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Decolourization potentiality of Actinobacteria on synthetic textile dyes and effluents - A comparative study

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Abstract

Decolourization of potentiality of the isolates of actinobacteria obtained from the soil polluted with textile dye effluents and three synthetic dyes namely red M5B, orange M2R and blue G was determined. Twenty five isolates of actinobacteria were isolated from the polluted samples of soil collected from Karur, Tamil Nadu. From the 25 isolates screened to determine the decolorization potentiality, only seven isolates were selected for further studies. The isolates of *Nocardiopsis* sp. K2 and *Streptomyces* sp. K6 showed more percentage loss of intensity of colour against red M5B than orange M2R and blue G. Effect of pH, temperature, incubation period, culture media, carbon and nitrogen sources was optimized for suitable conditions for maximal decolourization.

Key words: Decolourization, *Nocardiopsis* sp. K2, *Streptomyces* sp. K6, optimization, Textile dyes.

INTRODUCTION

Textile industry is one of the most important water intensive industries and discharge large volumes of waste water containing variety of pollutants and colouring matters (Chung, 1992). Waste water discharged out is highly coloured and contain complex mixtures of several dyes. The suspended particles content of the dye effluent varies depending on the dying process, but it is generally in the range of 10-200 mg/l (Nigam *et al*., 1996). All synthetic dyes used in the textile industry are resistant to conventional waste water treatment processes (Shaul *et al*., 1991). Several physico-chemical methods have been used to eliminate the coloured effluents in waste water (Reife, 1993). But they are generally expensive, limited applicability and cause the accumulation of a large quantity of sludge. Therefore, the development of systems using microorganisms for the treatment of textile dye effluents systems has become a viable alternative. Microbial decolourization processes have the advantage of being simple in design and low in cost when compared with conventional methods. It does not produce any hazardous toxic products and cause problems of biomagnifications (Robinson *et al*., 2001).

Several microorganisms have been used to decolourize effluents from different sources because of their ability to degrade under *in vitro* conditions (Salah Uddin *et al*., 2007). Among the microorganisms, bacteria are the most commonly used for various bioremediation processes. Nevertheless, need of the hour is to screen novel microorganisms for the effective degradation of environmental waste/pollutants. However, the reports on biodegradation of textile dyes by actinobacteria are scanty and their potentiality is not yet explored completely (Ayed *et al*., 2009**).** Actinobacteria play a vital role in the biodegradation processes during biological treatment. The present study deals with screening of the actinobacteria to determine the decolourizing potentiality against textile dye effluent and synthetic dyes, optimization of the suitable conditions for maximum decolourization, and characterization of the potent decolourizing organisms phenotypically.

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MATERIALS AND METHODS

Collection of soil sample and effluent and isolation

The soil sample and the real dye effluent from the first alkaline extraction stage were collected from dye polluted area of Karur District, Tamil Nadu, India and used in this study. Soil samples were serially diluted and the actinobacteria were isolated by spread plate technique on starch casein agar (SCA) medium (g/L; starch 10; casein 0.3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ $7H_2O$ 0.05; $CaCO_3$ 0.02; $FeSO_4$. $7H_2O$ 0.01; agar 18) supplemented with amphotericin B 50 µg/mL and cycloheximidine 50 µg/mL to reduce other bacterial and fungal contaminations. After incubation for 7 days at 30±2°C, the actinobacterial colonies were purified and maintained in SCA medium for further assay.

Screening and selection of actinobacterial isolates

All the isolates of actinobacteria were screened for determining the decolourization ability against both real textile dye effluent and synthetic dyes. Ten milliliter of spore suspension of each actinobacterial isolate was inoculated into flasks containing 100 mL effluent basal medium (Zhout Zimmerman medium) (g/L: yeast extract 5; glucose 5; NH_4 $2SO_4$ 0.5; K_2HPO_4 2.66; Na_2HPO_4 4.32; dye 100 mg) and incubated at 30±2°C for 8 days. Eight milliliter sample was withdrawn aseptically at different time intervals of 2, 4, 6 and 8 days. The samples were centrifuged at 8000 rpm for 15 min. Then the clear

supernatant was taken and its absorption was determined at the absorption maxima (at 465 nm) of textile dye effluent. The actinobacterial isolates with maximum decolourization ability were selected for further study.

Tolerance of actinobacteria against textile dyes

Plate assay

Maximum concentration of the synthetic dyes that could be tolerated by the isolates of actinobacteria was determined by the standard method described by Hernandez *et al*. (1994). One loop full of spore suspension of actinobacterial isolates was transferred to 1 mL of sterile distilled water and vortexes to get uniform suspension. An aliquot of 20 µL of this suspension was spotted on effluent basal agar plates with variable concentration (200, 400, 600 and 800 µg/mL) of the dyes. Growth of the actinobacteria on the plates was compared with their growth on control plates without dye after 8 days of incubation at 30±2°C. Actinobacteria which could tolerate relatively high concentration of dye were selected for further analyses.

Broth assay

Ten milliliter of the spore suspension of actinobacteria was inoculated in to 100 mL of effluent based medium containing different synthetic dyes namely orange M2R, blue G and red M5B, and incubated for 7 days at 30±2°C. The samples were centrifuged at 8000 rpm for 15 min at different time intervals of 2, 4, 6 and 8 days, and the clear supernatants were used for the spectrophotometric determination of the rate for color removal.

The percentage of decolourization was calculated using the following standard formula:

$$\% \text{ Decolourization} = \frac{\text{Initial OD} - \text{final OD X 100}}{\text{Initial OD}}$$

Optimization of decolourization potentiality
Effect of incubation period

The decolourization of effluent and synthetic dyes (200 µL/100 mL of liquid effluent basal medium) by actinobacteria was determined separately. The selected isolates of actinobacteria were inoculated into effluent basal medium and incubated at 30±2°C for 8 days. The samples were withdrawn at two day intervals and the decolourizing activity was determined spectrophotometrically at 465 nm.

Effect of PH

Initial pH of the effluent and synthetic dyes solution was adjusted to 3, 5, 7, 9 and 11 with 1N NaOH/HCL for decolorization assay. The effluent samples were centrifuged at 12,000 rpm for 5 min and filtered through Whatman No.1 filter paper. The inoculated medium was incubated at 30±2°C for 8 days. The decolourization rate was determined spectrophotometrically at 465 nm.

Effect of temperature

The decolourization effect of selected actinobacteria was determined by inoculating the culture in to the effluent basal medium and incubating at different temperature such as 20, 30, 40 and 50°C for 8 days. The samples were withdrawn after 8 days and the rate of decolourization was determined.

Effect of NaCl

The selected isolates were inoculated into effluent basal medium containing the concentrations of NaCl including 0.1, 2, 4, 6 and 8%. After 8 days, the samples were withdrawn for determination of decolourizing activity of actinobacteria as mentioned earlier.

Effect of carbon sources

Ten milliliter spore suspension of each actinobacterial isolate was added separately in to 100 mL of effluent basal medium supplemented with carbon sources such as glucose, starch and mannitol at 1% level. All the flasks were incubated at the 30±2°C for 8 days and the decolonization of the samples was analyzed as described earlier.

Effect nitrogen sources

Ten milliliter of spore suspension of each isolate was added into 100 mL of effluent basal medium, and was separately supplemented with three different nitrogen sources such as ammonium sulphate (1%), sodium nitrate (1%) and urea (0.5%). After 8 days of incubation, the decolourizing ability of the isolates was analyzed as described earlier.

Effect of consortium on decolourization

The isolates for the consortium development were selected based on decolourization efficiency (>55%) and the ability to degrade variety of dyes. The cultures of actinobacterial consortium was inoculated into effluent and synthetic dyes, and incubated for 8 days. After incubation, the decolonization efficiency was determined.

Characterization of potential decolourizers

Morphological characteristics

Based on the potentiality to remove the colours from the dye effluent and synthetic dyes only two isolates K2 and K6 were selected for further characterization. Morphological characteristics of the strains were observed using slide culture technique on SCA medium (Williams and Cross, 1971; Pridham and Tresner, 1974). After incubation of 7-10 days the slides were observed under the light microscope (Leitz Diaplom, Germany).

Biochemical, physiological and cultural characteristics

Biochemical characteristics of the strains were determined following the methods of International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). Production of H_2S, urease, catalase and oxidase, reduction of nitrate and hydrolysis of gelatin, casein, starch and lipid were determined. The ability to grow at various temperatures (25-50°C), range of pH (5-9) and in different concentrations of NaCl (1-4 g/L) on medium was also tested. Cultural characteristics of the strains were determined following the incubation period of 4-7 days at 30±2°C on culture media recommended by the ISP and other culture media namely, yeast extract malt extract agar (ISP medium 2), oat meal agar (ISP medium 3), glycerol-asparagine agar (ISP medium 5), peptone-yeast extract iron agar (ISP medium 6) and SCA (Himedia, Mumbai). After incubation, the growth, colour of spore mass, reverse side colour and production of diffusible pigments by the strains were observed.

Chemotaxonomy

Isomers of diaminopimelic acid (DAP) in cell wall hydrolysates and whole cell sugar of actinobacteria were determined by thin layer chromatography (TLC) following the standard methods of Boone and Pine (1968) and Lechevalier and Lechevalier (1970, 1980).

RESULTS AND DISCUSSION

In the present study, a total of 25 isolates were isolated from the dye effluent polluted soils of Karur. Morphology of the colony, spores and mycelia of all the isolates was determined on the basis of growth on SCA medium. The spore morphology of the isolates showed various structures such as spiral, filamentous, spirally twisted and elongated aerial mycelium. In the present study, white, grey, yellow and pink colour spore mass producing isolates were found predominantly. Similar type of study has been reported by Vijayakumar *et al*. (2015) from marine soils of East Coast of South India.

All the 25 soil actinobacteria were screened for their decolorizing activity of textile dye and some synthetic dyes (orange M2R, blue G and red M5B). Among them, 28% (n=7) of the isolates showed decolourization capability after 8 days when tested with both effluent and synthetic dyes. Of the seven isolates, three isolates (K2, K6 and K7) decolorized the dye effluent and four isolates (K2, K4, K5 and K6) produced zone of clearance around the mycelia against all the three synthetic dyes on effluent basal medium. All the four isolates were tolerant up to 400 µg/100 mL of synthetic dyes. It was found that maximum colour removal of about 61% and 59.2% was observed with strains K6 and K2 respectively for the textile dye effluent. About 72%, 69.3% and 47% of decolourization was recorded with the strain K6 against the synthetic dyes namely red M5B, Orange M2R and blue G respectively.

The colour removal ability of the microbes is often influenced by the load of microbial cells, components of medium and cultural conditions such as aeration, agitation, pH, temperature, salinity, time course, nitrogen and carbon sources, which often vary from organism to organism. In the present study, the actinobacteria isolated from dye effluent polluted soils exhibited the characteristic removal of colour from both effluent and synthetic dye which commenced from second day onwards. Maximum colour loss, 70 and 67.5%, was caused by the strains K2 and K6 respectively against the synthetic dyes red M5B on 8th day. But, both K2 and K6 strains decolourised the textile dye effluent at 60% level on 8th day (Fig. 1; 2). It has been reported that the rate of decolourization progressively increased up to 8 days, and after this period of time the rate of decolourization gets decreased (Asad *et al*, 2007).

Actinobacteria are marked by relative intolerance of acidity, usually preferring neutral or slightly alkaline soil for the growth (Flaig and Kutzner, 1960). Maximum colour removal of the dye effluent (54%) was observed by strains K6 at pH 11, whereas strain K2 showed only 40% level of colour loss at the pH 9 and 11. On the contrary maximum colour loss of three synthetic dyes (blue G 59%; orange M2R 54.8%; red M5B 48%) was observed with the strain K2 at pH 9. Similarly, maximum degradation of all three synthetic dyes was observed (red M5B 65%; blue G 61%; orange M2R 57%) with the strains K6 at pH11. In the present study, it was found that the optimum pH for the colour removal of the textile dye effluent and three synthetic dyes was slightly alkaline (pH's 9-11) (Fig. 3; 4). However, maximum colour loss was observed against untreated paper mill effluent at pH 4.5 (Hernandez *et al.*, 1994).

Temperature is a key factor that influences the growth, and all the physiological, biochemical and metabolic activities of the microorganisms. In the present study, among the 4 different temperatures tested for decolourization, maximum colour loss of the effluent was 36% at 40°C and 43% at 30°C was recorded with the isolates K2 and K6 respectively. But, the effluent inoculated with actinobacteria incubated at other temperatures (20, 40, 60°C) showed only 20-40% of decolourization activity. The three synthetic dyes were degraded moderate to minimum at all the temperature tested. Optimum decolourization temperature of the synthetic dyes was 30°C for both actinobacterial strains. Maximum colour loss of synthetic dye orange M2R (47% and 54.1%) was observed for both the strain K2 and K6 at 30°C (Fig. 5, 6). In spite of the fact that the red M5B decolorization was achieved over a temperature range of 25-40°C, the optimum colour removal by the tested bacterium was observed at 30-35°C, and a sharp decline was recorded beyond the optimum temperature (Mabrouk and Yusef, 2008). Thus, the present and

Table 1. Phenotypic features of potential decolourizing actinobacteria

Properties	*Nocardiopsis* sp. K2	*Streptomyces* sp. K6
Microscopical characteristics		
Gram staining	Gram positive	Gram positive
Acid fast staining	Non acid fast	Non acid fast
Spore morphology	Totally sporulated short chain aerial mycelium	Long chain
Presence of aerial mycelium	Present	Present
Presence of substrate mycelium	Present	Present
Cultural characteristics		
Yeast extract malt extract (ISP Medium 2)		
Aerial mycelium	Light grey	Grey
Substrate mycelium	Dark brown	Dark brown
Oat meal agar (ISP-3)		
Aerial mycelium	Dark grey	Light grey
Substrate mycelium	Dark brown	Brown
Glycerol asparaginase agar (ISP-5)		
Aerial mycelium	Dull grey	Grey
Substrate mycelium	Yellow	Dark brown
Peptone iron yeast agar (ISP-6)		
Aerial mycelium	Grey	Dull grey
Substrate mycelium	Creamy white	Brown
Starch casein agar		
Aerial mycelium	Grey	White
Substrate mycelium	Dull grey	Brown
Biochemical characteristics		
H_2S production	-	-
Nitrate reduction	-	+
Urease production	+	+
Gelatin hydrolysis	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Lipid hydrolysis	+	+
Catalase	-	-
Oxidase	-	-
Chemotaxonomic characteristics		
Whole cell sugar	-	-
Cell wall amino acid	-	-

- = negative; + = positive

previous observations could be attributed to the thermal deactivation of the decolourizing enzymes and production of low biomass.

The ability of microorganism to tolerate high concentrations of NaCl in media which could also influence the growth and metabolic activities of the actinobacteria is well known. In the present study, among the five different NaCl concentrations tested for the decolourization of textile dye effluent and synthetic dyes mediated by actinobacteria, the colour loss 50% and 43.2% was exhibited by K2 and K6 respectively, when compared the said effect over the media without NaCl, the rate of colour removal decreased with increase in the NaCl concentration. Both K2 and K6 strains showed maximum degradation of 3 synthetic dyes in

Table 2. Physiological features of potential decolourizing actinobacteria

Properties	*Nocardiopsis* sp. K2	*Streptomyces* sp. K6
Effect of temperature (°C)		
20	+++	+++
30	++++	++++
40	+++	+++
50	+	+
Effect of pH		
5	++	++
6	+++	+++
7	++++	++++
8	+++	+++
9	+++	+++
Effect of NaCl (%)		
0 (without NaCl)	++	++
1	+++	+++
2	++++	++++
3	+	+
4	-	-

- = nil; + = poor; ++ = fair; +++ = good; ++++ = excellent

the medium which did not have salinity, whereas the other salts concentrations showed negative effect as there was moderate to minimum colour loss. Remarkably, both the strains K2 and K6 removed the colour of blue G upto 65% and 56.1% respectively in media without NaCl (data not shown).

Carbon and nitrogen sources are essential nutrients for growth, survival and biological activities of the microorganisms. In the present study, it was found that the effluent basal medium supplemented with three different carbon sources showed dye effluent decolourization 63% and 68% with strains K2 and K6 respectively when they were grown in the media with glucose as carbon source, followed by 51% for K2 and 56% for K6 in starch containing broth and 35% for K2 and 32.3% for K6 in mannitol containing broth. Further, all the three synthetic dyes were decolourized maximally in the glucose containing culture broth (data not shown). Das *et al.* (1995) reported glucose as the best source of carbon for the effective decolourization of crystal violet for *Penicillium chrysogenum*. Similarly, it has also been reported that the glucose was best carbon source for maximum decolourization of synthetic dyes and textile dyes (Sani and Banerjee, 1999).

The expression of decolourization potentiality of the strains K2 and K6 was highest 63.2 and 66% respectively in the media supplemented with urea as the nitrogen source; moderate, 33 and 45% respectively in the medium with sodium nitrate as nitrogen source; and it was very less 12.7 and 19% respectively in the medium with ammonium sulphate. Similarly, all the synthetic dyes also lost their colour when they were tested with urea as a nitrogen source, whereas other nitrogen sources allowed only moderate to minimum decolourization by the actinobacterial strains K2 and K6 (data not shown). It has been reported that the maximum per cent decolourization (41.3%) was achieved by a bacterium for the red dye with urea as nitrogen source (Sivarajasekar *et al.*, 2008). Earlier, in several reports, yeast extract and peptone were used as nitrogen source resulting in higher decolourization of textile dyestuff, but they could not be used as cheap nitrogen source for industrial scale. Hence it becomes academic rather than their use in large scale. Thus the present study lends support to the view that urea could be used as a very good nitrogen source in the studies on decolourization.

Many researchers have mentioned that a higher degree of biodegradation and mineralization can be expected when co-metabolic activities within a microbial community complement each other. In such a 'consortium', the organism can act synergistically on a variety of dyes and dye mixture. One organism may be able to cause a biotransformation of the dye, which consequently renders it more accessible to another organism that otherwise is unable to attack the dye (Nigam *et al.*, 1996). However, in the present study, only one actinobacterial consortium was developed using two potent decolorizing agents (K2 and K6), and analyzed for effluent treatment. It was found that the textile effluent was decolourized by actinobacterial consortium at 58.7% level, followed by the synthetic dyes red M5B 65%, orange M2R 53.2% and blue G 51% (data not shown). The growth of cultures as consortium showed good growth in agitation culture rather than static culture, but the colour removal was best in static culture as there was 75-100% of colour removal from the 4 dyes and dye mixture (Xu and Leburn, 1999). Similarly, Kim *et al.* (2003) observed the reduction of COD and maximum decolourization of textile effluent when microbial combinations were used.

The two potential decolourizing actinobacteria are characterized based on the standard procedures of ISP. Both the strains were gram positive and non-acid fast. The strain K2 developed short chain spores on aerial mycelium, whereas the strain K6 developed short chain spores on aerial mycelium (Table 1). Both the strains K2 and K6 were cultured on five different culture media and the isolates showed variations in colour of aerial mycelium such as grey, white, pink and yellow and they also varied in the colour of substrate mycelium, but none of them produced diffusible pigments (Table 1). Biochemically, the isolate K2 showed positive response to urease activity and hydrolyses of starch, gelatin, casein and lipid. Similarly, the isolate K6 showed positive response to nitrate reduction, urease and hydrolyses of starch, gelatin, casein and lipid, and negative for catalase and oxidase. Both the isolates were

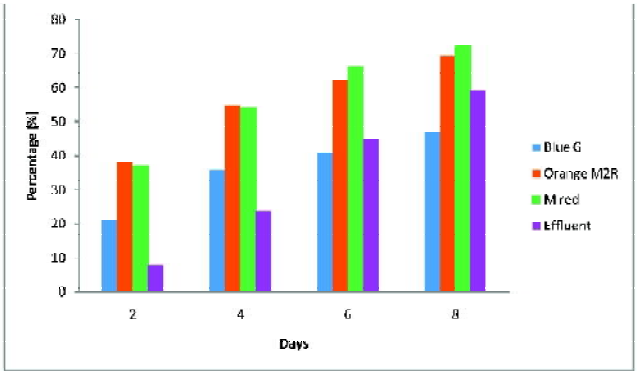

Fig. 1. Time course of decolourization by *Nocardiopsis* sp. K2

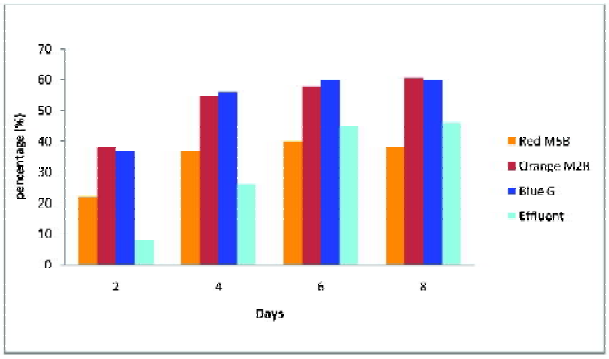

Fig. 2. Time course decolorization by *Streptomyces* sp. K6

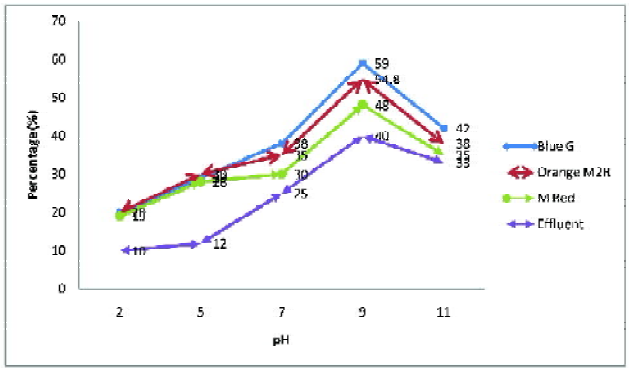

Fig. 3. Effect of pH on decolourization by *Nocardiopsis* sp. K2

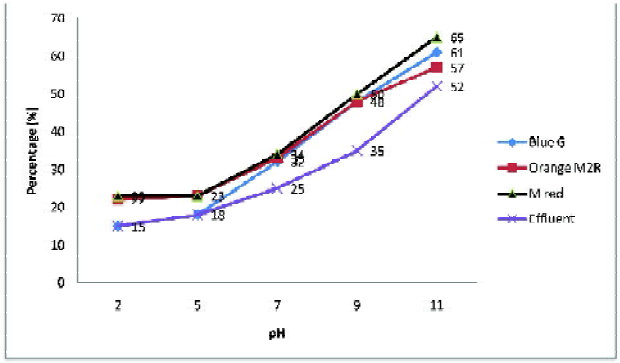

Fig. 4. Effect of pH on decolourization by *Streptomyces* sp. K6

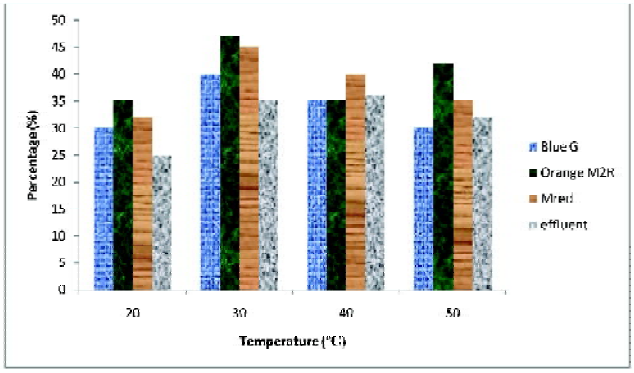

Fig. 5. Effect of temperature on decolourization by *Nocariopsis* sp.K2

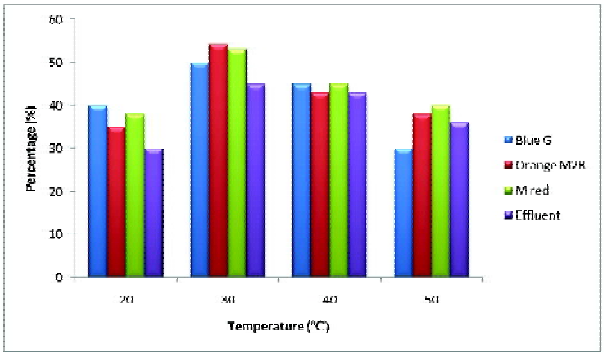

Fig. 6. Effect of temperature on decolourization by *Streptomyces* sp. K6

negative for H_2S production, catalase and oxidase, and the strain K2 did not reduce nitrate (Table 1). Significantly, the chemotaxonomy is one of the most useful techniques for the generic level identification of actinobacteria (Lechevalier and Lechevalier, 1980). The TLC analysis of the present study, showed that the strain K2 possessed mannose sugar while, the strain K6 contained diaminopemilic acid in their cell but no sugars were found in their cell wall (Table 1). Both isolates grew well at temperature 30°C, pH7.0 and NaCl concentration 2 g/L. The actinobacteria showed moderate to minimum growth in other temperatures, pHs and salinity conditions, the growth was completely absence at 4% salinity level (Table 2). Based on the phenotypic properties, both potential decolourizing actinoabcteria used in the present study were identified as *Nocardiopsis* sp. K2 and *Streptomyces* sp. K6. The findings of the present study are in line with the reports made by many workers (Sivakumar *et al.*, 2005; Remya and Vijayakumar, 2008; Vijayakumar *et al.*, 2007). On the basis of the above facts, the identified isolates of actinobacteria of the present study could potentially be used in the decolourization of dyes and dye effluents.

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