DISCOVERY

Degradation of Azo dyes by immobilized *Pseudomonas aeruginosa* and *Bacillus subtilis*

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ABSTRACT

Immobilized *Pseudomonas aeruginosa* and *Bacillus subtilis* are able to reduce azo group enzymatically and are used as a biocatalyst for the decolorization of waste water containing azo dyes. Cells of *Pseudomonas aeruginosa* and *Bacillus subtilis* were immobilized by entrapment in natural and synthetic polymerized matrices. Immobilized cells were less sensitive to agitation rates (dissolved oxygen levels) and pH. The time required for 50% conversion (t1/2) remained nearly the same for CGN- and PAA- immobilized cells during four cycles including the stable decolonization efficiency of these immobilized cells.

Keywords: Immobilization; Azo dye; Decolorization.

Abbreviations: LB – Luria–Bertani; YG - yeast extract –glucose medium; NaP - Sodium phosphate.

1. INTRODUCTION

Over the past decades, azo dyes have been the most commonly used synthetic dyes (Michaels et al., 1986; Chung et al., 1993; Carliell et al., 1995), all of which aquatics environment. Some natural microbial species, including Bactria, fungi, algae, are capable of removing the color azo dyes via biotransformation and biodegradation, or even mineralization (Chung et al., 1993; Glenn et al., 1983; Banal et al., 1996). Decolorization of azodyes by bacteria is typically initiated by azo reductase catalyzed reduction or cleavage of azo bonds under anaerobic environment. As a consequence, conventional aerobic wastewater treatment processes (such as activated sludge) usually could not efficiently remove the color of azodyes since compound are often recalcitrant aerobically (Chung et al., 1993; Carliell et al., 1995; Zimmermann et al., 1982). Apparently there is still a need to develop novel bio-logical decolorization process leading to a more effective cleanup of azodyes (Chung et al., 1993). However, practical uses of bacterial process for color removal have not been well documented.

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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 (Yung et al., 1996; Kourkoutas et al., 2004) rather than bacterial cells which also hold potential for decolorization (Chung et al., 1993; Glenn et al., 1983; Banal et al., 1996). In this study, cells of *P. aeruginosa and Bacillus subtilis* were immobilized in two natural gel matrices (calcium alginate and k-carrageenan) and synthetic polymer matrices (polyacrylamide). The decolorization kinetics of the immobilized cell was studied by investigating the effects if substrate concentration agitation rate temperature and pH on decolorization. Repeated batch operations were also preformed with the immobilized cells to justify repetitive use. Information obtained from this work is expected to become a useful reference for further development of effective decolorization bioprocesses utilizing immobilized bacterial cells as the biocatalyst.

Immobilized *Pseudomonas* species able to reduce azo group's enzymatic ally were used as a bio catalyst for the decolorization of wastewater containing azo dyes. Immobilized cells were less sensitive to agitation rates and pH as compared with suspended cells. After four repeated experiments, the decolorization rate of free cells decreased by nearly 45% while CA–GGN and PAA–immobilized cells retained over 75, 85 % of their original activity, respectively.

Early studies reported that the degradation of phenol by a defined mixed culture, consisting of *Pseudomonas putida, Cryptococcus elinovii* (Chung et al., 1993; Banal et al., 1996). The microorganisms were entrapped either in calcium alginate or chitosan alginate. The mixed immobilized cultures were able to store for up to six months without loss of phenol degradation capacity.

Recently, we attempted to determine the decolorization kinetics of a *Pseudomonas aeruginosa and Bacillus subtilis* strain able to decolorize a group of azo dyes efficiently in suspended cell system (Hu, 1994). How ever since enzymic reduction of azo groups is normally inhibited by dissolved oxygen (Chung et al., 1993), it is essential that bacterial decolorization is operated under nearly anaerobic condition. Oxygen–limiting environment appears to be unfavorable for the aerobic cellular growth of Pseudomonas and Bacillus and this limits the overall decolorization efficiency. This problem may be resolved by using immobilized–cell systems which not only increase the biomass concentration, but also enhance the stability, mechanical strength and reusability of the biocatalyst. Cell immobilization by entrapment with in natural or synthetic matrices is particularly suitable for Bactria decolorization of azo dyes since it creates a local anaerobic environment favorable to oxygen–sensitive decolorization. In contrast, despite the fact that the suspended–cell system allows better contact with the substrates, it may be less feasible in practical higher the density.

2. MATERIALS AND METHODS

2.1. Microorganism and cultivation

P. aeruginosa and *Bacillus subtilis* was isolated from a wastewater of dyeing factory. It was cultivated aerobically at 28°C in Luria–Bertani (LB) broth or yeast extract–glucose (YG) medium (1.25g/l of glucose (Difco) and 3g/l of yeast extract (Difco)) (Hu, 1994).

2.2. Immobilization of cells

2.2.1. Method for preparing immobilized cells

4% sodium alginate was dissolved in 50 ml of sterile liquid mineral medium and thoroughly stirred with 1 ml of the different micro organism. The final mixture was extruded through a needle and transfer to 200ml of 2M calcium chloride (2%) solution to yield 3 to 4mm (diameter) of alginate beads. After 20 min the beads were filtered out and stored in 0.5% $Cacl_2$ in the refrigerator for 12days. Before use, alginate matrix was coated with poly-acrylamide resin by immersion in 100 ml of 96% ethylalcohol with 15%w/w of Eudragit-100. Immobilized cells were recovered by draining of calcium chloride solution. The beads were allowed to dried by air for 2hrs and washed with sterile water.

2.2.2. Degradation by Immobilized Cells

0.2% dye solution was prepared and from that dye concentrations of 25ppm, 50ppm, and 100ppm were prepared and 4 grams of the immobilized cells were added. Absorbance values of the supernatant were noted and percentage of degradation was calculated. The beads from this experiment were washed with sterile distilled water and used as inoculums as before to see the effect of different dye concentration for the 1st wash of beads. This was followed for three washes of beads.

2.2.3. Mineral Salt Medium (MS Medium)

Media used for the degradation studies throughout the experiment was MS medium. The compositions (g/l) are:
K_2HPO_4 -1.0; $(NH_4)SO_4$-5.0; Nacl-1.0; $MgSO_4.7H_2O$-0.5; $FeSO_4.7H_2O$-0.1; $ZnSO4.7H_2O$-0.1; $CuSO_4.5H_2O$-0.1; $MnSO_4.7H_2O$-0.1; pH-7; Distilled water-1000 ml

2.3. Preparation of Stock Solutions

2.3.1. Dye Stock Solutions

0.2% of Direct Brown 2 stock solutions were prepared in 100 ml of sterile distilled water under sterile condition. Then the dye solution was filter sterilized. It was shaken well each time before use so that they are not settled.

2.3.2. Working Concentration of the Dyes from the stock solutions of dyes for 100ml of MS medium

25 ppm = 0.125ml; 50 ppm = 0.250 ml; 100 ppm = 0.500 ml

2.3.3. Measurement of dye concentration

The concentrations of Azo dyes in the samples were determined by measuring the absorbance of the supernatant at 590nm.

2.3.4. Scanning Of Dyes (Direct Brown 2) For Its Maximum Absorbance

Dyes were scanned spectrophotometrically in the wave length range of 400 – 800 nm to find out the wavelength showing maximum absorbance. It was found to be 490nm for Direct Brown 2. The structure of the dye was shown in Fig.1.

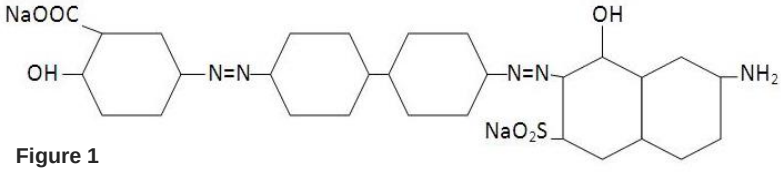

Figure 1
Structure of Direct Brown II

2.3.5. Reading Absorbance Value Spectrophotometrically At the Respective Wavelength

4ml of the samples from each experiment flask were taken separately and added with immobilized beads and were incubated. The supernatant constituting the dyes were taken and its absorbance values were read spectrophotometrically at 490nm (Direct Brown 2).

2.3.6. Estimation of Percentage of Degradation

The percentage of degradation was calculated using the formula:
Percentage of degradation = [(Initial absorbance – Final absorbance) / Initial absorbance] x 10.

2.4. Biodegradation of kinetic studies

2.4.1. Color removal with cell-free immobilization matrices

Cell-free immobilization particles were prepared following the procedures for immobilized cells except the cells. A desired amount of cell-free matrix particles (total volume = 3.5-4.5 cm3) was added into a concentration range of 25-100ppm. The residual dye concentration in the solution was detected as a function of time, until equilibrium was reached.

2.4.2. Batch decolorization operations with immobilized cells

Immobilized *P. aeruginosa,* and *Bacillus subtilis* (total biomass loading of ca 25 mg) were placed in a 50-ml decolorization medium containing 0.5% yeast extract and designated concentrations of the removal of dye color. Decolorization with an identical amount of the blank control. To evaluate the effects of operation and environmental factors on the efficiency of color removal, the batch decolorization experiments were carried dye concentrations (0-200mg/l). Temperatures (20-47°C), and pH values (5.0-9.0). Since the enzymic decolorization activity is strongly inhibited by agitation and the experiment was performed under the 'static –incubation' condition (i.e., neither aeration nor agitation was employed), except for the experiments investigating the effect of the agitation speed on decolorization. The immobilized cells of *P. aeruginosa,* and *Bacillus subtilis* (total biomass loading of ca 25 mg) were placed into a decolorization medium containing 5g/l yeast extract and 200 mg/l dye. The resulting solution was statically incubated at 28°C for decolorization. After complete color removal, the immobilized–cell particles were collected, and rinsed twice. For compaction, repeat the batch experiment procedures.

2.5. Azo dye degradation activity of the strain by consortium

2.5.1. Determination of azoreductase activity for crude cell extract at different pH

P. aeruginosa and *Bacillus subtilis* cells are suspended in buffer at a cell concentration of approximately with an ultrasonic processor, sonicated 60-75W for 5min (Sonics & Materials inc). 1 ml of cell extract (typically at 2-3 g protein /l) was added into 12ml of reaction mixture containing 0.1 M NaP buffer, 0.35mM NADH (sigma); the pH of the reaction mixture was adjusted in range of 5 to 9. The residual dye concentration in the reaction mixture was detected as a function of time, and the enzyme activity was determined from initial rate of dye disappearance. The crude cell extract was collected after removal of cell debris from the disrupted cell solution by centrifugation at 10000*g and 4°c for 8min. The azoreductase activity of the cell extract was concentrated by ammonium salt precipitation method and desalted by dialysis method.

The enzyme was purified by Affinity chromatography method and the protein was estimated by Lowry's method. The molecular weight of the protein was determined by SDS-PAGE. The purified enzyme was immobilized and used for degradation of Direct Brown 2.

3. RESULTS AND DISCUSSION

3.1. Non-enzymic color removed with cell-free matrices

Cell-free beads were examined for their non-enzymic color removed ability. This will served as a control for the results obtained from immobilized cell experiments (Fig.1 to 4). The adsorption ability of the three matrices was compared on the basis of the volume of the beads. Since, the same bead volume entraps a similar amount of biomass, but the weight of each bead made from different matrices were varies significantly. With a fixed initial dye concentration (200mg/l) and similar total volume of cell-free particles, dye concentration decreased only 2-5% with CGN between negatively-charged. Direct Brown-2 may form to impede the contact of the dye molecules with the matrices. In contrast, the PAA matrix is neutral electrically (Michaels et al., 1986; Carliell et al., 1995), and thus may have relativity higher affinity for the dye resulting in the better adsorption capacity. The density of cell-free bead (0.43 g dry matrix/cm^3) was higher than that of immobilized (0.083 g /cm^3) cell beads. Thus, it may also have higher adsorption capacity up to 9mg dye/cm^3, much higher than the maximal capacity for CA (0.9 mg dye/cm^3).

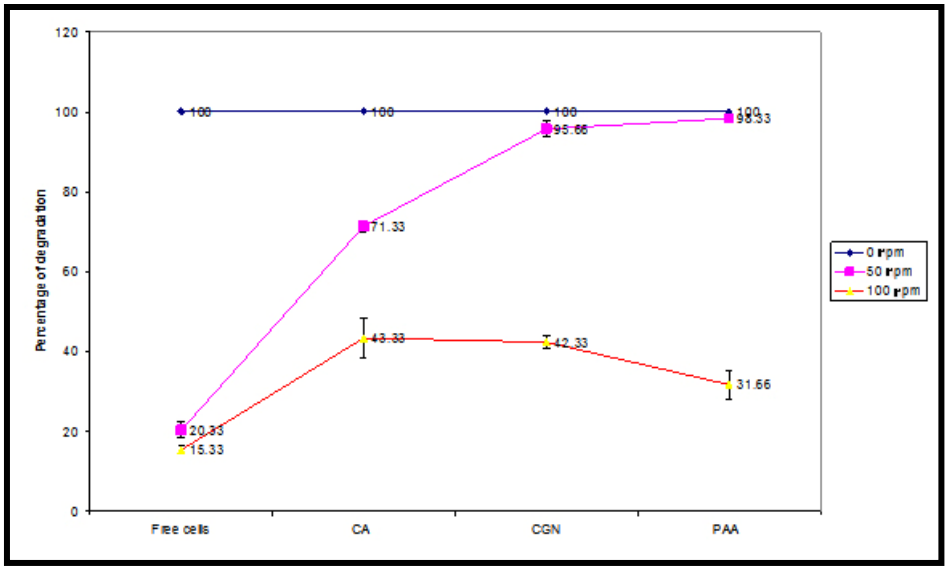

Figure 2
Percentage of degradation direct brown II by immobilized cells (concentration of dye (100 ppm))

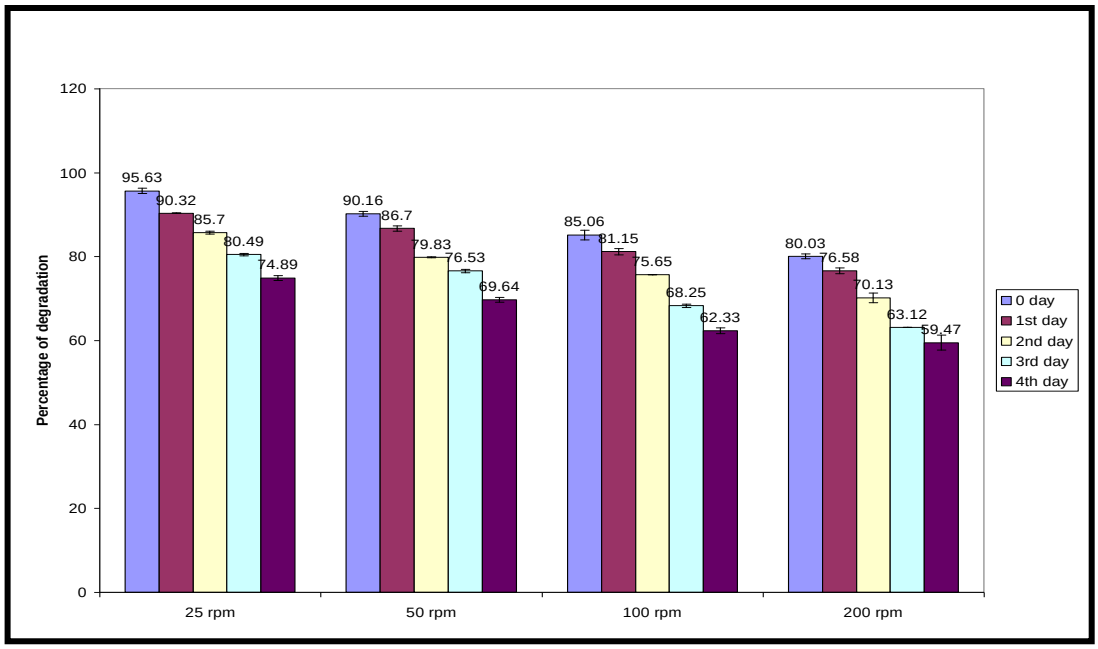

Figure 3
Percentage degradation of direct brown II by immobilized cells

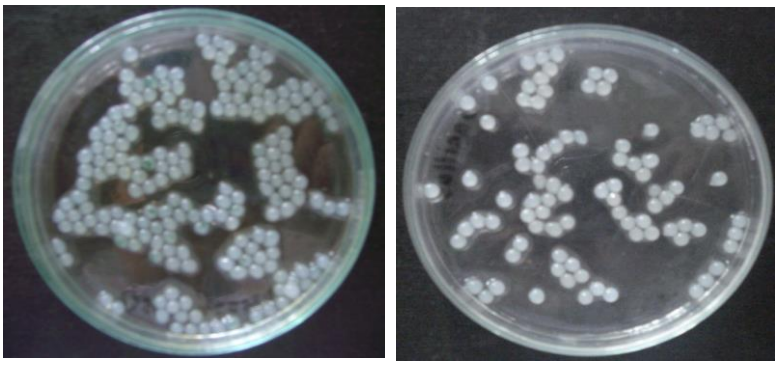

Figure 4
(A) Immobilized bacterial cells (B) control beads

3.2. Effect of dye concentration on decolorization of Direct Brown 2

Table 1 shows the dependence of dye concentration on the decolorization rate for free. The adsorption effect played a role in the decolonization dynamics primarily during the early stage enzymic transformation by cells would eventually dominate and lead to a complete color removal. The Michaelis-menten model is represented by: Udye = udye, max Cdye / Kdye + Cdye.

Table 1 Effect of dye concentration on decolorization of Director Brown 2

	Relative specific decolorization rate (%)			Equilibrium conversion (%)		
	0 rmp	**50 rmp**	**100 rmp**	**0 rmp**	**50 rmp**	**100 rmp**
Free cells	100	20	15	98	20	15
CA immobilized cells	100	71	43	96	95	82
CGN immobilized cells	100	96	42	98	97	69
PAA immobilized cells	100	98	31	97	95	64

Table 2 Effect of temperature and pH on decolorization of direct brown 2

	Free cell	CA immobilized cell		CGN immobilized cell		PAA immobilized cell	
	t ka (h)		t ½		t ½ (h)		t ½ (h)
Cycle 1	96	98	13	98	9	99	7
Cycle 2	92	86	13	97	8	98	5
Cycle 3	86	80	16	86	12	90	6
Cycle 4	85	74	22	89	10	80	7

Table 3 Percentage of degradation direct brown 2 by immobilized cells at different rpm

Type of immobilization	Relative specific decolorization rate (%)			
	0 rpm	50 rpm	100rpm	Control value
Free cells	100	20.33±2.02[a]	15.33±0.88[a]	6.15±0.75
SA immobilized cells	100	71.33±1.45[b]	43.33±4.91[c]	7.16±0.95[a]
CGN immobilized cells	100	95.66±2.02[c]	42.33±1.45[c]	6.90±0.87[a]
PAA immobilized cells	100	98.33±0.33[c]	31.66±3.48[b]	6.76±0.37[a]

Table 4 Percentage degradation of direct brown II by immobilized cells

Concentration of the dye (ppm)	Percentage of degradation					
	Day 0	Day1	Day2	Day3	Day4	Control beads
25	95.63±0.60[d]	90.32±0.08[d]	85.70±0.33[d]	80.49±0.28[d]	74.89±0.56[c]	7.16±0.95[a]
50	90.16±0.57[c]	86.70±0.65[c]	79.83±0.09[c]	76.53±0.45[c]	69.64±0.57[b]	6.90±0.87[a]
100	85.06±1.16[b]	81.15±0.75[b]	75.65±0.01[b]	68.25±0.40[b]	62.33±0.70[a]	6.76±0.37[a]
200	80.03±0.57[a]	76.58±0.64[a]	70.13±1.12[a]	63.12±0.01[a]	59.47±1.82[a]	7.20±0.52[a]

3.3. Effect of temperature and pH on decolorization of Direct Brown 2

Over the range of 30 to 45°C the specific decolorization rate of free and immobilized cells increased as the temperature increased the final conversion maintained 95% (Table 2). However, for free and immobilized cells, prolonged operations (over 15 h) at temperatures higher than 45°C led to a significant loss in cell viability and a sharp decreases in the decolorization activity (Fig.5 & 6). Therefore, to ensure the stability for long-term operation, decolorization with free and immobilized cells of *P. aeruginosa*. Bacillus *subtilis* exhibited better decolorization with free and immobilized cells of *P. aeruginosa*. Bacillus *subtilis* should not be undertaken at temperatures higher than 45°C. Free cells of *P.aeruginosa* and *Bacillus subtilis* exhibited better decolorization rates at higher pH, as the specific decolorization rate enhanced two fold for a pH increase from 5 to 9. In contrast to free cells, decolorization with the immobilized cells was less sensitive to pH with less than 20% deviation over the pH range of 5-9. This seems to suggest that the mass transfer barrier in the immobilized cells may impede the transport of bulk H^+ ions into the cells embedded within the polymeric matrices resulting in a significant pH effect for the immobilized cells. Since azo reductase activity of crude cell extract of *P. aeruginosa* and *Bacillus subtilis* did not vary appreciably over pH 5-9. The pH effect on the free cells was not related to kinetics of azo reductase, but may be more likely related to the transport of dye molecules across the cell membrane (Table 3 & 4), which was considered as the rate-limiting step for decolorization with Bacillus Subtilis.

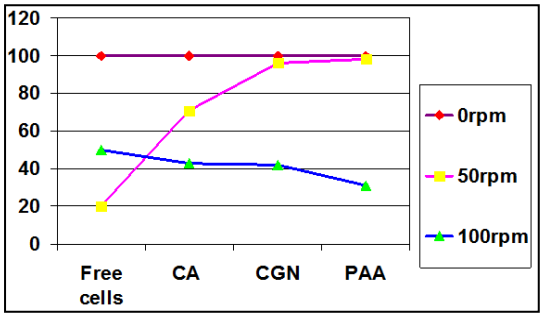

Figure 5
Relative Specific Decolorization Rate

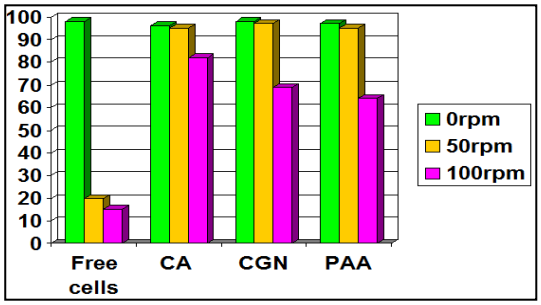

Figure 6
Equilibrium Conversion Rate

4. CONCLUSION

In comparison with the free cell, immobilized cells of *P. aeruginosa* and *Bacillus subtilis* were less sensitive to variation pH and dissolved oxygen and well better stability for the repeated use of the cell decolorization of direct brown 2. Although free cell *P. aeruginosa* and *Bacillus subtilis* shows a higher specific decolorization rate than those of immobilized cells. Immobilization greatly enhanced the biomass contrition efficiency which could therefore achieved higher total decolorization efficiency. Decolorization of PAA immobilized matix exhibited a different pattern, since PAA matrix had a high adsorption capacity of dye molecules resulting in an adsorption phase before the enzymic decolorization became dominate PAA immobilized cells add similar maximum specific decolorization rate to that re cell. The km value of PAA immobilized decolorization percentage is higher than that CA and CGN immobilized cells.

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Conflict of Interest

The authors declare no conflicts of interests any matter related to this paper.

Data and materials availability

All related data have been presented in this paper.

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