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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).