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Application of Calcium Alginate–Starch Entrapped Bitter Gourd (*Momordica charantia*) Peroxidase for the Removal of Colored Compounds from a Textile Effluent in Batch as well as in Continuous Reactor

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Abstract Calcium alginate–starch entrapped bitter gourd peroxidase has been employed for the treatment of a textile industrial effluent in batch as well as in continuous reactor. The textile effluent was recalcitrant to decolorization by bitter gourd peroxidase; thus, its decolorization was examined in the presence of a redox mediator, 1.0 mM 1-hydroxybenzotriazole. Immobilized enzyme exhibited same pH and temperature optima for effluent decolorization as attained by soluble enzyme. Immobilized enzyme could effectively remove more than 70% of effluent color in a stirred batch process after 3 h of incubation. Entrapped bitter gourd peroxidase retained 59% effluent decolorization reusability even after its tenth repeated use. The two-reactor system containing calcium alginate–starch entrapped enzyme retained more than 50% textile effluent decolorization efficiency even after 2 months of its operation. The absorption spectra of the treated effluent exhibited a marked difference in the absorption at various wavelengths as compared to untreated effluent. The use of a two-reactor system containing immobilized enzyme and an adsorbent will be significantly successful for treating industrial effluents at large scale, and it will help in getting water free from aromatic pollutants.

Keywords Alginate · Bitter gourd peroxidase · Concanavalin A · Entrapment · Immobilization · Starch

Abbreviations

BGP	bitter gourd peroxidase
Con A	concanavalin A
I-BGP	immobilized peroxidase
S-BGP	soluble BGP

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Introduction

The wastewater generated from textile processing industries contains higher amounts of suspended solids, dissolved solids, unreacted dyestuffs, and other auxiliary chemicals that are used at various stages of dyeing and other processes [1]. Strong color of the textile wastewater is the most serious problem because it affects water transparency and, in turn, the photosynthetic activity in water bodies [2]. Numerous physical, chemical, and biological approaches have been employed for the removal of colored compounds from industrial effluents, but these approaches have some inherent demerits such as high cost, formation of hazardous by products, and intensive energy requirement [3, 4]. In order to overcome these problems, environmentalists have searched alternative procedures for their remediation.

Enzyme-based procedures have attracted the attention of researchers as a potential and viable alternative to conventional methods due to their highly selective nature. Further, inhibition by toxic substances is minimal in enzymatic treatment, and the process can operate over a broad range of aromatic concentrations with low retention time [3]. Several limitations prevent the use of free enzymes as their stability and catalytic ability decreases with the complexity of the effluent [3–6]. Some of these limitations could be avoided by using enzymes in their immobilized form that can act as catalysts with long lifetime and high stability [5–8], thus, being a better alternative for wastewater treatment at large scale.

Entrapment of enzymes with different polymeric materials along with their application in treatment of various pollutants has been studied [9]. However, appropriate selection of entrapment material specific to the enzyme and optimization of process conditions is still under investigation. The cost of material and its stability are also taken into consideration.

Here an attempt has been made to use an alginate and starch hybrid gel for the entrapment of bitter gourd peroxidase (BGP) in its complexed form with concanavalin A (Con A). The effect of pH and temperature on the effluent decolorization by BGP has been evaluated. Immobilized BGP preparation has been investigated for its reusability and its textile effluent decolorization efficiency in stirred batch process as well as in continuous packed bed reactor.

Materials and Methods

Materials

Ammonium sulfate, calcium chloride, silica, and starch were procured from the SRL Chem. Pvt. Ltd. Mumbai, India; *o*-dianisidine HCl was received from IGIB, New Delhi, India. Hydrogen peroxide was obtained from Merck Chem. Ltd. Mumbai, India. Jack bean meal was purchased from Loba, Chem. Co. Mumbai, India. Sodium alginate was the product of Koch-Light, England. The untreated cotton textile effluent was obtained from the cotton industry located in Sector 7, Noida, and U.P., India. Bitter gourd was purchased from the local vegetable market. Other chemicals and reagents used were of analytical grade.

Ammonium Sulfate Fractionation of Bitter Gourd Proteins

Bitter gourd (100 g) was homogenized in 200 ml of 100 mM sodium acetate buffer, pH 5.0. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000×*g* on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The clear supernatant was subjected to salt fractionation by adding 20–80% (*w*/*v*) ammonium sulfate.

The solution was stirred overnight at 4 °C and the obtained precipitate was collected by centrifugation at 10,000×*g* on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The collected precipitate was redissolved in 100 mM sodium acetate buffer, pH 5.0, and dialyzed against the assay buffer [10].

Preparation of Con A-BGP Complex

Jack bean extract (10%, *w*/*v*) was prepared by adding jack bean meal (10.0 g) in 100 ml of 0.1 M sodium phosphate buffer, pH 6.2. The mixture was stirred at room temperature for 12 h. Insoluble residue was removed by centrifugation at 3,000×*g* for 30 min. The clear supernatant was collected and was used as source of Con A.

BGP (490 U) in ten batches was incubated with 10% jack bean extract (0.7 ml), and the final volume was adjusted to 2.0 ml with 100 mM sodium phosphate buffer, pH 6.2. The mixture was incubated at 37 °C for 12 h. The insoluble complex was collected by centrifugation at 3,000×*g* for 15 min at room temperature. The precipitate was washed thrice with sodium phosphate buffer, pH 6.2, to remove unbound protein. Con A-BGP complex (3,430 U) in 20.0 ml was crosslinked by using 0.5% glutaraldehyde for 2 h at 4 °C with constant shaking [11].

Entrapment of Crosslinked Con A-BGP into Calcium Alginate–Starch Beads

The crosslinked Con A-BGP complex (647 U) in five batches was mixed with sodium alginate (2.5%, *w*/*v*) and starch (2.5%, *w*/*v*), and volume was made up to 20 ml with assay buffer. The resulting mixture was slowly extruded as droplets through a 5.0-ml syringe with attached needle No. 20 into 0.2 M $CaCl_2$ solution. The beads were further gently stirred for 2 h and suspended in 0.1 M sodium acetate buffer, pH 5.0. The obtained calcium alginate–starch entrapped crosslinked Con A-BGP (2,560 U) was stored at 4 °C and further used [12].

Effluent Processing and Dilution

The textile effluent was collected from the industrial site situated in sector 7, Noida, (U.P.) India. The effluent was centrifuged and collected supernatant was further diluted with 100 mM sodium acetate buffer, pH 5.0 until the effluent exhibited optical density of approximate 0.550. The λ_{max} of the effluent was determined by using Cintra 10 *e* UV visible spectrophotometer.

In various experiments, textile effluent decolorization was calculated as follows:

$$\text{Decolorization}(\%) = \frac{\text{Absorbance of untreated effluent} - \text{Absorbance after treatment}}{\text{Absorbance of untreated effluent}} \times 100$$

Effect of pH on the Decolorization of Textile Effluent by BGP

Effect of pH on the BGP catalyzed decolorization of effluent was monitored in the buffers of different pH; glycine-HCl (pH 3.0), sodium acetate (pH 4.0–5.0), and Tris-HCl (pH 6.0–10.0). The molarity of each buffer was 100 mM. Effluent was treated with BGP (0.28 U ml^{-1}) in the presence of 1.0 mM 1-hydroxybenzotriazole (HOBT) and 0.72 mM H_2O_2 for 1 h at 37 °C. Untreated effluent in each buffer was considered as control (100%) for the calculation of percent decolorization at each pH.

Effect of Temperature on the Decolorization of Textile Effluent

The effect of temperature on the BGP-catalyzed decolorization of textile effluent was monitored at various temperatures (20–80 °C) in the presence of 1.0 mM HOBT and 0.72 mM H_2O_2 for 1 h in 100 mM sodium acetate buffer, pH 5.0. Decrease in color of textile effluent after treatment by soluble and immobilized BGP was monitored at specific wavelength maxima. Untreated effluent incubated at each temperature was considered as control (100%) for the calculation of percent decolorization at this temperature.

Effluent Decolorization Reusability of Immobilized BGP

The textile effluent (5.0 ml) was incubated by immobilized BGP (1.4 U) for 1 h at 37 °C in the presence of 1.0 mM HOBT and 0.72 mM H_2O_2 in sodium acetate buffer, pH 5.0. After, the reaction enzyme was separated by centrifugation and stored in the assay buffer for over 12 h at 4 °C. The similar effluent decolorization experiment was repeated ten times with the same preparation of immobilized BGP and each time with a fresh batch of diluted effluent. The decolorization of effluent was monitored at a specific wavelength of 580 nm. The percent decolorization was calculated by taking untreated effluent as control (100%).

Decolorization of Effluent in Batch Process

The textile effluent (250 ml) was independently treated by soluble and immobilized BGP (37.5 U) in 100 mM sodium acetate buffer, pH 5.0 in the presence of 1.0 mM HOBT and 0.72 mM H_2O_2 for varying times at 40 °C (Table 1). Aliquots were removed at various indicated times and were analyzed for the remaining color at 580 nm. Untreated effluent was considered as control (100%) for the calculation of percent decolorization.

Table 1 Decolorization of textile effluent in batch processes at various times.

Time (min)	Decolorization (%)	
	S-BGP	I-BGP
30	22.16±1.14	31.27[a]±1.24
60	28.43±1.98	39.69[a]±1.47
90	33.45±0.67	47.93[a]±1.37
120	41.98±1.11	56.32[a]±1.57
150	48.13±0.75	61.89[a]±1.65
180	47.67±1.23	71.24[a]±1.13
210	48.22±0.99	71.18[a]±0.54
240	48.59±1.20	71.67[a]±1.11

Textile effluent was incubated with BGP (37.5 U) in 250 ml of 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.72 mM H_2O_2 and 1.0 mM HOBT at 40 °C for 240 min

[a] denotes that the values ($P<0.05$) were statistically significant when batch process/decolorization % catalyzed by immobilized

BGP was compared to the batch process/decolorization % catalyzed by soluble BGP, with respect to time.

Experimental and Operational Procedure for the Treatment of Effluent in a Two-Reactor System

A two-reactor system was developed for the continuous removal of colored compounds from textile effluent. First column (10.0×2.0 cm) was filled with immobilized BGP (1,162 U) connected to another column contained activated silica (10.0×2.0 cm). Activated silica was prepared by incubating 5.0 g of silica in an oven (120 °C) for 12 h and washed thrice with 20 ml of distilled water. The industrial effluent was diluted by 100 mM sodium acetate buffer (pH 5.0) containing 1.0 mM HOBT and 0.75 mM H_2O_2, which exhibited O.D 0.550 (approximately). This mixture was passed through a two-reactor system at room temperature (30±2 °C) under the same experimental conditions as mentioned in the previous section. The flow rate of the column was maintained at 20 ml h^{-1}. After every 10 days, samples were collected and analyzed spectrophotometrically after centrifugation for the remaining color.

UV-Visible Spectral Analysis of Effluent Treated in a Two-Reactor System

Samples collected at various times from the two-reactor system were analyzed by UV-visible spectrophotometer for the intensity of color as well as for their spectra. The intensity of the effluent color was measured at 580 nm. Spectra for the control and effluent treated by BGP in reactor were taken on Cintra 10 *e* UV-visible spectrophotometer (200–700 nm).

Assay of Peroxidase Activity and Protein Estimation

The activity of peroxidase was determined from a change in the optical density ($A_{460\ nm}$) by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in the presence of 18 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 for 15 min at 37 °C [13].

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine HCl min^{-1} at 37 °C.

The protein concentration was determined using the procedure described by Lowry et al. [14]. Bovine serum albumin was used as a standard protein.

Data Analysis

All the experiments were performed in triplicates and twice. For each experiment, a reaction mixture without enzyme was prepared under the same conditions as mentioned in the text and was used to detect possible change of color, which was not due to enzyme activity. Decolorization of effluent by soluble and immobilized BGP was analyzed by one-way analysis of variance. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, <5%.

Results

The collected industrial effluent was suitably diluted, and its absorption spectrum was recorded. The spectrum of effluent exhibited a maximum absorption at 580 nm (Fig. 1). All the effluent decolorization measurements were carried out at this wavelength. The effluent was found to be stable upon exposure to H_2O_2, enzyme, HOBT, calcium alginate–starch beads, activated silica, respectively. It was also recalcitrant to the combined action of BGP

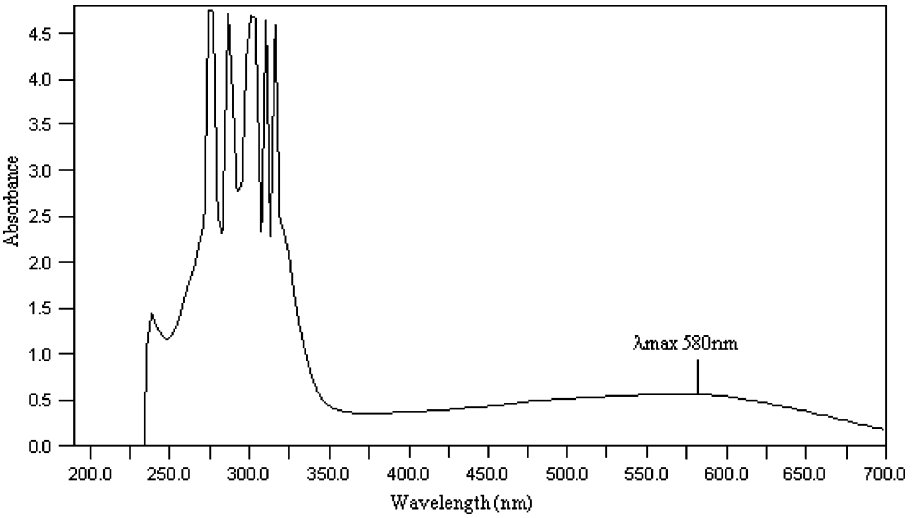


Fig. 1 UV-vis spectra of textile effluent

and H_2O_2. Thus, we found that the effluent decolorization was due to combined action of BGP, H_2O_2, and a redox mediator, HOBT.

Effect of pH

Entrapped BGP showed efficient effluent decolorization in the buffer of acidic pH 3.0–5.0. Effluent was decolorized maximally 26% and 50% by soluble and immobilized BGP at pH of 5.0 (Fig. 2). Subsequently, the color removal was significantly dropped from pH 6.0 onwards.

Effect of Temperature

Figure 3 demonstrates the effect of temperature on the decolorization of textile effluent by soluble and immobilized BGP. Immobilized and soluble BGP could decolorize maximally 52% and 26% effluent at 40 °C. However, above 40 °C, the rate of effluent decolorization was significantly decreased.

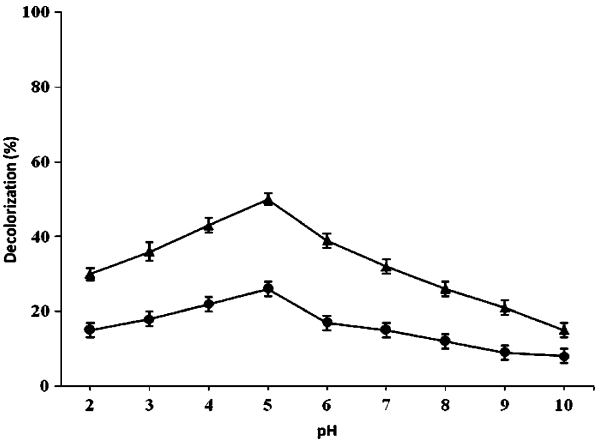


Fig. 2 Effect of pH on the decolorization of the textile effluent by BGP. The textile effluent (5.0 ml) was incubated with BGP (1.4 U) in the presence of 1.0 mM HOBT, 0.72 mM H_2O_2 at 37 °C for 1 h in the buffers of different pH. The morality of each buffer was 100 mM. *Symbols* indicate treatment of effluent by soluble (*circle*), and immobilized (*triangle*).

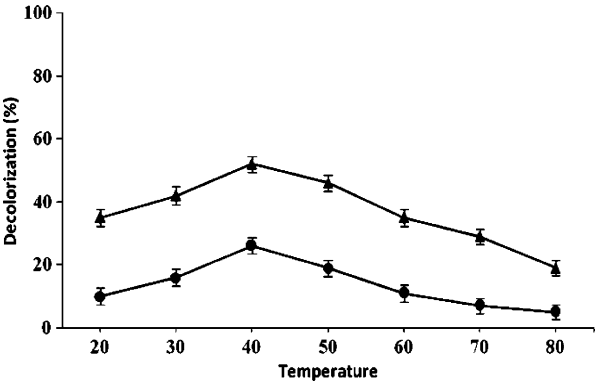


Fig. 3 Effect of temperature on the decolorization of textile effluent by BGP. The decolorization of textile effluent by soluble and immobilized BGP was carried out at various temperatures (20–80 °C). For *symbols*, please refer to legend of Fig. 2

Effluent Decolorization Reusability of BGP

In order to make immobilized BGP more practical in a reactor, it was necessary to investigate the reusability of immobilized BGP. The effluent decolorization reusability of immobilized BGP was continuously decreased on its repeated use (Fig. 4). Immobilized BGP retained 59% effluent decolorization activity after its tenth repeated use.

Decolorization of Effluent in Batch Process

Textile effluent was treated in a stirred batch process by BGP in the presence of 1.0 mM HOBT for various times at 40 °C resulted in a significant loss of effluent color. Immobilized BGP could decolorize 71% of effluent color within 3 h of incubation, while S-BGP could decolorize only 48% effluent under similar experimental conditions (Table 1).

Treatment of Effluent in a Two-Reactor System

The performance of the two-reactor system in terms of effluent decolorization has shown in Fig. 5. The effluent was quite effectively decolorized by immobilized enzyme present in a two-reactor system. However, immobilized BGP decolorized 51% of the initial color from textile effluent even after 60 days of the operation of reactor (Fig. 6).

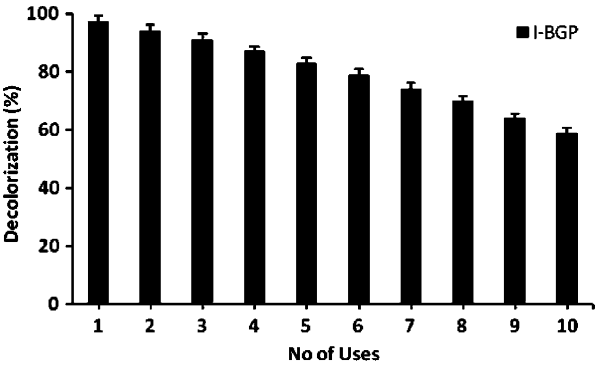


Fig. 4 Textile effluent decolorization reusability of immobilized BGP. Immobilized BGP was incubated with textile effluent for 1 h at 37 °C in triplicates. Decolorization of textile effluent was determined after incubation period. After reaction, the immobilized enzyme was collected by centrifugation and stored in assay buffer at 4 °C overnight. Next day, the similar experiment was repeated. This procedure was repeated for ten successive times

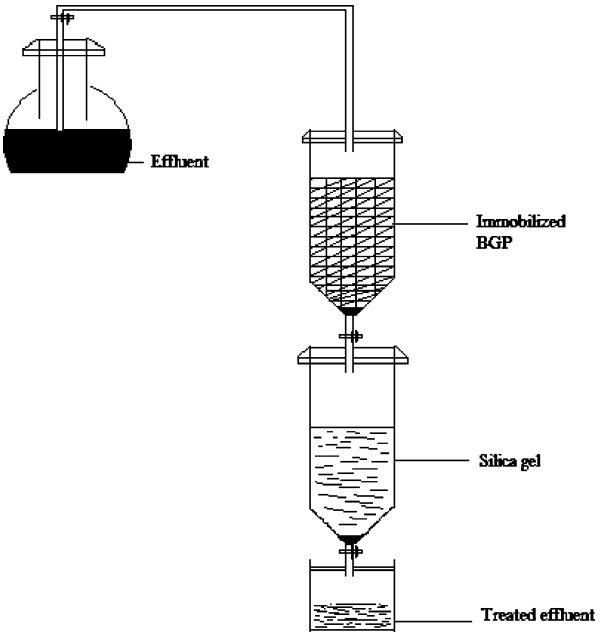


Fig. 5 Schematic representation of two-reactor system used for the decolorization and removal of colored compounds from textile effluent. A column (10.0×2.0 cm) filled with entrapped BGP (1,162 U) was connected to a second column containing activated silica. Textile effluent having O.D. approximately 0.550 was continuous passed into the reactor in the presence of 1.0 mM HOBT and 0.72 mM H_2O_2 for a period of 60 days at room temperature (30± 1). Effluent removal in two-reactor system was done under the same experimental conditions as described in text

Spectral Studies of Treated Effluent in Reactor

A UV-visible spectrum of textile effluent before and after enzymatic treatment in two-reactor system has been shown (Fig. 7). The decrease in absorbance peaks in UV-visible regions with respect to the number of days of operation showed remarkable variation in the absorbance at various wavelengths.

Discussion

Most of the studies on dye decolorization have been done in a defined media or synthetic wastewater where a single dye or their mixtures are usually present. However, industrial effluents are more complex due to presence of other contaminated substances along with colored compounds; under such conditions, the treatment of these pollutants is a difficult problem [15]. For the first time, an effort has been made to treat cotton textile industrial effluent by calcium alginate–starch gel entrapped BGP. In order to make this immobilized

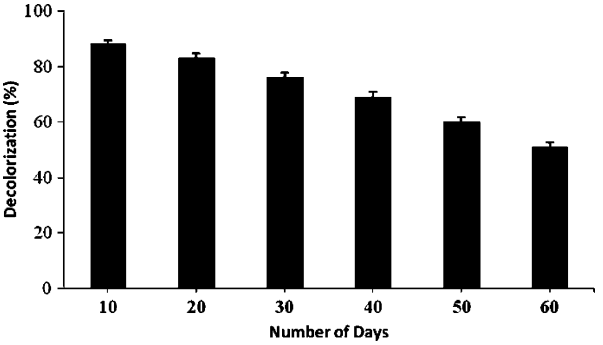


Fig. 6 Spectrophotometric monitoring of effluent decolorization by two-reactor system. Effluent was treated in a two-reactor system as described in text. Samples were taken after a gap of 10 days from the reactor and were analyzed spectrophotometrically at 580 nm for the loss of color from effluent

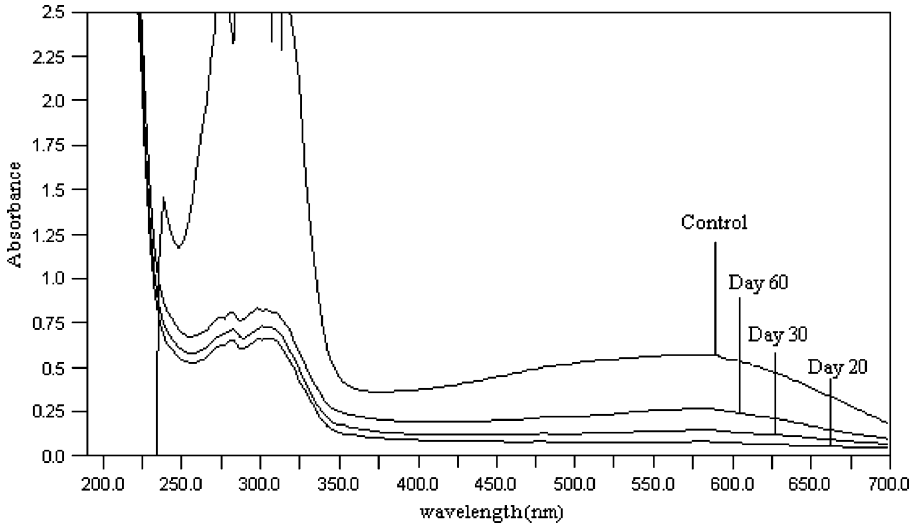


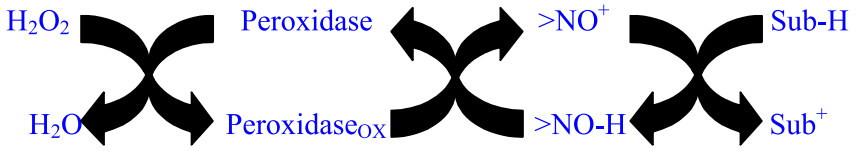
Fig. 7 Absorption spectra of textile effluent treated by immobilized BGP. Industrial effluent was treated in two-reactor system as described in text and their absorption spectra were recorded on Cintra 10 *e* spectrophotometer. The spectra in the figure are labeled

BGP preparation more successful for the large-scale treatment of aromatic pollutants present in complex form in industrial effluents, treatment of such compounds was optimized under various experimental conditions by BGP.

To rule out the possibility of effluent decolorization due to adsorption of effluent on alginate–starch beads, a controlled identical reactor filled with calcium alginate–starch beads without immobilized BGP was developed under identical conditions as mentioned in the text for the enzyme-filled reactor (Fig. 5). The effluent was also passed through this reactor for a period of 1 month. There was no change in the color of the effluent after passing through this reactor.

Thus, it is worth mentioning that the effluent was found to be stable upon exposure to treatment by either one of them; H_2O_2, enzyme, HOBT, calcium alginate–starch beads, silica gel. However, the textile effluent decolorization was a result of H_2O_2 dependent peroxidase catalyzed reaction in the presence of a redox mediator, HOBT. HOBT is N–OH group containing aromatic compound, and such compounds are oxidized by H_2O_2-mediated peroxidase, which in turn, acts as a charge-transfer mediator. Oxidized HOBT helped in the oxidation of recalcitrant aromatic compounds present in the textile effluent. These oxidized compounds either get adsorbed on an adsorbent or with time changed into insoluble polymers [16]. The prospect of using mediator (HOBT) is to offer important possibilities to either indirectly increase the range of compounds that can be oxidized through the direct action of enzyme or to offer multiple modes of attack on a substrate, thereby leading to enhanced conversion of target compound [8].

The schematic representation of enzymatic oxidation of recalcitrant substrate (Sub-H) by means of peroxidase and a >NO–H group containing redox mediator (HOBT) is given below;



In this study, the oxidation of effluents by H_2O_2 with peroxidase/N–OH type compounds (HOBT) has been described with optimal concentration of HOBT (1.0 mM) and this concentration was too less as compared to the redox mediators used in previous studies [17, 18]. Li et al. [19] have reported the use of HOBT and violuric acid at concentrations of 10 mM, which caused significant inactivation of laccase. It is evident from the literature that high concentrations of redox mediator may not be appropriate for wastewater treatment processes because of high cost of mediators, possibility of creating negative impacts on effluent toxicity, or in the environment upon their disposal into receiving waters [20].

Maximum decolorization of effluent by BGP was found at pH 5.0 (Fig. 2). This implies that the immobilized BGP preparation has a higher stability against the pH variation owing to the protection of enzyme by immobilization. However, it has been demonstrated that entrapment of enzymes in gel beads provides a microenvironment of the enzyme, which played an important role in the state of protonation of the protein molecules [12].

The color removal efficiency by free and immobilized enzyme was increased with increasing temperature up to 40 °C (Fig. 3). However, at lower temperatures, relatively higher color removal was observed for the immobilized BGP. Improvement in the thermal stability of calcium alginate–starch entrapped Con A-BGP complex may come from multipoint complexing of peroxidase with Con A. However, some earlier workers have described that the complexing of enzymes with lectins enhanced its thermal stability. This enhancement in thermal stability was due to several interactions between enzyme and Con A. Thus, the results imply that immobilization protects the enzyme denaturation at higher temperatures [7, 21].

The advantage of immobilized enzyme does not lie only in increasing the stability but also in its reusability. Calcium alginate–starch entrapped BGP retained remarkably very high effluent decolorization activity (59%) even after its tenth repeated use (Fig. 4). The activity loss of the entrapped BGP preparation after its tenth repeated use in effluent decolorization was appreciably much lower as compared to acid dye removal by polyacrylamide gel entrapped HRP where after fifth repeated reuse HRP retained only 50% dye decolorization efficiency [22]. Our observations have suggested that calcium alginate–starch entrapped BGP has more advantages in removing higher percentage of color from industrial effluent.

Immobilized BGP could decolorize more than 70% color of the effluent within 3 h of incubation in a batch process (Table 1). After 3 h, the increase in the rate of effluent decolorization was insignificant, which might be due to product inhibition. Thus immobilized enzymes were more effective in decolorization of industrial colored effluent as compared to soluble enzyme because they are protected against the inactivation caused by end product of the reaction [16].

In a two-reactor system, one of the reactor containing immobilized BGP and the other reactor filled with activated silica was operated for the continuous decolorization or removal of effluent. The reactor operated without operational problem and indicating high effluent removal efficiency (Fig. 6). Our results are in agreement with studies of earlier workers that the treatment of effluent containing aromatic compounds resulted in the formation of free radical, which could be easily removed by using an adsorbent in the second reactor [23, 24].

Removal of reaction product as insoluble complex is an important signal for the detoxification of aromatic compounds from wastewater. It has already been demonstrated that HRP and BGP can catalyze free-radical formation followed by spontaneous polymerization of a variety of aromatic compounds including phenols [16], chlorophenols [25], and dyes [7, 26, 27].

In order to confirm the decolorization and removal of colored compounds from textile effluent through two-reactor system, spectral analysis became an important aspect to show a loss in these compounds after treatment with immobilized enzyme present in one of the reactor and adsorbent in the second reactor, where all the free radical compounds, activated products get adsorbed. The decrease in absorbance peaks in UV-visible region provided strong evidence for the decolorization of aromatic pollutants from wastewater (Fig. 7). There was a complete decolorization of the chromophoric group and a significant reduction in the peak associated with the aromatic ring. The disappearance of absorption peak in the presence of HOBT in UV-visible region was due to the formation of free radicals, which get adsorbed on the silica gel present in the second reactor [16, 23].

Conclusions

On the basis of the results obtained in the present work, it can be concluded that the system employed here is highly suitable for use in dye decolorization. This system was quite simple to operate; the oxidation and removal of colored pollutant via this double reactor could be performed without any problem. Thus, it provides clear evidence regarding the suitability of such system for the application of immobilized enzymes at large scale for the treatment of water polluted with aromatic pollutants. It has been found that reactor prepared with such immobilized BGP could be continuously operated for longer times.

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