

Water Treatment from Phenol Derivatives by the Oxidoreductases Immobilized on Alginate Microspheres

Valentina Matveeva^{a,*}, Boris B. Tikhonov^b, Polina Yu. Stadolnikova^b, Alexander Sidorov^b, Olga V. Grebennikova^b, Viktor I. Panfilov^c, Esther Sulman^b

^aTver State University, Zhelyabova str., 33, 170100, Tver, Russian Federation

^bTver State Technical University, A. Nikitin str., 22, 170026, Tver, Russian Federation

^cD.I. Mendeleev University of Chemical Technology of Russia, Miusskaya sq., 9, 125047, Moscow, Russian Federation
matveeva@science.tver.ru

In this paper, the synthesis of novel effective biocatalysts based on oxidoreductases (peroxidase extracted from the horseradish roots; commercial glucose oxidase) immobilized on the sodium alginate microspheres was performed. To activate the carboxylic groups of the support, enzyme immobilization on the sodium alginate microspheres was carried out using carbodiimide and N-hydroxysuccinimide. This allows the successful crosslinking of enzyme and support to be reached. The investigation of catalytic properties of the synthesized biocatalysts was carried out in the oxidation of phenol and 4-chlorophenol in the presence of 4-amino antipyrine. The activity and stability of the synthesized biocatalysts was studied. The use of biocatalysts immobilized on the sodium alginate microspheres allows more than 95 % of phenol and 4-chlorophenol conversion to be achieved. The synthesized biocatalysts exhibit 25 - 30 % activity in comparison with the native enzyme in the oxidation of phenol and its derivatives. The biocatalysts were found to save their activity in the up to 10 multiple reuses.

1. Introduction

Phenol and its derivatives are extremely toxic water pollutants that can cause disorders of the central nervous system, eye mucous membranes, respiratory tract, skin, and have a carcinogenic effect (Michałowicz et al., 2007). Phenol containing wastewater is formed during the thermal conversion of solid fuel (on coke-chemical, shale-processing plants, and gas-generating stations), the production of plastics, synthetic fibers, dyes, paper, etc. (Anku et al., 2017). Phenolic compounds are widely used for the synthesis of various aromatic compounds, disinfection, wood impregnation, as pesticides, and for many other purposes (Al Hashemi et al., 2015). A wide application of phenols in industry and agriculture leads to the pollution of drinking water sources and causes an increase in the morbidity of the population (Babich et al., 1981). Besides, the decay of phenolic compounds is accompanied by a sharp absorption of oxygen from the water, which can lead to the death of fish and other representatives of flora and fauna (Zapor, 2004).

The toxic effect of phenolic compounds makes scientists to search for novel ecologically friendly methods for water purification (Cordova Villegas et al., 2016). Among the most effective methods for phenol utilization, the destructive (thermal oxidation, electrical oxidation, hydrolysis, and chemical oxidation) (Sarno et al., 2019) and regeneration techniques (extraction purification, distillation, rectification, adsorption, ion exchange purification, reverse osmosis, ultra-filtration, etherification, polymerization, polycondensation, biological purification and conversion of phenols into low-soluble substrates) (Al-Obaidi et al., 2018) can be emphasized. In spite of the wide range of the existing phenol utilization methods, most of them do not provide full removal of contaminants from the water and require significant costs for the organization of purification processes.

Biocatalytic oxidation of phenol and its derivatives using immobilized oxidoreductase enzymes is a prospective way to remove phenolic compounds from water (Kurnik et al., 2015). Horseradish peroxidase (C.E. 1.11.1.7) is an enzyme extracted from the root of *Armoracia rusticana*. This enzyme can oxidize the organic compounds by the hydrogen peroxide into free radicals which further oligomerize to the insoluble quinones (Veitch, 2004). To

decrease the process cost and avoid the use of high amount of peroxidase, another oxidoreductase enzyme – glucose oxidase (C.E. 1.1.3.4) can be used. Glucose oxidase catalyzes the oxidation of β -D-glucose to D-glucono- δ -lactone (δ -glucono-1,5-lactone) and H_2O_2 using molecular oxygen as an electron acceptor (Bankar et al., 2009). In the literature, the data on the successful immobilization of oxidoreductases on quartz glass, polymeric supports (i.g. polyacrylamide, polystyrene), polysaccharides (cellulose, agarose, alginates, carrageenan), ion-exchange resins, bentonite, and silica are described (Tikhonov et al., 2019). The use of biodegradable natural polymers as a support for the oxidoreductase immobilization can significantly increase the safety and sustainability of the phenol oxidation process (Bilal et al., 2019). Sodium alginate – a derivative of alginic acid- is a polysaccharide of brown seaweed of the *Laminaria* and *Macrocystis*. This polymer consists of the residues of β -D-mannuronic and α -L-guluronic acids in the pyranose form which are linked in linear chains by (1,4)-glycoside bonds. This compound is considered to be the highly effective support for enzyme immobilization (Malik et al., 2017). Sodium alginate is soluble in the alkaline medium, and has a limited solubility in water. It has high sorption capacity and can swell in aqueous solutions. These properties allow two main functions (catalytic and adsorptive) in the biocatalysts to be combined (Zhao et al., 2018). Different forms (films, spheres, gel) can be obtained from the sodium alginate using different methods (Asadi et al., 2018). The novelty of the current research is connected with the development of highly effective, stable and biodegradable biocatalyst for the oxidation of phenolic compounds. The activation of carboxylic groups on the alginate microsphere surface using carbodiimide and N- hydroxysuccinimide allows the covalent binding between the support and enzyme to be obtained. This significantly decreases the enzyme activity losses during the reaction. The developed biocatalyst has higher activity in phenol oxidation in comparison with the analogues. It provides the higher degree of oxidation and reduces the range of side-products, and allows the process cost to be reduced. The aim of this work is the synthesis and investigation of novel effective biocatalyst based on the glucose oxidase immobilized on sodium alginate microspheres. The activity and operational stability of the synthesized biocatalyst were studied in the oxidation of phenol and 4-chlorophenol.

2. Experimental

2.1 Materials

The following materials were used in the experiments: sodium alginate (Sigma-Aldrich, USA); calcium chloride ($CaCl_2$, Reachim, Russia); N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (carbodiimide, Sigma-Aldrich, USA); N-hydroxysuccinimide (Sigma-Aldrich, USA); horseradish root (*Armoracia rusticana*); glucose oxidase (Sigma-Aldrich, USA, 2000-1000 U/g); phenol (Sigma-Aldrich, USA); 4-chlorophenol (Sigma-Aldrich, USA); 4-aminoantipyrine (Sigma-Aldrich, USA); hydrogen peroxide (H_2O_2 , Kupavna-reactiv, Russia); glucose (Sigma-Aldrich, USA); distilled water; phosphate buffer solution (pH = 7.0, Nevareactiv, Russia).

2.2 Biocatalysts preparation

To obtain the peroxidase extract, 5 g of *Armoracia rusticana* root was ground and mixed with 50 mL of phosphate buffer solution (pH = 7.0) and stirred continuously for 1 h. The mixture was centrifuged at 5,000 rpm for 20 minutes and filtered using a microporous filter. The centrifugate was stored in a refrigerator at a temperature of 3 ± 1 °C.

Microspheres from sodium alginate were synthesized for enzyme immobilization. The synthesis was performed as follows: 10 mL of 1.5 wt. % sodium alginate solution was dropped into the 100 mL of 1.5 wt. % calcium chloride solution. The microspheres with a diameter of 2-2.5 mm were obtained. The microspheres were kept in the solution for 2 minutes and, then, were washed with distilled water.

For enzyme immobilization, the alginate microspheres were kept for 12 h in 50 mL of the solution consisted of 0.394 g of carbodiimide and 0.144 g of N-hydroxysuccinimide. Then, the microspheres were washed with water, treated by the enzyme extract for 6 hours and washed with water. The biocatalyst was stored in a refrigerator at a temperature of 3 ± 1 °C.

2.3 Chlorophenol utilization process

The estimation of the activity of the synthesized biocatalysts was carried out in a glass batch reactor in the oxidation of phenol and 4-chlorophenol in the presence of hydrogen peroxide and 4-aminoantipyrine (Figure 1). While using the bienzymatic system (horseradish peroxidase – glucose oxidase) the reaction of formation of hydrogen peroxide proceeds according to the mechanism presented in Figure 2. In this case, the glucose oxidation by glucose oxidase was used to obtain hydrogen peroxide. The biocatalyst activity was measured by the change in the absorbance at 506 nm.

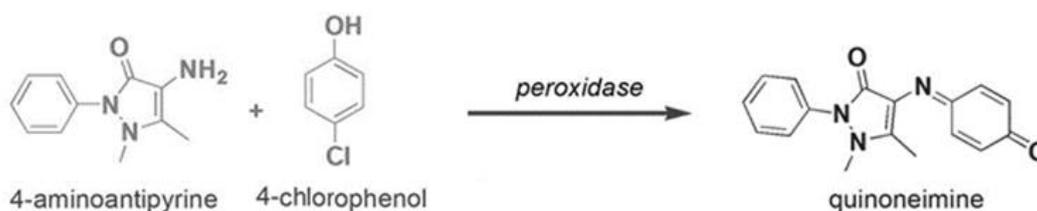


Figure 1: Oxidation of 4-chlorophenol in the presence of peroxidase, hydrogen peroxide and 4-aminoantipyrine

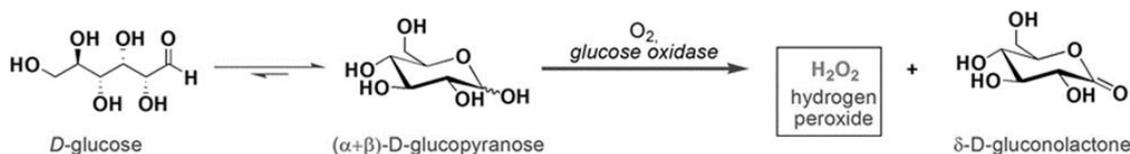


Figure 2: Oxidation of D-glucose by glucose oxidase

3. Results and discussion

To obtain the sodium alginate microspheres with the optimal mechanical and physical-chemical properties, the experiments on the variation of the concentrations of calcium chloride and sodium alginate were performed. It was found that a decrease in the concentration of sodium alginate less than 1.5 wt. % led to a significant increase in the diameter of the microspheres. The microsphere size increase negatively affects their strength. When increasing the concentration of sodium alginate over 1.5 wt. %, the reagent consumption increases due to the high viscosity of the solution. A decrease in the concentration of calcium chloride less than 1.5 wt. % led to a decrease in the strength of the resulting microspheres. When the calcium chloride concentration increased over 1.5 wt. %, no changes in the physical, chemical and mechanical properties of the microspheres were observed. The further increase in the reagent concentration is impractical.

The synthesized microspheres had the following size distribution: < 2 mm – 10.1 %; from 2 to 3 mm – 78.3 %; > 3.0 mm – 11.6 %.

The catalytic properties of the enzyme extract obtained from the root of *Armoracia rusticana* were studied in the oxidation of phenol and 4-chlorophenol varying the initial substrate concentration. Similar experiments were carried out with glucose oxidase and glucose as a source of hydrogen peroxide. The initial glucose concentration (5 mmol/L) was chosen to provide an excess of hydrogen peroxide.

The peroxidase containing extract was immobilized on the sodium alginate microspheres. For immobilization, an optimization of the biocatalyst component ratio was performed. The amount of carbodiimide and N-hydroxysuccinimide was calculated based on the number of carboxylic groups of sodium alginate (0.0394 g of carbodiimide and 0.144 g of N-hydroxysuccinimide per 1 g of sodium alginate). The activity of the biocatalysts with a different component ratio in the oxidation of phenol and 4-chlorophenol is presented in Table 1.

Table 1: Activity of catalysts (U/mg) with different component ratio (g of sodium alginate/g of horseradish root)

Substrate	10:1	4:1	2:1	4:3	1:1
Phenol	0.09	0.23	0.35	0.36	0.34
4-Chlorophenol	0.12	0.28	0.58	0.55	0.58

The biocatalyst with the following composition was optimal: 10 g of sodium alginate; 0.394 g of carbodiimide and 0.144 g of N-hydroxysuccinimide, 50 mL of distilled water, 50 mL of enzyme extract obtained from 5 g of *Armoracia rusticana* root. Further increase in the amount of enzyme extract did not lead to an increase in the activity of the synthesized biocatalyst. This can be explained by the limited number of free carboxylic groups on the microsphere surface.

The optimal biocatalyst was tested in the oxidation of phenol and 4-chlorophenol varying the initial substrate concentrations. The dependences of the absorbance at 506 nm from the time for the different substrate concentrations are presented in Figure 3.

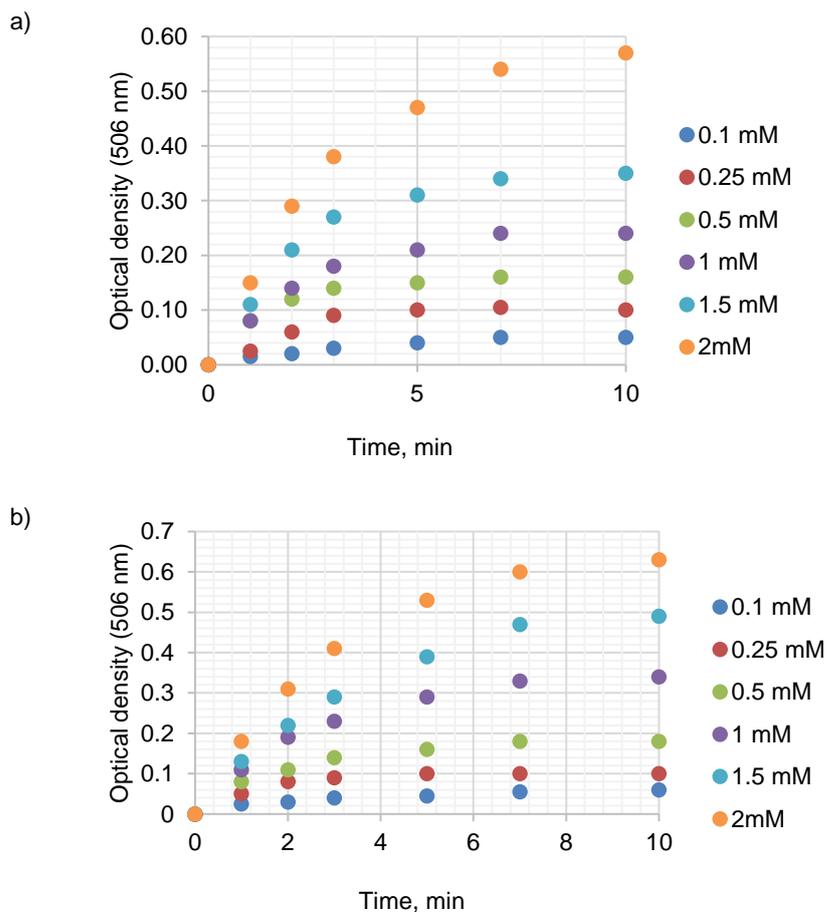


Figure 3: Kinetic curves of phenol (a) and 4-chlorophenol (b) oxidation by immobilized biocatalyst varying initial concentrations of substrates

The results of the experiments in the presence of glucose oxidase and glucose as a source of hydrogen peroxide are presented in Figure 4. The absorbance was recalculated into the reaction product concentrations using the molar absorption coefficient (for phenol – $0.275 \text{ L}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$; for 4-chlorophenol – $0.335 \text{ L}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$). Based on the product concentration dependence on time a linearization of the Michaelis-Menten equation in the Lineweaver-Berk coordinates ($1/V_0 - 1/C_0$) was performed. The kinetic parameters for the enzyme extract and the synthesized biocatalyst (maximum reaction rate V_m , Michaelis constant K_M , catalyst activity) were calculated (Table 2).

Table 2: Kinetic parameters of the synthesized biocatalysts (HRP – horseradish peroxidase; GOX – glucose oxidase; ALG – alginate microspheres)

Biocatalyst	$V_m, \text{mM}\cdot\text{s}^{-1}$		K_M, mM		Activity, U/mg	
	phenol	4-chlorophenol	phenol	4-chlorophenol	phenol	4-chlorophenol
HRP	0.054	0.048	0.93	0.51	1.07	1.25
HRP-GOX	0.051	0.050	1.06	0.65	0.95	1.12
ALG-HRP	0.012	0.009	1.24	0.68	0.35	0.58
ALG-HRP-GOX	0.011	0.085	1.33	0.71	0.31	0.53

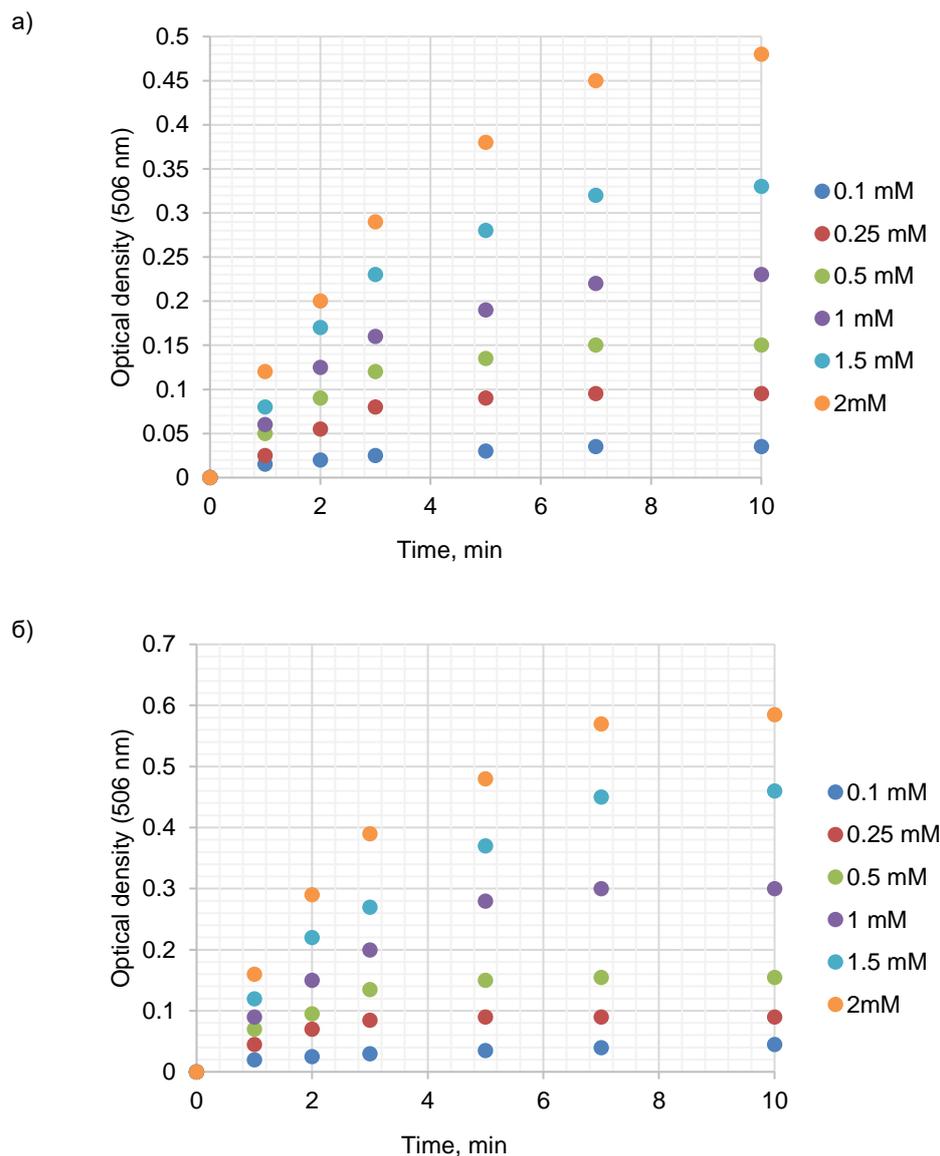


Figure 4: Kinetic curves of phenol (a) and 4-chlorophenol (b) oxidation by immobilized biocatalyst in the presence of glucose oxidase and D-glucose

The synthesized biocatalyst can effectively oxidize phenol and 4-chlorophenol in concentrations up to 2 mmol/L. The immobilized enzyme exhibits about 25 - 30 % of the activity of the native enzyme (see Table 2). The decrease in the activity can be explained by the reaction heterogenization and mass transfer limitation for the substrate molecule access to the catalyst active sites. In spite of the significant activity decrease, the synthesized biocatalyst can be used repeatedly for the effective utilization of phenols. The use of bienzymatic system (horseradish peroxidase – glucose oxidase) allows the high effectiveness in the phenol oxidation to be achieved. In this case, the use of hydrogen peroxide can be excluded due to the activity of glucose oxidase. Besides, the reaction products can be adsorbed on the microspheres and removed from the reaction medium that is confirmed by the slight spotting of the microsphere surface. These advantages allow the synthesized biocatalyst to be an effective alternative to the common methods of phenol utilization.

To estimate the stability of the synthesized biocatalyst on the base of horseradish peroxidase immobilized on the alginate microspheres, the experiments on the multiple reuses were performed (see Table 3). It is seen that the biocatalyst saves about 75 % of the initial activity after 10 consecutive cycles.

Table 3: Activity of the synthesized biocatalyst in 10 consecutive cycles

Substrate	Number of cycles									
	1	2	3	4	5	6	7	8	9	10
Phenol	0.35	0.33	0.31	0.3	0.28	0.28	0.27	0.27	0.27	0.26
4-Chlorophenol	0.58	0.54	0.51	0.49	0.47	0.45	0.44	0.43	0.42	0.43

4. Conclusions

Effective heterogeneous biocatalysts based on horseradish peroxidase and glucose oxidase immobilized on microspheres of sodium alginate were synthesized. It was shown that the developed biocatalysts allow the successful oxidation to be achieved with the phenol and its derivatives concentrations up to 2 mmol/L. The biocatalysts exhibit about 25 - 30 % of the activity of the native enzymes. In spite of the activity decrease, the developed biodegradable biocatalysts can be used repeatedly with a minimal loss in the effectiveness. The experiments showed a saving of up to 75 % of the initial biocatalyst activity after 10 cycles of reuse.

Acknowledgments

The study of synthesised biocatalysts was funded by the Russian Foundation for Basic Research (grant 18-08-00424).

References

- Al Hashemi W., Maraqa M.A., Rao M.V., Hossain M.M., 2015, Characterization and removal of phenolic compounds from condensate-oil refinery wastewater, *Desalination and water treatment*, 54(3), 660-671.
- 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.
- Anku W.W., Mamo M., Govender P., 2017, Phenolic compounds in water: sources, reactivity, toxicity and treatment methods. In: M. Soto-Hernandez, M. Palma-Tenango, M.del Rosario Garcia-Mateos (Eds.) *Phenolic Compounds-Natural Sources, Importance and Applications*, Chapter 17, In Tech Open, 420-443.
- Asadi S., Eris S., Azizian S., 2018, Alginate-based hydrogel beads as a biocompatible and efficient adsorbent for dye removal from aqueous solutions, *ACS Omega*, 3(11), 15140–15148.
- Babich H., Davis D.L., 1981, Phenol: A review of environmental and health risks, *Regulatory Toxicology and Pharmacology*, 1(1), 90-109.
- Bankar S.B., Bule M.V., Singhal R.S., Ananthanarayan L., 2009, Glucose oxidase - an overview, *Biotechnology Advances*, 27, 489–501.
- Bilal M., Iqbal H.M.N., 2019, Naturally-derived biopolymers: potential platforms for enzyme immobilization, *International Journal of Biological Macromolecules*, 130, 462-482.
- Cordova Villegas L.G., Mashhadi N., Chen M., Mukherjee D., Taylor K.E., Biswas N., 2016, A short review of techniques for phenol removal from wastewater, *Current Pollution Reports*, 2(3), 157–167.
- Kurnik K., Treder K., Skorupa-Klaput M., Tretyn A., Tyburski J., 2015, Removal of phenol from synthetic and industrial wastewater by potato pulp peroxidases, *Water, Air & Soil Pollution*, 226(8), 254.
- Malik D.J., Sokolov I.J., Vinner G.K., Mancuso F., Cinquerrui S., Vladislavjevic G.T., Clokie M.R.J., Garton N.J., Stapley A.G.F., Kirpichnikova A., 2017, Historical perspective formulation, stabilisation and encapsulation of bacteriophage for phage therapy, *Advances in Colloid and Interface Science*, 249, 100-133.
- Michałowicz J., Duda W., 2007, Phenols – Sources and Toxicity, *Polish Journal of Environmental Studies*, 16(3), 347-362.
- Sarno M., Spina D., Castaldo R., Cocca M., Gentile G., Ambrogio V., 2019, Catalytic removal of phenols with PtRu alloy/MoS₂ nanoparticles embedded in hyper-cross-linked polymers, *Chemical Engineering Transactions*, 73, 223-228.
- Tikhonov B., Sulman E., Stadol'nikova P., Sulman A., Golikova E., Sidorov A., Matveeva V., 2019, Immobilized enzymes from the class of oxidoreductases in technological processes: a review, *Catalysis in Industry*, 11, 251-263.
- Veitch N.C., 2004, Horseradish peroxidase: a modern view of a classic enzyme, *Phytochemistry*, 65, 249–259.
- Zapor L., 2004, Toxicity of some phenolic derivatives - in vitro studies, *International Journal of Occupational Safety and Ergonomics*, 10(4), 319-331.
- Zhao L., Wang J., Zhang P., Gu Q., Gao Ch., 2018, Absorption of heavy metal ions by alginate, *Bioactive Seaweeds for Food Applications*, 255-268.