

1 PROCESS BIOCHEMISTRY (ISSN: 1359-5113) (eISSN: 1873-3298) 50: (11) pp. 1835-1842. (2015)

2 [doi:10.1016/j.procbio.2015.08.008](https://doi.org/10.1016/j.procbio.2015.08.008)

3

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

7

8 **Laura Amina Dahili<sup>a</sup>, Ilona Kelemen-Horváth<sup>a</sup>, Tivadar Feczko<sup>a,b\*</sup>**

9 <sup>a</sup>Doctoral School of Molecular and Nanotechnologies, Research Institute of Chemical and Process  
10 Engineering, Faculty of Information Technology, University of Pannonia, Egyetem u. 10, H-8200  
11 Veszprém, Hungary, e-mails: [dahili@mukki.richem.hu](mailto:dahili@mukki.richem.hu), [kelemen@mukki.richem.hu](mailto:kelemen@mukki.richem.hu)

12 <sup>b</sup>Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences, Hungarian  
13 Academy of Sciences, Magyar tudósok körútja 2., H-1117, Budapest, Hungary

14 \*Corresponding author: e-mail: [tivadar.feczko@gmail.com](mailto:tivadar.feczko@gmail.com), phone: +36-88-623508, fax: +36-88-624038

15

16

17 **Abstract**

18

19 Horseradish peroxidase (HRP) is a promising catalyst in the enzymatic process of phenolic compounds  
20 removal from wastewaters. Enzyme immobilization provides important benefits in a biotechnological  
21 process. Fine particles with a high surface-to-volume ratio composed of ethyl cellulose (EC) polymer  
22 were generated by Nano Spray Dryer B-90 as supports for HRP. Carrier particles were prepared by spray  
23 caps with hole size of 7  $\mu\text{m}$ , 5.5  $\mu\text{m}$  and 4  $\mu\text{m}$ . Purified HRP and crude extract from horseradish were  
24 covalently bound to the carrier using a carbodiimide cross-linker. The attached HRP content, the effect of  
25 pH on the activity and the storage stability were investigated. 2,4-dichlorophenol, an extremely persistent  
26 chlorinated phenol was removed by the immobilized enzyme, and the effect of main process parameters  
27 such as  $\text{H}_2\text{O}_2$  and 2,4-dichlorophenol substrate concentrations were studied. After immobilization both the  
28 purified HRP and the horseradish extract performed better in the pH range of 4-10 and could preserve the  
29 activity substantially longer than the free enzyme. The immobilized enzyme was found to be  
30 outstandingly efficient (in optimal case close to 100 %) in the elimination of 2,4-dichlorophenol, which  
31 was also the consequence of the high adsorbing capacity of the fine particles. The reuse study proved the  
32 operational stability of HRP attached to EC even after 10 consecutive cycles.

33

34 **Keywords**

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

37

## 38 **1. Introduction**

39

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

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

53 Horseradish peroxidase is a heme-containing enzyme belonging to the class III of plant peroxidases. It has  
54 Fe(III) protoporphyrin IX as the prosthetic group, which plays an important role in its catalytic  
55 mechanism. Horseradish peroxidase possesses significant applications in life sciences, including  
56 bioassays, DNA-probes, biosensors, bioremediation of phenol and some of its derivatives [4].  
57 However, its industrial application is greatly limited by its low thermostability and low reactivity in organic  
58 media. HRP is also prone to suicide inactivation by the  $H_2O_2$  substrate in the applications such as  
59 diagnostics and biosensors as well as in wastewater treatment [5]. The relatively short lifetime of  
60 enzymes and their instability in harsh environment limit their applications. Thus, plenty of trials  
61 have been done on enzyme stabilization; such as entrapment of enzyme molecules in sol-gel,  
62 polymer matrixes and nanoporous materials, attachment of enzymes onto highly hydrophilic  
63 surfaces, e.g. chitosan, dextran and polyethylene glycol, and separation of enzyme layers from  
64 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  $\sim 1\text{--}2\ \mu\text{m}$ ) 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  $1\text{--}7\ \mu\text{m}$ , size  
74 range  $0.5$  to  $15\ \mu\text{m}$ , 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  $\text{Fe}_3\text{O}_4$  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  $\text{Fe}_3\text{O}_4$  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

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

100

## 101 **2. Materials and methods**

102

### 103 *2.1. Materials*

104

105 Purified HRP (223 U/mg) was purchased in dry solid form from Amresco (Solon, Ohio) and stored at -  
106 20°C until use. One unit of HRP activity was defined as the formation of 1.0 mg purpurogallin from  
107 pyrogallol in 20 s at 0.022 M initial pyrogallol concentration and 0.045 M H<sub>2</sub>O<sub>2</sub> in 3.0 ml distilled water.  
108 Crude extract was gained from horseradish plants and stored after extraction at -20°C.

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

116

### 117 *2.2. Extraction of horseradish*

118

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

127

### 128 *2.3. Spray drying with Nano Spray Dryer B-90*

129

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

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

145

146 *2.4. Analysis of the carrier particles*

147

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*

163

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

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

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

178

## 179 *2.6. HRP content determination*

180

181 Protein content of purified HRP cross-linked onto EC was measured by the Lowry-Folin assay. Total  
182 protein content was determined by the Folin-Ciocalteu phenol reagent via its reaction with tyrosine and  
183 tryptophan residues in proteins. Solution A contained 24.5 ml 4 % sodium carbonate and 24.5 ml 0.2 M  
184 sodium hydroxide. Solution B contained 0.5 ml 1 % copper sulfate and 0.5 ml 2.7 % potassium sodium  
185 tartrate. Solution A and B prepared daily were mixed and 2 ml of this mixture was added to the samples  
186 followed by vortexing. After 10 minutes, it was combined with 200  $\mu$ l of 2 N Folin-Ciocalteu phenol  
187 reagent (used in 1:1 dilution) and kept at room temperature for 30 minutes. Samples were centrifuged at  
188 13000 rpm for 5 minutes (Heraeus Biofuge pico) and the absorbance of the supernatant was measured at  
189 750 nm.

190

## 191 *2.7. Activity measurement*

192

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

198 The reaction was initiated by the addition of 1 mg/ml dispersed particles containing cross-linked HRP.

199 The absorbance was measured using a spectrophotometer (Pharmacia LKB-Biochrom) at 470 nm at room



200 temperature. The absorbance was monitored after 2 minutes and the obtained initial values (absorbance at  
201 0 s) were subtracted from the results during calculating the activity values, thus avoiding the light  
202 scattering effect of the particles. All measurements were performed in triplicate.

203

#### 204 *2.8. Effect of pH*

205

206 The activities of free and cross-linked HRP (purified and extracted) were measured at a pH range 4-10.  
207 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  
208 7.0-9.0) and 0.1 M sodium carbonate (pH 9.0-10.0) buffer solutions at room temperature.

209

#### 210 *2.9. Storage stability*

211

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

218

#### 219 *2.10. 2,4-dichlorophenol assay*

220

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

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

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

235 For reusability test samples containing 1.4 mM 2,4-dichlorophenol and 10 mg/ml carrier particles with  
236 crude extract from horseradish (0.087 U/ml) cross-linked on EC particles sprayed with a spray cap 4  $\mu\text{m}$   
237 were stirred with a magnetic stirrer at room temperature and hydrogen peroxide (2mM) initiated the  
238 reaction. After 2 hours, samples were ultracentrifuged with 30000 rpm for 15 minutes at 10°C and  
239 supernatant was analysed by HPLC. Remained particles were washed with TRIS buffer solution (0.02 M,  
240 pH6) after every cycle and reused, then, removal efficiency was assayed again. This procedure was  
241 repeated for 10 cycles. Control experiments were also performed under the same conditions without HRP  
242 and/or  $\text{H}_2\text{O}_2$  for 3 cycles.

243

### 244 **3. Results and discussion**

245

#### 246 *3.1. Particle size distribution and morphology*

247

248 Ethyl cellulose particle size was determined after the spray drying process by the laser diffraction method  
249 (Fig. 1). The majority of the EC particles were between 1 and 10  $\mu\text{m}$  by using 7  $\mu\text{m}$  and 5.5  $\mu\text{m}$  spray  
250 caps, whilst cc. 10 % of them were smaller than 1  $\mu\text{m}$  and most of them were below 3  $\mu\text{m}$  if generated by  
251 4  $\mu\text{m}$  spray cap hole size (Table 1).

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

261

### 262 *3.2. HRP immobilization*

263

264 The condition of HRP immobilization was optimized in our recent work [13]. In the present study we  
265 compared the HRP activity of particles prepared by the three different spray cap hole sizes. Both purified  
266 HRP and horseradish extract, respectively, were immobilized onto the ethyl cellulose support FTIR  
267 spectra were recorded for both the EC fine particles and the supports cross-linked by HRP. Successful  
268 attachment was proved by FTIR measurement that clearly showed the amide bond presence in the EC-  
269 HRP cross-linked sample (Fig. 3). Purified enzyme represented a higher residual activity after  
270 immobilization on particles prepared with any of the three spray caps (Fig. 4). The reason might be that  
271 extract of horseradish contains several other proteins as well, and the cross-linking procedure is not  
272 selective, which means that other proteins can also be attached to the activated sites of ethyl cellulose,  
273 thus competing with HRP. Although the crude extract was not analysed, some literature data is provided  
274 as an estimate of potentially fixed material. The average protein content of horseradish is 1.2 % (m/m)  
275 [15]. Lascu et al (1986) separated horseradish peroxidase by Sepharose CL-6B column from horseradish  
276 root [16]. They stated that probably every protein bound to the column, and 30-40 % of the bound material  
277 was found to be horseradish peroxidase.

278 As was expected, by decreasing the spray cap hole size the residual activity of HRP increased, since with  
279 a lower hole size, smaller particles with a higher specific surface area can be generated. However,  
280 comparing the size distribution of particles formed by spray cap hole size of 7  $\mu\text{m}$  and 5.5  $\mu\text{m}$ , the  
281 difference is much smaller than between those ones which were formed by 5.5  $\mu\text{m}$  or 4  $\mu\text{m}$  (Fig. 1).  
282 Moreover, according to the SEM images (Fig. 2) it can be concluded that spraying with a cap hole size of  
283 7  $\mu\text{m}$  resulted in significantly more particles with a doughnut shape than the one sprayed with 5.5  $\mu\text{m}$ . The  
284 ratio of doughnut- and spherical shapes, obtained after spraying with the three different spray caps, was  
285 calculated from minimum three SEM images, and average values were given in Table 2. This calculation  
286 showed that spraying with a 7  $\mu\text{m}$  hole size resulted in the highest ratio of doughnut-shaped carriers,  
287 which explains the higher amount of bound HRP related to carrier particles with a smaller size obtained  
288 by spraying using the 5.5  $\mu\text{m}$  hole size. The substantially higher residual activity of cross-linked HRP on  
289 the support obtained using a spray cap 4  $\mu\text{m}$  can be interpreted by the much lower size of formed particles  
290 related to the carriers prepared by the other two spray caps. While the residual activity of extracted HRP  
291 attached to the ethyl cellulose support ranged from 6 to 10 U/g, that of the purified enzyme was between  
292 10 and 28 U/g. These values are comparable with that of Leiriao et al. [17] who coupled HRP by  
293 glutaraldehyde and carbodiimide on hydrophilic polyacrylonitrile membrane and on silica and glass beads,  
294 respectively, and the HRP activity changed from 0.8 up to 28 U/g support. Similarly Lai and Lin [3]  
295 immobilized HRP to aminopropyltriethoxysilane-activated magnetite and reached 18 U/g maximal  
296 specific enzyme activity, while porous aminopropyl glass-immobilized HRP led to 42.7 U/g.  
297 The HRP mass attached to the support was investigated by the Lowry-Folin assay. It was studied  
298 exclusively with the purified HRP since horseradish extract also contains other proteins (Fig. 5), which  
299 makes it impossible to determine the HRP content of those supports crosslinked with crude extract of  
300 horseradish. Protein content was found to be similar on ethyl cellulose carriers prepared by spray caps of 7  
301  $\mu\text{m}$  and 5.5  $\mu\text{m}$  in accordance with the active HRP concentration, although it was slightly higher at  
302 support formed by a 5.5  $\mu\text{m}$  cap hole; nevertheless, taking into consideration the standard deviations the  
303 difference might be negligible. In agreement with active HRP measurement the highest HRP content (7  
304 mg/g) could be cross-linked to the fine particles formulated by the smallest spray cap. As a comparison

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

315

### 316 *3.3. pH working range*

317

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

331

332 *3.4. Storage stability*

333

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

345

346 *3.5. 2,4-dichlorophenol removal*

347

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

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

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

364 2,4-dichlorophenol removal efficiency was also studied as a function of substrate concentration. While  
365 adding 10 mg/ml solid support containing 0.093 U/ml HRP (H<sub>2</sub>O<sub>2</sub> concentration: 2 mM), the increasing  
366 substrate concentration resulted in a slight decrease in the removal efficiency (Fig. 9a). Although low  
367 enzyme concentration (2 mg/ml support with 0.012 U/ml HRP) represented a similar tendency (H<sub>2</sub>O<sub>2</sub>  
368 concentration: 1 mM), but with significantly lower removal efficiency (Fig. 10b). It can be concluded that  
369 both low substrate and low enzyme concentration caused inhibition in the elimination reaction. It is also  
370 clear that even the highest conversion of 2,4-dichlorophenol with 2 mg/ml support containing 0.012 U/ml  
371 HRP was also significantly lower than the removal obtained using 10 mg/ml particles containing 0.093  
372 U/ml HRP in the solution of the same substrate concentration.

373 The effect of carrier particle size and that whether the enzyme was purified or present in crude extract  
374 were analyzed. 10 mg/ml carrier was homogenized in the reaction mixture containing 300 mM H<sub>2</sub>O<sub>2</sub>  
375 concentration, which contained 0.067 U/ml and 0.096 U/ml active extracted and purified HRP,  
376 respectively. There was no significant difference between enzyme either immobilized on ethyl cellulose  
377 particles generated using a spray-cap with different hole sizes or obtained from different sources  
378 possessing substantially diverse activity (Fig. 11).

379 Repeated use of HRP attached to the fine EC particles was examined at the optimal removal conditions  
380 (Fig. 12). It was shown that even after 10 reuse the removal of the persistent substrate exceeded 60 %  
381 efficiency. Control tests without HRP enzyme were also performed in order to investigate the adsorption  
382 capacity of the fine support with high surface area. Surprisingly, the removal was very high without the  
383 enzyme (Fig. 13), however after 3 repeated batch cycle its value dropped to that level (cc. 65 %) which  
384 was reached by the immobilized enzyme even after 9 reusing. Moreover, it was found that there was not  
385 significant difference between removal efficiency of EC particles with or without H<sub>2</sub>O<sub>2</sub>, 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  $\mu\text{m}$ , 5.5  $\mu\text{m}$  and 4  $\mu\text{m}$ ) 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  $\text{H}_2\text{O}_2$   
402 concentrations. Chlorinated phenol and  $\text{H}_2\text{O}_2$  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

## 408 **Acknowledgements**

409

410 Ethyl cellulose supply is courtesy of Dow Deutschland Anlagengesellschaft mbH. Authors are grateful to  
411 László Hajba for the FTIR measurements.

412



413 **References**

414

415 [1] Gomez JL, Bodalo A, Gomez E, Bastida J, Hidalgo AM, Gomez M. Immobilization of peroxidases on  
416 glass beads: An improved alternative for phenol removal. *Enzyme and Microbial Technology*  
417 2006;39:1016–1022.

418 [2] Klibanov AM, Alberti BN, Morris ED, Felshin LM. Enzymatic removal of toxic phenols and  
419 anilines from waste waters. *Journal of Applied Biochemistry* 1980;2:414–21.

420 [3] Lai YC, Lin SC. Application of immobilized horseradish peroxidase for the removal of p-chlorophenol  
421 from aqueous solution. *Process Biochemistry* 2005;40:1167–1174.

422 [4] Khosravi A, Vossoughi M, Shahrokhian S, Alemzadeh I. Nano reengineering of horseradish  
423 peroxidase with dendritic macromolecules for stability enhancement. *Enzyme and Microbial*  
424 *Technology* 2012;50:10–16.

425 [5] Asad S, Torabi SF, Fathi-Roudsari M. Phosphate buffer effects on thermal stability and H<sub>2</sub>O<sub>2</sub>-  
426 resistance of horseradish peroxidase. *International Journal of Biological Macromolecules* 2011;48:566–  
427 570.

428 [6] D’Annibale A, Stazi SR, Vinciguerra V, Di Mattia E, Sermanni GG. Characterization of immobilized  
429 laccase from *Lentinula edodes* and its use in olive-mill wastewater treatment. *Process Biochemistry*  
430 1999;34:697–706.

431 [7] Caramori SS, Fernandes KF. Covalent immobilisation of horseradish peroxidase onto poly (ethylene  
432 terephthalate)–poly(aniline) composite. *Process Biochemistry* 2004;39:883–888.

433 [8] Lee SH, Heng D, Ng WK, Chan HK, Tan RBH. Nano spray drying: A novel method for preparing  
434 protein nanoparticles for protein therapy. *International Journal of Pharmaceutics* 2011;403:192–200.

435 [9] Chan HK, Kwok PCL. Production methods for nanodrug particles using the bottom-up approach.  
436 *Advanced Drug Delivery Reviews* 2011;63:406–416.

437 [10] Chang Q, Tang H. Immobilization of horseradish peroxidase on NH<sub>2</sub>-modified magnetic  
438 Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub> particles and its application in removal of 2,4-dichlorophenol. *Molecules* 2014;19:15768–  
439 15782.

- 440 [11] Chang Q, Tang H. Optical determination of glucose and hydrogen peroxide using a nanocomposite  
441 prepared from glucose oxidase and magnetite nanoparticles immobilized on graphene oxide.  
442 *Microchimica Acta* 2014;181:527-534.
- 443 [12] Huang J, Chang Q, Ding Y, Han X, Tang H. Catalytic oxidative removal of 2,4-dichlorophenol by  
444 simultaneous use of horseradish peroxidase and graphene oxide/Fe<sub>3</sub>O<sub>4</sub> as catalyst. *Chemical Engineering*  
445 *Journal* 2014;254:434-442.
- 446 [13] Dahili LA, Feczko T. Cross-linking of horseradish peroxidase enzyme to fine particles generated by  
447 nano spray dryer B-90. *Periodica Polytechnica Chemical Engineering* 2015;59:209-214.
- 448 [14] Iskandar F, Gradon L, Okuyama K. Control of the morphology of nanostructured particles prepared  
449 by the spray drying of a nanoparticle sol. *Journal of Colloid and Interface Science* 2003;265:296–303.
- 450 [15] Nutrient Data Laboratory, ARS, USDA. Nutrient content of ethnic and geographic specific foods,  
451 Southern Testing and Research Laboratories, 1995 Beltsville MD.
- 452 [16] Lascu I, Abrudan I, Muresan L, Presecan E, Vonica A, Proinov I. Salting-out chromatography on  
453 unsubstituted Sepharose CL-6B as a convenient method for purifying proteins from dilute crude extracts :  
454 Application to horseradish peroxidase. *Journal of Chromatography A* 1986; 357:436-439.
- 455 [17] Leiriao PRS, Fonseca LJP, Taipa MA, Cabral JMS, Mateus M. Horseradish peroxidase immobilized  
456 through its carboxylic groups onto a polyacrylonitrile membrane. *Applied Biochemistry and*  
457 *Biotechnology* 2003;110:1–10.
- 458 [18] Azevedo AM, Vojinovic V, Cabral JMS, Gibson, TD, Fonseca LP. Operational stability of  
459 immobilized horseradish peroxidase in mini-packed bed bioreactors. *Journal of Molecular Catalysis B*  
460 *Enzymatic* 2004;28:121–8.
- 461 [19] Pundir CS, Malik V, Bhargava AK, Thakur M, Kalia V, Singh S, Kuchhal NK. Studies on  
462 horseradish peroxidase immobilized onto arylamine and alkylamine glass. *Journal of Plant Biochemistry*  
463 *& Biotechnology* 1999;8:123-126.
- 464 [20] Fernandes KF, Lima CS, Lopes FM, Collins CH. Properties of horseradish peroxidase immobilised  
465 onto polyaniline. *Process Biochemistry* 2004;39:957–962.

466 [21] Feng Q, Tang, B, Wei, QF, Hou DY, Bi SM, Wei AF. Preparation of a Cu(II)-PVA/PA6 composite  
467 nanofibrous membrane for enzyme immobilization. *International Journal of Molecular Sciences*,  
468 2012;13:12734–12746.

469 [22] Bayramoğlu G, Arica MY. Enzymatic removal of phenol and p-chlorophenol in enzyme reactor:  
470 Horseradish peroxidase immobilized on magnetic beads. *Journal of Hazardous Materials* 2008;156:148–  
471 155.

472 [23] Fritzen-Garciaa MB, Monteiroa FF, Cristofolinia T, Acuña JJS, Zanetti-Ramosd BG, Oliveirae  
473 IRWZ, Soldif V, Pasag AA, Creczynski-Pasaa TB. Characterization of horseradish peroxidase  
474 immobilized on PEGylated polyurethane nanoparticles and its application for dopamine detection. *Sensors*  
475 and *Actuators B: Chemical* 2013;182:264–272.

476 [24] Kalaiarasan E, Palvannan T. Removal of phenols from acidic environment by horseradish peroxidase  
477 (HRP): Aqueous thermostabilization of HRP by polysaccharide additives. *Journal of the Taiwan Institute*  
478 *of Chemical Engineers* 2014;45:625–634.

479 [25] Roper JC, Sarkar JM, Dec J, Bollag JM. Enhanced enzymatic removal of chlorophenols in the  
480 presence of co-substrates. *Water Research* 1995;29:2720–2724.

481 [26] Laurenti E, Ghibaudi E, Ardisson S, Ferrari RP. Oxidation of 2,4-dichlorophenol catalysed by  
482 horseradish peroxidase: characterization of the reaction mechanism by UV-visible spectroscopy and mass  
483 spectrometry. *Journal of Inorganic Biochemistry* 2003;95:171–176.

484 [27] Pramparo L, Stüber F, Font J, Fortuny A, Fabregat A, Bengoa C. Immobilisation of horseradish  
485 peroxidase on Eupergit<sup>®</sup>C for the enzymatic elimination of phenol. *Journal of Hazardous Materials*  
486 2010;177:990–1000.

487 [28] Nazari K, Esmaili N, Mahmoudi A, Rahimi H, Moosavi-Movahedi AA. Peroxidative phenol  
488 removal from aqueous solutions using activated peroxidase biocatalyst. *Enzyme and Microbial*  
489 *Technology* 2007;41:226–233.

490  
491

492 Table 1. Volume mean diameters (D [4.3]) and d (0.1), d (0.5), d (0.9) values of nano spray dried ethyl  
 493 cellulose (EC) using spray cap hole size 7  $\mu\text{m}$ , 5.5  $\mu\text{m}$  and 4  $\mu\text{m}$ .

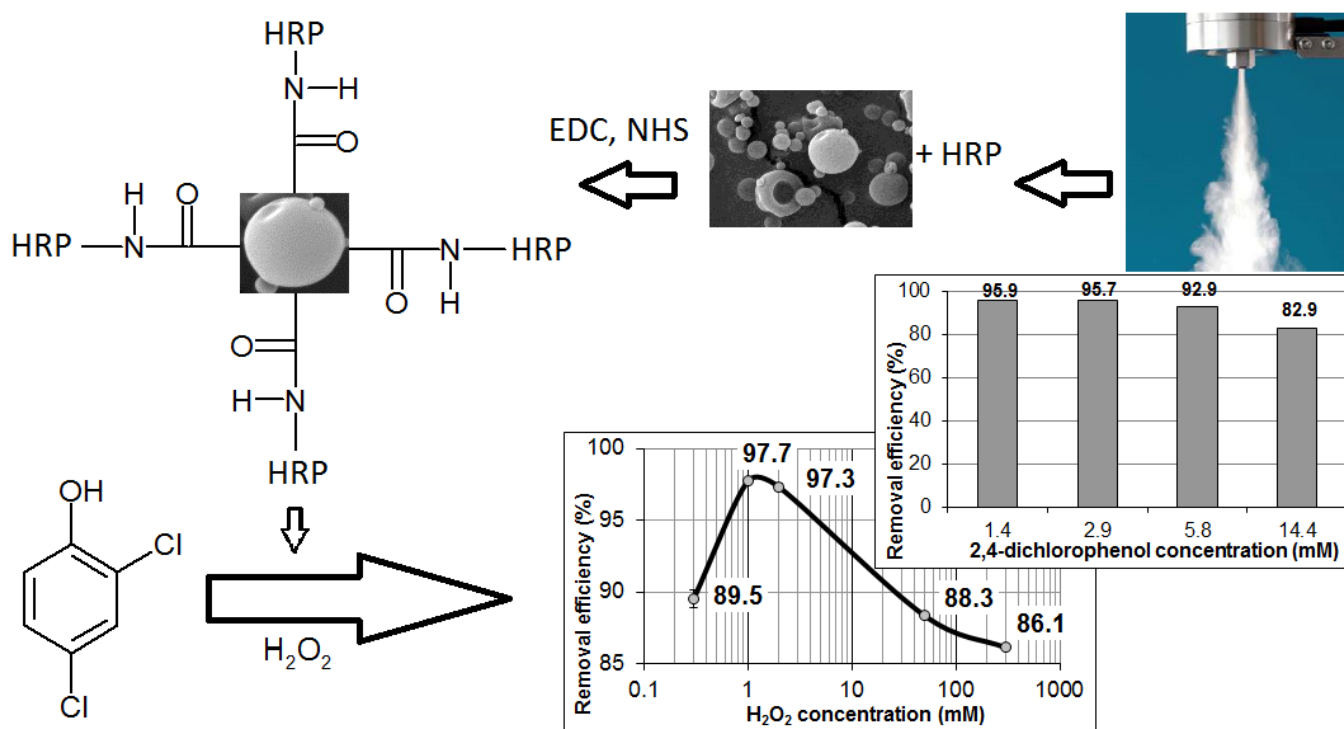
Sample Names	D [4.3]	d (0.1)	d (0.5)	d (0.9)
EC 7 $\mu\text{m}$	4.2	1.4	3.4	8.2
EC 5.5 $\mu\text{m}$	3.0	1.4	2.7	5.14
EC 4 $\mu\text{m}$	1.7	1.0	1.5	2.6

498 Table 2. Percentage of doughnut and spherical shape of nano spray dried ethyl cellulose carriers. Spray  
 499 caps were applied with hole size of 7  $\mu\text{m}$ , 5.5  $\mu\text{m}$  and 4  $\mu\text{m}$ .

Spray cap hole size	Doughnut shape (%)	Spherical shape (%)
7 $\mu\text{m}$	26	74
5.5 $\mu\text{m}$	8	92
4 $\mu\text{m}$	19	81

503

504 Graphical abstract



505

506

507 Figure captions:

508

509 Fig. 1. Particle size distribution of ethyl cellulose particles. The supports were nano spray dried using

510 spray caps with hole size of 7  $\mu\text{m}$ , 5.5  $\mu\text{m}$  and 4  $\mu\text{m}$ .

511 Fig. 2. SEM images of nano spray dried ethyl cellulose particles. For preparation spray cap hole size 7  $\mu\text{m}$   
512 (a, d), 5.5  $\mu\text{m}$  (b, e) and 4  $\mu\text{m}$  (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  $\mu\text{m}$ .

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  $\text{H}_2\text{O}_2$  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  $\mu\text{m}$ , respectively.

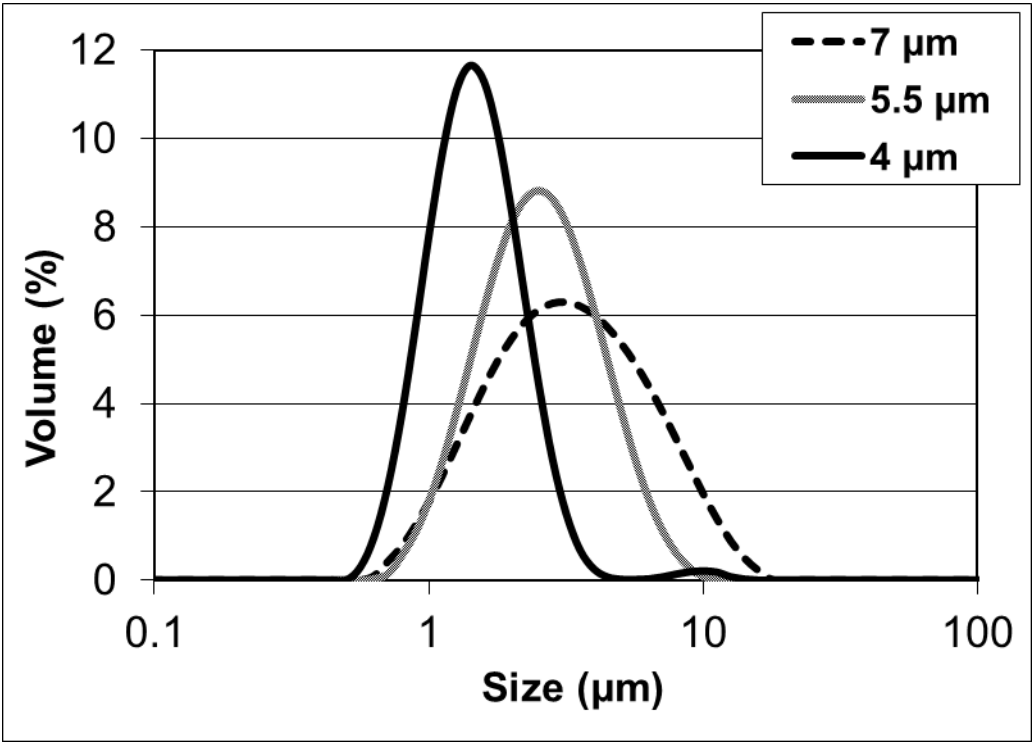
533 Fig. 12. 2,4-dichlorophenol removal efficiency (%) of extract from horseradish attached to ethyl cellulose  
534 particles in repeated batch operation.

535 Fig. 13. 2,4-dichlorophenol removal efficiency (%) of ethyl cellulose particles in repeated batch operation.

536

537

538 Fig. 1.

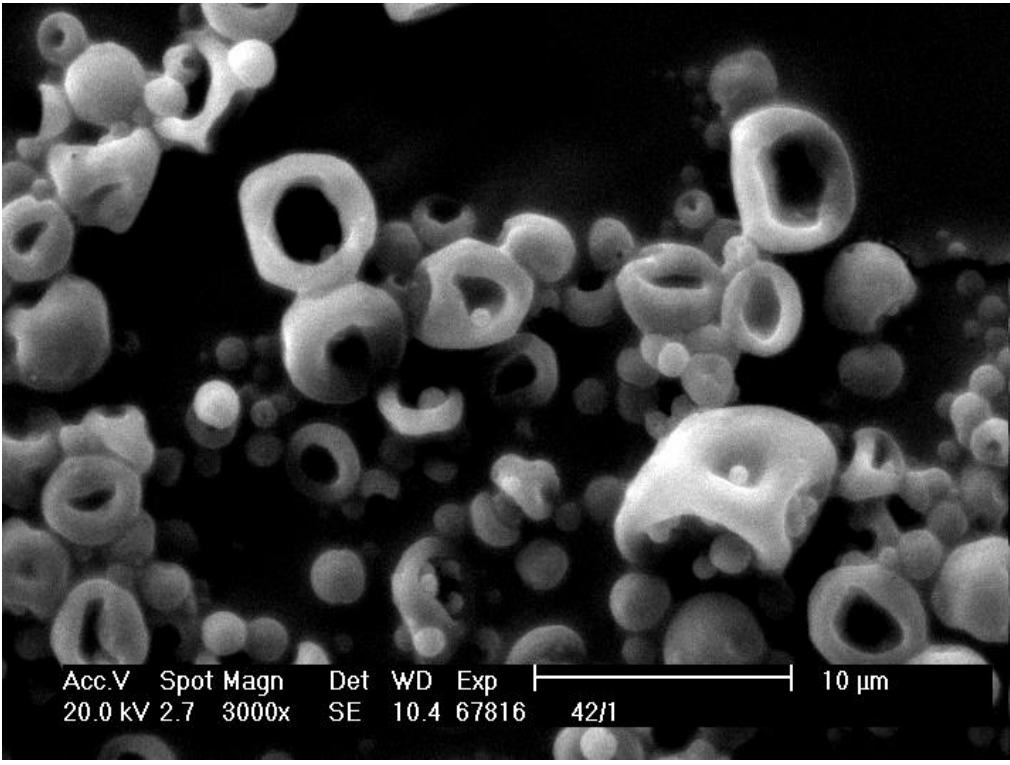


539

540

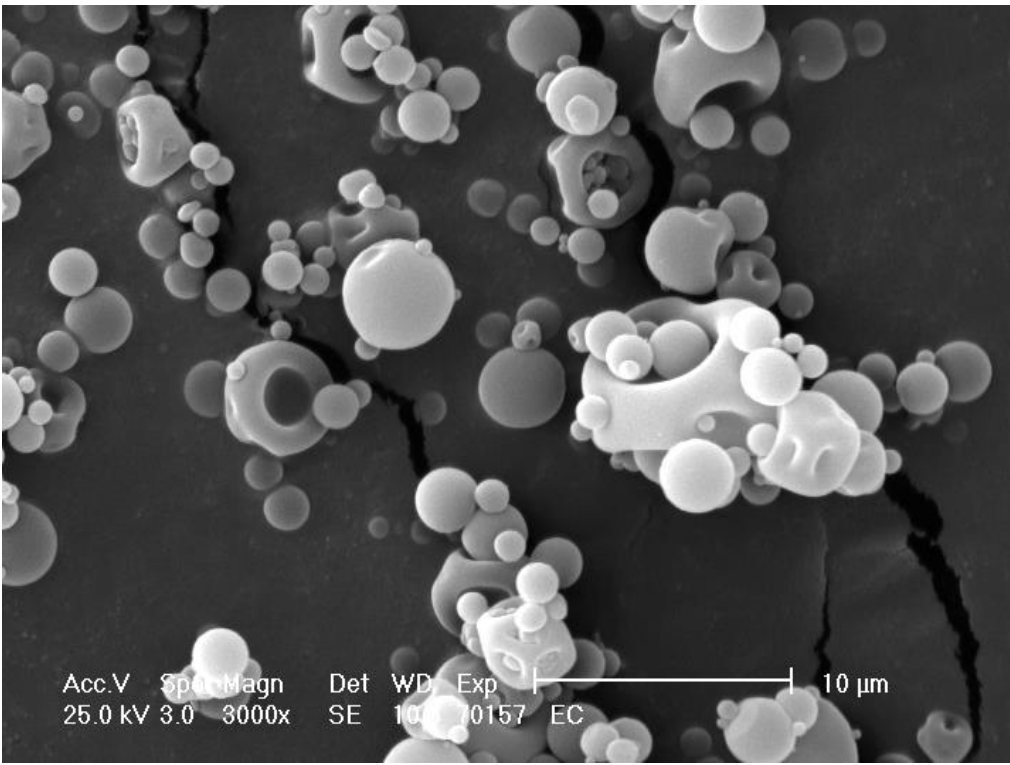
541

542 Fig. 2a



543

544 Fig. 2b

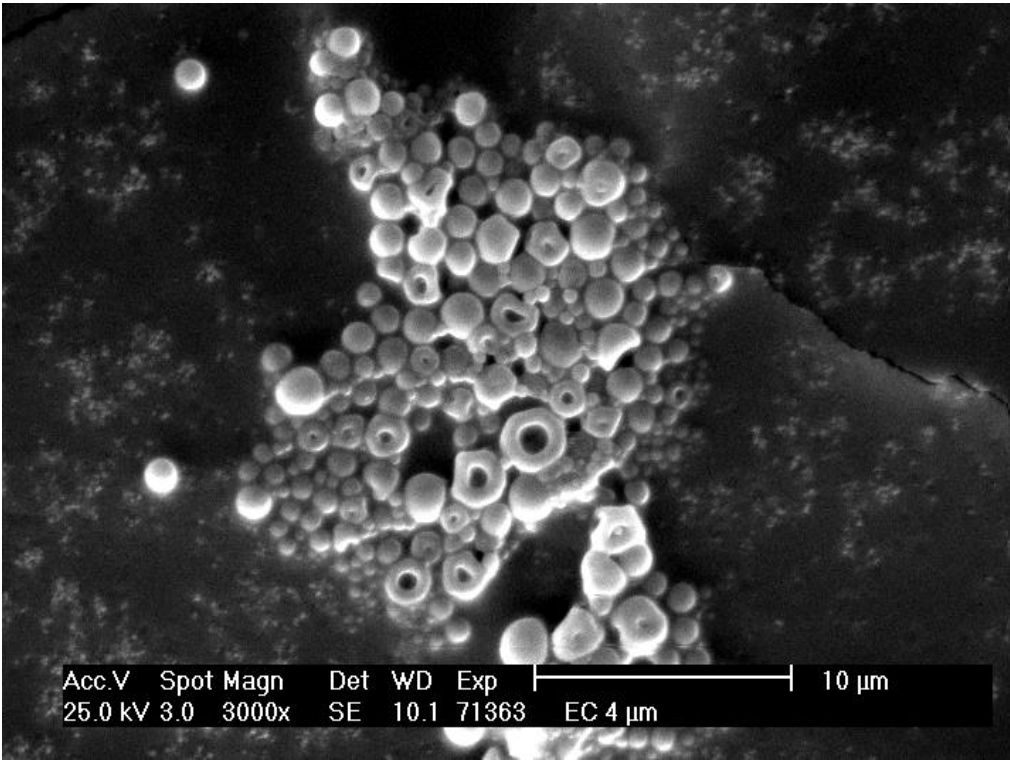


545

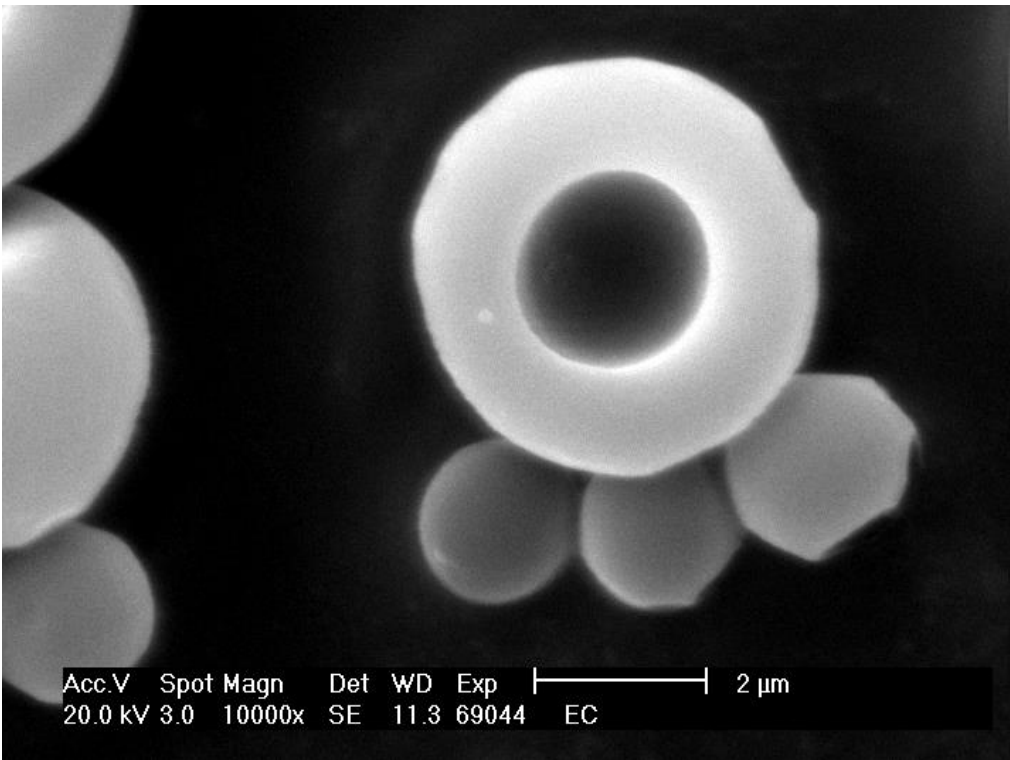
546

547

548 Fig. 2c

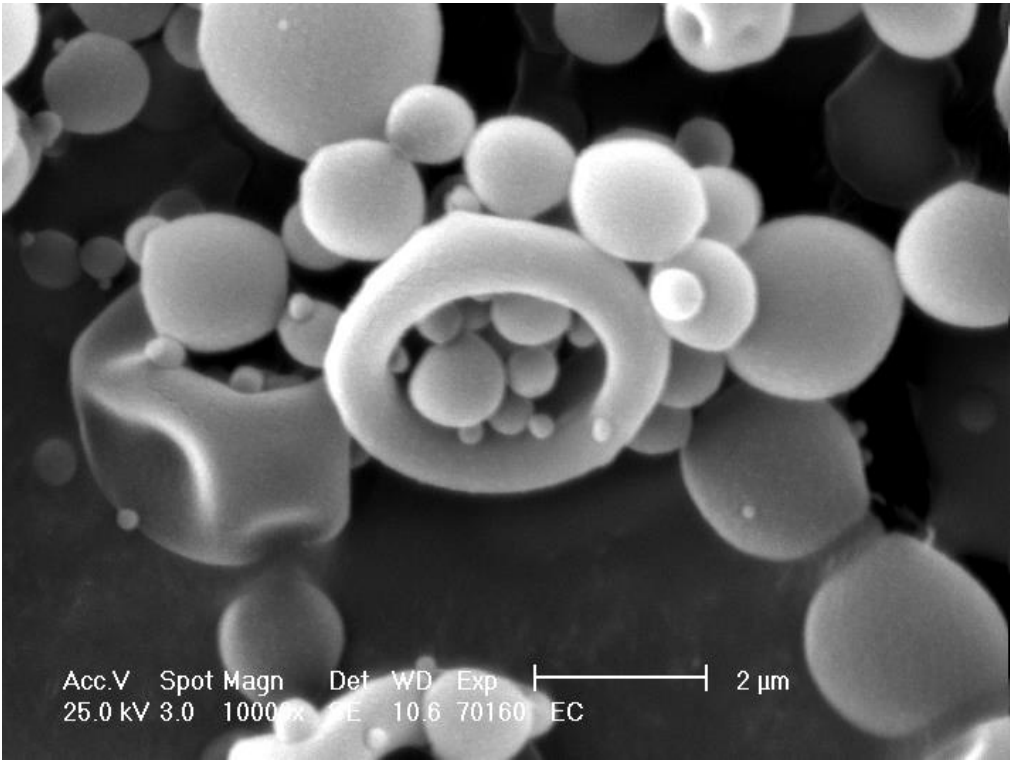


550 Fig 2d



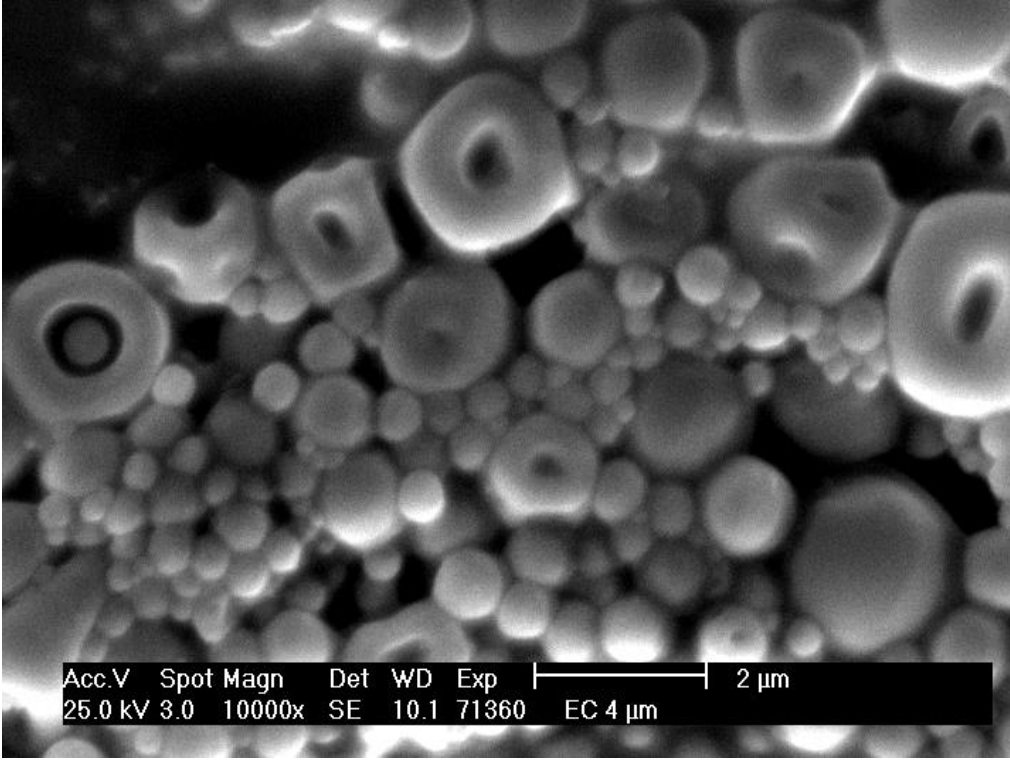


553 Fig 2e



554

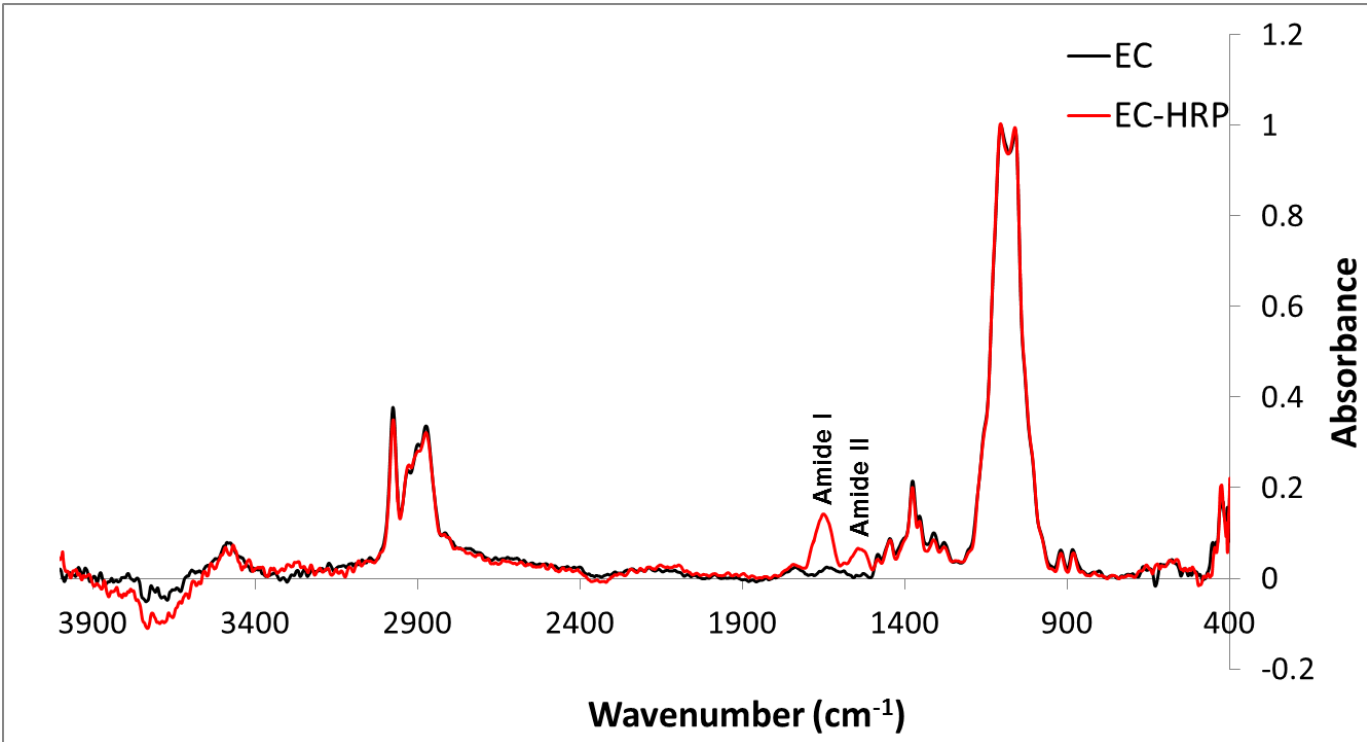
555 Fig 2f



556

557

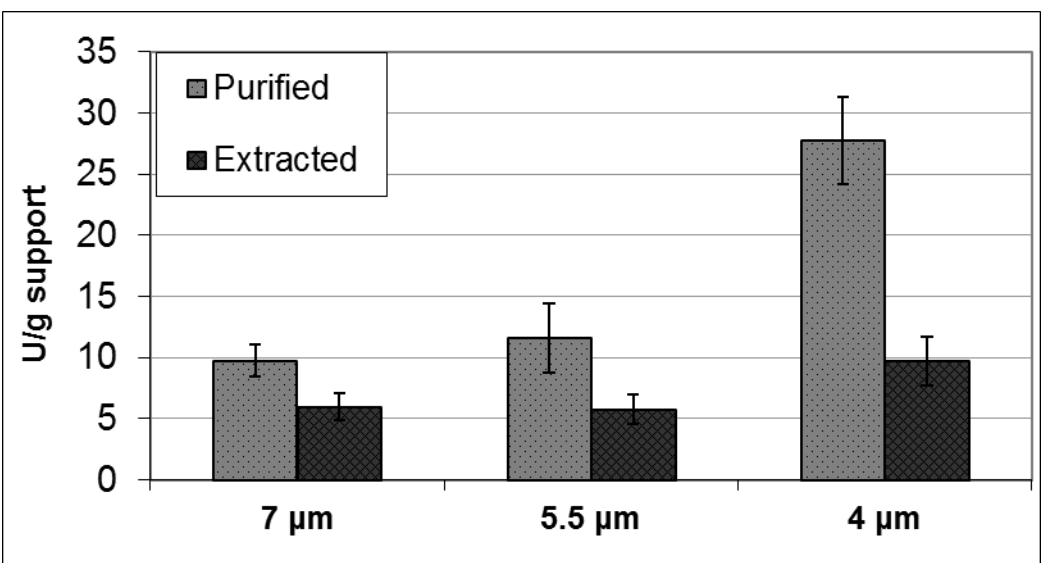
558 Fig. 3



559

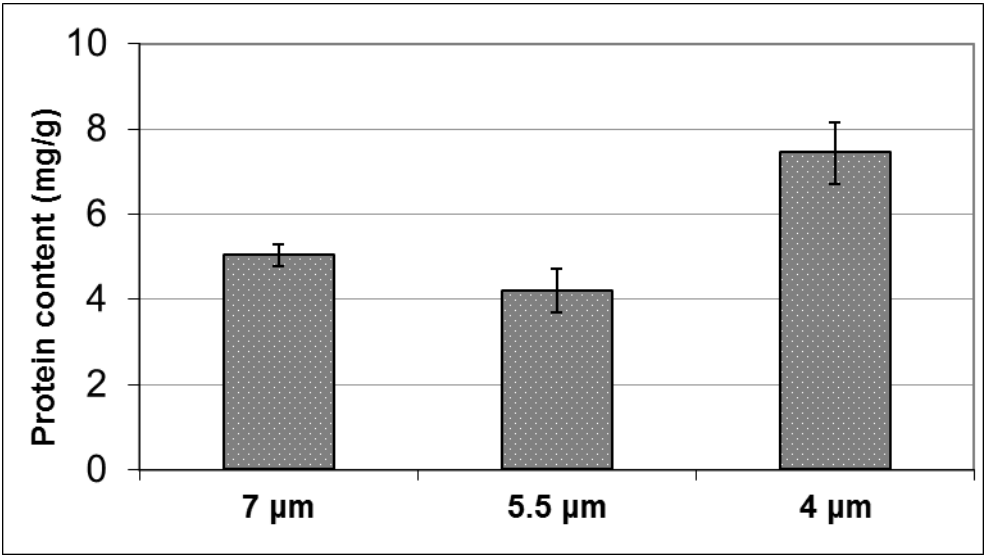
560

561 Fig. 4



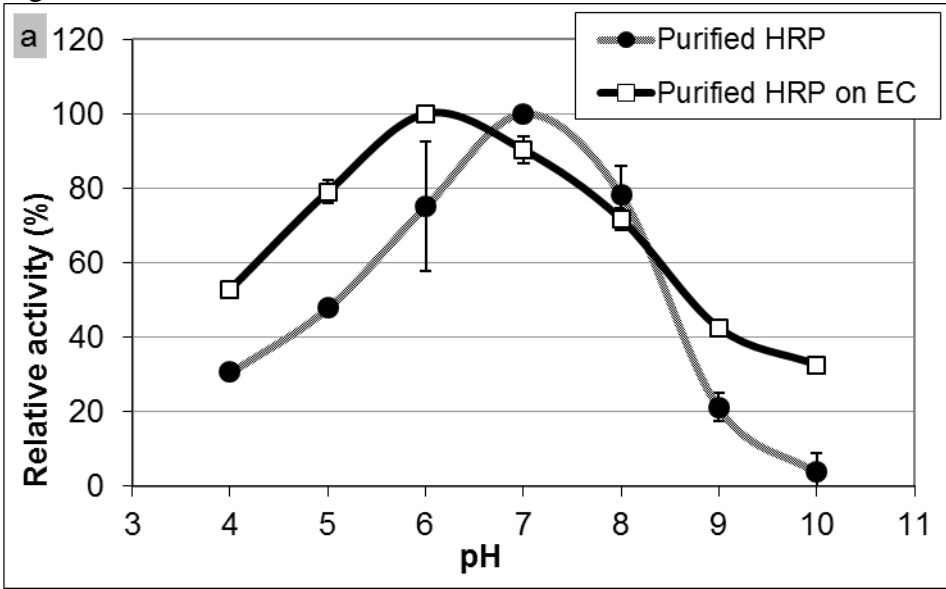
562

563



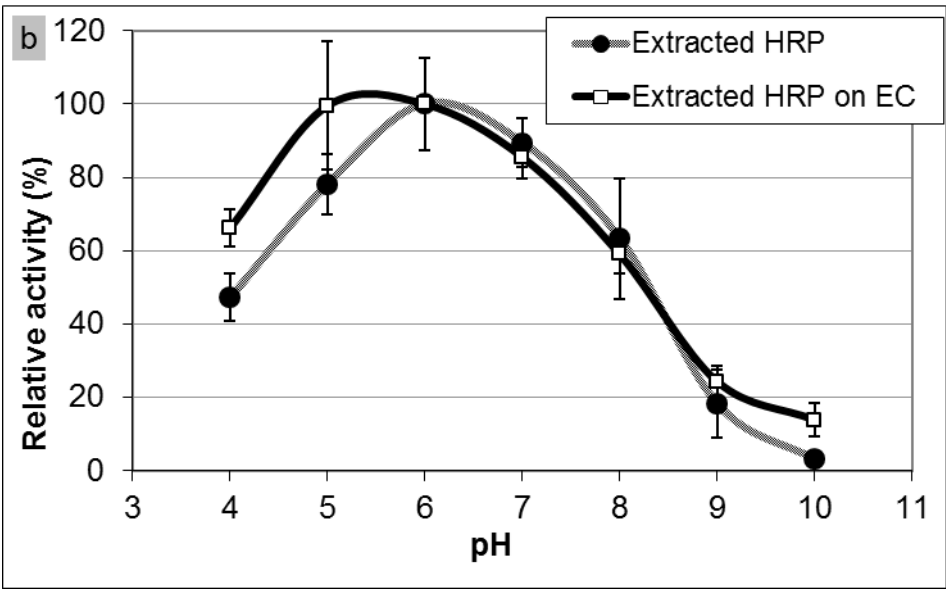
565

566 Fig. 6a



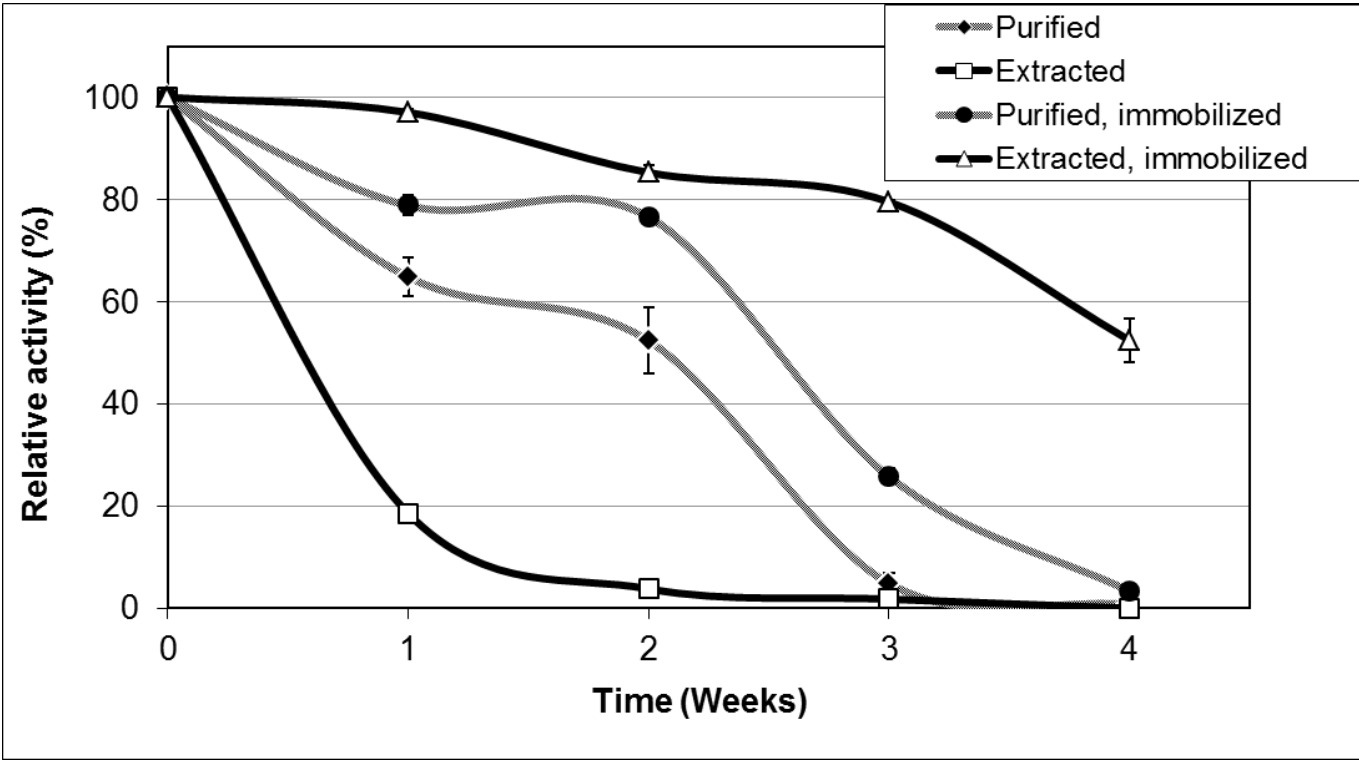
567

568 Fig. 6b



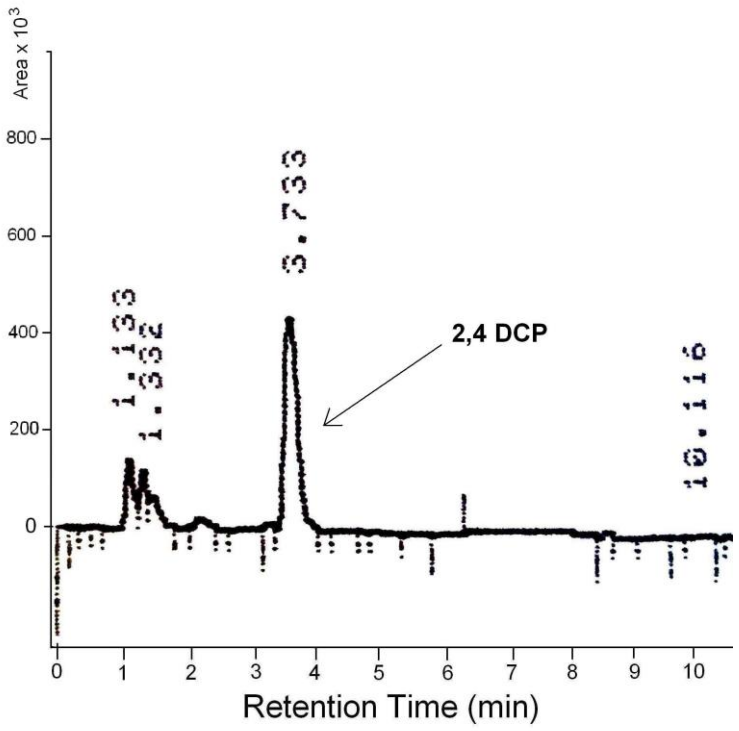
569

570 Fig. 7



571

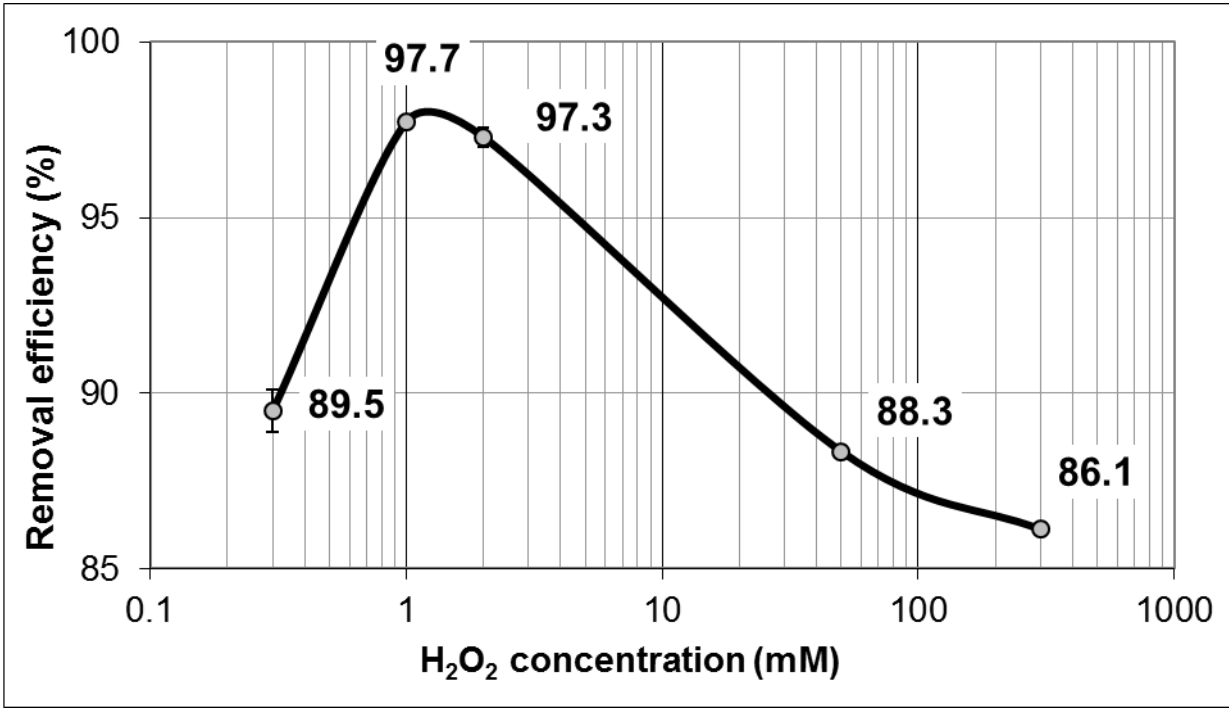
572 Fig. 8



573

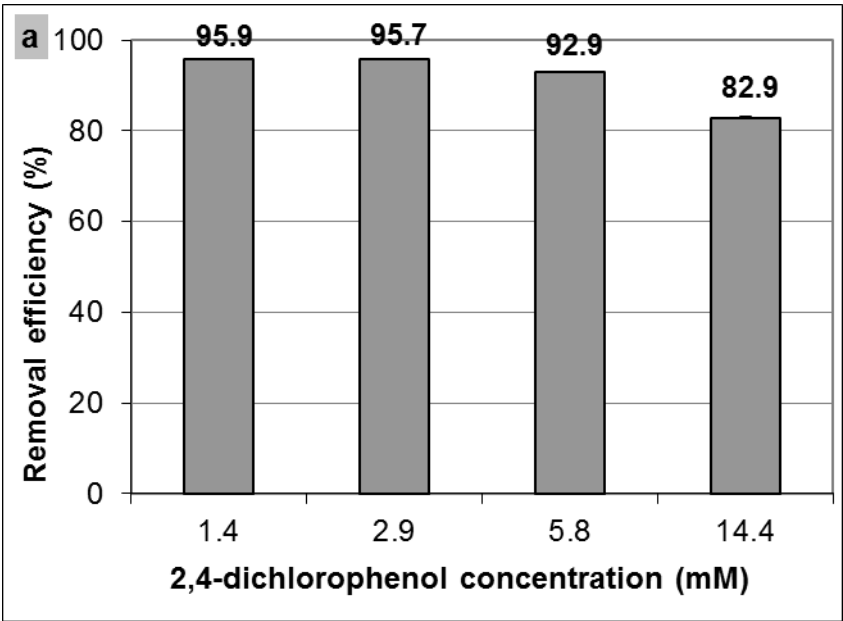
574

575 Fig. 9



576

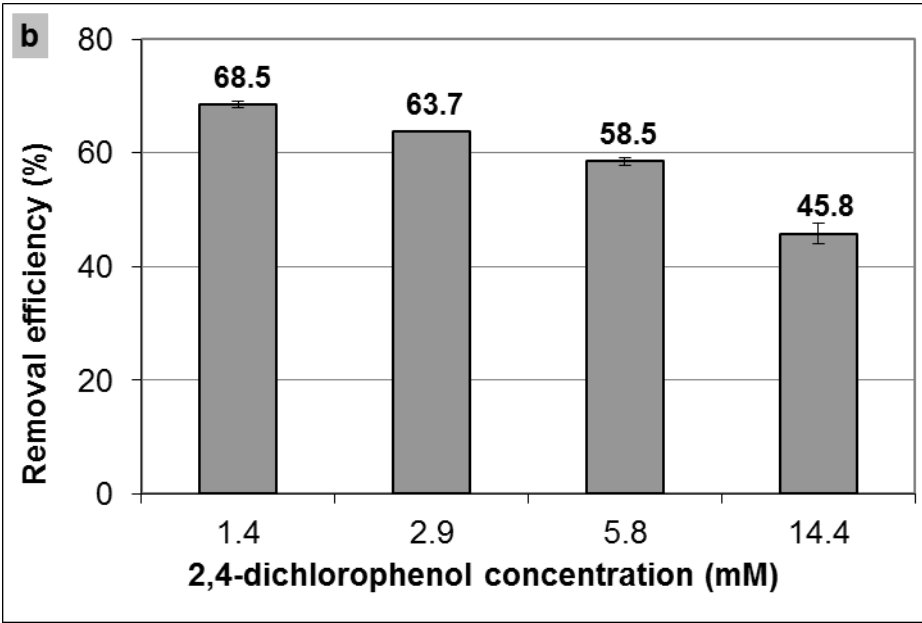
577 Fig.10a



578

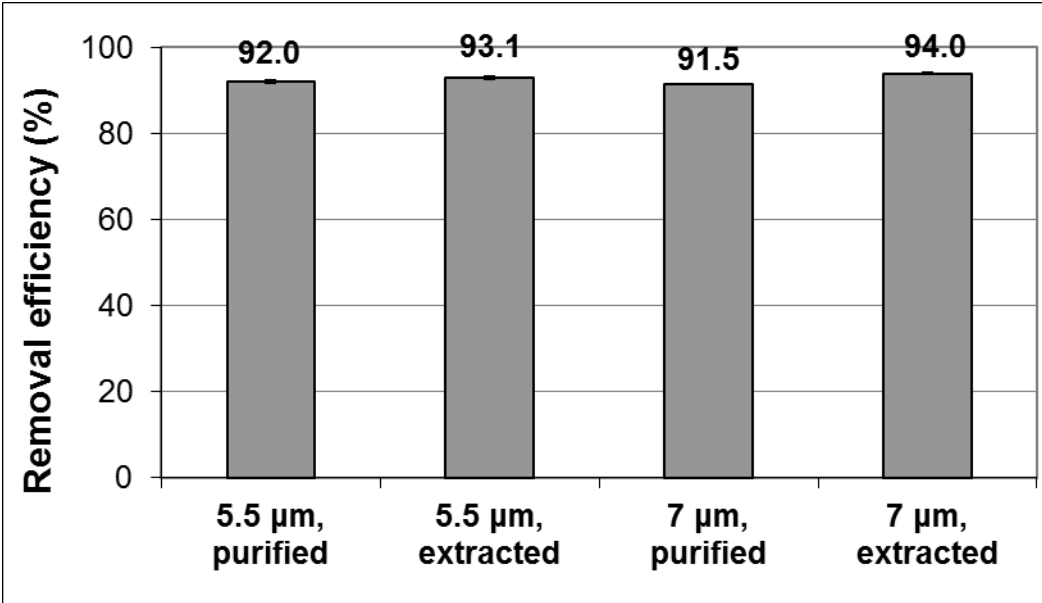
579

580 Fig. 10b



581

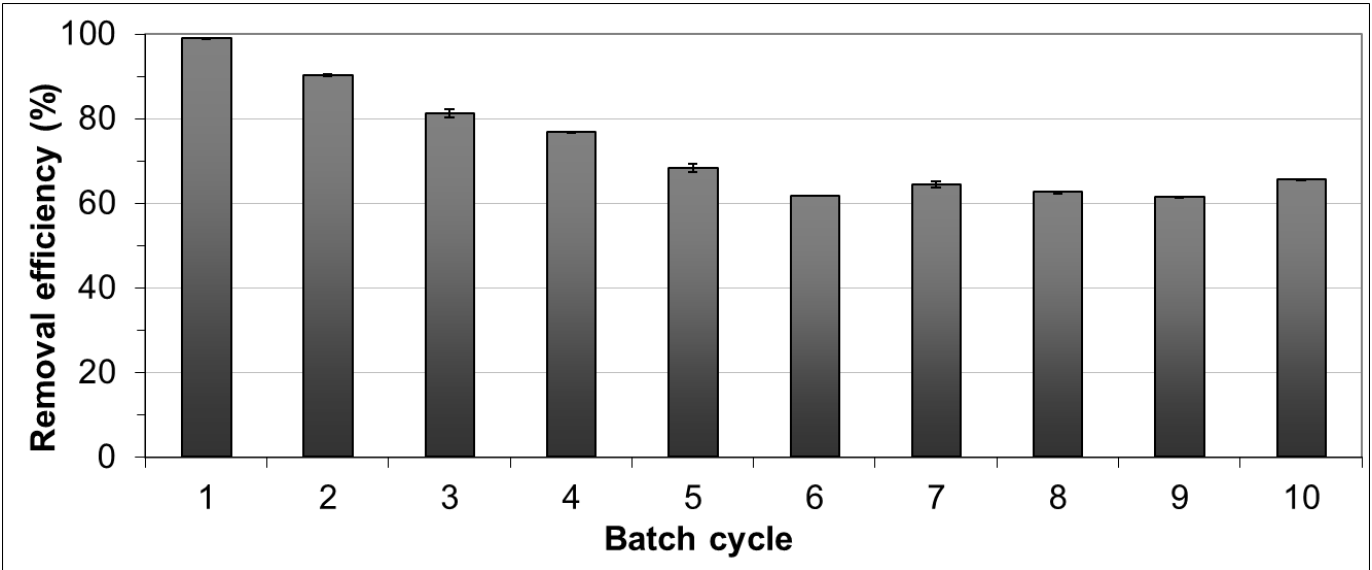
582 Fig. 11



583

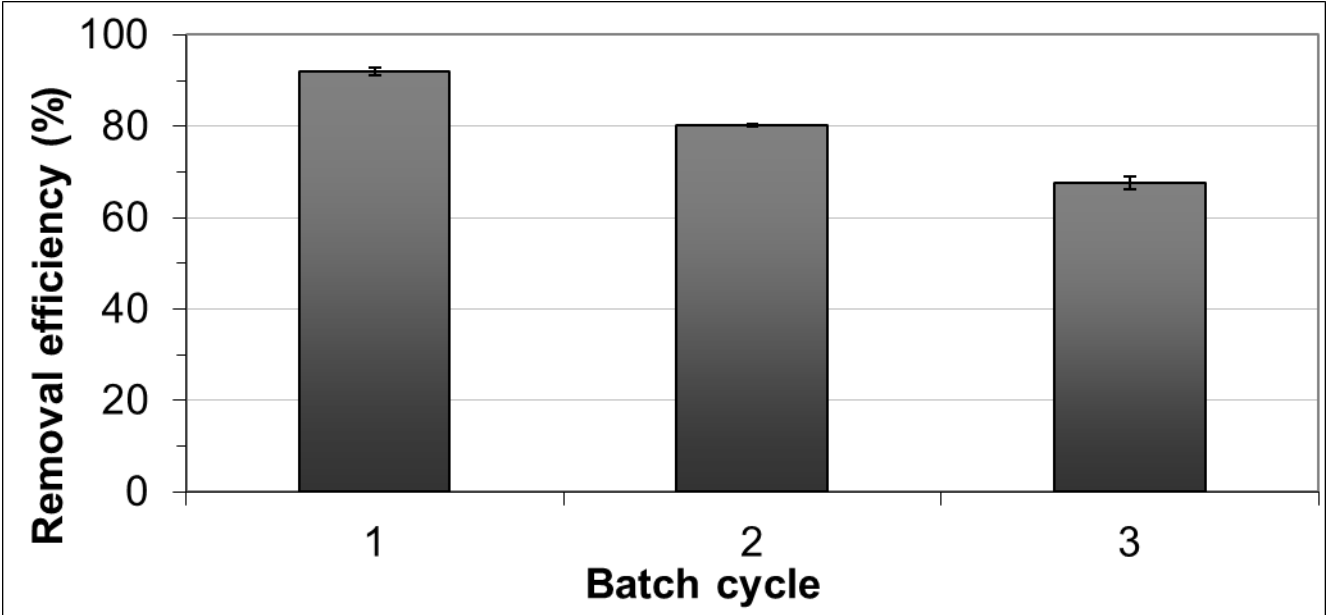
584

585 Fig. 12



586

587 Fig. 13



588

589

590

591

592

593