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Biodecolorization of azo dye mixture (Remazol Brilliant Violet 5R and Reactive Red 120) by indigenous bacterial consortium isolated from dye contaminated soil

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ABSTRACT

Aims: The present study investigated the biodegradation and removal of dye mixture (Remazol Brilliant Violet 5R and Reactive Red 120) using a new bacterial consortium isolated from dye-contaminated soil.
Methodology and results: Among the total 15 isolates screened, the two most efficient bacterial species (SS07 and SS09) were selected and identified as *Enterobacter cloacae* (MT573884) and *Achromobacter pulmonis* (MT573885). The removal efficiency of dye mixture by *E. cloacae* and *A. pulmonis* at an initial concentration of 100 mg/L was 82.78 and 84.96%, discretely. The bacterial consortium was developed using selected isolates and the optimum conditions for removing dyes were investigated. The maximum decolorization efficiency was achieved at pH 7; 35 °C; dye concentration, 100 mg/L; and initial inoculum concentration, 0.5 mL with mannitol and ammonium sulfate as carbon and nitrogen sources. The maximum removal efficiency of 91.3 ± 3.35% was achieved at the optimal conditions after 72 h of incubation.
Conclusion, significance and impact of study: Decolorization of azo dyestuff by the developed microbial consortia conforms to the zero-order reaction kinetics model. Consortia of *E. cloacae* and *A. pulmonis* was established as an effective decolorizer for the Remazol Brilliant violet 5R and Reactive Red 120 dye mixture with >90% color removal.

Keywords: Azo dyes, biodegradation, microbial consortium, RBV 5R, RR 120

INTRODUCTION

Rapid urbanization and industrialization have led to the discharge of a large amount of waste into the environment and creating more pollution. Textile, food, leather, dyestuff and dyeing industries are the primary sources of colored effluents that comprise numerous dyes (Tony *et al*., 2009). These effluents introduce different organic and inorganic components into the receiving aquatic bodies and soil (Kuppusamy *et al*., 2017). Discharge of untreated textile wastewater can cause a rapid diminution of dissolved oxygen in the receiving water bodies and lead to significant environmental damage. The sunlight penetration into deeper water bodies layers is significantly reduced, thereby interrupting photosynthesis, resulting in water quality deterioration and lower gas solubility (Rosu *et al*., 2018). Further rigorous irrigation of cultivated lands and grassland contamination with water polluted through dying effluents can cause severe pollution (Rosu *et al*., 2018; Benkhaya *et al*., 2020). It is well known that most of the dyes existing in the wastewater and its breakdown products are potentially toxic to life forms and are carcinogenic or mutagenic to humans (Roy *et al*., 2018).

Azo dyes are the primary group of colorants broadly used in the textile industry for dyeing processes. These dyes are an environmental concern because of their color, bio recalcitrance nature and potential toxicity (Mahmoud *et al*., 2017). Due to the complex structure of aromatic compounds with one or more (-N=N-) azo groups, these dyes exhibit a high level of resistance to degradation (Mahmoud *et al*., 2017; Benkhaya *et al*., 2020). Several physical, chemical and biological techniques can be employed for azo dye removal from industrial wastewater. Adsorption by conventional adsorbents like activated carbon, peat and silica gel are the most common and widely utilized technology in medium and small-scale industries (Rosu *et al*., 2018). Advanced technologies such as membrane filtration, ion exchange, reverse osmosis, electrokinetic coagulation, flocculation, froth flotation, ozonation and fenton oxidation have also been used for color removal from dying effluents (Kuppusamy *et al*., 2017; Karthik *et al*., 2020). However, it is still challenging to treat the effluents

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using these conventional methods mainly because of high chemical oxygen demand, intense color, pH and the excess amount of suspended solids, salts, sulfides, chlorine and heavy metals (Lade *et al*., 2012; Shah, 2014). Moreover, the physicochemical methods of effluent treatment have drawbacks such as high operation/maintenance cost, colossal energy requirements, chemical sludge generation and the emission of toxic substances (Roy *et al*., 2018; Ajaz *et al*., 2020).

Recently, there has been an increased interest in exploring the microbial treatment of azo dye-contaminated wastewater. It has the benefits of being eco-friendlier, less expensive, generates less sludge and a smaller amount of toxic substances, and consumes less water than many other substitutes (Shah, 2014). Diverse taxonomic groups of microbes such as bacteria, fungi, yeast and algae have been reported for their ability to decolorize azo dyes (Roy *et al*., 2018). Among the bioremediation techniques, wastewater treatment by using bacteria is emerging as one of the promising methods. Bacterial decolorization occurs due to microbial cell's adsorption or biodegradation of dye molecules (Banat *et al*., 1996). Enzymatic biotransformation initiates the decolorization of azo dyes while using bacteria, and it involves the reductive cleavage of azo bonds with the azo reductase enzyme (Lade *et al*., 2012). Several investigators have reported the ability of bacteria to decolorize dye effluent, both in pure and mixed cultures. There are notable advantages of using microbial consortia over pure cultures to suppress synthetic dyes (Li *et al*., 2019; Srinivasan *et al*., 2020).

When comparing the mixed culture with pure culture, the individual strains cannot completely degrade azo dyes. Biodegradation of azo dyes produces intermediate products such as aromatic amines, which vary in their resistance to biodegradation and can be mutagenic and carcinogenic (Lade *et al*., 2012). These intermediate compounds can limit the degrading bacterial growth and activity such that the treatment process becomes incompetent and unfeasible (Lade *et al*., 2012; Shah, 2012). In a microbial consortium, the individual strains may attack the dye molecule at different positions. They will also utilize metabolites produced by the co-existing strains for further reactions to occur. The degradation of aromatic amines will occur due to complementary organisms, making the process more effective and efficient (Ajaz *et al*., 2020). As a result of synergistic metabolic activity, biodegradation and mineralization by mixed microbial populations occur at a higher rate (Ajaz *et al*., 2020). Different microbial taxa in the groups may have diverse biological and ecological functions. Hence, the color removal efficacy will be greatly affected by the microbial community's structure and composition. Understanding the microbial community's structure and composition changes can offer critical data for optimizing the decolorization process (Srinivasan *et al*., 2020). Further maximum decolorization of the dyes can be achieved by optimizing conditions such as incubation time, pH, temperature, dye concentration and availability of suitable carbon or nitrogen sources (Ajaz *et al*., 2020).

Based on these considerations, the present study deals with the isolation and identification of bacterial strains from dye-contaminated soil and developing a bacterial consortium for efficient decolorization of a mixture of widely used textile azo dyes (Remazol Brilliant Violet 5R and Reactive Red 120). The decolorization parameters were optimized by the one-factor-at-a-time approach to attain maximum dye degradation by developed bacterial consortia.

MATERIALS AND METHODS

Sample collection

The dye-contaminated soil samples were collected from the common effluent treatment plant (CETP), Tirupur, Tamilnadu, India. The samples were collected aseptically in a screw-capped glass tube and transported to the laboratory as early as possible. Samples were preserved in the refrigerator at 4 °C for further studies.

Chemicals and culture medium

The textile azo dyes Remazol Brilliant Violet 5R (RBV 5R) and Reactive Red 120 (RR 120) were procured from SIGMA, India. The minimal salt media (MSM) used for enrichment and decolorization was prepared by adding the following components: Na_2HPO_4 (12.8 g/L), KH_2PO_4 (3 g/L), NH_4Cl (1 g/L), NaCl (0.5 g/L), 0.05 M $MgSO_4$ (10 mL/L), 0.01 M $CaCl_2$ (10 mL/L) and 20% glucose (30 mL/L) (Lalnunhlimi and Krishnaswamy, 2016). All the chemicals used in the study were of high purity and analytical grade procured from Hi-Media Laboratories, Mumbai. Furthermore, nutrient broth and nutrient agar media (Hi-Media Laboratories) were used for culture maintenance.

Enrichment, isolation and screening of dye degrading bacteria

Dye degrading bacterial strains were isolated from soil samples. About 10 g of soil sample was inoculated into 100 mL of MSM medium in 250 mL Erlenmeyer flasks and amended with 100 mg/L of filter-sterilized RBV 5R and RR 120. Individual bacterial isolates were obtained by plating the developed culture on an MSM agar medium comprising 100 mg/L of distinct azo dyes. The isolates which showed a zone of clearance around them were picked and cultured for further experiments. The preferred colonies were then purified by repeatedly streaking on a nutrient agar medium and further utilized for decolorization assay.

Decolourization assay

All decolorization assays were carried out in triplicates. A volume of 100 µL of precultured bacterial cultures was

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added to 10 mL of MSM containing 100 mg/L of RBV 5R, RR 120 and dye mixture (contains 100 mg/L of both RBV 5R and RR 120). The bio decolorization of individual and mixed dye by bacterial strains was observed for five days. Uninoculated MSM added with azo dyes was used as a control. The samples were withdrawn periodically to monitor the decolorization process, centrifuged at 10,000 rpm for 15 min, filtered through a syringe filter. Decolorization of colorants was monitored using UV-Vis spectrophotometer by measuring the absorbance at the corresponding λ_{max} of the dye (420 nm for BRV 5R, 535 nm for RR 120 and 535 nm for mixed dye) and was compared with the uninoculated control. The color removal efficiency of the bacterial cultures was determined using an Equation (1):

$$\text{Decolourization (\%)} = [(A_0 - A_t) \times 100]/A_0 \quad (1)$$

Where, A_0 = initial absorbance; A_t = final absorbance.

The measurement of decolorization of dyes by bacteria was performed at an interval of 24 h for five days and the results were tabulated. Amongst the isolates, the two most efficient dye decolorizing bacterial strains were further taken for compatibility analysis.

Compatibility analysis

Microbes involved in the consortia should not exhibit any antagonistic effect over other microbes in the consortia. The decolorization efficiency will be enhanced only when the organisms are compatible with each other. The compatibility analysis was done with the selected efficient strains as per Geethadevi *et al.* (2018). The test was performed as follows: two nutrient agar plates were taken, and each plate was bored with a well. The plates were smeared with one of the two selected cultures, and 10 µL of the supernatant from another culture was added to the well and vice versa. The plates were then kept for incubation at 37 °C for 24 h. The absence of any inhibition zone around the wells is considered as the strains were compatible. The test was repeated with permutations and combinations.

Identification of selected strains

The colony characteristics, Gram's reaction and cell morphology of the selected dye degrading bacterial isolates were identified via standard biochemical and microscopic techniques. The isolate's physiological and biochemical characteristics were evaluated by Voges-Proskauer, methyl red, indole, catalase, oxidase, urease, citrate utilization and hydrogen sulfide production tests. The isolate's ability to ferment several sugars, including glucose, lactose, sucrose and mannitol was also performed. The study of colony characteristics and the morphological characterization consist of smear preparation and Gram's staining was determined according to Bergey's Manual of Systemic Bacteriology (Biavati, 2012).

Both strains were genetically distinguished by employing a 16S rRNA sequencing study after extracting the genomic DNA using the EXpure Microbial DNA isolation kit. The standard primers (27F-5' AGAGTTTGATCTGGCTCAG-3' and 1492R-5' TACGGTACCTTGTTACGACTT-3') were utilized to amplify the 16S region of the rRNA gene. Then, polymerase chain reaction (PCR) amplification was performed using a thermal cycler system in the following order: initial denaturation (for 3 min at 94 °C), 30 cycles of denaturation (at the same temperature for 30 sec), annealing (at 60 °C for 30 sec), elongation (at 72 °C for 60 sec) and final extension (for 10 min at 72 °C). PCR product was then purified by Montage PCR Clean-up kit (Millipore) and sequenced using AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) in ABI PRISM® BigDyeTM Terminator Cycle Sequencing kits. After cautious collection and editing in the Bioedit software, the sequences were deposited in the NCBI database for accession numbers. The sequence obtained during 16S rRNA sequencing was investigated through the NCBI BLAST (http://www.ncbi.nlm.nih.gov) database to identify the similar strain through homologous sequences alignment. The corresponding sequences were downloaded and inferred using the Neighbor-joining method to determine the evolutionary antiquity. It was performed through online software: Muscle (v3.7), Gblocks (v0.91b), PhyML (v3.0 aLRT) and TreeDyn (v198.3) on the Phylogeny.fr platform. Finally, the gene sequences obtained in this study were deposited in the GenBank database.

Optimization of physio-chemical parameters

The optimum decolorization of azo dyes was performed by adopting the standard one-factor-at-a-time (OFAT) approach, i.e., modifying one individual parameter while keeping the other parameters at a fixed level. The experiments were conducted with a consortium of SS07 and SS09 in MSM medium supplemented with 100 mg/L of mixed dye at various pH, temperature, carbon and nitrogen sources. The medium was maintained at different pH (3, 5, 7, 9 and 11) by using 0.1 M HCl and 0.1 M NaOH solution. The influence of temperature was examined by incubating the culture in an incubator at 20, 25, 30, 35 and 40 °C. The effect of carbon sources was studied by adding 1% of carbon bases (fructose, lactose, sucrose, starch and mannitol) individually as a supplement to MSM. Nitrogen sources, such as yeast extract, peptone, tryptone, ammonium sulfate and urea, were added to MSM at a concentration of 0.5%.

To find a bacterial consortium color removal efficiency at higher dye concentrations, MSM was amended with different concentrations (50, 100, 150, 200 and 250 mg/L) of azo dyes. The dye removal efficiency was observed using a UV-Vis spectrophotometer after incubation periods of 24, 48, 72 and 120 h. To study the effect of inoculum concentration, 100 mL of sterile MSM broth was modified with 100 mg/L of RBV 5R and RR 120, and different volumes (0.5, 1, 1.5, 2 and 2.5 mL) of overnight

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cultures were inoculated. The measurement of decolorization was done at an interval of 24 h for five days. A separate flask without culture in dye amended MSM was used as a control.

Kinetic study

In this present study, reaction kinetic models (zero-, first-, second-order kinetics) were applied to investigate the decolorization of mixed dye by developed bacterial consortia. The kinetic equations of zero-, first-, second-order reaction were expressed as below Equations (2)–(4):

$$[A]_t = [A]_0 - k_0 t \quad (2)$$
$$ln\,[A]_t = ln\,[A]_0 - k_1 t \quad (3)$$
$$1/[A]_t = 1/[A]_0 + k_2 t \quad (4)$$

Where $[A]_0$ and $[A]_t$ are the concentration of dye at the initial time (t = 0) and reaction time (t); t is the reaction time; k_0, k_1 and k_2 are the kinetic rate constant of zero-, first-, second-order reaction, correspondingly. Kinetic rate constant and correlation coefficient were calculated by plotting a graph of t versus $[A]_t$, t versus $ln\,[A]_t$ and t versus $1/[A]_t$ for zero-, first- and second-order kinetic models, respectively.

FTIR analysis

The removal of azo dyes was studied by analyzing the changes in Fourier-Transform Infrared Spectroscopy (FTIR) spectra of dye mixture before and after treatment. FTIR analysis was performed using SHIMADZU FTIR 8400S (Japan) in the mid-IR region of 400 to 4000 cm^{-1}. In this study, all the samples were analyzed in triplicates and presented as the mean value of the three measurements.

Statistical analysis

All experiments were conducted in triplicate. The rate of decolorization was measured in percentage and expressed as mean ± standard deviation values. The data were analyzed using the Statistical Package for the Social Sciences (SPSS) Version 21.

RESULTS AND DISCUSSION

Isolation and selection of dye degrading bacterial isolates

Cultivable bacteria from the dye-contaminated soil sample were isolated using the spread plate of the MSM agar media containing azo dyes. The Petri-dishes were incubated for 24 h at 37 ± 2 °C and colonies with distinct morphology and decolorization zone were selected and purified by regular subculturing. The early screening of microbial populations for the decolorization of azo dyes indicates 15 isolates designated as SS01 to SS15. The isolated strains were then subjected to quantitative screening for their ability to decolorize RBV 5R (100 mg/L) and RR 120 (100 mg/L) by inoculating in MSM broth for five days. Table 1 shows the decolorization of azo dyes (RBV 5R and RR 120) by individual isolates. Among the 15 isolates, 13 strains (SS01–SS13) decolorized more than 65% of the RBV 5R and all the strains decolorized above 78% of the RR 120 dye in 72 h. Two unidentified strains designated as SS07 and SS09 were recognized as the most efficient decolorizer of both dyes. About 81 and 76% of the RBV 5R and 90 and 88% of the RR 120 dye were decolorized after 72 h of incubation by SS07 and SS09, correspondingly.

All 15 isolates were further grown on MSM media amended with mixed dye. They showed the ability to decolorize dye mixture in 48 h and the maximum decolorization was observed after 72 h of incubation at 35 °C. Similar to the decolorization of individual dyes, bacterial isolates labeled as SS07 and SS09 showed promising decolorization of 84.96 and 82.78% after 72 h (Table 1). Simultaneously, no other strain exhibited the potential to decolorize more than 80% of the dye mix even after incubation of 120 h. In addition, the discoloration gradually decreased after 72 h of incubation, which can be attributed to the efflux mechanism of the dye from the bacterial cells in order to reduce the dye toxicity. Similar observations have previously been reported by Hefnawy *et al.* (2017), Salem *et al.* (2019) and Selim *et al.* (2021). These two laboratory isolates (SS07 and SS09) were chosen for further studies as they attained maximum decolorization efficacy compared to all other strains.

Identification of dye-decolorizing bacteria

The two organisms which showed significant decolorization of the mixed dye were investigated for their morphological, cultural, physiological and biochemical features. Results of biochemical experiments have represented in Table 2. Through Gram's staining, it was found that both the isolates were Gram-negative straight rods. The isolate SS07 exhibited positive results for Voges-Proskauer, citrate, glucose, lactose, sucrose, mannitol and catalase metabolism tests and negative results for indole, methyl red, urease and oxidase tests. The indole production, methyl red, Voges-Proskauer and urease tests produced negative results for the SS09 bacterium. While citrate, glucose, catalase and oxidase test results were observed to be positive. The above results indicate that the isolates SS07 and SS09 are closely related to *Enterobacter* and *Achromobacter* genus.

Further, the 16S rDNA gene sequencing technique was performed to identify the newly isolated SS07 and SS09 bacterial strains. The 16S rDNA sequences of bacterial isolates SS07 and SS09 were deposited to NCBI and Gene Bank (Accession No: MT573884, MT573885). BLAST similarity search specified that the bacterial isolate SS07 was analogous to *Enterobacter* and SS09 was analogous to *Achromobacter*. A phylogenetic tree constructed with similar sequences showed that isolates

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Table 1: Percentage decolorization of Remazol Brilliant Violet 5R and Reactive Red 120 by bacterial strains isolated from contaminated soil.

Isolates	Percentage of decolorization (%)														
	Remazol Brilliant Violet 5R (420 nm)					Reactive Red 120 (535 nm)					Mixed dye (535 nm)				
	Time (h)					Time (h)					Time (h)				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
SS01	10.53	42.86	71.43	66.67	52.39	9.52	40.48	80.95	78.57	71.43	13.04	39.13	76.43	72.08	67.39
SS02	4.76	38.1	71.43	61.9	57.14	11.9	45.24	85.71	80.95	73.81	10.86	41.3	78.6	73.91	71.74
SS03	4.76	38.1	66.67	57.14	52.38	14.29	47.62	83.33	80.95	76.19	10.87	39.13	78.6	76.09	73.91
SS04	4.76	42.86	71.43	57.14	47.62	11.9	42.86	85.71	83.33	78.57	8.69	41.3	76.43	78.26	73.91
SS05	14.29	38.1	66.67	61.9	38.1	7.14	47.62	83.33	80.95	76.19	15.21	39.13	78.6	77.43	71.74
SS06	4.76	33.33	66.67	57.14	38.1	4.76	33.33	78.57	71.43	64.29	8.69	23.91	63.04	62.6	63.91
SS07	14.29	28.57	80.95	57.14	42.86	16.67	50	90.47	80.95	78.57	15.21	45.65	84.96	80.43	73.91
SS08	10.53	47.62	66.67	66.67	52.39	14.29	47.62	85.71	83.33	76.19	13.04	39.13	78.26	76.09	69.56
SS09	10.53	33.33	76.19	52.39	52.39	14.29	50	88.09	83.33	76.19	4.35	23.91	82.78	71.74	76.09
SS10	4.76	38.1	66.67	57.14	52.39	11.9	45.24	80.95	83.33	73.81	10.87	41.3	77.6	73.91	66.09
SS11	14.29	42.86	61.9	57.14	47.62	7.14	40.48	78.57	71.43	64.29	15.21	36.96	72.43	73.91	68.26
SS12	4.76	47.62	61.9	57.14	52.39	14.29	45.24	85.71	78.57	76.19	6.52	36.96	78.26	76.09	73.91
SS13	4.76	47.62	66.67	71.43	47.62	11.9	47.62	83.33	78.57	71.43	4.35	30.43	54.35	65.22	71.74
SS14	4.76	19.05	42.86	47.62	47.62	4.76	30.95	78.57	71.43	69.05	10.87	41.3	71.73	72.6	66.09
SS15	10.53	19.05	33.33	38.1	47.62	14.29	35.71	80.95	83.33	73.81	13.04	45.65	78.6	78.26	71.74

SS07 and SS09 belonged to *Enterobacter cloacae* and *Achromobacter pulmonis*, respectively (Figure 1).

Compatibility testing

The compatibility of the bacterial strains is crucial for the formation of consortia that can break down toxic dyes more efficiently. A compatibility analysis was carried out to verify that the selected strains were suitable for effective color removal when used as a consortium. Both *Enterobacter cloacae* and *Achromobacter pulmonis*, which were used in compatibility tests, showed thriving and vigorous growth in the nutrient agar medium. The main reason for this compatibility of the selected bacterial strains may be that they co-existed in a familiar environment for an extended period of time. In addition, a significant enhancement in the color removal rate was observed during the decolorization of mixed azo dyes by the developed consortia. The above results suggest that the isolates selected were compatible with each other. The consortium of adapted microorganisms uses more enzymes with a wide spectral range that could break down the complex organic compounds and the colorant content in the wastewater (Li *et al*., 2019).

Optimization of physio-chemical parameters

Effect of pH and temperature

The influence of pH on color removal must be studied in detail since the metabolic activities and transport of nutrients across the cell compartments are extremely dependent on the growth medium's pH (Ezhilarasu, 2016). The effect of pH was studied at different pH ranges between 3 and 11. A comparison of decolorization of mixed dye at various pH is presented in Figure 2A. The dye decolorization varies with pH and the optimal color removal was observed at pH 7 (82.54 ± 5.26%), followed

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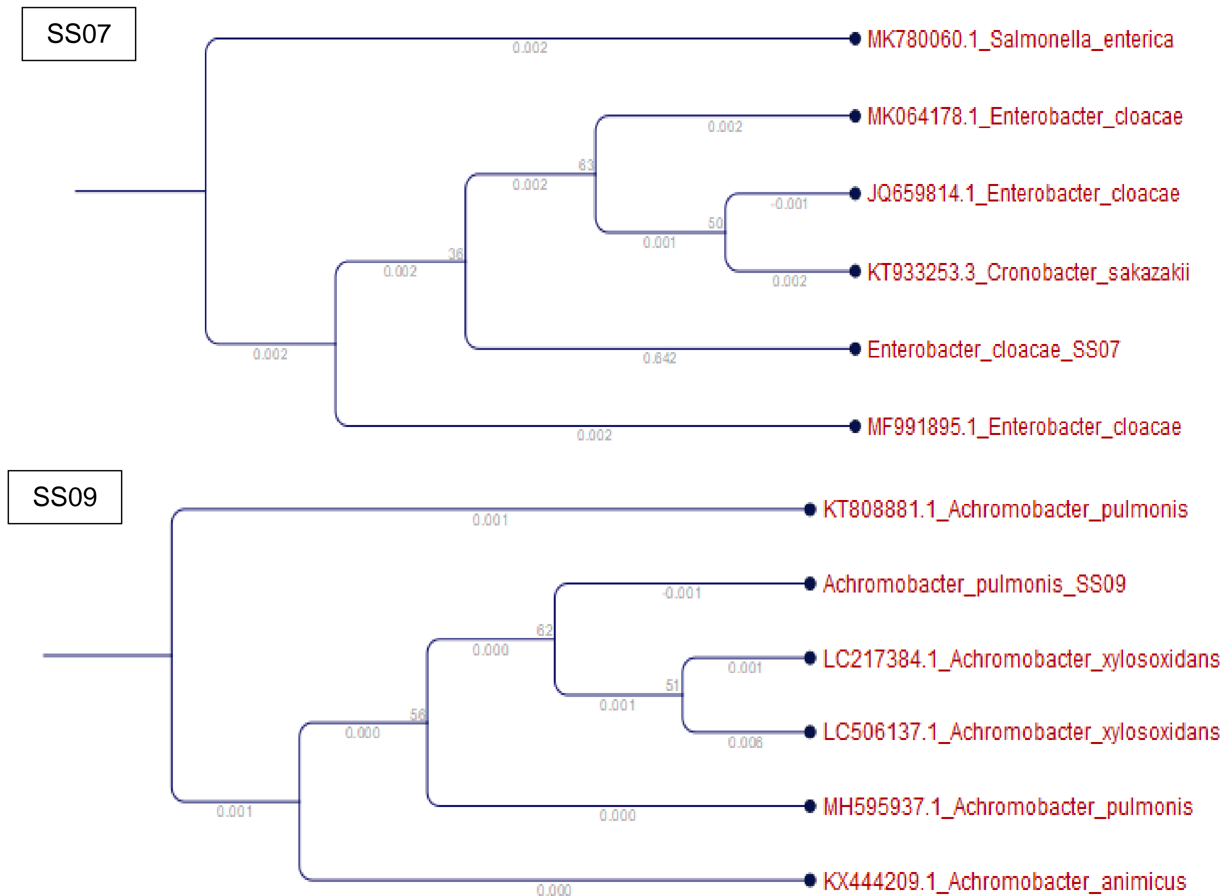

Figure 1: Phylogenetic tree of the isolated bacterial strains, identified through 16S rDNA sequencing of the selected strains showing the evolutionary relationship.

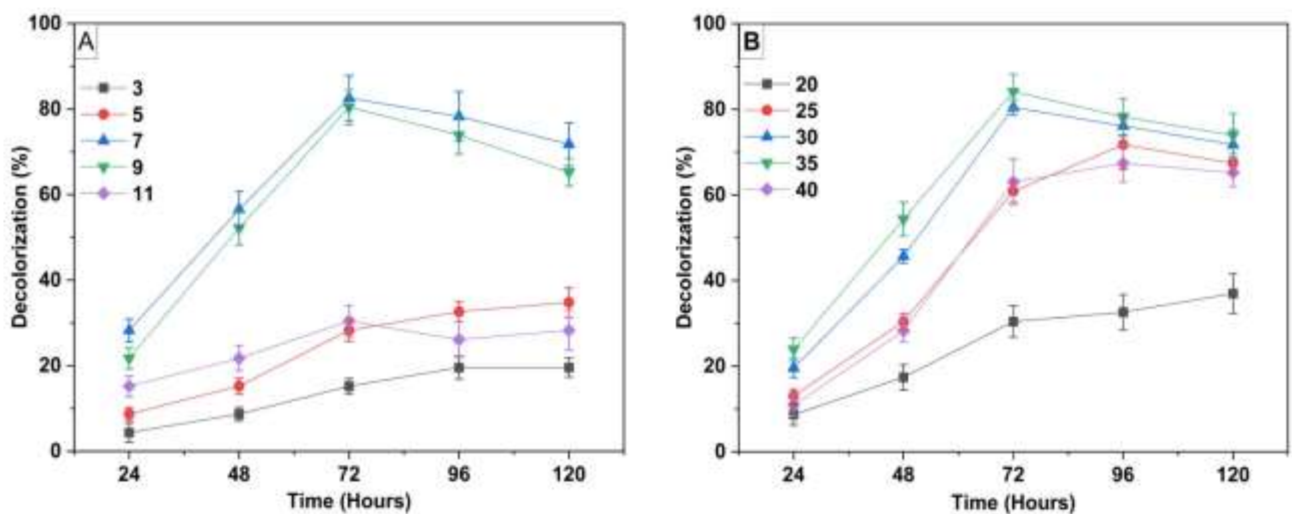

Figure 2: Effects of changes in the (A) pH and (B) temperature on decolorization ability (azo dye mixture of RBV 5R and RR 120 at 100 mg/L) of developed consortia. The values are presented as mean ± standard deviation.

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Table 2: Morphological and biochemical characteristics of bacterial isolates SS07 and SS09.

Test	SS07	SS09
Gram's staining	-	-
Shape	Straight rods	Straight rods
Indole production	-	-
Methyl red	-	-
Voges-Proskauer	+	-
Citrate utilization	+	+
Glucose fermentation	+	+
Lactose fermentation	+	+/-
Sucrose fermentation	+	+/-
Mannitol fermentation	+	+/-
Urease	-	-
Catalase	+	+
Oxidase	-	+

"+" indicates positive; "-" indicates negative; "+/-" indicates either positive or negative.

by pH 9 (80.43 ± 4.06%). Figure 2A shows that a threefold surge in the color removal percentage can be achieved by increasing pH from 5.0 to 7.0. The results suggest that neutral and slightly basic pH conditions would be more favorable for azo dye decolorization. At the optimum pH, the electrostatic force of attraction between the negatively charged microbial biomass and positively charged dye molecules enhances the binding, resulting in a substantial upsurge in dye removal (Li *et al*., 2019). Below the optimum pH, the competency between the H^+ ions with dye cations, triggering a diminution in color removal efficacy. At alkaline pH, dye molecules become negatively charged compounds due to the deprotonation of the azo bonds. As a consequence, there will be an obstruction in the decolorization of azo dyes. Whereas in acidic pH, the azo bond will be protonated, leading to decreased dye decolorization due to chemical structure changes (Hsueh and Chen, 2007; Rahman *et al*., 2019).

The growth and decolorizing ability of the consortium were largely dependent on the temperature. Since decolorization is a metabolic process, fluctuations in the temperature play a major role in treating dye effluent using bacteria (Shah, 2012). Over a range of temperatures from 25 to 35 °C, the bacterial consortium color removal activity was found to be increased along with the temperature (Figure 2B). Further, the marginal reduction was observed in decolorization while increasing temperature beyond 35 °C. This might be attributed to the loss of cell viability or thermal deactivation of decolorizing enzymes at higher temperatures (Chang and Kuo, 2000). Earlier studies accentuated that the ideal temperature for the growth of bacterial consortia and dye decolorization was ranged between 25 to 37 °C (Li *et al*., 2019). Shah (2014) reported that the optimal pH and temperature for removing Remazol Black B were 6 and 37 °C, respectively. Gunti *et al.* (2021) achieved the complete decolorization of Acid Orange 10 (200 mg/L) by *Bacillus subtilis* at pH 6 and 40 °C of temperature. Similar observations were conveyed on degrading Reactive Red 120 by *Bacillus cohnii* (Padmanaban *et al*., 2016) and by a bacterial consortium of *Stenotrophomonas acidaminiphila*, *Pseudomonas putida*, *P. fluorescence* and *B. cereus* (Khehra *et al*., 2005).

Effect of carbon and nitrogen sources

To enhance the decolorization performance of the bacterial consortium, additional carbon and nitrogen sources were supplied. As seen in Figure 3A, the maximum decolorization of mixed dyes was observed as 86.09 ± 4.56%, with mannitol as a carbon source. With the addition of other carbon bases, not as much color removal was recorded. The decolorization was negligible in the presence of starch (36.96 ± 3.78%), whereas modest when using fructose (58.7 ± 3.06%), lactose (63.04 ± 2.82%) and sucrose (73.91 ± 2.86%). Eskandari *et al.* (2019) achieved 75% of decolorization of Reactive Black-5 while utilizing glucose as a carbon source, which increased to 76.5% when using lactose carbon source. Nair *et al.* (2017) reported the highest decolorization in the existence of starch (95%) and very low decolorization while using sucrose (25%). In contrast, moderate activity was shown for glucose (55%) and lactose (80%).

Nitrogen bases are vital media complements for NADH's renewal that acts as an electron donor to reduce azo dyes by microorganisms (Mani and Hameed, 2019). In our study, ammonium sulfate appeared to be a suitable nitrogen base with a maximum decolorization of 87.36 ± 3.78% (Figure 3B), followed by yeast extract (82.61 ± 2.82%). Lalnunhlimi and Krishnaswamy (2016) highlighted that the decolorization of mixed azo dyes (Direct Blue 151 and Direct Red 31) was improved while using yeast extract (0.5%) and sucrose (1%). Eskandari *et al.* (2019) reported that yeast extract, peptone and ammonium dihydrogen phosphate are effective nitrogen sources for bacteria to treat colorants. In the treatment of light red dye, the maximum percentage of decolorization within 24 h by *Proteus mirabilis* isolate (70-75%) from textile effluent and *Klebsiella pneumonia* (30-35%) from sludge was observed in the presence of yeast extract. Whereas *P. mirabilis* removes 6-65% color from textile wastewater and *Shigella* sp. removes 70-75% from sludge in the presence of urea (Sethi *et al*., 2012). In line with that, several studies (Saratale *et al*., 2009; Lade *et al*., 2012; Nair *et al*., 2017; Guo *et al*., 2020) have shown that the supplement of additional carbon and nitrogen sources can enhance the degradation of azo dyes by bacterial strains since the dyes are deficient in nutrient sources.

Effect of initial dye and inoculum concentration

The ability of the bacterial consortium to decolorize the azo dyes at various concentrations (50, 100, 150, 200 and 250 mg/L) was investigated. Figure 4A illustrates a noticeable decrease in color removal due to an increase in dye's initial concentration. At a lower concentration (50 mg/L), about 91.65 ± 3.35% of the color was removed,

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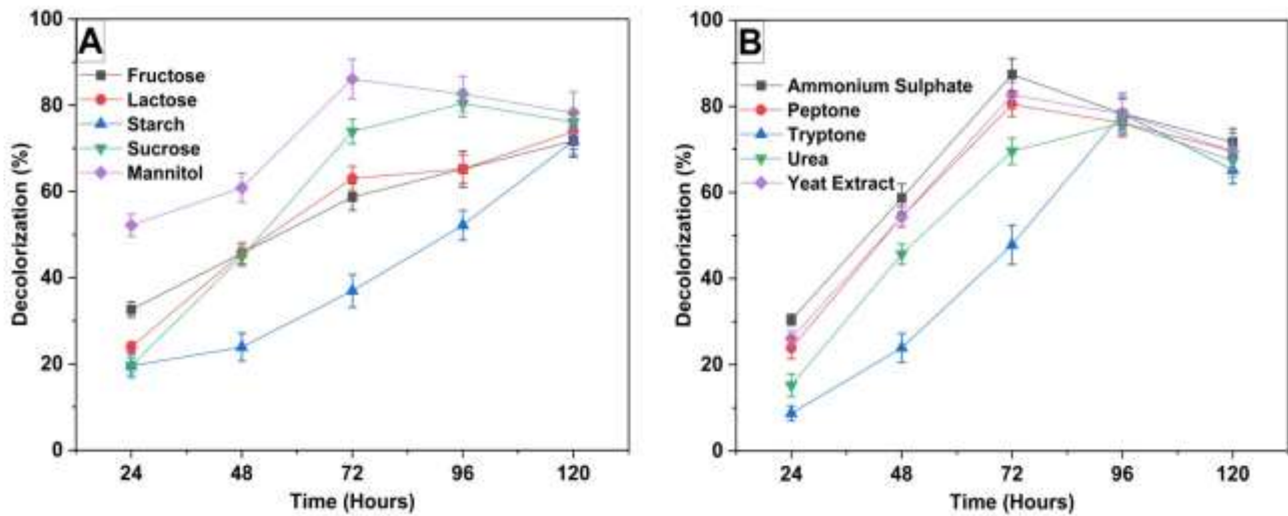

Figure 3: Effects of various (A) carbon and (B) nitrogen sources on of azo dye mixture (RBV 5R and RR 120 at 100 mg/L) decolorization by developed consortia. The values are presented as mean ± standard deviation.

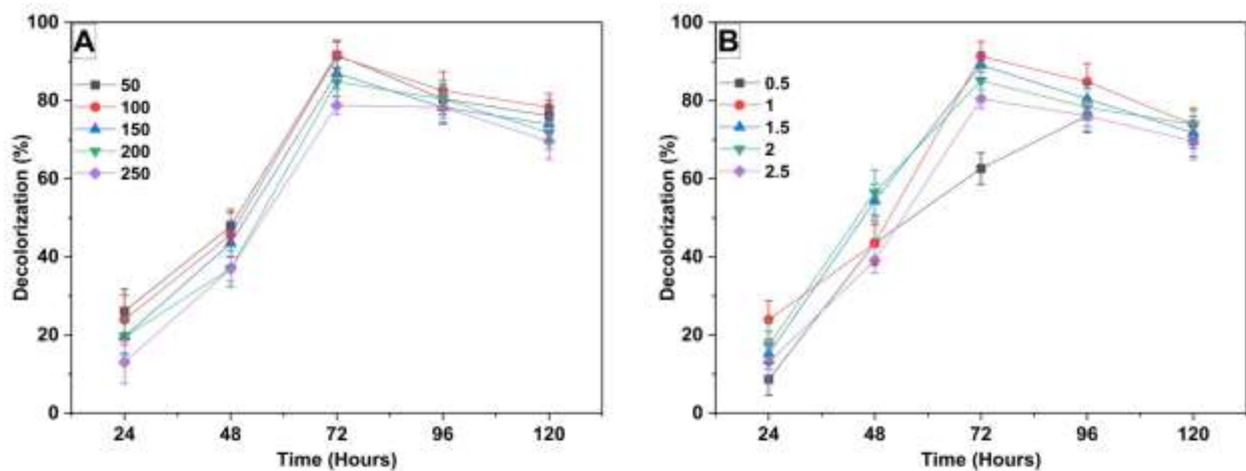

Figure 4: Influence of different (A) dye concentration and (B) inoculum size on decolorization of azo dye mixture (RBV 5R and RR 120 at 100 mg/L) by developed consortia. The values are presented as mean ± standard deviation.

which was slightly reduced to 91.30 ± 4.22% at 100 mg/L of mixed dye. A further increase in dye concentration results in a substantial reduction of color removal. This decrease may be due to the substrate inhibitory effect and toxicity of the dye at higher concentrations, while at lower concentrations, all dye molecules have an equal chance to interact with available binding sites in the azoreductase, resulting in higher rates of decolorization (Sethi *et al.,* 2012; Guadie *et al.,* 2017; Tahir *et al.,* 2021). Crystal violet was completely removed at concentrations of up to 20 µmol/L, 65.5% was removed at a concentration of 200 µmol/L, and only 13.2% was removed at a concentration of 500 µmol/L (Zablocka-Godlewska *et al.*, 2015). These observations were also supported by the recent findings of Martorell *et al.* (2018) and Vargas-de la Cruz and Landa-Acuña (2020). Figure 4B showed the influence of inoculation size on bacterial growth and dye decolorization. The rate of discoloration of the mixed dye was found to be directly related to the inoculum size, which gradually increased as the volume of the inoculation culture increased. The outcomes revealed that at least 500 µL of inoculum had to be added to attain optimal decolorization within 72 h. Further increasing inoculation size does not improve either decolorization efficiency or strain growth. Tahir *et al.* (2021) also showed similar results showing 61-85% dye decolorization rates for Mordant Black-11 dye upon increasing the inoculum volume from 0.5% to 2%. The results were consistent with those of some related researchers (Phugare *et al.*, 2011; Tan *et al.*, 2013; Martorell *et al.*, 2018).

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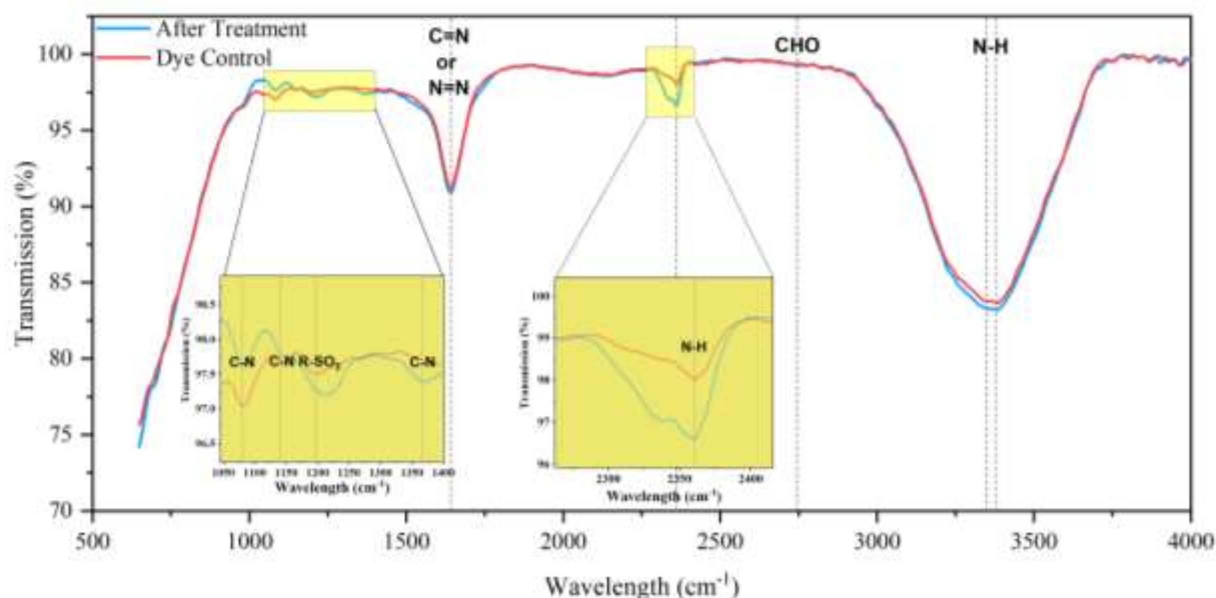

Figure 5: Variations in the FTIR spectra of azo dye mixture before (dye control) and after treatment with the bacterial consortium.

Table 3: The calculated zero-, first- and second-order decolorization rate constant and correlation coefficient (R^2) for Azo dye removal by bacterial consortia.

Dye concentration (mg/L)	Zero-order		First-order		Second-order	
	k_0 (mg/L/h)	R^2	k_1 (h^{-1})	R^2	k_2 (L/mg/h)	R^2
50	0.683	0.964	0.020	0.866	0.0044	0.790
100	1.404	0.960	0.020	0.863	0.0021	0.789
150	2.106	0.973	0.016	0.889	0.0009	0.811
200	2.717	0.952	0.015	0.857	0.0006	0.798
250	3.424	0.976	0.013	0.910	0.0003	0.840

Shift in FTIR spectra of dyes

The decolorization and biodegradation of azo dye were qualitatively monitored by FTIR spectra. A comparison between the FTIR spectrum of mixed dye before and after treatment with microbial consortia was given in Figure 5. FTIR spectra of dye mix (control) showed two peaks at 3348 and 3379, and the broad peak at 2360 cm^{-1} is attributed to the N-H amino compound stretching. A strong, sharp peak at the wavelength of 1643 cm^{-1} is characteristic of double-bonded nitrogen groups such as azo (N=N) and imino (C=N) functional groups. Two specific peaks of wavelength 1080 and 1196 cm^{-1} are shown in the FTIR spectrum due to C-N (aliphatic amines) stretching and $R-SO_3$ (sulfur compounds). The change in adsorption position and appearance of new peaks was observed after treatment (Figure 5). FTIR spectrum of azo dye mixture obtained after treatment with consortium showed a new peak at 2746 cm^{-1} for aldehyde stretching and two peaks at 1141 and 1211 cm^{-1} for C-N stretching. Further reduction in peak intensity at 1080 cm^{1} (belongs to sulfate ion) and strengthening the peak at 1365 cm^{-1} for C-N (aromatic tertiary amine) were also observed. The significant difference between the spectra of treated and untreated dye solutions implies the biodegradation of azo dyes.

Kinetics study

The results of kinetic studies are given in Table 3. The zero-order kinetic model's correlation coefficient (R^2> 0.95) is closer to unity than the first-, second-order kinetic models. Consequently, the results showed that dye removal follows zero-order reaction kinetics well. In tradition, zero-order reaction rate constant k_0 varies with reaction time (t) and is independent of the initial concentration. However, in this work, the decolorization rate was slightly increased from 0.683 mg/L/h (at initial concentration 50 mg/L) to 3.424 mg/L/h (at initial concentration 250 mg/L). These may be due to the availability of excess dye molecules to microbes, which enhance the decolorization rate. The present study results are consistent with the previously reported work of Sudha *et al.* (2018) and Tan *et al.* (2012).

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CONCLUSION

The bacterial consortia strain developed in this study can decolorize about 91.3 ± 3.35% of azo dye mixture (RBV 5R and RR 120) at 100 mg/L. At the start of the experiment, the constructed consortium reduced azo dye concentration from 100 mg/L to 23 mg/L within 72 h. Further, the optimization of physicochemical conditions effectually amplified the rate of decolorization. After using mannitol and ammonium sulfate as carbon and nitrogen sources and adjusting the pH to 7 and the temperature to 35 °C, the bacterial consortia managed to decolorize 91.3 ± 3.35% of the dye mix. The kinetic data indicate that the process was well described by zero-order kinetics. These investigations reveal the potential use of the developed bacterial consortia (*Enterobacter cloacae* and *Achromobacter pulmonis*) to treat dye effluent at an industrial scale efficiently. Further, field trials should be conducted to validate the efficiency of the developed consortia.

CONFLICT OF INTEREST

The authors declare they have no competing interests.

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