Immobilization and Reusability Efficiency of Laccase Onto Different Matrices Using Different Approaches

Jagdeep Singh*, Rajeev Kumar Kapoor

Department of Microbiology, Maharshi Dayanand University Rohtak, Haryana, India.

ABSTRACT

Laccase has been 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 different polymers, inorganic materials and glass beads. It was observed that the 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 1st cycle to 10th cycle.

Key Words: *Laccase, Immobilization, Reusability, Polymers, Glass Beads.*

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INTRODUCTION

Laccase

Laccase (EC Number 1.10.3.2) is the type of polyphenol oxidase enzyme. Laccases have been characterized as a family of copper-containing oxidases, and have also been recognized as multi-copper oxidases [1-4]. Researchers have shown that Laccase enzyme based catalysis causes reduction of the oxygen molecule to water. In this reaction, the oxidation of organic and inorganic substrate (methoxy-substituted mono- & diphenols, amino phenols and aromatic amines etc.) takes place by transfer of one electron [5-7]. There are different sources of Laccase enzyme such as; bacteria, plants and fungi. Most of the sources reported for these enzymes have been of fungal origin [8] and these enzymes have been produced extracellularly which made easier and faster procedure of enzyme purification. Among all the investigated fungal classes, white rot fungi have been reported of more common source of Laccase, such as; *Trametes versicolor*, *Phlebia radiata* and *Pleurotus ostreatus* [7, 9, 10]. Investigators have reported the isolation of Laccase from *Trichoderma* species like *T. atroviride* and *T. harzianum*, *T. longibrachiatum* [11-13]. Including this, Laccase producing fungi of the class basidiomycetes and ascomycetes have been isolated from the marine source such as; *Cerrena unicolor*, *Coriolopsis byrsina*, *Diaporthepha seolorum and Pestalotiopsis uvicola* [14]. Moreover, some strains of bacteria and plants have also been found to produce Laccase [15]. A considerable stability level of this extracellular enzyme in the extracellular space makes it suitable to be applied in numerous bioprocesses such as dye decolourization, biodegradation of xenobiotic pollutants, bleaching in paper and pulp industries, de-lignification of lignocellulosics and newspaper deinking etc. [4]. One of the most significant application of Laccase enzyme is the degradation of emerging contaminants called endocrine disrupting compounds (EDCs) from the polluted waste & drinking water [16-18].

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

Corresponding author: Jagdeep Singh
Address: Department of Microbiology, Maharshi Dayanand University Rohtak, Haryana, India.
E-mail: ✉ jagdeeprajendra@gmail.com
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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 an immobilized form. The third form of laccase has been used as the enzymes exhibit the number of features that make them privileged as compared to the conventional chemical catalysts. But effective applications of enzymes may be impeded by unfavorable properties of the enzymes like being non-reusable, highly sensitive to various denaturizing agents and also having deleterious sensory or toxicological impacts. Using immobilized enzymes can remove many of these unfavorable limitations [19]. Immobilization is achieved by fixing enzymes to or within solid supports. Immobilization procedures enable the reusability of biocatalysts and impact on a lot of parameters like general catalytic activity, efficacy of catalyst utilization, deactivation and regeneration of kinetics, and cost. Also, the immobilization process, waste disposal and final application of the immobilized enzyme catalyst have been the factors which lead to the toxicity of immobilization reagents [20].

Several methods like: adsorption, entrapment, crosslinking and covalent bonding can be used to immobilize the enzymes (Figure 1). Immobilizing by physical entrapment can be applied widely, and the original structure and function of the enzymes may be a little perturbed. The immobilization method selection is influenced by the different chemical properties and structure of enzymes, the distinct characteristics of substrates, and the reaction products. Thus, it has been very difficult to state the optimum immobilization method and support for any enzyme. Widely used system for enzyme entrapment is the immobilization within a poly acrylamide 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 which is able to react with the enzyme [21, 22].

Table 1: The supports and matrices employed for the 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	

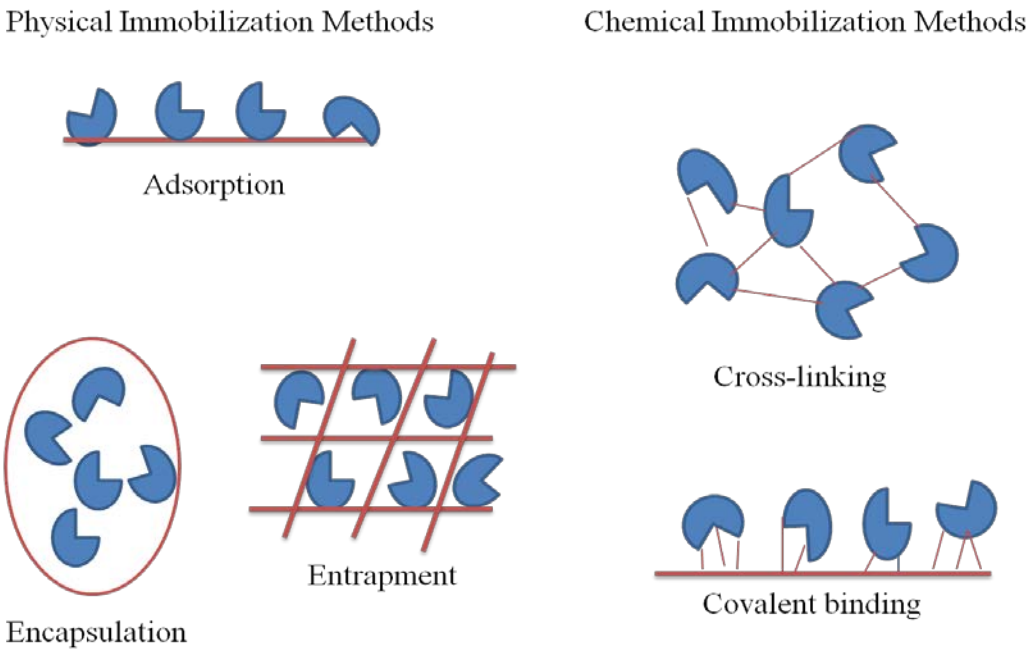

Figure 1: Drawings of physical and chemical enzymatic immobilization methods [23].

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 the adsorption, cross inking, covalent and entrapment methods. These procedures were employed with the natural polymer (Cotton) and synthetic polymers (Polypropylene, Polystyrene, Polyester, and Polyacrylamide), and inorganic materials (Glass bead).

Laccase Assay

Laccase activity was measured following the method of Vasdev and Kuhad (1994) [24]. 200 µL of culture filtrate was added to 800 µL of 5.0 mM guaiacol in 100.0 mM citrate phosphate buffer (pH 5.4). The reaction mixture was monitored for change in the absorbance for the first few minutes, or till the increase in the optical density (OD) was linear against time. A change in absorbance of 0.01 $min^{-1}mL^{-1}$ 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 [25]; and 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 the beads were washed five times with distilled water, and twice with CP buffer (pH 7.0).
6. 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.
7. The beads were washed five times with CP buffer, and twice with distilled water.
8. 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 the 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 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 through the following steps:

1. 9% Acrylamide and 1% Bis-acrylamide were dissolved in the 0.02M phosphate buffer (pH7.0)
2. Then, 1mL of partial purified enzyme was mixed under the vacuumed condition.
3. To this, 0.5% ammonium pre sulphate (freshly prepared) and of 50% TEMED were added for polymerization, the contents were stirred gently, and for setting of the gel, this solution was poured in to 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.
4. Enzyme activity was measured under the 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 [26, 27] with some modifications. The following steps were employed:

Immobilization by simple adsorption:

1. This procedure was employed with 1 gram of each natural and synthetic polymer. Polypropylene fibre piece, polystyrene beads, polyester and cotton thread were washed three times in ethanol under vacuum, and three times with distilled water.
2. 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, and stirred for 30 min.

3. The relative laccase activity was determined.

Adsorption followed by cross-linking:

4. Glutaraldehyde was used to treat the immobilizates; 50 mL of a 2.5% (w/v) aqueous solution was added, and the reaction was allowed to continue for an extra 10 min. Vacuum filtration was applied to separate the immobilizates.
5. The proportional 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 was 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 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 from the same-industrial immobilization enzyme must be active (high active in a unit of volume, U g1 or ml1), 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 enzymes. However, in different immobilization systems, the highest activity of immobilized enzyme was found on polypropylene filter pieces (95%) followed by the cotton threads (92%) and the glass beads (85%) (Figure 2).

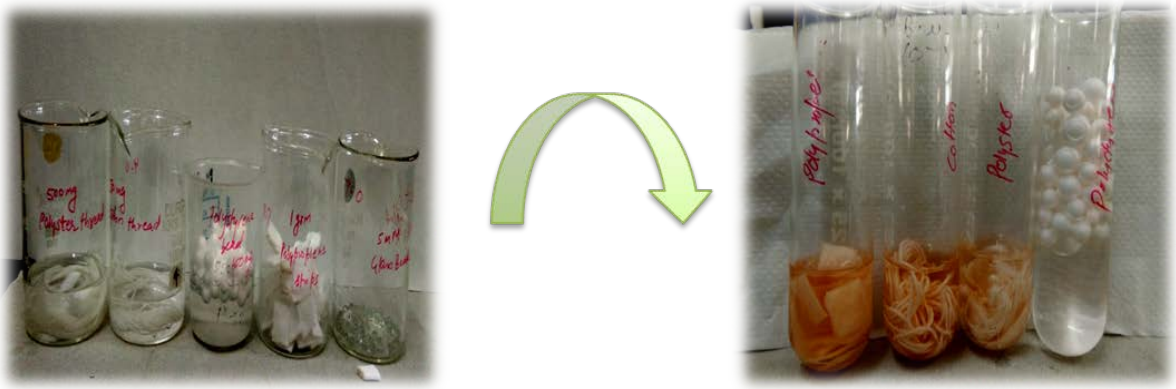

Figure 2: Immobilization of laccase on different polymers

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 95 % for polypropylene, 50 to 80 % for cotton thread, 52 % to 92 % for the polyester thread and for 25 to 45% 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

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Minovska et al. (2005) reported that polypropylene demonstrated a 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 concentration and pore size of the matrix used might have caused the immobilized laccase activity [28]. Due to the 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) 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 [29], laccase was immobilized on bacterial nano-cellulose (BC) by adsorption, and 83% activity was obtained with a BC size of 7 × 7 to 10 × 10 mm^2. Leonowicz et al. (1988) 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 the 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 compared to the immobilization on glass beads (72%).

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 has been shown in the figure 4 that the efficiency of immobilization on polypropylene pieces was reduced from first cycle (95%) to (15%) 10^{th} 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 very low (45%) in the first cycle, and it became zero in the 6^{th} cycle of use.

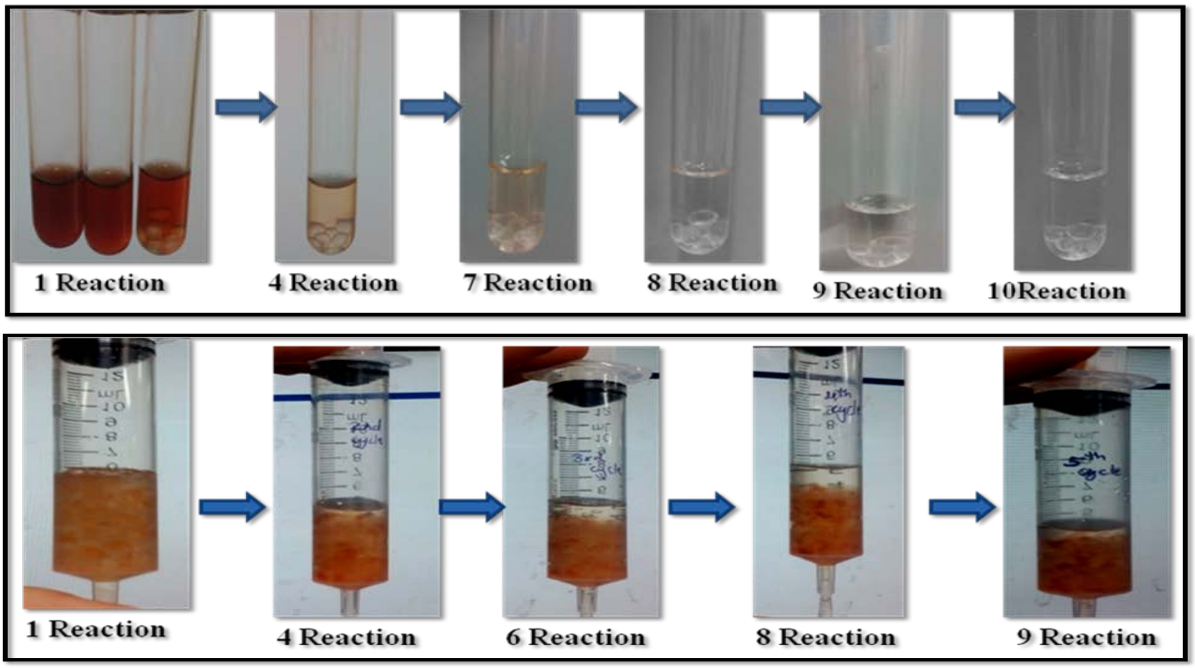

Figure 3: **Efficiency of reusability of immobilized laccase on a) glass beads b) polyacrylamide gel**

However, the 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 [25]. Furthermore, after 10 repeated cycles, the activity of amylase enzyme entrapped in polyacrylamide was 56 %. Sharma et al. (2018) also suggested that the gels of polyacrylamide and agar were fragile and cannot be used for repeated conversion of substrate into product [30]. However, the study of Sadighi et al. (2013) 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 [31]. Hong et al. (2017) reported the reusability of the immobilized laccase enzyme by reacting a batch of immobilized laccase with ABTS for 15 cycles [32]. Their data showed that the entrapped and covalently bonded enzymes after being reused for eight cycles represented the retained enzyme activities above 60%.

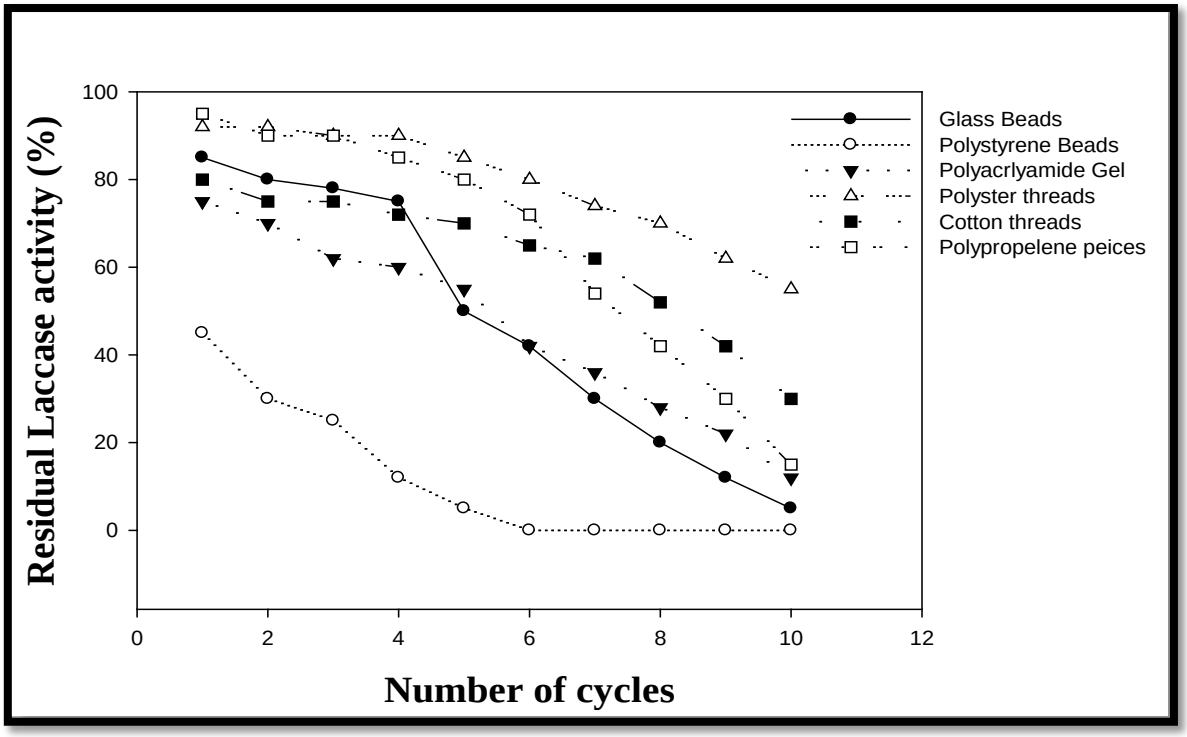

Figure 4: Reusability and stability of the laccase when immobilized on various support materials, and assayed for the guaiacol laccase activity

CONCLUSION

Enzymes based catalysis have been considered advantageous due to their high specificity, environment friendliness, and wide range of operational conditions. The immobilization of enzyme has offered further advantage to enzymes based catalysis as it has enhanced reusability, and made 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, laccase enzyme was immobilized to different polymers, inorganic materials, 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 the activity of enzyme was reduced in successive cycles for all the matrices. Though, immobilization has been a remarkable approach for cost effective enzymatic processes, it must be accompanied with the high residual activity with respect to free enzyme and maintenance of this activity up to the maximum number of cycles.

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REFERENCES

1. Agrawal, K., Chaturvedi, V., & Verma, P. (2018). Fungal laccase discovered but yet undiscovered. Bioresources and Bioprocessing, 5 (4),1-12.
2. Mehra, R., Muschiol, J., Meyer, A. S., & Kepp, K. P. (2018). A structural-chemical explanation of fungal laccase activity.Scientific Reports. 8. 10.1038/s41598-018-35633-8.
3. Claus, H. (2004). Laccases: structure, reactions, distribution. Micron, 35(1–2), 93–96.
4. Sitarz, A. K., Mikkelsen, J. D., & Meyer, A. S. (2015). Structure, functionality and tuning up of laccases for lignocellulose and other industrial applications. Critical Reviews in Biotechnology, 36, 70–86.
5. Gianfreda, L., Xu, F., & Bollag, J.-M. (1999). Laccases: A Useful Group of Oxidoreductive Enzymes. Bioremediation Journal, 3, 1–26.
6. Bourbonnais, R., & Paice, M. G. (1990). Oxidation of non-phenolic substrates. FEBS Letters, 267 (1), 99–102.
7. Bourbonnais, R., Paice, M. G., Reid, I. D., Lanthier, P., & Yaguchi, M. (1995). 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, 61, 1876–1880.
8. Kiiskinen, L.-L., Ratto, M., & Kruus, K. (2004). Screening for novel laccase-producing microbes. Journal of Applied Microbiology, 97, 640–646.
9. Niku-Paavola, M L, E Karhunen, P Salola, and V Raunio. 1988. "Ligninolytic Enzymes of the White-Rot Fungus Phlebia Radiata." Biochemical Journal 254,877–84.
10. Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., & Sannia, G. (2000). Copper Induction of Laccase Isoenzymes in the Ligninolytic Fungus Pleurotus ostreatus. Applied and Environmental Microbiology, 66, 920–924.
11. Holker, U, J Dohse, and M Höfer. (2002). "Extracellular Laccases in Ascomycetes Trichoderma Atroviride and Trichoderma Harzianum." Folia Microbiologica, 47 (4), 423–27.
12. Velazquez-Cedeno, M. A., Farnet, A. M., Ferre, E., & Savoie, J. M. (2004). Variations of Lignocellulosic Activities in Dual Cultures of Pleurotus ostreatus and Trichoderma Longibrachiatum on Unsterilized Wheat Straw. Mycologia, 96, 712.
13. Gochev, V. K., & Krastanov, A. I. (2007). Isolation of Laccase Producing Trichoderma Spp. Bulgarian Journal of Agricultural Science, 13, 171–176.
14. Verma AK, Raghukumar C, Verma P, Shouche YS, Naik CG. 2010. Four marine-derived fungi for bioremediation of textile mill effluents. Biodegradation 21: 217-233. DOI: 10.1007/s10532-009-9295-6.
15. Shraddha, Shekher, R., Sehgal, S., Kamthania, M., & Kumar, A. (2011). Laccase: Microbial Sources, Production, Purification, and Potential Biotechnological Applications. Enzyme Research, 2011, 1–11.
16. Zhou, X, Yang, Z, Luo, Z, Li, H, Chen, G (2019) Endocrine disrupting chemicals in wild freshwater fishes: Species, tissues, sizes and human health risks. Environmental Pollution. 244, 462-468.
17. Yu H, Caldwell DJ, Suri RP (2019) In vitro estrogenic activity of representative endocrine disrupting chemicals mixtures at environmentally relevant concentrations. Chemosphere. 215, 396-403.
18. Singh, J., Saharan, V., Kumar, S., Gulati, P., & Kapoor, R. K. (2017). Laccase grafted membranes for advanced water filtration systems: a green approach to water purification technology. Critical Reviews in Biotechnology, 38, 1–19.
19. Brijwani, K., Rigdon, A., & Vadlani, P. V. (2010). Fungal Laccases: Production, Function, and Applications in Food Processing. Enzyme Research, 2010, 1–10. doi: 10.4061/2010/149748.
20. Pannu, J., & Kumar Kapoor, R. (2014). Microbial Laccases: A Mini-Review On Their Production, Purification And Applications. International Journal of Pharmaceutical Archive-3, 528–536.
21. Asgher, M., Shahid, M., Kamal, S., & Iqbal, H. M. N. (2014). Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology. Journal of Molecular Catalysis B: Enzymatic, 101, 56–66.
22. Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. 3 Biotech, 3 (1), 1–9.
23. Wang, X., & Uchiyam, S. (2013). Polymers for Biosensors Construction. State of the Art in Biosensors - General Aspects. InTech. DOI: 10.5772/54428.
24. Vasdev, K., and R. C. Kuhad. 1994. "Decolorization of PolyR-478 (Polyvinylamine Sulfonate Anthrapyridone) By Cyathus Bulleri." Folia Microbiologica 39, 61–64.
25. Leonowicz, A., Sarkar, J. M., & Bollag, J.-M. (1988). Improvement in stability of an immobilized fungal laccase. Applied Microbiology and Biotechnology, 29, 129–135.
26. Huber, D., Pellis, A., Daxbacher, A., Nyanhongo, G., & Guebitz, G. (2016). Polymerization of Various Lignins via Immobilized Myceliophthora thermophila Laccase (MtL). Polymers, 8(8), 280.
27. Minovska, V., Winkelhausen, E., & Kuzmanova, S. (2005). Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. Journal of the Serbian Chemical Society, 70, 609–624.
28. Georgieva, S., Godjevargova, T., Mita, D. G., Diano, N., Menale, C., Nicolucci, C., Golovinsky, E. (2010). 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, 66 (1-2), 210–218.
29. Chen, L., Zou, M., & Hong, F. F. (2015). Evaluation of Fungal Laccase Immobilized on Natural Nanostructured Bacterial Cellulose. Frontiers in Microbiology, Nov 10, 6, 1245.
30. Sharma, J, R Mahajan, and VK Gupta. (2018). "Comparison and Suitability of Gel Matrix for Entrapping Higher Content of Enzymes for Commercial Applications." Indian Journal of Pharmaceutical Sciences, 72(2):223-8.

31. Sadighi, A., & Faramarzi, M. A. (2013). Congo red decolorization by immobilized laccase through chitosan nanoparticles on the glass beads. Journal of the Taiwan Institute of Chemical Engineers, 44, 156–162.

32. Hong, C. S., Lau, C. C. Y., Leong, C. Y., Chua, G. K., & Chin, S. Y. (2017). 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, 36 (5), 1–10.