, Adejoye and Fasidi (2010) and Sivakumar et al. (2010) reported that yeast extract stimulated higher production of laccase by *Schizophyllum commune* and *Ganoderma sp*., respectively.

3.11 Influence of Tween-80 Concentration

Surfactants, especially Tween-80, can increase the bioavailability of less soluble substrates for the fungi and stimulate growth of the fungal spores (Zheng and Obbard, 2001). *Pl. ostreatus* ARC280 was grown on the medium described by Tlecuitl-Beristain et al., 2008 supplemented with different concentrations of Tween-80 (0.1 – 0.75%; v/v) as a surfactant to define the optimal concentration needed to induce maximum laccase yield. The results obtained showed that, the enzyme production expressed as a relative activity reached its maximum value at a concentration of 0.1% (v/v Tween-80) and increased by about 44% than control (without Tween-80). In addition, it was found that increasing Tween-80 concentrations up to this value resulted in a gradual decrease in laccase formation (Fig. 7). The stimulatory effect of surfactants may be a consequence of its action on cell membranes causing increased permeability and /or by promoting the release of cell-bound enzymes (Zeng et al., 2006). Tween 80 with a concentration of 0.015 gl^{-1} simulated higher laccase production by *Pl. ostreatus* HP-1 as compared to the control (Patel et al., 2009). The specific

mechanism by which surfactants enhance extracellular enzyme production in filamentous fungi has not been elucidated.

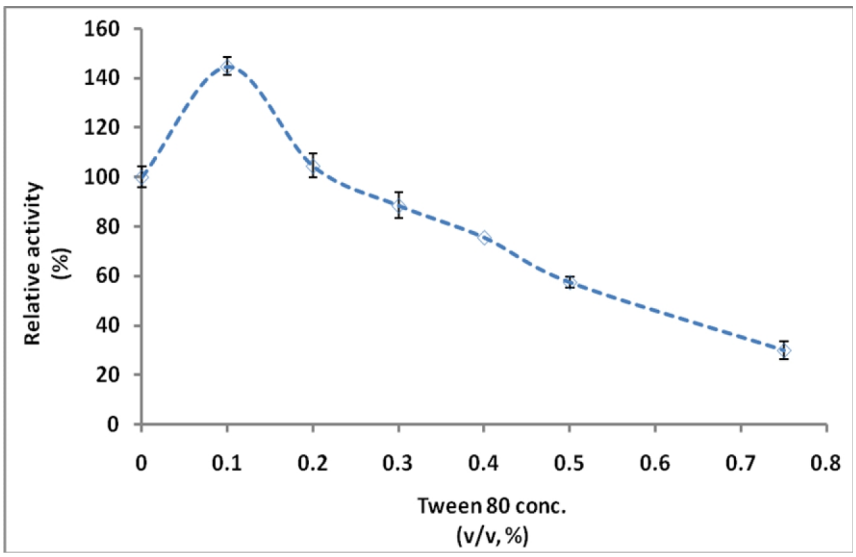

Fig. 7. Influence of Tween-80 concentration on the formation of *Pl. ostreatus* ARC280 laccase

3.12 Effect of Various Concentrations of Copper Sulfate

Copper as a micronutrient has a key role as a metal activator, induces both laccase transcription, and plays an important role in laccase production (Palmieri et al., 2000). In order to find out the suitable concentration of copper sulfate for induction maximum production of laccase from *Pl. ostreatus* ARC280, a series of concentrations of copper sulfate (50, 100, 250, 500, 750, 1000, 1250, and 1500µM) were used. Among the different concentrations tested, 100µM supported the maximum laccase production by *Pl. ostreatus* ARC280 with relative activity of 119.23% compared to the original medium containing 50µM $CuSO_4.5H_2O$. However, when the concentration of copper was increased beyond 100µM, significant decrease in fungal growth and laccase production was observed (Fig. 8). This may be attributed to an inhibitory effect of copper at higher concentrations. Copper sulfate at a concentration of 30µM supported the maximum laccase production by *Ganoderma sp*. (Sivakumar et al., 2010). On the other hand, Copper sulfate (667µM) addition increased up laccase production to 7-fold by *Colletotrichum truncatum* (Levin et al., 2007). Niladevi and Prema (2007) obtained maximum laccase activity when copper sulfate was used at a concentration of 1mM. Galhaup et al. (2002) and Stajić et al. (2006) reported that the addition of copper sulfate in various concentrations (1-10 mM) stimulates laccase production. It is worthy to mention that, the absence of copper sulfate in the medium resulted to a very low laccase formation. On the other hand, neither growth nor activity was noticed with all concentrations started from 1000µM (data not shown). Baldrian (2003) stated that higher copper concentrations may be toxic for fungi, affecting their growth and enzymatic activities.

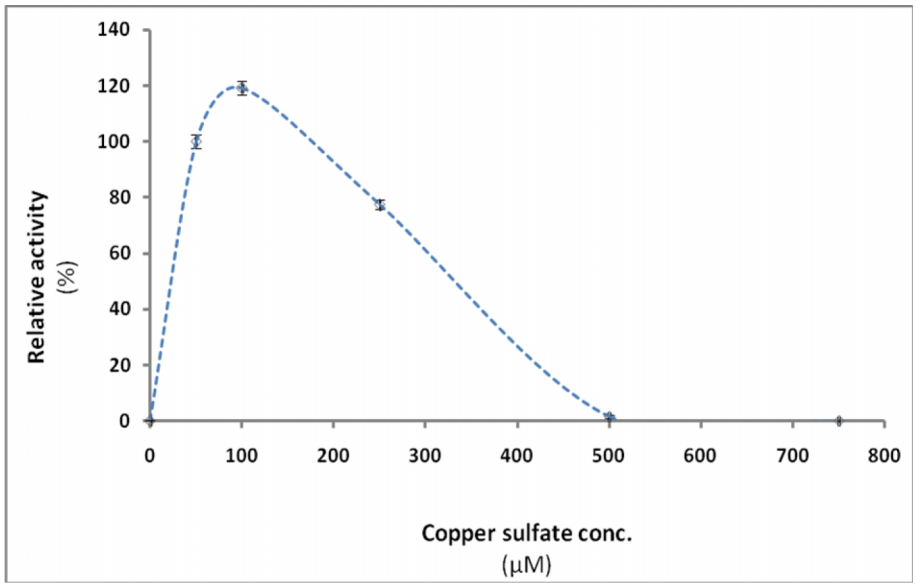

Fig. 8. Influence of $CuSO_4$ concentration on the formation of *Pl. ostreatus* ARC280 laccase

4. CONCLUSION

In view of the results obtained, it can be concluded that: Laccase production by *Pl. ostreatus* ARC280 has been shown to depend markedly on the composition of the culture medium, carbon, nitrogen content and inducer compounds and governed by parameters such as pH of the production medium and other nutritional parameters. In future, we are interested to study the properties of laccase and test the ability of this enzyme to degrade the various dyes as an important enzyme for various industrial applications and this strain seems to be a prospective organism for further biotechnological exploitation.

ACKNOWLEDGEMENTS

This work is supported by the National Research Centre (NRC), Cairo, Egypt. So authors wish to acknowledge it for the facilities that enabled them to accomplish this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

Adejoye, O.D., Fasidi, I.O. (2010). Effect of cultural conditions on biomass and laccase production in submerged medium by *Schizophyllum commune* (Fries), a Nigerian edible mushroom. EJEAF Che., 9(3), 600-609.

Annunziatini, C., Baiocco, P., Gerini, M.F., Lanzalunga, O., Sjögren, B. (2005). Aryl substituted N-hydroxyphthalimides as mediators in the laccase-catalyzed oxidation of lignin model compounds and delignification of wood pulp. J. Mol. Cata. B: Enzym, 32, 89-96.

Aríca, M.Y., Altıntas, B., Bayramoğlu, G. (2009). Immobilization of laccase onto spacer-arm attached non-porous poly (GMA/EGDMA) beads: Application for textile dye degradation. Bioresour. Technol., 100, 665–669.

Baldrian, P. (2003). Interaction of heavy metals with white rot fungi. Enzyme Microb. Technol., 32, 78–91.

Baldrian, P. (2004). Purification and characterization of laccase from the white rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Appl. Microbiol. Biotechnol., 63, 560–563.

Baldrian, P. (2006). Fungal laccases – occurrence and properties. FEMS Microbiol. Rev., 30, 215–242.

Bernardi, E., Minotto, E., Nascimento, J.S. (2008). Aproveitamento de resíduo de curtume como suplemento no cultivo de *Pleurotus ostreatus*. Arq. Inst. Biol., 72, 243-246.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C., Mougin, C. (2002). Crystal structure of a four-copper laccase complexed with an arylamine: Insight into substrate recognition and correlation with kinetics. Biochem., 41, 7325–7333.

Bollag, J.M., Leonowicz, A. (1984). Comparative studies of extracellular fungal laccases. Appl. Environ. Microbiol., 48, 849–854.

Bononi, V.L., Capelari, M., Mazieiro, R., Trufem, S.F.B. (1999). *Cultivo de Cogumelos Comestíveis*. Ícone. São Paulo, Brazil, 206.

Bora, P. (2003). Production of laccase by the phytopathogenic fungus *Rhizoctonia solani*. Doctor of Philosophy, Murdoch University, Perth, Western Australia.

Cavallazzi, J.R.P., Kasuya, C.M., Soares, M.A. (2005). Screening of inducers for laccase production by *Lentinula edodes* in liquid medium. Braz. J. Microbiol., 36, 383-387.

Chawachart, N., Khanongnuch, C., Watanabe, T., Lumyong, S. (2004). Rice bran as an efficient substrate for laccase production from thermotolerant basidiomycete *Coriolus versicolor* strain RC3. Fung. Divers., 15, 23-32.

Chefetz, B., Chen, Y., Hadar, Y. (1998). Purification and Characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl. Environ. Microbiol., 64(9), 3175–3179.

Claus, H. (2003). Laccases and their occurrence in prokaryotes. Arch. Microbiol., 179, 145–150.

D'Souza-Ticlo, D., Garg, S., Raghukumar, C. (2009). Effects and interactions of medium components on laccase from a marine-derived fungus using response surface methodology. Mar. Drugs, 7, 672-688.

Da Re, V., Papinutti, L., Villalba, L., Forchiassin, F., Levin, L. (2008). Preliminary studies on the biobleaching of loblolly pine Kraft pulp with *Trametes trogii* crude extracts. Enzyme Microb. Technol., 43, 164–168.

Das, N., Naskar, S., Chowdhury, P., Pasman, B., Adhikari, D. (2011). Experimental evidence for presence of a growth regulating extracellular laccase in some *Pleurotus* species. Research Journal of Microbiology, 6, 496-502.

Desai, S.S., Nityan, C. (2011). Microbial laccases and their applications: A review. Asian Journal of Biotechnology, 3, 98-124.

Dhaliwal, R.P.S., Garcha, H.S., Khanna, P.K. (1992). High laccase producing *Pleurotus florida* mutants. W. J. Microbiol. Biotechnol., 8, 39-41.

Difco Manual (1972). Difco Manual of dehydrated culture, media and reagents 9th ed., 245. Difco Laboratories, Detroit, Michigan, USA.

Elisashvili, V., Penninckx, M., Kachlishvili, E., Tsiklauri, N., Metreveli, E., Kharziani, T., Kvesitadze, G. (2008). *Lentinus edodes* and *Pleurotus* species lignocellulolytic enzymes activity in submerged and solid-state fermentation of lignocellulosic wastes of different composition. Bioresour. Technol., 99, 457-462.

Elshafei, A.M., Hassan, M.M., Haroun, B.M., Elsayed, M.A., Othman, A.M. (2012). Optimization of laccase production from *Penicillium martensii* NRC345. Advances in Life Sciences, 2(1), 31-37.

El-Shora, H.M., Youssef, M.M., Khalaf, S.A. (2008). Inducers and inhibitors of laccase from *Penicillium*. Biotechnology, 7, 35-42.

Filazzola, M.T., Sannino, F., Rao, M.A., Giangreda, L. (1999). Bioremediation and biodegradation: Effect of various pollutants and soil-like constituents on laccase from *Cerrena unicolor*. J. Environ. Qual., 28, 1929–1938.

Fu, Y., Viaraghavan, T. (2001). Removal of CI Acid Blue 29 from an aqueous solution by *Aspergillus niger*, Am. Assoc. Text. Chem. Color. Rev., 1, 36–40.

Galhaup, C., Goller, S., Peterbauer, C., Strauss, J., Haltrich, D. (2002). Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. Microbiol., 148, 2159–2169.

Gogna, E., Vohra, R., Sharma, P. (1992). Biodegradation of rose bengal by *Phanerochaete chrysosporium*. Lett. Appl. Microbiol., 14, 58–60.

Gomori, G. (1955). Preparations of buffers for the use in enzyme studies. Methods Enzymol., 1, 138-146.

Grönqvist, S., Buchert, J., Rantanen, K., Viikari, L., Suurnäkki, A. (2003). Activity of laccase on unbleached and bleached thermomechanical pulp. Enzyme Microb. Technol., 32, 439–445.

Halaburgi, V.M., Sharma, S., Sinha, M., Singh, T.P., Karegoudar, T.B. (2011). Purification and characterization of a thermostable laccase from the ascomycetes *Cladosporium cladosporioides* and its applications. Proc. Biochem., 46, 1146–1152.

Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W., Zupancic, S. (1996). Production of fungal xylanases. Biores. Technol., 58, 137-161.

Jaouani, A., Guillén, F., Penninckx, M.J., Martínez, A.T., Martínez, M.J. (2005). Role of *Pycnoporus coccineus* laccase in the degradation of aromatic compounds in olive oil mill wastewater. Enzyme Microb. Technol., 36, 478–486.

Jordaan, J., Leukes, W.D. (2003). Isolation of a thermostable laccase with DMAB and MBTH oxidative coupling activity from a mesophilic white rot fungus. Enzyme Microb. Technol., 33, 212–219.

Kaal, E.E.J., Field, J.A., Joyce, T.W. (1995). Increasing ligninolytic enzyme activity in several white-rot basidiomycetes by nitrogen-sufficient media. Bioresour.Technol., 53, 133–139.

Karamyshev, A.V., Shleev, S.V., Koroleva, O.V., Yaropolov, A.I., Sakharov, I.Y. (2003). Laccase catalyzed synthesis of conducting polyaniline. Enzyme Microb. Technol., 33, 556–564.

Kenney, J.F., Keeping, E.S. (1962). The standard deviation and calculation of the standard deviation in mathematics of statistics, Pt1,3rd ed. Princeton, NJ, Van Nostrand, 77-80.

Khammuang, S., Sarnthima, R. (2008). Laccase-aided antioxidant activity assay and antioxidant activity of selected Thai vegetables. Journal of Applied Sciences, 8, 2718-2724.

Khammuang, S., Sarnthima, R. (2009). Laccase activity from fresh fruiting bodies of *Ganoderma sp.* MK05: Purification and Remazol Brilliant Blue R decolorization. Journal of Biological Sciences, 9, 83-87.

Khlifi, R., Belbahri, L., Woodward, S., Ellouz, M., Dhouib, A., Sayadi, S., Mechichi, T. (2010). Decolourization and detoxification of textile industry wastewater by the laccase-mediator system. J. Haz. Mat., 175, 802–808.

Kiiskinen, L.L., Viikari, L., Kruus, K. (2002). Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. Appl. Microbiol. Biotechnol., 59, 198–204.

Kirby, N., Marchant, R., McMullen, G. (2000). Decolourization of synthetic textile dyes by *Phlebia tramellosa*. Fed. Eur. Microb. Soc. Microbiol. Lett., 188, 93–96.

Kramer, K.J., Kanost, M.R., Hopkins, T.L., Jiang, H., Zhu, Y.C., Xu, R., Kerwin, J.L., Turecek, F. (2001). Oxidative conjugation of catechols with proteins in insect skeletal systems. Tetrahedron, 57(2), 385–392.

Krishna, C. (1999). Production of bacterial cellulases by solid state bioprocessing of banana wastes. Bioresour. Technol., 69, 231- 239.

Kudanga, T., Nyanhongob, G.S., Guebitzb, G.M., Burton, S. (2011). Potential applications of laccase-mediated coupling and grafting reactions: A review. Enzyme Microb. Technol., 48, 195–208.

Leonowicz, A., Grzywnowicz, K. (1981). Quantitative estimation of laccase forms in some white–rot fungi using syringaldazine as a substrate. Enzyme Microbiol. Technol., 3, 55–58.

Levin, L., Ramos, A.M., Parisi, M., Gally, M. (2007). Screening of *Colletotrichum* (Ascomycota) isolates, causal agents of soybean anthracnose, for laccase production. Bol. Soc. Argent. Bot., 42(1-2), 71-77.

Lonsane, B.K., Ghild, N.P., Budeatman, S., Kamakrishna, S.V. (1985). Engineering aspects of solid-state fermentation. Enzyme Microb. Technol., 7, 258- 265.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265- 275.

Mansur, M., Suarez, T., Fernandez-Larrea, J.B., Brizuela, M.A., Gonzalez, A.E. (1997). Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197. Applied Environ. Microbiol., 63, 2637-2646.

Minussi, R.C., Miranda, M.A., Silva, J.A., Ferreira, C.V., Aoyama, H., Marangoni, S., Rotilio, D., Pastore, G.M., Durán, N. (2007). Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. Afr. J. Biotechnol., 6(10), 1248-1254.

Mohidem, N.A., Mat, H. (2009). The catalytic activity of laccase immobilized in sol-gel silica. Journal of Applied Sciences, 9, 3141-3145.

Munusamy, U., Sabaratnam, V., Muniandy, S., Abdullah, N., Pandey, A., Jones, E.B.G. (2008a). Biodegradation of polycyclic aromatic hydrocarbons by laccase of *Pycnoporus sanguineus* and toxicity evaluation of treated PAH. Biotechnology, 7, 669-677.

Munusamy, U., Sabaratnam, V., Muniandy, S., Abdullah, N., Pandey, A., Jones, E.B.G. (2008b). Characterisation of laccase from *Pycnoporus sanguineus* KUM 60953 and KUM 60954. Journal of Biological Sciences, 8, 866-873.

Nagai, M., Sato, T., Watanabe, H., Saito, K., Kawata, M., Enei, H. (2002). Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes* and decolorization of chemically different dyes. Appl. Microbiol. Biotechnol., 60, 327–335.

Niladevi, K.N., Prema, P., (2007). Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization. Bioresour Technol, 99, 4583-4589.

Osma, J.F., Saravia, V., Toca Herrera, J.L., Rodríguez Couto, S. (2007). Mandarin peelings: The best carbon source to produce laccase by static cultures of *Trametes pubescens*. Chemosphere, 67, 1677-1680.

Palmieri G., Giardina, P., Bianco, C., Fontanella, B., Sannia, G. (2000). Copper Induction of Laccase Isoenzymes in the Ligninolytic Fungus *Pleurotus ostreatus.* Appl. Environ. Microbiol., 66(3), 920–924.

Patel, H., Gupte, A., Gupte, S. (2009). Effect of different cultural conditions and inducers on production of laccase by a basidiomycete fungal isolate *Pleurotus ostreatus* HP-1 under solid state fermentation. Bioresour., 4(1), 268-284.

Pointing, S.B., Vrijmoed, L.L.P. (2000). Decolourization of azo and triphenylmethane dyes by *Pycnoporus sanguinues* producing laccase as the sole phenoloxidase. W. J. Microbiol. Biotechnol., 16, 317–318.

Prabu, P.C., Udayasoorian, C. (2005). Phenol metabolism by white rot fungus *Phanerochaete chrysosporium* isolated from Indian paper mill effluent enriched soil samples. Asian Journal of Plant Sciences, 4, 56-59.

Prabu, P.C., Udayasoorian, C., Balasubramanian, G. (2006). Isolation, molecular characterization and reactivity with 2,6 dichlorophenol of a laccase and isolation of laccase gene specific sequences from lignin degrading basidiomycete *Phanerochaete chrysosporium* (TL 1). Biotechnology, 5, 522-529.

Ravankar, M.S., Lele, S.S. (2006). Enhanced production of laccase using a new isolate of white-rot fungus WR-1. Proc. Biochem., 41, 581-588.

Sabu, A., Pandey, A., Daud, M.J., Szakacs, G. (2005). Tamarind seed powder and palm kernel cake: Two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. Bioresour. Technol., 96(11), 1223-1228.

Sharma, D.K., Tiwari, M., Behere, B.K. (1996). Solid state fermentation of new substrates for production of cellulase and other biopolymer hydrolyzing enzymes. Appl. Biochem. Biotechnol., 15, 495- 500.

Shulter, M.L., Kargi, F. (2000). Bioprocess engineering basic concept, Prentice Hall of India Pvt Ltd, New Delhi, India.

Sivakumar, R., Rajendran, R., Balakumar, C., Tamilvendan, M. (2010). Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma sp*. Int. J. Eng. Sci. Technol., 2(12), 7133-7141.

Stajić, M., Persky, L., Hadar, Y., Friesem, D., Duletic-lausevic, S., Wasser, S.P., Nevo, E. (2006). Effect of copper and manganese ions on activities of laccase and peroxidases in three *Pleurotus* species grown on agricultural wastes. Appl. Biochem. Biotechnol., 168, 87-96.

Subramanian, C.V. (1995). Mushrooms: Beauty, diversity, relevance. Curr. Sci., 69, 986-997.

Thurston, C.F. (1994). The structure and function of fungal laccases. Microbiol., 140(1), 19–26.

Timur, S., Pazarlýoglu, N., Pilloton, R., Telefoncu, A. (2004). Thick film sensors based on laccases from different sources immobilized in polyaniline matrix. Sens. Actu B: Chem., 97, 132–136.

Tlecuitl-Beristain S., Sánchez, C., Loera, O., Robson-Geoffrey, D., Díaz-Godínez, G. (2008). Laccases of *Pleurotus ostreatus* observed at different phases of its growth in submerged fermentation: Production of a novel laccase isoform. Mycological Research, 112, 1080-1084.

Verdin, A., Sahraoui, A.L.H., Durand, R. (2004). Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. International Biodeterioration & Biodegradation 53, 65 – 70.

Yoshida, H. (1883). Chemistry of Lacquer (Urishi) part 1. J. Chem. Soc. (Tokyo), 43, 472–486.

Zadrazil, F., Gonser, A., Lang, E. (1999). Influence of incubation temperature on the secretion of extracellular ligninolytic enzymes of *Pleurotus sp*. and *Dichomitus squalens* into soil. Proceedings of the Conference on Enzymes in the Environment. Activity, Ecology and Applicants, 12-16 July., Granada, Spain.

Zeng, G.M., Shi, J.G., Yuan, X.Z., Liu, J., Zhang, Z.B., Huang, G.H., Li, J.B., Xi, B.D., Liu, H.L. (2006). Effects of Tween 80 and rhamnolipid on the extracellular enzymes of *Penicillium simplicissimum* isolated from compost. Enzyme Microb. Technol., 39, 1451- 1456.

Zheng, Z.M., Obbard, J.P. (2001). Effect of nonionic surfactants on elimination of polycyclic aromatic hydrocarbons (PAHs) in soil slurry by *Phanerochaete chrysosporium*. J. Chem. Technol. Biotechnol., 76, 423–429.