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Studies on decolorization of malachite green using immobilized *Pseudomonas putida*

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ABSTRACT

The present study emphasizes on the effect of immobilization on the decolorization of malachite green using Pseudomonas putida. The bacteria were immobilized using calcium alginate entrapment method. The effect of pH, temperature, dye concentration, inoculum volume was studied using One Factor At a Time approach for the decolorization of malachite green using calcium alginate immobilized Pseudomonas putida. Malachite green was decolorized by around 97% with the immobilized bacterium and the optimal conditions were found to be pH 7, 30°C temperature, 200 mg/L dye concentration and 6 immobilized beads. The degree of decolorization using immobilized Pseudomonas putida cells was found to be higher than free Pseudomonas putida cells. The immobilized beads were fed into a packed bed reactor and used to treat malachite green batchwise. The use of the reactor to decolorize dye improved its scope for large scale decolorization of textile effluents. Thus the use of immobilized Pseudomonas putida in the decolorization of dyes is an environmental friendly method which offers many advantages like reusability, low cost product formation, enhanced thermal and chemical stability, lesser downstream processing steps and ease of handling.

Keywords: immobilization, malachite green, *Pseudomonas putida*, calcium alginate, entrapment, reactor

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INTRODUCTION

Immobilization is a technique of confining or anchoring the enzyme in a solid support material for their stability or functional reuse. The support material is called carrier matrix. The materials used are usually inert polymers or inorganic materials. Examples of support materials are glass, starch, cellulose, polyethylamide.
Immobilization has emerged since last decade as a very powerful tool to improve almost all enzyme properties like stability, activity, specificity and selectivity, and reduction of inhibition [1].

Immobilization of enzymes offers a variety of advantages over free enzymes. Immobilized enzymes have more stability, can be used repeatedly, more economical and involve lesser downstream processing steps [2-3]. Immobilization avoids enzyme aggregation and autolysis, increases flexibility of reactor design and facilitates the removal from the reaction medium [4-5], using it as a requirement of industrial utilization [6-7].

Immobilization of microbial cells is a best way to exploit their ability to degrade textile dyes. It has many advantages than a free cell. It prevents cell washout and a high cell density to be maintained in bioreactors. The catalytic stability of microbes is often improved by immobilization. This enables the microbial cells to degrade high

concentration of toxic compounds. Immobilization also enhances substrate uptake as there is ample amount of nutrient available at the solid- liquid interface [8].

Enzymes can be immobilized by physical adsorption, ionic binding, covalent binding, cross-linking and entrapment methods [9].

Entrapment is taken as the most preferable method because it prevents excessive loss of enzyme activity after immobilization, increases enzyme stability in microenvironment of matrix, protects enzyme from microbial contamination [10]). Physical entrapment in calcium alginate beads has shown to a relatively easy, rapid and safe technique [11] in comparison with other immobilization methods [12].

Discharge of dyes into the aquatic systems is a leading cause of environmental pollution. The textile dyes disturb the marine ecosystem, as they undergo chemical and biological changes [3] and their breakdown products are also toxic to most aquatic organisms [4,5]. It also greatly affects the photosynthesis of hydrophytes by limiting light penetration, thereby deteriorating gas solubility and water quality [6].

Malachite green, an N-methylated diaminotriphenylmethane dye, has been widely used as an antifungal agent in the fish farming industry and used for dyeing silk, wool, leather, ceramics, cotton. Malachite green is highly toxic to mammalian cells; it promotes hepatic tumor formation in rodents and also causes reproductive abnormalities in rabbits and fish. Hence, there are both environmental and human health concerns about the bioaccumulation of malachite green and leucomalachite green in terrestrial and aquatic ecosystems [13].

Bioremediation may be the most effective method for treating industrial dyes wastewater [14].

Thus far only few researchers have reported utilization of immobilized cell system for decolorization of waste water and most cases have focused on immobilization of fungal biomass

[15,16] rather than bacterial cells which also hold potential for decolorization [17-19].

The use of freely suspended microbial cells for dye removal is limited owing to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength of the biomass. Immobilized cells offer advantages over dispersed cells such as high cell density, strong endurance of toxicity, lower operating costs, simple maintenance management and lower residual sludge.

Fixed film bioreactors, packed bed bioreactors, anaerobic/ aerobic rotating biological contactors, fluidized bed reactors have been reported to be used in decolorization of dyes [20].

In this paper, the effect of pH, temperature, dye concentration and volume of inoculum was studied using One Factor At a Time (OFAT) approach for malachite green decolorization when immobilized *P.putida* cells were used and compared with the earlier reports when free *P.putida* cells were used. The immobilized *P.putida* cells were packed and used as a reactor to continuously decolorize the dye. The reusability of the immobilized cells and their capability to decolorize the dye were checked.

EXPERIMENTAL SECTION

2.1. Bacterial strain

Pseudomonas putida MTCC 102 was purchased from IMTECH, Chandigarh.

2.2. Chemicals

Malachite green of analytical grade (dye content 40%) was purchased from Hi-Media. Sodium alginate and calcium chloride were of analytical grade from Hi- media. All the other chemicals used in our experiments were of analytical grade.

2.3. Equipment

UV spectrophotometer of ELICO, India was used for measuring the decolorization efficiency.

2.4. Decolorization studies

100 mg/L of malachite green dye amended in nutrient broth was inoculated with 1 mL of *Pseudomonas putida* strain and incubated at 37^0C for 72 hours. After the incubation, the reaction mixture was centrifuged at 2000 rpm for 15 minutes and the supernatant was taken to measure the dye decolorization efficiency. Dye decolorization was measured by monitoring the decrease in absorbance of the dye in a double beam UV-Visible spectrophotometer (Elico, India) at 620nm [21, 22]. Dye decolorization efficiency is expressed as follows: equation 1,

$$\text{Decolorization efficiency (\%)} = \frac{[A_0 - A_t]}{[A_0]} * 100 \quad \text{---- (1)}$$

Where A_0 : initial absorbance

A_t : final absorbance

2.5. Immobilization of cells in Calcium-alginate

The entrapment of cells in alginate was performed [23]. Sodium alginate (3 % w/v) was dissolved in boiling water and (5% v/v) bacterial culture was added to 100 ml sodium alginate solution and mixed by continuous stirring. This sodium alginate/cell mixture was extruded drop by drop into a cold sterile 0.2 M $CaCl_2$ solution through syringe. For better hardness and stability, gel beads were resuspended into a fresh $CaCl_2$ solution and kept in refrigerator overnight.

2.5.1. Physiochemical parameters optimization

Immobilized *P. putida* were placed in a 5 ml nutrient broth medium containing designated concentrations of the dye. To evaluate the effects of operational factors on the efficiency of dye degradation, the batch decolorization experiments were carried out at varying pH (4-9), temperature (30-70°C) and individual dye concentration (100-300 mg/l) and inoculum size (1-6 beads). Since the decolorization activity is strongly inhibited by agitation the experiment was performed under the 'static –incubation' condition. The immobilized cells of *P. putida* were placed into a decolorization medium containing malachite green dye. The resulting solution was statically incubated at 25°C for decolorization [24].

These results were compared with the physiochemical parameter optimization results using free *P.putida* cells [13].

2.5.2. Packed bed Reactor

A packed bed reactor was designed to demonstrate the applicability of this system at higher scales. It was developed using a glass column of 24 cm height and 1.6 cm internal diameter. The available void volume was 12 ml. The bioreactor was then filled with alginate beads and inoculated with malachite green dye samples of 200 mg/ l. The dye sample was collected at different time intervals at the exit of the column and analyzed for dye concentration.

RESULTS AND DISCUSSION

3.1. Immobilization

Immobilized cells are more efficient in degradation than free cells. Immobilization prevents the washout of cells and allows high cell density to be maintained in the reactor. Immobilized cells can degrade higher concentrations of toxic compounds when compared to free cells (Plate 1) [24].

3.2. Physiochemical parameters optimization

3.2.1. Effect of pH

pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0 [22, 23, 24]. The optimum pH for malachite green decolorization was found to be 7 [13].

Immobilized beads showed higher decolorization at pH 7 similar to free cells (Figure 1).

3.2.2. Effect of temperature

The decolorization rate was found to be maximum at 30°C and found to decrease at higher temperatures. This might have occurred due to adverse effect of high temperature on the enzymatic activities [25]. Hence the optimum temperature was found to be 30°C [13].

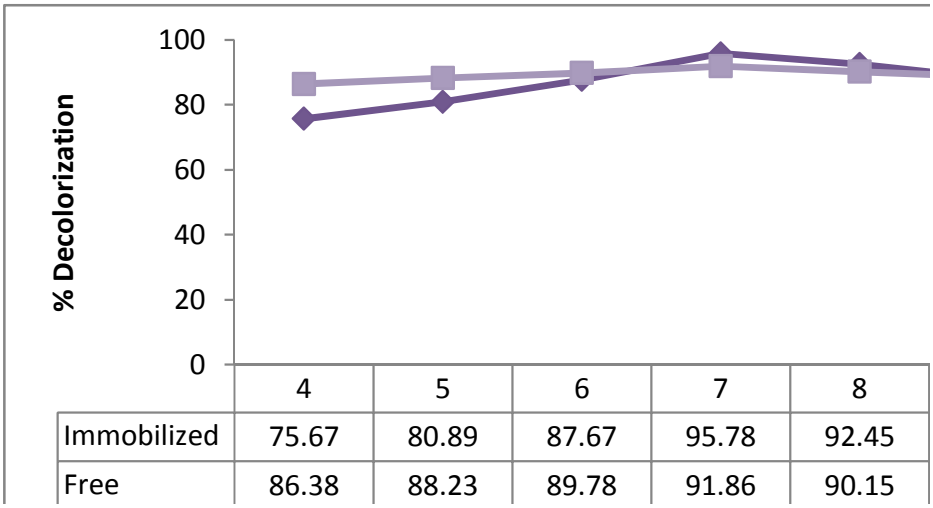
At any indicated temperature, decolorization by immobilized *P.putida* cells was significantly higher when compared to free cells. This improvement in the thermal stability of the calcium alginate entrapped cells may be attributed to the multipoint attachment of the cells to the matrix which provides enhanced resistance to the cells [26]. The optimum temperature was found to be 30°C (Figure 2).

3.2.3. Effect of dye concentration

The decolorization rate was found to increase with dye concentration upto 200 mg/mL beyond which the rate is lowered. The optimum dye concentration was hence found to be 200 mg/L. Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases. The decrease in decolorization efficiency might be due to the toxic effect of dyes [13, 27]. At any indicated temperature, decolorization by immobilized *P.putida* cells was significantly higher when compared to free cells. The optimum dye concentration was 200 mg/L (Figure 3).

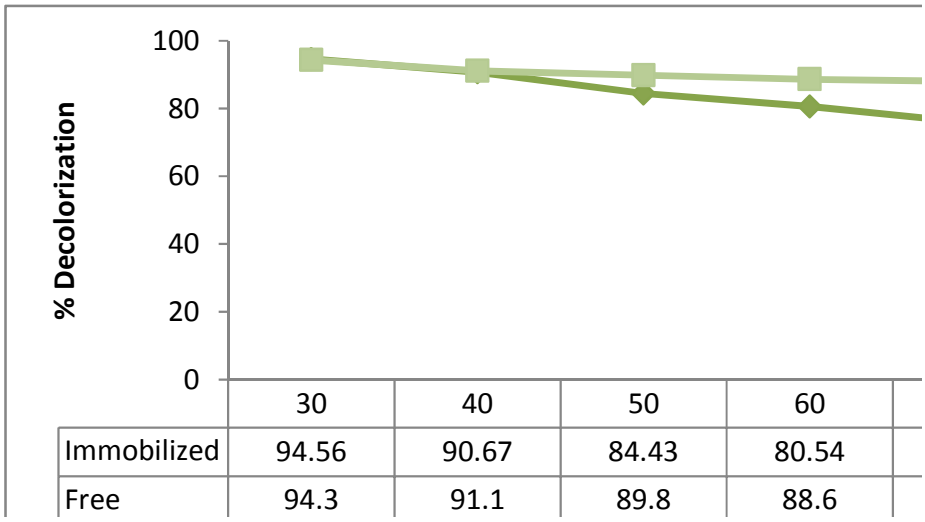
3.2.4. Effect of inoculum volume

The decolorization rate was found to increase with increase in inoculum volume. Beyond 0.5 mL the increase was not significant. Thus the optimum inoculum was found to be 0.5 mL (Figure 4) [13].



	4	5	6	7	8
Immobilized	75.67	80.89	87.67	95.78	92.45
Free	86.38	88.23	89.78	91.86	90.15

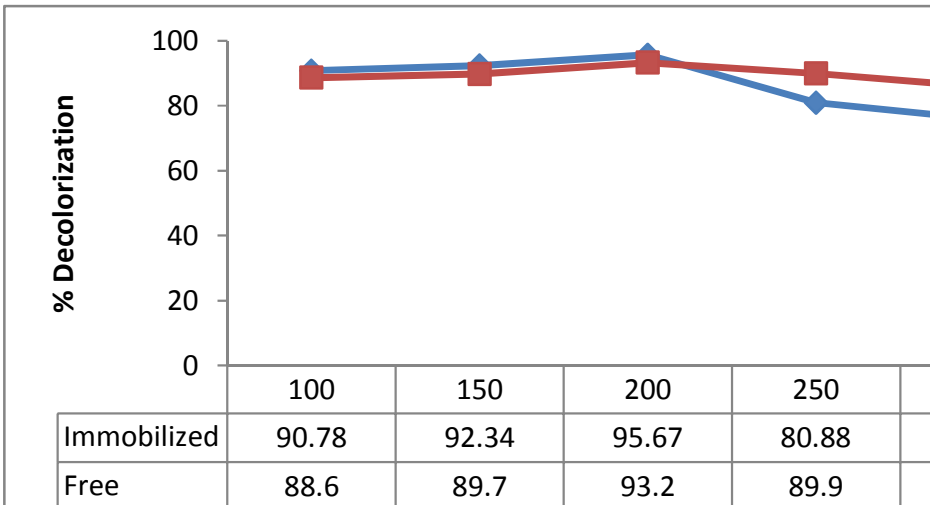
Figure 1. Effect of pH on malachite green decolorization using immobilized *Pseudomonas putida*



	30	40	50	60	
Immobilized	94.56	90.67	84.43	80.54	
Free	94.3	91.1	89.8	88.6	

Figure 2. Effect of temperature on malachite green decolorization using immobilized *Pseudomonas putida*

The decolorization rate increased with increase in inoculum size from 1 to 6 beads per reaction volume of 5 mL. Thus the optimum number of beads was 6 (Figure 5).



	100	150	200	250
Immobilized	90.78	92.34	95.67	80.88
Free	88.6	89.7	93.2	89.9

Figure 3. Effect of dye concentration on malachite green decolorization using immobilized *Pseudomonas putida*

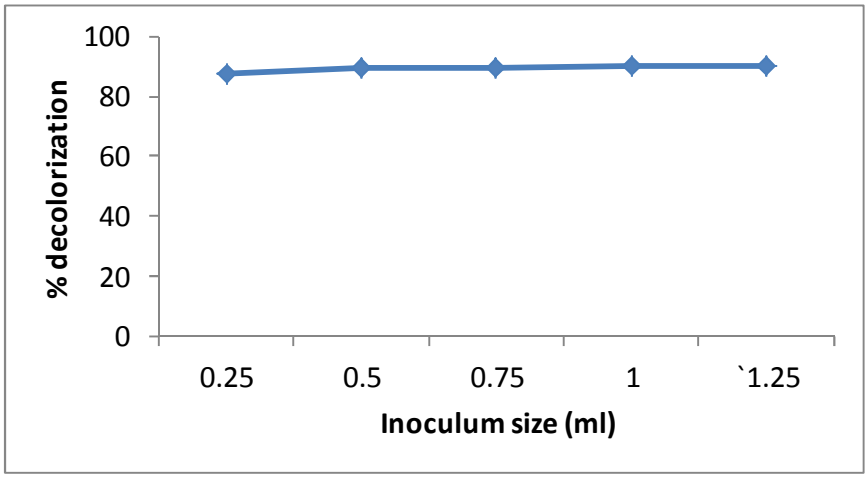


Figure 4. Effect of inoculum size on the decolorization of various dyes using *Pseudomonas putida*

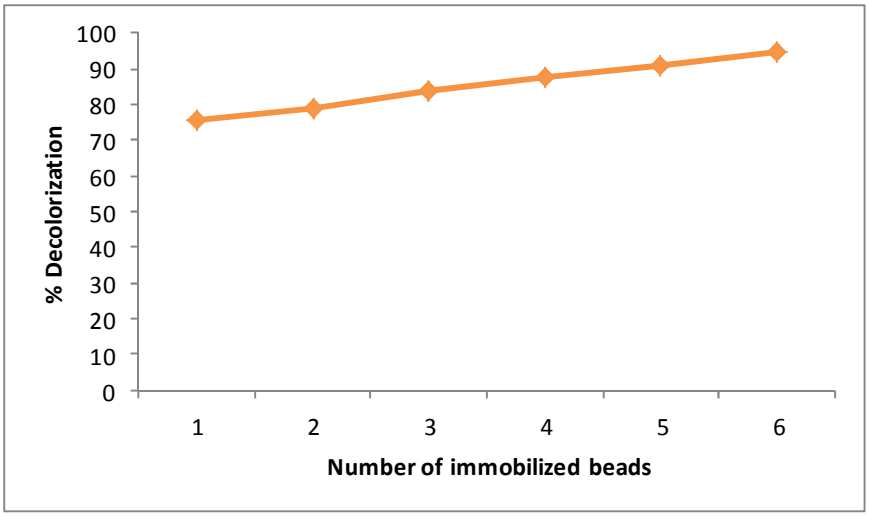


Figure 5. Effect of inoculum volume on malachite green decolorization using immobilized *Pseudomonas putida*

3.3. Reactor Studies

The application of a reactor for dye decolorization improves its scope for use in larger scale for treating industrial effluents. The reactor was used in batch mode and was found to decolorize the dye upto 97% (Plate 2). The immobilized bacterial beads were reused 5 times by washing in distilled water after each use. Beyond that, the decolorization % reduced gradually.

a. b.

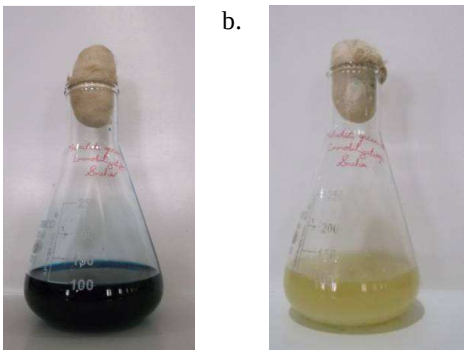


Plate 1. Malachite green and its decolorized product after treatment with immobilized *Pseudomonas putida* a: Malachite green dye (200 mg/l) amended in nutrient broth, b: Decolorized product after 72 hours of incubation with immobilized *Pseudomonas putida* at the optimum conditions

a. b.

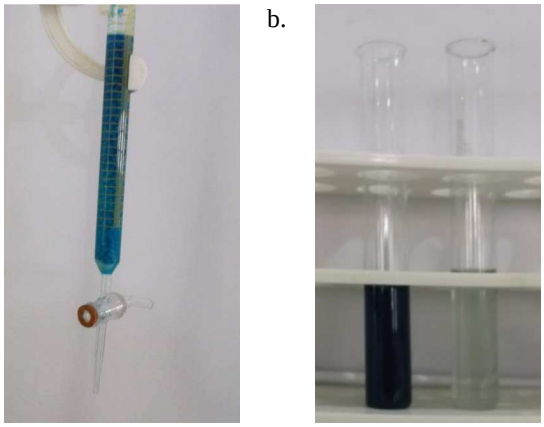


Plate 2. Reactor studies result a: Reactor filled with immobilized beads and incubated with malachite green dye 200 (mg/L) amended in nutrient broth b: left to right: test tube containing malachite green dye (200 mg/L) amended in nutrient broth and poured into the reactor and test tube containing the decolorized sample collected from the reactor outlet

CONCLUSION

It can be thus concluded that immobilization of cells is an appropriate method for large scale treatment of dye containing wastewater. Studies on physiochemical parameter optimization using One Factor At a Time approach proved that immobilization increased the efficiency of decolorization under the optimal conditions. Reusability of the cells increased when the cells were packed as a reactor. LC-MS and FTIR studies can be carried out to understand the mechanism behind the degradation of the dyes by the bacteria. Further studies on this bacterial strain could explore new tools and techniques to evolve viable and eco friendly solutions for the treatment of dyes in industrial effluents.

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