

Research Article

Novel *Castellaniella denitrificans* SA13P as a Potent Malachite Green Decolorizing Strain

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Triphenylmethane dyes represent a major group of dyes causing serious environmental hazards. Malachite Green is one of the commonly and extensively used triphenylmethane dyes although it is carcinogenic and mutagenic in nature. Various physicochemical methods have been employed for its elimination but are highly expensive, coupled with the formation of huge amount of sludge. Hence, biological methods being ecofriendly are good alternatives. In the present study, the novel bacterial isolate SA13P was isolated from UASB tank of tannery effluent treatment plant. Phylogenetic characterization of 1470 bp fragment of SA13P has revealed its similarity with *Castellaniella denitrificans*. This strain has been found to decolorize the dye (malachite green) at a concentration of 100 mg L⁻¹ (80.29%). Decolorization was done by living bacterial cells rather than adsorption. Growth conditions have also been optimized for the decolorization. Maximum decolorization was observed at a temperature of 37°C and pH 8.0. Also, it has been found that bacterization of seeds of *Vigna radiata* with *Castellaniella denitrificans* SA13P increases germination rate. We have reported for the first time that *Castellaniella denitrificans* SA13P may be used as a novel strain for dye decolorization (malachite green) and biological treatment of tannery effluent.

1. Introduction

Out of all the contaminants present in wastewater, coloured compounds are considered as the most undesirable components and are mainly contributed by dyes [1]. Dyes are aromatic water-soluble dispersible organic colorants, having impending applications in numerous industries. More than 10,000 different commercially available synthetic dyes (mainly pigments belonging to azo, anthraquinone, and triphenylmethane groups) are produced in over 0.7 million tons per year [2]. These are widely used in leather, textile, paper printing, color photography, rubber products, pharmaceuticals, food coloring, cosmetics, and many other industries [3]. The presence of these dyes in the aqueous ecosystems presents the serious environmental and health concerns [4, 5]. The majority of them remain unreacted and are directly lost in the effluents. Some of the dyes and dye degraded products are reported to be carcinogenic and mutagenic in nature [6–8]. Their disposal is becoming

troublesome worldwide. These are released into the environment as wastewaters and besides producing an aesthetically unacceptable coloring of water bodies, they cause decreased gas solubility in water. They also block the penetration of light to the lower depths of aquatic systems, resulting in inhibition of photosynthesis [9, 10]. Thus, these effluents must be treated before releasing into the natural environment.

Malachite green, a triphenylmethane dye, is extensively used as food additive, parasiticide, and fungicide in food, dyeing, and fish farming industry throughout the world [11]. This dye is resistant to biodegradation and has toxicological effects on the liver, lungs, and other organs of mammals as well as animals. Due to these characteristics, it has been banned in several countries and listed as a priority chemical for carcinogenicity testing by the US Food and Drug Administration [11, 12]. However, it is still used in many areas of the world due to its low cost, ready efficacy, and lack of suitable alternatives [13]. Therefore, the environmental

pollution caused due to its long-term and widespread usage has become a serious problem.

Several physicochemical methods, such as adsorption, precipitation, and photodegradation have been applied to remove dyes from wastewaters [14]. However, these methods are costly, inefficient, and produce large amounts of sludge, hence not widely used [2]. Scientists have been exploring the microbial diversity, particularly of contaminated areas in search for organisms that can degrade a wide range of pollutants [15]. Although numerous microorganisms can decolorize such dyes, only a few are able to mineralize these compounds into CO₂ and H₂O [16]. These include bacteria, fungi, and algae capable of decolorizing a wide range of dyes with high efficiency [17].

Microbial decolorization and degradation have appeared as an environmentally pleasant and cost-competitive alternative to chemical decomposition processes [18]. Several microorganisms capable of degrading malachite green including *Chlorella* sp., *Shewanella* sp., and *Pseudomonas* sp. have been extensively reported [19–22]. Apparently there is a need to expand novel biological decolorization processes leading to the more efficient clean-up of Malachite Green using a single microorganism. Thus the main objective of our study was to observe the degradation of Malachite Green in a successive process using exclusively a novel bacterium isolated from tannery effluent, its process optimization, and molecular characterization.

2. Materials and Methods

2.1. Dyestuff, Chemicals, and Microbiological Media. In order to obtain a high-performance bacterial decolorization, Malachite Green (commonly used triphenylmethane dye) was chosen for the screening of dye degrading bacteria. All the chemicals used were of highest purity and of analytical grade. Reagents and other fine chemicals were obtained from Himedia Laboratory, India.

2.2. Sample Collection. Tannery effluent samples were collected from the outlet of combined effluent treatment plant at Jajmau (Kanpur), India, which lies in Indo-Gangetic plains between the parallels of 88°22'E longitude and 26°26'N latitude. Jajmau (Kanpur) is the major hub of tanneries. The effluent released from tanneries is used for irrigation of agricultural land. Due to long-term irrigation, area is having highly contaminated sites and hence was used for collection of samples for the present investigations. The samples were collected in presterilized plastic and polyethylene bags and were stored at 4°C in laboratory.

2.3. Isolation. One mL of diluted sample was spread on respective nutrient agar plate and then incubated at 37°C for 4–5 days. Individual bacterial colonies which varied in shape, size, and color were picked and purified by streaking.

2.4. Screening of the Isolates. For primary screening, test tube containing 10 mL of Luria Bertani (LB) broth with malachite green (10 mg L⁻¹) was prepared. Sterilized broth

was inoculated with 1% inoculum and then incubated at 37°C for 5 days. Reduction in color was observed at an interval of 12 h.

For secondary screening, selected isolates from primary screening were further checked at higher concentration of dye (100 mg L⁻¹). Decolorization of dye was measured in terms of the decrease in optical density (at 610 nm) of the supernatant obtained after centrifugation (10,000 g for 20 minutes). Percent decolorization was calculated using the following formula [23]:

$$\text{Decolorization (\%)} = \left[\frac{(\text{OD}_i - \text{OD}_t)}{\text{OD}_i} \right] \times 100, \quad (1)$$

where OD_i refers to the initial absorbance and OD_t refers to the absorbance after incubation.

2.5. Optimization of Cultural Conditions for Dye Decolorization. The bacterial isolate showing significant decolorization activity in secondary screening was chosen for the optimization of cultural conditions. Decolorization process was optimized by using the traditional stepwise strategy, varying one factor at a time. Effect of temperature on decolorization was studied by inoculating media flasks containing 100 mg L⁻¹ dye at different temperatures (25°C to 45°C) for 4 days. The effect of pH on decolorization was observed by incubating flasks containing dye media (conc. 100 mg L⁻¹) and appropriate inoculum at different pH values (5.0 to 11.0) at optimum temperature for 4 days.

2.6. Comparison of Live and Autoclaved Cells for Decolorization. Two fresh culture broths of selected isolate were prepared and half of them were autoclaved. Both the autoclaved (inactive) and living cells were centrifuged at 7500 g for 4 minutes. To determine if extracellular by-products or bacterial cells are involved in decolorization, the supernatant and pellets of the living and nonliving cells were incubated with the dye; absorption was used as a measure of their decolorization activity [24].

2.7. Toxicity Test. Toxicity of tannery effluent before and after treatment was checked by seed germination experiment [25]. Mung bean (*Vigna radiata*) seeds were surface sterilized with 0.1% HgCl₂ for 2 minutes and then washed repeatedly with sterilized deionized water. Initially, the effect of various concentrations of untreated tannery effluent (25%, 50%, 75%, and 100%) was observed on seeds of mung bean. Seeds were spread on sterilized petri dishes lined with sterilized filter paper. Then the seeds were treated with equal volume (10 mL) of raw and treated tannery effluent. The petri dishes were kept at room temperature (25 ± 2°C) and the seed germination (%) was recorded after every 24 h. The plant growth measurements included shoot length and root length; wet shoot weight, wet root weight, dry shoot weight, and dry root weight were also calculated in 15th day old seedlings.

2.8. Molecular Characterization. Total genomic DNA of bacterial isolate was obtained using Charles and Nester [26]

method with slight modifications. Pure culture of bacteria was raised in 10 mL of nutrient broth for 18 h–24 h to obtain cell O.D of 0.6 at 600 nm. The bacterial pellet was washed in 1.5 mL of 0.85% NaCl, centrifuged for 2 minutes at 12,000 g, and was resuspended in 0.4 mL Tris-EDTA buffer ($T_{10}E_{25}$). Cell lysis was done by adding 20 μ L of 25% SDS, 50 μ L of 1% lysozyme, and 50 μ L of 5 M NaCl followed by incubation at 68°C for 30 minutes in a circulatory water bath. For protein precipitation, 260 μ L of 7.5 M ammonium acetate solution was added to the microcentrifuge tubes and tubes were kept on ice for 20 minutes followed by centrifugation at 12,000 g for 15 minutes at 20°C. Supernatant was carefully pipetted out in another fresh, sterile microcentrifuge tube to which 1 μ L RNase (4 mg mL^{-1}) was added followed by incubation at 37°C for 20 minutes. An equal volume of chloroform was added in the tubes and proper mixing was done by inverting the tube up and down several times. RNA was precipitated by centrifuging for 1 minute at 12,000 g. The top layer containing total cell DNA was pipetted out in fresh microfuge tube and was used for next step. DNA was precipitated by adding 0.8 volume of isopropanol followed by incubation on ice for 30 minutes and pelleted by centrifuging at 10,000 g for 15 minutes. DNA was further washed with 0.5 mL of 70% ethanol and spun down at 10,000 g for 1 minute. Pure DNA sample was then suspended in 20 μ L Tris-EDTA buffer ($T_{10}E_1$) or deionized water and stored at 4°C for further use.

2.8.1. Agarose Gel Electrophoresis. The genomic DNA sample of bacteria was quantified through agarose gel electrophoresis by analyzing their migration on 0.8% agarose gel prepared in 0.5 M Tris-borate-EDTA (TBE) buffer and run in an electrophoresis tank filled with the same concentration of TBE buffer. The genomic DNA was diluted with Tris-EDTA ($T_{10}E_1$) buffer so as to achieve a concentration of 50 ng in 10 μ L to be used as a template DNA in PCR amplification reaction.

2.9. Bacterial Identification by 16S rRNA Sequences and Phylogenetic Relationship. Identification was done by 16S rRNA analysis using universal primers. The sequences of the primers used for the amplification were 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (3'-ACGGCTACCTTGTACGACTT-5'). Sequence was analyzed at NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) using BLAST (N) program. Phylogenetic tree was constructed by maximum likelihood method using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software (v. 5.05) [27]. The final sequence was deposited at GenBank.

2.10. Statistical Analysis. All the experiments were performed in triplicate. Results were expressed as mean \pm SE (standard error) and analyzed using paired *t*-test (SPSS, v. 16). A *P* value less than 0.05 is considered as statistically significant.

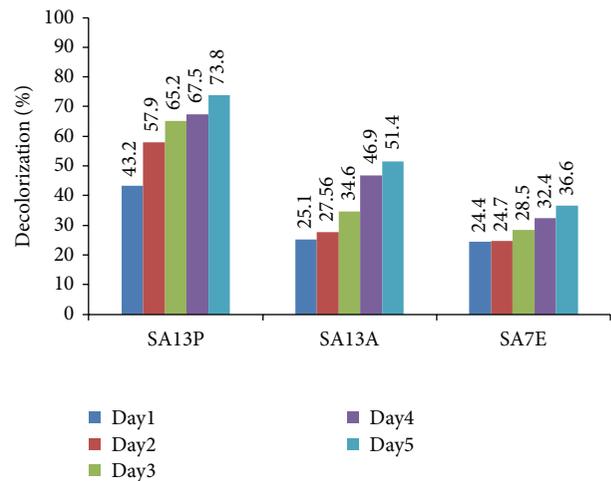


FIGURE 1: Graph comparing decolorization (%) of malachite green by various isolates.

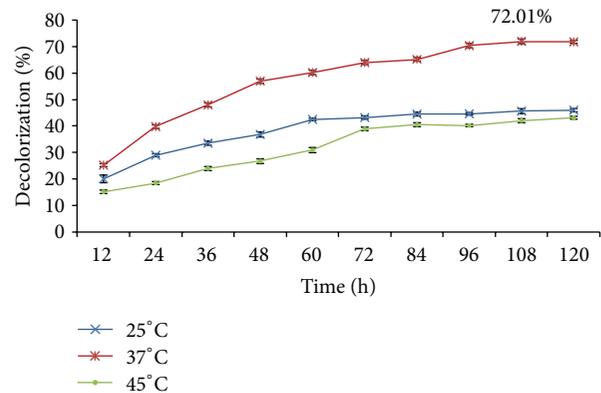


FIGURE 2: Effect of temperature on decolorization activity.

3. Results and Discussion

3.1. Isolation. Total of 136 different bacterial isolates were obtained. About 46 isolates were obtained from tannery effluent and remaining 90 were obtained from soils irrigated with treated tannery effluent.

3.2. Screening. Out of 136 isolates, only three bacteria (SA13A, SA13P, and SA7E) exhibited visible decolorization of Malachite Green (10 mg L^{-1}) and were selected for secondary screening (Table 1). During secondary screening extent of decolorization was measured and it was found that strain SA13P (Figure 1) exhibited maximum decolorization ($73.80\% \pm 0.25$) of Malachite Green in 5 days. Moreover, this value has been found to be highly statistically significant ($P < 0.05$) and hence strain SA13P was further selected for optimization of cultural conditions. Rate of decolorization is function of time. It has been reported earlier also that rate of decolorization is slow in 12 h and gets accelerated after 15 h indicating that organism requires some time to acclimatize in the environment [28].

TABLE I: Decolorization (%) of Malachite Green by various isolates.

Isolate(s)	Day 1	Day 2	Day 3	Day 4	Day 5
SA13P	43.20* \pm 0.10	57.90* \pm 0.05	65.20* \pm 0.05	67.50* \pm 0.16	73.80* \pm 0.25
SA13A	25.10 \pm 0.12	27.56 \pm 0.08	34.60 \pm 0.17	46.90 \pm 0.06	51.40 \pm 0.05
SA7E	24.40 \pm 0.12	24.70 \pm 0.16	28.50 \pm 0.21	32.40 \pm 0.12	36.60 \pm 0.17

All values are expressed as mean \pm SE, * $P < 0.05$ (significant in comparison to SA13A and SA7E).

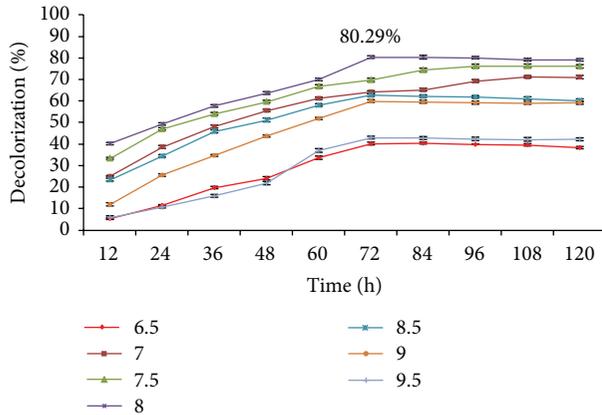
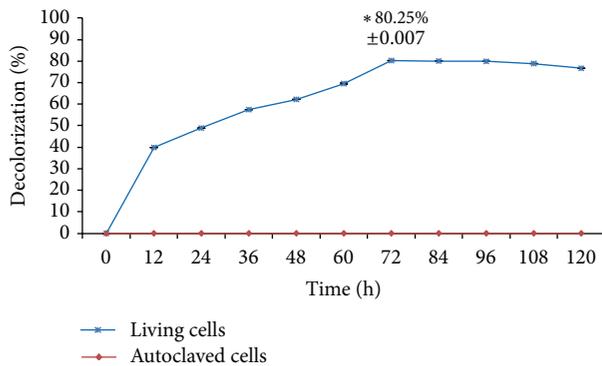
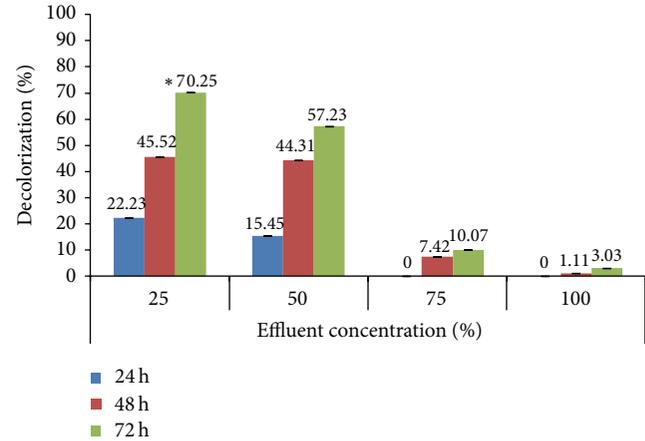


FIGURE 3: Effect of pH on decolorization.

FIGURE 4: Effect of living and autoclaved cells on decolorization. * $P < 0.05$ (significant as compared to autoclaved cells).

3.3. Effect of Temperature and pH on Decolorization. Strain SA13P exhibited strong decolorizing activity at 37°C. Although a lag phase was observed and the decolorization rate was comparatively low at 25°C, the decolorization extent increased to a similar level from 25°C to 37°C (Figure 2). Decolorizing activity was significantly suppressed at 45°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 45°C. Also the effect of pH was observed at optimum temperature. It has been found that the efficient decolorization was achieved between pH 7.0 and 8.0, with maxima (80.29% \pm 0.59; 72 h) at pH 8.0 (Figure 3). This may be due to the fact that the optimum pH for the growth of strain SA13P has a major effect on its efficiency of dye decolorization. Earlier Mali et al. [28] reported similar observations for decolorization of triphenylmethane and azo dye by *Pseudomonas* sp.

FIGURE 5: Effect of different concentrations of tannery effluent on seed germination. All values are expressed as mean \pm SE, * $P < 0.05$ (significant as compared to 50%, 75%, and 100% effluent).

pH between 6.0 and 8.0 was found to be optimum for decolorization. Wu et al. [29] also reported decolorization of triphenylmethane dyes by *Pseudomonas otitidis* strain WL-13. The optimum temperature was found to be 37°C and optimum pH reported was in range of 7.5 to 8.0. *Achromobacter xylosoxidans* MG1 strain has also been reported for malachite green decolorization. The optimum decolorization activity has been observed at pH 6.0 and 38°C in LB decolorization medium [30].

3.4. Effect of Living and Autoclaved Cells on Decolorization. It was observed that autoclaved cells exhibited no decolorization activity while decolorization of Malachite Green by living cells has appeared within 12 h of incubation. When the data was analyzed, highly statistical significance was observed ($P < 0.05$) in decolorization by living cells as compared to autoclaved cells. As seen in Figure 4, living cells of strain SA13P showed (39.87% \pm 0.012) and (48.88% \pm 0.005) decolorization activity after 12 h and 24 h of incubation. Maximum activity (80.25% \pm 0.077) was observed after 72 h of incubation. Activity decreased to (78.86% \pm 0.008) and (76.64% \pm 0.003) in 108 h and 120 h of incubation. Thus, it can be inferred from the above results that dye decolorization is by degradation process rather than adsorption on bacterial cells.

3.5. Toxicity Test. Seed germination of *Vigna radiata* was affected by different concentrations (25%, 50%, 75%, and 100%) of raw tannery effluent. Different concentrations of effluent bring significant change (Figure 5) in the rate of

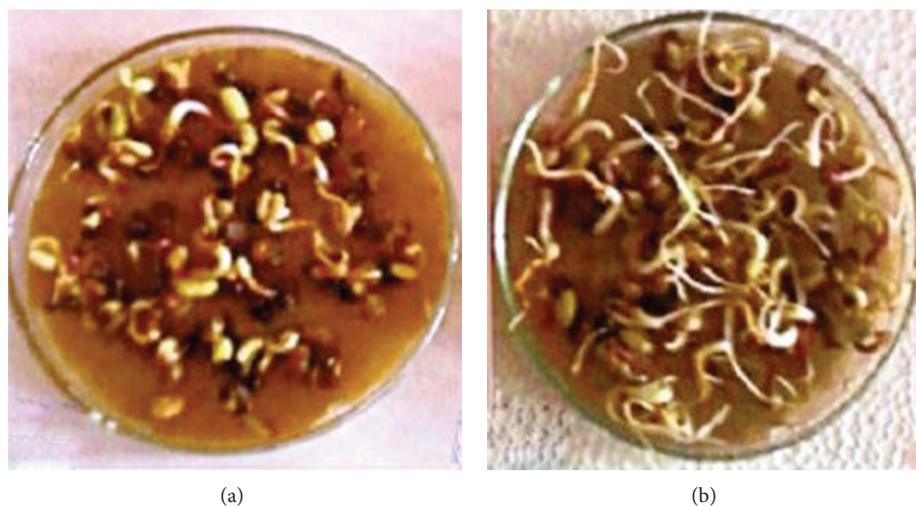


FIGURE 6: Seedling growth under (a) raw effluent and (b) treated effluent.

seed germination. At lower concentration (25%) germination was found to be 22.23% in 24 h but at higher concentration (50%) germination percentage was 15.45% (24 h) while concentration of 75% and 100% imposed lethal effect on seeds. Seed germination was found to be maximum after 72 h at all concentrations of effluent. The seed germination percentage was excellent at lower concentrations (up to 50%) of effluent but at higher concentrations (specifically at 100%), the seed germination was decreased abruptly. On the other hand, toxicity test of bioremediated effluent was performed at 100% concentration of effluent and various seed germination parameters were calculated (Table 2). Raw effluent showed $3.03\% \pm 0.12$ seed germination while seed treated with bioremediated effluent resulted in $83.1\% \pm 0.11$ germination. This increased germination rate has been found to be highly statistically significant ($P < 0.05$) for treated effluent. Similarly when data was analyzed for seedling length and seedling weight, statistical significant increase ($P < 0.05$) has been exhibited by bioremediated effluent in comparison to raw effluent. Seedlings growth of *Vigna radiata* with treated effluent as well as untreated effluent has been shown in Figure 6. The above observations indicated that effluent treated with strain SA13P resulted in significant seed germination as compared to raw effluent ($P < 0.05$). Similar results have also been reported for *Triticum aestivum* and *Phaseolus mungo* seeds. Malachite green in comparison to its degradation products have been found to significantly affect plumule and radical length indicating less toxicity of degraded products [31].

3.6. Molecular Characterization. Amplified DNA fragments were sequenced using Sanger Dideoxy method [32]. Forward and reverse sequence of isolate was joined using DNA Baser v 3.5.3 software and finally was identified as *Castellaniella denitrificans*. The 16S rRNA gene sequence obtained from the isolate was compared with other bacterial sequences by using NCBI mega BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for their pairwise identities. Isolate has shown 100% identity

TABLE 2: Various seed germination parameters of raw and treated effluents.

Germination parameter(s)	Raw effluent	Treated effluent
Germination (%)	3.03 ± 0.12	$83.1^* \pm 0.11$
Shoot length (cm/seed)	0.06 ± 0.01	$3.71^* \pm 0.23$
Root length (cm/seed)	0.16 ± 0.01	$3.01^* \pm 0.02$
Wet root weight (mg/seed)	2.51 ± 0.01	$47.10^* \pm 0.11$
Wet shoot weight (mg/seed)	18.0 ± 0.86	$85.42^* \pm 0.25$
Dry root weight (mg/seed)	0.50 ± 0.01	$2.51^* \pm 0.02$
Dry shoot weight (mg/seed)	5.01 ± 0.02	$10.0^* \pm 0.11$

All values are expressed as mean \pm SE, * $P < 0.05$ (significant as compared to raw effluent).

with *Castellaniella denitrificans* strain NKNTAU with maximum query coverage of 100%. The 16S rRNA sequence of the isolated bacterium was submitted in the Genbank (NCBI) with an accession number KC684873. Further, consensus sequences were aligned and compared with the available database in NCBI. The phylogenetic tree was constructed using MEGA 5.05 software to determine evolutionary relationships of the isolate (Figure 7). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model [33].

4. Conclusion

The present study reveals the ability of the *Castellaniella denitrificans* to decolorize Malachite Green. Results obtained from this work reveal that this bacterial isolate possesses significantly high decolorization efficiency (80.29%). In current study it was found that decolorization is dependent on the pH and temperature. The optimal values of temperature and pH for decolorization were found to be 37°C and 8.0, respectively. As per literature surveyed, this is the first compilation of data in conviction with Malachite Green decolorization by *Castellaniella denitrificans*. In another experiment, it was found

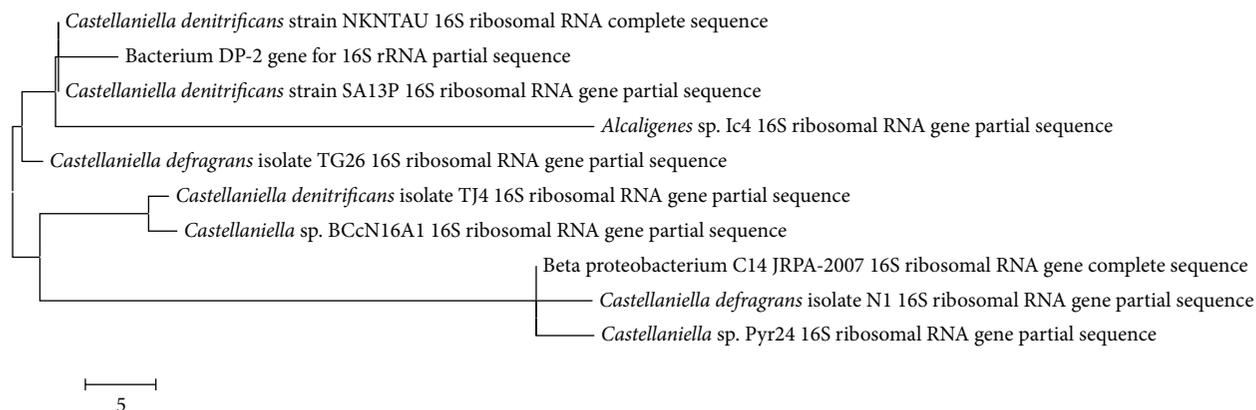


FIGURE 7: Molecular phylogenetic analysis of *Castellaniella denitrificans* SA13P by maximum likelihood method.

that viable cells are directly involved in dye decolorization by degradation mode rather than adsorption. Toxicity of treated and raw effluent was also studied by seed germination experiment and germination rate was found to be higher in effluent treated with putative strain. As *Castellaniella denitrificans* SA13P possess potential to degrade dye (Malachite Green), therefore may serve as potent dye decolorizer and bioremediating agent.

Conflict of Interests

We declare that there is no conflict of interests.

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