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Use of Magnetic Nanoparticles Fe_3O_4in the Synthesis of Biocatalysts Based on Horseradish Root Peroxidase

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In this study, magnetic nanoparticles Fe_3O_4synthesized by the polyol method were used to immobilize Horseradish root peroxidase. The surface of magnetic nanoparticles was pretreated with tetra-ethyl orthosilicate, 3-aminopropyltriethoxysilane, and glutaraldehyde. As a result, two biocatalysts were obtained Fe_3O_4/APTS/GA/HRP and Fe_3O_4/TEOS/APTS/GA/HRP. The activity of the synthesized biocatalysts was estimated spectrophotometrically in the oxidation reaction of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) with hydrogen peroxide. The optimal conditions for this reaction were selected (pH 6.0, temperature 45 °C). It has been determined that biocatalysts Fe_3O_4/APTS/GA/HRP and Fe_3O_4/TEOS/APTS/GA/HRP slightly decreased their activity during 5 consecutive experiments, by 19 and 15 %.

1. Introduction

One of the most important and pressing challenges facing the industry is moving towards greener and more sustainable manufacturing processes that minimize or avoid waste generation and the use of toxic and/or hazardous materials. Biocatalysis have many advantages in this regard. Today, enzymes are widely used in the production of pharmaceuticals, food, fine chemicals, flavors, and other products. The use of immobilized enzymes makes it possible to increase their activity, stability, easy release of the product or higher product quality achieved in fewer processing steps. Thanks to enzymatic processes, less waste is produced than with traditional synthetic routes. Enzymatic processes are more energy-efficient and provide higher purity products (Federsel et al., 2021).

The use of magnetic nanoparticles in biocatalysis, due to their unique properties, such as controlled particle size, large surface area, and ease of separating them and the reaction mixture by applying an external magnetic field, allows reuse of enzymes immobilized on magnetic nanoparticles for catalytic processes (Deepthiet al., 2014).Covalent immobilization between a nanocarrier and an enzyme decreases the steric hindrance compared with other immobilization techniques, such as encapsulation in gels, cross linked enzymes, entrapment, or a porous micro-carrier (Mariño et al., 2021).In addition, the use of nanoparticles for immobilization of enzymes contributes to an increase in the activity of the latter (Alsaiari et al., 2021).

Saleh et al. (2017) immobilized HRP on unmodified magnetic Fe_3O_4 nanoparticles. This biocatalyst retained 55 % of its original activity after 10 re-uses. The optimum pH value changed from 7.0 for soluble HRP to 7.5 for immobilized HRP, and the optimum temperature changed from 40°C to 50 °C. The immobilized HRP became more thermostable than the native enzyme. In addition, the oxidation of substrates of immobilized HRP was more efficient than native HRP. Tavares et al. (2020), applied a chemometric approach aimed at optimizing the process of immobilization of horseradish peroxidase (HRP) on magnetic iron nanoparticles, namely, Δ - FeOOH. Hydroxyl iron oxide (FeOOH) has a good surface with the presence of hydroxyl groups (OH-), which can facilitate the attachment of the enzyme. In addition, it has unique characteristics such as high surface area, magnetism, non-toxicity, and stability. The enzyme can successfully interact with ferroxite nanoparticles without the need for silica coatings and the addition of functional groups. The optimized process allows an efficient reusable biocatalyst to be obtained without costly operations. Immobilized HRP under optimal conditions showed a high oxidation efficiency of ferulic acid (82 %). In work Junhui et al. (2019), a

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multi-armed polymer (polyethylene glycol) based on magnetic graphene oxide was synthesized as a carrier for HRP immobilization. Compared to the free enzyme, thermal stability, storage stability, and service stability of immobilized HRP are improved. The immobilized enzyme retained its activity of more than 68.1% after 8 times of repeated use.

HRP immobilized on magnetic nanoparticles can be used in various oxidation processes. For example, Keshta B.E. et al. (2022) used HRP immobilized onto amine functionalized superparamagnetic iron oxide for the oxidative degradation of acid black-HC dye. Fe_3O_4 was prepared using the coprecipitation method and subsequently functionalized with 3-aminopropyltriethoxysilane. In the catalytic experiment, the immobilized HRP exhibited superior catalytic activity compared with that of free HRP. The immobilized enzyme was thermally stable up to 60 °C, while the free peroxidase was only stable up to 40 °C. Hojnik Podrepšek et al. (2020) report on the possibility of using immobilization of HRP and cholesterol oxidase enzymes on chitosan functionalized metal oxide micro- and nanoparticles to create biosensors.

Recently, we have already reported on the immobilization of peroxidase on magnetic particles and on the use of the synthesized biocatalyst in the oxidation of 2,3,6-trimethylpheol (Grebennikova et al.,2021).In this work, HRP on magnetic Fe_3O_4 nanoparticles synthesized by the polyol method was immobilized. The surface of the carrier before immobilization of the enzyme was modified and activated in two ways using tetra-ethyl orthosilicate, 3-aminopropyltriethoxysilane, and glutaraldehyde. Biocatalytic systems were tested in the oxidation reaction 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) with hydrogen peroxide.

2. Materials and methods

2.1 Materials

$FeCl_3 \cdot 6H2O$ (> 98 %, Khimmedservice, Russia), $FeSO_4 \cdot 7H_2O$ (> 98 %, Neva reagent, Russia), NaOH (> 98 %, Neva reagent, Russia), ethylene glycol (> 98 %, Component-reagent, Russia), succinic acid (> 98 %,Marbiopharm, Russia), tetra-ethyl orthosilicate (99 %, EKOS-1, Russia), urea (> 98 %, Indicator, Russia), glutaraldehyde (25 %, Panreac, Spain), horseradish root peroxidase (act.> 150 U / mg, RZ> 2.0, UK), ethanol (95 %, Medkhimprom, Russia), 3-aminopropyltriethoxysilane (> 98 %,SIGMA-ALDRICH, USA) were used to synthesize biocatalysts. Potassium phosphate (> 98 %, GRANCHIM, Russia) and sodium phosphate (> 98 %, GRANCHIM, Russia) were used to prepare buffer solutions. To test biocatalysts, we used 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (AlfaAesar, Germany) and hydrogen peroxide (37 %, Kupavnareaktiv, Russia).

2.2. Procedure for the synthesis of a biocatalyst based on immobilized HRP

2.2.1. Synthesis of magnetic nanoparticles (Fe_3O_4) by polyol method

There are many ways to synthesize magnetic nanoparticles. In this work, the most common method was chosen - polyol synthesis. The polyol method of obtaining allows you to control the size, texture, and shape of nanoparticles, and can also be used to obtain nanoparticles on a large scale (Jimenez et al., 2019).

The solvent that is mainly used in the polyol method of synthesizing metal oxide nanoparticles is ethylene glycol due to its strong reducing ability, high dielectric constant, and high boiling point (Shanmugam et al., 2020). Ethylene glycol is also used as a crosslinking reagent to bond with a metal ion to form metal glycolate, resulting in oligomerization.

In this work, the polyol method was carried out according to the well-known method (Chenget al., 2011). For this, 30 mL of ethylene glycol (C_2H_4 $(OH)_2$), 3 mmol - $FeCl_3 \cdot 6H_2O$, 1 mmol of succinic acid (HOOC –CH_2 - CH_2 - COOH) and 30 mmol of urea $(NH_2)_2CO$). Then the mixture was stirred for 30 min on a magnetic stirrer and then placed in a Teflon beaker of an autoclave (Parr Instr.) For 2 h at a temperature of 200 °C. After cooling the reaction medium, a black precipitate was separated using a neodymium magnet, which was then washed with ethyl alcohol several times. Then the sample was dried under vacuum at 60 °C for 6 h. Magnetic particles synthesized in this way are hydrophilic and highly magnetized.

2.2.2. Synthesis of biocatalysts using magnetic nanoparticles

To increase the stability and activity of biocatalysts, we used tetra-ethyl orthosilicate (TEOS), due to which the surface of the carrier has covered with OH-groups capable of interacting with other functional groups. In addition, the surface area of the carrier increases, which significantly increases the number of attached enzyme molecules.

To provide the surface of the support with reactive amino groups, magnetic nanoparticles were treated with 3-aminopropyltriethoxysilane (APTS), which can easily interact with surface hydroxyl groups.

For the strong binding of the enzyme to the carrier, glutaraldehyde (GA) was used, which is capable of forming azomethine bonds between the enzyme and amino groups located on the surface of the carrier.
In this work, two biocatalytic systems have synthesized. The first sample has obtained using APTES, GA, and HRP. To the obtained nanoparticles (0.5 g), 100 mL of ethanol, 1 mL of water, and 0.2 mL of APTS were added. The mixture has stirred for 5 h on a magnetic stirrer. Then the solution was washed several times with distilled water. To the resulting suspension were added 20 mL of water and 1 mL of GA. The modified and activated carrier was washed several times with phosphate buffer (pH 6.0) and then treated with 20 mL of HRP solution (0.001 g of HRP in 20 mL of phosphate buffer (pH 6.0)). The resulting biocatalyst was designated Fe_3O_4/APTS/GA/HRP.
A second biocatalyst sample has synthesized using TEOS. To the resulting mixture of nanoparticles (0.5 g), 1 mL of TEOS has added dropwise under mechanical stirring. The resulting suspension has left to stir for 3 h. After that, the resulting solution has washed with a magnet 5 times with distilled water and ethanol. Then the carrier has modified and activated with APTS, GA and HRP according to the method described above. The resulting biocatalyst has designated as Fe_3O_4/TEOS/APTS/GA/HRP.

2.2 Technique for carrying out kinetic experiments

Kinetic experiments were performed spectrophotometrically. The resulting biocatalytic systems and native HRP were tested in the oxidation of ABTS with hydrogen peroxide. The reaction was monitored by an increase in the optical density of the ABTS oxidation product (λ = 415 nm). The initial oxidation rate (V_0) was determined at various initial substrate concentrations (from 0.02 to 0.00125 M). To assess the effect of pH, a series of experiments were carried out with different pH values of the phosphate buffer (6.0 – 7.5). To determine the temperature optimum, experiments were carried out in the temperature range from 25 to 50 °C.

3. Results and discussions

The work of enzymes directly depends on the amount of substrate. To determine the optimal concentration of the substrate, experiments were carried out on the oxidation ABTS in the concentration range 0.00125 - 0.02 M in the presence of native HRP, Fe_3O_4/APTS/GA/HRP and Fe_3O_4/TEOS/APTS/GA/HRP (Figure 1).

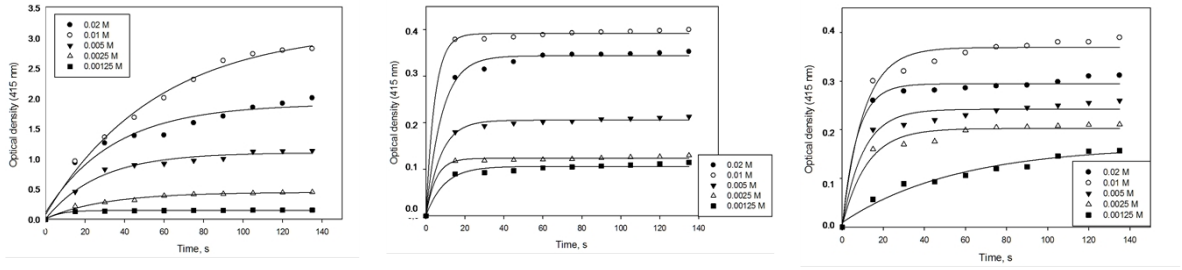

Figure 1: Dependence of optical density on time at various substrate concentrations: a) Native HRP, b) Fe_3O_4/APTS/GA/HRP and c) Fe_3O_4/TEOS/APTS/GA/HRP ($c_0(H_2O_2)$ = 1.5 M, T =25 ˚C, pH = 6.5, λ = 415 nm)

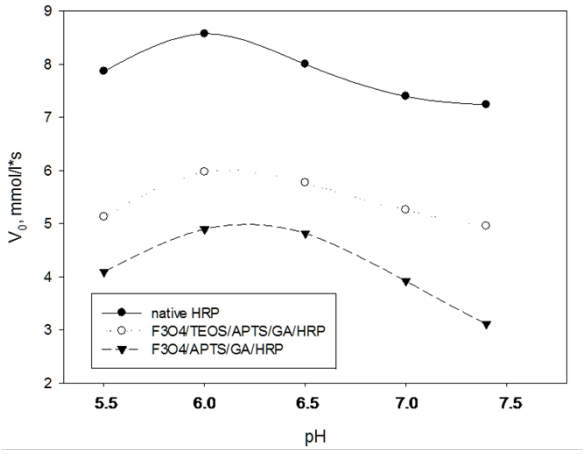

Figure 2: Dependence of the initial rate of the ABTS oxidation reaction at different pH values (c_{0ABTS} = 0.02 M, $c_0(H_2O_2)$ = 1.5 M, T =25 °C, λ = 415 nm)

Figure 1 shows that when all biocatalysts were used, the optimal substrate concentration was 0.01 M. that at high concentrations the moment comes when complete saturation of the active centers of the enzyme is reached. The determining factor of enzyme activity is the pH value. To select the optimal pH value in the ABTS oxidation reaction in the presence of synthesized biocatalysts, experiments were carried out in the pH range from 5.5 to 7.4 (Figure 2).

Figure 2 shows that at pH = 6.0, all biocatalysts achieved the best results. After analyzing the curves of the dependence of the initial rate of substrate oxidation on pH values, it can be concluded that immobilization on magnetic nanoparticles did not shift the pH optimum as compared to native HRP. As the pH moved away from the optimum (6.0), the initial reaction rate for all biocatalyst samples decreased, which is possibly related to the deformation of the active site of the enzyme (Buchholz et al., 2012).

Each enzyme exhibits maximum activity at a certain temperature because at high temperatures, the enzyme loses its catalytic activity due to denaturation. To determine the temperature optimum of the ABTS oxidation reaction in the presence of synthesized biocatalysts, experiments were carried out at temperatures: 25 ˚C; 30 °C; 35 °C; 40 °C; 45 °C; 50 °C (Figure 3).

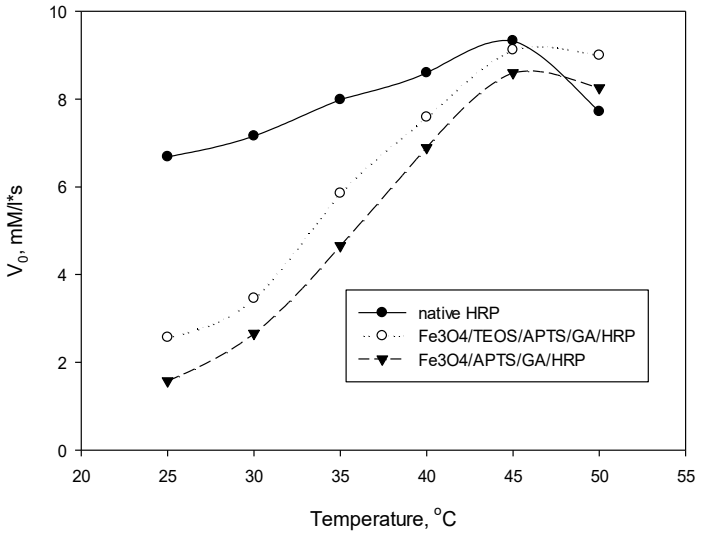

Figure 3: Dependence of the initial rate of the ABTS oxidation reaction on various temperatures (c_{0ABTS} = 0.02 M, $c_0(H_2O_2)$ = 1.5 M, pH = 6.0, λ = 415 nm)

Figure 3 shows that for Fe_3O_4/APTS/GA/HRP, Fe_3O_4/TEOS/APTS/GA/HRP, and native HRP, the best results were achieved at T = 45 °C. With increasing temperature, the initial rate of oxidation in the presence of biocatalysts based on HRP immobilized on magnetic nanoparticles does not decrease significantly. Native HRP when the temperature rises above 45 °C reduces the initial rate of substrate oxidation, which indicates a loss of enzyme activity, probably due to denaturation at higher temperatures (Singh et al., 2018). Using Fe_3O_4/APTS/GA/HRP and Fe_3O_4/TEOS/APTS/GA/HRP as biocatalysts, initial oxidation rate at temperature 45 °C with slightly lower compared to native HRP, which is associated with a decrease in the activity and mobility of the enzyme after immobilization. However, as a result of immobilization HRP thermal stability of the enzyme is observed, since the initial rate of substrate oxidation at temperatures above 45 °C higher for the immobilized enzyme, which is consistent with the literature data (Grebennikova et al., 2020).

To assess the reusability of biocatalysts for repeated use, Fe_3O_4/APTS/GA/HRP, Fe_3O_4/TEOS/APTS/GA/HRP were tested in five successive experiments at pH 6.0 and 45 °C. After each catalytic reaction, the biocatalysts were separated with a neodymium magnet and used in the next experiment. The results are shown in Figure 4.

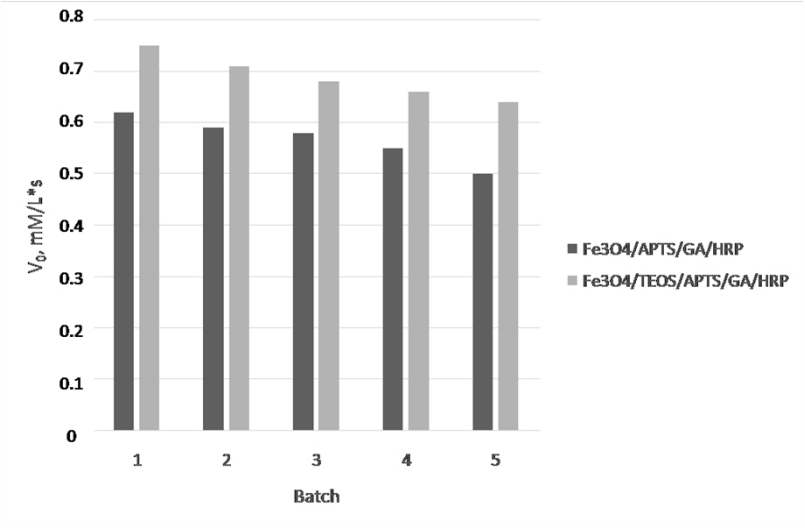

Figure 4: Initial oxidation rate of ABTS when reusing biocatalysts

From the presented data, it can be concluded that immobilized on magnetic nanoparticles HRP becomes stable after several recycles, which is explained by the stabilizing/activating effect of iron oxide as an enhancer of enzymatic activity. It can also be seen from Figure 4 that the biocatalyst Fe_3O_4/TEOS/APTS/GA/HRP is the most stable. The graph shows that after five consecutive catalytic cycles Fe_3O_4/TEOS/APTS/GA/HRP lost only 15 % of its activity, and the biocatalyst Fe_3O_4/APTS/GA/HRP - 19 %. The higher stability of the biocatalyst Fe_3O_4/TEOS/APTS/GA/HRP may be associated with the content of silicon dioxide in its composition, due to which the surface of the carrier is covered with OH- groups capable of interacting with other functional groups, providing strong covalent binding of the enzyme to the surface of the carrier (Samiur et al., 2021).

4. Conclusions

Immobilized on magnetic nanoparticles HRP has shown high efficiency in the oxidation of ABTS with hydrogen peroxide. We selected the optimal conditions for this reaction in the presence of synthesized biocatalysts (pH = 6.0, temperature 45 °C). The highest activity was exhibited by the biocatalyst Fe_3O_4/TEOS/APTS/GA/HRP, containing TEOS. In addition, when reused in 5 recycles, this biocatalytic system lost only 15 % of its activity, while the Fe_3O_4/APTS/GA/HRP biocatalyst lost 19 % of activity. The synthesized biocatalysts can be successfully utilize for the oxidation of various phenolic compounds, ensuring the efficiency of the process and the ease of separating the biocatalytic system from the reaction mixture.

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