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Research article

Microbial decolorization of Reactive Black 5 dye by *Bacillus albus* DD1 isolated from textile water effluent: kinetic, thermodynamics & decolorization mechanism

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HIGHLIGHTS

- Bacterial strain *Bacillus albus DD1* having potential to decolourise and degrade RB5 was isolated, identified and characterized.
- Effect of process parameters like pH, temperature, yeast extract as a co-substrate, inoculum size and initial dye concentration on the decolorization reaction was studied, the isolate showed growth and decolorization at alkaline pH 9 and 40 °C temperature, indicating its suitability for field application.
- More than 98% removal of RB5 was achieved within 38 h using the bacterial isolate, and a significant negative correlation existed between RB5 decolourization and bacterial growth.
- Bio-decolorization process followed the first order kinetics, rate constant and reaction rate for RB5 decolourization, was 0.0523 s^{-1} and 2.6 × 10^{-3} mol/m^3 sec, respectively.
- Reduction of azo bond and subsequent biodegradation of RB5 and were confirmed through LC-MS and 3,6,8-trihyroxynapthalene and phthalic acid were identified as degradation products.

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ABSTRACT

Reactive Black 5 is one of the most widely used dye in textile and other industries. It is one of the significantly toxic azo dye which poses a serious threat to the environment when discharged into water bodies. A bacterial strain having potential to decolourise and degrade RB5 was isolated from textile effluent, and further identified and characterized. On the basis of morphological, biochemical, and 16s rRNA sequence analysis, the isolate was identified as *Bacillus albus DD1*. It showed 98% removal of RB5 from aqueous medium within 38 h under optimum parameters, pH 7, temperature 40 °C, in the presence of 1% yeast extract as a co-substrate, and 25% inoculum size at the initial dye concentration of 50 mg/l. Kinetic study revealed the decolorization reaction is a first order non-spontaneous reaction. The rate constant and reaction rate for RB5 decolourization in presence of the isolate was 0.0523 s^{-1} and 2.6 × 10^{-3} mol/m^3 sec, respectively. Values for ΔH and ΔS of the decolourization reaction, determined by thermodynamic analysis, were estimated to be +20.80 kJ/mol and ΔS = −0.1 kJ/mol K, respectively. LC-MS analysis revealed that decolorization was due to degradation of RB5 by cleavage of azo-bond by the bacterium, with the formation of s 3,6,8-trihyroxynapthalene and phthalic acid as degradation products. Therefore, the bacterium *Bacillus albus* DD1 has potential for application in biological treatment of dye contaminated industrial waste water.

1. Introduction

Industrialization is seen as the economically critical factor for developing countries. However, the source of environmental pollution lies in the unsafe handling of industrial waste. A great amount of textile dyes, (approximately 10,000 synthetic dyes; 7 × 10^7 metric tonnes) are manufactured every year worldwide (Srivastava et al., 2021). Azo dyes are the biggest and most widely used class of synthetic dyes used in the

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textile industry (Samir Ali et al., 2019). When the dyes are released into the water, they become more stable due to the simpler molecular structure and become recalcitrant. Moreover, they are harmful for life forms due to their mutagenic and carcinogenic nature, due to the presence of one or more azo bonds (Droguett et al., 2020). For the removal of reactive azo dyes of RB5 from waste water a number of methods including membrane separation, electrocoagulation (Ghaffarian Khorram and Fallah, 2019); adsorption (Martínez-Sánchez et al., 2018; Naraghi et al., 2018; Wielewski et al., 2020); Oxidation (Vasconcelos et al., 2016); Ozonation (Zheng et al., 2016) are used. Among all these methods, membrane separation, electrocoagulation, direct precipitation or adsorption simply modify the pollutants from one phase to another. Thus, there wastewater treatment methods are substantially inadequate for removing synthetic dyes from wastewater as they are cost and labour intensive, low efficiency, limited versatility and interference with other waste water components (Lops et al., 2019). As a result, research is now progressing towards biological treatments methods as they are environmentally friendly and cost effective. Variety of microorganisms, including fungi, bacteria, yeasts and algae are used for the degradation and decolorization of various reactive dyes as they mostly degrade reactive azo dyes under varying environmental conditions (Bhatia et al., 2017; Eslami et al., 2019).

In this study, Reactive black 5 is used as a model dye as it is the one of the most widely used dye for the dyeing of cotton and other cellulosic fibres and is therefore highly consumption in industries (Şengil and Özacar, 2009). Among, these microbes most situated for field applications are bacteria and fungi as many of them are more rigorous and adaptable to the dynamic field conditions because of their diverse metabolic potential, high stress tolerance potential and ability to survive under unfavourable conditions by formation of dormant structures like spores and cysts. In the current study, we report decolourization of an azo dye RB5 by a halophilic and alkalophilic bacterial strain of *Bacillus albus DD1* isolated from textile effluent. The effect of various parameters on decolourization reaction, and kinetics and thermodynamic behaviour of the reaction was also studied. Attempts were also made to identify decolorization mechanism or pathway by LC-MS analysis studies.

2. Materials and methods

2.1. Azo dye and culture medium

The Reactive Black 5 (RB5, Azo dye) of analytical grade with CAS Number 17095-24-8 (Molecular formula $C_{26}H_{21}N_5Na_4O_{19}S_6$) was purchased from Sigma-Aldrich. For decolorization studies, a stock solution of RB5 of concentration 5000 mg/l was prepared in double distilled water and diluted before use. The composition of mineral salt medium used for decolorization study was (g per liter): KH_2PO_4, 1.00 g; Ca $(NO_3)_2.4H_2O$, 0.05 g; $(NH_4)_2SO_4$, 1.00 g; Na_2HPO_4, 2.78 g; $MgSO_4.7H_2O$, 0.5 g; Peptone, 2 g. All the chemical was of analytical grade. The pH of MSM was adjusted to 7 ± 0.2 using 0.1 M NaOH or 0.1 M HCl.

2.2. Isolation of dye decolorizing bacteria by enrichment method

Waste water effluent samples were collected from textile industry at Kanpur, UP, India, and stored at 4 °C till further use. Enrichment technique was performed to isolate bacteria capable of dye decolorization by adding 10 ml of effluent to 90 ml of MSM containing 50 mg/l RB5 in 250 ml of Erlenmeyer flask and incubated at 30 °C at 100 rpm shaking in an orbital shaker incubator for 100 h. After the completion of incubation time, 10 ml of MSM was used to inoculate another 90 ml MSM containing 50 mg/l RB5 and incubated under at same conditions (Kushwaha et al., 2017). Similarly, after three rounds of enrichment, aliquots were poured on solid MSM plates containing RB5 (50 mg/l) and incubated at 30 °C for 24–48 h. Following incubation, pure colonies capable of decolorizing RB5 were picked and streaked on nutrient agar plates to get pure culture and named DD1 strain.

2.3. Identification of the bacterial isolate

Morphological characterization of bacterial strain DD1 was done by observing colony characteristics on nutrient agar plate after 24 h of incubation at 30 °C. Gram's staining, endospore staining, capsule staining and motility test were performed to investigate bacterial morphology (McCaig et al., 1994). IMViC (Indole test, Methyl red test, Voges-Proskauer test and *Citrate utilisation* test), oxidase, catalase, starch hydrolysis, and sugar fermentation tests have been conducted according to the standard protocol for biochemical characterization. In addition, bacterial isolate molecular validation was performed by 16S rRNA gene sequencing (Mamiatis et al., 1985; Thompson et al., 1994). Colony PCR was performed on a plate of LB agar from 24 h fresh cells. The 16S rRNA gene was amplified by the use of 8F (5′-AGAGTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTGTTACGACTT-3′) universal primers. There were 5 μL 10X PCR buffers (Sigma), 0.5 μL Taq polymerase (Sigma), 10 mM dNTPs (Fermentas) and 20 μM primers (each) in the PCR mixture (50 μL) (Bachate et al., 2012). The PCR was performed in a DNA Engine thermal cycler (Biometric) using the following conditions initial denaturation at 95 for 5 min, 40 cycle consisting of 95 for 1 min (denaturation), 54 for 1 min, (annealing), 72 for 2 min (primer extension) and final extension 72 for 10 min.

2.4. Decolorization of RB5 using the bacterial isolate DD1

MSM (50 ml) containing 50 mg/L of RB5 dye in 250 ml of *Erlenmeyer flaskswas* inoculated with 10% of nutrient broth culture (1OD) incubated at 30 °C under both shaking (100 rpm) and static conditions, for 120 h. Medium without bacterial inoculum served as control. The experiment was run in triplicates. Media samples were withdrawn periodically and analyzed for dye concentration, cell biomass and cell density. Media aliquots were centrifuged at 7000 rpm for 20 min at 4 °C to separate the biomass which was weighted and reported as g/L, while cell count was estimated used serial dilution method using nutrient agar for bacterial cultivation and results were expressed as colony forming units (cfu/ml), the supernatant was used to estimate the concentration of RB5 by measuring absorbance at 597 nm.

Bacterial biomass separated by centrifugation was used to estimate the cell density. Biomass was washed twice with phosphate buffer (pH 7) and centrifuged at 7000 rpm for 20 min at 4 °C to separate the cell biomass which was suspended in Millipore water and used to measure OD at 600 nm.

2.5. Effect of different parameters on decolourization of RB5 by the bacterial isolate

Decolourization of RB5 by the strain was carried out under different set of conditions like shaking, pH, incubation temperature, initial dye concentration, presence of co-substrates, to study the effect of different parameters on it.

2.5.1. Shaking and static condition

Decolourization studies were performed at both shaking and static condition using MSM (pH 7.0) with 50 mg/L initial dye concentration and 10% inoculum. Shaking was provided by keeping the samples in a rotary shaking incubator at 100 rpm at 35 °C for 120 h while static samples were kept in incubator at same temperature. Samples were withdrawn periodically and quantified for the dye concentration and cell density as discussed pin previous sections.

2.5.2. Dye concentration

Decolourization experiment was conducted at 25, 50, 100, 200 and 400 mg/L initial dye concentration using 10% inoculum, pH 7.0 and 35 °C incubation temperature under static conditions.

2.5.3. pH

The pH of the MSM containing 50 mg/L RB5 was set at 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 M HCl or NaOH. Medium was inoculated using 10% inoculum (1OD), incubated at 35 °C for 120 h under static conditions.

2.5.4. Incubation temperature

Decolourization of RB5 by the isolate was studied at incubation temperature of 25 °C, 30 °C, 35 °C, 40 °C and 45 °C at initial dye concentration of 50 mg/L, 10% inoculum, pH 7.0 under static conditions.

2.5.5. Inoculum size

Decolourization studies were performed using different inoculum size including 5, 10, 15, 20, 25% of bacterial inoculum at 50 mg/l dye concentration, pH 7.0 and 40 °C incubation temperature, using 10% inoculum.

2.5.6. Presence of different co-substrates

In the MSM used for decolourization studies peptone was the co-substrate, it was replaced by different other co-substrates like glucose, sodium lactate, sodium formate, beef extract, sodium butyrate and yeast extract at the concentration of 2 g/L and used to study the effect of co-substrate on decolorization. Initial dye concentration was 50 mg/L, with 10% inoculum, pH 7.0, and incubation temperature of 40 °C.

2.5.7. Co-substrate concentration

Effect of concentration of yeast extract as co-substrate was investigated. MSM containing varying concentration of yeast extract (0.2, 0.4, 0.6, 0.8 and 1 g/L) was used for the experiment, other conditions being the same. Initial dye concentration was 50 mg/L, with 10% inoculum, temperature 40 °C and pH 7.0.

2.6. Study of kinetic and thermodynamic parameters of decolourization reaction

The rate of reaction and rate constant were calculated for the data obtained to fit a first-order expression ($Ct/C_\circ$ = e-kt), where Ct is the concentration of the chromium at time t, $C_\circ$ is its initial concentration, and k is the rate constant calculated as the slope of the line obtained by plotting ln ($Ct/C_\circ$) against time (t).

The Eyring-Polanyi equation was used here to analyze the rate of the dye decolorization reaction at different temperatures. The equation can be defined as:

$$\ln(k_1/T) = -(\Delta H/R)1/T + \ln (K_B/h + \Delta S/R) \quad (1)$$

$$\text{Hence, } \ln[(k_1 h)/(K_B T)] = -(\Delta H/R)1/T + \Delta S/R \quad (2)$$

where, k_1 is rate constant of first order reaction, $K_B = 1.38066 \times 10^{-23}$ J/K (Boltzmann constant), R = 8.31441 J/mol K (Universal gas constant), h = 6.6262×10^{-34} J/s (Planck constant), ΔH is change in enthalpy (J/mol) and ΔS is change in entropy (J/mol K) (Ahmad Farid et al., 2018; Zamani et al., 2016).

Further, after determining the value of ΔH and ΔS; by Van't Hoff equation ΔG (Gibb's free energy) can be determined by placing the values in equation below.

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

2.6.1. Decolorization of RB5 at optimum conditions

From the above studies, it was observed that the optimum conditions were pH 7, 1% yeast extract, 50 mg/L dye, 25% inoculum size and temperature 40 °C. Uninoculated MSM served as control in all the cases. Samples were withdrawn periodically and analysed for dye concentration and cell density. MSM without any co-substrate containing RB5 at the initial concentration of 50 mg/L was used. Other conditions were same as specified. In this experiment the potential of the isolate to used RB5 as the sole carbon source was checked, where yeast extract or any other carbon source was excluded in the medium. Visible spectra of the samples were recorded using a UV-Vis spectrophotometer.

2.6.2. Kinetic studies

Kinetic studies were implemented to estimate the rate of the reaction of dye decolorization by the bacterium.The rate of reaction and rate constant were calculated for the data obtained to fit a first-order expression

$$(Ct/C_o) = \text{e-kt}) \quad (4)$$

where C_t is the concentration of the chromium at time t, C_o is its initial concentration, and k is the rate constant calculated as the slope of the line obtained by plotting ln (Ct/C_o) against time (t). The half-life ($t_{1/2}$), of the dye was calculated using the following equation.

$$t_{1/2} = \ln (2)/k = 0.693/ k \quad (5)$$

$$\text{(RB5) Final} = \text{(RB5) Initial } e^{-kt} \quad (6)$$

Where; (RB5) Final is the final concentration of dye (mg/L), (RB5) Initial is the initial concentration of the dye (mg/L), k is the first-order rate constant, and t is the time (hrs.).

2.7. Statistical analysis

All the experiments were performed in triplicates and the results are expressed as mean ± SD. All the experimental data was statistically analyzed using student's *t test*, using the GraphPad Prism 5.

2.8. LC-MS analysis

The degradation products were identified by LC-MS analysis of culture samples inoculated with MSM at 0 hr and 24 h (Asses et al., 2018; Wanyonyi et al., 2019). Aliquots of samples were taken and biomass was separated by centrifugation at 7000 rpm for 20 min at 4 °C, followed by membrane filtration using membrane of 0.2 μm diameter. Mixture of methanol and Millipore water in the ratio of 7:3 (v/v) and 0.02 M phosphate buffer were used as the mobile phase in a gradient elution program. HPLC Accucore (C18, 150 2.1, 2.6 um) was equipped with a photo diode array detector at 254 nm for the LC-MS analysis, which was performed by LCMS -ACQTQD#QBB1152. As a mobile phase, methanol: water was employed (with a volumetric ratio of 50:50) with a flow rate of (0.1 ml/min), and the injected sample was 10 l. An ion-trap spectrometer equipped with electron spray ionization was used to transfer the sample to the MS detector (ESI, Mass spectra were extracted Thermo Finnigan LCQ-DUO, USA). It was discovered that mass spectra could be obtained at voltages of 4.5 kV, capillary temperatures of 275–280 °C, and sheath gas concentrations as high as 40 AU (arbitrary units).

3. Result and discussion

3.1. Isolation of RB5 decolorizing bacteria

A dye decolorizing bacterial strain was isolated after culture enrichment technique. Gram staining test revealed that it was Gram positive strain and produced small circular flat and dry colonies on nutrient agar, it also formed endospores and showed the presence of capsule (Table S1). Subsequently, on the basis of morphological and biochemical characteristics and 16s rRNA gene sequencing the bacterium was identified as *Bacillus albus* and designated *Bacillus albus* DD1. Phylogenetic tree of the bacterial isolate obtained using 16s rRNA sequences is provided in Figure S2 and the gene sequence is available at the NCBI gene bank with accession no. MT810121 (Figure S1) (https://www.ncbi.nlm.nih.gov/nuccore/MT810121).

3.2. Decolourization of RB5 using Bacillus albus DD1

Bacillus albus DD1 was found to decolourise Reactive Black 5 up to 71.5 ± 3.4% in comparison to 3.6 ± 0.4% in uninoculated control, in MSM after 120 h of incubation along with increase in cell biomass and optical density (initial concentration of RB5 50 mg/l). After 120 h the cell biomass and cell count were found to be 1.3 g/L and 2.4 log number of cells. A significant positive correlation coefficient existed between percent dye decolourization and cell density [$R = 0.75$, $P < 0.05$] and biomass [$R = 0.61$, $P < 0.05$]. This suggests that the decolourization reaction is linked to growth of the bacterium where RB5 is co-metabolised by the bacterium.

3.3. Effect of different process parameters on decolorization

The effect of several parameters such as shaking, pH, % inoculum, initial dye concentration, incubation temperature, presence and concentration of co-substrate was investigated in this study.

3.3.1. Effect of shaking and static conditions

The *Bacillus albus* isolate was able to decolorize RB5 up to 72.1 ± 3.4 and 27 ± 1.2% under the static and shaking conditions, respectively. Similar observations were reported by Bouraie et al., (2016); they reported the decolorization of RB5 (100 mg/l initial concentration) up to 76% in static conditions whereas 56% in shaking conditions within 24 h. Xie et al. (2020) also reported 90% decolorization of RB5 (100 mg/l) in static condition in 24 h. Several other researchers (Hamad, 2020; Montañez-Barragán et al., 2020) have also reported better decolorization of the dye under static culture as compare to shaking conditions. In an oxygen-rich environment, oxygen, rather than the azo groups of the dyes, serves as the terminal electron acceptor, resulting in little decolorization.

Better decolourization of RB5 in static conditions may be due to the inhibition of decolorization reaction caused by aeration and shaking; since, bacterial degradation of azo dye by azoreducatses usually begins with the reduction of azo bond by the enzymatic action under anaerobic condition. According to, Seyedi et al. (2020) under shaking conditions, oxygen prevents the azoreducatses from obtaining the electrons required for azo bond cleavage, whereas under static conditions, the electrons are readily available for the reaction. Therefore, static conditions were selected for further dye experiments.

3.3.2. Effect of dye concentration

Figure 1a shows the effects of initial dye concentration on the decolorization % of RB5 at various initial dye concentrations (50–400 mg/l) to decolorize the effects of *Bacillus albus DD1* up to 120 h. The results shows that 72.2 ± 3.2, 71.5 ± 2.5, 54.5 ± 1.2, 26.2 ± 1.5 and 11.34 ± 0.3% at the initial dye concentration of 25, 50,100, 200 and 400 mg/L respectively at an incubation tie of 120 h. According to the verdicts on increasing the initial dye concentration the level of decolorization during the same time interval starts decreasing. The highest decolorization 72.2 ± 3.2 and 71.52.5% was obtained at 25 mg/l and 50 mg/l whereas lowest decolorization (11.34 ± 0.3%) was observed at 400 mg/l. This may due to due to the impact of dye molecules in blocking the active sites of azoreducatses enzymes, or the toxic impact of dyes, may have caused the decrease in decolorization efficiencies (Seyedi et al., 2020). Another reason for a decrease in decolorization ability at higher concentration may be due to the toxicity of dye. The azo dyes have one or more sulfonic-acid groups on their aromatic rings which inhibit microbial development. Similar trends were observed and reported in several studies which concludes that the decolorization rate is decreased on increasing the dye concentration (Al-Tohamy et al., 2020; Garg et al., 2016; Nguyen et al., 2020; Wielewski et al., 2020).

3.3.3. Effect of pH

pH is an important component in a variety of microbial activity. In the present study, bacterial isolate showed highest decolorization efficiency at pH 7.0 (72.8 ± 1.9%) followed by pH 8.0 (73.2 ± 2.0%), pH 9.0 (73.2 ± 2.3%), pH 6.0 (50.3 ± 2.1%), and pH 5.0 (38.9 ± 1.9%), in 120 h. The results showed that there was no significant difference in the decolourization% at pH 7.0, 8.0 and 9.0, indicating that the bacterium was metabolically active and could bring about the degradation reaction at a pH range of 7–9 efficiently (Figure 1b). Similar results are were also reported by El Bouraie and El Din (2016); Javaid et al. (2018); Shah and Pate (2013); Zin et al. (2020). At lower pH up to 7, the enzyme's metabolic activity is most active and can easily bind the dye surface's active site, resulting in increased dye decolourization. As the pH value increased from 7–9 with an increased concentration of dye, the higher concentration of dye leads to negative effect on the decolorization (Hashem et al., 2018). Martorell et al. (2017) observed that dye cations cannot compete with H^+ ions at lower pH values, resulting in a decrease in decolourization efficiency. The electrostatic force of attraction between the negatively charged surface of biomass and the positively charged dye cations is strongest at high pH values. As a result, dye molecule transport across the cell membrane is recognized to be a rate-limiting phase in decolorization.

3.3.4. Effect of temperature

The incubation temperature for RB5 decolorization is an important parameter. It has a significant impact on microbial ability for the decolorization of dye wastewater. Low and high temperatures can alter bacterial reproduction and restrict enzyme-related metabolic processes, and high temperatures can even cause enzyme inactivation and bacteria mortality. The bacterial isolate *B. albus DD1* was mesophilic bacterium shows better decolorization ranging from 25 to 40 °C. Maximum dye decolourization was observed at the incubation temperature of 40 °C (73.1 ± 2.0%) at 35 °C (70.4 ± 3.1%), 30 °C (61.4 + 2.3%), 25 °C (40.0 ± 0.6%) and 45 °C (23 ± 1.3%). The decolorization was only 40% at 25 °C, which increases to 60% at 30 °C and further increases up to 75 % at 40 °C as shown in Figure 1c, however further temperature rise leads to the decrease in decolorization up to 23% due to the loss of the cell viability (Shah, 2016; Meng et al., 2019). Similar results are were also reported by El Bouraie and El Din (2016); Naraghi et al. (2018); Xie et al. (2020) in which decolorization of RB5 was found to be maximum at a temperature of 40 °C. Also, variation in the temperature largely affected the biodegradation activities of microorganisms. Under extreme conditions, microbial activity decreases drastically due to their slow reproduction rate and deactivation of enzymes which are responsible for catabolic action on dyes (Karim et al., 2018).

3.3.5. Effect of inoculum size

The degradation of dye was also depending largely on amount of bacterial inoculum. It was observed that increasing the amount of bacterial inoculum the % decolorization was also increased as shown in Figure 1d. Highest decolourization (74.7 ± 2.1%) was obtained with 25% inoculum followed by that with 20 (73.2 ± 1.3%), 15 (71.0 ± 2.0%), 10 (66.6 ± 3.3%) and 5% inoculum (54.9 ± 1.2%). On increasing the inoculum size, the growth of microorganisms and the synthesis of biomass would also increase; since, on lower inoculum sizes, the cells take longer time to divide into a sufficient number to exploit the generate enzymes and exploit the substrate (Ayed et al., 2017; Palanivelan et al., 2015; Roy et al., 2018). Bonugli-Santos et al. (2016) also reported that the decolorization potential increases with the increase in inoculum size to a certain extent, beyond which it decreases, as the enzyme production is reduced due to nutrition depletion caused by increased biomass, resulting in a decrease in metabolic activity of microorganisms.

3.3.6. Effect of different co-substrates

In Figure 2a the ability of *Bacillus albus DD1* on decolorization efficiency of RB5 in the presence of various co-substrates is shown in Figure 2a. Usually azo dyes often have a low carbon content hence in the absence of co-substrates the degradation of azo dyes by bacteria becomes very challenging (Al-Tohamy et al., 2020). Among various co-substrates,

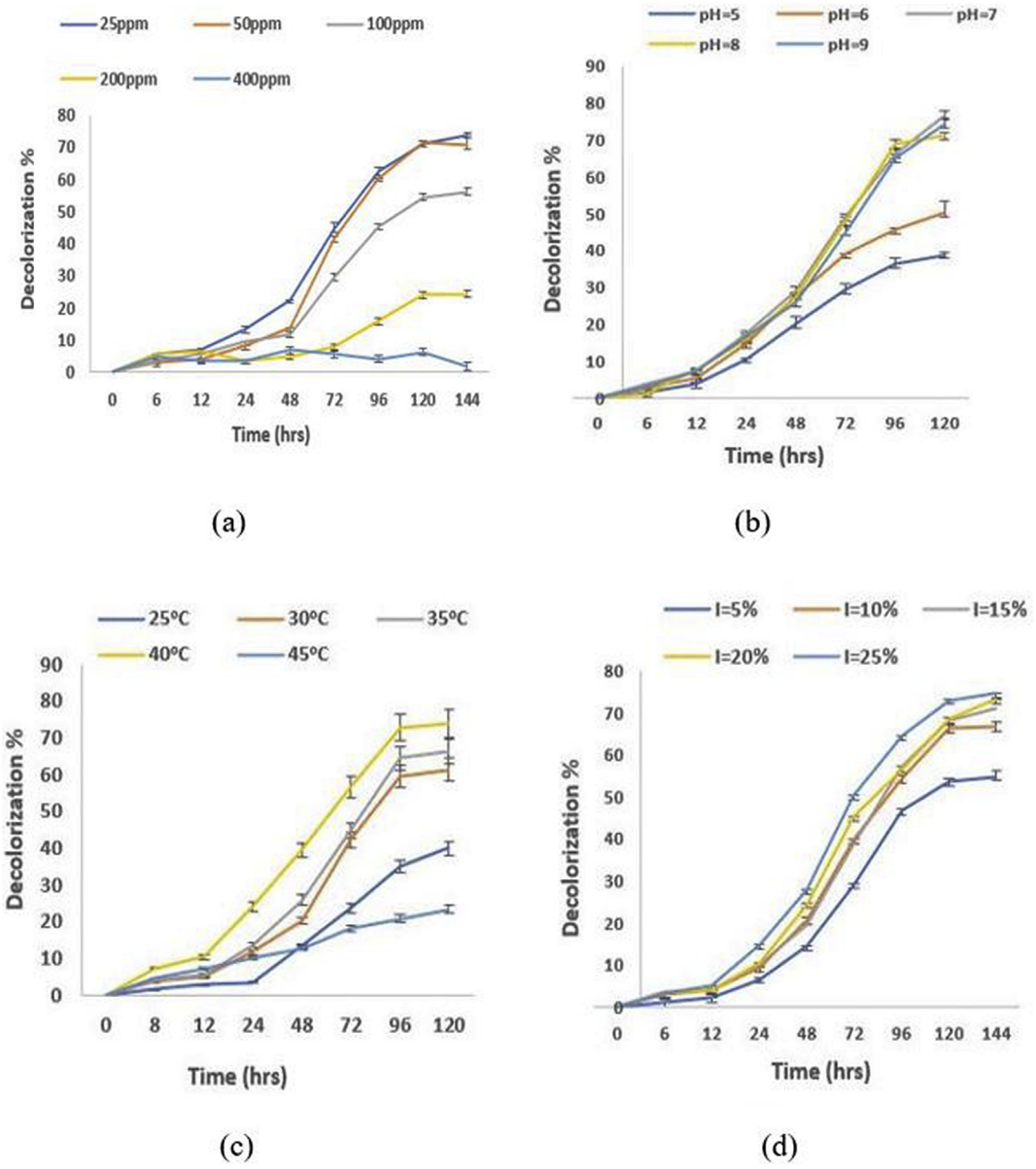

Figure 1. Effect of (a) initial dye concentration (b) pH (c) incubation temperature (d) inoculum size on decolorization of azo dye RB5.

the maximum decolourization was obtained with yeast extract followed by peptone (71.7 ± 2.6%), beef extract (69.01 ± 3.4%), glucose (68.8 ± 1.1%), sodium lactate (50.0 ± 2.3%), sodium butyrate (44.9 ± 2.5%), sodium formate (43.9 ± 2.5%) and sodium acetate (43.6 ± 2.6). Figure 2b shows the effect of yeast extract concentration on RB5 decolorization, it is evident that percent decolorization increased with increase in concentration of yeast extract. The maximum dye removal (92.3 ± 3.4%) was achieved at 1% concentration of yeast extract within 38 h, thus reducing the overall time required for decolourization. According to the findings of Al-Tohamy et al. (2020); Xie et al. (2020), yeast extract was the best co-substrate, which shows better decolorization for the removal of RB5 in comparison to glucose, xylose and lactose. Behzat (2015) reported that for NADH regeneration, which acts as an electron donor when azo bonds are reduced, yeast extract metabolism is thought to be significant. Similarly, Khan et al., 2015 reported that yeast extract can expedite decolorization rate by enhancing the enzyme system of the dyes.

3.3.7. *Effect of yeast extract concentration*

Figure 2b depicts the influence of yeast extract concentration on RB5 decolorization, it is obvious that % decolorization increased with increase in concentration of yeast extract. The % decolorization observed at different concentration of yeast extract was 71.8 ± 3.5 (0.2%), 79.01 ± 1.0 (0.4%), 81.8 ± 2.2 (0.6%), 82.7 ± 2.1 (0.8%) and 92.3 ± 3.4% (1%). Maximum color removal, 92.3 ± 3.4%, was achieved at 1% concentration of yeast extract within 72 h, thus lowering the overall time required for decolourization. Microorganisms for reactive dyes decolourization are greatly affected by their growth determined by the availability of nutrients (Louati et al., 2019). Nitrogen (yeast extract) is an important nutrient as a primary contributor to color degradation. The metabolism of yeast extract is believed to be a critical step in the regeneration of NADH, which serves as an electron donor when azo bonds are reduced (Saini et al., 2018). The explanation for low dye decolorization activity at lower concentration of yeast extract is that the low concentration could not meet the nutritional requirements of the bacterial isolate for growth.

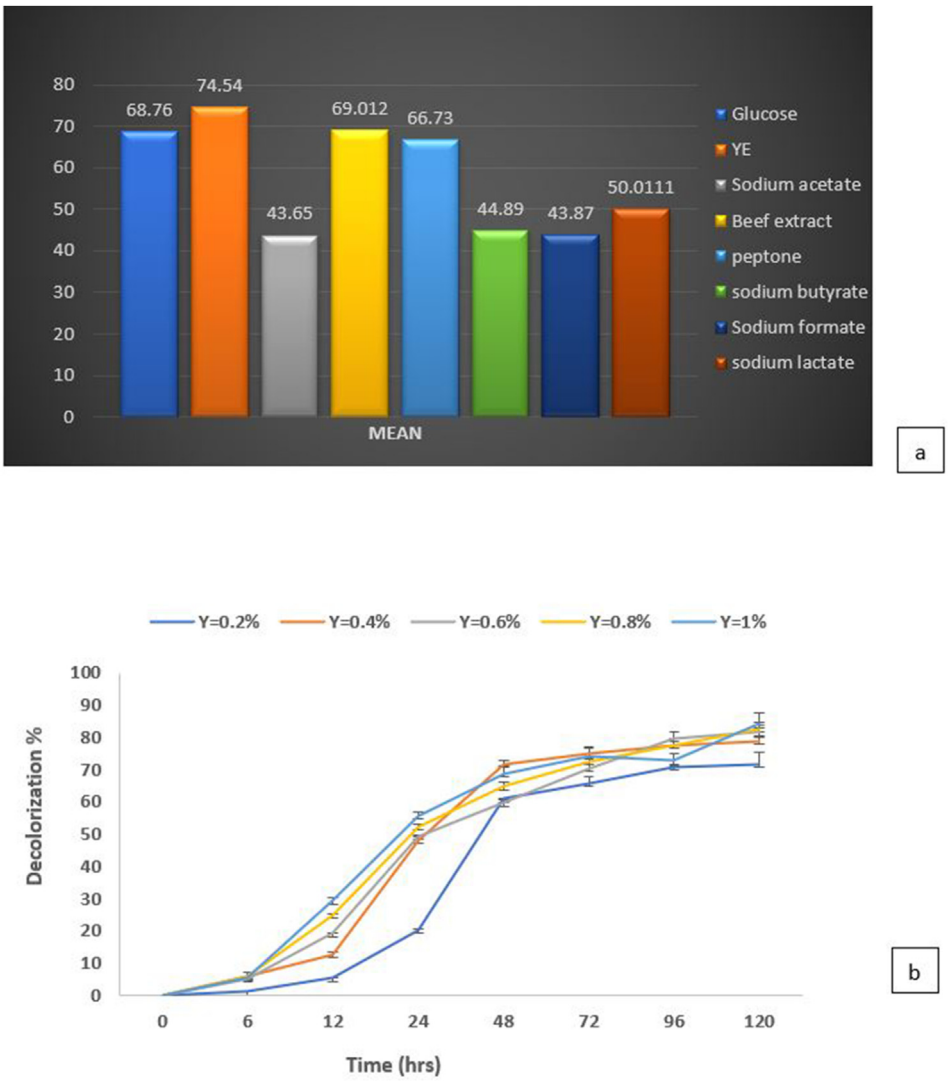

Figure 2. Effect of (a) different co-substrates (b) yeast extract concentration on decolorization of azo dye RB5.

The metabolism of yeast extract is necessary to bacteria in order to replenish NADH as an electron donor for breaking up azo bonds (Behzat, 2015). Azoreducatses, NADH dependent enzyme catalyses the reduction of azo compounds to amines, resulting in degradation of azo dye (Dick et al., 2016). In present investigation, it was shown that 1 % was optimum concentration for decolorization of dye by the bacterium and also it shortened the time of incubation for decolorization.

3.4. RB5 decolourization by Bacillus albus DD1 under optimum conditions

Under optimum conditions i.e., static incubation; pH 7; incubation temperature, 40 °C; inoculum size, 25%; yeast extract concentration, 1%, the isolate was found to decolorize the dye up to 98.5 ± 1.3% within 38 h. The bacterial isolate also showed 69.4 ± 2.2% decolourization, in absence of yeast extract or any other co-substrate indicating that it could use RB5 as sole carbon source for its growth along with its removal from the medium (Figure S2). Bacterial cell density was found to increase with the decrease in dye concentration suggesting that decolorization of RB5 by the isolate is growth limited and that the isolate can co- metabolize a significant (R ˂0.05) co-relation was found to exist between RB5 concentration and cell density. A comparison of microbial decolourization of RB5 as reported in literature is presented in Table 1. The table indicates that % degradation report in the present study is higher in less time duration or even comparable with the most of the earlier reports. Some of them have reported up to 100% decolourization of RB5 at high initial dye concentrations (Al-Tohamy et al., 2020; Ishchi and Sibi, 2020) but field applicability of the reported microbes is limited as efficient dye removal is reported at lower pH values (approx. 5.0), while the pH of textile waste water is towards alkalinity (Dubey et al., 2018; Srivastava et al., 2021).

3.5. Kinetics and thermodynamics study

The kinetics of the RB5 decolorization reaction, at optimum conditions of pH 7, 1% yeast extract, 50 mg/L dye, 25% inoculum size and temperature 40 °C, were evaluated using pseudo first order kinetic model. The -ln ($C/C_\circ$) versus t plot gives a straight line if the first order kinetics is applicable. For the determination of the kinetics of order, plots between -ln ($C/C_\circ$) and time are drawn. It is very clear from that the bio-decolorization reaction of RB5 followed the first order kinetics as -ln ($C/C_\circ$) versus time plot shows the linear relationship with good dispersion and correlation coefficient (R^2) of 0.8721. Balapure et al. (2014); Garg et al. (2016); Xie et al. (2020) also found the pseudo first order kinetics for the degradation of RB5. The first order rate constant and reaction rate for RB5 decolorization, in presence of *Bacillus albus DD1* was 0.0523 s^{-1} and 2.6 × 10^{-3} mol/m^3 sec, respectively. While, the half-life of RB5 ($t_{1/2}$) in presence of *B. albus* was found to be 13.53 h. The values of activation energy, E_a (calculated from slope and intercept of plot) and frequency factor, *A,* (calculated using ln *k* versus 1/T) (Eq. (4)), were found to be

Table 1. Comparative presentation of microbial decolorization of RB5 as reported in literature.

Dye (Initial concentration	Microbial Strain	Experimental parameters	Decolorization (time)	References
RB5 (50 mg/l)	*Bacillus albus*	pH 7.0, temperature 40 °C	98% (38 h)	Current study
RB5 (10 mg/l)	*Bacillus Cereus Strain KM201428*	pH 9.0, temperature 25 °C	97% (120 h)	Wanyonyi et al. (2019)
RB5 (50 mg/l)	*Phanerochaete chrysosporium*	pH 6.0, temperature 26 °C	92.4% (5 days)	Wielewski et al. (2020)
RB5 (50 mg/l)	*Aspergillus Niger*	pH 10.0, temperature 25 °C	94% (7 days)	Arfin et al. (2019)
RB5 (50 mg/l)	*Pseudomonas entomophilia BS1*	pH 5.0, temperature 37 °C	93% (120 h)	Khan and Malik (2016)
RB5 (100 mg/l)	*Aeromonas hydrophila*	pH 7.0, temperature 35 °C	76% (24 h)	El Bouraie and El Din (2016)
RB5 (100 mg/l)	*Peniophora sp. CBMAI 1063*	pH 6.0, temperature 28 °C	92% (4 days)	Bonugli-Santos et al. (2016)
RB5 (100 mg/l)	*Pseudomonas aeruginosa strain ZM130*	pH 9.0, temperature 25 °C	91.1 (180)	Maqbool et al. (2016)
RB5, DB 71 (200 mg/l each) DR1 (300 mg/l)	*Chlorella Vulgaris*	pH 5.0, temperature 40 °C	100 % (14 days)	Ishchi and Sibi (2020)
RB5 (200 mg/l)	*Staphylococcus sp. MEH038S Micrococcus luteus SEH038S*	pH 7.0, temperature 30 °C	90.8% (3 days)	Sadeghi et al. (2019)
RB5 (1500 mg/l)	*Sterigmatomyces halophilus* SSA1575	pH 5.0, temperature 30 °C.	100%	Al-Tohamy et al. (2020)

+27.95 kJ/mol and 2275 (1/Day), respectively (Figure 3a). The results are in good agreement with the recent studies on removal of RB5 (Xie et al., 2019; Zamani et al., 2016).

Furthermore, kinetic constants (0.0264, 0.0345, 0.0486, 0.0512, and 0.0523 s^{-1}) of the first order were also measured at various temperatures (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C), respectively. It also shows that the degradation model rate constant is increased with an increase in the temperature. Further, the thermodynamic analysis was carried out at various temperatures (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C). In kinetic studies, thermodynamic parameters are defined by Eyring-Polanyi equation (Eq. (1)) and first-order rate constants at such temperatures. The graph plot shown in (Figure 3b) between the (ln (K_1h/K_b. T) vs 1/T represented the value for ΔH and ΔS and they were determined to be +20.80 kJ/mol and ΔS = −0.1 kJ/mol K, respectively. A positive value of ΔH suggests that the reaction is endothermic and absorbed heat which contributes to the reactants being passed to products. The negative value of ΔS shows that the mechanism has been less disrupted and that most of the energy inputs are driven by reaction rather than distribution. The ΔG

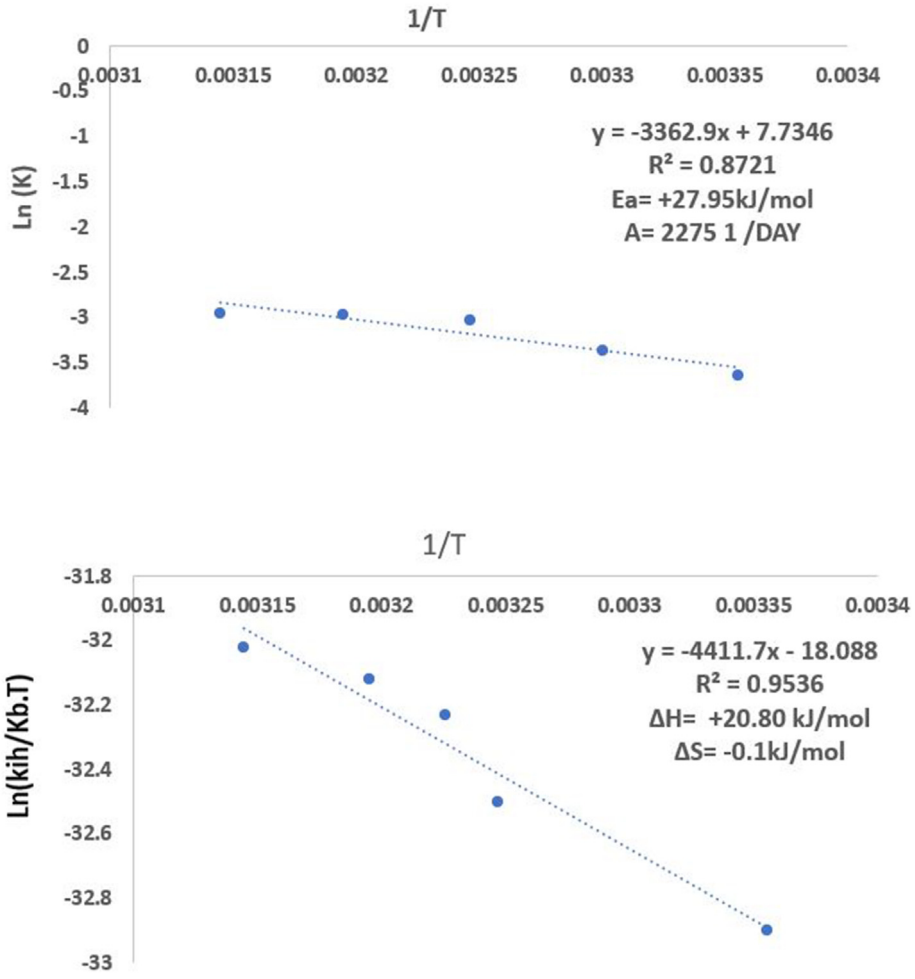

Figure 3. (a) First order kinetic model of RB5 decolorization (b) Eyring–Polanyi model for decolorization of RB5 at different temperature.

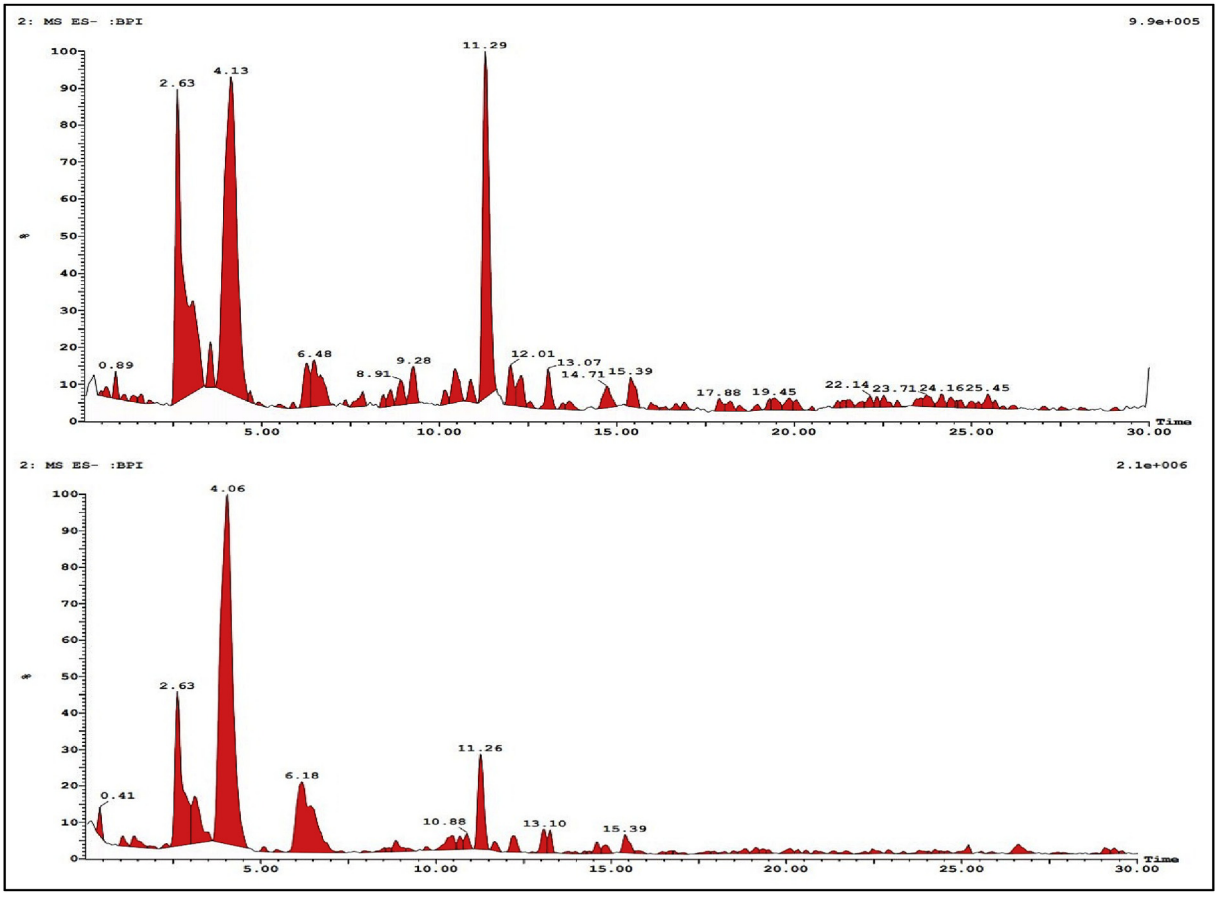

Figure 4. LC-MS chromatogram of RB5 and its degradation metabolites at (a) 0 h (b) 24 h.

value at the temperature 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C are 50.69, 51.19, 51.69, 52.19, 52.69 kJ/mol is also calculated. The positive value of ΔG indicates thermodynamically the non-spontaneous mechanism (Zamani et al., 2016).

4. UV-vis spectral analysis for biodegradation

The UV-vis spectra from 400–800 nm corresponding to untreated and treated dye solution of RB5 depicts that the peak obtained at 597 nm (corresponding to RB5) completely disappear after 72 h of incubation at optimum conditions by the bacterial isolate in inoculated samples in comparison to the respective control (Figure S3), which confirmed that the dye has been decolorized.

5. LC-MS analysis for biodegradation

LC-MS chromatograms at 0 h and 24 h showed a peak at retention time 11.29 min corresponding to unmetabolized RB5 dye (Figure 4). The relative intensity of peak corresponding to RB5 was reduced from 100% to around 28% in 24 h suggesting that the compound has been degraded due to bacteria metabolism. In addition, the spectra obtained at 24 h also showed the appearance of some new peak at retention time 6.18 min, 10.88 min corresponding to intermediate metabolites. The mass spectra of RB5 (retention time 11.29 min) showed molecular ion peak at 577 nm. The metabolites appear at retention time 6.18 min and 6.4 min on the basis of MS spectrum identified as 3,6,8-trihyroxynapthalene and phthalic acid showing molecular mass of (165 m/z) and (178 m/z) as shown in supplementary data. This suggest that the decolorization of RB5 dye was initiated by cleavage of azo bonds, leading to the formation of intermediates like 3,6,8-trihydroxynapthalene and phthalic acid which may further be metabolised by the bacterium.

6. Conclusions

Bacillus albus DD1 isolated from the textile waste water could decolourize azo dye RB5 up to 98.5% within 38 h by co-metabolizing it, in presence of 1% yeast extract, at pH 7.0, and incubation temperature of 40 °C. Co-Metabolism and degradation of RB5 by the strain was evident by significant increase in cell density along with the decolourization. Also, the bacterium could grow in presence of RB5 as the sole carbon source along with decolourization up to 69.4% in absence of any co-substrate. Biodegradation of RB5 was the decolourization mechanism as indicated by increase in cell biomass as well as the appearance of intermediate metabolic degradation products in LC-MS analysis. LC-MS analysis also revealed that the bacterium breaks the azo bonds of RB5 molecules and further degrades it into simpler compounds like 3,6,8-trihyroxynapthalene and phthalic acid (identified by LCMS). Hence, *Bacillus albus* DD1 has the potential for application in biological treatment of dye contaminated industrial waste water.

Declarations

Author contribution statement

Ankita Srivastava & Lalit Kumar Dangi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Radha Rani & Sushil Kumar: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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