

Sequential Microaerophilic-Oxic Phase Mineralization of Azo Dyes by a Monoculture of *Pseudomonas Aeruginosa* Strain AWF Isolated from Textile Wastewater

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Abstract A novel dye degrading bacterium capable of decolorizing and mineralizing four different dyes (Methyl red, Orange II, Direct red 80, and Direct blue 71) was isolated from textile industrial wastewater using the selective enrichment technique. The bacterium was identified as *Pseudomonas aeruginosa*. More than 80 % decolorization of Direct red 80 was obtained under microaerophilic conditions in 48 h, whereas only 10 % color removal was obtained under oxic conditions at the same time. Subsequent aeration of the decolorized medium resulted in the mineralization of the metabolic intermediates generated after azo bond cleavage by *P. aeruginosa* as confirmed by total organic carbon content and high-performance liquid chromatography analyses. The degradation products were characterized by Fourier transform infrared spectrometer and nuclear magnetic resonance techniques whereas the biotoxicity profile of the samples were evaluated using the brine shrimp lethality test assay. Data from this study provide evidence of dye mineralization and detoxification by a monoculture of *P. aeruginosa* in successive microaerophilic/oxic stages.

Keywords Azo dyes · Decolorization · Mineralization · *Pseudomonas aeruginosa* · Biototoxicity · Microaerophilic

1 Introduction

The toxicity, carcinogenicity, and mutagenicity of synthetic dyes and the ever-increasing legislative restrictions regarding effluent discharges make dye contamination of water bodies an environmental concern as well as a public health problem (Silveira et al. 2009). Color is usually the first contaminant to be recognized in water bodies and wastewater and besides the aesthetic problem, dyes also obstruct light, limit photosynthesis, and reduce oxygen mass transfer (Khalid et al. 2008a; Patel and Suresh 2008). Synthetic dyestuffs are extensively used in a number of industries, such as textiles, food, cosmetics, construction, rubber, and paper printing, with the textile industry being the largest consumer of dyes (Pandey et al. 2007). These industries consume about 34 million tonnes of the global dyestuff production worldwide and the more than 10,000 commercially available dyes in use today (Amoozegar et al. 2011; Cervantes and Dos Santos 2011). Large amounts of these dyes, because of inefficiency in dyeing operations, do not bind to the fibers and are therefore released into waste systems or the environment. Depending on the class of dye, their release into wastewaters can range from 2 % of the original concentration for basic dyes to as high as 50 % for reactive dyes (Boer et al. 2004; Khalid et al. 2008a; Tan et al. 2000). Hence, this large contaminant load in effluents of the textile industry, which is one of the greatest generators of liquid effluent pollutants because of the high quantities of water used in the dyeing processes, poses a pollution threat that must be reduced for safe discharge to the environment (Khalid et al. 2008b).

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The largest and most versatile class of dyes which constitute about 60–70 % of dyes applied in textile processing are the azo dyes (van der Zee et al. 2001). They have been used increasingly in industries because of their ease and cost effectiveness in synthesis, their stability, and the variety of colors available compared with natural dyes (Chang et al. 2004; Seesuriyachan et al. 2007) and are the most common synthetic dyes released into the environment (Chang et al. 2001; Zhao and Hardin 2007). They are toxic, resistant to temperature and light, very stable in acidic and alkaline conditions (El Ahwany 2008; Pinheiro et al. 2004), and have been reported to be resistant to biodegradation, with the potential for persistence and accumulation at high levels in the environment (Talarposhti et al. 2001). The current methods for azo dye removal in textile industrial wastewater, which are mainly physicochemical methods, result in color removal. However, these methods are costly, result in generation of concentrated sludge and cause secondary pollution problems due to use of chemicals in treatment (Anjaneyulu et al. 2005; dos Santos et al. 2007). These limitations have prompted the search for alternative novel color removal strategies for dye wastewater treatment.

Environmental biotechnology has been applied to treatment of dye contaminated wastewaters in recent times. Some bacteria have been reported to degrade azo dyes under anaerobic conditions (Ogugbue and Sawidis 2011; Singh et al. 2007). Some of these bacteria have been highly effective for initial treatment of several azo dyes, leading to the decolorization of the parent chemicals and their breakdown into colorless aromatic amines. However, a complete process requires removal of these aromatic compounds generated as metabolites during biodegradation. This has become pertinent since these biotransformation products have been shown to be toxic and in some cases carcinogenic and mutagenic (Tan et al. 1999). Purified azo dyes are usually not directly mutagenic or carcinogenic, except for some azo dyes with free amino groups in their structure (Cervantes and Dos Santos 2011). However, several aromatic amines readily produced as metabolic intermediates after reductive cleavage of azo dyes are known as mutagens and carcinogens (Chung 2000; Golka et al. 2004). de Aragão Umbuzeiro et al. (2005) had reported that 75 % of wastewaters from textile industries in the metropolitan area of Sao Paulo, Brazil demonstrated the most positive mutagenic responses among different types of industrial effluents analyzed. Unfortunately,

only a few bacteria are able to mineralize these compounds into CO₂ and H₂O (Junghanns et al. 2008). Thus, the isolation and identification of efficient dye-mineralizing bacterial species is important for developing effective biotechnological treatment regimes for azo dye wastewater since biological treatment strategies are environmentally friendly, cost-competitive, produce less sludge, and require less water consumption compared with physicochemical methods (Banat et al. 1996; Rai et al. 2005).

Prior to this study, it was believed that individual bacterial strains usually cannot degrade azo dyes completely, leaving the intermediate products which are often carcinogenic aromatic amines, and need to be further decomposed (Joshi et al. 2008). Hence, treatment systems composed of mixed microbial populations (consortium) were used to achieve biodegradation and mineralization of synthetic azo dyes due to the synergistic metabolic activities of the microbial community (Khehra et al. 2005). Modi et al. (2010) reported that more than 97 % of the azo dye was degraded by *Bacillus cereus* after 72 h of incubation while, the extents of decolorization in solution were 76 % in 54 h and 63 % in 72 h with *Micrococcus glutamicus* and *Pseudomonas oleovorans*, respectively (Saratale et al. 2009b; Silveira et al. 2009). In this study, decolorization by *Pseudomonas aeruginosa* was more rapid with Direct red 80 (76.69 % in 24 h). However, this study has demonstrated the capability of a monoculture of *P. aeruginosa* to effectively decolorize azo dyes and mineralize the resulting metabolic intermediates arising from dye cleavage in a sequential microaerophilic/oxic process. Dye mineralization was analyzed using high-performance liquid chromatography (HPLC), Fourier transform infrared spectrometer (FT-IR), and nuclear magnetic resonance (NMR) analytical techniques while the biotoxicity profile of the untreated and treated dye solutions was determined using the brine shrimp lethality test (BSLT) assay.

2 Materials and Methods

2.1 Chemicals

Azo dyes used in this study were purchased from Sigma-Aldrich Chemical Co. USA. They include Methyl red 97 % (CI, 13,020; λ_{\max} , 435 nm), Orange II sodium salt 85 % (CI, 15,510; λ_{\max} , 485 nm), Direct

red 80 25 % (CI, 35,780; λ_{\max} , 540 nm), and Direct blue 71 50 % (CI, 34,140; λ_{\max} , 587 nm). They were used as received without further purification. Stock solutions of the dyes were prepared by adding 10 g of dye powder in 1 L of deionized water and were sterilized by membrane filtration using a 0.02- μm pore size membrane filter. Figure 1 shows the chemical structure of Direct red 80 used as a model dye in the experiments. Other chemicals or reagents used were of analytical grade.

2.2 Isolation of Dye Decolorizing Bacterial Cultures

Textile industrial wastewater samples collected from a textile industry in Penang, Malaysia were used for the isolation of dye-degrading bacteria by the selective enrichment culture technique using Bushnell–Haas medium (BHM) amended with azo dye as sole source of carbon and energy. Methyl red dye was used to develop the dye degrading bacterial consortium. The composition of the BHM consisted of the following (in grams per liter): MgSO_4 , 0.2; K_2HPO_4 , 1.0; KH_2PO_4 , 1.0; CaCl_2 , 0.02; FeCl_3 , 0.05; and NH_4NO_3 , 1.0 whereas the modified version of the medium also contained glucose 5 % (*w/v*) and yeast extract 0.09 % (*w/v*) but was not used for the isolation procedure. Medium pH was adjusted to 8 using 0.1 M NaOH or HCl solution. Sterile BHM (90 mL) amended with 1 mL of stock solution that was included 1 g/L concentration of dye and contained in 250 mL Erlenmeyer flasks were inoculated with 10 mL of the wastewater and incubated under microaerophilic conditions at 35 °C for 7 days. Once decolorization was observed, repeated transfers (10 %) of established microbial culture were conducted in freshly prepared BHM amended with 1 mL of stock solution until dye decolorizing cultures showed stable growth and consistent decolorization. The enriched cultures from the flasks were inoculated onto nutrient agar amended with a 5-mL stock solution of Methyl red dye in plates by serial dilution followed by the spread plate technique and incubated for 48 h.

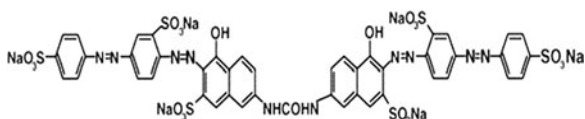


Fig. 1 Chemical structure of the model dye, Direct red 80 (CI, 35,780) used in this study

2.3 Screening of Decolorizing Bacterial Strains

The 12 isolated bacterial strains were further examined for their decolorization potentials in 250 mL Erlenmeyer flasks containing 100 mL of the modified BH medium amended with 50 mg dye/L, from these 12 isolated bacterial six of them were suitable for decolorization. These six morphologically distinct colonies that developed with halos around them on agar surface were selected and purified by streak plating on nutrient agar. Pure cultures of dye-degrading bacteria obtained were then screened for their broad spectrum azo dye biodegradability potentials.

The modified BH medium containing yeast extract and glucose was used here to determine the maximum rates of decolorization for these strains since previously, glucose and yeast extract had been shown to supplement the growth of azo dye degrading bacteria (31). The respective bacterial strains were inoculated into the media in different flasks containing the different dyes by adding inocula of uniform cell density ($\text{OD}_{600\text{ nm}}$, 0.6). The medium to inoculum ratio (*v/v*) was 9:1 yielding approximate cell densities of 10^7 CFU/mL. The flasks were hermetically sealed and incubated at 35 °C under static conditions. A similar set of treatments with cotton-stoppered flasks was set up and incubated on a shaker to monitor aerobic decolorization of the dyes. Color removal due to abiotic factors was also determined under similar conditions using uninoculated control flasks containing modified BH medium amended with each azo dye. The most effective decolorizer with a broad spectrum of azo dye decolorization was selected, identified and used for further studies. All experiments were performed in triplicate.

2.4 Bacterial Identification

Preliminary identification of the selected bacterial strain by morphological analysis was conducted by using the light microscope and FESEM (SEM Leo Supra 50 VP field emission). The genotype of the strain was identified by determining the 16S rDNA sequences of the strain. Extraction of DNA was carried out by using GF-1 DNA Extraction Kit by following the manufacturer's instructions. Polymerase chain reaction (PCR)-mediated amplification of the 16S rDNA gene from the extracted gene was carried out and the PCR product obtained was purified and concentrated using GF-1 Gel DNA Recovery Kit (Amann et al. 1995; Jaspers

and Overmann 2004). Nucleotide sequences of the 16 rDNA gene were determined by Vivantis (M) Sdn Bhd, Malaysia. The expected length of amplicon was 1,500 bp. Both forward and reverse sequences were compared with sequences in GeneBank database using BLAST search tool or RDPII (Ribosomal Database Project II) database to seek for homology sequences (NCBI 2012). Thereafter, top 10 corresponding sequences from the generated blast result were selected and used to perform multiple sequence alignment and phylogenetic tree construction using neighbor-joining (NJ) method to conclude the evolutionary history and identity of the unknown bacteria.

2.5 Decolorization and Mineralization Assay

Decolorization efficiency of the selected isolate was checked using the tetra-azo dye, Direct red 80 (50 mg/L), in modified BHM. Activated culture (10 %, v/v; OD_{660 nm}, 0.6) of the isolate was inoculated into Erlenmeyer flasks containing 200 mL of BHM and incubated at 35 °C for 24 h under microaerophilic conditions. Samples were withdrawn intermittently (every 4 h) during incubation and used for determination of dye decolorization by monitoring the absorbance of clarified samples. To determine dye adsorption on bacterial cells, the cell pellets after centrifugation were resuspended in an equal volume of methanol to extract the dye (Ogugbue et al. 2011). The suspensions were vigorously shaken in a vortex mixer, centrifuged and thereafter, checked for its absorbance. The fate of aromatic amines generated after the cleavage of azo bonds during dye decolorization was determined using the batch sequential microaerophilic/oxic culture experiments in BHM supplemented with Direct red 80. The decolorization phase of the experiment was started by inoculating the BH medium with the isolate and incubating at 35 °C for 48 h under microaerophilic conditions or until no color was observed. Subsequently, same flasks were incubated under shaking conditions (150 rpm) as previously described for another 24 h at 35 °C. Aerobiosis was introduced after static incubation to encourage degradation of aromatic amines generated after degradation of the poly azo dye since aromatic amines are recalcitrant to degradation under microaerophilic conditions (Gottlieb et al. 2003). Abiotic control flasks were also set up and kept under similar conditions as the inoculated ones. Samples were withdrawn intermittently (every 24 h) during incubation and used for determination of decolorization and

mineralization by monitoring samples' absorbance and total organic carbon (TOC) content. FT-IR and HPLC analyses were also carried out on withdrawn samples to determine dye degradation.

2.6 Analytical Methods

The residual color in the original and treated samples was analyzed by measuring the absorbance of the samples at 540 nm wavelength using a ultraviolet (UV)–visible double beam spectrophotometer (Shimadzu 160A, Japan). The samples withdrawn from experimental flasks at intervals were centrifuged at 12,000 rpm for 10 min to remove suspended particles and cells that may obstruct absorbance readings. The supernatants obtained after centrifugation were analyzed by measuring the decrease in absorbance at the wavelengths relevant to each dye with reference to the un-inoculated controls. Un-inoculated dye-free medium was used as blank. All assays were performed in triplicate and the extent of decolorization was calculated accordingly as follows:

$$\text{Decolorization (\%)} = \frac{(AB_0 - AB_1) \times 100}{AB_0}$$

Where AB_0 is the absorbance of the dye solution before decolorization and AB_1 is the absorbance of the dye solution after decolorization (Chen et al. 1999; Kumar et al. 1997; Sheth and Dave 2009). Growth was also monitored spectrophotometrically by checking the optical density of withdrawn samples before centrifugation. The optical density was studied at 660 nm after homogeneously mixing samples.

2.7 High-Performance Liquid Chromatography

The intermediates formed due to bacterial decolorization and mineralization of dye was analyzed by HPLC. The HPLC (1260 Agilent Technologies, USA) was equipped with a quaternary gradient pump system and a multi wavelength detector. The samples were eluted in gradient mode using a C18 column (5 μm; Agilent ZORBAX ODS). The mobile phase consisted of HPLC grade methanol and water in the ratio of 70:30. Samples were eluted at a flow rate of was 1.0 mL/min at room temperature for 15 min. The samples were filtered with a 0.2-μm membrane filter before injection and 10 μL of the filtered samples was injected into the HPLC

(Gomare et al. 2008). The eluates were monitored by HPLC at wavelength of 254 nm.

2.8 Determination of TOC

The TOC content of the dye containing samples was monitored during treatment under microaerophilic conditions and after agitation using a TOC analyzer (Shimadzu 5000A) with direct injection of the samples after centrifugation and filtration through a glass fiber filter (Whatman, USA). The mineralization of the dye was then quantitatively determined as TOC removal ratio, before and after the treatment process (Saratale et al. 2009a):

$$\text{TOC removal ratio (\%)} = \frac{\text{Initial TOC}_{(\text{zero h})} - \text{Observed TOC}_{(t)}}{\text{Initial TOC}_{(\text{zero h})}} \times 100$$

Where, $\text{TOC}_{(\text{zero h})}$ and $\text{TOC}_{(t)}$ are the initial TOC value (at zero h) and the TOC value after a particular reaction time (t), respectively.

2.9 Fourier Transform Infrared Spectroscopy and Nuclear Magnetic Resonance Spectroscopy

FT-IR analysis was carried out using Nicolet Is10 Spectrophotometer (Thermo Fisher Scientific, Madison, WI) and was used for investigating the changes in surface functional groups of the samples, before and after microbial decolorization. Liquid samples were loaded on the aperture of the liquid analyser and the changes in the percent transmission at different wavelengths were observed for treatment samples and compared with control dye in the mid-IR region of $400\text{--}4,000\text{ cm}^{-1}$ with 16 scan speeds. The resolution of the spectrometer was 4 cm^{-1} . The spectra were then subjected to baseline correction and the bands were studied to quantify the changes in the chemical structure of the dye.

Proton nuclear magnetic resonance ($^1\text{H NMR}$) spectra of samples before and after degradation were recorded on a Bruker Avance 400 MHz spectrometer at 500.13 MHz at 295.3 K. Samples, dried by the classical double-pulsed field gradient of echo sequence: WATER-GATE, were dissolved in D_2O , transferred to NMR tubes and the ^1H spectrum recorded to observe the structural transformation in the dye molecules during treatment. A total of 32 scans were collected (acquisition time, 3.17 s; spectral window of 10,330.578 Hz) and a 0.3-Hz line

broadening was applied. Fourier transformation and a baseline correction of spectra were carried out prior to spectra integration with Bruker software.

2.10 Scanning Electron Microscopy

Scanning electron microscopy (SEM) photomicrographs of bacterial cells were taken to determine morphological changes in cells during decolorization. Culture samples of *P. aeruginosa* before and after decolorization were centrifuged (3,000 rpm for 5 min), and the retrieved cells fixed in McDowell–Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2) at $4\text{ }^\circ\text{C}$ overnight. The samples were washed twice with phosphate buffer and then post-fixed with 1 % osmium tetroxide for 1 h. After post fixing, the cell pellets were washed twice with distilled water, dehydrated in graded ethanol and hexamethyldisilazane, and then air-dried in a dessicator at room temperature (McDowell and Trump 1976). With double-sided tape, the samples were mounted on to SEM specimen stubs, coated with gold in sputter system in a high vacuum chamber and examined in a field emission scanning electron microscope (FESEM; Leo Supro, Carl-Zeiss SMT, Oberkochen, Germany) at an acceleration voltage of 10 kV and a working distance of 8 mm.

2.11 Toxicity Test (Brine Shrimp Assay)

Brine shrimp (*Artemia salina*) lethality assay was conducted to determine the toxicity of the treated dye solutions based on the LC_{50} (lethal concentration) criterion. The assay was chosen as a toxicity level bioindicator because of its fast response to concentrated and diluted by-product solutions (Palácio et al. 2009). Brine shrimp eggs were hatched in a beaker filled with filtered artificial sea water under constant aeration. The artificial seawater was prepared from commercial sea salt 38 g/L (Sasidharan et al. 2008). After 48 h, the phototrophic nauplii were gently collected by pipette and distributed in tens into each well of 24-multiwell plates containing the samples. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. The treated samples were tested in triplicates in a concentration series (100, 50, 25, 12.5, and 6.25 mg/L). As a control assay, ten larvae of *A. salina* were also incubated in artificial sea water without dye. The plates were maintained at room temperature ($25\pm 2\text{ }^\circ\text{C}$) and light

regime under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths in each concentration and control (seawater) well was determined (Meyer et al. 1982). LC_{50} values with brine shrimp were obtained with 95 % confidence intervals from 24 h counts using the probit analysis method described by Litchfield and Wilcoxon (1949).

3 Results and Discussion

3.1 Isolation and Screening of Dye-Decolorizing Strains

Six bacterial isolates that showed potentials for azo dye decolorization were isolated from the wastewater of a textile manufacturing plant in Malaysia after acclimation for 6 weeks. The bacterial strains were selected based on their ability to form a clear halo on agar plate containing azo dye. Among the 12 isolates, one strain (AWF) was selected based on its ability to show the highest extent of dye decolorization during screening and was used for further studies. This selected strain was a Gram negative, motile rod identified as *P. aeruginosa* on the basis of its 16S rRNA gene sequence. The top 10 sequences from the generated BLAST result were selected and used to perform multiple sequence alignment and phylogenetic tree construction using NJ method to conclude the identity of the unknown bacteria (Fig. 2). The phylogenetic tree obtained show that the isolate is of the Gamma Proteobacteria class of bacteria and clustered within the family of *Pseudomonadaceae*. The isolate was identified as *P. aeruginosa* since the 16S rRNA gene of the isolate shared 99 % similarity with other strains of *P. aeruginosa* such as *P. aeruginosa* strain MMA83 and *P. aeruginosa* strain NL01.

3.2 Decolorization and Mineralization Studies

The isolate, *P. aeruginosa*, decolorized four different azo dyes (Methyl red, Orange II, Direct red 80 and Direct blue 71) in modified BHM amended with the respective dyes albeit, to varying degrees when incubated under microaerophilic conditions (Fig. 3). More than 80 % color removal was obtained for all the dyes tested in 48 h indicating the broad spectrum azo dye decolorizing activity of the isolate. Decolorization by this isolate was more rapid with Direct red 80 (76.69 % in 24 h) and hence, the dye was used for further studies. In

comparison with other strains reported in literature, *P. aeruginosa* showed a promising feasibility for dye decolorization; for example, Modi et al. (2010) reported a dye decolorization extent of 97 % in 72 h with *Bacillus cereus*, whereas with *M. glutamicus* and *P. oleovorans*, extents of color removal in solution were 76 % in 54 h and 63 % in 72 h, respectively (Saratale et al. 2009b; Silveira et al. 2009). The varying degrees of decolorization of the different dyes by the isolate may be attributed to the individual dye's structure and complexity. The nature and position of functional groups on the aromatic rings and their resulting interactions (steric hindrance) with the azo bond may also be responsible for the differences obtained (Ogugbue et al. 2011). No color changes were observed in the uninoculated control flasks under shaking or static conditions suggesting color removal from dye solution was not due to abiotic loss. Moreover, evaluation of the cell walls for adsorption of dyes by a simple cell pellet inspection indicated that the decolorization of the dyes was due to biodegradation rather than adsorption. No change in cell color was exhibited by the *P. aeruginosa* cells throughout the decolorization process as the cells maintained their original color. Blanquez et al. (2004) had proposed that the dye degradation mechanism consists of a first step of dye adsorption on microorganisms before their degradation within the cells. Adsorption levels most times are an indication of biotransformation efficiency or its absence as rapid dye biodegraders rarely show high adsorption rates upon decolorization and incubation for an extended time period (Ogugbue and Sawidis 2011). In this study, the adsorption step was not observed and this was probably due to the rate of dye reduction which was probably too rapid for bioaccumulation on cell walls to be noticed.

With Direct red 80, incubation under oxic conditions resulted in more than 50 % inhibition of color removal from solution though; optimal growth of the bacterium was obtained with aerobiosis (data not shown). The extent of decolorization obtained for Direct red 80 during aerobiosis was 10 % after 48 h as compared with 84.6 % color removal obtained under microaerophilic conditions (Fig. 4). This may be attributed to the inhibition of the bacterial enzyme, azoreductase, by oxygen. Azoreductase is the key enzyme responsible for reductive azo dye degradation in bacterial species (Franciscon et al. 2009). This enzyme which has been isolated from several bacterial species are inducible flavoproteins and have been shown to use both NADH and NADPH as

To evaluate the mineralization of the generated aromatic amines by the monobacterial culture, the mineralization extent (represented by percent TOC removal) was determined (Fig. 6). Bacterial azo dye biodegradation proceeds in two stages. The first stage involves reductive cleavage of the azo bonds ($-N=N-$), resulting in the formation of generally colorless but potentially hazardous—aromatic amines, whereas the second stage involves degradation of the aromatic amines (van der Zee and Villaverde 2005). Azo dye reduction usually requires anaerobic conditions, whereas bacterial biodegradation of aromatic amines is an almost exclusively aerobic process (Fig. 7). Thus, the aromatic amines which accounted for the consistent TOC levels after decolorization were mineralized and probably released as carbon (IV) oxide after incubation under oxic conditions. Hence, the mineralization of the aromatic amines into gaseous carbon (IV) oxide may explain the reduction in TOC levels after the oxic phase. Percentage TOC removal was just 15.63 % after the first 48 h of microaerophilic treatment whereas subsequent incubation under oxic conditions for 24 h led to a rapid TOC removal from 15.63 to 70.91 %. No changes in TOC levels were obtained in the controls and in filter-sterilized microaerophilic-treated cultures suggesting that the elimination of the degradation intermediates was not due to abiotic factors but rather due to the activities of the microorganism. *P. aeruginosa* showed good potentials for TOC removal compared with previously reported physicochemical methods viz. Fenton/UV-C process for Reactive black 5 (Lucas and

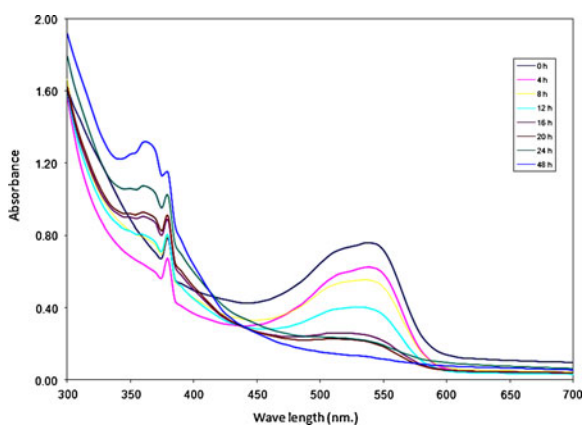


Fig. 5 UV-visible spectral signatures of Direct red 80 showing the decolorization profile at various time intervals during degradation by *P. aeruginosa* under microaerophilic conditions

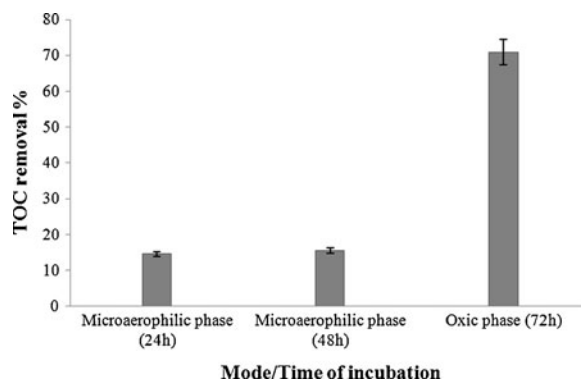


Fig. 6 TOC removal during sequential microaerophilic/oxic phase degradation of Direct red 80 by *P. aeruginosa*

Peres 2007) in which a TOC removal of 46.4 % was obtained. HPLC chromatograms (Fig. 8) obtained for the two phase process indicated the emergence of new peaks after microaerophilic incubation which was attributed to the generation of aromatic amines. These new peaks disappeared after oxic incubation but were still present in filter-sterilized microaerophilic process samples after prolonged incubation. These results thus, suggest the mineralization of Direct red 80 by *P. aeruginosa* during the sequential microaerophilic-oxic process. Data obtained were consistent with the UV-visible spectra and TOC data based on the reaction intermediates' emergence and disappearance timeline. The emergence of new peaks in HPLC chromatograms occurred simultaneously with decrease in absorbance peaks of the dye and the disappearance of the new peaks after the oxic phase also confirmed data on TOC indicating decrease in levels after that phase. Absence of any detectable products in the reaction mixture and decreasing TOC after reaction time had been stated as a supportive evidence for complete mineralization of dyes (Saratale et al. 2010).

Comparison of FT-IR spectrum of control dye with treated samples clearly indicate the biodegradation of parent dye compound by *P. aeruginosa* (Fig. 9) The FT-IR spectra of the control dye sample showed several peaks in the region where N-H and O-H stretching is normally observed ($3,300-3,500\text{ cm}^{-1}$). However, after microbial treatment under microaerophilic and oxic conditions, a significant reduction in absorption was observed in this region. The fact that no new peaks appeared between $3,300$ and $3,500\text{ cm}^{-1}$ (attributed to azo bonds and an OH group in the a-position relative to

Fig. 7 General overview of the fate of azo dyes and aromatic amines during anaerobic-aerobic treatment (van der Zee and Villaverde 2005)

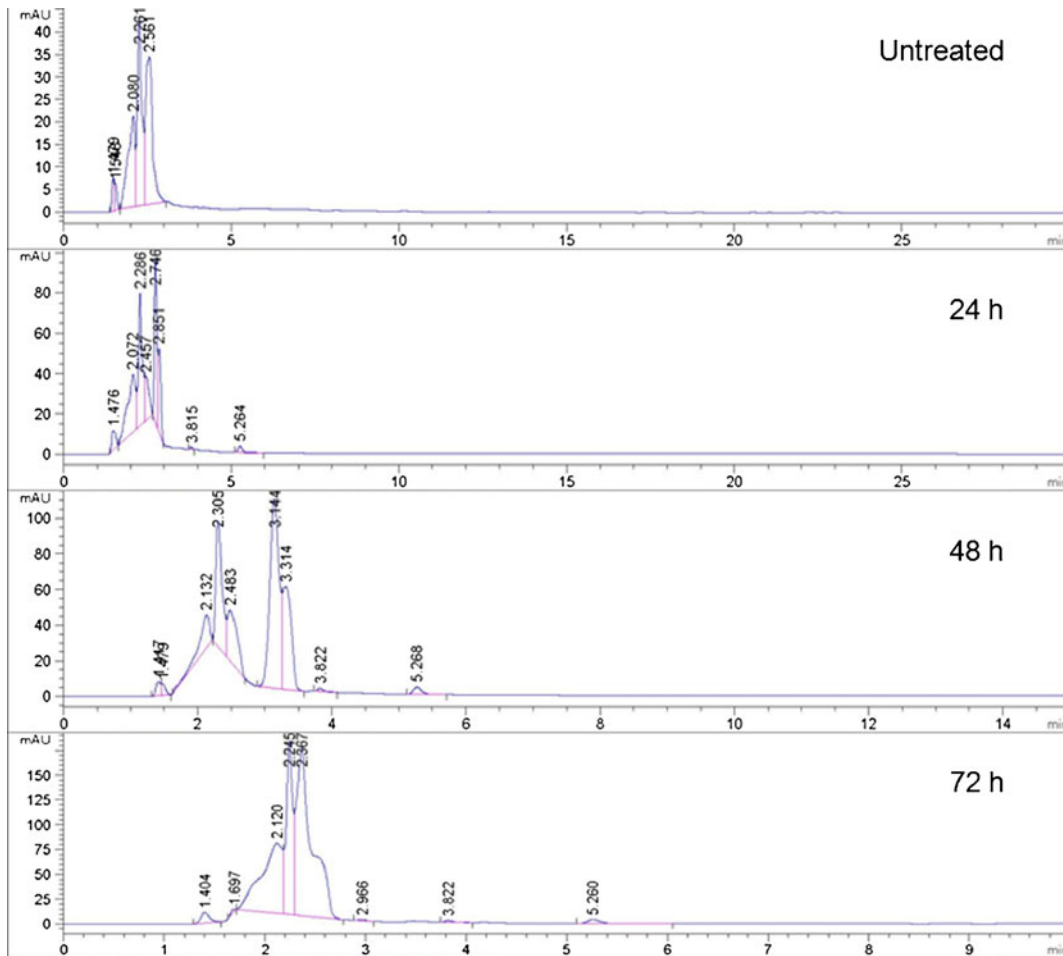
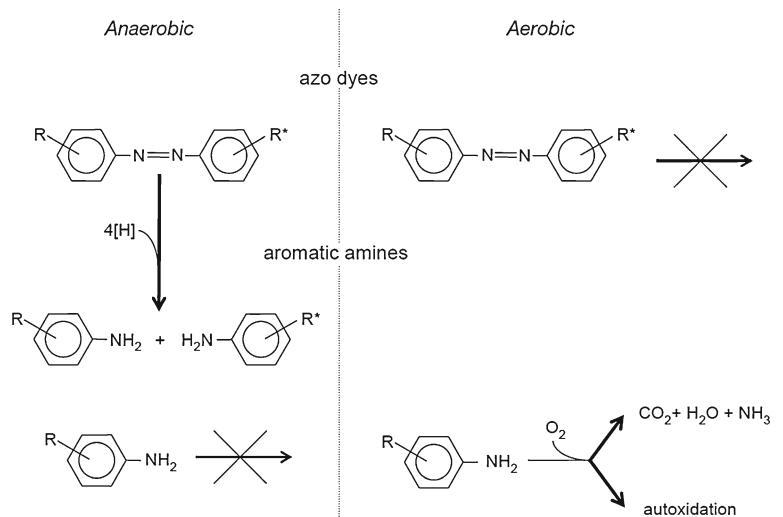


Fig. 8 HPLC chromatograms of azo dye Direct red 80 and metabolic intermediates obtained after sequential microaerophilic/oxic phase degradation by *P. aeruginosa*

Fig. 9 FT-IR spectrum of untreated Direct red 80 (a), and its degradation products after 24 (b), 48 (c), and 72 h (d) of treatment using *P. aeruginosa*

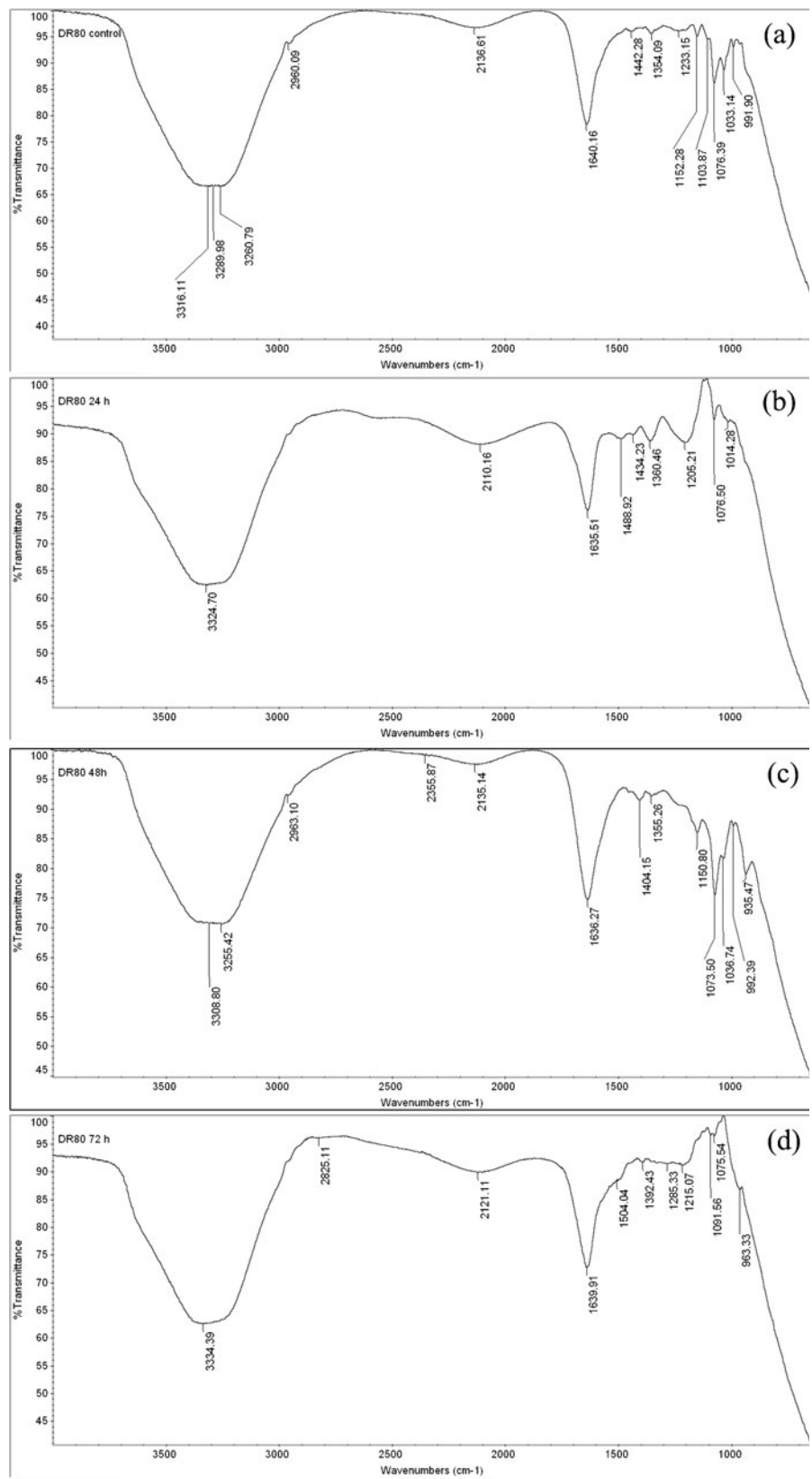
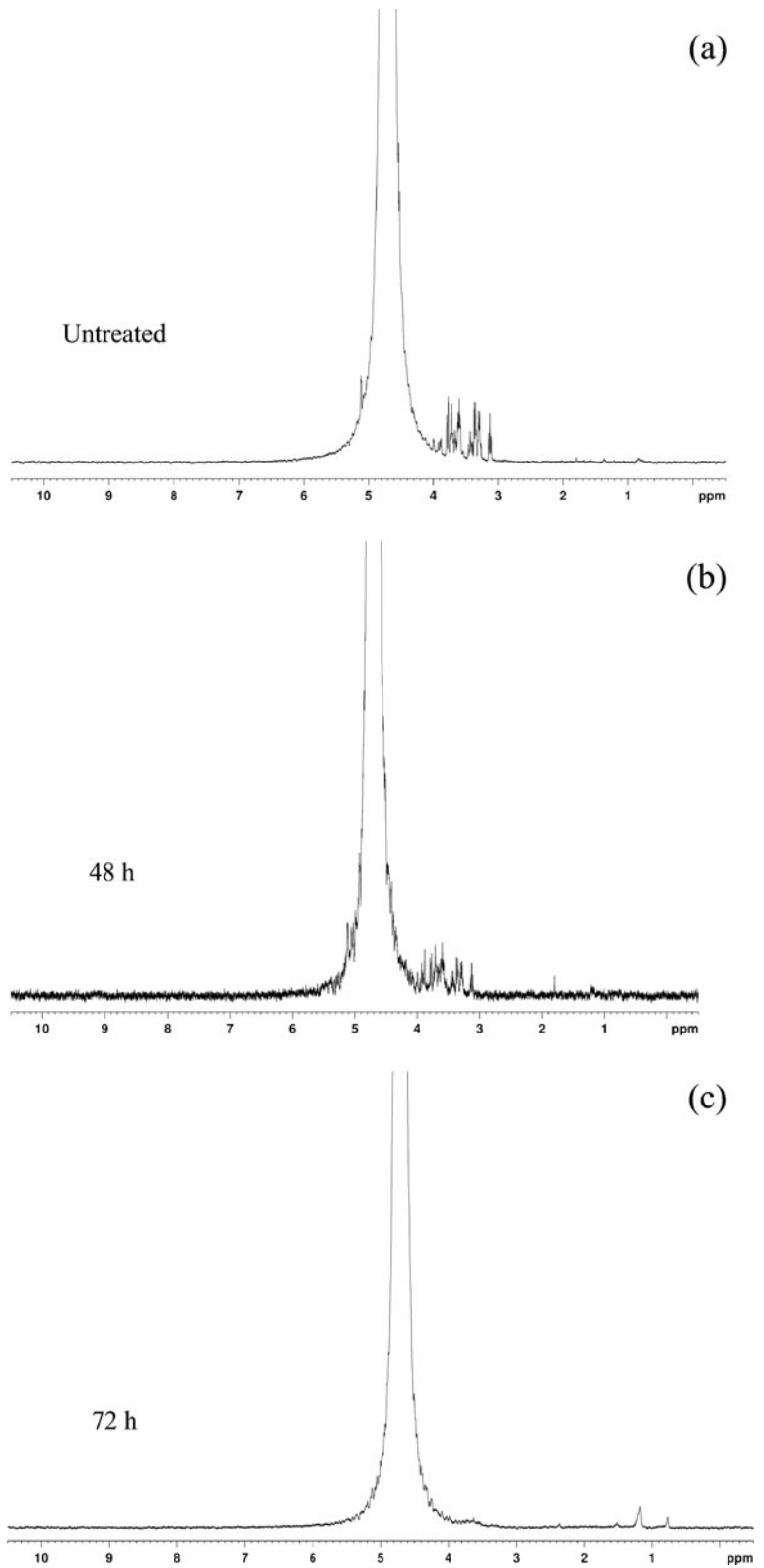


Fig. 10 ^1H NMR data for Direct red 80 before and after degradation by *P. aeruginosa*. **a** Untreated dye **b** after degradation under microaerophilic conditions and **c** after degradation under oxic conditions



the azo linkage) and in the region between 1,340 and 1,250 cm^{-1} ($-\text{NH}_2$) after the oxic treatment, suggested that the azo linkage could have been transformed into N_2 or NH_3 or incorporated into the biomass (Gavril and Hodson 2007). During microaerophilic treatment, bands located within the region of 1,340–1,020 cm^{-1} stretching representing amine groups within the dye structure were retained but disappeared from the spectrum of the oxic treated dye confirming the previous

HPLC chromatograms indicating removal of aromatic amines after oxic treatment. N–N stretching at 1,640 cm^{-1} represented azo linkages $-\text{N}=\text{N}-$ in parent dye compound and of $-\text{N}-\text{N}-$ stretching in α -substituted compounds which were attenuated after the oxic treatment. Moreover, a new band appeared in the C–H region at around 950–900 cm^{-1} associated with the formation of alkene groups which suggested that the products were undergoing irreversible chemical changes, probably due

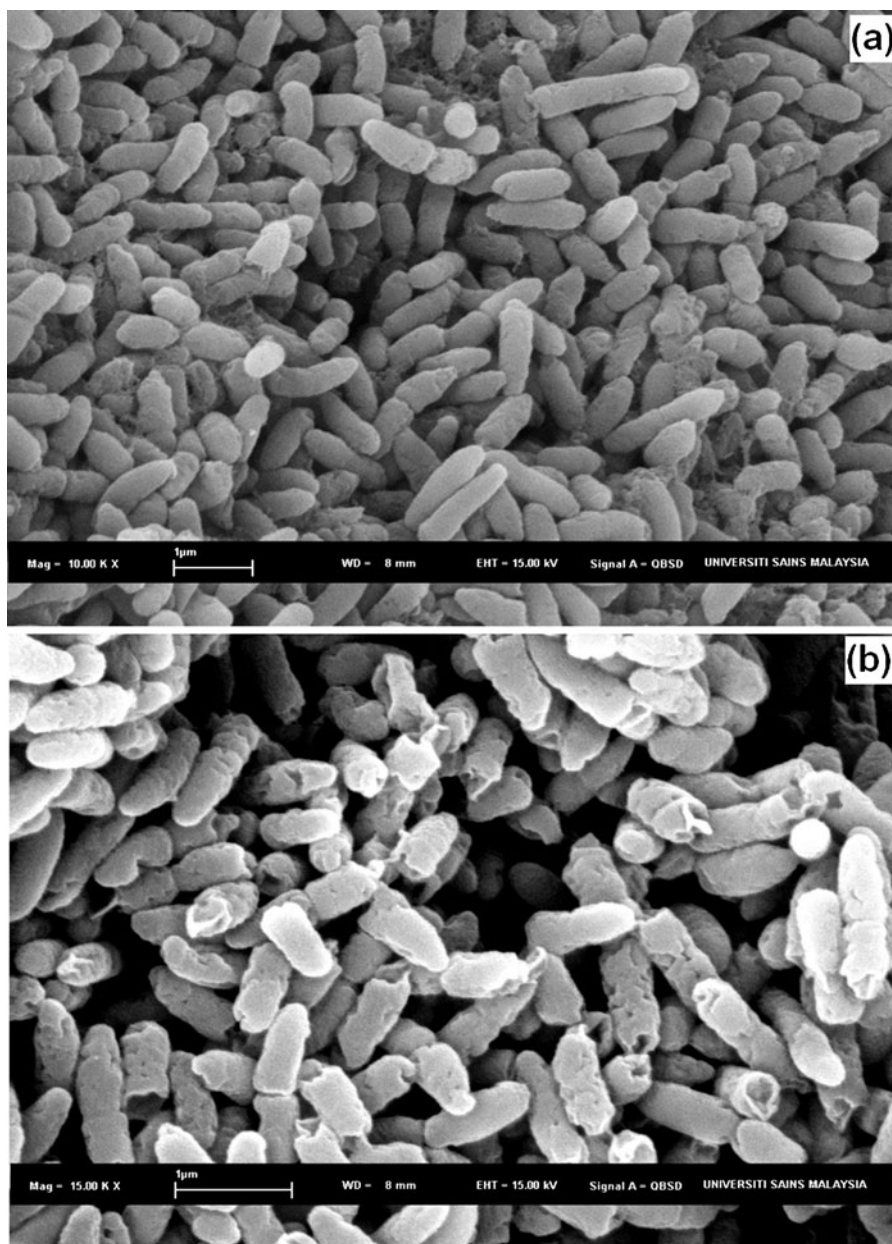


Fig. 11 SEM micrographs of *P. aeruginosa* before (a) and after (b) exposure to Direct red 80

to concomitant biodegradation reactions of the products formed during the reductive dye degradation as this new band disappeared during the oxic stage.

^1H NMR spectroscopy with solvent peak suppression was used for direct analysis of the reaction mixture. The ^1H NMR data showed a good correlation with UV–visible spectrophotometry data for Direct red 80 decolorization and the TOC data for degradation of aromatic amine intermediates during aerobiosis. In control samples, the spectrum showed signals between 3.0 and 4.0 ppm corresponding to the protons of the aromatic rings. However, the spectrum peaks became attenuated after treatment with the isolate under microaerophilic conditions with many of the major peaks resolved (Fig. 10) indicating the biodegradation of the tertiary amine. For the oxic phase-treated sample, the ^1H NMR spectrum showed signal attenuation in the aromatic region (3.0–4.0 ppm) suggesting complete loss of aromaticity from Direct red 80 as a result of its mineralization by *P. aeruginosa*. In the same spectrum new signals appeared in the high field region (between 0.5 and 2.0 ppm) indicating the formation of hydrocarbon aliphatic compounds.

Attempts at mineralization of azo dyes were made in the past using a consortium of bacteria. Treatment systems composed of mixed microbial populations were used to achieve biodegradation and mineralization of synthetic azo dyes due to the synergistic metabolic activities of the microbial community (Saratale et al. 2011a; Tony et al. 2009). In a microbial consortium, the individual strains may attack the dye molecule at different positions or may utilize metabolites produced by the co-existing strains for further decomposition (Jadhav et al. 2008; Saratale et al. 2009c). It was believed that individual bacterial strains usually cannot degrade azo dyes completely, leaving the intermediate products which are often carcinogenic aromatic amines, and need to be further decomposed (Joshi et al. 2008). However, this study has demonstrated the capability of a monoculture of *P. aeruginosa* to effectively decolorize Direct red 80 and mineralize the resulting metabolic intermediates arising from dye cleavage in a sequential microaerophilic/oxic process. This use of a pure culture system ensures reproducible data, and thus interpretation of experimental observations becomes easier. It is also possible to regulate the enzyme system of pure isolates of dye degrading bacteria to produce modified strains with enhanced enzyme activities using the tools of biochemistry and molecular biology (Saratale et al. 2011b).

3.3 Scanning Electron Microscopy

Scanning electron microscopy was performed to evaluate the changes in cellular morphology due to exposure of the bacterial cells to the dye for 72 h during degradation. Figure 11 displays the photomicrograph of the cells before and after exposure to Direct red 80. The untreated *P. aeruginosa* cells, prepared for SEM in dye-free medium, were about 1.2 μm long and displayed a smooth and intact surface.

However, incubation of the cells with the dye resulted in clear alterations of the cell surface structure, e.g., change from smooth to rough, decreased surface stiffness and formation of micelles, which also points to the destabilization of the peptidoglycan layer by the dye. The surface of the cells looked corrugated with some cells showing some dimples but their average length remained unaltered (Fig. 11). Hence, it may be hypothesized that the dye or their metabolic intermediates exerted some form of stress on the bacterial cells despite their ability to mineralize the dyes eventually. This observation indicates the resilience of the cells to shock loadings and its ability to perform as an effective decolorizer despite obvious stress signals attributable to the toxicity of the dye or its degradation intermediates.

3.4 Biotoxicity Assay

The toxicity of the dye and metabolic intermediates was assessed to determine the toxicity profile of the dye solution following the different phases of biological treatment. The relative sensitivity of the brine shrimps (determined as LC_{50}) to the dye and degradation

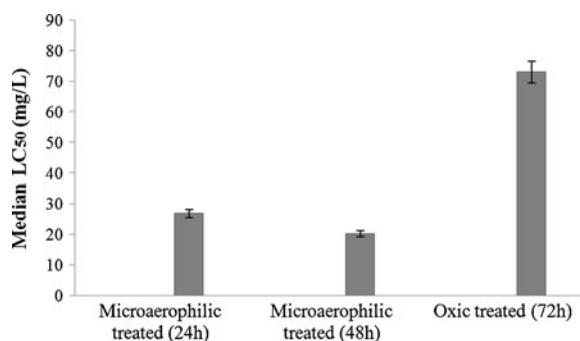


Fig. 12 Concentration of Direct red 80 and degradation metabolites needed to kill 50 % of test shrimp (LC_{50} values) before and after sequential microaerophilic-oxic treatment of dye solution using *P. aeruginosa*

metabolites was studied and results obtained presented in Fig. 12.

The LC₅₀ data indicated an increase in toxicity of the solution after 24 and 48 h of microaerophilic phase treatment in proportionality to color removal. However, a decrease in toxicity of the dye solution was obtained after treatment by *P. aeruginosa* under oxic conditions. The increase in toxicity after treatment under microaerophilic conditions may be attributed to the generation of aromatic amines. The first step in the biodegradation of azo dyes is cleavage of the azo bond, resulting in decolorization and the formation of aromatic amines. These aromatic amines are intermediates used in azo dye synthesis and appear only after their reduction. Most of these aromatic amines are known environmental contaminants because of their complicated construction, different varieties, higher chemical stability, and poor biodegradation (Khan and Banerjee 2010), and their presence in industrial wastewater creates serious environmental problem (Hildenbrand et al. 1999). These amines are more hydrophobic and thus, may easily cross the cell membranes, consequently being more toxic than the original dyes. This may explain the higher toxicity (low LC₅₀) obtained for the microaerophilic phase-treated dye solution. These results thus indicate that the elimination of color from a dye solution does not always translate into reduction in toxicity of the solution. An oxic phase treatment is thus, required to eliminate these toxic aromatic amines to reduce the harm to the environment when such wastewaters are discharged into water bodies.

4 Conclusions

The study investigated the ability of a monoculture of *P. aeruginosa* to decolorize and mineralize azo dyes during a sequential microaerophilic-oxic process. This strain decolorized the azo dye (Direct red 80) during the microaerophilic phase treatment with mineralization of the generated intermediates obtained during the oxic treatment process. The extent of decolorization rate was strongly affected by the presence of oxygen in medium, whereas, oxic conditions were required for the degradation of the generated aromatic amine intermediates. The percentage decolorization of Direct red 80 by *P. aeruginosa* under microaerophilic conditions was 84.6 % in 48 h while the extent of mineralisation during the oxic process in the subsequent 24 h was

70.91 %. UV–visible spectrophotometry was used to determine the extent of decolorization of the dye while HPLC, FT-IR, and NMR analytical techniques were used to prove the mineralization of the dyes. Toxicity bioassay using the BSLT clearly indicated inhibitory characteristics of intermediate metabolites after microaerophilic treatment suggesting increased toxicity upon azo dye decolorization under these conditions. However, further treatment of the solution under oxic conditions by *P. aeruginosa* led to decreased toxicity of the dye solution. This study has demonstrated the ability of a monoculture of *P. aeruginosa* to decolorize, mineralize and detoxify azo dye in a sequential microaerophilic-oxic process which gives it an added advantage for treatment of textile industry wastewater. The potential of this culture for application in treatment of real dye bearing wastewaters using appropriate bioreactors is currently being investigated in our laboratory.

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