GLOBAL JOURNAL OF ENVIRONMENTAL SCIENCES VOL. 5, NO. 1 , 2006: 19 - 25
 ISSN 1596 – 6194

INFLUENCE OF CHEMICAL STRUCTURES ON BIODEGRADATION OF AZO DYES BY *PSEUDOMONAS SP*

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(Received 10 August, 2005; Revision Accepted 22 November, 2005)

ABSTRACT

The influence of chemical structure on biodegradation of azo dyes- Mordant Black 17, Mordant Black 11, Direct Red 2 and Direct Red 28 by *Pseudomonas* sp. was investigated. Biodegradation indices monitored were decolourization, residual dye concentration, population density (TVC) and mineralization (CO_2 evolution). Decolourization was accompanied by concomitant decrease in residual dye concentration and increase in population of bacterial cells. Percentage decolourization was higher in media which contained monoazo (single azo bond) dyes compared to the media which contained diazo (double azo bonds) dyes. The results were attributed to the number of azo bonds and the electronic characters of the substituent groups. Oxidative catabolism of the dyes was inhibited by electron-withdrawing substituents (azo-, nitro-, and sulphonate) while, electron-donating substituents (methyl, amino and hydroxyl) enhanced oxidative catabolism. Biodegradation of the dyes was influenced by the aromatic substitution pattern.

Key words: azo dyes, biodegradation, *Pseudomonas* sp., influence of chemical structure

INTRODUCTION

Major classes of synthetic dyes include azo, anthraquinone and triarylmethane dyes (Padmavathy *et al.*, 2003) and many of these dyes and/or their intermediate metabolic by-products are potentially mutagenic, toxic and carcinogenic (NIOSH, 1980, Houk *et al.*, 1991, Levine 1991, Rafii and Cerniglia, 1995, Oranusi *et al.*, 2002, Méndez-Paz *et al.*, 2005). With increased use of a wide variety of dyes, pollution by dye wastewater is becoming a global environmental issue (Moreira *et al.*, 2004). In the textile industries, up to 50% of dyes are lost in effluents (Moreira *et al.*, 2004).

Dyes must have a high degree of chemical and photolytic stability (Brown and Anliker, 1988) and stability against microbial attack (Pagga and Brown, 1986). However, the more resistant a dye is to these agents, the more difficult it is to remove colour from wastewaters and to biodegradation (Stolz, 2001, Méndez-Paz *et al.*, 2005). Various physico-chemical technologies (adsorption, coagulation-flocculation advanced ozone oxidation, ionizing radiation, advanced chlorination and ultra filtration) are used in dye wastewater treatment (Chen *et al.*, 1999, Méndez-Paz *et al.*, 2005). These technologies are expensive and commercially unattractive (Arslan *et al.*, 2000) and merely transfer the pollutants from one phase to another. For example, chlorination has the disadvantage of producing organochloride by-products (Sarasa *et al.*, 1998).

There is an increasing global trend towards biological processes for treatment of dye wastewater. Biological processes offer low cost, low technology, an efficient alternative for simultaneous colour and organic matter removal (Méndez-Paz *et al.*, 2005)

Azo dyes are characterized by the presence of one or more azo groups (-N=N-) and are the largest class of synthetic dyes with greatest variety of colours (Chen *et al.*, 1999). Currently between 2000-3000 different types of azo dyes are used to dye various materials such as textiles, paper, cosmetics, food, leather and pharmaceuticals (Chen *et al.*, 1999, Stolz, 2001) which implies their wide occurrence in dye wastewater.

Azo dyes are recalcitrant to biodegradation (Shaul *et al.*, 1991, Chen *et al.*, 1999) and because of their toxic, carcinogenic and mutagenic potentials they are second to polymers in terms of new compounds submitted for registration in the U.S.A. under the Toxic Substances Control Act (Brown and DeVito, 1993).

However, microbial degradation of azo dyes under anaerobic conditions has been reported (Délee *et al.*, 1998, Bromby-Challenor *et al.*, 2000, Van der Zee *et al.*, 2001). The major setback of azo dye reduction under anaerobic conditions is the generation of potentially toxic aromatic amines which are not degraded further under these conditions (Stolz, 2001, Méndez-Paz *et al.*, 2005). Bacterial utilization of azo dyes as sole source of carbon and energy under aerobic conditions has been reported (Yatome *et al.*, 1993, Dykes *et al.*, 1994, Oranusi and Ogugbue 2001, Oranusi and Ogugbue, 2003). Under aerobic conditions, the aromatic amines are further metabolized leading to the detoxification of the amines (Brown and Labourer, 1983).

Microbial degradation of aromatic compounds under aerobic conditions is influenced by the presence of substituent groups on the aromatic moiety of the dye molecule (Knackmuss, 1981, Shaul *et al.*, 1991, Field *et al.*, 1995). We had earlier reported the utilization of azo dyes (Orange II and Direct Blue 71) by the *Pseudomonas* sp. used in this study as the sole source of carbon, energy and nitrogen (Oranusi and Ogugbue, 2001, Oranusi and Ogugbue, 2005). Bacterial azoreductases which mediate the reductive cleavage of azo bond in azo dye have been reported to be substrate-specific (Zimmermann *et al.*, 1984, Stolz, 2001).

In this study, we investigated the ability of *Pseudomonas* sp. to degrade azo dyes - Mordant Black 11, Mordant Black 17, Direct Red 2 and Direct Red 28 and the effect of the chemical structure of the dyes on their susceptibility to oxidative catabolism. It is hoped that the results will be of value in the potential use of this bacterium in the treatment of dye wastewaters which contain different azo dyes with varying chemical composition, and in the synthesis of new generation of azo dyes which are more susceptible to aerobic biodegradation while retaining their dyeing potential.

MATERIALS AND METHODS

Organism

Pseudomonas sp. was originally isolated from effluent from Textile Mill, Aba, Nigeria and adapted to growth on azo dyes (Orange II and Direct Blue 71) as sole source of carbon, energy and nitrogen (Oranusi and Ogugbue, 2001, Oranusi and Ogugbue, 2005). Stock cultures were maintained on nutrient agar (Oxoid) slants at 4^0C.

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Dyes

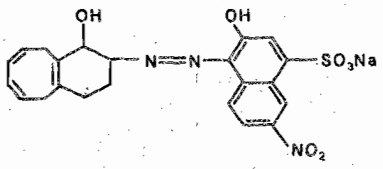
The dye substrates used were Mordant Black 17, Mordant Black 11, Direct Red 2 and Direct Red 28 (Aldrich Chemical Co., U.S.A.). These dyes are commonly used by various dye-based industries in Nigeria. Fig.1 shows the chemical structures of the dyes.

Stock solution of each dye was prepared in deionised water and sterilized by membrane filteration (0.2µm pore size, Acrodisc).

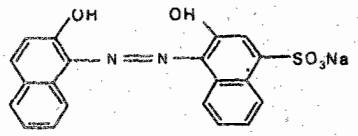
Media

The basal medium contained NaCl 2.0, $MgSO_4.7H_2O$ 0.42, KCl 0.29, K_2HPO_4 1.27, KH_2PO_4 0.85 in gL^{-1}, EDTA 0.5ml and deionised water 1,000ml. The pH of the medium was 7.0. Sterilization was by autoclaving. On cooling, 5ml of appropriate sterile stock solution of each dye was added into each of triplicate set of 250ml Erlenmeyer flask which contained 95ml of medium. Final dye concentration in each flask was 0.02mg ml^{-1}.

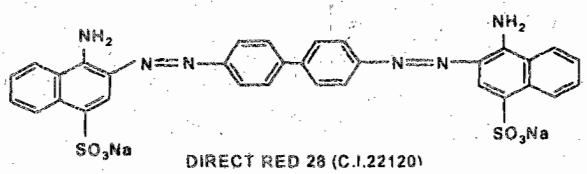
Medium which contained Mordant Black 17 was designated A, medium B contained Mordant Black 11, medium which contained Direct Red 2 was designated C while, medium D contained Direct Red 28.



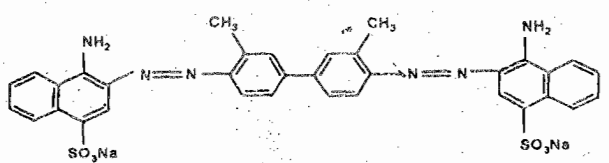
MORDANT BLACK 11 (C.I. 14645)



MORDANT BLACK 17 (C.I.15705)



DIRECT RED 28 (C.I.22120)



DIRECT RED 2 (C.I.23500)

FIG. 1 STRUCTURES OF AZO DYES USED (Adapted from Aldrich Catalogue, U.S.A.)

Standard inoculum

Cells from stock cultures were inoculated into 20ml of nutrient broth (Oxoid) contained in 250ml Erlenmeyer flask. Cultures were incubated at 28±2^{0}C for 24h with shaking at 150 r.p.m.

Culture conditions

Ten milliliters of the standard inoculum was inoculated into each of triplicate set of 250ml Erlenmeyer flasks which contained 100ml of appropriate medium (A,B,C, or D). Final cell density (ca. 8.00 x 10^6 CFU ml^{-1}). Controls for each medium type consisted of triplicate set of uninoculated flasks.

Cultures were incubated at 28±2^{0}C with shaking (200 r.p.m.). Samples were withdrawn at zero times and at specific time intervals for biodegradation assays (decolourisation, residual dye concentration and total viable count).

Biodegradation assays

Decolourisation

At zero time and at 12h intervals, samples (5ml) were aseptically withdrawn from each inoculated and control flasks and centrifuged. Centrifugation was at 6,000 r.p.m. in a bench centrifuge (Baird and Tatlock, England) for 30min. The resulting supernatant was carefully withdrawn and designated as follows: SA, SB, SC or SD for supernatants from medium A, B, C or D respectively.

The optical density (OD) of the supernatants was determined spectrophotometrically with spectrophotometer 6110 (Jenway, United Kingdom) at λ_{max} for each dye (Mordant Black II λ_{max} 503 nm; Mordant Black 17 λ_{max} 569nm; Direct Red 2 λ_{max} 500nm and Direct Red 28 λ_{max} 497nm). Percentage decolourisation was calculated:

$$\% \text{ Decolourisation} = \frac{OD_{initial} - ODf_{inal}}{OD_{initial}} \times \frac{100}{1}$$

where $OD_{initial}$ = optical density of culture supernatant immediately after inoculation,

OD_{final} = optical density of culture supernatant at each sampling

The cell pellets from each of the appropriate medium were suspended in physiological saline and centrifuged as above. The resulting supernatants were designated SAI, SBI, SCI or SDI for supernatant from A, B, C or D medium respectively. The OD of the supernatants was determined as for sample supernatant.

Residual Dye Concentration

The residual dye concentration in each of the above supernatants (sample and controls) was determined from a calibration curve of optical density against various concentrations for each dye.

Total Viable Count

One milliliter of culture broth was aseptically withdrawn at zero time and every 12h from each of the inoculated flasks. Samples were diluted by ten-fold serial dilution (10^{-1} – 10^{-5}) in physiological saline. Appropriate dilutions were plated out onto duplicate set of nutrient agar (Oxoid) plates by the pour plate method. Plates were incubated at 28 ± 2°C for 24 – 48h and the number of colonies which developed was counted. Results are expressed as colony – forming-units per ml (CFU ml^{-1}).

Mineralization

Mineralization experiment for each dye consisted of triplicate set of 150ml Biometer flasks with side arm (Mueller *et al.*, 1992). Each sidearm contained 20ml sterile 0.01M NaOH solution to trap evolved carbon IV oxide (CO_2). Into each flask was added 100ml of appropriate medium (A, B, C or D) and flushed with 100% sterile oxygen (O_2). Each flask was then inoculated with 10ml of standard inoculum (ca. 8.00 x 10^6 CFU ml^{-1}) and flushed with sterile O_2.

Controls consisted of duplicate set of uninoculated flasks. Incubation was at 28±2°C with shaking for 48h. Flasks were flushed with sterile O_2 for 5sec every 12h during incubation. Evolved CO_2 was determined volumetrically with 0.01M HC1 as titrant and phenolphthalein as indicator. Evolved CO_2 was calculated following the method outlined in APHA (1992).

RESULTS

The time course for changes in optical density (OD) of culture medium (decolourisation), residual dye concentration (RDC) and growth profile (total viable count, tvc) when *Pseudomonas* sp. was cultured on the various dye substrates as sole sources of carbon, energy and nitrogen are depicted in Figures 2 - 5. All four dyes were decolourised, though to varying extent as evidenced by decrease in OD of the culture supernatant. Decrease in OD (increase in decolouration) was accompanied by concomitant decrease in RDC and increase in tvc.

In medium A (Fig. 2) the optical density decreased from an initial value of 0.099 to a final value of 0.009 (90.91% decolourisation). Dye concentration at zero time was 20.00mgL^{-1} and 1.75 mgL^{-1} at 48h of incubation. Total viable count increased from an initial value of 8.05 x 10^6 CFU ml^{-1} to 6.00 x 10^8 CFU ml^{-1} after incubation.

In medium B (Fig. 3) the results obtained for the various indices were as follows: optical density decreased from 0.244 at zero time to 0.031 at 48h (86.16% decolourisation). Dye concentration decreased from an initial value of 20.00mgL^{-1} to a final value of 2.74mg L^{-1}. The population density at time zero was 8.20 x 10^6 CFU ml^{-1} while, the final population density was 4.35 x 10^8 CFU ml^{-1} after incubation.

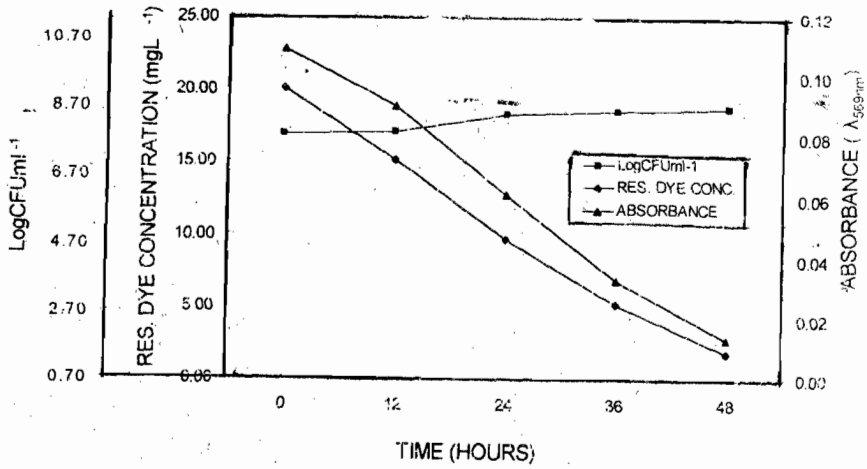


FIG. 2 GROWTH PROFILE OF *Pseudomonas* sp. WHEN CULTURED ON MORDANT-BLACK 17 AS SOLE SOURCE OF CARBON, NITROGEN AND ENERGY.

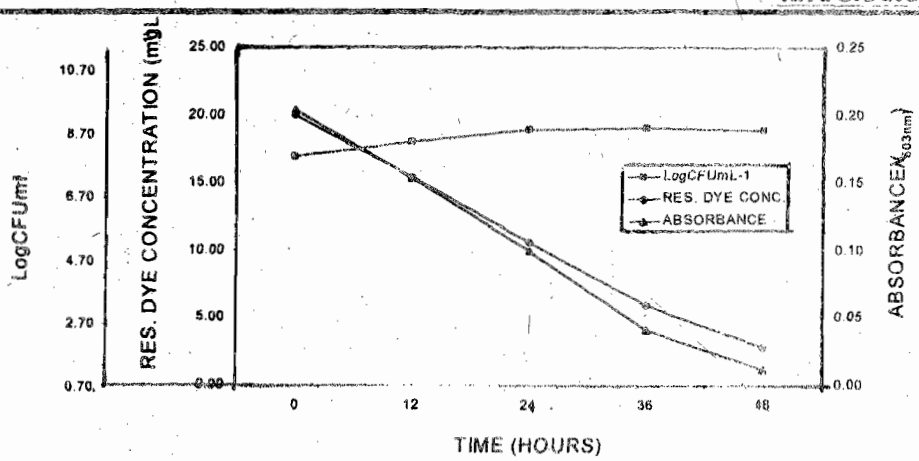


FIG. 3 GROWTH PROFILE OF *Pseudomonas* sp. WHEN CULTURED ON MORDANT BLACK 11 AS SOLE SOURCE OF CARBON, NITROGEN AND ENERGY.

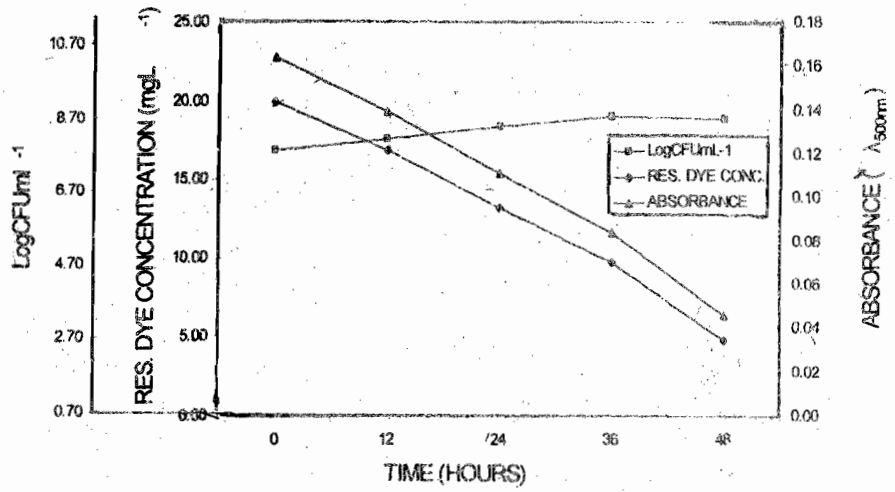


FIG. 4 GROWTH PROFILE OF *Pseudomonas* sp. WHEN CULTURED ON DIRECT RED 2 AS SOLE SOURCE OF CARBON, NITROGEN AND ENERGY.

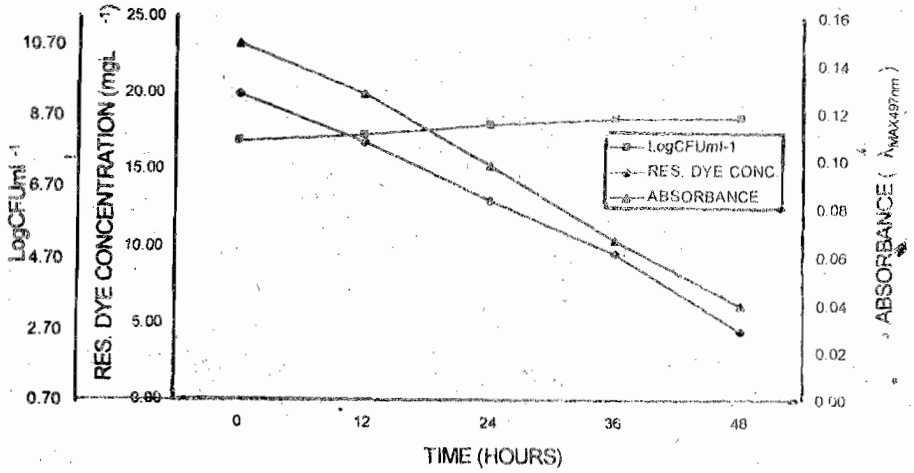


FIG. 5 GROWTH PROFILE OF *Pseudomonas* sp. WHEN CULTURED ON DIRECT RED 28 AS SOLE SOURCE OFCARBON, NITROGEN AND ENERGY.

When cultured in medium C (Fig. 4), the OD decreased from an initial value of 0.189 to 0.042 (77.78% decolourisation). Initial dye concentration was 20.00 mgL^{-1} while, the final concentration was 4.5[illegible] mgL^{-1}. The biomass increased from 8.00 x 10^6 CFU ml^{-1} at zero time to 3.05 x 10^8 CFU ml^{-1} at 48h of incubation.

The population density increased from an initial value of 7.80 x 10^6 CFU ml^{-1} to 7.55 x 10^8 CFU ml^{-1} at 48h incubation in medium D (Fig. 5). The OD decreased from 0.164 at zero time to 0.046 (71.95% decolourisation). The initial dye concentration was 20.00mgL^{-1} which decreased to 4.81mgL^{-1} after incubation.

There was neither change in optical density (no decolourisation) of the medium nor change on concentration of the dye substrates in all the control flasks. The optical density of the supernatant (SA1, SBI, SCI, and SDI) of the cell pellets was negligible (< 0.0001).

Figure 6 shows the data obtained on the quantity of evolved carbon IV oxide (CO_2). All four dyes were mineralized although to varying degrees.

The highest value of 8.24±0.68 was obtained in medium A which contained Mordant Black 17. The data obtained for the other dyes substrates were as follows: 8.03±0.55 (Mordant Black II); 6.00±0.32 (Direct Red 2) and 5.78±0.21 (Direct Red 28). Carbon dioxide was not detected in the control flasks

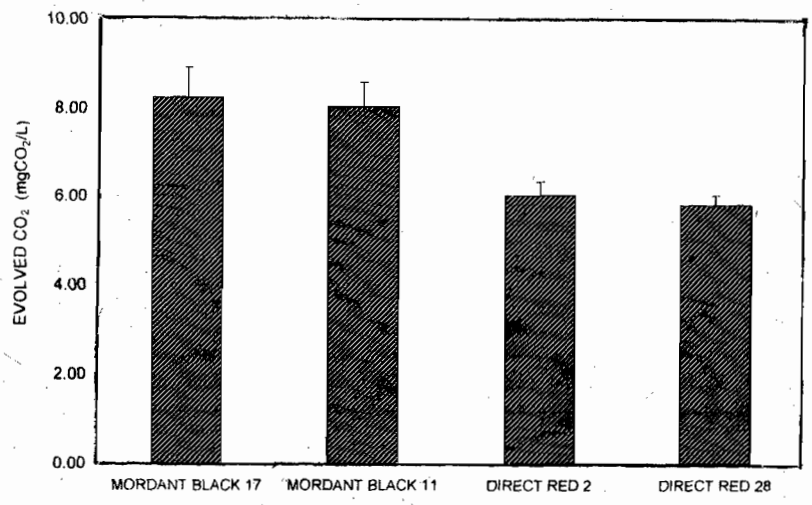


FIG. 6 CARBON-DIOXIDE (CO_2) EVOLVED WHEN *Pseudomonas* sp. WAS CULTURED ON THE AZO DYES AS SOLE SOURCE OF CARBON, NITROGEN AND ENERGY.

DISCUSSION

In this study, we considered azo dyes (Fig. 1) with different substituent groups. The results obtained on the various indices (Figs. 2 - 6) may be attributed to the electronic character of the substituent groups on the aromatic moiety of the dyes and the number of azo bonds. Oxidative catabolism of aromatic compounds is influenced by the substituent groups on the aromatic ring (Field *et al.*, 1995). Electron withdrawing substituent groups generate electron-deficiency which stabilize the aromatic ring thereby, inhibiting aerobic degradation while, electron-donating substituents destabilize the ring (electron-rich) which enhances oxidative degradation (Knackmuss, 1981). Decolourization (decrease in optical density of the culture media) is the result of the cleavage of the azo bond (chromophore group) which generates colourless amines (Ganesh *et al.*, 1994). Decolourization was also accompanied by concomitant decrease in residual dye concentration. The results confirmed that the dye substrates were not only transformed but were also degraded. Percentage decolourization was higher with monoazo dyes (90.91% Mordant Black 17, 86.16% Mordant Black 11) compared to diazo dyes (77.78% Direct Red 2, 71.95% Direct Red 28). The rate of cleavage of the single azo bond in monoazo dyes was faster than for the double bond in diazo dyes.

Microbial decolourization of azo dyes has been reported; *Pseudomonas* sp. (Oranusi and Ogugbue, 2001); *Cellulomonas* sp. (Oranusi and Ogugbue, 2003); *Proteus mirabilis* (Chen *et al.*, 1999) and *Trametes versicolor* (Moreira *et al.*, 2004)

There was neither decolourization nor change in the concentration of the dyes in all the control flasks. The results confirm that decolourization and decrease in concentration of the dyes in the inoculated flasks were due to metabolic activities of the organisms.

Dye molecules adsorbed to cell surfaces resulting in decolourization (Pagga and Brown, 1986, Yuxin and Jiang, 1998, Chen *et al.*, 1999). The optical density of the supernatant (SAI, SBI, SCI, SDI) was negligible (<0.0001). This confirms that decolourization and decrease in residual dye concentration (Figs. 2 - 5) was not due to adsorption.

The sole source of carbon and energy in all the media was the appropriate dye substrate. Ring opening of the aromatic moiety of the dye substrates provided the sole source of carbon and energy. The increase in total viable count and evolved carbon-dioxide (Figs. 2 - 5) and (Fig. 6) respectively, confirm that there was ring opening of the dye substrates. Bacterial utilization of azo dyes as sole source of carbon and energy has been reported (Yatome *et al.*, 1993, Dykes *et al.*, 1994, Coughlin *et al.*, 1999, Oranusi and Ogugbue, 2005).

The differences in data obtained on total viable counts (Figs. 2 - 5) and evolved carbon-dioxide (Fig. 6) may be attributed to the influence of the substituent groups. The electron-withdrawing groups common to the monoazo dyes are the azo and sulphonate groups, while the electron donating substituents are the hydroxyl groups (Fig. 1). In addition, Mordant Black 11 has a nitro group which is a strongly electron-withdrawing group. The electron withdrawing character of the nitro group enhances the stability of Mordant Black 11 which reduces its susceptibility to oxidative catabolism. The electron withdrawing substituents common to the diazo dyes are the azo and sulphonate groups while the common electron donating groups are the amino group (Fig. 1), In addition, Direct Red 2 has two methyl substituent groups (electron donating). The additional destabilizing effect of the methyl group enhances the degradability compared to Direct Red 28 (Figs. 2 - 6). Phenolic azo dyes with electron donating substituent groups (methyl or methoxy) were degraded by *Pyricularia oryzae* while unsubstituted phenolic dyes were not degraded (Chivukula and Renganathan, 1995)

Haug *et al.* (1991) reported that aromatic compounds with electron withdrawing sulphonate groups were resistant to biodegradation or are incompletely degraded.

This study has demonstrated that (i) the azo dyes were utilized as sole sources of carbon and energy under aerobic conditions by *Pseudomonas* sp. This organism either alone or under consortium cultivation has a potential in bioprocess treatment of dye wastewaters which contain a variety of azo dyes. (ii) Susceptibility to biodegradation was dependent on the substituent groups. Electron –withdrawing groups enhanced the stability of the aromatic ring which reduced susceptibility while, electron-donating substituents destabilized the aromatic ring resulting in enhanced susceptibility to biodegradation. The results may be of value in the synthesis of a new generation of azo dyes which are less recalcitrant to biodegradation while, retaining their dyeing potential. Research is continuing on degradation of other azo dyes with different aromatic substituent patterns by this bacterium.

ACKNOWLEDGEMENT

We are grateful for financial support from Elder G.O.Ogugbue to C.J. Ogugbue and Zitadel Laboratories Port Harcourt, Nigeria for technical assistance.

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