Immobilization of polyphenol oxidase on chitosan/organic rectorite composites for phenolic compounds removal
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ABSTRACT
Chitosan/organic rectorite (CTS/OREC) composites were prepared and characterized by Fourier transform infrared spectrometry and X-ray diffraction. Polyphenol oxidase (PPO) was immobilized on CTS/OREC by physical adsorption (APPO) and covalent binding (CPPO). Taguchi method was applied in the optimization of immobilization conditions resulting in the highest enzyme activity of $16.37 \times 10^3$ and $8.92 \times 10^3$ U/g for APPO and CPPO, respectively. APPO enzyme activity was higher than that of CPPO, while CPPO showed the higher enzyme loading capacity than that of APPO. The removal percentage of phenolic compound, including phenol (PH), 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP), by immobilized PPO was also explored. The results indicated that APPO was more efficient in phenolic compounds removal than CPPO. APPO contributed to a quick removal in the first hour, and the removal percentage of PH, 4-CP and 2,4-DCP could reach 69.3 ± 4.2%, 89.8 ± 2.5% and 93.8 ± 1.7% within 2 h, respectively. The order of removal percentage of phenolic compounds for both immobilized PPO was 2,4-DCP > 4-CP > PH. After 10 consecutive operations, the removal percentage of 2,4-DCP reached 73.2 ± 2.6% and 60.3 ± 1.5% for APPO and CPPO, respectively. The results introduced a novel support for PPO immobilization, and the immobilized PPO had great potential in wastewater treatment.

Key words | chitosan, immobilization, phenolic compounds, polyphenol oxidase, rectorite

HIGHLIGHTS
- Polyphenol oxidase (PPO) was immobilized on chitosan/organic rectorite composites.
- PPO was immobilized by physical and covalent binding.
- Optimal immobilization conditions were determined by Taguchi method.
- Catalytic properties of immobilized PPO on phenolic compounds were evaluated.
GRAPHICAL ABSTRACT

NOTATION

2,4-DCP 2,4-dichlorophenol
4-CP 4-chlorophenol
A activity unit per gram of support of immobilized polyphenol oxidase (U/g)
APPO polyphenol oxidase immobilized on chitosan/organic rectorite composites by physical adsorption
C₀ initial phenolic compounds concentration (mg/L)
Cₑ equilibrium concentration of polyphenol oxidase (mg/mL)
Cᵢ final concentration of polyphenol oxidase (mg/mL)
Cᵢ initial concentration of polyphenol oxidase (mg/mL)
Cᵣ remaining phenolic compounds concentration (mg/L)
CPPO polyphenol oxidase immobilized on chitosan/organic rectorite composites by covalent binding
CTAB cetyltrimethyl ammonium bromide
CTS chitosan
CTS/OREC chitosan/organic rectorite composites
Eₐ activation energy (kJ/mol)
Eₗ₋ₐ the mean-free energy of the adsorption of the Dubinin–Radushkevich model (kJ/mol)
FT-IR Fourier transform infrared spectrometry
ΔG° Gibbs free energy change (kJ/mol)
ΔGₑ–S Gibbs free energy of substrate binding (kJ/mol)
ΔGₑ–T Gibbs free energy of transition state formation (kJ/mol)
ΔH° free enthalpy change (kJ/mol)
GA glutaraldehyde
kₙᵣₜ turnover number (h⁻¹)
Kₑ Freundlich isotherm constant related to the adsorption capacity (mg/g) (mL/mg)ⁿ
Kₑ Langmuir isotherm constant (mL/mg)
Kₘ Michaelis–Menten constant (mg/L)
LC enzyme loading capacity (mg/g)
**L-DOPA**  L-3,4-dihydroxyphenylalanine  

**m**  weight of support (g)  

**n**  number of repetitions in a trial under the same conditions in Taguchi experiment  

**n_F**  Freundlich constant related to adsorption intensity  

**OREC**  organic rectorite  

**PH**  phenol  

**PPO**  polyphenol oxidase  

**q_{cal}**  calculated adsorption capacity of polyphenol oxidase at equilibrium (mg/g)  

**q_e**  adsorption capacity of polyphenol oxidase at equilibrium (mg/g)  

**q_{exp}**  experimental adsorption capacity of polyphenol oxidase at equilibrium (mg/g)  

**q_m**  maximum adsorption capacity of polyphenol oxidase predicated by isotherms (mg/g)  

**R**  gas constant (8.314 J/mol/K)  

**R^2**  coefficient of determination  

**RP**  phenolic compounds removal percentage (%)  

**ΔS^*_o**  free entropy change (/mol/K)  

**S/N**  signal-to-noise in Taguchi method  

**S/N_p**  predicated signal-to-noise in Taguchi method  

**T**  absolute temperature (K)  

**XRD**  X-ray diffraction  

**U**  enzyme activity unit  

**U_i**  immobilized enzyme activity (U)  

**V**  reaction volume (mL)  

**V_{max}**  maximum rate of the reaction (mg/L/h)  

**y**  measured enzyme activity in Taguchi experiment  

**GREEK LETTERS**  

**β_{D-R}**  Dubinin–Radushkevich isotherm constant (mol^2/J^2)  

**ε**  Polanyi adsorption potential of the Dubinin–Radushkevich model  

**χ^2**  non-linear chi-square test  

**INTRODUCTION**  

Phenolic compounds have been listed as priority pollutants by the US Environmental Protection Agency due to their acute toxicity and bio-recalcitrant nature. The permissible level of phenol is restricted to 1 μg/L in surface waters (Phipps et al. 1981). Hence, preventing phenolic compounds from contaminating industrial effluents is very important.  

Conventional physical, chemical and physico-chemical technologies for the phenolic compounds removal from industrial effluents, including extraction, adsorption, chemical oxidation and electrochemical methods, have faced some limitations such as low efficiency, high cost, high energy requirements, applicability to limited concentration range and formation of hazardous by-products that are even more toxic than the original phenols. Compared with physico-chemical technologies, biological technologies are considered as ecofriendly, cost-effective and viable alternatives for treatment of phenolic compounds. However, biological technologies have their own limitations such as inability to treat high concentration phenolic compounds, lower degradation rate and production of sludge. An enzymatic method, which may be an alternative method, has great potential in the treatment of phenolic compounds because of mild reaction conditions, high specificity and selectivity, biodegradability and is environmentally friendly (Jun et al. 2019).  

Polyphenol oxidase (PPO), which is widely distributed throughout the scale from microorganisms, to plants, animals, and human beings, is a type of copper-containing oxidoreductase and has bifunctional activity: ortho-hydroxylation of monophenols to diphenols by a mono-oxygenase activity and sequential oxidation of diphenols to quinones by an oxidase activity. The ability of PPO to use atmospheric oxygen as the electron acceptor, along with their broad substrate specificities (oxidize a large group of phenolics and/or polyphenolic components to their corresponding nontoxic quinones) has allowed it to be used for the removal of toxic and hazardous phenolic contaminants from waste-water and effluent.  

The important reason that PPO has not been exploited at an industrial level is that it requires large amount of the enzyme to achieve high removal efficiency due to enzyme inactivation and difficulty in reuse. Enzyme immobilization is one of the most important technologies, which significantly improves enzyme operational stability and reusability, and thereby improves the efficiency and reduction of the cost of the process (Jun et al. 2019).  

In order to preserve activity and improve stability of immobilized enzyme, choosing the appropriate immobilization strategy and support are very important. The immobilization strategy is one of the critical factors that can affect the properties of immobilized enzymes. A different strategy applied for the same enzyme immobilization...
can change the enzyme structure but it usually does not improve the biocatalysts properties. Hence, a compromise should be made between retention of high catalytic activity and operational benefits when choosing the immobilization strategy.

Support material serves an important role in catalytic efficiency of immobilized enzyme technology because the microenvironment of the support material can influence the physical and chemical structures of the support, the interaction nature between enzyme and support, the binding positions and also the number of bonds. For environmental use, except to fulfill the basic requirements, immobilization support should be nontoxic and environmentally friendly. Clay minerals, including bentonite, montmorillonite, rectorite, smectite, kaolinite, etc., are hydrous magnesium or aluminum phyllosilicates abundant in nature (An et al. 2015). The interlayer cations of clay can be exchanged easily by either inorganic or organic cations. Organically modified clay possesses additional functional groups, increased adhesion area, hydrophobicity and lowered steric hindrance (Shen & Gao 2019). Consequently, organic clay improves properties for enzyme immobilization, including increased loading amount, improved biocompatibility and operational stability (An et al. 2015).

Chitosan (CTS) is a second most abundant biopolymer and one of the most widely used support materials for enzyme immobilization owing to its a number of advantages, both from a technological and ecological point of view, such as low cost, easy availability, biodegradability, biocompatibility, hydrophilicity and adsorption properties, etc. (Benucci et al. 2018). The existence of numerous amine and hydroxyl groups in the chitosan structure enable effective binding of different enzymes without the involvement of any cross-linking agents.

In this present study, CTS was intercalated into organic rectorite (OREC) and chitosan/organic rectorite (CTS/OREC) composites were used as immobilization support for PPO. Rectorite (REC) was modified by cetyltrimethyl ammonium bromide (CTAB) to obtain OREC through cation exchange, which exhibited larger interlayer distance and aspect ratio than REC (Deng et al. 2012). To the best of our knowledge, this is the first study on the enzyme immobilization onto CTS/OREC composites. The aim of this study was to evaluate the potential application of the CTS/OREC as novel support for enzyme immobilization. PPO was immobilized onto CTS/OREC by physical adsorption or covalent binding. The optimization of immobilization conditions were studied using Taguchi method for attainment of the cost-effective process. The application of immobilized PPO for removing phenol (PH), 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) from aqueous solution was studied. The effect of immobilization strategy and chemical structure of substrate on the catalytic properties of immobilized PPO was discussed.

### MATERIALS AND METHODS

**Materials**

REC was purchased from Hubei Mingliu Inc. Co. (Wuhan, China). CTS (weight-average molecular weight 328 kDa, degree of deacetylation 85.6%) was purchased from Shandong Aokang Biotechnology Co. (Shandong, China). Cetyltrimethyl ammonium bromide (CTAB) was supplied by Tianjin Guangfu Fine Chemical Research Institute. PPO (EC 1.14.18.1, from mushroom as lyophilized powder) and l-3,4-dihydroxyphenylalanine (l-DOPA) were obtained from Sigma-Aldrich (St. Louis, USA). All other reagents were of analytical grade and used without further purification.

**Preparation of CTS/OREC composites**

OREC was prepared by ion-exchange reaction. REC (1 g) was dispersed in deionized water (100 mL). Subsequently, 2 g CTAB was added and the mixture was stirred at 90 °C for 5 h. The product was centrifugated, washed with deionized water and dried at 80 °C, and the sample OREC was obtained.

CTS (1 g) was dissolved in 1 L acetic acid solution (1%, v/v). The CTS solution was slowly added to OREC suspension at 60 °C and stirred for 48 h. CTS/OREC composites with initial CTS to OREC weight ratios of 8:1, 4:1, 1:1 and 1:4 were prepared. The formed composites were washed with deionized water and dried at 60 °C to obtain CTS/OREC1, CTS/OREC2, CTS/OREC3 and CTS/OREC4, respectively.

**Characterization of enzyme immobilization supports**

FT-IR spectra were recorded with KBr pellets on Fourier transform infrared spectrometer Nicolet iN10 + iZ10 (Nicolet Instrument Corporation, Madison, USA) with a range of 4,000–500 cm⁻¹. X-ray diffraction (XRD) patterns were conducted using an X-ray diffractometer (D8Advance, Bruker, Germany) with the CuKα radiation (λ = 0.15416 nm).
Immobilization of PPO

Physical adsorption

CTS/OREC (0.02 g) was immersed in 2 mL buffer solution containing a certain amount of PPO and shaken at room temperature to obtain adsorbed PPO (APPO). The buffers used were citrate-phosphate buffer (50 mM, pH 4–5) and phosphate buffer (50 mM, pH 6). APPO was thoroughly washed with phosphate buffer (50 mM, pH 7) and stored at 4 °C until further use.

Covalent binding immobilization

CTS/OREC (0.02 g) was activated using glutaraldehyde (GA) solution (10 mL) for 2 h at room temperature. The GA-activated supports were thoroughly washed with deionized water. Then, they were immersed in 2 mL buffer solution containing a certain amount of PPO and shaken for a certain time at room temperature to obtain covalent binding PPO (CPPO). The buffers used were citrate-phosphate buffer (50 mM, pH 4–5) and phosphate buffer (50 mM, pH 6–7). CPPO was thoroughly washed with phosphate buffer (50 mM, pH 7) and stored at 4 °C until further use.

Taguchi experiment

$L_{16}(3^4)$ and $L_{16}(4^2)$ orthogonal array were used for the experimental design in order to determine the optimization PPO immobilization conditions using physical absorption and covalent binding, respectively. The selected factors and their levels are listed in Table 1.

Because the aim of present study was to maximize enzyme activity, the optimal combination of PPO immobilization conditions can be obtained from the signal-to-noise (S/N) ratio following Equation (1) for the ‘larger is better’ (LIB):

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

where $n$ and $y_i$ is the number of repetitions in a trial under the same conditions and the measured enzyme activity, respectively.

| Enzyme | Trial no. | A Support | B pH | C time | D E/S ratio* | E GA concentration | Mean S/N ratio |
|--------|-----------|-----------|------|--------|--------------|-------------------|---------------|
| APPO   | 1         | 1 (CTS/OREC1) | 1 (4) | 1 (1 h) | 1 (15 mg/g) | 75.87             |
|        | 2         | 1         | 2 (5) | 2 (2 h) | 2 (20 mg/g) | 82.95             |
|        | 3         | 1         | 3 (6) | 3 (3 h) | 3 (25 mg/g) | 73.66             |
|        | 4         | 2 (CTS/OREC2) | 1    | 2      | 3            | 77.51             |
|        | 5         | 2         | 2    | 3      | 1            | 81.37             |
|        | 6         | 2         | 3    | 1      | 2            | 77.97             |
|        | 7         | 3 (CTS/OREC3) | 1    | 3      | 2            | 75.55             |
|        | 8         | 3         | 2    | 1      | 3            | 74.63             |
|        | 9         | 3         | 3    | 2      | 1            | 74.50             |
| CPPO   | 1         | 1 (CTS/OREC1) | 1 (4) | 1 (1 h) | 1 (5 mg/g) | 74.29             |
|        | 2         | 1         | 2 (5) | 2 (2 h) | 2 (10 mg/g) | 76.33             |
|        | 3         | 1         | 3 (6) | 3 (3 h) | 3 (15 mg/g) | 78.64             |
|        | 4         | 1         | 4 (7) | 4 (4 h) | 4 (20 mg/g) | 75.74             |
|        | 5         | 2 (CTS/OREC2) | 1    | 2      | 3            | 77.42             |
|        | 6         | 2         | 2    | 1      | 4            | 76.31             |
|        | 7         | 2         | 3    | 4      | 1            | 77.60             |
|        | 8         | 2         | 4    | 3      | 2            | 74.95             |
|        | 9         | 3 (CTS/OREC3) | 1    | 3      | 4            | 74.59             |
|        | 10        | 3         | 3    | 1      | 4            | 76.65             |
|        | 11        | 3         | 3    | 4      | 1            | 75.24             |
|        | 12        | 3         | 4    | 2      | 1            | 75.62             |
|        | 13        | 4 (CTS/OREC4) | 1    | 4      | 2            | 75.58             |
|        | 14        | 4         | 2    | 3      | 1            | 75.47             |
|        | 15        | 4         | 3    | 2      | 4            | 73.93             |
|        | 16        | 4         | 4    | 1      | 3            | 73.43             |

*S/E ratio: weight ratio of enzyme and support.
The enzyme activity of immobilization PPO at the optimal level obtained from the Taguchi experiments was predicted. The theoretically predicted signal-to-noise (S/N_p) can be calculated as (Srivastava et al. 2007):

$$S/N_p = S/N_0 + \sum_{i=1}^{n} (S/N_i - S/N_0)$$ (2)

where $S/N_0$ and $S/N_i$ are the total mean S/N ratio and individual S/N ratio at the optimal level, respectively, and $n$ is the number of repetitions in a trial under the same conditions.

Confirmation experiments were carried out at the optimum conditions in order to verify the results obtained from Taguchi experiments. The design of experiments, optimization of the process and analysis of variance (ANOVA) were done by Qualitek-4 statistical software.

**Evaluation of enzyme loading capacity**

The amount of the PPO loaded on support was assayed by the Bradford method (Bradford 1976). It was calculated in relation to enzyme loading capacity ($LC$, mg/g) by the following equation:

$$LC = \frac{(C_i - C_f)V}{m}$$ (3)

where $C_i$ and $C_f$ are the initial and final concentration of the PPO (mg/mL), respectively; $V$ is the PPO solution volume (mL); and $m$ is the weight of support (g).

**Adsorption isotherm**

Since the immobilization process was carried out using adsorption, the adsorption isotherms were plotted to better understand the mechanisms behind PPO binding to CTS/OREC. Adsorption experiments were performed with variation of PPO concentration (0.05–0.30 mg/mL) under optimal conditions. The adsorption capacity, $q_e$ (mg/g) is given by Equation (4):

$$q_e = \frac{(C_i - C_e)V}{m}$$ (4)

where $C_i$ and $C_e$ are the initial and equilibrium concentration of PPO (mg/mL), respectively; $V$ is the PPO solution volume (mL); and $m$ is the weight of support (g).

Isotherm models were modelled by a non-linear regression method using a trial-and-error procedure of the solver add-in with Microsoft Excel. The coefficient of determination ($R^2$) and the non-linear chi-square test ($\chi^2$) were used to test the best fit of the isotherm model to the experimental data, as shown in the following equation:

$$R^2 = \frac{\sum (q_{cal} - q_{exp})^2}{\sum (q_{cal} - q_{exp})^2 + \sum (q_{cal} - q_{exp})^2}$$ (5)

$$\chi^2 = \frac{\sum (q_{exp} - q_{cal})^2}{q_{cal}}$$ (6)

An $R^2$ value closer to 1 and a smaller $\chi^2$ value indicate that the data from isotherm models are similar to the experimental data.

**Activity assay of immobilized PPO**

The enzyme activity was assayed as described by Bayramoğlu et al. (2015) using L-DOPA as substrate. Free PPO solution (0.3 mL, 1.0 mg/mL) or immobilized PPO (0.02 g) was added to a solution of 5.0 mL L-DOPA in phosphate buffer (3.0 mL, 50 mM, pH 7), which was saturated with pure oxygen for 2 min at 25 °C to start the reaction. An increase in absorbance at 475 nm was recorded over the first 10 min. The enzyme activity unit (U) is described as the amount of enzyme that generates 1.0 μmol of dopachrome min/mL at 25 °C. All experiments were performed in triplicates.

The activity unit per gram of support (A) was used to define the activity of immobilized PPO:

$$A (U/g) = \frac{U_I}{m}$$ (7)

where $U_I$ is the immobilized enzyme activity (U); $m$ is the support weight (g).

**Removal of phenolic compounds**

If there were no special instructions, the phenolic compounds removal experiments were carried out as following: immobilized PPO (0.02 g) was added to 10 mL (100 mg/L) phenolic compounds solutions at 30 °C to initiate the reaction. At the predetermined reaction time, immobilized PPO was separated by filtration. To measure the concentration of remained phenolic compounds in the solution, the treated solutions were filtered through a
0.45 μm membrane and the filtrate was analyzed using the colorimetric method, where phenolic compounds (1.0 mL) react with 4-aminoantipyrine (4-AAP, 1.0 mL, 2.0 mM in 100 mM phosphate buffer solution of pH 10) and potassium ferricyanide reagent (1.0 mL, 6 mM in 100 mM phosphate buffer solution of pH 10) to form a red quinone-type dye. After 5 min, absorbance was measured at 510 nm (Gómez Carrasco et al. 2012). The absorbance data were converted to phenolic compounds concentration using a calibration curve.

The phenolic compounds removal percentage (RP) was assessed by the following equation:

\[
\text{Removal percentage (\%)} = \frac{C_0 - C_t}{C_0} \times 100
\]  

where \(C_0\) and \(C_t\) are the initial and remaining phenolic compounds concentration (mg/L), respectively.

**Effect of pH**

The effect of pH on the removal of phenolic compounds was determined at different pH. The buffers used were citrate-phosphate buffer (50 mM, pH 4–5), phosphate buffers (50 mM, pH 6–8) and glycine–NaOH buffer (50 mM, pH 9). All experiments were performed in triplicate.

**Effect of temperature**

The effect of temperature was investigated in the range of 10–50 °C in phosphate buffer at their optimal pH, respectively. The activation energy \(E_a\) was calculated from the slope of the Arrhenius plot:

\[
\text{Slope} = - \frac{E_a}{R}
\]  

where \(R\) is the gas constant (8.314 J/mol/K).

**Effect of substrate concentration**

The kinetic constants, Michaelis–Menten constant \(K_m\) and maximum rate of the reaction \(V_{\text{max}}\), were determined by measuring initial rates of the reaction with different substrate concentrations (50–150 mg/L) in phosphate buffer under optimum assay conditions and calculated from a Lineweaver–Burk plot. The turnover number \((k_{\text{cat}})\) was calculated as described by Zhang & Neau (2002) as the following equation:

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[E]}
\]  

where \([E]\) is the PPO concentration.

Gibbs free energy change \((\Delta G^\circ)\), free enthalpy change \((\Delta H^\circ)\), free entropy change \((\Delta S^\circ)\), free energy of substrate binding \((\Delta G_{E-S})\) and transition state formation \((\Delta G_{E-T})\) were determined as described by Wehaidy et al. (2019) as follows:

\[
\begin{align*}
\Delta H^\circ &= E_a - RT \\
\Delta G^\circ &= -RT \ln \frac{k_{\text{cat}}T}{K_m} \\
\Delta S^\circ &= \frac{\Delta H^\circ - \Delta G^\circ}{T} \\
\Delta G_{E-S} &= -RT \ln \frac{1}{K_m} \\
\Delta G_{E-T} &= -RT \ln \frac{k_{\text{cat}}}{K_m}
\end{align*}
\]

**Effect of reaction time**

The effect of reaction time on the removal of phenolic compounds was assayed by performing the enzyme catalysis reaction for different time intervals at the optimum reaction pH and temperature.

**Reusability**

To assess the reusability of immobilized PPO, 2,4-DCP was used as the substrate. Repeated batch reaction was performed 10 times, each for 4 h, using the same immobilized PPO. After each reaction, immobilized PPO was separated and washed with phosphate buffer (50 mM, pH 7). The RP for 2,4-DCP was measured, and then the immobilized PPO was transferred into a fresh substrate solution.

**RESULTS AND DISCUSSION**

**Characterization of CTS/REC and CTS/OREC**

Figure 1(I) shows the FT-IR spectra of REC, OREC and CTS/OREC2 composites. The two pairs of peaks at
1,026.8/1,055.3 and 908.9/937.5 cm\(^{-1}\) are characteristic for REC (Luo et al. 2013). The broad adsorption band at 3,647.0, 3,434.4 and 1,633.9 cm\(^{-1}\) are assigned to the Al–OH hydroxyl stretching vibration, H–OH stretching vibration and the bending vibration of H\(_2\)O, respectively (Wu et al. 2013). For OREC, most peaks did not change comparison with REC, indicating that the basic crystal structure did not change after REC was modified with CTAB. In the spectrum of OREC, the peaks at 2,925.3, 2,850.4 and 1,472.4 cm\(^{-1}\) were associated with the C–H\(_2\) asymmetric and symmetric stretching vibration, and the bending vibration of C–H of CTAB (Chen et al. 2019), which revealed that OREC was successfully prepared. The spectrum of CTS/OREC exhibited the characteristic peaks of CTS and OREC. The broad absorption band at 3,345.1 cm\(^{-1}\) was attributed to the overlapping of the –OH stretching vibration of OREC and N–H bonding to –OH of CTS. The characteristic peak at 1,590.3 cm\(^{-1}\) was ascribed to –NH\(_2\) deformation vibration in the CTS (Paluszkiwicz et al. 2011). The bending vibration of C–H of CTAB in OREC at 1,472.4 cm\(^{-1}\) shifted to 1,469.7 cm\(^{-1}\) in CTS/OREC. The results indicated that electrostatic interactions and the hydrogen bond existed between OREC and CTS in CTS/OREC.

The XRD patterns of CTS/OREC composites with different weight ratios of CTS and OREC are shown in Figure 1(II). With the increasing of weight ratio of CTS to OREC, the diffraction peak of the resulting CTS/OREC composites shifted from 2.91° of CTS/OREC1 to 2.20° of CTS/OREC4, which indicated the \(d\)-spacing increased from 3.03 nm to 4.02 nm based on the Bragg diffraction equation. The results suggested the successful intercalation of CTS into OREC interlayer.

**Optimization of enzyme activity of immobilized PPO by Taguchi method**

The mean S/N ratios response for immobilized PPO activity obtained according to Equation (1) are listed in Table 1. For APPO, experiment number 2, the combination condition \(A_1B_2C_2D_2\), which meant support (A) in level 1, immobilization pH (B) in level 2, immobilization time (C) in level 2 and weight ratio of enzyme and support (E/S ratio, D) in level 2, had the highest S/N ratio 82.95 among the nine experiments. For CPPO, experiment number 3, \(A_1B_3C_3D_3E_3\), which meant support (A) in level 1, immobilization pH (B) in level 3, immobilization time (C) in level 3, weight ratio of enzyme and support (E/S ratio, D) in level 3 and GA concentration (E) in level 3, achieved the highest S/N ratio with 78.64 among the 16 experiments.

Because the obtained optimal immobilization conditions based on the Taguchi results were not arranged in the initial orthogonal experiment (as shown in Table 1), a confirmation experiment was carried out based on the predicted one. The predicted S/N\(_p\) ratios computed by Equation (2) and confirmed S/N ratio are listed in Table 2. The results suggested the immobilization process can be improved by the combination of the suitable immobilization conditions. As shown in Table 2, APPO and CPPO reached the highest enzyme activity at \(16.37 \times 10^3\) and \(8.92 \times 10^3\) U/g at optimal preparation conditions, and LC of APPO and CPPO were 17.3 and 26.5 mg/g, respectively.
As shown in Table 2, the enzyme activity of APPO was higher than that of CPPO, while CPPO showed a higher LC than that of APPO. Previous literatures indicated a large amount of enzyme bound on the support could limit the accessibility of substrate to the active sites of immobilized enzyme, resulting in a reduction of enzyme activity (Knezevic et al. 2006). Thus, the lower enzyme activity of CPPO may be due to the change of the native conformation of enzyme and high LC (Hou et al. 2014).

**Isotherms of adsorption**

The interaction between PPO and CTS/OREC composites can be described by adsorption isotherm at equilibrium. To determine the adsorption process, the three isotherm models, i.e., Langmuir, Freundlich and Dubinin–Radushkevich, were used to fit the experimental data of PPO adsorption. The isotherm model, the theoretical parameters of isotherm at the optimum conditions, along with $R^2$ and $\chi^2$ were calculated and are illustrated in Table 3. Compared with the three kinds of isotherm models, the Langmuir isotherm model had the highest $R^2$ and lowest $\chi^2$, indicating that Langmuir isotherm model is the most fitting of the experimental data of PPO adsorption process. Langmuir isotherm assumes that the monolayer adsorption takes place only at homogenous sites on the adsorbent surface with uniform energy level. The results indicated that monolayer coverage of the enzyme molecules is the main adsorption mechanism due to homogeneous distribution of active sites on the surface of CTS/OREC. The values of $1/n_F$ of the Freundlich isotherm imply the type of isotherm and can be classified as irreversible ($1/n_F = 0$), favourable ($1/n_F < 1$) and unfavourable ($1/n_F > 1$). The value of $1/n_F$ was found to be 0.38, supporting the favourable nature of the PPO adsorption process. The values of apparent free energy from the Dubinin–Radushkevich isotherm model was less than 8 kJ/mol, indicating that physical adsorption was involved during the adsorption process.

**Removal of phenolic compounds**

**Control experiments**

The influence of the immobilization support on phenolic compounds removal was investigated and the results are shown in Figure 2. As shown in Figure 2, initially, RE

| Enzyme | Initial condition | S/N ratio | Optimum conditions | Predicated S/N ratio | Experimental S/N ratio | A ($10^3$ U/g) | LC (mg/g) |
|--------|------------------|-----------|--------------------|----------------------|-----------------------|----------------|-----------|
| APPO   | $A_1B_2C_2D_2$   | 82.95     | $A_2B_2C_2D_2$    | 84.41                | $A_2B_2C_2D_2$        | 16.37         | 17.3      |
| CPPO   | $A_1B_3C_3D_3E_3$| 78.64     | $A_2B_3C_4D_3E_3$ | 79.06                | $A_2B_3C_4D_3E_3$     | 8.92          | 26.5      |

**Table 2**  | Confirmation test results for multi-response with initial and optimal adsorption condition

**Table 3**  | Determination of adsorption isotherms using Langmuir, Freundlich, and Dubinin–Radushkevich isotherm models

| Isotherm model          | Equations                                  | Parameter | Values  |
|-------------------------|--------------------------------------------|-----------|---------|
| Langmuir                | $q_e = \frac{q_mK_LC_e}{1 + K_LC_e}$       | $q_m$     | 20.23   |
|                         |                                            | $K_L$     | 223.82  |
|                         |                                            | $R^2$     | 0.9860  |
|                         |                                            | $\chi^2$  | 0.268   |
| Freundlich              | $q_e = K_FC_e^{1/n_F}$                     | $K_F$     | 76.52   |
|                         |                                            | $1/n_F$   | 0.38    |
|                         |                                            | $R^2$     | 0.9260  |
|                         |                                            | $\chi^2$  | 1.099   |
| Dubinin–Radushkevich    | $q_e = q_m\exp(-\beta_{D-R}e^2)$           | $q_m$     | 62.37   |
|                         |                                            | $\beta_{D-R}$ | 0.012   |
|                         | $\epsilon = RT\ln\left(1 + \frac{1}{C_e}\right)$ | $E_{D-R}$ | 6.43    |
|                         | $E_{D-R} = \frac{1}{\sqrt{2\beta_{D-R}^2}}$ | $\chi^2$  | 12.42   |

**Figure 2**  | Removal efficiency of phenolic compounds by immobilization support CTS/OREC. Reaction condition: phenolic compounds concentration 100 mg/L, temperature 30 °C, pH 7, CTS/OREC 0.02 g.
increased with the increasing of adsorption time and RE reached 7.8 ± 0.4%, 13.5 ± 0.3% and 15.5 ± 0.6% for PH, 4-CP and 2,4-DCP after 2 h, respectively. As the reaction time progressed there was very limited increase in RE. Although the RE of phenolic compounds increased with the increasing numbers of chlorine groups in the substrate, the effect of the structure of phenolic compounds was not significant. The results revealed that phenolic compounds removed by support via adsorption were limited. Therefore, the phenolic compounds removal was attributed to immobilized PPO.

**Effect of pH**

The solution pH is a major factor affecting not only the enzyme ionization state which decided its tertiary structure, but also the ionization state of the substrates. The effect of pH values on the removal of phenolic compounds are shown in Figure 3. As shown in Figure 3(a), for APPO, the optimum pH value for PH removal was observed at pH 7. The optimal pH decreased to 6 and 5 for 4-CP and 2,4-DCP, respectively. The results indicated that, for APPO, the chemical structure of substrates affected the optimal pH of immobilized PPO, which decreased with the increasing numbers of chlorine groups in substrate. As shown in Figure 3(b), for CPPO, the highest RPs were obtained at pH 6 for all three kinds of substrate, suggesting that the optimal pH for phenolic compounds removal was not related to the chlorine group on the aromatic ring.

The RP of phenolic compounds also depended on their chemical structure. For APPO, the maximum RP was 69.2 ± 1.4%, 89.3 ± 1.6% and 93.1 ± 2.6% for PH, 4-CP and 2,4-DCP, respectively. For CPPO, the maximum RP was 49.4 ± 3.4%, 68.4 ± 1.4% and 75.0 ± 2.6% for PH, 4-CP and 2,4-DCP, respectively. Compared with APPO, the decreased RP of phenolic compounds caused by CPPO can be explained by the decreasing of enzyme activity due to the change of the native conformation of enzyme. The RP order at the optimal pH was 2,4-DCP > 4-CP > PH for both immobilized PPO, which agreed with the results of other investigators where phenols with chlorine atoms at ortho- and para-positions were more susceptible to PPO (Espín et al. 2000).

**Effect of temperature**

The effect of temperature on phenolic compounds removal was performed at optimal pH and temperatures from 10 to 50 °C. Figure 4(a) and 4(b) show that, for both immobilized PPO, the RPs of phenolic compounds initially increased and then decreased with the increasing temperature, and the optimal temperature was 30 °C for all three kinds of substrate. The lower RP at low temperature may be due to the low solubility of the phenolic compounds. The RP decrease at temperatures higher than the optimal was possibly because of the thermal deactivation when temperature exceeds the tolerance level. In addition, dissolved oxygen in the reaction system decreases as the temperature increases, which may be another reason for the RP decrease (Aktas & Tanyolac 2005).

The Arrhenius plots of APPO and CPPO for different phenolic compounds were linear as shown in Figure 4(c) and (d), respectively. In the case of APPO, the calculated $E_a$ values for PH, 4-CP and 2,4-DCP were 42.9, 33.2 and 32.0 kJ/mol, respectively. For CPPO, $E_a$ for PH, 4-CP and 2,4-DCP were 48.6, 44.0 and 39.7 kJ/mol, respectively. The energy barrier for 2,4-DCP catalysis was lowest, which...
indicated that the activation energy required to form the activated complex reduced (Avwioroko et al. 2019) and 2,4-DCP would be the most easily catalyzed among the three kinds of substrate for both immobilized PPO.

Compared with CPPO, APPO showed the lower $E_a$ for all three kinds of substrate, implying that APPO improved the catalytic efficiency by decreasing $E_a$ required to achieve activated complex (Agrawal et al. 2020). The lower $E_a$ values also suggested that the catalytic efficiency of APPO was restricted by mass-transfer rather than kinetic limit (López-Cruz et al. 2006).

Effect of substrate concentration

The kinetic parameters of $K_m$, $V_{max}$, $k_{cat}$, $V_{max}/K_m$ and $k_{cat}/K_m$ for immobilized PPO are shown in Table 4. The $K_m$ defines the affinity of enzyme towards the substrate. For APPO, 2,4-DCP showed the lowest $K_m$ value of 216.9 mg/L, which was lower than that of 4-CP of 239.0 and PH of 275.4 mg/L. The results indicated that the substitution of chlorine atoms at the aromatic ring increased the affinity of immobilized PPO towards the substrate. In the case of $V_{max}$, $V_{max}/K_m$, $k_{cat}$ and $k_{cat}/K_m$ values, the order PH < 4-CP < 2,4-DCP was obtained. The results suggested that the catalytic efficiency improved as the chlorine atoms substituted in the aromatic ring and 2,4-DCP was the best substrate for APPO. For CPPO, the values of all the above kinetic parameters followed the same order as APPO.

Comparing the two kinds of immobilized PPO, for CPPO, the higher $K_m$ but lower $V_{max}$, $V_{max}/K_m$, $k_{cat}$ and $k_{cat}/K_m$ were obtained because the same substrate was
used. The higher $K_m$ value of CPPO showed that the substrate had lower accessibility to the enzyme active sites and lower flexibility of the enzyme molecule, which may be due to covalent binding providing strong binding interaction between PPO and support. In additional, the increased catalytic efficiency of APPO may be due to the increase of $V_{\text{max}}$, which was sufficient to compensate the increase of $K_m$. It can be said that $V_{\text{max}}$ played an important role in the catalytic activity of APPO but the contribution of $K_m$ was less.

The $\Delta G^\circ$ is the thermodynamic parameter for the actual feasibility and extent of a catalytic reaction. A lower $\Delta G^\circ$ value indicates that the conversion of transition state into product is more spontaneous (Riza et al. 2007). The $\Delta H^\circ$ value is related to the energy necessary to form an activated complex. The lower $\Delta H^\circ$ value suggests that the formation of an activated complex is very efficient (George & Sugunan 2014). The $\Delta S^\circ$ is correlated to the order degree of activated complex. The negative and lower $\Delta S^\circ$ values suggests that the structure of activated complex is more ordered (Riza et al. 2007).

As shown in Table 4, for both immobilized PPO, the $\Delta G^\circ$ values differed among the three kinds of substrate, although the differences were generally small. The lowest $\Delta G^\circ$ value was obtained for 2,4-DCP, indicating that the conversion of transition state into product was easiest but the effect of chemical structure of substrate on the $\Delta G^\circ$ values was very limited. The $\Delta G^\circ_{E-S}$ and $\Delta G^\circ_{E-T}$ values followed the same order as $\Delta G^\circ$ values. The results indicated that although the ground state of 2,4-DCP fitted less tightly into the active site cleft compared with PH and 4-CP, the most important reason for high catalytic efficiency of 2,4-DCP can be attributed to the efficiently form of activated complex. The $\Delta H^\circ$ values of the different substrates showed the order as 2,4-DCP $< 4$-CP $<$ PH, which meant that the chlorine atoms at the aromatic ring in the substrate affect the activated complex stability. The $\Delta S^\circ$ (in absolute value) followed the opposite order as $\Delta H^\circ$ suggesting that the activated complex of PH was more ordered.

In the case of immobilized PPO, for the same substrate, the $\Delta G^\circ$, $\Delta G^\circ_{E-T}$, $\Delta G^\circ_{E-S}$, $\Delta H^\circ$ values for APPO were lower than CPPO, while the results of $\Delta S^\circ$ (in absolute value) were opposite. It provided an evidence that the higher catalytic efficiency of APPO due to the more spontaneous for formation of the activated complex (Riza et al. 2007). The results also proved that the immobilization strategy significantly influenced the properties of the resulting immobilized PPO and finally determined its catalysis properties. In this study, compared with covalent binding, physical adsorption was a better method for immobilizing PPO.

**Effect of reaction time**

To investigate the effect of reaction time on the removal of phenolic compounds by immobilized PPO, RPs were carried out at different time intervals. As shown in Figure 5(a), for APPO, RPs of phenolic compounds increased with the increase in reaction time and reached 69.3 ± 4.2%, 89.8 ± 2.5% and 93.8 ± 1.7% for PH, 4-CP and 2,4-DCP after 2 h.

| Parameter                  | APPO          | CPPO          |
|----------------------------|---------------|---------------|
| $E_a$ (kJ/mol)             | PH            | 4-CP          | 2,4-DCP       |
| $K_m$ (mg/L)               | 275.4         | 239.0         | 216.9         |
| $V_{\text{max}}$ (mg/L/h)  | 7.23          | 9.17          | 11.00         |
| $k_{\text{cat}}$ (h$^{-1}$) | 0.26          | 0.58          | 0.51          |
| $k_{\text{cat}}/K_m$ ($10^{-3}$) | 7.59       | 11.09         | 14.66         |
| $\Delta G^\circ$ (kJ/mol)  | 72.39         | 71.79         | 71.33         |
| $\Delta H^\circ$ (kJ/mol)  | 40.38         | 30.68         | 29.48         |
| $\Delta S^\circ$ (J/mol/K) | −105.65       | −135.68       | −138.13       |
| $\Delta G^\circ_{E-S}$ (kJ/mol) | 14.15    | 13.80         | 13.55         |
| $\Delta G^\circ_{E-T}$ (kJ/mol) | 12.30   | 11.34         | 10.64         |

**Table 4** | Kinetic and thermodynamic parameters for different substrate catalysis of free and immobilized PPO

The $\Delta G^\circ$, $\Delta G^\circ_{E-T}$, $\Delta G^\circ_{E-S}$, $\Delta H^\circ$ values for APPO were lower than CPPO, while the results of $\Delta S^\circ$ (in absolute value) were opposite. It provided an evidence that the higher catalytic efficiency of APPO due to the more spontaneous for formation of the activated complex (Riza et al. 2007). The results also proved that the immobilization strategy significantly influenced the properties of the resulting immobilized PPO and finally determined its catalysis properties. In this study, compared with covalent binding, physical adsorption was a better method for immobilizing PPO.

**Effect of reaction time**

To investigate the effect of reaction time on the removal of phenolic compounds by immobilized PPO, RPs were carried out at different time intervals. As shown in Figure 5(a), for APPO, RPs of phenolic compounds increased with the increase in reaction time and reached 69.3 ± 4.2%, 89.8 ± 2.5% and 93.8 ± 1.7% for PH, 4-CP and 2,4-DCP after 2 h.
respectively. With the prolonging of reaction time to 6 h, RPs slight increased to 72.3 ± 3.1%, 91.4 ± 2.0% and 94.3 ± 1.8%, respectively. The removal reaction was followed by a very slow removal process, possibly due to the decrease in the concentration of phenolic compounds. As reported by previous literatures, the decline of conversion of phenolic compounds can also be due to the accumulation of the catalytic products which inhibited the catalytic process (Liu et al. 2012).

For CPPO (Figure 5(b)), RPs initially increased rapidly and 70.9 ± 2.5% and 78.1 ± 2.6% of 4-CP and 2,4-DCP were obtained at 4 h, respectively. After that, the RP increased slowly and reached 78.1 ± 2.9% and 83.6 ± 3.1% at 8 h, respectively. For PH, RP increased continually in the first 6 h and RE reached 62.3 ± 3.1%, but RP increased slightly with the prolonging of reaction time, and finally RP reached 66.7 ± 2.7%. For any enzymatic process, it is desired to have high catalytic efficiency and shortened reaction time. The shortened reaction time obtained from APPO compared with CPPO might be attributed to the higher PPO activity. Phenolic compounds can diffuse into the active sites of APPO more easily than into those of CPPO and the reaction equilibrium is reached faster.

Reusability

Reusability of immobilized enzyme is very important and a prerequisite for industrial application. The reusability of immobilized PPO is shown in Figure 6. With the recycling number increasing, the RP of 2,4-DCP decreased for both immobilized PPO. After 10 consecutive operations, the RP of 2,4-DCP was 73.2 ± 2.6% and 60.3 ± 1.5% for APPO and CPPO, respectively. During the enzymatic reaction, the reaction product could accumulate on the support, which might hinder the active sites of the enzyme and affect adversely the reaction of the next cycles. The active site of the immobilized PPO might also be distorted due to the frequent encountering of the substrate on the same active site, resulting in the delay of the catalytic efficiency partially or entirely. In the investigated range, CPPO showed the higher reusability than APPO. For APPO, the enzyme activity loss could be due to the weakened bond strength between the enzyme and support, and hence PPO leached out from the support during reuse and washing operations. Covalent binding offered strong chemical
bonding between PPO and support which improves the stability of the immobilized PPO. The decrease in enzyme activity of CPPO could be attributed to deactivation during each cycle.

**CONCLUSION**

The study is the first report of immobilization PPO on CTS/OREC composites. The immobilization conditions were optimized successfully by Taguchi method. A different immobilization strategy determined the microenvironments of the enzyme, and the immobilized PPO showed different enzyme activity and properties. APPO exhibited the higher enzyme activity compared with CPPO. As the two kinds of immobilized PPO were used to removal phenolic compounds from aqueous solution, for the three kinds of investigated substrate, 2,4-DCP showed the higher removal efficiency. Moreover, to highlight the significance of the presented study and to emphasize the improvement of the phenolic compounds removal by immobilized PPO on CTS/OREC composites, the results were compared with those of other recently published studies (Table 5). It should be emphasized that the type of support has a significant impact on the efficiency of phenolic compounds removal and most of the summarized catalytic processes require a longer reaction time and lower phenolic compounds concentration compared with our study. The phenolic compounds removal process presented in our study was effective in mild conditions. We believe that this work presents a new approach to the synthesis of highly efficient biocatalyst with high catalytic activity, high stability, low cost and recyclability from the cheap and environmentally friendly sources. All the results demonstrated that the PPO immobilized on CTS/OREC composites possessed a great potential for application in phenolic compounds removal.

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**Table 5** Comparison of immobilized PPO on various supports for phenolic compounds removal

| Supports                                      | Immobilization strategy | Substrate (concentration) | Reaction time (h) | Optimum pH | Optimum temp. (°C) | RP (%) | References                  |
|-----------------------------------------------|-------------------------|---------------------------|-------------------|------------|--------------------|--------|-----------------------------|
| Chitosan-clay composite beads                 | Covalent binding        | Phenol (10 mg/L)          | 6                 | 7.0        | 25                 | 100    | Dinçer *et al.* (2012)      |
| 3-aminopropyl triethoxysilane modified diatom biosilica | Covalent binding        | Phenol (20 mg/L)          | 12                | 7.0        | 45                 | 87     | Bayramoğlu *et al.* (2013) |
| Silicon gel                                   | Covalent binding        | Phenol (20 mg/L)          | 12                | 7.0        | 45                 | 74     |                            |
| Calcium aluminosilicate                       | Covalent binding        | Phenol (100 mg/L)         | 75                | 6.8        | Room temp.        | 35.5   |                            |
| Sodium aluminosilicate                        | Covalent binding        | Phenol (100 mg/L)         | 8/3               | 8.0        | Room temp.        | 34     |                            |
| Polyacrylonitrile beads                       | Covalent binding        | Phenol (1 mmol/L)         | 12                | 7.0        | 40                 | 96     | Wu *et al.* (2017)          |
| Amine-functionalized graphene oxide           | Adsorption              | Phenol (1 g/L)            | 5                 | 5.0        | 30                 | 99.5   | Pravin *et al.* (2018)      |
| Chitosan/organic rectorite composite          | Adsorption              | Phenol (100 mg/L)         | 2                 | 7.0        | 30                 | 69.3   | Present study               |
|                                               |                         | 4-CP (100 mg/L)           | 2                 | 6.0        | 30                 | 89.8   |                            |
|                                               |                         | 2,4-DCP (100 mg/L)        | 2                 | 5.0        | 30                 | 93.8   |                            |
|                                               | Covalent binding        | Phenol (100 mg/L)         | 8                 | 6.0        | 30                 | 66.7   |                            |
|                                               |                         | 4-CP (100 mg/L)           | 8                 | 6.0        | 30                 | 78.1   |                            |
|                                               |                         | 2,4-CP (100 mg/L)         | 8                 | 6.0        | 30                 | 83.6   |                            |

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