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Tentacle carrier for immobilization of potato phenoloxidase and its application for halogenophenols removal from aqueous solutions

Nikola Lončar*, Zoran Vujčić

Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, Belgrade, Serbia

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ABSTRACT

Halogenated compounds represent one of the most dangerous environmental pollutants, due to their widespread usage as biocides, fungicides, disinfectants, solvent and other industrial chemicals. Immobilization of a protein through coordinate bonds formed with divalent metal ions is becoming an attractive method due to its reversible nature, since the protein may be easily removed from the support matrix through interruption of the protein–metal bond hence giving inherently cleaner and cheaper technology for wastewater treatment. We have synthesized novel 'tentacle' carrier (TC) and used it for immobilization of partially purified potato polyphenol oxidase (PPO). The obtained biocatalyst TC-PPO showed pH optimum at 7.0–8.0 and temperature optimum at 25 °C. Immobilized PPO shows almost 100% of activity at 0 °C. TC-PPO was more resistant to the denaturation induced by sodium dodecyl sulphate (SDS) detergent as compared to its soluble counterpart and was even slightly activated at SDS concentration of 1%.

TC-PPO was tested in the batch reactor for 4-chlorophenol and 4-bromophenol removal. More than 90% removal was achieved for both halogenophenols at concentration of 100 mg/L from aqueous solution. For both halogenophenols TC-PPO works with over 90% removal during first three cycles which decrease to 60% removal efficiency after six cycles each of 8 h duration.

1. Introduction

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 enzyme is required. Enzyme immobilization improves the operational stability and half-life of the enzyme therefore reducing the treatment cost [1]. Enzyme immobilization procedures often require purified enzyme and expensive supports and reagents [2]. The purpose of this procedure is to allow reuse of enzymes for many reaction cycles. However, this implies that immobilized enzyme preparation is more expensive. Novel immobilization protocols are still needed in order to achieve massive implementation of enzymes as catalysts of the complex chemical processes under the benign experimental and environmental conditions. There is always a search for cheaper support and enzymes for preparing an immobilized enzyme preparation for the aforementioned applications.

Covalent-type linkages between molecule and matrix are generally considered best, however many of the recommended immobilization procedures require pre-derivatisation of the matrix, extended periods for coupling reactions and specialized conditions [3]. In some cases there is considerable loss of activity during the immobilization procedure, in some cases up to 85% as recently reported by Pramparo et al. [4]. We were searching for a method in which matrix derivatisation after preparation could be avoided and where virtually instantaneous coupling could be achieved under mild and easy-to-handle conditions. To accomplish this, we employed the chelating properties of copper – which is often used for purification by immobilized metal-affinity chromatography (IMAC) and immobilization of tyrosinases from different sources [5]. By exploiting this property, high enrichment of enzyme was obtained due to its higher binding affinity to the support relative to other proteins. A similar approach has been applied for direct immobilization of tyrosinase on Celite, D-sorbitol cinnamic ester and copper alginate gel [6–8]. Sepharose and agarose derivatives containing metal chelating groups such as iminodiacetic acid (IDA) or nitriloacetic acid (NTA) are all suitable supports [9–11]. The high cost of commercially available chelating-agarose and cellulose is their biggest limitation. Additionally, short spacer arms restrict the mobility of the bound enzyme and impair the apparent catalytic activity. This opens up the possibility for creating new supports, with longer chelating arms (tentacles) that can offer more fluidity for the enzyme and higher availability, subsequently increasing the enzyme substrate contact.

* Corresponding author. Tel.: +381 11 3282 393; fax: +381 11 2636 061.
E-mail addresses: nloncar@chem.bg.ac.rs (N. Lončar), zvujcic@chem.bg.ac.rs (Z. Vujčić).

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Both laboratory and industrial scale separations of proteins rely mostly on ion-exchange resins. These resins may be utilized for a certain number of separation cycles after which their efficiency diminishes. In the present study, this reused ion-exchange (IEX) chromatography matrix – diethylaminoethyl (DEAE) cellulose fibers – were selected as the raw, starting material for preparation of support material for immobilization of PPO. DEAE cellulose is a derivative of the natural polymer cellulose and it possesses a desirable hydrodynamic structure since fibrilar structures have a very large active surface. It was chosen over other materials because of its biodegradability, hydrophilicity and presence of hydroxyl groups, cost and natural origin [12,13]. The tentacle carrier (TC) was synthesized by activation of amino groups of DEAE cellulose with epichlorohydrine followed by introducing IDA groups, which were subsequently charged with copper ions. Binding of enzyme during the immobilization procedure is achieved through coordinative binding of N, O and S atoms of enzyme amino acid residues to copper ions.

Horseradish and turnip peroxidases, both soluble and immobilized by different procedures, have been successfully employed in phenol removal [14]. Although those sources of different peroxidases are available in abundance, they are seasonal and rather expensive plants. On the contrary, PPO from potatoes is the enzyme with most potential for biotechnology due to its exceptionally low price since it can be purified from the potato food industry waste [6,15]. It has been shown previously [16] that PPO activity was the greatest at the tuber exterior, including the skin and cortex tissue 1–2 mm beneath the skin, which opens up possibilities for the exploitation of potato peels. This process may be in conjunction with the potato chips industry where excess proteins in effluents are a major problem. It was shown that enzymatic treatment efficiency is independent of the enzyme purity. Moreover a crude or partially purified preparation is protected from inactivation due to the significant quantity of other proteins present [14]. This feature leads to a significant reduction in overall treatment costs; hence we decided to partially purify PPO using only one chromatographic step under oxygen free conditions, with the purpose of enriching phenoloxidase activity. The immobilization procedure consists of an overnight incubation of enzyme preparation with the produced TC to give TC-PPO. The catalyst obtained by this procedure has been tested in batch reactors for removal of 4-chlorophenol (*p*-CP) and 4-bromophenol (*p*-BP) from synthetic wastewater.

2. Materials and methods

2.1. Reagents

Potato (*Solanum tuberosum*) tubers were obtained from the local market. Commercially available DEAE-cellulose (Sigma) was used in this study. However, it was a reused DEAE-cellulose, i.e. not appropriate for protein purification anymore since after many uses it does not provide required resolution in chromatography of proteins. Phosphate buffers, sodium chloride, acetic acid, L-3,4-dihydroxyphenylalanine (L-DOPA), Tris, sodium hydroxide, epichlorohydrin, sodium carbonate, iminodiacetic acid, copper sulphate, SDS, *p*-CP, *p*-BP, 4-aminoantipyirine, potassium ferricyanide, sodium hydrogen carbonate and ethanol used were of the highest available purity. They were purchased unless otherwise stated, from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA). Sephadex G-25 Coarse and QAE Sephadex A-50 were purchased from GE Healthcare Life Sciences.

2.2. Preparation of TC-carrier

5 g of preswollen reused DEAE-cellulose was resuspended in 20 ml of 3 M NaOH and 2 ml of epichlorohydrin. Activation was carried on stirrer for 2 h. Activated DEAE-cellulose was washed and transferred into solution made of 2 g NaOH, 3 g Na_2CO_3 and 4.3 g IDA, incubated 2 h at 50 °C and then left overnight. After washing with 500 ml of water, produced tentacle carrier was charged with copper (1 g $CuSO_4 \cdot 5H_2O$ in 25 ml H_2O), washed with water again and then with 7% acetic acid. TC-carrier was then washed with 10 volumes of water followed by washing with 10 volumes of 20% ethanol solution. TC-carrier was stored at 4 °C until further use.

2.3. Carrier characterization

Water content has been determined in 10 replicative measurements by taking 200 mg of semidry TC-PPO and drying it at 60 °C using Eppendorf Vacuum Concentrator Model 5301 until constant weight. Amount of bound epoxy group prior to introduction of IDA has been determined by method described by Axen et al. [17]. Amount of incorporated Cu(II) ions is determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) by iCap 6500Duo, equipped with a CID86 chip detector (Thermo Scientific, UK). Instrumental operating conditions for ICP-AES are RF power: 1150 W; plasma view: axial; nebulizer gas flow: 0.50 L/min; auxiliary gas flow: 0.50 L/min; coolant gas flow: 12 L/min; analysis pump rate: 50 rpm). TC was previously dissolved in 10 ml of 65% HNO_3, 0.2 ml of H_2O_2 using ETHOS 1, Advanced Microwave Digestion System, (MILESTONE, Italy). Microwave digestion was done in a two step program, first being heating to 200 °C over 15 min and second being held at 200 °C for 20 min. Sample was then diluted to 25 ml. Blank was prepared same way without sample.

2.4. Partial purification of PPO

Potato (*S. tuberosum*) tubers were obtained from the local market and were kept at 3 °C for 12 h. Thereafter, whole tubers were homogenized in 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 6 cm × 60 cm Sephadex G25 Coarse column. 60 g of preswollen QAE Sephadex A-50 was equilibrated with 10 volumes of 10 mM Na-phosphate buffer, pH 7.3. Equilibrated and deaerated ion-exchanger was added to extract and mixed with stirrer 30 min in oxygen-free atmosphere. After that matrix was washed with 3 volumes of starting buffer and enzyme was eluted with 0.75 M NaCl in starting buffer in 500 ml. Obtained enzyme preparation was stored at −20 °C until use.

2.5. Polyphenol oxidase activity assay

PPO activity was determined using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate at 25 °C by measuring the initial rate of dopachrome formation [18].

2.6. Immobilization

150 mg of dry TC (1 g of semidry) carrier was equilibrated with 25 ml of 0.15 M potassium-phosphate buffer pH 7.0 with 0.5 M NaCl to prevent nonselective ionic adsorption. TC carrier was then resuspended in 30 ml of enzyme preparation (192,000 U). Same carrier was added to 0.5 M NaCl as blank probe. Mixtures were left for 12 h on IKA orbital shaker at 400 rpm. Biocatalysts were removed by centrifugation at 14000 × *g* and TC was washed six times with 30 ml of 0.9% NaCl (untill total unbound enzyme was eluted).

2.7. Immobilized PPO activity assay

The activity of immobilized enzyme was assayed using a modified version of the method of Kwon and Kim [18], since

immobilizate has to be removed by centrifugation from L-DOPA solution prior to spectrophotometric measurement. Twenty milligrams of air dried biocatalyst was added to 1 ml of 9.3 mM L-DOPA in 50 mM Tris HCl buffer pH 7.0 at 25 °C. The mixture was shaken for 3 min and immobilizate was removed by centrifugation at 14000 × g. Increase in absorbance due to dopaquinone formation in resulting supernatant was measured at 475 nm. The blank sample contained 20 mg of dried blank carrier instead of biocatalyst, besides the other component of activity assay mixture. Specific activity of immobilized PPO is defined as increase in absorbance of 0.001 per min per gram of semidry biocatalyst under the given assay conditions.

2.8. Determination of pH optimum

To determine the pH optimum against L-DOPA, 10 mg of TC-PPO and 50 μl of soluble PPO was incubated with 750 μl of appropriate 50 mM buffer (acetate, pH 4.3–5.8; Tris–HCl, pH 7.0–8.0; potassium phosphate, pH 9.0–10.0) for 30 min and 250 μl of 9.3 mM L-DOPA was added. Further steps are same as described in Section 2.7.

2.9. Determination of temperature optimum

To determine the temperature optimum against L-DOPA, 10 mg of TC-PPO and 50 μl of soluble PPO were resuspended in preincubated 750 μl of 50 mM sodium phosphate buffer pH 7.0 at appropriate temperature (0–95 °C) after which 250 μl of 9.3 mM L-DOPA preincubated at same temperature was added. Further steps are same as described in Section 2.7. For soluble enzyme above described procedure for PPO activity assay was used. Highest activity was designated as 100%.

2.10. Treatment of soluble and immobilized PPO with detergents

Soluble and immobilized enzymes were incubated with SDS detergent (0.25–1.0%) in 50 mM sodium phosphate buffer pH 7.0 at 25 °C for 100 min. Remaining PPO activity was determined as described in Section 2.7. The activity of the untreated preparation was taken as a control (100%).

2.11. Preliminary application study of immobilized PPO using batch reactor

Removal of phenolic compounds from synthetic wastewater was investigated with 30 mg dry biocatalyst (310,000 U/g) that was added to a 20 ml of 100 mg/L solutions of *p*-CP and *p*-BP dissolved in 50 mM sodium phosphate buffer pH 7.0 and incubated on orbital shaker to allow continuous oxygenation. Phenol solution was oxygenated by air sparging for 10 min prior to use.

2.12. Quantitative assay of phenol compounds

Treated solutions were sampled at different time scale and filtered through 0.45 μm filter and then tested for remaining phenol content using 4-aminoantipyrine (AAP) assay [19]. The concentrations of phenols were measured using a reaction 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 was proportional to the concentration of phenols in the assay solution.

2.13. UV spectrometry analysis of TC-PPO treatment products

In order to confirm the oxidation of *p*-BP and *p*-CP by TC-PPO, spectral analysis was performed. An UV spectrum was recorded using Cintra40 UV–vis Spectrometer in 200–380 nm range. This was done for *p*-BP and *p*-CP before and after treatment with TC-PPO as described in Section 2.11. The diminution in absorbance peak of treated sample in UV region was taken as an evidence for the removal of both compounds.

2.14. 2.14. p-CP and p-BP removal reusability of TC-PPO

Reusability of TC-immobilizate was tested by repeating the above described batch reactor experiment six times. Between these cycles immobilizate was collected by filtration and washed three times in sodium phosphate buffer pH 7.0.

2.15. Statistics

All experimental results reported in the next sections were based on averaging results of repeated experimental runs (triplicates), with the standard deviation ranging from 2 to 6% of the reported average.

3. Results and discussion

3.1. Synthesis of 'tentacle' carrier

Previously it was described that during synthesis of DEAE-cellulose matrix, there is always few percent of residual amino groups besides diethylaminoethyl groups that are primarily derivatised by epichlorohydrin used in this study [20]. This happens because DEAE groups are somewhat sterically hindered and hence less reactive and epichlorohydrin activation leaves some of the DEAE groups unreacted, thus obtaining the mixed IMAC/IEX carrier. Epichlorohydrine spacer arm is introduced in DEAE cellulose *via* creating bond with nitrogen atom as shown in Fig. 1a. Under these conditions OH groups of cellulose are less reactive than NH_2 because most of OH groups are already spent during synthesis of DEAE-cellulose, and those left are less reactive due to steric hindrance.

Chelating group, IDA is irreversibly coupled to epoxy-activated DEAE cellulose (Fig. 1a–c.). Copper ions reversibly form a coordinate bond with the chelating group thus creating tentacle Cu-carrier (Fig. 1d).

3.2. Carrier characterization

Water content has been determined to be 85%. Amount of bound epoxy group prior to introduction of IDA has been determined to be 0.144 mmol/g of dry support. Copper content measured by ICP-AES is 7.3110 mg/g of dry support.

3.3. Partial purification of PPO

One of the problems for the purification of polyphenoloxidase from plant material is presence of phenolics since these compounds are oxidized to quinones by PPO, which could react covalently with the enzyme, resulting in aggregation of enzyme [18]. Strategy applied in this work for minimizing these unwanted effects is immediate desalting of crude extract using industrial Sephadex G25 (coarse granulation for fast chromatography). Oxygen-free atmosphere in later steps prevents oxidation of enzyme with remaining phenolics, since other substrate – oxygen is not available. QAE Sephadex is a well known industrial chromatographic matrix. Its wide spread use is consequence of its high capacity for protein binding and cheap price. Purification steps were designed to exploit maximum yield from capturing chromatography methodology with respect to time required for this process. Care has been taken to optimize purification conditions so the process can be

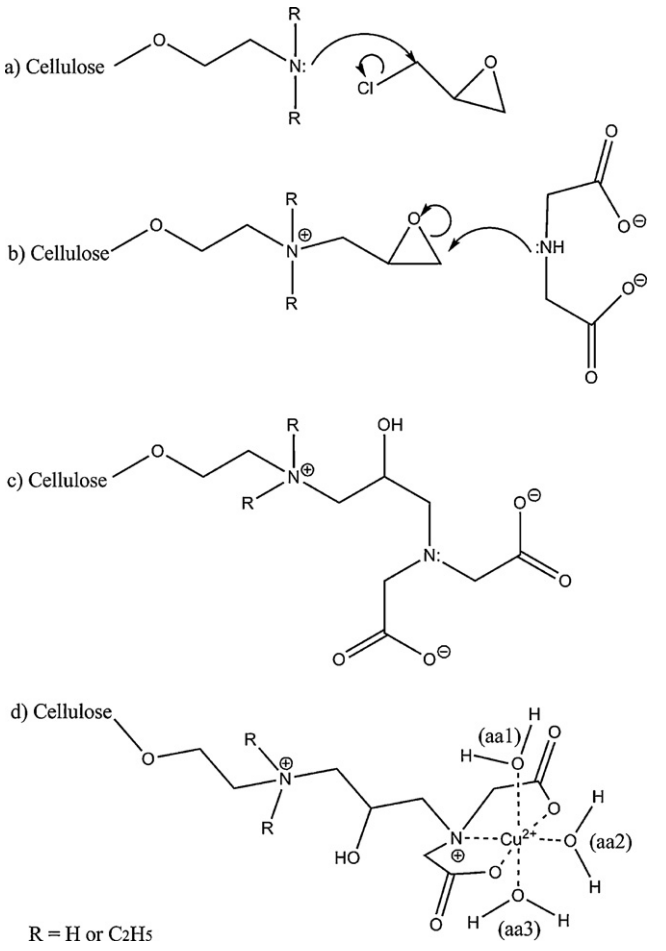

Fig. 1. Synthesis of tentacle carrier. Epichlorohydrin activation of DEAE cellulose is followed by introducing IDA functional groups. Copper ions are bound by coordinative bonds of IDA and three molecules of water replaced during immobilization process by N, O and S atoms of amino acid residues present on surface of protein.

easily scaled-up to industrial level. For this purpose batch chromatography and cheap phosphate buffer have been chosen. This procedure gives a yield of around 70% polyphenoloxidase activity as compared to starting extract. The partially purified PPO showed 8500 U/ml under given assay conditions.

3.4. Immobilization

Protein immobilization is achieved by reversible binding of copper and certain amino acid residues of the protein (aa1–aa3 in Fig. 1d). At this stage, the metal ions can be viewed as electron-pair acceptors (Lewis acids) and the N, 0, and S atoms of the amino acid side chain ligands as electron-pair donors (Lewis bases). These amino acids must be located on the surface of the protein molecule in order to be accessible to the metal ion chelate. This support offers more fluidity and hence substrate accessibility to bound protein as compared to conventional support of this type with much shorter spacer arm.

Using partially purified preparation of PPO under given immobilization conditions biocatalyst with activity of 310,000 U/g of dry biocatalyst was produced.

3.5. Effect of pH and temperature

Experiments were carried out to assess the effect of temperature and solution pH on the activity of TC-PPO. All other parameters were kept constant while varying the temperature from 0 to 95 °C and pH from 4.3 to 10.

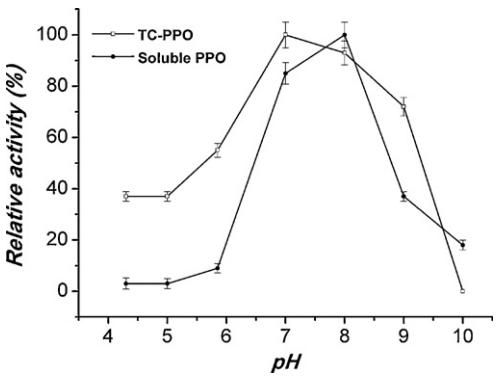

Fig. 2. Effect of the solution pH on the oxidation rate of L-DOPA by soluble and immobilized PPO.

The experimental results for the effect of initial solution pH on the concentration of substrate and oxidation rate of L-DOPA are shown in Fig. 2. These results reveal that TC-PPO is active towards L-DOPA at wider pH range in contrast to soluble PPO. pH optimum for TC-PPO is 7.0–8.0. This is very convenient since pH close to neutrality is desirable for the treatment of halogenophenols effluents. Since the pK_a values of *p*-BP and *p*-CP at 25 °C are 9.37 and 9.41, respectively [21], the removal efficiency of TC-PPO will decrease at pH close to 9.5 and this is attributed to the formation of the *p*-BP and *p*-CP conjugated bases that do not permit the phenol compounds to act as hydrogen donors.

A plot of the TC-PPO activity versus temperature is shown in Fig. 3. Determination of temperature optimum showed similar trends for both soluble and immobilized PPO. Clearly, the activity of potato PPO and consequently its ability to oxidize phenolic substrates is optimized at about 25 °C. Higher temperatures seemed to negatively affect the activity of PPO; however, immobilized enzyme has 34% of residual activity at 90 °C. Exposure to lower temperatures is expected to slow down the enzymatic activity, but immobilized PPO has shown almost 100% of activity at 0 °C under assay conditions described in Section 2.9. This is interesting characteristic since it avoids necessity for temperature regulation in large reactors, which further leads to energy saving.

3.6. Effect of SDS detergent

Wastewater also gets contaminated with detergents released from industry and municipal sewage so there is a need to investigate biocatalyst efficiency in presence of detergent [22]. The stability of TC-PPO and soluble PPO in presence of anionic detergent

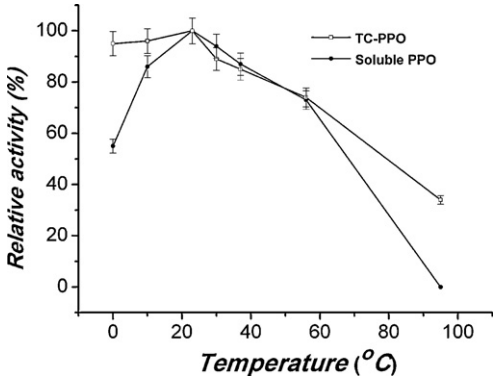

Fig. 3. Effect of the solution temperature on the oxidation rate of L-DOPA by soluble and immobilized PPO.

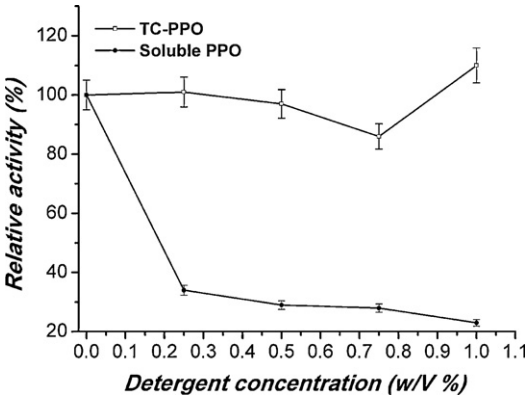

Fig. 4. Effect of SDS on soluble and immobilized PPO.

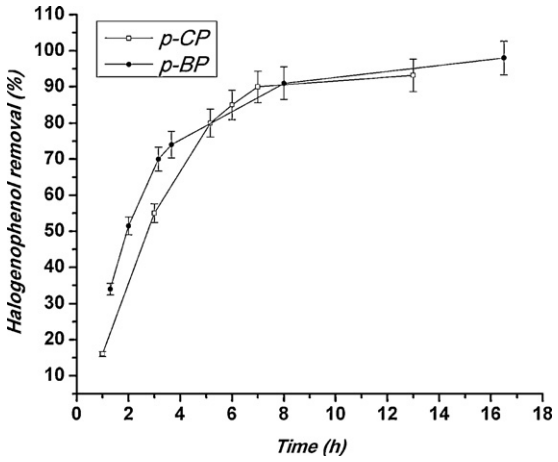

Fig. 5. Removal of 100 mg/L halogenophenols from aqueous solution. Aliquots are taken at different time points and phenol concentration measured.

SDS was examined. Soluble and immobilized potato PPO preparations were exposed to various concentrations of SDS (0.1–1.0%, w/v). Khan et al. [6] conducted investigation of Celite immobilized PPO and showed that immobilized enzyme was more resistant to the denaturation induced by SDS detergent as compared to its soluble counterpart. Our results comply with this as considerable stabilization is obvious as well as slight activation at SDS concentration of 1% as shown in Fig. 4. Activation by submicellar concentrations of SDS is well documented for some plant, fungal and bacterial PPOs [23–25]. On the other side, SDS denatures most proteins by conformational change and thus decreases activity of soluble enzyme. Activation of immobilized PPO may be explained by limited conformational change as a consequence of binding of small amounts of SDS which induce or initiate activation of enzyme. Limited conformational change is direct characteristic of immobilized enzyme. Incubation of soluble enzyme with 1.0% SDS for 1 h resulted in a loss of 77% of its original activity under identical incubation conditions.

3.7. Preliminary application study of TC-PPO

As model pollutants we have chosen *p*-BP and *p*-CP because they are used as disinfectants in home, hospitals and farms [26] and they had been used previously as model system [2,27,28]. During experimental removal of these two compounds from aqueous solution similar results were obtained for both *p*-CP and *p*-BP. In preliminary study for removal of 100 mg/L *p*-BP from aqueous solution we showed that more than 90% removal was achieved after 8 h as shown in Fig. 5. *p*-CP was removed by same rate. This experiment has been carried out at constant temperature held at 25 °C. pH of solution remained same during whole treatment experiment. However, color formation was observed to change from transparent at the beginning of the process to lightly brownish after 2–3 h. This is followed by dark brown/black precipitate formation of polymers formed from oxidized halogenophenols. This precipitate partly adsorbs on TC-PPO and partly remains in solution.

In order to confirm the oxidation of *p*-BP and *p*-CP by TC-PPO, spectral analyses were performed on filtered samples. The diminution in absorbance peak of treated sample in UV region was a clear evidence for the removal of treated halogenophenols (data not shown).

By this treatment halogenophenol concentration was lowered below 10 mg/L which is an upper limit value set by water control regulations for phenolic compounds present in wastewater released in open water [29]. Almost equally successful was the process that exploited soluble bitter gourd peroxidase published by Ashraf and Husain [27]. However, some biocatalysts obtained with commercial carriers such as Eupergit, have shown much less effectiveness in phenol compounds removal. It has been shown that PPO and HRPO immobilized on Eupergit carriers were able to remove only 45% of *p*-BP and 50% of phenol [4,30], and this fact is one of the reasons for creating new carriers.

3.8. Reusability of TC-immobilizate

The main goal of enzyme immobilization is the industrial reuse of enzymes for many reaction cycles so it was necessary to investigate the reusability of immobilizate for the removal of *p*-CP and *p*-BP. For both *p*-BP and *p*-CP TC-PPO works with over 90% removal during three cycles, each of 8 h duration. After six times of repeated tests the efficiency of *p*-CP and *p*-BP removal by TC-PPO immobilizate decreased to 55% and 60%, respectively (Fig. 6).

As described in Section 3.7 accumulation of dark precipitates on immobilizates was observed. Accumulation of dark precipitate gradually increased after each cycle and this can be explanation for a reduction of the removal efficiency in latter cycles since precipitate interacts with bound PPO by hydrophobic interactions thus preventing its movement and possibly inhibits it as pseudo substrate. Further studies should examine application of continuous reactors which will prevent accumulation of reaction product on this biocatalyst. Previously it was reported that colored products generated from phenol compounds by tyrosinase react with amino group containing coagulants such as chitosan

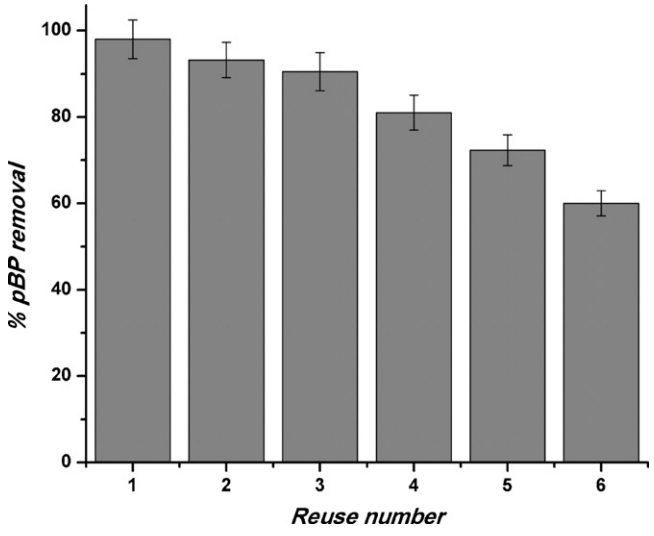

Fig. 6. The effect of repeated use on the activity of TC-PPO.

and polyethylenimine [31]. In order to decrease time necessary to complete treatment usage of coagulants in batch reactor may be explored because removal of reaction products will provide driving force for reaction to continue at the same rate as at the beginning. Better alternative to coagulants usage is application of TC-PPO in continuous packed bed airlift reactors since this will decrease contact time between catalyst and reactive quinones thus prolonging number of possible reuses. This is a subject of our further study.

4. Conclusions

From the industrial point of view simplicity and cost-effectiveness are the key demands for immobilized enzymes and these requirements are fulfilled with low cost PPO and low cost TC. Moreover, TC can be reused until enzyme activity decreases and after that carrier can be stripped with EDTA and charged with new dose of enzyme. Presented method of immobilization in overall is very economical procedure for the immobilization of PPO and immobilizate showed good potential for halogenophenol removal by polymerization. Thus, the proposed process can be considered as a first step in further exploration of possible carriers for application of immobilized PPO for phenol and phenol derivatives removal from wastewater.

Acknowledgements

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