

DOI: <http://dx.doi.org/10.21123/bsj.2020.17.3.0720>

Decolorization of Phenol Red Dye by Immobilized Laccase in Chitosan Beads Using Laccase - Mediator - System Model

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Received 25/7/2018, Accepted 11/4/2019, Published 1/9/2020



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Abstract:

This work describes the enhancement of phenol red decolorization through immobilizing of laccase in chitosan and enzyme recycling. Commercial laccase from white rot fungus, *Trametes versicolor* (Tvlac), was immobilized in to freshly prepared chitosan beads by using glutaraldehyde as a cross linker. Characterization of prepared chitosan was confirmed by FTIR and scanning electron microscope (SEM). Tvlac (46.2 U/mL) immobilized into chitosan beads at 0.8 % glutaraldehyde (v/v) within 24 hrs. Synthetic (HBT) and natural (vanillin) mediators were used to enhance dye decolorization. It was found that 89 % of phenol red was decolorized by chitosan beads within 180 min. in the absence of enzyme and mediator, while decolorization percentage of the dye was completed (100%) at 120 min. when chitosan immobilized laccase was applied. Moreover, the decolorization was completed within 25 and 50 min. in the presence of chitosan immobilized laccase and of HBT or vanillin respectively. On the other hand, the recycling of chitosan immobilized laccase was still decolorize phenol red and continued up to ninth cycle to reach 70% of dye decolorization .

Key words: Chitosan, Decolorization, Immobilize, Laccase, Mediators, Phenol red.

Introduction

Fungi from the Basidiomycetes, known as white rot fungi, are a heterogeneous group of microorganisms that are able to degrade a wide variety of recalcitrant pollutants, including various types of dyes. Laccase based decolourisation treatments are potentially preferable to bioremediation technologies since the enzyme is produced in larger amounts. Laccases belong to the group of phenol oxidases; these copper containing enzymes are oxidative enzymes detected in many plants but mainly produced by numerous fungi (1). Wastewater cleared from textile and dye stuff industries therefore have to be treated due to rising communal worry over their harmfulness, carcinogenicity and impact on water bodies (2). The many changed and complex molecular structures of dyes, however, make dye wastewater difficult to be treated by conventional biological and physico-chemical processes and, therefore, novel treatment technologies requisite to be investigated. (3). Laccase Mediator Systems (LMS) have been extensively studied in latest years, with three types

of mediators having been proposed, and with the NOH-type and phenol-type having been originate to be active in the decolourisation of dyes. Furthermore, a laccase-mediator system has been used in decolourising indigo carmine. Mediators might be added to the substrates of interest to overcome eventual limitations. Chemical and natural mediators work as intermediate substrates for laccase. Thus, the mediators are oxidized and the formed radicals can interact with the bulky or high redox potential substrate hereby widening the substrate range of laccase (4). The several changed and complex molecular structures of dyes, however, make dye wastewater problematic to be treated by conventional biological and physico-chemical processes and, therefore, advanced treatment technologies need to be investigated. (5). Chitosan, is the natural basic polysaccharide, and involves wide care from medicinal researchers because of its respectable biocompatibility and low toxicity (6). Through using chitosan to combine cells or enzymes, greater enzyme action can be booked (7).

While entrapment is comparatively low-cost and simple, the provision swells and cracks easily, foremost the leak of the cells and enzymes, thus lessening the enzyme activity and immobilization efficiency (8). That cross linker can upsurge the strength of the provision by creating three-dimensional grid structure which is the most widely used cross linker (9). There are also some problems: the response circumstances are comparatively problematic to control in cross-linked provision preparation, and the cross linking reaction frequently reason structural denaturation of enzyme molecules resultant in harmful effects for active places (10). The immobilization of laccase onto chitosan beads to improve its ability to damage synthetic dyes; immobilized Tvlac was evaluated by squalor of many synthetic dyes (11). The present work aims to use freshly prepared chitosan beads to entrap laccase from *T. versicolor* after characterization by FTIR and SEM. The chitosan immobilized laccase was applied in the decolorization of phenol red dye in the presence of natural and synthetic mediators. Moreover, the number of recycling of chitosan beads immobilized laccase was investigated.

Materials and Methods:

Materials

Chitosan of low molecular weight was supplied by ABCO Laboratories (Eng. Ltd., Gillingham, England). Commercial laccase from *T. versicolor*, 1-hydroxybenzotriazole (HBT) and vanillin were provided by Fluka Chemicals Company (Switzerland). Glutaraldehyde and phenol red dye were purchased from Aldrich.

Chitosan Beads Preparation

One gram of chitosan powder was dissolved in 40 mL of acetic acid (1 % v/v). The solution is stirred for 1 hr till a homogenous solution is obtained. The subsequent solution was poured drop wise into a coagulant batch of 2M NaOH to produce sphere-shaped beads. Then, the beads of 1- 2 mm in diameters were collected by filtration process. Thereafter, the collected beads were washed several times with deionized water till impartiality is

accomplished, and dried at room temperature to constant weight.

Immobilization of Laccase in Chitosan Beads

Glutaraldehyde was used as a crosslinker for Laccase immobilization. One gram of glutaraldehyde-crosslinked chitosan beads was mixed with 2 ml of 46.2 U/ml laccase and shake at 150 rpm for 24 hrs. at room temperature. Then, the beads were washed with deionized water and dried at room temperature, and stored in dry conditions until use.

Decolorization Phenol Red in the LMS Model

Chitosan immobilization laccase was used to decolorize 300 μ M phenol red prepared in acetate buffer Ph 4.6. Chitosan beads (250 mg) were immersed in 5ml of phenol red in the presence of 100 mM HBT or vanillin as synthetic and natural mediators. The beads were incubated at 25 °C for 30,60,90,120,180,210 and 240 min. with stirring (150 rpm). Decolorization percentage is determined as in the following equation[1] .

$$\text{Decolorization \%} = \frac{A_i - A_o}{A_i} * 100\%$$

Where A_i is the initial absorbance of the synthetic dye, and A_o is the final absorbance of the dye after decolorization.

FTIR Analysis

Fourier transformation of infrared (FTIR) spectra investigated were measured by Perkin-Elmer Fourier transformation of infrared spectrophotometer ((model 2000) over the wavenumber range of 400-4000 cm^{-1} .

Scanning Electron Microscope (SEM)

The diameter and morphology of chitosan beads were examined by scanning electron microscopy (SEM) (Inspect S50, Holand). Sample was dispersed on glass slide and silver paste used as filament. Then viewed using an accelerating voltage of 15 kilovolt at different magnifications.

Results and Discussion

Preparation and Characterization of Chitosan

The results obtained show that the chitosan beads used as natural polymeric resins perform well with glutaraldehyde as cross linker and laccase enzyme to removing phenol red dye. (Fig .1) explains the stages of prepare the beads laccase entrapment.

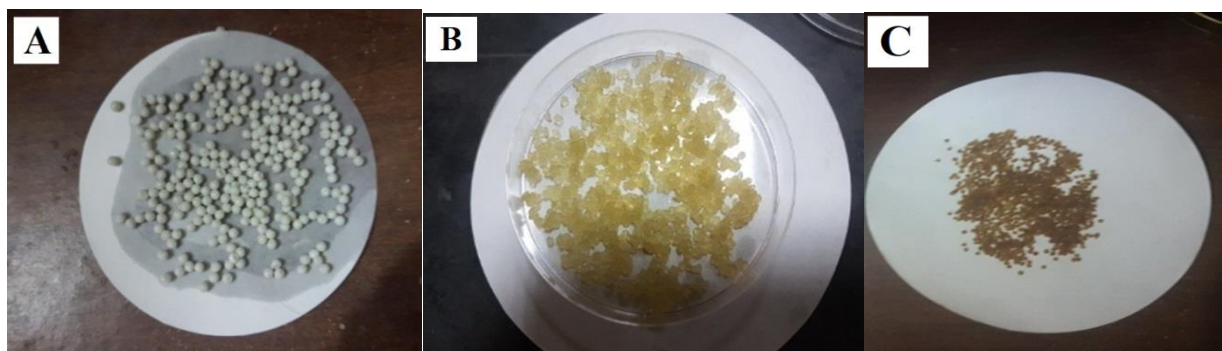


Figure 1. Stages of Prepare Beads Laccase immobilized : (A) Chitosan Beads; (B) Chitosan Beads with Glutaraldehyde and (C) Chitosan Beads Laccase immobilized.

Chitosan beads surface was also observed by SEM. The images present beads with regular sphere-like in shape and approximate diameter of 1mm (Fig.2) The surface of chitosan beads was

more smooth and more regular, whereas the surface of chitosan laccase enzyme beads was rough and irregular.

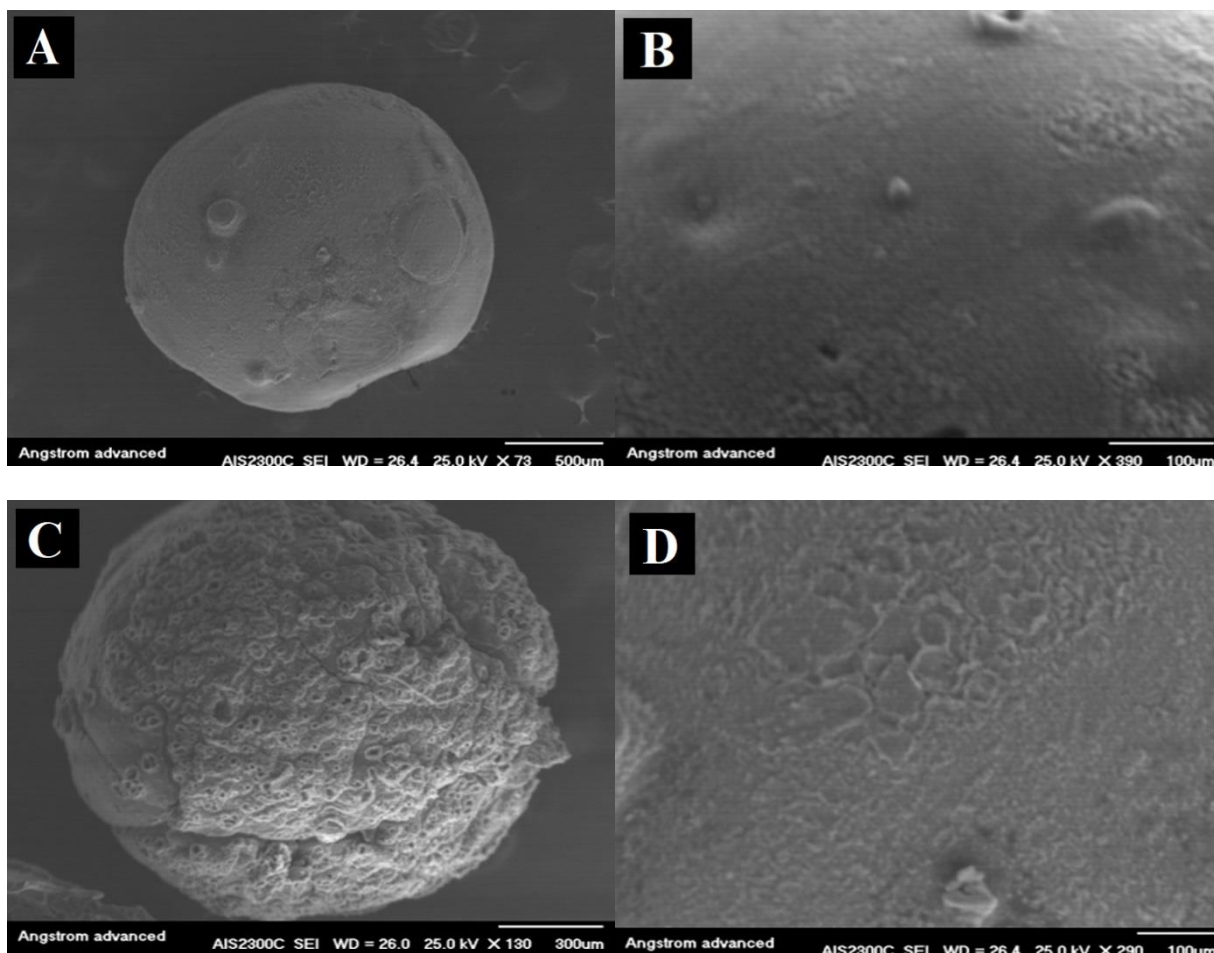


Figure 2. SEM Images for : (A) Chitosan Beads; (B) Surface of Chitosan Beads; (C) Chitosan Beads Immobilized Laccase and (D) Surface of Chitosan Beads Immobilized Laccase Enzyme.

Moreover, chitosan FTIR spectra before and after cross-linking with glutaraldehyde and chitosan immobilized laccase were determined (Fig 3.A). Chitosan as an immobilized carrier contains an amino group in its structure and is produced by the deacetylation of chitin. Glutaraldehyde reacts with the amino group of chitosan to form a Schiff

base. Generally, the reaction process between the amino group and an aldehyde group is modified by the effect of the electrophilicity of the carbonyl group in the aldehyde. The results show that the chitosan spectra include 1651cm^{-1} band, which corresponds to (-NH_2 in plane) bending vibrations. The spectrum of glutaraldehyde pretreated chitosan

shows a peak at 1657 cm^{-1} can be attributed to an imine bond ($\text{N}=\text{C}$) and the second at 1599 cm^{-1} is associated with an ethylenic bond ($\text{C}=\text{C}$). These results proved overlapping matching to $-\text{NH}$ and $-\text{C}=\text{N}-$ extending in the newly shaped Schiff base (Fig. 3.B). On the other hand, the carbonyl peak at 1599 cm^{-1} disappears upon creation of the imine bond (Fig. 3.C). The formation designates the attendance of covalently-bound enzyme in the chitosan beads and that agrees with what is published in (11).

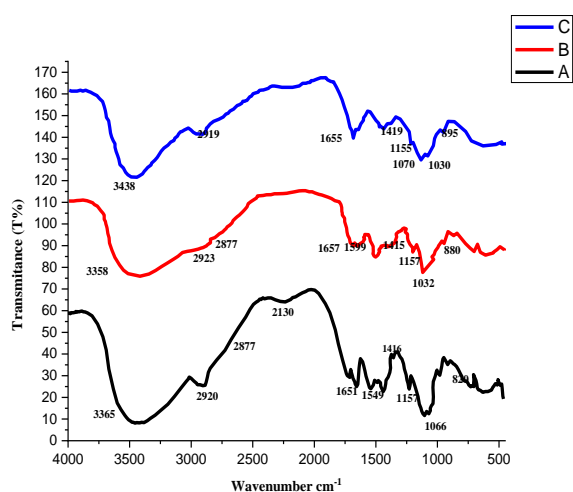


Figure 3. FTIR Spectra of : (A) Chitosan Beads; (B) Chitosan Beads with Glutaraldehyde and (C) Chitosan Beads Immobilized Laccase Enzyme.

Decolourization of Phenol Red Dye

Decolorization percentages of phenol red by chitosan beads, chitosan beads immobilized laccase and chitosan beads immobilized laccase in the presence, as LMS model, or absence of two mediators were determined. The results showed that decolorization of dye by chitosan beads was 89% within 180 minutes. Chitosan is one of the world's most plentiful and low-cost biopolymers that possesses several properties as an ideal adsorbent for removing pollutants from wastewater. Originally, chitin was boiled in potassium hydroxide to produce an acid-soluble product called chitosan. An ideal adsorbent for dye removal possesses the following properties: large surface area and high adsorption capacity, has suitable pore size and volume, easy accessibility, cost effectiveness, mechanical stability, compatibility, ease of regeneration, environmentally friendly, high selectivity to remove a wide range of dyes and does not require high processing, while it reaches 100% within 120 minutes by chitosan immobilized laccase. The presence of (100) mM vanillin or mM HBT mM vanillin or mM HBT enhanced

decolorization by chitosan immobilized laccase to 100% within 50 and 25 minutes, respectively, when incubated at 30°C (Fig.4). Phenol red decolorization to some extents depends on the mediator types, while the elimination efficiency of the dye depended on its chemical structures (12). Phenol red exhibits a deliberate rate of oxidation by laccase. This is possibly because the phenol red molecule has three benzene rings and this brands it difficult for the enzyme to admit the molecular core. The use of laccase-mediator systems (LMS) can upsurge the oxidation rate by acting relatively like a vehicle shuttle to allow the enzyme to spread sites that might not otherwise be accessed, and the routes by which the mediators act for increasing the oxidation of recalcitrant compounds are either through electron transfer (ET) and hydrogen abstraction or through hydroxyl radicals and superoxide anion radicals.

Thus, decreasing the time for and improving the extent of the decolourisation (13).

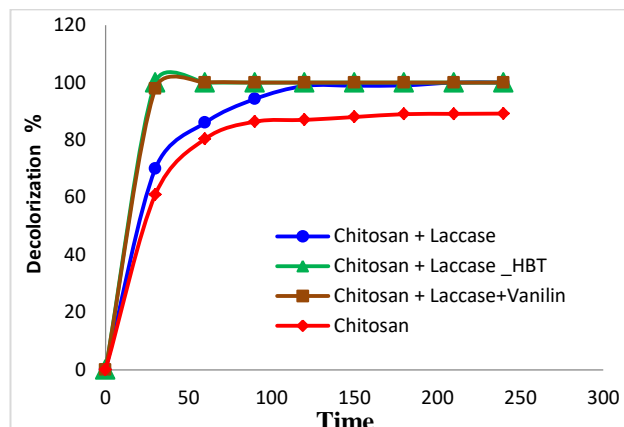


Figure 4. Decolourization of Phenol Red by Chitosan Immobilized Laccase in the LMS Model. Absorbance of Decolorization Determined by Spectrophotometer at 570 nm After Incubation at 30 C in $\text{pH } 4.6$.

Reusability of Immobilized Laccase on Chitosan Beads

Immobilization of laccase in chitosan beads were reused for (9) times and their phenol red decolorization against its initial decolourization efficiency was measured. This test was carried out by filtration of chitosan beads immobilized laccase after each cycle, followed by washing with 0.1M citrate phosphate buffer (4.6) and reintroducing into another fresh reaction medium. This procedure was repeated for 9 times. The laccase activity at optimum (4.6) and temperature 30°C at the first measurement was 100%. It was seen in (Fig. 5) the decolourization of laccase chitosan beads reduced in the number of cycle and reached to 70% at cycle number nine. The viability of restoration of laccase entrapment and frequent use is one of the most

important indicators for reduction. Rendering to the statistics of the reusability of laccase entrapment, afterward four cycles of continuous use, the relative activity of laccase was $\geq 80\%$ and it stayed $\geq 60\%$ after ninth cycle. The comparative activity immobilized laccase in chitosan beads decreases with increasing the use of, plummeting to about 70% of its original activity after 9 cycles. This reduction in enzyme action could be credited to inactivation and damage of enzyme molecules throughout process.

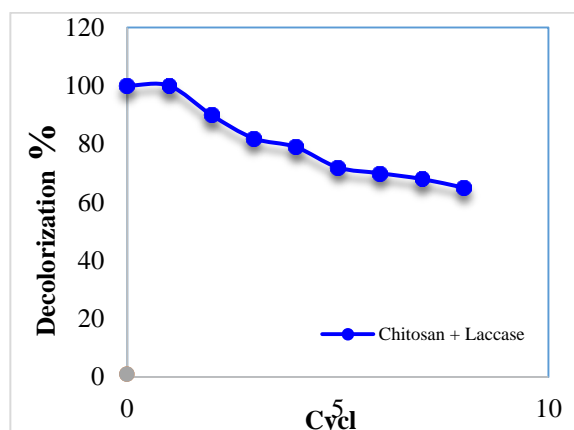


Figure 5. Decolourization of Phenol Red Correlation with Recycle Number of Chitosan Beads Immobilization with Enzyme Laccase and (HBT) Moderator.

Conclusion:

Immobilization of laccase in chitosan beads was performed in this study to be used in the decolorization of phenol red dye. The application of LMS enhanced the decolorization percentage and reduced the time of dye decolorization. No differences in the decolorization efficiency when natural and synthetic mediators were applied in the LMS model. Moreover, chitosan beads that immobilized laccase could be reused many times in the treatment of phenol red dye.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Technology.

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قصر صبغة الفينول الاحمر بواسطة اللاكبيز المقيد في حبيبات الكايتوسان باستخدام نظام (LMS) الوسيط

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الخلاصة :

يصف هذا العمل قصر الصبغة الحمراء من خلال تقييد انزيم اللاكبيز بالكايتوسان. يتكون انزيم اللاكبيز التجاري من فطر العفن الابيض (Tvlac) حيث يقيد بطريقة سهلة التحضير بحبيبات الكايتوسان المنشط بالكلوترالديهيد. لقد تم تعزيز توصيف الكايتوسان المحضر باستخدام تقنيات الـ FTIR والـ SEM. لقد قيد (46.2 وحدة/ مل) من انزيم Laccase تماما في حبيبات الكايتوسان المحضره بنسبة 0.8% (v:v) من الكلوترالدهيد خلال (24 ساعة، استخدمت العوامل الوسطية الصناعية (HBT) والطبيعية (vanillin) لتعزيز عملية قصر صبغة الفينول الاحمر من خلال انزيم اللاكبيز المقيد. حيث وجد ان عملية القصر لحبيبات الكايتوسان تصل الى قيمتها العظمى 89% خلال 180 دقيقة في حين تصل عملية القصر لحبيبات الانزيم المقيد بلكايتوسان الى (100%) خلال 120 دقيقة ولكن باستخدام HBT والـ vanillin كمواد وسطية تصل عملية القصر 100% في 25 دقيقة و 50 دقيقة على التوالي. ومن ناحية اخرى اعاد استخدام حبيبات الانزيم المقيد بلكايتوسان اعطت نتيجة قصر 100% عند اول دورة ومن ثم بدت تتناقص تدريجيا النتائج مع عدد الدورات الى ان تصل الى فعالية 70% بعد 9 مرات للإعادة الاستخدام في عملية قصر الصبغة.

الكلمات المفتاحية: الكايتوسان، قصر الصبغة، تحميل، انزيم اللاكبيز، المساعد، الفينول الاحمر.