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Investigation on the Removal of 2,4-Chlorophenol using Horseradish Peroxidase Immobilized on NanoHybrid

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Herein, in this report, was developed a nano-hybrid of Fe_3O_4/Ag/GO nanoparticles (NPs), constituted of graphene oxide (GO) supporting flower-like Fe_3O_4/Ag NPs in the presence of citric acid surfactant. The citric acid-functionalized Fe_3O_4/Ag/GO nanoparticles were used for Horseradish peroxidase (HRP) immobilization. The immobilized HRP can be tested to remove 2,4-dichlorophenols in the presence of H_2O_2, the degradation of 2,4-DCP at pH 6 at 25 °C and in the presence of H_2O_2 (0.8 mM) was for free and immobilized HRP of 40 % and 77 %, respectively. Moreover, immobilized HRP showed a high removal efficiency of ~94% in the presence of 0.6 mM of H_2O_2.

1. Introduction

Chlorophenols, which are chlorinated aromatic compounds, are widely used in the manufacture of plastics, pharmaceuticals, printing and dyeing materials, pesticides, wood preservatives, and petrochemicals (Zhang et al., 2009). They are a significant group of pollutants because of their high toxicities. In particular, some of them have carcinogenic, teratogenic, and mutagenic effects. In 1987, the US Environmental Protection Agency categorized chlorophenols as priority pollutants and set a permissible upper limit of 0.5 mg/L in public water supplies (Elghniji et al., 2012; Jia et al., 2012).
Because of increasing public health concerns and stricter regulations on treatment and disposal, it becomes more and more essential to develop new efficient methods for removing these compounds from wastewaters. The reported methods for removing phenolics from wastewaters include activated carbon adsorption, microbial degradation, chemical oxidation, corona discharge, and most recently, enzymatic degradation.
A potential alternative to conventional methods suggested by many researchers was biological treatment through the use of enzymes.
In particular, a protein isolated from the roots of horseradishes, Horseradish peroxidase (HRP) is the widely used catalyst in enzymatic reactions (Chang et al., 2014, Magorio et al., 2012). In the presence of hydrogen peroxide, HRP can be used to remove chlorophenols and other pollutants, which have been labeled as “priority pollutants” by the US Environmental Protection Agency (EPA), from infect water. On the other hand, HRP showed several limitations during the reaction such as low stability, i.e. in various conditions it can suffer for fast deactivation (Huang et al., 2005).
A possible solution for the stabilization of HRP may be achieved by multiple approaches such as chemical crosslinking, medium engineering, enzyme immobilization (Sarno et al., 2017; Sarno et al 2019a), or protein engineering. The immobilized enzyme should retain the same functionality and have the advantages of better storage stability, thermal stability, and ease of operation compared with those of the free protein in solution (Sharma et al., 2017). Nanomaterials such as nanoparticle (NPs), nanocarbon, etc… represent favorable supporting materials for enzyme immobilization, thanks to their specific surface area and active enzyme loading (Feng et al., 2011). Another promising candidate for the enzyme immobilization was graphene oxide (GO) due to the large specific surface area. Moreover, it can be easily modified and dispersed in the water thanks to a variety of reactive oxygen functional groups on its surface (Zhang et al ., 2010; Chang et al., 2014). Graphene oxide can be used as support for nanoparticle dispersion and stabilization (Jiang et al.,

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2011; Zhang et al., 2010). Herein, in this report, was developed a new nano-hybrid of Fe_3O_4/Ag/GO nanoparticles, constituted of GO supporting flower-like Fe_3O_4/Ag NPs in the presence of citric acid surfactant. The citric acid-functionalized Fe_3O_4/Ag/GO nanoparticles, synthesized via a solvothermal process, can immobilize HRP to remove 2,4-chlorophenols in the presence of H_2O_2. The effect of reaction time, H_2O_2 concentration, and the reusability on the removal of 2,4-dichlorophenols were also evaluated.

2. Material and Method

2.1 Material

Ferric chloride hexahydrate ($FeCl_3$•$6H_2O$, 98%), urea (100.5%), ethylene glycol (EG), citric acid (99.9%), ethanol, silver nitrate ($AgNO_3$), 2,4-dichlorophenol, Horseradish peroxidase (~150 U/mg), potassium ferrycyanide, sodium bicarbonate ($NaHCO_3$) and 4-aminoantipine were purchased from Aldrich Chemical Co. All chemicals were of analytical grade. The graphene oxide (GO) was acquired from Graphene Supermarket.

2.2 Synthesis of Nano-hybrid

0.075 g of GO, 0.81 g of Fe_3Cl_3·6 H_2O, 0.001 g of $AgNO_3$, 1.80 g of Urea and 0.1 g of citric acid were dispersed in 30 ml of ethylene glycol. The solution was homogenized by sonication for 5 min and the mixture was shifted into a Teflon-lined stainless steel autoclave. The synthesis was carried out for 4 h at 200°C. After 4h the reaction mixture was cooling to room temperature and the black material was washed with ethanol. Finally, the material was dried for 12 h at 60°C.

2.3 HRP Immobilization

0.1 mg of Fe_3O_4/Ag/GO NPs was added at 0,1, 0,2, 0,4 mg/ml of HRP in buffer solution (pH 6.0, 0.1 M) and mixed for 3 h at 4°C. After coupling time the immobilized enzymes were separated by an external magnetic field. The immobilized HRP were collected and cleaned three times with buffer phosphate (pH 6.0, 0.1 M) to remove free enzymes. The immobilized HRP was stored at 4°C in the buffer solution (pH 6.0, 0.1 M) for further measurements. The immobilization efficiency was determined using the Bradford method (Bradford, 1976).

2.4 Catalytic experiments

The experiments were carried out in triplicate. Immobilized HRP (0.1 mg/ml) was added to an aqueous solution of 2,4-dichlorophenol (10 mL, 70 mg/L); the pH was adjusted to 6.0 using sodium phosphate buffer. In particular, the mixture was mixed at a speed of 200 rpm at 25 °C. The degradation test was initiated by the addition of H_2O_2 (0.4, 0.6, and 0.8 mmol/L) after 30 min of mixed to achieve adsorption-desorption equilibrium. The reaction degradation was observed for 180 min and at time intervals, 2 mL aliquots of the reaction solution were recovery and they immobilized were immediately recovered by magnetic separation.

2.5 Characterization and analysis

The surface morphology of Fe_3O_4/Ag/GO was characterized on a transmission electron microscope (TEM FEI Tecnai electron microscope operating at 200 KV) equipped with an EDX probe.
The concentration of 2,4-DCP was determined with a spectrometer method (Sarno et al., 2019b) on an EVOLUTION 60s spectrophotometer (Thermo Scientific). The frequency of 2,4 DCP was analyzed by measuring the absorbance of the solution at 510 nm.
The oxidation intermediates of 2,4 DCP were analyzed on GC-MS analyzer (Thermo Scientific) being equipped with HP-5 column (0.25 µm×0.25 mm×30 m). The temperature program of GC started at 50°C and was held for 1 min. Then the column was sequentially heated at a rate 7 °C/min to 180°C, held for 1 min and then heated at a rate of 10°C/min to 280°C and held for 1 min. Injector and detector temperature were at 230°C. Helium was employed as a carrier gas with a constant flow of 1 ml/min.

3. Result and Discussion

3.1 Characterization of Nano-hybrid

The morphology of Fe_3O_4/Ag NPs on GO was characterized by TEM (Figure 1). The Fe_3O_4/Ag NPs are well-dispersed on GO. From TEM image we can observe small dark NPs core surrounded by a fringed light-colored shell, see the scheme in Figure 1.

The surface morphology of the nano-hybrid was studied using SEM (FESEM LEO1525) as shown in Figure 2. As revealed by the images, graphene oxide appears to be arranged in thin petals, each crumpled and wrinkled as it is typical of GO. These nano-sheets are folded and it is possible to distinguish the edges of individual sheets, including kinked and wrinkled areas. The nanoparticles, grew on GO, are also visible, homogeneously dispersed on the nano-sheets surface.

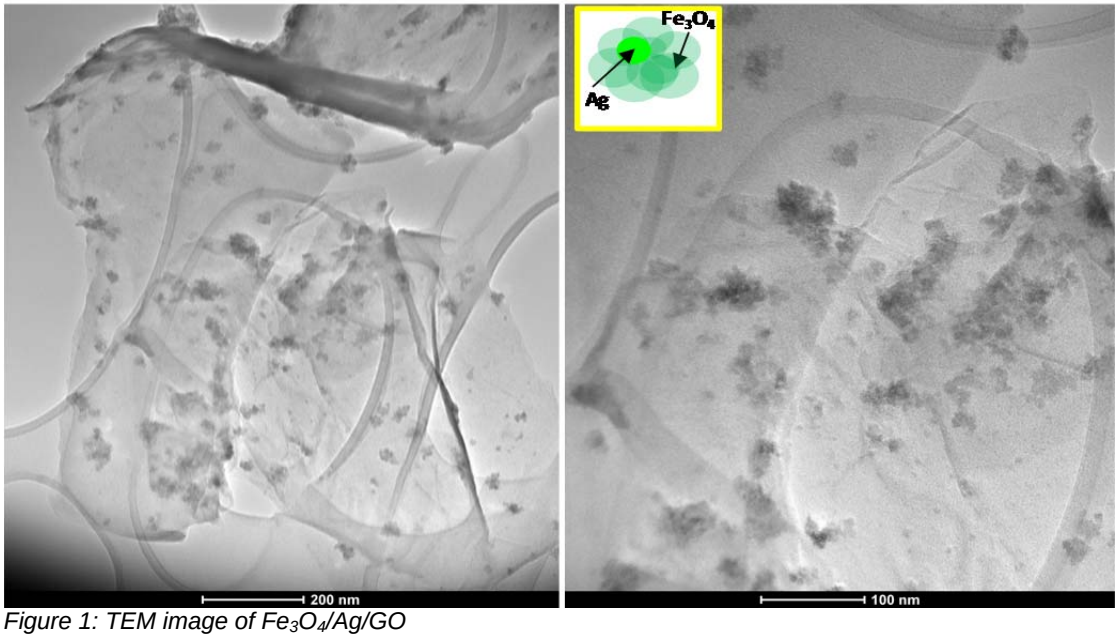

Figure 1: TEM image of Fe_3O_4/Ag/GO

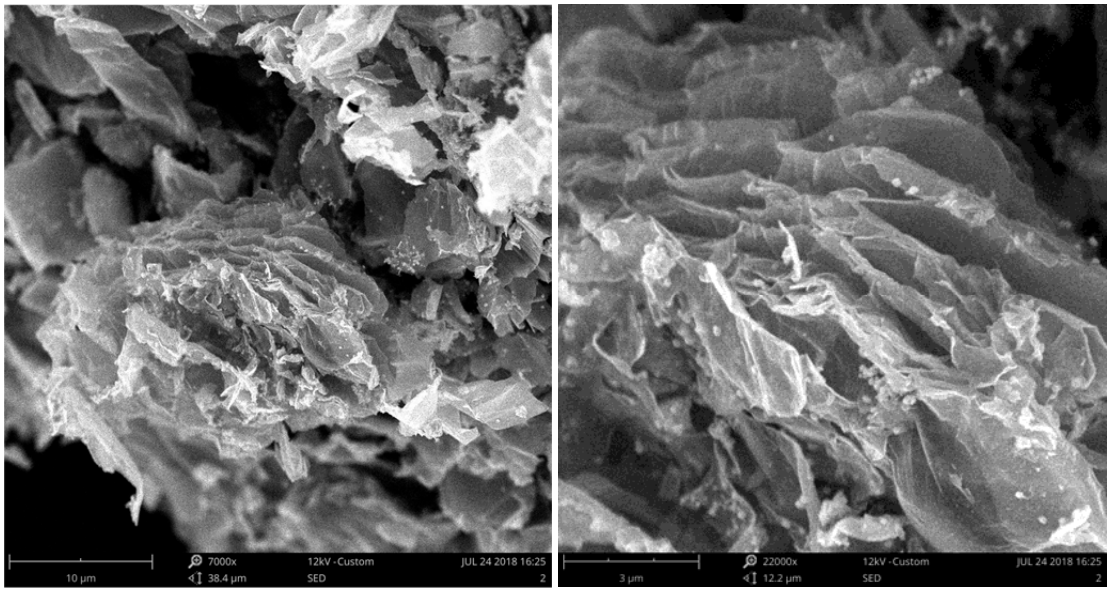

Figure 2: SEM image of Fe_3O_4/Ag/GO NPs

3.2 Effect of free and immobilize HRP on the degradation of 2,4-dichlorophenol

Figure 3 showed the degradation of 2,4-DCP (70 mg/L) at pH 6 and 25 °C in the presence of H_2O_2 (0.8 mM) for free and immobilized HRP.

The degradation of 2,4-DCP was very slow for free HRP, about 40 % removal after 180 min. The degradation of 2,4-DCP was enhanced in the system of Fe_3O_4/Ag/GO@HRP, leading to a removal of 2,4-DCP of about 77% in 180 minutes. This significant enhancement of the 2,4-DCP degradation suggests that there is a synergistic effect of HRP and Fe_3O_4/Ag/GO.

For comparison, at the same operating conditions the 4-chlorophenol (4-CP) was removed, too. The removal efficiency of 89 %, due to the intrinsic much more simplicity of this molecule was observed. In particular, due to the larger difference between the work function of Ag and magnetite on the respect of the Ag/Fe_3O_4 heterojunction (Sarno et al., 2019b), more favorable enzyme immobilization and activity was achieved.

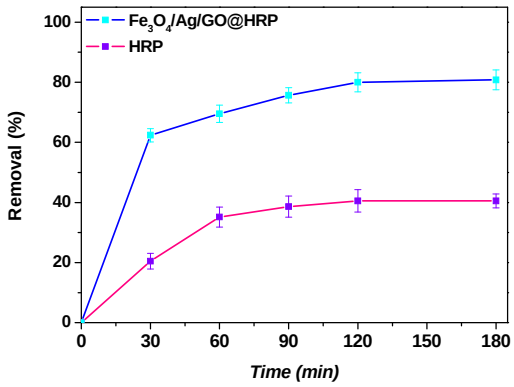

Figure 3. Effect of time on 2,4 dichlorophenol degradation for free and immobilized HRP. Immobilization conditions: coupling temperature, 4 °C; coupling pH, 6; lipase concentration, 0.1 mg/ml; time, 3 h. Degradation test conditions: reaction temperature, 25 °C; catalyst concentration, 0.1 mg/ml; H_2O_2 concentration, 0.8 mM; 2,4-DCP concentration, 70 mg/L. Each point represents the mean of three experiments ± S.E.

3.3 Degradation mechanism: catalytic effect of H_2O_2

The addition of H_2O_2 is fundamental in activating the enzymatic cycle (Huang et al., 2005). First of all, it produces peroxidase radical intermediates, which attack the 2,4-DCP (S) compounds to form free radicals ($S^{\cdot}$), as shown in Figure 4. In detail, the catalytic cycle of HRP is initiated by H_2O_2. The enzyme is first oxidized by peroxide to compound I, after which compound I abstract one electron from a 2,4-DCP molecule to form compound II and generates a phenoxyl radical. Finally, compound II oxidizes a second 2,4-DCP molecule, releasing another phenoxyl radical and returning the enzyme to its native state, thereby completing the cycle. In particular, the silver inclusions, because of the different work functions with magnetite (Sarno et al., 2019a) determining favorable electron mobility vs Ag, assist the electron migration from the enzyme to the residual GO hydrophilic moieties, transferring electrons to H_2O_2 and resulting in the formation of a highly oxidized compound I of HRP. The generated phenoxyl radicals react with each other to form oligomers, while the soluble coupling products can contribute to serve as phenolic substrates and undergo further oxidative coupling until larger polymers that precipitate from the solution are formed.

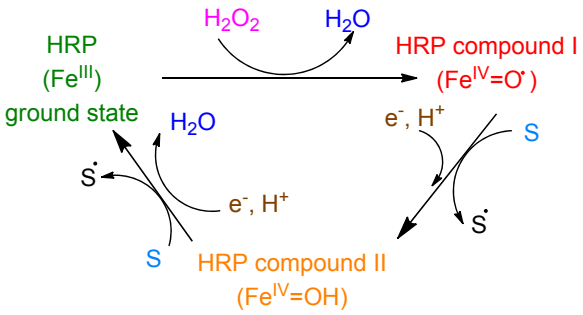

Figure 4. Catalytic cycle of HRP

The effect of initial concentrations of H_2O_2 was investigated on the degradation of 2,4-DCP for the system Fe_3O_4/Ag/GO@HRP (see Figure 5). As shown in Figure 5, as the H_2O_2 initial concentration increased from 0.4 to 0.6 mmol/L, the removal efficiency of 2,4-DCP increased from 68% to 94%. On the other hand, when the H_2O_2 concentration is higher than 0.6 mmol/L, the 2,4-DPC degradation efficiency gradually decreases,

probably due to an excessive amount of H_2O_2 that leads to enzyme deactivation. Moreover, the presence of excess free phenoxyl radicals can degrade HRP, leading to an inactive state.

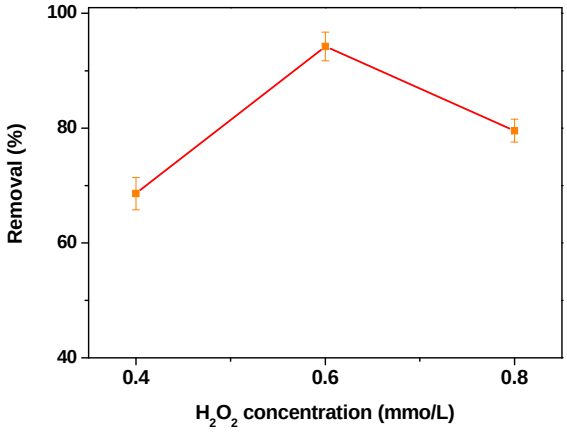

Figure 5. Effect of H_2O_2 concentration on 2,4-dichlorophenol degradation. Immobilization conditions: coupling temperature, 4 °C; coupling pH, 6; lipase concentration, 0.1 mg/ml; time, 3 h. Degradation test conditions: reaction temperature, 25 °C; catalyst concentration 0.1 mg/ml; reaction time, 3h; 2,4-DCP concentration, 70 mg/L; pH, 6; Each point represents the mean of three experiments ± S.E.

In order to clarify the reaction mechanism, the intermediates of 2,4 dichlorophenol oxidation, after 90 min of reaction with Fe_3O_4/Ag/GO@CA catalyst were analyzed and identified by GC-MS. By GC-MS analysis, some intermediated, such as 2-chloro-1,4-benzoquinone, dimer and a smaller amount of trimer, were identified. According to the GC-MS measurement, a possible mechanism of 2,4-DCP was shown in Figure 6.

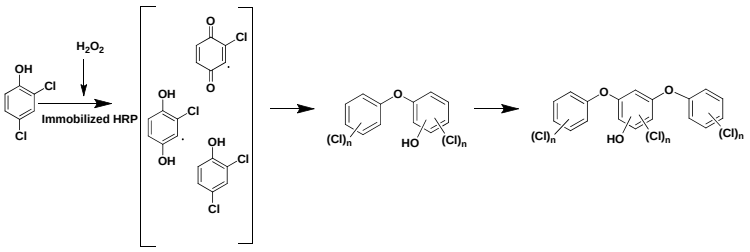

Figure 6. Possible pathways for the oxidative catalytic removal of 2,4-DCP using immobilized HRP.

3.4 Recycling and reusability of immobilized HRP

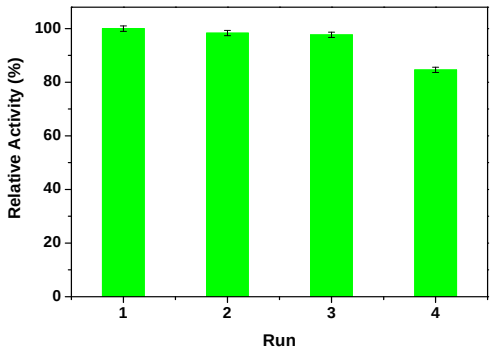

Figure 7. Recycling and catalytic stability of immobilized HRP. Immobilization conditions: coupling temperature, 4 °C; coupling pH, 6; lipase concentration, 0.1 mg/ml; time, 3 h. Degradation test conditions: reaction temperature, 25 °C; catalyst concentration 0.1 mg/ml; H_2O_2 concentration, 0.6 mmol/L, 2,4- DCP concentration, 70 mg/L; pH, 6; reaction time, 3 h. Each point represents the mean of three experiments ± S.E.

The reusability test of immobilized HRP for degradation 2,4-DCP for four successive cycles was shown in Figure 7. After the degradation test, the immobilized enzyme was separated using an external magnetic field and washed several times with a buffer solution and reused. The immobilized HRP retains >96% of the initial activity for the first three cycles. The mildly declined activity after three cycles can be attributed to the formation of free radicals generated during enzymatic oxidation of the 2,4-dichlorophenol (Cheng et al., 2006), and the accumulation of the reaction products produced during degradation than blocked the active sites of HRP (Huang et al., 2014).

4. Conclusions

Magnetic Fe_3O_4/Ag/GO MNPs were synthesized using a solvothermal method. HRP has physically immobilized on the Fe_3O_4/Ag/GO nano-hybrid thanks to citric acid moieties. The immobilized HRP was used for degradation of 2,4-dichlorophenol in the presence of H_2O_2 activating species. When the H_2O_2 concentration in the batch reaction system was ~0.6 mM, maximum removal was achieved within 3 h. The immobilized HRP was more stable during operation and storage compared to the free counterpart. Finally, the high operational stability obtained with the immobilized HRP indicates that it could successfully be used in a large scale system for the enzymatic degradation of 2,4-DCP.

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