Influence of the Support Modification Methods on the Chlorophenol Utilization by the Peroxidase Immobilized on Titanium Dioxide

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In the current work, the synthesis of the effective multicomponent biocatalysts on the base of horseradish peroxidase immobilized on the modified titanium dioxide for the utilization of chlorophenolic water contaminants was performed. The influence of the support modification methods on the biocatalyst activity and stability was studied. The immobilization of horseradish peroxidase on the unmodified titanium dioxide was found to be ineffective due to the high losses in the enzyme amount during the reaction as well as the biocatalyst particles aggregation (the activity reduces more than 95 %). The titanium dioxide surface modification by the sodium alginate and carbodiimide (23.5 % activity in comparison with the native enzyme); hydrochloric acid, chitosan and glutaric dialdehyde (33.7 % activity in comparison with the native enzyme); and hydrochloric acid, chitosan, glutaric dialdehyde and aminopropyltriethoxysilane (42.5 % activity in comparison with the native enzyme) were found to be the most effective methods. Besides, the last two biocatalysts showed high stability in 10 consecutive cycles. The synthesized biocatalyst can be effectively used for the removal of chlorophenols from the wastewater until the concentrations of 10 ppm.

1. Introduction

Chlorophenols are among the most dangerous pollutants of water resources that have harmful effects on humans and animals due to high toxicity and carcinogenicity (Igbinosa et al., 2013). Purification of water from chlorinated phenols remains an important environmental challenge, primarily due to the large volumes of its global production and use (Olaniran et al., 2011).The main sources of chlorophenols are industrial wastewater (chemical, petrochemical, cellulose, wood processing industry, basic organic synthesis, production of paints and varnishes, synthetic resins, plasticizers, adhesives, antiseptics, pesticides, and pesticides, etc.) (Igbinosa et al., 2013). Another possible source of chlorinated phenol are the processes of water treatment (chlorination of water, where the chlorinating agents interact with the natural phenols and humic substances in the water) (Xiong et al., 2019). Phenol chlorinated derivatives have an intense unpleasant odor even in the smallest concentrations. In addition, the presence of chlorophenol compounds in the aqueous environment contributes to the formation of condensation of even more toxic substances – chlorinated biphenyls and dioxins (Nobuyasu et al., 2003). This causes the accumulation of the pollutants in the soil, disrupts the biocenosis and worsens the quality of natural water.

Reverse osmosis, adsorption or chemical oxidation to CO_2 and H_2O or water-insoluble polymers are the methods most commonly used for water purification from chlorophenols, however, none of the known methods leads to the complete removal of chlorophenols from water (Al-Obaidi et al. 2018). The major disadvantages of the traditional methods of wastewater purification are the wide range of the side products formed as well as the high cost of the purification processes.

In this regard, in recent years, researchers are looking for effective alternative methods of chlorophenols disposal. The enzymatic oxidation of chlorinated phenol by immobilized enzymes of the oxidoreductase class (EC 1) is one of the most promising methods (Bayramoglu et al., 2008). Horseradish peroxidase (EC 1.11.1.7)

is an enzyme that catalyzes the oxidation of phenolic substrates to o-quinones by hydrogen peroxide (Veitch, 2004). Immobilization of this enzyme allows its multiple uses in the industrial processes, significantly increasing its operational stability (Datta, 2013).The literature data describes several immobilized biocatalytic systems based on horseradish peroxidase synthesized for utilization of phenols and chlorophenols, for example on aldehyde glass (Bodalo et al., 2008), magnetic poly(glycidylmethacrylate-co-methylmethacrylate) beads (Bayramogluetal., 2008), magnetic Fe_3O_4/SiO_2particles (Chang et al., 2014), organogel-silica composite (Soomro et al., 2016).

In this work, titanium dioxide (TiO_2), a nanocrystalline oxide material with unique properties, is used as a support for the immobilization of horseradish peroxidase. Having high electrostatic characteristics, the titanium ion polarizes adsorbed water molecules, which leads to the appearance of OH-groups on its surface, which allows covalent binding of enzymes to the surface of particles (Morozov, 2014). The main problem with the use of titanium dioxide as a support for enzyme immobilization is the increased tendency of TiO_2 particles to aggregate due to local surface charges (Tshuto et al., 2017). To improve the stability of TiO_2 suspension, various methods can be used: the formation of an acidic medium (the surface of the particles is protonated, thus, the similarly charged particles interfere with the aggregation process), the addition of organic disaggregating reagents and modifiers, etc. (Guiot et al., 2013).

The focused modification of the surface of titanium dioxide in order to increase its binding with the enzyme molecules and improve the stability of the biocatalytic system allows effectively application of the biocatalytic removal of chlorophenols as an alternative to the traditional methods and leads to the decrease in the purification cost as well as the decrease in the range of the formed side products.

The purposes of this paper were the synthesis of the novel effective multicomponent biocatalyst on the base of the horseradish peroxidase immobilized on the modified titanium dioxide, and investigation of the influence of different methods of titanium dioxide surface modification on the activity and operational stability of the synthesized biocatalyst in the oxidation of 4-chlorophenol.

2. Experimental

In the Experimental section, the materials used in the current work, as well as the methods of biocatalyst synthesis and chlorophenols utilization in the presence of the synthesized multienzyme system, are given.

2.1 Materials

In this work the following chemicals and materials were used as received: titanium dioxide (TiO_2, «LDChim»); hydrochloric acid (HCl, «Kupavnareaktiv»); chitosan (low viscosity, Fluka); 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich); glutaric dialdehyde (25%, Acros Organics); KH_2PO_4); sodium alginate (Sigma-Aldrich); N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (carbodiimide, Sigma-Aldrich); sodium hydroxide (NaOH, «Reachim»); roots of Armoracia Rusticana; 4-chlorophenol (Sigma-Aldrich); 4-aminoantipyrine (Sigma-Aldrich); hydrogen peroxide (H_2O_2, «Kupavnareaktiv»); distilled water (H_2O); phosphate buffer solutions («Nevareaktiv», pH = 1.65; 4.01; 6; 7; 9.18; 12.43)

2.2 Biocatalysts preparation

In order to obtain the horseradish peroxidase (HRP) from the plant feedstock, the fresh root core of horseradish (Armoracia Rusticana) was grained. The peroxidase was extracted in a phosphate buffer solution pH = 7 (4 g of root core in 30 mL of buffer) for 1 h at a constant stirring. The mixture was centrifuged at 5000 rpm for 20 min and subsequently the centrifugate was filtered using a microporous filter. The resulting solution has peroxidase activity. Before the experiments, the extract was stored at a temperature of 3±1°C.

The following samples were synthesized:

(1) The sample of TiO_2 (0.5 g) was held in the enzymatic solution containing HRP (1 h).

(2) The sample of TiO_2 (0.5 g) successively aged in 0.1 M hydrochloric acid solution (1 hour), 0.2 % solution of chitosan (1 h), 2 % solution of the glutaricdialdehyde(24 h). The obtained modified support was held in the enzymatic solution containing HRP (1 h).

(3) The sample of TiO_2 (0.5 g) successively aged in 0.1 n hydrochloric acid solution (1 h), 0.2 % solution of chitosan (1 h), 5 % solution of 3-aminopropyltriethoxysilane (1 h), 2 % solution of the glutaric dialdehyde(24 h). The obtained modified support was held in the enzymatic solution containing HRP (1 h).

(4) The sample of TiO_2 (0.5 g) successively aged in 0.1 n hydrochloric acid solution (1 h), 0.2 % solution of sodium alginate (1 h), 5 % solution of 3-aminopropyltriethoxysilane (1 h). The obtained modified support was held in the enzymatic solution containing HRP and carbodiimide (0.1 %) (1 h).

All stages of the biocatalysts preparation were carried out with an intermediate washing with distilled water and filtering.

2.3 Chlorophenol utilization process

The activity of the synthesized biocatalysts was determined in a thermostat glass reactor in the oxidation reaction of 4-chlorophenol in the presence of hydrogen peroxide and 4-aminoantipyrine to increase the optical density of the reaction mixture at a wavelength of 506 nm (Figure 1).

Figure 1: Oxidation of 4-chlorophenol in presence of hydrogen peroxide and 4-aminoantipyrine

The optical density of the reaction mixture was recalculated in the concentration of reagents and conversion using a predetermined 4-chlorophenol molar absorption coefficient. The kinetic parameters of the extract and biocatalyst (activity, Michaelis constant (K_m) and reaction rate limit (V_m)) were determined by the method of double inverse coordinates (Cho et al., 2018) from the initial reaction rate varying the initial concentration of 4-chlorophenol.

3. Results and Discussion

The activity of the enzyme extract obtained from Armoracia Rusticana root according to Section 2.2. was studied in the 4-chlorophenol oxidation in the presence of hydrogen peroxide and 4-aminoantipyrine. The oxidation reaction kinetic curves varying the initial 4-chlorophenol concentration are presented in Figure 2.

Figure 2: The kinetic curves of the 4-chlorophenol oxidation reaction in the presence of free enzymatic extract varying the initial concentration of 4-chlorophenol

For the enzymatic extract, the activity was 0.53 U/g regarding the initial plant feedstock (at a concentration of 4-chlorophenol 1.05 mM), K_m – 0.14 mM, V_m – 0.0046 mM·s^{-1}

As it was shown in the experiments, in the case of HRP immobilization on unmodified titanium dioxide (sample (1)) biocatalyst activity decreased significantly: activity – 0.025 U/g regarding to the original plant material (at a concentration of 4-chlorophenol 1.05 mM), K_m – 1.46 mM, V_m – 0.00006 mM·s^{-1}. This is primarily due to the fragile fixation of HRP on the surface of titanium dioxide containing a sufficiently large amount of OH-groups (Zhu et al., 2009), which led to significant enzyme losses in the process of biocatalyst synthesis.

While the immobilization of HRP was performed on titanium dioxide modified by chitosan and glutaric dialdehyde (sample (2)), a significant increase in the activity of heterogeneous biocatalyst was observed: activity - 0.18 U/g regarding to the original plant material (at a concentration of 4-chlorophenol 1.05 mM), K_m – 0.25 mM, V_m – 0.00024 mM·s^{-1}. Treatment of titanium dioxide with hydrochloric acid, chitosan, and glutaric dialdehyde allowed stabilization of titanium dioxide particles, preventing their adhesion due to the poly-cationic properties of chitosan

(Yang et al., 2016). In order to increase the adsorption capacity of the support and to ensure the presence of functional amino groups on the support surface, further crosslinking with amino groups of the enzyme using glutaric dialdehyde was carried out. However, the loss of enzyme during the synthesis of the biocatalyst remained significant.

Immobilization of HRP with the additional use of APTES (sample (3)) solved this problem. The activity of this sample was 0.23 U/g regarding the initial plant material (at a concentration of 4-chlorophenol 1.05 mM), K_m – 0.14 mM, V_m – 0.00032 mM·min^{-1}. When APTES added to the system, first, the APTES ethoxy groups and the hydroxyl groups on the surface of titanium dioxide (the OH- group content on the TiO_2 surface is increased by treating the surface of titanium dioxide with hydrochloric acid) react with condensation, forming a strong Ti-O-Si bonds (Yang et al., 2014), secondly, the content of amino groups on the surface of the support increases, which significantly strengthens the enzyme fixation on the surface of the modified support compared with the biocatalytic system without the use of APTES. Thus, the combination of two methods of modifying the surface of titanium dioxide significantly increases the efficiency of crosslinking and, accordingly, the activity and operational stability of immobilized HRP.

Sample (4) synthesized using sodium alginate and pre-activation of carboxyl groups by HRP carbodiimide (Kazenwadel et al., 2015), showed a slightly lower efficiency, compared with samples (2) and (3) – activity 0.12 U/g regarding to the initial plant material (at a concentration of 4-chlorophenol 1.05 mM), K_m – 0.31 mM, V_m – 0.00019 mM·s^{-1}. The decrease in activity and stability is due to the insufficient strength of the crosslinking of HRP carboxyl groups with support modified by amino groups, which leads to enzyme losses during the synthesis of the biocatalyst. Thus, according to the results, the optimal method of titanium dioxide surface modification for the immobilization of HRP was the joint use of chitosan and APTES.

The kinetic parameters of immobilized biocatalysts are slightly lower than those of the enzymatic extract, which is primarily due to the heterogeneity of the system and the limited access of substrate molecules to the active HRP centers. Experiments have shown that the immobilized biocatalyst is stable and practically does not lose its activity in 10 consecutive experiments - loss of 26.5 and 21% of initial activity, for samples (2) and (3), respectively. Also from the above data, it is clear that the immobilization did not lead to a significant increase in the Michaelis constant, and, accordingly, the deterioration of the enzyme affinity to 4-chlorophenol.

The scheme of the process of synthesis of the optimal biocatalyst is shown in Figure 3.
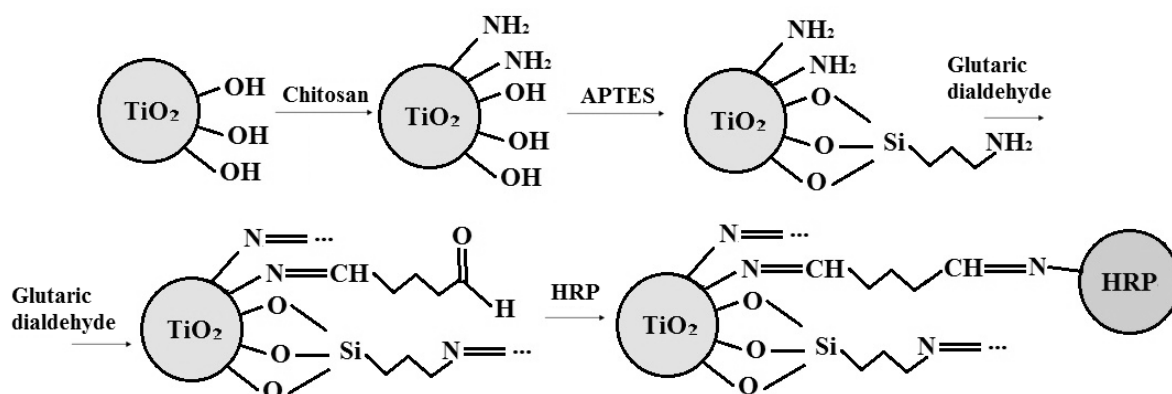

Figure 3: Optimal biocatalyst synthesis scheme

The results of the experiments on the variation of 4-chlorophenol concentration in the oxidation reaction in the presence of an optimal biocatalyst (sample (3)) are shown in Figure 4.

For the sample Eq (3) the experiments on the variation of reaction mixture pH were also performed (Figure 5). As can be seen from Figure 5, the activity of the immobilized biocatalyst at pH = 7 was found to be significantly higher than that for the other pH values. This value is congruent to the pH optimum for the native HRP.
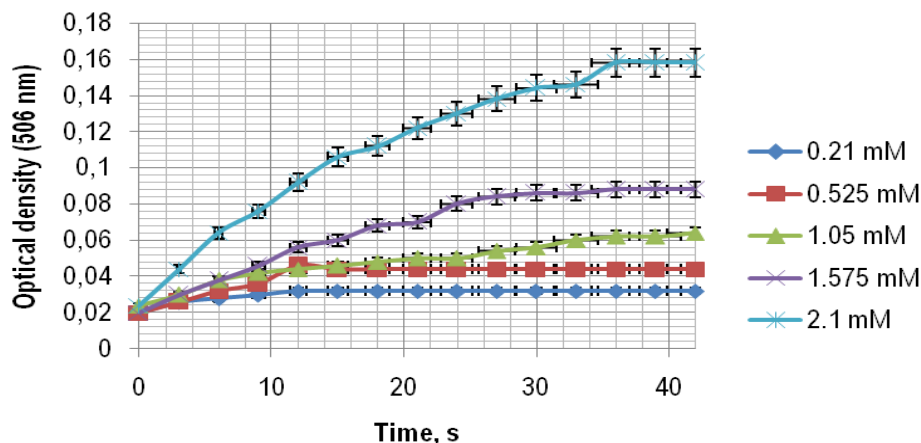
Figure 4: Kinetic curves for 4-chlorophenol oxidation in the presence of the optimal biocatalyst varying the initial 4-chlorophenol concentration
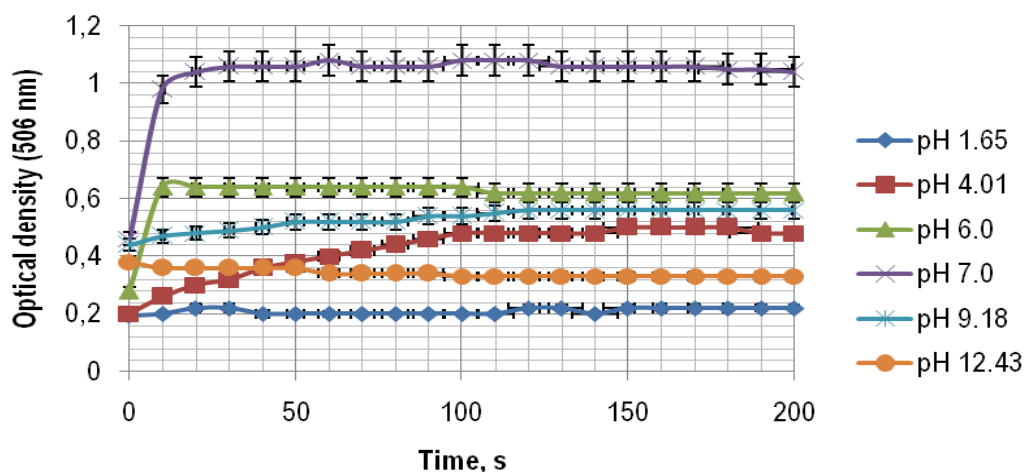

Figure 5: Kinetic curves for 4-chlorophenol oxidation in the presence of the optimal biocatalyst varying the reaction mixture pH

4. Conclusions

Multicomponent biocatalysts based on horseradish peroxidase (EC 1.11.1.7) immobilized on titanium dioxide modified by various combinations of components (HCl, APTES, chitosan, sodium alginate, glutaric dialdehyde, carbodiimide) were synthesized. The activity of biocatalysts was studied in the oxidation reaction of 4-chlorophenol with hydrogen peroxide in the presence of 4-aminoantipyrine. Experiments have shown that the optimal way to modify the surface of titanium dioxide for the immobilization of HRP is the joint use of HCl, APTES, chitosan and glutaric dialdehyde (maintenance of 42.5 % of the initial activity, 21 %loss of activity after 10 consecutive experiments). The high efficiency of this method of immobilization is ensured primarily by a stronger fixation of the HRP on the surface of titanium dioxide. The optimal pH value of the reaction mixture for the optimal biocatalyst was determined as 7.0. The results obtained in this work allow the conclusion about the prospective application of the synthesized biocatalyst in the processes of chlorophenol removal from the wastewater in the concentrations up to 10 ppm.

References

Al-Obaidi M.A., Kara-Zaitri C., Mujtaba I.M., 2018, Statistical-based modelling and optimization of chlorophenol removal from wastewater using reverse osmosis process, Chemical Engineering Transactions, 70, 2023-2028.

Bayramoglu G., AricaM. Y., 2008, Enzymaticremoval of phenol and p-chlorophenol in enzyme reactor: Horseradish peroxidase immobilized on magnetic beads, Journal of Hazardous Materials, 156, 148-163.

Bodalo A., Bastida J., Maximo M.F., Montiel M.C., Gómez M., Murcia M.D., 2008, A comparative study of free and immobilized soybean and horseradish peroxidases for 4-chlorophenol removal: protective effects of immobilization, Bioprocess and Biosystems Engineering, 31, 587–593.

Chang Q., Tang H., 2014, Immobilization of horseradish peroxidase on NH_2-modified magnetic Fe_3O_4/SiO_2particles and its application in removal of 2,4-Dichlorophenol, Molecules, 19, 15768-15782.

Cho Y.-S., Lim H.-S., 2018, Comparison of various estimation methods for the parameters of Michaelis–Menten equation based on in vitro elimination kinetic simulation data, Translational and Clinical Pharmacology, 26(1), 39-47.

Datta S., Christena L.R., Rajaram Y.R.S, 2013, Enzyme immobilization: an overview of techniques and support materials, Biotechnology, 3, 1–9.

Guiot C., Spalla O., 2013, Stabilization of TiO_2 nanoparticles in the complex medium through a pH adjustment protocol, Environmental Science & Technology, 47(2), 1057-1064.

Igbinosa E.O., Odjadjare E.E., Chigor V.N., Igbinosa I.H., Emoghene A.O., Ekhaise F.O., Igiehon N.O., Idemudia O.G., 2013, Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective, The Scientific World Journal, Article ID 460215, 11 pages.

Kazenwadel F., Wagner H., Rapp B.E., Franzreb M., 2015, Optimization of enzyme immobilization on magnetic microparticles using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a crosslinking agent, Analytical Methods, 7, 10291-10298.

Morozov A. N., 2014, Synthesis and catalytic properties of the nanostructured coverings of dioxide of the titan, PhD Thesis, Moscow, Russia, 2014.

Nobuyasu S., Takashi O., Masako U., Takumi T., Nobuyuki T., 2003, Temporal change of polychlorinated dibenzo-p-dioxins, dibenzofurans and dioxin-like polychlorinated biphenyls source in paddy soils, Journal of Environmental Chemistry, 1, 117–131.

Olaniran A.O., Igbinosa E.O., 2011, Chlorophenols and other related derivatives of environmental concern:Properties, distribution and microbial degradation processes, Chemosphere, 83, 1297–1306.

Soomro R., Memon N., Bhanger M.I., Denizli A., 2016, Horseradish peroxidase immobilized into an organogel-silica composite for the transformation of chlorophenols to biodegradable organic acids, Hacettepe Journal of Biology and Chemistry, 44 (3), 361–374.

Tshuto T., Kitoto E., Ranamane L., Chirwa E.M.N., 2017, Simultaneous degradation of phenol and reduction of chromium (VI) using UV/TiO_2 photocatalysis, Chemical Engineering Transactions, 57, 895-900.

Veitch N.C., 2004, Horseradish peroxidase: a modern view of a classic enzyme, Phytochemistry, 65, 249–259.

Xiong J., Ma Y., 2019, Catalytic Hydrodechlorination of Chlorophenols in a Continuous Flow Pd/CNT-Ni Foam Micro Reactor Using Formic Acid as a Hydrogen Source, Catalysts, 9(77), 1-11.

Yang C., Yang C., 2014, Preparation of TiO_2 particles and surface silanization modification for electronic ink, Journal of Materials Science: Materials in Electronics, 25, 3285-3289.

Yang J.M., Tsai R.-Z., Hsu Ch.-Ch., 2016, Protein adsorption on polyanion/polycation layer-by-layer assembled polyelectrolyte films, Colloids and Surfaces B: Biointerfaces, 142, 98-104.

Zhu Y., Cao H., Tang L., Yang X., Li C., 2009, Immobilization of horseradish peroxidase in three-dimensional macroporous TiO_2 matrices for biosensor applications, Electrochimica Acta, 54, 2823–2827.