Biodegradation of benzidine based azodyes Direct red and Direct blue by the immobilized cells of *Pseudomonas fluorescens* D41

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Benzidine based azodyes are proven carcinogens, mutagens and have been linked to bladder cancer of human beings and laboratory animals. The textile and dyestuff manufacturing industry are the two major sources that released azodyes in their effluents. The dye, Direct blue contains two carcinogenic compounds namely benzidine (BZ), 4-amino biphenyl (4-ABP), while the dye Direct red has benzidine (BZ). Among 40 isolates of *Pseudomonas fluorescens* screened, one isolate designated as D41 was found to be capable of extensively degrading the dyes Direct blue and Direct red. Immobilized cells of *P. fluorescens* D41 efficiently degraded Direct red (82%) and Direct blue (71%) in the presence of glucose.

Benzidine (BZ) based azodyes are widely used in dye manufacturing, textile dyeing, color paper printing and leather industries\(^1\). The potential toxicity, mutagenicity and carcinogenicity of azodyes are well documented\(^2\). In 1980, the National Institute of Occupational Safety and Health (NIOSH) published the studies of carcinogenicity of BZ based dyes in experimental animals and epidemiological studies of workers exposed to dyes\(^3\). BZ dyes have long been recognized as a carcinogen in human urinary bladder\(^4\) and tumorigenic in a variety of laboratory animals\(^5\). A higher incidence of hemorrhagic cystitis, recurrent papillomas and cancer of the urinary bladder in workers employed in the manufacturers benzidine based dyes with an average of 12 years of employment. The effect appears to be due to the nitro (-NO\(_2\)) and amino (-NH\(_2\)) groups of the intermediates. The hydrogen substitution in 1 and 4 position of benzene ring have a high affinity for binding carcinogenic substances and the route of entry of benzidine into different target organs of rats\(^7\). The NIOSH declared BZ based dyes to be carcinogenic due to their biotransformation to BZ. Since BZ is used as a reactant in dye synthesis workers could be directly exposed to the carcinogen. Also exposure could occur via generation of BZ during the bleaching of dyed fabrics and as a result of \(\cdot\)r/\(\cdot\)e metabolism\(^8\). Experimental studies with rats, dogs, and hamsters have shown that benzidine and BZ congener based dyes which excrete potentially carcinogenic aromatic amines and their N-acetylated derivatives in their urine\(^9,11\). Bioremediation approaches provide method of detoxification or degradation of carcinogenic compounds and wide spectrum of aromatic compounds from the environment\(^12\). Here, we report the use of immobilized cells of *Pseudomonas fluorescens* D41 for the degradation of benzidine-based dyes Direct blue and Direct red.

Materials and Methods

Benzidine based azodyes namely Direct blue (Diazoised 1-amino 2-naphthol-4-sulphonic acid with B-naphthol) and Direct red (Tetra azoised Benzidine with napthionic acid) were purchased from Ecolab, Madurai. The media components were obtained from Hi-Media Bombay, India.

Microorganisms

Rhizosphere soil samples were collected from various sources, and screened for organisms capable of degrading dyes in minimal agar plates containing (g/l): (NH\(_4\))\(_2\)SO\(_4\), 0.5; KH\(_2\)PO\(_4\), 2.66; Na\(_2\)HPO\(_4\), 32; Agar, 20 and dyes (50 ppm). Colonies were selected on the basis of their ability to form clear zone on agar plate. They were subsequently grown in the same liquid medium containing the selected dye.

Immobilization of cells

The seed cultures of *P. fluorescens* D41 was grown in nutrient broth and the cells from the late log phase culture were harvested by centrifuging at 10,000 rpm for 10 minutes, and the cells were washed and suspended in 0.1% (w/v) NaCl. To 75 ml 0.1% (w/v)

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NaCl solution, 3.5% (w/v) of sodium alginate was added, dissolved by constant agitation and then sterilized. To the sterile sodium alginate slurry, cell suspension of *P. fluorescens* D41, 25 ml of 0.1% (w/v) NaCl were added and mixed thoroughly without forming any air bubble in the slurry. The slurry containing the cells was extended as drops through a tube of (2mm diameter) into 4% CaCl₂ solution. The drops formed into spherical beads of 2 mm size. The gel beads were kept in 4% CaCl₂ solution at 5°C for about an hour for complete gelation. Then the beads were washed with sterile distilled water and used.

**Decolourization study**

The decolourization (%) was also calculated by the modified method of Yatome et al. Near the decrease in absorbance at 610 and 525 nm respectively monitored as decolourization. The uninoculated medium was maintained as control. Decolourization activity was calculated and expressed in terms of percentage:

\[
\text{Decolouration} \% = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100
\]

**Experimental design**

Immobilized cells of *P. fluorescens* D41 and the dye Direct blue (50 ppm) or Direct red were added to the 500 ml Erlenmeyer flasks incubated at 30°C for 24 hr with mild agitation. At intervals samples withdrawn, 2 μl injection were analyzed by GC-MS.

The capillary gas chromatograph was equipped with a standard Schimadzu GC-17A injector operated in the splitless. The injection port temperature was 270°C. The oven was initially maintained at 60°C for 1 min and heated at 6°C/min to 250°C, where it remained for 30 min, separation was achieved on DB5 - 60 m, 0.32 μg 60-m capillary column operated with 7 lb/m² of helium carrier gas. The capillary column was installed directly in to the Schimadzu GC-17A Mass Selective Detector Mass Spectrophotometer via the standard GC-MS interface oven. The interface oven temperature was maintained at 280°C. The mass spectrometer was operated in the 70-V electron impact mode. Source temperature was 270°C. The analyzer was scanned from 50 - 300 M / Z.

**Results and Discussion**

**Screening of bacterial strains degrading dyes**

Soil samples were diluted and plated on the nutrient agar containing different benzidine dyes (50 ppm). Colonies exhibiting clearing zone around them were scored. One of the bacterial strain exhibited clear
zone in the medium containing benzidine dyes namely Direct blue and Direct red. Later, we have identified this *Pseudomonas fluorescens* strain and designated as *Pseudomonas fluorescens* D41. The efficiency of this strain for the decolorization of dyes were measured. This strain efficiently decolorized the dye Direct red (82%) and the dye Direct blue (71%). The dye Direct blue contains two carcinogenic aromatic compounds namely Benzidine (BZ), 4-amino biphenyl (4-ABP) and Direct red has single carcinogenic compound Benzidine (BZ). The GC-MS profile of Direct blue and Direct red is shown in Fig. 1. The compound with the retention time of 35 mts and the mass spectral fragmentation pattern M⁺m/z 184 was identical to the authentic BZ. After the growth of the *P. fluorescens* D41 in the medium containing dye, the metabolites were extracted from the spent medium by the following method. The treated sample is reduced with sodium dithionate, extracted once in diethyl ether, after drying the content is reconstituted in methonol. The metabolites identified in the extract were 4-amino biphenyl (4-ABP) with the retention time of 26.6 min and mass spectral fragmentation pattern M⁺m/z 169 and identical to authentic compounds Benzidine with the retention time of 35 min and the Mass spectral fragmentation pattern M⁺m/z 57 was identified. The structures of Direct blue and Direct red and the identified metabolites was shown in (Fig. 2). BZ was the product of the reduction of two azo (-N=N-) bonds of Direct blue, whereas 4-ABP was ostensibly formed upon monodeamination of BZ.

![Chemical structures of the dye Direct blue and Direct red and their possible reactive benzidine group. A. Direct blue B. Direct red C. Possible metabolites of benzidine group.](image-url)

**Dye degradation by viable cells of *P. fluorescens* D41**

The cells grown in the dye containing medium was separated by centrifugation and the spent medium and pellet were independently inoculated into the fresh medium containing the dye and the decolourization efficiency was examined. Decolourization of dyes was observed in the medium inoculated with the cell pellet while there was no decolourization noticed in the medium inoculated with the spent medium. These results suggested the direct involvement of bacterial cells in the decolourisation of the azo dyes.

Dye decolourisation may take place through dye removal by simple adsorption of the dye at the cell surface or degradation of the dyes by the cells. Cells would become deeply colored if the dyes were removed by adsorption but cells remain colorless if they degrade the dyes. There was no change in the color of the pellet of the culture of *P. fluorescens* D41 after their growth in dye containing medium. To find out optimum cell mass concentration for the decolourization of the azo dyes, the cell mass concentration from 2.4 to 24 g/l was used. There was no significant decolourization of the dye was observed with the cell mass of 2.4 g/l. About 40-50% decolourization was obtained with the cell mass of 4.8 to 7.2 g/l. Greater than 60% decolourization of both dyes were obtained with the cell mass of 12 to 24 g/l in 4hr (Fig. 3). There was considerable delay in decolourization in the pellet with low cell density. Similarly, when the heat-killed cells were incubated overnight in the medium containing dyes, there was no significant color reduction. Colour removal was achieved Direct red (65%) and Direct blue (55%) with in 4 hr only by the use of actively growing bacterial cells (Fig. 4). Therefore, the decolourization of the dye was through bacterial degradation of the dyes.

**Dye degradation by immobilized cells of *P. fluorescens* D41**

Several bacterial strains are known to degrade a wide range of compounds and thereby detoxify them. Application of whole cell immobilization technology is a promising method of exploiting this ability of the bacteria in degradation of the dyes. Immobilization prevents washout of cells and allows a high cell density to be maintained in a continuous reactor. The catalytic stability is often improved upon immobilization, microorganisms may tolerate and degrade higher concentrations of toxic compounds than do their non-immobilised counterparts. To investigate the degradation and decolourization percentage, free cells and
immobilized cells of P. fluorescens D41 with various inoculum sizes, (5 to 15% v/v) were used. Maximum decolourization efficiency was obtained by the (15% v/v) (Fig. 4) immobilized cells, and shown that immobilised systems have an advantage over a free cell system (Figs 5, 6) resulting in sustained degradation ability. A number of reports suggested that the certain bacteria could utilize specific azo dye and certain sulfonated azo compounds as sole carbon and energy

Fig. 3—Effect of cell mass concentrations of P. fluorescens D41 on decolourization of the dye Direct blue and Direct red. The different cell mass (●) 2.4 g/l; (X) 4.8 g/l; (●) 7.2 g/l; (●) 12 g/l; (●) 24 g/l of P. fluorescens D41 was inoculated in to the minimal medium supplemented with 50 ppm of Direct blue (A) and Direct red (B) and 2 g/l glucose incubated at 37°C for 4 hr. The residual dye concentration was calculated by measuring absorbance of culture supernatant at 610 nm (Direct blue) and 525 (Direct red).

Fig. 4—Effect of free and immobilized cells of P. fluorescens D41 on decolourization of the dyes Direct red and Direct blue. P. fluorescens D41 free cells (A, C) and of immobilized cells (B, D) were inoculated into the minimal medium supplemented with 50 ppm of Direct blue (C, D) and Direct red (A, B) incubated at 37°C for 4 hr. The residual dye concentration was calculated by measuring absorbance of culture supernatant at 610 nm (Direct blue) and 525 (Direct red). A - Free cells on degradation of Direct red; B - Immobilized cells; C - Free cells on degradation of Direct blue D - Immobilized cells.

Fig. 5—Biodegradation of dye-Direct blue by P. fluorescens D41. A- Control B- Free cells C- Immobilized cells.
source. The enzyme azo benzene reductase were purified and characterised from Pseudomonas strain K24\textsuperscript{18}. Several authors employed different microorganisms for degradation of various chemicals and toxic compounds such as pentachlorophenol, 3,4-dichloroaniline, 2-chlorophenol \textsuperscript{19-22}. From the present study, it can be concluded that the complete transformation of carcinogenic compounds, benzidine (BZ), and 4-amino biphenyl (4-ABP) to non-toxic compounds can be achieved with immobilized cells of \textit{P. fluorescens} D41.

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