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Scale up of 2,4-dichlorophenol removal from aqueous solutions using *Brassica napus* hairy roots

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ABSTRACT

Chlorophenols are harmful pollutants, frequently found in the effluents of several industries. For this reason, many environmental friendly technologies are being explored for their removal from industrial wastewaters. The aim of the present work was to study the scale up of 2,4-dichlorophenol (2,4-DCP) removal from synthetic wastewater, using *Brassica napus* hairy roots and H_2O_2 in a discontinuous stirred tank reactor. We have analyzed some operational conditions, because the scale up of such process was poorly studied. High removal efficiencies were obtained (98%) in a short time (30 min). When roots were re-used for six consecutive cycles, 2,4-DCP removal efficiency decreased from 98 to 86%, in the last cycle. After the removal process, the solutions obtained from the reactor were assessed for their toxicity using an acute test with *Lactuca sativa* L. seeds. Results suggested that the treated solution was less toxic than the parent solution, because neither inhibition of lettuce germination nor effects in root and hypocotyl lengths were observed. Therefore, we provide evidence that *Brassica napus* hairy roots could be effectively used to detoxify solutions containing 2,4-DCP and they have considerable potential for a large scale removal of this pollutant. Thus, this study could help to design a method for continuous and safe treatment of effluents containing chlorophenols.

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1. Introduction

Chlorophenols are among the most frequent persistent pollutants found in the environment. They are highly toxic due to their chlorine substituents and their toxicity increases with the degree of chlorination of the phenol ring [1]. They have been extensively used in the production of pesticides, herbicides and wood preservatives. Thus, they are frequently found not only in industrial effluents but also in soil and groundwater. In addition, in several countries, waste solutions generated in various industrial processes are discharged into natural water streams without a previous treatment, producing serious environmental problems.

The World Health Organization (WHO) [2] has established limit concentrations for chlorophenols in drinking-water less than 1 mg/L, whereas in our country, Argentina, law 24,051 of hazardous residues established guide levels for drinking water and surface waters of 0.3 and 4 μg/L respectively, for 2,4-DCP. However, higher concentrations than those mentioned were frequently found in contaminated environments, where the reported levels of chlorophenols ranged from 150 μg/L to 200 mg/L and even more [3–5]. Therefore, the removal of such compounds from water and soils is of relevant significance.

As a consequence, various physico-chemical treatments have been proposed to remediate polluted sites. However, these treatments are complex and, sometimes, they produced more toxic compounds than the former. This situation is triggering the development of new treatment technologies for these wastewaters with high 2,4-DCP concentrations, especially environmentally friendly technologies and which do not produce toxic by-products.

Some biological methodologies were described for 2,4-DCP degradation including the use of algae [1]; fungi [6] and bacteria [7]. Another approach was based on the use of several oxidative enzymes, belonging to the class of oxidoreductases, such as peroxidases from different plant and fungal origin [8,9]. These enzymes catalyzed the oxidation of phenolic compounds with H_2O_2 forming free-radicals. Then, products undergo non-enzymatic reactions leading to the formation of insoluble polymers that can be removed by sedimentation or filtration. In addition to this previously described approach, plant tissues like roots [10] and hairy root cultures of various plant species had been used for removal of phenolic compounds [11–13].

Despite the fact that the application of hairy root cultures for decontamination of several organic compounds was previously proposed by us and other authors [11–15], the scale up of such

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process was poorly studied. So, the aim of this work was to study the performance of a degradation process using *B. napus* hairy roots and H_2O_2 in a stirred tank reactor and to establish some operational conditions. The study was focused on the evaluation of some variables involved in the oxidative process to remove 2,4-DCP from water. Moreover, a biossay for the determination of potential toxic effects of treated solutions was also applied and evaluated.

2. Material and methods

2.1. Materials

Hairy root cultures of *B. napus* were obtained by inoculating sterile leaf explants with *Agrobacterium rhizogenes* strain LBA 9402 as it was previously described [16]. They were subcultured every 30–35 d in Murashige–Skoog (MS) liquid medium [17] enriched with vitamins [16].

All substances used in the experiments were of Analytical Reagent Grade. The concentration of H_2O_2 was determined spectrophotometrically using $\varepsilon_{240\,nm} = 43.6\,M^{-1}\,cm^{-1}$.

For toxicity test, *Lactuca sativa* L. seeds were used and they were kindly provided by Eng. Nelida Granvall from INTA – La Consulta (Mendoza province, Argentine).

2.2. Removal of 2,4-DCP: optimization of inoculum size

Hairy roots, at stationary phase of growth (30 d of culture), were cut into pieces and placed into Erlenmeyers flasks containing 100 mg/L of 2,4-DCP and 5 mM H_2O_2, at pH 6.5. Different amounts of root biomass were used (0.1–0.4 g) in order to find the optimal quantity of roots. Incubations were initiated by the addition of H_2O_2 and performed in an orbital shaker at 100 rpm, and $25 \pm 2\,^{\circ}C$. After incubation the reaction solutions were taken and saved for the determination of the residual amount of 2,4-DCP as it was described.

Different kinds of controls were carried out in order to estimate enzymatic and/or non-enzymatic mechanisms of 2,4-DCP removal. These controls were performed as follows: Controls (a) and (b), 2,4-DCP solution (100 mg/L) without roots and with or without H_2O_2. They were used for evaluation of 2,4-DCP auto-oxidation, 2,4-DCP losses by evaporation, as well as the possible reaction between both reactants. The other control was (c), which contained 0.1 g of roots in 100 mg/L of 2,4-DCP solution, but without H_2O_2; to evaluate enzymatic processes catalysed by oxidases which do not require H_2O_2 as substrate. Finally, two controls (d and e) were performed in the same conditions, but with 0.1 g of autoclaved roots (30 min, 121 °C), with and without H_2O_2 (5 mM) respectively, to analyse physical mechanisms of elimination.

After incubation for 30 min in an orbital shaker at 100 rpm, at $25 \pm 2\,^{\circ}C$ the reaction solutions were separated and all of them were taken and stored for the determination of the residual amount of 2,4-DCP as described above.

All assays were carried out in triplicate and the results are the mean of three independent experiments.

2.3. 2,4-DCP and hydrogen peroxide determination

The residual amount of 2,4-DCP was measured through an spectrophotometric assay, as follows. Aliquots of 5 mL of each sample were mixed with 25 μL of 4-aminoantypirine (2%, w/v), 25 μL of 6 M NH_4OH and 50 μL of potassium ferricyanide (8%, w/v). After 5 min, 2.5 mL of chloroform was added and the absorbance was determined at 510 nm. The determination limit of this method is 1 μg/L.

The results were expressed as percentage of the initial concentration. The results of 2,4-DCP removal were also expressed as removal efficiency, which is defined as the ratio between the concentration of 2,4-DCP removed and the initial concentration of this pollutant in the incubation medium, and is usually employed to quantify the degree of water purification achieved in the assay [18].

Hydrogen peroxide concentrations were monitored using the procedure described by Sergiev et al. [19]. Briefly, samples of 500 μL were taken and mixed with 500 μL of 10 mM potassium phosphate buffer, pH 7. Then, 1 mL of 1 M KI was added and the mixture was homogenised. The absorbance was measured at 390 nm, in a Beckman spectrophotometer, and the data were transformed to H_2O_2 concentration using a calibration curve, which was carried out with known concentrations of H_2O_2.

2.4. Experimental system and equipment

Removal of 2,4-DCP was performed in a 3 L stirred-tank bioreactor (Applikon instruments) with the adequate instrumentation for the measurement and control of the operating parameters, such as pH and temperature values. The pH was continuosly measured with an autoclave-sterilizable Phoenix sensor with a Cole Palmer controler 29041-02. There was a single sparger with an impeller in central shaft of the vessel compartment for aeration and proper mixing of medium. During the experiment, an autoclavable nylon mesh (pore size 1.5 mm) was tightened just around the Rushton turbine in order to protect roots from shear damage by direct contact with impellers (Fig. 1). So, the roots remain floating around the mesh during the experiment.

The reactor was used with a working volume of 1 L of pollutant solution. The removal reaction was carried out at room temperature and agitation speed of 200 rpm, by adding a root inoculum of 10 g, which was selected based on previous results, of biomass optimization (data not shown). Roots, used for these experiments, were previously grown in 250 mL Erlenmeyers flasks until they reached stationary phase of growth (30 d). A solution containing 100 mg/L of 2,4-DCP was added to the reactor. Then, roots and 5 mM H_2O_2

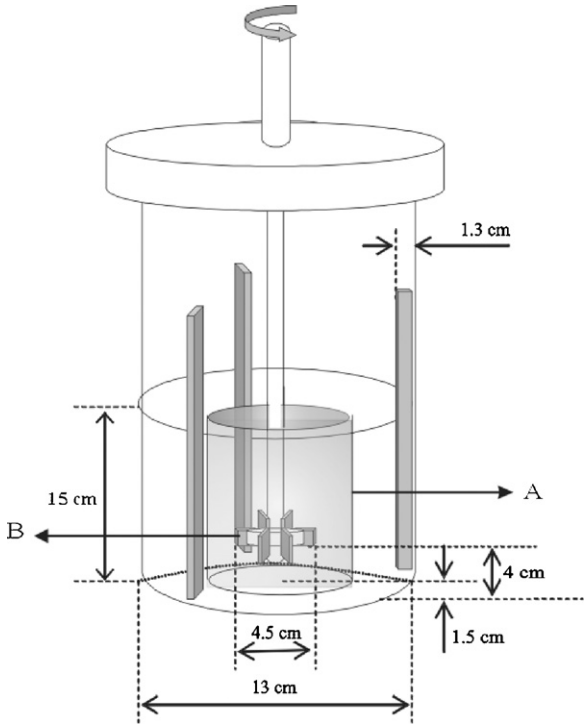

Fig. 1. Diagrammatic representation of the bioreactor used for the scale-up of 2,4-DCP removal. (A) Autoclavable nylon mesh; (B) single sparger with an impeller.

were added. Temperature, pH, and 2,4-DCP removal were routinely monitored.

2.5. Re-use of roots assay

B. napus hairy roots were re-used for 2,4-DCP removal, for six consecutive cycles. The experiment was performed using 10 g of fresh hairy roots, which were incubated with 1 L of 100 mg/L of 2,4-DCP solution and 5 mM H_2O_2 for 30 min. Then, the solution was removed and replaced by a fresh solution of the same composition plus 5 mM H_2O_2. This procedure was repeated once every 30 min, for 3 h consecutively.

2.6. Toxicity assay

The solutions obtained after the removal process were tested applying a toxicity test, with *L. sativa* L. seeds. This test was selected because it was proposed for use in toxicity studies by US EPA and the US Food and Drug Administration (US FDA). The use of *L. sativa* is suggested for its high germination rate and sensitivity to contaminants and also for its low genetic variability. An experiment based on following germination of seeds and early growth of seedlings, was conducted. Twenty seeds were placed in Petri dishes containing filter paper, in aseptic conditions. Then, seeds were treated with 5 mL solutions containing different concentrations of 2,4-DCP (2–100 mg/L) and with post-removal solutions (after treatment with roots). These solutions were prepared and added to the seeds in order to determine EC50.

A solution containing 1 and 5 mM H_2O_2 was tested as control, in order to estimate its toxicity. In addition, control seeds were exposed to distilled water.

The time of incubation (5 d) was chosen according to standard procedures [20]. The seeds were germinated and seedlings grew in growth chamber under controlled conditions (mean air temperature 25 °C, relative air humidity 80%) and in darkness. After this time, the number of germinated seeds, and the parameters of early growth (length of primary root and hypocotyl) were measured. The experiment was done by triplicate. Results are the mean of three independent experiments. Interpretation of toxicity data was conducted according to the methods described in the guidelines of US EPA. Probit program version 1.4 was employed to calculate EC50 values and corresponding 95% confidence intervals depending on the raw data distribution for assessing the quality of the treatment applied. The percentages of relative seed germination; hypocotyl elongation (HE); root elongation (RE) and germination index (GI), were calculated according to standard methods using Eqs. (1)–(4) [21].

$$\text{relative seed germination (SG)} = \frac{\text{seeds germinated with 2,4-DCP}}{\text{seeds germinated with water}} \times 100 \quad (1)$$

$$\text{relative hypocotyl elongation (HE)} = \frac{\text{mean hypocotyl length with 2,4-DCP}}{\text{mean hypocotyl length with water}} \times 100 \quad (2)$$

$$\text{relative root elongation (RE)} = \frac{\text{mean root length with 2,4-DCP}}{\text{mean root length with water}} \times 100 \quad (3)$$

$$\text{germinatrion index (GI)} = \frac{\text{relative seed germination} \times \text{relative root elongation}}{100} \quad (4)$$

The germination index combines germination and root growth and consequently reflects the toxicity more completely. The root elongation is the percentage of root length compared to control and it can be an indication of the presence of stress effects or other non-acute toxicological effects in the plant evolution.

3. Results and discussion

3.1. Removal of 2,4-DCP

Hairy roots offer several advantages for phytoremediation studies, as they can grow rapidly in hormone-free medium, providing a greater surface area of contact between contaminant and tissue. They also produce large quantities of exudates which are composed of enzymes and some metal chelating compounds that may detoxify or sequester harmful organic and inorganic pollutants [22]. In particular, *B. napus* hairy root cultures are characterized by high growth rates under defined conditions and, after 30-day culture, approximately 20 times increase in root biomass was obtained. In previous works we have demonstrated the capability of these hairy roots for phenolic compound removal and the role of peroxidases in the removal process [14,23].

In the present work we have employed *B. napus* hairy roots in order to scale-up the removal of 2,4-DCP. It is important to note that the concentration of 2,4-DCP used in this study is relevant in environmental conditions because such concentrations can exist in heavily contaminated sites and wastewaters [3–5,24].

For scale-up experiments, a 10 g inoculum of fresh weight of actively growing hairy roots was placed in the reactor, which contained 2,4-DCP solution. Roots were placed out of the basket designed to protect roots from the effect of shear stress. Reaction was started with 5 mM H_2O_2 addition. This H_2O_2 concentration was selected according to our previous results [14]. In this work we determined that H_2O_2 must be added to the reaction mixture to obtain an efficient detoxification and the optimal H_2O_2 concentration was 5 mM, for solutions containing 100–500 mg/L of 2,4-DCP. We also determined that only a little additional removal could be obtained by adding H_2O_2 in concentrations greater than 15 mM and, moreover, an excess of H_2O_2 must be avoided because it would cause enzyme inactivation [14].

The residual amount of 2,4-DCP in the reactor, during 30 min of treatment is showed in Fig. 2. After 30 min residual 2,4-DCP was only 2.1 ± 0.25%, which reveals the high removal efficiency of *B. napus* hairy roots. The pH and remanent H_2O_2 were measured at different times of treatment (Fig. 2). Throughout the experiment,

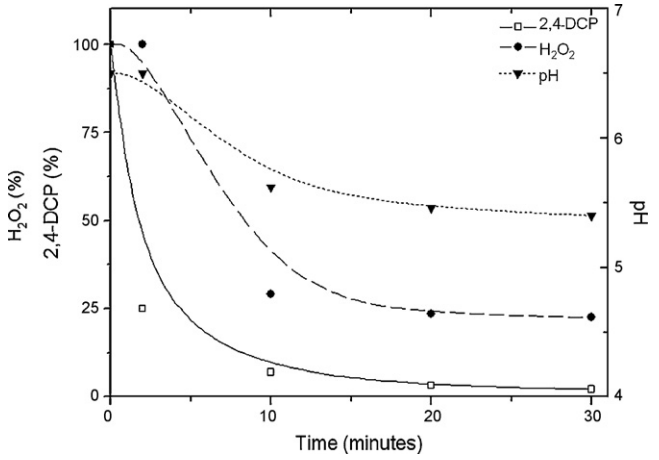

Fig. 2. Evolution of 2,4-DCP removal, pH values and H_2O_2 concentration during 2,4-DCP removal.

Table 1
Residual 2,4-DCP of different control assays.

Controls	Residual 2,4-DCP (%)
2,4-DCP with H_2O_2	97.4 ± 2.2
2,4-DCP with autoclaved HR	67.7 ± 4.9
2,4-DCP with autoclaved HR and H_2O_2	72.0 ± 7.4
2,4-DCP with HR	64.7 ± 5.9
2,4-DCP with HR and H_2O_2	2.1 ± 0.25

Note: The assays were carried out with 100 mg/L of 2,4-DCP and 5 mM H_2O_2. HR: *B. napus* hairy roots. Data represents mean values ± standard error (S.E.), based on three independent determinations.

Table 2
Re-use of *B. napus* hairy roots for six consecutive cycles, using solutions initially containing 100 mg/L 2,4-DCP and H_2O_2 5 mM, and replaced every each cycle.

Number of cycles	Residual 2,4-DCP (%)	Residual H_2O_2 (%)
1	2.1 ± 0.3	22.5 ± 0.7
2	4.6 ± 0.3	27.7 ± 0.8
3	5.0 ± 0.5	25.7 ± 0.7
4	8.2 ± 0.5	30.8 ± 3.6
5	14.5 ± 1.5	27.2 ± 1.1
6	13.8 ± 1.5	26.0 ± 1.0

the pH value decreased, from 6.5 to 5.4 as a consequence of the pollutant dehalogenation. The decrease in the pH value did not affect the removal process, because *B. napus* hairy roots are able to remove 2,4-DCP in a wide pH range (4–9), as was previously described by Agostini et al. [14]. This change in pH value could be associated with the enzymatic transformation of 2,4-DCP and the chloride ions liberation from this halogenated compound, as a consequence of polymerization, which finally formed HCl. Dehalogenation of chlorinated phenols was also found using peroxidases from different sources [9,25] and with other oxido-reductases [26]. It involves the chloride substituent release as a result of an oxidative coupling reaction, which is the initial step leading to further decomposition of these organic compounds. Dehalogenation may also be related to coupling of chlorophenols to already bound substrates [27]. This reductive dechlorination would represent a real detoxification since the toxicity of the chlorinated phenols frequently decreases with decreasing number of chlorine substituents. Therefore, it is possible that the ability of *B. napus* hairy roots to perform reductive dechlorination can be exploited as part of a remediation technology.

The high removal efficiencies (98%) obtained in a reaction medium supplemented with 5 mM H_2O_2 suggested a participation of peroxidases in the transformation of chlorophenolic compounds, as was previously described [14,23]. However, in order to evaluate other possible mechanisms associated with 2,4-DCP removal, several controls were carried out (Table 1). There were no removal in the controls containing 2,4-DCP with 5 mM H_2O_2, but without roots indicating that no chemical reaction between both reactants occurred. However, 2,4-DCP removal efficiency was recorded as 32% in autoclaved (dead) *B. napus* hairy roots. This could be attributed to the non-specific sorption of the pollutant by the roots, which is a physical process rather than a biological process [11,28]. Moreover, a 35% removal efficiency was obtained when the roots were incubated with the pollutant in the absence of H_2O_2 (Table 1). From this data it could be speculated that many factors such as sorption or the activity of phenoloxidases, like laccases, had a little influence in the removal process. These results are in agreement with those described by other authors [11,13,15], which reported that peroxidases are the main oxidoreductases involved in phenolic compounds transformation.

In reuse studies, as was indicated in Section 2, the reactor was operated in batch mode, with an initial 2,4-DCP concentration of 100 mg/L and 5 mM H_2O_2. In these conditions, approximately complete 2,4-DCP removal was achieved after 30 min and the levels of 2,4-DCP decreased to approximately 2% of the initial concentration (Table 2). At the end of this batch period, the solution was removed, and replaced by fresh 2,4-DCP solution, plus hydrogen peroxide and the same roots were used, for a new cycle of 30 min. This operation was repeated for six consecutive cycles. Under these conditions 2,4-DCP levels decayed and the removal efficiencies were high. At the end of the experiment we could demonstrate that *B. napus* hairy roots were able to remediate 2,4-DCP even in six reuse cycles with a slightly decrease in efficiency (from 98 to 85%). During the experiment a decrease in H_2O_2 levels was registered in each cycle, which was due to the enzymatic removal catalysed by peroxidases (Table 2). However, H_2O_2 was not totally consumed in the enzymatic reaction and levels from 22.5 to 30.8% remained in the reaction medium. In addition, no mechanical damage was observed macroscopically in the roots.

Thereafter with increasing time period, hairy roots turned brown. These results were in agreement with ours obtained previously, in a low scale performed with Erlenmeyer flasks [14] and with hairy roots of other plant species [15,29]. This fact suggested that the polymeric product, obtained after 2,4-DCP removal by *B. napus* hairy roots, precipitated in the surface of roots, as was also described in tobacco hairy roots by Talano et al. [30], which is advantageous from a biotechnological point of view.

In terms of technical complexity, the operation of the reactor with hairy root cultures required no additional separation stage. Consequently, the polymers generated by the enzymatic reaction catalysed by peroxidases contained in the roots, remained entrapped in the root tissues. Therefore, it is easy to separate the reaction product contrarily to other proposed methods, like the use of pure enzymes, which require another stage of separation, because polymers remain in the reaction medium [31].

3.2. Toxicity assay

Depending on the phenols treated or the biological system used in effluent treatments, the reaction products can be more toxic than the parent phenol. Moreover, the particular treatment conditions may significantly influence the quality and quantity of the reaction products that are formed. In this way, the degradation products formed during catalytic oxidation of phenols are mainly polymeric compounds, probably less toxic than the original ones [32,33]. However, it has been reported that intermediary toxic products can be formed during oxidative treatment and they can persist after this treatment. Consequently, the potential formation of persistent or toxic intermediates should be evaluated, if the proposed method of 2,4-DCP removal fulfilled the criteria for discharge to the later stage of the treatment process or into the environment. So, it is important to apply a toxicity test in order to evaluate the potential toxicity of the remaining solution. According to that, the solutions obtained after the removal process performed with *B. napus* hairy roots were tested for their toxicity applying a toxicity test, using *L. sativa* L. seeds. Thus, germination of seeds and early growth of seedlings were registered. Germination is normally known as a physiological process beginning with water imbibition by seeds and culminating with root emergence [34]. However, there are different definitions of seed germination according to its root length: emergence of root, >1 mm or >5 mm [34]. In this study, seeds showing emergence of radicle or cotyledon coming out of the seed coat were recorded as being germinated.

Fig. 3 shows results of the acute toxicity test, using *L. sativa* L. seeds. As it could be seen, relative seed germination (SG) was strongly inhibited by the presence of 100 mg/L of 2,4-DCP. The EC50-5d value based on germination test was 52 mg/L. However, the post-removal solution (PR) was less toxic than the parent, because no inhibition of *L. sativa* germination was observed. Germi-

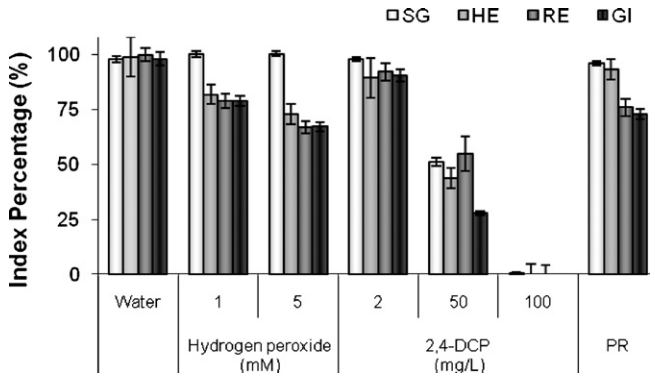

Fig. 3. Effect of 2,4-DCP (2–100 mg/L); H_2O_2 (1 and 5 mM) and post-removal (PR) solutions, on SG, HE, RE and GI indexes. Water was used as control. The error bars represent standard error of the mean.

nation was often reported to be little affected by chemical pollution and germination studies alone would not predict the effects on the subsequent growth of the species tested [35]. Thus, we also determined seedling growth of *L. sativa* plants, which was expressed on the basis of hypocotyl and roots lengths represented by the HE and RE indexes. According to Kordan et al. [34], root elongation could be, beyond seed germination, a convenient indicator of the environmental toxicity. As shown in Fig. 3, it was evident that increased concentrations of 2,4-DCP strongly inhibited both the length of root and shoot of seedlings. The EC50 values based on root and hypocotyl length were 33.5 and 26 mg/L, respectively. Therefore, plant growth was a more sensitive endpoint than germination as was shown in Fig. 3. Post-removal solutions showed a decrease in HE, RE and GI indexes, which could mean that the solutions had slight toxic effects. However, similar results were obtained in experiments performed with 1 and 5 mM H_2O_2, which were carried out to control the toxicity of this reactant. Thus, we suppose that this toxic effect could be attributed to a residual concentration of H_2O_2 which remains in the reaction medium, in PR solutions. In order to verify that, we carried out similar removal experiments with *B. napus* hairy roots using a low H_2O_2 concentration (4 mM) in the reaction medium. This procedure was repeated for six consecutive cycles. Then, we calculated SG, HE, RE, and GI indexes for post-removal solutions derived from cycles 1, 3 and 6. Fig. 4 shows that in the assayed conditions post-removal solutions were not toxic compared with controls treated with water. After removal, the values of remanent H_2O_2 were approximately 0.65 ± 0.05 mM. So, the importance of an adequate selection of H_2O_2 concentration

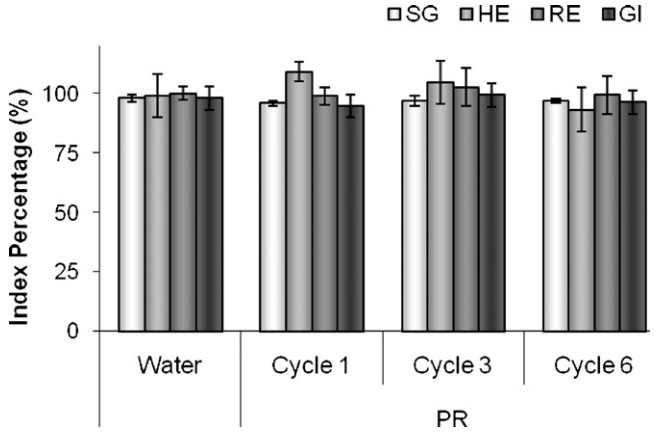

Fig. 4. Effect of post-removal solutions using 4 mM H_2O_2 (from cycles 1, 3 and 6), on SG, HE, RE and GI indexes. Water was used as control. The error bars represent standard error of the mean.

not only to reach high removal efficiencies but also to avoid residual toxicity effects has been highligthed in our study.

The results of the toxicity test applied to post-removal solutions are clearly in agreement with our previous experimental data on the high removal efficiency (98%) of *B. napus* hairy roots. Therefore, we suggest that *B. napus* hairy roots could be effectively used to detoxify solutions containing 2,4-DCP, on a large scale process.

4. Conclusion

In the present investigation, fast growing *B. napus* hairy roots were used to successfully remediate 2,4-DCP. In order to scale up the process, a reactor with a mesh was used to separate the roots from the impeller, to avoid the mechanical damage of the roots during reaction. High removal efficiencies were obtained (98%), in a short time (30 min). The pH value decreased in the reaction medium, from 6.5 to 5.4 as a consequence of the pollutant dehalogenation. However, the removal process was not affected, because these hairy roots are able to remove 2,4-DCP in a wide pH range (4–9). Therefore, our results showed, in laboratory scale studies, that *B. napus* hairy root cultures have considerable potential for the removal of 2,4-DCP, from aqueous solutions, with a good performance. Roots could be re-used for 2,4-DCP removal almost for six consecutive cycles, showing high removal efficiencies; which decreased from 98 to 86%, in the last cycle. Post-removal solutions showed no toxicity, as was demonstrated using an acute toxicity test, with *L. sativa* L. seeds. Thus, this study helps to design a method for continuous and safe treatment of phenolic effluents on a large scale.

Acknowledgements

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