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Counterfeit of Immobilized Enzymes in Packed Bed Reactor

Megala S[1], Priyadarsan S[2], Shreeprada A M[3], Swarupa Dhar[4], Rohith[5]

[1]Assistant professor, Dept of Chemical Engineering, MVJ College of Engineering, Bengaluru, Karnataka, India
[2345]UG student, Dept of Chemical Engineering, MVJ College of Engineering, Bengaluru, Karnataka, India

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Abstract - *Textile industrial effluents, contain high dye concentrations. Without effective treatment, the discharge of textile effluent to the environment affects aquatic life and makes water unfit for any kind of usage. Thus, the aim of this work is to develop an effective treatment method for industrial dye effluent. Adsorption is one of the most efficient techniques for decontaminating industrial dye effluent, and its performance is dependent on the adsorbent's characteristics. The development of immobilised laccase enzymes as adsorbents in a packed bed reactor is more economical, effective, and environmentally friendly.*

In the present study, laccase enzyme extracted from fungi was immobilized by entrapment method in sodium-alginate gel and cross-linked with copper sulphate pentahydrous. By determining the optimum operating conditions for reusability, flow rate, bed height, concentration, and pH, a batch decolorization of dye solution was performed in a packed-bed reactor.

The method led to very effective laccase immobilization and also imparted significant stability to the enzyme. Then immobilized laccase enzyme were characterized by BET and SEM, and the results showed that laccase could be well immobilized on sodium alginate and revealed that, the immobilized laccase enzyme beads exhibited noticeable levels of porosity. In a batch decolorization, 73-100% decolorization was achieved for the prepared dye solution. Crystal violet dye was found to be highly decolorized up to 100% followed by Methylene Blue (99.40%), Congo Red (98.18%), Rhodamine B (97.7%) and Methyl orange (92.73%).

Key Words: **Adsorption, Decolorization, Dye effluent, Immobilized Laccase Enzyme, Packed Bed Reactor**

1.INTRODUCTION

Textile processing is one of the oldest and most complex industry and its production has been improved due to continuous development and automation, causing a major pollution problem around the world. The rheology of wastewater discharged by textile mills is highly complex in nature, sometimes difficult for a specialist to grasp and understand it. Out of 70% total water phase across the world, only a negligible part of it is in pure form[1]. The main damage caused by the textile industry is due to improperly treated waste discharged into water bodies, which are responsible for 80% of the total emissions caused by the industry. Dye is the substance that is used to impart color to a substrate. Different types of dyes are used in the textile industry such as acid dyes, reactive dyes, basic dyes, and azoic dyes. One of its properties is the ability to impart color to a given substrate because of the presence of chromophoric groups in its molecular structures. Decolorization of dyes from textile effluents depend on enzyme origin, presence of other compounds in solution and also a process condition. Enzymes are biodegradable, do not produce toxic by-products, and exhibit high specificity towards target dyes, allowing for selective and efficient decolorization. Enzymes such as laccases, peroxidases, azoreductases, and lipases are commonly used to decolorize dyes. Additionally, enzymes can improve the overall performance of dye decolorization processes, resulting in higher color removal efficiency and improved wastewater quality. Compared to free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes. Enzyme immobilization refers to the process of attaching or confining enzymes to a solid support matrix, thereby enhancing their stability, activity, and reusability. Immobilized enzymes have numerous applications in various fields, including biocatalysts, biofuel production, pharmaceuticals, food processing, and environmental remediation. In recent years, a wide range of interest and high attention has been directed toward exploring the potential of immobilized enzymes. The enzyme immobilization methods can be classified according to the interaction between the enzyme and the support matrix, such as adsorption, entrapment, encapsulation, covalent bonding etc.

1.1 Packed Bed Reactor

The operation of a packed bed reactor involves passing the wastewater through the packed bed, where the solid particles serve as an adsorbent for the decolorization process. The design and operation of a packed-bed reactor for dye decolorization depend on various factors, such as the type and concentration of dyes, particle size and shape, flow rate, and contact time. These parameters play a major role in reactor efficiency. Optimal conditions need to be determined through experimental studies and process optimization.

2. MATERIALS AND METHODS

2.1 Materials

A fungi-based laccase enzyme was purchased from the vendor. Textile dyes, Sodium alginate, and all other chemical reagents were obtained from the Chemical Engineering Department at MVJ College of Engineering, Bengaluru.

2.2 Selection of Enzyme

Table -1: Properties of the Enzyme

Sl. No	Properties	Azoreductases	Peroxidases	Lipase	Laccase
1	Max. Velocity (Vmax) µM/sec	10.6	2.16	0.7	1527.7
2	Michaelis Menten constant (Km) µM	125	0.27	1.25	0.8158
3	Temperature °C	55	60	40	65
4	Molecular weight kDa	20	44	50	50-140
5	Flow rate ml/min	1.5	0.5	0.5	$8.3x10^{-3}$
6	pH	5-7	5-10	4.5-7.5	5.5-7.5

Based on the observation table 1, laccase exhibits the highest Vmax among all the other enzymes. Vmax is inversely proportional to Michaelis Menten Constant (Km), and laccase also demonstrates a low Km value. This indicates that laccase tightly binds to the substrate's active site compared to the other enzymes. Laccase also displays superior temperature stability, with a maximum temperature of 65°C at which it remains highly active. However, its activity decreases beyond this temperature, and it becomes unstable after reaching 80°C.

With a molecular weight of 50-140 kDa, laccase ensures stability and efficiency. The flow rate required is also low, and it works efficiently within a pH range of 5.5-7.5, indicating its suitability for neutral conditions. In conclusion, laccase emerges as the most efficient and effective enzyme for immobilization in our project due to its highest Vmax, low Km value, and high temperature stability. Immobilization can further enhance its activity at higher temperatures [2, 3, 4, 5]

2.2 Immobilization of Laccase Enzyme by Entrapment Method

The Entrapment of laccase in alginate gel was performed by adding 1 gramme of laccase enzyme to a 3% sodium alginate solution under continuous stirring at room temperature for one hour, where the formation of the alginate-laccase enzyme mixture takes place. The sodium alginate solution was prepared by adding 3 grammes of alginate powder to 100 ml of distilled water. The higher the concentration of sodium alginate, the smaller the pore size of the beads, leading to lower immobilization efficiency. The formed alginate-laccase enzyme mixture was taken in a syringe and then added dropwise into chilled 0.2M $CaCl_2$ and $CuSO_4.5H_2O$ solutions, respectively. The process is called cross-linking, and the resulting beads were left to harden overnight at 4 to 10 °C. The beads were washed several times with deionized water to remove any unencapsulated enzyme and then stored at a cold temperature[6].

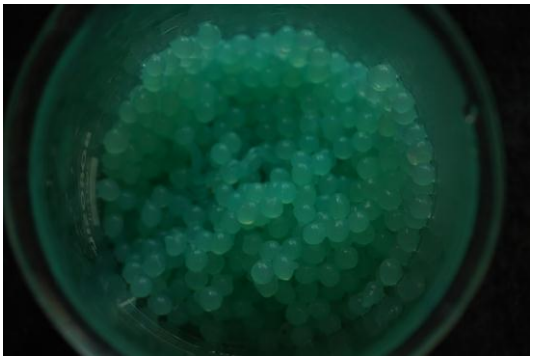

Fig -1: Immobilized laccase enzyme

2.3 Preparation of Stock Solution

A 500 ml stock solution of known concentration (50 ppm) was prepared by dissolving 25 mg of dye in 500 ml of distilled water. Then, a 30-ppm dye solution was prepared by adding 60 ml of the stock solution to a 100 ml standard flask and diluting it up to the mark.

2.4 Characterization of Immobilized Laccase Enzyme

To investigate the optimal conditions for the treatment of dye solution using immobilized laccase enzyme in a packed bed reactor, a study was conducted. The study focused on various parameters and their efficiency in dye decolorization. Specifically, their recyclability, morphological characteristics, surface area analysis, and the effects of concentration, pH, bed height, and flow rate were analyzed.

2.4.1 Effect of Flow Rate

The adsorption performance was investigated for pre-planned flow rates of 3, 6, 9, 12, and 15 ml/min, using a centrifugal pump with a variable flow resistor. The objective was to observe the effect of these flow rates on the adsorption process. Throughout the experiment, the bed height was maintained at approximately 10 cm, while the inlet concentration remained constant at 30 ppm

2.4.1 Effect of Bed Height

In order to assess the impact on the adsorption performance, the study investigated for different bed heights of 5.0, 7.5, and 10.0 cm. The aim was to observe how these varying bed heights affected the adsorption process. During the experiment, the flow rate was maintained at approximately 3 ml/min, while the inlet concentration remained constant at 30 ppm.

2.4.3 Effect of Inlet Concentrations

To analyze the sway on the adsorption performance, the study explored various inlet concentrations of 5, 10, 15, 20, 25, 30, 40, 50 ppm. The objective was to observe how these different inlet concentrations affected the adsorption process. Throughout the experiment, the bed height was maintained at approximately 10 cm, while the flow rate remained constant at 3 ml/min.

2.4.4 Effect of pH

In order to assess the impact of pH on the adsorption performance of immobilized laccase enzyme, the research examined the pH of the dye solution by employing various buffer tablets to adjust it towards both the alkaline and acidic ends of the spectrum. During the experiment, the flow rate was maintained at approximately 3 ml/min and a constant bed height of 10 cm, while the inlet concentration remained constant at 30 ppm.

2.4.5 Reusability Test

The reusability of immobilized enzyme was measured by using the same immobilized enzyme for nine cycles of 100 ml each. Throughout the experiment, the flow rate was maintained at approximately 3 ml/min and a constant bed height of 10cm, while the inlet concentration remained constant at 30 ppm. The immobilized enzyme was removed and washed with buffer tablets of pH (phosphate and acetic buffer), and then the immobilized enzyme was transferred to a fresh 0.2M copper sulphate pentahydrate solution.

2.5 Batch Decolorization of Dye solution in Packed Bed Reactor by an Immobilized Laccase Enzyme

A batch decolorization of a dye solution was performed in a packed-bed reactor with the aid of a dropping funnel with a height of 13 cm and an internal diameter of 2.4 cm. The adsorbent (immobilized laccase enzyme) was packed in the reactor with glass wool at various stages to prevent the loss of adsorbent during the process. The adsorbent and glass wool were arranged alternatively (Glass wool-beads-Glass Wool-Beads-Glass wool). During the experiment, the bed height was consistently maintained at approximately 10 cm. The initial absorbance of the dye was measured at its respective wavelength and recorded for each dye using a bio-photometer. The dye solution was pumped into the reactor using a centrifugal pump, with the flow rate set at approximately 3 ml/min. A constant bed height of 10 centimeters was maintained. The inlet concentration of the dye was kept constant at 30 ppm in an alkaline condition. After treatment, the decolorized water was filtered using Whatman filter paper, and the final absorbance was determined. The percentage of removal efficiency for the dye was calculated based on the difference in absorbance at λ max, according to [7].

$$\text{Decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Fig -2: Experimental setup

3. RESULTS AND DISCUSSION

3.1 SEM Analysis

The immobilized laccase enzyme beads were morphologically analyzed using SEM, with an image scale bar ranging from 1millimeter to 2 micrometers and a magnification range of 27 to 9000. The morphological analysis revealed that the immobilized laccase enzyme beads exhibited a spherical shape and exhibited noticeable levels of porosity and binding sites on their surfaces.

3.2 BET Analysis

The surface areas of the immobilized laccase enzyme beads were measured at 77.3K using a Nova Station B instrument. The surface area was calculated based on the BET nitrogen adsorption isotherm. The BET analysis shows that the immobilized laccase enzyme beads have a surface area of 5.661 m^2/g, a pore volume of 0.007 cc/g, and a pore diameter of 2.571 nm.

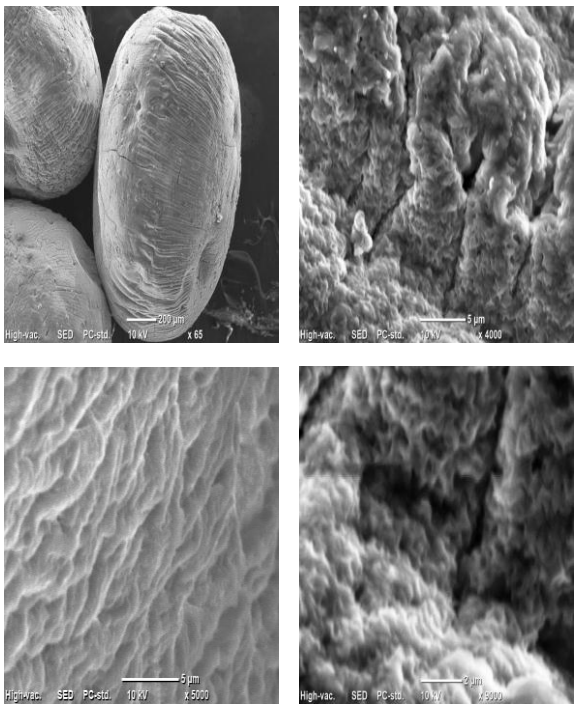

Fig -3: SEM images of immobilized laccase enzyme

3.3 Effect of Flow Rate

The effect of varying flowrate was examined with a bed height of 10cm and an initial concentration of 30 ppm. the result obtained are displayed in chart 1. At the lowest flow rate of 3 ml/min, the decolorization efficiency was found to be the highest for crystal violet, achieving a complete decolorization of 100%. Following crystal violet, Rhodamine B showed a decolorization efficiency of 99.19%, Methylene Blue exhibited a decolorization efficiency of 98.76%, Congo Red demonstrated a decolorization efficiency of 98.06%, and Methyl Orange achieved a decolorization efficiency of 97.91%. The percentage of decolorization decreased as the flow rate increases to 6, 9, 12, and 15 ml/min. The results indicate that reducing the flow rate in the packed-bed reactor increases the contact time between the immobilized laccase enzyme and the dye. This extended contact time at lower flow rates enhances the efficiency of the enzymatic degradation process, leading to improved decolorization outcomes.

3.4 Effect of Bed Height

A study was conducted on the effects of different bed heights (5 cm, 7.5 cm, and 10 cm) in a packed bed reactor. the result obtained are displayed in chart 2. The study maintained a constant flow rate of 3 ml/min and an inlet concentration of 30 ppm. It is observed that an increase in bed height results in more adsorbent in the reactor, which leads to higher outlet flow resistance. When the bed height is increased from 5 cm to 10 cm, the removal percentage of dye shows improvement in decolorization. The decolorization efficiencies of different dyes exhibited notable improvements: Congo Red's efficiency increased from 86.24% to 98.06%, Crystal Violet's efficiency increased from 95.07% to 100%, Methyl Orange's efficiency increased from 72.19% to 93.29%, Methylene Blue's efficiency increased from 88.47% to 98.76%, and Rhodamine dye's efficiency increased from 82.56% to 97.41%.

3.5 Effect of Inlet Concentration

Eight different initial feed concentrations (5, 10, 15, 20, 25, 30, 40, and 50 ppm) of dye solution were used to evaluate the effect of initial inlet concentration where the bed height and flow rate were kept constant at 10 cm and 3 ml/min, respectively. The results obtained are shown in chart 3. It is observed that as the concentration increased, the efficiency of decolorization was reduced to 74.28% against Methyl Orange, followed by Rhodamine B (80.64%), Methylene Blue (86.71%), Congo Red (89.46%), and Crystal Violet (98.05%). The result indicates that the concentration directly affects the removal efficiency, leading to a reduction in adsorption capacity.

3.6 Effect of pH

The effect of varying pH was examined with a bed height of 10 cm, a flow rate of 3 ml/min, and an initial concentration of 30 ppm. The results obtained are displayed in chart 4. It shows that the dye solution was effectively decolorized in all pH variation. However, the highest efficiency in decolorization of dyes is observed under alkaline conditions except Rhodamine B dye. In acidic conditions, the dye solutions negatively impact the activity of the immobilized

laccase enzyme, leading to decreased reusability and reduced shelf life.

3.7 Reusability Test

Testing the reusability of immobilized enzymes is important in any industry because it may reduce the cost of using enzymatic processes. The results obtained are displayed in chart 5. It was observed that enzymes retain their activity after repeated cycling. After the fifth cycle, the beads started reducing their efficiency of decolorization.

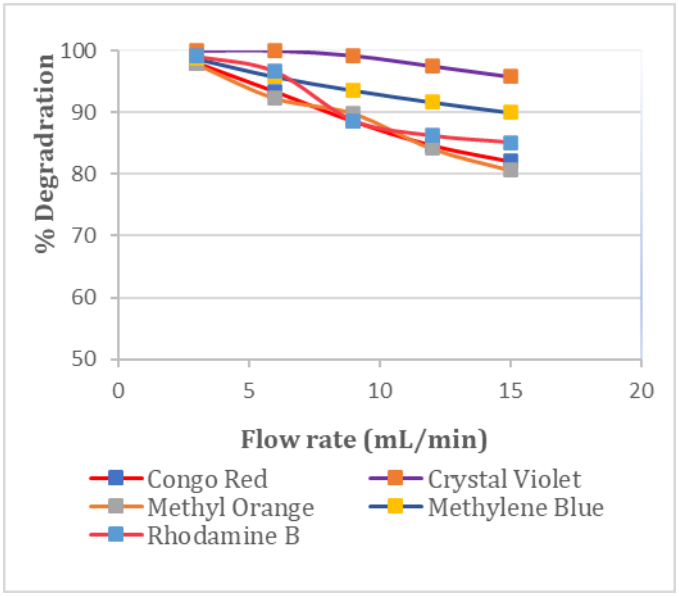

Chart -1: Effect of different flow rate

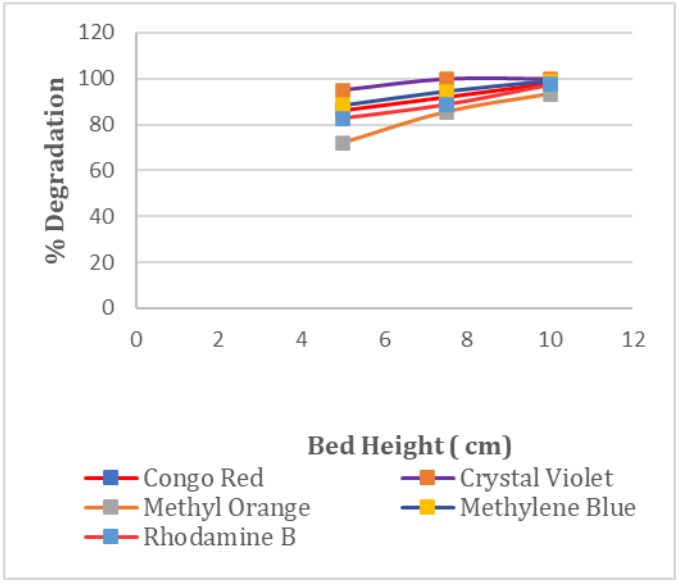

Chart -2: Effect of different bed height

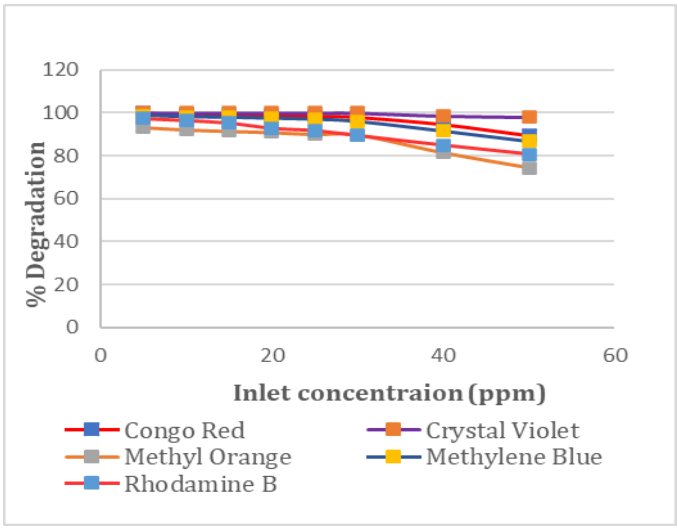

Chart -3: Effect of different inlet concentration

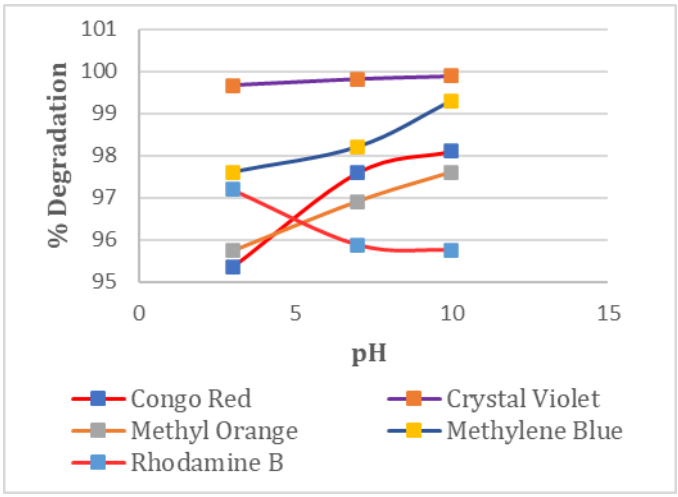

Chart -4: Effect of different pH values

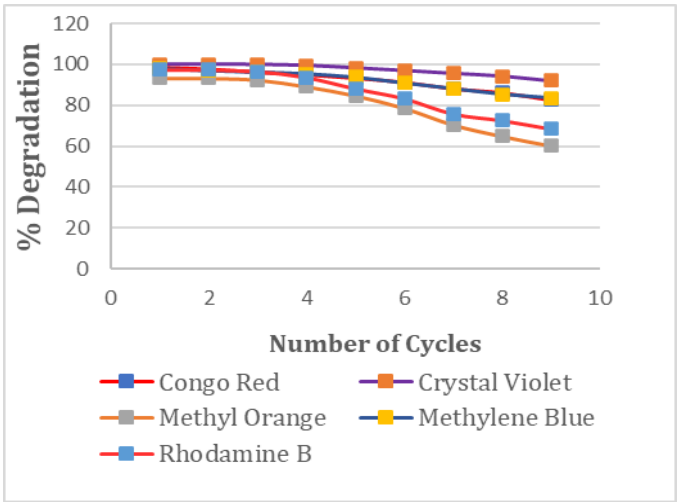

Chart -5: Effect of repeated uses of immobilized laccase

3.8 Batch Decolorization of Dye solution in Packed Bed Reactor by an Immobilized Laccase Enzyme

By considering various parameters such as flow rate, concentration, pH, bed height we conducted an experimental study. Throughout the experiment, the flow rate was maintained at approximately 3 ml/min and a constant bed height of 10cm, while the inlet concentration remained constant at 30 ppm in alkali condition. Laccase immobilized using sodium alginate demonstrated the highest decolorization efficiency of 100% for crystal violet dye, followed by 99.40% for methylene blue, 98.18% for Congo Red, 97.7% for Rhodamine dye, and 92.73% for methylene orange during the first cycle. It is noteworthy that crystal violet and methylene blue, which are classified as basic dyes and have minimal reactivity in the environment, exhibited comparable decolorization efficiencies during subsequent cycles. Conversely, Congo Red, Rhodamine B, and Methyl Orange, which are toxic dyes with high water solubility, displayed lower decolorization efficiencies with each successive cycle, indicating a rapid decline in their reusability.

Table -2: Decolorization efficiency of different dye solutions by an immobilized laccase using batch decolorization in a packed bed reactor

Sl. No	Dye solution	Wave length	Initial Absorbance	Final Absorbance	% Decolorization
1	Crystal violet	595	0.074	0.000	100
2	Methylene blue	550	0.674	0.004	99.40
3	Congo red	550	0.215	0.039	98.18
4	Rhodamine b	550	2.957	0.067	97.7
5	Methyl orange	490	0.798	0.058	92.73

4. CONCLUSION

This work investigated the effectiveness of an immobilized laccase enzyme, obtained from a vendor, for the batch decolorization of dye solution in a packed-bed reactor. The experiment showed that the adsorption process of immobilized laccase enzyme depends on flow rate, bed height, initial dye concentration, and pH condition. The experiment demonstrated that a decrease in flow rate, a higher bed height, a lower initial dye concentration, and alkaline pH conditions favour the adsorption process of the immobilized laccase enzyme. During the experimentation, it was observed that the decolorization process required a significant amount of time, additionally, maintaining a low flow rate and concentration was necessary. As an increase in these parameters negatively affected the decolorization efficiency. To overcome these challenges and enable large-scale decolorization in industrial settings, it is proposed to combine the immobilized laccase enzyme with a photochemical reactor. This integration would result in reduced treatment time, improved flow rate, increased adsorption capacity, enhanced recyclability and reusability, and an extended shelf life of the immobilized laccase enzyme.

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