Batch, repeated batch and continuous degradation of Reactive Black 5 and Reactive Red 120 dye by immobilized bacteria

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This study presents batch degradation of Reactive Black 5 (RB5) and Reactive Red 120 (RR120) dyes using free cells of *Aeromonas punctata* and *Pseudomonas aeruginosa*, resulting 100% degradation up to 100 ppm and 150 ppm of respective dye. Immobilized cells showed 100% degradation of RB5 dye (up to 150 ppm) and RR120 dye (up to 200 ppm). On repeated use of immobilized beads, both *A. punctata* and *P. aeruginosa* showed 100% degradation of respective dye (at 150 ppm conc.) up to 10 cycles. During continuous degradation, degradation efficiencies of both cultures decreased with increase in dye concentration and flow rate. Degradation efficiency of *P. aeruginosa* was better than *A. punctata* with different flow rate and dye concentration.

**Keywords:** Ca-alginate, Degradation, Immobilization, Reactive Black 5 dye, Reactive Red 120 dye

**Introduction**

Synthetic dyes are extensively used in textile, paper & printing industries as well as in dye houses. They contain azo, nitro and sulfo groups, and are not easily amenable for microbial attack. Reactive Black 5 (RB5) dye has been used in textile industries for dying of cotton, woollen and nylon fabrics worldwide, and is toxic and cause allergic reactions of respiratory tract. Reactive Red 120 (RR120) dye is most suitable for dyeing cellulose fibers, and may cause sensitization by inhalation and skin contact. Degradation studies on RB5 and RR120 are limited using immobilized cells (ICs). This study presents batch, repeated batch and continuous degradation of RB5 and RR120 dyes.

**Experimental Section**

**Materials, Isolation and Screening**

Dyes (RB5, RR120) and other chemicals were procured from Hi-media Pvt Ltd, Mumbai, India. Soil samples from GKVK, Bangalore and Lalbagh, Bangalore, textile industrial effluents, and sewage samples in and around Bangalore were collected in sterile containers and used for isolation of cultures. Samples (1 g or 1 ml) were inoculated into 100 ml of mineral salts medium (MSM) (pH 7) separately with 50 ppm of respective dye. Flasks were incubated at 37°C for 90 days with intermittent addition of dye for enrichment of cultures. Pure cultures of each isolate were maintained on MSM with 1.5% agar. A loopful of cultures was inoculated separately into 100 ml MSM with 50 ppm of respective dye and 50 ppm of glucose. Every 24 h screening for degradation of dyes were carried out by measuring UV-vis spectrophotometer reading at 597 nm for RB5 and 520 nm for RR120.

**Identification and Inoculum Preparation**

*Aeromonas punctata* (accession no. JN561149) and *Pseudomonas aeruginosa* (accession no. JN561148), isolated from textile industrial effluent, were inoculated into MSM with 50 ppm of respective dye and 50 ppm of glucose. Flasks were incubated at 37°C for 24 h. Then, cultures (100 µl) were inoculated into agar MSM and incubated overnight. Then, inoculums (CFU/ml) were obtained by counting visible colonies on agar medium.

**Immobilization with Calcium-Alginate Beads**

Alginate entrapment of *A. punctata* and *P. aeruginosa* was performed by reported method. Calcium-alginate bead (CAB) formation was carried out...
using 3% cell pellet in 3% sodium alginate solution using ice cold 0.2 M CaCl₂ solution and stored at 4°C until further use.

Batch Degradation using Free Cells (FCs) and Immobilized Cells (ICs)
For degradation with FCs, 5 ml of inoculum (8 x 10¹⁰ CFU/ml) of *A. punctata* and *P. aeruginosa* were added to separate flasks containing 100 ml of MSM (pH 7) with 100 mg/l each of glucose and yeast extract. For degradation with ICs, 5 g (wet wt) of CABs with ICs were placed in 250 ml of conical flask containing 100 ml of minimal MSM (pH 7) with 100 mg/l each of glucose and yeast extract. Flasks were incubated at 37°C and 150 rpm. Degradation efficiencies of two cultures were checked by varying dye concentration (50 - 500 mg/l) with an interval of 50 mg/l.

Repeated Batch Degradation
To observe long term stability of dye degradation by ICs, CABs were used for repeated batch degradation. After each cycle of incubation (24 h), CABs were washed in buffer and transferred to fresh flask containing media. Spent media were used for degradation analysis. To check cell leakage of CABs, degradation samples (1 ml) after each cycle was plated onto nutrient agar media. After overnight incubation, visible colonies were counted and cell leakage values (CFU/ml) were found out.

Continuous Degradation Studies
Continuous treatments of dyes were carried out in a continuous flow reactor filled with immobilized CABs. Degradation process was carried out by continuous supply of sterile minimal MSM containing respective dyes using peristaltic pump (Micilns PP10-4C, India). To check efficiencies of *A. punctata* and *P. aeruginosa* under immobilized condition, continuous treatment of respective dyes was carried out by varying dye concentration as well as flow rate (ml/h) of media.

For continuous treatment, a cylindrical glass column (4 cm x 50 cm, vol 650 ml) with inlet and outlet facilities was used. The bottom of column was packed with a glass wool (diam, 4 cm) followed by a porous glass-frit. Then reactor was packed with respective ICs to a height of 30 cm, and attached to a reservoir containing minimal MSM with respective dyes. Medium was fed into the column continuously using peristaltic pump (Micilns PP10-4C, India) through a side arm present near the bottom of column. Medium after respective dye degradation was continuously removed from side arm situated just above the packed bed. Detention time was calculated as void volume / flow rate (ml/h). Degradation rate (R) = (Ci-Ce) x D, where Ci and Ce are concentrations of respective dyes in influent and effluent respectively, and D (dilution rate) = flow rate (ml/h) / void volume of reactor (ml).

LC-MS Analysis
LC-MS analysis was carried out by LCMS-2010SA, Shimadzu, Japan using column C18 (4.6 mm x 250 mm). Mobile phase was methanol:water (50:50 v/v). Flow rate (0.6 ml/min) and UV detector at 254 nm was used. Injection volume of dye and its degraded product was 10 µl. Mass spectra were obtained using an ion – trap mass spectrometer fitted with an electron spray (ESI, Thermo Finnigan LCQ-DUO, USA) interface operated in negative ionization mode with a spray voltage of 4.5 kV, at a capillary temperature of 275°C, sheath gas at 40 AU (arbitrary unit) and auxiliary gas at 26 AU.

Treatment of Textile Effluents
Efficiencies of *A. punctata* and *P. aeruginosa* in removal of color from textile effluents were checked further. CABs with cells of *A. punctata* and *P. aeruginosa* were mixed in equal weight and bioreactor column was filled with the same. Inlet tube was connected to flask containing untreated textile effluents and efficiencies of cultures were checked at different flow rate. Efficiency was checked based on visible colour difference of effluent before and after treatment.

Results
Batch, Repeated and Continuous Degradation of Dyes
Under batch degradation using FCs and ICs, *A. punctata* showed 100% degradation (up to 100 ppm of dye conc.) in presence of FCs, whereas immobilized *A. punctata* showed 100% degradation (up to 150 ppm of dye conc.). *P. aeruginosa* was better than *A. punctata* in degradation efficiency with both FCs and ICs. FCs of *P. aeruginosa* showed 100% degradation (up to 150 ppm of dye conc.), whereas ICs showed 100% degradation (up to 200 ppm of dye conc.). Further increase in dye concentration resulted in decreased efficiencies of both FCs and ICs of *A. punctata* and *P. aeruginosa*. At 500 ppm of respective dye concentration, efficiencies of *A. punctata* showed 30% degradation and that of *P. aeruginosa* showed 40% degradation of dye.
immobilized *A. punctata* showed 74% degradation (Fig. 1a) and that of *P. aeruginosa* showed 78% degradation (Fig. 1b).

Repeated use of immobilized beads with *A. punctata* and *P. aeruginosa* showed 100% degradation of respective dye at 150 ppm concentration up to 10 cycles. Further use of CABs resulted in decreased efficiencies of ICs of both cultures. Decrease in efficiency was more in case of *A. punctata* than that of *P. aeruginosa*. By the end of 25 cycles, immobilized *A. punctata* showed 68% degradation of RB5, whereas *P. aeruginosa* showed 80% degradation of RR120 dye (Fig. 2).

Stability of *P. aeruginosa* immobilized beads was better than that of *A. punctata*. By the end of 90 days, $5 \times 10^3$ CFU/ml of cell leakage was recorded in beads immobilized with *P. aeruginosa*. Beads immobilized with *A. punctata* showed $6 \times 10^4$ CFU/ml of cell leakage by the end of 60 days.

Efficiencies of both immobilized beads during continuous degradation were better at lower flow rate and lower dye concentrations. *A. punctata* (Fig. 3a) showed 100% degradation of RB5 up to 1600 ppm of
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cont.
Fig. 4—LC-MS spectra of degradation product of: a) Reactive Black 5 dye; b) Reactive Red 120 dye
dye at 10 ml/h flow rate (detention time, 360 min). At 100 ml/h flow rate (detention time, 36 min), it showed 100% degradation of dye only with 200 ppm of dye concentration. With 2000 ppm of dye concentration, A. punctata showed 89% degradation of RB5 at 10 ml/h flow rate and 40% degradation at 100 ml/h flow rate. P. aeruginosa (Fig. 3b) showed 100% degradation up to 2000 ppm of RR120 dye concentration and 10 ml/h flow rate, whereas 100% degradation was recorded at 100 ml/h flow rate with 200 ppm of dye concentration. RR120 dye at 2000 ppm concentration was degraded by P. aeruginosa up to 100% at 10 ml/h and 50% at 100 ml/h flow rate.

LC-MS Analysis
LC-MS analysis of dyes and their degraded product confirmed degradation of RB5 by A. punctata and RR120 dye by P. aeruginosa. The peak present in dyes spectra were absent in degraded products, indicating that entire dye was decomposed to colorless (low mol wt) fragments by respective cultures. LC-MS analysis of RB5 degraded sample (Fig. 4a) showed presence of a compound (mol wt 173, retention time 3.12 min), interpreted as 2-nitroso-1-napthol. LC-MS analysis of RR120 dye degraded product (Fig. 4b) showed a peak of unidentified compound (mol wt 747, retention time 2.83 min).

Treatment of Textile Effluents
When efficiencies of immobilized A. punctata and P. aeruginosa in treating textile effluent were carried out, they were able to show degradation efficiencies up to 50 ml/h flow rate.

Discussion
ICs are better than FCs in their efficiencies of dye degradation. Immobilization prevents washout of cells and allows high cell density to be maintained in a continuous reactor. ICs can tolerate and degrade higher concentrations of toxic compounds as compared to FCs. In present study on batch degradation, efficiencies of immobilized A. punctata and P. aeruginosa were better than FCs. In similar observations, P. fluorescens D41 was used for degradation of Direct Red and Direct Blue dyes. Under repeated batch degradation of RR120 and RB5 dyes, efficiency of P. aeruginosa was better in dye degradation than A. punctata. Gradual increase in cell leakage was recorded up to 90 days. Similarly, during continuous degradation studies, gradual decrease in degradation efficiency was recorded with increase in flow rate as well as increase in dye concentration. Decrease in degradation% at higher flow rate could be due to lesser retention time, because of which dye and cells are in contact only for a short period. Similar reports have been recorded by Usha et al. When CABs with immobilized A. punctata and P. aeruginosa were mixed and used for effluent treatment, efficiencies of beads were better up to 50 ml/h flow rate. Immobilization of cells in CABs provides both aerobic and anaerobic condition for cells to act on dyes. A combination of aerobic and anaerobic steps is reported to efficiently degrade textile dyes.

Conclusions
A. punctata and P. aeruginosa can be used for degradation of textile dyes. ICs of both cultures have better degradation efficiencies than FCs. Degradation pathways for RB5 and RR120 by A. punctata and P. aeruginosa need to be established.

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