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ORIGINAL PAPER

Biodegradation of azo dye Direct Orange 16 by *Micrococcus luteus* strain SSN2

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Abstract In the present study, the decolorization and degradation of azo dye Direct Orange 16 (DO-16) by a potential bacterial isolate isolated from textile effluent were evaluated. Through 16S rRNA sequence matching, the potential isolate was identified as *Micrococcus luteus* strain SSN2. The effects of various factors (pH, temperature, salt and dye concentration) on decolorization were investigated. The strain SSN2 had the ability to decolorize DO-16 with 96 % efficiency at pH 8, 37 °C and 3 % NaCl in a short time of 6 h under static conditions. DO-16 decolorization was assessed by UV–Vis spectrophotometer with gradual decrease of dye peak intensity at 430 nm (λ_{max}). Analytical techniques (thin-layer chromatography, Fourier transform infrared spectroscopy and high-performance liquid chromatography) further confirmed that biodegradation of DO-16 was due to reduction of the azo bond. The phytotoxicity assay (with respect to seeds of *Vigna mungo* and *Vigna radiata*) demonstrated the less toxic nature of the DO-16-degraded products compared to the toxic azo dye.

Keywords Dye · Bacterial strain · Decolorization · Degradation · Phytotoxicity

Introduction

In the current scenario of globalization and industrialization, man has progressed a lot in setting up various kinds of industries, viz. textile industry, paper industry, food industry. The dyes form an integral part of these industries. The textile industry uses approximately 1.3 million tons of dyes and dye precursors, almost all of which are manufactured synthetically. The maximum amount of dye is utilized by the textile industry for its various dyeing processes (Chang et al. 2001). Among the different kinds of dyes used, the most common being azo, anthraquinone and deoxidizing dyes. Azo dyes are synthetic colorants and as a part of their structure contain one or more azo groups (–N=N–). They are an important and largest class of synthetic dyes extensively used in textile industries (Vandevivere et al. 1998). Nearly 60–70 % of all textile dyestuffs contain azo dyes, thus making them the largest group to be emitted into the environment (Saratale et al. 2011). During the dyeing process, approximately 10–15 % of the dyes used remain unbound and are released with the wastewater; they are resistant to fading, water and many chemicals due to their complex structures (Robinson et al. 2001). The presence of color in water poses a serious threat to the environment, affecting water recreational value, light penetration and thereby reducing photosynthesis and dissolved oxygen (Dafale et al. 2010). A number of physicochemical methods are available for the treatment of textile dyeing effluents containing azo dyes (Rajkumar et al. 2007; He et al. 2008; Kusvran et al. 2011; Ju et al. 2009; Zhou et al. 2013). However, these methods have their own limitations such as high cost, sludge generation and low efficiency (Patidar et al. 2012). Bioremediation or biological methods have attracted considerable attention as they are eco-friendly, efficient and low cost characteristics (Deng et al. 2008). Different groups of microorganisms, including bacteria, yeasts, filamentous fungi and microalgae, have been reported to decolorize various kinds of dyes (Daneshwar et al. 2007; Jadhav and Govindwar, 2006;

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Jasinska et al. 2012; Li et al. 2009). Although bacterial decolorization of dyes has been extensively studied (Pandey et al. 2007; Hong et al. 2007; Kudlich et al. 1997), focus has always been on the isolation of new especially indigenous bacteria capable of degrading wide range of dyes. In this study, we focused on the isolation and identification of dye decolorizing microorganism from textile effluent. We investigated the potential of *Micrococcus luteus* strain SSN2 to decolorize and degrade the azo dye Direct Orange 16 (DO-16). The strain SSN2 was checked for its ability to decolorize DO-16 at various pH, temperature, salinity and increasing dye concentration under static conditions. The biodegradation of DO-16 was evaluated by thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR) and high-performance liquid chromatography (HPLC). Phytotoxicity study carried out upon two common plant seeds *Vigna radiata* and *Vigna mungo* demonstrated the less toxic nature of degraded metabolites. The current work was carried out at VIT University, Vellore, Tamil Nadu, India on July 10, 2013.

Materials and methods

Chemicals

Azo dye (DO-16) was purchased from a local market and used for the study without further purification. HPLC grade methanol was purchased from Sigma Co. Ltd. All other chemicals were of analytical grade.

Analytical procedures

Decolorization was analyzed using a UV–Vis spectrophotometer (Hitachi U-2800), and biodegradation was monitored by thin TLC, FTIR (Thermo Nicolet AVATAR 330) and HPLC (Waters, Model No. 501).

Isolation, screening and identification of potential dye decolorizing bacteria

Textile effluent was collected from textile industry near Ranipet (Latitude 12° 56′ 0″N, Longitude 79° 20′ 0″E), Tamil Nadu, India. Isolation of bacteria was carried out by serial dilution technique. Distinct colonies were selected and repeatedly streaked to ensure purity on LB agar. Preliminary screening for dye decolorization was carried out in 100-ml conical flask with 50 ml of LB broth. The flasks were inoculated with a loop full of isolated pure isolates and kept in shaker at 130 rpm for overnight growth. DO-16 (5 mg/L) was added to the overnight grown strains and incubated under static conditions until decolorization occurred. The bacterium showing higher potential was selected for further decolorization experiments. The potential DO-16 decolorizing isolate was identified using 16S rRNA sequence analysis. The generated 16S rRNA sequence was analyzed at NCBI server at Blast-n site (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignment was done by using CLUSTALW program V1.82 at European Bioinformatics site (http://www.ebi.ac.uk/clustalw). Ambiguous positions and gaps were excluded during calculations. The phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using Kimura-2-parameter distances in Mega 5 package (Tamura et al. 2011). The stability among the clades of a phylogenetic tree was assessed by taking 1,000 replicates of the data set with a cut off value of 50. The nucleotide sequence data obtained in the study was submitted to the GenBank database under accession number HF562858.

Decolorization assay

A loopful of *M. luteus* SSN2 was inoculated in 100 ml of LB broth and was kept under shaking at 130 rpm for overnight. To the overnight grown culture, DO-16 (100 mg/L) was added and incubated under static conditions. At regular intervals, 4 ml of sample was withdrawn and centrifuged at 10,000 rpm for 15 min. Supernatant was collected and analyzed by UV–Vis spectrophotometer at 430 nm (λ_{max} of DO-16). All experiments were carried out in triplicate. Decolorization rate was calculated and expressed as percentage decolorization by the formula:

$$\%\text{Decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Effect of physicochemical parameters on DO-16 decolorization

Decolorization of the DO-16 by *M. luteus* SSN2 was carried out at different physicochemical parameters like pH (6, 7, 8 and 9), temperatures (32, 37, 40 and 45 °C), dye concentration (100, 200, 300, 400 and 500 mg/L) and salt concentration (1, 2, 3, 4 and 5 % NaCl).

Degradation analysis by TLC, FT-IR and HPLC

The degraded metabolites were extracted from supernatant using equal volume of ethyl acetate. Thin-layer chromatography was performed on a silica gel plate (2.5 × 7.5 cm), silica gel G, incorporating 13 % calcium sulfate for binding. The developing solvent system used was methanol–chloroform in the ratio 60:40. The bands of aromatic compounds were observed under UV detector

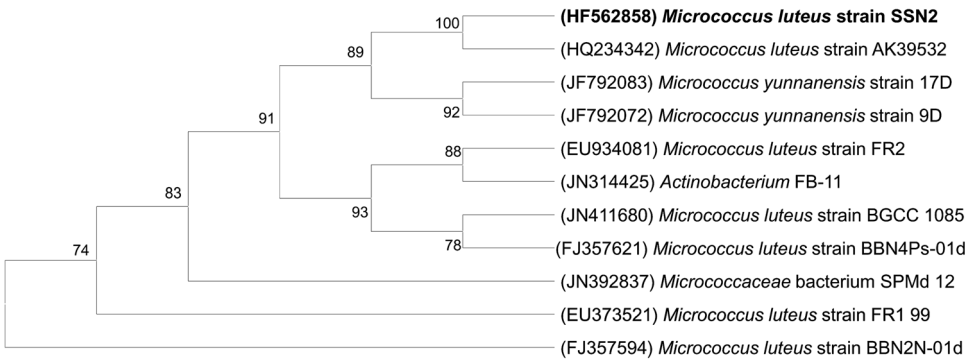

Fig. 1 Phylogenetic tree of *M. luteus* strain SSN2 constructed by neighbor joining algorithm

(254 nm). The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator for FTIR analysis. The FTIR analysis was carried out in the mid-IR ranging from 400 to 4,000 cm^{-1} with a 16 scan speed. The sample was mixed with spectroscopically pure KBr in the ratio of 1:9, and analysis was carried out. Changes in % transmittance were observed at different wavelengths. The sample was evaporated in rotary vacuum and was dissolved in methanol. HPLC analysis (Waters model No. 1525) was carried out on C18 column (symmetry, 4.6 × 250 mm). The mobile phase used was methanol–water (50:50), and UV detector was set at 430 nm.

Phytotoxicity study

The toxicity of azo dye and its degraded metabolite was studied on the seeds of *V. radiata* and *V. mungo*. 100 ppm of DO-16 dye and degraded metabolite was used to determine the toxicity. Control was maintained with distilled water. The seeds were grown for 7 days with fixed volume of the dye (DO-16), degraded metabolite and distilled water. After a period of 7 days, difference in root and shoot length was measured in all the sets.

Statistical analysis

All the tests were performed in triplicate, and data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test. The results were presented as the mean ± standard deviation (SD).

Results and discussion

Isolation, screening and identification of DO-16 decolorizing bacteria

A total of ten distinct bacterial isolates were obtained on LB agar plates from the textile effluent. Preliminary screening for decolorization revealed the potential of isolate SSN2 in effectively decolorizing DO-16. The isolate SSN2 decolorized DO-16 (100 mg/L) with 96 % rate at pH 8, 37 °C and in the presence of 3 % NaCl under static conditions in 6 h. The potential isolate was identified as *M. luteus* strain SSN2 based on 16S rRNA gene sequence analysis (1,439 bp). BLAST analysis of the 16S rRNA sequence comparison showed that the strain SSN2 had the closest phylogenetic affiliation to the genus *Micrococcus*. The homology result indicated that the strain *M. luteus* sp. SSN2 was in the phylogenetic branch of *Micrococcus* genus and showed 100 % sequence match with *M. luteus* strain AK39532 (Fig. 1).

Decolorization analysis by UV–Vis

UV–Vis spectral analysis of DO-16 decolorization by *M. luteus* sp. SSN2 is depicted in Fig. 2. The characteristic DO-16 peak observed at 430 nm (λ_{max}) at the beginning of static incubation (0 h) with strain SSN2 decreased gradually overtime during decolorization (6 h). Decolorization of DO-16 was confirmed by the decrease in the intensity of dye peak overtime. Similar result was observed during the decolorization of malachite green (MG) by *Micrococcus* sp. strain BD 15 (Du et al. 2013).

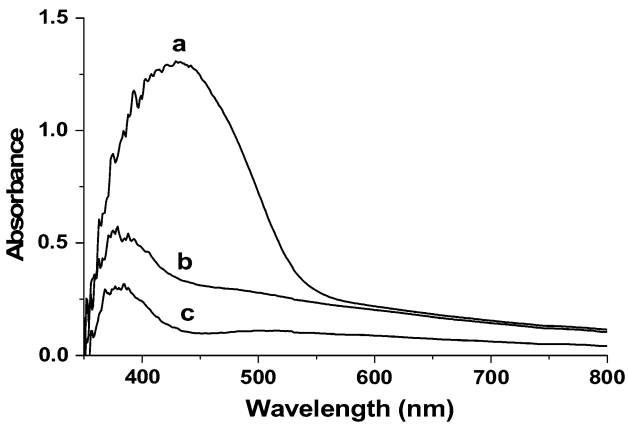

Fig. 2 UV–Vis spectral analysis of DO-16 at different time **a** 0 h, **b** 3 h and **c** 6 h

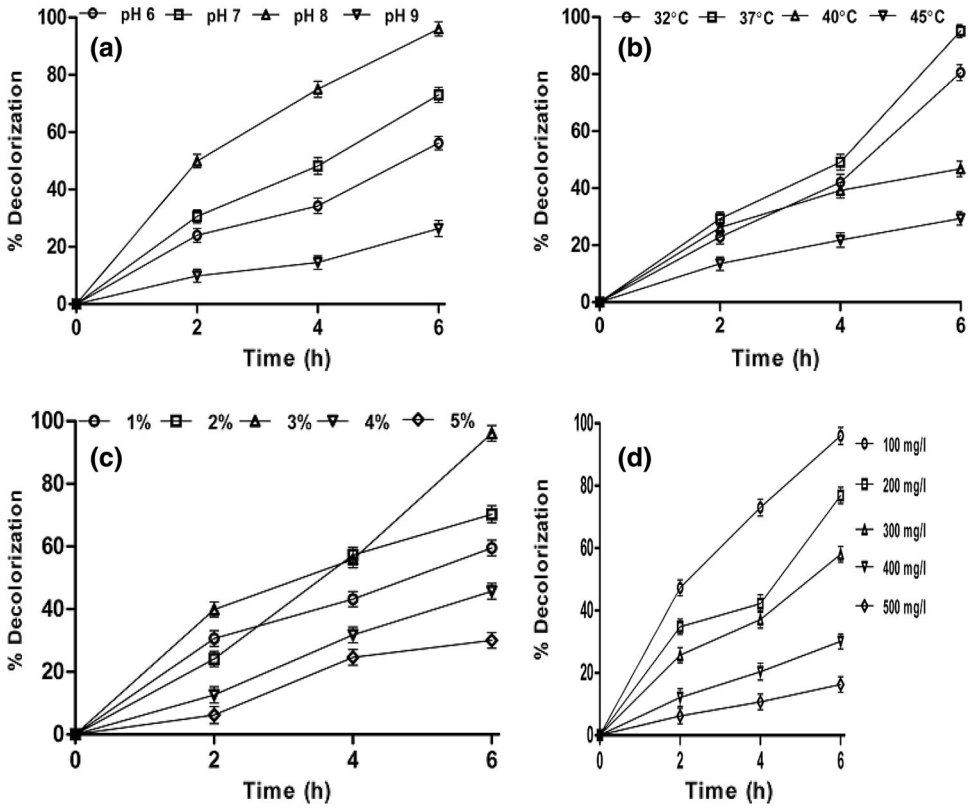

Fig. 3 Decolorization of DO-16 at different **a** pH, **b** Temperature, **c** Salinity and **d** increasing dye concentration by strain SSN2 under static conditions

Effect of physicochemical parameters on DO-16 decolorization

It was observed that the decolorization rate of DO-16 increased with rise in pH. The best decolorization was achieved at pH 8, with 96 % decolorization after 6 h under static conditions (Fig. 3a). Similar results were observed in *Micrococcus glutamicus* NCIM-2168 and *Micrococcus* strain R3 during the decolorization of reactive green 19A, Scarlet R (Saratale et al. 2009a, 2009b) and methyl red (Olukanni et al. 2009). The rate of decolorization by strain SSN2 was 56 and 73 % at pH 6 and 7, respectively. At pH 9, decolorization was found to be decreased with rate of 26 %. *Micrococcus* sp. strain SSN2 showed higher decolorizing ability at 37 °C with 96 % reduction of DO-16 under static conditions in 6 h (Fig. 3b). Similar result with temperature was noticed in *Bacillus cohnii* MTCC 3616 and *Deinococcus radiodurans* R1 (Prasad and Rao 2013; Lv et al. 2013). DO-16 decolorization was about 80 % at 32 °C. Decolorization rate of DO-16 was found to decrease substantially at 40 °C and 45 °C exhibiting 46 and 29 % rate. This inhibition is most likely due to deactivation of enzymes responsible for decolorization or loss of cell viability (Panswad and Luangdilok 2000; Cetin and Donmez 2006). Similar effect of temperature was seen in *Bacillus* sp. YZU1 (Wang et al. 2013). Rate of DO-16 decolorization increased with the increase in NaCl concentration. At 1 and 2 % NaCl, the strain SSN2 decolorized DO-16 by about 59 and 70 %, respectively. Optimal decolorization (96 %) of DO-16 was obtained

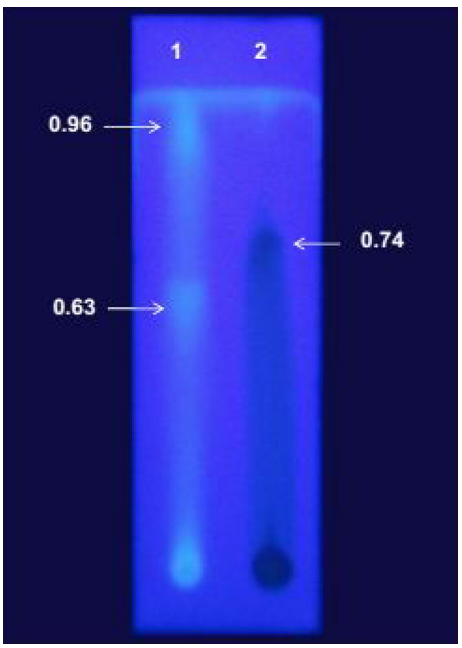

Fig. 4 Thin-layer chromatography (*TLC*). *Lane 1*: TLC resolution of ethyl acetate extracted metabolites of DO-16. *Lane 2*: TLC resolution of azo dye DO-16

with 3 % NaCl in 6 h (Fig. 3c). However, with increase in NaCl concentration to 4 and 5 %, the strain SSN2 showed decreased decolorization rates of about 45 and 30 %. This decrease in decolorization rate at higher salt concentrations could be attributed to stress leading to plasmolysis as many bacterial cells cannot withstand high salt concentrations (Zilly et al. 2011). Similar inhibition was noticed with *Bacillus* sp. YZU1 decolorizing reactive black 5 (Wang et al. 2013). The effect of initial dye concentration on DO-16 decolorization by strain SSN2 is shown in Fig. 3d. Maximum decolorization of 96 % was achieved at 100 mg/L concentration of DO-16 in 6 h under static conditions. Gradual decrease in decolorization was observed at 200, 300 and 400 mg/L dye concentration with rates of about 76, 58 and 30 %, respectively. Strain SSN2 exhibited negligible decolorization rate of 16 % at a higher (500 mg/L) concentration of DO-16. Similar result of decreased decolorization at higher dye concentration was reported earlier (Sani and Banerjee 1999). The decrease in decolorizing ability of strain SSN2 at higher dye concentration clearly indicates the toxic nature of the azo dye (Sponza and Isik 2004).

Degradation analysis by TLC, FTIR and HPLC

The TLC chromatograms of the parent dye and its extracted metabolites by the organic phase before and after decolorization by the strain SSN2 under UV light showed that the degraded sample had two additional bands (Fig. 4, Lane 1) with R_f values of 0.63 and 0.96. A band having an R_f value of 0.74 in the chromatogram indicated the azo dye (Lane 2). The disappearance of the dye band in the chromatogram of decolorized sample indicates complete decolorization of DO-16. Similar result was obtained in the

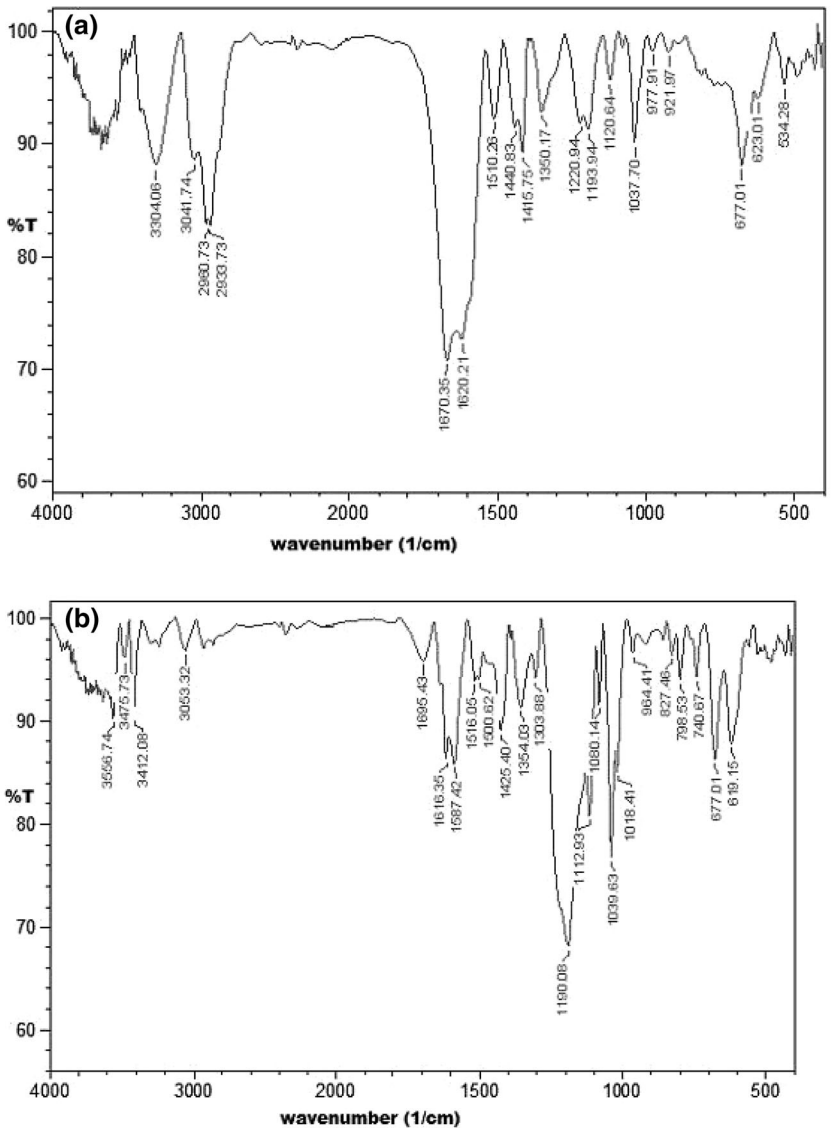

Fig. 5 **a** FTIR spectra of DO-16, **b** FTIR spectra of degraded sample

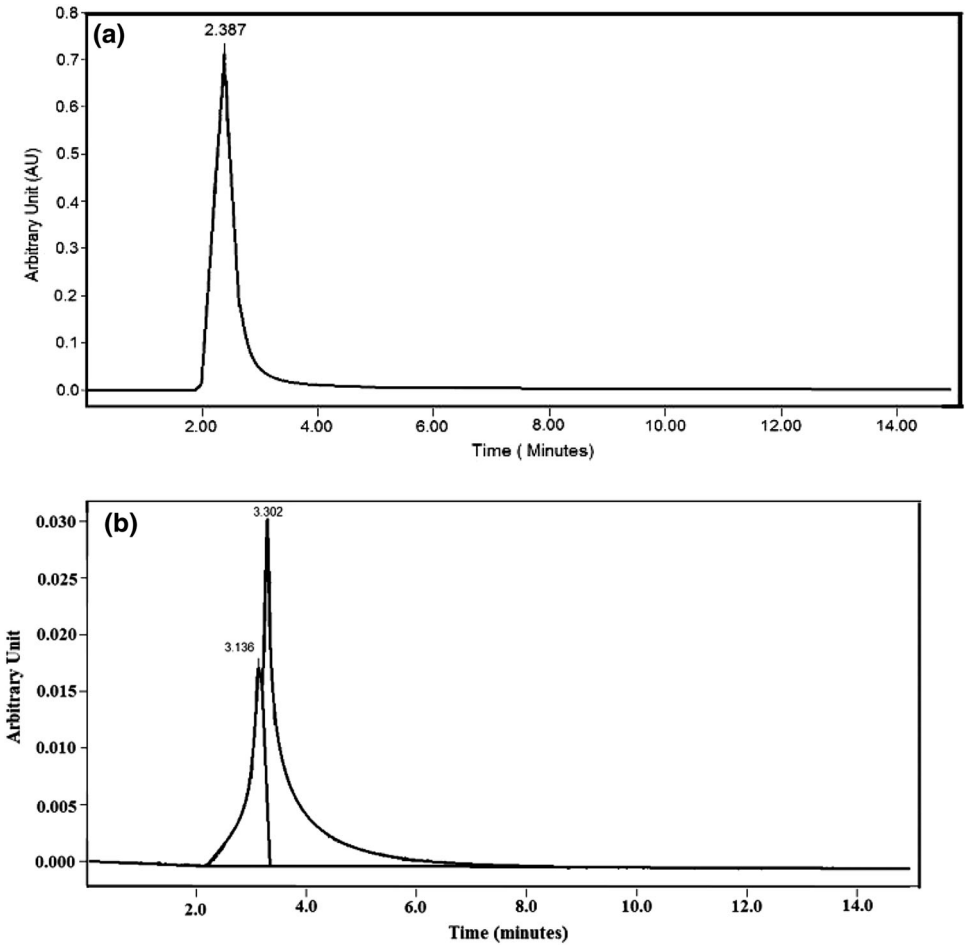

Fig. 6 **a** HPLC elution profile of DO-16, **b** HPLC chromatogram of degraded metabolites

Table 1 Phytotoxicity comparison of DO-16 and its extracted metabolites

Parameters	*Vigna mungo*			*Vigna radiata*		
	Water	DO-16 (100 ppm)	Extracted metabolites (100 ppm)	Water	DO-16 (100 ppm)	Extracted metabolites (100 ppm)
Germination (%)	100	30	100	100	30	100
Shoot (cm)	8.20 ± 0.23	3.26 ± 0.2**	5.23 ± 0.43*	11.01 ± 0.33	2.10 ± 0.81**	8.36 ± 0.44
Root (cm)	3.00 ± 0.25	00.50 ± 0.10**	1.45 ± 0.13	3.23 ± 0.10	0.43 ± 0.11**	1.45 ± 0.14*

Values are mean of three experiments ± SEM, significantly different from the control (seeds germinated in water) at * $P < 0.05$; ** $P < 0.01$, by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test

TLC report of Remazol Black b degradation by strain D2 (Asad et al. 2006). The FT-IR spectra of azo dye DO-16 and its degraded metabolite are depicted in Fig. 5a and Fig. 5b. In the IR spectrum of DO-16 dye, –N=N– stretching and C=O stretching were observed at 1,620 and 1,670 cm^{-1}, respectively, whereas in the IR spectra of degraded sample, band for –N=N– stretch shifted to 1,587 cm^{-1} with less intensity, and band for C=O stretch shifted to 1,695 cm^{-1} indicating degradation of the dye. In untreated dye, the bands around 2,933–2,960 cm^{-1} are most likely due to the presence of C–H stretch which were absent in the spectrum of degraded dye product. The tertiary amine group, represented by the bands around 1,193–1,220 cm^{-1} was also not found in degraded dye products although they are present in the IR spectrum of untreated dye. At the same time, new bands appeared at 1,018–1,112 cm^{-1} indicating the presence of sulfonate ion in the degraded dye. The spectrum of degraded dye showed a band at 1,190 cm^{-1}, a secondary alcohol group which was absent in the spectrum of untreated dye. All these changes in IR band frequencies clearly suggest that DO-16 has been degraded by strain SSN2. The HPLC analysis

report of the dye sample withdrawn at the beginning of static incubation (0 h) showed a single peak of azo dye at a retention time of 2.387 min (Fig. 6a). After a period of 6 h as the degradation proceeded, the HPLC chromatogram of the degraded sample showed the presence of two major peaks at 3.136 and 3.302 min, respectively (Fig. 6b). Absence of the dye peak and appearance of peaks at different retention times is indicative of biodegradation of azo dye.

Phytotoxicity

The phytotoxicity test with seeds of *V. radiata* and *V. mungo* showed toxicity of azo dye (DO-16). The dye (100 ppm) treated seeds of *V. radiata* and *V. mungo* exhibited 30 % germination. The average shoot and root length of *V. mungo* were 3.26 ± 0.2 and 0.50 ± 0.10 cm and 2.10 ± 0.81 and 0.43 ± 0.11 cm in *V. radiata* treated with DO-16. Distilled water-treated seeds showed average shoot and root length of 8.20 ± 0.23 cm and 3.00 ± 0.25 in *V. mungo* and 11.01 ± 0.33 cm and 3.23 ± 0.10 in *V. radiata*. Seeds treated with degraded metabolites exhibited 100 % germination. The mean shoot and root lengths of seeds exposed to degraded metabolites are 5.23 ± 0.43 cm and 1.45 ± 0.13 for *V. mungo* and 8.36 ± 0.44 cm and 1.45 ± 0.14 for *V. radiata,* respectively (Table 1). Similar result of phytotoxicity was observed in a study with azo dye direct red-22 and its degraded metabolites with seeds of *V. radiata*, *S. bicolor* and *V. mungo* (Prasad and Rao 2013). Another study reported the similar toxic effects on the root and shoot lengths of seeds of *V. mungo*, *V. radiata* and *S. bicolor* exposed to azo dye Direct Blue-1 compared to the less toxicity by the degraded metabolites (Prasad et al. 2013).

Conclusion

In the present study, the potential of *M. luteus* strain SSN2 isolated from textile effluent was assessed to rapidly decolorize and degrade the azo dye DO-16. The bacterial strain SSN2 showed maximum decolorization of DO-16 (100 mg/L) at pH-8, 37 °C, 3 % NaCl under static conditions. The degradation of DO-16 was confirmed by various analytical techniques. The phytotoxicity test revealed the less toxic nature of the degraded metabolite compared to the toxic azo dye DO-16. Hence, the isolate *M. luteus* strain SSN2 could be a promising candidate for the efficient decolorization of various dyes.

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