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LACCASE PRODUCTION BY POTENT FUNGAL ISOLATE *TRICHODERMA* SP PUF 2 THROUGH SUBMERGED FERMENTATION AND ITS APPLICATION IN DYE DECOLORIZATION

Priyanka Ghosh[1], Arpan Das[2] and Uma Ghosh[1*]

[1]Department of Food Technology & Biochemical Engineering, Jadavpur University, Kolkata-700032, West Bengal, India

[2]Department of Microbiology, Maulana Azad College, Kolkata-700013, West Bengal, India

ABSTRACT ■ Laccases are copper-containing lignolytic enzymes catalyze the oxidation of various phenolic and inorganic compounds. Besides degrading lignin, laccase has many potential applications including textile dye bleaching, effluent detoxification, soil bioremediation, etc. However, for application upto the industrial scale, significant amounts of laccase must be produced in order to efficiently compete with the traditional oxidizing agents like peroxides. In the present study, a laccase producing fungus, PUF 2 was isolated from soil and based on morphological characters, it was identified as *Trichoderma sp*. The laccase production ability by the strain was optimized under submerged condition. Highest laccase activity of 4.82 U/ml was observed after 5 days of fermentation at 30 °C, P^H 5.0, when dried pineapple peel (concentration in culture media) was used as carbon source. During the study on decolourization ability, it was found that the strain (*Trichoderma* sp PUF 2) successfully decolorized some tested commercial dyes like methyl red, bromophenol blue, crystal violet and phenol red, with an indication of its potentiality towards different industrial applications.

Key words: Laccase, Submerged fermentation, Agro waste, Dye decolorization, *Trichoderma* sp

INTRODUCTION

Laccase is 1, 4-benzenediol: oxygen oxido-reductases (EC 1.10.3.2) group of enzyme found in higher plants and microorganisms (Saraiva et al., 2012). It contains 4 copper ions per molecule that carryout electron oxidation of phenolic and its related compound and reduce oxygen to water (González et al., 2006). Due to the ability to oxidizing various phenolic and non-phenolic substrates, laccase has been the focus of an increased amount of attention with regard to its potential biotechnological applications, in fields including delignification, pulp bleaching, contaminated water treatment, and the detoxification of phenolic compounds (Srebotnik and Hammel 2000, Widsten and Kandelbauer 2008, Medhavi and Lele 2009. Laccase is also useful in a wide variety of applications in enzyme immunoassays as a marker enzyme, in the design of various biosensors and in energy transformation systems (Ghindilis, 2000). However, such determinations would clearly require large quantities of laccase. Fungal sources, at this point, are considered to be the most

* **Corresponding author** : e-mail: ughoshftbe@yahoo.co.in

promising sources for the production of such large quantities of laccase. Discovery of novel laccase producer is very important for industrial applications, besides development of genetically modulated high yielding producer. The enzymatic catalysis of laccases in different industrial applications such as textile dye bleaching, pulp bleaching and bioremediation could serve as a more environmentally benign alternative than the currently used chemical processes. Hence, the present research work aimed to isolate a potent laccase producing fungi, optimization of submerged conditions for laccase production and study the decolorizing ability for commercial dyes.

MATERIALS AND METHODS:

Isolation of laccase producing microorganisms:

Different soil samples from different places of West Bengal, India, were collected. The laccase producing fungi were screened based on the growth on Czapek-dox agar media containing guaiacol (Kiiskinen et al., 2014). Plates were observed for growth and development of reddish hallow zone around the colony. The organisms showing faster growth was selected as potent strains. Selected strain was stained with Lactophenol cotton blue and observed under microscope.

Basel medium and cultural conditions for submerged fermentation (SmF):

Potent strain was cultivated in Olga liquid medium containing 3g peptone, 10g glucose, 0.6 gm KH_2PO_4,0.001g $ZnSO_4$, 0.4g K_2HPO_4, 0.0005g $FeSO_4$, 0.05g $MnSO_4$ and 0.5g $MgSO_4$ per L, and kept in an incubator shaker at 200 rpm, 30ºC for 9 days (Udayasoorian and Prabu, 2005). 5ml of broth was collected from the third day; fungal mycelium was separated from the broth by filtering ~~it~~ through Whatman No. 1 filter paper. The filtrate collected was used for enzyme assay.

Investigation of process parameters:

The process parameters investigated in the current study were selection of suitable agro-waste and its concentration for laccase production, optimization of fermentation period (0-9 days), incubation temperature (25°C - 40°C) and fermentation pH (range 4.0–8.0). All the experiments were repeated in triplicates.

Study of dye decolorizing activity by the laccase:

For this purpose, fungal strain was grown in PDA plates containing 0.01% different dyes like methyl red, crystal violet, phenol red etc. The plates were incubated at 30°C for 5 days. Dye decolourization was determined by the clearing zone around the colony (Gnanasalomi and Gnanadoss 2013).

Laccase assay:

Laccase activity was measured by the method of Desai et al. (2011). Briefly, the reaction mixture contained 3ml acetate buffer (10mM pH 5.0), 1ml guaiacol (2mM) and 1ml enzyme source and enzyme blank contained 1ml of distilled water instead of enzyme source. The mixture was incubated at 30ºC for 15min. Absorbance of the solution was taken at 450nm using UV spectrophotometer. The laccase activity in U/ml is calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm by following this equation:

$E.A = (A \times V) / (t \times e \times v)$,

where E.A = Enzyme Activity (U/ml), A = Absorbance at 450nm, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and e = Extinction Coefficient ($M^{-1}cm^{-1}$).

Assay of Fungal Biomass:

Fungal biomass was assayed according to the method of Ramachandran et al. (2005). Glucosamine (Sigma) was used as the

standard. The results are expressed as μg glucosamine produced per ml of fermented broth.

RESULTS:

Screening and identification of laccase producing fungi:

Through plate screening method, 36 numbers of fungal isolates with laccase activity were primarily selected from different soils and plants samples from different places of West Bengal like Howrah, Namkhana and frezerganj. The results are summarized in Table 1. Out of these, one fungal strain (PUF2), isolated from forest soil sample, showed fast growth and higher laccase activity was selected for further studies. Based on macroscopic and microscopic characteristics (fig. 1), the strain was identified as *Trichoderma* sp. This strain was used for laccase production throughout the experiment.

Effect of different agro-waste on laccase production:

To study the effect of agro-wastes on laccase production, different substrates like wheat bran, rice bran, tea waste, orange peel, cucumber peel, banana peel, potato peel, sugarcane baggase, ribbed gourd peel, saw

Table1. Distribution of laccase producing fungi in different environmental samples

Sample collection place	*No. of samples*	*Number of isolates*
Howrah	Undisturbed forest soil (10) Dry bark and leaves of forest trees (10)	4 3
Frezerganj	Undisturbed forest soil (11) Soil sample from sea belt (5)	4 3
Namkhana	Undisturbed forest soil (11) Soil sample from sea belt (5	2 2
Howrah	cattle farms (5)	5
Howrah	Shaw mill (2)	2
Howrah	Soil around paper factory (8)	5
Howrah	Compost (6)	6

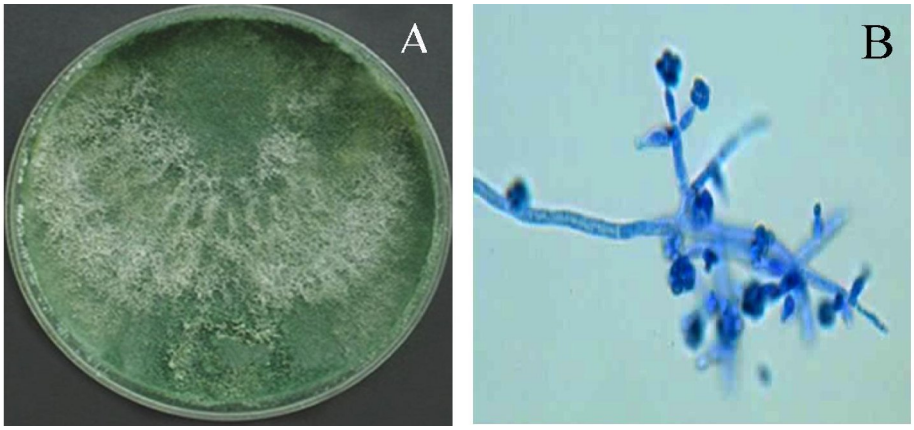

Fig 1. Macroscopic (A) and Microscopic (B) character of isolated fungal strain PUF 2

dust, corn cobs, pineapple peel etc. were used in the fermentation medium as sole carbon source. Amongst all the carbon sources tested, pineapple peel was found to be the most suitable for the production of laccase (2.27 U/ml), followed by cucumber peel and potato peel (Fig. 2). The concentration of pineapple peel was further varied and result indicated that 3% (w/v) substrate concentration supported maximum laccase yield of 2.83U/ml.

Optimization of incubation period for laccase production:

The effect of incubation period on laccase production by PUF 2 is shown in Fig 3. The laccase activity steadily increased with increasing incubation period and attained maximum (3.22 U/ml) on the 5th day of incubation. On longer incubation, laccase activities gradually decreased, while fungal biomass continued to increase from the 2nd to the 7th day of incubation and then became nearly constant.

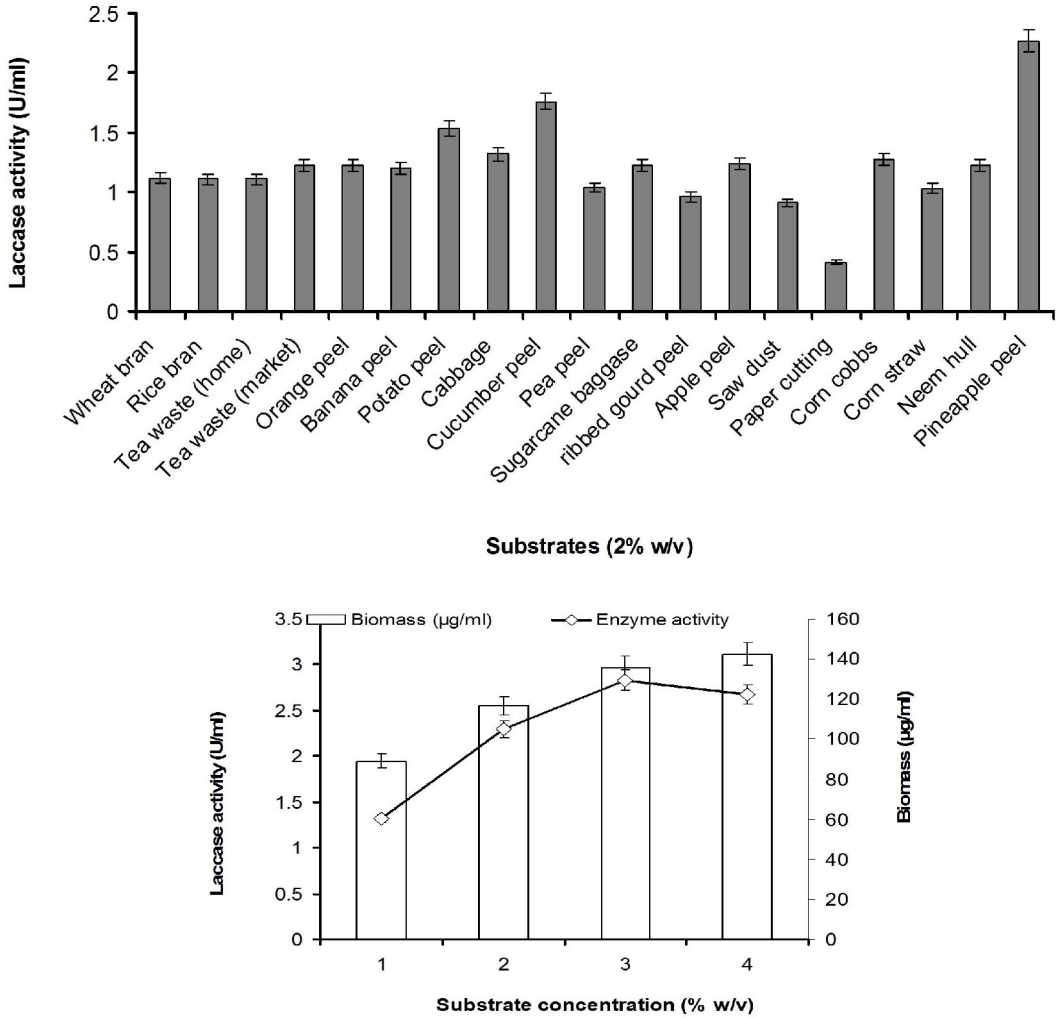

Fig 2. Optimization of suitable agrowaste as sole carbon sources for laccase production by fungal strain PUF2 under SmF.

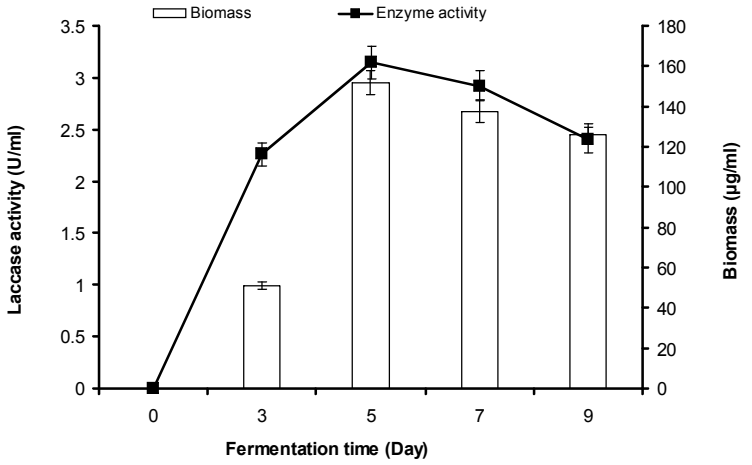

Fig 3. Optimization of fermentation time for laccase production by fungal strain PUF2 under SmF

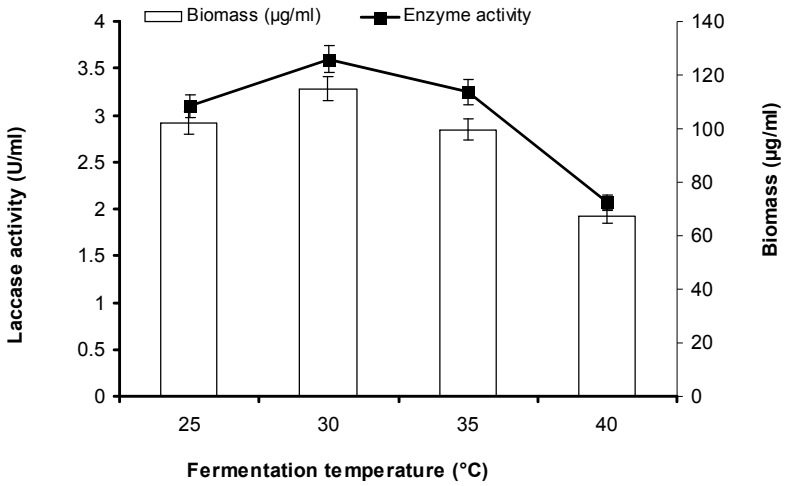

Fig 4. Optimization of fermentation temperature for laccase production by fungal strain PUF2 under SmF

Optimization of incubation temperature for laccase production:

The effect of fermentation temperature on mycelial growth and laccase yield was studied and the result is shown in fig 4. The result indicated that significant amount of laccase was produced in the temperature range of 25 to 35°C with temperature maxima at 30°C, after which enzyme yield was gradually decreased. The production of fungal biomass was also varied in accordance with the laccase yield.

Optimization of medium pH for laccase production:

The effect of fermentation pH on laccase production by the fungal strain PUF 2 is shown in Fig 5. With the change in the pH of the fermentation medium, a significant variation in laccase yield was observed. The results showed maximal laccase activity (4.82 U/ml) and fungal growth at pH 5.0, below and above which, both enzyme activity and biomass yield was decreased.

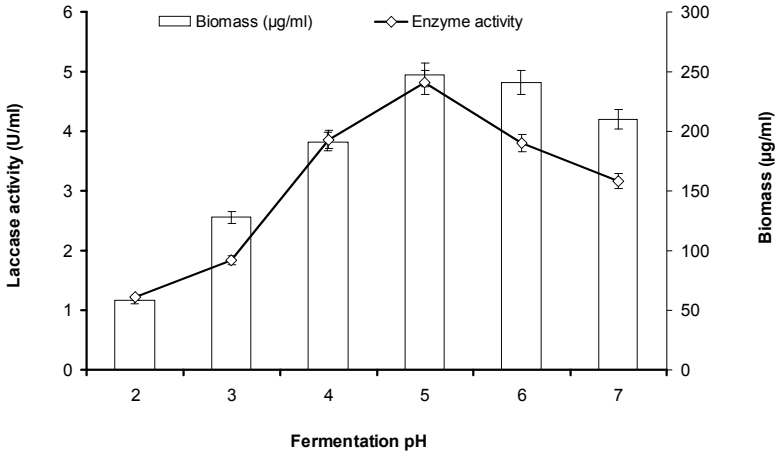

Fig 5. Optimization of fermentation pH for laccase production by fungal strain PUF2 under SmF

Study on dye decolourization activity:

In the current experiment, the ability of the laccase from the isolated fungal strain PUF2 to decolorize different commercial dyes was assessed. The result shown in Table 2, indicates that crude laccase from strain PUF 2 was capable to decolorize several dyes like bromophenol blue, methyl red, crystal violet and phenol red effectively. No zone of dye degradation was observed in case of brilliant green.

Table 2. Decolorization of different commercial dyes by fungal strain PUF 2

Name of dye	result
Brilliant green	-
Methyl red	+
Bromophenol blue	++
Crystal violet	+
Phenol red	+

DISCUSSION:

Laccases are one of the oldest enzymes ever described which have been detected in several organisms like insects and bacteria, but they are especially abundant in fungi. Fungi are the only microorganisms able to degrade the wood components most efficiently (Gianfreda et al., 1999). In the current experiment different laccase producing fungal strains were isolated from different environmental samples (Table 1). The potent fungal strain PUF 2 was isolated from forest soil. Rich abundant of laccase specific substrates in the forest soil (due to decomposition of plant phenolic compounds) can favours the dominancy of laccase producing fungi. The selected PFU 2 strain was identified based on its colony morphology as well as microscopic visualizations (spore and hyphae). The organism produced white to green cushions of sporulating filaments (Fig 1). The microscopic observations showed the arrangements of conidia in balls at the tips of filaments. These colony characteristics and microscopic visualizations were in close similarity with the genus *Trichoderma*; hence the isolated strain was identified as *Trichoderma* sp PUF2 (Ahmed and Siddiqui, 2015). Several group of fungi like *Trametes Hirsuta*, *Pleurotus sajor-caju*, *Lentinus edodes*, *Schizophyllum* sp were isolated from soil that are capable of producing laccase (Kalra and Shavez, 2014; Patrick et al., 2011; Vetchinkina et al., 2008; Mahajan et al.,2015). It is well

known that fungal enzyme production is greatly influenced by different physico-chemical fermentation parameters (Yaghoubi et al., 2008). Hence the effect of different influencing factors on laccase production by strain *Trichoderma* sp PUF2 were analyzed. Fungal strain PUF 2 was grown on various complex agro-wastes (replacing glucose in Olga medium) to determine their effect on the induction of laccase enzymes (Fig 2). Amongst all the carbon sources tested, pineapple peel was found to be the best carbon source for laccase production followed by cucumber peel. Agro-wastes are generally considered as the cheap substrates rich in carbohydrates, proteins and minerals which can support fungal growth (Lorenzo et al., 2002). Utilization of agro-waste as substrate in the fermentation media is also favorable from economic view point (Moldes et al., 2004). During the study it was found that after 5 days of fermentation, maximum laccase and fungal biomass production was achieved (Fig 3). It suggests that the enzyme production is dependent on biomass and late exponential phase of fungus growth. As laccases are part of the primary metabolite, produced during the exponential phase of growth, and at the onset of the death phase, the enzyme secretion starts decreasing (Xiao et al., 2003). Fermentation temperature and pH are considered as important factors for fungal growth and enzyme production. Temperature significantly influence the growth, development and in general, metabolic activities of an organism. Hence, it was essential to optimize temperature for maximum laccase production. Fig.4 showed that PUF 2 produced maximum laccase activity at 30 °C, hence proving its mesophilic nature. The decrease in laccase production below this temperature might be due to lower transport of substrate across the cells at lower temperature, causing lower yield of the product (Viswanath et al., 2014). At higher temperature, the maintenance energy requirement of cellular growth was high due to thermal denaturation of the enzymes of the metabolic pathway, resulting in lower production of the metabolites. The effect of fermentation pH in Fig 5 revealed that maximal laccase activity was found at pH 5.0. A further increase in pH reduced the laccase activity. The reason for decreasing production at higher pH was probably due to proteolytic inactivation of the enzyme (Ghosh et al., 2017). Hence, it suggested that slightly acidic pH values favoured laccase production, with further increase of pH, the laccase activity decreased gradually. The H^+ concentration in the fermentation medium thereby had a profound effect on the enzyme production.

The majority of synthetic dyes are not biodegradable and highly resistant to light and temperature, hence are dangerous for the environment. So before draining it into the environment, the dye effluent should be treated appropriately. The treatment of the dyes with physico-chemical process including coagulation/ flocculation, membrane filtration and activated carbon adsorption is usually expensive, complicated and produced a large quantities of sludge (Murugesan et al., 2007). So, biological decolourization method by microbial enzymes can be considered as an alternative method to dye degradation and colour removal (Adosinda et al., 2003; Manjinder et al., 2005).

Laccase is such type of enzyme that has the ability to decolorize different commercial dyes. In the current experiment, the isolated fungus PUF2 decolorized several dyes like methyl red, crystal violet, phenol red, to which are shown in table 3. The decolorization of dyes is a useful indicator of the potential capability to degrade pollutants,

such as aromatic polycyclic hydrocarbons and polychlorobiphenyls.

From different environmental samples one fungal strain *Trichoderma* sp PUF2 was isolated as potent laccase producer. The effect of physico-chemical parameters on the production of laccase by the isolated fungal strain PUF 2 was studied using "one factor at a time" design. Different parameters like agro-waste (pineapple peel) as carbon source and its concentration (4% w/v), fermentation time (5days), temperature (30ºC) and initial pH (pH-5) of the medium were found to influence the laccase production significantly. By using this optimal fermentation medium, the laccase yield was increased up to 4.82 U/ml. The strain was also capable of decolorizing several dyes, which is a useful indicator of the potential capability to degrade different types of aromatic polycyclic pollutants. For industrial application, high yield of laccase is required. So further optimization is needed to be performed through solid state fermentation and decolorization efficiency will be analyzed using some commercial textile dyes.

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REFERENCES

Adosinda, M., Martins, M., Nelson, L., Armando, J.D. (2003): Comparative studies of
fungal degradation of single or mixed bioaccessible reactive azo dyes, Chemosphere.52: 967–973.

Ahmed, S and Siddiqui, H.A. (2015): Screening and assessment of laccase producing
Trichoderma species isolated from different environmental samples, J. Anim. Plant Sci. 25: 606-610.

Desai, S.S., Tennali, G.B., Nityanand, C.N., Anup, A.C., Deshpande, G and Murtuza,
B.P.A. (2011): Isolation of laccase producing fungi and partial characterization of laccase, Biotechnol. Bioinf. Bioeng. 1: 543-549.

Ghindilis, A. (2000): Direct electron transfer catalysed by enzymes: application for
biosensor development, Biochem. Soc. Trans. 28: 84-89.

Ghosh, P and Ghosh, U. (2017): Statistical optimization of laccase production by
Aspergillus flavus PUF5 through submerged fermentation using agro-waste as cheap substrate, Acta Biologica Szeged. 1: 25-33.

Gianfreda, L., Xu, F and Bollag, J.M. (1999): Laccases: a useful group of
oxidoreductive enzymes, Bioremed. J. 3: 1–25.

Gnanasalomi, V.D.V and Gnanadoss, J.J. (2013): Molecular characterization and
phylogenetic analysis of laccase producing fungal isolates with dye decolourizing potential Research in Biotechnology, Res. in Biotechnol. 4: 01-08.

González Arzola, K., Polvillo, O., Arias, M.E., Perestelo, F., Carnicero,A., González-
Vila, F.J and Falcon M.A. (2006): Early attack and subsequent changes produced in an industrial lignin by a fungal laccase and a laccase-mediator system: an analytical approach, Appl. Microbiol. Biotechnol. 73: 141–150.

Kalra, K and Shavez, M. (2014): Production of Laccase from Banana Skin by
Trametes Hirsuta In Solid State and Submerged Fermentation, Asian J.
Biochem. Pharm. Res. 4: 221-231.

Kiiskinen, L.L., Ratto, M and Kruus, K. (2004): Screening for novel laccase producing
microbes. J. Appl. Microbiol. 97: 640-646.

Lorenzo, M., Moldes, D., Rodrý´guez Couto, S and Sanroma´n, A. (2002):
Improvement in laccase production by employing different lignocellulosic wastes in submerged cultures of Trametes versicolor, Bioresour. Technol. 82: 109–113.

Mahajan, R., Sharma, N.R and Joshi, M. (2015): Optimization of Lignocellulose
Degrading Enzyme Laccase from Basidiomycetes Using One Variable at a Time Approach, Res. J.

Pharm. Biol. Chem. Sci. 6: 275-281.
Manjinder, S.K., Harvinder, S.S., Deepak, K.S., Bhupinder, S.C and Swapandeep, S.C.
(2005): Decolorization of various azo dyes by bacterial consortium, Dyes and Pigments. 67: 55–61.
Medhavi,V and Lele, S. (2009): Laccase:properties and applications, Bioresour. 4:
1694–1717.
Moldes, D., Lorenzo, M and Sanroma´n, M.A. (2004): Different proportions of laccase
isoenzymes produced by submerged cultures of Trametes versicolor grown on lignocellulosic wastes, Biotechnol. Lett. 26: 327–330.
Murugesan, K., Dhamija, A., Nam, I.H., Kim, Y.M and Chang, Y.S. (2007):
Decolourization of reactive black 5 by laccase: Optimization by response surface methodology, Dyes and Pigments. 75: 176–184.
Patrick, F., Mtui, G., Mshandete, A. M and Kivaisi, A. (2011): Optimization of laccase
and manganese peroxidase production in submerged culture of Pleurotus
sajor-caju, African J. Biotechnol. 10: 10166-10177.
Ramachandran, S., Roopesh, K., Nampoothiri, K.M., Szakacs, G and Pandey, A.
(2005): Mixed substrate fermentation for the production of phytase by Rhizopus spp. using oilcakes as substrates, Process Biochem. 40: 1749-1754.
Saraiva, J., Tavares, A.M and Xavier, A.R. (2012): Effect of the inducers veratryl
alcohol, xylidine, and lignin sulphonates on activity and thermal stability and inactivation kinetics of laccase from Trametes versicolor, Appl. Biochem. Biotechnol. 167: 685–693.
Srebotnik, E and Hammel, K.E. (2000): Degradation of nonphenolic lignin by the
laccase/1-hydroxybenzotriazole system, J. Biotechnol. 81: 179–188.
Udayasoorian, C and Prabu, P.C. (2005): Biodegradation of phenols by ligninolytic
fungus Trametes versicolor, J. Biol. Sci. 5: 824-827.
Vetchinkina, E.P., Pozdnyakova, N.N and Nikitina, V.E. (2008): Laccase and Lectin
Activities of Intracellular Proteins Produced in a Submerged Culture of the
Xylotrophic Basidiomycete Lentinus edodes, Curr. Microbiol. 57:381–385.
Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P and Narasimha, G. (2014):
Fungal laccases and their applications in bioremediation, Enzym. Res. ID 163242.
Widsten, P and Kandelbauer, A. (2008): Laccase applications in the forest products
industry: a review, Enzyme Microb. Technol. 42: 293–307.
Xiao, Y.Z., Tu, X.M., Wang, J., Zhang, M., Cheng, Q., Zeng, W.Y and Shi, Y.Y.
(2003): Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete Trametes sp. strain AH28-2, Appl. Microbiol. Biotechnol. 60: 700–707.
Yaghoubi, K., Pazouki, M and Shojaosadati, S.A. (2008): Variable optimization for
biopulping of agricultural residues by Ceriporiopsis subvermispora, Bioresour. Technol. 99: 4321-4328.