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**Jagdeep Singh**Department of Microbiology,  
Maharshi Dayanand University  
Rohtak, Haryana, India**Rajeev Kumar Kapoor**Department of Microbiology,  
Maharshi Dayanand University  
Rohtak, Haryana, India

## Immobilization and reusability efficiency of Laccase onto different matrices using different approaches

**Jagdeep Singh and Rajeev Kumar Kapoor**

**Abstract**

Laccase is recognized as lignocellulose oxidase enzyme which is involved in bioremediation, chemical synthesis, Bio-bleaching of paper pulp, bio-sensing, textile finishing and wine stabilization. The immobilization of enzymes offers several advantages for enzyme-based catalysis because the storage and operational stabilities of enzymes are frequently enhanced. Including this, the reusability of immobilized enzymes exhibits a key advantage with respect to free enzymes. In this study, various immobilization approaches were employed with the different polymers, inorganic materials and glass beads. It was observed that enzyme immobilized to glass beads exhibited the highest residual activity of 92% which was followed by the polypropylene (86%). Including this, the efficiency of reusability of immobilized laccase was also evaluated up to 10 successive cycles and it was observed that the efficiency of immobilized enzyme was reduced from 1<sup>st</sup> cycle to 10<sup>th</sup> cycle.

**Keywords:** laccase, immobilization, reusability, polymers, glass beads

**Introduction**

Laccase (EC Number 1.10.3.2) is the type of polyphenol oxidase enzyme. Laccases are also characterized as a family of copper-containing oxidases and are also recognized as multi-copper oxidases (Agrawal *et al.*, 2018; Mehra *et al.*, 2018; Claus 2004; Sitarz *et al.*, 2015) [1, 17, 27, 7, 18]. Researchers showed that Laccase enzyme based catalysis cause reduction of the oxygen molecule to water. In this reaction oxidation of the organic and inorganic substrate (methoxy-substituted mono- & diphenols, amino phenols and aromatic amines etc.) takes place by transfer of one electron (Gianfreda *et al.*, 1999; Bourbonnais & Paice 1990; Bourbonnais *et al.*, 1995) [10, 4, 5]. There are different sources of Laccase enzyme such as; bacteria, plants and fungi. Most of the sources reported for these enzymes are the fungal origin (Kiiskinen *et al.*, 2004) [15] and these enzymes are produced extracellularly which makes the easier and faster procedure of enzyme purification. Among all the investigated fungal classes, white rot fungi are reported the more common source of Laccase, such as; *Trametes versicolor*, *Phlebia radiata* and *Pleurotus ostreatus* (Bourbonnais *et al.*, 1995; Niku-Paavola *et al.*, 1988; Palmieri *et al.*, 2000) [18, 19, 20]. Investigators have reported the isolation of Laccase from *Trichoderma* species like *T. atroviride* and *T. harzianum*, *T. longibrachiatum* (Holker *et al.*, 2002; Velazquez-Cedeno *et al.*, 2004; Gochev & Krastanov 2007) [12, 30, 11]. Including this, Laccase producing fungi of the class basidiomycetes and ascomycetes have been isolated from the marine source such as; *Cerrena unicolor*, *Corioliopsis byrsina*, *Diaporthepha seolorum* and *Pestalotiopsis uvicola* (Verma *et al.*, 2009) [1]. Moreover, some strains of bacteria and plants also have been found to produce Laccase (Shraddha *et al.*, 2011) [25]. A considerable stability level of this extracellular enzyme in the extracellular space make them suitable for their applications in numerous bioprocesses such as dye decolorization, biodegradation of xenobiotic pollutants, bleaching in paper and pulp industries, de-lignification of lignocellulosics and newspaper deinking etc. (Sitarz *et al.*, 2015) [27]. One of most significant application of Laccase enzyme is the degradation of emerging contaminants called endocrine disrupting compounds (EDCs) from the polluted waste & drinking water ( Zhou *et al.*, 2019; Yu *et al.*, 2019; Singh *et al.*, 2017) [25, 24, 27, 28].

**Immobilization of Laccase**

Researchers have used Laccases in different forms for the various biotechnological applications especially in the degradation of a wide spectrum of contaminants particularly EDCs. The efficiency of the enzyme has been tested: (a) in a free form, (b) with co-substrates to promote the reaction and raise the redox potential as a "Laccase-mediator system" (c) and in

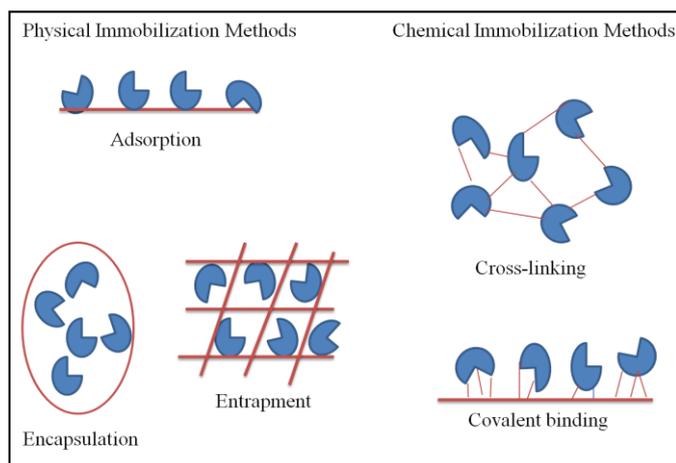
**Correspondence****Jagdeep Singh**Department of Microbiology,  
Maharshi Dayanand University  
Rohtak, Haryana, India

an immobilized form. The third form of laccase is being used as the enzymes exhibit a number of features that make them advantageous as compared to conventional chemical catalysts. But effective applications of enzymes may be hampered by undesirable properties of the enzymes such as their non-reusability, high sensitivity to several denaturing agents and presence of adverse sensory or toxicological effects. Many of these undesirable limitations may be overcome by the use of immobilized enzymes (Brijwani *et al.*, 2010) [5]. Immobilization is achieved by fixing enzymes to or within solid supports. Immobilization procedures enable the reusability of biocatalyst and influence several parameters such as overall catalytic activity, the effectiveness of catalyst utilization, deactivation and regeneration of kinetics, cost. Also, the toxicity of immobilization reagents should be considered in connection with the immobilization process, waste disposal and final application of the immobilized enzyme catalyst (Pannu and Kapoor 2014) [21]. Enzymes may be immobilized using a variety of methods

such as; adsorption, entrapment, crosslinking and covalent bonding (Figure 1). Immobilization by physical entrapment has the benefit of wide applicability and may provide relatively small perturbation of the enzyme native structure and function. The immobilization method selection is influenced by the different chemical properties and structure of enzymes, the distinct characteristics of substrates and reaction products. Thus, it becomes very difficult to state the optimum immobilization method and support for any enzyme. Widely used system for enzyme entrapment is the immobilization within a polyacrylamide gel which is obtained by polymerization/cross-linking of acrylamide with the enzyme. Some examples of water-insoluble supports for enzyme immobilization are cyanogens bromide-activated sepharose and Sephadex. It has been suggested that the chemically activated method for sepharose CL-6B with an epoxide is efficient to obtain an aldehyde activating group in the support able to react with the enzyme (Asgher *et al.*, 2014; Datta *et al.*, 2013) [20, 2].

**Table 1:** The supports and matrices employed for immobilization of enzymes

Synthetic polymers	Natural Polymers	Inorganic Materials
Amberlite	Alginate	Zeolites
Glutaraldehyde	Chitosan and Chitin	Ceramics
Polyethylene glycol	Collagen	Celite
Polyvinyl chloride	Gelatin	Silica Glass
Cyclodextrin	Cellulose	Activated carbon
Polyurethane microparticles	Starch	Charcoal
Polyaniline	Sepharose	



**Fig 1:** Drawing of physical and chemical enzymatic immobilization methods (Wang and Uchiyam 2013).

## Material and Methods

### Chemicals and Reagents

Guaiacol, Nitric acids were purchased from Sigma Chemicals (St Louis, USA). Acrylamide and Bis-acrylamide, TEMED, glutaraldehyde Acrylamide, bis-acrylamide, N, N, N, N-Tetramethyl-Ethylenediamine TEMED, ammonium persulphate and other chemicals and reagents were purchased from HiMedia, Sisco Research Laboratories (SRL), E-Merck, etc. All the chemicals used in the present investigation were of analytical reagent (AR) grade. Commercially purchased *Trametes versicolor* Laccase from Sigma chemicals was used in the study.

### Immobilization of Laccase

Immobilization of laccase was carried out by adsorption, cross-linking, covalent and entrapment methods. These

procedures were employed with the natural polymer (Cotton) and synthetic polymers (Polypropylene, Polystyrene, Polyester, and Polyacrylamide), an inorganic material (Glass bead).

### Laccase Assay

Laccase activity was measured following the method of Vasdev and Kuhad (1994) [29]. 200  $\mu\text{L}$  of culture filtrate was added to 800  $\mu\text{L}$  of 5.0 mM guaiacol in 100.0 mM citrate-phosphate buffer (pH 5.4). The reaction mixture was monitored for change in absorbance for the first few minutes or till the increase in optical density (OD) was linear against time. A change in absorbance of  $0.01 \text{ min}^{-1} \text{ mL}^{-1}$  at 470 nm was defined as 1 unit of laccase activity (U).

### Immobilization of laccase by the glutaraldehyde-linker method on glass beads

Immobilization on the glass beads was carried out according to the already testified method (Leonowicz *et al.*, 1988) [5] and the following steps were employed:

1. Aminopropylation of the 5mm glass beads was done by first heating for 1 h in 10% nitric acid at 80 to 90°C and then extensively washed with water.
2. Acid-washed glass (1g) was then heated for 3h at 70°C with 20 ml of 10% aqueous APES solution (pH 3.4).
3. The aminopropyl-glass beads were washed with water on a sintered glass filter, air dried and then dried overnight in an oven at 95 to 100°C.
4. The aminopropyl-glass beads were again washed with water before the next treatment of 5ml of 5% glutaraldehyde prepared in 0.1M CP buffer (pH 7.0).
5. The suspension was degassed under vacuum for 1hr and then beads were washed five times with distilled water and twice with CP buffer (pH 7.0).

- Glass beads were suspended in 2.0 ml of 0.1M CP buffer and mixed with 1.5 ml of 100 U laccase solutions. The suspension was stirred for 24 h at 4°C.
- Beads were washed five times with CP buffer and twice with distilled water.
- Enzyme assay was done by using guaiacol substrate. During both the enzyme binding and the glutaraldehyde steps, the reaction was first allowed to proceed under reduced pressure to remove air from the pores of the carrier. Each experimental variation was carried out in triplicate. The amount of laccase bound to the carrier was determined both indirectly, from enzyme content of the reaction supernatant and washings, and by direct measurement. Direct measurement of the enzyme was determined from the activity of the immobilized enzyme, on the basis of the specific activity of the soluble form.

**Entrapment Immobilization with Polyacrylamide Gel**

Entrapment of enzyme by polyacrylamide gel was done according to the already testified method (Mina *et al.*, 2012) with some modifications. Following steps were employed:

- 9% Acrylamide and 1% Bis-acrylamide were dissolved in the 0.02M phosphate buffer (pH7.0)
- Then, 1mL of the partial purified enzyme was mixed under vacuumed condition.
- To this, 0.5% ammonium pre sulfate (freshly prepared) and of 50% TEMED were added for polymerization, the contents were stirred gently and for the setting of the gel, this solution was poured into Petri plates for 10 minutes at 20°C. The gel was then cut into square blocks and stored in 100mM potassium phosphate buffer pH 6.5.
- Enzyme activity was measured under standard assay conditions.

**Immobilization by simple adsorption followed by cross-linking on polypropylene, polystyrene beads, polyester and cotton threads**

This type of immobilization of Laccase was carried out according to the previously reported method (Huber D.*et.al.*, 2016; Minovska *et al.*, 2005) with some modifications. Following steps were employed:

**Immobilization by simple adsorption:**

- This procedure was employed with 1 gram of each natural and synthetic polymer. Polypropylene fiber piece, polystyrene beads, polyester and cotton thread were washed three times in ethanol under vacuum and three times with distilled water.
- Washed polymers were then dispersed in the immobilization buffer (100mM citrate phosphate buffer pH 7) and laccase was added to a final protein concentration of 1% w\_w\_1 based on the total amount of matrix taken stirred for 30 min.
- The relative laccase activity was determined.

**Adsorption followed by cross-linking**

- The immobilizates were then treated with glutaraldehyde; 50 mL of a 2.5% (w/v) aqueous solution were added and the reaction was allowed to proceed for an additional 10 min. The immobilizates were separated by vacuum filtration.
- The relative laccase activity was determined.

**Reusability of immobilized Laccase**

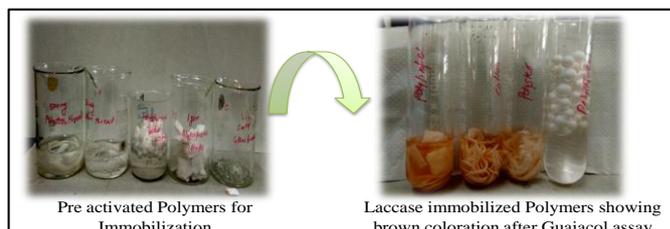
Immobilized laccase was repeatedly used for enzyme assay for several batches of reaction (10 cycles) to check the degree of reusability.

**Results and Discussion**

**Immobilization of laccase enzyme**

Immobilization of enzymes is carried out to enhance the economics of biocatalytic processes. Immobilization allows reusing the enzyme for an extended period of time and enables its use in many biotechnological applications like in purification of water, paper & pulp industry, detoxification of industrial effluents and food industry. Although the best methods of immobilization might differ from enzymes to the enzyme. From application to application and from carrier to carrier, depending on the peculiarities of each specific application, criteria for assessing the robustness of the immobilized enzyme participate remaining the same-industrial immobilization enzyme must be active ( high active in a unit of volume, U g<sup>-1</sup> or ml<sup>-1</sup>), highly selective (to reduce side reaction), high stable ( to reduce cost by effective reuse), cost-effective (low cost contribution thus economically attractive), safe to use (to meet safety regulations) and innovative (for recognition as intellectual property).

In the present study, the laccase was immobilized on various supports, namely, glass beads, polyacrylamide gel, polypropylene, polystyrene, polyester, polyacrylamide and cotton. Table 1 clearly shows that immobilization of enzyme reduced its activity in comparison to free enzyme. However, within the various immobilization systems, the highest activity of the immobilized enzyme was observed on glass beads (92%), polypropylene filter pieces (86%) followed by cotton threads (84%) and (Figure 2).



**Fig 2:** Immobilization of laccase on different polymers

Polypropylene, polystyrene beads, polyester and cotton threads were used again in a modified adsorption method in order to improve the immobilization efficiency. After coupling them with glutaraldehyde, the immobilization efficiency was enhanced from 61 to 86% for polypropylene, 50 to 84% for cotton thread, and 52 to 75% for the polyester thread and for 25 to 40% for the polystyrene beads (Table 2).

**Table 2:** Activity immobilization yield of laccase on various supports

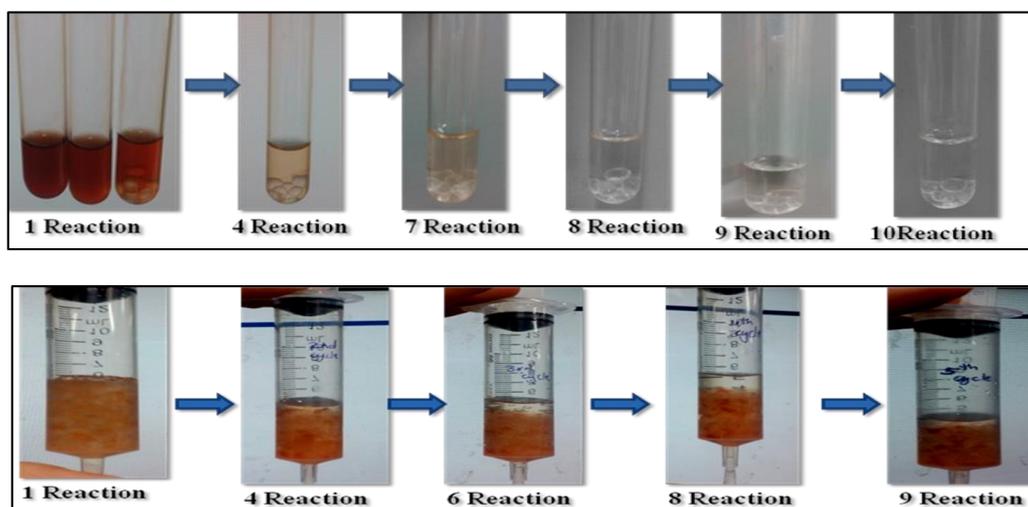
Immobilization supports	Relative enzyme activity (%)
Control	100
Glass beads	92
Polyacrylamide Gel	75
Polypropylene small pieces	86
Polystyrene beads	40
Polyester thread	75
Cotton thread	84

Minovska *et al.* (2005) [18] reported that polypropylene demonstrated high enzymatic activity. The polypropylene immobilizates exhibited a lipolytic activity of 333 U/g support at a protein loading of 8.1 mg/g (40.3 % protein adsorbed) and a moderate efficiency of 61%. The better activity of this immobilizate might be due to the stronger adsorption of the hydrophobic enzyme onto the polypropylene surface. The enhancement in the immobilization efficiency has been reported from 61 to 98 % for polypropylene after coupling adsorption with glutaraldehyde. The observed variation in the immobilized laccase activity may be due to the concentration and pore size of the matrix used. Georgieva *et al.* (2010) [18] Due to a higher degree of immobilization laccase was able to oxidize a wide range of phenolic substrates including BPA at a higher rate. Huber *et al.* (2016) [2] carried out the laccase immobilization on Polypropylene (PP) beads and reported that around 50% of the enzyme was bound to the PP beads after 8 h of reaction, while after 24h the percentage increased to 57%. In another study (Chen *et al.*, 2015) [5] laccase was immobilized on bacterial nano-cellulose (BC) by adsorption and 83% activity was obtained with a BC size of  $7 \times 7$  to  $10 \times$

$10 \text{ mm}^2$ . Leonowicz *et al.* (1988) [16] found a 90% of the original activity after immobilization on glass beads with increased stability on a wider pH and temperature range and also reported that reuse of enzyme was greatly improved as compared to those of the free laccase. The laccase from *Pleurotus florida* immobilized on various polymers and glass beads and the laccase entrapped in the polyacrylamide gel showed 78% immobilization yield as compare to the immobilization on glass beads (72%).

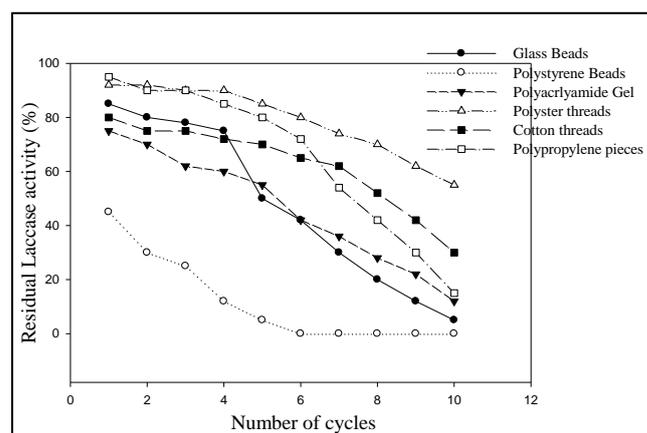
**The efficiency of reusability of immobilized laccase**

In this study, the efficiency of reusability of immobilized laccase on glass beads and different polymers was evaluated for up to 10 successive cycles figure 3. It is shown in figure 4 that the efficiency of immobilization on polypropylene pieces was reduced from the first cycle (95%) to (15%) 10<sup>th</sup> cycle. Similarly for glass beads, polyacrylamide gel, polyester and cotton threads the efficiency reduced from 85 to 5%, 75 to 12%, 92 to 55% and 80 to 30% respectively but for polystyrene beads the efficiency was in very low (45%) in first cycle and becomes zero in the 6<sup>th</sup> cycle of use.



**Fig 3:** Efficiency of reusability of immobilized laccase on a) glass beads b) polyacrylamide gel

However, immobilization of laccase enzymes by covalent bonding on porous glass beads that were activated with 3-aminopropyltriethoxysilane and glutaraldehyde gave a retained activity of 65% after 6 cycles (Leonowicz *et al.*, 1988) [16]. Furthermore, after 10 repeated cycles, the activity of amylase enzyme entrapped in polyacrylamide was 56 %. Sharma *et al.* (2010) [15] also suggested that the gels of polyacrylamide and agar were fragile and cannot be used for repeated conversion of substrate into product. However, the study of Sadighi *et al.* (2013) [12] showed that 96% of the activity of the laccase from *Aspergillus oryzae*, immobilized on glass beads was still retained after 25 reusing rounds of treatment. Hong *et al.* (2017) [1] reported the reusability of immobilized laccase enzyme by reacting a batch of immobilized laccase with ABTS for 15 cycles. Their data showed that the retained enzyme activities of entrapped and covalently bonded enzyme after being reused for eight cycles were well above 60%.



**Fig 4:** Reusability and stability of the laccase when immobilized on various support materials and assayed for the guaiacol laccase activity

## Conclusion

Enzymes based catalysis are considered advantageous due to high specificity, environment-friendly and wide range of operational conditions. Immobilization of enzyme offers a further advantage to enzyme-based catalysis as it enhances reusability and makes the process cost-effective. A viable enzyme immobilization method must maintain high catalytic efficiency and maintain the activity until many reaction cycles. In this study, the laccase enzyme was immobilized to different polymers, inorganic material, and glass beads in order to find out suitable immobilized matrices. In this study varying residual activity of immobilized laccase was observed and it was also found that activity of the enzyme was reduced in successive cycles for all the matrices. Though immobilization is a remarkable approach for cost-effective enzymatic processes but it must be accompanied by high residual activity with respect to free enzyme and maintenance of this activity up to a maximum number of cycles.

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## References

1. Agrawal K, Chaturvedi V, Verma P. Fungal laccase discovered but yet undiscovered. *Bioresources and Bioprocessing*. 2018; 5:1-12
2. Asgher M, Shahid M, Kamal S, Iqbal HMN. Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. *Journal of Molecular Catalysis B: Enzymatic*. 2014; 101:56-66.
3. Bourbonnais R, Paice MG. Oxidation of non-phenolic substrates. *FEBS Letters*. 1990; 267:99-102.
4. Bourbonnais R, Paice MG, Reid ID, Lanthier P, Yaguchi M. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Applied and Environmental Microbiology*. 1995; 61:1876-1880.
5. Brijwani K, Rigdon A, Vadlani PV. Fungal Laccases: Production, Function, and Applications in Food Processing. *Enzyme Research*, 2010, 1-10.
6. Chen L, Zou M, Hong FF. Evaluation of Fungal Laccase Immobilized on Natural Nanostructured Bacterial Cellulose. *Frontiers in Microbiology*. 2015; 6:1245.
7. Claus H. Laccases: structure, reactions, distribution. *Micron*. 2004; 35(1-2):93-96.
8. Datta S, Christena LR, Rajaram YRS. Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*. 2013; 3-19.
9. Georgieva S, Godjevargova T, Mita DG, Diano N, Menale C. Non-isothermal bioremediation of waters polluted by phenol and some of its derivatives by laccase covalently immobilized on polypropylene membranes. *Journal of Molecular Catalysis B: Enzymatic*. 2010; 66:210-218.
10. Gianfreda L, Xu F, Bollag JM. Laccases: A Useful Group of Oxidoreductive Enzymes. *Bioremediation Journal*. 1999; 3:1-26.
11. Gochev VK, Krastanov AI. Isolation of Laccase Producing *Trichoderma Spp.* *Bulgarian Journal of Agricultural Science*. 2007; 13:171-176.
12. Holker UJ, Dohse Höfer M. Extracellular Laccases in Ascomycetes *Trichoderma Atroviride* and *Trichoderma Harzianum*. *Folia Microbiologica*. 2002; 47:423-27.
13. Hong CS, Lau CCY, Leong CY, Chua GK, Chin SY. A comparison of entrapped and covalently bonded laccase: Study of its leakage, reusability, and the catalytic efficiency in TEMPO-mediated glycerol oxidation. *Biocatalysis and Biotransformation*. 2017; 35:1-10.
14. Huber D, Pellis A, Daxbacher A, Nyanhongo G, Guebitz G. Polymerization of Various Lignins via Immobilized *Myceliophthora thermophila* Laccase (MtL). *Polymers*. 2016; 8:280.
15. Kiiskinen LL, Ratto M, Kruus K. Screening for novel laccase-producing microbes. *Journal of Applied Microbiology*. 2004; 97:640-646.
16. Leonowicz A, Sarkar JM, Bollag JM. Improvement in stability of an immobilized fungal laccase. *Applied Microbiology and Biotechnology*. 1988; 29:129-135.
17. Mehra R, Muschiol J, Meyer AS, Kepp KP. A structural-chemical explanation of fungal laccase activity. *Scientific Reports*. 2018; 8:17285.
18. Minovska V, Winkelhausen E, Kuzmanova S. Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. *Journal of the Serbian Chemical Society*. 2005; 70:609-624.
19. Niku-Paavola ML, Karhunen E, Salola P, Raunio V. Ligninolytic Enzymes of the White-Rot Fungus *Phlebia Radiata*. *Biochemical Journal*. 1988; 254:877-84.
20. Palmieri G, Giardina P, Bianco C, Fontanella B, & Sannia G. Copper Induction of Laccase Isoenzymes in the Ligninolytic Fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology*. 2000; 66:920-924.
21. Pannu J, Kumar Kapoor R. Microbial Laccases: A Mini-Review On Their Production, Purification And Applications. *International Journal of Pharmaceutical Archive*. 2014; 3:528-536.
22. Poonkuzhali K, Palvannan T. Comparison of biopolymers for immobilization of laccase: Ecotoxicity assessment of azo dye. *Indian Journal of Biotechnology*. 2013; 12:395-401.
23. Sadighi A, Faramarzi MA. Congo red decolorization by immobilized laccase through chitosan nanoparticles on the glass beads. *Journal of the Taiwan Institute of Chemical Engineers*. 2013; 44:156-162.
24. Sharma JR, Mahajan Gupta VK.. Comparison and Suitability of Gel Matrix for Entrapping Higher Content of Enzymes for Commercial Applications. *Indian Journal of Pharmaceutical Sciences*. 2018; 72: 223.
25. Shraddha Shekher R, Sehgal S, Kamthania M, Kumar A. Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications. *Enzyme Research*, 2011, 1-11.
26. Singh J, Saharan V, Kumar S, Gulati P, Kapoor RK. Laccase grafted membranes for advanced water filtration systems: a green approach to water purification technology. *Critical Reviews in Biotechnology*. 2017; 38:1-19.
27. Sitarz AK, Mikkelsen D, Meyer AS. Structure, functionality and tuning up of laccases for lignocellulose and other industrial applications. *Critical Reviews in Biotechnology* 2015; 36:70-86.
28. Sun WQ, Payne GF. Tyrosinase-containing chitosan gels: A combined catalyst and sorbent for selective phenol removal. *Biotechnology and Bioengineering*. 2000;

51:79-86.

29. Vasdev K, Kuhad R. Decolorization of PolyR-478 (Polyvinylamine Sulfonate Anthrapyridone) By *Cyathus Bulleri*. *Folia Microbiologica*. 1994; 39:61-64.
30. Velazquez-Cedeno MA, Farnet AM, Ferre E, Savoie JM. Variations of Lignocellulosic Activities in Dual Cultures of *Pleurotus ostreatus* and *Trichoderma Longibrachiatum* on Unsterilized Wheat Straw. *Mycologia*. 2004; 96:712.
31. Wang X, Uchiyam S. Polymers for Biosensors Construction. State of the Art in Biosensors - General Aspects. In Tech, 2013, DOI: 10.5772/54428
32. Yu H, Caldwell DJ, Suri RP. In vitro estrogenic activity of representative endocrine disrupting chemicals mixtures at environmentally relevant concentrations. *Chemosphere*. 2019; 215:396-403.
33. Zhou X, Yang Z, Luo Z, Li H, Chen G. Endocrine disrupting chemicals in wild freshwater fishes: Species, tissues, sizes and human health risks. *Environmental Pollution*. 2019; 244:462-468.