DEGRADATION OF DYES CONTAMINATED AN AQUEOUS SOLUTION USING LACCASE SUPPORTED ON LOCAL PORCELAINTE

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ABSTRACT

This research explores an efficient decolorization approach of aqueous solution containing Reactive Navy Blue (RNB), Reactive Red (RR), Reactive Turquoise Blue (RTB), and Reactive Black (RB) dyes by employing laccase immobilized on granular Porcelanite (PC). The process of immobilizing the laccase enzyme from Fenugreek seeds onto Porcelanite was achieved through a covalent method and the immobilization ratio was reached to 98%. PC function groups and surface texture was examined using Scanning electron microscope (SEM) and Fourier transform infrared (FT-IR) techniques. The impact of several process parameters, including pH, particle amount, dye concentration, and temperature, were investigated. The treatment successfully led to maximum decolorization rates of 95.50% for RB, 90.19% for RNB, 85.63% for RTB, and 80.82% for RR after only 24 hours, in which the best conditions were (5, 1.5 g, 50 mg/l,25 ◦C, respectively) . Interestingly, the coefficient of determination (R^2) for RB, RNB, RTB, and RR dyes was 87%, 82%, 87%, and 72%, respectively, indicating high model predictivity of the behavior of dyes post-decolorization, indicating enhanced wastewater remediation via laccase immobilization.

Keywords: support materials, enzyme immobilization, laccase, porcelanite.

تحلل الأصباغ الملوثة للمحاليل المائية باستعمال اللاكييز المقيد على البورسلين المحلي

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المستخلص

يهدف هذا البحث لايجاد طريقة فعالة لإزالة اللون من المياه الملوثة بالاصباغ مثل الأزرق الداكن التفاعلي (RNB)، والأحمر التفاعلي (RR)، والأزرق الفيروزي التفاعلي (RTB)، والصبغة السوداء التفاعلية (RB) عن طريق استعمال اللاكييز المثبت على البورسلين الحبيبي (PC). تم تحقيق عملية تثبيت إنزيم اللاكييز المستخلص من بذور الحلبة على البورسلين من خلال طريقة التثبيت التساهمية وبلغت نسبه التقييد 98%. تم تشخيص البورسلين الحبيبي وملمس السطح باستخدام تقنيات التحليل الطيفي للأشعة تحت الحمراء FTIR والفحص المجهري الإلكتروني SEM. درس تأثير العديد من المتغيرات على عملية الازالة، مثل الرقم الهيدروجيني للمحلول وكمية حبيبات البورسلين المضافة وتركيز الصبغة ودرجة الحرارة. بينت النتائج الى زيادة معدلات إزالة اللون بنسبة 95.50% لـ RB، و90.19% لـ RNB، و85.63% لـ RTB، و80.82% لـ RR بعد 24 ساعة من الخلط حيث كانت أفضل الظروف هي 5، 1.5 غم، 50 ملغم/لتر، 25 على التوالي. ومن المثير للاهتمام، أن قيم معامل الارتباط(R^2) للأصباغ RB وRNB وRTB وRR كان 87%، 82%، 87%، و72% على التوالي، مما يشير إلى تنبؤ نموذجي عالي لسلوك الأصباغ بعد إزالة اللون، مما يشير إلى تحسين معالجة مياه الصرف عن طريق تثبيت اللاكييز.

الكلمات المفتاحية: المواد الداعمة، تثبيت الإنزيم، اللاكييز، البورسلين.

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INTODUCTION

The growing industrial production approaching 70 million tons annually leads to high synthetic or azo dyes contamination causing severe water pollution (18,50). Due to their necessity in production, these xenobiotic chemicals are extensively employed across numerous industries, such as paper, textiles, food consumables, plastics, cosmetics, and candles (8). Even though industries have made efforts to integrate wastewater treatment processes into their production cycles, it is concerning that around 90% of textile dyes processed in sludge sewage treatment plants remain non-degradable (1). Consequently, an alarming quantity beyond 150 thousand tons of dyes is discharged without proper treatment causing severe risk to the ecosystems annually (50). Various water treatment methods are used to filtrate effluents containing phenolic or azo contaminants, such as adsorption, precipitation, agglomeration/sedimentation flotation, chlorine decontamination, membrane filtration, bio flocculation, ion pair extraction, electrolysis, ultrasonic mineralization, ion exchange, sonication, photocatalysis, ozonation, and advanced oxidation processes (4,18,50). However, these processes often present challenges regarding operational complexity, reliance on costly feedstocks, requirement of sophisticated instrumentation and control schemes, limited versatility, and susceptibility to other wastewater pollutants. They can also lead to the formation of genotoxic or dangerous byproducts (26,41). Treatments with enzyme-based biocatalysts have emerged as an attractive alternative that degrades synthetic dyes without introducing toxicity and are used to overcome current limitations of treatment methods. Enzyme-based biocatalysts can process distinctive pollutants, reduce treatment time, and improve cost-effectiveness (33). Laccase enzymes are commonly used for treating azo dyes (37). Their structure has four copper electrons, which allows them to oxidize phenolic compounds into easily disposed phenoxyl radicals (18, 28). Laccases catalyze diverse phenolic and non-phenolic aromatic substrates (39,49). However, laccase activity has several limitations, including high chemical sensitivity (resulting in low recovery from reaction media), instantaneous disposability (raising concerns about long-term stability and suitability for large-scale treatments), and reduced activity in harsh environments (28,49). Interestingly, immobilizing enzymes on substrates makes them an excellent solution with improved stability and reusability (11,16). Minerals are used to support the development of biocatalytic systems. Among many minerals used for enzyme immobilization, clay minerals, such as halloysite, bentonite, kaolinite, sepiolite and montmorillonite,, are mainly used (3,35). They are highly available, biocompatible, and require minor processing or purification (13). They have functional groups on their surface (i.e., COOH, -OH, -SH, -NH2, and C=O) that assist in forming covalent bonds with enzymes (25). Thus, laccases have better activity when immobilized on minerals using processes like adsorption, entrapment, encapsulation, and covalent attachment (13,37). Dye decolorization using laccase immobilizers on Iraqi Porcelanite Rocks has not been reported, which can be a great area to explore, which may lead to mitigating the problem of dye non-degradability in the Iraqi wastewater and reduce its risk to the overall ecosystem. The Historical data design of the Response Surface Methodology (HHD-RSM) is used to affords the researcher the opportunity to define the design points using all or some of the available experimental data, which is commonly used to improve the outcome of dye decolorization. HHD-RSM is commonly optimized to create quadratic models that present the statistical relationship among several variables by bringing together several mathematical and statistical methods. In particular, HHD-RSM helps choosing the ideal trial conditions of a system or deciding which area fulfills the operating requirements (46). In this paper, laccases were immobilized onto Iraqi PCs, and their ability to decolorize different types of dyes was studied. FTIR and SEM techniques were used to examine PC properties before and after immobilization (dye decolorization). The performance of the developed immobilizing laccase-based system (referred to as Porcelanite-Lac) was optimized using a Batch study. A polynomial relationship was established between all the variables to

investigate the primary objective of the removal efficiencies using RSM. Various statistical analyses were used to assess the best fit of the experimental data with the developed prediction model.

MATERIALS AND METHODS

Reactive dyes

Four dyes, Reactive Navy Blue (RNB), Reactive Turquoise Blue (RTB), Reactive Red (RR), and Reactive Black (RB), were provided by the Department of Dying and Printing, Al-Kut Textile Factory. 1 gm of each dye was dissolved in 1 litter of distilled water and diluted to the needed concentrations. At the ideal wave length for each dye, the absorbance of each was measured as shown in Table 1.

Table 1. Wave length of each dye

Type of dye	RB	RNB	RTB	RR
wave length (λ_{max})	597	596	667	517

Chemicals

Three buffer solutions, namely phosphate-buffered ($C_{l2}H_3K_2Na_3O_8P_2$, BDH, England), sodium acetate (99% $C_2H_3NaO_2$ BDH, England,) tris-base ($C_4H_{11}NO_3$, Hi Media, India), and o-tolidine,3-aminopropyltriethoxysilane,acetone, Gluteraldehyde were bought from Hi-media.

Extraction and preparation of laccase

Following the methodology reported elsewhere (40), Laccase was extracted from commercially available Fenugreek seeds. The enzyme demonstrated its highest specific activity, reaching 5340.38 units/mg of protein, when extracted using a sodium phosphate buffer with a concentration of 0.02 M and pH 8.0. The extraction process involved a weight-to-volume ratio of 1:40 and a duration of 210 minutes.

Laccase Assay

The activity of enzyme was measured by the method described by Kalral et al. (17), the activity of laccase was calculated using O-tolidine as a substrate source. Through a spectrophotometer, the oxidation of O-tolidine was detected via measuring the increase of absorbance at 366 nm (ε366 = 27,600 M-1 cm-1). One unit of enzyme activity was defined as the quantity of enzyme required to oxidize 1 μmol of substrate per minute. Protein concentration was analyzed according to the method described by Bradford (6).

Preparation of granular porcelanite

The General Company for Geological Survey and Mineralogy provided the samples used in this research. The porcelanite samples were crushed and milled before undergoing multiple washes with distilled water. Subsequently, they were dried in a drying oven (UNB 100, memmert, Germany) at 110°C for 24 hr to ensure complete moisture removal and achieve a constant weight. sieving, and the portion consisting of particles 75 μm was gathered for experimentation.

Evaluation of the adsorption capacity of PC before immobilization

When conducting decolorization studies using immobilized enzymes, it is crucial to evaluate the support material's adsorption capacity. Typically, color removal combines enzymatic and adsorption methods .This was accomplished by adding 1g of PC individually in all sampling of 100 ml volume, initial concentration (50mg/l) placed in 250 ml glass bottles and shaking at 200 rpm using orbital shaker (Rotamax, Heidolph, Germany) at room temperature for 24h. Samples were first centrifuged at 6500rpm for 5 minutes using a centrifuge (CAPP /model CRC-656 /Denmark) before being measured for their absorbance value at the maximum wavelength of each dye by single wavelength spectrophotometer (Thermo-genesys10UV,USA) (2). The percentage of discoloration after the adsorption process was defined as:

$$\text{Dye removal (\%)} = \frac{A_o - A_e}{A_o} * (100) \ldots (1)$$

Where A_0 and A_e are the absorbances before and after the adsorption process, respectively.

Laccase immobilization

The laccase's immobilization on PC was performed following a reported protocol (32,43) with slight modifications. Briefly, 5g of PC was boiled in 5M HNO_3 for 1 h with continuous stirring. HNO_3 was eliminated through centrifugate at 6500 rpm, and the resulting particles were thoroughly washed with distilled water- until the supernatant reached a pH of 6.0. Next, PC was activated by mixing at 45°C overnight with 5 ml of a 2% 3-aminopropyltriethoxysilane prepared in acetone. In the next step, the silanized particles were treated with 5 ml of a 5% glutaraldehyde

solution for 30 minutes. Excess glutaraldehyde was eliminated by subjecting the sample to a vacuum incubator overnight. Thorough rinsing with distilled water was performed on the particles. To complete the immobilization process, 10 ml of crude laccase (diluted at a 1:1 ratio, containing 55.05 mg of enzyme protein) was introduced to the particles and left at 4°C overnight. The resulting suspension underwent several rounds of washing with a buffer until no laccase activity was detected and named as PC-Lac. The activity of the immobilized enzyme was determined using the following equations:

Immobilized Enzyme activity=Total enzyme activity–non-immobilized enzyme activity …..(2)

Enzyme Immobilization (%) = (Immobilization enzyme units /Total enzyme units)×100 …….(3)

Batch study and parametric optimization

To optimize the process parameters, a series of experiments were carried out in batch placed in 250 ml glass bottles and shaking at 100 rpm with varying pH (2.0–10.0), particle amount of (0.5-2) g, dye concentration of (10-50) mg/L, and temperature (10–60^{o} C). One parameter was varied at a time, keeping all other parameters constant. All experiments were conducted at degradation time (24 hr) (24). The percentage of discoloration was calculated using Eq.(1).

Verifying performance of dye decolorization using the design of experiment (DOE)

For the verification of the dye decolorization process using PC-Lac, response surface methodology (RSM) and historical data design (HDD) on Design expert was used. The historical data fed into DOE was the same as the real experimental data, including four independent variables: pH, particle amount, initial dye concentration, and temperature. The corresponding values for these variables are presented in Table 2. The dye decolorization percentage was considered the dependent variable or response in this verifying study. By systematically varying the levels of the independent variables according to the HHD, the relationship between these variables and the decolorization efficiency of the dyes can be analyzed and optimized in building models.

Table 2. Variables and levels applied for the Porcelanite -laccase (Porcelanite -Lac) performance.

Variable	unit	-1	+1
pH	---	**2**	**10**
Particle amount	g	**0.5**	**2**
Concentration	**mg/L**	**10**	**50**
Temperature	C	**10**	**60**

Design Expert software (Version 13, Stat-Ease) was used to perform the experimental design and statistical analysis. Response Surface Method (RSM) was used to establish a polynomial relationship, enabling the consideration of the treatment process in the decolorization process. The proposed polynomial equation is expressed as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \sum_{i=1}^{k-1} \sum_{i=2}^{k} \beta_{ij} x_i x_j + e_i \quad \ldots (4)$$

In the given equation, the response variable is represented by Y. The term β0 serves as the intercept (offset), while the coefficients βi, βii, and βij correspond to the first-order, quadratic, and interaction effects, respectively. The indices i and j refer to specific parameter numbers, and the residual error is denoted by e_i.

RESULTS AND DISCUSSION

Evaluation of the adsorption capacity of PC before immobilization.

The efficiency of dyes removal using PC alone was tested to explore his capacity to adsorb the four dyes. This has been done to extinguish between adsorption and degradation. The maximum removal was 1.81, 9.44, 10.21, and 5.08% for RNB, RTB, RR, and RB, respectively, which is attained at 30 mg/L starting concentration. The dye adsorption on the support was equal to or less than 10% of the color removal. The results are shown in Figure (1). The PC showed low removal efficiency. The direct use of the raw materials as adsorbents has been found to be limited due to leaching of organic substances such as tannin, lignin, cellulose and pectin into the solution, and low surface area and consequently need chemical modification to enhance their capacities (27). Therefore, PC will be tested as supported for the enzyme to prepare them for degradations of the four dyes.

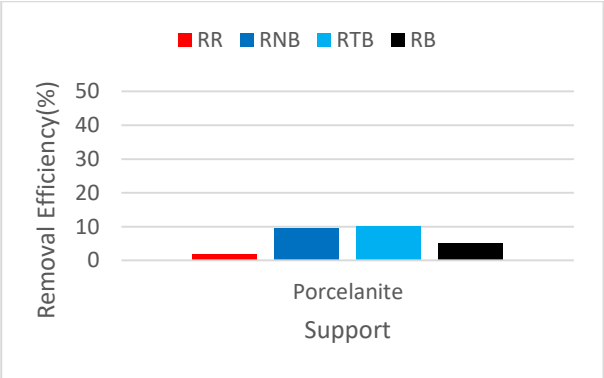

Figure 1. Removal efficiency of dyes using, PC for 3 hr , 200 rpm, initial concentration = 50 mg/L and 25°C

Immobilization of laccase by covalent methods: Crude fenugreek seeds laccase was immobilized by covalent using porcelanite. The immobilization ratio of the porcelanite reaching reached to 98%. This may be attributed to that; PC have the higher surface area. Therefore, the higher surface area of the support leads to higher enzyme loading on the support matrix (29). No previous studies have used porcelanite, this kind is low cost, easy availability, and the process is very simple, which makes it good for large-scale industrial applications.

Parameters optimization for porcelanite-Lac performance

The process parameters were optimized by changing one parameter while holding the others constant. The following experiments investigating different parameters were carried out to evaluate the dye removal performance of the immobilized enzymes of Porcelanite-Lac from simulated wastewater.

Effect of pH : To examine the pH influence on dye removal efficiency for RB, RNB, RTB, and RR, respectively, parameters like particle amount, temperature, contact time, initial concentration, and agitation speed were kept constant. These parameters were set at 1g, 24 hours, 25°C, 100 rpm, and 30 mg/L, respectively. However, the pH of the samples varied within the range of 2, 4, 5, 6, 8, and 10. To adjust the pH accordingly, 0.1N HCl and/or 0.1N NaOH solutions were employed, and a pH meter (WTW brand, Model 3110, Germany) was utilized for accurate measurements. The objective was to identify the optimum pH that yielded the highest dye removal efficiency. Once the optimal pH was determined through these experiments, it would be employed for subsequent investigations. The results are presented in Figure 2. The optimal pH value was 5 for all dyes, resulting in maximum removal efficiencies of 90.69%, 86.56%, 81.05%, and 76.07% for RB, RNB, RTB, and RR, respectively. The dye removal rate is known to decrease in extremely acidic or alkaline conditions, which disturb and inhibit laccase activity (44). Each immobilizing enzyme has an optimal pH that plays a significant role in their enzymatic reactions (20). The enzyme acid-base characteristics and the substrate also impact the relationship between the pH level and the activity of immobilized enzymes. Other factors may also affect immobilized enzyme activity, making it challenging to quantitatively analyze the relationship between pH and enzyme activity (19).

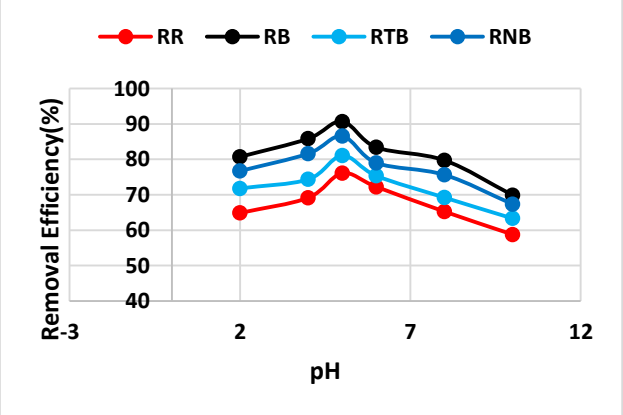

Figure 2. Impact of pH on dyes removal at particle amount = 1 g, initial concentration = 30 mg/L, 25°C, and 100 rpm for 24 hr.

Effect of particle amount

Different amounts of particles, specifically 0.5g, 1g, 1.5g, and 2g, were measured precisely using an electrical balance (Sartorius Lab - TE214S, Germany) to evaluate the impact of particle amount on dye decolorization efficiency using PC-Lac. The dye concentration was maintained at a constant level of 30 mg/L throughout the experiments. The contact time was set at 24 hours, and the temperature was maintained at 25°C. The outcomes are depicted in Figure 3. The finding indicated that dye decolorization increased with an increase in the particle amount due to the catalytic effect of the immobilized enzyme (19). After determining the optimum amount to be 1.5g, this quantity was consistently utilized in all subsequent degradation

experiments. With this amount, the removal efficiencies for RB, RNB, RTB, and RR were measured to be 95.08%, 90.06%, 85.10%, and 80.70%, respectively. It is evident that when the particle amount increases, the number of available immobilized enzyme binding sites also increases, leading to a higher percentage of dye degradation.

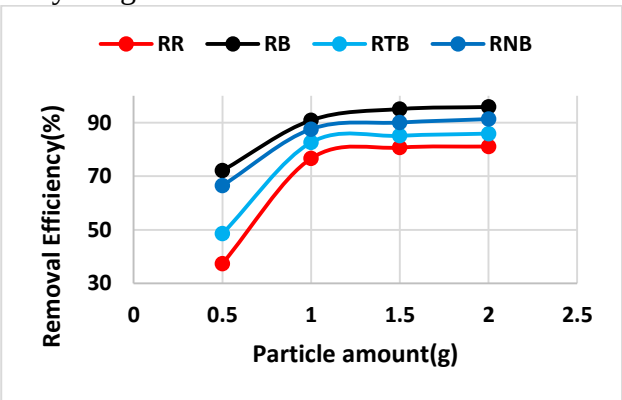

Figure 3. Impact of particle amount on dyes removal at pH =5, initial concentration = 30 mg/L 25°C, and 100 rpm for 24 hr.

Effect of initial concentration

The impact of initial dye concentration on dye removal efficiency was evaluated based on the optimal particle amount and pH determined in the previous experiments. The initial dye concentration was systematically varied across the 10, 20, 30, 40, and 50 mg/L range. This broad range of concentrations facilitated a comprehensive examination of the correlation between initial dye concentration and dye removal efficiency. According to Figure 4, the decolorization efficiency of PC-Lac exhibited an upward trend with increasing dye concentration, reaching a peak decolorization efficiency of 95.58%, 90.67%, 85.73%, and 81.98% for RB, RNB, RTB, and RR, respectively, at a dye concentration of 30 mg/L. However, further increases in dye concentration beyond 30 mg/L resulted in a gradual decline in dye removal. As a result, the experimental conditions determined that 30 mg/L was the threshold concentration for achieving optimal removal of all dyes by PC-Lac. These findings demonstrate that the concentration of the aqueous phase dye affected the enzyme activity. The reaction's speed increases until it reaches its maximum when the substrate concentration gradually increases while the enzyme concentration remains constant. Any additional substrate addition after reaching equilibrium had no effect on the pace of the reaction, and increasing the dye concentration resulted in less dye decolorization because more dye molecules were present (19). However, excessively high pollutant concentrations can lead to laccase inhibition, thereby reducing the removal rate (47). The decolorization of dye is dependent on the chemical structure of the dye and the initial dye concentration (15).

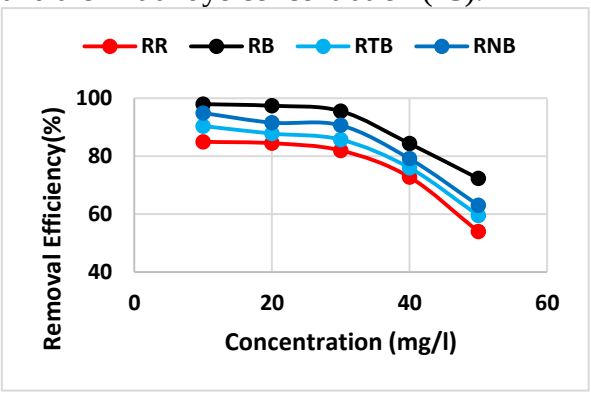

Figure 4. Impact of initial concentration on dyes removal at pH=5, particle amount = 1.5 g 25°C, and 100 rpm for 24 hr.

The decolorization of the four dyes were found not equal, this is maybe due to the differences in redox potentials and the affinity of their steric structure with the active site present in the enzyme (37).

Effect of temperature

An ideal temperature is required to facilitate effective dye degradation because temperature change substantially impacts enzyme activity. With temperatures ranging from (10, 20, 25, 30, 40, 50, and 60^{o}C) using a thermostatic shaker (Shaking Incubator, Zhicheng, China). Other parameters were taken from the previous experiments, while the agitation speed and contact time were fixed at 24 hours and 100 rpm, respectively, to find the removal efficiency. Results presented in Figure 5 demonstrate that dye degradation increased significantly up to 95.50%, 90.19%, 85.63%, and 80.82 % at 25^{o}C for RB, RNB, RTB, and RR, respectively. However, beyond this temperature, decolorization began to decline, reaching values as low as 62.28%, 58.89%, 54.10%, and 50.09% at 60°C for RR, RB, RTB, and RNB, respectively. According to numerous studies, the ideal temperature ranges for immobilized enzymes are typically between 25^{o}C and 40^{o}C (12, 42, 22).

The enzyme's catalytic activity is severely hindered at lower temperatures, leading to a lower removal rate (9). Pollutant degradation declines as the temperature rises, exceeding the optimal temperature (45). High temperatures severely harm laccase oxidative efficiency by causing conformational changes in laccase structure, denaturation, and decreased degradation efficiency (48).

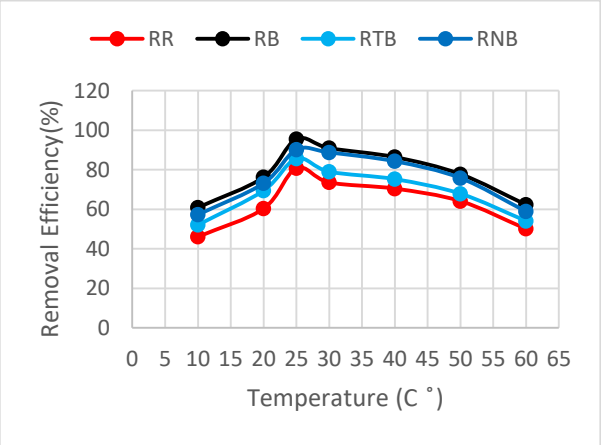

Figure 5. Impact of temperature on dyes removal at pH=5, particle amount = 1.5 g, initial concentration = 30 mg/L, and 100 rpm for 24 hr.

FTIR analyses

Fresh PC samples loaded with dyes were mixed separately with spectroscopic-grade KBr and formed into pellets under a pressure of approximately 1 MPa. Pellets had a diameter of around 10 mm and a thickness of 1 mm. Subsequently, the PC samples were scanned using FTIR within the 4,000-400 cm^{-1} range. The spectra of pristine PC and PC-Lac before and after decolorization of RR, RB, RTB, and RNB dyes are shown in Figure 6. These spectra of the tested PC showed different absorption peaks, demonstrating its complex nature. Prominent absorption peaks in the spectra include distinct bands at approximately 455 cm^{-1}, representing the Si–OH stretches. Another notable peak observed was at 790.81 cm^{-1}, representing the presence of the PH group. Additionally, a band at 1095.50 cm^{-1} resembles Si-O-Si, and peaks at 1442.75 cm^{-1} and 1654.92 cm^{-1} correspond to the presence of C=O groups. Furthermore, sharp peaks at 2870.08 cm^{-1}, 3248.13 cm^{-1}, and 3614.60 cm–1 represent the stretching vibrations of O-H bonds (23,36).

The results showed significant changes in the PC-Lac spectrum following the laccase immobilization, suggesting alterations in the functional groups of PC (Table 3). Specifically, the PC-Lac spectrum exhibited a noticeable increase in peak intensity around 1120 cm−1. Alongside, the emergence of a new peak at approximately 1370 cm^{-1} suggests the presence of OH groups (7,21). A decrease in peak intensity at 1120 cm^{-1}, which had initially increased during digestion, was also observed, suggesting that laccase formed covalent bonds with the available OH groups in PC post-immobilization. The bands corresponding to functional groups shifted towards higher transmission, with percentage changes of 191.33%, 153.62%, 438.4%, and 42.78% for PC-Lac loaded with RB, RNB, RTB, and RR dyes, respectively. Therefore, the degradation order of dyes removed from the surface of the PC is as follows: RB > RNB > RTB > RR.

Table 3. Functional groups before and after PC was loaded with RNB, RTB, RR and RB dye

Wave Number, cm^{-1}	Type of bond	Wavenumber (cm-1) after degradation (RB)	(RNB)	(RTB)	(RR)
3616.71	-OH	3614.99	3614.33	3614.31	3616.33
3434.81	-OH	3434.57	3434.76	3434.31	3428.81
2870.08	-OH	2743.76	2745.98	2855.39	2747.65
1651.13	C=O	1644.54	1638.23	1638.27	1655
1389.25	C=O	1355.97	1388.96	1387.87	1095.94
1105.34	Si-O-Si	1093.65	1099.94	1105.12	1095.94
793.63	-PH	788.09	791.98	790.11	793.05
486	Si–OH	480.05	479.15	478.79	475.83
Wave Number, cm^{-1}	**Type of bond**	**Difference in wavenumber (cm^{-1}) (RB)**	**(RNB)**	**(RTB)**	**(RR)**
3616.71	-OH	1.72	2.38	2.4	0.38
3434.81	-OH	0.24	0.05	0.5	6
2870.08	-OH	126.32	124.1	14.69	122.43
1651.13	C=O	6.59	12.9	12.86	-3.87
1389.25	C=O	33.28	0.29	1.38	293.31
1105.34	Si-O-Si	11.69	5.4	0.22	9.4
793.63	-PH	5.54	1.65	3.52	0.58
486	Si–OH	5.95	6.85	7.21	10.17
Sum of differences in wavenumber (cm^{-1}), after-before degradation =		191.33	153.62	42.78	438.4

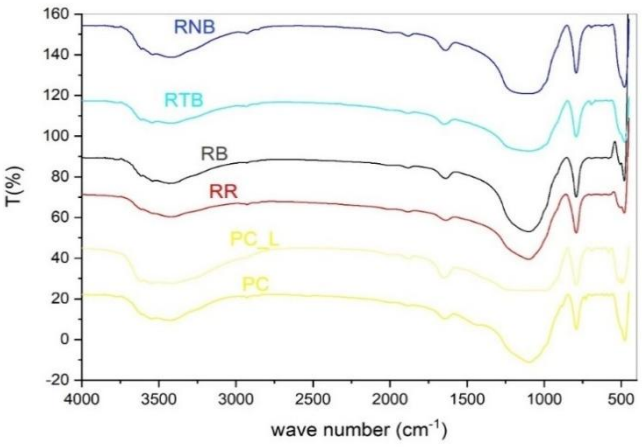

Figure 6. FTIR spectra analysis of (a) raw Pc and (b) Pc -loaded laccase, (c)RR-loaded Pc, (d)RB-loaded Pc, (e) RTB-loaded Pc, (f) RNB-loaded Pc.

SEM analysis

The surface morphology of PC and PC-Lac was evaluated by SEM. Before laccase immobilization (Figure 7), the surface of the PC exhibited a smooth appearance. Upon immobilization, the surface of PC-Lac showed an uneven texture, which can be because of acid treatment and the binding of enzyme macromolecules. Adsorbent particles can adsorb micropollutants from the solution, thereby providing an extended timeframe for degradation in the presence of laccase (38).

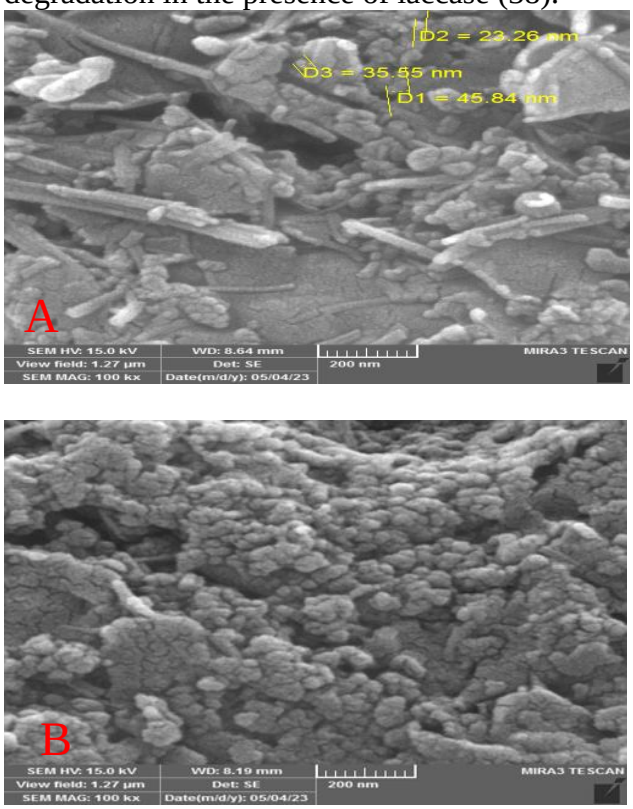

Figure 7. SEM micrographs of PC A: before laccase immobilization and B: after laccase immobilization

Statistical analysis

Analysis of Variance (ANOVA) test was used to assess the regression models to identify the most significant variables that fit the model (Tables 4 and 5). The ANOVA test identifies variables with undetectable impact on the model. The test ensures that model characteristics, including p-values, F-values (Fisher variation ratio), sum of squares, lack of fit values, and adequate precision, meet predetermined acceptable criteria. To evaluate the overall performance of the models, metrics, such as the coefficient of determination (R^2), adjusted R^2, and predicted R^2 are used. The R^2 measures the alignment between experimental and expected results, ranging from 0 to 1, where 0 indicates no correlation among the data (34). The ANOVA results for each model, as shown in Table 4, indicate that the Model F-values of 28.76, 19.39, 27.76, and 11.47for RB, RNB, RTB, and RR dyes, respectively, signify the significance of the models. The probability of achieving F-values of this magnitude solely due to random variation is merely 0.01%. P-values below 0.0500 indicate the statistical significance of the model terms, as observed in this study with terms A, B, C, and D. Consequently, these models can be effectively employed to explore and optimize the design space (30,10).

Table 4. Estimated regression coefficients and corresponding F-value and significance level.

	(RB)		(RNB)	
Source	**F-value**	**p-value**	**F-value**	**p-value**
Model	**28.76**	**< 0.0001**	**19.39**	**< 0.0001**
A-pH	**2.39**	**0.1404**	**2.79**	**0.1129**
B-Dose	**58.04**	**< 0.0001**	**39.06**	**< 0.0001**
C-Con.	**33.55**	**< 0.0001**	**19.66**	**0.0004**
D-Temp.	**26.96**	**< 0.0001**	**19.63**	**0.0004**
Lack of Fit	**7.89**	**0.0573**	**4.25**	**0.1296**
	(RTB)		**(RR)**	
Source	**F-value**	**p-value**	**F-value**	**p-value**
Model	**27.76**	**< 0.0001**	**11.47**	**0.0001**
A-pH	**4.66**	**0.0454**	**0.3033**	**0.5890**
B-Dose	**24.30**	**0.0001**	**16.78**	**0.0008**
C-Con.	**38.44**	**< 0.0001**	**15.25**	**0.0011**
D-Temp.	**54.74**	**< 0.0001**	**17.17**	**0.0007**
Lack of Fit	**22.84**	**0.0127**	**68.86**	**0.0025**

The R^2 values for the dye removal percentages for the models were determined to be 0.8712%, 0.8202%, 0.8672%, and 0.7296% for RB, RNB, RTB, and RR, respectively (Table 5). These values indicate a strong agreement between the predicted and experimental data, suggesting a good fit for the

models. Additionally, the precision of the model, which evaluates the signal-to-noise ratio, presented high reliability. Precision values of RB, RNB, RTB, and RR dye removal percentages were 18.5444, 14.7911, 19.9862, and 11.7679, respectively; it is considered desirable for a ratio greater than 4. This model can be used to navigate the design space. The statistical analysis in Table 5 provides further insights into the decolorization of reactive dyes using immobilized laccase extracted from Fenugreek seeds. The adjusted R^2 values of 0.8409, 0.7779, 0.8360, and 0.6660% for RB, RNB, RTB, and RR dyes, respectively, indicate the significance of the applied models. Additionally, the low coefficient of variation (C.V.%) values of 3.72, 4.25, 3.82, and 6.48% for RB, RNB, RTB, and RR dyes, respectively, signify the high reliability and predictability of the experiments (5,31).

Table 5 Statistical analysis of the results for decolorization the reactive dyes by immobilized laccase extracted from Fenugreek seeds

	RB	RNB	RTB	RR
Std. Dev.	3.21	3.52	3.02	4.69
Mean	86.31	82.90	79.01	72.33
C V %	3.72	4.25	3.82	6.48
R^2	0.8712	0.8202	0.8672	0.7296
$R^2_{adi.}$	0.8409	0.7779	0.8360	0.6660
R^2_{Pre}	0.7829	0.6970	0.7954	0.5479
A_{deq} Precision	18.544	14.7911	19.9862	11.7679

The mathematical expressions of the relationship between the removal efficiency of RB, RNB, RTB, and RR dyes, in terms of coded factors, are represented by equations:

RB% =96.02704-0.760351 pH+16.63879 Dose-0.588673Con. -0.361280 Temp. …(5)

RNB% =92.07715-0.900528 pH+14.95795 Dose-0.493857 Con. -0.337905 Temp. …(6)

RTB% =91.62980+0.996993 pH+10.11194 Dose-0.591782 Con. -0.483508 Temp. …(7)

RR% =86.21847+13.04856 pH-0.394919 Dose-0.578801Con. -0.420573 Temp. …(8)

Conclusions

The immobilized laccase enzyme extracted from Fenugreek seeds on Iraqi Porcelanite showed high decolorization efficiency towards RB, RNB, RTB, and RR dyes, operated under optimum process parameters obtained through batch experiments. Response Surface Methodology and the statistical design of trials with four parameters were used to create models (Equations) and verify the color removal of the reactive textile dyes. The critical process conditions, such as the effect of pH, particle amount, initial dye concentration, temperature, and their interactions, were optimized for the decolorization. The findings demonstrated that the experimental value and anticipated decolorization are congruent. This proposed eco-friendly technology may be implemented to process textile waste, notably for water recycling.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

REFERENCES

1. Abadulla E, T. Tzanov, S. Costa, KH. Robra, A. Cavaco-Paulo, and G M. Gübitz. 2000. Decolorization and detoxification of textile dyes with a laccase from Trametes hirsuta. Applied and Environmental Microbiology. Aug;66(8):3357-62. doi: 10.1128/AEM.66.8.3357-3362.2000

2. Ali A, 2019. Treatment of wastewater contaminated with dyes using modified low-cost adsorbents. Desalination and Water Treatment, 140, pp.326-336, https://doi.org/10.5004/ dwt.23513

3. An N, C. Zhou, X. Zhuang, D. Tong, and W. Yu, 2015. Immobilization of enzymes on clay minerals for biocatalysts and biosensors. Applied Clay Science. Elsevier Ltd.https://doi.org/10.1016/j.clay.2015.05.029

4.Atieh M, 2014. Removal of Phenol from Water Different Types of Carbon – A Comparative Analysis. APCBEE Pro-cedia, 10,136–141. https://doi.org/10.1016/j.apcbee.2014.10.031.

5. Aziz G, S. Hussein, M. M-Ridha, S. Mohammed, K. Abedd, M. Muhamad, and H Hasan, 2023. Activity of laccase enzyme extracted from Malva parviflora and its potential for degradation of reactive dyes in aqueous solution. Biocatalysis and Agricultural Biotechnology 50. https://doi.org/10.1016/j.bcab.2023.102671.

6. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of

protein-dye binding. Analytical Biochemistry Journal. 72: 248-254.

7.Brányik, T, A. Vicente, R. Oliveira, and J. Teixeira, 2004. Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. Biotechnology and Bioengineering. 5;88(1):84-93.
doi: 10.1002/bit.20217. PMID: 15389484

8.Brüschweiler BJ,and C. Merlot,2017. Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. Regulatory Toxicology and Pharmacology.;88:214-226.
doi: 10.1016/j.yrtph.2017.06.012

9.Dai Y, J. Yao, Y. Song, X. Liu, S. Wang, and Y. Yuan, 2016. Enhanced performance of immobilized laccase in electrospun fibrous membranes by carbon nanotubes modification and its application for bisphenol A removal from waterJournal of Hazardous Materials. 2016 Nov 5;317:485-493.
doi: 10.1016/j.jhazmat

10. Dil E, M Ghaedi, A. Ghaedi, A. Asfaram, A. Goudarzi, S. Hajati, and V. Gupta, 2016. Modeling of quaternary dyes adsorption onto ZnO-NR-AC artificial neural network: Analysis by derivative spectrophotometry. Journal of Industrial and Engineering Chemistry, 34, 186–197. https://doi.org/10.1016/j.jiec.2015.11.010.
doi: 10.1016/j.biortech.2017.03.093.

11.Dong, Z.; Z Liu,.; J.Shi,; Tang,; H.Xiang, X.; F.Huang,; M.-M.Zheng. 2019. Carbon Nanoparticle-Stabilized Pickering Emulsion as a Sustainable and High-Performance Interfacial Catalysis Platform for Enzymatic Esterification / Trans-esterification. ACS Sustainable Chemistry & Engineering., 7, 7619–7629.
DOI:10.1021/acssuschemeng.8b05908

12.Farias S, DA. Mayer, D de Oliveira, de Souza SMAGU,and AAU. de Souza.2017. Free and Ca-Alginate Beads Immobilized Horseradish Peroxidase for the Removal of Reactive Dyes: an Experimental and Modeling Study. Applied Biochemistry and Biotechnology. Aug;182(4):1290-1306. doi: 10.1007/s12010-017-2399-2.

13.Fernández-Fernández M, MÁ. Sanromán, and D. Moldes. 2013.Recent developments and applications of immobilized laccase. Biotechnology Advances. 1808-25. doi: 10.1016/j.biotechadv.2012.02.013.

14.Ghiaci M, H. Aghaei, S. Soleimanian, M. Sedaghat, 2009. Enzyme immobilization Part 1. Modified bentonite as a new and efficient support for immobilization of Candidarugose lipase. Applied Clay Science., 43, 289–295.DOI:10.1016/j.clay.2008.09.008.

15.Gupta, V., S. Khamparia, I. Tyagi, D. Jaspal, and A. Malviya, 2015. Decolorization of mixture of dyes: A critical review. Global Journal of Environmental Science and Management, 1, 71-94.
10.7508/gjesm.2015.01.007.

16.Huang W-C, W. Wang, C. Xue, and X. Mao, 2018. Effective Enzyme Immobilization onto a Magnetic Chitin Nanofiber Composite. ACS Sustainable Chemistry & Engineering., 6, 8118–8124.
https://doi.org/10.1021/acssuschemeng.8b01150

17. Kalral, K. R., Chauhan, M. Shavez, and S. Sachdeva, 2013. Isolation of laccase producing Trichoderma Spp. and effect of pH and temperature on its activity. International Journal of Chemistry and Technology Research. 5(5): 2229-2235.

18. Kanagaraj J, T. Senthilvelan, and R. Panda, 2015. Degradation of azo dyes by laccase: biological method to reduce pollution load in dye wastewater. Clean Technologies and Environmental Policy 17, 1443–1456. https://doi.org/10.1007/s10098-014-0869-6.

19. Katuri K, S. Venkata Mohan, and P. Sarma, 2009. Laccase-membrane reactors for decolorization of an acid azo dye in aqueous phase: Process optimization. Water Research 43, 3647–3658.
doi:10.1016/j.watres.2009.05.028.

20.Lehninger A, D. Nelson, and M. Cox, 2000. Principles of Biochemistry (p. 1200). Worth Publishers Inc.: N.Y.

21.LI, Qing zhu and et al. 2009. Lead desorption from modified spent grain. Transactions of Nonferrous Metals Society of China (English Edition), 19(5), 1371–1376. https://doi.org/10.1016/S1003-6326(08)60452-5.

22.Lu Y, Q. Yang, and Y. Chen, 2017. Enhanced Activity of Immobilized Horseradish Peroxidase by Carbon Nanospheres for Phenols Removal. Clean -

Soil, Air, Water 45(2): 1600077. doi:10.1002/clen.201600077.
23. Ma Q. Y, TJ Logan, and S. J. Traina,.1995. Lead immobilization from aqueous solutions and contaminated soils using phosphate rocks. Environmental Science & Technology. 29(4):1118-26. doi: 10.1021/es00004a034.
24. Mahmoodi N. M, M. Arabloo, and J. Abdi, 2014. Laccase immobilized manganese ferrite nanoparticle: synthesis and LSSVM intelligent modeling of decolorization. Water Research. 67:216-26. doi: 10.1016/j.watres.2014.09.011.
25. Mbouguen, J. K.; E. Ngameni;and A. Walcarius,. 2006. Organoclay-enzyme film electrodes. Analytica Chimica Acta, 578, 145–155. doi: 10.1016/j.aca.2006.06.075.
26.Moilanen U, J. Osma, E. Winquist, M. Leisola, and S. Couto, 2010. Decolorization of simulated textile dye baths by crude laccases from Trametes hirsuta and Cerrena unicolor. Engineering in Life Sciences., 10, 242 247. https://doi.org/10.1002/elsc.200900095.
27.Noeline, B. F, D. M Manohar, ,and T. S Anirudhan, 2005. "Kinetic and equilibrium modeling of lead(II) sorption from water and wastewater by polymerized banana stem in a batch reactor [J]. Separation and Purification Technology.Vol. 45,pp. 131−140.
28.Osma J, 2009. Production of Laccases by the White-Rot Fungus Trametes Pubescens for Their Potential Application to Synthetic Dye Treatment. doctoral Dissertation; Universitat Rovira I Virgili: Tarragona.
29.Pandey, D., A. Daverey, and K. Arunachalam. 2020. Biochar: Production, properties and emerging role as a support for enzyme immobilization. Journal of Cleaner Production. Elsevier Ltd. https://doi.org/10.1016/j.jclepro.2020.120267
30.Ridha M, S. Hussein, Z. Alismaeel, M. Atiya,and G. Aziz, .2020. Biodegradation of reactive dyes by some bacteria using response surface methodology as an optimization technique. Alexandria Engineering Journal. 59(5), 3551–3563. https://doi.org/10.1016/j.aej.2020.06.001.
31. Ridha, A. I., and M. J. M-Ridha,. 2023. Determination of the optimum conditions for removal of congo red Dye by peroxidase enzyme plant. Al-Khwarizmi Engineering Journal, 19(2), 15–25. https://doi.org/10.22153/kej.2023.03.001
32. Robbinson P, P. Dunnil, and M. D. Lilly. 1971. Porous glass as a solid support for immobilization or affinity chromatography of enzymes. Biochimica et Biophysica Acta (BBA) - Enzymology,24:659–61. DOI: 10.1016/0005-2744(71)90160-4.
33. Roriz M. S, J. F. Osma, J. A. Teixeira, and S. Rodríguez Couto, 2009. Application of response surface methodological approach to optimise Reactive Black 5 decolouration by crude laccase from Trametes pubescens. Journal of Hazardous Materials.; 169: 691–696. doi: 10.1016/j.jhazmat.2009.03.150 PMID: 19409701.
34.Sadoon zahraa, and M. M., Ridha., 2020. Removal of reactive dyes by electro coagulation process from aqueous solution, Journal of Engineering, 26(2), pp. 14–28. doi: 10.31026/j.eng.2020.02.02
35.Sedaghat M, M. Ghiaci, H. Aghaei,and S Soleimanian-Zad, 2009. Enzyme immobilization. Part 4. Immobilization of alkaline phosphatase on Na-sepiolite and modified sepiolite. Applied Clay Science. 46, 131–135. https://doi.org/10.1016/j.clay.2009.07.021
36.Soejoko DS and M. O. Tjia, 2002. Infrared spectroscopy and X-ray diffraction study on the morphological variations of carbonate and phosphate compounds in giant prawn (Macrobrachium rosenbergii) skeletons during its moulting period Journal of Materials Science. 38, 2087–2093. doi:https://doi.org/10.1023/A:1023566227836.
37. Sridharan, R., Krishnaswamy, V., Archana, K. M. et al. Integrated approach on azo dyes degradation using laccase enzyme and Cul nanoparticle. SN Appl. Sci. 3, 370 (2021). https://doi.org/10.1007/s42452-021-04164-9
38.Taheran M, M. Naghdi, S. K. Brar, E. Knystautas, M Verma, RY Surampalli,and JR Valero, 2016. Development of adsorptive membranes by confinement of activated biochar into electrospun nanofibers. Beilstein Journl of Nanotechnology. 7:1556-1563. doi: 10.3762/bjnano.7.149.
39. Thurston C, 1994. The structure and function of fungal laccases. Microbiology,

140,19–26. https://doi.org/10.1099/13500872-140-1-19.
40. Tlaiaa Y.S, S.I Hussein, and M.J M-Ridha. 2023. "Evaluation the properties of purified laccase extracted from some local plants under the optimum conditions". Iraqi Journal of Agricultural Sciences 54 (4):1101-12. https://doi.org/10.36103/ijas.v54i4.1802.
41.Van der Zee F. P, and S. Villaverde. 2005 Combined anaerobic-aerobic treatment of azo dyes--a short review of bioreactor studies. Water Research. Apr;39(8):1425-40. doi: 10.1016/j.watres.2005.03.007.
42.Wang, Shuai and et al. 2016. Oxidative removal of phenol by HRP-immobilized beads and its environmental toxicology assessment. Ecotoxicology and Environmental Safety, 130, 234–239. https://doi.org/10.1016/j.ecoenv.2016.04.022.
43.Weetall, H. H., and A. M. Filbert, 1974 Porous Glass for Affinity Chromatography Applications. Methods in Enzymology 34, 59–72. doi:10.1016/S00766879(74)34007-4.
44.Xu R, C. Chi, F. Li, and B. Zhang, 2013. Laccase-polyacrylonitrile nanofibrous membrane: highly immobilized, stable, reusable, and efficacious for 2,4,6-trichlorophenol removal. ACS Applied Materials and Interfaces, 5(23), 12554–12560.https://doi.org/10.1021/am403849q.
45.Xu R, Y. Si, and B. Zhang, 2014. Triclosan removal by laccase immobilized on mesoporous nanofibers: Strong adsorption and efficient degradation. Chemical Engineering Journal 255, 63–70. doi:10.1016/j.cej.2014.06.060.
46.Xu, L.; Sun, J.; Qaria, M.A.; Gao, L.; Zhu, D. Dye Decoloring Peroxidase Structure, Catalytic Properties and Applications: Current Advancement and Futurity. Catalysts 2021, 11, 955. https://doi.org/10.3390/catal11080955
47.Yaohua, G., X.ping, J. feng, and S. keren, 2019. Co-immobilization of laccase and ABTS onto novel dual-functionalized cellulose beads for highly improved biodegradation of indole. Journal of Hazardous Materials, 365, 118–124. https://doi.org/10.1016/j.jhazmat.2018.10.076
48. Zdarta J, K. Antecka, R. Frankowski, A. Zgoła-Grześkowiak, H. Ehrlich, and T Jesionowski. 2018.The effect of operational parameters on the biodegradation of bisphenols by Trametes versicolor laccase immobilized on Hippospongia communis spongin scaffolds. Science of The Total Environment. Feb 15;615:784-795. doi: 10.1016/j.scitotenv.2017.09.213
49. Zhang, Di and et al. 2017. Laccase immobilized on magnetic nanoparticles by dopamine polymerization for 4-chlorophenol removal. Green Energy Environment., 2, 393–400. doi:10.1016/j.gee.2017.04.001
50. Zou H, and Y. Wang. 2017. Azo dyes wastewater treatment and simultaneous electricity generation in a novel process of electrolysis cell combined with microbial fuel cell. Bioresource Technology. Jul;235:167-175.