Decolorization of Methyl Orange (As a Model Azo Dye) by the Newly Discovered *Bacillus Sp*

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ABSTRACT: *A bacterial strain (strain PS) was isolated from the textile effluents carrying Serilene Black BNFS® (C.I. Disp. Blk. Mix) disperse dye. The isolate was able to decolorize the dye without the need for any exogenous carbon source. Full sequencing of its 16S rRNA indicated that Bacillus sp strain PS is related to Bacillus cereus groups. Silica- gel-thin layer chromatography of Serilene black dye showed that its main component is a blue dye. FT- IR analysis of this blue fraction showed that its structure corresponds to azoic dyes. Thus Bacillus sp, strain PS was used to decolorize methyl orange as a model azo dye, which it did after 2 days of incubation under aerobic conditions on a shaker incubator (30°C, 140 rpm). Comparing TLC and GC-MS analyses with the authentic sample main showed that its decomposition product is N, N-dimethyl 1, 4- phenylene diamine. Experiments with N, N-dimethyl 1, 4-phenylene diamine as a co-substrate in mineral medium showed that this component disappeared after 7 day incubation. These observations confirm that the decomposition of Serilene dye occurs in a manner similar to that of methyl orange.*

KEY WORDS: *Azo dye, Bacillus, Decolorization, Methyl orange, Textile effluent.*

INTRODUCTION

Azo dyes are the largest class of dyes commercially used in the textile industries[1]. Most of these compounds are highly resistant to microbial attack, and are therefore hardly removed from effluents by conventional biological wastewater treatments, such as activated sludge [2]. Not all dyes currently used may be degraded and /or removed with physical and chemical processes and sometimes the degradation products formed are even more toxic [3]. The traditional textile finishing industry consumes about 100 liter of water to process about 1 kg of textile material. The new closed-loop technologies such as the reuse of micro-bially or enzymatically treatment of dyeing effluents could help reducing this enormous water consumption [3]. Enzymes, such as lignin peroxidase, manganes peroxi-dase, and laccase, are involved in lignin degradation, while reductases participate in the decolorization of the azo dyes [4-6].

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Bacterial decolorization under aerobic conditions usually results in adsorption of dyestuffs on bacteria rather than their oxidation [7]. White-rot fungi can degrade a wide variety of recalcitrant compounds by their extra-cellular enzyme systems [8]. However, it is difficult to keep them in functional form in the activated sludge systems, because of their special nutritional and environmental requirements. Moreover, bacterial degradation is much faster than fungal degradation of textile dyestuffs [9].

The disperse dye and dyestuffs are most heavily used and are thus of particular environmental concern because of their potential for formation of toxic aromatic amines and their low rate of degradation during aerobic waste treatment as well as their resistance to chemical oxidation [10]. Serilene Black BNFS® is a disperse black azo dye used for dying of polyester fabrics in textile mills in Iran. Thus, in this study our attention was focused on decolorization and biodegradation of methyl orange (MO) as a model compound for the azo dyes in order to understand the mechanism involved and the specific enzymes that are responsible for the azo dye metabolism by bacteria.

MATERIALS AND METHODS

Material

All chemicals and biochemicals (including culture media) of the highest available purity were obtained from the Merck, GmbH (Darmstadt, Germany) and Sigma, Inc, (St Louis, Mo, USA). The disperse dye (Serilene Black® (SB)) was a gift from the representative of the Yorkshire, Ltd (Sydney, Australia) in Iran. Real dying effluents containing this dye were obtained from this supplier and plant effluents from a textile mill in the city of Kashan and stored at 4 ºC until required.

Methods

Wastewater samples were taken from the dying effluents of a textile plant in the city of Kashan and used for isolation of bacteria. A modified method of *Norteman et al.,* [11] was used for isolating and screening of the aerobic bacteria. Taxonomic identification including biochemical characterization and phylogenic analysis were performed by DSMZ (Deutsche Sammlung Von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) under Id number: 03-1484.

Culture conditions

A loop full of 24 h nutrient agar culture was transferred into a series of 250 ml erlenmeyer flasks each containing 50ml of sterile nutrient broth medium. The flasks were incubated at 32 ºC in an orbital shaker (140 rpm, 24h) (Orbital incubator model SI50, Stuart Scientific, UK).

Decolorization experiment

Three ml of the SB dye (Serilene Black) (stock solution 1000mgL^{-1}) or methyl orange (MO) was added to 500 ml erlenmeyer flasks each containing 100 ml mineral salt (0.235 NaH_2PO_4; 0.07Mg SO_4, $7H_2O$; 0.014 $CaCl_2$; 0.001 $FeCl_3$, $6H_2O$ (g/l) (with or without carbon and nitrogen sources) and autoclaved. These flasks were inoculated either with the cell biomass or the overnight culture of the isolated strain and placed on the shaker incubator (3 days and 140 rpm, 32ºC). After suitable intervals, samples were removed and processed for determining the extent of decolorization and chemical analysis. Decolorization of the SB dye and methyl orange were measured following the method described by *Nigam et al*. [12].

Chemical analyses

For chemical analysis of the biodegradation products, 10 Liter of dye (SB or MO) treated or untreated were centrifuged at 4000g (ser. No. 331093. Code: 775, MSG, England), and the supernatants filtered through 0.45µm filters to remove the suspended cells and particles. For extraction, the pH of cell free solutions was adjusted oat 8.5 (using 10% NaOH) and dissolved in dichloromethane (1:1). The two phases were separated with a decanter after shaking. The organic phase dried with anhydrous sodium sulfate and the evaporated in a rotary evaporator after filtration.

Organic solid residues were dissolved in a small amount of dichloromethane and used first for thin-layer chromatography on silica gel (EtAc 30 : 70 hexane as mobil phase) and then for FT-IR, GC-Ms analyses. The same procedure was carried out with N, N-dimethyl 1, 4-phenylene diamine- dihydrochloride that results from the azo bond cleavage. The Infrared spectra were recorded on a Shimadzu-4300 FT-IR (Kyoto Japan). Mass spectra of the samples were determined by a Finnigan Mattsa 70 (USA) mass spectrometer.

Chromatography of Serilene black

Serilene black is a mixed dispersed dye. For detection of its components, separation was carried out by silica gel (G60-F257) thin-layer chromatography using dichloromethane as the mobile phase. The main component was separated by preparative thin layer chromatography. The main component was dissolved in dichloromethane by removing the corresponding band from the TLC plate and pouring it in the said solvent. For preparation of large amounts of the main component, column chromatography was carried out on a silica gel column and eluted by hexane, followed by hexane: CH_2Cl_2 (90 : 10) and finally by hexane: CH_2Cl_2 (80 : 20). Identification of the major component was carried out via FT-IR analyses.

Nucleotide sequence accession number

The nucleotide sequences was done by Institute of microbiology, Vienna university for the partial 16s-rRNA from *Bacillus Sp strain PS* and have been deposited in the EMBL Nucleotide Sequence Database under accession no: Aj515146**.**

RESULTS AND DISCUSSION

Isolation/ identification of the microbial strain

20 pure bacterial cultures were isolated from samples taken from the textile effluent containing the Serilene black dye. Decolorization tests showed that only one of the strains was able to decolorize Serilene under aerobic conditions without presence of exogenous carbon source [13]. Biochemical and phylogenic examination performed by DSMZ identification service in Germany showed that the PS strain is related to the Bacillus cereus group [13].

Chromatography of Serilene black

The TLC experiment showed several spots each with a different color, the blue one being the main component. FT-IR analysis of this component shows an absorption band at 1571.057.2 cm^{-1} confirming its azo structure (Fig. 1).

Decolorization of Methyl Orange

To determine the catabolic pathway of the blue azoic component of SB, methyl orange (MO) was chosen as a model compound. The strain PS was able to grow in mineral medium containing MO in the presence of glucose.

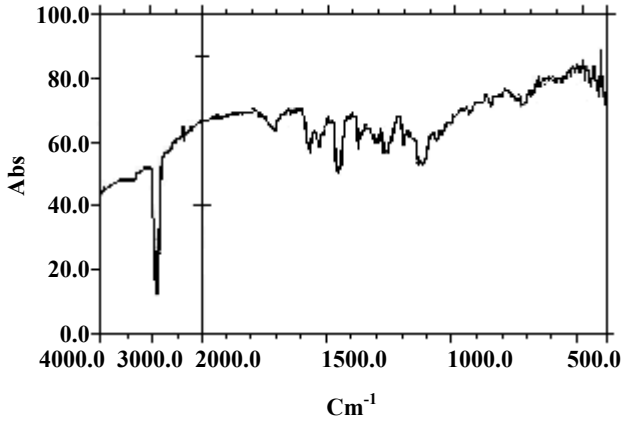

Fig. 1: FT-IR spectra of the main component of Serilene Black showing a band at 1571.057.2 cm^{-1} corresponding to azo functional group.

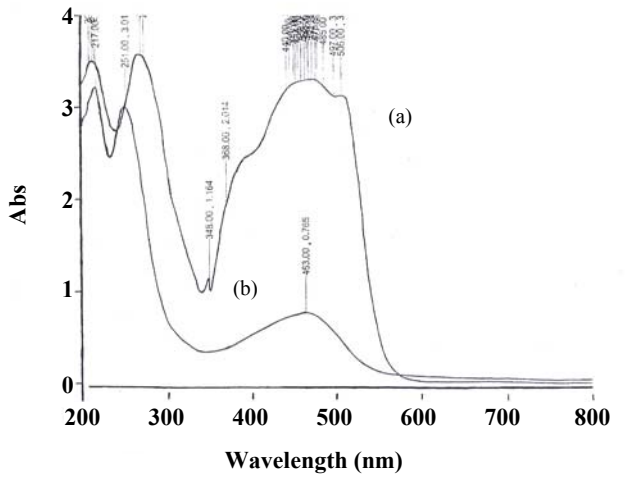

Fig. 2: UV-VIS spectra of the a) Methyl orange in first time of incubation and b) Biotreated methyl orange (MO) after 2 days incubation at 32°C (by overnight).

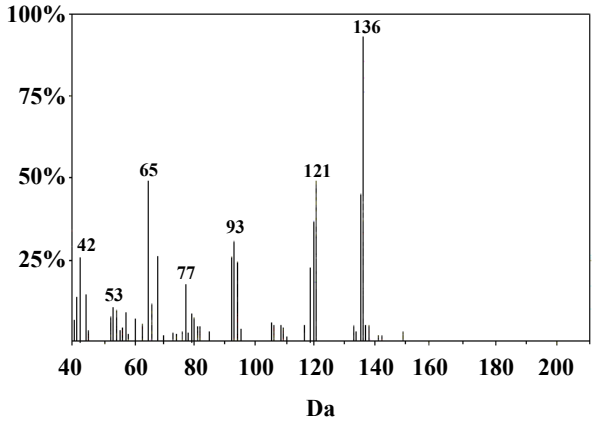

Fig. 3: GC-Ms spectra showing the products (N, N-Dimethyl, 1, 4-phenylene diamine) formed after decolorization of methyl orange by strain PS.

Decolorization of MO occurred after 2 days of incubation at 32 ºC under aerobic conditions (Fig. 2). There have been some reports which suggest the decolorization of certain sulfonated azo dyes occur under aerobic conditions after 2 months [14]. Another report indicates that azo dyes are essentially nondegradable by bacteria under aerobic conditions [15].

Kulla et al. [17] describe a degradative pathway for sulfonated azo dyes by Pseudomonas strain previously adapted to grow on the corresponding carboxylated azo dyes [14].

Our experiments show that *Bacillus sp strain PS* is able to decolorize MO under aerobic conditions.

The TLC results and GC-Ms spectra show that the strain PS can convert about 98 % of MO to N, N-dimethyl, 1, 4-phenylene diamine (Fig. 3). Others have reported that anaerobic cleavage of the azo linkage by the reductase enzymes is the initial step in the biodegradation of the azo dyes [16].The aerobic degradation of MO observed here suggests, in accordance with *Kulla*, that despite the presence of oxygen, the initial degradation step appears to be a reduction of the azo linkage by an oxygen-insensitive azo reductase [17].

In the presence of glucose, the N, N-dimethyl, 1, 4-phenylene diamine dihydrochloride disappeared by PS strain after 7 days of incubation on a shaker incubator (32ºC and 140 rpm).

Therefore it is suggested that the expected reduction products of MO are completely metabolized as there are no traces observed in the TLC plate. Thus it seems that this is the first demonstration of a complete biological mineralization of a sulfonated azo dye by *Bacillus cereus* group.

Haug [18] reported an anaerobic-aerobic degradation process of the sulfonated azo dye mordant yellow 3 by a bacterial culture. Therefore it seems likely that the mechanism proposed for decolorization of MO is similar to that of the main component of SB. The proposed pathway for the biodegradation of methyl orange is schematically depicted in Fig. 4.

We are currently attempting to identify the gene(s) and the expressed product(s) of the strain PS which are responsible for the formation of the mediators involved in biodegradation of MO and the main component of SB.

Acknowledgments

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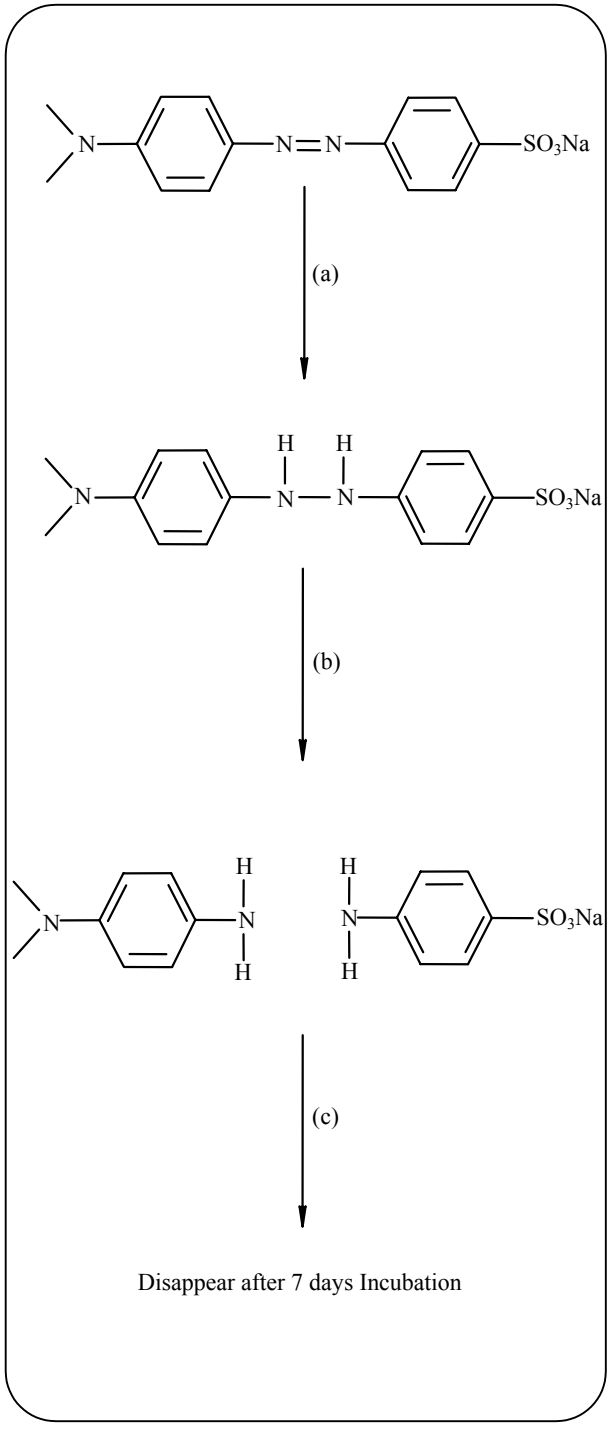

Fig. 4: Proposed pathway for degradation of methyl orange by Bacillus Sp strain PS. a) Methyl orange; b) N, N-dimethyl, 1, 4-phenylene diamine Dihydrochloride; c) Sulfanilic acid sodium salt.

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