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Mechanism of Navitan Fast Blue S5R degradation by *Pseudomonas aeruginosa*

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Abstract

The mechanism by which *Pseudomonas aeruginosa* degraded Navitan Fast Blue S5R was studied using TLC, FTIR, HPLC and GC–MS analysis. Degradation started with the reduction of azo bonds producing metanilic acid and peri acid whose presence was confirmed by HPLC. Aniline, the desulfonated product from metanilic acid and salicylic acid could also be detected by HPLC. GC–MS analysis of the degradation products confirmed the presence of aniline and revealed the presence of β-ketoadipic acid. Based on these products a probable pathway has been proposed for the degradation of Navitan Fast Blue S5R by *Pseudomonas aeruginosa*.

Keywords: Azo dye; Pathway; Aromatic amines; Salicylic acid; β-Ketoadipic acid

1. Introduction

Modern dyes are required to have high degree of chemical and photolytic stability in order that they maintain their structure and color. They are designed to resist breakdown attributable to time and exposure to sunlight, water, soap and other parameters such as bleach and perspiration. The color fastness, stability and resistance of dyes to degradation have made color removal from waste waters difficult as they are not readily degraded under aerobic conditions (O'Neill et al., 1999).

The sulfonic acid groups that are introduced to increase the water solubility of the dye and the azo group (chromophoric group) confer resistance to microbial attack, making them recalcitrant to oxidative decolorization (Kulla et al., 1983; Coughlin et al., 1999). Biodegradation of azo dyes was carried out by a variety of microorganisms including anaerobic and aerobic bacteria (Yatome et al., 1981; Chung and Kuo, 2000), fungi (Ollikka et al., 1993), actinomycetes (Burk and Crawford, 1998) and algae (Acuner and Dilek, 2003). While degradation by actinomycetes and fungi is an oxidative activation of dyes resulting in the formation of cationic species, making the molecule vulnerable to the nucleophilic attack of water (Goszczynski et al., 1994), bacterial degradation of azo dye is often initiated under anaerobic conditions by an enzymic biotransformation step that involves the cleavage of azo linkages with the aid of azoreductase utilizing reduced coenzyme as electron donor (Zimmermann et al., 1982). Under aerobic conditions, azo dyes have been considered practically nondegradable by bacteria (Michaels and Lewis, 1986). However, Kulla (1981) reported the aerobic degradation of certain sulfonated azo dyes by *Pseudomonas* strains. Despite the presence of oxygen the initial degradation appeared to be the reduction of the azo linkage by an oxygen insensitive azo reductase.

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It has been reported that complete mineralization of dyes is possible only if anaerobic reduction is followed by aerobic oxidation of the amines formed in the reductive steps (Tan et al., 1999; Rajaguru et al., 2000). Under anaerobic conditions, a number of bacteria are known to catalyse the reductive cleavage of the azo bond, which leads to the formation of aromatic amines. These amines are well known for their mutagenic and carcinogenic potential (Chung et al., 1981; Chung, 1983). Certain azo dyes, which are known to form carcinogenic amines on reductive cleavage of the azo bonds, have already been banned (Reife et al., 1998).

Hence, an attempt has been made to study the aerobic degradation of Navitan Fast Blue S5R, a commercial diazo dye used in leather and textile industries by *Pseudomonas aeruginosa* and to identify the degradation products by various spectroscopic and chromatographic methods so as to enable us to elucidate a possible mechanism or pathway by which the dye has been degraded. To our knowledge, this is the first report on the mechanism by which this commercially important dye is degraded by *P. aeruginosa*.

2. Materials and methods

Biodegradation studies were carried out in 3 l Hoffkin's flasks containing 1 l of the culture medium with 100 mg l^{-1} Navitan Fast Blue S5R. The flasks were inoculated with 2% inoculum containing approximately 2×10^8 cells per ml of *P. aeruginosa*. The composition of the culture medium and culture conditions were described elsewhere (Valli Nachiyar and Suseela Rajakumar, 2003). The culture medium containing degradation products of Navitan Fast Blue S5R was drawn at regular intervals, centrifuged at 10 000 rpm for 15 min. The supernatants were analyzed by HPLC (Valli Nachiyar and Suseela Rajakumar, 2003) and the lyophilized supernatants were analyzed by thin layer chromatography (TLC) and FTIR. Portion of the supernatant was extracted thrice with equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and then the solvent was evaporated in a rotary evaporator. The ethyl acetate extract was subjected to HPLC and GC–MS analyses.

GC–MS analysis of the ethyl acetate extract was performed by using Perkin Elmer Autosystem XL-GC with Turbomass MS spectrometer. The column used was PE5 Elite Series 30 m × 0.250 i.d. with the film thickness of 1 μm and the column oven was programmed between 120 and 260 °C at the rate of 10 °C per minute with the injection temperature of 280 °C. Mass spectra were recorded under scan mode in the range of 40–400 amu.

3. Results and discussion

TLC analysis of the lyophilized culture medium containing the degradation products of Navitan Fast Blue S5R on fluorescent silica plates (polygram sil G/UV, Germany) using the solvent system chloroform:ethanol in 9:1 ratio revealed a spot with the R_f value of 0.12 in the sample incubated for 48 h. Analysis of this spot by FTIR spectroscopy after extraction with chloroform showed peaks corresponding to C–H stretch at 2917 and 2841 cm^{-1} and C=O stretch at 1727 cm^{-1} characteristic of aliphatic carboxylic acid.

HPLC analysis using C-18 reverse phase column equipped with UV–Visible spectrophotometer was used to identify the degradation products of Navitan Fast Blue S5R in the culture supernatant. We have reported the presence as well as the amount of metanilic acid produced during degradation of Navitan Fast Blue S5R in our previous report (Valli Nachiyar and Suseela Rajakumar, 2003). The compound having the peak at 3.4 min reported earlier was found out to be salicylic acid. Nearly 22.8 mg l^{-1} of salicylic acid could be detected when 100 mg l^{-1} of Navitan Fast Blue S5R was degraded after 24 h of incubation under shake culture condition, which disappeared after 48 h of incubation. Peri acid was detected with the R_T value of 2.3 with the UV detector set at 265 nm. The concentrations of peri acid were 24.7 and 32.7 mg l^{-1} after 24 and 48 h of incubation respectively and disappeared completely after 72 h of incubation.

HPLC analysis carried out on the ethyl acetate extract of the culture medium containing the degradation products of Navitan Fast Blue S5R (100 mg l^{-1}) after 48 h revealed the presence of aniline with the R_T value of 10.5.

Mass spectra corresponding to the peak with R_T value of 3.89 indicates a fragmentation pattern with m/z 93, 92, 91 and 65 characteristic of aniline (Fig. 1a and b). The presence of aniline was confirmed by injecting authentic aniline as standard. Even though, the molecular ion (m/z 160) for β-ketoadipic acid was not seen in the mass spectra, presence of fragments with m/z 101 and 115, strongly suggested its presence indicating alpha cleavage of the molecular ion (Fig. 1c). The strong m/z 43 signal in the obtained spectrum is characteristic of a keto group and, in conjunction with other detected fragments provides strong evidence for the presence of keto group (Silverstein et al., 1974). The low R_T value for β-ketoadipic acid may be explained in two ways. It may be acetylated during extraction with ethylacetate or it may be acetylated by the enzymes of bacteria (Narro et al., 1992).

Quantification of CO_2 (Abubacker et al., 2001) and NH_3 (Jefferey et al., 1989) produced during degradation showed that nearly 74% of carbon in the dye and 68% of nitrogen in the dye was converted to CO_2 and NH_3 respectively.

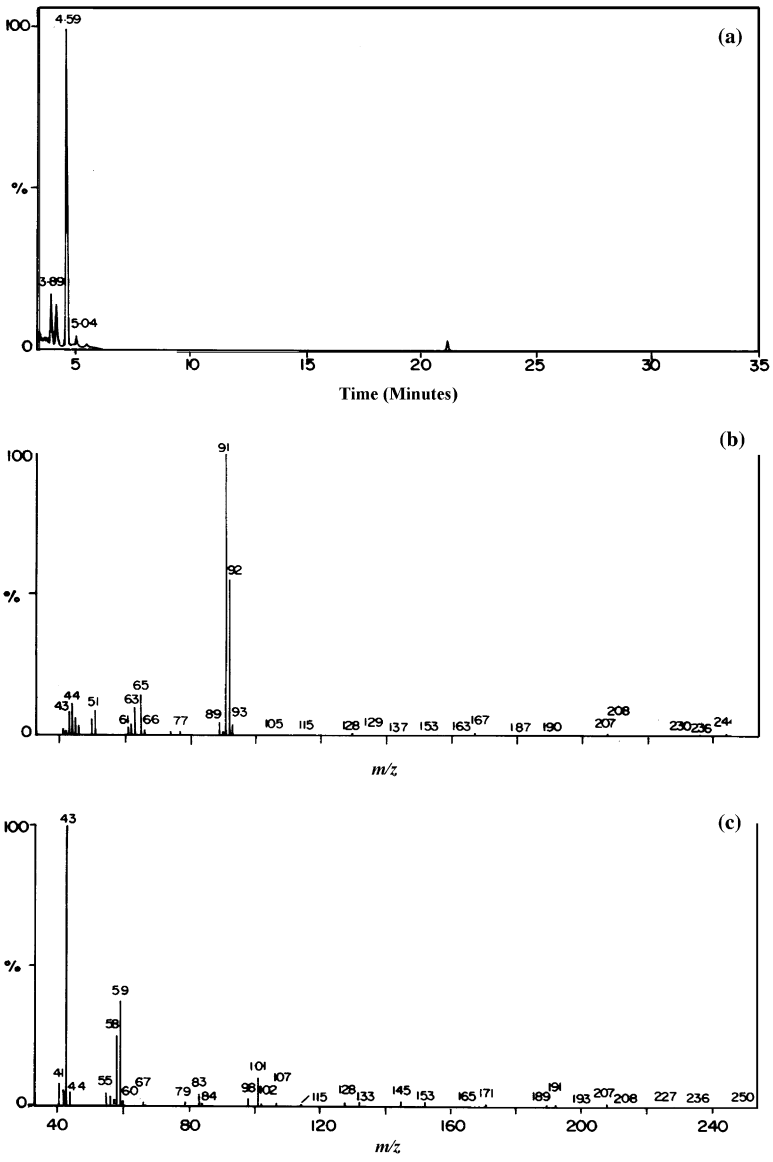

Fig. 1. GC–MS analysis of an ethylacetate extract of the culture filtrate containing the degradation products of Navitan Fast Blue S5R degraded by *P. aeruginosa* after 48 h incubation. (a) Gas chromatogram; (b) mass spectra for the peak with R_T value of 3.89 min; (c) mass spectra for the peak with R_T value of 4.59 min.

This investigation provides strong evidence that the initial step in the degradation of Navitan Fast Blue S5R by *P. aeruginosa* was cleavage of azo bond and corroborates with previous reports on bacterial degradation of azo dyes (Haug et al., 1991; Blümel et al., 1998). The presence of salicylic acid shows that the naphthalene part of the dye must have undergone degradation. It has been reported that salicylic acid was identified as an intermediate compound from microbial degradation of naphthalene (Aranha and Brown, 1981).

The evidence for the formation of β-ketoadipic acid as identified from the GC–MS spectra indicates that aniline and salicylic acid formed might have undergone oxidative deamination to catechol using molecular oxygen and oxygenases, which through ortho pathway of reaction involving hydroxylation and decarboxylation reactions resulted in the formation of β-ketoadipic acid. This result corroborates well with the findings of Lyons et al. (1984) who have identified the formation of *cis–cis* muconic acid, β-ketoadipic acid and succinic acid which ultimately enters the TCA cycle resulting in the release of aniline carbon as CO_2. Similarly the salicylic acid formed would have been converted to catechol by decarboxylation followed by hydroxylation

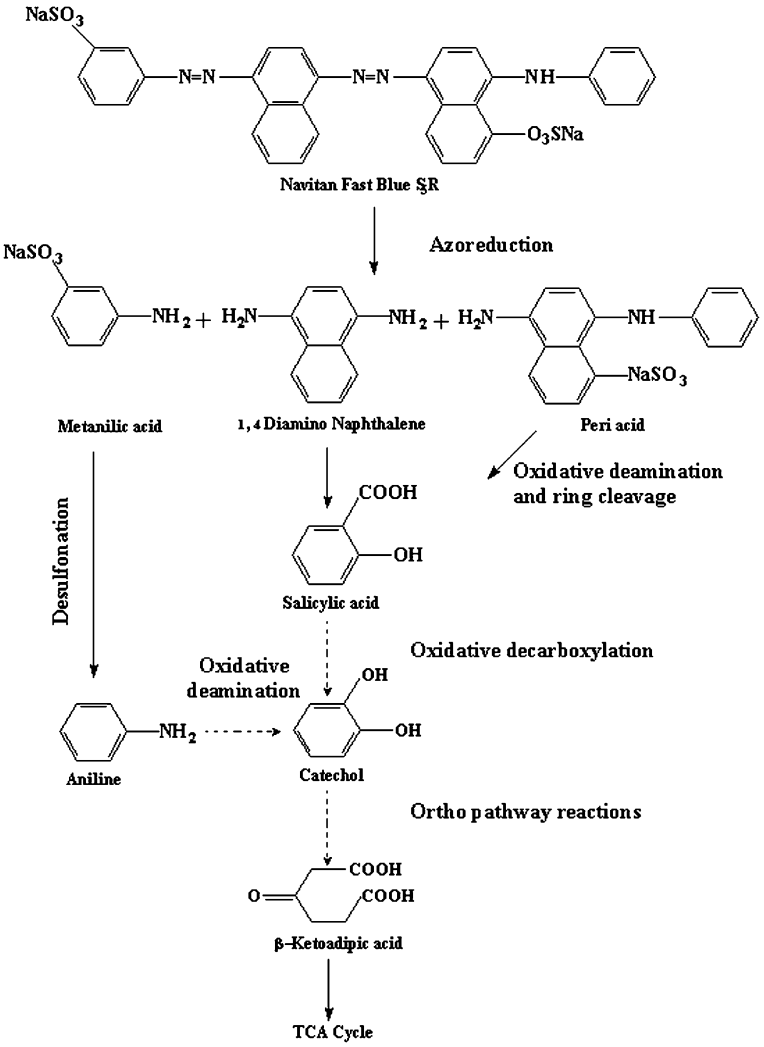

Fig. 2. Proposed pathway for the degradation of Navitan Fast Blue S5R by *P. aeruginosa*.

(Chakrabarthy, 1972). Catechol could not be detected in the GC–MS since this is a difficult intermediate to isolate (You and Bartha, 1982; Lyons et al., 1984) whereas, β-ketoadipic acid identified in the GC–MS indicates that this metabolite might have been formed from metanilic acid or peri acid or diaminonaphthalene or from all the three of them, which finally enters the TCA cycle resulting in the release of CO_2.

Based on the above findings probable mechanism for the degradation of Navitan Fast Blue S5R by *P. aeruginosa* has been proposed, as shown in Fig. 2.

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