International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 11 (2014) pp. 105-115**
http://www.ijcmas.com

Original Research Article

Isolation, characterization and screening of actinomycetes from textile industry effluent for dye degradation

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Keywords

Textile industry effluent, Bioremediation, Indigenous microorganisms, Actinomycetes, *Streptomyces* spp., Azo dye degradation

A B S T R A C T

The principle aims of this study were to isolate dye degrading actinomycetes from textile industry effluent, to characterize the obtained isolates and to screen them for azo dye degradation. Experiments included collection of soil samples; isolation of actinomycetes by serial dilution and spread plate technique on ISP2 medium; characterization for their microscopic, physiological and biochemical properties; and screening for azo blue and azo orange dye degradation. Soil samples collected from two locations around textile dye industry at Palakkad, Kerala were used for isolation. Isolates obtained were identified as *Streptomyces* spp. on the basis of their morphological, physiological and biochemical properties. The two isolated *Streptomyces* spp., being indigenous in nature presented significant ability to degrade azo blue and azo orange dyes. However precise identification requires further studies including molecular characterization. Optimization of conditions to yield better degradation and further studies with these organisms will provide insights into metabolic pathways, responsible enzymes, and their role in bioremediation of textile industry effluents.

Introduction

Textile industry is one of the oldest and largest industries of India. Synthetic dyes are coloring agents mainly used in textile industries which generate a huge amount of wastewater in the process of dyeing. It is estimated that these industries discharge around 280,000 tons of dyes worldwide every year into the environment (Tom Sinoy *et al*., 2011). Discharge of these colored effluents into rivers and lakes results in reduction of dissolved oxygen concentration, thus creating anoxic condition and leading to the acute toxic effects on the flora and fauna of the ecosystem. In addition to being aesthetically displeasing, the release of colored effluents in water bodies reduces the photosynthesis as it impedes penetration of light in water

(Slokar and Le Marechal, 1998; Strickland and Perkins, 1995). The toxicity of effluent is because of the presence of dye or its degraded products which are mutagenic or carcinogenic. Pollution caused by dye effluent is mainly due to durability of the dyes in the wastewater (Jadhav *et al.,* 2007). Therefore, industrial effluents, like textile wastewater containing dyes must be treated before their discharge into the environment. The color removal of textile wastewater is a major environmental concern (Thakur *et al.,* 2012). Color can be removed from wastewater by chemical and physical methods including absorption, coagulation – flocculation, oxidation and electrochemical methods. These methods are quite expensive, have operational problems and generate huge quantities of sludge (Kapdan and Kargi, 2002; Sandhaya *et al.,* 2005). Thus, there is a need to find alternative treatments that are effective in removing dyes from large volumes of effluents and are low in cost. Biotreatment offers a cheaper and environmentally friendlier alternative for color removal in textile effluents (Bhargava and Jahan, 2012). The ubiquitous nature of microorganisms makes them invaluable tools in effluent biotreatment (Olukanni *et al.,* 2006). Strains of bacteria, fungi and algae can be used extensively in bioremediation of textile effluents. These microorganisms produce both constitutive and inducible enzymes to bioremediate chemical compounds present in wastewater (Gupta *et al.,* 2011). Though majority of bioremediation studies have been concentrated upon bacterial cultures not much work has been done using actinomycetes.

Actinomycetes represent a group of relatively abundant and metabolically diverse bacteria in soils (Labeda and Shearer, 1990). Actinomycetes now are being recognized for their degradative capacity of highly recalcitrant compounds. Actinomycetes have been shown to specifically degrade hydrocarbons (McCarthy and Williams, 1992), chlorinated solvents (Wackett *et al.,* 1989), explosives (Pasti-Grigsby *et al.,* 1996), plasticizers (Klausmeier and Osman, 1976) and azo dyes (Zhou and Zimmermann, 1993). The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. In this context, to develop a practical bioprocess for treating dye-containing wastewater it is important to isolate indigenous microbial strains (Chen *et al.,* 2003). Owing to these aspects, the present investigation was aimed to isolate indigenous microbes for remediative purposes from soil in contact with textile industry effluent. In the present study we isolated actinomycetes strains and by referring the Bergey's manual a series of biochemical tests were performed to identify the organisms (Holt *et al.,* 1994).

Materials and Methods

Sample collection

The samples were collected in the month of February from top 4.0 cm soil profile, near the effluent pipe of textile dye industry, where most of the microbial activity takes place. Samples were also collected from the soil near lake into which the effluent is released in Palakkad district. Soil sample (approx. 500g) was collected by using sterile spatula into clean, dry and sterile polythene bags along with, marking pen, rubber band and other accessories. The site selection was done taking care of various characteristics such as organic matter, moisture content, particle size and color of soil and avoids contamination as far as possible. Samples were stored in iceboxes and transported to the laboratory where they were kept in refrigerator at $4^{\circ}C$ until analysis.

Soil pH

Soil pH was determined according to the procedure described by Akpor *et al.* (2006) using Horiba make D-51 pH meter - Measuring object & amp; The material was separated on the ¼ in. (6.3 mm) sieve and the minus ¼ in. (6.3 mm) material was used for testing. An aliquot of 0.1g soil was placed into a glass beaker and mixed with 0.1 ml of distilled water to obtain soil slurry and then covered with watch glass. The sample was allowed to stand for a minimum of one hour, stirring every 10 to 15 minutes. This allows the pH of the soil slurry to stabilize at the room temperature. The temperature of the sample was measured and the temperature controller of the pH meter was adjusted to that of the sample temperature. The pH value was then recorded according to the instruction of the manufacturer.

Isolation of actinomycetes from soil sample

Each sample was divided in to two parts. One part was used as wet sample and the second part was placed in dryer for one week to be used as dry sample. The samples were serially diluted up to the 10^{-3} dilution. Aliquot of 0.2 ml of each dilution was inoculated in duplicate on to the plates of the ISP2 medium by the spread plate technique (Shirling and Gottlieb, 1966). All the inoculated plates were incubated at 37°C for 7 days. By the pure culture technique, strains of actinomycetes were isolated using medium incorporated with 0.5 mg nystatin and nalidixic acid as antifungal and antimicrobial agent respectively.

Phenotypic characteristics

It includes some basic tests: aerial mass color, reverse side pigment, melanoid pigments, spore chain morphology and spore morphology (Shirling and Gottlieb, 1966).

Aerial mass color: For the grouping and identification of actinomycetes the chromogenicity of the aerial mycelium is an important character. The colors of the mature sporulating aerial mycelium can be white, gray, red, green, blue and violet (Prauser, 1964). When the aerial mass color falls between two colors series, both the colors are recorded. In the cases where aerial mass color of a strain showed intermediate tints, then in that place both the color series are noted (Shirling and Gottlieb, 1966; Gayathri and Muralikrishnan, 2013). In this study the cultures were grown on starch casein agar (Tadashi, 1975) at 40°C for five days.

Reverse side pigments: The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and non distinctive or none (-). Colors observed for distinctive were greenish brown, brownish black or distinct brown pigment. A color with low chroma such as pale yellow, olive or yellowish brown was included in the latter group (-). In this study starch casein agar medium was used to grow the actinomycetes at 40°C for five days.

Melanoid pigments: The grouping was made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colors) on the starch casein agar medium. The strains were grouped as melanoid pigment produced (+) and not produced (-) (Pridham and Gottlieb, 1948). For the melanoid pigment observation the inoculated plates where kept under incubation for 4 to 5 days. The strains which show cultures forming a greenish brown to

brown to black diffusible pigment or a distinct brown pigment modified by other color were recorded as positive (+), total absence of diffusible pigment were recorded as negative (-) for melanoid pigment production (Gayathri and Muralikrishnan, 2013).

Spore chain morphology: The species with spore bearing hyphae are reported to be of three types: Flexible- Rectiflexibiles (RF), Open loops - Retinaculiaperti (RA) and Spira - Spirals (S). Characteristic of the spore bearing hyphae and spore chains was determined by the direct microscopic examination of the culture area, by the standard protocol of cover slip culture technique. Plates containing sterile ISP2 medium were prepared. After solidification, medium was scooped out from the central portion of the plate by a sharp scalpel making a rectangular area. Then three sterile coverslips were placed on the hollow rectangular space. Slowly actinomycetes spores were inoculated at the edge of the coverslips touching the medium. The plates were incubated at 28±2°C for 5 days and examined periodically taking out the coverslips (Gayathri and Muralikrishnan, 2013). Adequate magnification (40X) was used to establish the presence or absence of spore chains and to observe the nature of spore chains.

Spore surface morphology: Spore surface features were observed under Zeiss EVO-LS scanning electron microscope (SEM). The cover slip culture technique prepared for observation under the light microscope was also used for this purpose. The electron grid was cleaned and adhesive tape placed on the surface of the grid. The spore structures in actinomycetes were reported to be any of the four types-: smooth (sm), spiny (sp), warty (wa) and hairy (ha).

Physiological and biochemical characteristics

Assimilation of carbon sources: The ability of two actinomycete strains to use various carbon compounds as source of energy was studied by following the method recommended in International *Streptomyces* Project using carbon utilization medium with slight modification (Pridham and Gottlieb, 1948). Chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials were used. Stock solution of D-glucose, L-arabinose, sucrose, D-fructose, D-xylose, raffinose, D-mannitol, cellulose, rhamnose, inositol at 10% (w/v) was prepared separately in sterile water and sterilized by filtering through 0.22µm pore size membrane filters. In 100ml of basal mineral salt agar, 10ml of this sterilized carbon source was mixed to give a final concentration of 1% (w/v).

Plates were streaked and incubated at 37°C for 7 to 10 days. Growth were observed by comparing them with positive (D-glucose) and negative control (no carbon source). Results were recorded as follows: Strong utilization (++) referring to, growth equal to or greater than positive control; Positive utilization (+) designed to, growth apparently better than the negative control, but less than the positive control; Doubtful utilization (±) defined for, growth slightly better than negative control and apparently less than the positive control; Negative utilization (-) for growth similar to or less than the negative control (Seow *et al.,* 2010).

Sodium chloride tolerance: Different concentrations of sodium chloride were added to the starch casein agar medium to check the sodium at the final concentrations of 0, 5, 10, 15, 20, 25 and 35% (w/v). The

isolate was streaked on the agar medium, incubated at 37°C for 7–15 days and the presence or absence of growth was recorded on 7th day onwards.

Ability to grow at different pH: This test was carried out on ISP2 medium with pH 5, 6, 7, 8, 9 and 10 adjusted by using 1 N HCl and 1 N NaOH. Duplicate slants were prepared for each strain at each pH value. After inoculation and incubation of 10 days, readings were taken for each strain.

Utilization of cellulose: Carboxy methyl cellulose (CMC) was added to the ISP2 medium at the final concentration of 1% (w/v). The plates were inoculated and incubated for 7–15 days. A control plate without carboxy methyl cellulose (CMC) was used as standard. After 7 days of incubation onwards the plates were observed for the growth of actinomycetes.

Hydrogen sulphide production: For this test, the tryptone - yeast extract agar (Tadashi, 1975) slants were inoculated and incubated for 7 days at 37°C (Shirling and Gottlieb, 1966). After incubation the slants were observed for the presence of the characteristic greenish-brown, brown, bluish-black or black color indicative of H_2S production. The incubated tubes were compared with uninoculated controls.

Gelatin liquefaction: Liquefaction of gelatin is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyze this protein to amino acids. Once this degradation occurs, even at 4°C it will not restore the gel characteristic.

Gelatin deep tubes with 12% (w/v) gelatin were inoculated and incubated at 37°C. Observation was made after 7 days. The extent of liquefaction was recorded after keeping the tubes in cold conditions (4°C) for an hour. Cultures that remain liquefied were indicative of gelatin hydrolysis.

Hydrolysis of starch: The degradation of starch requires the presence of the extracellular enzyme amylase. For this test, cultures were grown for 5–7 days on ISP2 medium supplemented with 1% (w/v) of starch. The plates were flooded with Lugol's iodine solution after incubation and the development of clear zone around the culture streaks were recorded as the hydrolysis of starch.

Coagulation of milk: Milk coagulation was studied with skimmed milk (Himedia). The skimmed milk tubes were inoculated and incubated at 37°C. The extent of coagulation was recorded from 7th to 10th day of incubation.

Lipolytic activity: The strains were inoculated on to the ISP2 medium and incubated at 37°C for 10 days. After incubation the plates were treated with 1% (w/v) Tween 20 (Himedia) and lipolytic activity was evidenced by halo formation around the colony.

Evaluation of decolorisation capacity of the isolates with increased dye concentration

Decolorisation capacity of the two isolated strains were compared by preparing, various concentrations of dye from wastewater effluent (50, 100, 200, 300 and 400 l/L) (Zhao and Hardin, 2007). Decolorisation rate was also investigated under shaking condition. A fixed inoculum of 20 % (2 ml) was used in the study. The pH was maintained at 7.2. The percentage decolorisation was determined over 48 h period. The decolorisation was measured

both in the case of Azo blue and Azo orange dyes respectively using double beam UV-vis spectrophotometer (AU – 2701, Systronics, India) at 623 and 544 nm respectively.

Results and Discussion

Soil pH

The pH value was 6.3 for the sample near the effluent pipe and 6.8 for the sample near the lake.

Isolation and phenotypic characterization of actinomycetes

One strain was isolated from each of the two soil samples and they were named as isolate 1 (I1) from the soil sample collected near the effluent pipe and isolate 2 (I2) from the soil sample collected near the lake. The characteristics of both the isolates are presented in table 1. Both the isolated strains were Gram positive. The colour of the substrate mycelium was white for I1 and gray for I2. No pigment on the reverse side or melanoid pigment was produced by isolate I1 whereas isolate I2 generated colored pigment on reverse side and but it was negative for melanoid pigment. On examination under microscope, isolate I1 exhibited spira spore chain morphology and isolate I2 showed retinaculum spore chain. Spore surface morphology was studied under the scanning electron microscope (SEM) at different magnifications of 500, 3000, 10,000 and 20,000. Isolate I1 showed a smooth surface while isolate I2 exhibited spiny surface.

Physiological and Biochemical Characteristics

The ability of two actinomycetes strains in utilizing various carbon compounds as source of energy is mentioned in table 2. Isolate I2 was capable of utilizing all the carbon sources except arabinose whereas isolate I1 could not utilize sucrose, raffinose, fructose, rhamnose and arabinose.

Sodium chloride tolerance was observed only at 5% concentration and no growth at higher concentrations in both the isolates as shown in table 2. Both the strains showed pH tolerance at different ranges of pH 5–9 as shown in table 2. However, growth of isolate I2 was not observed at pH 5.

The results of biochemical tests are mentioned in table 3. Both the isolates were positive for catalase test, cellulose degradation, H_2S production, starch hydrolysis and caseinase activity. Isolate I1 was negative whereas isolate I2 was positive for lypolytic activity, while both the isolates were negative for gelatin liquefaction.

Based on phenotypic, physiological and biochemical characteristics the two isolates were identified to be *Streptomyces* spp., by matching the results obtained with the keys given for actinomycetes in ISP (International *Streptomyces* Project).

Decolorisation capacity of the isolates

Isolates I1 and I2 are positive for azo blue and azo orange dye degradation (Fig. 1a, b). The optical density was measured and shown in Fig. 2 and 3. Isolate I1 degraded azo blue more quickly within 48 h than Isolate I2, which is evidenced by decrease in O.D. (Fig. 2). The capacity of the isolates to degrade the Azo orange dye is shown in Fig. 3. It was noticed that Isolate I2 degraded azo orange efficiently than Isolate I1 within 48 h.

Table.1 Phenotypic characterization of the actinomycetes isolates

Sl. No.	Phenotypic Characteristics	Isolate I1	Isolate I2
1.	Gram Staining	Gram Positive	Gram Positive
2.	Substrate Mycelium	White	Gray
3.	Reverse side pigments	Absent	Present
4.	Aerial mass colour	White	Grey
5.	Melanoid pigments	present	Absent
6.	Spore chain morphology	Spirals	Retinaculum
7.	Spore surface morphology (SEM)	Smooth surface	Spiny surface

Table.2 Physiological characterization of the actinomycetes isolates

Sl. No.		Isolate -I1	Isolate -I2
	Carbon sources		
1	Negative control	+	+
2	Positive control	+	+
3	Xylose	+	+
4	Inositol	+	+
5	Sucrose	-	+
6	Raffinose	-	+
7	Fructose	-	+
8	Rhamnose	-	+
9	Manitol	+	+
10	Arabinose	-	-
	Sodium chloride tolerance		
11	Negative control	-	-
12	5 %	+	+
13	10 %	-	-
14	15 %	-	-
15	20 %	-	-
16	25 %	-	-
17	30 %	-	-
	Growth at different pH		
18	Negative control	-	-
19	pH-5	+	-
20	pH-6	+	+
21	pH-7	+	+
22	pH-8	+	+
23	pH-9	+	+

+ Growth; - No Growth

Table.3 Biochemical characterization of the actinomycetes isolates

Sl. No.	Biochemical characteristics	Isolate -I1	Isolate -I2
1	Cellulose degardation	+	+
2	H_2S Production	+	+
3	Gelatin liquefaction	-	-
4	Starch hydrolysis	+	+
5	Coagulation of milk	+	+
6	Lipolytic activity	-	+

+ Positive; - Negative

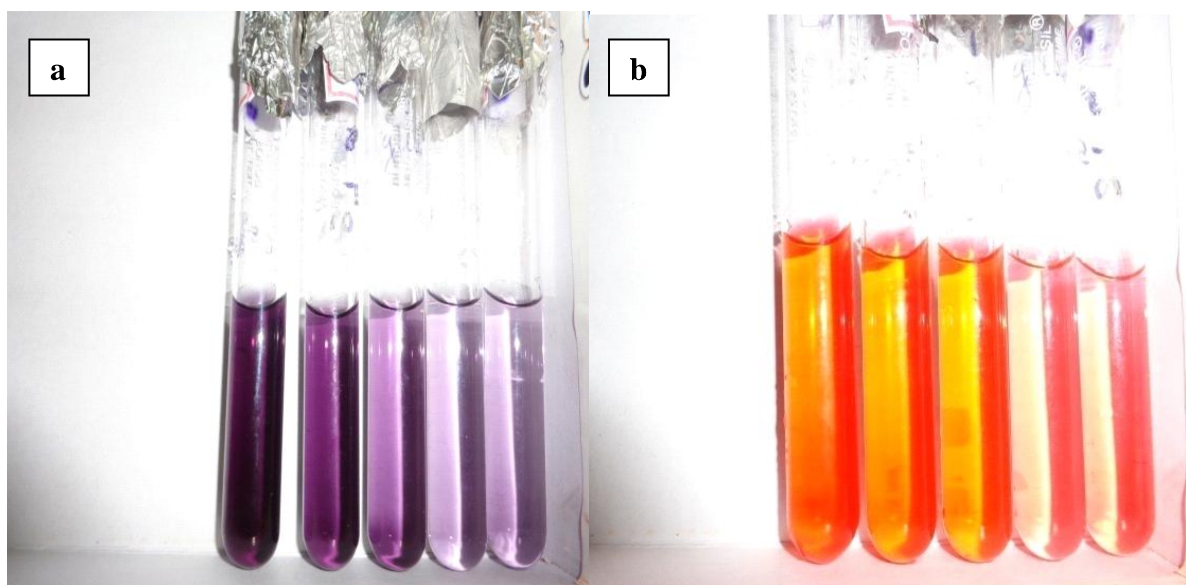

Figure.1 (a) Azo blue decolorisation (b) Azo orange decolorisation
(in the order of 0h, 12h, 24h, 36h & 48h from left to right)

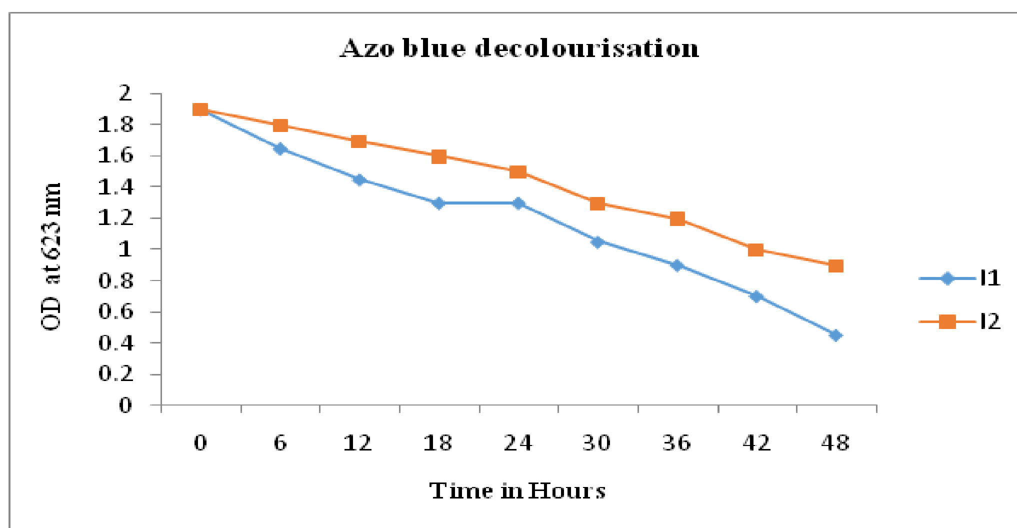

Figure.2 Azo blue decolorisation by actinomycetes isolates I1 and I2

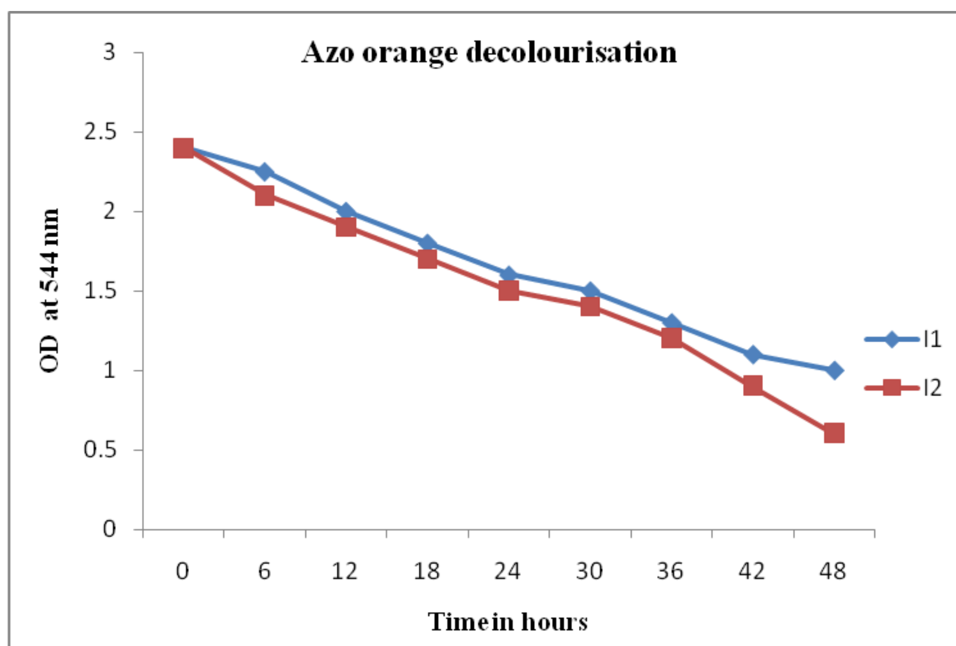

Figure.3 Azo orange decolorisation by actinomycetes isolates I1 and I2

Textile industry in India is a fast growing industry and its wastewater is rated as the most polluting among all industrial sectors considering both volume and composition of effluent (Hussain *et al.,* 2004). The release of dyes into the environment constitutes water pollution. Tight government legislation is forcing textile industries to treat their waste effluent to an increasingly high standard (Jadhav *et al.,* 2007). The treatment systems based on using microorganisms capable of decolorizing / degrading these recalcitrant compounds offer an environment-friendly clean-up process and are presently gaining prominence (Gupta *et al*., 2011).

Most of the dye degradation and effluent treatment systems are based on anaerobic digestions and focused on bacterial cultures. In the present work two actinomycetes strains were isolated from polluted soil collected from in and around textile dye industry at Palakkad, Kerala. The identification of the isolates as actinomycetes was done by conducting tests like aerial mass color, reverse side pigment, melanoid pigments, spore chain morphology and spore morphology. Characterization of the isolates was done by physiological and biochemical tests like carbon assimilation, ability to grow under various pH and salinity. Isolate I1 showed its potential to grow at broad range of pH from pH 5 to pH 9, whereas the isolate I2 proved to prefer slightly acidic to high alakaline pH (pH 9) for its growth and shows no growth below pH 6. Gelatin hydrolysis was not shown by any strain. But the amylase activity was shown by both the isolates. All these results are in par with the results obtained by previous works done by many researchers. The results indicated that the two strains belong to actinomycetes, specifically to the genus *Streptomyces*. However, precise identification requires further molecular characterization. Bacteria including actinomycetes have been identified by many workers based on these characteristics (Kuberan *et al.,* 2011; Pillai *et al.,* 2011).

The isolates were employed for the degradation of the azo blue and azo orange dyes and found to be effective decolourizing agents. From the results it is evident that both the isolates could decolourize the effluent significantly within 48 h. The effectiveness of microbial treatment systems depends upon the survival and adaptability of microorganisms during the treatment processes and indigenous microorganisms definitely have an edge over microbes from other alien sources in survival and adaptability (Chen *et al.,* 2003). Bioremediation using indigenous microorganisms is one of the most effective and efficacious method, which has no principally any harmful environmental effects (Najirad *et al.,* 2012).

In conclusion, the two actinomycetes strains (Isolate I1 and I2) isolated in the present study are indigenous microbes having significant dye degrading ability and could be employed for better bioremediation of textile effluents. However, further research is required to better understanding and effective employment of these isolates.

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