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Removal of aqueous phenol and phenol derivatives by immobilized potato polyphenol oxidase

NIKOLA LONČAR[1#], NATAŠA BOŽIĆ[2#], IVAN ANĐELKOVIĆ[1], ALEKSANDRA MILOVANOVIĆ[2#], BILJANA DOJNOV[2#], MIROSLAVA VUJČIĆ[2#], GORAN ROGLIĆ[1#] and ZORAN VUJČIĆ[1*#]

[1]*Faculty of Chemistry, University of Belgrade, Studentski trg 12–16, Belgrade and* [2]*Institute of Chemistry, Technology and Metallurgy – Center of Chemistry, University of Belgrade, Studentski trg 12–16, Belgrade, Serbia*

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Abstract: Phenols containing halogens, which tend to deactivate the aromatic nuclei, constitute a significant category of highly toxic and difficult-to-degrade pollutants, which arise from a wide variety of industries. The main purpose of this study was to obtain an inexpensive immobilized enzyme for the removal of phenols. Partially purified potato polyphenol oxidase (PPO) was immobilized onto different commercial and laboratory produced carriers. Three of the obtained biocatalysts, with the highest PPO activities, namely Eupergit C250L––PPO; Celite–PPO and CelluloseM–PPO, were tested in a batch reactor for the removal of phenol, 4-chlorophenol and 4-bromophenol. In the case of 2.5 mM substrates with Eupergit C250L–PPO, an around 45 % removal of 4-bromophenol was achieved, while the removals 4-chlorophenol and phenol were 35 and 20 %, respectively. The reusability of Eupergit C250L–PPO for the removal of 4-chlorophenol was tested. After eight repeated tests, the efficiency of 4-chlorophenol removal by Eupergit C250L–PPO immobilisate had decreased to 55 %.

Keywords: polyphenol oxidase; potato; phenol; immobilization; Eupergit.

INTRODUCTION

Increased production of plastics, dyes, pesticides and other chemicals has resulted in the generation of hazardous chemical wastes and hence environmental pollution. A few persistent pollutants, including several pesticides, are carried in air and water over several hundred kilometers, affecting the wildlife and general population.[1] These pollutants are non-biodegradable and are known to have carcinogenic, mutagenic or chronic toxic effects.

* Corresponding author. E-mail: zvujcic@chem.bg.ac.rs
# Serbian Chemical Society member.
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Chlorinated organic compounds, in particular, are found to be resistant to biochemical degradation. Monochlorophenols, among others phenol compounds, serve as intermediates in the production of pesticides.[2] These are also used as antimicrobial agents in a wide array of products, such as adhesives, oils, textiles and pharmaceutical products. Phenols are present in the wastewater of various industries, such as refineries up to 6–500 mg L^{-1}, coking operations (28–3900 mg L^{-1}), coal processing (9–6800 mg L^{-1}) and the manufacture of petrochemicals (2.8–1220 mg L^{-1}).[3,4] Other sources of wastewater streams containing phenols are pharmaceuticals, plastics, wood products, paint and pulp, and paper industries (0.1–1600 mg L^{-1}).[3,4] The release of phenol-containing wastewater into open water is forbidden without prior treatment because of the toxicity of phenol and its derivatives. Due to the toxic nature of some of these compounds, the Environmental Protection Agency, EPA, has set a water purification standard of less than 1 μg L^{-1} of phenol in drinking waters.[5] Moreover, EPA studies have shown that the usage of chlorine for disinfection of phenol-containing water may yield toxic 2-chlorophenol.[5]

Biological treatment methods are generally cheaper than physical or chemical treatment methods for lowering phenol concentrations.[6] The microorganisms used are usually aerobes, including *Pseudomonas* sp., *Alcaligenes* sp., *Azotobacter* sp., *Rhodococcus* sp. and *Cryptococcus* sp.[7,8] Most of these studies were performed with pure cultures. The inherent toxicity of chlorophenols, or of the intermediates produced during their degradation, compromises the ability of such pure cultures to completely mineralize the chlorophenols present in wastewater. In addition, bromophenols are actually used as effective disinfection reagents. Therefore, biological treatment techniques, if used alone, have a serious limitation in treating non-biodegradable/toxic chemicals. Another alternative is to use enzymes, such as polyphenol oxidases and peroxidases. Nevertheless, the major obstacle in the commercial application of soluble enzymes for environmental purposes is their limited operational stability, which means that a continuous supply of large amounts of fresh and partially purified enzyme is required.

However, enzyme immobilization is an excellent technique to overcome this problem due to its high storage stability and better control of the catalytic process.[9] The potential advantages of enzymatic treatment as compared with conventional treatments include among others: application to recalcitrant materials, operation at high and low contaminant concentrations over a wide pH range, including extreme values (pH 2 or 11), and temperature and salinity ranges, and easy control of the process.

A number of oxidative enzymes from bacteria, fungi and plants have been reported to play an important role in numerous waste treatment applications. Peroxidases and polyphenol oxidases can act on specific recalcitrant pollutants by precipitation or transforming to other products and permitting a better final treat-

ment of the waste. Improvement of the enzyme operational stability and half-life and thereby a reduction in treatment cost was accomplished through enzyme immobilization.[10]

For the treatment of large volumes of waste-waters, reactors containing immobilized enzymes are desirable because of the high cost of enzymes. Enzyme immobilization techniques usually provide, in addition to the desired reuse of the enzyme, unexcelled advantages such as product separation and continuous operation.[11] Cheaper supports and enzymes for the preparation of immobilized enzyme preparations for such applications are always been sought after. Polyphenol oxidase (PPO) from potato is an exceptionally cheap enzyme because it can be purified from potato waste of the food industry. It was shown previously[12] that PPO activity was the greatest at the exterior of the tuber, including the skin and cortex tissue 1 to 2 mm beneath the skin.

Therefore, in this work, the possible use of several commercial carriers, as well as a few non-conventional carriers, for potato PPO immobilization was examined. Three of them, Eupergit C250L, Celite and CelluloseM, which had the highest percentage of bound PPO activity, were tested in batch reactors for the removal of phenol, 4-chlorophenol (CP) and 4-bromophenol (BP) from synthetic wastewater.

EXPERIMENTAL

Reagents

Potato *(Solanum tuberosum*) tubers were obtained from the local market. Commercially available carriers: Eupergit C250L, Celite, Cellulose (MTM®, Green Sand and Birm), as well as laboratory modified carriers: MTM® enriched with 10 % Ti, cellulose enriched with 1 % Ti, cellulose enriched with 0.7 % Ti, cellulose enriched with 0.25 % Ti and CelluloseM prepared by Meng *et al.*[13] were used in this study. All employed reagents and solvents were of the highest available purity but at least analytical grade. They were purchased unless otherwise stated from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA).

Preparation of crude extract

Potato tubers were kept at 3 °C for 12 h. Potato tubers were chilled to 3 °C. Thereafter, whole tubers were homogenized in a commercial juicer. The homogenate (1 L) was centrifuged at 3500 rpm at 4 °C. 600 mL of clear supernatant was desalted against 10 mM Na phosphate buffer pH 7.3 using a Sephadex G25 coarse column.

Purification of polyphenol oxidase

60 g of preswollen QAE Sephadex A-50 was equilibrated with 10 mM Na phosphate buffer, pH 7.3. The equilibrated ion-exchanger was added to the extract and mixed with a magnetic stirrer for 30 min in an oxygen-free atmosphere. Then the matrix was washed with starting buffer and the enzyme was eluted with 500 mL of 0.75 M NaCl in starting buffer. The partially purified enzyme preparation was stored at –20 °C until use.

Polyphenol oxidase activity assay

PPO activity was determined using L-DOPA as the substrate at 25°C by measuring the initial rate of dopachrome formation.[14] The standard assay mixture contained 0.1 ml of en-

zyme in 1.5 ml of 9.3 mM L-DOPA in 50 mM Tris-HCl pH 7.0. The absorbance at 475 nm was measured using a Philips UV–VIS–NIR PU 8630 spectrophotometer. One unit of PPO activity was defined as the amount of enzyme that catalyzes an increase in absorbance of 0.001 per min at 25 °C.

Immobilization of polyphenol oxidase onto the various carriers

10 mg of each carrier was measured in triplicate and added into 200 µL of enzyme preparation (8471 U ml^{-1}). 10 mg of each carrier was added in 0.9 % NaCl as a blank probe. Mixtures were left for 24 h on IKA orbital shaker at 400 rpm. Biocatalysts were removed by centrifugation at 14000 rpm and washed six times with 0.9 % NaCl. For further study, three biocatalysts with the highest activity were scaled up to 1 g and produced using the above-described procedure.

Immobilized polyphenol oxidase activity assay

The activity of the immobilized enzyme was assayed using a modified version of the method of Kwon and Kim.[14] Ten milligrams of partially dried biocatalyst (enzyme + support) was added to 800 µL of 2.325 mM L-DOPA in 50 mM Tris HCl buffer pH 7.0 at 25 °C. The mixture was shaken for 3 min and then centrifuged at 14000 rpm. The increase in the absorbance due to the formation of dopaquinone in the resulting supernatant was measured at 475 nm. The blank sample contained 10 mg of blank carrier instead of biocatalyst besides the other components of activity assay mixture. This was necessary because some carriers: MTM®, MTM® enriched with 10 % Ti, Green Sand and Birm, possess an inherent ability to oxidise L-DOPA. The specific activity of the immobilized PPO is defined as an increase in absorbance of 0.001 per min per gram of immobilisate under the given assay conditions.

Preliminary application study of the immobilized PPO using a batch reactor

Removal of phenolic compounds from synthetic wastewater was investigated with 100 mg of semidry biocatalyst that was added to 3 mL of 2.5 and 10 mM solutions of phenol, CP and BP and incubated for 5 h on an IKA orbital shaker to allow continuous oxygenation. After 5 h, the solutions were tested for the remaining phenol content using the 4-aminoantipyrine (AAP) assay.[15] The concentrations of phenols were measured using a colorimetric assay in which the phenolic compounds react with 2.08 mM AAP and 8.34 mM potassium ferricyanide in 0.25 M sodium bicarbonate solution to form a red quinone-type dye that absorbs light with a peak wavelength of 510 nm. The extent of color generation at 510 nm after a 6-min incubation time is proportional to the concentration of phenols in the assay solution. Absorbance readings were converted to phenolic concentrations using calibration lines.

pH optimum of soluble PPO and Eupergit C250L–PPO immobilizate

To determine the optimum pH of soluble and immobilized PPO activity against L--DOPA, 0.1 ml of the soluble enzyme and 10 mg of partially dried biocatalyst and a series of 50 mM buffers in the pH range from 3.0 to 10.5 were used (acetate, pH 3.0–5.0; phosphate, pH 6.0–10.5) and the residual activity was determined under the above-described enzyme assay conditions.

The reusability of Eupergit C250L–PPO immobilizate for 4-chlorophenol removal

The reusability of Eupergit C250L–PPO immobilizate for CP removal was tested by repeating the above-described 5 h incubation experiment 8 times. Between these 8 cycles, the immobilizate was collected by centrifugation and washed three times in assay buffer.

RESULTS AND DISCUSSION

Potato is an inexpensive source of enzymes for biotechnology.[16] Horseradish and turnip peroxidases have been employed in phenol removal.[17] The sources of peroxidases are available in abundance in Serbia, but these are seasonal and expensive plants; thus, using potatoes which are available during the whole year represent an advantage. PPOs from potato are the most suitable enzymes with respect to their availability and cost.[18] The efficiency of the enzymatic treatment was found to be independent of the enzyme purity and, therefore, it was possible to utilize a crude or partially purified preparation that is protected from deactivation due to the significant quantity of protein present instead of a purified one.[17] This feature leads to a significant reduction in treatment costs.

The polyphenol oxidases were partially purified using ion-exchange chromatography on QAE Sephadex with a yield of 69 %. The results of the purification are presented in Table I.

TABLE I. Partial purification of polyphenol oxidase from potato tuber

Property	Crude extract	Desalted crude extract	QAE-Sephadex eluted fraction
A_{475}	1.000	1.060	0.860
V / mL	600	650	480
Activity, U ml^{-1}	10000	10600	8600
Total activity, U ml^{-1}	6000000	6890000	4128000

In spite of the intrinsic ability to oxidize L-DOPA, judging by the instantaneous change in the color of the reaction mixture after mixing the carrier with the substrate, MTM®, MTM® enriched with 10% Ti, Birm and Green Sand were used for the immobilization, since the oxidizing ability of the carrier was subtracted by the use of a blank. Titanium modified cellulose beads (90 μm) with different amounts of bound titanium (0.25, 0.7 and 1.0 %, w/w) and titanium cellulose prepared according to Meng *et al.*[13] were also used in this study. In addition, some commercially available carriers, Eupergit C250L, Celite and unmodified cellulose, were also tested. MTM®, MTM® enriched with 10 % Ti, Birm and Green Sand were found not appropriate carriers for PPO immobilization due to the negligible or very low bound PPO activity. Furthermore, regarding the cellulose carriers, only CelluloseM showed a reasonably high bound activity of PPO, while the other four cellulose materials had very similar (low) values for the activity towards L-DOPA. Significant activities were detected with Celite–PPO, CelluloseM–PPO and Eupergit C250L–PPO biocatalysts (Fig. 1). The Eupergit C250L–PPO biocatalyst had the highest activity according to its specific activity.

As model pollutants, phenol, BP and CP were chosen. During the experimental removal of these three compounds from aqueous solution, the best results were obtained with PPO immobilized on Eupergit C250L. Comparison of the im-

mobilizates gave “ladder” histograms in the case of 2.5 mM substrates (Fig. 2) and a similar histogram with lower values in the case of 10 mM substrates (Fig. 3).

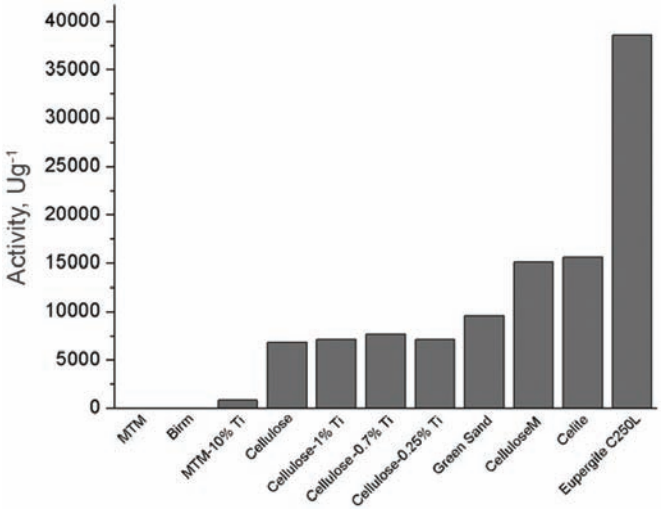

Fig. 1. Comparison of activity of produced biocatalysts towards 2.325 mM L-DOPA. Values represent specific activity of each produced biocatalyst (U g^{-1} of dry carrier).

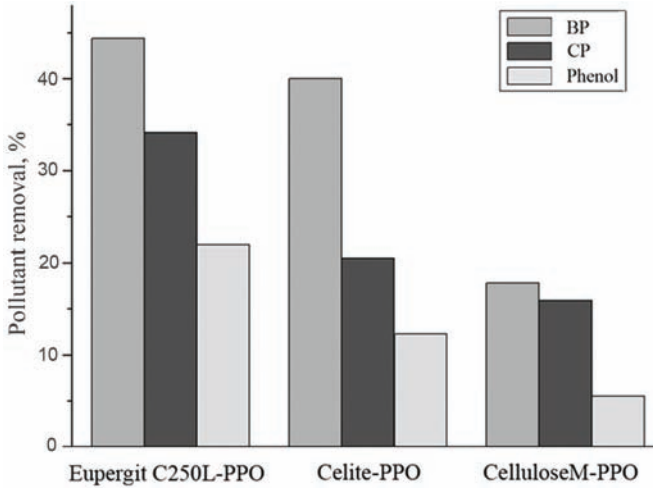

Fig. 2. Removal of 2.5 mM of 4-bromophenol, 4-chlorophenol and phenol after 5 h in a batch reactor.

Removal was the highest with the Eupergit C250L–PPO immobilizate, then with Celite–PPO immobilizate and the lowest with CelluloseM–PPO immobilizate. Although the removal of phenol can be considered as poor, due to low monophenolase activity of potato PPO,[19] it is comparable with the results obtained

by others for immobilized laccase activity.[20] In the case of 2.5 mM substrates with Eupergit C250L biocatalyst, around 45 % removal of BP was achieved, while 35 % and 20 % of the CP and phenol were removed, respectively. In the study of Levy *et al*.,[21] in which horseradish peroxidase immobilized on cellulose was used for the removal of BP, only 17 % removal was obtained with an initial BP concentration of 0.2 mM. Usually, a higher percent removal was obtained when a lower initial pollutant concentration was used. Bearing this in mind, it can be concluded that the Eupergit C250L–PPO biocatalyst used in the present study showed high efficiency for the removal of BP and CP. When 10 mM concentrations were used, the biocatalysts still showed removal abilities but it seems that in possible application, dilution of such wastewater would be useful.

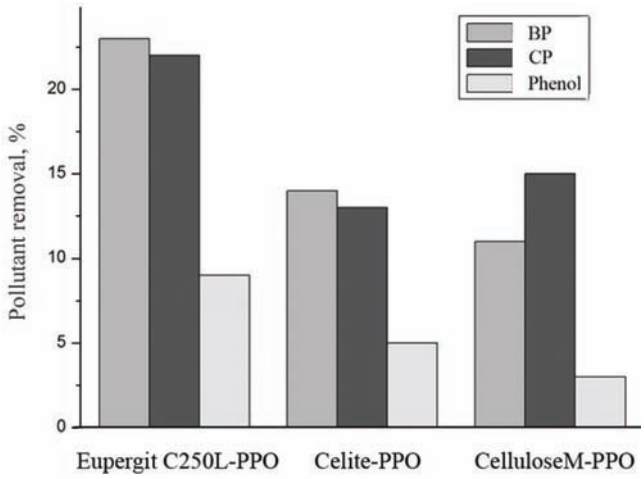

Fig. 3. Removal of 10 mM 4-bromophenol, 4-chlorophenol and phenol after 5 h in a batch reactor.

The optimum pH of free and Eupergit C250L–PPO immobilizate was studied since the pH value is one of the most influential parameters altering enzyme activity in an aqueous medium. The optimum pH values for free PPO and Eupergit C250L–PPO immobilizate were 7–9 and 8–9, respectively (Fig. 4). The difference in the optimal pH values of the soluble and immobilized enzyme might be a consequence of a change in the ionic environment of the carrier around the active sites of the enzyme.

In order to attain a better practical employment of the Eupergit C250L–PPO immobilizate in a batch reactor, it was necessary to investigate the reusability of the immobilizate. The reusability of Eupergit C250L–PPO for the removal of CP was considered. After eight repeated tests, each of 5 h duration, the efficiency of CP removal by Eupergit C250L–PPO immobilizate had decreased to 55 % (Fig. 5). The formation and accumulation of dark precipitates on immobilizates were

observed. Further studies should examine the application of continuous reactors, which would prevent the accumulation of reaction products on the biocatalyst. The reusability of an immobilized enzyme is one of its important advantages, which influences the cost of industrial applications.[22] Similar studies in which immobilized peroxidase had retained 40 % of its activity after eight repeated applications was considered as a significant reusability.[23]

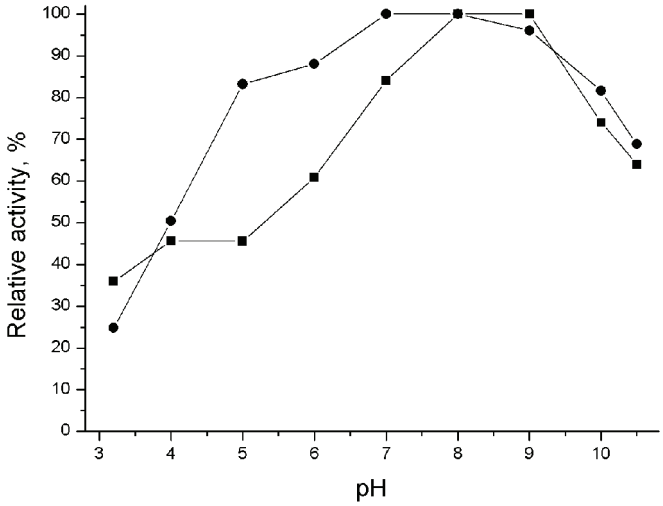

Fig. 4. Effect of pH on the activity of soluble PPO (●) and Eupergit C250L–PPO immobilizate (■).

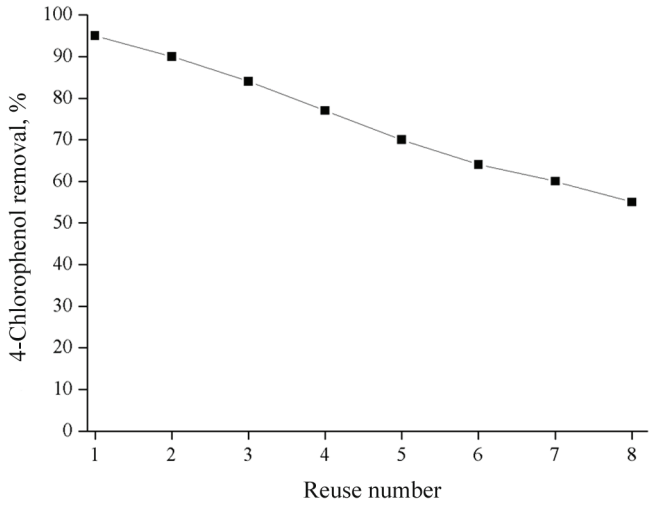

Fig. 5. 4-Chlorophenol removal reusability of Eupergit C250L–PPO immobilizate.

Khan *et al.*[18] investigated PPO immobilized on Celite and showed that the immobilized enzyme was more resistant to denaturation induced by pH, temperature, urea, the detergents SDS and Triton X-100 and Tween 20, and the water-miscible organic solvents acetonitrile, dimethylformamide, dioxane and *n*-propanol as compared to its soluble counterpart. In conjunction with these results, the present results give rise to a spectrum of possible carriers for PPO and other enzymes that might be used for wastewater treatment.

CONCLUSIONS

PPO enzymes from a low purity source, *i.e.*, partially purified potato preparation, showed good potential for phenol removal by polymerization. The experimental results indicated that phenol conversion in synthetic wastewaters is possible using Eupergit C250L, Celite and CelluloseM carriers. The Eupergit C250L––PPO biocatalyst used in this study showed high efficiency for the removal of BP and CP and significant reusability for removal of CP after 8 cycles of application. The presented immobilization method is a very economic procedure for the immobilization of PPO. Thus, the proposed process can be considered as a first step in further explorations for possible PPO carriers for the removal of phenol and phenol derivatives from wastewater.

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ИЗВОД

УКЛАЊАЊЕ ФЕНОЛА И ФЕНОЛНИХ ДЕРИВАТА ИЗ ВОДЕ ИМОБИЛИЗОВАНОМ ПОЛИФЕНОЛ-ОКСИДАЗОМ ИЗ КРОМПИРА

НИКОЛА ЛОНЧАР[1], НАТАША БОЖИЋ[2], ИВАН АЊЂЕЛКОВИЋ[1], АЛЕКСАНДРА МИЛОВАНОВИЋ[2], БИЉАНА ДОЈНОВ[2], МИРОСЛАВА ВУЈЧИЋ[2], ГОРАН РОГЛИЋ[1] и ЗОРАН ВУЈЧИЋ[1]

[1]*Хемијски факултет, Универзитет у Београду, Студентски трг 12–16, Београд и* [2]*Институт за хемију, технологију и металургију – Центар за хемију, Универзитет у Београду, Студентски трг 12–16, Београд*

Халогеновани феноли имају дезактивирано ароматично језгро и чине значајну категорију веома токсичних и тешко разградивих загађивача у разним индустријским гранама. Главни циљ овог рада је био добијање јефтиног имобилизованог ензима за уклањање фенола. Делимично пречишћена полифенол-оксидаза (ПФО) из кромпира је имобилизована на различитим комерцијалним и лабораторијски синтетизованим носачима. Од добијених биокатализатора, три са највећим активностима ППО, названи Еупергит Ц250Л–ПФО; Целит–ПФО и ЦелулозаМ–ПФО, тестирани су у реактору за уклањање фенола, 4-хлорфенола и 4-бромфенола. У случају 2,5 mM супстрата са Еупергит Ц250Л–ПФО, постигнуто је око 45 % разградње 4-бромфенола, док су 4-хлорфенол и фенол разграђени 35, односно 20 %. Тестирана је и способност вишеструке употребе Еупергит Ц250Л–ПФО имобилизата за уклањање 4-хлорфенола. Након осам поновљених циклуса ефикасност Еупергит Ц250Л–ПФО имобилизата за уклањање 4-хлорфенола је пала на 55 %.

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