



Decolorization and degradation of methyl orange by *Bacillus stratosphericus* SCA1007



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ABSTRACT

The present study deals with complete decolorization and degradation of sulfonated azo dye (Methyl orange) by *Bacillus stratosphericus* SCA1007. These classes of dyes are difficult to degrade by conventional treatment methods. To overcome this problem, an environment friendly and efficient method was developed to degrade this recalcitrant dye. *Bacillus stratosphericus* SCA1007 could carry out complete decolorization of Methyl orange in a wide range up to 150 mg/L of dye concentration, at temperature 35 °C and pH 7 under static condition along with yeast extract in minimal salt media. This is the first study demonstrating complete decolorization of Methyl orange by *Bacillus stratosphericus* SCA1007 under optimized conditions within 12 h of incubation. The degradation of dye was studied by UV-vis and FTIR. Toxicity studies were performed on *Escherichia coli* and *Vigna radiata* to confirm the non-toxic nature of the degraded products.

1. Introduction

The industrial revolution which made mass production possible was a turning point in the history of mankind. However, industrialization did not come without any cost. While on the one hand, it made humans more affluent and facilitated economic growth of nations, on the other it also resulted in environmental pollution covering the entire spectrum from water to land and air. Over the years, with global warming and climate change coming to the fore, there is greater concern and call for mitigating environmental pollution.

Changing economic patterns and growing industrialization have resulted in pollution of our water bodies and deterioration in quality of water. The worsening appearance of rivers and the deteriorating quality of their waters led to the worsening of citizens' health (Haseena et al., 2017). Various human activities have resulted in contamination of water bodies. One of the major sources of such contamination is effluents released by industries. In recent years, there is an increased interest in analysis of causes of environmental pollution, one of which has been industries using dyes. Because of inherent toxic nature of dyes, they pollute water. The dyeing industry has been listed by Central Pollution Control Board as heavily polluting industries (Rajan 2014).

Colored effluent is the first sign of water pollution (Ali, 2010). Discharge of colored effluent pollutes the receiving water bodies. Uses of this water has serious implications on environment and human

health (Akarslan and Demiralay, 2015). The untreated effluents make their way to agricultural land making serious impact on human (Li et al., 2017). The discharge of these colored effluents reduces the light penetrating capacity of water, thus affecting the photosynthetic activity of aquatic flora, thereby affecting source of food of the aquatic ecosystem (Elumalai and Saravanan, 2016).

Azo dyes are group of synthetic dyes, which are majorly used in paper printing, textile industry, food, leather and tanning industry, paper making and cosmetic industry (Popli and Patel, 2015). It is estimated 10–15% of the total produced dye is lost during their synthesis and dyeing processes (Pirkarami and Olya, 2017). Methyl Orange is a sulphonated azo dye. It is also used as pH indicator in laboratories. It is brightly colored and highly soluble in water, which makes it difficult to remediate through conventional treatment system. Dyes having benzidine group attached are tumorigenic and carcinogenic due to their biotransformation to benzidine compound (Pillai, 2017).

Different physical-chemical methods used are filtration, adsorption, precipitation, chemical oxidation, coagulation, electrolysis and photodegradation for remediation of dyes waste water, but these methods have major disadvantages like limited versatility, high cost, low efficiency, interfering with other wastewater components (Sharma and Roy, 2015). Because of chemical instability of these pollutants, traditional wastewater treatment techniques have been proven ineffective in remediation of waste water having synthetic dyes. To develop efficient,

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environmentally friendly technologies to remediate dyes in wastewater to the permissible limit at affordable cost is of prime importance (Megha et al., 2015). The removal of synthetic dyes from industrial effluent through microorganisms have considerable benefits like low processing costs, non-toxic products or complete mineralization (Bhatia et al., 2017).

Dye decolorization is mainly followed by two processes: (i) adsorption of dyes by microbial biomass (ii) degradation of dyes by microbial cells. In adsorption, the dye does not break down to smaller fragments thus keeping intact, the original structure of the dye. But in degradation process, the parental structure of the dye is destroyed, or split into fragments or in some cases converted into CO₂, H₂O and inorganic salts (Zhou and Zimmermann, 1993). Diverse microorganism like fungi, bacteria, yeast and algae can decolorize and can completely mineralize many azo dyes (Garg and Tripathi, 2017). These includes *Aeromonas hydrophila*, *Citrobacter* sp, *Enterobacter* sp, *Aspergillus niger*, *Alternaria solani*, *Candida krusei*, *Gloeocapsa*, *Pleurocapsoides* (Ali, 2010). Bacterial decolorization is generally faster than fungi, therefore more preferred. However, a few reports are available related to decolorization by gram positive bacteria (Ali, 2010).

To the best of our knowledge this is the first report on dye decolorization by a gram-positive strain *Bacillus stratosphericus* SCA1007 within 12 h of incubation under optimized culture conditions. Moreover, the studied bacterium was found to be highly tolerant to high salinity conditions.

2. Material and methods

The chemicals under study complied with prescribed analytical grade. Methyl Orange was procured from Sigma Aldrich (Bangalore, India). Nutrient media (in g/L: Yeast extract 5, Peptone 10, Sodium chloride 5 and minimal salt media (in g/L: Yeast extract (1%), NaCl 1.0, CaCl₂ 0.1, MgSO₄.7H₂O 0.5, Na₂HPO₄ 1.0, KH₂PO₄ 1.0, agar (solid media) and carbon source (1%) was obtained from HiMedia (Mumbai, India).

2.1. Batch decolorization analysis

Soil samples were collected from local dyeing area of Bihar and Jharkhand and were serially diluted followed by spreading of sample over nutrient agar plate. Biochemical, morphological and molecular characterizations (by 16S rRNA gene sequencing by Xcelris Labs, Ahmedabad, India) were performed. 16S rDNA sequences of bacterial isolate SCA1007 have been submitted to GenBank under accession numbers KY992944 and was identified as *Bacillus stratosphericus* (<https://www.ncbi.nlm.nih.gov/nuccore/KY992944>).

The sulphonated azo dye decolorization was demonstrated in 250 ml flask having 100 ml of minimal salt media. *Bacillus stratosphericus* SCA1007 was inoculated in the reaction media. The inoculum size was fixed to optical density 1.0 (λ_{\max} = 600 nm, 1.50 X 10⁶ cells/ml) and incubated under shaking condition (120 rpm) at 35 °C. Thereafter, ambient culture conditions were used in successive experiments. Aliquots (2 ml) was withdrawn after incubation and centrifuged to separate the bacterial biomass. The absorbance of culture supernatant was recorded. λ_{\max} of test dye was determined. Decrease in Methyl orange concentration, determined by the calibrated curve of dye (methyl orange) concentration versus absorbance, was recorded. Decolorization activity was studied by using absorbent (D), to calculate percentage decolorization or the concentration of dye removed (I) according to following formulae (Dos et al., 2007):

$$D(\%) = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

$$I(\%) = \frac{A_1 - A_2}{A_1} \times 100$$

Where,

A₁ Dye Concentration (mg/L) of in control sample

A₂ Residual Methyl orange concentration (mg/L) in media

Bacteria growth in culture flask was determined by calculating optical density at 600 nm at regular interval. To study the impact of different physical factors, the batch decolorization experiments were performed under static culture conditions, temperatures (4–55 °C), initial pH (4–8) and dye concentration (25–400 mg/L). Guaiacol Agar plate assay was done to determine the presence of laccase enzyme (Kiiskinen et al., 2004; López et al., 2006). Screening was done on nutrient agar plate supplemented with 0.5 mM guaiacol and incubated at 35 °C for 48 h. All the experiments were performed in triplicates. Standard deviation was calculated from three independent experiments.

Products obtained after a fixed period of incubation were extracted and mixed with an equal quantity of ethyl acetate. Anhydrous sodium sulphate (Na₂SO₄) was used to dry the extracted product further in the rotary vacuum evaporator. Fourier transform infrared (FTIR) analysis of extracted product was done using Shimadzu corporation (Japan) spectrophotometer in the mid-IR region of 500–4500 cm⁻¹ with scan speed of 16 min⁻¹.

2.2. Toxicity study

Microbial and phytotoxicity study of Methyl Orange dye and its degraded metabolite was studied to check the toxicity of textile effluent. Dye and its degraded products obtained were dissolved in 5 ml of distilled water. This final volume was utilized for microbial toxicity study in relation to *Escherichia coli*, while same concentration of dye was taken as control. Microbial growth inhibition zone was recorded after incubation period of 24 h at 35 °C (Shah et al., 2013b). Phytotoxicity study was carried out at room temperature using *Vigna radiata*. Surface sterilization of seeds was done using mercuric chloride solution to inhibit growth of fungus. 10 seeds of *Vigna radiata* were taken in petri plate. These were watered with 10 ml sample of Methyl Orange (150 mg/L) and its degradative products (Parshetti et al., 2006). Control was set using water. After 7-days, germination rate (%) and length of the root and shoot were recorded. The mean was calculated with the standard deviation (±), which was statistically analyzed by Student's t-test.

3. Result and discussion

3.1. Decolorization analysis

Novel strain of *Bacillus stratosphericus* SCA1007 was found to completely decolorize Methyl Orange within 12 h of incubation under static condition. Decolorization of Methyl Orange was confirmed by UV-Visible spectral analysis (Fig. 1). Decolorization of Methyl Orange was found to be more due to degradation than adsorption. Bioadsorption and degradation are two processes followed by decolorization. Dye bioadsorption may be evident from examination of the bacterial cells. Bacterial cell mass with bioadsorption property will be colored, whereas with degradation property, cell will be colorless. Methanol extract of cell pellets after 12 h incubation with dye indicates that the dyes were not adsorbed on to cell biomass (data not shown). This observation concludes that decolorization of dye was majorly because of degradation, then bioadsorption to bacterial biomass. Presence of colored bacterial biomass, after 6 h of incubation may be demonstrated by fact that bioadsorption is often the preliminary step in the mechanism involved in transportation of dye into the cytoplasm of cell which ultimately leads to biodegradation of dye by viable cells (Ogugbue and Sawidis, 2011). The growth of bacteria and time taken to decolorize Methyl Orange by *Bacillus stratosphericus* SCA1007 are shown in

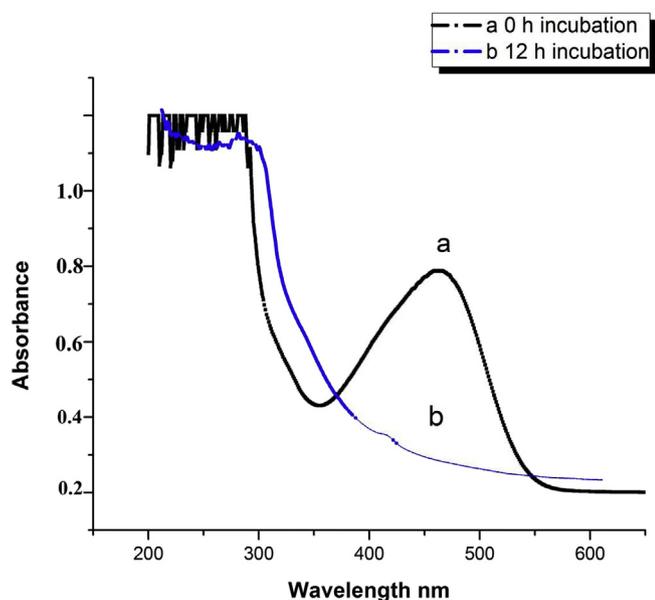


Fig. 1. UV/visible spectra of Methyl orange dye before (a) and after decolorization (b) by *Bacillus stratosphericus* SCA1007.

(Fig. 2). Laccase enzyme been produced by *Bacillus stratosphericus* SCA1007 which is confirmed by appearance of reddish-brown zone around bacteria. We believe laccase enzyme may attribute to bio-conversion of Methyl Orange.

Maximum decolorizing ability of *Bacillus stratosphericus* SCA1007 was achieved by optimizing various factors like dye concentrations (25–400 mg/L). Percentage decolorization was found inversely proportional to dye concentration at concentrations above 150 mg/L. This is due to the increase in toxicity of dyes which results in inhibition of cellular activities and cell growth (Shah et al., 2013a). For industrial use of an isolate, it is important to identify microorganisms which show efficiency to degrade high concentration of dye. It has been reported that industrial effluent usually contains dyes with concentration varying from 10 to 50 mg/L (Padamavathy et al., 2003) and in this case, *Bacillus stratosphericus* SCA1007 showed the potential to decolorize dye up to a concentration of 150 mg/L (Fig. 3). This confirms that an adequate amount of color removal can be achieved by the selected strain.

The impact of pH on decolorization potential of the bacterial isolate was examined, over a pH range of 4–11. It was observed that the color removal increased from pH 4 to 7. Above pH 8 and below pH 4,

decolorization was insignificant by *Bacillus stratosphericus* SCA1007. Best decolorization of Methyl Orange was found to be at neutral pH 7 (Fig. 4). pH related transportation of dye particles through the cell membrane might be considered as a rate limiting factor for decolorization of dye (Chang and Lin, 2001). H^+ ions inhibit dye cations at $pH < 4$, thereby causing reduction in color removal efficiency. Whereas at $pH > 4$, the biomass surface gets negatively charged and thus creates a center of attraction for positively charged dye cations electrostatically (Daneshvar et al., 2007). Results obtained in our study were in accordance with previous studies (Shobana and Hangam, 2012; Won et al., 2004).

It has been observed that, change in temperature has significant influence on decolorization of Methyl Orange and was studied over the range of 5 °C–55 °C. The optimum temperature for maximum decolorization of Methyl Orange dye by *Bacillus stratosphericus* SCA1007 was found to be 35 °C (Fig. 5). Increasing the temperature to 45 °C and above, results in a drastic decrease in decolorization efficiency of microorganism. The result obtained is in accordance with a previous study which showed that 35 °C is the optimum temperature for maximum decolorization of Methyl Orange by *Pseudomonas* sp. (Patel et al., 2013). Decrease in decolorization efficiency at elevated temperature might be because of decrease in cell viability or catabolic enzymes denaturation (Chang and Lin, 2001; Saratale et al., 2011). Since, temperature beyond ambient range may require additional energy source, rendering the process to be less cost effective (Ogugbue and Sawidis, 2011), it is important to determine that the selected microbes can grow in natural environmental conditions.

Since industrial effluents containing dyes have high amount of dissolved salts, hence it is important to test the salinity tolerance of the selected microorganism for effective decolorization of dye (Kuhad et al., 2004). Experiment was conducted for a wide range (1%–7%) of saline culture condition. Complete decolorization was seen up to 3%. In the range of 4%–7% salinity, there was decline in dye decolorization (Fig. 6). Decrease in decolorizing activity by microorganism was because of high salt concentration which leads to plasmolysis or loss of cellular activity. *Bacillus stratosphericus* SCA1007 could efficiently decolorize Methyl Orange over a wide range (1%–5%), unlike in case of *Klebsiella* sp which showed only decolorization of dyes up to 80% (Cui et al., 2014).

Dye degradation is very slow process because of limited availability of carbon and nitrogen due to complex structure of dye. Supplying additional carbon and nitrogen might increase the degradation process of target molecules (Sihag et al., 2014). To enhance decolorization potential of *Bacillus stratosphericus* SCA1007, different nitrogen sources were supplied individually in MSM excluding yeast extract under

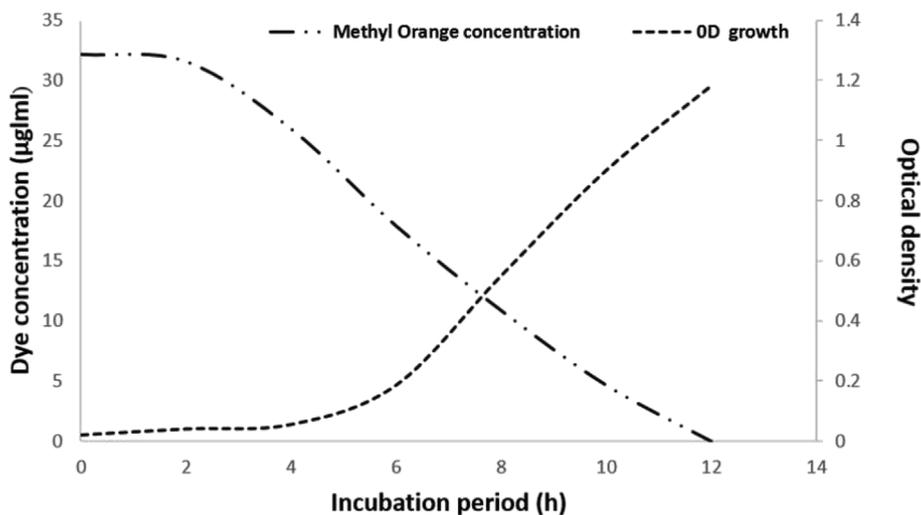


Fig. 2. Line graph showing decrease in dye concentration with increase in cell density of isolate *Bacillus stratosphericus* SCA1007.

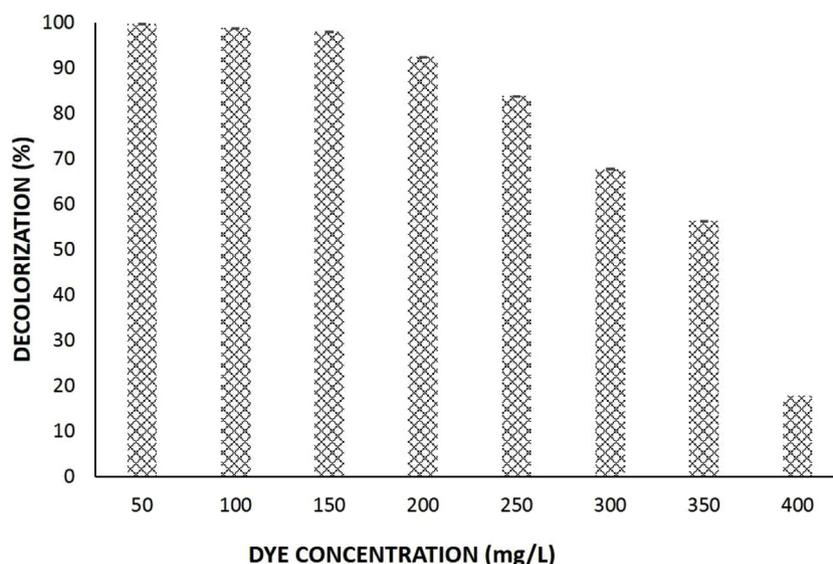


Fig. 3. Decolorization of Methyl Orange dye at different concentration of dye by *Bacillus stratosphericus* SCA1007. Values are mean of triplicate determination. Error bars represent standard deviation.

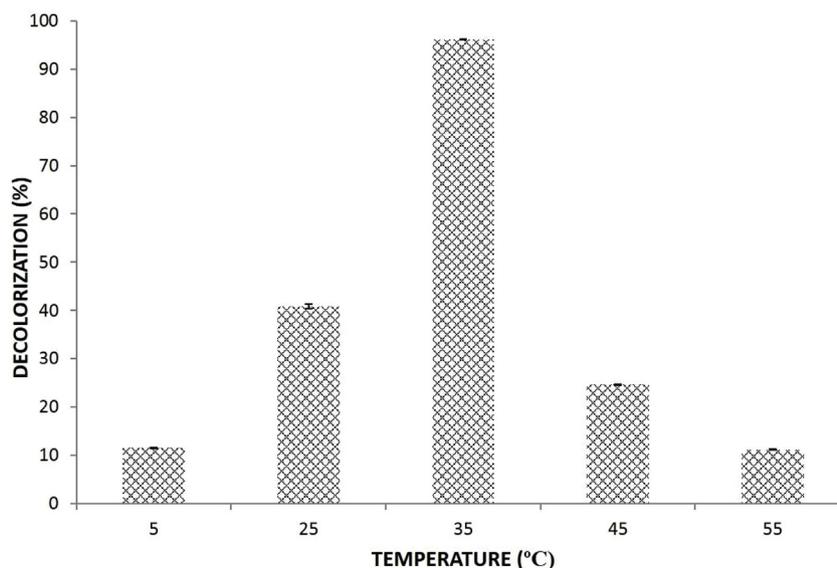


Fig. 4. Decolorization of Methyl Orange dye at different incubation temperature by *Bacillus stratosphericus* SCA1007. Values are mean of triplicate determinations. Error bars represent standard deviation.

optimized conditions of concentration of dye, pH, temperature and salinity. Different nitrogen sources like potassium nitrate, ammonium nitrate, sodium nitrate, urea, beef extract, yeast extract, tryptone and peptone were used. With the supplementation of yeast extract, complete decolorization was observed within 12 h of incubation under optimized conditions. Different carbon sources like glucose, sucrose, starch, maltose, dextrose, mannitol, fructose and glycerol were individually tested along with yeast extract. There was no significant change with the supplement of carbon source (Data not shown). Yeast extract is an organic nitrogen source, an essential media supplement for generation of NADH in the media which acts as electron donor for decolorization of dye by bacteria (Hu, 1994).

3.2. Degradation analysis

The FTIR spectrum of Methyl Orange and its extracted product after 12 h of incubation at 35 °C by *Bacillus stratosphericus* were compared. The spectrum of control dye showed peak at 698.23, 624.94 and

570.93 cm^{-1} for C–S stretching vibration. Sulphonic nature of dye was confirmed by peak at 1365.60 cm^{-1} . Peak at 1604.77 cm^{-1} showed N=N stretching vibration. Stretching at 821.61 cm^{-1} showed distributed benzene ring. Ring vibration was confirmed by peak at 1037.70 cm^{-1} and 945.12 cm^{-1} . Peak at 1519.91 and 1446.61 cm^{-1} for C=C–H in plane C–H bend, Peak at 2900.94 cm^{-1} showed asymmetric stretching of by CH_3 . Bending vibration of N–H was observed at 3460.30 cm^{-1} .

The FTIR spectrum of product extracted after treatment with *Bacillus stratosphericus* SCA1007 after 12 h incubation showed changes in peak as compared to control (dye) peak. The peak was at 3217.27 cm^{-1} for N–H stretching. Asymmetrical CH_3 stretching vibration was indicated by peak at 2927.94 cm^{-1} . The peak at 1670.35 and 1450.47 cm^{-1} showed stretching vibration for C–N and CH_3 respectively. The changes observed in IR spectrum of degraded product suggest degradation of Methyl Orange by *Bacillus stratosphericus* SCA1007 (Fig. 7).

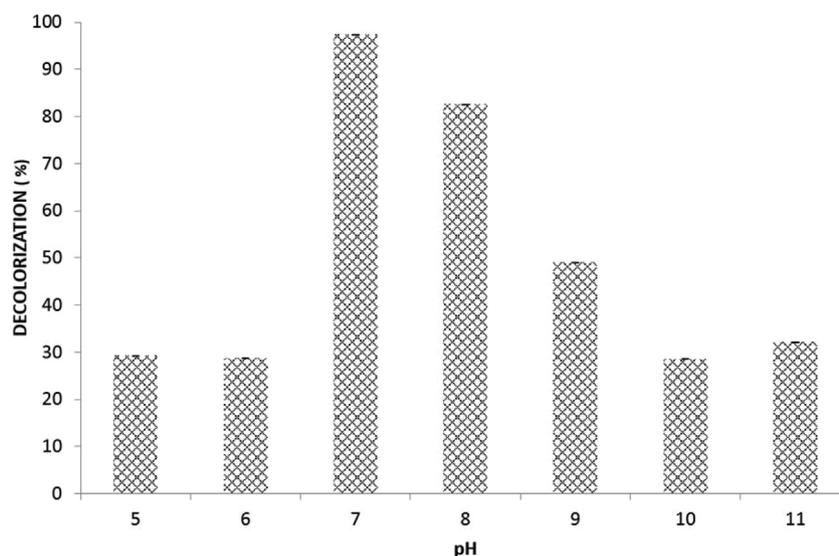


Fig. 5. Decolorization of Methyl Orange dye at different pH by *Bacillus stratosphericus* SCA1007. Values are mean of triplicate determinations. Error bars represent standard deviation.

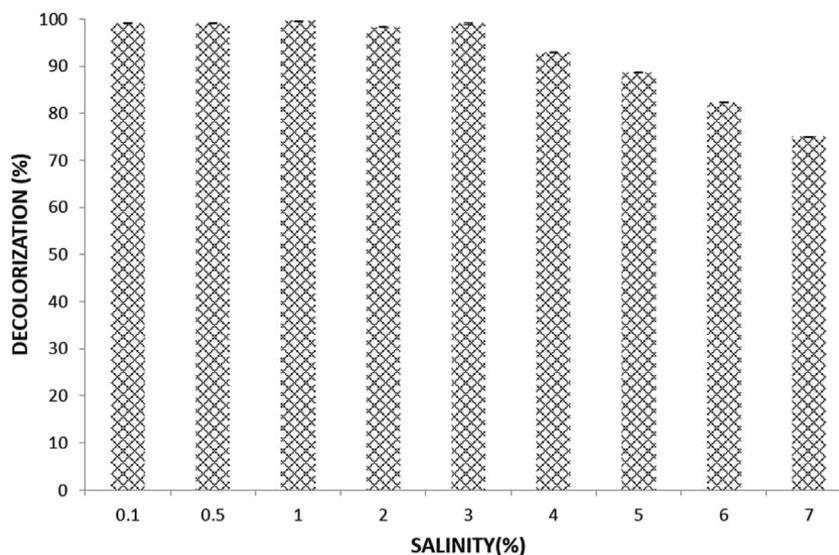


Fig. 6. Effect of salinity on the decolorization study by *Bacillus stratosphericus* SCA1007. Error bars represent standard deviation.

3.3. Toxicity study

The discharge of dye containing wastewater and dye treated wastewater on land may affect soil fertility directly, which is directly related to agricultural productivity. Thus, for the safety of environment it is very important to assess the toxicity of dye pre and post degradation. For microbial toxicity study, zone of inhibition measuring 2 mm was observed with control Methyl Orange dye, whereas the degraded metabolite did not show any growth inhibition.

Phytotoxicity study was carried out on seeds of *Vigna radiata* towards dye and their degraded products. Seeds which were grown in presence of dye showed 70% germination. The mean of root length and shoot length of *Vigna radiata* was found to be 1.60 ± 0.23 cm and 5.95 ± 0.34 cm. Seeds treated with distilled water showed 100% germination. The mean of root length and shoot length of *Vigna radiata* was found to be 4.72 ± 0.45 cm and 12.81 ± 0.34 cm. Seed treated with degraded products showed 90% germination. The average of root length and shoot length of *Vigna radiata* was found to be 2.80 ± 0.11 cm and 11.52 ± 0.19 cm (Table 1, Fig. 8). Similar result of phytotoxicity was observed in case of *Triticum aestivum* and *Oryza*

Sativa with Reactive Red 141 and their degraded products (Telke et al., 2008).

4. Conclusion

In this study bacterium strain *Bacillus stratosphericus* SCA1007 was isolated from local dyeing area. *Bacillus stratosphericus* SCA1007 could completely decolorize Methyl Orange through the process of degradation rather than adsorption. Toxicity study of Methyl Orange dye degraded by bacterium and optimization of various physical factors affecting the decolorization was investigated. This isolate could also tolerate salinity as high as 3%. These observations demonstrate that the bacterium is adaptive in nature and can degrade dye. However, efficiency of the strain needs to be demonstrated for its application in treatment of real sample.

Conflicts of interest

The authors declare that they have no conflict of interest.

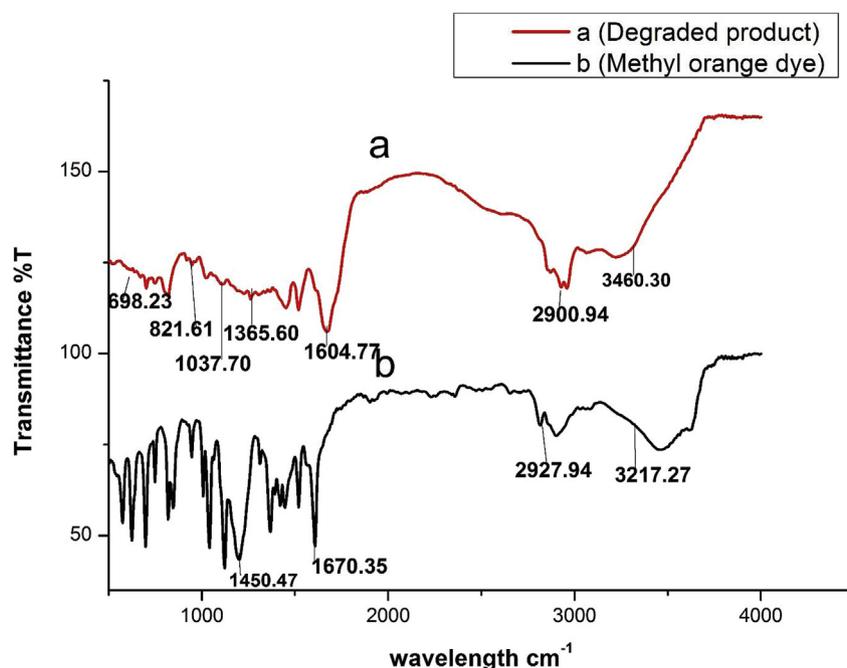


Fig. 7. The FTIR spectrum of Methyl orange 16 dye (b) and metabolite extracted (a) after 12 h of incubation at 35 °C.

Table 1

Study of the toxic effect of Methyl Orange and its degraded metabolites on *Vigna radiata* *.

Parameter studied	Water	Methyl Orange dye	Degradative product of Methyl orange
Germination %	100	70	90
Length of root (cm)	4.72 ± 0.45	1.60 ± 0.23	2.80 ± 0.11
Length of shoot (cm)	12.81 ± 0.34	5.95 ± 0.34	11.52 ± 0.19

*The mean was calculated with the standard deviation (±), which was statistically analyzed by Student's t-test (with > 95 percentage confidence level; p < 0.05).



Fig. 8. Toxicity study of Methyl Orange dye and its degraded metabolites on germination of *Vigna radiata* seeds.

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