



Biodecolorization of Azo Dye Acid Blue 113 by Soil Bacterium *Klebsiella variicola* RMLP1

Pradeep Kumar Singh¹, Pankaj Singh², Rajat Pratap Singh³ and Ram Lakhan Singh^{1,4*}

¹Department of Biochemistry, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India; drrlsingh@rediffmail.com

²Department of Biotechnology, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India

³Department of Biotechnology, Guru Ghasidas University, Koni, Bilaspur - 495009, Chhattisgarh, India

⁴Vice-Chancellor, Nilamber-Pitamber University, Medininagar, Palamu - 822101, Jharkhand, India

Abstract

The present study was aimed to isolate a new bacterial strain for the degradation/decolorization of azo dye Acid Blue 113 (AB 113). The physico-chemical method is inadequate for degradation of azo dyes; therefore, an environmental friendly and competent method such as use of the biological organism was studied for decolorization of AB 113. Bushnell and Hass (BHM) medium containing AB 113 dye were used to perform the decolorization study. 16S rRNA gene sequencing approach was used for identification of bacterial isolate as a *Klebsiella variicola*. The optimum process parameters for the decolorization of AB 113 were found at pH 8, 35°C temperature and 100 mg/L dye concentration during 72 h incubation. Glucose and ammonium sulphate was the carbon and nitrogen source suited well for the decolorization of dye. The results proved that the *Klebsiella variicola*, offer huge ability in treating textile wastewater containing the color AB 113.

Keywords: Acid Blue 113, Azo Dye, Decolorization, *Klebsiella Variicola*, 16S rDNA

1. Introduction

Synthetic dyes are xenobiotic, aromatic compounds which provide permanent color to various materials. These dyes offer broad range of color shades and consume minimum energy during their appliance in the textile, food, paper, paint, varnish, cosmetics and pharmaceutical industries¹. On the basis of structure of chromophore, 20 different types of dye groups are available². The textile processing industries produces huge quantity of azo dye³. It was estimated that globally 7x10⁵ metric tons of textile color is generated each year and 70% of this vast amount is contributed by azo dyes⁴. About 10-15% of the azo dyes used in dyeing process is unbound and are likely to be discharged into water bodies⁵. Azo dyes are cyclic organic preparations comprise one or more azo bond (–N=N–). These bonds are accountable for recalcitrant nature of dyes and give resistant capacity towards its natural degradation. Discharge of these dyes into environment decreases light dispersion into water which minimize the photosynthetic process of aquatic species⁶. These dyes are also mutagenic⁷ and carcinogenic to human and other aquatic animals⁸. Number of physico-chemical techniques for example, flocculation, ion exchange,

membrane filtration, coagulation, photo-oxidation, electrolysis and ozonation are used for textile wastewater treatment but, they have some limitations such as generation of large quantities of toxic chemical sludge and high operational and maintenance cost⁹. As physico-chemical techniques are associated with some limitation, there is a need to develop some more efficient and cost-effective methods for the removal of these dyes from wastewater. The biological methods include bacterial decolorization, fungal decolorization, phytoremediation, phytoremediation and enzymatic methods¹⁰. Thus, biological microorganisms such as bacteria, fungi, algae, and plants are successfully used in the decolorization and degradation of these dyes. These microbial based decolorization and degradation methods have some decisive advantages such as low operating cost, efficient and production of less sludge and eco-friendly nature¹¹. The major benefits of biological methods are low preparation techniques and easy maintenance of microbes¹². The decolorization process by fungi and algae attributed to adsorption rather than degradation which result in retention of dye in the environment. Bacterial degradation of dyes primarily begins under anaerobic conditions by an enzyme mediated step¹³. The ensuing degradation products for

*Author for correspondence

example toxic aromatic amines are further cleaved by multi-step route either aerobically or anaerobically¹⁴.

This study aims to isolate and identify the potential of bacterial strain for decolorization of Acid Blue 113 dye and optimization of various nutritional and physical conditions.

2. Materials and Methodology

2.1 Dyes and Chemicals

The dye Acid Blue 113 (AB 113) and other azo dyes used in this study were purchased from Sigma-Aldrich Chemicals. All the other chemicals and media components were of highest purity and of an analytical grade.

2.2 Medium Used for Culture Studies

Bushnell and Haas medium (BHM) having MgSO_4 0.2g/L, K_2HPO_4 1.0g/L, CaCl_2 0.02g/L, FeCl_3 0.05g/L, NH_4NO_3 1.0g/L, yeast extract (0.05% w/v) and glucose (0.1% w/v) was employed in this study.

2.3 Isolation and Screening of Decolorizing Bacteria

Soil samples were collected from the different sites of Tanda, Ambedkar Nagar, Uttar Pradesh (India). These soil samples along with BHM amended with dye AB 113 (100 mg/L) were employed to isolate and screen the dye decolorizing strain. Soil suspension (10% w/v) was prepared and 10 ml aliquots were transferred into an Erlenmeyer flask having 100 ml of dye containing media. After several times repeated transfers into fresh dye containing media, dye decolorization was achieved. The decolorized samples were serially diluted, and dilutions were subsequently plated on BHM agar plates containing 100 mg/L AB 113 dye. The isolates showing visible decolorization zones with distinctive morphology were streaked on 100 mg/L AB 113 dye containing BHM agar plates. The bacterial isolate that showed the highest decolorizing ability of AB 113 dye was designated as BT-9.

2.4 Bacterial Identification by 16s rRNA Gene Amplification and Sequencing with BLAST Analysis

A fresh single colony of bacterial strain BT-9 was inoculated in 50 ml of Luria Bertani (LB) broth and maintained at 37°C temperature in a shaker incubator for 24 hrs. After centrifugation at 8000 rpm of broth culture, the genomic DNA was extracted by Qiagen genomic DNA purification kit by using cell pellet. The purified DNA was used for the amplification

of 16S rRNA gene. A 50 µl reaction of prepared Master Mix (Promega) and 50 ng genomic DNA of isolate BT-9 was added along with 10 picomols of each primer (27f-1492R) and mixed properly¹⁵. The Polymerase chain reaction was performed in a thermal cycler (BioRad My-cycler) and after the completion of the PCR, amplicons were stored at -20°C for further qualitative assessment by Agarose gel electrophoresis. Amplification product (2 µl) mixed with 6X gel loading dye was loaded on 1.2% agarose gel and electrophoresis was run for 4 hrs at 60 V. 1 kb DNA marker ladder (Promega) was used for the assessment of amplicon size and agarose gel was visualized in gel-documentation system (BioRad) and the image was saved.

16S rRNA gene PCR product was purified (Qiagen) and processed for DNA sequencing. DNA sequencing was performed by Sanger dideoxy sequencing (BigDye Terminator Sequencing Kit V3.0) on ABI 3130XL Genetic Analyzer (Applied Biosystems USA). DNA sequence was checked for quality and a performed nucleotide BLAST (BLASTn, NCBI, USA) analysis. Neighbor-Joining method used to conduct evolutionary analysis by using the MEGA 6 software¹⁶.

2.5 Effects of Various Process Parameters on AB 113 Decolorization

Effects of various process parameters such as incubation condition (shaking and static), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), temperature (20-40 °C interval), carbon source (glucose, fructose, sucrose, maltose, starch and lactose), nitrogen source (peptone, ammonium sulphate, ammonium nitrate, yeast extract, and ammonium chloride), dye AB 113 concentration (100, 200, 300, 400, 500 and 600 mg/L) on decolorization of AB 113 were investigated. All the experiments were carried out in triplicate in 15 ml screw capped tubes under static condition.

2.6 Decolorization of Various Textile Dyes by *Klebsiella variicola* RMLP1

Decolorization efficiency of *Klebsiella variicola* RMLP1 was tested with structurally different azo dyes. In this experiment five different azo dyes, namely Direct Blue 71, Reactive Red 120, Acid Orange, Congo Red and Metanil Yellow with concentration (100 mg/L) were used under the optimized process parameters.

2.7 Decolorization Manner of *Klebsiella variicola* RMLP1 Strain on Acid Blue 113

To study the decolorization manner of *Klebsiella variicola* RMLP1 strain on Acid Blue 113, heat killed and live bacterial cells were used. A bacterial cell suspension was autoclaved at 121°C for 30 minutes. The culture medium containing autoclaved bacterial suspension was used to test the decolorization limit.

Culture medium having live bacterial suspension was used as a control. Both heat killed and live bacterial cells containing medium were used to perform decolorization activity under the static condition for 72h of incubation. After incubation, samples were centrifuged at 10,000 rpm for 10 min at 4°C. To spot any alteration of dye structure following decolorization, UV-Visible spectrophotometer (Labtronics) was used to scan decolorized sample supernatant from 200 nm to 800 nm.

2.8 Measurement of Decolorization Efficiency

To assess the decolorization efficiency, samples were collected at every 12 h intervals. The samples were centrifuged at 10,000 rpm for 10 min at 4°C and were studied by quantification of culture supernatant absorbance at 566 nm. The decolorization potential was mentioned as percentage of decolorization¹⁷.

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

2.9 Statistical Study

All experiment was performed in triplicate. Microsoft Excel was used to calculate standard deviation (SD) and results presented as mean $\pm$ SD value.

3. Results

3.1 Isolation and Identification by Sequencing 16S rRNA Gene

Dye decolorizing bacterial strain was isolated from soil sample collected from wastewater contaminated sites of Tanda, Uttar Pradesh, India. BT-9 bacterial strain which showed highest potential to decolorize AB 113 dye was isolated for further study by using BHM medium.

16S rRNA gene sequence approach was used for identification of bacterial strain BT-9. Bacterial strain BT-9 was identified (with 99% sequence similarity) as *Klebsiella variicola* after the nucleotide BLAST (BLASTn, NCBI, USA) analysis. The DNA sequence was submitted to the GenBank database (NCBI, USA) and accession number (KY794214) was received. Phylogenetic analysis was performed by MEGA 6 software¹⁶. 16S rRNA gene sequences from 10 closely related *Klebsiella* were downloaded from NCBI database. All 10 DNA sequences along with strain RMLP1 were used for phylogenetic analysis and DNA sequence from *Rickettsia* was used as out group. Dendrogram showed that strain RMLP1 is closely related to the *Klebsiella variicola* (Figure 1).

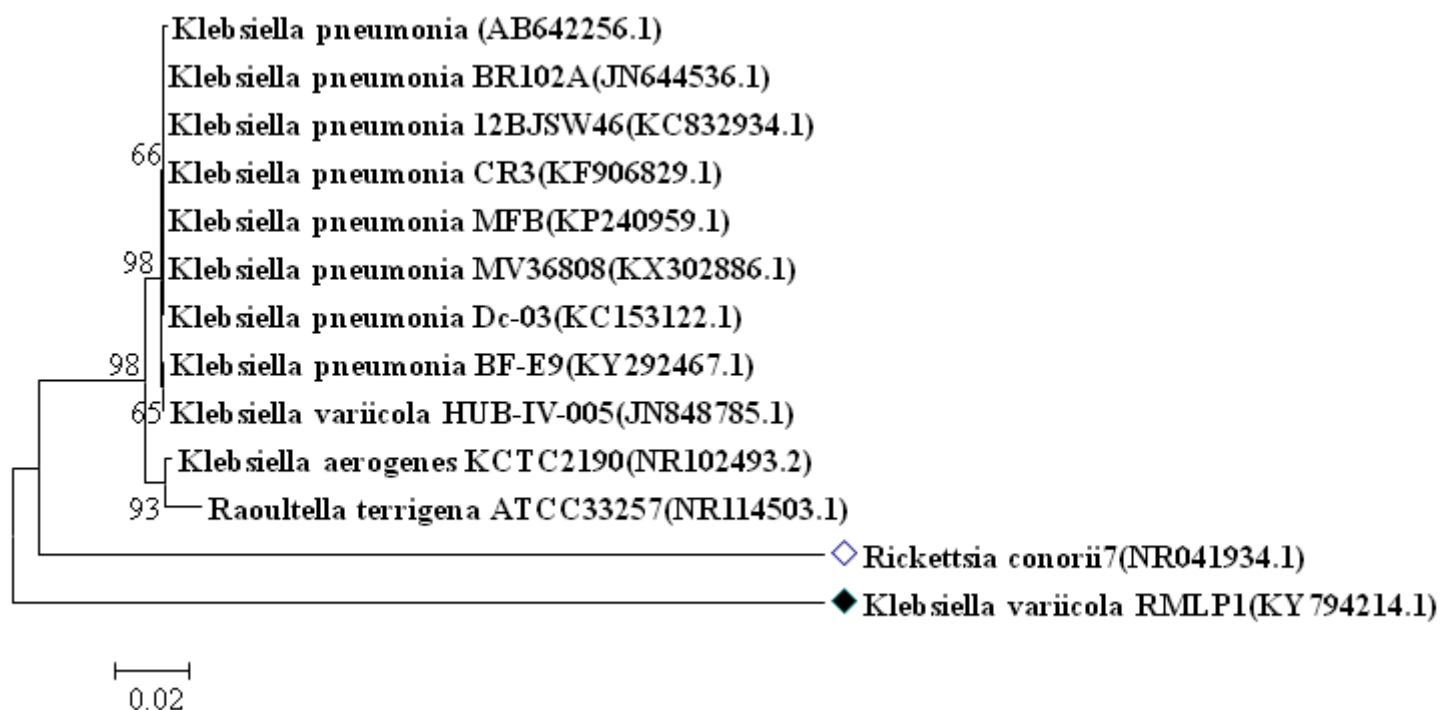


Figure 1. Dendrogram of the *Klebsiella variicola* RMLP1 strain.

3.2 Effect of Incubation Condition

Physiological characteristics of the cells such as growth and development are highly influenced by presence and absence of oxygen. It was believed that oxygen inhibits the dye decolorization process as it possesses high redox potential. The *Klebsiella variicola* RMLP1 strain showed 80.66% color removal of Acid Blue 113 in static condition while only 31.03% color removal observed in shaking environment (Figure 2).

3.3 Effect of pH and Temperature

For effective growth and metabolic activity of bacteria, pH and temperature are two very important physical factors. The optimum pH and temperature is a demand of bacterial cell which contribute in successful decolorization of dyes. In natural environment pH occurs in a range of 5 to 9. Since pH is considered as a rate limiting factor as it responsible for transport of dye across the bacterial cell membrane for dye decolorization. The maximum decolorization (86.48%) of AB 113 dye by *Klebsiella variicola* RMLP1 strain was found at pH 8 (Figure 3). A further deviation in either side of pH 8 leads to reduction of decolorization percentage.

Klebsiella variicola RMLP1 strain showed increased decolorization of AB 113 dye as temperature increases from 20-35 °C (Figure 4). Beyond 35°C temperature decolorization efficiency of the strain RMLP1 was considerably decreases. Thus, optimal temperature was found to be 35°C with 90.53% color removal of AB 113 dye by *Klebsiella variicola* RMLP1 strain.

3.4 Effect of Carbon and Nitrogen Source

Carbon and nitrogen source are necessary for the successful color removal by bacteria. These sources are involved in enhancement of metabolic functioning and bacterial growth and concurrently facilitate the decolorization process by reduction of azo bond. The bacterial strain *Klebsiella variicola* RMLP1 showed maximum decolorization of AB 113 by using these sources as a co-substrate. Maximum decolorization (91.01%) was achieved with carbon source glucose. The sucrose, fructose, maltose, lactose and starch were seem to be less suitable source of carbon with 74.13%, 48.92%, 29.46%, 49.98% and 22.59% decolorization of AB 113 dye, respectively (Figure 5).

The extent of color removal by *Klebsiella variicola* RMLP1 strain was tested with various nitrogen sources such as peptone, yeast extract, ammonium chloride and ammonium sulphate. The highest rate of decolorization (93.43%) was achieved with the ammonium sulphate, whereas 73.43%, 91.19%, 50.49% and 46.75% decolorization of AB 113 dye respectively was observed, with the ammonium nitrate, yeast extract, peptone and ammonium chloride nitrogen sources (Figure 6).

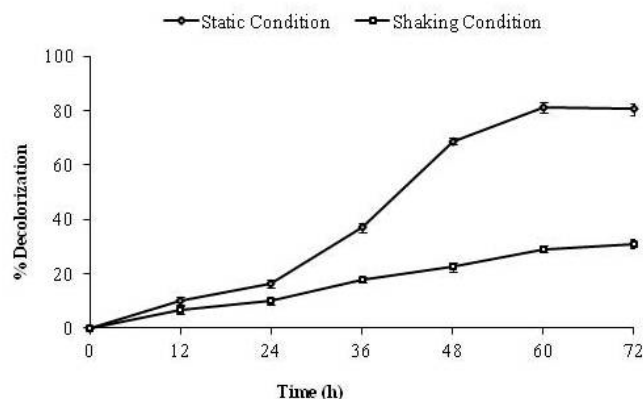


Figure 2. Effect of incubation conditions.

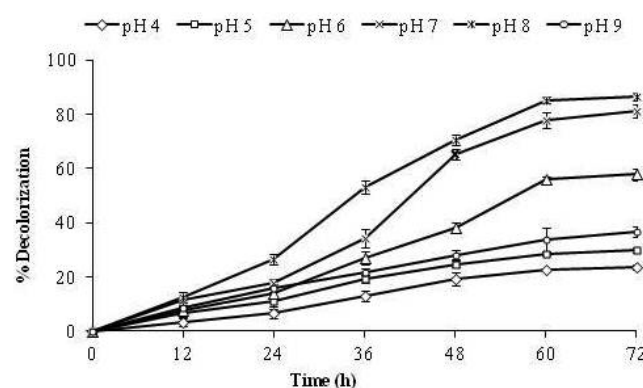


Figure 3. Effect of pH.

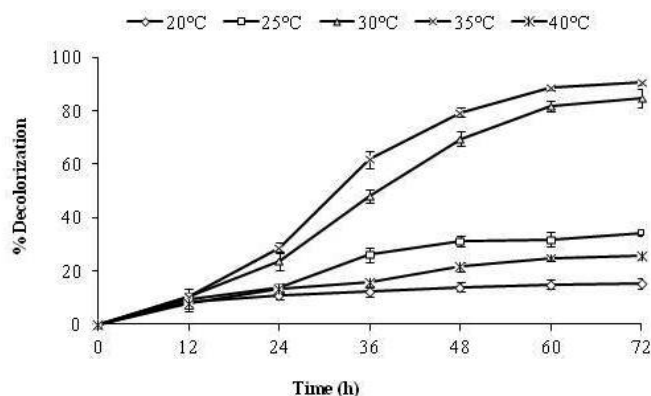


Figure 4. Effect of Temperature.

3.5 Effect of AB 113 Dye Concentration

The rate of dye decolorization largely depends on dye concentration present in a medium. The *Klebsiella variicola* RMLP1 strain showed low color removal of AB 113 dye at higher concentration of dye. The highest decolorization up to 92.75% of AB 113 was achieved by RMLP1 strain at 100 mg/L concentration of dye. Further increase from 200 to 600 mg/L of dye concentration leads to reduction in decolorization rates (Figure 7).

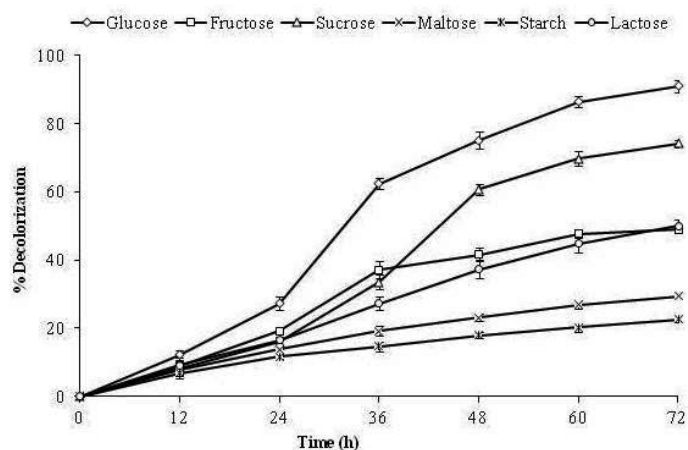


Figure 5. Effect of various carbon sources.

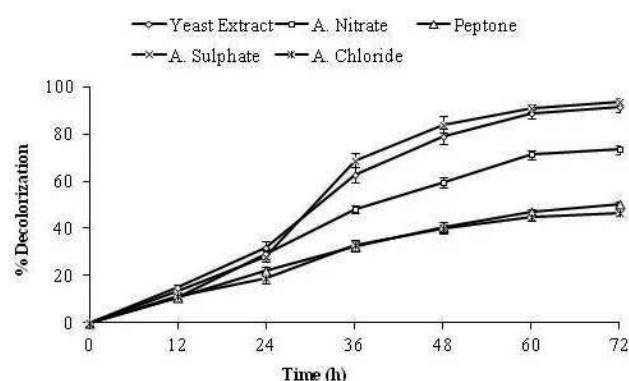


Figure 6. Effect of various nitrogen sources.

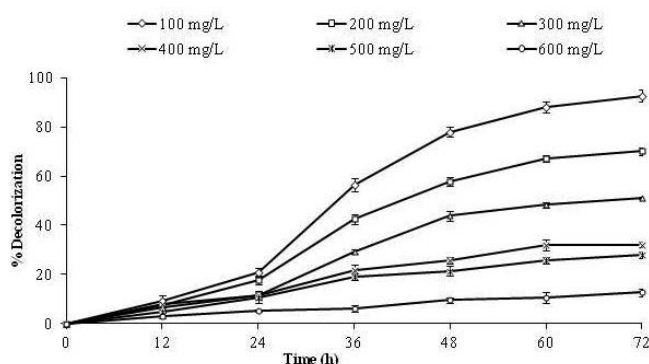


Figure 7. Effect of AB 113 dye concentration.

3.6 Decolorization of Various Textile Dyes by *Klebsiella variicola* RMLP1

All test dyes were decolorized up to different extent by *Klebsiella variicola* RMLP1 at its optimized physico-chemical and nutritional conditions for decolorization of AB 113 dye. The highest decolorization of 88.68% was achieved for Direct Blue 71. Other four dyes including Reactive Red 120, Acid Orange,

Congo Red and Metanil Yellow were decolorized up to 79.54%, 62.01%, 51.06% and 29.25%, respectively. The difference in the color removal extent of various dyes attributed to structural complexity of test dyes¹⁸. The decolorization efficiency of *Klebsiella variicola* RMLP1 against all the azo dyes tested in this study showed that *Klebsiella variicola* RMLP1 might be utilized in decolorization of recalcitrant dye containing wastewater.

3.7 Decolorization Manner of *Klebsiella variicola* RMLP1 Strain Against Acid Blue 113

Decolorization of azo dyes may be due to adsorption¹⁹ or degradation²⁰. During adsorption bacterial cells only adsorbed dyes onto its surface, whereas degradation process involved in generating new compounds via reduction of azo bond by bacterial enzymes. If the decolorization is due to degradation either the major absorbance peak of visible region will entirely disappear or a new peak will emerge²¹. The medium having autoclaved bacterial cells (heat killed) achieved only 16.92% decolorization after 72 h incubation with blue colored cell pellets. This low decolorization extent might be due to the adsorption phenomenon showed by heat killed bacterial cells. In the control experiment, 88.41% decolorization of AB 113 was observed after 72 h of incubation (Figure 8). The spectral scan from 200 to 800 nm of supernatants after treatment proved that absorbance peak maxima at 566 nm in visible region completely disappeared in control culture (Figure 9). The change of absorption pattern in UV and visible region proved that the AB 113 dye molecular structure was altered after decolorization. The azo bond was the primary chromophore of AB113 which was responsible for blue color of dye. The change in visible spectra indicated that the primary chromophore was destroyed due to the cleavage of azo bond during the decolorization reaction. These findings suggested that the decolorization of AB 113 dye by *Klebsiella variicola*

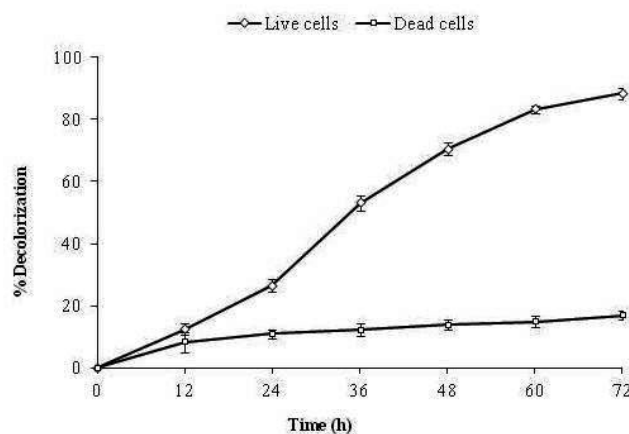


Figure 8. Decolorization of AB 113 by live cells and dead cells (heat-killed) of *Klebsiella variicola* RMLP1 strain.

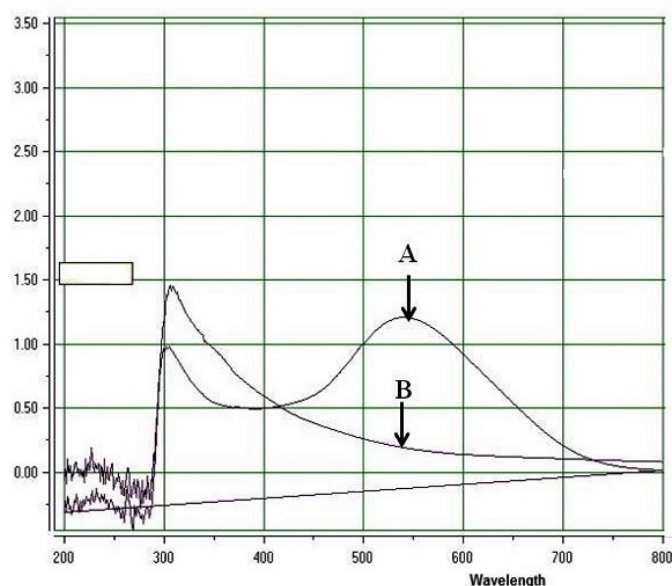


Figure 9. Variation in the UV-visible spectra of AB 113 before and after decolorization by *Klebsiella variicola* RMLP1 strain (A, 0 h; B, 72 h).

RMLP1 was due to the biodegradation rather than adsorption. Many researchers concluded that the biodegradation instead of adsorption is the mode for dye decolorization by bacteria which supports our study^{22,23}.

4. Discussion

The bacterial strain *Klebsiella variicola* RMLP1 efficiently decolorized the AB 113 dye under static condition. Ghodake *et al.*²⁴ have found similar result for decolorization of Amaranth dye by using bacterium *Acinetobacter calcoaceticus* under static condition. Isik and Sponza²⁵ observed 100% color removal of dye Congo Red with *Pseudomonas* sp. in static condition as compared to non-static condition, where 45% decolorization of Direct Black 38 was reported. It was also reported that *Citrobacter* sp. CK3 successful removal of dye Reactive Red 180 by more than 96% under static conditions whereas in shaking condition only 13% color removal was achieved²⁶. In addition, Singh *et al.*²⁷ also studied the potential of *Staphylococcus hominis* RMLRT03 strain for decolorization of dye Acid Orange under static and shaking condition and found that 85.52% and 32.47% color removal respectively. Higher concentration of oxygen under shaking condition inhibits the reductive enzyme accountable for breakdown of azo linkage²⁸.

The optimal pH for decolorization of AB 113 by *K. variicola* RMLP1 strain was found at pH 8. A similar trend of dye decolorization efficiency (89.06%) by *Pseudomonas* sp. was also reported at same pH²⁹. Likewise, Das *et al.*³⁰ achieved more than 95% decolorization of Remazol Navy Blue by *Bacillus*

pumilus HKG212 at pH 8. Similarly, 96% decolorization of Direct Orange 16 was achieved under static condition by *Micrococcus luteus* strain SSN2 at pH 8³¹.

The optimal temperature for decolorization of dye AB 113 by RMLP1 strain was found to be 35°C temperature. Similar to our result, Arulazhagan³² found that bacterial culture *Bacillus subtilis* showed 60% decolorization of Reactive Red M8B at 35°C. Likewise, *Bacillus halodurans* MTCC 865 degraded up to 90% of Acid Black 24 at 37°C in static condition³³. Furthermore, Tripathi and Srivastava³⁴ demonstrated that 37°C was the most favorable temperature for Orange 10 dye removal with *P. putida* MTCC 102.

Generally, the rate of decolorization of azo dyes was enhanced up to the most favorable temperature by several bacterial sp., after that slight change in temperature leads to reduction of decolorization activity. This decrease in decolorization extent at higher temperature was due to the denaturation of azoreductase enzyme.

Glucose was found as the best carbon source for AB 113 dye decolorization by RMLP1 strain. Similar to our result, Carolin *et al.*³⁵ also noticed nearly 95% decolorization of Methyl Orange in the presence of glucose using *Micrococcus yunnaenensis* strain. Wang *et al.*²⁶ demonstrated that *Citrobacter* CK3 sp., was efficiently remove dye Reactive Red 180 when added glucose in culture medium. It was believed that glucose is preferential carbon source, supports the dye decolorization, and also promotes bacterial growth and development which leads to increased dye decolorization³⁶.

In our study ammonium sulphate is found best source of nitrogen for decolorization of AB 113 dye. Similar to our finding, Garg *et al.*³⁷ reported ammonium sulphate as good source of nitrogen for bacterium *P. putida* SKG-1 to achieve maximum decolorization of dye Acid Orange 7. Equally, Carolin *et al.*³⁵ found effective decolorization of Methyl Orange dye by *M. yunnaenensis* in presence of yeast extract and peptone mediated nitrogen supplement. The culture medium supplemented with organic/inorganic nitrogen source involved in production of NADH, which facilitates the decolorization of azo dyes by bacterial system via donating the electrons³⁸.

The strain RMLP1 showed highest decolorizing activity at dye concentration of 100 mg/L while above this concentration of dye the rate of decolorization was gradually reduced. The decrease in decolorization extent was attributed to toxicity of dye to bacteria by inhibition of metabolic activity. Ghodake *et al.*²⁴ also reported that the dye concentration 100 mg/L of Amaranth was removed up to 71% in 72 hrs by *Acinetobacter calcoaceticus* but beyond this decolorization activity was not effective. Moreover, Carolin *et al.*³⁵ reported the highest decolorization rate of Methyl Orange by *M. yunnaenensis* strain at 100 mg/L and further increase in dye concentration leads to decrease in decolorization rate.

5. Conclusion

In this study, a dye decolorizing bacterial strain, *Klebsiella variicola* RMLP1 was isolated from soil contaminated with textile effluent. The biodecolorization activity of *Klebsiella variicola* RMLP1 was mediated through degradation means but not by adsorption. Biodegradation of AB 113 by *Klebsiella variicola* RMLP1 strain was not earlier described in any other studies. *Klebsiella variicola* RMLP1 has immense potential for bioremediation of number of azo dyes frequently used in various industries as it possessed potent decolorizing activity.

6. Acknowledgement

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