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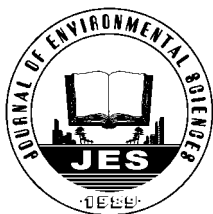
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Evaluating the effectiveness of marine actinobacterial extract and its mediated titanium dioxide nanoparticles in the degradation of azo dyes

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ABSTRACT

Aim of the present study was to synthesize titanium dioxide nanoparticles (TiO₂ NPs) from marine actinobacteria and to develop an eco-friendly azo-dye degradation method. A total of five actinobacterial isolates were isolated from Chennai marine sediments, Tamilnadu, India and analyzed for the synthesis of TiO₂ NPs using titanium hydroxide. Among these, the isolate PSV 3 showed positive results for the synthesis of TiO₂ NPs, which was confirmed by UV analysis. Further characterization of the synthesized TiO₂ NPs was done using XRD, AFM and FT-IR analysis. Actinobacterial crude extract and synthesized TiO₂ NPs was found efficient in degrading azo dye such as Acid Red 79 (AR-79) and Acid Red 80 (AR-80). Degradation percentage was found to be 81% for AR-79, 83% for AR-80 using actinobacterial crude extract and 84% for AR-79, 85% for AR-80 using TiO₂ NPs. Immobilized actinobacterial cells showed 88% for AR-79 and 81% for AR-80, dye degrading capacity. Degraded components were characterized by FT-IR and GC-MS analysis. The phytotoxicity test with 500 µg/mL of untreated dye showed remarkable phenotypic as well as cellular damage to *Tagetes erecta* plant. Comparatively no such damage was observed on plants by degraded dye components. In biotoxicity assay, treated dyes showed less toxic effect as compared to the untreated dyes.

Introduction

Dyes play an important role in human history from ancient time. There are different classes of dyes in which azo dyes are one of the most important class. Azo dyes are the largest synthetic chemicals which are mainly used in food, textile, plastic, leather, printing, cosmetics, and pharmaceutical industries and also as chemical indicators. Azo dyes are compounds that possess R-N=N-R' functional group, in which R and R' can be either alkyl or aryl (Chudgar, 1985). Azo dye comes in different forms which include oxy-azo, polyazo, amido-azo, tetra-azo and diazo compounds (Chen et al., 2010). Nearly 65%–75% of synthetic dyes commercially utilized as colourants are azo

dyes. As azo dyes have very poor exhaustion properties, all the remaining dye particles which are unbound to the fiber get released into the environment and that will ultimately lead to bioaccumulation. Presence of dyes in effluent gives the indication of water pollution which leads to reduced dissolved oxygen content (Chen et al., 2003a). Thus, there is a great need to remove dyes from industrial effluent before its discharge into surrounding environment (Moller and Wallin, 2000). Most of the presently used chemical and physical effluent treatment techniques do not decolorize the coloured effluent completely, besides these methods are too expensive and have operational difficulties (Praveen et al., 2009). Thus, there is an urgent demand for the development of effective methods to treat these toxic textile industry effluents.

One of the advanced technologies used for the purpose is photocatalytic decolorization (Zhao and Zhang,

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2008). Photocatalysis is defined as a process by which a semiconducting material absorbs light of energy equal to or more than its band gap, thereby generating electrons and holes, which can then further generate free-radicals in the system for oxidation of substrate. The resulting free-radicals are efficient oxidizers of the organic matter. The photocatalytic decolorization of many dyes has been broadly and highly explored in many previous studies using different nanoparticles (Loannis and Triantafyllos, 2004; Yizhong, 2000). Photocatalysis has been proved and considered as a cost effective alternative for the purification of different types of dye-containing effluent (Radia and Abdelaziz, 2008; Sushil et al., 2009). Due to its non-toxicity, chemical stability and photosensitivity nature, titanium dioxide nanoparticles (TiO_2 NPs) have been investigated for the degradation of several environmental pollutants (Chun et al., 2006). It was revealed that the TiO_2 NPs shows high photocatalytic activity in the presence of UV irradiation as compared to natural solar radiation. Use of TiO_2 NPs in effluent treatment can be considered as an economical effluent treatment process. TiO_2 NPs-assisted photocatalysis (UV/ TiO_2) resulted in the high degree of toxicity removal as compared to other methods. Approximately 93% toxicity removal has been reported by the process of TiO_2 NPs-assisted photocatalysis (Sharma et al., 1991; Machado et al., 2008). In order to increase the rate of biodegradation of dyes, the use of immobilization technology towards decolorization system has received increasing attention. Immobilized nanoparticles and bacterial cells show more promising results compared to free cells in the dye degradation process (Lachheb et al., 2002).

Biological systems have achieved considerable importance over the existing chemical and physical techniques used in the treatment of the dye containing effluent because of its environment friendly, cost effective nature (Beydill et al., 1998). The ability of bacteria to degrade dye and mainly azo dyes has been successfully investigated (Carliell et al., 1995). Aerobic condition is considered to be suitable for the degradation of azo dye using bacteria (Wuhrmann et al., 1980; Liang et al., 2008; Pagga and Taeger, 1994). Actinomycetes are a group of bacteria which possess many important and interesting features (Bhagabati et al., 2004). In the last decades, marine actinobacteria are increasingly being isolated from marine environments demonstrating that actinobacteria are ubiquitous in marine sediments. They are producers of a large number of natural products, many of them with clinical, pharmaceutical (Bull et al., 2000) or agricultural application. Marine actinobacteria are surmised to have different characteristics, as marine environmental conditions are extremely different from terrestrial ones. It is therefore assumed that marine actinomycetes might produce different types of bioactive compounds (Fenical et al., 1999; Gesheva et al., 2005). Karthik et al. (2012) reported first marine actinobacterial mediated TiO_2 NPs synthesis. So far, only one report was

available for marine actinobacterial mediated TiO_2 NPs synthesis. Hence, the present work was focused on the biosynthesis of TiO_2 NPs using marine actinobacteria and to elucidate its azo dye (Acid Red 79 (AR-79) and Acid Red (AR-80)) degrading ability.

1 Materials and methods

1.1 Dyes and chemicals

Pure form of azo dye AR-79 and AR-80 with maximum absorption at 560 nm and 540 nm respectively was acquired from a local textile industry of Tamil Nadu, India. All the chemicals and media used in the study were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Hi Media Laboratories, Mumbai, India.

1.2 Isolation of marine actinobacteria and biosynthesis of TiO_2 NPs

Marine sediments were collected from Chennai region (Latitude: $11^{\circ}00'N$, Longitude: $78^{\circ}00'E$), Tamil Nadu, India. Isolation of actinobacteria was done using Starch-Casein agar by spread plate technique after serial dilution in 50% sea water. All agar plates were supplemented with 50 $\mu\text{g/mL}$ of nystatin to avoid fungal contamination and incubated at room temperature for one month. Powdery and leathery colonies were isolated, purified and stored at 4°C until further use (Karthik et al., 2010).

For the synthesis of TiO_2 NPs, isolated actinobacterial colonies were inoculated in soluble starch broth, production medium (soluble starch 25 g, glucose 10 g, yeast extract 2 g, CaCO_3 3 g, trace elements 1 mL, distilled water 1000 mL) and incubated for 7 days at 28°C in shaker incubator. After incubation, crude sample was centrifuged and pellet was discarded. To the supernatant $\text{TiO}(\text{OH})_2$ was added aseptically and kept for incubation for 24 hr at room temperature.

1.3 Characterization of nanoparticles

After incubation, 1 mL of supernatant was withdrawn from $\text{TiO}(\text{OH})_2$ added culture broth and absorbance was taken using UV-Visible spectrophotometer (U-2800, Japan) in the range of 400–600 nm. Qualitative testing of supernatant by UV-Visible spectrophotometer, confirmed the reduction of $\text{TiO}(\text{OH})_2$ and synthesis of TiO_2 NPs.

Biosynthesized TiO_2 NPs was heat dried and powder form was obtained. X-ray diffraction measurements were carried out on D8 advance diffractometer (BRUKER, Germany). The scanning was done in the region of 2θ from 30° to 80° at 0.02 min and the time constant was 2 sec. The mean particle diameter of TiO_2 NPs were calculated from the XRD pattern using the Scherrer equation:

$$D = K\lambda/\beta_{1/2}\cos\theta \quad (1)$$

where, K is the shape constant λ is the wavelength of the X-ray, $\beta_{1/2}$ and θ are the half width of the peak and half of the Bragg angle, respectively.

The powdered sample was diluted in sodium dodecyl sulphate solution and then subjected for ultrasonification. The prepared sample was coated onto glass slide and then used for the analysis. Atomic force microscopy (AFM) analysis of the TiO_2 NPs was performed under ambient condition using Nano Surf Easy Scan2, Switzerland.

Two milligrams of TiO_2 NPs powder sample was mixed with 200 mg KBr (FT-IR grade). The mixture was ground subsequently for 3–5 min. Pellet was then prepared using die-set and qwik-handi press. The pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000–450 cm^{-1} in FT-IR spectroscopy (AVATAR 300 FT-IR, Thermo Nicolet, USA).

1.4 Azo dye degradation

The rate of azo dye degradation was determined by calculating the rate of discoloration of the azo dyes using the formula: Discolouration = Initial absorbance–Final absorbance/Initial absorbance $\times 100\%$. Experiment was carried out in three sets for both the dyes. Three milliliters of dyes (500 $\mu\text{g/mL}$) was taken in all the test tubes. One set was kept as control, second and third was treated with actinobac terid extract (AE) and TiO_2 NPs at different concentrations (100–500 $\mu\text{g/mL}$) respectively and incubated for 24 hr. Minimum effective concentration of AE and TiO_2 NPs was standardized as 200 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ respectively.

Further dye degradation experiment was carried out same as above with AE 200 $\mu\text{g/mL}$ and TiO_2 NPs 100 $\mu\text{g/mL}$. The degradation of the dye solution was confirmed by measuring absorbance of the treated dye samples after 10, 20, 30 and 60 min using UV-spectrophotometer at 540 and 560 nm corresponding to the maximal absorption of the dye AR-79 and AR-80 respectively (Fujishima and Honda, 2006). All the analyses were carried out in triplicates. Data were presented as mean \pm standard deviation. Statistical correlations between groups were calculated using two way ANOVA.

Immobilization of AE was done by dissolving sodium alginate in broth containing AE. The final solution with 3% sodium alginate was prepared by mixing properly. The AE alginate mixture was dripped in chilled 0.05 mol/L calcium chloride solution from a height of 20 cm. The beads were allowed to harden for an hour. Dye solution was treated with immobilized AE in the same way as described above.

Degraded components of dye were extracted using chloroform as solvent. Both the treated dyes, DA-79 and DA-80 were mixed with chloroform separately, and then components were extracted using separating funnel and

allowed to dry. Dried components were scraped out and the powder form was used for FT-IR and GC-MS analysis for characterization of degraded components (DA-79 and DA-80).

1.5 Phytotoxicity assay using *Tagetes erecta*

The phytotoxicity study was carried out with *Tagetes erecta* in order to assess the toxicity of dyes and its degraded products. Different sets of plants were arranged and treated with AE, TiO_2 NPs, AR-79 dye solution, AR-80 dye solution, and solutions of DA-79 and DA-80. Treated plants were allowed to grow in their natural environment for one week. After the period of one week, plants were compared with that of control for different phenotypic as well as cellular parameters.

1.6 Biototoxicity using brine shrimp hatchability test

The brine shrimp hatchability assay was used to determine cytotoxicity of AR-79 and AR-80 as well as DA-79 and DA-80 (Karthik et al., 2013). Shrimp eggs were allowed to hatch in glass tubes filled with sea water. One set of test tubes were kept as control and others were treated with different concentrations (25, 50, 75, 100 $\mu\text{g/mL}$) of AE, TiO_2 NPs, AR-79, AR-80, DA-79, DA-80 respectively. All the test tubes were incubated at 28°C under continuous lighting and aeration for 72 hr and observed at regular intervals to check the biototoxicity effect. Live shrimps were then observed under microscope and toxicity effect was calculated by counting the number of survivors.

1.7 Identification of potential marine actinobacteria

The genus of isolate (PSV-3) with good dye degrading activity against azo dyes was identified using cell wall composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). The species was identified based on methods described by Shirling and Gottlieb (1966) and the key of Nonomura (1974).

2 Results and discussion

2.1 Isolation and characterization of TiO_2 NPs synthesizing actinobacterial strain

A total of five actinobacterial isolates (PVS-1 to PSV-5) were isolated. Among the isolated marine actinobacteria, only one isolate (PSV-3) was found to be potential for synthesis of TiO_2 NPs. Malarkodi et al. (2013) reported eco-friendly synthesis of TiO_2 NPs using *Planomicrobium* sp. The absorbance of TiO_2 NPs was found to be in the range of 400–420 nm. The crystalline nature of TiO_2 NPs was analyzed using XRD. The XRD spectrum was matched with JCPDS card No.89-8303 which exhibits the TiO_2 NPs peaks at 2θ as 27.44°, 32.04°, 45.06°, 56.64°

and 76.37° . The mean particle diameter of TiO_2 NPs was calculated from the XRD pattern (**Fig. 1**) using the Scherrer equation. The average particle size obtained from XRD data was found to be about 58.3 nm.

2.2 AFM and FT-IR

TiO_2 NPs were found to be of spherical in shape of about 37.54 nm length at 15 μm image length (**Fig. 2**). Similarly, the extracellular synthesis of TiO_2 NPs has been investigated using the marine actinobacteria, *Streptomyces* sp. LK-3. The particle size and morphology was characterized using UV-Visible, XRD, AFM, SPM techniques and found to be in the range of 3.5–92 nm with spherical and oval shape (Karthik et al., 2012). The different peaks shown in the **Fig. 3** represent different functional groups like aldehyde, alkenes, nitro and alkynes groups. The peaks at 2065.76 cm^{-1} represents the C–H aldehyde stretching,

1637.56 cm^{-1} as C=C conjugate, 1384.89 cm^{-1} as NO_2 conjugate and 644.22 cm^{-1} as alkynes. As TiO_2 NPs is metal it will never possess any functional groups, thus presence of various functional groups showed the TiO_2 NPs synthesizing ability of the isolate PSV-3.

2.3 Dye degradation

2.3.1 AE and TiO_2 NPs

AE as well as TiO_2 NPs treated AR-79 and AR-80 showed significant dye degradation. The level of degradation was found to be increasing with increase in time interval. Degradation was indicated by the discoloration of the dye. The discoloration of azo dyes occurs when the free radicals of TiO_2 NPs binds with the positively charged dye. The degradation of the AR-79 and AR-80 was confirmed by UV-Visible analysis. The maximum degradation percentage obtained for AR-79 and AR-80 was found to be 84%

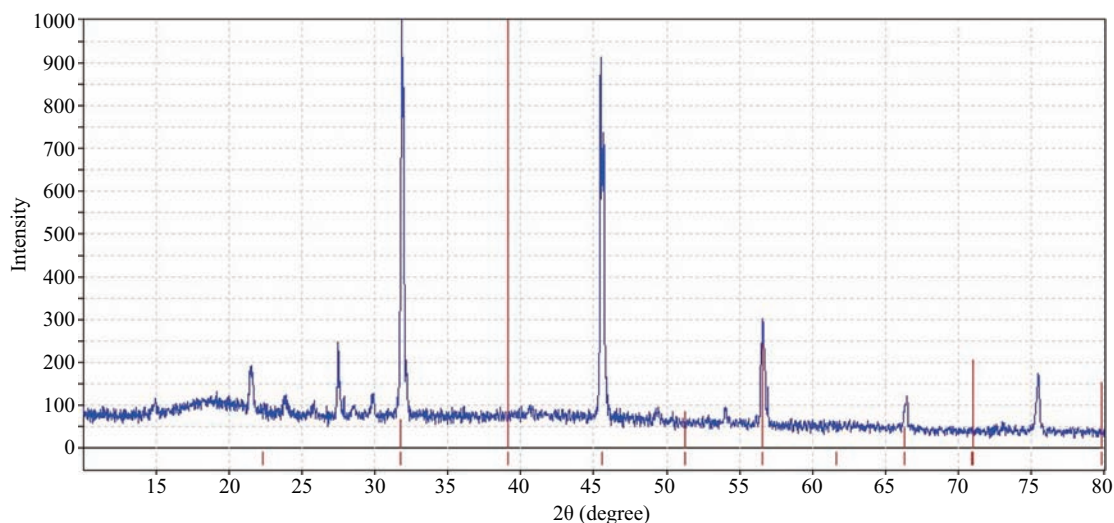


Fig. 1 XRD pattern of TiO_2 NPs.

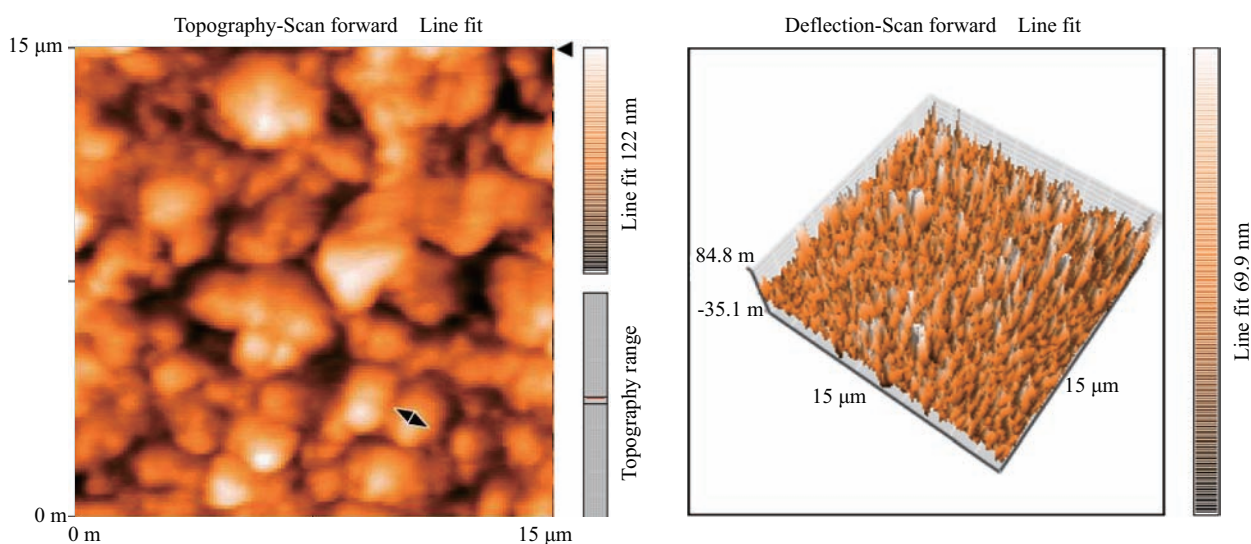


Fig. 2 AFM topography of TiO_2 .

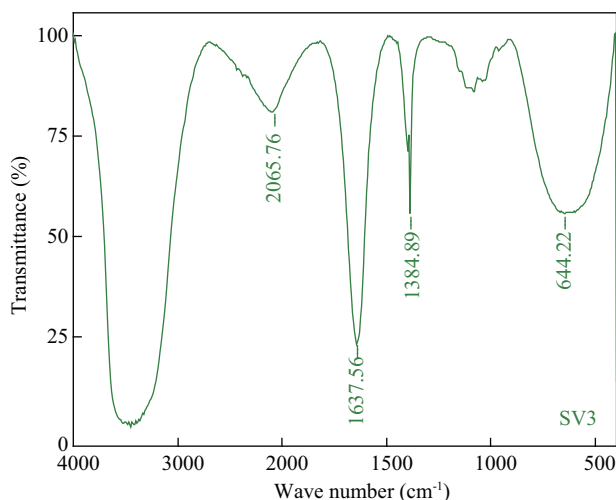


Fig. 3 FT-IR spectrum of TiO_2 .

and 85% respectively using TiO_2 NPs (Fig. 4a and b). Similarly, Arun and Bhaskara Rao (2012) reported 40% to 60% of azo dye degradation by *Bacillus cohnii* MTCC 3616. Likewise, Yogendra et al. (2011) reported 53% to

91% azo dye degradation using calcium aluminate NPs and Jaquadale et al. (2012) also reported 80% photocatalytic degradation of Navy Blue HE2R by surface modified TiO_2 NPs. Hence, obtained results indicate that AE and TiO_2 NPs possess the potential of azo dye degradation.

Azo dye solution with immobilized beads of AE showed increased level of degradation. Maximum rate of degradation was found to be 88% for AR-79 and 81% for AR-80 (Fig. 4c). Likewise previous studies on immobilized microbial consortium have shown 75% of azo dye degradation (Chen et al., 2003b). According to one of the latest report hydrogel supported TiO_2 NPs can be used for textile dye degradation several times without lose of efficiency (Harikumar et al., 2013). Hence, immobilized AE beads were found to be more effective in azo dye degradation. Marine actinobacteria can be thus considered as an imperative biological system for dye degradation process and also for the synthesis of TiO_2 NPs.

2.3.2 Gas chromatography mass spectroscopy

Graphs obtained after GC-MS analysis are shown in Fig. 5. Number of peaks, indicating the abundance of components

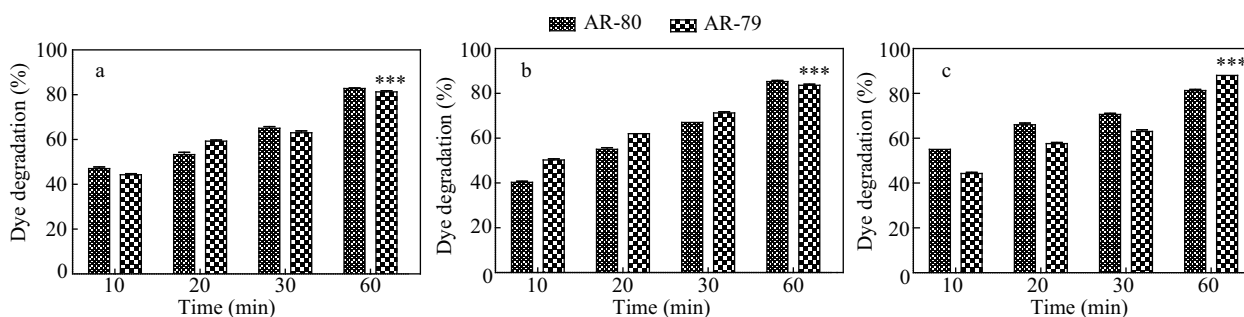


Fig. 4 Degradation of Acid Red-80 (AR-80) and Acid Red-79 (AR-79) using actinobacterial extract (AE) (a) and actinobacterial mediated TiO_2 NPs (b) and immobilized AE (c).

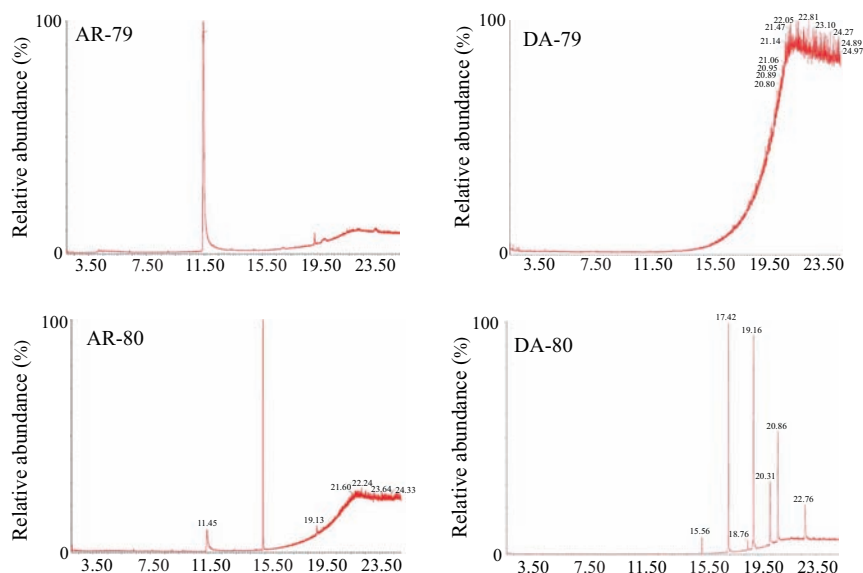


Fig. 5 GC-MS result of AR-79, degraded AR-79 (DA-79), AR-80, and degraded AR-80 (DA-80).

was found to be increased considerably in treated dye sample as compared to untreated dye sample. Thus increased number of components confirmed the degradation of azo dyes into various small and non toxic components like 1-heptacosanol, hexacosanol, acetic acid, chloro-hexadecyl ester etc. Thus, PSV-3 was found to be efficient in converting toxic azo dyes into non-toxic components.

2.4 Phytotoxicity assay using *Tagetes erecta* plants

There were significant differences between the control and the test plants. Phenotypic changes such as in shoot length, root length, number of leaves, branching etc. was observed (**Table 1**). Cytotoxic effect was clearly visible in transverse sections of treated plants in comparison to control plant as shown in **Fig. 6**.

2.5 Biototoxicity assay using brine shrimp hatchability test

The test results indicates that the dyes AR-79 and AR-80 are toxic on shrimps whereas degraded dye components possess non-toxic effect on shrimps. In the brine shrimp hatchability assay, the degree of inhibition was observed to be directly dependent on the concentration of the dye (AR-79 and AR-80), DA-79, DA-80, AE and TiO₂ NPs.

Table 1 Phenotypic parameters considered for phytotoxicity test for plant

Samples	Length (cm)	No. of leaves	Root length (cm)	Branching
Control	22	57	6.5	8
DA-79	16	78	7.5	10
DA-80	18.7	60	4.5	10
AR-79	12.7	43	4.7	5
AR-80	12	36	3.5	7
AE	20	63	6	9
TiO ₂ NPs	14.2	49	3	12

Degraded dye components exhibited very low toxicity effect towards the shrimps. The hatching rate of shrimp was increased to a greater extent from 25 to 100 µg/mL when the shrimps were treated with DA-79 and DA-80 compared to that of the shrimps treated with AR-79 and AR-80. It was observed that at higher concentration of dyes and the total number of brine shrimps were detrimental. The reduction of egg hatching at high concentration (100 µg/mL) when treated with dye may be because of the presence of some toxic substances that are inhibitory to brine shrimp.

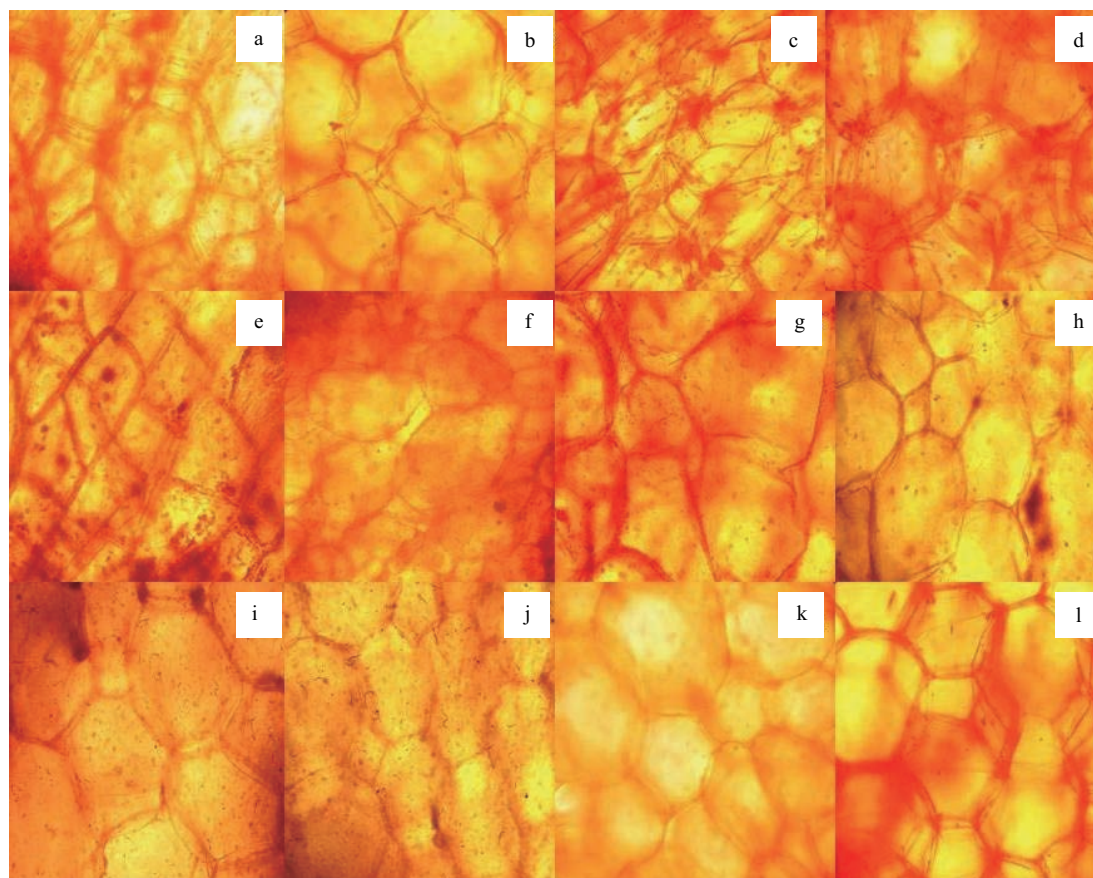


Fig. 6 Biototoxicity assay using *Tagetes erecta* plants (a) stem with DA-79, (b) stem with DA-80, (c) stem with AR-79, (d) stem with AR-80, (e) root with AR-79, (f) root with AR-80, (g) root with AE, (h) stem with AE, (i) root with TiO₂ NPs, (j) stem with TiO₂ NPs, (k) control root, and (l) control stem.

Table 2 Morphological characteristics of isolate PSV-3

Characteristics	PSV-3	<i>S. bluensis</i>	Characteristics	PSV-3	<i>S. bluensis</i>
Aerial mass colour	Brownish white	W (B)	Utilization of sole carbon sources		
Melanoid pigment	–	–	Arabinose	+	+
Reverse side pigment	–	–	Xylose	+	+
Soluble pigment	–	–	Inositol	+	+
Spore chain	Spiral	Spiral	Mannitol	+	+
			Fructose	+	+
			Rhamnose	+	+
			Sucrose	+	+
			Raffinose	+	+

2.6 Identification of Actinobacteria

The isolate PSV-3 possess LL-DAP and contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype I. The micromorphological observations of the isolate PSV-3 reveal that the strain belongs to the genus *Streptomyces*. The physiological characteristics of the isolate PSV-3 are given in **Table 2** and compared with those of the *Streptomyces* species given in the key of Nonomura (1974). Short, compact spiral shaped spore chains were observed under microscope (100x). Thus, PSV-3 was identified as *Streptomyces bluensis*.

3 Conclusions

Conventional dye-degrading treatments require huge expense as well as incessant input of chemicals which is uneconomical and may cause further environmental damage. Therefore, eco-friendly treatments need to be developed. In the present study, biosynthesis of TiO₂ NPs was successfully done with the help of marine actinobacteria. The synthesized TiO₂ NPs showed prominent dye degrading capacity compared to previous results. Moreover the products of dye degradation showed non-toxic nature towards biological test samples. Thus, our study suggests that marine actinobacteria as well as synthesized TiO₂ NPs possess a significant azo dye degrading capacity and can be applied in bioremediation of textile and other industrial effluents containing toxic dyes.

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