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BIODEGRADATION OF AZO DYES BY IMMOBILIZED AND FREE CELLS OF *PSEUDOMONAS SP.* UNDER FED-BATCH CONDITIONS

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ABSTRACT

Degradation of azo dyes-Reactive Yellow 2, Direct Orange 31, Direct Blue 14 and Reactive Red 4 by suspended free cells and immobilized cells of *Pseudomonas* sp. was studied under three cycle batch conditions. Degradation (percentage dye loss) was higher in the suspended free cells than in the immobilized cells during the first cycle. First cycle data for suspended free cells were: 77.50% (Reactive Yellow 2), 78.50% (Direct Orange 31), 58.00% (Direct Blue 14) and 52.85% (Reactive Red 4). Corresponding data for the immobilized cells were: 70.60% (Reactive Yellow 2), 65.90% (Direct Orange 31), 48.00% (Direct Blue 14) and 36.05% (Reactive Red 4). During the second and third cycles, degradation was higher in immobilized cell cultures than in suspended free cells. Data for immobilized cells during the second and third cycles were: 73.95% and 78.62% (Reactive Yellow 2), 69.74% and 73 95% (Direct Orange 31), 49.85% and 51.30% (Direct Blue 14), 38.85% and 41.12% for Reactive Red 4. Corresponding data for suspended cell cultures were: 65.10% and 53.70% (Reactive Yellow 2), 64.10% and 52.80% (Direct Orange 31), 46.10% and 32.10% (Direct Blue 14), 41.90% and 30.74% (Reactive Red 4). It was concluded that degradation was enhanced by immobilization and is a feasible microbial alternative to physico-chemical and/or anaerobic/aerobic systems for the treatment of dye wastewaters.

KEY WORDS: Biodegradation, azo dyes, *Pseudomonas* sp., immobilization, cycle batch conditions.

INTRODUCTION

Major classes of synthetic dyes include azo, anthraquinone and triarylmethane dyes (Padmavathy *et al.*, 2003). Synthetic dyes are used extensively in textile, food, leather, pharmaceutical, cosmetics, paper printing and colour photography (Chen. *et al.*, 1999, Padmavathy *et al.*, 2003). Usually, dyes have a high degree of chemical and photolytic stability (Brown and Anliker, 1988) and stability against microbial attack (Pagga and Brown, 1986). Moreira *et al.* (2004) report that 50% of dyes are lost after dyeing processes and are discharged in wastewaters into the environment. Weber and Adams (1995) report that attempts to model the fate and transport of dyes in the environment have not been successful. Dyes are recalcitrant to biodegradation (Chen *et al.*, 1999, Kim and Shoda, 1999).

Dyes are potentially toxic, carcinogenic and mutagenic (NIOSH, 1980, Houk *et al.*, 1991, Brown and DeVito, 1993). The discharge of dye wastewaters into the environment is currently recognised globally as an environmental problem (Padmavathy *et al.*, 2003, Moreira *et al.*, 2004, Mendez-Paz *et al.*, 2005). Azo dyes are characterised by one or more azo bonds and are the largest class of synthetic dyes with the greatest variety of colours (Chen *et al.*, 1999). Azo dyes constitute 60 – 70% of all dyes produced globally (Carliell *et al.*, 1995), hence their wide occurrence in dye wastewaters.

Various physico-chemical techniques in use for treatment of dye wastewaters include adsorption, precipitation, chemical oxidation, photo degradation, chlorination and membrane filtration. All these techniques have serious restrictions and are economically unattractive because of high cost, formation of hazardous by-products or intensive energy requirement. For example, chlorination generates toxic organochlorides (Sarasa *et al.*, 1998, Chen *et al.*, 1999, Stolz, 2001).

Biological processes provide a low-cost and efficient alternative to physico-chemcial methods (Stolz, 2001, Mendez – Paz *et al.*, 2005). Microbial degradation of azo dyes under anaerobic conditions (Carliell *et al.*, 1994, Bragger *et al.*, 1997, Van der Zee *et al.*, 2001) and under aerobic conditions (Ghosh *et al.*, 1992, 1993, Oranusi and Ogugbue, 2001, 2003) has been reported. Anaerobic degradation generates toxic aromatic amines which are not further degraded under anaerobic condition (Mendez-Paz *et al.*, 2005). Aerobic degradation of aromatic amines has been reported (Nortemann *et al.*, 1994). Sequential and simultaneous anaerobic/aerobic systems have been used to degrade azo dyes (Tan *et al.*, 1999, 2000). These systems are operated with suspended free cells in submerged cultures. Conditions in such cultures are constantly changing which will affect the process.

An extension of biological processes is cell immobilisation. Immobilised cells have enhanced more stable activity than free cells, and are protected against toxins, bacteriophages. They can also survive long periods of time, retain their physiological activity and immobilised at high cell densities (King and Goosen, 1993, Levinson *et al.*, 1994, Tanaka *et al.*, 1994). Degradation of various toxic compounds by immobilised cells have been reported on nitriles and amides (Chapatwala *et al.*, 1993), simazine (Martin-Montalvo *et al.*, 1999) and naphthalene (Manohar and Karegoudar, 1998). There are few reports on degradation of dyes by immobilised cells (Kudlich *et al.*, 1996, Mielgo *et al.*, 2002).

The aim of this work was to study the biodegradation of four azo dyes by immobilised cells of *Pseudomonas* sp. and compare with that obtained with suspended free cells; and in this way assess the method that has a higher potential for the degradation of the dyes tested.

MATERIALS AND METHODS

Microorganism

Pseudomonas sp. was originally isolated from a dye-house effluent (Oranusi and Ogugbue, 2001) and adapted to growth on azo dyes (Orange II and Direct Blue 71). Stock cultures were preserved on nutrient agar (Oxoid) slants at 4°C in a refrigerator.

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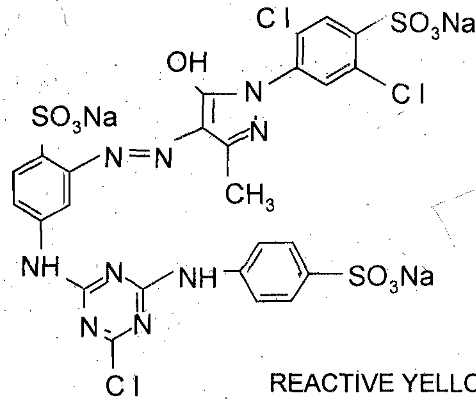
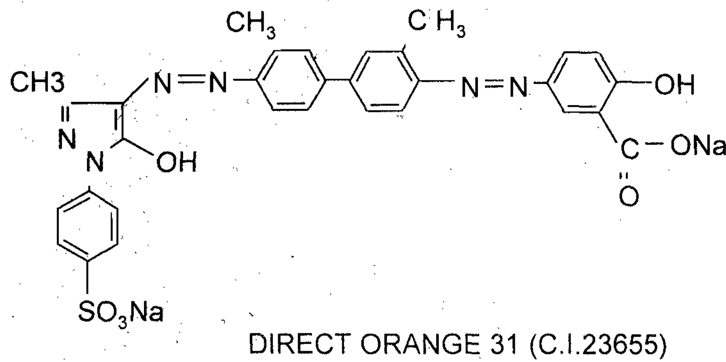
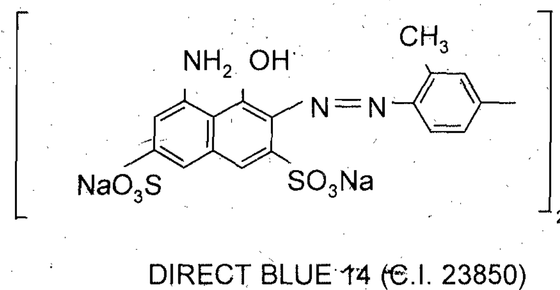
Dyes

The azo dyes used were Reactive Yellow 2, Direct Orange 31, Direct Blue 14 and Reactive Red 4 (Aldrich Chemical Co., U.S.A.). The chemical structures of the dyes arr shown in Fig. 1. Stock solution of each dye was prepared

Direct Blue 14 and Medium D contained Reactive Red







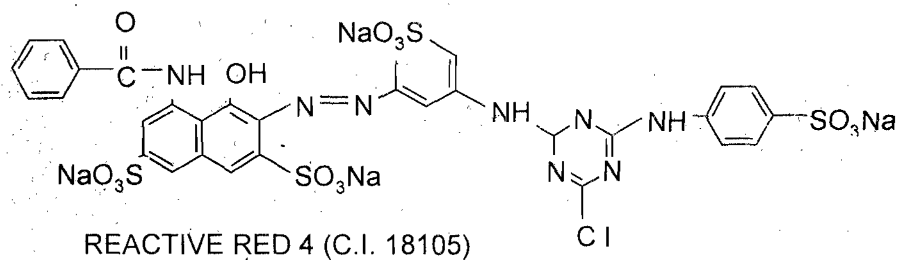


FIG.1 STRUCTURES OF AZO DYES USED. (Adapted from Aldrich catalogue, USA)

Inoculum development

Cells from stock cultures were inoculated in 50ml nutrient broth (Oxoid) contained in replicate 250ml Erlenmeyer flasks. Incubation was at 28 ± 2^{0}C with shaking (200 rpm) for 24h. Cells were harvested by centrifugation at 6,000 rpm for 15 min in a refrigerated centrifuge at 10^{0}C. Cell pellets were suspended in 20ml phosphate – saline buffer (PSB) at pH 7.0 and recentrifuged and later suspended in duplicate 150ml flasks each containing 20ml PSB. Flasks were coded FA and FB.

Loopful of culture from each flask was inoculated into 10ml nutrient broth and incubated at 28 ± 2^{0}C for 24h. There was increased turbidity of the medium. This confirmed that the cells were viable and were used for immobilisation and suspended free cells studies.

Growth on agar no. 1

A loopful of the standard inoculum was inoculated onto triplicate set of agar No. 1 plates by the spread plate method. The plates were incubated at 28 ± 2 ^{0}C for 72h. There was no growth on the plates. This showed that the organism was non-agarolytic.

Influence of concentration of agar

Choice of 1% (w/v) agar was based on the results of the preliminary experiments conducted with 0.5%, 1.0%, 1.5% and 2.0% (w/v) agar. Agar at 0.5% (w/v) could not form solid gel beads. There was negligible degradation with 1.5% and 2.0% (w/v) agar gel beads. This was attributed to diffusional limitation of both nutrients and oxygen into the gel beads.

Immobilization

The procedure was adopted from the methods of Knaebel *et al.* (1997) and Sofer (1997). Four grams of agar No. 1 (Oxoid) was added into 200ml phosphate buffer at pH 7.0 contained in 2-L Erlenmeyer flask. Sterilisation was by autoclaving. After sterilisation the agar solution was kept molten (45 – 50^{0}C) in a water bath. Ten millilitres of cell suspension from FA flask was mixed with 10ml of the molten agar by gentle stirring with glass rod for 15min to obtain 1% (w/v) agar solution. The resulting agar/cell suspension was extruded with a hypodermic syringe (diameter 1.0mm) into sterile 600ml vegetable oil (Food grade Turkey brand) contained in 2-L flask maintained at 45 – 50^{0}C in a water bath and mixed with gentle stirring for 10min. The macroparticle agar gel beads (approx. 2mm diameter) formed were hardened in a refrigerator at 10^{0}C for 24h. Excess oil was decanted. Residual oil on the beads was removed by repeated gentle washing with a mixture of phosphate buffer solution/Tween 80 until no oil sheen was visible on the surface of the supernatant. Tween 80 was washed off with phosphate buffer. The immobilized cells were suspended in the appropriate growth medium (A, B, C or D) until used. Prior to use, a loopful of culture from each medium type (A, B, C or D) was streaked onto nutrient agar plates. Plates were incubated in deionised water and sterilised by membrane filtration (membrane filter 0.2 µm pore size, Acrodisc, France)

Media

Four types of media were used. The basal medium containing the following in gL^{-1}: NaCl 2.0, $MgSO_4.7H_2O$ 0.42, KCl 0.28, K_2HPO_4 1.27, KH_2PO_4 0.85, $NaNO_3$ 0.42 mixed with EDTA 0.5ml and deionised water 1,000ml. The pH was 7.0. The medium was dispensed in 95ml amounts into replicate sets of 250ml Erlenmeyer flasks and sterilised by autoclaving. On cooling, 5ml of appropriate sterile stock solution of each dye was added into each of six replicate set of the flasks (final dye concentration 0.02mg ml^{-1}). The flasks were coded: medium A contained Reactive Yellow 2; Medium B contained Direct Orange 31, medium C contained at 28 ± 2^{0}C for 24 – 48h and observed for growth. Colonies developed on the plates. This confirmed the viability of the cultures.

Biodegradation studies

Fed-batch cultivation was carried out in three cycles. Retention time for each cycle was 48h.

First Cycle

Three 250ml Erlenmeyer flasks each containing 100ml of appropriate medium (A, B, C or D) was inoculated with 10ml immobilized cells (ca. 1.50 x 10^6 CFUml^{-1}). Controls consisted of 10ml cell-free agar gel beads. Controls were incubated at 28 ± 2^{0}C with shaking (150 rpm).

Ten millilitres of standard inoculum (Flask B) was inoculated into each of triplicate set of 250ml Erlenmeyer flasks which contained 100ml of appropriate medium (A, B, C or D). Controls consisted of duplicate set of uninoculated medium.

Immobilized cell cultures and suspended free cell cultures and controls were incubated at 28 ± 2^{0}C with shaking (150 rpm). At zero time and at 48h of incubation, 5ml sample was withdrawn from each flask for determination of percentage dye loss. Samples were centrifuged at 6,000rpm for 30min in a bench centrifuge (Baird and Tatlock, England). The optical density of the resulting supernatant was determined spectrophotometrically in a spectrophotometer 6110 (Jenway, England) at λ_{max} for each dye (λ_{max} 404nm Reactive Yellow; λ_{max} 428nm Direct Orange 31, λ_{max} 607nm Direct Blue 14 and λ_{max} 517nm Reactive Red 4). Dye concentration was obtained from the calibration curve of OD against concentration for each dye.

Second Cycle

After 48h of incubation, the culture broth from the first cycle was withdrawn. The cells (immobilized and free cells) were washed twice with physiological saline. After washing, 100ml of sterile fresh medium (A, B, C or D) was added into each flask (immobilized and free cells). Cultures and controls were treated as for the first cycle.

Third Cycle

At the end of 48h of incubation, the culture broth from the second cycle was treated as for second cycle.

Leakage of cells from immobilized cells

At the end of each cycle, aliquots (1ml) were withdrawn from each of the immobilised culture flasks and ten-fold serial dilutions were made in physiological saline. Dilutions were plated out onto nutrient agar and incubated at 28 ± 2^{0}C. The number of colonies which developed was counted and multiplied by the reciprocal of the dilution factors and expressed as colony-forming-units per ml (cfu ml^{-1}). Growth was not observed from the first cycle and second cycle samples for all four media. The data obtained for the third cycle samples were all < 3.0 x 10^2 cfu ml^{-1}. The results showed that leakage of cells into the medium was negligible and degradation of the dyes was carried out by the immobilized cells.

RESULTS AND DISCUSSION

Cells of *Pseudomonas* sp. were successfully immobilized in agar matrix. The immobilized and suspended free cells degraded the four azo dyes tested. Data obtained on percentage dye loss are presented in Figs. 2 - 5. The concentration of the dyes in all media remained the same in all the control flasks.

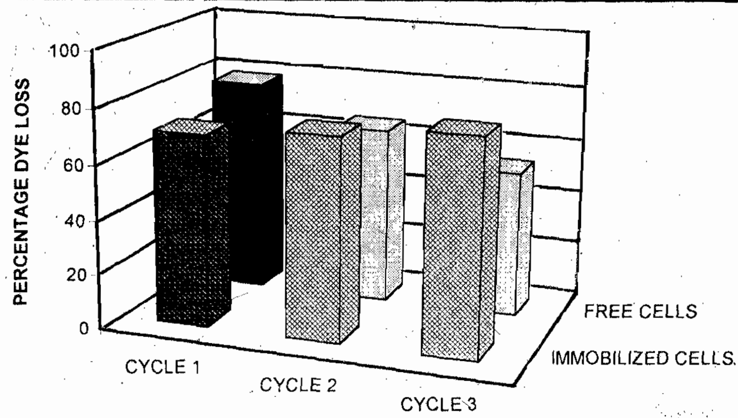


Fig. 2 PERCENTAGE DYE LOSS WHEN IMMOBILIZED AND FREE CELLS OF *Pseudomonas* sp. WERE CULTURED ON REACTIVE YELLOW 2. INCUBATION WAS AT 28 ± 2°C FOR 48h

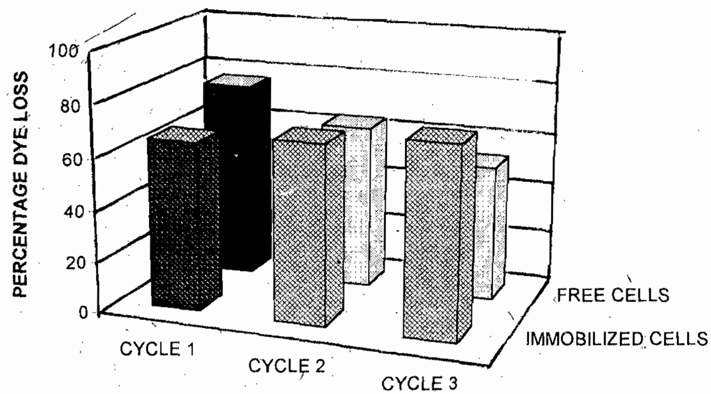


Fig. 3 PERCENTAGE DYE LOSS WHEN IMMOBILIZED AND FREE CELLS OF *Pseudomonas* sp. WERE CULTURED ON DIRECT ORANGE 31. INCUBATION WAS AT 28 ± 2°C FOR 48h.

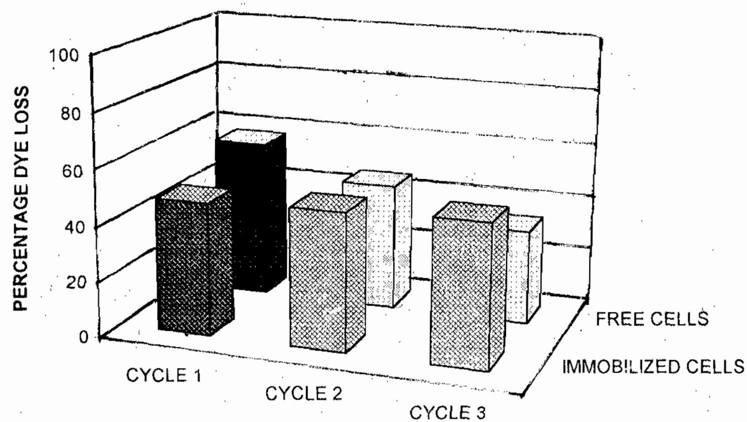


Fig. 4 PERCENTAGE DYE LOSS WHEN IMMOBILIZED AND FREE CELLS OF *Pseudomonas* sp. WERE CULTURED ON DIRECT BLUE 14. INCUBATION WAS AT 28 ± 2°C FOR 48h.

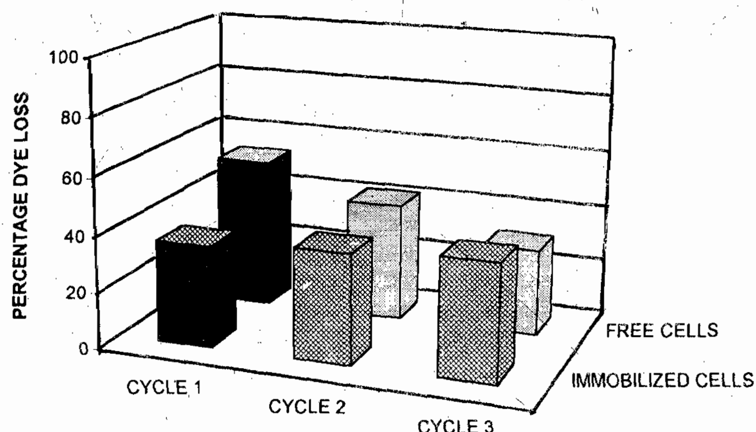


Fig. 5 PERCENTAGE DYE LOSS WHEN IMMOBILIZED AND FREE CELLS OF *Pseudomona* sp. WERE CULTURED ON REACTIVE RED 4. INCUBATION WAS AT 28 ± 2°C FOR 48h.

During the first cycle, biodegradation (percentage dye loss) was higher in suspended free cell cultures compared to the immobilized cell cultures. In immobilized cultures, the efficiency of degradation increased steadily from the first cycle through the second and third cycles. For example, in medium A which contained Reactive Yellow 2 percentage dye loss was 70.60 for first cycle, 74.35 for second cycle and 78.62 for third cycle. In suspended free cell cultures, degradation was highest for first cycle and least for the third cycle. For example, data for medium A which contained Reactive Yellow 2 was as follows: 77.50% (first cycle); 65.10% (second cycle) and 53.70% (third cycle).

Availability of nutrients and oxygen to immobilised cells is hindered by diffusional limitations which can lead to a reduction in some aspects of cellular activity (Karsten *et al.*, 1993, Omar, 1993a and b). The results obtained with immobilized cultures and free cell cultures (Figs. 2 - 5) showed that the effect of diffusional limitation was negligible. This may be due to the agar gel strength (1% w/v) used and the size of the gel beads (diameter approx. 2mm). Knaebel *et al.* (1997) have reported that the problem of diffusion is negligible when gel bead diameter is between 200µm – 3mm.

Bacterial azoreductases mediate the reductive cleavage of the azo bond of azo dyes. Zimmermann *et al.* (1982, 1984) have reported that azoreductases are either oxygen- sensitive or oxygen-insensitive. The results obtained (Figs. 2 - 5) showed that the azoreductase of this organism is oxygen-insensitive as degradation occurred in both types of cultures.

Metabolism of azo dyes generates various by-products including aromatic amines. These amines have been reported to be toxic (Houk *et al.*, 1991, Yongjie-He and Bishop, 1994, Young and Yu, 1997). The cultural conditions in the immobilized cultures are relatively stable as a result of diffusion of metabolic by-products into the medium. In suspended free cell cultures, the cultural conditions will change as by-products accumulated. Oranusi and Ogugbue (2004, 2005) have reported that the pH in cultures of organisms which metabolised azo dyes drifted to the alkaline range. The pH change may affect the activity of the azoreductase enzyme. The relative stability in immobilized cultures might have contributed to the higher degradation compared to the free cell cultures (Figs. 2 – 5).

Enhanced and more stable activity obtained with immobilized cells compared with free cells was attributed to the protection offered by the gel beads (Lakhwala and Sofer, 1991, Tanka *et al.*, 1994).

The *Pseudomonas* sp. used in this study has been reported to degrade azo dyes- Orange II and Direct Blue 71 (Oranusi and Ogugbue, 2001). This study has demonstrated (i) the dye substrate degradation spectrum of the organism and (ii) that immobilized bacterial cells is a promising technology for the treatment of dye wastewaters. The technology offers a feasible alternative to current sophisticated physico-chemical and anaerobic/aerobic systems because of ease of operation, low technology and low cost especially for our local small and medium scale dye-based industries.

Further studies on the biodegradation of other dyes by immobilized bacterial cells are recommended, especially on various polymer matrices (agar, alginate, polyacrylamide, exudates from various varieties of raffia palm and the mesocarp of cassava wastes).

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