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CHARACTERIZATION OF A NEWLY ISOLATED AZO-DYE DEGRADING *KLEBSIELLA* SP. STRAIN RGUDBI01

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ABSTRACT

Azo-dyes such as crystal violet are commonly used ingredient in all types of dying industries which more often eventually reach water and soil due to washing and disposal practices. They are non-biodegradable inside higher organisms and are less biodegradable due to their xenobiotic character. However, some of the microbes exhibit their extensive potential to degrade such recalcitrants. This study reports the isolation and characterization of a newly isolated *Klebsiella sp*. strain RGUDBI01 from petroleum-contaminated soil samples. The species-level identification was done by 16S rDNA gene sequencing (ON945611.1). The isolate exhibited 88% azo-dye degradation just after 3 days of incubation under optimized conditions. The treated samples exhibited a significant increase in the growth of *Cicer arietinum* seeds compared to the control group. This result suggests that the dye-contaminated samples, after treatment, displayed non-cytotoxic behavior. The strain also showed its potential to tolerate and withstand a wide range of pH, salinity, and substrate concentration which supports its futuristic environmental application. In addition to the above, the strain could also produce biosurfactants with $\upsilon_{C=C}$, $\upsilon_{C=C-H}$, and υ_{C-C} functional groups which were evaluated based on FTIR spectral analysis.

Keywords: Azo-dye degradation; biosurfactant production; *Klebsiella* sp.; petroleum degradation; water treatment

INTRODUCTION

The inclination towards urbanization and industrialization has hampered the backbone of the environment in the worst possible ways, one of which can be seen with the humongous release of industrial wastewater into the soil and water (**Lotito *et al*., 2014**). Industrial wastewater is a hazardous combination of xenobiotic compounds including petroleum hydrocarbons, heavy metals, pesticides, dye, dyestuff, *etc*. Among industrial sectors, the dyeing industry is considered one of the frontrunners that pollute the environment after the petroleum industry (**Lotito *et al*., 2014**). The majority of the globally produced dyes are azo dyes, ranging from 60-70% on an annual scale (**Benkhaya *et al*., 2020**). Azo dyes are among the most commercially widespread aromatic compounds with one or more –N=N– groups in their chemical structure (**Ajaz *et al*., 2020**) and they have a production rate of 70,000 tn/y of the total global dye production (Maroudas *et al*., 2021). Complex modifications, such as the presence of aryl rings belonging to benzene or naphthalene, and electron withdrawing groups, aid in their highly toxic and recalcitrant nature. The degree of degradation of the azo group depends on the electron density around the –N=N– bond. Electron-withdrawing groups, such as –NH_2 and –OH, decrease the electron density and facilitate the reduction of the azo group, resulting in the release of an aromatic amine (**Das, 2016; Chen, 2006**). Azo dyes are often linked with carcinogenicity and mutagenicity, and they are a group of highly toxic compounds depleting both flora and fauna, depending on the time of exposure to the dyes (**Sarkar *et al*., 2017; Mahmood *et al*., 2016**). The easy solubility and intense coloration of azo dye such as crystal violet [4-[bis[4-(dimethylamino)phenyl]methylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium; chloride], Aniline Yellow (4-phenylazoaniline), Solvent Yellow 3 (o-Aminoazotoluene), and Sudan 1 (1-phenylazo-2-naphthol), *etc*., not only hampers the aesthetic of water and lowers the penetration of sunlight leading to a decrease in photosynthesis but also effects the pollution indicators like biological oxygen demand (BOD) and chemical oxygen demand (COD) (**Mahmood *et al*., 2016**). One of the most widely used dyes, Crystal violet which has a very intense color and easy inhalation is linked to several health issues like skin and digestive tract irritation, renal and respiratory failure, *etc* (**Mani and Bharagava, 2016**). Hence, efficient degradation of dye is of utmost importance and a precaution towards the upcoming disaster.

Various treatment approaches have been introduced for dye degradation which include physical, chemical, and biological methods (**Sarkar *et al*., 2017**). The physical and chemical treatment approaches broadly include a bio-electrochemical system, Advance Oxidation Process (AOP), membrane separation, *etc* (**Shindhal *et al*., 2020**). On the other hand, the biological approaches mainly include microbial remediation (**Sarkar *et al*., 2017; Shindhal *et al*., 2020**). However, physical and chemical methods have significant drawback as they are cost-intensive, requiring a large number of chemicals and producing sludge (**Sarkar *et al*., 2017**).

Microorganisms with their versatile structure have depicted a great potential to decolorize azo-dyes in a cost-effective and eco-friendly manner (**Sreedharan *et al*., 2019**). Bacteria such as *Enterobacter Cloacae*, *Bacillus* sp, *Klebsiella pneumoniae*, and *Pseudomonas stutzeri* (**Moawad *et al*., 2010; Jaiswal and Gomashe, 2017; Mustafa *et al*., 2021; Joshi *et al*., 2020**), algae such as *Chlorella* and *Oscillatoria, etc* (**Saratale *et al*., 2011**), fungi such as *Trametes versicolor* and *Aspergillus niger*, *etc* (**Cano *et al*., 2012; Asses *et al*., 2018**), and yeasts such as *Candida tropicalis*, and *Scheffersomyces spartina, etc* (**Das *et al*., 2011; Tan *et al*., 2016**) have shown a cost-effective way for textile dye degradation. It is important to mention that most of the azo-dyes are hydrocarbon derivatives and some of them are produced from petroleum (Sajid 2022). Among others, various species of *Klebsiella* are also known for their potential to degrade complex petroleum hydrocarbon and some of the azo-dyes very effectively by producing biosurfactants but are not reported for their potential to degrade crystal violet which is one of the most widely used azo-dyes (Garg and Dixit 2020). It may also be noted the Northeastern part of India is one of the biodiversity hotspots in the world with mostly unexplored environmentally important microbes. Considering these facts, the current research was initiated to characterize the azo-dye degradation potential of a newly isolated biosurfactant producing *Klebsiella* sp. strain RGUDBI01 from a petroleum contaminated site located in Guwahati, Assam.

MATERIALS AND METHODS

Isolation and screening of dye degrading microbes

Soil samples were collected from petroleum oil logged sites located across the Guwahati city of Assam. The soil samples were diluted six-fold in 0.5% saline and inoculated on Bushnell and Haas (BH) agar (composition in g/L: $MgSO_4$- 0.2, $CaCl_2$- 0.02, KH_2PO_4- 1.0, K_2HPO_4- 1.0, NH_4NO_3- 1.0, $FeCl_3$- 0.05, agar-agar- 20.0) supplemented with 200 µL crystal violet (CV) at 37 ºC for 72 h. The microbial colonies obtained after incubation were sub-cultured on nutrient agar for further use.

Precisely, 150 mL of BH broth taken in individual capped 250 ml Erlenmeyer flasks maintained at a concentration of 50, 100, 150,... 250 mg/mL of crystal violet (CV) were inoculated with previously cultured 1 mL (OD600nm=1.0) of pure

culture of the best isolate. These were then maintained at 37 ºC and 135 rpm for 72 h to assess its potential to decolorize CV. Suitable controls were maintained using un-inoculated tubes. The decolorization of CV by the isolated strain was quantified based on the following formula (**Meerbergen *et al.*, 2017**):

$$\text{Decolorization capacity (\%)} = \frac{(\text{Initial absorbance} - \text{final absorbance})}{\text{Initial absorbance}} \times 100$$

Based on the above, the optimum dye concentration for the degradation was confirmed and the same was maintained with different parameters in further studies. The same experimental conditions were repeated using different pH (6,7,…10) and salinity [3,6,…12% (*w/v*) of NaCl] for the determination of the optimum pH and salinity for CV degradation.

Extraction and characterization of bacterial biosurfactant

The best isolate was inoculated in BH broth supplemented with 2% (*v/v*) used motor oil as a carbon source followed by incubation at 37 ºC maintained at 135 rpm for 72 h. The cell-free extract was collected by sedimenting the cells at 10,000 rpm for 10 min at 4 ºC followed by the cold acetone extraction method (**Basumatary *et al*., 2020**). The extracted biosurfactants were freeze-dried and spectrophotometrically characterized by FTIR followed by biochemical characterization (saponification test, and molish test) for the determination of their chemical nature.

Molecular identification of the isolate

The bacterial isolate was identified based on 16S rDNA gene sequencing. Briefly, the total bacterial genomic DNA was extracted and purified by the SDS lysis method followed by the amplification of the 16S rDNA gene by using 27F and 1492R primers. The amplicons were sequenced by using ABS system. The consensus sequence was generated and the phylogenetic tree was constructed considering the top ten hits based on NCBI BLAST results by MEGA10TM software based on the neighbor joining method at a bootstrap value of 10000 by forcing *Phytobacter massiliensis* JC163 (Genbank accession: NR125600.1) as outgroup.

Cytotoxicity assay with reference to seed germination

Microbial-treated CV samples were used to see their effect on *Cicer arietinum* seed germination (n=30). Briefly, moist cotton beds were prepared in glass petri dishes by soaking the cotton with 20, 40, and 60 mL of microbial-treated CV samples. These were then inoculated with *Cicer arietinum* seeds and covered with lids allowed to germinate and grow in the dark at room temperature. The germination rate and the growth of the seedlings were recorded till 72 h at an interval of 24 h. Positive and negative controls were maintained by using 400 mg/L of CV in BH broth and un-inoculated BH broth respectively.

Statistical analysis

All the results obtained in the current study were given suitable statistical treatment and are expressed in mean ± S.D. form. Student's *t*-test was performed to analyze the significance of the results obtained.

RESULTS

A total of 54 unique bacterial colonies were screened based on their ability to degrade crystal violet dye in the current study. The phylogenetic tree indicated that the isolated bacterial strain shares 62% homology with *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* strain 01A030 (GenBank accession no. NR134062.1) and hence the isolate was identified as *Klebsiella* sp. strain RGUDBI01 (Fig. 1). The GenBank accession no. ON945611.1 was successfully received in support of the submission in the NCBI Genbank database.

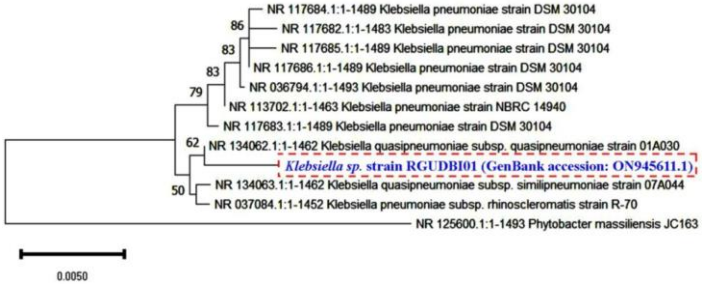

Figure 1 The phylogenetic tree shows the evolutionary relationship of the identified strain (*Klebsiella* sp. strain RGUDBI01) with the 10 most closely related species based on BLAST hits. The tree was constructed by Neighbour-joining method forcing *Phytobacter massiliensis* JC163 as an out group with 1000 bootstrap value.

The FTIR spectrum shows the analysis of crude bacterial biosurfactant (Fig. 2). The peak at 1017.73 cm^{-1} interprets the presence of the υ_{C-H} bend. The band at 979.60 cm^{-1} interprets the presence of $\upsilon_{C=C-H}$ bend which falls under the vinyl-related compound region. The peak at 856.43 cm^{-1} indicates the presence of υ_{C-C} stretch.

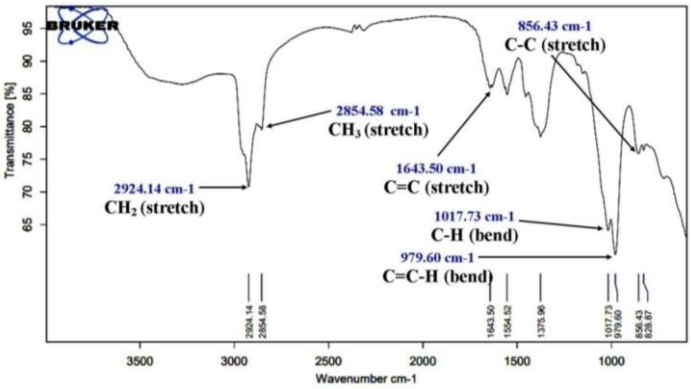

Figure 2 FTIR spectra show the presence of different functional groups in the crude biosurfactant.

It was observed that *Klebsiella* sp. strain RGUDBI01 could degrade 200 mg/L crystal violet dye within 72 h (Fig. 3). However, in higher concentrations, the dye degradation rate was remarkably increased from the 48th h of incubation. The maximum degradation rate at 200 mg/L concentration of the dye was evaluated to be 88.76%. At low salinity, *i.e*., from 3-9% (*w/v*) concentration, the CV decolorization rate was found very high which decreased drastically at 12% salinity (Fig. 4).

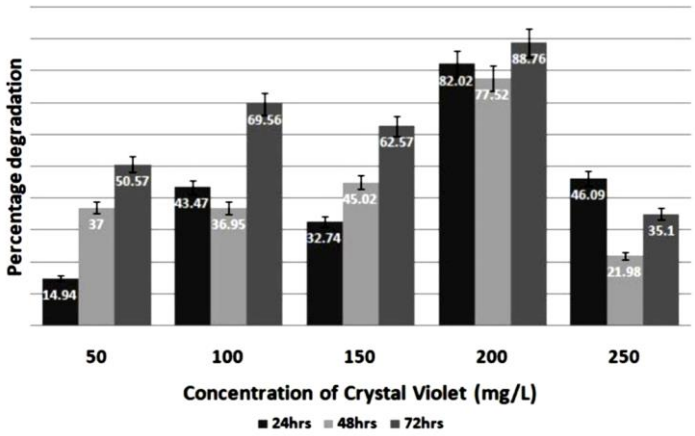

Figure 3 Degradation of crystal violet by *Klebsiella* sp. strain RGUDBI01 at different concentrations and time intervals.

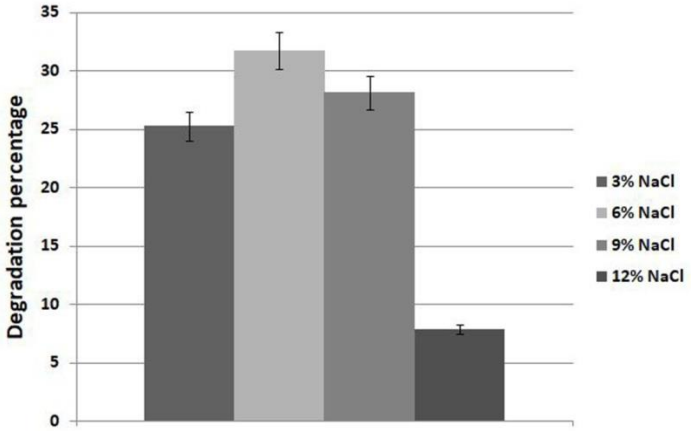

Figure 4 Crystal violet degradation at different salinity levels.

With the increase in salinity, the decolorization rate decreases abruptly. The highest degradation rate by *Klebsiella* sp. strain RGUDBI01 was observed at a NaCl concentration of 6% (*w/v*), with a degradation rate of 31.70%. It was observed that a salinity concentration between 3-9 g/L is suitable for decolorization of crystal violet. The growth rate of the seedlings was also found to increase in the

treated samples with the increase in the time of incubation (Fig. 5) indicating the non-cytotoxic effect of the bacterial-treated CV solution.

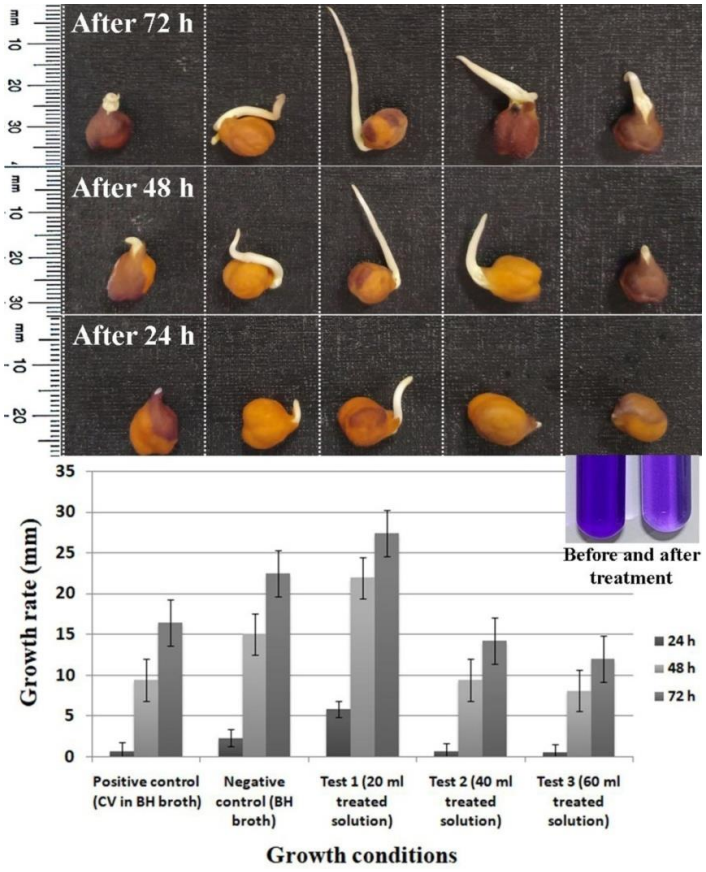

Figure 5 Results of seed germination assay on microbial treated CV (crystal violet) solutions against suitable control. The inset figures show the decolorization of dye after microbial treatment and the respective growth of seedlings against the statistical data.

DISCUSSION

Azo-dye is the most abundantly produced dye in the world and the dyeing industry is considered one of the leading contributors to environmental pollution (**Lotito *et al.*, 2014**). Azo-dyes and their intermediates are often associated with carcinogenic and mutagenic potentials (**Sarkar *et al.*, 2017**). The discharge of azo-dye effluents into the water also degrades water quality through various mechanisms (**Mahmood *et al.*, 2016**). In this context, bioremediation using microbes has depicted an excellent degradation potential in azo-dye (crystal violet) degradation. Bioremediation is suggested in this study as it is cost-effective and an environmental approach to achieving our goal and this process is not backboned by huge drawbacks like excessive chemicals, man force, and most importantly sludge formation. In this study, azo-dye degrading isolates were screened from soil samples contaminated with petroleum hydrocarbon, considering the fact that some of the azo-dyes are the derivatives of petroleum hydrocarbon only. The selection criteria for further experimentation were based on their ability to decolorize crystal violet dye. Among the isolates, the most effective strain was identified as *Klebsiella* sp. strain RGUDBI01 through 16S rDNA gene sequencing. This best strain was identified based on 16S rDNA gene sequencing using 27F and 1492R primers. Genus *Klebsiella* is a member of the family Enterobacteriaceae, and domain bacteria are a group of gram-negative facultative anaerobes (**Cui *et al.*, 2014**). The members of the family Enterobacteriaceae like *Klebsiella* and *Escherichia coli* are omnipresent in soil and water and have been isolated from diverse habitats including wastewater and industrial-activated sludge (**Meerbergen *et al.*, 2017**). The genus *Klebsiella* is often linked to opportunistic infections in mammals and humans (**Meerbergen *et al.*, 2017**). However, it is worth noting that several studies on the genus *Klebsiella* have recognized its futuristic potential in hydrocarbon degradation (Ozyurek and Bilkay 2018; You et al., 2018) and azo-dye degradation (**Meerbergen et al., 2017; Zablocka-Godlewska *et al.*, 2015; Dixit and Garg, 2015**) due to its common presence in soil. These evidences also advocate its environmental friendliness and cheap approach in the field of bioremediation.

The FTIR analysis of the biosurfactant produced by the newly isolated *Klebsiella* sp. strain RGUDBI01 showed more than five prominent peaks in the spectra indicating the compound is a complex organic molecule with a small mass and molecular weight (Nandiyanto *et al*., 2019). The single bond area *i.e.*, 2500-4000 cm^{-1} contains prominent peaks. The sharp peak at 2924.14 cm-1 indicates the presence of asymmetric υ_{CH2} stretch and absorption bands in the 2935 and 2860 cm-1 is identified as linear aliphatic compounds. A narrow band at 2854.58 cm^{-1} indicates the presence of aliphatic υ_{CH3} and narrow bands below 3000 cm^{-1} indicate aliphatic compounds in the single bond area region. The absence of peaks between 3000 and 3200 cm^{-1} shows that there is no aromatic structure and there is no specific peak for aldehyde between 2700 and 2800 cm^{-1} indicating the absence of aldehyde. No peak in the 2000-2500 cm^{-1} indicates the absence of a triple bond compound in the crude sample. In the double bond region *i.e.*, 1500-2000 cm-1, the functional group can be imino (C=N), carbonyl (C=C), and azo (N=N). The peaks between 1680-1630 cm^{-1} belong to the amide class of carbonyl compounds and in the spectra, the peak at 1643.50 cm^{-1} indicates the presence of $\upsilon_{C=C}$ stretch. In the fingerprint region 600-1500 cm^{-1} which is specific and unique for every compound, three peaks were observed.

Many microbes produce biosurfactants which are surface-active compounds widely used in bioremediation (**Mishra *et al.*, 2021**) because of their characteristic ionic nature, environmental compatibility, low toxicity, and high emulsifying activity, *etc* (**Mishra *et al.*, 2021; Das *et al.*, 2017**). Biosurfactants produced by a strain of *Klebsiella pneumoniae* are known to exhibit very high emulsifying activity against hydrocarbons helping in the biodegradation of hydrocarbon pollutants (**Nwaguma *et al.*, 2016**). Considering these facts, this newly isolated *Klebsiella* sp. strain RGUDBI01 may be recommended not only for the bioremediation of azo-dye contaminants but also for petroleum bioremediation.

The present dye degradation result indicates that *Klebsiella* sp. strain RGUDBI01 is quite tolerant to crystal violet and capable of decolorizing and degrading the dye, even at relatively high concentrations. The degradation percentage of azo-dye is greatly dependent on the chemical structure of the dye itself (**Pinheiro *et al.*, 2022**). Studies have shown that the dyes with simple molecular structure and low molecular weight such as Methyl Red, Orange I, and Methyl Orange, *etc*. as easy candidates for degradation as compared to complex molecular structure compounds such as Eriochrome Red B and Ponceau S, *etc* (**Cui *et al.*, 2014**).

Germination of seeds in the azo-dye contaminated medium after bioremediation may be considered an important bio-indicator to assess toxicity. Crystal violet in BH was treated with the isolated strain and the effect of the treated samples was observed in the germination *Cicer arietinum* seeds. The germination rate in the treated samples (test-1) was observed to be 90%, compared to only 40% in the positive control (non-treated crystal violet in BH broth). The enhanced growth in *Klebsiella* sp. strain RGUDBI01 may be attributed to its possession of a structural gene called *AcdS*, which codes for 1-aminocyclopropane-1-carboxylatedeaminase (ACCD). This enzyme functions as a plant growth promoter also can protect plants from abiotic stresses such as salinity, temperature fluctuations, and drought, *etc* (**Singh *et al.*, 2015**). In light of the above, the futuristic application of the newly isolated *Klebsiella* sp. strain RGUDBI01 in the bioremediation of azo-dye-contaminated soil and water cannot be ruled out.

CONCLUSIONS

Degradation of azo-dye in water and soil through cost-effective and environment benign methods is the need of the hour. The newly isolated strain *Klebsiella* sp. RGUDBI01 exhibited 88% crystal violet dye degradation in liquid culture just after 3 days of incubation. Crystal violet samples treated with Klebsiella sp. strain RGUDBI01 significantly promoted the growth of *Cicer arietinum* seeds compared to the respective control, indicating the non-cytotoxic behavior of the isolated. The isolated microbe could tolerate a wide range of pH, salinity, and substrate concentration, which may help exploring its potential in field trials.

Conflict of interest: The authors declare that there is no conflict of interest.

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