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A potential tissue culture approach for the phytoremediation of dyes in aquaculture industry

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ABSTRACT

Brilliant green has been used as an effective compound to control external fungal and protozoan infections of fish though is carcinogenic and teratogenic. Present study focuses on the efficient degradation of brilliant green using callus cultures of the plant, *Tecoma stans* var. *angustata*, showing a peroxidase activity of 3.07 IU g^{-1}. Callus cultures retained 86% activity after immobilization in calcium alginate. Dye degradation parameters were initially optimized using batch cultures. A packed bed reactor was constructed using the immobilized beads and different concentrations of the dye from 8.5 to 45 mg l^{-1} along with H_2O_2 were given in an up-flow mode. *K*m and *V*max for the dye degradation were 0.01924 g l^{-1} and 0.035 g l^{-1} h^{-1}. The bioreactor could degrade 94% of the dye (concentration–35 mg l^{-1}) at a residence time of 42.0×10^{-3} h. Percentage of degradation varied depending on the flow rate, residence time and dilution rate. TLC and reverse phase HPLC analysis showed that the dye was completely degraded to minor non toxic metabolites via complete degradation of the aromatic rings and by cleavage of functional groups. The current study is a preliminary work that can be used for application in Aquaculture and allied Industries where biodegradation of brilliant green is required.

1. Introduction

Triphenylmethane dyes are used extensively in textile, food, cosmetic, paper, medical and leather industries. Brilliant green, also called emerald green or malachite green G is a triarylmethane dye of the malachite-green series used in dilute solution as a topical antiseptic. Brilliant green is readily absorbed by fish and fish eggs during waterborne exposure where it acts as a respiratory poison, damaging the cell's ability produce energy to drive vital metabolic processes and persist in edible fish tissues for extended periods of time [1]. Its worldwide use in aquaculture will probably continue due to its relatively low cost, ready availability, and efficacy and hence, potential human exposure to brilliant green could result from the consumption of treated fish and from working in the dye and aquaculture industries. Different methods are available to treat the wastewater containing dyes of which most of them are chemical which are costly and less efficient and produce large amount of sludge [2]. Photocatalytic degradation of Brilliant Green (BG) has also been studied by many scientists [3,4]. Studies on brilliant green degradation with ozone microbubbles has shown the formation of a large number of intermediate compounds during oxidation and has reported only 80% degradation [5]. Biological processes are getting more and more attention since it is economic and environment friendly and result in negligible sludge formation as it can possibly lead to complete degradation of the dye molecules to carbon dioxide and water [6]. So many works are being carried out around the world on the use of bacterial and fungal peroxidases in dye decolorization [7]; but the aging of fungal mycelium and the risk of contamination by bacteria under non-sterile conditions have hindered its application. Plant peroxidases can function as an alternative for the degradation of brilliant green in such situations as these are environment friendly and mild and can overcome most of the disadvantages related to other methods [8,9]. But, almost all the works done in the field of dye and effluent treatment are based on commercial enzymes like HRP [10] which is not appreciable due to the prohibitive cost of the enzyme.

Major limitation of plant peroxidase is the low yield and high cost of production compared to the bacterial and fungal enzymes. Reduction in enzyme cost can be achieved through continuously reusing the enzyme after immobilizing on various supports and by decreasing the purification cost [11]. Immobilization of a biocatalyst is a well-accepted method for better process control, reduced

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operational cost and for continuous removal of toxic metabolites [12]; but most of the dye and effluent treatment studies reported have used pure commercial horse radish peroxidase (HRP) enzyme for degradation studies and also the supporting material for immobilization is also much costly [13].

In the present study, 2,4-dichlorophenoxy acetic acid was used for the proliferation of callus in the plant, *Tecoma stans* var. *angustata*. The friable callus without any further purification was immobilized in calcium alginate beads which were used for the degradation of brilliant green and the degradation was optimized under different conditions as batch cultures and finally a packed bed reactor was constructed for the continuous degradation of the dye. Here, the plant callus is protected from the possible toxic effects of the dye by entrapment in alginate beads. Immobilization of callus in alginate beads has rendered improved enzyme stability under extreme conditions of temperature and pH and imparts reusability subsequently making it good for using in continuous processes. Moreover, most of the reported dye effluent treatments with biological systems also focus on decolourization studies rather than degradation thereby leaving smaller aromatic compounds which are not environment friendly [14]. The present study is a cost effective method of brilliant green degradation where the enzyme in immobilized callus shows good optimization parameters compared to the enzyme in free callus and bears good storage stability and reusability making it better than most of the reported immobilized systems for bioremediation.

2. Materials and methods

2.1. Chemicals

ABTS (2,2′-Azino-bis(3-ethylbenz-thiazolin-6-sulfonic acid) was purchased from Sigma chemicals, USA and H_2O_2 from BDH, England. Brilliant green was purchased from Aldrich (Milwaukee, WI).

2.2. Sources of peroxidase enzyme

The plant, *Tecoma stans* var. *angustata* belonging to family Bignoniaceae was collected from the campus of Regional Research Laboratory (CSIR), Trivandrum, India.

2.3. Tissue culture and callus initiation

Middle parts of the leaf segments with midrib (7 × 10 mm) were washed well in 10% labolene for 10 min followed by a treatment in 0.1% mercuric chloride for 5 min and inoculated onto media containing different concentrations of hormones on full-strength MS medium [15]. 3.0% (w/v) sucrose was used as the carbon source and 0.8% (w/v) agar as inert solidifying agent, and incubated at 25 °C under a photo period of 12/12 h. Hormone combination used for the callus initiation was 1.0 mg l^{-1} BA (benzyladenine) and 0.05 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and the callus initiated was continuously sub cultured in medium containing only 2,4-D (0.05 mg l^{-1}).

2.4. Immobilization, and activity retention studies

4% (w/v) sodium alginate and 6% (w/v) callus cells in distilled water were mixed well using magnetic stirrer. The mixture was extruded through a needle into 0.06 M $CaCl_2$ solution to yield calcium alginate beads of 3–4 mm diameter, that were kept in curing solution of 0.025 M $CaCl_2$ in refrigerator. The beads were washed well with sterile water before use.

In order to find out the enzyme activity of the immobilized callus, the beads were given an incubation period of 10 min in the appropriate buffer prior to it being introduced into the cuvette at definite intervals of time and the increase in absorbance per minute was monitored. Activity retained by the immobilized enzyme was calculated using the formula

$$\text{Activity Retention}(\%) = \frac{\text{Immobalised Enzyme Activity}}{\text{Initial Activiity of the enzyme in callus}} \times 100$$

Peroxidase activity of callus before and after immobilization was assayed by the method of Bergmeyer using ABTS as substrate [16]. Storage of the callus and the immobilized callus were done in sterile ¼ strength MS medium devoid of growth regulators.

2.5. Dye degradation and optimization studies

Callus as such as well as the immobilized callus in calcium alginate was used for the degradation studies. To obtain maximum degradation, parameters like pH, enzyme and H_2O_2 concentration were standardized by trial-and-error method. The spectrum of the dye was scanned by a UV spectrophotometer (UV 2100, Shimadzu, Japan) in the range of 290–690 nm. The percentage of degradation was calculated from the difference between the initial and final λ_{max} value (600) of the dye.

The experiments were carried out at a constant temperature (30 ± 2 °C) by varying the process parameters such as pH and concentration of dye, enzyme and H_2O_2 initially. The kinetics was carried out in a series of vials at pH 6.5, for the degradation of 17 mg l^{-1} dye, using an enzyme of 2.7×10^{-3} units and H_2O_2 dose of 0.1 mM. Residual dye concentration of each vial was estimated after every half an hour to study the optimum contact time.

Optimization of pH was carried out at constant concentrations of enzyme, H_2O_2 and dye (2.7×10^{-3} IU, 0.1 mM and 26 mg l^{-1} respectively) and the percentage of degradation was observed after 2 h. Different concentrations of the dye (8.5 mg l^{-1}, 17 mg l^{-1}, 26 mg l^{-1} and 34 mg l^{-1}) were treated at pH 6.5 with enzyme of 2.7×10^{-3} IU using 0.1 mM H_2O_2 to study the optimum concentration of dye that can be degraded under the specified conditions. In order to find out the optimum concentration of H_2O_2, the concentration of the same was varied (0.03, 0.06, 0.10 and 0.13 mM) in the reaction mixture at constant dye concentration (17 mg l^{-1}), pH (6.5) and enzyme concentration (2.7×10^{-3} IU). 0.0013, 0.0027, 0.0041 and 0.0055 IU (as beads) of enzyme was given separately to a mixture of 17 mg l^{-1} dye and 0.1 mM H_2O_2 at pH 6.5, to find out the optimum enzyme concentration for the degradation.

All the above stated experiments were done at batch reactor stage at 30 ± 2 °C, repeated thrice with 3 replicates each.

2.6. Construction of a packed bed reactor (PBR) for continuous dye degradation

Schematic representation of PBR shown in Fig. 1. PBR was constructed as per Roy and Abraham [17]; but with some modification. The free callus as well as the immobilized callus were stored in ¼ strength MS medium without sucrose and plant growth regulators after every 10 h of continuous run to get it rejuvenated, after which it was again fed with the dye solution. The bioreactor was made up of a glass column with 10 cm height and 3 cm internal diameter with a working volume of 71 ml 3 g callus (activity of 9.3 IU), immobilized in calcium alginate was packed in the reactor. Different dye concentrations like 8.5, 17, 25, 35 and 45 mg l^{-1} were given along with 0.1 mM H_2O_2 in an up-flow mode at pH 6.5 and 30 ± 2 °C at different flow rates from 0.3 ml min^{-1} to 5 ml min^{-1}. The percentage of degradation was observed after it reached a steady state (around 2 h). K_m and V_{max} of the bioreactor for the degradation of the dye was calculated by plotting the dye concentrations per reaction rate against the dye concentrations (Hanes-Woolf plot).

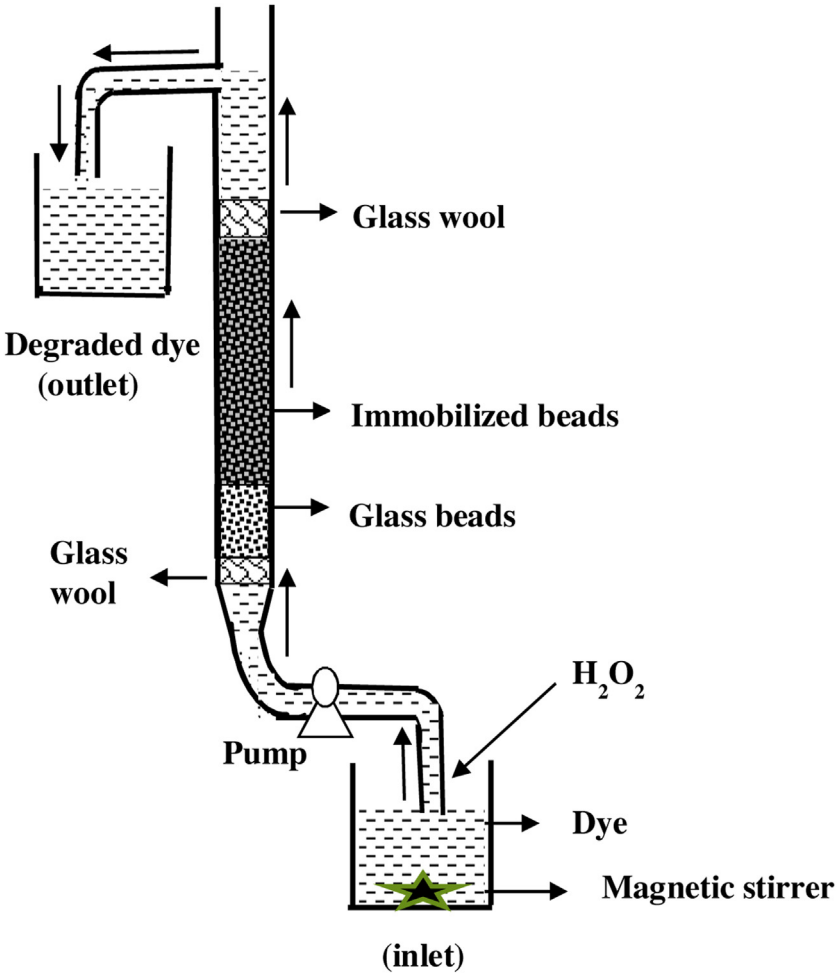


Fig. 1. Schematic diagram of the packed bed reactor constructed using immobilized callus cultures.

Bioreactor parameters like dilution rate (D) and residence time (R) were calculated using the formulae,

$$\text{Dilution rate(D)} = \frac{\text{Flow rate}}{V_0}, \quad V_0 \text{ is the void volume}$$

$$\text{Residencetime(R)} = \frac{1}{D}$$

2.7. Determination of the dye adsorption capacity of immobilized beads

Calculated using the formula, $q = \frac{V(Ci-Cf)}{M}$
q = Dye uptake capacity (mg of dye/g of immobilized beads)
V = Volume of dye solution (litres)
Ci = Initial concentration of dye ($mg\,l^{-1}$)
Cf = Equilibrium concentration of the dye in solution ($mg\,l^{-1}$)
M = Concentration of alginate in solution ($g\,l^{-1}$)

2.8. Analysis of degradation

The dye samples after and before treatment with the enzyme were analysed by TLC using the solvent system, Hexane and Ethyl acetate (4:6).

2.9. HPLC analysis

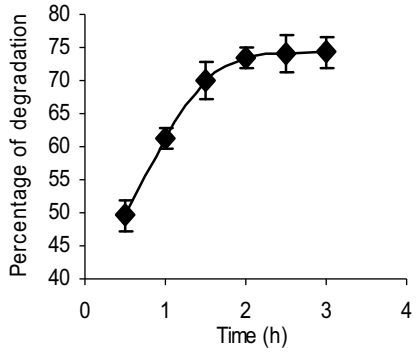
High performance liquid chromatography (HPLC) (Shimadzu LC-8A) with a reverse phase C18 column 250 × 4.6 mm (particle size–5 μM) was employed to understand the dye removal during the enzyme catalyzed treatment. The separated components were detected at 600 nm. Isocratic mobile phase consisted of 80:20 acetonitrile with acetate buffer (pH 4.5) flowed at 1 ml min^{-1}. 50 μl aliquots of control sample (dye without degradation) and the sample after degradation were injected into the column at 30 ± 2 °C after diluting with acetonotrile.

2.10. Analysis of chemical oxygen demand (COD)

COD is considered as one of the important quality control parameters of effluents. COD was calculated in the sample before and after degradation studies [18].

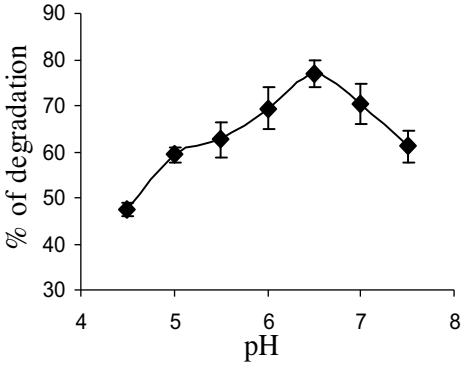
2.11. Study of reusability of immobilized callus

In order to study the longevity of immobilized beads and to study the reusability of the same in degradation study, the beads were kept in sterile ¼ strength MS medium devoid of growth regulators and carbon source for half an hour after every cycle.



[1]pH 6.5, Enzyme–2.7 X 10^{-3}, H_2O_2–0.1 mM, dye–26 mg l^{-1}

Fig. 2. Optimum contact time for the degradation of brilliant green by the immobilized callus of *Tecoma*, as batch cultures[1].
[1]pH 6.5, Enzyme–2.7 × 10^{-3}, H_2O_2–0.1 mM, dye–26 mg l^{-1}.



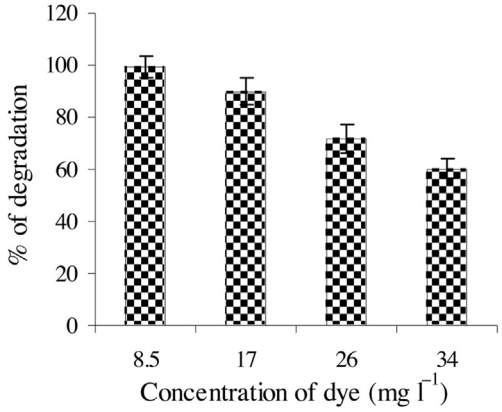
[2]Enzyme–2.7 X 10^{-3}, H_2O_2–0.1 mM, dye–26 mg l^{-1}.

Fig. 3. Optimum pH for the degradation of 17 mg l^{-1} brilliant green by the immobilized callus of *Tecoma* as batch cultures[2].
[2]Enzyme–2.7 × 10^{-3}, H_2O_2–0.1 mM, dye–26 mg l^{-1}.

3. Results and discussion

3.1. Enzyme optimization studies of batch cultures

Peroxidase activity as well as callus proliferation and type of callus varied depending on the type and concentration of hormones used. Optimum biomass growth as well as enzyme activity was obtained on the 20th day of inoculation by the combined effect of 1.0 mg BA l^{-1} and 0.05 mg 2,4-D l^{-1} (Data not shown). In order to reduce the cost of the procedure, medium provided with only 2,4-D, a very cheap weedicide that functions as plant growth regulator was used for the further proliferation of the callus. The callus invoked in 0.05 mg l^{-1} 2,4-D showed a peroxidase activity of 3.1 IU g^{-1}. When the callus was used without immobilization, dye was easily adsorbed on the surface making desorption and degradation comparatively difficult. Callus retained 86% of its activity after immobilization and retained around 97% of that activity even after 7 cycles of reuse. It was calculated that 5 g of friable callus got immobilized in 100 g of calcium alginate beads; each bead weighing around 500 mg. Entrapment is the most widely used technique for the immobilization of whole cells, and alginate is a suitable matrix material because it is nontoxic and the method used for its gelation and biocompatibility [19]. Immobilization protects the callus from the adverse effects of the external environment.

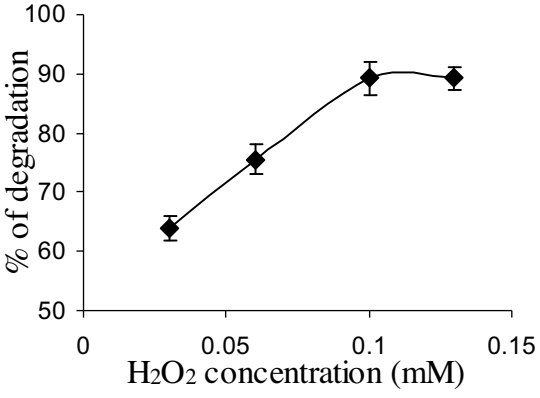


Enzyme–2.7 X 10^{-3}, H_2O_2–0.1 mM, pH–6.5

Fig. 4. Degradation of different concentrations of dye using the immobilized callus of *Tecoma.*
Enzyme–2.7 × 10^{-3}, H_2O_2–0.1 mM, pH–6.5.

3.2. Dye degradation studies of batch cultures

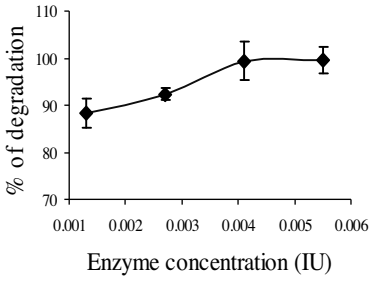
76% of 26 mg l^{-1} dye was degraded within a contact time of 2 h (Fig. 2). Under the specified conditions, the pH optimum for the degradation of brilliant green was 6.5 where the degradation percentage was increased to 82 (Fig. 3). Gradual decrease in the degradation percentage was observed when the pH was either increased or decreased. The percentage of degradation varied at different initial dye concentrations. 8.5 mg l^{-1} dye showed a degradation of 99.3% whereas 17 mg l^{-1} showed around 90% degradation (Fig. 4). Concentration of H_2O_2 is a very significant factor as it contributes to the catalytic cycle of peroxidase, which accepts the aromatic compound to carry out its oxidation to a free radical form. Optimum dye degradation of 89% was attained when 0.1 mM of H_2O_2 was used and further increase in concentration did not increase the degradation percentage (Fig. 5). An enzyme concentration of 0.041 IU was found to degrade nearly 100% of 17 mg l^{-1} dye under the specified conditions (Fig. 6).



pH 6.5, enzyme–2.7 X 10^{-3}, dye–17 mg l^{-1}

Figure 6

Fig. 5. Effect of H_2O_2 concentration on the degradation of the dye, brilliant green using immobilized callus cultures of *Tecoma* as batch cultures.
pH 6.5, enzyme–2.7 × 10^{-3}, dye–17 mg l^{-1}.



pH 6.5, H_2O_2 concentration–0.1 mM, dye concentration–17 mg l^{-1}

Fig. 6. Effect of enzyme concentration on the degradation of the dye, brilliant green by the immobilized callus of *Bignonia* as batch cultures.
pH 6.5, H_2O_2 concentration–0.1 mM, dye concentration–17 mg l^{-1}.

Table 1
Parameters of dye degradation using PBR.

Flow rate, F ($l\,h^{-1}$)	Dilution rate, D (h^{-1})	Residence time, R (h)
0.018[a]	66.7	15×10^{-3}
0.03	111.1	9×10^{-3}
0.06	222.2	4.5×10^{-3}
0.12	444.4	2.25×10^{-3}
0.18	666.7	1.50×10^{-3}
0.24	888.9	1.12×10^{-3}
0.3	1111.1	9.0×10^{-3}

[a] 0.3 ml min^{-1}.

3.3. Bioreactor studies

3 g immobilized callus in calcium alginate showing an activity of 9.3 IU was found to degrade the dye very efficiently. Changes in parameters like dilution rate (D) and residence time (R) with flow rate are shown in Table 1. The beads showed the dye adsorption initially followed by degradation.

3.3.1. Dye adsorption

Even q at 30 min was found to be only 0.0093 and it was reduced to 0.0004 after about 2 h. The values clearly depict that the rate of adsorption of dyes on the alginate beads was comparatively negligible and reduction in q value with increase in time was due to desorption and degradation of the dye by the callus immobilized in the alginate beads. So, in our study, though both adsorption and degradation occurred simultaneously, we can neglect the former and can claim that the dye decolourization is predominantly by degradation by the peroxidase enzyme. (But, dye desorption was comparatively difficult when the callus was directly used, as the adsorbed dye interfered with enzyme activity and ultimately affecting degradation).

3.3.2. Dye degradation

Initial adsorption of dye on calcium alginate was followed by degradation by the peroxidase enzyme present in the immobilized callus. Concentrations up to 25 mg l^{-1} were degraded completely at a flow rate of 1 ml min^{-1} (data on 8.5 and 17 mg l^{-1} not shown). A concentration of 35 mg l^{-1} dye was completely degraded at a flow rate of 0.3 ml min^{-1}. Around 80% degradation was observed when a concentration of 45 mg l^{-1} was treated at a flow rate of 0.5 ml min^{-1} and significantly no reduction was observed when the flow rate was further reduced (Table 2), indicating the need for further increasing any other parameter kept constant. K_m and V_{max} values of the bioreactor for the degradation of different concentrations of the dye were 0.01924 g l^{-1} and 0.035 g l^{-1} h^{-1} respectively from the Hanes-Woolf plot (Fig. 7). Shaffiqu et al. [20] have reported a degradation percentage of 54 in an hour when 25 mg l^{-1} brilliant green was treated with *Ipomea palmata* peroxidase (0.04 IU) along with 2 mM H_2O_2 at pH 8. Difference in parameters including pH in enzymes in different sources can be attributed to the difference in an optimum condition requirement of enzyme from different source plants for the dye degradation. Most of the available methods of dye removal depend on adsorption which results in the increase in pH due to the release of some ions from the adsorbents into the outer solution [21]. In the present study, the adsorbed dye was also completely degraded by the enzyme produced in the system. We have focussed on dye degradation rather than mere removal or decolourization and the pH optimization studies have been done based on degradation. Moreover, optimum pH for the peroxidase enzyme activity is different from that of degradation study because enzyme optimization studies are usually done based on the standard assay procedure conditions using ABTS as substrate whereas in the degradation study, the dye itself functions as the substrate. Optimum pH even varies before and after immobilization also. (refer supplementary data).

Table 2
Degradation percentages of brilliant green at different flow rates in PBR.

Dye concentration (mg l^{-1})	Flow rate (ml min^{-1})	Degradation (%)
25	1	99.97 ± 0.5
	2	86.6 ± 2.5
	3	76.5 ± 1.9
	4	67.6 ± 2.03
	5	47 ± 1.9
35	0.3	99.1 ± 0.6
	0.5	92.3 ± 1.1
	1	89.7 ± 3.2
	2	76.2 ± 1.8
	3	66.3 ± 2.2
	4	45.7 ± 1.6
45	0.3	82.4 ± 3.0
	0.5	79.3 ± 2.4
	1	63.4 ± 1.6
	2	45.2 ± 0.9

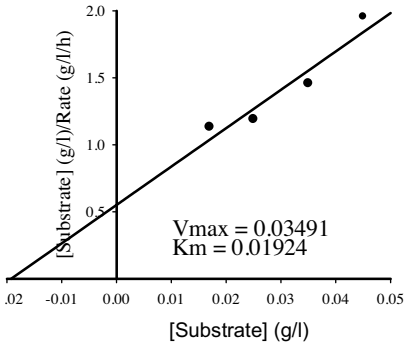
3.4. Analysis of degradation, COD studies

The degradation products were analysed by TLC and the degraded components moved along with the solvent front leaving only traces of the original dye behind and did not give any aggregates or precipitates. This is contrary to the reports that the treatment of phenols and aromatic amines by peroxidases and tyrosinases result in the formation of large insoluble aggregates [22]. Very few reports are available on the degradation products or intermediates of triphenyl methane dyes [23]. HPLC profiles of the control sample showed a peak at a retention time of 6.7 min (Fig. 8A). Sample from 50% dye degradation showed an additional peak at a retention time of 3.7 (Fig. 8B). A large number of small peaks in addition to the one at 3.7 were observed when sample showing 75% dye degradation was analysed (Fig. 8C). The final sample after complete degradation showed only some minor peaks showed no major peak, confirming the complete degradation of the aromatic groups (Fig. 8D).

There was gradual increase in COD removal when each of the aforementioned sample was analysed for COD. When the final sample after complete degradation was tested, the COD removal was found to be enhanced to ~99%, whereas the colour removal was 100%.

3.5. Reusability of immobilized callus

The immobilized beads could be recycled about 7 times with around only 10% loss of activity. This result is much better than triphenyl methane dye degradation studies done by Kumar et al.



Enzyme concentration–9.3 IU, H_2O_2 concentration–0.1 mM, pH–6.5, flow rate–1 ml min^{-1}

Fig. 7. Hanes-Woolf plot for the degradation of brilliant green using the bioreactor.
Enzyme concentration–9.3 IU, H_2O_2 concentration–0.1 mM, pH–6.5, flow rate–1 ml min^{-1}.

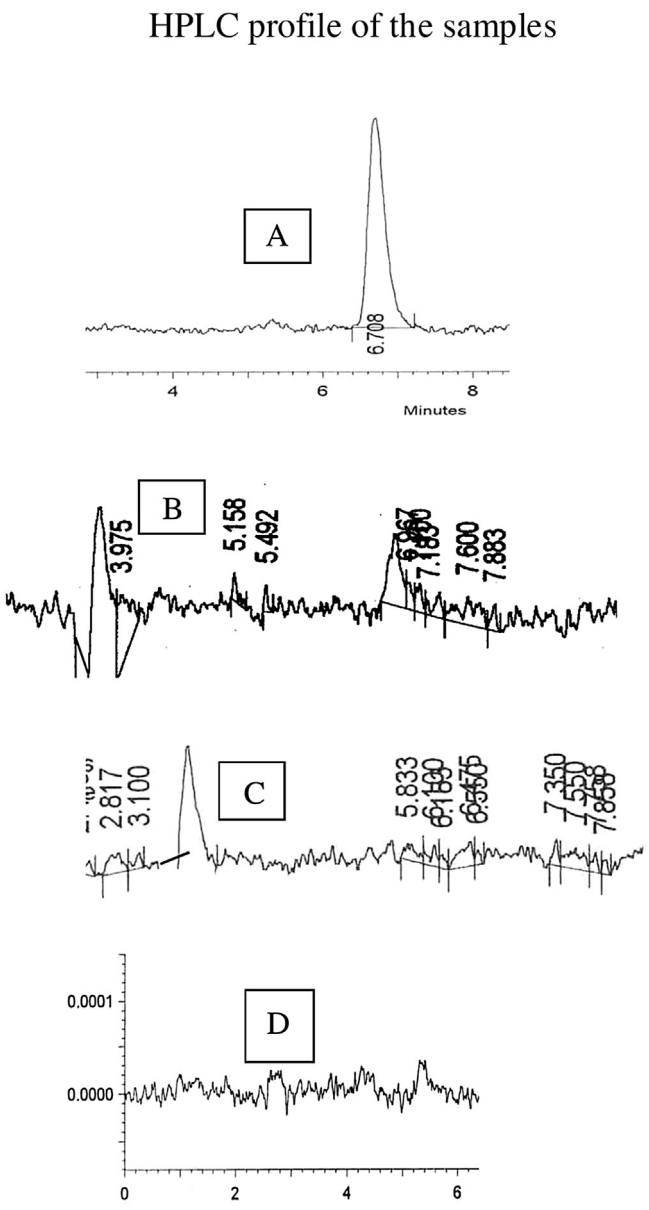


Fig. 8. HPLC profile of the samples.
A–Control (dye), B–Sample showing about 50% degradation, C–sample showing 75% degradation, D–completely degraded sample

[24] using pure laccase enzyme by crosslinking technology. The calcium alginate was found to be a good mild medium to protect the inner callus without affecting the enzyme activity and the incubation given in the medium was found to rejuvenate the callus in beads.

4. Conclusion

Biological effluent and dye treatment methods are much better than physicochemical dye removal methods like adsorption, chemical precipitation and flocculation, oxidation by ozone, chlorine and hydrogen peroxide, reduction, electrochemical treatments and ion-pair extraction etc. Though a large number of works are going on regarding bioremediation of wastewater using enzymes, only a few reports are there on enzyme mediated degradation of organic pollutants [25]. Most of such works are based on decolourization rather than degradation which results in the formation of more dangerous aromatic intermediates [26]. One of the major challenges associated with peroxidase catalyzed removal of phenols and other aromatic compounds is the susceptibility of the enzyme to inactivation by various side reactions of the treatment [27]. Though there are a few numbers of reports on the peroxidase enzymes from plants for their ability to catalyze the removal of aromatic compounds from wastewaters, the studies mainly focus on commercial horseradish peroxidase, soybean peroxidase (SBP) [25] etc. that are very costly. In our study, we used the callus cultures of an easily available plant that uses only very low concentration of 2,4-D as plant growth regulator for callus proliferation. We have also optimized the degradation parameters that will be very much useful for carrying out the scale-up studies.

Hydrogen Peroxide is a chemical widely used in aquaria as an antibacterial and anti-algal agent. Use of required quantity of peroxidase enzyme in the aquaria can nullify the adverse effects of Brilliant Green considering it as a reducing substrate for the enzyme in the presence of Hydrogen Peroxide. The present study throws light on the potential application of a cheap source of peroxidase enzyme for use in aquaria to deactivate the adverse effect of the dye.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2016.08.001.

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