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Optimization of color, lignin, and total phenol removal from pulp and paper wastewater using immobilized laccase: a Taguchi approach

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This study investigated the optimization of color, lignin, and total phenol removal from pulp and paper wastewater with immobilized laccase from *Trametes versicolor* on titanium dioxide nanoparticles. A Taguchi L9 orthogonal array design was used to efficiently investigate the impacts of four essential factors: catalyst concentration, pH, reaction temperature, and reaction time, each of which varied over three levels. The immobilized laccase's performance was examined by assessing the reduction in color (Pt–Co), lignin (mg L^{-1}), and total phenols (mg L^{-1} GAE). The results demonstrated remarkable variations in the reduction of pollutants, highlighting the significance of the selected parameters. The best combination of factor values for simultaneous elimination was found using S/N ratio analysis, with time having the greatest impact. The regression models for lignin removal showed the strongest predictive power, with R^2 values of 98.23% for free laccase and 94.41% for immobilized laccase. All of the models were validated to be statistically significant. Immobilized laccase outperformed free laccase with removal efficiencies of 94.01% for color, 95.45% for lignin, and 94.25% for total phenols compared to the lower removal rates of 64.46%, 58.32%, and 52.65% observed respectively for the pollutants. This study demonstrates the Taguchi method's effectiveness in optimizing pulp and paper wastewater treatment with immobilized laccase, offering valuable insights for efficient and cost-effective pollutant removal.

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Water impact

Water shortages and industrial waste management are major issues in many South African rural communities, including parts of Durban. This study provides a potentially inexpensive and beneficial solution for these problems. Enzyme-based bioremediation may be more practical than chemical heavy and energy-intensive treatment methods. It promotes safer disposal by drastically reducing hazardous pollutants like color, lignin, and total phenols, minimizing health hazards and environmental deterioration.

1. Introduction

The pulp and paper industry is a major source of pollutants and produces over half a million tons of pulp and paper sludge annually in South Africa.[1,2] Most of the sludge is either disposed of in landfills or burned.[2] The sludge can be washed from these landfills to surface water by natural processes. Like the majority of developing countries worldwide, South Africa primarily utilizes surface water for residential, recreational and agricultural applications in its rural areas.[3,4] This is due to either limited potable water supplies or insufficient water supply infrastructure.[4] The majority of wastewater treatment plants in South Africa discharge their waste directly into rivers or streams that are used by the neighboring villages for sustenance. An investigation by Stryftombolas[5] was conducted to determine how the local paper mill and other industrial operations affected the lower Thukela River's ecological health. The impact on the water quality manifested as high amounts of suspended solids, high sodium levels, color changes, higher temperatures and excessive chemical and biological oxygen demands (CODs and BODs). Another study by Edokpayi *et al.*[6] demonstrated that wastewater treatment facilities in the Vhembe district of South Africa rarely treat wastewater to acceptable standards. The sediments of the majority of the rivers have high cadmium levels, which could be extremely dangerous for aquatic life. The Mvudi and Mudidaba rivers had levels of Cd and Pb above the advised threshold. These two were the most polluted of the five rivers investigated. While the majority of the trace metal levels in some of the

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rivers met the regulatory limit for irrigation and domestic water use, the levels found in this study were higher than the threshold levels of water used in aquatic farming. Therefore, there may be a serious risk to human health when using such water for household purposes. In another study by Osode and Okoh,[3] the effects of the final effluent of a wastewater treatment plant on the physicochemical characteristics of the receiving watershed in a South African suburb in the Eastern Cape Province was evaluated between August 2007 and July 2008. The parameters of water quality were examined using guidelines by the South African Department of Water Affairs and Forestry. Physiochemical properties like electrical conductivity (EC) (237–325 μS cm^{-1}), turbidity (7.7–62.7 NTU), phosphate (0.1–4.0 mg L^{-1}), and COD (5–211 mg L^{-1}) did not meet the approved standards. It seems traditional wastewater treatment techniques are no longer effective to remove the pollutants before discharge to larger water bodies. High-cost use, production of secondary sludge, and use of harsh chemicals have made these treatment techniques extraneous.[7,8] Therefore, alternative technologies are necessary to reduce the adverse effects of these pollutants on the environment and also promote waste recycling for industrial purposes. One of these alternative techniques is the use of biocatalysts like laccase enzymes. Although laccase immobilization has been extensively researched, most of them cover the decolorization of textile dyes. Its application for the intricate, diverse matrix of pulp and paper wastewater remains mostly underexplored. The high molecular weight of lignin and the presence of a wide variety of phenolic inhibitors make this effluent a special challenge.[9,10] Considering this background, this paper aims to investigate the use of laccase in its free and immobilized forms to remove environmental pollutants from pulp and paper industrial wastewater. The findings will help develop new, cost-friendly, efficient and environmentally friendly water treatment techniques to guide wastewater treatment plants like those in the rural areas into improving effluent treatments before discharging into larger water bodies. This will also serve as a guide to further investigations into biocatalyst-based water treatment methods.

1.1. Laccase and enzyme immobilization

Laccases are made up of four copper atoms that are classified into three types based on UV-visible and electron paramagnetic resonance (EPR) spectroscopy: type 1 (T1), also known as the blue site; type 2 (T2), also known as the normal site; and type 3 (T3), also known as the binuclear site.[11] Laccase removes pollutants through a catalyzed oxidation reaction that occurs in three phases: (i) the substrate is oxidized by attaching to the laccase's active site, which loses an electron and produces a radical. T1 copper is reduced by receiving electrons from its substrate. The radicals then undergo further processes such as self-coupling or polymerization to generate less dangerous and readily removed chemicals. (ii) Electrons are then transported from T1 to the T2/T3 trinuclear cluster. (iii) Finally, electrons at the T2/T3 trinuclear cluster convert molecular oxygen to water.[11,12] Several investigations on industrial wastewater have reported that laccase is effective at removing challenging pollutants. For this investigation, laccase from *Trametes vesicolor* was physically adsorbed onto titanium dioxide nanoparticles (TiO_2 NPs) to immobilize it. The selection of laccase from *Trametes versicolor* is motivated by a strong scientific justification based on its individual features and synergistic bioremediation potential explained by several documented literature reviews.[12–15] *Trametes versicolor* is a widely known and thoroughly studied white-rot fungus that produces large amounts of laccase. As a result, laccase is commercially available and easy to acquire in large quantities for industrial use and research. *Trametes versicolor* laccases are well-known for being more stable than certain other laccases throughout a wide range of pH and temperature conditions, making them appropriate for the fluctuating nature of industrial effluent.[16]

Titanium dioxide nanoparticles (TiO_2 NPs) were chosen as the preferred support material because of its many beneficial qualities, which have been documented by several researchers.[17,18] Nanoparticles such as zinc oxide, titanium dioxide, copper oxide, and iron oxide have been immobilized onto polymeric membranes to eliminate environmental pollutants and conduct anti-biofouling investigations.[19–22] Titanium dioxide is renowned for its mechanical strength, resistance to microbial deterioration, and chemical inertness. This strong physical shielding prolongs the immobilized enzyme's operational lifespan and significantly increases its reusability over several treatment cycles by protecting it from denaturation brought on by harsh wastewater conditions (such as pH fluctuations, the presence of inhibitors, proteolysis, *etc.*) and mechanical stress.[23] The large surface area for enzyme binding makes it possible to have a high density of immobilized laccase, which can result in a large concentration of accessible active sites inside the reactor when linked optimally. Overall catalytic effectiveness of the system in breaking down contaminants is maintained or even improved by this increased enzyme loading capacity.[17,23] Laccase can be immobilized on TiO_2 NPs to increase its pH and heat stability, increasing its spectrum of operation and efficiency in a range of applications.[17] The process is more economical and effective because of the robust nature of TiO_2 NPs, which enable the immobilized enzyme to be used repeatedly.[17] Furthermore, there are very few reports on the use of cutting-edge nanostructured materials like TiO_2 NPs as a laccase carrier in this particular application, despite the availability of many supports. The remarkable chemical stability and large surface area of TiO_2 have not yet been completely utilized for the treatment of pulp and paper industrial effluent.

1.2. Process optimization for pollutant removal

For this current study, the Taguchi method (TM) was employed to analyze the data using L9 orthogonal array (OA),

signal-to-noise (S/N) ratio and analysis of variance (ANOVA) tools to achieve optimum degradation of the target pollutants in the pulp and paper effluent. Additionally, this was used to identify the factors that have the greatest influence on the response variable. The L9 orthogonal array of the Taguchi method has been chosen for the current study based on previously described investigations.[24,25] The catalyst concentration, wastewater pH, reaction temperature, and reaction time were the parameters chosen. Ultimately, this study seeks to rectify the critical gaps in water treatment by creating an innovative biocatalyst through the immobilization of laccase onto TiO_2 NPs, utilizing it for the concurrent elimination of color, lignin, and total phenols from pulp and paper wastewater, and implementing the rigorous Taguchi statistical design method to methodically enhance the process for optimal degradation efficiency.

2. Experimental

2.1. Analytical materials

Commercially available laccase from *Trametes vesicolor* was purchased from Sigma-Aldrich (Darmstadt, Germany) with batch number 38429-10G. The minimal activity of laccase is >0.5 U mg^{-1} which represents 0.5 units per milligram of protein. Titanium dioxide nanoparticles (TiO_2 NPs) with batch number 637254-500G were also purchased from Sigma-Aldrich for the laccase immobilization. The TiO_2 NPs have a particle size of <25 nm and a purity of 99.7% (trace metals basis). All other analytical grade chemical reagents used for the experiment were sourced locally. They include sodium hydroxide (NaOH; 10% m/v), acetic acid (CH_3OOH; 5% v/v), Folin–Ciocalteu's (FC) phenol reagent (Sigma-Aldrich), sodium carbonate (Na_2CO_3; 20% m/v) and gallic acid ($C_7H_6O_5$).

2.2. Effluent sample collection

Pulp and paper mill effluent samples were collected from a paper industry in the south of Durban, South Africa, with gatekeeper permission. The final treated effluent was collected in sterilized plastic jerry cans from the pulp paper mill's discharge drain site after sulphite pulping. The effluent contained phenols and lignin in substantial amounts. The laboratory's effluent was refrigerated at 4 °C until it was needed for further analysis.

2.3. Effluent characterization and pollutant measurements

The characteristics of the pulp and paper mill effluent used for the study were determined by testing for the components, focusing on color, lignin, and total phenols. The different tests were conducted based on standard methods adopted from the American Public Health Association (APHA)[26] and different research papers and publications (refer to the SI document). The results were presented by implementing triplicate analysis (±SD).

2.4. Experimental procedure

For the study, 100 ml of wastewater sample was characterized and pre-treated to remove suspended solids before using for each experimental run according to the design by the TM. Laccase with a concentration range of 1–5 mg ml^{-1} was dissolved in sodium acetate buffer solution (37.5 mM and pH 4.5) to maintain the pH throughout the reaction time. The mixture of the sample and laccase was placed under constant stirring at 600 rpm in a newly designed batch reactor at a temperature range of 35–55 °C and a pH range of 3–5. The temperature was maintained using a temperature control system and the wastewater was periodically checked using a thermometer. The pH was monitored and checked constantly every 30 minutes. It was adjusted accordingly using tiny drops of NaOH (10% m/v) and CH_3OOH (5% v/v) to maintain the required pH throughout the reaction time for each experimental run. The sample mixture was collected after the reaction time range of 4–6 hours depending on the data for each experimental run and the concentration of the pollutants (color, lignin, and total phenols) measured to determine the efficiency of the laccase-catalyzed pollutant removal.

2.4.1. Immobilization of laccase on TiO_2 NPs. For immobilization, a ratio of 1 : 10 of TiO_2 NPs was used depending on the concentration of the laccase for each experimental run. To achieve immobilization by physical adsorption, laccase and TiO_2 NPs were dissolved in sodium acetate buffer (37.5 mM and pH 7.0) to create a uniform suspension. The mixture was stirred gently using a magnetic stirrer for 4 hours to allow the laccase enzyme to absorb into the surface of the nanoparticles. The mixture was then centrifuged at 2500 rpm for 45 minutes to separate the laccase–TiO_2 complexes. The resulting complex was washed with acetate buffer solution to remove unbound enzymes and stored at 4 °C until it was required for further analysis.

2.5. Experimental design and optimization using the Taguchi method

Using the technique developed by Taguchi, a comparative study was carried out employing the laccase in free and immobilized forms. The statistical analysis and design of the experiment (DOE) were conducted using MINITAB 19. To identify the ideal conditions for laccase performance in free and immobilized forms, this design uses a typical orthogonal matrix L9 as adopted by Praveen *et al.*[27] to investigate four independent factors at three levels. The pH, reaction time, catalyst concentration, and reaction temperature are the four variables that were considered (refer to the SI document). One of the main goals of this research was to reduce pollutants as much as possible using the larger-the-better criterion. The S/N ratio was calculated for each experimental run using the formula for this method:[24]

$$\frac{S}{N} = -10 \log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_1^2}\right) \quad (1)$$

where n represents the number of iterations performed and y represents the percentage yield for a particular experimental run.

During this study, the percentage yield (y_i) of pollutant removal for each experimental run was estimated using the formula below.

$$y_i(\%) = \left(\frac{C_i - C_f}{C_i}\right) \times 100 \quad (2)$$

where C_i is the initial concentration of the pollutant and C_f is the final concentration after treatment.

2.6. Morphological analyses of immobilized laccase

Scanning electron microscopy (SEM) was used to determine if laccase immobilization on TiO_2 NPs was successful. Images captured by SEM were used to examine the morphology of the produced membranes. The elemental composition and spatial distribution of elements in the catalyst membrane was also estimated using X-ray fluorescence (XRF) and elemental mapping of energy dispersive X-ray spectroscopy (EDX) respectively. Laccase immobilization on TiO_2 NPs was also verified by Fourier transform infrared (FTIR) spectrum analysis at a wavelength of 550–4000 cm^{-1}.

3. Results and discussion

3.1. Characterization of pulp and paper mill effluent

The characteristics of the pulp and paper effluent sample were measured using methods adopted from research publications (refer to the SI document). The average values for the three measurements in Table 1 illustrate the characteristics of the pulp and paper mill effluent employed in the current study. The concentration of each pollutant before and after treatment is displayed in Table 2, along with the degradation rate, which measures the percentage of each pollutant that was eliminated.

3.2. Biocatalyst characterization

The physicochemical properties of the biocatalyst were assessed using XRF and EDX for elemental composition, SEM for surface morphology, and FTIR to identify chemical bonds, thereby confirming the successful immobilization of the enzyme.

Table 1 The characteristics of the pulp and paper mill effluent

Characteristics (units)	Value[a]
pH	4.86 ± 0.01
Colour (Pt–Co)	679 ± 26.19
COD (mg L^{-1})	2134 ± 6.00
BOD5 (mg L^{-1})	452.37 ± 11.84
Lignin (mg L^{-1})	273.01 ± 6.15
Total phenols (mg L^{-1} GAE)	200.53 ± 1.55
Total solids (mg L^{-1})	550 ± 18.03

[a] All values are mean (n = 3) ± SD.

3.2.1. Biocatalyst composition. On a dry basis, XRF was used to determine the chemical composition of the immobilized biocatalyst. The result showed that the immobilized enzyme contained a high amount of titanium (48.30%) from the TiO_2 NPs. This is considered a favorable base which serves as the immobilization support for the laccase due to its high surface area and biocompatibility.[17] Oxygen, which is a part of both the laccase enzyme and TiO_2 NPs, was detected in high amounts (43.68%). The key function of the oxygen in the immobilized laccase is to serve as the final electron acceptor in the catalytic oxidation of substrates.[11] The remaining 8.02% was mainly carbon (7.74%), which is a primary element in the amino acids that make up the protein structure of the laccase enzyme, and copper (0.28%), which facilitates the transfer of electrons from the substrate to oxygen.[11] Carbon helps build and stabilize the active site of the enzyme for catalysis and substrate binding.[17] To gain a better understanding of the composition of the immobilized enzyme, the chemical structures of the free laccase and TiO_2 NPs were also examined prior to immobilization (refer to the SI document). The main components of laccase were found to be carbon (61.44%) and oxygen (38.19%), with copper, calcium and iron making up the remaining 0.37%. TiO_2 NPs were mostly composed of titanium (34.35%) and oxygen (58.48%), with trace levels of carbon (6.80%) and copper (0.37%) found. These might be attributed to contamination from handling and storage during sample preparation as well as exposure to the environment.

3.2.2. Biocatalyst morphology. The SEM image of *Trametes versicolor* laccase immobilized on TiO_2 NPs using sodium acetate buffer solution is displayed in Fig. 1(A). The image shows an uneven and rough surface, which suggests that laccase is embedded on the surface of TiO_2 NPs. The Lac–TiO_2 complex appears aggregated, retaining the texture of unmodified TiO_2. However, the immobilization process may have caused the aggregation to be more noticeable or distinct because laccase served as a bridge, causing the particles to cross-link. The porous appearance of the aggregates indicates a large surface area, which is advantageous for laccase catalytic processes.[28] To further comprehend the Lac–TiO_2 complex, the SEM images of laccase and TiO_2 NPs prior to immobilization were examined and are shown in Fig. 1. The laccase enzyme coating the nanoparticles is probably the cause of the rough texture, as it modifies the fine crystal clusters and uniformity observed in the unmodified TiO_2 NPs. The porous nature promotes improved substrate accessibility and aids the immobilized enzyme in maintaining its activity.[17] The average size of TiO_2 NPs was around 25 nm as stated in the data sheet of purchase. It had a rounded and reasonably regular form prior to immobilization. Laccase resulted in a slight increase in particle size which may result in modifications to the overall morphology of the nanoparticles as it appears as an extra layer on the particles. A study by Isanapong *et al.*[29] showed similar morphological observations. Similar surface

Table 2 Characteristics of the pulp wastewater before and after treatment with free and immobilized laccase

Characteristics	Before treatment	After treatment (free)	Degradation rate (%)	After treatment (immobilized)	Degradation rate (%)
Color (Pt–Co)	679 ± 26.19	241.33 ± 3.51	64.46	40.67 ± 2.52	94.01
Lignin (mg L^{-1})	273.01 ± 6.15	113.79 ± 1.64	58.32	12.42 ± 4.86	95.45
Total phenols (mg L^{-1} GAE)	200.53 ± 1.55	94.94 ± 0.41	52.65	11.54 ± 0.31	94.25

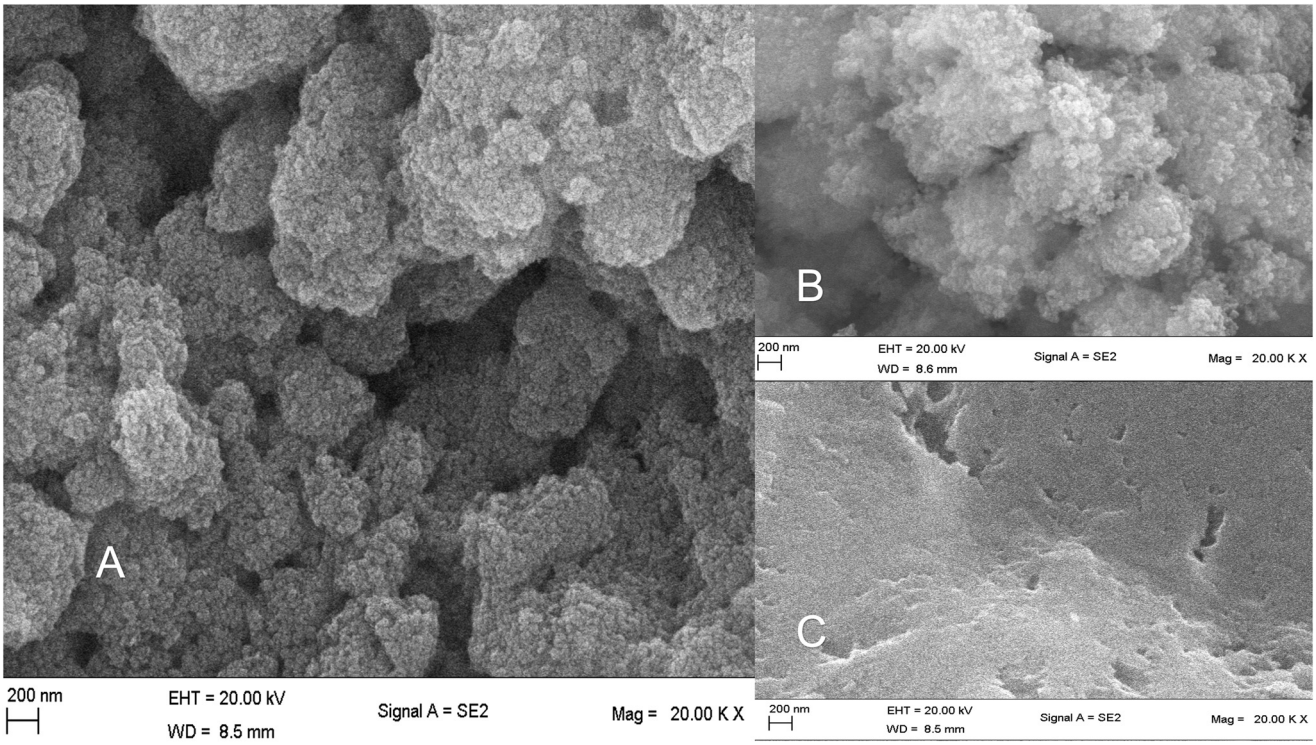

Fig. 1 SEM images comparing the surface morphology of (A) laccase immobilized on TiO_2 NPs, (B) unmodified TiO_2 nanoparticles, and (C) *Trametes versicolor* laccase, revealing structural differences and similarities.

morphological features were noted in another study by Xu *et al.*[18] that used dextranase on TiO_2 NPs, thus validating TiO_2 NPs as a desirable choice for immobilization support.

3.2.3. Biocatalyst major component phase. The elemental composition of the immobilized complex was deduced from the EDX spectrum of the laccase immobilized on TiO_2 NPs by physical adsorption as shown in Fig. 2(a). The presence of titanium was indicated by a noticeable peak at about 4.5 keV. This peak corresponds to the titanium in the TiO_2 NPs, which acts as the immobilization support for laccase enzyme. Oxygen is represented by a peak at about 0.5 keV. Oxygen is present in the laccase enzyme's active sites and protein structure, as well as in the TiO_2 structure. The presence of carbon is indicated by a peak close to 0.3 keV. This element is mainly linked to the organic nature of laccase enzyme because proteins contain carbon. The enzyme immobilization is confirmed by the presence of copper peaks around 1.0 keV, 8.0 keV and 9.0 keV. The presence of copper in the spectrum is very important as it signifies the presence of the active sites of the multi-copper oxidase laccase in the sample.

The spatial distribution of the various elements present in the sample is shown by the elemental mapping images obtained from EDX analysis as presented in Fig. 2(b) and (c). These images illustrate the uniformity of the distribution and aid in confirming that laccase was successfully immobilized on TiO_2 NPs. The effective immobilization of laccase onto the TiO_2 support was validated by EDX elemental mapping. An even nanoparticle basis was revealed by the homogeneous distribution of titanium (yellow) and oxygen (green). On the other hand, the less consistent carbon mapping (blue) demonstrated that the enzyme was adsorbed onto the surface of the nanoparticles instead of creating a continuous layer. Significantly, the presence of dispersed copper dots (red), which is an essential part of the laccase active sites, offered definitive proof of the enzyme's successful immobilization.

3.2.4. FTIR biocatalyst analysis. The FTIR spectra displayed in Fig. 3 show laccase immobilized on TiO_2 NPs as well as the free laccase and TiO_2 NPs, respectively. A wide band at about 3300 and 3400 cm^{-1} denotes N–H stretching resulting from laccase proteins and O–H stretching associated with hydroxyl groups arising from

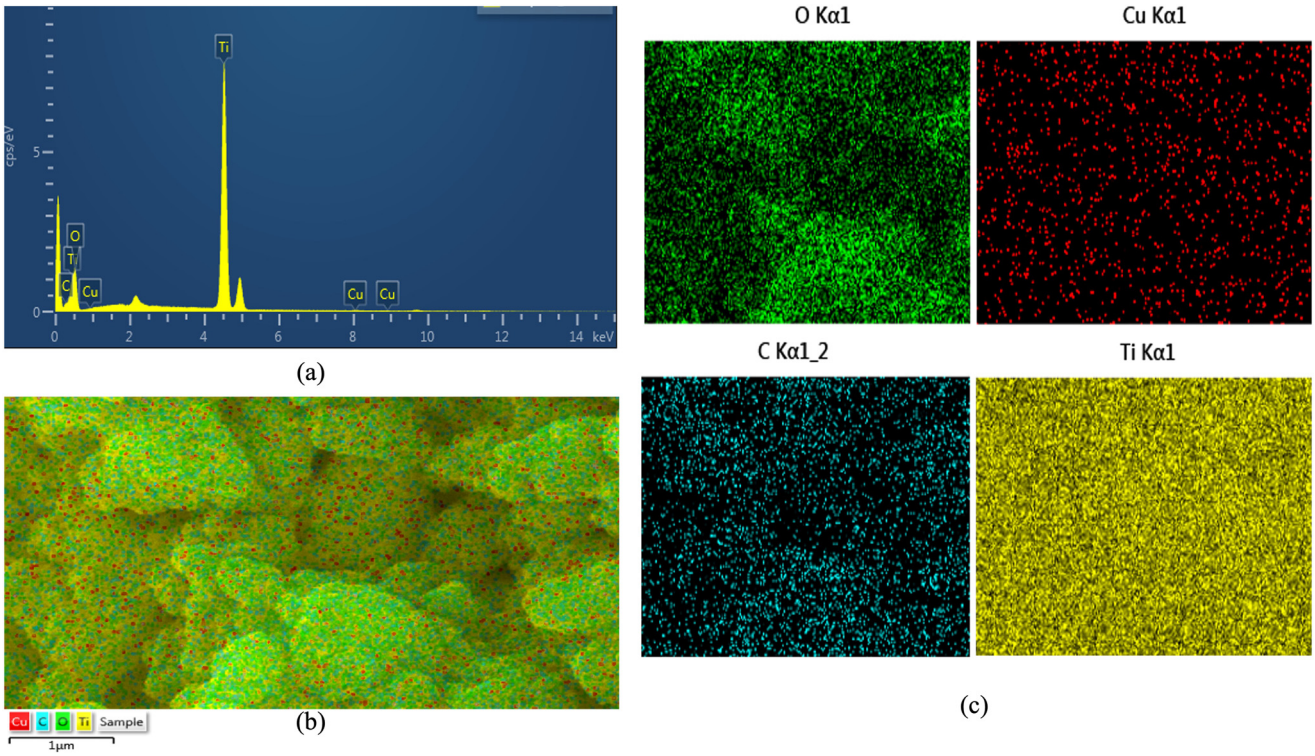

Fig. 2 EDX analysis of laccase immobilized on TiO_2 NPs, showing (a) the EDX spectrum of the elemental composition, (b) the spatial distribution of elements, and (c) the elemental mapping of major components, confirming the presence and distribution of titanium, oxygen, copper, and carbon.

adsorbed water. The O–H vibrations are associated with the hydroxyl groups on the surface of the TiO_2 NPs and water molecules. The N–H stretching shows that the peptide backbone of the enzyme (proteins) contains amino groups. Similar peaks have been reported by different researchers, thereby corroborating the current study.[24,29,30] Weak peaks that indicate C–H stretching vibrations are found between 2880 and 2900 cm^{-1}. The presence of the laccase enzyme's organic framework is shown by these peaks, which are linked to aliphatic C–H bonds in its structure.[30] The successful immobilization of laccase on the TiO_2 surface is confirmed by the appearance of a significant peak in the region 1650–1620 cm^{-1}, which corresponds to the amide I band. The amide I band is caused by C═O stretching vibrations in the peptide bonds of the enzyme's protein backbone and reflects the characteristic secondary structure of the enzyme.[24] There is a peak in the 1380 and 1400 cm^{-1} region. This is equivalent to the amide II band, which is linked to the protein backbone's N–H bending and C–N stretching vibrations.[24,31] This also confirms that laccase is present on the surface of the nanoparticles. There are significant peaks in the 800–400 cm^{-1} region. The Ti–O–Ti stretching vibrations from the titanium dioxide lattice are characterized by these peaks as observed from the TiO_2 spectrum.[31] Nonetheless, further changes or broadening in this region would suggest that TiO_2 and the laccase enzyme interacted during adsorption. This implies surface adhesion or bonding *via* weak chemical interactions or physical adsorption.

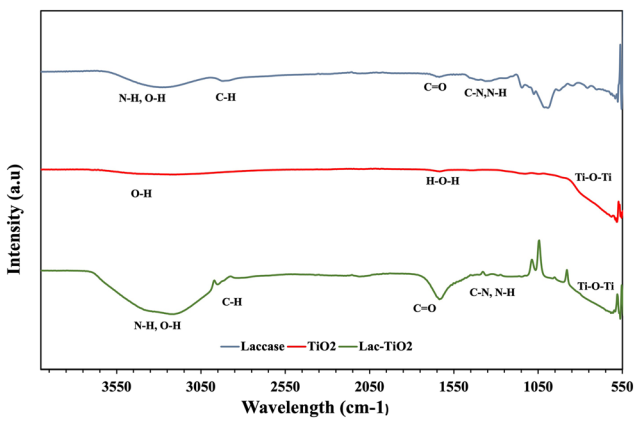

Fig. 3 A comparison of the FTIR spectra of immobilized laccase, TiO_2 NPs, and free laccase, emphasizing changes in functional groups (N–H, C–H, C═O, Ti–O–Ti) signifying successful laccase immobilization on TiO_2 NPs.

3.3. Data presentation of the Taguchi design

The results from studies that have been conducted to ascertain the efficiency of pollutant removal using Taguchi's design are shown in Tables 3 and 4. The experimental results were evaluated by converting data to S/N ratios using the

Table 3 Experimental results showing removal efficiency (RE) and S/N ratios for pollutants using free laccase

	Factors and their levels				Color		Lignin		Total phenols	
Run	A	B	C	D	% RE	S/N ratio	% RE	S/N ratio	% RE	S/N ratio
1	1	3	35	4	56.3	35.00	52.2	34.35	50.4	34.06
2	1	4	45	5	53.8	34.61	56.7	35.08	35.9	31.09
3	1	5	55	6	58.8	35.38	58.7	35.37	52.5	34.41
4	3	3	45	6	66.3	36.43	67.9	36.64	51.2	34.18
5	3	4	55	4	59.4	35.47	50.0	33.98	39.6	31.96
6	3	5	35	5	58.2	35.29	55.1	34.82	42.4	32.55
7	5	3	55	5	55.8	34.94	59.1	35.43	44.3	32.93
8	5	4	35	6	60.5	35.64	67.6	36.59	50.7	34.11
9	5	5	45	4	55.2	34.84	50.6	34.08	52.1	34.34

Table 4 Experimental results showing removal efficiency (RE) and S/N ratios for pollutants using immobilized laccase

	Factors and their levels				Color		Lignin		Total phenols	
Run	A	B	C	D	% RE	S/N	% RE	S/N	% RE	S/N
1	1	3	35	4	91.9	39.27	84.4	38.52	88.2	38.91
2	1	4	45	5	87.0	38.79	76.4	37.66	91.5	39.23
3	1	5	55	6	84.2	38.51	55.1	34.82	91.2	39.20
4	3	3	45	6	86.0	38.69	74.0	37.38	85.9	38.68
5	3	4	55	4	87.0	38.79	73.3	37.30	89.4	39.03
6	3	5	35	5	91.3	39.21	83.9	38.48	94.2	39.48
7	5	3	55	5	81.3	38.20	52.9	34.47	78.8	37.93
8	5	4	35	6	84.8	38.57	78.1	37.86	90.1	39.10
9	5	5	45	4	96.3	39.67	90.3	39.11	93.4	39.41

Taguchi OA in the MINITAB 19.0 software tool (Version 19.2020.1.0) and Microsoft excel (refer to the SI document).

3.3.1. Selection of optimum conditions for pollutant removal. The process parameters were optimized using the S/N ratio to determine the level with the highest value, suggesting optimal performance with limited fluctuations. The delta value, which is the difference between a factor's highest and lowest S/N ratios,[32] was used to rank factors according to their impact on the pollutant. The factor with the highest delta value was deemed the most significant, making it easier to effectively determine the variables with the strongest impact (refer to the SI document).

3.3.2. Analysis of variance (ANOVA). ANOVA analysis was utilized to determine the factors influencing the output parameters of the responses. Additionally, it was used to evaluate their contributions to the reductions in color, lignin, and total phenols as shown in Tables 5–7. In Taguchi analysis involving limited replication, it becomes crucial to ascertain the percentage contribution of each factor. It measures how much of the overall variation in the S/N ratios can be attributed to each of the factors. The percentage contribution was calculated using the formula:[33]

$$\text{Percentage contribution} = \frac{SS_A}{SS_T} \times 100 \quad (3)$$

Table 5 ANOVA for color using free and immobilized laccase

Free laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	42.836	21.4178	38.8
pH	2	6.976	3.4878	6.32
Temperature	2	0.309	0.1544	0.28
Time	2	60.282	30.1411	54.60
Total	8	110.402		100

Immobilized laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	0.616	0.3078	0, 36
pH	2	36.436	18.2178	21, 43
Temperature	2	58.242	29.1211	34, 26
Time	2	74.729	37.3644	43, 95
Total	8	170.022		100

where SS_A is the adjusted sum of squares for a factor, while SS_T is the total sum of squares. Time played a significant factor with the highest percentage contributions in almost all cases studied. In certain cases, other factors like catalyst concentration, pH and temperature were also significant as observed from the results, although to a lesser extent.

3.3.3. Regression analysis. Linear regression models were generated using Minitab 19.0 to predict the reduction of color, lignin, and total phenols. These models, designed for both free (1) and immobilized (2) laccase, connect pollutant reduction with four key factors: catalyst concentration (A), pH (B), temperature (C), and reaction time (D) as provided in eqn (4)–(9).

$$\text{Color (1)} = 50.2 + 0.217A - 1.03B - 0.017C + 2.45D \quad (4)$$

$$\text{Color (2)} = 107.99 - 0.058A + 2.10B - 0.258C - 3.37D \quad (5)$$

Table 6 ANOVA for lignin using free and immobilized laccase

Free laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	15.75	7.874	4.47
pH	2	37.9	18.948	10.75
Temperature	2	11.7	5.848	3.32
Time	2	287.16	143.581	81.46
Total	8	352.5		100

Immobilized laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	40.14	20.07	3.10
pH	2	66.5	33.25	5.13
Temperature	2	866.54	433.27	66.83
Time	2	323.52	161.76	24.95
Total	8	1296.7		100

Table 7 ANOVA for total phenols using free and immobilized laccase

Free laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	32.607	16.303	10.73
pH	2	91.327	45.663	30.05
Temperature	2	8.527	4.263	2.81
Time	2	171.42	85.71	56.41
Total	8	303.88		100

Immobilized laccase

Source	Degree of freedom	Sum of squares	Mean square	% Contribution
Catalyst concentration	2	14.196	7.098	8.21
pH	2	117.696	58.848	68.10
Temperature	2	33.829	16.914	19.57
Time	2	7.109	3.554	4.11
Total	8	172.829		100

$$\text{Lignin (1)} = 38.51 + 0.808A - 2.467B - 0.118C + 6.900D \quad (6)$$

$$\text{Lignin (2)} = 143.7 + 0.45A + 3.00B - 1.085C - 6.80D \quad (7)$$

$$\text{Total phenols (1)} = 38.8 + 0.69A + 0.18B - 0.118C + 2.05D \quad (8)$$

$$\text{Total phenols (2)} = 87.06 - 0.717A + 4.317B - 0.2183C - 0.633D \quad (9)$$

The coefficient of determination (R^2) was used in the study to assess the performance of the regression models, with different outcomes.[34] Lignin models demonstrated a robust fit with the highest R^2 values of 98.23% for free laccase and 94.41% for immobilized laccase. The R^2 values for the color and total phenol models were lower but still acceptable. The predictability of the models for forecasting pollutant removal was validated using residual plots, which showed that the model coefficients were statistically significant and the errors were normally distributed as shown in Fig. 4.

3.3.4. Confirmation test. The ideal conditions determined by the S/N ratio analysis were used to conduct the confirmation test, which is the final step in the Taguchi method. The model result was then confirmed by comparing the outcomes with the predicted results from the Minitab software as shown in Table 8. The research demonstrates that the Taguchi technique is a reliable and accurate optimization tool, as the prediction error for pollutant reduction utilizing both free and immobilized laccase was consistently less than 20%, which is the benchmark according to previous studies.[35–37]

3.4. Effect of process parameters on pollutant removal efficiency

When comparing the application of free and immobilized laccase for the degradation of the pollutants, the key findings revealed significant differences in their optimal conditions.

3.4.1. Effect of process parameters on color. The ideal concentration of free laccase for color removal was observed to be 3 mg mL^{-1} as shown in Fig. 5(a). Performance was

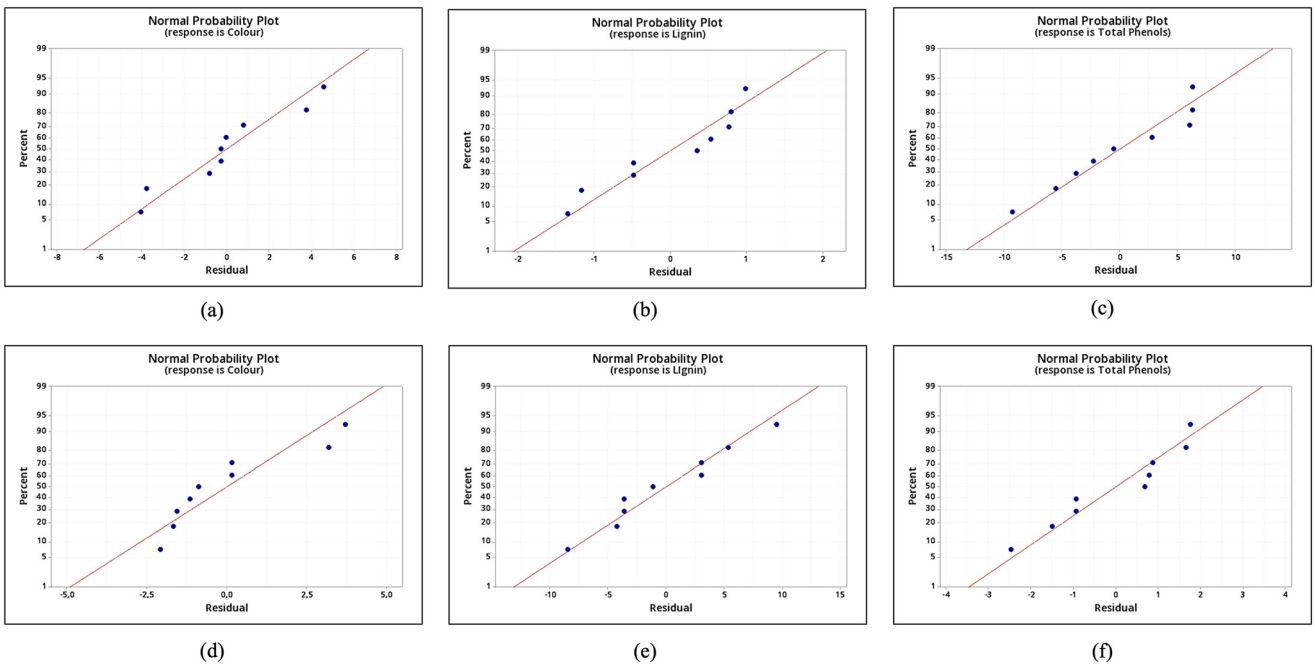

Fig. 4 Residual normality plots for color, lignin, and total phenols using free and immobilized laccase. These figures compare the effects of free and immobilized laccase treatments, evaluating residual normality for color, lignin, and total phenol removal. A normal distribution is indicated by data points along the red line. The plots for free laccase are illustrated in (a), (c), and (e) for color, lignin, and total phenols, respectively, while immobilized laccase are shown in (b), (d), and (f).

Table 8 Confirmation test results for pollutant removal using free (1) and immobilized (2) laccase

Pollutant	Optimal conditions	Actual mean (%)	Predicted mean (%)	Error (%)
Color (1)	A2:B1:C1:D3	64.5	52.63	18.34
Color (2)	A2:B3:C2:D1	94.01	96.93	3.11
Lignin (1)	A3:B1:C2:D3	58.32	55.83	4.26
Lignin (2)	A2:B2:C1:D1	95.45	92.17	3.44
Total phenols (1)	A3:B3:C1:D3	52.65	57.63	9.45
Total phenols (2)	A1:B3:C1:D1	94.25	96.10	1.97

actually reduced by increasing the concentration to 5 mg mL^{-1}, most likely as a result of inhibitory effects like steric hindrance.[38] The immobilized laccase displayed consistent performance at low to moderate concentrations as shown in Fig. 5(b). At high concentrations, the enzymes on the surface of the nanoparticles were overcrowded, possibly causing a mild inhibition.[39] The optimal pH ranges for both enzyme forms were opposites of each other. Free laccase worked best under severely acidic (pH 3) conditions and performed worse as the pH increased. However, at a pH of 5, immobilized laccase performed noticeably better. This demonstrates a key advantage of immobilization: it broadens the enzyme's ideal pH range and makes it more stable under less acidic conditions.[29] Both forms of enzymes performed better at lower temperatures and were sensitive to high ranges. Free laccase remained stable up to 45 °C, but its activity dropped at 55 °C, most likely due to thermal denaturation.[29] The immobilized enzyme's effectiveness likewise sharply declined at 55 °C. The main takeaway is that high temperatures remain a limiting issue even though immobilization enhances thermal stability up to 45 °C. The most significant factor for free laccase was reaction time as color removal was best achieved at 6 hours. On the other hand, the immobilized laccase operated considerably more quickly, achieving its peak efficiency in just 4 hours. This is a noteworthy discovery because it suggests that the immobilization matrix improves the interactions between the enzyme and the substrate, resulting in a quicker and more effective process.[17]

3.4.2. Effect of process parameters on lignin. The performance of free laccase in lignin degradation improved gradually as the catalyst concentration increased up to 5 mg mL^{-1} as shown in Fig. 5(c), indicating that greater enzyme availability improves efficiency. Immobilized laccase, on the other hand, performed best at a concentration of 3 mg mL^{-1} as shown in Fig. 5(d), with efficiency decreasing as concentration increased. This shows that immobilization resulted in more efficient enzyme activity. In terms of pH, free laccase performed best under acidic conditions (pH 3),

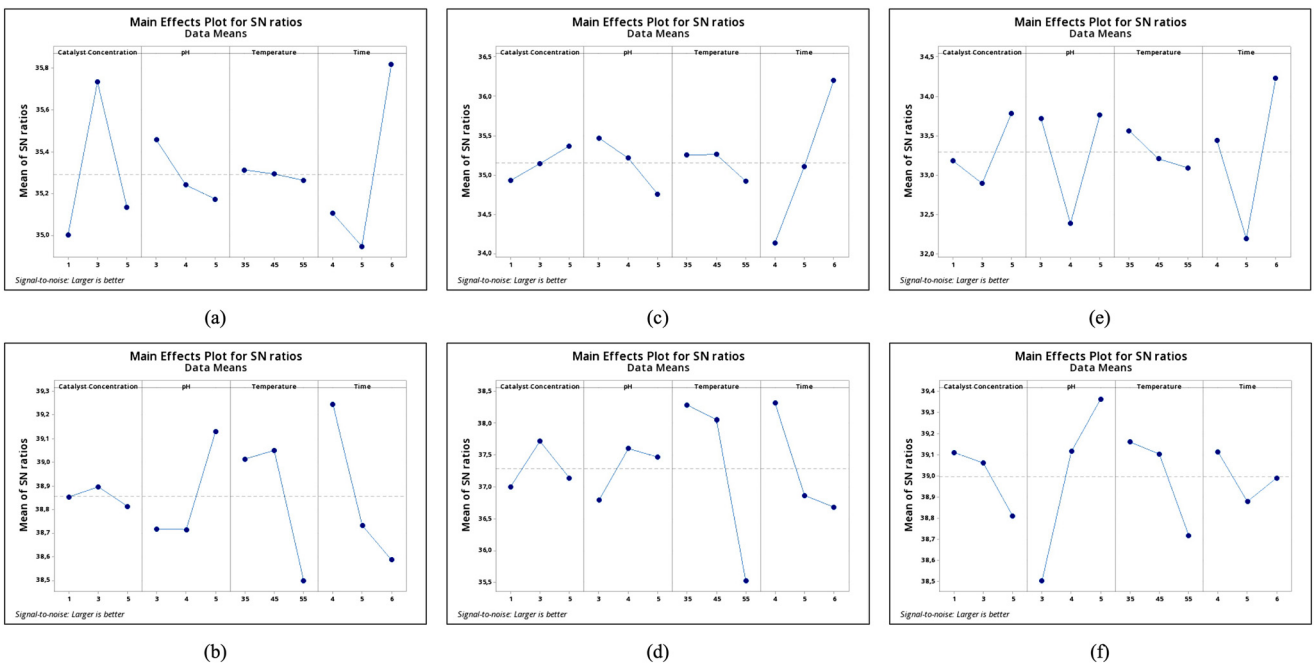

Fig. 5 S/n ratio plots of free and immobilized laccase for reduction in color, lignin, and total phenols. These plots illustrate how process parameters (catalyst concentration, pH, temperature, and time) affect the efficiency of pollutant removal as measured using S/N ratios. By comparing free and immobilized laccase, the optimum conditions for reducing each pollutant are identified. Each factor's relative impact on color, lignin, and total phenol elimination is displayed in the plots. The color, lignin, and total phenol plots for free laccase are displayed in (a), (c), and (e), respectively, whereas the immobilized laccase plots are displayed in (b), (d), and (f).

whereas immobilized laccase had a broader tolerance, peaking at pH 4 and remaining effective at pH 5, indicating that immobilization increased the enzyme's pH stability.[19] Both free and immobilized laccase demonstrated a similar temperature response, with maximum activity at 35–45 °C and a considerable decline in performance above 55 °C which can be attributed to thermal denaturation. Nevertheless, the immobilized laccase showed a less rapid decline in efficiency, suggesting superior heat stability. The performance of free laccase improved steadily up to 6 hours in terms of reaction time. Incredibly, the efficiency of immobilized laccase declined from 4 to 6 hours, suggesting that its improved stability and faster substrate interaction allow it to achieve peak performance in shorter time.[29]

3.4.3. Effect of process parameters on total phenols. For free laccase, performance was enhanced by increasing the catalyst concentration to 5 mg mL^{-1} as shown in Fig. 5(e), showing that more enzyme is required for successful phenol degradation. Immobilized laccase, on the other hand, exhibited exceptional stability, with efficiency remaining fairly stable across the tested concentrations (1 mg mL^{-1} to 5 mg mL^{-1}) as shown in Fig. 5(f), implying that immobilization reduces the enzyme's sensitivity to concentration variations.[40] Regarding pH, free laccase performed best at pH 5 and was extremely sensitive to a decrease at pH 4, indicating an affinity for a less acidic environment. Immobilized laccase, on the other hand, showed a greater pH tolerance, exhibiting steady activity from pH 3 to pH 5, indicating improved pH stability.[41] The performance of both free and immobilized laccase decreased dramatically when the temperature rose from 35 °C to 55 °C because of denaturation. The decline in the performance of immobilized laccase was less noticeable, suggesting that immobilization provided protection against thermal stress. Furthermore, the efficiency of both enzymes decreased at 5 hours and then sharply increased at 6 hours, demonstrating a non-linear relationship with reaction time. This implies that both free and immobilized laccase benefit from a longer reaction time for optimum phenol degradation, enabling more thorough substrate breakdown.

4. Conclusion

This study successfully demonstrated the treatment of pulp and paper effluent with a novel biocatalyst: laccase immobilized on TiO_2 NPs. Immobilization significantly improved the degradation of the target pollutants, according to the Taguchi optimization method. Degradation rates of 94.01% for color, 95.45% for lignin, and 94.25% for total phenols were attained by the immobilized laccase. These results were significantly better compared to the lower removal rates of 64.46%, 58.32%, and 52.65% observed respectively for the pollutants when using free laccase. Analysis of variance (ANOVA) determined reaction time as the most consistently influential parameter, and the accuracy of the optimized model was validated by confirmation tests. While further research on the reusability of the biocatalyst is necessary to assess its long-term economic feasibility, this study confirms immobilized laccase on TiO_2 NPs as a highly successful and promising strategy for the bioremediation of effluent from the pulp and paper industry.

Author contributions

Peterson Thokozani Ngema: supervision, resources, writing – review & editing, funding acquisition, validation, project administration. Thobeka Pearl Makhathini: supervision, conceptualization, writing – review & editing, resources, validation, funding acquisition. Toritsegbone Erik Tite: writing – original draft, visualization, methodology, formal analysis, data curation, software, conceptualization, investigation.

Conflicts of interest

There are no conflicts to declare.

Data availability

Supplementary information: The SI file provides detailed methodological and data-driven information that complements the main article. The document includes: the schematic and description of the custom-designed batch reactor used for the analysis, as well as the experimental set-up; the detailed procedures for effluent characterization and pollutant measurements, including images of the processes; Excel sheet with process parameter calculations and plots; the Taguchi L9 design table; detailed S/N ratio response tables and the confirmation test results. See DOI: **https://doi.org/10.1039/D5EW00677E**.

The data supporting this article have been included as part of the SI.

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References

1 M. E. Gibril, P. Lekha, J. Andrew, B. Sithole, T. Tesfaye and D. Ramjugernath, Beneficiation of pulp and paper mill sludge: production and characterisation of functionalised crystalline nanocellulose, *Clean Technol. Environ. Policy*, 2018, **20**(8), 1835–1845.

2 A. Chimphango, S. O. Amiandamhen, J. F. Görgens and L. Tyhoda, Prospects for Paper Sludge in Magnesium Phosphate Cement: Composite Board Properties and Techno-Economic Analysis, *Waste Biomass Valoriz.*, 2021, **12**(9), 5211–5233.

3 A. N. Osode and A. I. Okoh, Impact of discharged wastewater final effluent on the physicochemical qualities of a receiving watershed in a suburban community of the eastern cape province, *Clean: Soil, Air, Water*, 2009, **37**(12), 938–944.
4 J. N. Edokpayi, J. O. Odiyo and O. S. Durowoju, *Impact of Wastewater on Surface Water Quality in Developing Countries: A Case Study of South Africa. Water Quality*, IntechOpen, Croatia, 2017, pp. 401–416.
5 I. C. Stryftombolas, *Ecotoxicological Assessment of the Impact of Paper and Pulp Effluent on the Lower Thukela River Catchment, KwaZulu-Natal, South Africa and the Toxicological Assessment of Similar Effluent from Two Other Mills*, University of Johannesburg (South Africa), 2010.
6 J. N. Edokpayi, M. Nkhumeleni, A. M. Enitan-Folami and F. C. Olaniyi, Water quality assessment and potential ecological risk of trace metals in sediments of some selected rivers in Vhembe district, South Africa, *Physics and Chemistry of the Earth, Parts A/B/C*, 2022, **126**, 103111.
7 M. Alshabib and S. A. Onaizi, A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges, *Sep. Purif. Technol.*, 2019, **219**, 186–207.
8 S. I. Khan, A. Zarin, S. Ahmed, F. Hasan, A. O. Belduz and S. Canakci, *et al.*, Degradation of lignin by bacillus altitudinis sl7 isolated from pulp and paper mill effluent, *Water Sci. Technol.*, 2022, **85**(1), 420–432.
9 A. Kumar, N. K. Srivastava and P. Gera, Removal of color from pulp and paper mill wastewater- methods and techniques- a review, *J. Environ. Manage.*, 2021, **298**, 113527.
10 R. Toczyłowska-Mamińska, Limits and perspectives of pulp and paper industry wastewater treatment – A review, *Renewable Sustainable Energy Rev.*, 2017, **78**, 764–772.
11 A. Rostami, A. Abdelrasoul, Z. Shokri and Z. Shirvandi, Applications and mechanisms of free and immobilized laccase in detoxification of phenolic compounds — A review, *Korean J. Chem. Eng.*, 2022, **39**(4), 821–832.
12 T. E. Tite, P. T. Ngema and T. P. Makhathini, Exploration of Free and Immobilized Biocatalysts for the Treatment of Paper Industry Effluents, *Sep. Purif. Rev.*, 2024, 1–18.
13 L. Arregui, M. Ayala, X. Gomez-Gil, G. Gutierrez-Soto, C. E. Hernandez-Luna and M. Herrera de Los Santos, *et al.*, Laccases: structure, function, and potential application in water bioremediation, *Microb. Cell Fact.*, 2019, **18**(1), 200.
14 Y. Adamian, L. Lonappan, K. Alokpa, S. N. Agathos and H. Cabana, Recent Developments in the Immobilization of Laccase on Carbonaceous Supports for Environmental Applications - A Critical Review, *Front. Bioeng. Biotechnol.*, 2021, **9**, 778239.
15 Z. Chen, W. D. Oh and P. S. Yap, Recent advances in the utilization of immobilized laccase for the degradation of phenolic compounds in aqueous solutions: A review, *Chemosphere*, 2022, **307**(Pt 3), 135824.
16 W. Latif, C. Ciniglia, M. Iovinella, M. Shafiq and S. Papa, Role of White Rot Fungi in Industrial Wastewater Treatment: A Review, *Appl. Sci.*, 2023, **13**(14), 8318.
17 A. Kolodziejczak-Radzimska, J. Zembrzuska, K. Siwinska-Ciesielczyk and T. Jesionowski, Catalytic and Physicochemical Evaluation of a TiO(2)/ZnO/Laccase Biocatalytic System: Application in the Decolorization of Azo and Anthraquinone Dyes, *Materials*, 2021, **14**(20), 6030.
18 Y. Xu, H. Wang, Q. Lin, Q. Miao, M. Liu and H. Ni, *et al.*, Immobilization of Dextranase Obtained from the Marine Cellulosimicrobium sp. Y1 on Nanoparticles: Nano-TiO(2) Improving Hydrolysate Properties and Enhancing Reuse, *Nanomaterials*, 2023, **13**(6), 1065.
19 J. Hou, G. Dong, Y. Ye and V. Chen, Laccase immobilization on titania nanoparticles and titania-functionalized membranes, *J. Membr. Sci.*, 2014, **452**, 229–240.
20 I. Munnawar, S. S. Iqbal, M. N. Anwar, M. Batool, S. Tariq and N. Faitma, *et al.*, Synergistic effect of chitosan-zinc oxide hybrid nanoparticles on antibiofouling and water disinfection of mixed matrix polyethersulfone nanocomposite membranes, *Carbohydr. Polym.*, 2017, **175**, 661–670.
21 C. Iriarte-Mesa, S. Diaz-Castanon and D. G. Abradelo, Facile immobilization of trametes versicolor laccase on highly monodisperse superparamagnetic iron oxide nanoparticles, *Colloids Surf., B*, 2019, **181**, 470–479.
22 A. Waheed, U. Baig and M. A. Ansari, Fabrication of cuo nanoparticles immobilized nanofiltration composite membrane for dye/salt fractionation: Performance and antibiofouling, *J. Environ. Chem. Eng.*, 2022, **10**(1), 106960.
23 D. Ren, Z. Wang, S. Jiang, H. Yu, S. Zhang and X. Zhang, Recent environmental applications of and development prospects for immobilized laccase: a review, *Biotechnol. Genet. Eng. Rev.*, 2021, **36**(2), 81–131.
24 R. R. C. Monteiro, A. L. B. de Oliveira, F. L. de Menezes, M. C. M. de Souza, P. B. A. Fechine and J. C. S. dos Santos, Improvement of enzymatic activity and stability of lipase A from Candida antartica onto halloysite nanotubes with Taguchi method for optimized immobilization, *Appl. Clay Sci.*, 2022, **228**, 106634.
25 F. Deniz, Optimization of Biosorption Conditions for Color Removal by Taguchi DOE Methodology, *Environ. Prog. Sustainable Energy*, 2012, **32**(4), 1129–1133.
26 American Public Health Association, American Water Works Association, *Standard methods for the examination of water and wastewater*, APHA Press, Washington, DC, 19th edn, 1995.
27 N. Praveen, U. S. Mallik, A. G. Shivasiddaramaiah, R. Hosalli, C. D. Prasad and S. Bavan, Machinability Study of Cu-Al-Mn Shape Memory Alloys using Taguchi Method, *J. Inst. Eng. (India): Ser. D*, 2024, 231–243.
28 S. Sanguanpak, W. Shongkittikul, A. Wannagon and C. Chiemchaisri, Effects of TiO2 on the laccase enzyme immobilization and the bisphenol-A removal of the ceramic membranes, *Desalin. Water Treat.*, 2017, **93**, 163–170.
29 J. Isanapong, K. Lohawet and P. Kumnorkaew, Optimization and characterization of immobilized laccase on titanium dioxide nanostructure and its application in removal of Remazol Brilliant Blue R, *Biocatal. Agric. Biotechnol.*, 2021, **37**, 102186.

30 H. M. N. Iqbal, G. Kyazze, T. Tron and T. Keshavarz, Laccase from Aspergillus niger: A novel tool to graft multifunctional materials of interests and their characterization, *Saudi J. Biol. Sci.*, 2018, **25**(3), 545–550.
31 I. C. Arvizu, R. B. García-Reyes, M. A. Pech-Canul and A. García-González, Evaluating the role of laccase immobilized TiO2 for potential use as biocathode in a microbial electrolysis cell, *Biochem. Eng. J.*, 2023, **194**, 108883.
32 C. Hamzaçebi, Taguchi Method as a Robust Design Tool, *Quality Control - Intelligent Manufacturing, Robust Design and Charts*, IntechOpen, 2021, pp. 111–130.
33 S. Kumar, P. Satsangi and D. Prajapati, Optimization of process factors for controlling defects due to melt shop using Taguchi method, *Int. J. Qual. Reliab. Manag.*, 2013, **30**(1), 4–22.
34 P. Sivaiah and D. Chakradhar, Modeling and optimization of sustainable manufacturing process in machining of 17-4 PH stainless steel, *Measurement*, 2019, **134**, 142–152.
35 M. H. Cetin, B. Ozcelik, E. Kuram and E. Demirbas, Evaluation of vegetable based cutting fluids with extreme pressure and cutting parameters in turning of AISI 304L by Taguchi method, *J. Cleaner Prod.*, 2011, **19**(17–18), 2049–2056.
36 T. Kıvak, Optimization of surface roughness and flank wear using the Taguchi method in milling of Hadfield steel with PVD and CVD coated inserts, *Measurement*, 2014, **50**, 19–28.
37 S. A. Alavi-Borazjani, L. A. C. Tarelho and M. I. Capela, Parametric optimization of the dark fermentation process for enhanced biohydrogen production from the organic fraction of municipal solid waste using Taguchi method, *Int. J. Hydrogen Energy*, 2021, **46**(41), 21372–21382.
38 R. Saravanan, F. Gracia and A. Stephen, Basic principles, mechanism, and challenges of photocatalysis, in *Nanocomposites for visible light-induced photocatalysis*, ed. M. M. Khan, D. Pradhan and Y. Sohn, Springer, Cham, Switzerland, 2017, pp. 19–40.
39 J. Hou, G. Dong, B. Luu, R. G. Sengpiel, Y. Ye, M. Wessling and V. Chen, Hybrid membrane with TiO2 based bio-catalytic nanoparticle suspension system for the degradation of bisphenol-A, *Bioresour. Technol.*, 2014, **169**, 475–483.
40 H. Sa, M. Michelin, T. Tavares and B. Silva, Current challenges for biological treatment of pharmaceutical-based contaminants with oxidoreductase enzymes: Immobilization processes, real aqueous matrices and hybrid techniques, *Biomolecules*, 2022, **12**(10), 1489.
41 G. Olcucu, O. Klaus, K.-E. Jaeger, T. Drepper and U. Krauss, Emerging solutions for in vivo biocatalyst immobilization: Tailor-made catalysts for industrial biocatalysis, *ACS Sustainable Chem. Eng.*, 2021, **9**(27), 8919–8945.

