Immobilization of horseradish peroxidase onto kaolin by glutaraldehyde method and its application in decolorization of anthraquinone dye

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Abstract

The problem of environmental pollution becomes more worrisome day by day, primarily due to the large amounts of wastewater contaminated with various harmful organic compounds, discharged untreated or partially clean into the environment. Feasibility of use of horseradish peroxidase (*Amoracia rusticana*) in the synthetic dyes decolorization was approved by many researchers. Among a number of supports used for the immobilization, it was found that natural clay, kaolin, has excellent features which are a precondition for obtaining biocatalysts with the excellent performances. For this reason, a horseradish peroxidase was immobilized onto kaolin using glutaraldehyde as a cross-linking agent. Obtained biocatalyst was applied in the decolorization of anthraquinone dye C.I. acid violet 109. Under determined optimal conditions (pH 4.0, hydrogen peroxide concentration 0.6 mM, dye concentration 30 mg L^{-1}, temperature 24 °C) around 76% of dye decolorization was achieved. Reusability study showed that resulting biocatalyst was possible to apply four times in the desired reaction with relatively high decolorization percentage.

Keywords: horseradish peroxidase, immobilization, wastewater, dyes.

The global water crisis has become worrisome problem because it is leading worldwide cause of deaths and diseases [1]. The textile industry is the main consumer of water in the production process, and therefore generates a large amount of wastewater. Wastewater from the textile industry is colored, rich in suspended particles, heavy metals, organic matter and other pollutants and often untreated or partially treated discharged into the environment. Complex composition of textile wastewater requires special treatment methods and many of them are high in energy demands and expensive. Around 2–20% of untreated wastewater is directly discharged in to the environment causing direct contamination of ground and surface water [1,2]. Different types of dyes (azo, anthraquinone and reactive) are applied in the textile processing technology and they represent the biggest problem in the textile wastewater. Most of them, like anthraquinone dyes, have steadfast structure based on the fused aromatic rings which make them difficult to remove and wastewater stay colored for a long period. Colored wastewater leads to impaired photosynthesis, inhibition of growth, development and reproduction of aquatic organisms [3,4]. Besides, the presence of dyes and their degradation products cause serious human health problems such as hemorrhage nausea, reproductive system, liver, brain and central nervous system damage [5]. Traditional methods for wastewater treatment can be divided into three groups: physical, chemical and biological and because of the complexity and variability in the composition of the wastewater they cannot adequately respond to stringent regulations for water purity [6]. Stringent standards for the discharge of wastewater into the environment motivated researchers to develop alternative methods for the treatment of wastewaters. Usage of oxidoreductive enzymes (manganese, lignin, horseradish, turnip and tomato peroxidase) is on the first place, because they show merit in converting complex chemical compounds under mild environmental conditions with high efficiency [5]. Horseradish peroxidase (EC: 1.11.1.7, HRP) applied in the treatment of wastewater can act on the harmful compounds by converting them into innocuous products or changing the characteristics of a given waste making it more favorable for the treatment [7]. The wastewater is characterized with high temperatures, high or low pH, high ionic strength, high reactant concentrations and presence of inhibitors which can all alter the catalytic conformation of the enzyme. Because of this it is necessary to convert enzyme into immobilized form which leads to increased stability, ease of handling, possibility to reuse and a consequent decrease in running cost [8,9].

In this paper in order to develop a sustainable and eco-friendly biocatalyst for the decolorization of col-

ored wastewater a simple method for the immobilization was applied. HRP was immobilized onto kaolin using glutaraldehyde (GA) as a cross-linking agent. Influence of the initial glutaraldehyde concentration as well as the initial enzyme concentration on the enzyme coupling yield and the activity of resulting biocatalyst was examined. The obtained immobilized biocatalyst was tested in the decolorization of the model dye C.I. acid violet 109 (AV 109).

EXPERIMENTAL

Materials

Anthraquinone dye C.I. acid violet 109 was provided by Lanaset (Lanaset Violet B). Horseradish peroxidase, glutaraldehyde and pyrogallol were obtained from Sigma–Aldrich (USA). Kaolin was supplied from Carlo Erba, and hydrogen peroxide was purchased from Merck (Darmstadt). All other chemicals used in this study were of analytical grade.

Methods

Support activation

In order to increase the reactivity of the support, prior to immobilization, kaolin was activated using thermal treatment [10–12]. Thermal activation was accomplished after heating the kaolin paste (50% of moisture) in the oven for 2 h at 550 °C.

The effect of the initial glutaraldehyde concentration on the immobilized mass and enzyme activity

100 mg of thermally activated support was suspended in 0.5 mL of different glutaraldehyde solution concentrations 0.12–1.0% and gently mixed at room temperature for 1 h [13]. After the reaction was finished excess of glutaraldehyde was washed several times with distilled water. Prepared support was used for the HRP immobilization.

Immobilization of HRP

Immobilization of HRP was carried out by immersing 100 mg of GA–kaolin into 0.5–5.0 mg mL^{-1} of HRP solution in phosphate buffer (100 mM, pH 7.0) and suspension was incubated for 4h at room temperature. Biocatalyst was separated from the supernatant and washed with immobilization buffer until no activity was detected in the washings. Immobilized enzyme was stored at 4 °C until use.

Measurement of the enzyme activity

Activity of the free and immobilized HRP was measured using standard substrates, hydrogen peroxide and pyrogallol. The reaction mixture for measuring the activity of free enzyme consisted of: 1 mL of 13 mM pyrogallol in the potassium-phosphate buffer pH 7.0, 10 μL of 3% hydrogen peroxide and 10 μL of diluted enzyme solution. The reaction rate was monitored

spectrophotometrically at 420 nm by following the change in absorbance values every 30 s for 3 min. The reaction mixture for measuring the activity of immobilized HRP consisted of: 3 mL of 13 mM pyrogallol prepared in the potassium phosphate buffer pH 7.0, where few milligrams of immobilized biocatalyst were suspended. After adding 30 μL of hydrogen peroxide, magnetic stirrer was set on the maximum and reaction rate was recorded each 60 s for 3 min in the samples obtained after centrifugation. One unit of the activity was defined as the amount of peroxidase that will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 7.0 and 20 °C [14].

Protein content determination

Immobilization yield was calculated after protein content determination in the initial solution, supernatant and washings. Protein content was determined using modified method of Lowry [15]. The difference between the offered, protein in the supernatant and washings represent the mass of bound protein.

Dye decolorization in batch process

Optimal pH was evaluated by incubating the free and immobilized HRP with equal activity in AV 109 solution (30 mg L^{-1}) at pH 3.0–12.0 under 25 °C. Influence of hydrogen peroxide concentration on the decolorization was examined by varying the hydrogen peroxide concentration in the range 0.05–2.0 mM. In the third set of experiments, dye concentration influence was examined by varying the dye concentration in the range 10–50 mg L^{-1}, on the decolorization. In order to compare the properties, all experiments were performed with both forms of free and immobilized HRP under similar reaction conditions. In defined time intervals aliquots were collected from the reaction mixtures and residual dye amount was followed using UV––Vis spectrophotometer (UV Shimadzu 1700, Shimadzu Corporation, Kyoto, Japan) at maximum wavelength for the tested dye (λ_{max} 590 nm, AV 109).

Decolorization percentage was calculated using following mathematical expression [16]:

$$\text{Decolorization} = \frac{100\left(A_0 - A_t\right)}{A_0} \qquad (1)$$

where A_0 is the initial absorbance of the untreated dye solutions (control) and A_t is the absorbance of the dye solution after enzymatic treatment.

RESULTS AND DISCUSSION

The influence of the initial glutaraldehyde concentration

Support activation using glutaraldehyde was studied intensively because the method is simple, inexp-

ensive and efficient [17]. Glutaraldehyde used in the activation step has a role of a spacer arm and may prevent direct contact of the enzyme with the support. However, the excess of the glutaraldehyde can randomly attach the amino groups of the enzyme resulting in the hindering of the enzyme active center and inactivation [18]. Because glutaraldehyde is prone to cross linking and forming dimmers its initial concentration can greatly affect the amount of free aldehyde groups on the activated support at thus the immobilization and the activity yield. Influence of the initial glutaraldehyde dose on the protein loading and the activity of the immobilized preparation is presented in Figure 1.
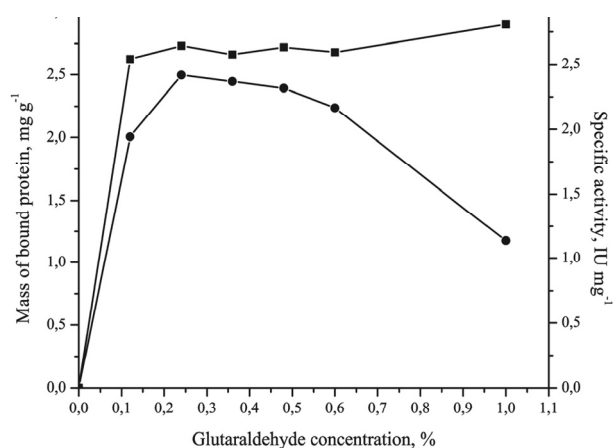

Figure 1. Initial glutaraldehyde concentration (GA) influence on mass of bound protein (square) and specific activity of immobilized preparation (circle).

It can be seen from the Figure 1 that, in the tested range, increasing the initial glutaraldehyde concentration did not have effect on the protein loading which is almost constant (2.7 mg g^{-1}), while the increase in GA concentration up to 0.24% leads to increase in the activity of the immobilized enzyme (2.4 IU mg^{-1}). Further increase resulted in the reduction of the activity which falls more than double at GA concentration of 1%. The explanation for such behavior lays in the fact that high concentration of aldehyde groups can lead to multi-point covalent attachment that can change the active enzyme conformation and transform it into inactive form [19]. Similar results were given in the literature. Chen *et. al* immobilized β-galactosidase using GA and obtained the most active biocatalyst at GA concentration of 0.3%. Increase the GA concentration above 0.3% GA significantly reduced the obtained preparation activity, as a consequence of multipoint attachment between enzyme and support [13].

Immobilization of HRP

After activation, support was used for the immobilization of HRP. Herein, in order to examine the sup-

port capacity and the activity of immobilized preparations, offered enzyme concentration was varied.
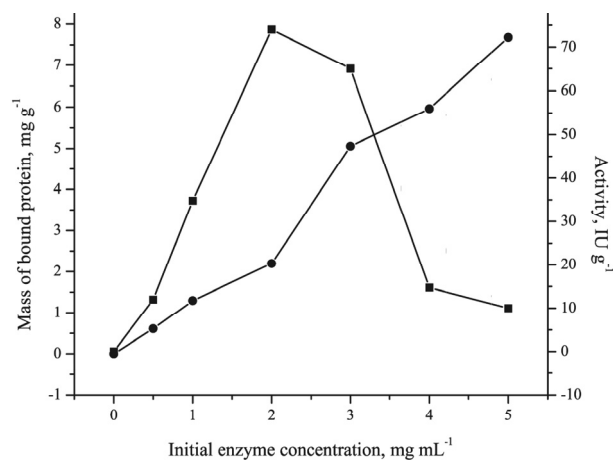

Figure 2. Initial enzyme concentration influence on mass of bound protein (circle) and specific activity of immobilized preparation (square) (reaction conditions: reaction time 4 h, pH 7.0, glutaraldehyde concentration 0.24%, temperature 24 °C).

It is evident from the Figure 2 that, in the investigated range, increasing the offered mass of protein resulted in almost linear increase of protein loading. This indicates a large number of available aldehyde groups on the support surface. However, the increase in the mass of bound proteins does not necessarily mean the activity increase. From the Figure 2 it can be observed that the increase in the offered enzyme concentration from 0.5–2.0 mg mL^{-1} leads to activity increase up to 74.2 IU g^{-1}. Further increase in the offered enzyme concentration resulted in a sharp activity decrease and was negligible for the initial concentration of 5.0 mg mL^{-1}. Due to increase in the mass of loaded protein enzyme molecules become more densely packed which increases the possibility of steric hindrances and limits the mass diffusion as well [17]. Also, at higher protein loading activity of the immobilized preparation was significantly impaired mainly due protein-protein interactions. The protein–protein interactions are the consequence of deficiently space between adsorbed proteins which leads to structural rearrangements and activity decrease [20,21].

Dye decolorization in batch process

Optimum pH

Obtained immobilized biocatalyst with the specific activity of 2.4 IU mg^{-1} was used for the optimization of process parameters for the AV 109 decolorization in the batch reactor. As a batch reactor glass vial with magnetic stirrer was used. Because of its intrinsic influence on the acidic-basic behavior of the substrate and protonation state of histidine and arginine involved in the catalytic cycle, pH is one of the most important

parameters for the efficient performing of the desired reaction [23]. Optimum pH for the decolorization of AV 109 with the free and immobilized HRP was performed by measuring the decolorization at different pH values using the same amount of the free and immobilized HRP and similar reaction conditions. The experimental pH region from 3.0 to 12.0 is chosen because pH conditions of textile effluent are in that range [24]. The activity of the immobilized and free biocatalyst under different pHs is depicted in the Figure 3.
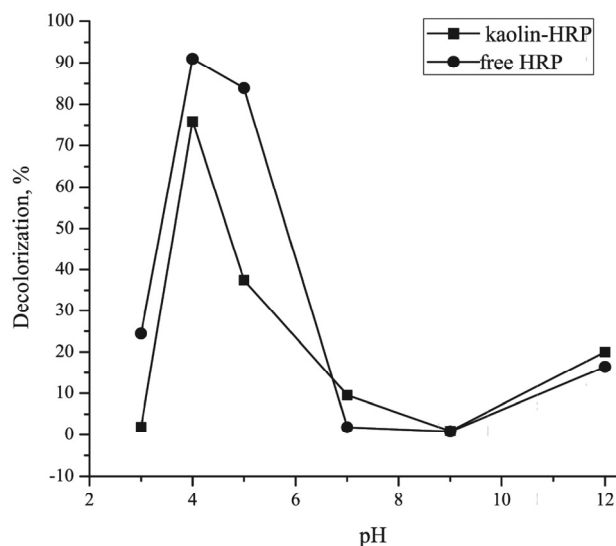

Figure 3. pH influence on the decolorization of AV 109 by free and kaolin-HRP (dye concentration 30 mg L^{-1}, temperature 24 ℃, hydrogen peroxide concentration 0.4 mM, enzyme concentration 0.1 U).

From the Figure 3 it can be seen that the optimum pH for the immobilized as well as for the free peroxidase corresponds to pH 4. A small degree of decolorization below pH 4.0 can be interrelated with the loss of heme group, which occurs much faster in this pH range, and the conversion of HRP into catalytically inactive form [25]. Under defined conditions after 60 min immobilized HRP oxidized 75.9%, meanwhile free oxidized 95% of AV 109 dye. This can be correlated with the steric effect of the support which hides the enzyme active center and the substrate may be difficult to approach. The obtained optimal pH corresponds to the reported value for HRP decolorization of Lanaset Blue 2R, where 59% of dye decolorization was achieved at pH 4.0 [26]. As it can be observed from the Figure 3, an increase in pH above 6.0 significantly decreased the decolorization. Similar results were obtained after applying HRP in the decolorization of Remazol Blue and Red Cibacron, where above pH 6.0 HRP activities were significantly smaller [27]. Performances of the immobilized biocatalyst are extremely dependent on the carrier selected for the immobilization. Thus, for example, Mohan *et al.* showed that HRP optimum pH

for decolorization of Acid Black 10 BX dye was 2.0, but Karim *et al.* approved that pH 6.0–8.0 was optimal for azo dye removal from textile effluent, when HRP was immobilized in acrylamide gel and β-cyclodextrin-chitosan complex, respectively [28,29]. Besides, free HRP exhibited highest activity in acidic pH, meanwhile immobilized biocatalyst showed stability against alkaline conditions unlike free HRP whose activity was negligible under these conditions. Also a sharp decline in the activity under alkaline conditions to pH 12.0 and thereafter abrupt increase can be seen on the Figure 3. This arise from the structural changes in the distal pocket of the heme cavity where movement of distal histidine leads to its binding to the metal ion or from the changes in spin state of iron, from high to low [30,31].

Optimum hydrogen peroxide dose

It is clear from the Figure 4 that catalytic yield is favored by the low hydrogen peroxide concentration. Increase in the H$_2$O$_2$ concentration above 0.6 mM induced a slight decrease in the decolorization efficiency. Inhibitory effect of coadjutant was noticed by other researchers too. For example, HRP immobilized in β-cyclodextrin-chitosan matrix showed the highest activity with 0.6 mM hydrogen peroxide concentration; where upon the decolorization percentage of tested azo dye was reduced significantly [32]. This phenomenon could be explained with the fact that excess of hydrogen peroxide leads to higher generation of intermediate species that inhibit the activity of the enzyme [7]. Besides horseradish peroxidase, other peroxidases used in the decolorization reactions also showed increased sensitivity against hydrogen peroxide [16,7,33].
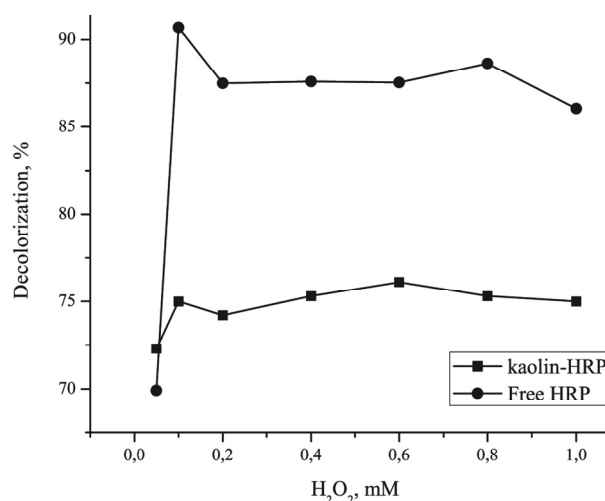

Figure 4. Initial H$_2$O$_2$ dose influence on the AV 109 decolorization by free and kaolin-HRP (dye concentration 30 mg L^{-1}, temperature 24 ℃, pH 4.0, enzyme concentration 0.1 U).

However, Figure 4 also shows a higher sensitivity of free HRP than its immobilized counterpart against suicide effect of the peroxide. The optimum dose for performing the decolorization of AV109 using immobilized HRP was 0.6 mM, meanwhile free HRP exhibited highest activity at peroxide concentration of 0.1 mM. This suggests that the immobilization protected active center and the enzyme is active in a much wider range of hydrogen peroxide concentrations.

Optimum dye dose

Figure 5 represents the dependence of decolorization percentage of the dye concentration. It is evident that the dye had inhibitory influence on HRP, too. For both, free and immobilized enzyme optimal concentration for the highest decolorization percentage (92.3% for free HRP and 76.5% for kaolin-HRP) was 30 mg L^{-1}. Decolorization decreased thereafter, but the inhibitory influence of the dye is much more pronounced on the free than on the kaolin-HRP. Up to a concentration of 100 mg L^{-1} immobilized enzyme retained almost constant decolorization potential (around 70%) while steep decreased for the free enzyme can be observed and the decolorization percentage falls to 21.3%. Immobilization had positive influence on the HRP since under determined conditions the free HRP retained 23%, while the kaolin-HRP retained 92% of the activity.
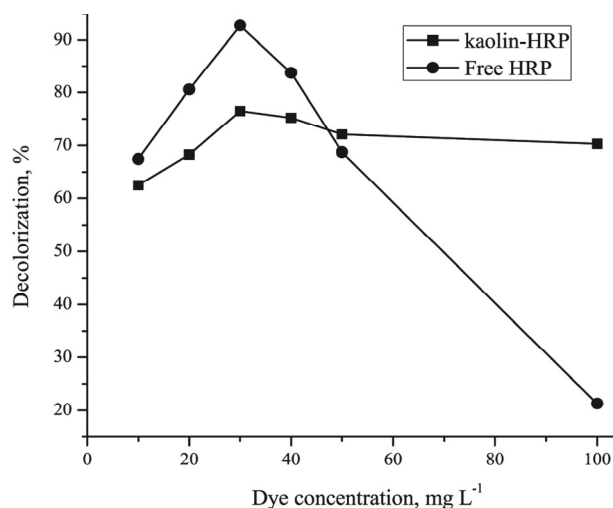

Figure 5. Initial dye concentration influence on the AV 109 decolorization by free and kaolin-HRP (hydrogen peroxide concentration 0.1 mM and 0.6 mM for free and kaolin-HRP, respectively, temperature 24 °C, pH 4.0, enzyme concentration 0.1 U).

The negative effect of the high dye concentration was observed in the decolorization reactions by other peroxidases [34,35]. Lower substrate concentrations were very effective in the suppression of the enzyme inactivation. Explanation for this can be found in the HRP catalytic cycle. Namely, in the HRP catalytic cycle

there are tree similar forms of the enzyme HRP E, E-I and E-II so there is the same possibility for dye binding for E-I as well as for E-II and form dead-end complex. With dye concentration increase the possibility for inactivate complex formation also increases [36–38]. When enzyme is immobilized by GA method, this formation of inactivate complex is almost absent.

It can be concluded that the optimal conditions are: enzyme concentration 0.1 U, pH 4.0, hydrogen peroxide and dye concentration 0.6 mM and 30 mg L^{-1}, 1 h of contact time. Under the optimal conditions the immobilized biocatalysts decolorized 76.5%, meanwhile free HRP 92.3% of AV 109 dye.

Reusability study

In addition, the possibility of application of obtained biocatalyst at industrial scale was evaluated trough reusability study. Results are presented in the Figure 6.
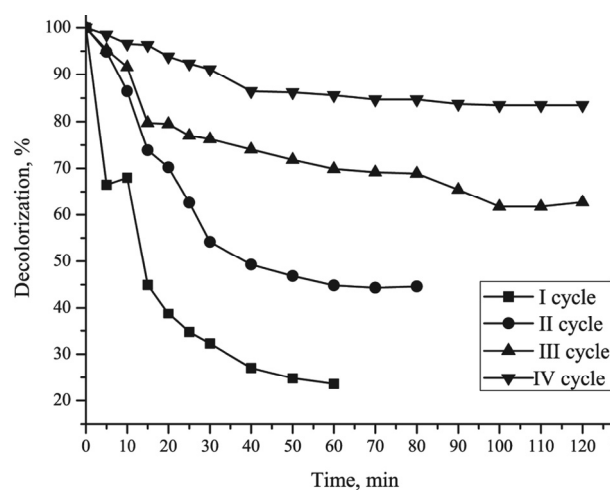

Figure 6. Reusability study (enzyme concentration 0.1 U, dye concentration 30 mg L^{-1}, pH 4.0, hydrogen peroxide concentration 0.6 mM, temperature 24 °C).

Biocatalyst applied under determined optimum conditions after first cycle was separated from the reaction mixture, rinsed with the reaction buffer and assessed for its left over catalytic activity. It was possible to apply the immobilized HRP four times in the reaction; where after each cycle progressive decrease in the decolorization was observed. For example, after fourth cycle immobilized HRP retained 15% of its original activity. Jiang *et.al* found that HRP encapsulated in phospholipid-templated titania retained around 50% of the activity after 6 consecutive cycles, meanwhile mesoporous materials did not show such good properties in terms of reusability and the immobilized enzyme was possible to apply only twice in the decolorization of dye Remazol Brilliant Blue R [39,40]. This behavior was explained by forming the reaction products layer across the enzyme active site, which thus became inaccessible for the substrate [40]. Immobil-

ization of HRP onto kaolin by GA method approved to be a good immobilization technique because 0.1 IU of immobilized HRP decolorized 55.7 mg L^{-1}, meanwhile free HRP only 27.7 mg L^{-1}.

CONCLUSION

After multiple set of experiments listed above, the kaolin has proven to be a good choice as the support for the immobilization of HRP. Obtained biocatalyst is characterized by good performances in terms of continuous use and the retained activity. The high degree of tested dye decolorization and improved properties in terms of pH, the impact of dye and hydrogen peroxide, indicated the possibility of this biocatalyst application as a clean alternative in wastewater treatment. In order to apply the immobilized enzyme in the continuous wastewater treatment plants further experiments should be focused on the stability increase.

Acknowledgement

REFERENCES

[1]   M. Rizwana, M. Darshan, D. Nilesh, Phytoremediation of textile wastewater using potential wetland plant: Eco sustainable approach, Int. J. Interdiscip. Multidiscip. Stud. **1** (2014) 130–138.

[2]   D.H.Y. Yanto, S. Tachibana, K. Itoh, Biodecolorization of textile dyes by immobilized enzymes in a vertical bio-reactor system, Procedia Environ. Sci. Eng. Manage. **20** (2014) 235–244.

[3]   M.B. Ibrahim, N. Poonam, S. Datel, M. Roger, Microbial decolorization of textile dye-containing effluents: a review, Bioresour. Technol. **58** (1996) 217–227.

[4]   S. Wijetunga, X.F. Li, C. Jian, Effect of organic load on decolourization of textile waste-water containing acid dyes in up flow anaerobic sludge blanket reactor, J. Hazard. Mater. **177** (2010) 792–798.

[5]   Q. Husain, Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review, Rev. Env. Sci. Biotechnol. **9** (2010) 117–140.

[6]   Md. A. Arafath, M. Hossain, S.S. Alam, R. Sourav, Studies on the adsorption efficiency and kinetics of dye removal from textile effluent using some natural bio-adsorbent, Int. J. Sci. Eng. Technol. **2** (2013) 853–856.

[7]   F. Jamal, S. Sinhg, T. Qidwai, D. Singh, P.K. Pandey, G.C. Pandey, M.Y. Khan, Catalytic activity of soluble versus immobilized cauliflower (*Brassica oleracea*) bud peroxidase-concavalin A complex and its application in dye color removal, Biocatal. Agric. Biotechnol. **2** (2013) 311–321.

[8]   K. Poonkuzhal, T. Palvannan, Comparison of biopolymers for immobilization of lacasse: Ecotoxicity assess-ment of azo dye, Indian J. Biotechnol. **12** (2013) 395–401.

[9]   A. Mugdha, M. Usha, Enzymatic treatment of waste-water containing dyestuffs using different delivery systems, Sci. Rev. Chem. Commun. **2** (2012) 31–40.

[10] A.M. Rashad, Metakaolin as cementitious material: History, scours, production and composition – A comprehensive overview, Constr. Build. Mat. **41** (2013) 303–318.

[11] G. Kakali, T. Perraki, S. Tsilvis, E. Badogiannis, Thermal treatment of kaolin: the effect on mineralogy on the pozzolanic activity, Appl. Clay Sci. **20** (2001) 73–80.

[12] B. Plešingerova, G. Sučik, M. Fabian, Surface area change of kaolin causing annealing, Acta Metallurg. Slov. **17** (2011) 169–176.

[13] H. Chen, Q. Zhang, Y. Dang, G. Shu, The effect of glutaraldehyde cross-linking on the enzyme activity of immobilized β-galactosidase on chitosan bed, Adv. J. Food. Sci. Technol. **5** (2013) 932–935.

[14] T. Cvetić, A. Sabovljević, J. B. Pristov, M. Sabovljević, M, Effects of day length on photosynthetic pigments and antioxidative metabolism of *in vitro* cultured moss *Atrichum undulatum* (Hedw.) P. Beauv. (*Bryophyta*), Bot. Serb. **33** (2009) 83–88.

[15] E.F. Hartree, Determination of protein: A modification of the Lowry method that gives a linear photometric response, Anal. Biochem. **48** (1972) 422–427.

[16] N. Šekuljica, N. Prlainović, A. Stefanović, M. Žuža, D. Čičkarić, D. Mijin, Z. Knežević-Jugović: Scientiffic World J. (2015), Article ID 371625.

[17] J. Zhu, G. Sun, Lipase immobilization on glutaraldehyde-activated nanofibrous membranes for improved enzyme stabilities and activities, React. Funct. Polym. **72** (2012) 839–845.

[18] H.J. Chae, E.Y. Kim, Man-Jin In, Improved immobilization yields by addition of protecting agents in glutaraledhyde induced immobilization of protease, J. Biosci. Bioeng. **89** (2000) 377–379.

[19] J.L. House, E.M. Anderson, W.K. Ward, Immobilization techniques to avoid enzyme loss from oxidase-based biosensors: A one year study, J. Diab. Sci. Technol. **1** (2007) 18–27.

[20] A. Sethuraman, G. Belfort, Protein structural perturbation and aggregation on homogenous surfaces, Biophys. J. **88** (2005) 1322–1333.

[21] A. Sethuraman, G. Vendanthan, T. Imoto, T. Przybycien, G. Belfort, Protein unfolding and interfaces: Slow dinamics of alpha-helix to beta-sheet transition, Proteins **56** (2004) 669–678.

[22] E. Laurenti, E. Ghibaudi, S. Ardissone, R.P. Ferrari, Oxidation of 2,4-dichlorophenol catalyzed by horseradish peroxidase: characterization of the reaction mechanism by UV-visible spectroscopy and mass spectrometry, J. Inorg. Biochem. **95** (2003) 171–176.

[23] A. Cordoba, I. Magario, M.L. Ferreira, Evaluation of hematin-catalyzed Orange II degradation as a potential alternative to horseradish peroxidase, Inter. Biodeteor. Biodegr. **73** (2013) 60–72.

[24] T. Thongsook, D.M. Barrett, Purification and partial characterization of broccoli (*Brassica oleracea Var. Italica*) peroxidases, J. Agric. Food. Chem. **53** (2005) 3206–3214.

[25] S.M.A.G.U. de Souza, E. Forgiarini, A.A.U. de Souza, Toxicity of textile dyes and their degradation by the enzyme horseradish peroxidase (HRP), J. Hazard. Mater. **147** (2007) 1073–1078.

[26] K. Knutson, S. Kirzan, A. Ragauskas, Enzymatic bio-bleaching of two recalcitrant paper dyes with horse-radish and soybean peroxidase, Biotechnol. Lett. **27** (2005) 753–758.

[27] S.V. Mohan, K.K. Prasad, N.C. Rao, P.P. Sarma, Acid azo dye degradation by free and immobilized horseradish peroxidase (HRP) catalyzed process, Chemosphere **58** (2005) 1097–1105.

[28] Z. Karim, R. Adnan, Q. Husain, A β-cyclodextrin-chitosan complex as the immobilization matrix for horseradish peroxidase and its application for the removal of azo dyes from textile effluent, Int. Biodeterior. Biodegrad. **72** (2012) 10–17.

[29] T.K. Das, S. Mazumdar, S. Mitra, Heme CD as a probe for monitoring local structural changes in heme-proteins: Alkaline transition in heme proteins, Proc. Indian Acad. Sci. (Chem. Sci.) **107** (1995) 497–503.

[30] A. Mandan, S. Modi, A. Saxena, D.V. Behere, S. Mitra, Nature of distal amino acid residue responsible for acid-alkaline transition in HRP: Circular dichroism studies, Proc. Indian Acad. Sci. (Chem. Sci.) **102** (1990) 431–503.

[31] M. Celebi, M. Altikatoglu, Z.M. Akdeste, H. Yildrim, Determination of decolorization properties of Reactive Blue 19 using horseradish peroxidase enzyme, Turkish J. Biochem. **37** (2012) 200–206.

[32] S. Akhtar, Q. Husain, Potential applications of immo-bilized bitter gourd (*Momordica charantia*) peroxidase in the removal of phenols from polluted water, Chemo-sphere **65** (2006) 1228–1235.

[33] M.Z. Alam, M.F. Mansor, K.C. Jalal, Optimization of decolorization of methylene blue by lignin peroxidase produced from sewage sludge with *Phanerocheate chryosporium*, J. Hazard. Mater. **162** (2009) 708–715.

[34] G. Yu, X. Wen, R. Li, Y. Qian, In vitro degradation of a reactive azo dye by crude lignolytic enzymes from nonimmersed culture of *Phanerocheate chryosporium*, Process Biochem. **41** (2006) 1987–1993.

[35] A.N.P. Hiner, E.L. Raven, R.N.F. Thorneley, F. Garcia-Canovas, J.N. Rodrigez-Lopez, Mechanisms of com-pound I formation in heme peroxidases, J. Inorg. Chem. **91** (2002) 27–34.

[36] F. Deyhimi, F. Nami, Peroxidase-Catalyzed Electrochem-ical Assay of Hydrogen Peroxide: A Ping-Pong Mech-anism, Int. J. Chem. Kinet. **44** (2012) 699–704.

[37] U. Bandyopadhyay, S. Adak, R.K. Banerjee, Role of active site residues in peroxidase catalysis: Studies on horse-radish peroxidase, PINSA **B65** (1999) 315–330.

[38] Y. Jang, W. Tang, J. Gao, L. Zhou, Y. He, Immobilization of horseradish peroxidase in phospholipid-templated titania and its application in phenolic compounds and dye removal, Enzyme Microb. Technol. **55** (2014) 1–6.

[39] M. Guo, L. Fuping, L. Minyao, L. Tuoping, P. Jun, N. Wang, P. Liang, C. Zhang, Purification of recombinant lacasse from *Trametes Versicolor* in *Pichia methanolica* and its use for the decolorization of anthraquinone dye, Biotechnol. Lett. **30** (2008) 2091–2096.

IZVOD

IMOBILIZACIJA PEROKSIDAZE IZ RENA GLUTARALDEHIDOM NA KAOLIN I PRIMENA U DEKOLORIZACIJI ANTRAHINONSKIH BOJA

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Očuvanje životne sredine, predstavlja jedan od vodećih izazova s obzirom na količinu otpadnih voda koje se svakodnevno generišu. Opšte je poznato da je voda jedan od polaznih uzroka mnogih bolesti, te je stoga akcenat stavljen na razvoj ekološki prihvatljivih, ali i održivih metoda prečišćavanja. Umesto tradicionalnih metoda prečišćavanja koje ne mogu da se primene na širok spektar jedinjenja, čijom primenom nastaje sekundarni problem zagađenja u obliku aktivnog mulja, koriste se enzimi. U ovom radu pažnja je usmerena na primenu peroksidaze biljnog porekla u dekolorizaciji sintetičkih boja. Sintetičke boje se primenjuju u svim granama industrije (kozmetičkoj, prehrambenoj, farmaceutskoj, tekstilnoj) koje zahtevaju velike količine procesne vode, koja napušta proizvodni proces kao otpadna voda bogata bojama, površinski aktivnim materijama, deterdžentima, teškim metalima itd. Otpadna voda sadrži sintetičke boje koje su toksične, kancerogene i mutagene i u cilju zaštite vodenih sistema i čoveka neophodno je adekvatno prečišćavanje pre ispuštanja u vodotokove. U ovom radu je ispitana mogućnost imobilizacije peroksidaze iz rena na prirodnu glinu, kaolin, koja je široko rasprostranjena u zemljištu, kako bi se dobio biokatalizator sa potencijalnom primenom u dekolorizaciji sintetičkih boja. Pre samog postupka imobilizacije, kaolin je obrađen termički (2 h, 550 °C) kako bi se izvršila fazna transformacija i dobio znatno reaktivniji nosač. Metakaolin, nastao termičkom obradom kaolina je tretiran različitim početnim koncentracijama glutaraldehida, kako bi se ispitao uticaj koncentracije glutaraldehida na masu vezanih proteina i aktivnost dobijenog imobilizata. Nakon optimizacije koncentracije glutaraldehida, ispitan je uticaj koncentracije enzima na masu vezanih proteina i aktivnost. Nakon imobilizacije, ispitana je potencijalna primena biokatalizatora u dekolorizaciji sintetičke boje AV 109. Dobijeni biokatalizator se pokazao znatno efikasnijim u pogledu stabilnosti u sredinamarazličitih pH, naročito alkalnoj; znatno je manje osetljiv na dejstvo inhibirajućih supstrata, vodonik-peroksida i boje. Pored toga, ispitivanjem reciklacije imobilisane peroksidaze, utvrđeno je da je ovaj biokatalizator moguće primeniti i u kontinualnom režimu.

Ključne reči: Peroksidaza iz rena • Imobilizacija • Otpadna voda • Boje