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Clean Production of 2,3,5-Trimethylhydroquinone using Peroxidase-Based Catalyst

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The selective oxidation of organic compounds to obtain vitamins is one of the key processes in chemistry and biotechnology. The use of toxic compounds such as chromium and manganese oxides for these purposes strongly affects the environment. We propose a novel bio-catalyst supported on magnetically separable nanoparticles based on the immobilized peroxidase. Biocatalysts based on immobilized enzymes received considerable attention due to important applications in syntheses of value-added chemicals, pharmaceuticals and drug intermediates with great catalytic efficiency and high yields of target molecules. In this work, we studied the oxidation of 2,3,6-trimethylphenol to 2,3,5-trimethylhydroquinone (an intermediate of vitamin E) using hydrogen peroxide. The effects of initial substrate concentration, temperature, and pH were studied. The optimum conditions for the process of oxidation 2,3,6-trimethylphenol in the presence of magnetically separable biocatalyst were found: initial substrate concentration – 1.5 mmol/L; temperature – 40 °C; pH – 6.5. The developed biocatalyst showed high activity and selectivity in 2,3,6-trimethylphenol oxidation to 2,3,5-trimethylhydroquinone.

1. Introduction

In recent years, the issue of creating environmentally-friendly catalysts for the oxidation of phenol-substituted compounds to the corresponding quinones, which are raw materials for the production of vitamins, has been a particularly acute issue (Sauxa et al., 2013). These processes are of great importance not only in laboratory conditions, but also in the pharmaceutical industry (Koreniuk et al., 2016). So, for example, 2,3,5-trimethylhydroquinone is one of the intermediate products for the production of vitamin E. Currently, vitamin E is one of the most important vitamins, since it has an antioxidant property, giving an anti-aging effect, preventing cardiovascular diseases and cancer. Due to its properties, it is widely used in food, as an antioxidant, in medicine and cosmetology, providing huge demand. Large-scale production of vitamin E is an economically expensive process (Roslan et al., 2016).

2,3,5-trimethylhydroquinone in industry is obtained by oxidation of 2,3,6-trimethylphenol to 2,3,5-trimethylbenzoquinone with molecular oxygen in the presence of a $CuCl_2$ catalyst, from which the desired product is then obtained. The high activity of the $CuCl_2$ catalyst is achieved by a high chlorine content. However, the chlorine content in amounts close to stoichiometric leads to the formation of toxic chlorophenols, which in their toxic effect are similar to dioxins. Their isolation and destruction leads to certain technological difficulties and additional economic costs (Li et al., 2009). Obviously, this process is not environmentally friendly. An environmentally friendly method for the oxidation of 2,3,6-trimethylphenol is the subject of study by many scientists. So, for example, Kholdeeva et al. (2016) studied the reaction of liquid-phase oxidation of compounds of the phenolic and naphthol series with hydrogen peroxide in the presence of mesoporous titanium-silicate catalysts. They achieved high conversion and selectivity for the target product reached 100 %. Another example of highly selective oxidation of 2,3,6-trimethylphenol with H_2O_2 was proposed by Roslan et al. (2016). Scientists used (meso-tetra-(p-sulfonatophenyl)-porphyrinato) copper as an oxidation catalyst. They achieved 100% selectivity at a temperature of 60 °C. Hu et al. (2017) proposed aerobic water-based oxidation of 2,3,6-trimethylphenol to trimethyl-1,4-benzoquinone using copper (II) nitrate and using oxygen dioxide as an oxidizing

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agent. Such a catalyst also showed high activity and selectivity. Palacio et al. (2020) achieved 90 % and 99 % conversion using 2,3,3-trimethylphenol 15 % phosphomolybdic acid and 15 % vanadophosphomolybdic acid on TiO_2 matrix as oxidation catalysts. Yerramreddy et al. (2019) proposed the oxidation of 1,2,4-trimethylbenzene with phthaloyl peroxide, which was obtained in situ by the oxidation of phthalic anhydride with aqueous hydrogen peroxide (30 % H_2O_2). 2,3,5-trimethylbenzoquinone was obtained with a high degree of purity and a yield of 92 % at 95 % conversion of the substrate at 120 °C for 2.5 h in the absence of solvent. Another method of aerobic oxidation of substituted phenols, including 2,3,6-trimethylphenol, was proposed by Yang et al. (2019). They achieved 91 % conversion and 89 % selectivity for trimethyl-1,4-benzoquinone at 30 °C for 3 h at an oxygen pressure of 0.2 MPa.One of the most important challenges facing industry today is the transition to more environmentally friendly and sustainable production processes that minimize or even avoid the generation of waste and the use of toxic and / or hazardous materials. Biocatalysis has many advantages in this regard, since enzymatic processes produce less waste than conventional synthetic routes, are more energy efficient and provide products with higher purity, and enzymes are universal nanoscale biocatalysts that can be used in many applications, including industrial biocatalysis. However, the use of enzymes insoluble form is often hampered by their price, instability and difficulty in their recovery and reuse. These disadvantages are eliminated by immobilizing the enzyme on solid carriers, since the immobilized biocatalyst shows improved stability during storage and operation and can be easily separated from the products in the reaction mixture and reused. The reuse of the enzyme in sequential catalytic systems also significantly reduces the costs of the biocatalyst, which otherwise would not be economically feasible using the native form (Sulman et al., 2019).

In this work, it is proposed to use immobilized horseradish root peroxidase (HRP) as an alternative environmentally friendly biocatalyst for the oxidation of 2,3,6-trimethylphenol (TMP).In this case, the stage of formation of the intermediate 2,3,5-trimethylbenzoquinone is excluded, which undoubtedly should affect the economic aspect. As a result of oxidation in the presence of such a biocatalyst, the contamination of target products with toxic impurities, as well as the release of environmentally unsafe wastewater, is excluded.Inorganic carriers of SiO_2, Al_2O_3 and magnetic nanoparticles were chosen as carriers of the enzyme. The latter were synthesized by the polyol method. For the synthesized biocatalysts, optimal oxidation conditions (initial TMP concentration, temperature, and pH) were selected under which they allowed the maximum yield of the target product to be obtained.

2. Materials and methods

2.1 Materials

Ethyleneglycol (99.9 wt. %), ferric chloride (III) 6-aqueous (99 wt. %), amber acid (99.9 wt. %), urea (99.8 wt. %), ethanol (95 wt. %), 3-aminopropyl triethoxysilane (>98 wt. %), SiO_2 (99.9 wt. %), Al_2O_3 (99.9 wt. %), chitosan (molecular weight *300,000, degree of deacetylation *75–80 %), sodium hydroxide (99.5 wt. %), hydrochloric acid (37 wt. %), glutaraldehyde solution (25 wt. %) peroxidase (Great Britain, RZ > 2.0, act. > 150 u/mg, demineralized, lyophile powder) were used for the biocatalyst synthesis. 2,3,6,-trimethylphenol (95 wt. %), hydrogen peroxide (50 wt. %) were used to test the biocatalyst obtained.

2.2 Methodology for the preparation of a biocatalyst based on peroxidase immobilized on SiO_2, Al_2O_3

Samples of $Al_2O_3$and SiO_2 carriers weighing 1 g each were calcined at 300 °C for 3 h, after which their surface was modified. For this, Al_2O_3 or SiO_2 was introduced into a solution of sodium polystyrenesulfonate (PSS) (volume - 10 mL, concentration 0.25 g/L), stirred for 60 min, filtered, washed with distilled water to pH 7 and dried under vacuum at 60 ºC (24 h). Then, the modified carriers were coated with a polyelectrolyte complex containing readily available amino groups, which can be used for intermolecular crosslinking with HRP. The next stage of modification is the treatment of samples with a solution of chitosan (0.15 g/L) in 50 mL of 0.01 M acetic acid with stirring for 60 min, followed by filtration, washing with distilled water and drying under vacuum at 60 ºC. For the strong binding of the enzyme to the carrier, the obtained samples (1 g) were treated with a solution of glutaraldehyde (volume - 50 mL, concentration varied from 0.2 g/L) with stirring for 60 min. The carrier prepared in this way was placed in an HRP solution (10 mL, 0.15 mg/mL), stirred, as before, for 60 min, the resulting biocatalyst sample was filtered off, washed and dried at 25 °C in vacuum.

As a result of this method of HRP immobilization, the following biocatalysts were obtained: Al_2O_3/PSS/Chit/Glut/HRP and SiO_2/PSS/Chit/Glut/HRP.

2.3 Methods for the preparation of a biocatalyst based on peroxidase immobilized on magnetic nanoparticles

In a beaker were completely dissolved in 30 mL of ethylene glycol: $FeCl_3 \cdot 6H_2O$ (3 mmol), succinic acid (1 mmol) and urea (30 mmol) with vigorous stirring with a magnetic stirrer (30 min). The resulting solution was transferred into a Teflon glass (50 mL volume) of a stainless steel autoclave (Parr Instrument, USA) and kept at 200 °C for

2 to 4 h. After cooling to room temperature, a black precipitate was separated by a magnet, which was washed with ethanol several times until a clear solution was obtained. Then it was dried under vacuum at 60 °C for 6 h.Fe_3O_4 nanoparticles are capable of adsorbing ions and molecules in solution due to the large number of free bonds on their surface. Nanoparticles dispersed in a neutral aqueous solution have Fe and O atoms capable of adsorbing OH and H groups, as a result of which the surface of the nanoparticles is abundantly coated with OH groups (Ma et al., 2003). In turn, the OH-group is able to interact with APTS (see Figure 1). To modify APTS magnetic nanoparticles, 150 mL of ethanol and 1 mL of water were added to their 25 mL of colloidal ethanol solution. APTS condensation reaction will more efficiently take place in a polar solvent (Demin et al., 2011). APTS of various concentrations (2 μL/mL) was added to the resulting mixture. The solution was stirred for 5 h on a magnetic stirrer. Then the solution was washed 5 times with ethanol and 5 times with distilled water. Treated APTS nanoparticles were stored for further use in water.
HRP was covalently immobilized onto pre-modified magnetic Fe_3O_4 nanoparticles. The Fe_3O_4/APTS magnetic nanoparticles washed with distilled water were treated with a HRP solution (0.15 mg/mL). The mixture was stirred at 4 °C for 6 h. As a result of this immobilization method, the following biocatalyst was obtained: Fe_3O_4/APTS/HRP.

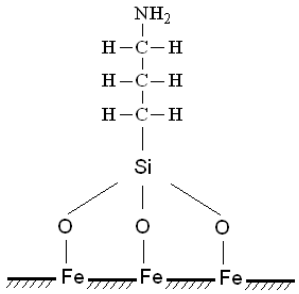


Figure 1: Magnetite particle surface modification for biocatalyst synthesis

2.4 Methodology for the oxidation of TMP

The oxidation of TMF to TMHC was carried out according to the scheme shown in Figure 2.

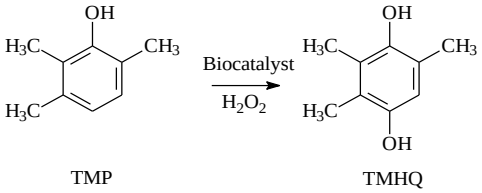


Figure 2: Reaction diagram of the TMP oxidation process

The oxidation of TMP was carried out in a glass reactor equipped with a stirrer and a heating jacket. The catalyst (0.1 g) and the substrate solution (30 mL) were placed in the reactor. The pH of the solution was adjusted with phosphate buffers. During the reaction, hydrogen peroxide equivalent to the substrate content was periodically added to prevent substrate inhibition. At certain intervals, samples were taken of the reaction mixture. After the experiment, the biocatalyst was separated by filtration (for Al_2O_3/PSS/Chit/Glut/HRP and SiO_2/PSS/Chit/Glut/HRP). When using Fe_3O_4/APTS/HRP, the biocatalyst was separated by a neodymium magnet.Analysis of the reaction mixture was performed by high-performance liquid chromatography (HPLC). An Ultimate 3000 (Dionex) HPLC system equipped with an ultraviolet sensor, an API-2000 mass spectrometer (Applied Biosystems), a Luna C18 analytical column (7 μm) with a theoretical number of plates of 40,000 and a size of 150 × 4 mm was used in the analysis. A solution of acetonitrile: water in a ratio of 50:50 was used as the mobile phase. The eluent flow rate was 0.5 mL/min at 7 MPa and 30 °C. Detection was carried out by a UV detector at a wavelength of 254 nm. Additional products and intermediate reaction products were not found in the reaction mixture, which can be explained by the high substrate specificity of HRP.

3. Results and discussions

To determine the optimal substrate concentration, experiments on the oxidation of TMP (0.5; 0.75; 1.0; 1.5; 2.0 and 2.5 mmol/L) were carried out in the presence of native HRP. Kinetic curves of product yield versus initial substrate concentration are presented in Figure 3.The optimal concentration of TMP was 1.5 mmol/L as when using native HRP, since at this concentration the maximum yield of the product is achieved. This may be due to the fact that the enzymatic reaction is able to be inhibited by a high concentration of the substrate.
It is known that Fe_3O_4 nanoparticles affect the rate of TMP oxidation, catalyzing this reaction, since they have a peroxidase-like effect due to iron atoms on their surface, which first form an intermediate complex with a substrate before catalysis (Yu et al., 2009). The high affinity of the nanoparticles to the substrate can lead to higher catalytic activity. The electrostatic interaction between the substrate and the surface of the nanoparticles can increase their affinity. In this regard, experiments were carried out on the oxidation of TMP in the presence of nanoparticles synthesized by the polyol method.

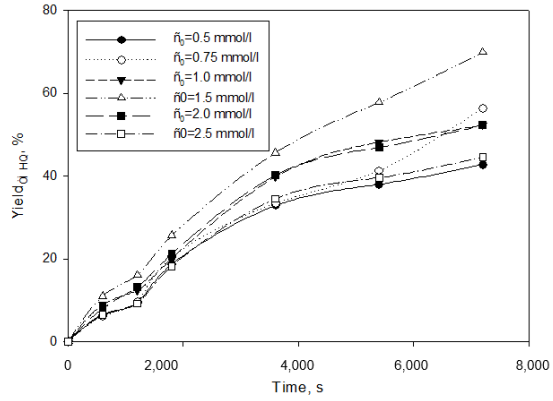


Figure 3: Kinetic curves of the dependence of the TMBH yield at various initial substrate concentrations (pH = 6.5, temperature 40 °C, C(H_2O_2) = 1.5 mol/L)

Experiments were conducted to optimize the conditions of the oxidation of TMP with native HRP; magnetic nanoparticles of Fe_3O_4; HRP immobilized on SiO_2, Al_2O_3 and Fe_3O_4. The kinetics of enzymatic catalysis uses the calculation of the initial reaction rates at various concentrations of the substrate, since the initial phase of the reaction most fully characterizes its course due to the absence of possible inhibition of target and side reactions and inhibition caused by substrate consumption by the products.
To determine the optimum temperature for the process of oxidation of TMP in the presence of synthesized biocatalysts, the reaction of oxidation of TMP was carried out at temperatures of 30 °C, 40 °C, 45 °C, 50 °C, 55 °C. The experimental results are shown in Figure 4.

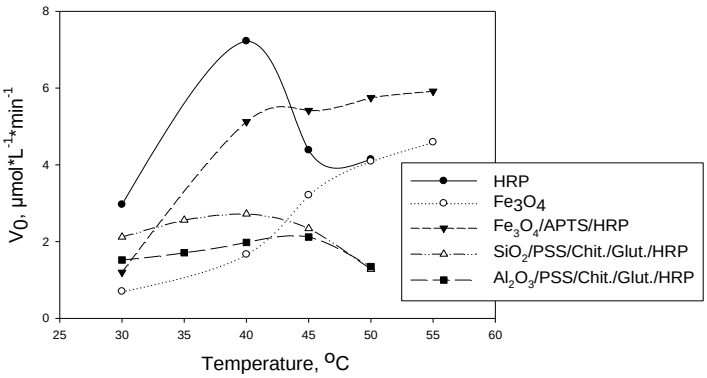


Figure 4: The initial reaction rate of the oxidation of TMP at various temperatures (c_0^{TMP} = 1.5 mmol/L, c_0 (H_2O_2) = 1.5 mol/L, pH 6.5, c_{kat} = 0.2 g/L)

Figure 4 shows that with increasing temperature, the activity of HRP immobilized on magnetic Fe_3O_4nanoparticles does not decrease, the enzyme becomes thermostable. This is likely to occur as a result of strong binding of the protein molecule to the carrier, as a result of which the native conformation of the enzyme does not undergo strong changes and the denaturation of the protein molecule is difficult. At temperatures above 40 °C, the enzyme is deactivated, the optimum temperature for conducting the TMP oxidation process in the presence of HRP-based biocatalyst is 40 °C immobilized on Fe_3O_4. Since, at a higher temperature, nanoparticles, rather than an enzyme, are more involved in the oxidation of TMP.For other catalyst samples, the optimum temperature of the TMP oxidation process is also 40 °C. All further experiments were carried out at a given temperature.
To assess the effect of pH, experiments were conducted in the pH range from 6.0 to 7.4 (Figure 5).

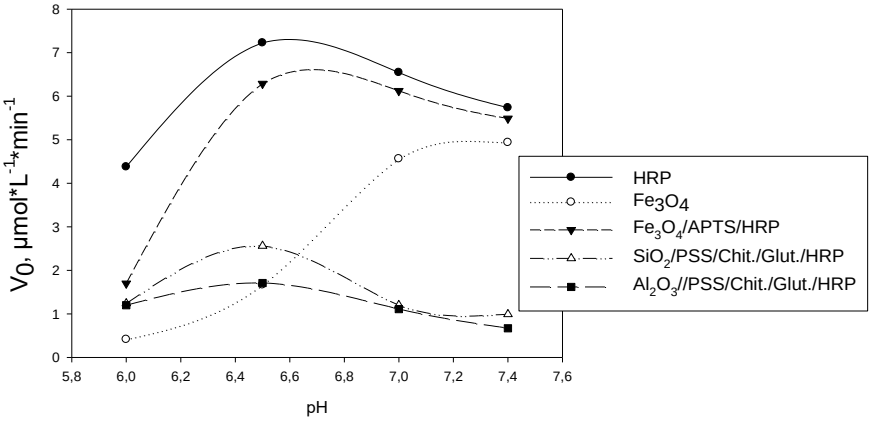
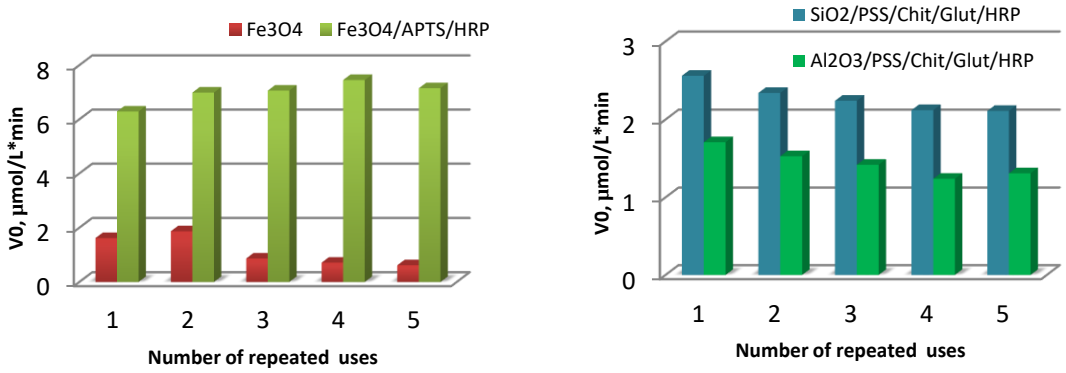


Figure 5: The initial reaction rate of the oxidation of TMP at various pH values (c_0^{TMP} = 1.5 mmol/L, c_0 (H_2O_2) = 1.5 mol/L, temperature 40 °C, c_{kat} = 0.2 g/L)

Kinetic curves show that the optimal pH for the TMP oxidation process in the presence of an HRP-based biocatalyst (for all carriers) and native HRP is 6.5. This suggests that the immobilization of HRPonto magnetic nanoparticles did not cause a pH optimum shift, as compared to native HRP. While using magnetic nanoparticles Fe_3O_4without an enzyme, the reaction rate increases with increasing pH.
To evaluate the stability of the synthesized biocatalysts, 5 successive oxidation experiments were performed with the same samples (Figure 6).



(a) (b)

Figure 6: Evaluation of stability of HRP immobilized on a) Fe_3O_4 and Fe_3O_4/APTS/HRP; b) SiO_2/PSS/Chit/Glut/HRP and Al_2O_3/PSS/Chit/Glut/HRP (c_0^{TMP} = 1.5 mmol/L, c_0 (H_2O_2) = 1.5 mol/L, temperature 40 ^{0}C, c_{kat} = 0.2 g/L)

As a result of the stabilization of the active sites of the enzyme, the immobilized HRP becomes the most stable. Figure 6b shows that the activity of HRP-based biocatalysts immobilized on SiO_2 and Al_2O_3 decreases slightly.

In the case of HRP immobilized on Fe_3O_4 (Figure 6a), the initial reaction rate first increases slightly, and then decreases. An increase in the activity of the biocatalyst can be caused by an increase in the number of active sites of the enzyme. A similar effect was described by the authors (Pestovsky et al., 2013) during the immobilization of HRP in a hydrogel. The decrease in activity can be explained by leaching of the enzyme from the surface of the carrier.

4. Conclusions

The biocatalysts synthesized in the work based on immobilized HRP can be successfully used in the environmentally friendly synthesis of vitamin E intermediate. The presence of the enzyme allows the TMP oxidation process to be carried out efficiently under mild conditions (temperature 40 °C and pH 6.5). HRP immobilized on magnetic nanoparticles showed the highest initial oxidation rate (6.28 $\mu mol \times L^{-1} \times min^{-1}$) compared to immobilization on SiO_2 and Al_2O_3(2.56 $\mu mol \times L^{-1} \times min^{-1}$and 1.71$\mu mol \times L^{-1} \times min^{-1}$). Such a biocatalyst can be easily separated from the reaction medium using a neodymium magnet, which greatly simplifies the process of synthesizing biologically active compounds.The synthesized biocatalysts slightly decreased their activity after 5 consecutive oxidation experiments with the same samples.

Acknowledgements

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