

Application of Diethylaminoethyl Cellulose Immobilized Pointed Gourd (*Trichosanthes dioica*) Peroxidase in Treatment of Phenol and α -Naphthol

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Abstract

Recently, enzymatic treatment using peroxidases in removal of aromatic compounds has gained importance. In this study pointed gourd peroxidase was salt fractionated and direct immobilization of these proteins on diethylaminoethyl cellulose for oxidation of phenol and α -naphthol has been investigated. The activated diethylaminoethyl cellulose was quite effective in high yield immobilization of peroxidases from pointed gourd and it could bind ~576 units per g of the matrix. Immobilized pointed gourd peroxidase on this anion exchanger showed very high effectiveness factor 'E' as 0.91 with an activity yield of 91%. Immobilized PGP (I-PGP) as compared to soluble counterparts (s-PGP) were more effective and removed 79%, 88% and 54% oxidation of phenol and α -naphthol by 75%, 81% and 61% at 30, 40 and 50°C respectively, with a treatment time of 140 min. In the absence CdCl₂ s-PGP as well as I-PGP exhibited upto 93% of oxidation of these compounds; whereas the presence of CdCl₂ negatively affected the removal of phenol and α -naphthol. The reactor worked well continuously for over one month for effectively oxidizing/ removing phenol and α -naphthol by 54% and 61% respectively. Thus, such immobilized enzyme systems in reactor have a great future and could be exploited for treating organic pollutants present in industrial effluents.

Keywords: Pointed gourd; Peroxidase; Diethylaminoethyl; Phenol; α -Naphthol; Polyethylene glycol; Batch reactor; Continuous reactor

Introduction

The presence of different aromatic compounds in wastewater from several industrial effluents is of great environmental concern. Phenolic compounds and its derivatives have their sources from a number of chemical industries such as petroleum, coal conversion, resins, textile dyes and paper processing [1]. Most phenolic compounds are toxic, pose health risk and have been classified as hazardous pollutants that can accumulate in the food chain [2-4]. α -Naphthol is a toxic hydroxylated metabolite of polycyclic aromatic hydrocarbon naphthalene widely used in the manufacture of plastics, rubber, synthetic fibers and dyes [5-7]. Due to their potential lethality, wastewater must be treated for the removal of these compounds/derivates prior to its final discharge into environment.

A number of conventional strategies are available for treating these aromatic compounds which includes microbial degradation, solvent extraction, chemical oxidation, incineration, adsorption on activated charcoal and etc. [8-12]. These methods are effective in removing the pollutants with limitations of being expensive, time consuming, and low efficiency, applicability to a limited concentration range and formation of soluble toxic by-products [13-15].

The enzymatic removal of aromatic compounds from wastewater by using peroxidase and hydrogen peroxide has found wide importance and acceptability [10,16-18]. Enzymatic method has certain advantages over conventional methods of treatment which includes: applicability over a broad range of pH, temperature, contaminant concentration, action on recalcitrant materials and simplicity in controlling the process [4,19]. Peroxidases (EC 1.11.1.7) are a group of heme-containing enzymes that have wide spectrum substrate specificity [20]. These enzymes have been isolated from many species of plants, animals and microorganisms [21]. A number of peroxidases in soluble forms have been employed in treatment of phenolic compounds including dyes, turnip roots, mustard, bitter gourd [1,22-27]. However, an effective use of enzymes was limited due to their non-reusability, sensitivity to various denaturants and high cost [28]. Some of these constraints may be overcome by immobilizing the soluble enzymes on various supports

[29,30].

Peroxidases have the ability to act on a number of aromatic compounds in the presence of hydrogen peroxide. The function of the latter is to oxidize the enzyme into its catalytically active form which in turn is capable of reacting with the phenolic contaminants [21]. During the reaction process these enzymes get inactivated which might be due to the free radical formation during enzymatic reaction. These radicals adsorb on the enzymes active site and block the substrate binding sites [12]. Nevertheless, the enzyme inactivation can be curtailed by using additives which includes borate, Polyethylene Glycol (PEG), gelatin etc. [27,31].

This study is an attempt to investigate the feasibility and reusability of using pointed gourd peroxidase (PGP) immobilized on DEAE (diethylaminoethyl) cellulose for the removal of phenol and α -naphthol from synthetic wastewater. Further, the removal of phenol and α -naphthol by immobilized PGP (I-PGP) was done in batch process as well as in a continuous vertical bed-reactor in presence of PEG.

Materials and Methods

Materials

Bovine serum albumin, o-dianisidine HCl, DEAE cellulose, ammonium sulphate, polyethylene glycol, α -naphthol, phenol and

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glutaraldehyde was procured from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. The pointed gourds were procured from local market.

Extraction and purification of soluble PGP (s-PGP)

Pointed gourd (350 g) was homogenized in a blender with 300 mL of 100 mM sodium acetate buffer, pH 5.5. The peroxidase protein was extracted as described previously [32].

Protein and peroxidase activity measurement

The protein was estimated using Lowry's method with bovine serum albumin as standard [33]. Peroxidase activity was measured as change in the optical density (460 nm) at 37°C by estimating the initial rate of oxidation of 6.0 mM o-dianisidine HCl by 18.0 mM H₂O₂. One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1mmol of o-dianisidine HCl in the presence of H₂O₂ per min at 37°C into colored product ($E_m = 30,000 \text{ M/L}$).

Treatment and activation of DEAE cellulose

The activation of DEAE cellulose was done using method described elsewhere [34,35]. Briefly 6.0 g of DEAE cellulose was gently stirred and allowed to swell overnight in 150 mL of distilled water. With a Buchner Funnel the swollen DEAE cellulose was filtered and incubated with 120 mL of 0.5 N HCl for 1 h. DEAE cellulose was collected by filtration and was washed with distilled water continuously till it attained pH 7.0. 125 mL of 0.5 N NaOH was added to HCl treated DEAE cellulose and stirred on a magnetic stirrer for 1 h at 4°C; was washed again with distilled water till it attained neutral pH. Further, it was suspended and stored in 100 mL of distilled water at 4°C.

Adsorption and cross-linking of PGP on DEAE activated cellulose (I-PGP)

PGP (6680 units) was added to 6.0 g of DEAE cellulose and stirred overnight at 4°C. Unadsorbed PGP was removed by extensive washing with the assay buffer. The preparation was treated with 0.3% (v/v) glutaraldehyde for 2 h at 4°C with constant stirring. Cross-linking was performed in presence of o-dianisidine HCl. Ethanolamine was added to a final concentration of 0.01% (v/v) to stop cross-linking. The solution was allowed to stand for 90 min at room temperature and the pellet was collected by centrifugation at 3000 \times g for 30 min on a cooling centrifuge 4°C [36,37].

Measurement of phenol and α -naphthol concentration

Phenol concentration was measured spectrophotometrically using 4-antiaminopyrene (4-AA) and potassium ferricyanide. Under alkaline conditions a red quinone-type dye is formed that absorbs at 510 nm upon completion of the reaction [38,39]. The mixture assay of 1 ml was formed in the order described by Saboora and Hejir [40] (750 μ l of 0.25 mM NaHCO₃ buffer, 50 μ l of aqueous phenol sample and 100 μ l of 20.8 mM 4-AAP and mixed vigorously). 100 μ l of potassium ferricyanide was added and mixed again and after 9 minutes of the ferricyanide addition, absorbance was measured at 510 nm. The oxidative degradation and removal of α -naphthol from polluted water was monitored at 293 nm. The percent oxidation and removal of α -naphthol was calculated by taking untreated α -naphthol polluted water as control (100%).

Influence of cadmium chloride (CdCl₂) on peroxidase (s-PGP and I-PGP) mediated removal of phenol and α -naphthol

Phenol (5 mM; 10 mL) and α -naphthol (0.5 mM, 10 mL) was

treated independently by soluble and immobilized PGP (2.6 U /mL) in 100 mM sodium phosphate buffer, pH 6.5, in presence of 0.8 mM H₂O₂, 0.15 mg/mL PEG-10000 and CdCl₂ (0.3-3.0 mM) for 2 h at 40°C. The reaction was stopped by heating in boiling water for 7 min. The insoluble product was removed by centrifugation at 3000 g for 20 min. Measurement of phenol and α -naphthol was done as described previously.

Reusability of I-PGP in treatment of phenol and α - naphthol

Phenol (5 mM, 50 mL) and α - naphthol polluted water (0.5 mM, 50 mL) was independently incubated with I-PGP (50 U) in sodium phosphate buffer, pH 6.5 in the presence 0.8 mM H₂O₂ and 0.15mg/mL (PEG-10000) for 3 h at 40°C. After the reaction, enzyme was separated by centrifugation and stored in the assay buffer for over 12 h at 4°C. Similar and independent experiments were repeated six times with the same preparation of I-PGP but with an addition of fresh batch of phenol and α -naphthol polluted water. The oxidative degradation and removal of phenol and α -naphthol from polluted water was monitored at 510 nm and 293 nm respectively as described previously.

Independent treatment of phenol and α -naphthol in a stirred batch process

Phenol (5 mM, 100 mL) and α -Naphthol (0.5 mM, 100 mL) was treated with s-PGP and I-PGP (100 U) independently in the presence of 0.8 mM H₂O₂ and 0.15 mg/mL (PEG-10000) for 5 h at three different temperatures (30, 40 and 50°C) in presence of 100 mM sodium phosphate buffer, pH 6.5 with constant stirring. Aliquots were taken from the reaction mixtures at varying times and the reaction was stopped by heating in boiling water for 7 min. The insoluble product was removed by centrifugation at 3000 g for 20 min. The oxidative degradation and removal of phenol and α -naphthol from polluted water was monitored at 510 nm and 293 nm respectively as described previously.

Independent treatment of phenol and α -naphthol in a continuous reactor filled with I-PGP

A vertical packed bed-reactor system was developed for the continuous oxidation of phenol and α -naphthol. The column (15.0 \times 2.0 cm) was filled with DEAE cellulose immobilized PGP (2200 U) and equilibrated with 100 mM sodium phosphate buffer, pH 6.5. The working volume of the reactor was 25.7 mL. The phenol (5 mM) and α -naphthol polluted water (0.5 mM) containing 0.8 mM H₂O₂ and 0.15 mg/mL of PEG-10000 were continuously passed through the reactor independently at room temperature. The flow rate and residence time of the column was maintained as 18 mL/h. Samples from the column outlet were collected and analyzed using UV-visible spectrophotometer for the remaining phenol and α -naphthol at 510 nm and 293 nm respectively.

Data analysis

Each value represents the mean for three independent experiments performed in duplicates.

Results

Adsorption and crosslinking of PGP on DEAE cellulose

DEAE cellulose adsorbed 576 units of peroxidase per g of the matrix (Table 1). The effectiveness factor 'E' of the DEAE immobilized peroxidase preparation was 0.91 and the activity yield was 91%. A sufficiently high effectiveness factor of DEAE immobilized pointed

Adsorption of PGP on DEAE	
PGP loaded (A) units	6680
PGP activity in the unbound (B) units	2900
Activity of adsorbed PGP per g of DEAE cellulose (units)	
Calculated value (A-B=C) (X)	630
Actual Value (Y)	576
Effectiveness factor (ϵ) (Y/X)	0.91
Activity yield ($\epsilon \times 100$) (%)	91

Table 1: Peroxidase activity was measured as described in text. Experiments were performed in duplicate. Each value represents the mean of three independent experiments. Average standard deviation was <5%.

gourd peroxidase (I-PGP) preparation indicated that the I-PGP preparation was an effective catalytic system.

Influence of CdCl_2 on oxidation of phenol and α -naphthol

In the absence CdCl_2 s-PGP as well as I-PGP exhibited upto 93% of oxidation of these compounds; whereas the presence of CdCl_2 of negatively affected the removal of phenol and α -naphthol (Table 2). On increasing the concentration of CdCl_2 (0.3 to 3.0 mM) the oxidation/removal of phenol and naphthol decreased from 86 to 55% and 87 to 61% respectively by I-PGP. It was also observed that s-PGP catalyzed the oxidation/removal phenol and naphthol from 79 to 43% and 84 to 42% respectively, which was relatively lower to that of I-PGP suggesting more susceptibility towards the presence of cadmium chloride (Table 2).

Reusability of I-PGP for oxidation of phenol and α -naphthol

Figure 1 shows the reusability data of I-PGP in the catalytic oxidation of these compounds. It was observed that ~40% oxidation was effective with I-PGP on the 7th repeated use which declined further to ~25% on the 10th use.

Removal of phenol and α -naphthol in a stirred batch process by s-PGP and I-PGP at different temperatures

Treatment of phenol and α -naphthol was done using s-PGP and I-PGP in a stirred batch process at temperatures of 30, 40 and 50°C (Table 3). It was observed that after 140 min of incubation the s-PGP oxidized/ removed phenol 73%, 76% and 54% at 30, 40 and 50°C respectively. However, under similar conditions of incubation I-PGP was able to catalyze 79%, 88% and 65% oxidation of phenol. In case of removal of α -naphthol 66%, 70% and 48% oxidation was achieved by s-PGP after 200 min of treatment at 30, 40 and 50°C respectively. However, under similar conditions of treatment I-PGP catalyzed the oxidative removal of α -naphthol by 75%, 81% and 61% at respective temperatures of 30, 40 and 50°C. It was observed that I-PGP was sustainable in these temperatures even on prolonging the incubation time to 320 min.

Treatment of phenol and α -naphthol by I-PGP in a continuous vertical reactor

Figure 2 shows the catalytic removal of phenol and α -naphthol by immobilized PGP present in a vertical packed bed-reactor. The reactor worked well continuously up to 35 days for effectively oxidizing/removing phenol and naphthol to 54% and 61% respectively. On increasing the operational duration the performance of the reactor decreased and removal of 16% and 19% was achieved on the 70th day for phenol and α -naphthol respectively.

Discussion

Protein/enzyme immobilization is a tricky approach. Although a

number of protocols are available for stably immobilizing the proteins, direct immobilization of partially purified proteins for sustainable removal of toxic compounds from industrial effluents still needs to be explored [41]. Several earlier investigators have described that the immobilization of enzymes on DEAE cellulose support resulted in the stabilization of enzymes against various forms of denaturation [42-44]. The work reported unfolds the effort to directly immobilize ammonium sulphate fractionated peroxidase from pointed gourd on DEAE cellulose. DEAE is an ion exchanger employed to purify diverse proteins from various sources [43]. PGP was adsorbed efficiently to the extent of 576 units per gram of DEAE cellulose. This immobilized preparation (I-PGP) was active, efficient and exhibited a high effectiveness factor ($\epsilon=0.91$) and an activity yield of 91% (Table 1). The effectiveness factor of an immobilized enzyme system reflects the efficiency of the immobilization procedure as it is a measure of internal diffusion [16]. In the case of PGP, the yield of immobilization on DEAE was sufficiently better over other methods of immobilization of peroxidases [45,46].

Several earlier investigators have also reported the use of DEAE cellulose support for high yield and stable immobilization of enzymes and proteins [44,47].

Peroxidase mediated polymerization has proven to be very effective

Cadmium Chloride (mM)	Percent removal of			
	Phenol		α -Naphthol	
	s-PGP	I-PGP	s-PGP	I-PGP
0.3	79	86	84	87
0.6	72	81	78	85
0.9	67	78	74	81
1.2	63	71	70	75
1.8	58	68	63	71
2.4	51	62	56	65
3.0	43	55	42	61

Table 2: Influence of cadmium chloride on the removal of Phenol and α -Naphthol by s-PGP and I-PGP. Phenol (5.0 mM, 10.0 mL) and α -Naphthol (0.5 mM, 10.0 mL) was treated by s-PGP and I-PGP (2.6 U/mL) in sodium phosphate buffer (100 mM, pH 6.5) in the presence of varying concentration of cadmium chloride (0.3 to 3.0 mM), H_2O_2 (0.8 mM) at 40°C for 2 h

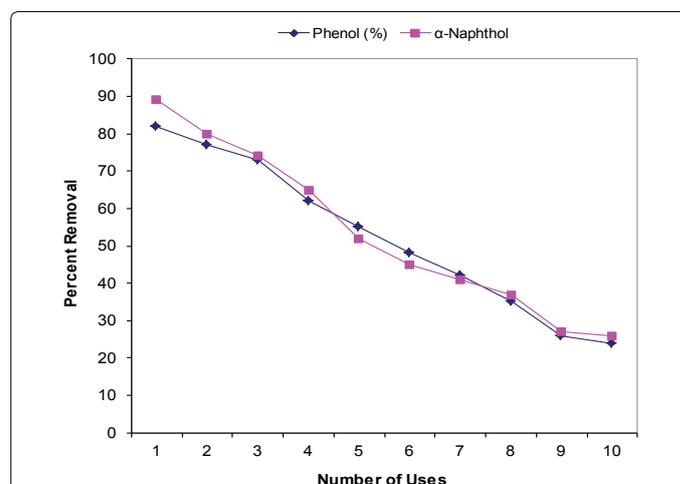


Figure 1: Reusability of I-PGP for oxidation of phenol and α -naphthol. Phenol (5 mM, 50) and α -Naphthol polluted water (0.5 mM, 50 mL) was treated with I-PGP (50 U) for 2 h at 40°C. After reaction, immobilized enzyme was collected by centrifugation at 3000 g and stored in assay buffer overnight at 4°C. Similar experiment was repeated 10 times with fresh batch of phenol and α -naphthol polluted water.

Time (min)	Removal of phenol (%) at						Removal of phenol (%) at					
	30°C		40°C		50°C		30°C		40°C		50°C	
	s-PGP	I-PGP	s-PGP	I-PGP	s-PGP	I-PGP	s-PGP	I-PGP	s-PGP	I-PGP	s-PGP	I-PGP
20	36	31	46	41	29	28	34	37	43	40	26	27
40	42	36	51	55	31	33	39	41	49	53	29	32
60	54	55	63	67	39	53	45	58	59	68	37	47
80	58	64	67	74	45	57	51	62	63	72	46	53
100	65	68	69	79	51	60	63	67	68	78	53	61
120	74	79	78	89	56	65	72	76	74	83	55	67
140	73	79	76	88	54	65	71	75	74	81	52	65
200	69	78	71	85	52	63	66	75	70	81	48	61
260	68	78	70	85	49	63	64	74	64	80	43	61
320	68	78	70	85	49	63	64	74	64	80	43	61

Table 3: Removal of phenol and α -naphthol in a stirred batch process by s-PGP and I-PGP at different temperatures. Phenol (5.0 mM, 100.0 mL) and α -Naphthol (0.5 mM, 100.0 mL) was treated by s-PGP and I-PGP (50 U) independently in the presence of H_2O_2 (0.8 mM) and 0.15 mg/mL (PEG 10000) at temperatures of 30, 40 and 50°C for varying time intervals.

in eliminating aromatic compounds like various phenols from both synthetic and real wastewaters [21,27,48]. Peroxidases, in the presence of hydrogen peroxide (H_2O_2), which acts as electron acceptor, are able to catalyze the oxidative polymerization of phenolic compounds to form insoluble polymers [49]. The native enzyme is oxidized by peroxide (H_2O_2) to an active intermediate enzymatic form which accepts an aromatic compound into its active site and carries out its oxidation. A free radical (AH•) is produced and released into solution leaving the enzyme in different state. This compound then oxidizes a second aromatic molecule, releasing another free radical product and returning the enzyme to its native state, thereby completing the cycle. Free radicals formed during the cycle diffuse from the enzyme into the bulk solution where they react to form water-insoluble polyaromatic products.

In the present work PGP immobilized on DEAE support was studied to treat water contaminated with phenols and α -naphthol. Immobilization of enzymes imparts merits over the free enzyme like stability over prolonged storage and reuse. Moreover, it may also improve the catalytic properties of enzyme [28,50]. Glutaraldehyde cross-linked DEAE cellulose adsorbed PGP preparation, efficiently catalyzed the oxidation and removal of phenol and α -naphthol.

In addition to aromatic pollutants wastewater is also loaded with several types of heavy metals; therefore we also evaluated the catalytic performance on oxidation of phenol/naphthol by s-PGP and I-PGP in the presence of heavy metals (Table 1). Cadmium is one of the most toxic metals and its influence on enzymatic catalysis was examined. It was observed that I-PGP was more tolerant and catalytically active at high concentrations of cadmium chloride in oxidation of these compounds whereas the s-PGP lost activity to a varying degree. One of the probable reasons for the decline in enzyme activity is the interference/ binding of metal ion to the catalytically exposed moieties and consequently the loss of structural integrity of PGP [51]. I-PGP were perhaps more resistant to alteration in their structures and hence under similar conditions performed better.

PEG is of particular interest as an additive because it significantly reduces the cost of treatment and has been declared as a non-toxic compound, for human consumption. Polyethylene glycol is biodegradable and as an additive improved the removal efficiency by protecting the enzyme during the reaction [52-56]. In this study the presence of PEG remarkably improved the catalytic performance of I-PGP in the oxidation of phenol and α -naphthol. A decrease in the peroxidase activity during the removal process showed that all peroxidases are susceptible to an oxidative inactivation in the

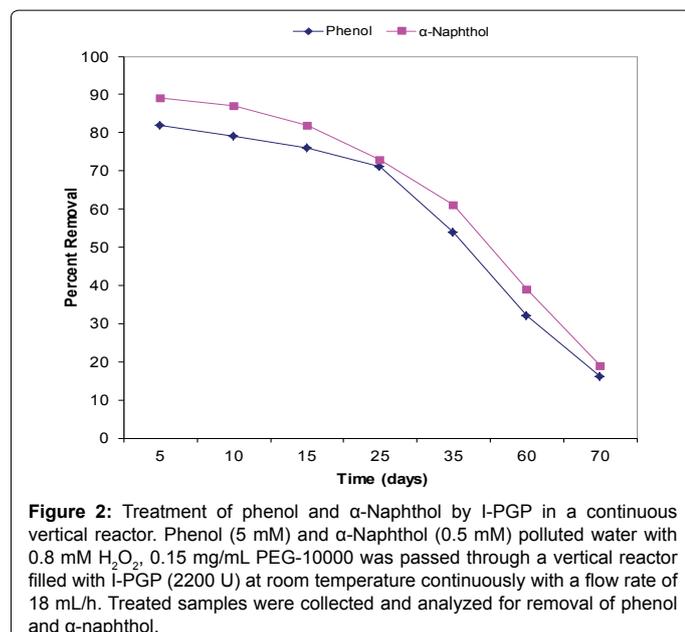
presence of hydrogen peroxide [57]. Different catalytic-mediated pathways, like heme destruction, and oxidation of essential amino acid residues appears to be responsible for this oxidative self-inactivation. As a result of this inactivation, enzyme is required in large amounts for a successful elimination of these phenolic compounds. Cheng et al., [55] showed that PEG improved the phenol removal efficiency of HRP by forming a protective layer around the active centre of enzyme which prevents the attack of free phenoxy radicals formed in the catalytic cycle. The adsorption of the reaction product on the active sites of enzyme molecules is prevented as most of the phenoxy radicals couple with PEG due to their greater affinity with PEG than the enzyme [39,40,56,58]. PEG-10000 provided a greater protection to PGP at the lowest concentration of PEG (in mg/L). Result showed a dramatic increase in the oxidation and removal of these compounds at concentration of 150 mg/L (data not shown).

As compared to soluble enzyme the immobilized peroxidase was much more effective in removing phenol and α -naphthol in a batch process. One of the reasons for better performance of immobilized preparations is shielding of a number of reactive free amino groups, which are exposed in s-PGP and consequently more susceptible to attack by free radical products [39]. Our earlier work support the observation of immobilized PGPs catalytic performance on various compounds usually present in industrial effluents [17].

DEAE cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it leads to desorption of enzyme from the support. To evaluate the efficiency of I-PGP on a large scale for the removal of phenol and α -naphthol, a vertical continuous reactor system was designed and operated continuously with a flow rate of 18 mL/h. The reactor performed continuous oxidative removal of these compounds to different degrees upto to 35 d without any operational problem like clogging which may be of concern since precipitate is formed during the enzymatic reaction (Figure 2). The oxidation of phenolic contaminant in the reactor is inversely proportional to the flow rate of reactor. A decrease in the removal rate at lower residence times can be due to the insufficient contact time between the phenolic compound and the peroxidases.

Conclusion

One of the remarkable properties of the immobilized enzyme over its soluble counterpart was that it can be separated from the reaction mixture and hence, can be reused repeatedly to transform its substrate. It is expected that DEAE cellulose adsorbed PGP preparation have a great future in the treatment of organic pollutants present in industrial



effluents.

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