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Efficient Decolorization of Dye Acid Blue 113 by Soil Bacterium *Bacillus subtilis* RMLP2

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Abstract

In this study, a bacterial strain was isolated from soil and tested for the decolorization of azo dye Acid Blue 113 (AB 113). Decolorization of azo dyes by means of physico-chemical method is not environmentally friendly thus an alternative method based on bacteria was employed for decolorization of AB 113. The color removal studies were performed using Bushnell and Hass medium amended with AB 113 dye. Bacterial isolate *Bacillus subtilis* RMLP2 was identified by 16S rRNA gene sequence analysis. The effect of various physico-chemical parameters such as incubation condition, pH, temperature, carbon source, nitrogen source and dye concentration on decolorization of AB 113 by *Bacillus subtilis* RMLP2 were studied. The bacterial isolate showed the remarkable higher percentage (92.71%) of color removal of dye AB 113 at 100 mg/L concentration, 35^{0}C, pH 7 during 72 h of incubation period under static condition. Yeast extract and glucose was found as best nitrogen and carbon source for efficient decolorization of dye. These results confirmed that the *Bacillus subtilis* has enormous ability to degrade dye AB 113 present in textile effluents.

Keywords: Acid Blue 113, Azo Dye, *Bacillus subtilis*, Decolorization, 16S rDNA

1. Introduction

Our sacred environment is mainly polluted by the discharge of millions of liters of wastewater containing synthetic dyes[1]. Dyes are described by presence of chromophore group in their complex structures and are mainly classified as anthraquinone, phthalocyanine and azo dyes[2]. Around 2,000 diverse xenobiotic azo dyes are widely employed in several industries like textiles, leather, cosmetics, food and printing[3,4]. Textile industries utilized around 70% of azo dyes as a coloring material during dyeing process[5]. These dye contains typical one or more (-N=N-) azo bond[6,7]. Worldwide, it was reported that approximately 280,000 tons of textiles dye are discharged along with industrial wastewater every year[8]. Wastewater released from textile industries was proved to be toxic and mutagenic to aquatic inhabitants once discharged into the water ecosystem[9,10]. Hence, the release of textile wastewater into the water reservoir is of major environmental concern[11,12]. Azo dyes also induce allergic reactions and their degradative metabolites are extremely mutagenic and carcinogenic to humans[13]. The parent compound (dye) was quietly found less toxic as compared to their degradative metabolites, which was recalcitrant and more injurious[14]. The textile industry wastewater consists of dyes, stabilizers, detergents and additives, employed at various steps of dyeing process. These agents are accumulated in aquatic bodies and leads to diminished penetration of sunlight thereby inhibiting photosynthesis and respiration in flora and fauna[15,16]. The wastewaters are characterized by different factors like Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), high pH, Suspended Solids (SS) and intense color quality[17]. The complex aromatic structures of dye contribute its resistance against the sunlight, temperatures

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and detergents[18]. The treatment of textile wastewater prior to their discharge into the environment is essential to avoid its negative impact. Conventional methods, for example, adsorption, ozonation, electrocoagulation, flocculation, ion exchange, reverse osmosis and membrane filtration have been utilized to treat the dye containing wastewater[11,19,20]. Some synthetic molecules for example nanosized gels[21], nanoparticles[22] and metal containing photoactive clay[23] have been developed for dye removal from wastewater. These methods were specific towards dyes, expensive and generate massive quantity of sludge after treatment[24]. This makes researcher to search some other viable alternatives method for wastewater treatments[25]. Biological methods including use of bacteria, algae and fungi are most appropriate and extensively tested treatment method due to less production of sludge, cost effectiveness and eco-friendly nature. These microorganisms possess enzyme system to decolorize and degrade the azo dyes under definite environmental conditions[26]. Numerous bacteria have been reported for efficient degradation of azo dyes[27,28].

In present study, we studied the potential of *Bacillus subtilis* RMLP2 bacterial strain for the efficient decolorization of azo dye Acid Blue 113 (AB 113). The effects of various nutritional and physical parameters on biodecolorization of AB 113 were also studied.

2. Material and Methods

2.1 Dyes and Chemicals

The azo dyes (AB 113, Reactive Red 120, Direct Blue 71, Congo Red, Acid Orange and Metanil Yellow) tested in present study were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., St. Louis, Missouri, USA. Other chemicals and media constituents were of an analytical grade and of highest purity.

2.2 Culture Medium

Bushnell and Haas Medium (BHM) prepared by using $MgSO_4$ 0.2 g/L, $CaCl_2$ 0.02 g/L, NH4NO3 1.0 g/L, $FeCl_3$ 0.05 g/L, K_2HPO_4 1.0 g/L supplemented with yeast extract (0.05% w/v) and glucose (0.1% w/v) were utilized throughout the experiments. The pH of culture medium was maintained at 7.0.

2.3 Isolation and Identification of Bacterial Strain

To isolate potent dye decolorizing bacterial strain, samples (soil) were collected from different dye spilled areas of dyeing houses of Tanda, Uttar Pradesh (India). Soil suspension was prepared by mixing 10 g of soil sample in 100 ml of BHM containing AB 113 dye and was utilized for isolation of potent decolorizing bacteria. Furthermore, 10% (v/v) aliquot of soil suspension was transferred into Erlenmeyer flask (250 ml) containing 100 ml BHM amended with AB 113 dye and kept at 30^{0}C under static and shaking condition. After a number of repetitive transfers into new dye containing media considerable dye decolorization extent was achieved. The decolorized sample was 10 times serially diluted and 100 microlitre aliquot of dilution were spread on BHM (containing AB 113 dye of 100 mg/L concentration) agar plates. Bacterial isolates showing clear zone of decolorization were repeatedly streaked to get pure culture on BHM (containing 100 mg/L AB 113 dye) agar plates for further purification. After purification bacterial isolates were preserved at 4^{0}C in slant and were further screen for their decolorization capacity against dye AB 113 (100 mg/L) in liquid medium. The bacterial isolate having maximum decolorization extent was designated as BT-2.

2.4 16S rRNA Gene Amplification of Bacterial Isolates for Identification and Phylogenetic Relationship

50 ml of Luria Bertani (LB) broth was used to inoculate freshly isolated single colony of isolate BT-2 and kept at 37^{0}C for 24 h in a shaker incubator (150 rpm). After centrifugation of broth, DNA purification kit was utilized for extraction of genomic DNA from cell pellet. The 16S rRNA gene amplification done by using purified bacterial genomic DNA. A reaction master mix (Promega) of 50 µl was prepared which contains 50 ng purified DNA. 10 picomols of both primers (27f-1492R) were added in master mix and mixed thoroughly[29]. Amplification of 16S rRNA gene was done by polymerase chain reaction using a thermal cycler (BioRad-My cycler). The amplification product (amplicons) was preserved at -20^{0}C. These amplicons were further used for qualitative analysis via agarose gel electrophoresis. Amplicons (2 µl) were mixed in loading dye (6X) and loaded on agarose gel (1.2%). The electrophoresis was run at 60 V for 4 h. The gel documentation system (BioRad) was used to visualize agarose gel and the size of amplicon was assessed by using DNA marker ladder (1 kb Promega).

The PCR product of 16S rRNA gene was purified (Qiagen) and processed for DNA sequencing. Sanger di-deoxy sequencing (Big Dye Terminator Sequencing Kit V3.0) was used for DNA sequencing on ABI 3130XL Genetic Analyzer (Applied Bio systems USA). Quality of DNA sequence was evaluated and performed nucleotide BLAST (BLASTn, NCBI, USA) analysis. Neighbor-Joining methods and Mega 6.0 software were employed for phylogenetic analysis[30].

2.5 Effects of Various Physico-chemical Parameters

For efficient decolorization of AB 113 dye, the effect of various process parameters such as static and shaking condition, temperature (20^0C, 25^0C, 30^0C, 35^0C, 40^0C interval), pH (4, 5, 6, 7, 8 and 9), carbon source (glucose, maltose, fructose, lactose, sucrose and starch), nitrogen source (ammonium nitrate, yeast extract, peptone, ammonium chloride and ammonium sulphate), dye concentration (100 mg/L - 600 mg/L) on decolorization of Acid Blue 113 were investigated. All experiments were performed in triplicates. The experiments were conducted in 15 ml screw capped tubes containing 10 ml of decolorizing mixture. The tubes were inoculated with 100 microlitre inoculum and kept under static condition at 30^0C.

2.6 Decolorization of Various Textile Dyes

The decolorization potential of *Bacillus subtilis* RMLP2 was tested under pre-optimized conditions for various structurally diverse azo dyes. Five different azo dyes namely, Reactive Red 120, Direct Blue 71, Congo Red, Acid Orange and Metanil Yellow with concentration of 100 mg/L were tested under the pre-optimized conditions.

2.7 Decolorization Manner of *Bacillus subtilis* RMLP2 Strain on Acid Blue 113

To investigate the decolorization manner of dye Acid Blue 113 by *Bacillus subtilis* RMLP2 strain, live and heat killed (autoclaved) bacterial cells were used. The culture medium inoculated with live bacterial cells suspension was treated as a control. An autoclaved (at 121^0C for 30 min) bacterial cell suspension was added into the culture medium to examine decolorization limit of Acid Blue 113. Both of these experiments were used to perform decolorization activity for incubation time of 72 h in static condition. After end of incubation time, decolorization mixture was centrifuged for 10 min at 10,000 rpm and 4^0C. To detect any alteration in structure of Acid Blue 113 in the decolorization mixture following decolorization, supernatant was scanned by using a UV-Vis spectrophotometer (Labtronics) between 200 nm and 800 nm wavelength.

2.8 Measurement of Dye Decolorization

To determine the decolorization percentage, decolorized mixture was analyzed at every 12 h of interval. The decolorized mixture was centrifuged for 10 min at 10,000 rpm and 4^0C. Supernatant was utilized for measurement of the decolorization by detecting change in absorbance at 566 nm by UV-Vis spectrophotometer[31]. The following formula was used to calculate the percentage of decolorization[32].

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

2.9 Statistical Analysis

The experiments in this study were performed in the replicates of three. The results obtained have been expressed in terms of mean ± SD value and standard error was calculated by using Microsoft Excel (version Windows 2007).

3. Results

3.1 Isolation and Identification of Bacterial Strain

Isolation of potent dye degrading bacterial strain was accomplished from soil samples collected from different dye spilled areas of dyeing houses of Tanda, Uttar Pradesh (India). A bacterial strain BT-2 which showed efficient decolorization activity towards dye Acid Blue 113 was isolated by using BHM supplemented with yeast extract and glucose. The strain BT-2 is a gram positive, rod shaped, endospore forming, oxidase negative and catalase positive bacterium.

16S rRNA gene sequencing was performed for identification of bacterial strain BT-2. The BT-2 bacterial strain was identified (with 99% sequence similarity) as *Bacillus subtilis* RMLP2 after the nucleotide BLAST (BLASTn, NCBI, USA) analysis. The sequence was then submitted to GenBank database (NCBI, USA) with accession number KY794215. 16S rRNA gene sequences from 10 closely related *Bacillus* were downloaded from NCBI database. All 10 DNA sequences along with strain RMLP2 were used for phylogenetic analysis and DNA sequence from E. coli was used as out group. Phylogenetic analysis was done by using MEGA 6 software[30]. Dendrogram showed that strain RMLP2 is closely related to the *Bacillus subtilis* (Figure 1).

3.2 Effect of Incubation Condition

Presence or absence of oxygen has profound influence on growth and activity of bacterial cells. Oxygen may be either favorable or inhibitive for bacterial decolorization of dyes. *Bacillus subtilis* RMLP2 strain decolorized the Acid Blue 113 dye up to 82.32% in static condition whereas only 34.00% decolorization was achieved in shaking environment (oxygen rich) during 72 h incubation (Figure 2).

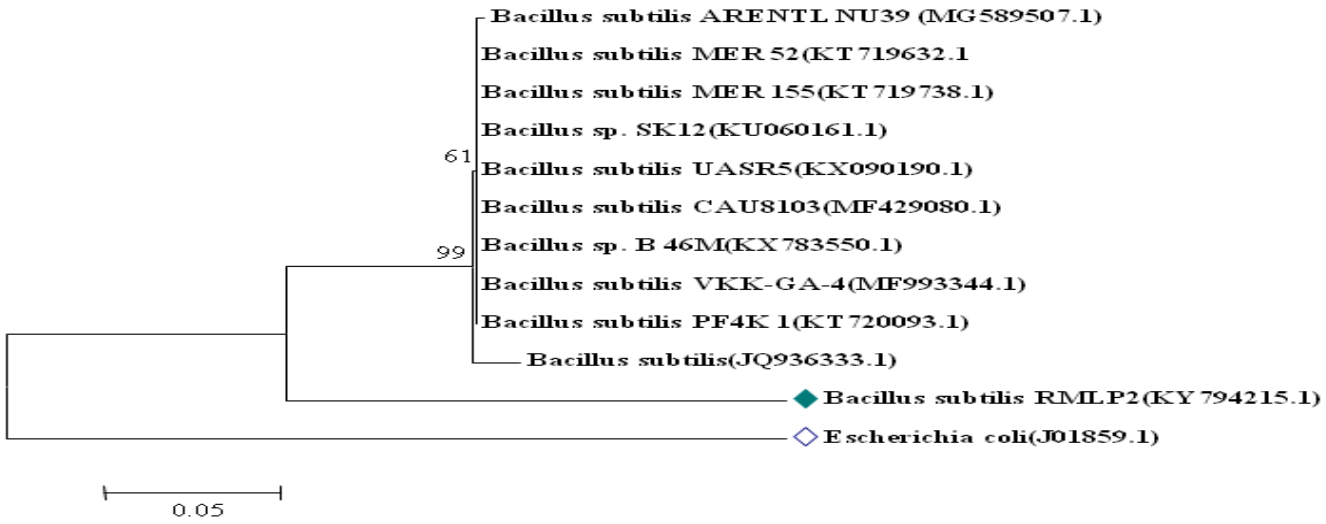


Figure 1. Phylogenetic tree of the *Bacillus subtilis* RMLP2 strain.

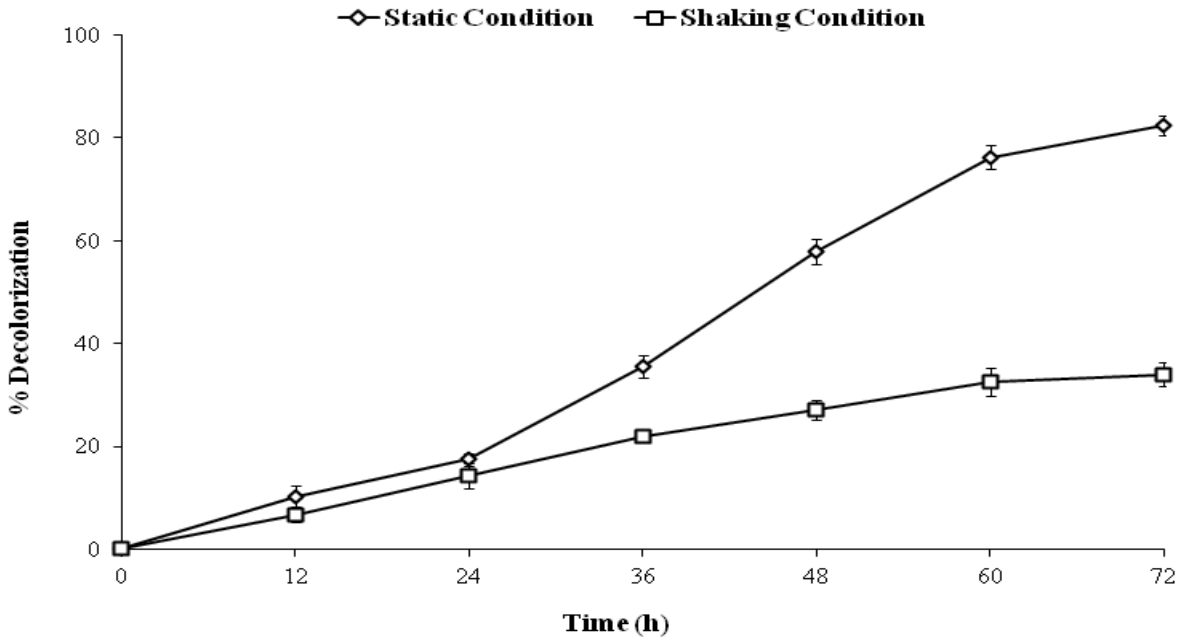


Figure 2. Effect of incubation condition on decolorization of AB 113 dye.

3.3 Effect of pH

The pH is an essential factor which influences the reproduction, microbial growth and bioremediation activity of microorganisms. The medium pH has a direct relation with the dye decolorization rate. Bacterial strain successfully removes the color in between pH 6 and 10. The role of pH on decolorization of AB 113 was studied at 100 mg/L, 30^{0}C under static condition for 72 h. The maximum decolorization (84.17%) can be obtained for AB 113 dye by *Bacillus subtilis* RMLP2 strain at optimum pH 7 under static condition (Figure 3). The decolorization rate gets dropped in either side of pH from the optimum level.

3.4 Effect of Temperature

The efficient growth and metabolism of bacterial cells immensely relies on the temperature which ultimately leads to significant dye decolorization. The consequence of

temperature on AB 113 dye decolorization was monitored at pH 7, 100 mg/L, under static condition for 72 h. It was recorded that, upon increase in temperature from 20 to 35°C, the rate of color removal was increased from 29.66% to 90.53% for AB 113 dye by strain RMLP2 and upon additional increase in temperature beyond 35^{0}C, the color removal was decreased to 44.27%. Thus the optimum temperature for color removal of AB 113 dye by strain RMLP2 was found to be 35^{0}C with 90.53% decolorization (Figure 4).

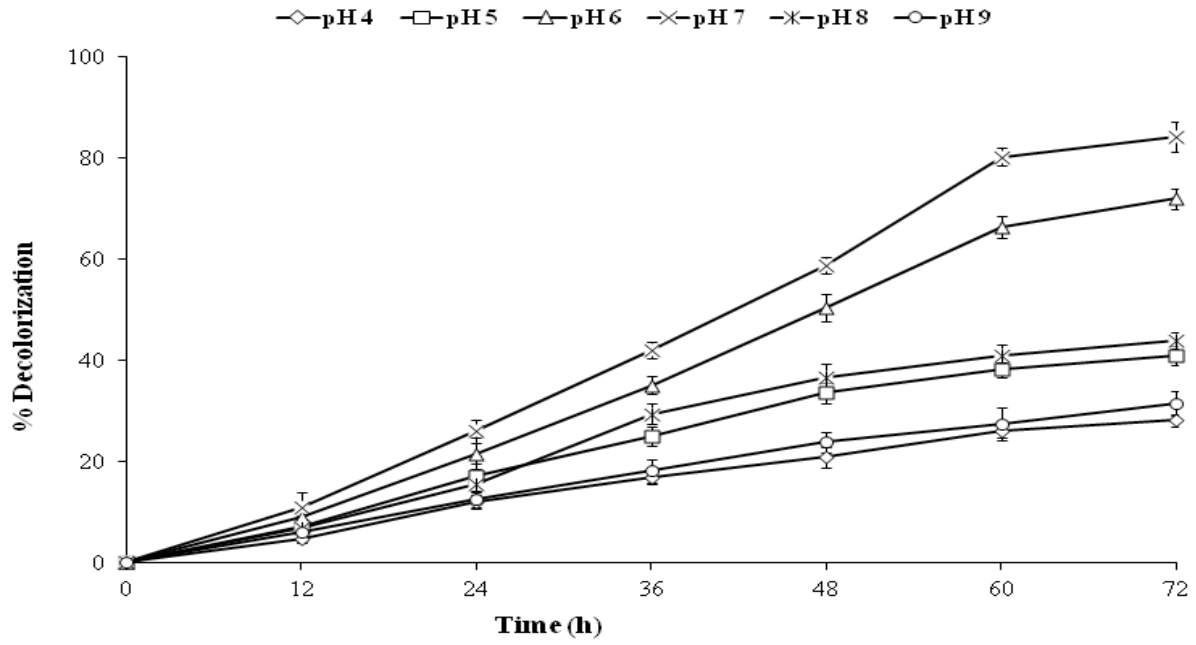


Figure 3. Effect of pH on decolorization of AB 113 dye.

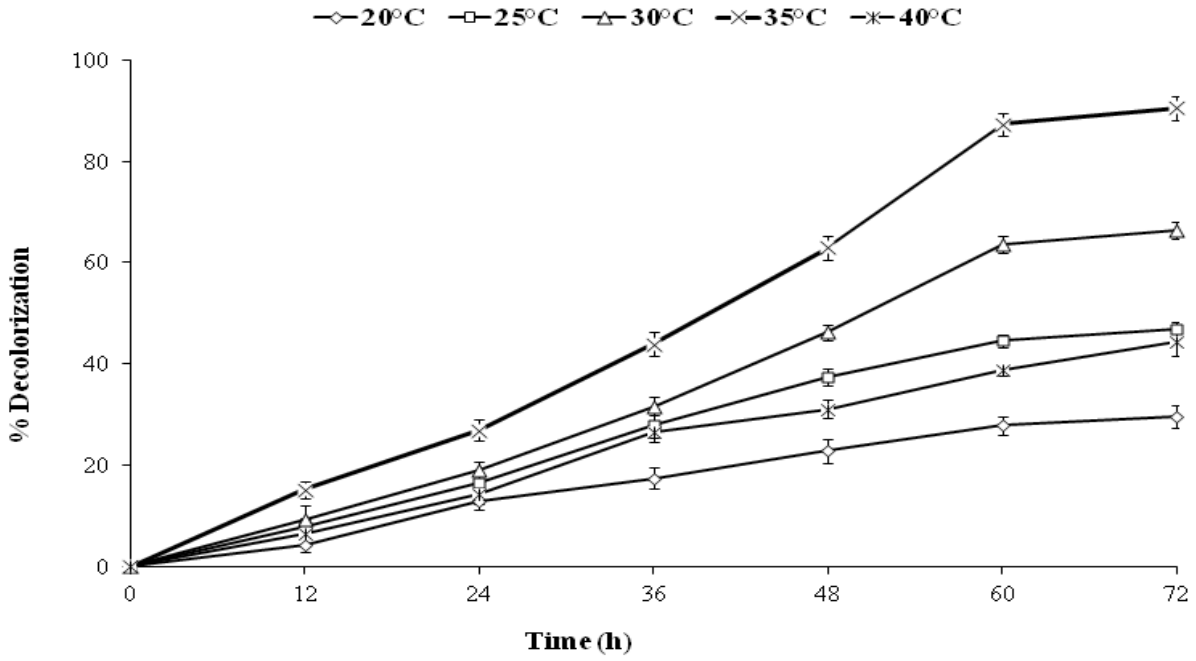


Figure 4. Effect of temperature on decolorization of AB 113 dye.

3.5 Effect of Carbon and Nitrogen Source

In the absence of any nutritional supplement (carbon or nitrogen sources) the bacterial decolorization of dyes is very difficult. These supplements (act as co-substrate) required by bacteria for its growth and metabolic activity and facilitate the transfer of electrons between donor and acceptor. The oxidation of electron donors leads to the reduction of azo bond resulting in decolorization of dye. The effect of carbon sources such as glucose, glucose, maltose, fructose, lactose, sucrose and starch, and nitrogen sources such as ammonium nitrate, yeast extract, peptone, ammonium chloride and ammonium sulphate on decolorization of AB 113 dye by bacterial isolate RMLP2 was studied in BHM at pH 7 and 35^{0}C temperature under static condition for 72 h. Among the different carbon sources tested, glucose was found as the best carbon source which facilitates 91.37% of decolorization of AB 113. The other carbon source like maltose, fructose, lactose, sucrose and starch were less suitable and facilitates and facilitates 68.21%, 72.05%, 42.25%, 65.42% and 27.91% decolorization of AB 113 respectively (Figure 5).

Similarly the maximum decolorization (91.85%) of dye AB 113 was achieved in presence of yeast extract by strain RMLP2. The other nitrogen sources like ammonium nitrate, peptone, ammonium chloride and ammonium sulphate was less suited for strain RMLP2 and achieved 68.74%, 67.58%, 42.97% and 60.48% decolorization of AB 113 respectively (Figure 6).

3.6 Effect of Dye Concentration

The consequence of variable dye concentration on rate of dye removal by *Bacillus subtilis* RMLP2 strain on AB 113 was measured at pH 7, temperature 35^{0}C under static condition. The strain RMLP2 showed that gradual increase in dye concentration (100 to 600 mg/L) leads to significant reduction in the percent decolorization. The maximum decolorization (92.71%) of AB 113 was achieved at 100 mg/L dye concentration during 72 h of incubation (Figure 7).

3.7 Decolorization of Various Textile Azo Dyes by *Bacillus subtilis* RMLP2

The *Bacillus subtilis* RMLP2 strain have the potential to decolorize the various azo dyes up to variable extent under pre-optimized conditions. Among the different dyes, the bacterial strain RMLP2 showed maximum decolorization (82.15%) of Direct Blue 71. Other four dyes including Reactive Red 120, Congo Red, Acid Orange and Metanil Yellow were removed up to 57.65%, 52.48%, 64.50% and 68.69%, respectively.

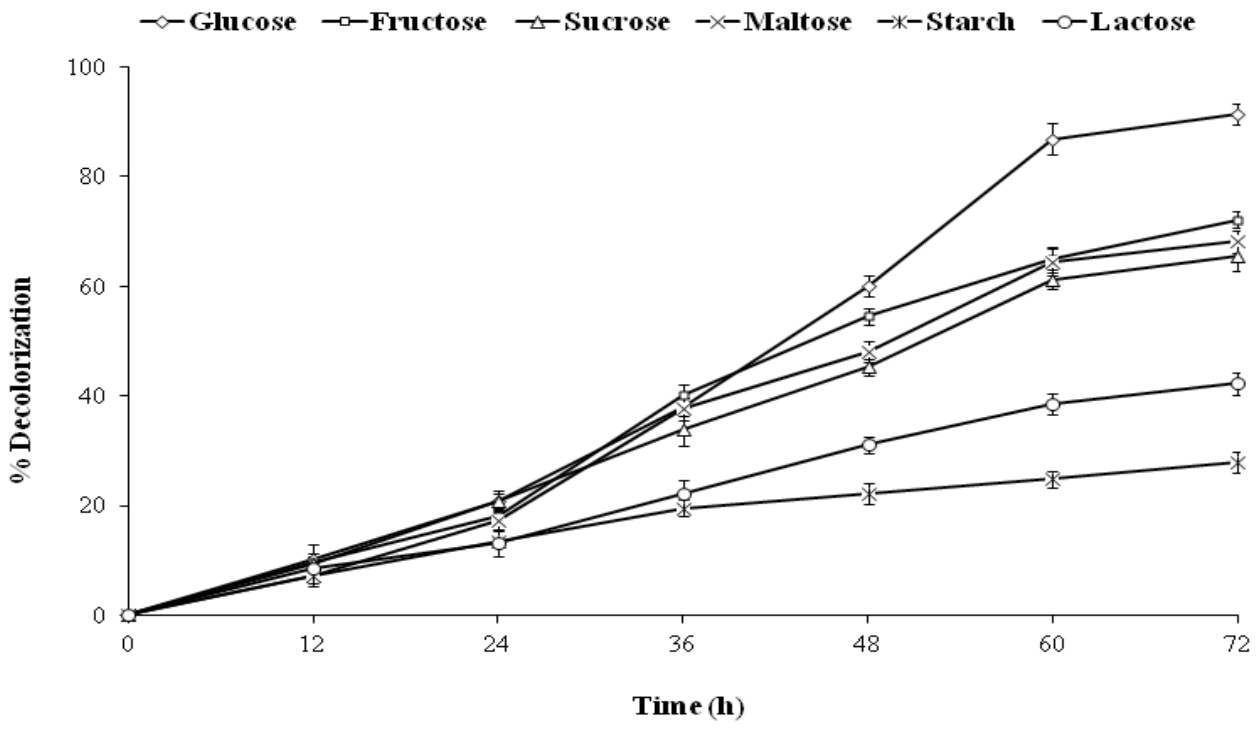


Figure 5. Effect of different carbon source on decolorization of AB 113 dye.

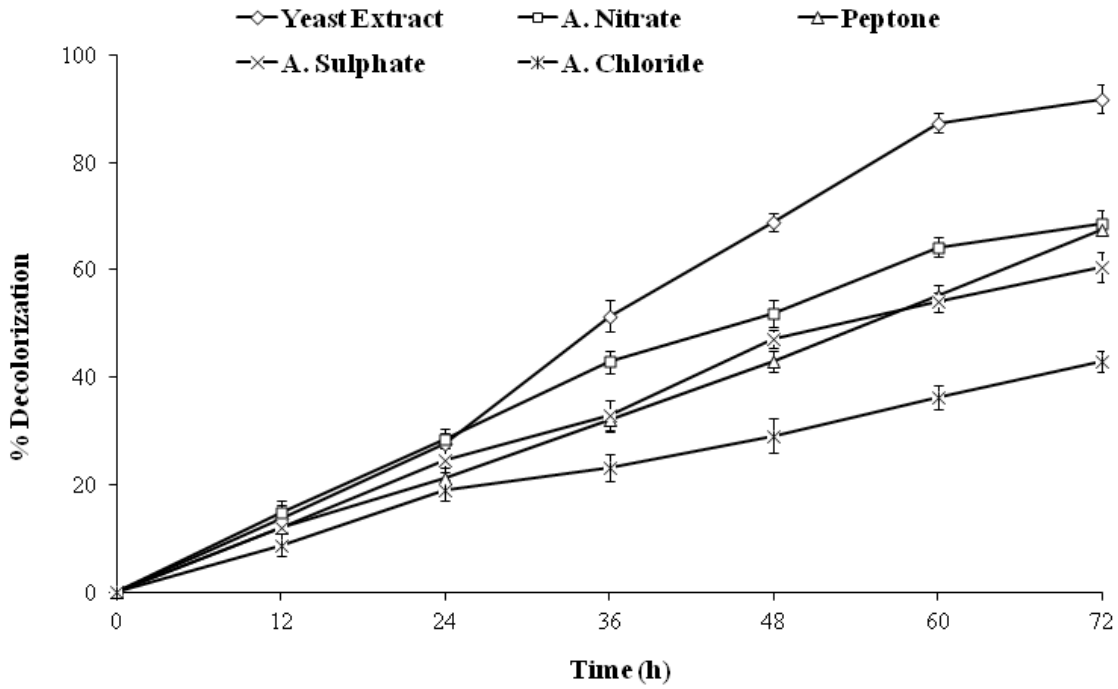


Figure 6. Effect of different nitrogen source on decolorization of AB 113 dye.

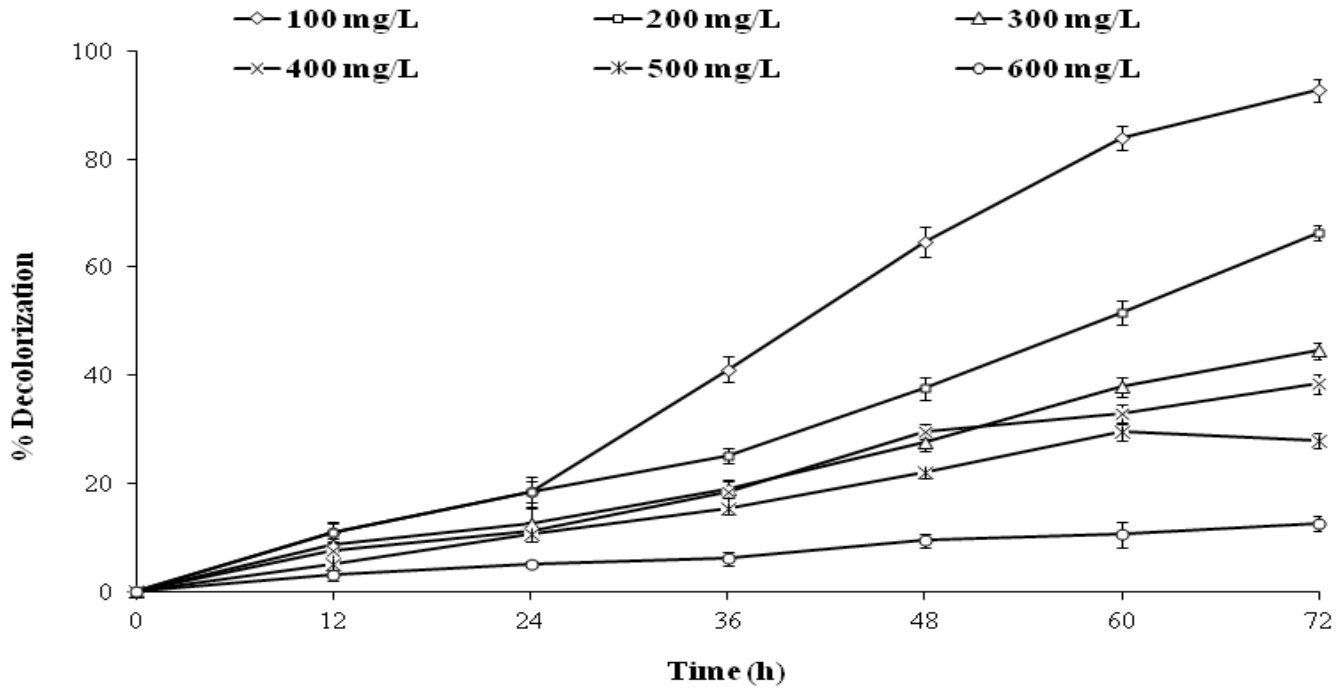


Figure 7. Effect of AB 113 dye concentration on decolorization of AB 113 dye.

3.8 Decolorization Manner of *Bacillus subtilis* RMLP2 Strain against Acid Blue 113

The basic mechanism for biodecolorization of azo dyes involved either adsorption[33] or degradation[34]. Adsorption involves the physical attachment of dyes on the surface of degraders but dye remains in the medium thus it is not a useful process while in degradation process of dye, the degraders utilizes their dye specific enzymes to break the azo bond and produces new compounds which may or may not be harmful. In the degradation process of dye, the major absorbance peak present in visible region would totally disappear or a new peak may emerge[35]. The culture medium inoculated with heat killed (dead) bacterial strain showed only 25.26% decolorization of AB 113 after 72 h of incubation. In the control experiment (live cells) the rate of decolorization was found up to 89.83% after 72 h of incubation (Figure 8). The UV-Vis spectral scan (200-800 nm) of supernatant of decolorized mixture showed maximum absorbance peak at 566 nm in visible region was totally disappeared in control experiment (Figure 9). This change of absorption pattern indicated that the decolorization of AB 113 was due to biotransformation and primary chromophore was destroyed during the process of decolorization.

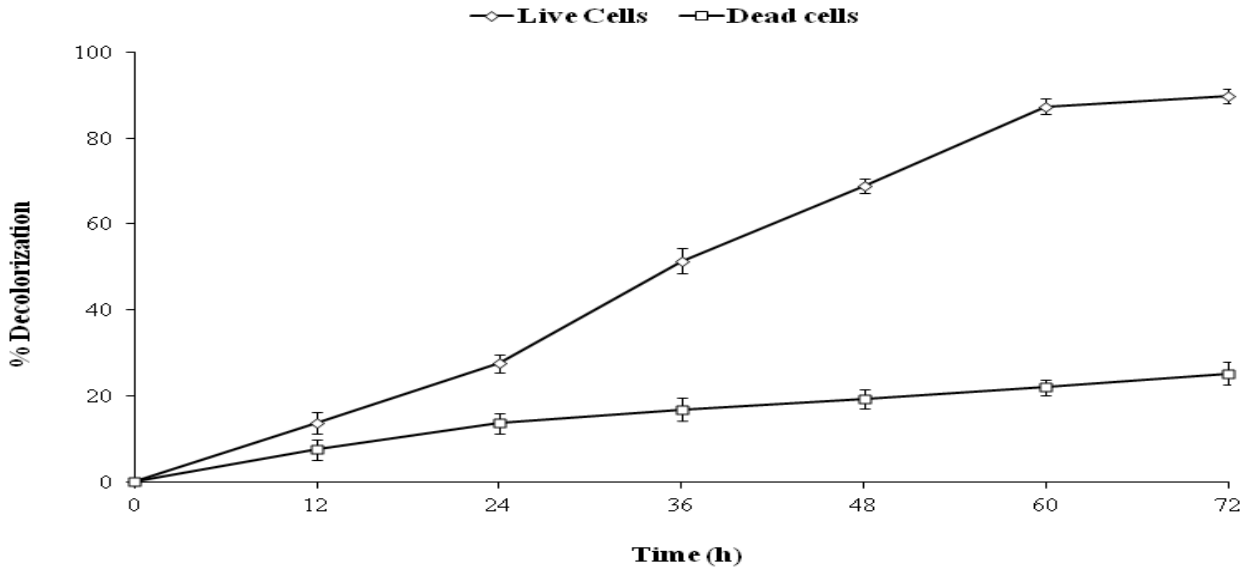


Figure 8. Decolorization of Acid Blue 113 dye by live cells and dead cells (heat-killed) of *Bacillus subtilis* RMLP2.

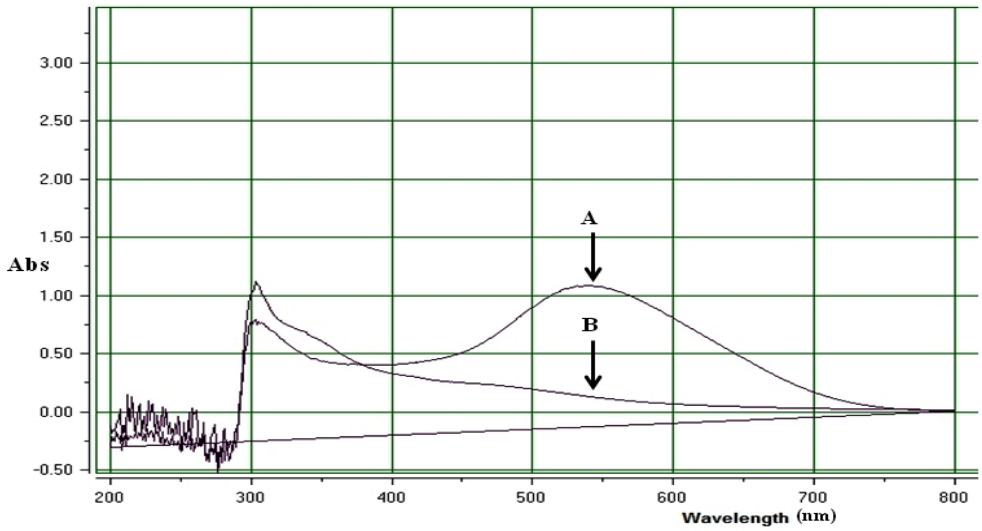


Figure 9. Variation in the UV-visible spectra of Acid Blue 113 dye before and after decolorization by *Bacillus subtilis* RMLP2 (A, 0 h: B, 72 h).

4. Discussion

The bacterial strain *Bacillus subtilis* RMLP2 was found to be potent decolorizer of the dye AB 113 under static condition. Unnikrishnan *et al.*[36] found maximum decolorization (88.55%) of Reactive Red 198 under static condition by using a dye tolerant bacterium A. *baumannii*. The decolorization (75.5%) of Remazol Black B by *Pseudomonas aeruginosa* KY284155 was observed under static condition after 48 h of incubation while only 4.26% color removal was observed under shaking condition[37]. Singh *et al.*[5] have observed the decolorization of Acid Orange up to 85.52% by *Staphylococcus hominis* RMLRT03 in static condition and only 32.47% decolorization under shaking condition. The static condition is preferred over shaking condition as availability of oxygen hinders the release of electron from electron donor (NADH) and compete with the azoreductase enzyme[38].

The maximum decolorization of AB 113 by *Bacillus subtilis* RMLP2 strain was recorded at pH 7. Similarly, Singh *et al.*[5] achieved the highest decolorization (94%) of azo dye Acid Orange at pH 7 with *Staphylococcus hominis* RMLRT03. Olukanni *et al.*[18] reported the highest color removal by *Micrococcus* R3 strain of dye Methyl Red at 6.0-8.0 pH range. The medium pH is the deciding factor for the entry of dye molecule across the cell membrane.

In the present study optimum temperature to achieve maximum decolorization of AB 113 was 300C. Shah *et al.*39 noticed that the optimum temperature for decolorization of azo dye Reactive Black by bacterial strains *Klebsiella oxytoca* and *Bacillus subtillis* was 32°C. Moreover, Arulazhagan40 attained the 60% color removal of Reactive Red M8B at 35^{0}C by culture *Bacillus subtilis*. It was found that slight alteration of temperature from the optimum level leads to inhibitory effect on rate of bacterial multiplication and growth as well as the enzymes activity[32].

The most suitable carbon source found for maximum decolorization of AB 113 dye by RMLP2 strain was glucose. Several reports indicate that the additional carbon source promote the effective decolorization of dye. Joe *et al.*[41] reported that the color removal of Reactive Black 5 and Reactive Red 3B-A by *Clostridium bifermentans* strains was enhanced by the addition of carbon source glucose to the culture medium. Similarly, *Micrococcus yunnaenensis* strain showed 95% decolorization of Methyl Orange by utilizing the glucose as additional carbon source[42]. Glucose not only provides energy to support growth and development of microbes but also act as electron donor which is responsible for breakdown of azo bond[43].

In present study yeast extract was found to be a best nitrogen source for AB 113 dye decolorization. Carolin *et al.*[42] demonstrated the decolorization of Methyl Orange dye by bacterium *M. yunnaenensis* in presence of additional nitrogen source yeast extract and peptone. Similar result was also reported by Telke *et al.*[44] where efficient color removal of Reactive Red 141 dye was achieved by *Rhizobium radiobacter* in presence of yeast extract as a co-substrate. The organic/inorganic nitrogen supplements are the source of NADH (an electron donor) which is responsible for breakdown of typical azo bonds by microbial system[45].

The best color removal of AB 113 dye was achieved at 100 mg/L concentration of dye and beyond this concentration rate of decolorization gradually decreases. Similar to our study, maximum color removal of Crystal Violet was observed at 150 mg/L concentration in 72 h. The degradation rate of Crystal Violet was decreased with increase in concentration of dye above from 150 mg/L46. Furthermore, Carolin *et al.*[42] achieved maximum color removal of Methyl Orange dye by strain *M. yunnaenensis* at 100 mg/L and additional increase in dye concentration showed reduction in decolorization rate. The decrease in color removal is attributed to an increased lethal effect of the dyes to the bacteria or it may mask the active sites of degradative enzymes produced by bacteria[5]. The strain RMLP2 also showed the ability to decolorize various azo dyes with remarkable decolorization percentage. The difference in decolorization extent of various dyes is due to complex structure of these dyes[32]. In this study, the decolorization potential of *Bacillus subtilis* RMLP2 was tested for different azo dyes and the results indicated that the strain RMLP2 could be applied for removal of toxic dye containing effluent.

The result of UV-visible spectral scan suggested that the bio-removal of AB 113 dye by *Bacillus subtilis* RMLP2 was due to the biodegradation rather than adsorption. Fan *et al.*[47] concluded similar result by demonstrating that the dye removal by bacteria was due to the biodegradation not by adsorption.

5. Conclusion

In this study, an efficient dye decolorizing bacterial strain *Bacillus subtilis* RMLP2 was isolated and identified from soil contaminated with dyes. Bacterial strain *Bacillus subtilis* RMLP2 has the potential to decolorize the azo dyes AB 113. The *Bacillus subtilis* RMLP2 showed maximum decolorization (92.71%) of AB 113 at pH 7, 35^{0}C temperature and 100 mg/L dye concentration along with glucose and yeast extract as nutritional supplements under

static condition. The mechanism behind the decolorization of AB 113 by *Bacillus subtilis* RMLP2 was degradation rather than adsorption. The decolorization potential of *Bacillus subtilis* RMLP2 against AB 113 and other azo dyes proved that the strain have immense potential and may be used for eco-friendly treatment of different harmful dyes and textile effluent.

6. Acknowledgements

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7. Conflict of Interest

The authors declared no conflict of interest.

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