

Research Article

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Bioremediation of Remazol Black B by newly isolated *Bacillus endophyticus* LWIS strain

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Abstract

Azo group containing most of the dyes are released into untreated wastewater lacking of any pretreatment and pollute water and soil environments. To thwart pollution of our prime resources, elimination of these dyes containing impurity is of enormous significance. For this purpose, wastewater samples were brought to gather from dye-contaminated sites of India. Total 14 bacterial isolates were isolated through enrichment and then tested for their potential to remove Remazol Black-B azo dye in liquid medium. Isolate LWIS1, LWS2 & LWS3 having competence to degrade Remazol Black-B proficiently were screened on modified mineral salt medium. LWIS1 was competent to absolutely remove the dye from the liquid medium in 10 h. The isolate LWIS 1 exhibited the most excellent performance at the dye concentration of 150 mg L⁻¹ medium (pH 7.5) and at temperature 37°C. Similarly, yeast extract proved to be the best carbon source for decolorization purpose. The results imply that the isolate LWIS1 could be used for the removal of the reactive dyes from textile effluents.

Keywords: Azo dyes; Remazol Black-B; Wastewater; Yeast extract

Introduction

Effluent discharged from the textile industries has variable characteristics in terms of pH, dissolved oxygen, organic, and inorganic chemical content, etc. Together with industrialization, awareness towards the environmental problems arising due to effluent discharge is of critical importance. Pollution caused by dye effluent is mainly due to durability of the dyes in waste water [1]. Existing effluent treatment procedures utilize pH neutralization, coagulation followed by biological treatment, but they are unable to remove recalcitrant dyes completely from effluents. This is because of the color fastness, stability, and resistance of dyes to degradation [2].

Bioremediation is the microbial clean up approach, microbes can acclimatize themselves to toxic wastes and new resistant strains develop naturally, which can transform various toxic chemicals to less harmful forms. Several reports have suggested that the degradation of complex organic substances can be brought about by bacterial enzymes [3-8]. Different dyes used in textile industry usually have a synthetic origin and complex aromatic molecular structures which make them more

stable and more difficult to be biodegraded. Due to their ease of manufacturing methodology, azo dye accounts for almost 80% of annual production of commercial dyes all over the world. There are over 10,000 commercially available dyes with a production of over 7×10⁵ tons per year [9]. Azo dyes, containing one or more azo bond (-N=N-), account for 60-70% of all textile dyestuffs used. It is estimated that about 10-15% of the total production of colorants is lost during their synthesis and dyeing Processes [10,11]. Whereas, in the case of reactive dyes almost 50% of the initial dye load is found in the dye bath effluents.

Colored industrial effluent is the most obvious indicator of water pollution and the discharge of highly colored synthetic dye effluents is aesthetically displeasing and cause considerable damage to the aquatic life. Although several physical-chemical methods have been used to eliminate the colored effluents in wastewater, they are generally expensive, produce large amounts of sludge. More often these conventional modes of treatment lead to the formation of some harmful side products. Interest is therefore now focused on the microbial biodegradation of dyes as a better alternative [12].

Some microorganisms, including bacteria, fungi and algae, can degrade or absorb a wide range of dyes [13]. The biological mode of treatment of dye bath effluents offers distinct advantages over the conventional modes of treatment. This method is more economical and leads to less accumulation of relatively harmless sludge. Most importantly, biological treatment of dye bath effluents is eco friendly. It causes mineralization of dyes to simpler inorganic compounds which are not lethal to life forms. The basic step in the decolorization and degradation of azo dyes is breakdown of azo bonds, leading to removal of color. Azo dyes are known to undergo reductive cleavage whereas the resultant aromatic amines are metabolized under aerobic conditions [14]. So for complete mineralization of azo dyes the microbial population forming part of treatment system should be able to work efficiently. In view of these problems the most potent bacterial culture was selected in this study for maximum decolorization of Remazol Black B.

Materials and Methods

Waste water sample collection and analysis

Ankleshwar, Gujarat, India is one of the most industrialized cities in India. It is ideal for waste water sample collection. The samples were collected from the activated sludge of the common effluent treatment plant of Ankleshwar, Gujarat, India. Samples were collected from the aeration tank of common effluent treatment plant. The Temperature and pH was analyzed at the site. The temperature was analyzed using laboratory grade thermometer and pH was analyzed by using pH meter (Hanna digital pH meter). The sample was transferred to laboratory at 4 °C as per the standard methods [24]. The physicochemical parameters such as Color, Biological Oxidation Demand, Chemical Oxygen Demand, Total Suspended Solids, and Total Dissolved Solids were analyzed as soon as the sample was brought to the laboratory (Table 1). Sample color was analyzed by spectrophotometer (SHIMADZU UV-1700). BOD was determined by employing evaporation method by dissolve oxygen meter while chemical oxygen demand was measured by instrument directly.

Table 1: Physico-Chemical Characteristics of the effluent collected from common effluent treatment plant.

Sr. No	Parameter	Unit	Effluent
1	Color	-	Blackish Blue
2	Smell	-	Pungent
3	pH	-	8.2
4	COD	ppm	12800
5	BOD	ppm	3400
6	TSS	ppm	1800
7	TDS	ppm	28600
8	NH ₄ -N	ppm	658

Enrichment and isolation of dye decolorizing bacteria

The bacterial strains were isolated from waste water containing activated sludge. Inoculums developed from the isolate LWS1 was first enriched using a modified MSM medium with Remazol Black-B dye as the source of C and N [20]. Remazol Black B dye was added to a concentration of 150 mg L⁻¹. The cultures containing 200 ml MSM with a dye broth in 500 ml Erlenmeyer flasks were inoculated with 10 ml volume of activated sludge. The flasks were incubated at 37°C for 5 days in static conditions. After incubation, the cell suspensions from each vial were plated on MSM agar medium and incubated at 37 °C for 24 h. Bacterial colonies that appeared on the agar medium were picked, washed gently with sterile water and re suspended in flasks containing fresh MSM broth enriched with the dye. Approximately 14 colonies actively growing were selected for purification.

Purification of bacterial isolates

The selected isolates were purified by streaking on agar containing MSM medium at 20 g L⁻¹ concentration. Streaking was done thrice in Zig-zag manner. The purified cultures were preserved in a refrigerator for subsequent study.

Screening

Using modified MSM medium, primary screening was done to obtain proficient bacterial strains able to decolorize the azo dye. For this purpose, 14 isolates gained ability to decolorize Remazol Black-B from all samples was selected. The decolorization ability of each isolate was tested in liquid medium. Remazol Black B amended media were incubated at 37 °C for 24 h with respective bacterial inoculum. After 24 h, harvesting of the cells was done by centrifugation at 10,000 rpm (REMI R-23, India) for 10 minutes. Then decolorization was measured with that facilitate of spectrophotometer (SHIMADZU-1700, Japan) at 597 nm. Uninoculated media were run as blanks to check abiotic decolorization. The three most efficient bacterial isolates (LWIS 1, 2 & 3) from the concluding screening were again tested for their decolorization potentials in test tubes at different time intervals. 0.5% yeast extract as a co-substrate were added in ten milliliters sterilized MSM broth containing azo dye (Remazol Black B) at 150 mg L⁻¹ concentration.

Uniform cell density of the inocula were added with respective bacterial strains at (OD: 0.6) 597 nm. Azo dye containing MSM medium with yeast extract in uninoculated test tubes were incubated under parallel environment to make sure for abiotic decolorization of dye. Decolorization was measured after every 6 hrs interval up to 24 hrs at 597 nm by spectrophotometer as described by Khalid et al. [20].

Environmental parameters optimization

Distinctive manner of parameters like substrate concentration, temperature and pH were optimized during the

performing tests for distinct carbon sources (Mannitol, Yeast extract, glucose, maltose) at 5 g L⁻¹ concentration were explored as co- substrate in the dye removal development. Optimization studies have involved the selection of the concentration of the dye (50, 75, 100, 125, 150, 200 and 250 mg L⁻¹), the temperatures (25, 30, 35, 40, 45°C) and pH (5, 6, 7, 8, 9). Isolated strains LWIS 1, 2 & 3 have been with in order to optimize their color removal competence. As culture conditions were the unchanged as used in color removal experiment i.e., 100 mg L⁻¹ of Remazol Black-B azo dye with MSM was used. Uninoculated blanks were run to make sure the abiotic decolorization throughout the experimentation.

Identification of selected isolate by 16s rRNA gene sequencing method

For 16S rRNA gene sequencing, DNA was isolated using the bacterial DNA isolation kit (Fungal/Bacterial/DNA Miniprep Kit, Zymo Research). The 16S rRNA gene was amplified by PCR using universal bacterial primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR product was purified using a QIA quick PCR purification kit (Qiagen) and sequenced using an ABI PRISM model 3700 automatic DNA sequencer and Big Dye Terminator cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequences of isolates were subjected to NCBI BLAST sequence similarity search Altschul et al. (1990) and EzBioCloud Kim et al. (2012) to identify the nearest taxa. Phylogenetic and molecular evolutionary analyses were done by MEGA version 6(reference). The 16s rRNA gene sequence was submitted to GENE BANK database.

Statistical analysis

Data were entered in a Microsoft® Excel 2007 spreadsheet.

Results

To scrutinize the color potency depth of the azo dye Remazol Black-B decolorizing activity by an effectual bacterial isolates, liquid medium was used. Based on the relative aptitude of the decolorization of different isolates, three most excellent performing bacterial isolates (LWIS1, 2 & 3) with more than 90% of decolorizing activity were preferred for more experiments (Data not shown).

Biodecolorization of remazol black-b by selected bacterial isolates

Microbial decolorization of Remazol Black-B by the most promising selected bacterial isolates (LWIS1, 2 & 3) was corroborated by performing one more experiment in liquid medium at distinctive time frame (Figure 1). It was surveyed that distinctive bacterial isolates had patchy aptitude to eliminate Remazol Black-B in actively growing cultures. The most competent bacterial isolate to decolorize Remazol black-B colorless was by isolate LWIS1 with 98% color degrading efficiency in 10 h incubation interlude while left over isolates

exhibited utmost decolorization in 16h. Isolate 2 was the second largely skilled bacterial isolate and it removes the color of Remazol Black-B up to 94% in 16 h. Similarly, isolate 3 had decolorization potential of 86%.

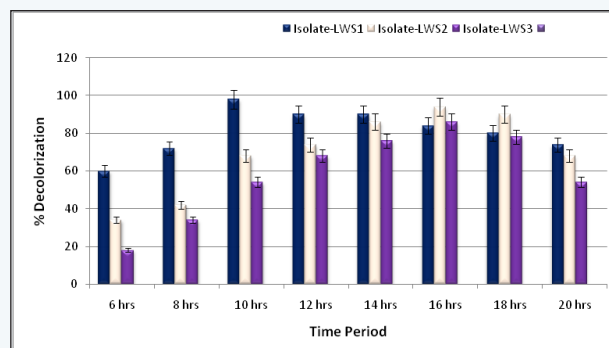


Figure 1: Biodecolorization of Remazol Black -B.



Figure 1.1: Bioremediation of Remazol Black B by isolate LWIS1.



Figure 1.2: Bioremediation of Remazol Black B by isolate LWS2.

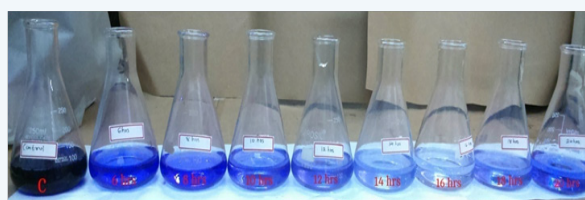


Figure 1.3: Bioremediation of Remazol Black B by isolate LWS3.

Factors affecting biodecolorization of Remazol Black-B in liquid medium

Potential of selected isolates (LWIS1, 2, and 3) was further explored for the optimization of assorted incubation/ environmental circumstances for removal of the azo dye in

liquid medium. It was apparent (Figure 2) that Remazol Black-B azo dye decolorization piercingly improved up to 100 mg L⁻¹ of substrate concentration and utmost removal was observed at 100 mg L⁻¹ of substrate concentration. Then, there was a slow but sure decline in the azo dye decolorization. Isolate LWIS1 was the most proficient azo dye removal strain with more or less complete removal of the color i.e., 100% decolorization at 100 mg L⁻¹ and minimum decolorization was recorded at 50 mg L⁻¹ while after 100 mg L⁻¹ substrate concentration, again LWIS1 showed a decreasing trend. Isolate 2 was the second at the rank with 90% decolorization at 100 mg L⁻¹. But, isolate 3 showed different trends from the other isolates; it indicated enhanced decolorization up to 200 mg L⁻¹ (82%).

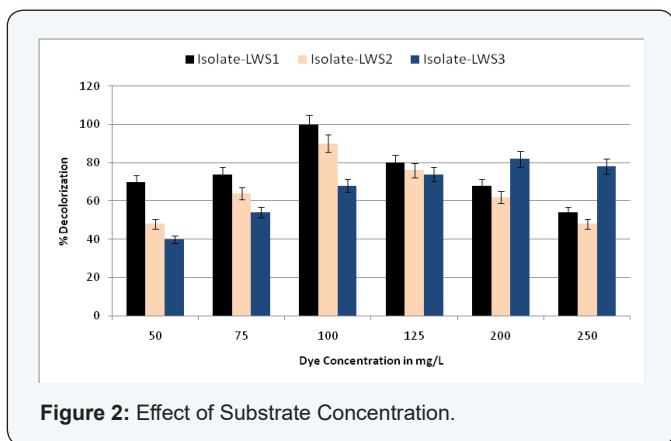


Figure 2: Effect of Substrate Concentration.

Carbon source effect

Sound effects of distinctive carbon sources such as maltose, mannitol, glucose and yeast extract were assessed on Remazol Black-B decolorization by bacterial isolates (Figure 3). It was noted that utmost decolorization take place with 5% yeast extract in all selected strains (85 to 98%) that was pursued by glucose in which decolorization takes place in the range of 20 to 25%. However, slightest decolorization was seen in the case of mannitol (10 to 15% and maltose (up to 18%).

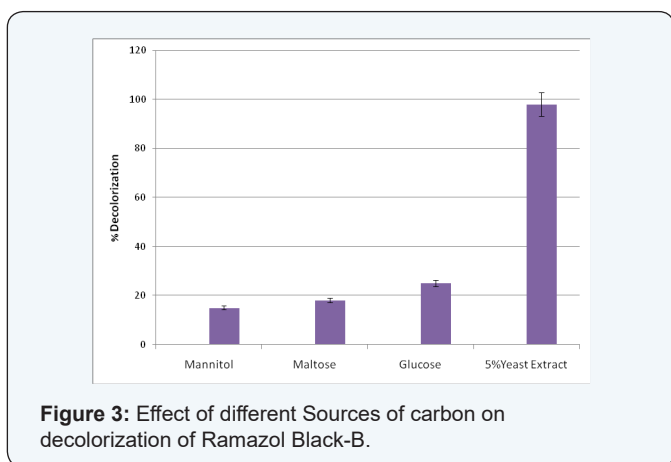


Figure 3: Effect of different Sources of carbon on decolorization of Ramazol Black-B.

pH effect

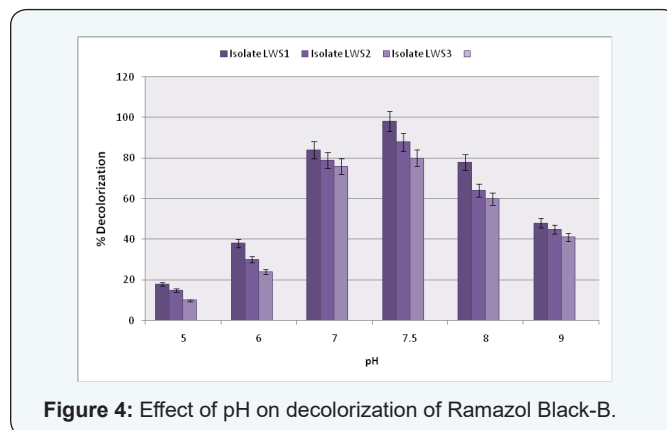


Figure 4: Effect of pH on decolorization of Ramazol Black-B.

For pH optimization study, pH ranging from 5 to 9 were exploited and all selected isolates were inoculated at these levels (Figure 4). To begin with increase in pH from 5 to 7, decolorization improved and utmost decolorization takes place at 7.5 pH. Likewise, auxiliary raise in pH from 7 to 9 had pessimistic outcome on decolorization aptitude of a mixture of isolates. The peak decolorization was observed with the isolate LWIS1 (98%) at pH 7.5 though least decolorization take place at pH 9. Comparable tendency in lingering isolates 2 and 3 were examined at pH 7.5. Overall, it was noted that all the bacterial isolates demonstrated most favorable decolorization from pH 5 to 7.5.

Incubation temperature effect

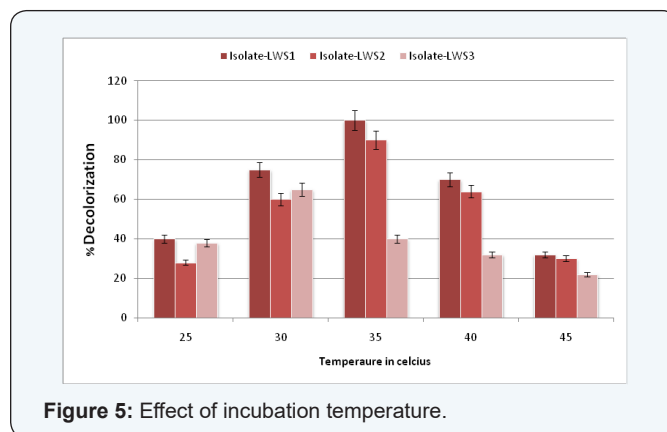


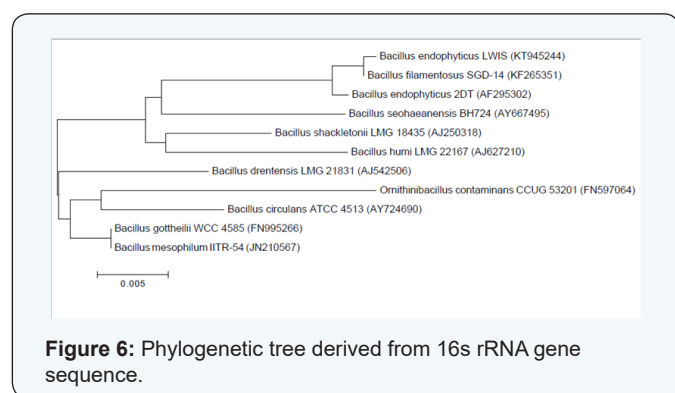
Figure 5: Effect of incubation temperature.

Five levels (25, 30, 35, 40 and 45°C) of the temperature used for the assessment of optimum biodecolorization Remazol Black - B by selected bacterial isolates. It is apparent (Figure 5), when the temperature is raised from 25 to 35°C there was an inconsistent trend in decolorization due to different isolates. Isolate LWIS 1 and showed gradual increase in decolorization, while isolate 3 indicated maximum decolorization at 25°C. Other two bacterial isolates (LWIS1 and 2) with a gradual increase from 25 to 35°C showed maximum decolorization at 35 C. As the temperature is further raised from 35°C to 45°C, there

was sharp decline in decolorization capacity in all isolates increased. Utmost decolorization was detected with the isolate 1 (98%) at 35°C and is followed by isolate 2 (94%) at the similar temperature. Least decolorization was observed at 45°C in all selected isolates.

Identification

Among three selected bacterial isolates LWIS1 exhibited maximum decolorizing potential against azo dyes, so it was identified by 16s rRNA gene sequencing approach. Sequence analysis of 16s rRNA gene showed that isolate LWIS1 had 98.89% similarity with the species *Bacillus endophyticus*. Phylogeny tree (Figure 6) based on Mega 6 indicated that isolate LWIS was a *Bacillus endophyticus* strain. The 16s rRNA gene sequence submitted in Gene Bank with accession number KT945244.



Discussion

Industrial effluent is very much unstable and it fluctuates repeatedly in an ample assortment depending upon the course practiced. Most of the south Asian countries have rigorous environmental trouble due to hasty industrialization development. This observable fact is very widespread where the polluting industries like paper, pulp, textile, dyeing, leather, tanning processing, sugar manufacturing flourish as clusters. Together with these, the textile industries are huge industrial patrons of waters as well as producers of wastewater. The effluent discharged by this industry headed to grim pollution of groundwater and soils and eventually has an effect on the livelihood of the poor [21].

During the dying process a significant amount of dyes and other chemicals lost in the wastewater. It is estimated that the dye loses between 10 - 15 % [22]. Dye is generally non-toxic to the environment, but the color waters there may impede high penetration influenced by the aquatic life and the usage limit [23]. Decolorization of industrial effluent has been a foremost anxiety in waste water that instigates from textile and dye stuff plant with a unremitting discharge of immense quantity of residual dyes to the environment. A well-organized management of the effluent is an environmental friendly approach for medication of textile effluent.

The microbial degradation of dye molecules in the environment is likely to be slow, which signifies that it is promising for high intensity of dye to keep on and probably accumulate. Because of the poor biodegradability of dyes, unadventurous process of biological treatments is unproductive in the management of dye containing wastewater. Biological decolorization is used in both aerobic and anaerobic environments. A number of reports discourage decolorization of the azo dye by microorganism under anaerobic conditions since it leads to the formation of the corresponding aromatic amines. The efficiency of microbial discolorization depends on the adaptive capacity and activity of selected microorganisms. In recent decades, many microorganisms are capable of degrading azo dyes, including bacteria, fungi and yeast. The discharge of waste water color in the river by the textile industry represents a serious environmental problem and a public health problem.

The main part of the wastewater contains azo dyes which are increasingly used in industries because of their ease and cost effective in the synthesis compared with natural dyes. Relative effectiveness of bacteria isolated for decolorization Remazol Black- B clearly implies that these can be effectively used for removing Remazol Black- B from industrial wastewater contaminated. Azoreductase is reported to be the key enzyme expressed in bacterial azo dyes degrading and catalyses the reductive cleavage of the azo bond [24, 20]. Azoreductase activity had been identified in a number of bacterial species recently, such as *Staphylococcus aureus*, *Shewanella putrefaciens*, *Shewanella* and *Pseudomonas spp.* [24- 26].

It was denoted that enhance in substrate concentration from its best possible level had pessimistic effect on decolorization capacity of isolated isolates. Explorations with distinctive dye concentrations in additional experiments also reported higher net color removal efficiencies at lower dye concentrations [27-29]. Dwindle in color removal ability at high substrate concentration might be due to the toxicity of the dye and co contaminants [30]. On aromatic ring of azo dye structure one or more sulphonic-acid groups are generally endorse, which might be active as detergents to hold back the growth of microbes [30]. One more explanation of the toxicity at elevated concentration may be due to the presence of heavy metals or metal complex dyes and/or the occurrence of non hydrolyzed reactive groups which may impede the bacterial growth [29]. In the same way, diminution in color removal at stumpy concentration of the substrate might be due to the shrink in enzyme capability to be acquainted with the substrate proficiently.

While in case of diverse carbon sources experimented yeast extract corroborated to be the most excellent amongst tested carbon source. Our results were in accordance with the research performed by Guo et al. [31] in which the bacterial strains grew well and absolutely decolorized K-2BP where either peptone

or yeast extract was contemporary in the medium; however, sucrose, glycerol, glucose, starch and lactose resulted in poorer rates of growth and decolorization of these dyes. Supplementary studies also accounted the utmost color removal of azo dyes in the presence of yeast extract by bacteria [32]. In case of variable pH, decolorization was on upper side at pH 7.5.

Whereas at alkaline pH conditions declined the decolorization efficiency of all the tested isolates were observed. So, from this investigation, it could be bring to a close that neutral pH sustained bacterial movement to decolorize Remazol Black-B in liquid medium [33-34]. Temperature is one more incredibly significant parameter for anaerobic treatment of wastewater. Selected isolates were mesophilic bacteria because they all showed better decolorization in the temperature range of 25 to 35 °C. Similar results were also reported by Guo et al. [31]. The mesophilic range is traditionally used [35-37] since it is generally thought that maintaining high temperature would be uneconomical, while degradation within the psychrophilic range is too slow. Overall, one of the selected isolate [1] of bacteria was able to completely remove color of the dye in 18 h. However, these isolates should be tested at large scale treatment system to examine their potential for bioremediation of dye-polluted wastewaters.

Conclusion

The current study divulges that the selected three isolates can be exploited lucratively for decolorizing Reactive Black B dye. The cultures exhibited maximum decolorization ability at pH between 7.5 for all the three isolates and 35 °C for 1, 2 & 25 °C for 3. Moreover, 5 g/L yeast extract was found to be optimum for decolorization. In conclusion, isolate LWS1 was found as one of the most efficient among others for bioremediation of Remazol Black B dye, which can be studied further for bioremediation of dye-polluted waters including rate of degradation of azo dyes other than the selected Black dye through an application of Bioaugmentation.

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