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2,4-dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horseradish immobilized to nano spray dried ethyl cellulose particles

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Abstract

Horseradish peroxidase (HRP) is a promising catalyst in the enzymatic process of phenolic compounds removal from wastewaters. Enzyme immobilization provides important benefits in a biotechnological process. Fine particles with a high surface-to-volume ratio composed of ethyl cellulose (EC) polymer were generated by Nano Spray Dryer B-90 as supports for HRP. Carrier particles were prepared by spray caps with hole size of 7 μm, 5.5 μm and 4 μm. Purified HRP and crude extract from horseradish were covalently bound to the carrier using a carbodiimide cross-linker. The attached HRP content, the effect of pH on the activity and the storage stability were investigated. 2,4-dichlorophenol, an extremely persistent chlorinated phenol was removed by the immobilized enzyme, and the effect of main process parameters such as H_2O_2 and 2,4-dichlorophenol substrate concentrations were studied. After immobilization both the purified HRP and the horseradish extract performed better in the pH range of 4-10 and could preserve the activity substantially longer than the free enzyme. The immobilized enzyme was found to be outstandingly efficient (in optimal case close to 100 %) in the elimination of 2,4-dichlorophenol, which was also the consequence of the high adsorbing capacity of the fine particles. The reuse study proved the operational stability of HRP attached to EC even after 10 consecutive cycles.

Keywords

Horseradish peroxidase, ethyl cellulose carrier, nano spray drying, immobilization, 2,4-dichlorophenol removal

1. Introduction

Most of the phenolic compounds that are present in the waste streams of a wide variety of industrial operations are toxic and some are carcinogens. They get into the food chain and generate important environmental problems. Their removal involves the use of microorganisms, adsorption by active carbon, or chemical oxidation. However, these methods frequently have disadvantages, such as low efficiency, high cost or the generation of products which are even more toxic than the original ones. An alternative for treating wastewaters containing phenolic compounds is enzymatic treatment by peroxidase. In the presence of the peroxidase catalyst, phenols are oxidized to generate the corresponding radicals; the radicals spontaneously react to rapidly form insoluble polymeric phenolic aggregates [1].

An enzymatic process with horseradish peroxidase (HRP) for the removal of phenols from wastewaters was first described by Klibanov et al. [2]. It has already been shown that crude HRP is as effective as purified HRP in catalyzing phenol removal; the significant inactivation of HRP during the process results in the use of a large quantity of HRP to ensure efficient phenol removal [3]. However, the high-efficiency elimination of chlorinated phenols from wastewater is still a challenge.

Horseradish peroxidase is a heme-containing enzyme belonging to the class III of plant peroxidases. It has Fe(III) protoporphyrin IX as the prosthetic group, which plays an important role in its catalytic mechanism. Horseradish peroxidase possesses significant applications in life sciences, including bioassays, DNA-probes, biosensors, bioremediation of phenol and some of its derivatives [4]. However, its industrial application is greatly limited by its low thermostability and low reactivity in organic media. HRP is also prone to suicide inactivation by the H_2O_2 substrate in the applications such as diagnostics and biosensors as well as in wastewater treatment [5]. The relatively short lifetime of enzymes and their instability in harsh environment limit their applications. Thus, plenty of trials have been done on enzyme stabilization; such as entrapment of enzyme molecules in sol–gel, polymer matrixes and nanoporous materials, attachment of enzymes onto highly hydrophilic surfaces, e.g. chitosan, dextran and polyethylene glycol, and separation of enzyme layers from sample solutions using polymeric membranes and ion-exchange polyion membranes [4].

65 The main objective of enzyme immobilization is to maximize the advantages of enzyme catalysis [6]. An
66 important aspect of this is the possibility of reaction interruption by removing the immobilised enzyme,
67 controlling these systems over product formation, which is not possible, when the enzyme is dissolved in
68 the reaction mixture [7].
69 Conventional spray drying is not suitable for the production of submicron-sized particles, because they are
70 too small to be collected by the cyclone (cutoff diameter ~ 1–2 μm) of the spray dryer [8]. Recently, a new
71 lab-scale equipment has become available (Büchi Nano Spray Dryer B-90) which is capable of capturing
72 even submicron particles by an electrostatic collector. In addition, a piezoelectrically driven vibrating
73 mesh atomiser is employed, which allows the production of finer droplets (median diameter 1–7 μm, size
74 range 0.5 to 15 μm, depending on the mesh aperture size) with narrow span membranes [8]. Ethyl
75 cellulose (EC) is an ecofriendly polymer which, according to our knowledge, has not been used as a
76 support for HRP so far.
77 Fine microparticles with an additionally porous structure should allow even faster and more efficient
78 intraparticle processing as the presence of pores goes along with a higher surface-to-volume ratio, which
79 allows efficient access, especially of macromolecular reactants such as proteins which would encounter
80 serious diffusion limitation to enter densely structured microparticles [9]. Chang and Tang [10] prepared
81 Fe_3O_4 nanoparticles by a co-precipitation method and coated them with a silica layer as carrier for HRP.
82 The activity during the fixing of the enzyme did not decrease substantially, and the immobilized enzyme
83 was significantly more stable against heating and pH variation in comparison with the free HRP. The
84 maximum 2,4-dichlorophenol conversion efficiency was around 80 %, and the catalytic performance of
85 the immobilized enzyme was high even after 4 cycles. A composite of graphene oxide and nano Fe_3O_4 as
86 an artificial enzymatic catalyst combined with HRP provided an outstanding synergistic removal of 2,4-
87 dichlorophenol (93%) [11,12].
88 In our recent work [13] we produced nano spray dried ethyl cellulose and poly(lactic co-glycolic acid) fine
89 particles, respectively, and found the optimal conditions for HRP cross-linking to these supports. In that
90 study purified HRP was immobilized, which due to the high cost of enzyme purification, may make
91 enzymatic processes economically infeasible. In the present paper we compare the 2,4-dichlorophenol

converting performance of purified HRP and crude extract from horseradish after attaching to ethyl cellulose supports prepared by nano spray drying using each of the available spray caps. The physical and chemical properties, the working range and storage stability of the immobilized purified as well as crude enzymes were extensively studied; moreover, the removal of persistent 2,4-dichlorophenol was also investigated. The main novelty of the work is the utilization of a new method for the preparation of fine particles of novel composition with high specific surface area for HRP enzyme and horseradish extract immobilization, which complexes were found to be especially effective in the elimination of a persistent chlorinated phenol.

2. Materials and methods

2.1. Materials

Purified HRP (223 U/mg) was purchased in dry solid form from Amresco (Solon, Ohio) and stored at -20°C until use. One unit of HRP activity was defined as the formation of 1.0 mg purpurogallin from pyrogallol in 20 s at 0.022 M initial pyrogallol concentration and 0.045 M H_2O_2 in 3.0 ml distilled water. Crude extract was gained from horseradish plants and stored after extraction at -20°C.

Ethyl cellulose (viscosity: 4 mPa s, 5 wt% in 80:20 toluene/ethyl alcohol, 25 °C) was a kind gift from Dow Deutschland Anlagengesellschaft mbH (Germany). Dichloromethane (DCM) was purchased from Scharlab (Hungary). Guaiacol was purchased from Cayman Chemical Company (USA). 30% hydrogen peroxide was obtained from VWR International LLC. N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC) (~98%), N-hydroxysuccinimide (NHS) (98%) and Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich. 2,4-dichlorophenol was bought from Fluka. Acetonitrile (Scharlau, HPLC grade) was purchased from Scharlab Ltd. (Hungary).

2.2. Extraction of horseradish

Home-grown, 1 year old horseradish plants were harvested in autumn (28th October) and stored in a refrigerator till the extraction, which was achieved after a day. Roots were cleaned with a brush in cold water eliminating soil and other contamination. 400 g horseradish root was ground with a centrifugal juicer device (Moulinex) that allows the extract to pass through a strainer basket while leaving most of the pulp behind. After grinding the crude extract was ultracentrifuged (Sorvall Discovery 90SE) at 32000 rpm for 50 minutes to separate 65 ml supernatant from the sediment including the pulp particles. The supernatant containing crude extract was removed and kept in a freezer at -20°C for further usage. No medium or agent were added to the extract.

2.3. Spray drying with Nano Spray Dryer B-90

Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland) utilizes piezotechnology to produce fine particles. The piezodriven spray nozzle generates ultra-fine droplets with a narrow size distribution, which are successively dried. The formed solid particles are electrostatically charged and collected at the surface of the cylindrical collecting electrode by electrical field. The main advantage of the Nano Spray Dryer is the novel electrostatic particle collector for the highest yields of fine particles. Two different setups can be established depending on the solvent type. The long version of the drying chamber is needed for aqueous solutions due to the time of evaporation, while the short version of the device is used for organic solvents. The device was operated in closed-mode configuration with the short version of the drying chamber.

Ethyl cellulose was dissolved in DCM to form 1 % (w/v) solution for spray drying using spray caps with 7 μm, 5.5 μm and 4 μm hole sizes, which were vibrated at 60 kHz ultrasonic frequency. Nitrogen was used as drying gas; the flow rate was set to 100 L/min. The relative spray rate was 0.2 l/h (100 %). During spray drying over 40°C, EC precipitation was experienced on the spray head due to the relatively high temperature that developed in the glass chamber; therefore, spray head temperature was kept under 40°C by cooling the EC solution with an ice bath to avoid precipitation and yield loss.

146 *2.4. Analysis of the carrier particles*

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148 The particle size of the spray dried powders was determined by the laser diffraction method (Mastersizer
149 2000, Malvern Instruments, UK). Before, the particles were first dispersed in distilled water (1 mg/ml)
150 and sonicated for 2 minutes. For the sample dispersion during size analysis the Malvern Hydro 2000SM
151 dispersion unit was used at a speed of 1200 rpm. The average particle sizes were expressed in equivalent
152 volume mean diameters (D [4. 3]). The d (0.1), d (0.5), d (0.9) values mark the sizes, below which 10 %,
153 50 % and 90 %, respectively, of the particles are present.
154 The morphology of the spray dried particles was characterized by scanning electron microscopy (SEM).
155 Spray dried EC particles were dispersed in distilled water (0.1 mg/ml), a drop was pipetted onto the grid
156 and dried overnight at room temperature. Then samples were vacuum-coated for 3 minutes with a mixture
157 of gold and palladium and examined with a Philips XL-30 Environmental Scanning Electron Microscope
158 (ESEM) at 20kV/25kV.
159 The ratio of particles with doughnut- and spherical shapes was visually calculated from minimum three
160 SEM images which contained more than 50 particles.

161

162 *2.5. Immobilization of HRP*

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164 HRP was covalently immobilized after the activation of EC. 50 mg of prepared carrier was dispersed in 5
165 ml distilled water (DW). 10 mg/ml of EDC and 10 mg/ml NHS based on previous results were added to
166 the samples, and stirred with a magnetic stirrer for 3 hours at room temperature. After the reaction time,
167 particle dispersions were placed into Falcon tubes and centrifuged (Heraeus Biofuge Primo R) with 8500
168 rpm for 20 minutes. The supernatant containing the non-attached EDC and NHS was removed. After three
169 times washing with distilled water and centrifuging, 2 ml of purified HRP (1 mg/ml) and 2 ml of
170 horseradish extract were added to the samples containing the carbodiimide activated particles at 4 °C.
171 After immobilization, non-cross-linked HRP, excess reagents and cross-linking by-products were removed
172 by three times washing with TRIS buffer (0.02 M, pH 6) and centrifuging samples at 8500 rpm for 20

minutes. Retained particles, containing cross-linked HRP were kept in the TRIS buffer dispersion (10 mg/ml) in a freezer for further usage.

The FTIR spectra were recorded with Varian Scimitar FTS2000 spectrometer (64 scans, 4 cm-1 resolution) equipped with liquid nitrogen cooled MCT detector and Pike GladiATR (with germanium micro-ATR element) accessory.

2.6. HRP content determination

Protein content of purified HRP cross-linked onto EC was measured by the Lowry-Folin assay. Total protein content was determined by the Folin-Ciocalteu phenol reagent via its reaction with tyrosine and tryptophan residues in proteins. Solution *A* contained 24.5 ml 4 % sodium carbonate and 24.5 ml 0.2 M sodium hydroxide. Solution *B* contained 0.5 ml 1 % copper sulfate and 0.5 ml 2.7 % potassium sodium tartrate. Solution *A* and *B* prepared daily were mixed and 2 ml of this mixture was added to the samples followed by vortexing. After 10 minutes, it was combined with 200 μl of 2 N Folin-Ciocalteu phenol reagent (used in 1:1 dilution) and kept at room temperature for 30 minutes. Samples were centrifuged at 13000 rpm for 5 minutes (Heraeus Biofuge pico) and the absorbance of the supernatant was measured at 750 nm.

2.7. Activity measurement

HRP activity was assayed using guaiacol chromophore and hydrogen peroxide (H_2O_2) as substrate while the guaiacol was peroxidated to tetraguaiacol. In the 3 ml reaction mixture the final concentration of H_2O_2 was 0.001 M and the concentration of guaiacol was 0.002 M. The assay system was carried out in a cuvette that contained 0.5 ml of 6 mM hydrogen peroxide as a substrate and 1.5 ml of 4 mM guaiacol in distilled water at room temperature.

The reaction was initiated by the addition of 1 mg/ml dispersed particles containing cross-linked HRP. The absorbance was measured using a spectrophotometer (Pharmacia LKB-Biochrom) at 470 nm at room

temperature. The absorbance was monitored after 2 minutes and the obtained initial values (absorbance at 0 s) were subtracted from the results during calculating the activity values, thus avoiding the light scattering effect of the particles. All measurements were performed in triplicate.

2.8. Effect of pH

The activities of free and cross-linked HRP (purified and extracted) were measured at a pH range 4-10. The effect of pH on HRP stability was analyzed by 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 9.0-10.0) buffer solutions at room temperature.

2.9. Storage stability

Purified (223 U/mg, 0.5 μg/ml) HRP and extract (103 U/mg, 3.3 μg/ml) from horseradish were stored at room temperature for 4 weeks at pH 6 in order to study the storage stability of the enzyme. The storage stability of the purified and extracted HRP was also investigated after cross-linking to the ethyl cellulose support. 10 mg of each of the immobilized enzyme samples were redispersed in 5 ml buffer (pH 6). The immobilized concentrations of purified and extracted enzyme were 28 U/g and 10 U/g, respectively. Activity was measured spectrophotometrically weekly.

2.10. 2,4-dichlorophenol assay

The phenol concentration of the samples was analysed and quantified by HPLC (High Performance Liquid Chromatography, Hewlett Packard). Experiments were performed on a 150 mm × 4 mm reverse phase column (particle size 5 μm, C18 BDS HYPERSIL, Thermo Scientific) at room temperature. The detection of phenol was carried out with a UV–vis detector at 283 nm after determining this value as the maximal absorbance of 2,4-dichlorophenol spectrophotometrically with Biochrom 4060 (Pharmacia LKB). The

mobile phase was a 50/50 vol. mixture of acetonitrile and 10 mM phosphoric acid at a flow rate of 1 ml/min.

Samples containing 2,4-dichlorophenol and cross-linked purified HRP or horseradish extract, immobilized onto carriers spray dried by using a spray cap hole size 5.5 μm or 7 μm were stirred with a magnetic stirrer at 200 rpm at room temperature. The reaction started when hydrogen peroxide was pipetted into the reaction mixture. After 2 hours, samples were centrifuged at 13000 rpm for 20 minutes at 10^{o}C. 20 μl of the supernatant was directly injected into the HPLC column and the removal efficiency was determined. The 2,4-dichlorophenol concentration was determined using a calibration curve. All measurements were performed in triplicate.

For reusability test samples containing 1.4 mM 2,4-dichlorophenol and 10 mg/ml carrier particles with crude extract from horseradish (0.087 U/ml) cross-linked on EC particles sprayed with a spray cap 4 μm were stirred with a magnetic stirrer at room temperature and hydrogen peroxide (2mM) initiated the reaction. After 2 hours, samples were ultracentrifuged with 30000 rpm for 15 minutes at 10^{o}C and supernatant was analysed by HPLC. Remained particles were washed with TRIS buffer solution (0.02 M, pH6) after every cycle and reused, then, removal efficiency was assayed again. This procedure was repeated for 10 cycles. Control experiments were also performed under the same conditions without HRP and/or H_2O_2 for 3 cycles.

3. Results and discussion

3.1. Particle size distribution and morphology

Ethyl cellulose particle size was determined after the spray drying process by the laser diffraction method (Fig. 1). The majority of the EC particles were between 1 and 10 μm by using 7 μm and 5.5 μm spray caps, whilst cc. 10 % of them were smaller than 1 μm and most of them were below 3 μm if generated by 4 μm spray cap hole size (Table 1).

The morphology of nano spray dried EC particles was visualised by scanning electron microscopy (Fig. 2). Particles showed spherical and partially doughnut-like morphology using hole size 7 μm, 5.5 μm and 4 μm spray caps. As can be seen in the SEM images, all of the smaller particles had a spherical shape and some of the bigger particles had doughnut shape, which was found with every spray cap. Doughnut-like particles are the result of the loss of structural stability of the sprayed droplets because of macro- and micro-hydrodynamic effects. In our case the destabilization of the initial shape of the bigger droplets occurs due to the relatively high viscosity that affects the morphology of the particles [14]. However, doughnut shape is not disadvantageous in the immobilization process, since it issues in higher specific surface area compared to spherical shape.

3.2. HRP immobilization

The condition of HRP immobilization was optimized in our recent work [13]. In the present study we compared the HRP activity of particles prepared by the three different spray cap hole sizes. Both purified HRP and horseradish extract, respectively, were immobilized onto the ethyl cellulose support FTIR spectra were recorded for both the EC fine particles and the supports cross-linked by HRP. Successful attachment was proved by FTIR measurement that clearly showed the amide bond presence in the EC-HRP cross-linked sample (Fig. 3). Purified enzyme represented a higher residual activity after immobilization on particles prepared with any of the three spray caps (Fig. 4). The reason might be that extract of horseradish contains several other proteins as well, and the cross-linking procedure is not selective, which means that other proteins can also be attached to the activated sites of ethyl cellulose, thus competing with HRP. Although the crude extract was not analysed, some literature data is provided as an estimate of potentially fixed material. The average protein content of horseradish is 1.2 % (m/m) [15]. Lascu et al (1986) separated horseradish peroxidase by Sepharose CL-6B column from horseradish root [16]. They stated that probably every protein bound to the column, and 30-40 % of the bound material was found to be horseradish peroxidase.

As was expected, by decreasing the spray cap hole size the residual activity of HRP increased, since with a lower hole size, smaller particles with a higher specific surface area can be generated. However, comparing the size distribution of particles formed by spray cap hole size of 7 μm and 5.5 μm, the difference is much smaller than between those ones which were formed by 5.5 μm or 4 μm (Fig. 1). Moreover, according to the SEM images (Fig. 2) it can be concluded that spraying with a cap hole size of 7 μm resulted in significantly more particles with a doughnut shape than the one sprayed with 5.5 μm. The ratio of doughnut- and spherical shapes, obtained after spraying with the three different spray caps, was calculated from minimum three SEM images, and average values were given in Table 2. This calculation showed that spraying with a 7 μm hole size resulted in the highest ratio of doughnut-shaped carriers, which explains the higher amount of bound HRP related to carrier particles with a smaller size obtained by spraying using the 5.5 μm hole size. The substantially higher residual activity of cross-linked HRP on the support obtained using a spray cap 4 μm can be interpreted by the much lower size of formed particles related to the carriers prepared by the other two spray caps. While the residual activity of extracted HRP attached to the ethyl cellulose support ranged from 6 to 10 U/g, that of the purified enzyme was between 10 and 28 U/g. These values are comparable with that of Leiriao et al. [17] who coupled HRP by glutaraldehyde and carbodiimide on hydrophilic polyacrylonitrile membrane and on silica and glass beads, respectively, and the HRP activity changed from 0.8 up to 28 U/g support. Similarly Lai and Lin [3] immobilized HRP to aminopropyltriethoxysilane-activated magnetite and reached 18 U/g maximal specific enzyme activity, while porous aminopropyl glass-immobilized HRP led to 42.7 U/g.

The HRP mass attached to the support was investigated by the Lowry-Folin assay. It was studied excusively with the purified HRP since horseradish extract also contains other proteins (Fig. 5), which makes it impossible to determine the HRP content of those supports crosslinked with crude extract of horseradish. Protein content was found to be similar on ethyl cellulose carriers prepared by spray caps of 7 μm and 5.5 μm in accordance with the active HRP concentration, although it was slightly higher at support formed by a 5.5 μm cap hole; nevertheless, taking into consideration the standard deviations the difference might be negligible. In agreement with active HRP measurement the highest HRP content (7 mg/g) could be cross-linked to the fine particles formulated by the smallest spray cap. As a comparison

Azevedo et al. [18] assembled mini-packed bioreactors for the immobilization of purified HRP on aminated controlled pore glass by using different procedures with glutaraldehyde and EDC cross-linker. Protein concentration was determined using Folin phenol reagent. A higher protein content was obtained when HRP was covalently immobilised using glutaraldehyde (21 mg/g), while the amount of HRP immobilised with EDC was five times lower, 3.9 mg/g. Pundir et al. [19] immobilized horseradish peroxidase onto zirconia coated arylamine and alkylamine glass beads by diazotization and glutaraldehyde coupling. High conjugation yield was achieved; 28 mg/g enzyme was attached to arylamine glass beads and 16 mg/g was immobilized to alkylamine beads. However, the immobilized enzyme content determination was an indirect method based on the loss of protein from solution during enzyme immobilization, which can have significantly higher error than direct method.

3.3. pH working range

The effect of pH on the working stability was investigated at the pH range of 4.0-10.0 (Fig. 6). At pH 9 the activity of the free and cross-linked enzyme was measured in both TRIS and sodium carbonate buffer in order to take into consideration the various ionic strengths of the used buffers. There was not significant difference between the activity values experienced with different buffers. The carrier particle size did not affect the pH dependence of the residual activity either. A displacement was observed of the optimum pH by one pH unit towards acidic values. Optimum pH was detected at pH 6 shifted from pH 7 comparing purified and immobilized enzyme on EC with free enzyme, and at pH 5 shifted from pH 6 in the case of horseradish extract. Immobilized enzyme activity was generally improved in both the acidic and the alkaline pH ranges, and this improvement was more considerable in the presence of the purified enzyme. The enhanced pH working range was a benefit of HRP immobilization also recognised by other groups. HRP immobilization on porous aminopropyl glass beads [3] and on polyaniline [20] via glutaraldehyde bonding eventuated that the optimum pH range was enhanced and shifted one and two units, respectively, towards the acidic range.

3.4. Storage stability

The storage stability of the enzyme was investigated for 4 weeks at room temperature. The control enzyme kept in the freezer at -20 ^{o}C did not show any significant change after 4 weeks (data not shown). Both immobilized purified HRP and crude extract from horseradish showed much higher stability as a function of time (Fig. 7). Nevertheless, the activity of extracted HRP decreased most rapidly, and the cross-linked extracted enzyme preserved its activity to the highest extent after 4 weeks (> 50 %). Both immobilized and free purified HRP lost most of their activity after 4 weeks, although cross-linked enzyme showed substantially higher stability till the third week of the study. The reason might be the forming complex that can be stabilized by electrostatic, ion-dipole or hydrophobic interactions, which was achieved during cross-linking controlling the three-dimensional structure of the enzyme and limiting the freedom of conformational changes, resulting in increasing stability [21]. The finding of higher storage stability after immobilization is in good agreement with that of other groups [22-24].

3.5. 2,4-dichlorophenol removal

One of the main advantages of the HRP immobilization is the enhancement of phenol and its derivatives removal efficiency. Roper et al. [25] degraded around 15 % and 45 %, respectively, of 2,4-dichlorophenol using HRP without and with a co-substrate. Laurenti et al. [26] published 25-30 % of 2,4-dichlorophenol degradation achieved by free HRP. They also analysed the reaction products, and identified the formation of dimer (2-chloro-6-(2,4-dichlorphenoxy)-1,4-benzoquinone), and rendered the presence of trimers and tetramers probable, as evidence of the catalytic polymerization of the pollutant. Nevertheless, according to published data on removal of phenols and chlorophenols (e.g. [10,12,27]) the elimination can be much more efficient using immobilized enzyme.

In order to optimize the 2,4-dichlorophenol removal by immobilized HRP, the effect of main parameters was investigated. The retention time for 2,4-dichlorophenol was found to be 3.7 min (Fig. 8). One of the most important factors in the peroxidative removal of phenol derivatives from aqueous solutions is the

concentration of H_2O_2, which has got an optimal value above which the suicide-peroxide inactivation may take place [28]. At 10 mg/ml HRP carrier concentration with 0.087 U/ml HRP and 1.4 mM 2,4-dichlorophenol concentration, the H_2O_2 concentration was varied between 0.3 and 300 mM. The highest phenol derivative removal was obtained using 1 mM H_2O_2 (Fig. 9); however, the suicide-peroxide inactivation was not too high.

2,4-dichlorophenol removal efficiency was also studied as a function of substrate concentration. While adding 10 mg/ml solid support containing 0.093 U/ml HRP (H_2O_2 concentration: 2 mM), the increasing substrate concentration resulted in a slight decrease in the removal efficiency (Fig. 9a). Although low enzyme concentration (2 mg/ml support with 0.012 U/ml HRP) represented a similar tendency (H_2O_2 concentration: 1 mM), but with significantly lower removal efficiency (Fig. 10b). It can be concluded that both low substrate and low enzyme concentration caused inhibition in the elimination reaction. It is also clear that even the highest conversion of 2,4-dichlorophenol with 2 mg/ml support containing 0.012 U/ml HRP was also significantly lower than the removal obtained using 10 mg/ml particles containing 0.093 U/ml HRP in the solution of the same substrate concentration.

The effect of carrier particle size and that whether the enzyme was purified or present in crude extract were analyzed. 10 mg/ml carrier was homogenized in the reaction mixture containing 300 mM H_2O_2 concentration, which contained 0.067 U/ml and 0.096 U/ml active extracted and purified HRP, respectively. There was no significant difference between enzyme either immobilized on ethyl cellulose particles generated using a spray-cap with different hole sizes or obtained from different sources possessing substantially diverse activity (Fig. 11).

Repeated use of HRP attached to the fine EC particles was examined at the optimal removal conditions (Fig. 12). It was shown that even after 10 reuse the removal of the persistent substrate exceeded 60 % efficiency. Control tests without HRP enzyme were also performed in order to investigate the adsorption capacity of the fine support with high surface area. Surprisingly, the removal was very high without the enzyme (Fig. 13), however after 3 repeated batch cycle its value dropped to that level (cc. 65 %) which was reached by the immobilized enzyme even after 9 reusing. Moreover, it was found that there was not significant difference between removal efficiency of EC particles with or without H_2O_2, which can be

386 explained by that EC is capable to adsorb the pollutant alone effectively, nevertheless, the attached
387 enzyme made the elimination perfect. The substantial adsorption of the particles is probably the reason for
388 the more efficacious removal compared to the 2,4-dichlorophenol conversion generally described in the
389 literature.
390
391 **Conclusions**
392
393 Horseradish peroxidase was successfully attached to fine ethyl cellulose particles formed by the nano
394 spray drying method. Decreasing the hole size of spray caps (7 μm, 5.5 μm and 4 μm) resulted in
395 diminishing particle size and due to a higher surface-to-volume ratio increased the cross-linked enzyme
396 content. A higher amount of purified HRP was cross-linked to the solid support than HRP from crude
397 extract, because the latter one also contains several other proteins which can also be attached to the active
398 sites of the carrier. The immobilization of both purified HRP and crude extract from horseradish improved
399 substantially the pH tolerance and the storage stability compared to the free enzyme. 2,4-dichlorophenol
400 substrate could be efficiently eliminated with a maximum of 97.7 % at 10 mg/ml HRP carrier
401 concentration containing 0.087 U/ml HRP and at 1.4 mM 2,4-dichlorophenol and 1 mM H_2O_2
402 concentrations. Chlorinated phenol and H_2O_2 concentrations had significant influence on the substrate
403 conversion, while particle size and enzyme source (purified or extracted) affected the elimination
404 negligibly. Reusability of the immobilized enzyme was satisfactory even after 10 cycles. Control
405 measurements showed that the ethyl cellulose fine particles are capable to adsorb significant amount of
406 2,4-dichlorophenol, which definitely contributed to the excellent removal achieved.
407
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409
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Table 1. Volume mean diameters (D [4. 3]) and d (0.1), d (0.5), d (0.9) values of nano spray dried ethyl cellulose (EC) using spray cap hole size 7 µm, 5.5 µm and 4 µm.

Sample Names	D [4.3]	d (0.1)	d (0.5)	d (0.9)
EC 7 µm	4.2	1.4	3.4	8.2
EC 5.5 µm	3.0	1.4	2.7	5.1
EC 4 µm	1.7	1.0	1.5	2.6

Table 2. Percentage of doughnut and spherical shape of nano spray dried ethyl cellulose carriers. Spray caps were applied with hole size of 7 µm, 5.5 µm and 4 µm.

Spray cap hole size	Doughnut shape (%)	Spherical shape (%)
7 µm	**26**	**74**
5.5 µm	**8**	**92**
4 µm	**19**	**81**

Graphical abstract

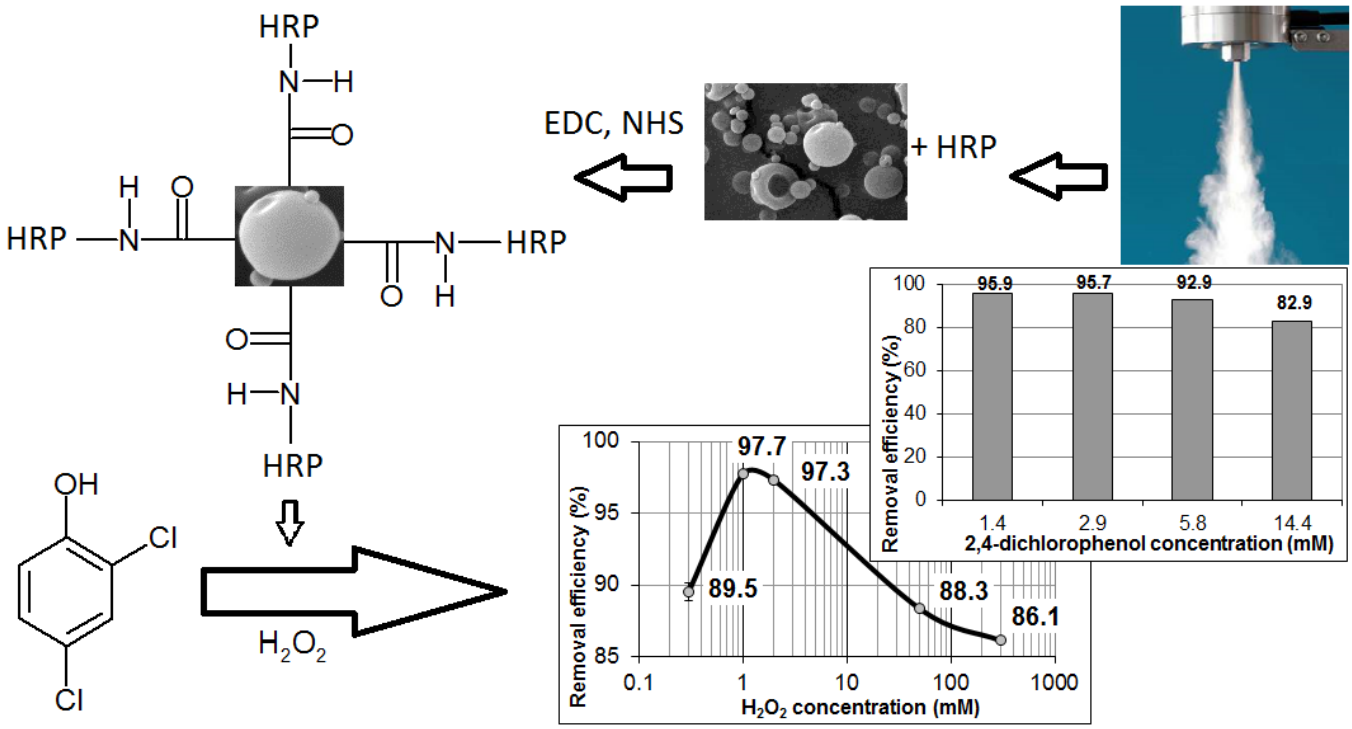

Figure captions:

Fig. 1. Particle size distribution of ethyl cellulose particles. The supports were nano spray dried using spray caps with hole size of 7 µm, 5.5 µm and 4 µm.

511 Fig. 2. SEM images of nano spray dried ethyl cellulose particles. For preparation spray cap hole size 7 μm
512 (a, d), 5.5 μm (b, e) and 4 μm (c, f) were used, and the images were taken with 3000x (a, b, c) and 10000x
513 (d, e, f) magnifications.
514 Fig.3. FTIR of ethyl cellulose particles (EC) and horseradish peroxidase immobilized on EC carriers (EC-
515 HRP).
516 Fig. 4. Residual activity of purified and extracted horseradish peroxidase cross-linked onto nano spray
517 dried ethyl cellulose.
518 Fig. 5. Protein content (mg/g carrier) of immobilized horseradish peroxidase on ethyl cellulose support.
519 The carriers were formed using spray caps with hole sizes of 7, 5.5 and 4 μm.
520 Fig. 6. Effect of pH on activity of purified (a) and extracted (b) horseradish peroxidase (HRP). Free and
521 on ethyl cellulose (EC) particles immobilized HRP were compared.
522 Fig. 7. Storing stability of free and immobilized horseradish peroxidase (HRP). Both purified HRP and
523 horseradish crude extract were investigated.
524 Fig. 8. HPLC spectrum of 2,4-dichlorophenol.
525 Fig. 9. 2,4-dichlorophenol removal efficiency (%) as a function of H_2O_2 concentration (mM) by
526 immobilized horseradish peroxidase.
527 Fig. 10. 2,4-dichlorophenol removal efficiency (%) by immobilized horseradish peroxidase (HRP) as a
528 function of 2,4-dichlorophenol concentration (mM). The immobilized HRP concentrations were 0.093
529 U/ml (a) and 0.012 U/ml (b).
530 Fig. 11. 2,4-dichlorophenol removal efficiency (%) of immobilized horseradish peroxidase (HRP).
531 Purified HRP and crude extract from horseradish (extracted) were immobilized onto particles generated
532 by spray cap with hole size of 5.5 and 7 μm, respectively.
533 Fig. 12. 2,4-dichlorophenol removal efficiency (%) of extract from horseradish attached to ethyl cellulose
534 particles in repeated batch operation.
535 Fig. 13. 2,4-dichlorophenol removal efficiency (%) of ethyl cellulose particles in repeated batch operation.
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537

538 Fig. 1.

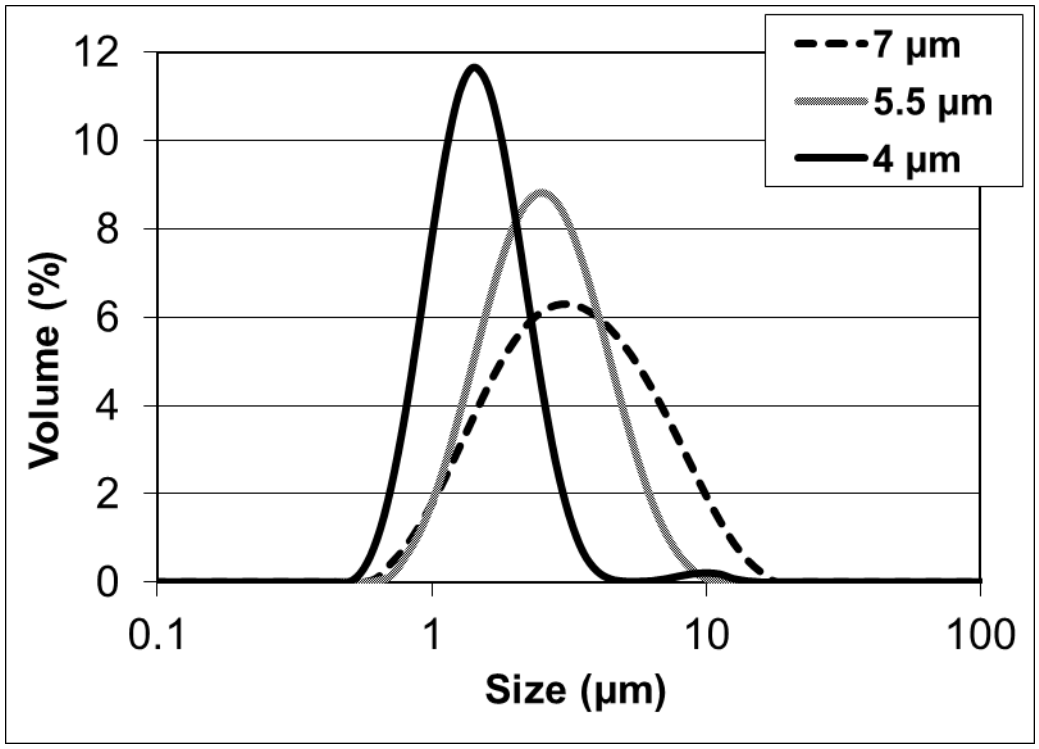

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Fig. 2a

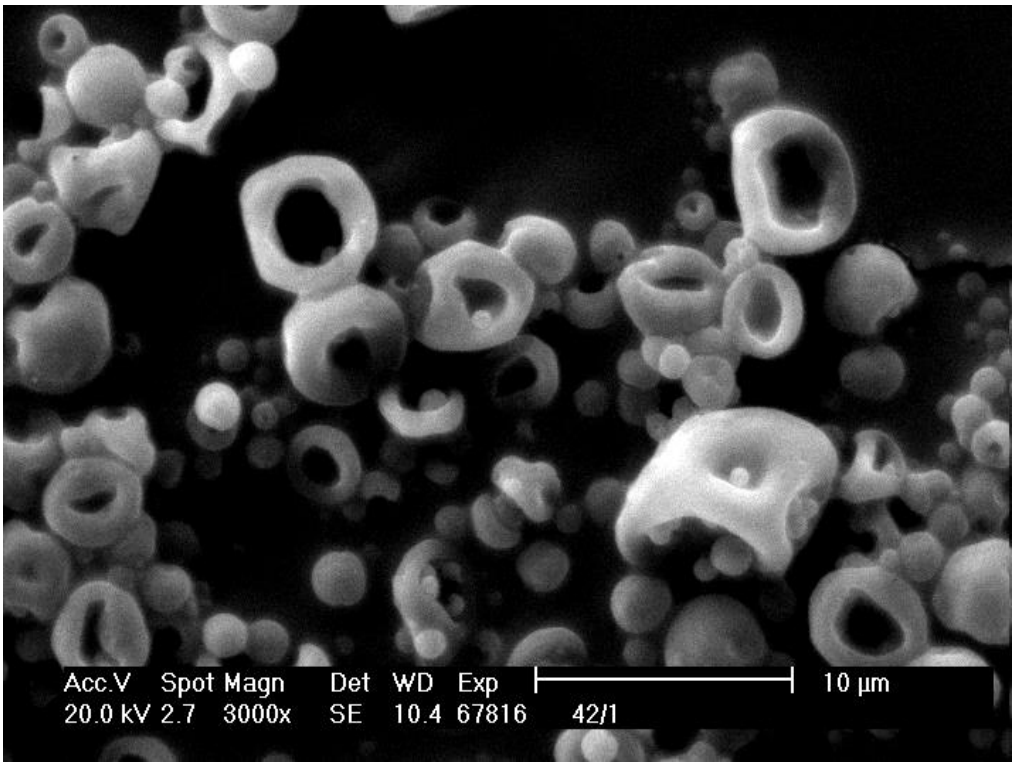
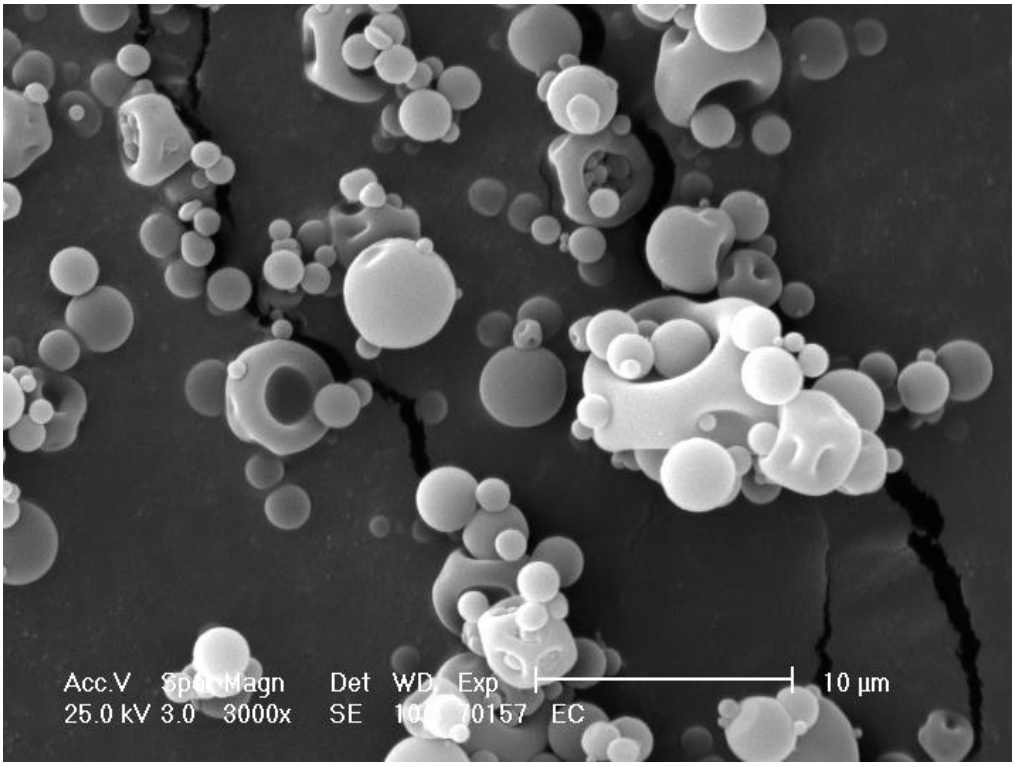

Acc.V Spot Magn Det WD Exp
10 µm
20.0 kV 2.7 3000x SE 10.4 67816 42/1

Fig. 2b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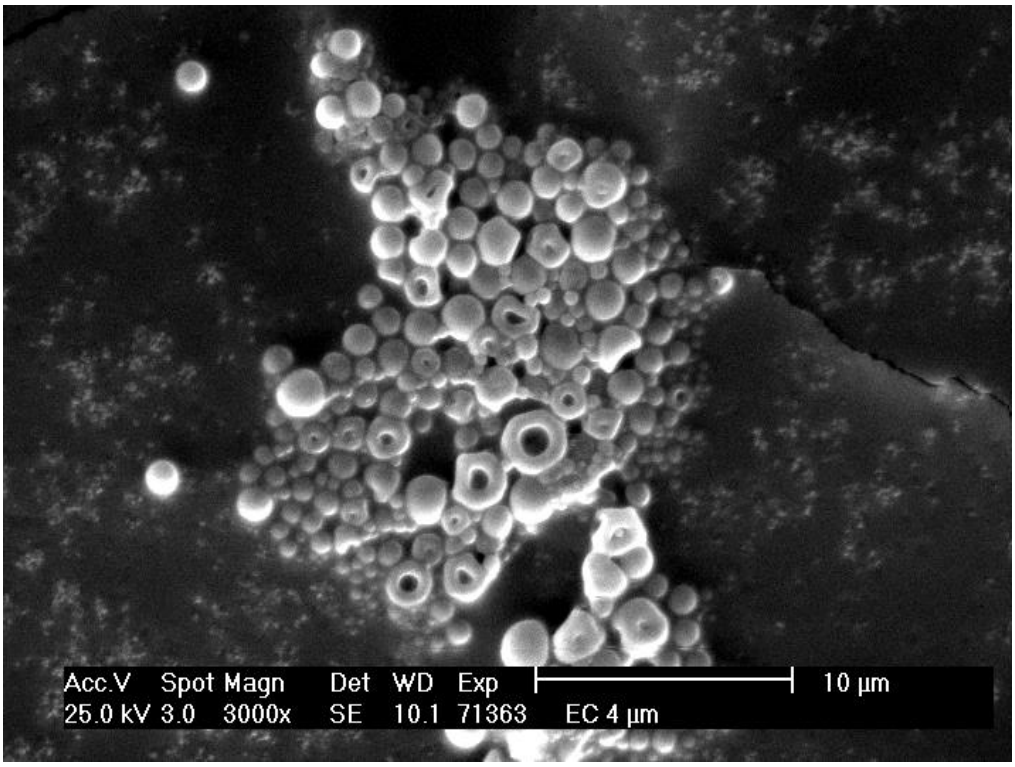
10 µm
25.0 kV 3.0 3000x SE

548 Fig. 2c



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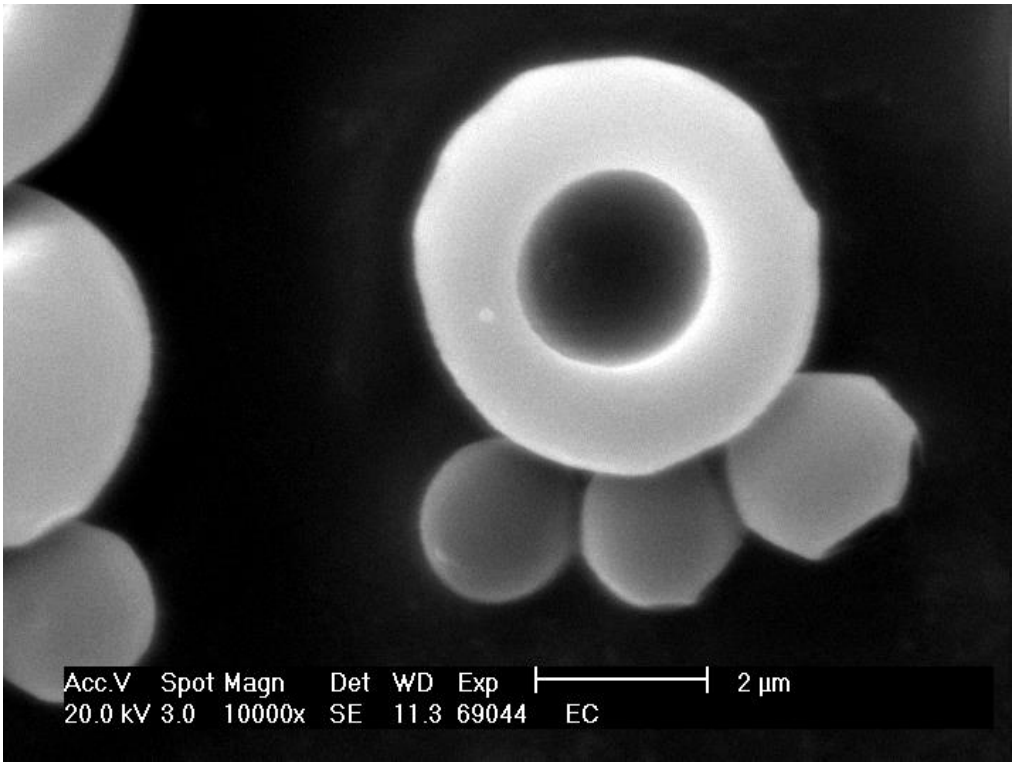
550 Fig 2d



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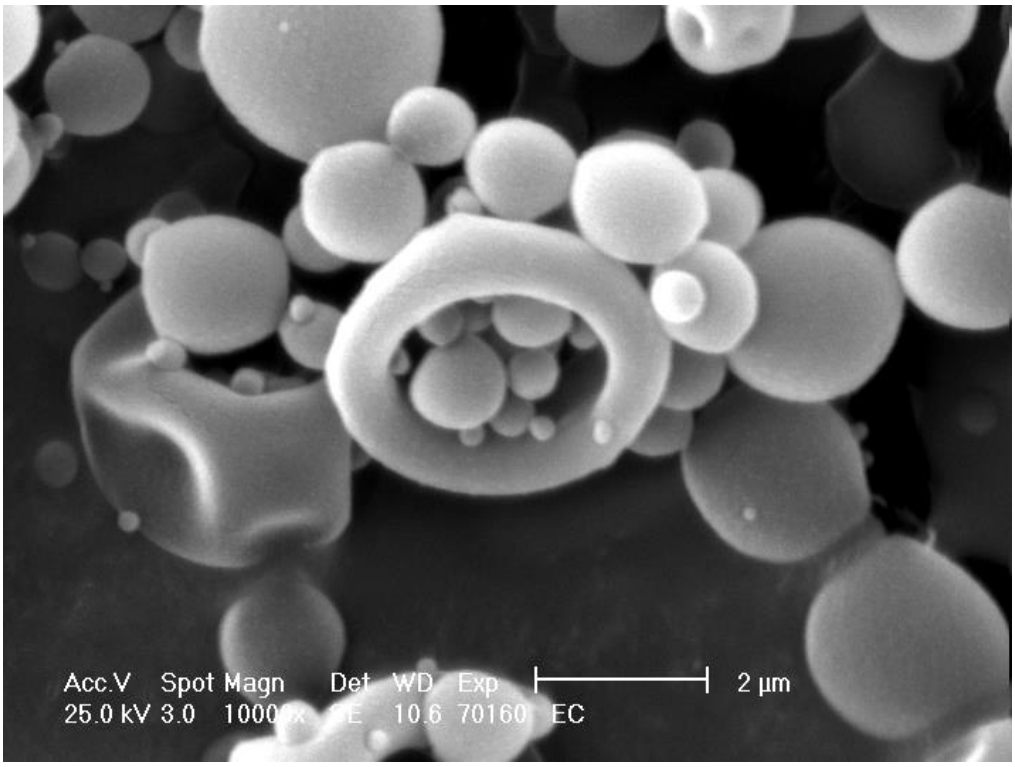
553 Fig 2e



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555 Fig 2f

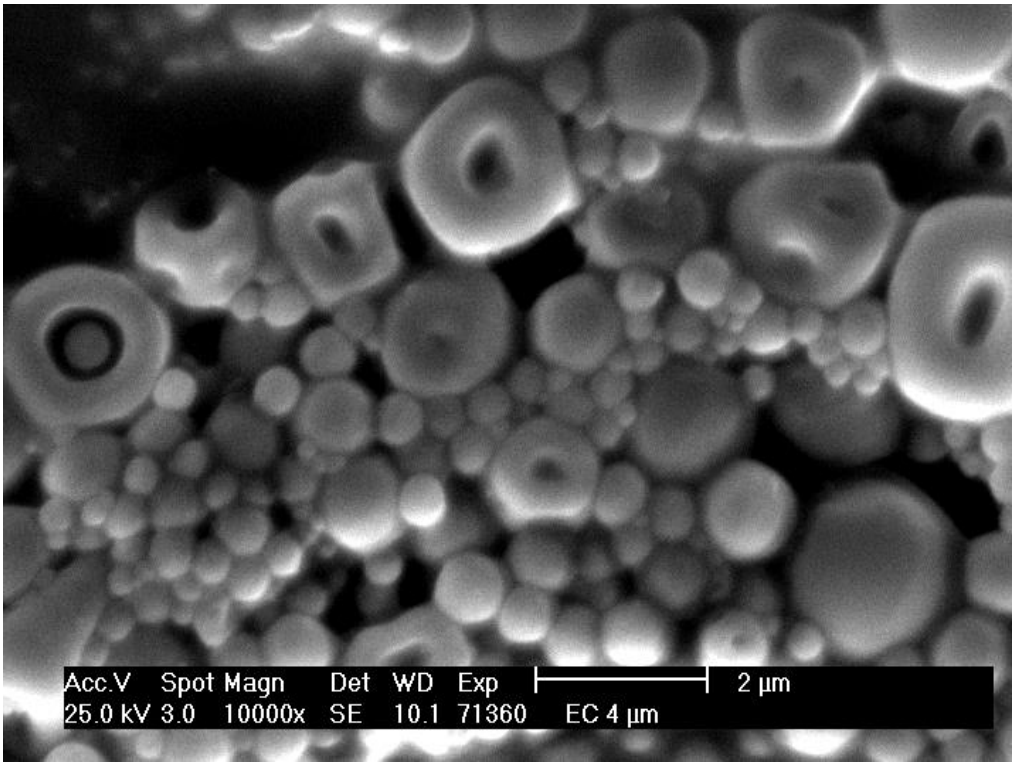

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Fig. 3

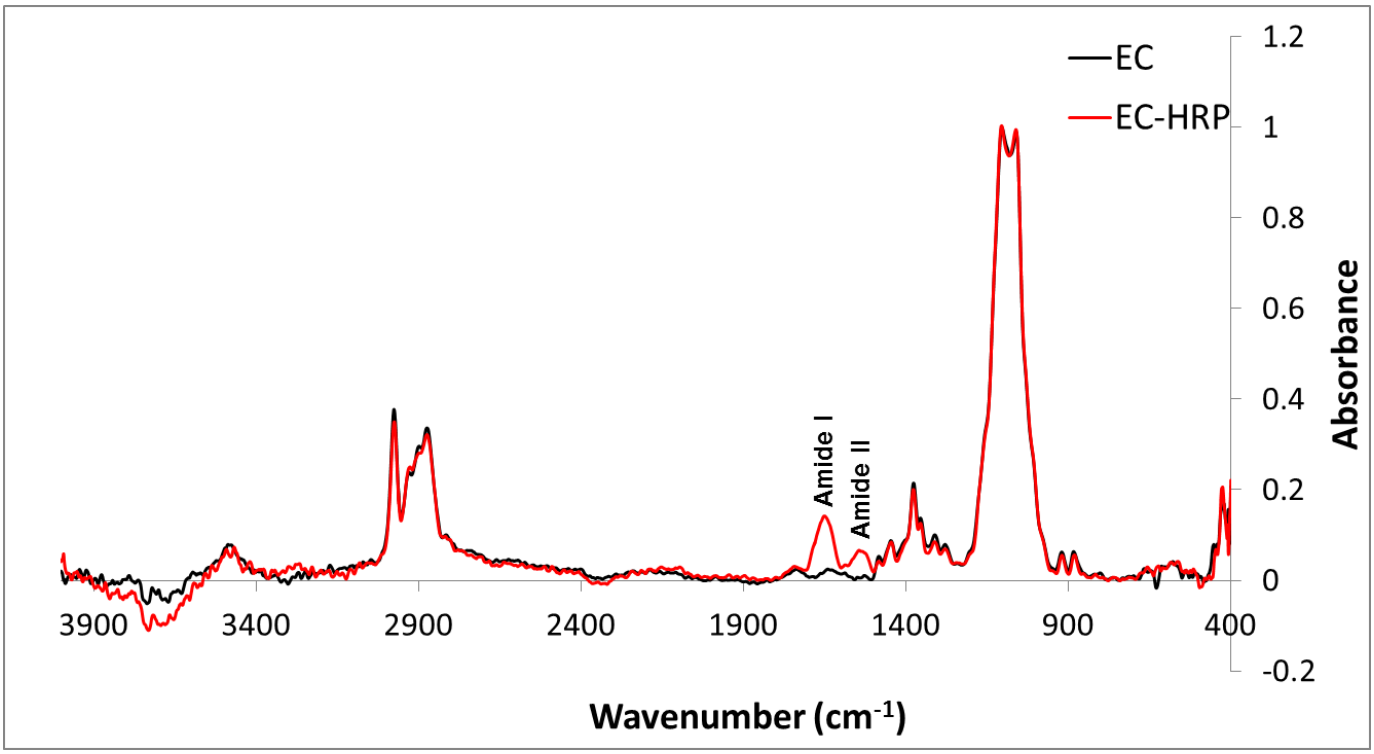
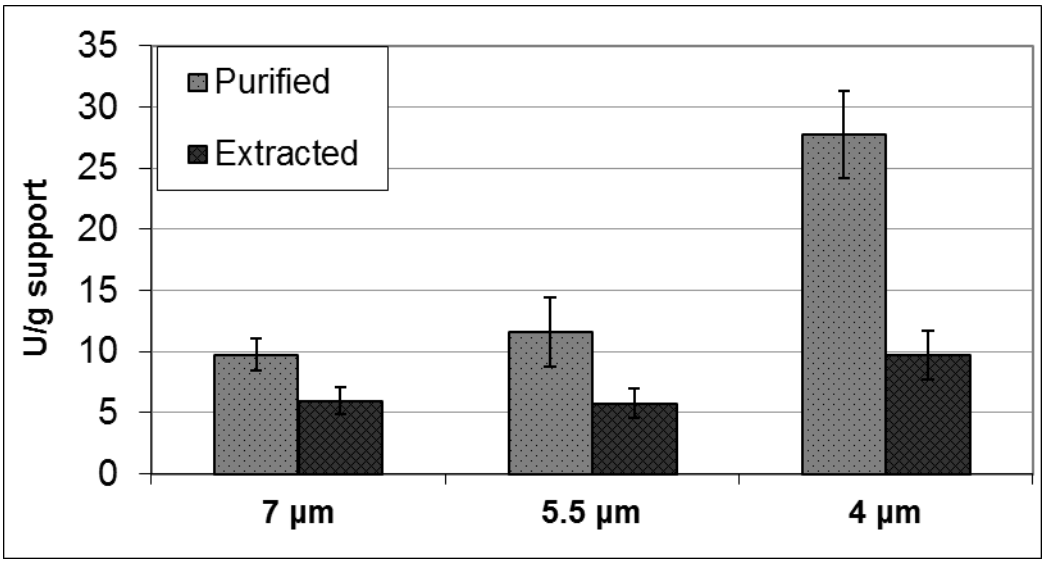
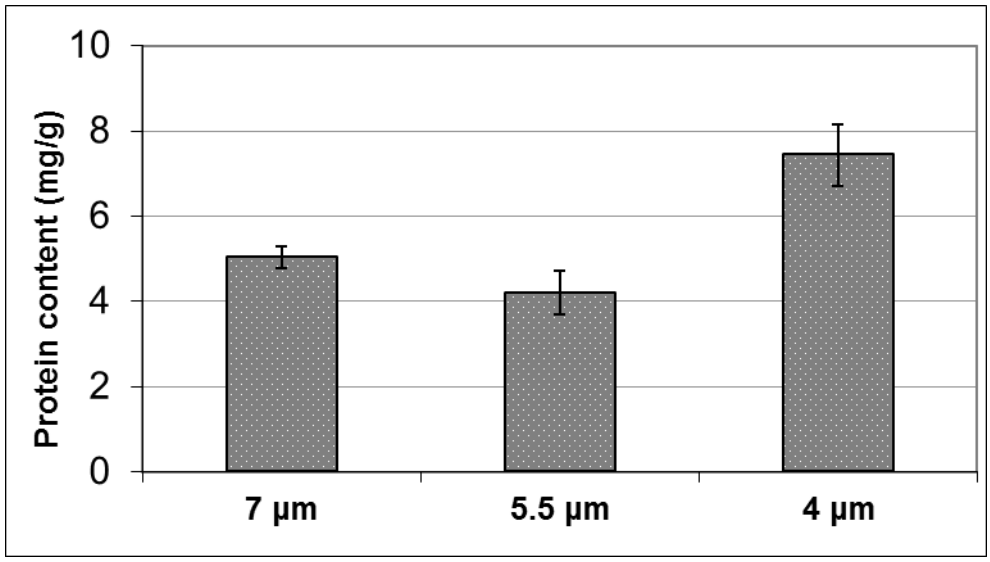

EC
EC-HRP
Amide I
Amide II
Absorbance
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
3900
3400
2900
2400
1900
1400
900
400
Wavenumber (cm-1)

Fig. 4


Purified
Extracted
U/g support
35
30
25
20
15
10
5
0
7 µm
5.5 µm
4 µm

564 Fig. 5



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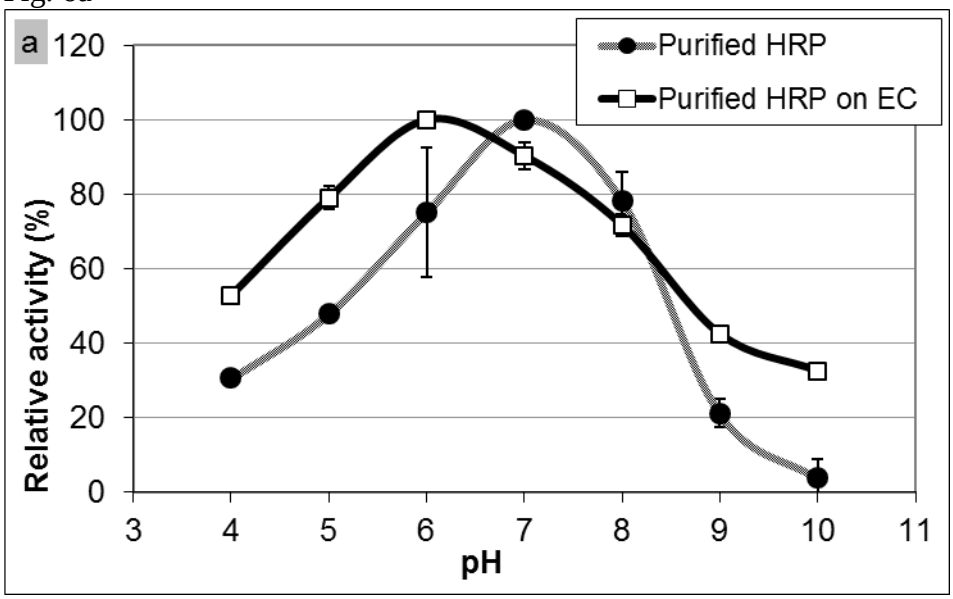
566 Fig. 6a



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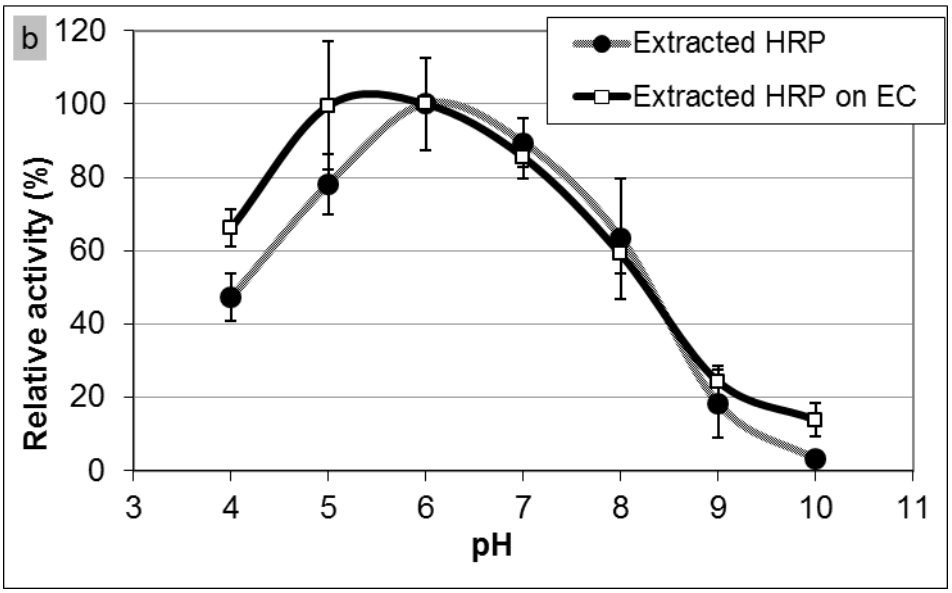
568 Fig. 6b



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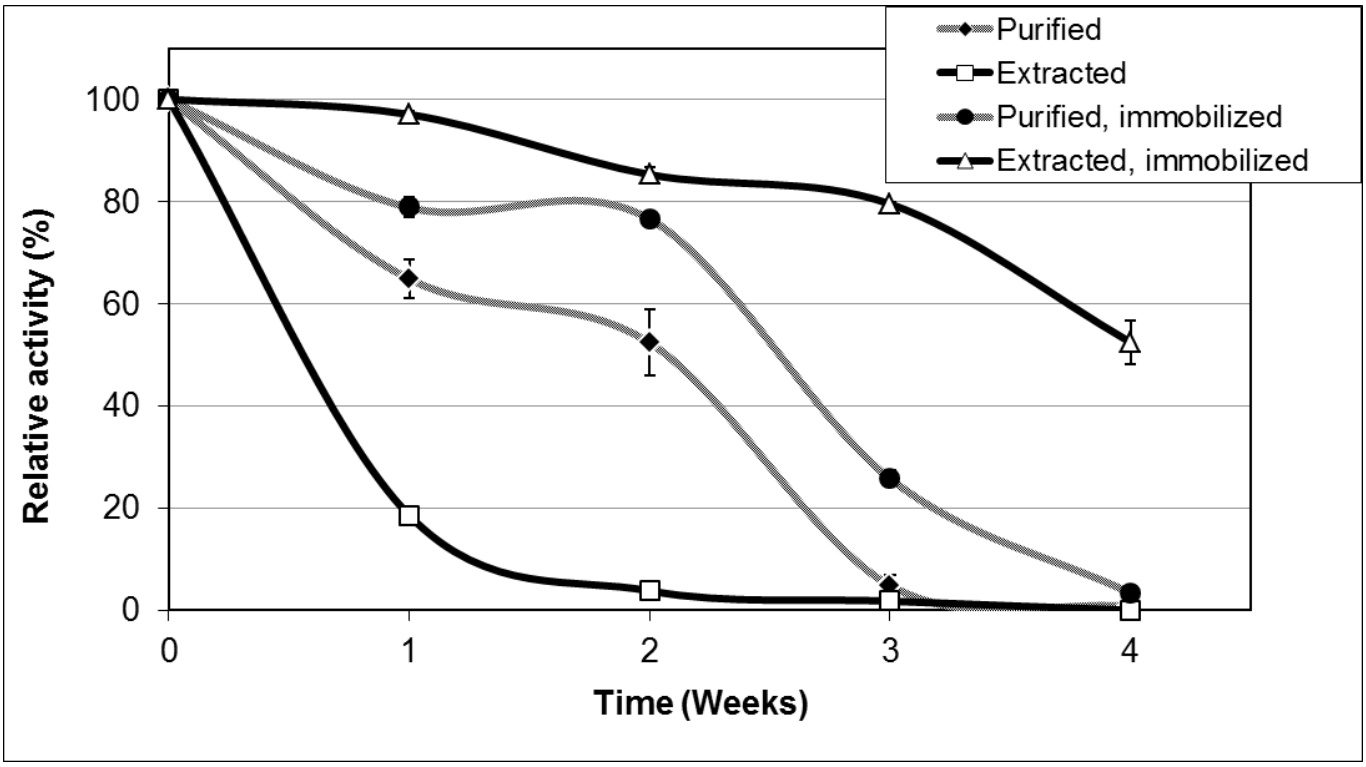
570 Fig. 7



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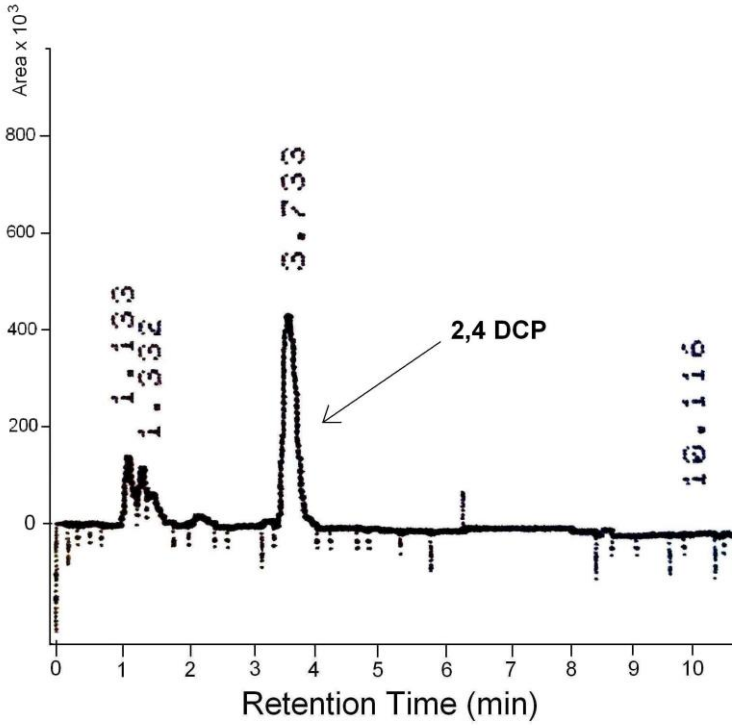
572 Fig. 8



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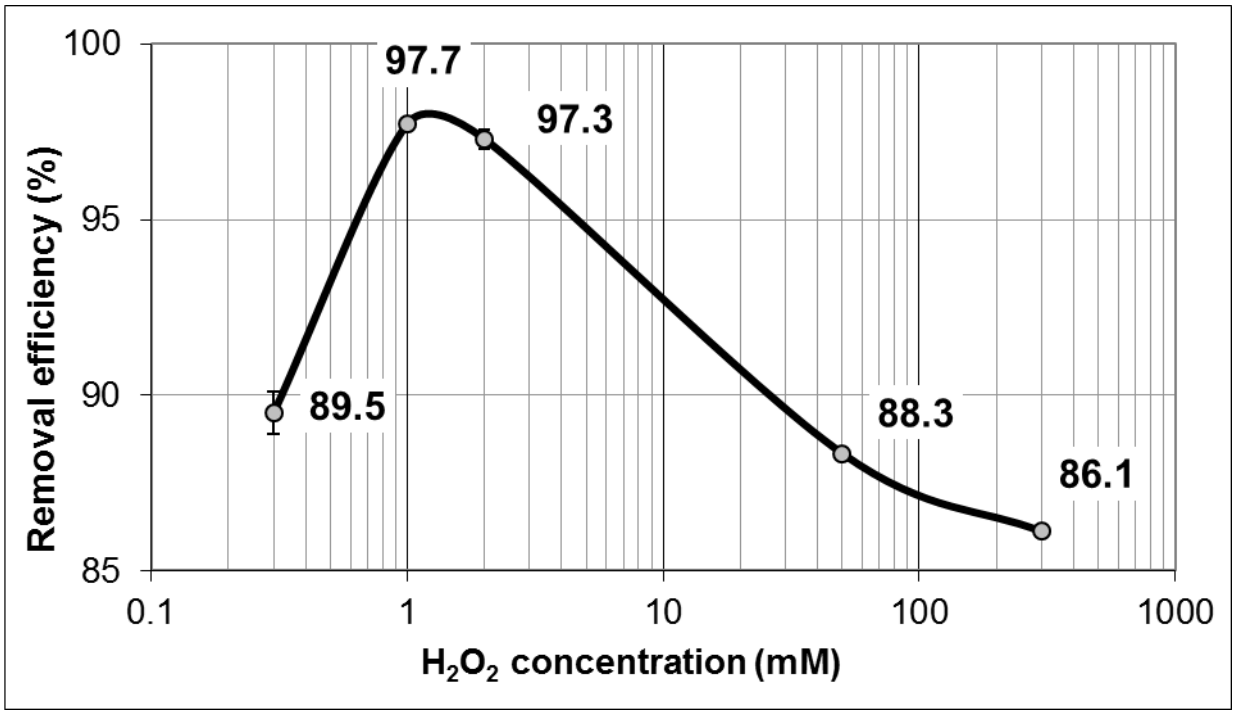
575 Fig. 9



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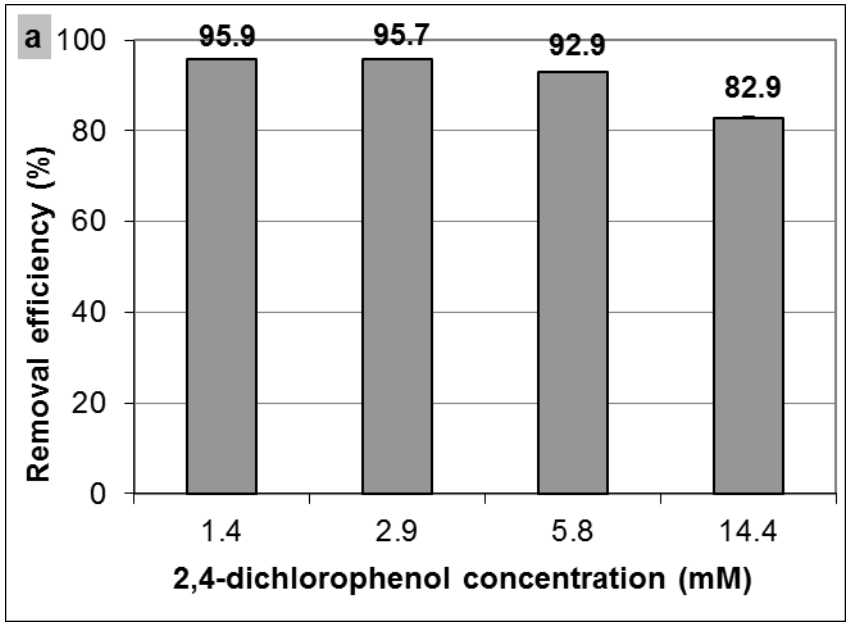
577 Fig.10a



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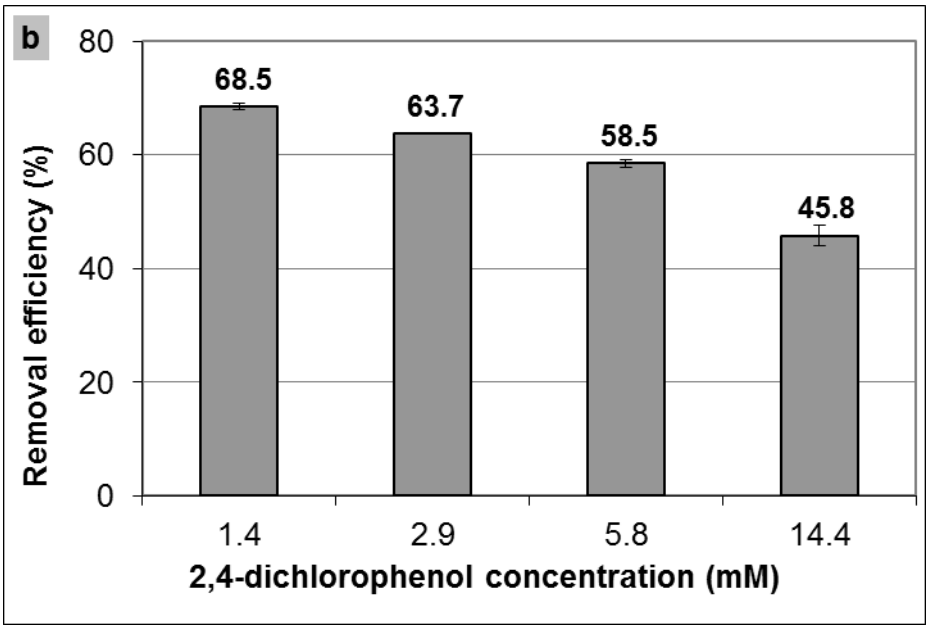
580 Fig. 10b



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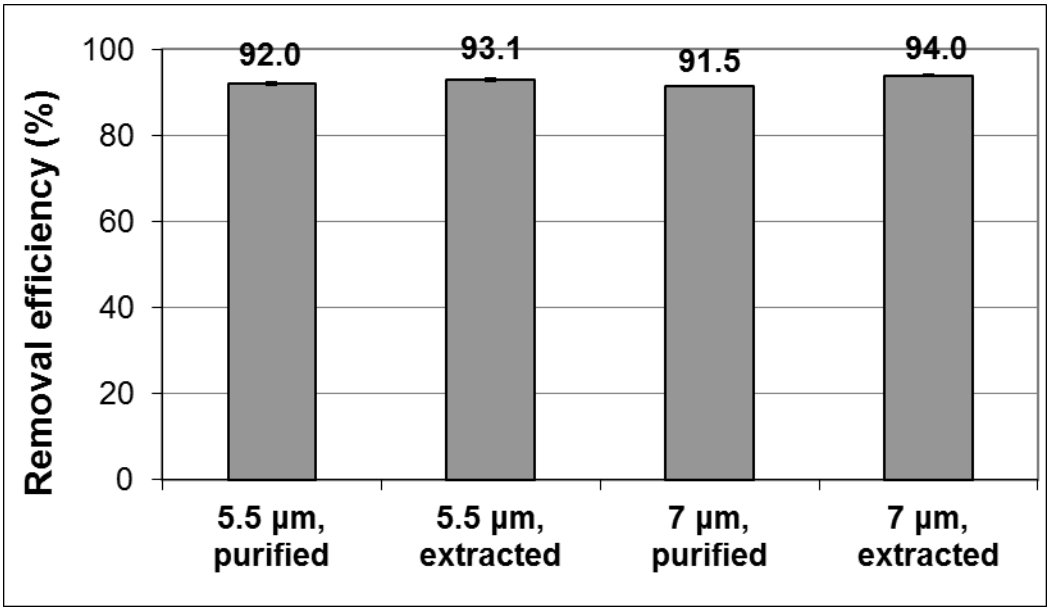
582 Fig. 11



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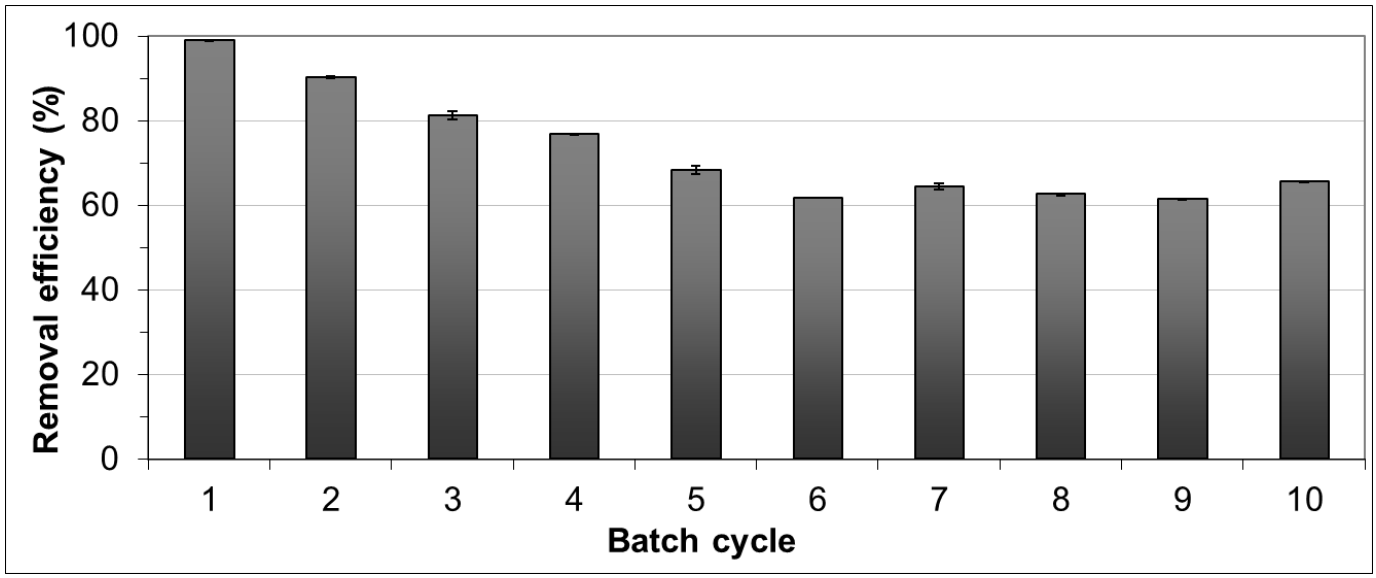

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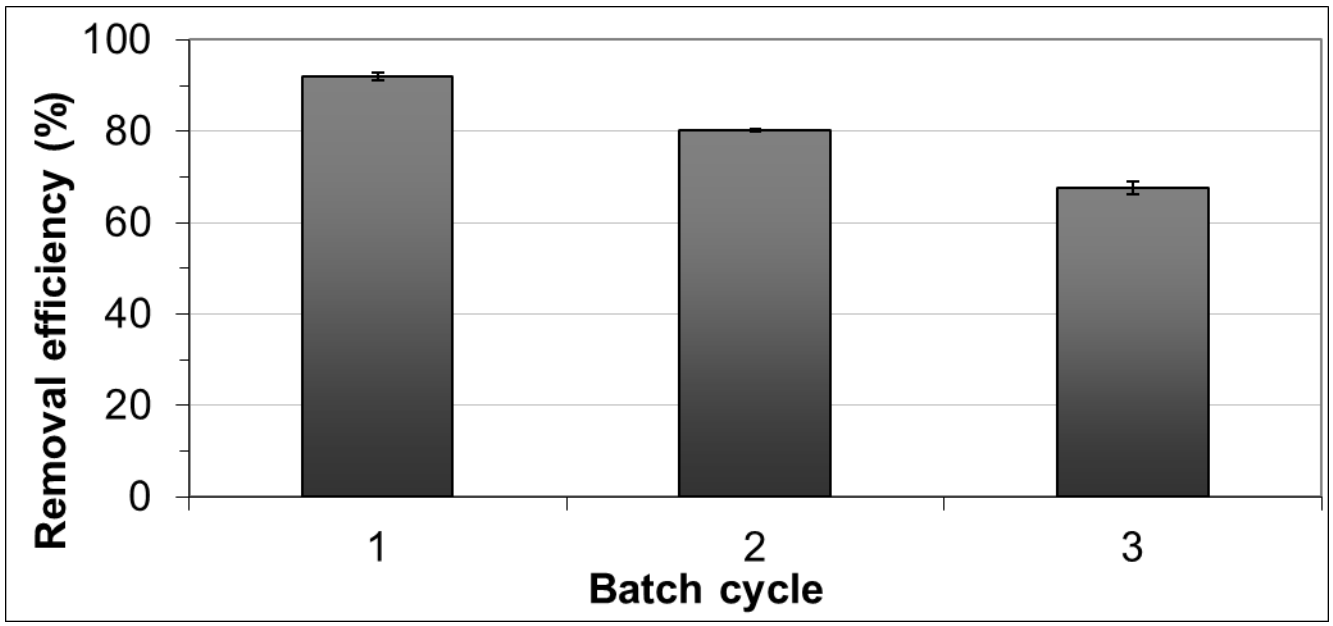
587 Fig. 13



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