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Efficient industrial dye decolorization by *Bacillus* sp. VUS with its enzyme system

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ABSTRACT

This work presents role of different enzymes in decolorization of industrial dye Orange T4LL by *Bacillus* sp. VUS. *Bacillus* sp. strain VUS decolorized dye Orange T4LL, under static anoxic condition in 24 h. During decolorization of Orange T4LL a significant induction in the activities of lignin peroxidase, tyrosinase, and reductases (NADH-DCIP, azo, and riboflavin) was observed. The biodegradation was monitored by Ultraviolet–visible spectroscopy, Fourier transform infrared spectroscopy, and high performance liquid chromatography. The final products 4-methyl-2-*o*-tolylazo-benzene-1,3-diamine and [3-(phenyl-hydrazono)-cyclohexa-1,4-dienyl]-methanol were characterized by gas chromatography–mass spectroscopy. Phytotoxicity, COD, and BOD revealed non-toxicity of degraded products. Phytotoxicity study demonstrated non-toxicity of the biodegraded products for crop plants with respect to *Triticum aestivum* and *Sorghum bicolor*. *Bacillus* sp. VUS with its enzyme system could be a useful tool for textile effluent treatment.

1. Introduction

Textile dyes are widely used in textiles, leather, plastics, cosmetics, and food industries (Selvam et al., 2003). Approximately 10,000 commercial dyes and dyestuffs are used in the coloring industries (Robinson et al., 2001). As more than 10% of the dyestuff used during these dyeing processes does not bind to the fibers, large amount of dyes are released into the environment, therefore resulting in serious environmental pollution (Pearcea et al., 2003). Particularly in the case of azo dyes, effluent treatment becomes a serious issue because of their negative impact on water ecosystems and human health. Azo dyes are characterized by the presence of at least one azo bond (–N=N–) bearing aromatic rings and have high photolytic stability and resistance towards major oxidizing agents to avoid degradation of dyes (Reife and Othmer, 1993).

Dye wastewaters are usually treated using physicochemical methods (Brent et al., 2006; Hasnat et al., 2007). Among them, coagulation and adsorption are the most commonly used methods. These methods do not destroy the dyes, they rather result in phase transfer of the pollutant and hence ultimate sludge disposal remains an unsolved problem (Sokolowska-Gajda et al., 1996).

Biological treatment of dye decolorization is an alternative method and is now an expanding technology. As compared to physicochemical methods, biological systems for the treatment of textile-printing wastewater are more favorable due to their cost effectiveness, lower sludge production, and ecological sociability. A variety of microorganisms can reduce azo dyes, including obligatory anaerobic strains (Beung-Ho and Weon, 1992), facultative anaerobic strains (Kudlich et al., 1997), and some intestinal anaerobes (Brown, 1981). It is generally considered that azo reduction by bacteria occurs under anaerobic condition. Bacteria can subsequently mineralize some aromatic amines aerobically (Stolz, 2001). In present study, we have shown that *Bacillus* sp. VUS has the ability to degrade direct azo dye Orange T4LL with non-toxic products and can be used as textile effluent treatment tool.

To verify the efficiency of this strain, we used different dyes, as used in textile industries (reactive, sulfonated azo dyes, and disperse dyes), for study in our previous published reports (Dawkar et al., 2008, 2009a, 2009b, 2009). Our previous results have shown that *Bacillus* sp. VUS have the ability to degrade textile dyes asymmetrically and reductively. Activity of enzymes like lignin peroxidase, laccase, and azo reductase was induced depending on the structure of dye/and metabolites formed during decolorization. In bacterial decolorization of dyes the presence of functional groups like hydroxyl groups lower the decolorization efficiency of bacteria. But it is not with *Bacillus* sp. VUS, since this bacterium is having oxidative and reductive enzymes. All our previous studies indicate that the major dye degradation mechanism was dependent on oxidative enzymes and little on reductive

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enzymes. Therefore, barrier presence in hydroxyl group overcome by oxidative enzymes like lignin peroxidase and laccase. In present study, we have shown that *Bacillus* sp. VUS has the ability to degrade direct azo dye Orange T4LL with non-toxic products and can be used as textile effluent treatment tool.

2. Materials and methods

2.1. Microorganism and culture conditions

Bacillus sp. strain VUS was isolated from textile effluent contaminated soil and deposited at publicly accessible culture collection center at National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India under accession no. NCIM 5342 (Dawkar et al., 2008). Pure culture of *Bacillus* sp. VUS was maintained on nutrient agar slants. Composition of nutrient agar was (g L^{-1}): NaCl 5, peptone 5, beef extract 3; agar 25.

2.2. Dyestuffs and chemicals

All chemicals used were of the highest purity and of the analytical grade. 2,2′-Azinobis (3-ethylbezthiazoline-6-sulfonate) (ABTS) was purchased from Sigma-Aldrich, USA. Tartaric acid, *n*-propanol, and catechol were purchased from Sisco Research Laboratories, India. Common name of the dye has been used for convenience. The dye was procured from Manpasand textile industry, Ichalkaranji, India.

2.3. Decolorization experiments

2.3.1. Decolorization at static and shaking condition

Bacillus sp. strain VUS was grown for 24 h at 40 °C in 250 mL Erlenmeyer flasks containing 100 mL yeast extract medium to study the effect of static and shaking condition on decolorization. After 24 h, 50 mg L^{-1} dye was added in inoculated flask and incubated at static as well as shaking condition at 40 °C for 120 rpm on orbital shaker. The aliquot (3 mL) of the culture media was withdrawn at different time intervals, centrifuged at 8000*g* for 20 min. Decolorization was monitored by measuring the absorbance of culture supernatant at 420 nm. Change in pH was also studied in the same sample. Growth of microorganism in dye containing medium was determined by the gravimetric method after drying at 80 °C until constant weight.

2.3.2. Decolorization at different dye concentration

In order to examine the effect of initial dye concentration on the decolorization at static anoxic condition 100, 150, 200, 250, 300, 350 and 400 mg L^{-1} of Orange T4LL dye was added in 24 h grown culture of *Bacillus* sp. VUS in yeast extract medium. The percent decolorization was measured at different time interval. All decolorization experiments were performed in three sets. Abiotic controls (without microorganism) were always included.

The percent decolorization (Saratale et al., 2006) and average decolorization rate (Khehr et al., 2005) were measured at different time interval. All decolorization experiments were performed in triplicate. Abiotic controls (without microorganisms) were always included.

The average decolorization rate (ADR) was calculated as follows:

$$\text{Average decolorization rate (ADR)} = \frac{C \times \%D \times 1000}{100 \times t}$$

where *C* is the initial concentration of dye (mg L^{-1}) and *%D* the dye decolorization (%) after time *t*.

2.4. Enzyme activities

2.4.1. Preparation of cell free extract

Bacillus sp. VUS was grown in 100 mL yeast extract medium at 40 °C, centrifuged at 8000*g* for 20 min. These cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) for sonication (sonics-vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining temperature at 4 °C and giving 10 strokes, each of 30 s with 2 min interval. This extract was used without centrifugation as enzyme source.

2.4.2. Enzyme assays

Laccase activity was determined by taking 10% 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in 0.1 M acetate buffer (pH 4.9) at room temperature. Oxidized ABTS was measured at 420 nm. The volume of reaction mixture maintained was 2 mL (Hatvani and Mecs, 2001). Tyrosinase activity was determined in a reaction mixture containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4). The formed catechol quinone was measured at 410 nm at room temperature by keeping volume of the reaction mixture at 2 mL (Yuri et al., 2006). Lignin peroxidase (LiP) activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture containing 100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H_2O_2 in 2.5 mL reaction mixture (Shanmugam et al., 1999). In all assay procedures blank contained all components except enzyme. One unit of enzyme activity was defined as a change in absorbance U mg^{-1} $min^{-1}$of enzyme.

NADH-DCIP reductase activity was determined by using a procedure reported earlier (Salokhe and Govindwar, 1999). The assay mixture contained 100 μM DCIP, 28.57 mM NADH in 50 mM potassium phosphate buffer (pH 7.4), and 0.1 mL of enzyme solution (sonicated cells suspension) in a total volume of 5.0 mL. The DCIP reduction was calculated using the extinction coefficient of 19 mM cm^{-1}. In azoreductase assay, methyl red (MR) dye reduction was calculated using the extinction coefficient 23.36 mM cm^{-1}. The assay mixture contained 0.350 mM NADH and 110 μM MR in a total volume of 1.0 mL.

Riboflavin reductase (RFR) NAD(P)H: flavin oxidoreductase was measured by a modification of Fontecave et al. (1987) method. In this aerobic assay, the flavin reductase catalyzes the reduction of riboflavin, and the reduced riboflavin is immediately reoxidized by oxygen. Cell extract was added to a solution (final volume, 1 mL) containing 100 μM of Tris–HCl (pH 7.5), 25 μM of NADPH, and 0.003 U riboflavin. The decrease in absorbance was measured at 340 nm spectrophotometrically. Reaction rates were calculated by using a molar extinction coefficient of 6.3 mM cm^{-1}. Protein concentrations were estimated by using Biuret method.

2.5. Biodecolorization and biodegradation analysis

Decolorization was monitored by UV–vis spectroscopic analysis (Hitachi U-2800) whereas biodegradation was monitored by high performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR). Identification of metabolites was carried out by gas chromatography–mass spectroscopy (GC–MS). To carry out all these analytical techniques, 100 mL decolorized sample were taken, centrifuged at 10000*g* and extraction of metabolites was carried from supernatant using equal volume of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. HPLC analysis was carried out (Waters model no. 2690) on C_{18} column (symmetry, 4.6 × 250 mm) with methanol:acetonitrile (1:1) as mobile phase with a flow rate of 1.0 mL min^{-1} and UV detector at 420 nm.

The biodegraded Orange T4LL was characterized by Fourier transform infrared spectroscopy (Shimadzu, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid-IR region of 400–4000/cm with 45 scan times. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. Rotary vacuum evaporated sample (extracted after 24 h decolorization period) was dissolved in methanol and GC–MS analysis of metabolites was carried out using a Shimadzu 2010MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min increased up to 200 °C with 10 °C min^{-1} rose up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

2.6. Phytotoxicity study

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye at the concentration of 1997.71 mg L^{-1}. Tests were carried out according to Parshetti et al. (2006), on two kinds of seeds commonly used in the Indian agriculture: *Triticum aestivum* and *Sorghum bicolor*.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test was used. Readings were considered significant when *P* was ≤ 0.05.

3. Results

3.1. Effect of static and shaking condition on decolorization

Decolorization of direct Orange T4LL was 90% at static condition and 0% at shaking condition. Growth was observed more at shaking (35 mg L^{-1}) as compared to static condition (29 mg L^{-1}) (Fig. 1). To confirm whether this decolorization was due to microbial action or change in pH, the change in pH was not observed. Appearance of peak shifts towards ultraviolet region was observed as the decolorization of direct Orange T4LL progressed. These observations suggest that the decolorization