J. Microbiol. Biotechnol. (2017), 27(4), 768–774
https://doi.org/10.4014/jmb.1606.06002

jmb

2,4-Dichlorophenol Enzymatic Removal and Its Kinetic Study Using Horseradish Peroxidase Crosslinked to Nano Spray-Dried Poly(Lactic-Co-Glycolic Acid) Fine Particles

Laura Amina Dahili[1], Endre Nagy[1], and Tivadar Feczkó[1,2,3]*

[1]*Doctoral School of Molecular and Nanotechnologies, Research Institute of Chemical and Process Engineering, University of Pannonia, H-8200 Veszprém, Hungary*
[2]*Department of Medicine 1, Goethe University Frankfurt, 60590 Frankfurt am Main, Germany*
[3]*Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences, H-1117 Budapest, Hungary*

Received: January 2, 2017
Revised: February 2, 2017
Accepted: February 2, 2017

First published online
February 7, 2017

**Corresponding author*
Phone: +49-69-6301-87667;
Fax: +49-69-6301-6301;
E-mail: tivadar.feczko@gmail.com

pISSN 1017-7825, eISSN 1738-8872

Horseradish peroxidase (HRP) catalyzes the oxidation of aromatic compounds by hydrogen peroxide via insoluble polymer formation, which can be precipitated from the wastewater. For HRP immobilization, poly(lactic-co-glycolic acid) (PLGA) fine carrier supports were produced by using the Nano Spray Dryer B-90. Immobilized HRP was used to remove the persistent 2,4-dichlorophenol from model wastewater. Both extracted (9–16 U/g) and purified HRP (11–25 U/g) retained their activity to a high extent after crosslinking to the PLGA particles. The immobilized enzyme activity was substantially higher in both the acidic and the alkaline pH regions compared with the free enzyme. Optimally, 98% of the 2,4-dichlorophenol could be eliminated using immobilized HRP due to catalytic removal and partly to adsorption on the carrier supports. Immobilized enzyme kinetics for 2,4-dichlorophenol elimination was studied for the first time, and it could be concluded that competitive product inhibition took place.

Keywords: Horseradish peroxidase, immobilization, 2,4-dichlorophenol substrate, enzyme kinetics

Introduction

2,4-Dichlorophenol (2,4-DCP) is a persistent intermediate of 2,4-dichlorophenoxyacetic acid herbicide [1]. Chlorophenol toxicity has been proven both in vitro and in vivo. Oral, percutaneous, and intraperitoneal LD_{50} values in rats were found to be 580–4,000, 2,000, and 430 ppm, respectively [2].

For chlorophenol removal, chemical and biocatalyzed methods are generally applied. However, these methods possess drawbacks, such as low efficiency and high cost. Moreover, the energy and source efficiency require the development of environmentally friendly mild processes [3]. An alternative for treating chlorophenol-containing wastewaters is based on the oxidation of 2,4-DCP by pure enzymes. It includes many advantages compared with other methods, such as the easier handling and storing of enzyme, and furthermore, the conventional methods are not selective [4].

Horseradish peroxidase (HRP) is widely utilized in life sciences, including bioassays, DNA probes, and biosensors. HRP catalyzes the oxidation of aromatic compounds by hydrogen peroxide or alkyl hydroperoxide. Via the formation of the corresponding radicals, they spontaneously react and insoluble polymers rapidly form, which can then be easily precipitated from the wastewater. It was applied in the removal of toxic phenol derivatives from wastewater [5, 6].

The protection of commercial enzyme stability is not an easy task. Water accelerates the degradation of enzymes, and thus, their drying can increase the shelf-life. Dehydration is a stress that also reduces the enzyme activity [7]. Enzyme immobilization onto solid supports provides several advantages, such as reusability, easy separation of the

enzyme from products, rapid stopping of reactions, a greater variety of bioreactor design, and improvement of the enzyme's stability [8, 9] and efficiency [10]. On immobilization, enzymes are often stabilized, and hence less sensitive to denaturing agents. Very recently, the stability of laccase [11] and sorbitol dehydrogenase [12] was extremely increased after immobilizing them onto Fe_2O_3 supports. All these advantages allow the economical improvement of enzymatic processes. Mainly, the following methodologies for enzyme immobilization have been reported: via binding to or encapsulation in an inorganic or organic polymer, or by crosslinking them to a support [13]. The "enzyme grafting onto polymeric nanoparticles approach" became significant because of the achievable large surface-to-volume ratio.

The spray drying method is extensively applied to gain powders from liquids. Patel *et al*. [14] prepared Fe_2O_3 yolk-shell particles using the spray drying method and immobilized enzymes such as HRP, glucose oxidase, and laccase with extremely high enzyme loading (292–398 mg/g), and increased their stability substantially. The Nano Spray Dryer (Büchi B-90) produces smaller particles than conventional spray dryers by a piezoelectrically driven vibrating-mesh atomizer, and is capable of capturing them efficiently using an electrostatic collector [15].

In the present paper, both purified and crude HRP were attached to nano spray dried PLGA supports, and furthermore we investigated their capability of 2,4-DCP removal. The size, the HRP content, the pH working range of the immobilized purified as well as crude enzymes, and the 2,4-DCP removal efficiency were studied. To the best of our knowledge the kinetics of 2,4-DCP elimination by immobilized HRP has not been evaluated so far, and thus, we made an attempt to estimate the enzyme kinetics.

Materials and Methods

Materials

Purified HRP lyophilisate (223 U/mg) was purchased from Amresco (USA) and stored at –20°C. HRP activity was determined by the producer via the conversion of pyrogallol to purpurogallin. Poly(lactic-co-glycolic acid) (PLGA; 50:50, Mw = 8,000; Resomer RG 502H) containing free carboxyl end-groups was obtained from Boehringer Ingelheim, Germany. Dichloromethane (DCM), acetonitrile (HPLC grade), and guaiacol were purchased from Scharlab (Hungary) and Cayman Chemical Company (USA), respectively. The 30% hydrogen peroxide was obtained from VWR International LLC (Hungary). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) (~98%), *N*-hydroxysuccinimide (NHS) (98%), catalase from bovine liver (2,000–5,000 units/mg), and the Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich (Hungary). 2,4-DCP was bought from Fluka (Hungary).

HRP Extraction

HRP was successfully extracted from young horseradish plants harvested in autumn. The roots were cleaned in cold water to eliminate soil and other contaminations. After cutting and grinding the horseradish with a centrifugal juicer machine, the crude extract was ultracentrifuged (Sorvall Discovery 90SE) at 158,000 ×*g* for 40 min to separate the supernatant from the sediment containing pulp pieces. Supernatant containing crude HRP extract was separated and it was kept in a freezer at –20°C for further usage.

Carrier Preparation by Nano Spray Dryer B-90

Fine carrier particles were prepared by a Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Switzerland) [16]. The 1% (w/v) PLGA in DCM was spray dried by nitrogen gas with different spray nozzles of cutoff 7, 5.5, and 4 μm, respectively. The device was operated in closed-mode configuration with the short version of the drying chamber, which is appropriate for organic solvents considering the short time of evaporation. The piezoelectric actuator was driven at 60 kHz ultrasonic frequency. The flow and spray rates were 100 l/min and 0.2 l/h (100%), respectively.

Size and Morphology Analyses

PLGA particles were dispersed in distilled water (1 mg/ml) by sonication at amplitude 40% for 2 min (Vibra-Cell VCX-130; Sonics, USA), and then the size distribution was measured by a laser diffraction method (Mastersizer 2000; Malvern Instruments, UK). The morphology of spray dried PLGA particles was analyzed by scanning electron microscopy (SEM). Before imaging, the samples were covered with gold and palladium under vacuum for 3 min and screened with a Philips XL-30 Environmental Scanning Electron Microscope (ESEM) at 25 kV.

HRP Immobilization

HRP enzyme was crosslinked to nano spray-dried carrier particles after the activation of the support with EDC and NHS reagents according to the recently optimized conditions [16]. To verify the successful immobilization qualitatively, FTIR spectra were taken by a Varian Scimitar FTS2000 spectrometer (64 scans, 4 cm^{-1} resolution) equipped with a liquid nitrogen-cooled MCT detector and Pike GladiATR (with germanium micro-ATR element) accessory. The total protein concentration of purified HRP crosslinked on PLGA was quantified by the Lowry-Folin method [17].

Activity Measurement

Guaiacol chromophore (2 mM) and 1 mM hydrogen peroxide (H_2O_2) substrates were added to 1 mg/ml free or to carriers-attached HRP in order to measure the activity of the enzyme. The color change was determined by a spectrophotometer (Pharmacia LKB-Biochrom, USA) at 470 nm after 2 min, and the absorbance values were corrected with that measured before the reaction in

order to exclude the particle light scattering. The pH dependence of HRP stability was investigated in the pH range of 4.0–10.0 (0.1 M citrate: pH 4.0–6.0; Tris: pH 7.0–9.0; and 0.1 M sodium carbonate: pH 10) buffers.

2,4-Dichlorophenol Removal and Enzyme Reusability

Specified amounts of 2,4-DCP were dissolved in distilled water, and then the immobilized enzyme and H_2O_2 were added and agitated at room temperature for 2 h. Then, the samples were centrifuged at 18,300 ×*g* for 20 min. The substrate content was studied by HPLC (Hewlett Packard, series 1050, USA) applying a 150 mm × 4 mm reverse phase column (particle size 5 µm, C18 BDS HYPERSIL; Thermo Scientific, USA) and operating with a UV–vis detector at 283 nm. The mobile phase was composed of a 50/50 (v/v). mixture of acetonitrile and 10 mM phosphoric acid.

Demonstrating the reusability of immobilized enzyme, the PLGA particles containing HRP were easily separated from the reaction mixture, whereas the free enzyme could not be reused. The reusability was studied as a function of substrate concentration. 2,4-DCP (1.4 and 0.7 mM) and 10 mg/ml extracted HRP crosslinked to PLGA particles were stirred at room temperature, and H_2O_2 was added to initiate the removal as described above. After each cycle, the suspensions were ultracentrifuged with 158,000 ×*g* for 15 min to remove the supernatant for the investigation of the removal efficiency by HPLC. The remaining particles were washed with Tris buffer solution (0.02 M, pH = 6) and reused. Control samples without HRP and/or H_2O_2 were examined through three cycles.

Enzyme Kinetics

The reaction mixture was defined as a solution containing 0.74–4.42 mM 2,4-DCP in Tris buffer (pH 6.0) and was prepared shortly before use. The immobilized catalyst and the H_2O_2 concentrations were 10 mg/ml (20.4 U/g) and 2 mM, respectively. The reaction mixture was stirred with a magnetic stirrer at room temperature, and the reaction was started by adding H_2O_2 to the mixture. In predetermined time points, an aliquot of 1 ml was taken and added to 1 ml of catalase solution (1 mg/ml) to immediately stop the reaction. These dispersions were centrifuged at 18,300 ×*g* for 20 min at 10°C. The 2,4-DCP concentration of samples was quantified by HPLC as described above.

The effect of 2,4-DCP concentration on the initial activity of immobilized HRP was studied to determine the kinetic parameters. The apparent values (Michaelis constant, $K_{m,}$ and maximum reaction rate, V_{max}) were determined directly from the Michaelis–Menten model Eq. (1) by means of the graphical method using the starting reaction rate data, where V is the reaction rate at the beginning of the process, and S denotes the substrate concentration. Then, nonlinear regression analysis was applied to determine the reaction inhibition, using the K_m and V_{max} values obtained by the graphical method.

$$V = \frac{V_{max}S}{K_m + S} \quad (1)$$

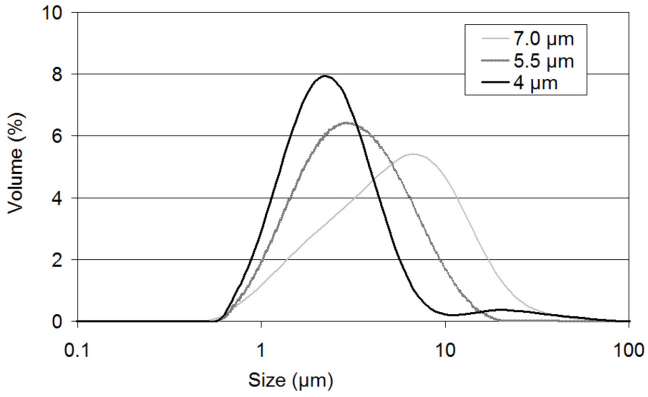

Fig. 1. Particle size distribution of nano spray-dried poly(lactic co-glycolic acid) particles.
The carrier particles were prepared by a Nano Spray Dryer B-90 using 7, 5.5, and 4 µm spray caps. The size distributions were determined by the laser diffraction method (Mastersizer 2000; Malvern Instruments, UK).

Results and Discussion

Particle Size Distribution and Morphology of Carrier Particles

The size analysis of produced particles was investigated after the spray-drying process by the laser diffraction method. The average particle sizes were expressed in equivalent volume mean diameters (D [4. 3]). The d (0.1), d (0.5), and d (0.9) values mark the sizes that correspond to 10%, 50%, and 90%, respectively, of the particles. The size of the generated particles was mainly influenced by the applied nozzle hole size during the spray drying process (Fig. 1). The smaller the spray cap hole size, the higher surface-to-volume ratio can be achieved, and consequently the amount of attached enzyme is enhanced. Particles generated by the 7 µm spray cap hole size showed a wider size distribution with a diameter of 1 to 16 µm. However, the majority of PLGA particles were between 1 and 8 µm by using 5.5 and 4 µm spray caps (Table 1).

The morphology of PLGA particles was analyzed by SEM (Fig. 2). The particles represented regular spherical shape, with a smooth surface, using any of the spray caps.

Table 1. Volume mean diameters (D [4. 3]) and d (0.1), d (0.5), d (0.9)) values of nano spray-dried poly(lactic co-glycolic acid) carriers using spray cap hole sizes 7, 5.5, and 4 µm.

Spray cap hole size	D [4.3] (µm)	d (0.1) (µm)	d (0.5) (µm)	d (0.9) (µm)
7.0 µm	7.5	1.7	5.7	15.2
5.5 µm	4.1	1.4	3.2	7.9
4 µm	3.8	1.2	2.4	5.4

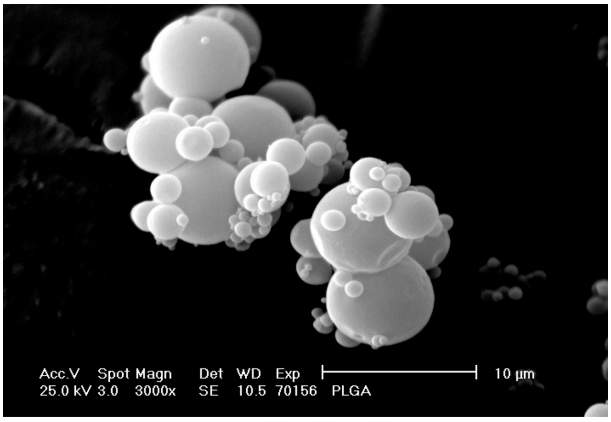

Fig. 2. Nano spray-dried poly(lactic co-glycolic acid) particles imaged by SEM using spray cap hole size 5.5 µm with 3,000× magnification.
SEM images were taken with a Philips XL-30 Environmental Scanning Electron Microscope.

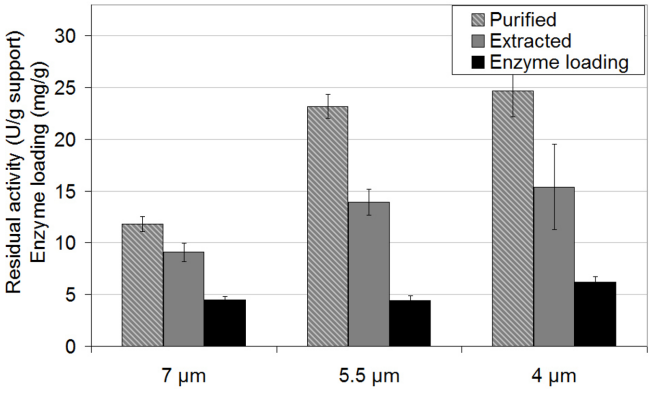

Fig. 4. Residual activity (purified and extracted) of horseradish peroxidase crosslinked onto poly(lactic co-glycolic acid) nano spray-dried by spray caps of size 7, 5.5, and 4 µm.
Gray bars represent residual activity (U/g support), black bars denotes enzyme loading (mg/g carrier). Error bars indicate the standard deviations of three parallel measurements.

HRP Immobilization on PLGA Supports

The PLGA particles were spray dried with three different spray cap hole sizes (7, 5.5, and 4 µm). Both extracted and purified HRPs were covalently attached to the PLGA particles by EDC crosslinker. The HRP attachment to the PLGA particles via amide bonds can be observed in the FTIR spectrum (Fig. 3).

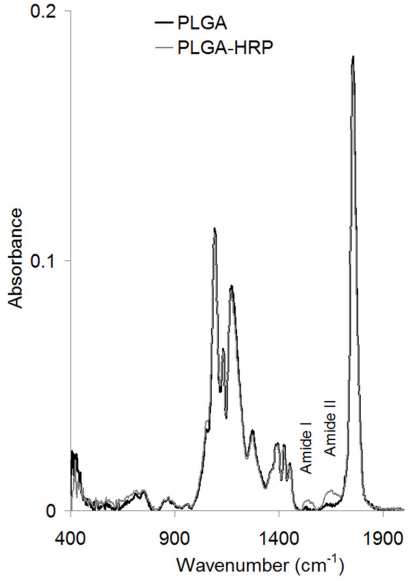

Fig. 3. FTIR of poly(lactic co-glycolic acid) (PLGA) particles and horseradish peroxidase (HRP) crosslinked to PLGA carriers (PLGA-HRP).
FTIR spectra were obtained by a Varian Scimitar FTS2000 spectrometer.

The purified enzyme preserved higher residual activity compared with the extracted one after the immobilization (Fig. 4), similar to our recent results found with ethyl cellulose-HRP particles [18]. The residual activity of HRP was proportionally increased using the smaller spray cap hole size, due to the smaller surface-to-volume ratio. The bigger particles with lower surface area resulted in decreased residual activity in accordance with the size measurements. The residual activity of extracted HRP attached to PLGA ranged from 9 to 16 U/g and that of purified enzyme was between 11 and 25 U/g. The measured activity of immobilized purified HRP was similar to that attached to ethyl cellulose, found in our recent work [18], except for the PLGA carriers of 5.5 mm, in which it was about double than that determined for ethyl cellulose supports. The performance of crosslinked extracted HRP was significantly higher in all of the PLGA batches.

The immobilized total protein mass was investigated by Lowry-Folin assay. As expected, the enzyme loading was enhanced by reducing the spray cap hole size (Fig. 4); that is, the highest enzyme loading (6.2 mg/g) was achieved by the smallest spray cap. These values did not show significant variance in comparison with the ones measured in HRP-ethyl cellulose composites [18]. This finding, combined with the fact that HRP generally kept a more substantial part of its activity after crosslinking, suggests that the HRP attached to the carboxylic groups of PLGA results in a more favorable composite than the HRP bound to the hydroxyl groups of ethyl cellulose.

pH Working Range

An optimal range of enzyme operation is an important issue in order to avoid protein denaturation. The HRP working stability was investigated at the pH range of 4.0–10.0. The carrier particle size did not show correlation with the enzyme activity as a function of pH. The optimum pH remained at the same value as that for the free enzyme (pH 6) for both purified and extracted HRP after crosslinking onto PLGA supports (Fig. 5). The enzyme activity was slightly improved by immobilization of the extracted HRP throughout the studied pH range, and this improvement was more substantial using purified enzyme.

2,4-Dichlorophenol Removal

In our recent work [18], we found that the optimal H_2O_2 concentration was 1 mM in the carrier-supported catalytic activity. The substrate concentration is also a relevant factor, which can inhibit the elimination reaction. Evidently, the higher the 2,4-DCP concentration, the lower the efficiency (Fig. 6). Optimally, 98% of the 2,4-DCP could be removed by immobilized HRP at a substrate concentration of 1.47 mM. The removal efficiency was investigated as a function of time at five different concentrations (Fig. 6), and these data were used for the kinetics analysis. At 2,4-DCP concentrations of 0.74 and 1.10 mM, the elimination was close to 100% at the end of the reaction, whereas at increasing concentrations, it reduced significantly. Huang *et al*. [19] reached similarly high removal efficiency using HRP and graphene oxide/Fe_3O_4 catalysts simultaneously; 2,4-DCP (50 mg/l) in the presence of 0.7 mmol/l H_2O_2. HRP immobilized on Fe_3O_4/SiO_2 magnetic nanoparticles performed a maximum 80% removal of 0.2 mM 2,4-DCP [10].

The loss of activity is generally associated with a rigid conformation of the enzyme molecule after covalent binding, which leads to stereochemical hindrance in the formation of the enzyme-substrate complex [20]. The low removal efficiency of biocatalysts is also attributed to the interaction between the forming phenoxy radicals and enzyme active site [21].

As one of the most important benefits of immobilized enzyme, its reusability was evaluated. HRP crosslinked to PLGA particles was reusable up to four cycles, with higher than 30% efficiency at a substrate concentration 1.47 mM (Fig. 7). Nevertheless, by decreasing the initial 2,4-DCP concentration from 1.47 to 0.74 mM, the HRP removal efficiency could be retained above 30% for nine repeated uses. Chang and Tang [10] found significantly higher removal efficiency (65%) after four uses, but nevertheless at substantially lower substrate concentration (0.2 mM). It might be attributed to the accumulation of the reaction products of the elimination reaction, which may cover the enzyme and affect the removal capacity of HRP in further cycles.

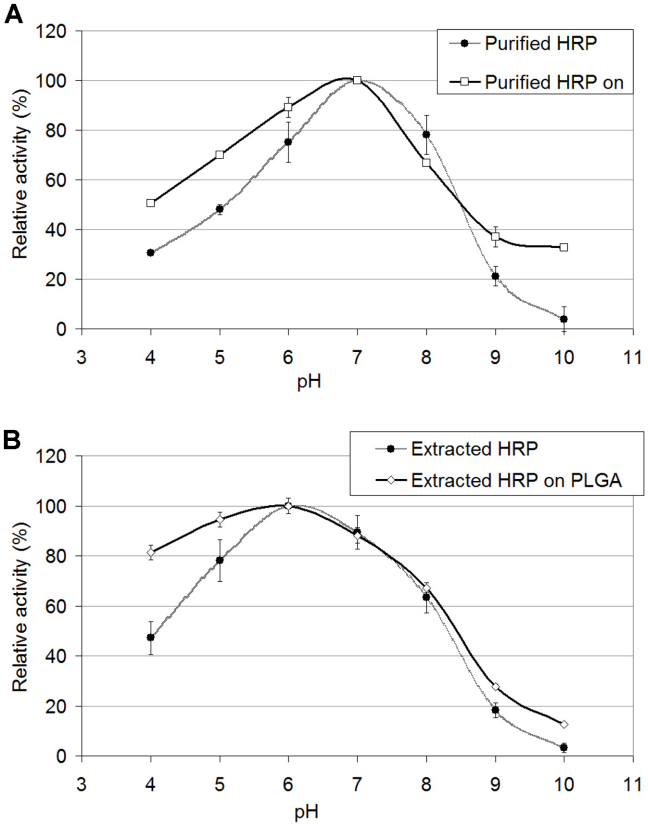

Fig. 5. pH dependence of purified (**A**) and extracted (**B**) horseradish peroxidase (HRP).
The pH was adjusted using 0.1 M citrate (pH 4.0–6.0), 0.02 M Tris (pH 7.0–9.0), and 0.1 M sodium carbonate (pH 10.0) buffers. Error bars indicate the standard deviations of three parallel measurements.

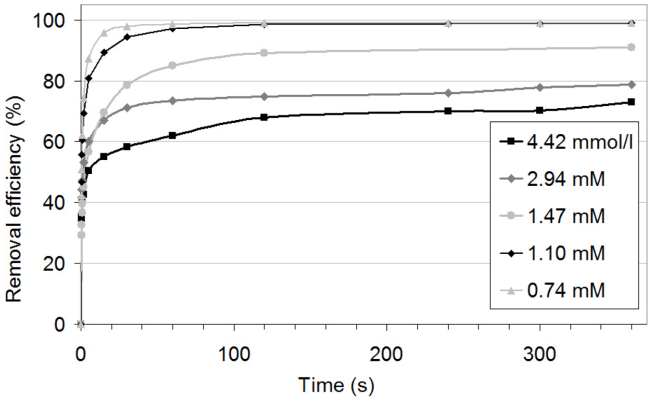

Fig. 6. Time plots of 2,4-dichlorophenol removal efficiency.
The initial substrate concentrations varied in the range of 0.74–4.42 mM.

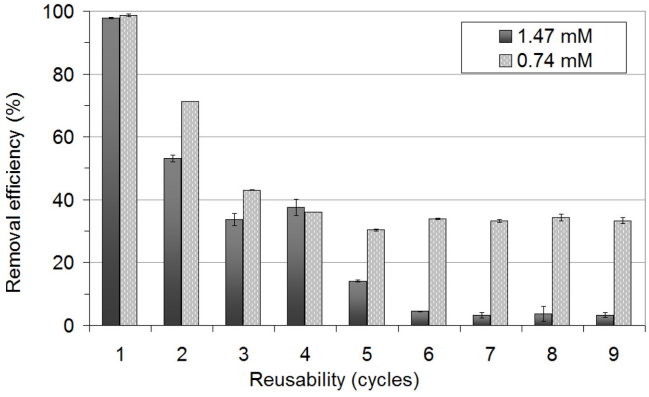

Fig. 7. Reusability of horseradish peroxidase (HRP) immobilized on poly(lactic co-glycolic acid) (PLGA) particles.
The initial concentrations of 2,4-dichlorophenol were 1.4 and 0.7 mM. Extracted HRP (10 mg/ml) crosslinked to PLGA particles was applied. Error bars indicate the standard deviations of three parallel measurements.

Control samples that did not include HRP enzyme were also studied in order to determine the substrate adsorption of the PLGA particles. The results showed that the neat PLGA particles are able to adsorb a significant amount of 2,4-DCP (35–40%). Comparing with our recent results obtained for HRP immobilized on ethyl cellulose fine carriers [18], although the removal efficiency was found to be lower with enzyme attached to PLGA particles from the second reuse, the more substantial ratio of substrate elimination was attributed to the adsorption with ethyl cellulose supports.

Kinetics of Immobilized HRP

In a particulate catalytic system, one has to study whether the diffusion inside the catalytic particle can affect the conversion. At an approximate average particle size of 5 μm, the diffusion time can be predicted from the following Eq. (2):

$$t_{\mathrm{diff}} = \frac{R^2}{D} = \frac{(5\times10^{-6}\,\mathrm{m})^2}{1.6\times10^{-9}\,\mathrm{m^2/s}} = 15.6\times10^{-3}\,\mathrm{s} = 0.0156\,\mathrm{s} \quad (2)$$

That means that the internal diffusion will not substantially influence the biochemical reaction. Another important point is the effect of the external mass transfer resistance. In a well-mixed phase, the thickness of the boundary layer is about 20–40 μm. Accordingly, the external resistance can have a negative effect on the process exclusively at the starting time point, which is negligible.

For an enzyme-catalyzed process, the basic parameters can be determined from the Lineweaver-Burk graphical method [22]. The reaction rate was determined by calculating its starting value as a function of the inlet substrate concentration and $1/V$ vs. $1/S$ for 2 min. The slope of the straight line provides the value of K_m/V_{max}, while the intersection point gives the V_{max}. Thus, the kinetic parameters were found to be V_{max} = 33.3 mM/min and K_m = 14 mM.

In order to check the predicted results, comparing with that of the measured ones, the following differential equation is to be solved:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -V \quad (3)$$

We have recalculated the substrate concentration change in time using the kinetic constant obtained by the Lineaweaver-Burk equation (V_{max}, K_m) including an additional inhibition constant, and the predicted concentration data were compared with the measured ones. It was found that the calculated data gave a much higher product concentration than the measured data as a function of time. This tendency was valid for the other inlet substrate concentrations as well. That is why we recalculated the kinetic parameters applying the potential inhibition kinetics; namely, competitive, uncompetitive, or non-competitive product inhibition, or mixed inhibition [22]. In our system the equation of competitive product inhibition approximated the measured values the most comprehensively. The following kinetic parameters were obtained: V_{max} = 33.3 mM/min, K_m = 14 mM, and K_p = 0.5 mM, in which K_p means the inhibition constant determined by the product. The calculated conversion data in the first 1 min harmonized with the measured ones, but that of the second minutes differed tendentiously. This tendency was also valid for the other inlet substrate concentrations. The conversion data increased in the second half of this time period, which means additional inhibition occurs probably due to the decreasing H_2O_2 concentration. However, we did not investigate the kinetics at higher H_2O_2 concentration because of the risk of suicide peroxide inactivation. Nevertheless, in future studies, it will be an important task to clarify the reason for the further inhibition of the catalytic process.

In summary, spherical poly(lactic-co-glycolic acid) carriers were generated by Nano Spray Dryer B-90, and HRP was attached to the fine supports. Decreasing the hole size of the spray caps (7, 5.5, and 4 μm) resulted in higher activity of the enzyme covalently fixed to the supports, due to the increasing surface-to-volume ratio. The activity of the immobilized enzyme was enhanced at both acidic and alkaline pH compared with the free enzyme. The substrate removal and enzyme reusability experiments showed that

the fixed enzyme is a promising tool for HRP-catalyzed removal of 2,4-DCP. The substrate was efficiently eliminated with a maximum of 98.0%, and most of the removal capacity was attributed to the catalytic effect of the enzyme, in contrast to our recent composite composed of HRP immobilized on ethyl cellulose; nevertheless, the adsorption of the PLGA-HRP fine particles played a role as well. Reusability of the immobilized enzyme was high until three batch cycles. To our best knowledge, HRP immobilized on a PLGA carrier has been used for the first time for efficient removal of a persistant chlorophenol. Nevertheless, a more important novelty of this work is the study of immobilized enzyme kinetics for 2,4-DCP elimination. The enzyme kinetic study denoted competitive product inhibition and probable hindrance by the reduction of the second substrate (H_2O_2) concentration.

Acknowledgments

The Authors are grateful to László Hajba for the FTIR measurements, and acknowledge Ilona Kelemen-Horváth's help in the HPLC analysis.

References

1. Laurenti E, Ghibaudi E, Ardissone S, Ferrari RP. 2003. Oxidation of 2,4-dichlorophenol catalysed by horseradish peroxidase: characterization of the reaction mechanism by UV-visible spectroscopy and mass spectrometry. *J. Inorg. Biochem*. **95:** 171-176.
2. Law MP, Law SW. 2002. Hazard assessment of 2,4-dichlorophenol. Available from http:// www.meti.go.jp/ english/report/downloadfiles/gED0307e.pdf. Accessed on November 5, 2016.
3. Perazzini R, Saladino R, Guazzaroni M, Crestini C. 2011. A novel and efficient oxidative functionalization of lignin by layer-by-layer immobilised horseradish peroxidise. *Bioorg. Med. Chem*. **19:** 440-447.
4. Caramori SS, Fernandes KF. 2004. Covalent immobilisation of horseradish peroxidase onto poly (ethylene terephthalate)–poly(aniline) composite. *Process Biochem*. **39:** 883-888.
5. Song HY, Liu JZ, Weng LP, Ji LN. 2009. Activity, stability, and unfolding of reconstituted horseradish peroxidase with modified heme. *J. Mol. Catal. B Enzym.* **57:** 48-54.
6. Zhang T, Xu XL, Jin YN, Wu J, Xu ZK. 2014. Immobilization of horseradish peroxidase (HRP) on polyimide nanofibers blending with carbon nanotubes. *J. Mol. Catal. B Enzym.* **106:** 56-62.
7. Namaldi A, Calik P, Uludag Y. 2006. Effects of spray drying temperature and additives on the stability of serine alkaline protease powders. *Dry Technol.* **24:** 1495-1500.
8. Jiang Y, Wu Y, Li H. 2015. Immobilization of *Thermomyces lanuginosus* xylanase on aluminum hydroxide particles through adsorption: characterization of immobilized enzyme. *J. Microbiol. Biotechnol.* **25:** 2016-2023.
9. Jin L, Li Y, Ren XH, Lee JH. 2015. Immobilization of lactase onto various polymer nanofibers for enzyme stabilization and recycling. *J. Microbiol. Biotechnol.* **25:** 1291-1298.
10. Chang Q, Tang H. 2014. Immobilization of horseradish peroxidase on NH_2-modified magnetic Fe_3O_4/SiO_2 particles and its application in removal of 2,4-dichlorophenol. *Molecules* **19:** 15768-15782.
11. Patel SKS, Kalia VC, Choi JH, Haw JR, Kim IW, Lee JK. 2014. Immobilization of laccase on SiO_2 nanocarriers improves its stability and reusability. *J. Microbiol. Biotechnol.* **24**: 639-647.
12. Kim T-S, Patel SKS, Selvaraj C, Jung W-S, Pan C-H, Kang YC, Lee JK. 2016. A highly efficient sorbitol dehydrogenase from *Gluconobacter oxydans* G624 and improvement of its stability through immobilization. *Sci. Rep.* **6:** 33438.
13. Jiang Y, Tang W, Gao J, Zhou L, He Y. 2014. Immobilization of horseradish peroxidase in phospholipid-templated titania and its applications in phenolic compounds and dye removal. *Enzyme Microb. Technol*. **55:** 1-6.
14. Patel SKS, Choi S-H, Kang Y-C, Lee J-K. 2016. Large-scale aerosol-assisted synthesis of biofriendly Fe_2O_3 yolk-shell particles: a promising support for enzyme immobilization. *Nanoscale* **8:** 6728-6738.
15. Chan HK, Kwok PCL. 2011. Production methods for nanodrug particles using the bottom-up approach. *Adv. Drug Deliver. Rev.* **63:** 406-416.
16. Dahili LA, Feczkó T. 2015. Cross-linking of horseradish peroxidase enzyme to fine particles generated by nano spray dryer B-90. *Period. Polytech. Chem. Eng*. **59:** 209-214.
17. Patel SKS, Singh M, Kumar P, Purohit HJ, Kalia VC. 2012. Exploitation of defined bacterial cultures for production of hydrogen and polyhydroxybutyrate from pea-shells. *Biomass Bioenergy* **36**: 218-225.
18. Dahili LA, Kelemen-Horváth I, Feczkó T. 2015. 2,4-Dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horsesradish immobilized to nano spray dried ethyl cellulose particles. *Proc. Biochem.* **50:** 1835-1842.
19. Huang J, Chang Q, Ding Y, Han X, Tang H. 2014. Catalytic oxidative removal of 2,4-dichlorophenol by simultaneous use of horseradish peroxidase and graphene oxide/Fe_3O_4 as catalyst. *Chem. Eng. J.* **254:** 434-442.
20. Leiriao PRS, Fonseca LJP, Taipa MA, Cabral JMS, Mateus M. 2003. Horseradish peroxidase immobilized through its carboxylic groups onto a polyacrylonitrile membrane. *Appl. Biochem. Biotechnol*. **110:** 1-10.
21. Alemzadeh I, Nejati S. 2009. Phenols removal by immobilized horseradish peroxidase. *J. Hazard. Mater.* **166:** 1082-1086.
22. Degroot LJ. 1967. Stimulation and inhibition of thyroid iodinating enzyme systems. *Biochim. Biophys. Acta* **136:** 364-374.