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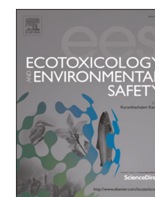

Yeast extract promotes decolorization of azo dyes by stimulating azoreductase activity in *Shewanella* sp. strain IFN4

Muhammad Imran [a,b,c,*], Muhammad Arshad [c], Fayek Negm [d], Azeem Khalid [e], Baby Shaharoona [f], Sabir Hussain [g], Sajid Mahmood Nadeem [h], David E. Crowley [a]

[a] Department of Environmental Sciences, University of California Riverside, Riverside 92521, USA
[b] Soil Science Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad 38000, Pakistan
[c] Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Faisalabad 38040, Pakistan
[d] Department of Botany and Plant Sciences, University of California Riverside, Riverside 92521, USA
[e] Department of Environmental Sciences, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan
[f] Department of Soil, Water and Agricultural Engineering College of Agricultural and Marine Sciences, Sultan Qaboos University, 123, Oman
[g] Department of Environmental Sciences & Engineering, Government College University, Faisalabad 38040, Pakistan
[h] University of Agriculture Faisalabad, Sub-campus Burewala, Pakistan

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ABSTRACT

Biological treatment of azo dyes commonly requires a combined anaerobic–aerobic process in which initial decolorization is achieved by reductive cleavage of azo bonds on the parent molecule. The present study was conducted to examine the relative importance of co-substrates for driving reductive decolorization of azo dyes by *Shewanella* sp. strain IFN4 using whole cells and enzyme assays. Results showed that the dye decolorization by strain IFN4 was faster in medium containing 1 g L^{-1} yeast extract (YE) as compared to nine other co-substrates. Moreover, only YE stimulated azoreductase activity (increased from 1.32 to 4.19 U/mg protein). Increasing the level of YE up to 8 g L^{-1} resulted into 81% decolorization of the dye in 1 h along with an increase in azoreductase activity up to 6.16 U/mg protein. Among the components of YE, only riboflavin stimulated the decolorization process as well as enzyme activity. Moreover, strain IFN4 demonstrated flavin reductase activity, and a significant correlation (r^2=0.98) between flavin reduction and dye reduction by this strain emphasized the involvement of flavin compounds in the decolorization process. The results of this study show that YE serves both as a source of reducing equivalents and an electron shuttle for catalyzing dye reduction.

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1. Introduction

Contamination of water resources by wastewater from the dye products industry is a common problem in developing countries where these industries are usually located. Currently, over 100,000 different synthetic dyes are used commercially (Revenkar and Lele, 2007), of which about 60–70% are azo dyes. These dyes are characterized by the presence of one or more azo groups (–N=N–) that contribute to the electron transfer properties of aromatic ring compounds that serve as chromophores in the dye molecules (Carliell et al., 1996). Following the dying process, substantial amounts of azo dyes (15–50%) are typically released in wastewater effluents (McMullan et al., 2001; Pratum et al., 2011). This wastewater is of concern as some dyes and their degradation products are mutagenic and carcinogenic (Carneiro et al., 2010). The presence of dyes in water bodies is also aesthetically unpleasant and reduces the rate of photosynthesis of aquatic plants (Roy et al., 2010). Moreover, dye-containing textile wastewater is commonly used for irrigation, which is problematic as some azo dyes are toxic to plants (Ayed et al., 2011). Application of dyes to soils via wastewater effluents also facilitates the possibility for food chain transfer when dye molecules or their metabolites are taken up by plants. Azo dyes are quite stable and degrade slowly in soils (Imran et al., 2015b). Although the impacts of dyes on soil biological processes are not yet well understood, azo dyes have been shown to alter the composition of microbial communities and levels of soil enzyme activities (Topac et al., 2009; Imran et al., 2015b). For all of these reasons, low cost, effective treatment systems are urgently needed to reduce environmental contamination from the dye products industry.

Bioreactor treatment systems for azo dyes have been developed using both bacteria and fungi (Verma and Madamwar, 2003; Elisangela et al., 2009). These systems still require further

* Corresponding author at: Soil Science Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.
E-mail address: imran1631@gmail.com (M. Imran).

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optimization to achieve effective treatment at low cost with short treatment times. Most azo dye degrading bacterial strains have relatively low growth and activity when cultivated on azo dyes as a sole carbon source (Saratale et al., 2010). Hence, bacterial decolorization of dyes often requires use of a co-substrate that is added to the wastewater to promote bacterial growth (Padmavathy et al., 2003; Senan and Abraham, 2004). Using a two-step process, dye degradation is initiated by the reductive cleavage of azo bond under anaerobic conditions, which leads to the production of colorless aromatic amines as initial degradation products. These metabolites are subsequently oxidized under aerobic environments (Supaka et al., 2004).

Bacteria decolorize the colored wastewater mainly through a class of enzymes referred to as azoreductases, which catalyze reductive cleavage of azo bonds by utilizing reducing equivalents (NADH or NADPH) generated from the metabolism of organic compounds. The substrate used to generate reducing equivalents can either be the dye molecule itself or a supplemental organic carbon source (co-substrate) that is added to support bacterial growth (Ong et al., 2012). In addition to serving as a labile carbon source, some co-substrates also can directly participate in azo dye decolorization by shuttling electrons from reducing equivalents generated inside the cells to the dye molecules. As azo bond cleavage often is a rate-limiting step in dye degradation (Rau and Stolz, 2003), the first step in which dyes are decolorized can be facilitated by addition of redox mediators to shuttle electrons from the cell to azo dye molecules in the external medium (Rau et al., 2002; Dos Santos et al., 2004, 2007; Jiao et al., 2009). A large number of different organic compounds have been tested as co-substrates (Oranusi and Ogugbue, 2005; Prasad and Rao, 2011). From these studies, many researchers have reported fast decolorization of azo dyes using YE as a co-substrate (Nachiyar and Rajakumar, 2003; Moosvi et al., 2007; Telke et al., 2008; Hussain et al., 2013; Anwar. et al., 2014; Imran et al., 2014), which suggest that YE may has multiple functions in promoting dye degradation, providing a carbon substrate for growth, vitamins, and additional compounds that may serve as electron shuttles.

The present study was conducted to investigate the role of YE in azo dye decolorization by *Shewanella* sp. strain IFN4, a well characterized, highly efficient dye degrading bacterium. Initially, nine co-substrates were compared with YE for their abilities to stimulate the decolorization of dyes by intact cells of strain IFN4. Additional studies examined the azoreductase activities of redox enzymes contained in fractions from cell homogenates. Based on the ability of YE to promote dye reduction, various individual components of YE were further investigated for their role in decolorization of azo dyes. Strain IFN4 also was tested for flavin reductase activity, and the relationship between flavin reduction activity and azo dye reduction was measured to explore the possible role of flavin compounds in degradation of azo dyes. Lastly, identification and localization of various enzymes with azoreductase activity in strain IFN4 was carried out and a mechanism for dye decolorization by *Shewanella* sp. strain IFN4 in the presence and absence of YE is proposed.

2. Materials and methods

2.1. Chemicals

Azo dyes including Reactive Black-5 (RB-5, dye content 55%), Direct Red-81 (DR-81, dye content 50%) and Acid Red-88 (AR-88, dye content 75%) were purchased from Sigma Aldrich, USA. The coenzymes, NADH and NADPH, used as electron donors for measuring azoreductase activity in cell fractions were obtained from Calbiochem, USA. Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), glycerol and all other chemicals were also of analytical grade.

2.2. Decolorization of azo dyes by Shewanella sp. strain IFN4

The bacterium *Shewanella* sp. strain IFN4 (accession number: KF840161) used in this study was isolated from the textile effluent (Imran et al., 2014). The ability of this strain to decolorize the selected azo dyes (DR-81, AR-88 and RB-5) and a mixture of these dyes was tested in liquid mineral salt medium (MSM) with and without YE (1 g L^{-1}). The composition of the MSM used was (g L^{-1}): NaCl (1.0), $CaCl_2 \cdot 2H_2O$ (0.1), $MgSO_4 \cdot 7H_2O$ (0.5), KH_2PO_4 (1.0), NH_4SO_4 (2.0), Na_2HPO_4 (1.0). The experimental conditions were medium pH: 7.2, incubation temperature: 28 °C, microorganism quantity: 1 mL inoculum (0.8 optical density measured at 600 nm) per 10 mL dye containing medium, static incubation with caped tubes (partially anaerobic condition). The concentrations of individual dyes and their mixture were maintained at 200 mg L^{-1} medium. To prepare a mixture of dye solution, each dye was added at the same ratio (1:1:1, v/v). The bacterial inoculum was added to test tubes containing MSM and each dye or the dye mixture was added and incubated for 4 h under the above described conditions. Un-inoculated MSM containing dye was incubated under similar conditions as an abiotic control. All the experiments were performed with 3 replicates per treatment and the experiments were repeated twice.

To measure azo dye decolorization, 1 mL aliquot was taken and centrifuged at 10,000g for 15 min at 4 °C to remove the bacterial cells. The supernatant was analyzed spectrophotometrically at λ_{max} of each dye and compared to supernatant collected from an un-inoculated control sample. The percentage of decolorization was calculated as follows (Hussain et al., 2013).

$$\text{Decolorization (\%)} = \frac{A - B}{A} \times 100$$

where A and B represent the absorbance of the un-inoculated control and the sample inoculated with strain IFN4, respectively.

2.3. Decolorization of azo dyes by strain IFN4 in the presence of different co-substrates

The effect of YE on decolorization of the dye mixture was compared with those of nine other co-substrates including glucose, starch, sucrose, fructose, lactose, mannitol, peptone, tryptone and beef extract. Each co-substrate was added to MSM at the rate of 1 g L^{-1}. MSM without any co-substrate was used as control. The percent decolorization achieved with each co-substrate was monitored under optimal incubation conditions at 35 °C and in medium adjusted to pH 8.5 (Imran et al., 2014), while other experimental conditions were same as described earlier. The decolorization was measured after 4 h incubation. The same experimental conditions were maintained in all subsequent studies involving intact bacterial cells.

Based on the ability of YE to stimulate dye decolorization, experiments were conducted with a range of YE concentrations from 0–10 g L^{-1} to ascertain the maximum decolorization rate. The possible role of specific components of YE, including riboflavin, pyridoxine and thiamine in azo dye decolorization was also investigated. The impact of these compounds on azo dye decolorization was assessed individually as well as in combination with YE (1 g L^{-1}). Each chemical was tested at concentration of 25 mg L^{-1}.

2.4. Quantification of azoreductase activity in cell fractions

Many different redox enzymes are able to catalyze azo dye

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