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Immobilization of fungal laccase on glutaraldehyde cross-linked chitosan beads and its bio-catalytic potential to degrade bisphenol A

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ABSTRACT

Bisphenol A is an endocrine disrupting compound that is continuously released into the environment. In this study, a laccase from *Trametes versicolor* was covalently immobilized onto high quality chitosan beads as carrier support chemically cross-linked with glutaraldehyde. Chitosan beads (average 2.0 mm diameter) developed using 2.5% (w/v) chitosan and functionalized with 2.0% (v/v) glutaraldehyde for 3 h yielded maximum immobilization efficiency (~84.7%). The surface topology of laccase-attached chitosan support was envisaged and compared with control beads by scanning electron microscope (SEM). The immobilized biocatalyst showed good operational stability, retaining 71.24% of its original activity after 10 repeated catalytic cycles with reference to its native form. Storage stability profile exhibited the superiority of the laccase-immobilized chitosan beads presenting over 90% of activity after preserving for 28 days at 4 °C, whereas free enzyme showed only 47.3% activity under the same conditions. In addition, the chitosan-based biocatalytic system achieved almost complete removal of bisphenol A from the aqueous solution after 150 min of the transformation process. Conclusively, these results proposed the use of the chitosan hydrogel beads immobilized laccase as a promising and environmentally friendly biocatalyst for the degradation of environmental pollutants, particularly the removal of phenolic compounds in wastewater.

1. Introduction

The use of biocatalysts as a substitute to chemical catalysts promotes the development of green industrial processes due to their exceptional characteristics including catalytic efficiency, low toxicity, biodegradability, high specificity, and mild reaction conditions (Amin et al., 2017; Asgher et al., 2017a; Bilal et al., 2017; Soozanipour et al., 2019). Laccases are multi-copper oxidases that have a great ability to oxidize an array of compounds, including aromatic amines, mono-, di-, and polyphenols, methoxy phenols, and ascorbates using molecular oxygen as co-substrates (Thurston, 1994). At contemporary, laccases have attracted profound research significance for numerous biotechnological applications, such as delignification of plant biomass for biofuel production, biopulping, biobleaching, bioremediation and detoxification of textile dyes/effluents, oxidation of organic pollutants, stabilization of fruit juices, biosensors, beverage, textile, animal feed, paper and pulp, degradation of antibiotics, steroids, and a rage of phenolics due to their high activity and substrate non-specificity (Asgher et al., 2014, 2017b; Barrios-Estrada et al., 2018a; Ma et al., 2018).

In spite of diverse biotechnological potentialities, the free laccases on an industrial scale have presented several disadvantages such as low stability under process conditions, activity inhibition and non-reusability, rendering their applications more expensive. Thermostable enzymes are desirable catalysts as these can withstand harsh industrial conditions of extreme pH and elevated temperatures environment (Shin et al., 2002). The catalytic properties of enzymes can be improved with the advancement in enzyme modification and tailoring techniques and tools. Therefore, biocatalysts with desired phenotypes can be constructed through immobilization, recombinant DNA, and genetic engineering approaches. Enzyme modification through different immobilization strategies is considered as the most effective and straightforward method in green biotechnology that allowing for the reuse of laccase, facile recovery, longer half-lives, stabilizing enzyme activity and structure, and diminished proteases activity (Bilal et al., 2018a,b,c).

Utilization of inexpensive supporting matrices and optimized immobilization processing conditions may boost up the applicability of

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several enzymes in immobilized forms. A variety of carrier supports has been examined in recent past to potentiate the traditional enzyme immobilization technology. Chitosan is a natural biopolymer of 2-amino-2-deoxy-β-D-glucose units connected by β-1,4-glycosidic bonds. It acquired a wider acceptance as support for enzyme immobilization because of its good biocompatibility, biodegradability, low cost, easy modification, physiological inertness, film-forming ability and a great affinity for proteins (Krajewska, 2004; Bilal et al., 2018c; Bilal and Iqbal, 2019a). Chitosan is typically obtained from partially deacetylated chitin, which exists in the cell wall of fungi and outer skeleton of insects, crustaceans, mushrooms, and beetles (Muxika et al., 2017). Among various enzyme immobilization methods, covalent attachment to a support material presents the advantages of tight enzyme fixation, minimum leaching and negligible product contamination with protein (Urrutia et al., 2018; Bilal and Iqbal, 2019b). Moreover, multipoint covalent immobilization between the support and enzyme molecule may provide a high stabilization to biocatalyst (Bilal et al., 2019). Covalent coupling of biocatalysts in the chitosan matrix is generally accomplished by the reaction of polymeric amino groups with the cross-linking reagent i.e. glutaraldehyde (Žuža et al., 2017; Bilal et al., 2019).

Bisphenol A [2, 2-bis (4-hydroxyphenyl) propane, BPA] is a widely used synthetic chemical to manufacture epoxy resins and polycarbonate plastics for food packaging purposes (Brugnari et al., 2018). However, the US Environmental Protection Agency (EPA) has declared it as an emerging endocrine disruptor due to toxic, carcinogenic, mutagenic, and reproductive effects in humans as well as wildlife (Mohapatra et al., 2011; Bilal and Iqbal, 2019c). Therefore, the presence of BPA poses a serious threat to the ecosystem and public health. Several free and immobilized laccases have shown the potential of BPA degradation (Upadhyay et al., 2016; de Freitas et al., 2017; Lassouane et al., 2019). In the present work, laccase immobilization was carried onto chitosan beads using glutaraldehyde as a bi-functional activating agent. Use of scanning electron microscopy (SEM) confirmed the effective enzyme immobilization. The developed immobilized-biocatalyst was applied for the degradation of bisphenol A from the aqueous solution to evaluate its industrial suitability.

2. Materials and methods

2.1. Chemicals

Chitosan, calcium chloride anhydrous, acetic acid, and glutaraldehyde were obtained from Sinopharm Company. Laccase from *Trametes versicolor*, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), BPA were supplied by Sigma-Aldrich. The highest purity grade reagents and chemicals were used, and all necessary solutions were prepared in deionized water throughout the experiment.

2.2. Immobilization of laccase using chitosan beads

For the development of the immobilization matrix, chitosan was well dissolved in 1.5% acetic acid solution by mild heating at 50 °C with continuous stirring. Varying concentrations of chitosan [2.0–4.0% (w/v)] were tried to develop beads of better mechanical strength. The resultant chitosan mixture was extruded dropwise into 1 M KOH solution by a syringe and hardened in this solution for 4 h at room temperature. Good quality beads of uniform size and shape were collected, filtered, and activated by incubating with varying concentrations of glutaraldehyde solution (1–4%, v/v) at room temperature. Any unattached glutaraldehyde from the beads surface was removed by thoroughly washing the activated chitosan beads with 50 mM Na-malonate buffer (pH 4.5). Finally, the beads were incubated with laccase enzyme for 24 h at 4 °C for immobilization. Fig. 1 illustrates a schematic process for laccase immobilization using chitosan beads. After designated reaction time, the beads were washed thrice with the same buffer and used for enzyme activity measurement at 280 nm. Immobilization efficiency (IE) for chitosan-immobilized enzyme was calculated using the relation as given in Eq. (1):

$$\text{Immobilization efficiency (\%)} = \frac{\text{Total activity of immobilized enzyme}}{\text{Total activity of free enyme}} \times 100 \quad \text{Eq. 1}$$

2.3. Characterization of surface morphology

The surface morphologies of control and enzyme-incorporated chitosan beads were envisaged using SEM (JSM 7800F, JEOL) to evaluate the presence of enzyme molecules inside these beads. For this, chitosan matrix derived dehydrated bead samples were mounted on metal grids with a double-sided adhesive tape followed by a gold coating for 2.0 min. High definition images were captured at operating pressure and deposition current of 7 X 10^{-2} bar and 20 mA, respectively, to examine the surface morphologies of each sample.

2.4. Recyclability and storage stability

For inspecting the repeated usability of the immobilized enzyme, the enzyme-immobilized chitosan beads used in one cycle were filtered, washed with Na-malonate buffer (pH 4.5), and employed to the subsequent batch for substrate oxidation. The percentage residual activity of immobilized enzymes was monitored at regular intervals using the standard assay protocol. The storage stability was assayed by preserving the free as well as immobilized forms of the enzymes in Na-malonate buffer (pH 4.5) at 4 °C for 28 days and residual enzyme activity was measured. The activity of the enzymes in the first run was denoted as 100%.

2.5. Degradation of bisphenol A

In order to investigate the degradation capability, BPA at a final concentration of 10 mg/L was separately treated by free and chitosan-immobilized laccase in triplicate Erlenmeyer flasks (250-mL capacity) at 30 °C for 150 min with continuous shaking in dark conditions. Sample aliquots were collected from the reaction flasks at varying time intervals during the degradation process and the BPA removal was measured spectrophotometrically following Eq. (2). In additions, the control experiments using chitosan beads with no incorporated laccase enzyme were also arranged in parallel to detect any possible adsorption of BPA on the chitosan beads.

$$\text{BPA removal (\%)} = \frac{([\text{BPA}]\text{initial} - [\text{BPA}]\text{time})}{[\text{BPA}]\text{time}} \times 100 \quad \text{Eq. 2}$$

2.6. Enzyme activity assay

Laccase activity was examined by determining the oxidation of 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonate) in Na-malonate buffer at room temperature (Wolfenden and Willson, 1982). A typical reaction combination (2.1 mL) includes 1.0 mL of Na-malonate buffer (50 mM), 1.0 mL of ABTS (1.0 mM) as an assay substrate and 100 µL of laccase enzyme solution. After an incubation period of 10 min, the change in absorbance was documented spectrophotometrically at 420 nm (ε_{420} 36000 M^{-1} cm^{-1}). Blank test-tube consists of the assay mixture excluding enzyme solution was also carried out in parallel.

2.7. Data analysis

Data were statistically analyzed using the statistical software package (SPSS Statics 21). The means and standard errors of means (mean ± S.E.) were calculated for three replicates. The significance of difference was analyzed by one-way analysis of variance (ANOVA)

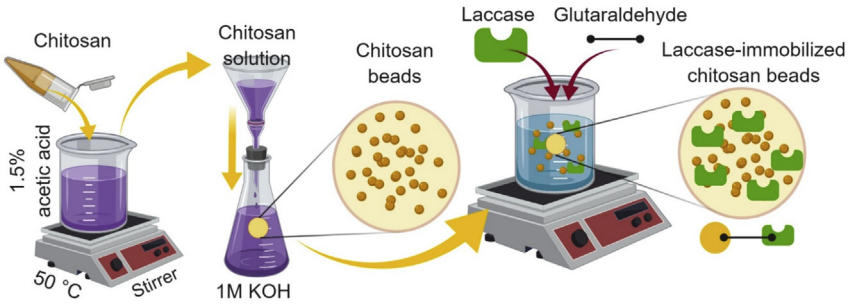

Fig. 1. A schematic process for laccase immobilization using chitosan beads.

along with Tukey-Kramer multiple comparison test.

3. Results and discussion

3.1. Immobilization of laccase on chitosan beads

The laccase enzyme was covalently immobilized onto glutaraldehyde activated chitosan beads. The glutaraldehyde-activated chitosan beads provided a biocompatible support surface leading to a maximum immobilization efficiency (IE) of 84.7%. At first, varying concentrations of chitosan solution ranging from 2.0 to 4.0% were used to develop beads with desired mechanical strengths. Chitosan microspheres developed with a chitosan solution of 2.5% (w/v) were observed most appropriate for laccase attachment (Fig. 2). In a second step, the beads were treated with different glutaraldehyde concentrations (a cross-linking agent) to evaluate the extent of enzyme immobilization, followed by enzyme coupling to the active sites of the support material. Results in Table 1 portrays that the beads treated with 2.0% glutaraldehyde for 3 h activation time furnished the highest IE. During glutaraldehyde reaction with chitosan, generation of aldehyde groups on the support surface may react with an amino group of the enzyme as well as other functional moieties on the surface (phenols, thiols, and imidazoles) (Barbosa et al., 2014). Additionally, the glutaraldehyde reaction with amino polysaccharide chitosan also facilitates the cross-linkage of various polymeric chains, leading to improved mechanical resistance of support and circumventing its solubilization in the aqueous acidic environment because of its cationic nature (Krajewska, 2004). Though the explicit chemistry of the reaction is not sufficiently elucidated, the reaction mechanism between cross-linker (i.e. glutaraldehyde) and amino groups of the enzyme may implicate Schiff bases, Michael addition, and nucleophilic substitution (Barbosa et al., 2014; Bilal et al., 2018c). At a lower concentration of glutaraldehyde, lesser aldehyde groups were generated resulting in lower IY. It was observed that initially increasing the concentration of glutaraldehyde promoted the IE, but at higher concentration beyond 2%, the IE was diminished that may be ascribed to steric hindrance because of numerous cross-linking points between enzyme molecules and on the chitosan surface (Asgher et al., 2017a; Kumari and Kayastha, 2011).

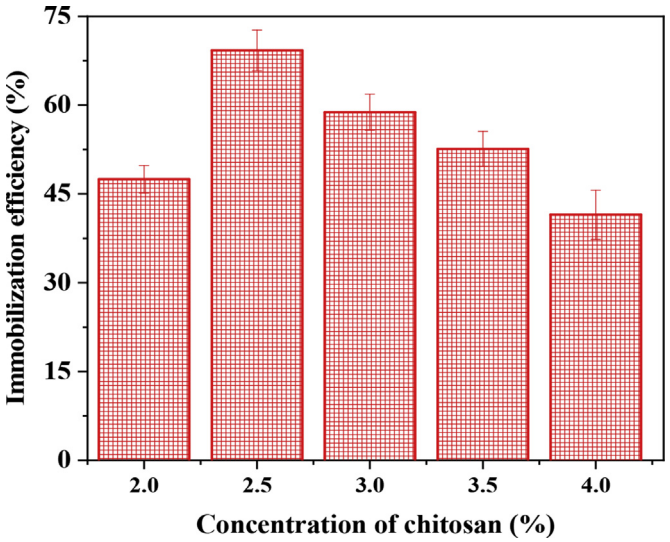

Fig. 2. Influence of different chitosan concentrations on laccase immobilization efficiency of chitosan beads.

Table 1
Immobilization parameters optimization for laccase immobilization on chitosan beads.

Glutaraldehyde (%)	Activation time (h)	Immobilization (%)
0.0	3.0	61.0 ± 3.16^{f}
	6.0	58.2 ± 1.87^{g}
1.0	3.0	67.4 ± 2.66^{e}
	6.0	68.0 ± 3.15^{e}
2.0	3.0	84.7 ± 2.78^{a}
	6.0	79.4 ± 3.29^{b}
3.0	3.0	76.3 ± 2.69^{c}
	6.0	72.0 ± 1.58^{d}
4.0	3.0	70.2 ± 3.18^{d}
	6.0	66.0 ± 2.19^{e}

Data are presented as a mean ± standard deviation from three replicated experiments. Different lowercase letters indicate significant differences at $P < 0.05$.

3.2. Characterization of chitosan beads surface with and without laccase

Scanning electron microscope analyzed the surface characterization of dried chitosan microspheres with and without bound laccase, and results are portrayed in Fig. 3. In contrast to control beads, substantial modifications on the surface topologies of chitosan matrix with immobilized laccase as envisaged from SEM images corroborated the laccase attachment on the surface of chitosan beads. The chitosan hydrogel beads with a smooth surface were envisaged without laccase immobilization. Nevertheless, the beads surface revealed a clearly modified outer surface with a marked extent of irregular aggregates presumably due to the coupling of enzyme molecules on the chitosan biopolymers surface after laccase immobilization. Earlier, the surface topology of laccase-conjugated chitosan capsules was found to be different from that to pristine chitosan beads without coupled enzyme. The coarseness/roughness of capsules surface was evidently increased after laccase immobilization than without enzyme coupled beads that exhibited an even and smooth surface (Asgher et al., 2017a). Rehman and coworkers (2014) reported that the surface of agar-agar support was also altered following pectinolytic enzyme entrapment. Before enzyme encapsulation, the pores were detected on the superficial surface of the agar-agar matrix and were covered with enzyme molecules after the immobilization process. Similarly, significant variations on the

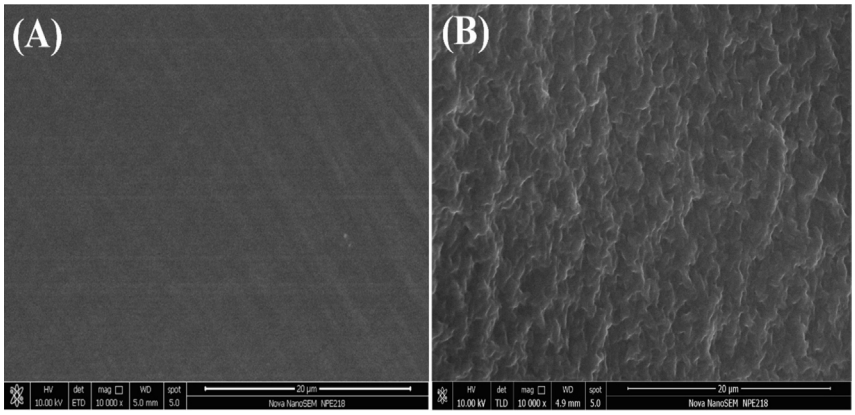

Fig. 3. Scanning electron micrograph (surface view) presenting the surface morphologies of the A) chitosan beads without enzyme attachment and B) laccase-incorporated chitosan beads.

outer surface of biopolymers with immobilized laccase have also been observed when laccase was entrapped in various biopolymers such as agar-agar, gelatin, and polyacrylamide (Asgher et al., 2017b).

3.3. Operational stability studies

The recycling ability of chitosan-coupled laccase was monitored by conducting a series of 10 substrate-oxidation cycles using ABTS as an assay substrate (Fig. 4). After three and five reusability cycles, the chitosan-bound laccase maintained 94.45 and 89.31% residual activity. Notably, more than 70% remaining activity was observed for immobilized laccase even after the completion of ten substrate-oxidation runs. Retention of this marked efficiency of chitosan-conjugated laccase to catalyzing the oxidation of the substrate in numerous successive reactions advocates its use as a green catalyst in numerous industrial bioprocesses. The activity reduction of chitosan-supported laccase by extending the number of catalytic cycles could be ascribed to release of enzyme particles from the chitosan network during excessive washing after each catalytic cycle. Additionally, the solution environment might induce some conformational changes in the enzyme molecule during continuous reprocessing, leading to diminished enzymatic catalytic performance. Therefore, it can be inferred that laccase immobilization onto chitosan beads impart substantial enzyme resistance, and consequently retaining its catalytic stability in many continuous oxidation cycles.

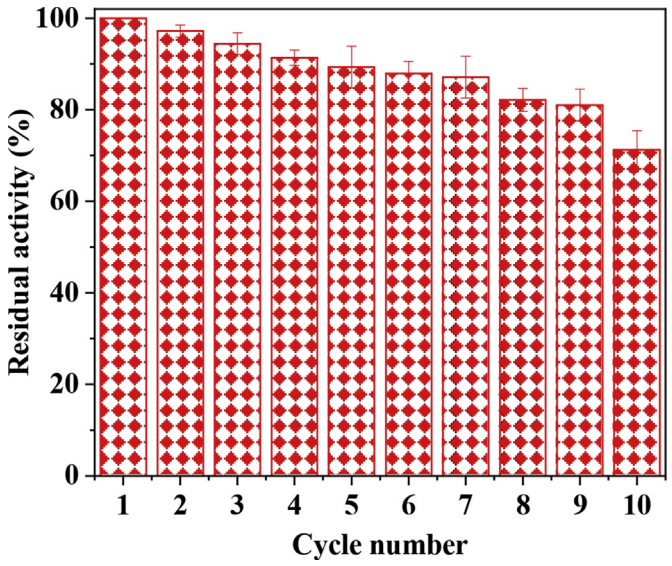

Fig. 4. Recycling portrayal of immobilized laccase for substrate oxidation.

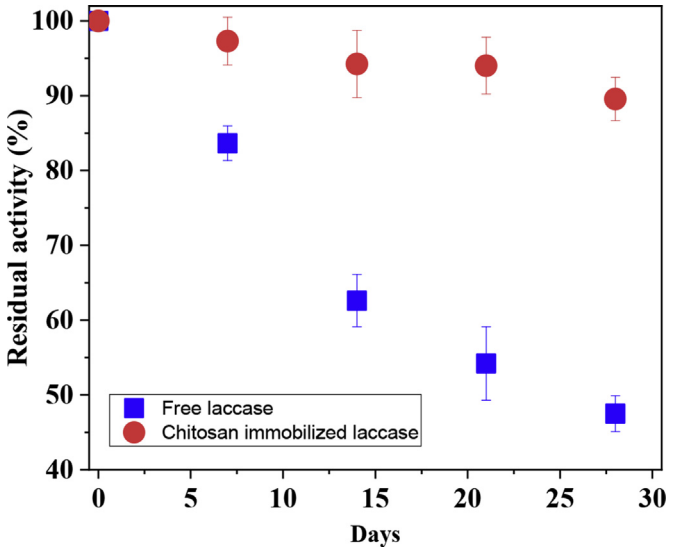

Fig. 5. Storage stability profile of chitosan-immobilized laccase with regard to soluble biocatalyst upon storage at 4 °C.

3.4. Storage stability studies

The free and chitosan-coupled laccases were preserved at 4 °C for four weeks to evaluate their storage stabilities, and the residual activity profile was recorded after every week (Fig. 5). Results showed that the storing stability of the chitosan-immobilized enzyme was substantially increased with reference to its free counterpart. At first and second week, the free and carrier-bound laccase showed a residual activity of 83.6, 62.5, and 97.3, 94.2, respectively. After four weeks, the carrier-supported enzyme preserved above 90% of its preliminary activity, whereas only 47.5% activity was found by the soluble enzyme under the identical conditions. This considerably increased storage stability due to immobilization is speculated to be a noteworthy feature for enzyme exploitation under extended storage times in industrial sectors. Generally, enzyme molecule readily starts losing its activity and stability once detached from its natural biocatalytic environment. Chitosan immobilization appears the most consistent approach to retaining the long-term enzyme catalytic efficiencies by providing a protective biocompatible microenvironment. Enhanced storage stability has also been reported for laccase, lignin and manganese peroxidase, catalase, pepsin, and lipase immobilized on functionalized chitosan microspheres than their non-immobilized forms (Çetinus and Öztop, 2003; Bilal et al., 2016; Kaushal et al., 2018).

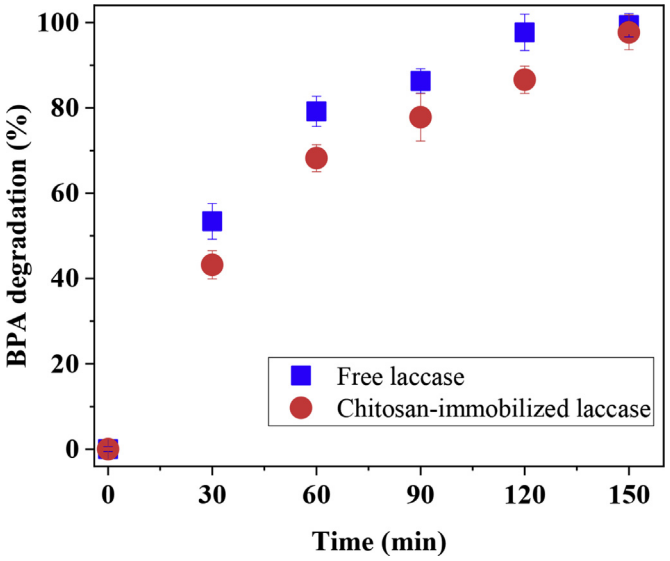

Fig. 6. Time-course degradation efficiency of bisphenol A by the free and chitosan-immobilized laccases.

3.5. Application of free and immobilized laccases for bisphenol A degradation

The free and chitosan-immobilized laccases were applied for the degradation and elimination of recalcitrant environmental pollutant i.e. BPA from the aqueous solution. Results revealed that both the free as well as immobilized biocatalysts were capable to effective removal of BPA. After one and 2 h of transformation process, the elimination of BPA was recorded to be 79.2, and 97.7%, and 43.2 and 86.6% from the solution by the soluble and covalently immobilized laccase derivative, respectively (Fig. 6). Notably, BPA was completely transformed and eliminated (more than 99%) by the free and immobilized laccases after 150 min of incubation. Additionally, negligible or no adsorption of BPA on chitosan beads without enzyme incorporation clearly indicate that the BPA degradation predominantly attributed to the contribution of only laccase enzyme. In an earlier study, Barrios-Estrada et al. (2018b) described the transformation of BPA by an indigenously isolated and purified laccase from *Pycnoporus sanguineus* (CS43) as well as a commercial laccase from *T. versicolor*. Both forms of laccase were covalently immobilized onto a ceramic membrane using glutaraldehyde as a coupling agent to develop laccase-assisted membrane biocatalytic system. Remarkably, both forms of the immobilized laccase attained a complete degradation of BPA in less than 24 h with a removal rate of 79.0 and 204.8 mol/min/U for *T. versicolor* and *P. sanguineus* (CS43), respectively. Similarly, Lassouane and coworkers, (2019) developed a novel Ca-alginate beads immobilized *T. pubescens* laccase biocatalytic system to degrade and transform BPA from the aqueous solution. The new immobilized biocatalyst led to complete removal of BPA in a short time period of 2 h under the optimal operating conditions of pH 5.0, 30 °C, at a final BPA and enzyme concentration of 20 mg/L and 1500 U/L, respectively. Moreover, it showed a potential recycling efficiency for removing BPA in 10 continuous batch reactions with the retention of more than 70% of its original activity after the last cycle. Under optimal immobilization and degradation environment, laccase immobilized on novel biopolymeric support, *Hippospongia communis* spongin-based scaffold, efficiently removed bisphenol F, BPA, and bioremoval-resistant bisphenol S from the solution. The scaffold-immobilized biocatalyst removed 100% of bisphenol F and BPA, and higher than 40% of bioremoval-resistant bisphenol S. It also presented an excellent repeatability and storage ability, preserving more than 80% of its original biocatalytic performance after 50 days of preservation (Zdarta et al., 2018).

4. Conclusions

In this investigation, laccase enzyme was immobilized on glutaraldehyde-cross-linked chitosan beads with a high immobilization efficiency of 84.7%. Effective incorporation of the enzyme on chitosan biopolymeric network was confirmed by SEM analysis. With regard to free enzyme, the immobilized laccase showed enhanced storage stability and substrate-oxidation potential in a repeated batch system. Furthermore, the developed biocatalysts presented a good performance for the degradation and removal of bisphenol A from an aqueous solution. In conclusion, the proposed immobilization approach has led to develop immobilized laccase with improved catalytic features and a promising candidate for elimination of bisphenol A from the aqueous solution.

Conflicts of interest

Authors declare that they have no conflict of interest.

Acknowledgments

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