Application of immobilized peroxidase for the removal of *p*-bromophenol from polluted water in batch and continuous processes

Humaira Ashraf and Qayyum Husain

ABSTRACT

Concanavalin A layered calcium alginate-cellulose beads adsorbed and cross-linked peroxidase of *Momordica charantia* was employed for the treatment of *p*-bromophenol polluted water. Immobilized peroxidase showed remarkably higher storage stability and retained about 78% phenolic compound removal efficiency over a period of one-month's storage at 4 °C. After a fourth repeated use immobilized enzyme retained nearly 50% *p*-bromophenol removal efficiency. *p*-Bromophenol removal by immobilized enzyme was ~84% in the presence of 0.1 mM $HgCl_2$. A significantly higher concentration of *p*-bromophenol was removed by immobilized enzyme in the presence of water-miscible organic solvents as compared to free enzyme. In stirred batch processes nearly 91%, 94% and 83% of *p*-bromophenol was removed in 3 h at 30, 40 and 50 °C, respectively. Immobilized enzyme present in two different reactors and operated at flow rates of 10 and 20 ml h^{-1} retained 75 and 65% *p*-bromophenol removal efficiency even after one month of their continuous operation. Absorption spectra for treated and untreated *p*-bromophenol exhibited a marked difference in absorbance at various wavelengths. Hence, it is concluded that reactors filled with immobilized enzymes can successfully be operated for the treatment of huge volumes of effluent containing various types of aromatic pollutants.

Key words | batch process, *p*-bromophenol, peroxidase, polyethylene glycol, wastewater

Humaira Ashraf
Department of Biochemistry,
Faculty of Life Sciences,
Aligarh Muslim University,
Aligarh 202002,
India

Qayyum Husain (corresponding author)
Faculty of Applied Medical Sciences,
Jazan University,
Jazan,
Kingdom of Saudi Arabia
E-mail: *qayyumbiochem@gmail.com*;
qayyumhusain@yahoo.co.in

ABBREVIATIONS

BGP	bitter gourd peroxidase
DMSO	dimethylsulfoxide
SI-BGP	surface immobilized bitter gourd peroxidase
PEG	polyethylene glycol
p-BP	*p*-bromophenol
S-BGP	soluble bitter gourd peroxidase

INTRODUCTION

A large number of chemical pollutants is generated by various industries worldwide annually. Such compounds, even if present in very small quantities, can impart negative effects on both nature and human health (Bayramoglu & Arica 2008). Phenols form one of the important constituents of these industries and, henceforth, find their way into the environment through industrial effluents. Most phenolic compounds are not only phytotoxic but also mutagenic, antimicrobial, as well as carcinogenic (Zilly *et al.* 2002; Casa *et al.* 2003). Therefore, it is essential to remove them from industrial wastewater before their final discharge into the surroundings.

Several physicochemical and biological approaches have been employed for the removal of phenolic compounds from industrial effluents (Kumar *et al.* 2004; Lai & Lin 2005; Bodalo *et al.* 2008). However, conventional procedures have faced some serious drawbacks such as formation of hazardous by-products, incomplete purification, prohibitively high purification costs, low efficiency and applicability only to a limited concentration range (Husain 2006; Cohen

doi: 10.2166/wrd.2011.017

et al. 2009). The focus on the microbial degradation of various phenolic compounds has resulted in the isolation, culture, adaptation and enrichment of a number of microorganisms that can grow on the compound as a sole carbon and energy source (Agarry *et al*. 2008). The biodegradation of phenolic compounds is not limited to the activity of a few microorganisms; it occurs frequently within bacteria, fungi, eukaryotic micro algae and higher plants (Semple & Cain 1997). However, microbial treatment has also exhibited some limitations such as high cost of production of microbial culture, limited mobility and metabolic inhibition (Husain 2010). Degradation of the aromatic compounds by bacteria, fungi and algae being attributed to secondary metabolic inhibition therefore requires appropriate growth conditions, which could be accomplished by additional loads of chemicals. Also the expression of the enzymes involved in the aromatic compound degradation is not constant with time but dependent on the growth phase of the organisms and is influenced by inhibitors that might be present in the effluent (Wesenberg *et al*. 2003). Moreover, algae and fungi take several days to detoxify the compounds present in wastewater (Sumathi & Manju 2000; Pinto *et al*. 2002). Henceforth, the use of peroxidases from plant and microbial sources has attracted the attention of enzymologists owing to their stable nature and relatively easy availability (Magri *et al*. 2005; Husain 2006; Husain *et al*. 2009).

Nevertheless, the use of soluble enzymes is limited due to their reusability, stability, sensitivity to various denaturants and applications in continuous reactors (Gomez *et al*. 2006; Husain & Husain 2008; Ashraf & Husain 2010). Such limitations can be overcome by using immobilized enzymes. Several supports like celite, sephadex and sepharose have been employed for the immobilization of peroxidases (Husain & Husain 2008; Husain *et al*. 2009; Husain 2010). Immobilization on calcium alginate-cellulose beads is straightforward and a large surface area is available for contact with the substrate (Matto & Husain 2009a, b; Ortega *et al*. 2009).

Oxidation of phenols by peroxidases in the presence of H_2O_2 resulted in the formation of free radicals, which might inactivate the enzyme. In this regard, use of various protective additives, such as borate, gelatin and polyethylene glycol (PEG), have been suggested to decrease enzyme inactivation, while being inexpensive, biodegradable and non-toxic (Bodalo *et al*. 2008; Ashraf & Husain 2009; Cohev-Yaniv & Dosoretz 2009). Studies have shown the addition of PEG is an effective procedure for protecting enzymatic activity and increasing aromatic compounds treatment efficiency (Yamada *et al*. 2010).

In this study, an attempt was made to employ bitter gourd peroxidase (BGP) immobilized on the surface of concanavalin A (Con A) layered calcium alginate-cellulose beads for the remediation of water contaminated with *p*-bromophenol (*p*-BP) in the presence of an additive, PEG. The operational stability of soluble and surface immobilized bitter gourd peroxidase (SI-BGP) for the treatment of *p*-BP has been compared in the presence of mercury (II) chloride ($HgCl_2$), *n*-propanol and dimethylsulfoxide (DMSO). *p*-BP removal reusability and storage stability of SI-BGP have been compared with its free form. SI-BGP was also used in stirred batch processes at various temperatures as well as in continuous packed-bed reactors for the removal of *p*-BP.

MATERIALS AND METHODS

Materials

o-Dianisidine-HCl was obtained from the Centre for Biochemical Technology, CSIR, India. Ammonium sulfate, PEG, *p*-BP, DMSO, $CaCl_2$ and cellulose were purchased from SRL Chemicals Pvt. Ltd (Mumbai, India). Glutaraldehyde was obtained from Thomas Baker Chemicals Pvt. Ltd (Mumbai, India). Sodium alginate was the product of Koch-Light Lab (Colnbrook, UK). Jack-bean meal was purchased from DIFCO (Detroit, USA). Bitter gourd was bought from a local vegetable market. All other chemicals and reagents employed in this study were of analytical reagent grade and were used without any further purification.

Ammonium sulfate fractionation of bitter gourd proteins

Bitter gourd (100 g) was homogenized in 200 ml of 0.1 M sodium acetate buffer at pH 5.5. The homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at a speed of $10{,}000 \times g$ on a Remi C-24 Cooling Centrifuge for 20 min. at 4 °C. The obtained clear supernatant was subjected to salt fractionation by adding 10–90% (w/v)

$(NH_4)_2SO_4$. The mixture was continuously stirred overnight at 4 °C for complete precipitation of proteins. The precipitate was collected by centrifugation at 10,000 × *g* on a Remi C-24 Cooling Centrifuge, redissolved in assay buffer and dialysed against the same buffer in order to remove traces of ammonium sulfate (Fatima & Husain 2008).

Preparation of jack-bean extract and layering of calcium alginate-cellulose beads by Con A present in extract

Jack-bean meal (10 g) was stirred in 100 ml of 0.1 M *tris*-HCl buffer, pH 6.2 overnight on a magnetic stirrer at room temperature. The clear extract obtained after centrifugation at 3,000 × *g* for 10 min. was used as a source of Con A for subsequent investigations (Matto & Husain 2009b).

A solution of sodium alginate (2.5%) and cellulose (2.5%) was prepared in 10 ml of 0.1 M sodium acetate buffer, pH 5.5. The mixture was slowly extruded as droplets through a 5.0 ml syringe with attached needle No. 20 into 0.2 M $CaCl_2$ solution to form calcium alginate-cellulose beads. The beads were further gently stirred for 2 h in $CaCl_2$ solution and washed by 0.1 M sodium acetate buffer, pH 5.5 and stored at 4 °C in the assay buffer for further use. Five hundred beads were incubated overnight with jack-bean extract (10 ml) at room temperature (30 ± 2 °C) and washed using the assay buffer.

Adsorption of BGP on Con A layered calcium alginate-cellulose beads and its cross-linking by glutaraldehyde

BGP (1,000 U) was incubated overnight with Con A layered calcium alginate-cellulose beads. The obtained immobilized enzyme was washed with the assay buffer and cross-linked with 0.1% (v/v) glutaraldehyde for 2 h at 4 °C. Ethanolamine was added to a final concentration of 0.01% (v/v) to stop further cross-linking. The solution was then allowed to stand for 90 min. at room temperature. The cross-linked beads were washed with the assay buffer and stored at 4 °C for further use (Matto & Husain 2009b).

Effect of heavy metal, $HgCl_2$, on *p*-BP oxidation

p-BP (0.4 mM, 5.0 ml) was independently treated by soluble and immobilized peroxidase (0.4 U ml^{-1}) in 0.1 M sodium acetate buffer in the presence of 0.75 mM H_2O_2, 0.1 mg ml^{-1} PEG and $HgCl_2$ (0.1–0.5 mM) for 2 h at 40 °C. The reaction was stopped by heating in boiling water for 5 min. The insoluble product was removed by centrifugation at 3,000 × *g* for 15 min. The oxidative degradation and removal of *p*-BP from polluted water was monitored at a wavelength of 280 nm in the UV. The percentage oxidation was calculated by taking untreated *p*-BP polluted water (containing all the reagents that were present in treated solution except the enzyme) as control (100%).

Effect of organic solvents on *p*-BP oxidation

p-BP (0.4 mM, 5.0 ml) was independently treated by soluble and immobilized BGP (0.4 U ml^{-1}) in the presence of increasing concentrations of water-miscible organic solvents: *n*-propanol/DMSO (2.0–30%, v/v) in 0.1 M sodium acetate buffer in the presence of 0.75 mM H_2O_2 and 0.1 mg ml^{-1} PEG for 2 h at 40 °C. Further reaction was stopped by heating in boiling water for 5 min. The insoluble product was removed by centrifugation at 3,000 × *g* for 15 min. The percentage oxidation was calculated by taking untreated *p*-BP polluted water as control.

Reusability of SI-BGP

p-BP polluted water (0.4 mM, 5.0 ml) was incubated with immobilized enzyme (0.4 U ml^{-1}) in sodium acetate buffer in the presence of 0.75 mM H_2O_2 and 0.1 mg ml^{-1} PEG for 2 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. The experiment was repeated four times with the same preparation of immobilized peroxidase but with an addition of a fresh batch of *p*-BP polluted water. The percentage removal was calculated by taking untreated compound as control (100%).

Storage stability of soluble and immobilized peroxidase

The soluble and immobilized enzyme preparations were stored at 4 °C in a refrigerator. Appropriate aliquots in triplicate of both the enzyme preparations were withdrawn over an interval of 5 days for a period of one month and were assayed for *p*-BP removal efficiency.

Treatment of *p*-BP in stirred batch processes

p-BP (0.4 mM, 100 ml) was treated by soluble and immobilized BGP (30 EU) independently in the presence of 0.75 mM H_2O_2 and 0.1 mg ml^{-1} PEG for 3 h at three different temperatures (30, 40 and 50 °C) in the presence of 0.1 M sodium acetate buffer, pH 5.5, with constant stirring. Aliquots were taken from the reaction mixtures at varying times and the reaction was stopped by heating in boiling water for 5 min. The insoluble product was removed by centrifugation at $3{,}000 \times g$ for 15 min.

Treatment and removal of *p*-BP through a continuous reactor filled with immobilized enzyme

Two packed-bed reactors (10.0×2.0 cm) were filled with immobilized enzyme (500 U) and equilibrated with 0.1 M sodium acetate buffer, pH 5.5. The working volume of both the reactors was 12.6 ml. The *p*-BP polluted water (0.4 mM) containing 0.75 mM H_2O_2 and 0.1 mg ml^{-1} PEG was continuously passed through the reactors working at two different flow rates, 10 and 20 ml h^{-1} at room temperature with residence time of the former (when allowing for the effect of porosity on the working volume) maintained at 0.17 h and the latter at 0.08 h, respectively. Samples from the column outlet were collected at the interval of 5 days and were analysed using UV–visible spectrophotometry at 280 nm.

Determination of peroxidase activity and protein concentration

Peroxidase activity was measured by the change in the optical density at 460 nm and at 37 °C by estimating the initial rate of oxidation of 6.0 mM *o*-dianisidine HCl by 18.0 mM H_2O_2 (Matto & Husain 2009a). Immobilized enzyme was continuously stirred for the entire duration of assay. The assay was highly reproducible with immobilized enzyme.

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyses the oxidation of 1 μmol of *o*-dianisidine HCl in the presence of H_2O_2 per min at 37 °C into coloured product ($\varepsilon_m = 30{,}000\ M^{-1}\ cm^{-1}$).

The protein concentration was estimated using the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. One millilitre of a suitably diluted aliquot of protein sample was taken. To this, 5.0 ml of freshly prepared alkaline copper reagent was added. The alkaline copper reagent was prepared by mixing copper sulfate (1%, w/v), sodium potassium tartarate (2%, w/v) and sodium carbonate (2%, w/v) in 0.1 NaOH in the ratio of 1 : 1 : 100. After incubation for 10 min. at room temperature, 0.5 ml of 1.0 N Folin's reagent was added. The contents were mixed and colour intensity was read after 30 min. against the reagent blank at 660 nm.

The percentage removal of *p*-BP was defined as:

$$\frac{\text{Absorbance of untreated} - \text{Absorbance of treated}}{\text{Absorbance of untreated}} \times 100$$

Data analysis

Each value represents the arithmetic mean for three independent experiments performed in duplicate. The average standard deviation of all the obtained values for a particular experiment was less than 5%. The data expressed in various studies was plotted using Sigma Plot-10.0 and expressed as mean ± SD. Analysis was carried out by one-way ANOVA. *P*-values <0.05 were considered statistically significant.

RESULTS

Immobilization of BGP

In this study, Con A layered calcium alginate-cellulose beads were used as carrier for the immobilization of BGP. Ammonium sulfate fractionated and dialysed bitter gourd proteins were adsorbed onto the beads and cross-linked by glutaraldehyde. Con A layered calcium alginate-cellulose beads retained nearly 63% of the original peroxidase activity. However, a marginal loss of 6% activity was observed upon cross-linking (Table 1).

Effect of mercury and organic solvents

Figure 1 shows the effect of mercury on the removal of *p*-BP by soluble and immobilized enzyme. Immobilized peroxidase had successfully removed 69% of *p*-BP in the

Table 1 | Immobilization of BGP on Con A layered cellulose-alginate beads

Methods	Activity expressed (%)
BGP adsorbed on Con A layered calcium alginate-cellulose beads	63 ± 1.36
Cross-linked BGP adsorbed on Con A layered calcium alginate-cellulose beads	57 ± 1.21

Each value represents the mean for three independent experiments performed in duplicate, with average standard deviations <5%.

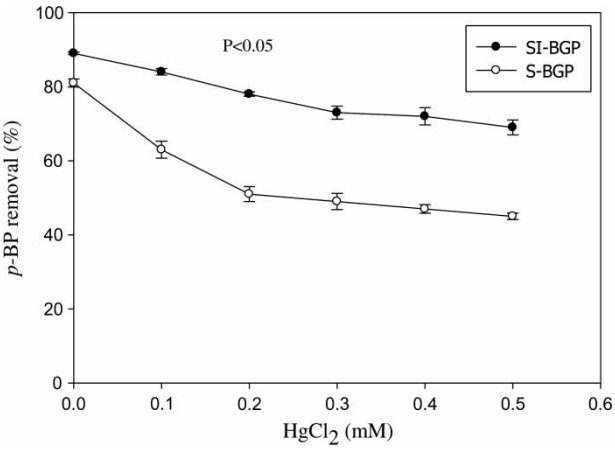

Figure 1 | Effect of $HgCl_2$ on BGP catalysed *p*-BP removal. *p*-BP (0.4 mM) was treated by peroxidase (0.4 U ml^{-1}) in the presence of 0.1 mg ml^{-1} PEG with increasing concentrations of $HgCl_2$ (0.1–0.5 mM), 0.75 mM H_2O_2 in 0.1 M sodium acetate buffer, pH5.5, at 40 °C for 2 h. Symbols indicate oxidative removal of *p*-BP by soluble (○) and immobilized (•) peroxidase.

presence of 0.5 mM $HgCl_2$ while its soluble counterpart oxidized only 45% of the compound.

The effect of organic solvents on the oxidation of *p*-BP by both enzyme preparations has been depicted in Table 2. Immobilized peroxidase retained 37% *p*-BP removal efficiency in the presence of 30% (v/v) DMSO, whereas soluble bitter gourd peroxidase (S-BGP) removed only 18% of the compound. In the presence of 30% (v/v) of *n*-propanol, immobilized enzyme removed 43% *p*-BP while S-BGP showed a marginal removal of 14%.

Reusability studies and storage stability

Reusability of immobilized peroxidase was a necessary aspect to be studied to make its applicability more practical. The enzyme retained 50% *p*-BP removal efficiency even after its fourth repeated use (Figure 2).

Figure 3 shows the storage stability of soluble and immobilized enzyme in terms of its ability to catalyse the oxidation of *p*-BP over a period of one month when stored at 4 °C. Immobilized peroxidase was capable of removing 78% *p*-BP from polluted water even after 30 days' storage at 4 °C whereas the free enzyme retained only 56% of its removal efficiency.

Treatment of *p*-BP in stirred batch processes

The removal of *p*-BP was performed by soluble and immobilized peroxidase in stirred batch processes at three different temperatures (Table 3). It was observed that maximum *p*-BP removal by both enzyme preparations was at 40 °C. The removal of *p*-BP by soluble and immobilized enzyme preparations was 81 and 91% at 30 °C, respectively, at 180 min.

Table 2 | Effect of organic solvents on the removal of *p*-BP

	***p*-BP removal (%)**			
	DMSO		***n*-propanol**	
Organic solvent (%, v/v)	**S-BGP**	**SI-BGP**	**S-BGP**	**SI-BGP**
2.0	67 ± 1.45	78* ± 1.14	63 ± 1.11	82* ± 1.48
4.0	60 ± 1.02	73* ± 0.95	60 ± 1.34	76* ± 1.89
6.0	51 ± 1.56	68* ± 1.47	57 ± 1.57	69* ± 1.32
8.0	42 ± 1.84	59* ± 0.88	51 ± 0.97	64* ± 2.14
10.0	34 ± 2.14	51* ± 1.68	43 ± 2.27	59* ± 2.35
20.0	24 ± 2.32	44* ± 2.15	30 ± 1.83	51* ± 1.68
30.0	18 ± 1.64	37* ± 1.45	14 ± 2.21	43* ± 1.87

Each value represents the mean for three independent experiments performed in duplicate, with average standard deviations <5%. Analysis was carried out by one-way ANOVA where * denotes that values ($P < 0.05$) were statistically significant when compared with S-BGP with respect to DMSO and *n*-propanol.

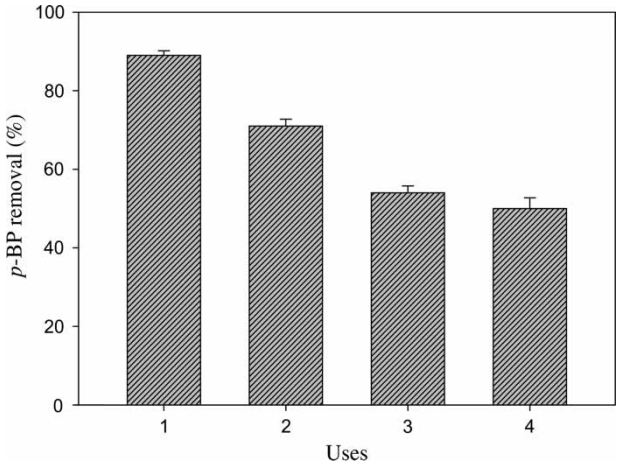

Figure 2 | *p*-BP removal reusability of SI-BGP. *p*-BP (0.4 mM) was treated by peroxidase (0.4 U ml^{-1}) in the presence of 0.1 mg ml^{-1} PEG, 0.75 mM H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5, at 40 °C for 2 h. Immobilized enzyme was collected by centrifugation after the reaction and stored overnight in assay buffer at 4 °C. This procedure was repeated four successive times with the same immobilized enzyme preparation but with an addition of a fresh batch of *p*-BP polluted water.

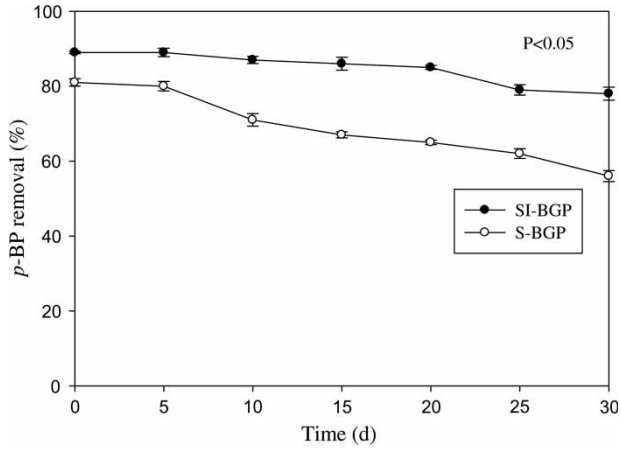

Figure 3 | Storage stability of soluble and immobilized BGP for the oxidation of *p*-BP. *p*-BP (0.4 mM, 5.0 ml) was assayed with 0.4 U ml^{-1} of the original activity of both the preparations of peroxidase over a gap of five days for a period of one month in the presence of 0.1 mg ml^{-1} PEG, 0.75 mM H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5, at 40 °C for 2 h. Symbols indicate oxidative removal of *p*-BP by soluble (o) and immobilized (•) peroxidase.

However, *p*-BP removal by immobilized peroxidase was higher at all the investigated temperatures as compared to free enzyme.

Continuous oxidation and removal of *p*-BP in a packed-bed reactor

The oxidative polymerization and removal efficiency of *p*-BP by immobilized peroxidase present in packed-bed reactors was about 96 and 88% after 5 days of their continuous operation at the flow rates of 10 and 20 ml h^{-1}, respectively (Table 4). As the time of reactor operation increased, the oxidation of *p*-BP continuously decreased and after 30 days the corresponding *p*-BP reactor removal efficiencies correspondingly became 75% and 65%. Figure 4 demonstrates the absorption spectrum for both treated and untreated *p*-BP with respect to the number of days of reactor operation run at a flow rate of 10 ml h^{-1}. The diminution in absorbance peaks of enzymatically treated *p*-BP in the UV region confirmed significant removal of the compound.

Table 3 | Removal of *p*-BP in stirred batch processes by soluble and immobilized peroxidase

	***p*-BP removal (%)**					
	Temperature (°C)					
	30		**40**		**50**	
Time (min)	**S-BGP**	**SI-BGP**	**S-BGP**	**SI-BGP**	**S-BGP**	**SI-BGP**
15	50 ± 0.66	46* ± 0.70	56 ± 1.87	53* ± 0.66	43 ± 1.76	41* ± 1.82
30	56 ± 1.39	61* ± 2.16	60 ± 1.62	69* ± 1.35	49 ± 1.17	55* ± 1.44
45	59 ± 1.85	67* ± 0.94	64 ± 0.75	74* ± 2.11	54 ± 1.33	63* ± 0.91
60	68 ± 2.58	80* ± 1.37	69 ± 1.90	81* ± 2.04	61 ± 1.78	71* ± 1.24
90	73 ± 1.93	84* ± 1.16	75 ± 1.41	87* ± 0.83	64 ± 0.91	76* ± 1.28
120	79 ± 0.65	88* ± 1.21	80 ± 1.87	91* ± 1.23	68 ± 1.18	79* ± 1.47
180	81 ± 1.28	91* ± 1.09	85 ± 0.73	94* ± 1.12	72 ± 2.12	83* ± 1.18

Each value represents the mean for three independent experiments performed in duplicate, with average standard deviations <5%. Analysis was carried out by one-way ANOVA where * denotes that values ($P < 0.05$) were statistically significant when compared with S-BGP at three different temperatures.

Table 4 | Removal of *p*-BP by immobilized peroxidase through a continuous reactor

	p-BP removal (%)	
Time (days)	10 ml h^{-1}	20 ml h^{-1}
5	96 ± 1.16	88 ± 1.25
10	92 ± 1.48	83 ± 1.63
15	86 ± 1.14	75 ± 2.10
20	83 ± 1.32	71 ± 1.76
25	79 ± 2.19	67 ± 2.34
30	75 ± 1.85	65 ± 1.78

Each value represents the mean for three independent experiments performed in duplicate, with average standard deviations <5%.

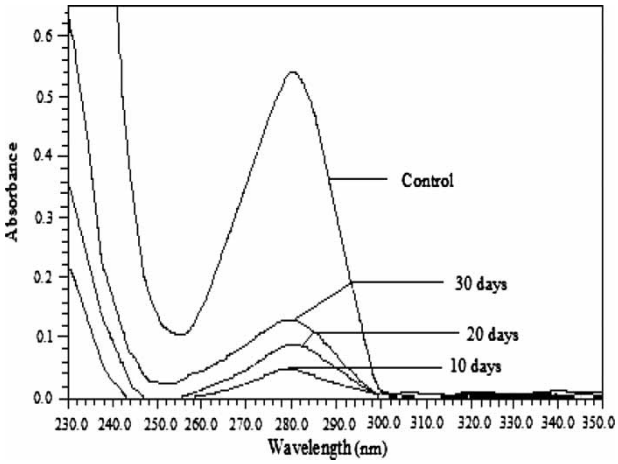

Figure 4 | UV spectra of *p*-BP contaminated wastewater. *p*-BP polluted water (0.4 mM) containing 0.1 mg ml^{-1} PEG and 0.75 mM H_2O_2 was passed continuously through the vertical-bed reactor (10.0 × 2.0 cm) filled with immobilized peroxidase (500 U) at room temperature. The flow rate of the column was maintained at 10 ml h^{-1}. Samples from the column outlet were collected after an interval of 5 days and analyzed spectrophotometrically and their spectra were also recorded after centrifugation.

DISCUSSION

Peroxidase-based treatment of aromatic compounds is gaining tremendous importance in comparison to other conventional methods due to their numerous advantages. However, easy availability, cost effectiveness and significantly higher stability in comparison to other peroxidases makes BGP a choice for the present study (Fatima *et al.* 2007; Fatima & Husain 2007, 2008).

Industrial wastewater is more complex with regard to the presence of other contaminated substances in addition to the aromatic compounds. It contains various types of heavy metals; therefore the oxidation of phenolic compounds by peroxidases in the presence of a heavy metal forms an important part of the study. Our findings showed that the inhibition of SI-BGP catalysed oxidation of phenolic compounds in the presence of $HgCl_2$ was quite low as compared to soluble enzyme (Figure 1). The improved stability against heavy metal inhibition in case of immobilized enzyme might be a consequence of structural changes introduced to the enzyme by immobilization whereas the interference in the structural integrity of soluble enzyme as a result of the binding of metal ion to its active site might have resulted in a decline in enzyme activity (Krajewska 1991; Coyle *et al.* 1999; Ashraf & Husain 2010).

Industrial wastewater may also contain various types of water-miscible organic solvents in addition to phenolic compounds, which might also interfere with the enzymatic removal efficacy. Our results revealed that immobilized peroxidase showed a greater efficacy in removing aromatic pollutants in the presence of organic solvents compared to its soluble counterpart (Table 2). A lower water requirement or enhanced rigidity to the enzyme structure was described by some earlier workers as a possible reason for the stabilization of immobilized enzymes against various forms of water-miscible organic solvents (Xin *et al.* 2005). The advantage of using immobilized enzyme also lies in increasing its stability and reusability. Unlike S-BGP, immobilized enzyme could be easily separated from the reaction mixture and reused. Studies showed that after four repeated cycles, the *p*-BP removal efficiency by immobilized enzyme decreased to 50% (Figure 2). The reaction products might have adsorbed onto the surface of immobilized enzyme and thus it resulted in a loss of catalytic activity (Bayramoglu & Arica 2008; Husain & Husain 2008). However, the data were significant in comparison to horseradish peroxidase immobilized on aluminium-pillared interlayered clay and white radish peroxidase immobilized on diethyl amino-ethyl cellulose, which only retained 4 and 37% removal efficiency after four and six repeated uses (Cheng *et al.* 2006; Ashraf & Husain 2010). The storage stability, among other factors, is an important character which determines the applicability of the immobilized enzyme on the industrial scale. *p*-BP removal efficiency of SI-BGP was decreased to 78% after a month of continuous storage at 4 °C. A significant decrease in the activity of immobilized horseradish peroxidase was also reported on a prolonged storage (Cheng *et al.* 2006).

The removal of *p*-BP in batch processes at various temperatures showed that immobilized enzyme was effective in removing a higher concentration of *p*-BP from wastewater (Table 3). Maximal removal of *p*-BP in a batch process by the enzyme was within a period of 3 h; however, no further increase in the removal of *p*-BP was reported on prolonged incubation. SI-BGP was more effective in removing a higher level of *p*-BP as compared to S-BGP (Table 3). This result was in agreement with several earlier studies where the immobilized enzymes have shown a remarkable removal of aromatic pollutant from polluted water (Husain 2006, 2010; Husain & Husain 2008; Husain *et al.* 2009). Shielding of reactable free amino groups in immobilized enzyme might have prevented its inactivation while in the case of soluble enzyme such groups were more exposed and hence more susceptible to product-mediated inactivation (Husain 2006; Bayramoglu & Arica 2008; Bodalo *et al.* 2008).

To evaluate the efficiency of SI-BGP on a large scale for the treatment of *p*-BP, vertical packed-bed reactor systems were designed and operated continuously with flow rates of 10 and 20 ml h^{-1}. The reactors were continuously operated without any operational problem and showed significant *p*-BP removal efficiency for the entire duration of operation. The extent of oxidation of phenolic compounds in the reactor varied inversely as the reactor flow rate. Several workers have shown a decrease in the degradation of phenolic compounds at a higher flow rate, which implied a lower residence time of phenolic compound inside the reactor (Bayramoglu & Arica 2008; Ashraf & Husain 2010). This decrease in removal rate at lower residence times might be due to insufficient contact time between the phenolic compound and the peroxidases. As shown in Table 4, a packed-bed reactor operated at a flow rate of 10 ml h^{-1} exhibited greater efficiency in terms of the removal of *p*-BP compared to the reactor operated with a flow rate of 20 ml h^{-1}. Confirmation of the oxidation of *p*-BP in the reactor was provided by a decrease in absorbance of the peaks in the UV region (Figure 4).

CONCLUSIONS

It is concluded that SI-BGP showed greater potential for the oxidative polymerization and subsequent removal of phenols from polluted water in comparison to S-BGP. Further studies showed that reactors constructed with such immobilized peroxidase preparations could be continuously used for the removal of phenolic compounds without any reactor blockage. This study has provided a relatively inexpensive technique for the large-scale removal of phenolic compounds from wastewater.

ACKNOWLEDGEMENTS

Aligarh Muslim University (Aligarh, India) is gratefully acknowledged for a UGC-BSR scholarship to one of us (HA).

REFERENCES

Agarry, S. E., Durojaiye, A. O. & Solomon, B. O. 2008 Microbial degradation of phenols: a review. *Int. J. Environ. Poll.* **32**, 12–28.

Ashraf, H. & Husain, Q. 2009 Removal of α-naphthol and other phenolic compounds from polluted water by white radish (*Raphanus sativus*) peroxidase in the presence of an additive, polyethylene glycol. *Biotechnol. Bioprocess Eng.* **14**, 536–542.

Ashraf, H. & Husain, Q. 2010 Use of DEAE cellulose adsorbed and crosslinked white radish (*Raphanus sativus*) peroxidase for the removal of α-naphthol in batch and continuous process. *Int. Biodeter. Biodegrad.* **64**, 27–31.

Bayramoglu, G. & Arica, M. Y. 2008 Enzymatic removal of phenol and p-chlorophenol in enzyme reactor: horseradish peroxidase immobilized on magnetic beads. *J. Hazard. Mat.* **156**, 148–155.

Bodalo, A., Bastida, J., Maximo, M. F., Montiel, M. C., Gomez, M. & Murcia, M. D. 2008 A comparative study of free and immobilized soybean and horseradish peroxidases for 4-chlorophenol removal: protective effects of immobilization. *Bioprocess Biosys. Eng.* **31**, 587–593.

Casa, R., D'Annibale, A., Pieruccetti, F., Stazi, S. R., Sermanni, G. G. & Lo Cascio, B. 2003 Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* **50**, 959–966.

Cheng, J., Yu, S. M. & Zuo, P. 2006 Horseradish peroxidase immobilized on aluminium-pillared interlayered clay for the catalytic oxidation of phenolic wastewater. *Water Res.* **40**, 283–290.

Cohen, S., Belinky, P. A., Hadar, Y. & Dosoretz, C. G. 2009 Characterization of catechol derivative removal by lignin peroxidase in aqueous mixture. *Biores. Technol.* **100**, 2247–2253.

Cohev-Yaniv, V. & Dosoretz, C. G. 2009 Fate of horseradish peroxidase during oxidation of monobrominated phenols. *J. Chem. Technol. Biotechnol.* **84**, 1559–1566.

Coyle, C., Duggan, P. & Godinho, M. 1999 The development of a phytoremediation technique for the detoxification of soils contaminated with phenolic compounds using horseradish peroxidase (*Armoracia rusticana*): preliminary results. *Int. J. Phytoremed.* **1**, 189–202.

Fatima, A. & Husain, Q. 2007 A role of glycosyl moieties in the stabilization of bitter gourd (*Momordica charantia*) peroxidase. *Int. J. Biol. Macromol.* **41**, 56–63.

Fatima, A. & Husain, Q. 2008 Purification and characterization of a novel peroxidase from bitter gourd (*Momordica charantia*). *Protein Pept. Lett.* **15**, 377–84.

Fatima, A., Husain, Q. & Khan, R. H. 2007 A peroxidase from bitter gourd (*Momordica charantia*) with enhanced stability against organic solvent and detergent: a comparison with horseradish peroxidase. *J. Mol. Catal. B: Enzym.* **47**, 66–71.

Gomez, J. L., Bodalo, A., Gomez, E., Bastida, J., Hidalgo, A. M. & Gomez, M. 2006 Immobilization of peroxidases on glass beads: an improved alternative for phenol removal. *Enzyme Microb. Technol.* **39**, 1016–1022.

Husain, M. & Husain, Q. 2008 Applications of redox mediators in the treatment of organic pollutants by using oxidoreductive enzymes: a review. *Crit. Rev. Environ. Sci. Technol.* **38**, 1–41.

Husain, Q. 2006 Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water: a review. *Crit. Rev. Biotechnol.* **26**, 201–221.

Husain, Q. 2010 Peroxidase mediated decolorization and remediation of wastewater containing industrial dyes: a review. *Rev. Environ. Sci. Biotechnol.* **9**, 117–140.

Husain, Q., Husain, M. & Kulshrestha, Y. 2009 Remediation and treatment of organopollutants mediated by peroxidases: a review. *Crit. Rev. Biotechnol.* **29**, 94–119.

Krajewska, B. 1991 Urease immobilized on chitosan membrane. Inactivation by heavy metal ions. *J. Chem. Technol. Biotechnol.* **52**, 157–162.

Kumar, A., Shashi, K. & Surendra, K. 2004 Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* 1194. *Biochem. Eng. J.* **22**, 151–159.

Lai, Y. C. & Lin, S. C. 2005 Application of immobilized horseradish peroxidase for the removal of *p*-chlorophenol from aqueous solution. *Process Biochem.* **40**, 1167–1174.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. 1951 Protein measurement with folin-phenol reagent. *J. Biol. Chem.* **193**, 265–275.

Magri, M. L., Miranda, M. V. & Cascone, O. 2005 Immobilization of soybean seed coat peroxidase on polyaniline: synthesis optimization and catalytic properties. *Biocatal. Biotransf.* **23**, 339–346.

Matto, M. & Husain, Q. 2009a Decolorization of textile effluent by bitter gourd peroxidase immobilized on concanavalin A layered calcium alginate-starch beads. *J. Hazard. Mat.* **164**, 1540–1546.

Matto, M. & Husain, Q. 2009b Calcium alginate–starch hybrid support for both surface immobilization and entrapment of bitter gourd (*Momordica charantia*) peroxidase. *J. Mol. Catal. B: Enzymat.* **57**, 164–170.

Ortega, N., Perez-Mateos, M., Pilar, M. C. & Busto, M. D. 2009 Neutrase immobilization on alginate-glutaraldehyde beads by covalent attachment. *J. Agric. Food Chem.* **57**, 109–115.

Pinto, G., Pollio, A., Previtera, L. & Temussi, F. 2002 Biodegradation of phenols by microalgae. *Biotechnol. Lett.* **24**, 2047–2051.

Semple, K. T. & Cain, R. B. 1997 Degradation of phenol and its methylated homologues by *Ochromonas danica*. *FEMS Microbiol. Lett.* **152**, 132–139.

Sumathi, S. & Manju, B. S. 2000 Uptake of reactive textile dyes by *Aspergillus foetidus*. *Enzyme Microb. Technol.* **27**, 347–355.

Wesenberg, D., Kyriakides, I. & Agathos, S. N. 2003 White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Adv.* **22**, 161–187.

Xin, J.-Y., Zhao, Y.-J., Shi, Y.-G., Xia, C.-G. & Li, S.-B. 2005 Lipase-catalyzed naproxen methyl ester hydrolysis in water saturated ionic liquid: significantly enhanced enantioselectivity and stability. *World J. Microbiol. Biotechnol.* **21**, 193–199.

Yamada, K., Ikeda, N., Takano, Y., Kashiwada, A., Matsuda, K. & Hirata, M. 2010 Determination of optimum process parameters for peroxidase-catalysed treatment of bisphenol A and application to the removal of bisphenol derivatives. *Environ. Technol.* **31**, 243–256.

Zilly, A., Souza, C. G. M., Barbosa-Tessmann, I. P. & Peralta, R. M. 2002 Decolorisation of industrial dyes by a Brazilian strain of *Pleurotus pulmonarius* producing laccase as the sole phenol-oxidizing enzyme. *Folia Microbiol.* **47**, 273–277.

First received 15 February 2011; accepted in revised form 8 May 2011