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# Ecotoxic potential of a presumably non-toxic azo dye

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ABSTRACT

Microbes have potential to convert non-toxic azo dyes into hazardous products in the environment. However, the role of microbes in biotransforming such presumably non-toxic dyes has not been given proper attention, thereby, questions the environmental safety of such compounds. The present study assessed salinity driven microbial degradation of an unregulated azo dye, Acid orange 7 (AO7), under moderately halophilic conditions of textile effluent. The halophilic microbial consortium from effluent decolorized ~97% AO7 (50–500 mg $L^{-1}$). The consortium efficiently decolorized the dye at different pH (5–8) and salinity (5–18% NaCl). The 16S rRNA sequence analyses confirmed the presence of *Halomonas* and *Escherichia* in the consortium. The FTIR and GC-MS analyses suggested microbial consortium degrade AO7 following symmetric and asymmetric cleavage and yield carcinogenic/mutagenic aromatic byproducts viz. aniline, 1-amino-2-naphthol, naphthalene, and phenyldiazene. In contrast to AO7, the biodegraded products caused molecular, cellular and organism level toxicity. The degraded products significantly reduced: radicle length in root elongation assay; shoot length/biomass in plant growth assays; and caused chromosomal abnormalities and reduced mitotic index in *Allium cepa* bioassay. We demonstrated that under saline conditions of textile effluent, halophilic microbes convert a presumably non-toxic azo dye into hazardous products. The study calls to review the current toxicity classification of azo dyes and develop environmentally sound regulatory policies by incorporating the role of environmental factors in governing dye toxicity, for environmental safety.

## 1. Introduction

Azo dyes, characterized by a chromophoric azo (-N=N-) bond, account for ~70% of the 9.9 million tons of industrial dye colorants used annually with a global turnover of USD 30.42 billion (Balapure et al., 2015; Gürses, 2016; GVR, 2017). These dyes have become an industrial choice worldwide due to low cost, ease of preparation, versatility, fastness, and intensity of colors (Rawat et al., 2016). Several industries like textile, leather, printing, fast food, and cosmetics flourish due to the prevalent use of azo dyes. Though, these industries contribute notably to a nation's economy, they have become an environmental challenge due to the injudicious discharge of untreated or partially treated effluent into the water, which is often directly used for irrigation (Solaraj et al., 2010). In fact, dye pollution comprises of 1/5th of the world's total wastewater pollution (Kant, 2012).

In developing nations, the hub of global dyeing industries, the common effluent treatment plants (CETP) either do not function or work with reduced efficiency, and do not comply with the environmental safety (Mathur et al., 2007). In fact, the treatment primarily focuses on the decolorization of effluent, considering the color induced decrease in light compensation point, oxygen level, and biological productivity of the water bodies as the major environmental concerns. However, the reductive cleavage of azo dyes which leads to dye decolorization also produces aromatic amines, which show mutagenic, carcinogenic, genotoxic, and teratogenic effects (Bergsten-Torralba et al., 2009; Brüschweiler et al., 2014; Brüschweiler and Merlot, 2017; Dellai et al., 2013; Pielesz et al., 2002). Also, azo dyes degrade in the environment on interacting with abiotic (hydrolysis, photolysis, and oxidation) and biotic (microbial activity) factors (Rawat et al., 2016), forming toxic byproducts. The current toxicity classification and the effluent management policies do not take into account such concealed nature of azo dyes thereby, puts a question mark on the assumed environmental safety of the ~5 million tons of dye annually released in the environment.

Inadequate legislative measures for the approval of dyes for industrial use and management of dye effluent have led to the uncontrolled spread of potentially toxic dyes in the environment (Umbuzeiro et al., 2005). Dye pollution specifically challenges a large

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part of the global population (~40%) living in developing nations like China, India, and Bangladesh, which are collectively catering to ~50% of the world's textile dyeing demands (Ghaly et al., 2013). European countries, like Germany, Spain, Sweden, France, Denmark, Portugal, have adopted strict environmental legislation to regulate the use of dyes and management of the effluent (Hessel et al., 2007). However, in developing nations, the problem becomes severe due to poor environmental legislation, substandard work conditions, and inefficient waste treatment systems (Rawat et al., 2016). Further, potentially toxic dyes receive approval based on toxicity assays involving parent compound tested under standard conditions. These studies did not consider the characteristic property of the textile effluents, especially the salinity, which has implications on the structure and function of microbial communities, and therefore governs the environmental fate of such azo dyes (Allègre et al., 2006; Correia et al., 1994).

To reexamine the criteria to assess environmental safety of azo dyes, we selected Acid orange 7 dye (AO7; C.I. 15510), which is a presumably non-toxic azo dye. Textile and cosmetic industries use AO7 widely. As per European Commission norms (EC No 1272/2008), AO7 is safe to the environment but a mild irritant to human skin and eye. It has been widely used as a model dye for bioremediation studies under standard laboratory conditions (Coughlin et al., 2002; Mutafov et al., 2007) and multistaged bioreactor environments (Coughlin et al., 2003; Fernando et al., 2014). The studies have shown the biotransformation of AO7 into different byproducts or even complete mineralization (Barragán et al., 2007; Fernando et al., 2014), however, the microbe-mediated environmental fate and consequence of AO7 dye at industrially relevant salinity level have not yet been ascertained. Therefore, the present work evaluated the impact of halophilic bacteria purified from industrial effluent on the fate of a non-toxic unregulated AO7 dye under moderate saline conditions of the dye water bath. To achieve this goal the objectives were: (i) to purify and identify AO7 degrading halophilic microbial consortium from the textile effluent and characterize the dye degraded products generated under saline condition and, (ii) to assess the molecular, cellular and organism level toxicity potential of microbe-mediated AO7 degraded products and determine a putative mechanism of AO7 degradation. The study demonstrated the ecotoxic potential of an unregulated non-toxic dye and put a question mark on the assumed environmental safety of the millions of tons of dyes and degraded products released into the environment globally.

## 2. Materials and methods

### 2.1. Culture media preparation and sample collection

Moderately Halophilic (MH) media [($gL^{-1}$) NaCl 81; $MgCl_2$ 7; $MgSO_4{\cdot}7H_2O$ 9.6; $CaCl_2$ 0.36; KCl 2; $NaHCO_3$ 0.06; NaBr 0.026; Peptone 5; Yeast Extract 10; Glucose 1] was used to isolate bacteria from the dye effluent of textile industry under moderately halophilic conditions (Amoozegar et al., 2011). The environmentally relevant NaCl concentration (81 g $L^{-1}$) was selected considering the surplus use of NaCl in dye bath recipe, usually ranging from 60 to 100 g $L^{-1}$ (Allègre et al., 2006).

In the present study, the dye degradation assays were conducted on the industrial grade Acid Orange 7 (AO7) dye. The purity of industrial grade AO7 dye was authenticated by analyzing and comparing its gas chromatogram with that of AO7 dye obtained from Sigma–Aldrich, Germany (CAS: 633-96-5) (Fig. S1).

To isolate the dye degrading microbes, effluent samples of textile dye industry were collected from a small-scale industrial cluster of North-East Delhi (28.664018N–77.271167E) located along the bank of the river Yamuna. The samples were stored at 4 °C and processed within 24 h of the collection. The effluent sample was inoculated (5% v/v) into the 250 mL Erlenmeyer flask containing 50 mL MH broth medium spiked with filter sterilized (0.2 μm) AO7 dye to make the final concentration of 50 mg $L^{-1}$. The culture was kept under agitated condition (150 rpm) at 30 °C for the acclimatization of the microbes for 14 days. The acclimatized microbial consortium was used for bacterial characterization and dye decolorization studies.

### 2.2. Identification of the microbial consortium

The bacterial species present in the acclimatized microbial consortium isolated from the dye effluent of textile industry were identified using 16S rRNA sequence analyses (Sharma et al., 2011). A 50 μL aliquot from 14-day acclimatized microbial consortium was serially diluted (up to $10^{-5}$ times) and spread over MH agar plates supplemented with AO7 (100 mg $L^{-1}$) and kept overnight. Morphologically distinct isolated colonies were picked and purified using repeated colony transfer on fresh media plates for 4–5 times. Purified bacterial isolates were used for isolation of genomic DNA using guanidium thiocyanate EDTA Sarkosyl (GES) method (Pitcher et al., 1989). Polymerase chain reaction (PCR) was performed for amplification of 16S rRNA gene using universal primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACGGYTACCTTGTTACGACTT-3´), for 30 cycles. The amplified products were analyzed by agarose (1%, low EEO) gel electrophoresis and then purified from the gel using QIAprep Miniprep Kit (QIAGEN) according to the manufacturer's protocol. The sequencing reactions were performed using 27F and 1492R primers (Macrogen Lab, Korea). The homologous sequence was searched in GenBank database using NCBI BLAST. The bacterial isolates were identified, and the sequences were submitted to NCBI GenBank under the accession numbers KY621547 and KY780498.

### 2.3. Microbe-mediated dye decolorization in varied environmental conditions

Dye degradation potential of the microbial consortium was assessed by inoculating acclimatized microbes (1% v/v) in 30 mL MH broth infused with sterile AO7 dye (50 mg $L^{-1}$). To study the effect of oxygen on dye decolorization the cultures were raised under agitated (150 rpm) and static condition at 30 °C for 72 h. Subsequently, the cultures were centrifuged at 10000 × *g* for 15 min to separate the bacterial cells. The supernatant was used to analyze microbe-mediated dye decolorization by measuring the change in the maximum absorbance of AO7 dye ($OD_{483\ nm}$). The percent dye decolorization was calculated as: {(initial $OD_{483\ nm}$ - final $OD_{483\ nm}$)/ initial $OD_{483\ nm}$}*100. Three replicates were maintained along with an uninoculated MH broth as a control.

The effect of the characteristic properties of textile effluent i.e. varying dye concentrations, pH, and salinity, on microbial biodegradation of AO7 was determined. The microbial consortium was tested for dye decolorization using 100, 250, and 500 mg $L^{-1}$ dye concentrations. The samples were observed over a period of 5 days and percent decolorization was calculated by estimating $OD_{483\ nm}$ every 24 h by aseptically collecting aliquot from the culture flask. The decolorization efficiency of the microbial consortium was assessed at pH range of 3–8. The MH broth was supplemented with filter sterilized AO7 (100 mg $L^{-1}$), and the pH of the media was adjusted using 1 N HCl/NaOH. Similarly, the effect of varying NaCl concentration on decolorization of AO7 (100 mg $L^{-1}$) was analyzed at 5%, 8%, 11%, 15% and 18% using NaCl (w/v). The decolorization assays were conducted at 30 °C under static conditions. Three replicates were maintained along with an uninoculated MH broth as a control.

### 2.4. Extraction and identification of microbe-mediated dye degradation products

The AO7 biodegraded products, formed after dye decolorization, were extracted using solvent extraction method. The dye degradation experiment was set up in MH broth supplemented with AO7 (100 mg $L^{-1}$) as outlined in Section 2.3. After 96 h, the bacterial cells