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Biodecolorization of Azo Dye by Bacteria *Alcaligenes faecalis Sub Sp. Phenolicus* Isolated from a Bark-Beetle Tunnel Developed in *Peltophorum Pterocarpum* Plant

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Abstract

This study assessed the decolorization of reactive red 120 (RR120) by *Alcaligenes faecalis* subsp. phenolicus strain isolated from the bark borer insect (Indarbela tetraonis) tunnel developed in Peltophorum pterocarpum. The optimal parameters for the dye of decolorization 0.1 mg/L of dye were pH 7, temperature 35°C, fructose (0.4% w/v) as the carbon supply (0.4% w/v), peptone (0.2% w/v) as the nitrogen source (0.4% w/v), 12 hours of static conditions, and 0.3 ml of inoculums. Cell suspension, sodium alginate (3%, w/v), and PVA (5%, w/v) immobilized cell beads (10 beads 0.5 mm in size) were used in the batch continuous reactor for complete bio-decolorization of RR120. The batch reactor was subjected to 5 cycles of batches for 3 days of constant use. Under optimal conditions, the batch mode achieved more than 99% dye decolorization and fabric color removal in less than 48 hours of contact. When the control and dye-decolorized media were analyzed using UV spectroscopy, the absorbance of the control medium was higher than that of the decolorized media. GC-MS and FTIR analysis revealed the basic compounds and functional groups of the parent RR120 dye. This strain decolored 76.51% of AB 113, 96.8% of orange II, 98.47% of congo red, 98.3% of RR120, 97.92% of phenol red individual dyes, and 94.72% of the dye mixture at 12 hours. A. faecalis subsp. Phenolicus strains produced positive results in the qualitative analytical test of exopolysaccharides (EPS) and plant growth-promoting rhizobacteria (PGPR) production. The RR120 was decolorized in the presence of heavy metal ions by A. faecalis sub-sp. Phenolicus bacteria.

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Introduction

Globally, there are more than 10,000 commercially available dyes, and each year, more than 0.7 million tons of dyes, including acidic, basic, reactive, and diazoic dyes, are manufactured. There has been a greater growth of industrial dyes due to the widespread use of chemically synthesized dyes in pulp, dyeing, textiles, paper, and other sectors. The most dangerous chemical types are dyes, mostly found in industrial wastewater.[2] Every year, improper dyeing techniques result in the loss of approximately 200,000 tonnes of unbound azo dyes throughout the processes of dying and finishing in the effluents. Around 15% of dye-containing effluent discharges untreated throughout the world into the environment.[3]

The textile sector uses the majority of azo dyes because they are versatile. Resistance to disintegration and degradation, as well as biological and photocatalytic stability, are the advantages of this dye. Aromatic amines, byproducts of the biodegradation of azo dyes, are produced during azo bond (-N=N-) cleavage and are frequently linked to carcinogenicity, genotoxicity, and chronic and acute biotoxicity.[4] They include single or several azo groups, which can resist degradation and build up at large concentrations with a substantial amount of persistence in the environment.[5] As azo dyes are xenobiotics, they are regarded as the main environmental contaminants.[6] Among the most common and widely used azo dyes that are difficult to remove are reactive dyes.[4] The transparency of dye-laden wastewater can cause acute and long-term toxicity, which also prevents aquatic plants and fauna from undergoing photosynthesis and reduces their oxygen in take.[3] These discharged dyes negatively affect aesthetic qualities, decrease dissolved oxygen, and disrupt aquatic ecosystems by blocking sunlight.[7] Furthermore, many aromatic amines and azo dyes are poisonous and carcinogenic, affecting aquatic organisms and human health via the food chain. Efficiently degrading azo dyes from dye-containing effluent has thus become an urgent challenge8. Due to the carcinogenic nature of textile dyes and their by-products, their removal is needed for the treatment of wastewater.[2]

The options for treatment for azo dye include physical, chemical, and biological degradation. However, because of their complete degradation, economic efficiency, and sustainability, biological approaches are the most often used.[7,9] Enzymatic approaches, phytoremediation, fungal decolorization, and bacterial decolorization are examples of biological techniques. The dye breaks down into many small compounds by microorganisms, which then disperse into water, carbon dioxide, and occasionally inorganic chemicals.[10] In recent years, Klebsiella sp. Y3 has been shown to decolorize various azo dyes6. Microbes of the genera Rhizobium, Azospirillum, and Pseudomonas are capable of degrading difficult chemical substances. They also function as PGPR (plant growth-promoting rhizobacteria), producing phytohormones and hydrogen cyanide (HCN).

The enzymatic phase, which consists of cleaving azo links with the help of an azoreductase and a donor of electrons, typically starts the degradation of the azo dye by bacteria. The resulting compounds, like some aromatic amines, can be broken down by the bacteria's oxygenase and hydroxylase.[11] It has been reported that several PGPRs not only encourage the growth of plants but also improve their environment through the removal of different pollutants. Compared to chemical and physical methods, microbial biodegradation is an effective, eco-friendly, and cost-effective approach. The advantages of rapid multiplication and degradation, demonstrated by bacteria such as *Acinetobacter baumannii* (which degrades Reactive Red 198), *Bacillus fermus* (which degrades Direct Blue 14), and *Dermacoccus abyssi* (which degrades Brilliant Black BN and Brilliant Crocein MOO), have drawn particular attention to bacterial degradation of textile dyes.[12]

Material and Methods

Sample Collection, Dyes, and Chemicals

The tunnel developed by the bark borer insect (*Indarbela tetraonis*) in the trunk of the *Peltophorum pterocarpum* plant was used as a sample for the isolation of microorganisms for the decolorization and degradation of reactive red 120 (RR120) dyes. The samples were collected in a sterile zigzag polybag. The geographical coordinates for the sampling site were latitude (22.12821°) and longitude (82.13816°). RR120, phenol red, acid blue 113, Congo red, and Orange II, dyes were purchased from Sigma Aldrich. Bushnell Hass broth medium (BHM) was prepared as follows: (g/L^{-1}) $FeCl_3$ 0.05, $CaCl_2$ 0.02, $MgSO_4$ 0.2, and NH_4NO_3 1.0, K_2HPO_4 1.0, supplemented

with glucose (0.4% w/v), 1% RR120 dye and yeast extract (0.2% w/v) media pH 7.0.[13]

Isolation, Screening, and Characterization of Rr120 Dye-Degrading Bacteria

A total of 3 (WR-1, WR-2, and WR-3) bacteria that break down reactive red 120 dyes were isolated from the bark borer tunnel (Figure 1A). A 100 ml flask containing 40 ml of BHM broth medium with 100 mg/L RR 120 dye, was filled with 5 g of the sample. The flask was then shaken at 120 rpm for 3 days at 35°C. Static anaerobic environments were maintained for three days at 35°C after 3 g of sample was added to 30 ml of BHM broth medium. Additionally, the decolorized samples (Figure 1b) were serially diluted, and plating was performed using dilutions of 10–6 and 10–7 on BHM agar supplemented with 100 mg/L Reactive Red 120. The isolated bacterial cultures were screened based on the percentage of decolorization. All isolated cultures were screened based on degradation efficiency, for which WR-2 was selected (Table 1). By staining and biochemical tests, the culture and colony morphology of the WR-2 isolates were identified. The genomic DNA of the positively isolated WR-2 was extracted for molecular characterization, and the 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3') universal primers were utilized for analyzing the 16S rRNA gene nucleotide sequence. An automated DNA sequence (ABI3730XL) was used to sequence the products. The EzBioCloud database was used for identification after the Lasergene package was used for assembly.

Table 1: Decolorization percentage of dye RR120 by the isolated bacterial cultures

No.	Bacterial isolates	Decolorization percentage		
		24 Hours	48 Hours	72 Hours
1.	WR-1	93.66±4.27	96.14±1.06	95.29±1.64
2.	WR-2	97.80±0.94	97.81±1.38	96.96±1.13
3.	WR-3	17.87±1.77	35.49±3.39	54.17±1.33

Fig. 1: a) Sampling site for isolation of bacteria, b) RR120 dye decolorized media by WR-2 (triplicate set)

Decolorization Studies

BHM-containing media was added as a supplement. A screw cap tube containing 30 ml of BHM supplemented with 1 ml culture solution of bacteria grown throughout the night was added for inoculation. Medium without dye solution was used as the blank, and the dye-containing uninoculated media was used as the control. For 48–72 hours, at 37°C, the tubes were incubated. At 12-hour intervals during the incubation period, 5 ml of culture media was removed, and the bacterial cell mass was separated after centrifugation of mixture at 10,000 rpm for

10 minutes at 4°C. The absorbance of the culture supernatants at the RR120 dye maximum absorption wavelength (524 nm) was measured using UV-Vis spectroscopy to assess the decolorization potential. The formula was used to determine the percentage of decolorization, which was used to express the decolorizing efficiency.[14]

$$\text{Decolorization \%} = \left\{ \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \right\} \times 100$$

Microbial Inoculums Preparation

BHM broth was used to cultivate the isolates. Following the inoculation, the 30 ml screw-cap tubes were placed at 35 °C in a static incubator until they reached their log phase, at which point they were used as inoculums (1.5×108 cells/ml).

Effect of Aeration, Inoculum Concentration, and Incubation Time

The decolorization of RR120 was investigated in both static and continuously shaking environments. In 100 ml flasks, 30 ml of BHM with RR 120 dye (100 mg/L^{-1}) was included for the decolorization study under continuous shaking conditions. Then inoculated with a bacterial strain and maintained at 35 °C for 12–72 hours while being shaken at 150 rpm. In 30 ml screw-capped tubes, the inoculation medium was taken out, and the plates were placed in an incubator under static conditions for 12–72 hours at 35°C. Decolorization was studied at different incubation times (12 to 72 hours). The experiments were carried out with the previously described inoculation mixture in a static culture environment. The effect of different inoculum concentrations on decolorization was assessed at different concentrations (0.3–3 ml) of BHM. The BHM was then incubated under ideal static culture conditions for 12 hours.[14]

Taguchi Experimental Design

The 5 variables pH range 4-8, temperature 25°C-45°C at 5°C intervals, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L RR120 dye Con; starch, lactose, sucrose, glucose, and fructose carbon sources (0.4% w/v); urea, ammonium sulfate, peptone, ammonium nitrate, and yeast nitrogen sources (0.2% w/v) were mutually exclusive and were used in the Taguchi design of the experiments. RR120 dye (100 mg/L^{-1}) was added to the BHM media. A suitable range of parameter alterations is included due to the selection of the 5 parameter levels. Tests were set up using the L_{25} orthogonal array of TOM after the parameters that were effective at 5 different levels were selected. As previously mentioned, performing a large number of experiments is necessary for the full factorial design. When more factors are involved, the process becomes more complicated and lengthy. Taguchi recommended an orthogonal array technique, a particularly constructed method that reduces the number of tests needed to observe the full parameter space, as a solution to this problem. Thus, Taguchi suggested measuring performance traits that vary from the desired desired outcome using the loss function. The signal-to-noise (S/N) ratio is then obtained from the outcome of this loss function. To analyze the S/N ratio, three performance category parameters are usually used. The three categories are smaller-the-better, larger-the-better, and nominal-the-best.[15]

Immobilization of *Alcaligenes Faecalis Subsp. Phenolicus*

A. faecalis subsp. *phenolicus* was cultured in LB media for 24 h at 35 °C and pH 7 before being immobilized. The cell pellets were extracted by centrifuging them for 10 minutes at 4°C at 5000 rpm. They were then washed with phosphate buffer solution (50 mM, pH 7.0) and suspended. The obtained cell suspension was mixed with sodium alginate (3%, w/v) and PVA (5%, w/v) in an even mixture. Using a syringe, the mixture was dripped dropwise into $CaCl_2$ (3%, w/v). As soon as SV-PVA cells were dripped into $CaCl_2$ (3%, w/v), beads (0.5 mm, 10 beads) were formed, which were kept in a shaker for 30 minutes. After restoring the cells for 48 hours in heterotrophic media, the produced beads were sterilized with distilled water and then kept in a $CaCl_2$ (3%, w/v) solution at 4oC for further use. Bacteria-free beads were made for the control experiments.[16, 17]

Batch-Continuous Decolorization

The studies were carried out in 30 ml screw-cap tubes that had 30 ml of RR120 dye, which included *A. faecalis* subsp. *phenolicus* immobilized 10 beads (0.5 mm size) and BHM broth medium. Batch decolorization tests were performed under static anaerobic conditions, 100 mg l^{-1} dye with pH 7 and 35°C temperature . to evaluate the impact of operating and environmental factors on the efficiency of dye removal.[18] After 12 hours, the beads were

transferred to a fresh medium so that nutrients continued to be provided, and batch decolorization of the dye continued to be performed.

Evaluation of Azo Dye and Azo Dye Mixture Decolorization by *A. faecalis* Subsp. *Phenolicus*

The *A. faecalis* subsp. *phenolicus* strain was used for this purpose, and each strain was inoculated individually in BHM broth medium before being incubated at 35-37°C. *A. faecalis* strains were utilized in studies to lessen azo dyes and mixed dyes (100 mg/l) from BHM media. Additionally, the percentage of dye decolorization at different incubation times was measured for the medium. Using a UV- 1900 UV–Vis spectrophotometer, the absorbance of the culture supernatants was measured at the maximum absorption wavelength of the azo dye mixture (525.6 nm), RR120 (524 nm), congo red (500 nm), phenol red (550 nm), orange II (484 nm) and acid blue 113 (570 nm).[19]

Fabric Decolorization

Fabric purchased from the local market in Bilaspur, Chhattisgarh, consisted of two sets of 100% cotton fabric (red and blue) cut into small, uniform pieces. The cloth pieces were properly packaged and autoclave-sterilized. The aseptic conditions of sterilized BHM broth media (30 ml) were used to separate tubes containing pieces of red and blue sterilized cloth. An overall quantity of 0.3 milliliters of inoculum was transferred to the test sample tubes, while the control medium remained uncultured. Anaerobic conditions were maintained for 2 days while all tubes were cultured at 35–37 °C.[20]

Impact of Heavy Metals on the Rr120 Decolorizing Potential of *A. faecalis* Subsp. *Phenolicus*

Cu, Pd, Zn, Ni, and Cd metal ions were used in a study to determine the efficacy of heavy metal tolerance. Separate additions of the heavy metals ranging from 1 to 6 mg/l were made to the dye decolorization reaction mixtures to examine the impact of ions from heavy metals on the biodegradation ability of Reactive Red 120. Additionally, all experiment was run in triplicate, and the previously indicated parameters were used when determining the decolorization rate. The dye decolorization rate measured in the lack of a bacterial strain was used as a control.[21,22]

Enzyme Assay

A. faecalis subsp. *phenolicus* strain was grown on LB broth media. For 24 hours, the culture tubes were incubated at 35 °C. After that, they were harvested by centrifuging the cells for 15 minutes at 4°C and 10000 rpm and then suspending them in pH 7.4 50 mM phosphate buffer. After centrifugation of homogenate for 15 minutes at 10,000 rpm, the supernatant was used as a source of crude enzymes.[23] Biotic and abiotic cultures were used as native and positive controls, respectively.[24] The presence of tyrosinase, laccase, and manganese peroxidase in the cell-free extract was tested. 0.01% guaiacol in a 2 ml reaction mixture sample was mixed with 0.1 M phosphate buffer (pH 7.4) for laccase screening. Reactions between the released laccases and guaiacol resulted in a reddish-brown colour.[25] The color change in the tubes containing 0.5 ml of the reaction solution with 0.5 ml of sodium tartrate buffer, 0.5 ml of guaiacol, and 10 mM H_2O_2 indicated the presence of manganese peroxidase. When the two substances and secreted manganese peroxidase react, a brownish-red color appears. The activity of tyrosinase was measured in a pH 7.4 0.1 M phosphate buffer with 2 ml of a reaction mixture containing 0.01% catechol. All enzyme screenings were performed at 30°C using reference blanks that lacked any component other than the enzyme. The enzyme screening test was carried out three times.[26]

Eps-Producing Strain Study

To create the EPS, the strain was introduced into a commercial MRS medium at 37°C for 48 hours. After adding twice as much chilled ethanol and centrifuging it for 24 to 48 hours while it was chilled at 4°C, the precipitates were obtained.[27, 28]

Pgpr-Producing Strain Study

NH_3 and indole acetic acid (IAA) production conformation tests have been studied as PGPR trait indices. The IAA generation of the bacterial strains was determined using the Gordon and Weber (1951) method.[29] The IAA precursor L tryptophan was added to the LB broth and the *A. faecalis* sub sp. *phenolicus* strain was then added. IAA content and supernatant were measured after 48 hours of incubation. 1 ml of Salkowski reagent was introduced into the supernatants, which were then left at room temperature for 30 minutes to quantify

IAA. The intensity of the generated pink color was checked.[30] Rhizobacteria isolated from *A. faecalis* subsp. *phenolicus* were evaluated for the production of hydrogen cyanide (HCN)

The culture was grown on Kings medium broth supplemented with 4.4 g/L glycine, further, the culture tubes were incubated at 35°C for four days. The formation of HCN is indicated through the development of an orange or red color. Peptone broth medium was prepared for NH_3 production. To produce ammonia, this strain of rhizobacterium was cultivated in peptone broth and incubated for 48 to 72 hours at 35°C. After adding 0.5 mL of Nessler's reagent to the bacterial suspension following incubation. A color change from brown to yellow signifies the generation of ammonia.[31]

Decolorization and Degradation Analysis

FTIR, GCMS, and UV-visible spectroscopy studies were conducted on the Reactive Red 120 azo dye model to confirm the decolorization and degradation of the *A. faecalis* subsp. *phenolicus* strain. Bacterial strains were cultured in BHM growth media supplemented with 50 mg/L RR120 dye for 12 hours at 35–37 °C. Control and test samples were analyzed following centrifuge at 10,000 × g for 10 minutes at 4°C to study the UV–Vis, GC–MS, and FTIR results. According to Agrawal *et al*, the peak variations within the treated and control dyes were measured utilizing a UV-Vis spectrophotometer.[32] Based on earlier research, the decolorized broth was extracted two times with ethyl acetate in double quantities. To examine the composition alterations within the molecules of the dye found in the effluents when the dye biodegradation procedure, 16-scan speed FTIR dye and extracted metabolite spectra in the 400–4000 cm–1 mid-IR range were obtained. After diluting the sample residue with 1 ml of HPLC-grade methanol, GC-MS evaluation was performed immediately. Under oven conditions, the injector temperature in the GC was maintained at 300°C with split-way oven temperature and hold time (80°C-2min, 100°C-2min, 210°C-5min, 230°C-4min, 250°C-2min, 300°C-10min). A solvent delay of 3 minutes was selected, and helium was utilized as the carrier gas. The MS was run in total ion current (TIC) mode, scanning at 70 electron volts (eV) between 50 and 450 m/z. By comparing the mass spectra in the NIST library database, the dye decolorization metabolites were identified.[33]

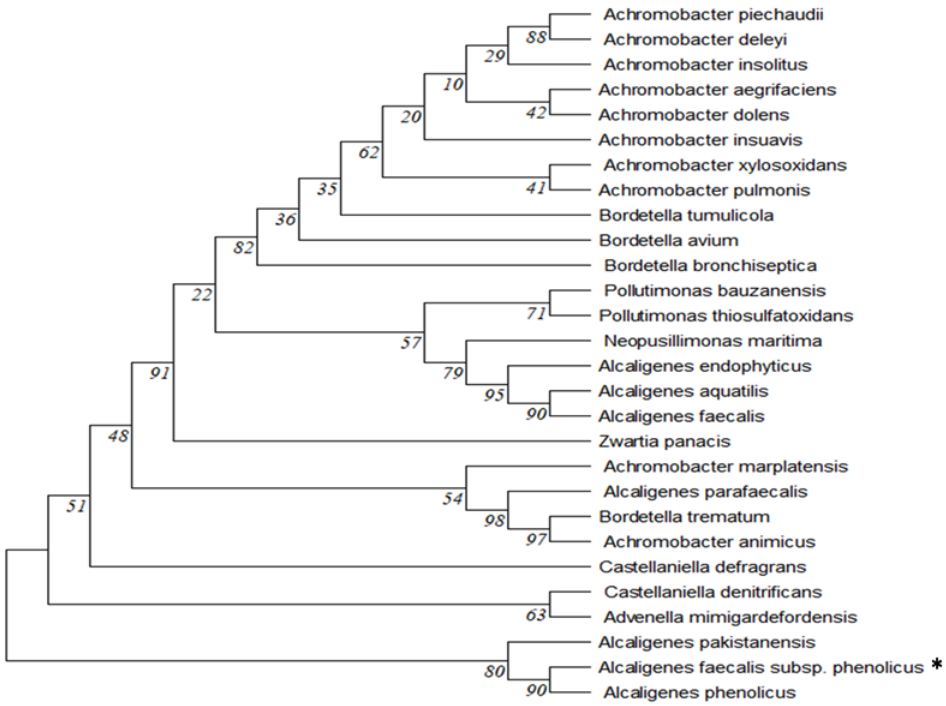


Fig. 2: a) : Phylogenetic tree of *A. faecalis* subsp. *Phenolicus* with different bacterial species

Results

Identification of Selected Isolate

The Bark borer, *Indarbela tetraonis*, of the Guru Ghasidas Vishwavidyalaya Bilaspur, campus contained the bacterial strain WR-2, which significantly degraded RR120 dye after 12 h. According to the result of the biochemical test of selected WR-2 strains, catalase, methyl red, citrate utilization, and mobility tests were found positive, and urease, indole, starch and gelatin hydrolysis, mannitol salt agar, phenylalanine deamination, gas, and H2S production test were found negative. The 16s RNA sequencing results showed that strain WR-2 was linked closely to Alcaligenes *faecalis* sub sp. *phenolicus* DSM 16503(T) accession number AUBT01000026 at 98.84%. The 16S rRNA gene sequences of the WR-2 strain from the NCBI and Mega 11 databases were used to construct a phylogenetic tree (Figure 2). The sequence has been uploaded to Gen Bank (MTCC) with accession number 13437.

Optimization of the Impact of Agitation Shaking and Static, Time, and Inoculum Size in Rr120 Decolorization

Shaking and Static conditions for cell growth are shown in Figure 3a. At the cell growth stage, oxygen has an important effect on physiological properties. Oxygen can either promote or prevent azo dyes from being broken and degraded by microbes. Under static conditions, the *A. faecalis* subsp. *phenolicus* strain decolored RR120 96%; however, under shaking conditions, only 32.47% decolorization was observed (Figure 3b). Under anaerobic and static conditions, *A. faecalis* subsp. *phenolicus* could completely decolorize RR 120 (100 mg/l) after 12 hours of incubation (Figure 3b). *A. faecalis* subsp. *phenolicus* was shown to decolorize RR120 dye in 0.3–3 ml of inoculums (Figure 3c). The decolorization rate increased significantly with a subsequent increase in inoculum size above the optimal value.

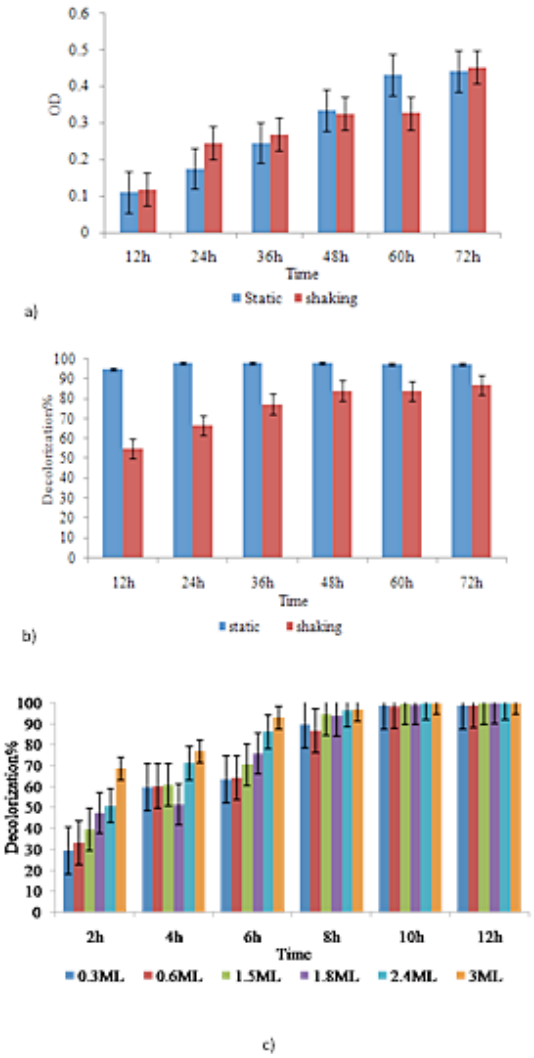


Fig. 3: Impact of a) growth, b) static and shaking conditions; c) inoculum size on RR120 decolorization by *A. faecalis* subsp. *phenolicus*

Taguchi Analysis

The use of statistical systems for support, such as the Taguchi design, has resulted from the parametric understanding of RR120 biodegradation by the *A. faecalis* subsp. *phenolicus* strain and an attempt to quantify the effect of individual parameters on the notable degradation of RR120. This experimental design aimed to determine the optimal parameters for RR120 degradation and a high degradation percentage in a short time. The S/N ratio is used by the Taguchi method to measure the level that deviates from the intended value. The S/N ratios differ based on the type of characteristic. The L_{25} array type of TOM was used in this work to assess five-level process parameters: temperature, carbon, nitrogen source, pH, and dye concentration. The significance of the process parameters' impact on one another increases. The collected observations were entered into the Taguchi Minitab software 19 after the tests recommended by the L25 orthogonal array were completed in triplicate under the

different situations stated in the orthogonal table (Table 2), and the necessary decolorization percentage, S/N ratio and means (Table 3) were calculated. A higher S/N ratio should be taken into consideration when calculating the degradation percentage. The software used a statistical approximation using the mean of the S/N ratio (a higher ratio indicates better performance) to identify and introduce the ideal conditions for each physical and chemical factor (pH, initial dye concentration, carbon, nitrogen source, and temperature). When the experiment was performed in triplicate under optimal conditions, the final results were 99% accurate, according to the software.[34] The mean ratios are shown in Figure 4. The ideal dye concentration was 1 (0.1 mg), the initial pH was 2 (7), the temperature was 3 (35°C), the carbon source was 4 (fructose), and the nitrogen source was 5 (peptone). Ultimately, 99.66% dye removal can be obtained under these conditions.[18] The signal and noise in the Taguchi method signify the acceptable and undesirable values for the respective output characteristic. The Taguchi approach uses the S/N ratio to measure the quality feature that deviates from the ideal quantity. The S/N response graph for RR120 solution decolorization is displayed in Figure 4.

Table 2: L25 Orthogonal observation using the Taguchi method for RR120 dye degradation

Exp.	pH	Tem.	Carbon	Nitrogen	Dye conc. mg/L^{-1}	Decolori -zation %	SNRA1	Mean1
L1	4	25	Glucose	Yeast extract	0.1	63.975	-36.1202	63.975
L2	4	30	Fructose	Peptone	0.2	78.655	-37.9145	78.655
L3	4	35	Sucrose	Ammonium sulphate	0.3	46.08	-33.2702	46.08
L4	4	40	Lactose	Ammonium nitrate	0.4	44.185	-32.9055	44.185
L5	4	45	Starch	Urea	0.5	29.71	-29.4581	29.71
L6	5	25	Fructose	Ammonium sulphate	0.4	57.73	-35.228	57.73
L7	5	30	Sucrose	Ammonium nitrate	0.5	25.11	-27.9969	25.11
L8	5	35	Lactose	Urea	0.1	94.55	-39.5132	94.55
L9	5	40	Starch	Yeast extract	0.2	39.965	-32.0336	39.965
L10	5	45	Glucose	Peptone	0.3	64.875	-36.2415	64.875
L11	6	25	Sucrose	Urea	0.2	69.75	-36.8709	69.75
L12	6	30	Lactose	Yeast extract	0.3	59.86	-35.5427	59.86
L13	6	35	Starch	Peptone	0.4	86.645	-38.7549	86.645
L14	6	40	Glucose	Ammonium Sulphate	0.5	41.46	-32.3526	41.46
L15	6	45	Fructose	Ammonium nitrate	0.1	92.845	-39.3552	92.845
L16	7	25	Lactose	Peptone	0.5	75.845	-37.5985	75.845
L17	7	30	Starch	Ammonium sulphate	0.1	90.27	-39.1109	90.27
L18	7	35	Glucose	Ammonium nitrate	0.2	99.665	-39.9709	99.665
L19	7	40	Fructose	Urea	0.3	95.455	-39.596	95.455
L20	7	45	Sucrose	Yeast extract	0.4	95.24	-39.5764	95.24
L21	8	25	Starch	Ammonium nitrate	0.3	78.06	-37.8486	78.06
L22	8	30	Glucose	Urea	0.4	94.41	-39.5004	94.41
L23	8	35	Fructose	Yeast extract	0.5	91.75	-39.2521	91.75
L24	8	40	Sucrose	Peptone	0.1	94.18	-39.4792	94.18
L25	8	45	Lactose	Ammonium sulphate	0.2	94.215	-39.4824	94.215

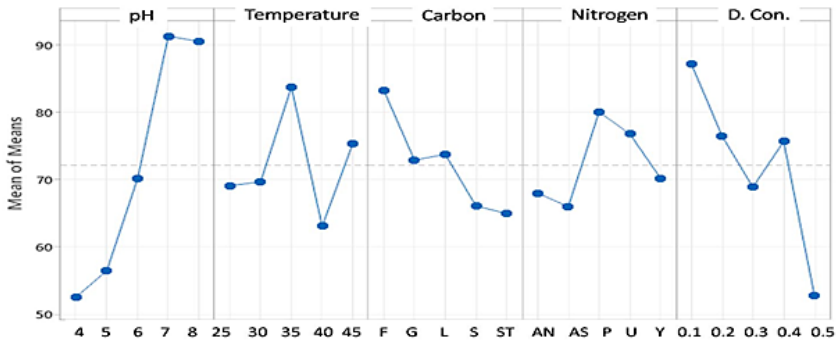
Repeated-Batch Decolorization

Complete decolorization was observed using immobilized bacterial beads for up to 25 cycles in anaerobic environments. After 5 cycles, the rate of decolorization decreased significantly; however, with the bacterial beads immobilized in this study,

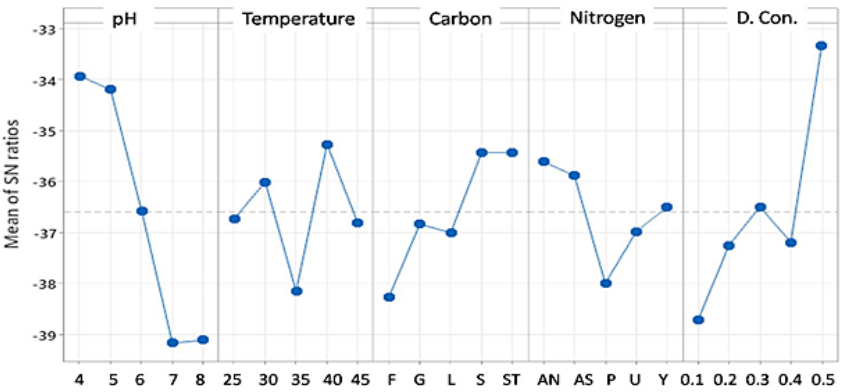
more than 95% decolorization was still observed in subsequent cycles (Table 4). For RR120 decolorization, the immobilized bacteria-mediator beads demonstrated excellent reusability.

Table 3: Response observation for means value and signal-to-noise ratio

Level	pH		Temperature		Carbon		Nitrogen		Dye conc.	
	Means	S/N	Means	S/N	Means	S/N	Means	S/N	Means	S/N
1	52.52	-33.93	69.07	-36.73	83.29	-38.27	67.97	-35.62	87.16	-38.72
2	56.45	-34.2	69.66	-36.01	72.88	-36.84	65.95	-35.89	76.45	-37.25
3	70.11	-36.58	83.74	-38.15	73.73	-37.01	80.04	-38	68.87	-36.5
4	91.3	-39.17	63.05	-35.27	66.07	-35.44	76.78	-36.99	75.64	-37.19
5	90.52	-39.11	75.38	-36.82	64.93	-35.44	70.16	-36.51	52.77	-33.33
Delta	38.77	5.24	20.69	2.88	18.36	2.83	14.09	2.38	34.39	5.38
Rank	1	2	3	3	4	4	5	5	2	1



a)



b)

Standard: F-Fructose, G-Glucose, S-Sucrose, L-Lactose, S-Starch; Y-Yeast, P-Peptone, A-Ammonium Sulfate, A-Ammonium nitrate, and U-Urea.

Fig. 4: a) Main effects for means of means b) Main effects for SN ratios means of SN ratios

Table 4. Decolorization percent of RR120 by using immobilized beads

Cycle	Decolorization percent	
	Immobilized cell beads (Cell-SA-PVA)	Control (cell Free beads) (SA-PVA)
1 Cycles	96.39±1.82	8.66±1.24
2 Cycles	97.55±0.87	8.66±0.47
3 Cycles	97.61±0.61	5.66±1.69
4 Cycles	97.95±0.56	10±2.16
5 Cycles	97.84±1.31	7.33±1.69

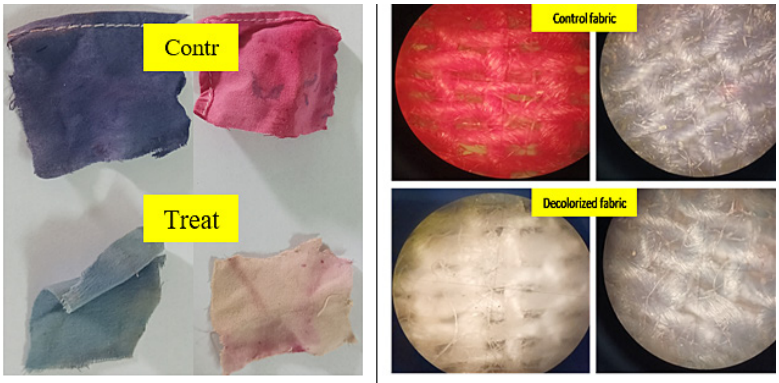


Fig. 5: Fabric decolorization observation (A), (B) Microscopic images

Decolorization of Azo Dye and Dye Mixtures

The bacterial strain was subsequently analyzed using a BHM medium supplemented with 300 mg/l concentrations of the corresponding dyes for the decolorization of AB 113, orange II, congo red, RR120, and phenol red individual dye and dye mixtures. For all dyes, there were differences in the rate of decolorization. It decolored 76.51% of AB 113, 96.8% of orange II, 98.47% of congo red, 98.3% of RR120, 97.92% of phenol red individual dyes, and 94.72% of the dye mixture at the 12-hour mark of the incubation period. It decolorized 99.1% of AB 113 after 24 hours of incubation. The nature and position of the substituent in aromatic rings, as well as the reaction between the azo link and various dyes, are the main factors influencing the variations in the dye decolorization pattern. These variations also affect structure, complexity, and specificity, according to reports.

Enzyme Assay

The difference between the production of enzymes found in the control and the cells produced after decolorization is indicated by the results. It is likely that biotransformation enzymes, such as laccase and MnP, are primarily responsible for the decolorization of cells. For every microbe, there may be differences in the appropriate contributions of MnP and laccase to the decolorization of dyes. According to previous studies, a significant enhancement in the activity of these enzymes during decolorization suggests that these enzymes are involved in the dye breakdown process.

PGPR Activity

The production of HCN, IAA, and NH3 was found to have good potential in the *A. faecalis* subsp. *phenolicus* strain. After 48 hours of incubation under static conditions, the cells were observed.

EPS Production

The extracellular polysaccharide that was isolated for this investigation, however, is a white powder. The pale yellow color of the powder could have resulted from bacteria. No powder was obtained in the control tubes because of the EPS production capacity of

the *Alcaligenes faecalis subsp. phenolicus* strain was compromised.

Fabric Decolorization

The fabric was separated from each tube, then washed 3 times with phosphate buffer, and dried at room temperature. Comparative observation and Raman Microscopic (Figure 6 a) and b) were performed on the color of the control fabrics and bacteria-treated fabrics. Compared with that of the control, the color of the bacterial-treated fabric became colorless. The fabric decolorization test showed the capability of bacteria to remove dye from the colored fabric.

Microscopic Images

Metal Tolerance Activity

According to the amount of time needed for the RR120 azo dye to completely decolorize, the impacts of heavy metal ions on the strain's ability to decolorize dye were studied. Figure 7 shows the results obtained from experiments carried out with Cu, Pd, Zn, Ni, and Cd heavy metal ions present at various concentrations. The *A. faecalis* subsp. *phenolicus* strain required 12 hours to completely decolorize Reactive Red 120 when no heavy metal ions were present. Remarkably, in the presence of metal tolerance, the time needed for the rate of 100% decolorization was reduced by around 72 hours. When the amount of metal ions, viz. Zn, Cu, Pd, and Ni, increased from 2% to 6%, there was a minor decrease in the amount of 72 h needed for RR120 to decolorize 100% of the media sample. >72 h required for 100% dye decolorization at a concentration of 1-6% for the metal ion Ni. Textile effluents with heavy metal ions may have issues with low biodegradability, extending the time required for biological treatment.

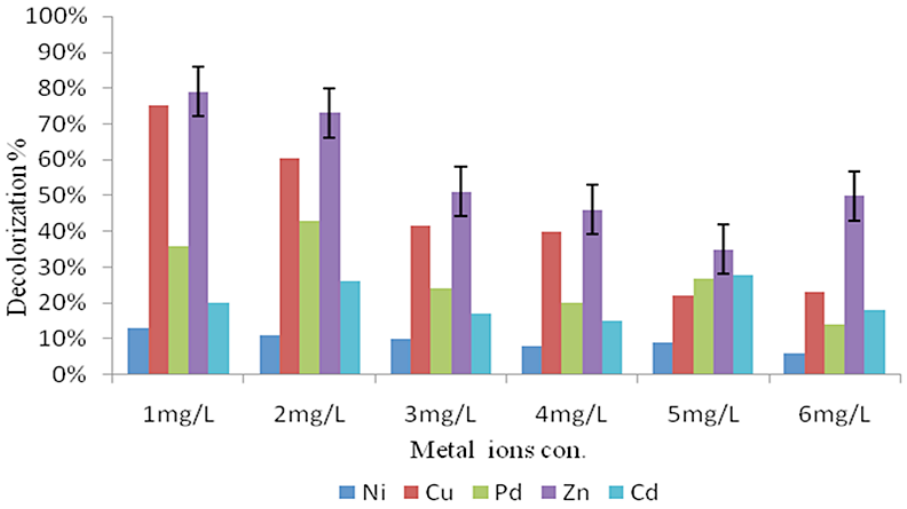


Fig. 6: Metal ion effects on the percentage of dye degradation

UV, FTIR, GCMS Analysis

The most common and basic technique for analyzing dye decolorization is UV-Vis spectroscopy. Figure 8 clearly shows how the decolorization of the RR120 azo dye results in the elimination of the sharp peak at the maximum wavelength of RR120 (λmax 524 nm), showing that the dye within the medium was destroyed by the bacteria after a 12-hour incubation period. Additionally, FTIR is often used as a suitable technique to examine the degradation of dyes. FTIR is a useful analytical technique in dye degradation research because it can be used to determine the type and degree of interactions that take place inside azo dyes with many functional groups which are degraded by bacteria. Therefore, the biodegradation method was studied using the mid-IR 400–4000 cm^{-1} range of FT-IR spectroscopy in addition to the previously discussed analytical methods. After mixing the samples with spectroscopically pure KBr, the analyses were completed. The presence of the dye compound is indicated by a medium peak in the control RR120 dye spectrum that revealed an O-H stretch of the phenolic group at 1381.38 cm^{-1} and a C = N stretch of the amine at 1130.61 cm^{-1} and

1028.09 cm^{-1}.When the FTIR spectrum of RR120 was compared to that of its degraded metabolites after 24 hours, it was evident that *A. faecalis* subsp. *phenolicus* biodegraded the dye. Additionally, the FTIR spectra of the extracted metabolites revealed a moderate peak at 3353.43 cm^{-1} corresponding to the formation of aliphatic primary amines through N–H stretching, with a strong peak at 1555.37 cm^{-1} corresponding to the N–O stretch and the formation of nitro compounds. Halo compound C-I stretching is indicated by the medium peak at 534.52 cm^{-1}. The results are displayed in Figure 9A and B. In RR120 dye, ten peaks were found during GC-MS analysis. RR120 dye components were identified Dodecane, 2, 6, 11-trimethyl-$$ 2, 6, 11-Trimethyldodecan, Tetrapentacontane, Tetrapentacontane, Bis (2-ethylhexyl) phthalate $$ Phthalic acid, and Hexatriacontane $$ n-Hexatriacontane. The broken-down metabolites of Reactive Red 120 were identified by GC-MS analysis (Figure 10, Table 5) and organic solvent extraction as pentanoic as acid (molecular mass 116, m/z 116, retention time 3.297, area 24.38%), pentanoic acid (molecular mass 116, m/z 116, retention time 5.253, area 0.35%), 2-piperidinone (molecular mass 99, m/z 99, retention time 13.617, area 39.28%), methyl tetradecanoate (molecular mass 242, m/z 242, retention time 23.934, area 0.27%), 1,4-diazabicyclo[4.3.0] nonan-2,5-dione (molecular mass 168, m/z 168, retention time 24.307, area 3.35%), cyclo(L-prolyl-L-valine) (molecular mass 196, m/z 196, retention time 25.490, area 12.77%), cyclo(L-prolyl-L-valine) (molecular mass 196, m/z 196, retention time 25.981, area 1.94%), and pyrroline This set off a series of events that eventually produced a stable intermediate.

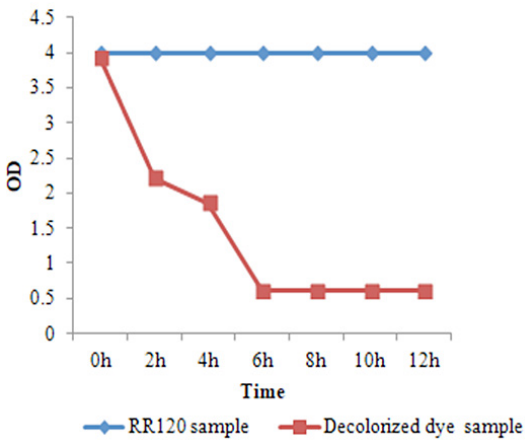


Fig. 7: UV-spectroscopy analysis

Discussion

According to a study by Rehaman *et al*, 74% and 85% of the PGPR *Azospirillum* sp. and Pseudomonas sp. decolorized reactive blue dye at 37°C, respectively, within 20 days.[35] Maniyam *et al* noted that the decolorization effectiveness of the methyl red dye was greater in a static situation than under shaking conditions for two Rhodococcus strains UCC 0016 and UCC 0008.[36] In this study, the PGPR *A. faecalis* sub sp. *phenolicus* was able to decolorize RR120 (0.1 mg/l), which reached 99% at pH 7, 35°C, 1% inoculum, and under static conditions within 12 h. Among the 8 azo dyes, namely, reactive blue R, Janus Green B, ponceau S, cibacron brilliant Red, Evans Blue, Alura Red AC, and brilliant green Bacillus subtilis was determined to be the most effective bacterium. The decolorization rate ranged between 71.8% and 100%.[37] *A. faecalis* subsp. *phenolicus* was able to degrade many azo dyes (76.51% of AB 113, 96.8% of orange II, 98.47% of congo red, 98.3% of RR120, and 97.92% of phenol red individual dyes and 94.72% of dye mixture), according to this work. According to the results, using a carbon source increased Enterococcus faecium's methyl orange decolorization by 100% and in Bacillus cereus by 75%.[38] According to this study, using a fructose and peptone carbon nitrogen source and 0.1% dye contraction increased the decolorization of RR120 by *A. faecalis* subsp. *phenolicus* 100%.To decolorize Congo red dye Goswami *et al*, combined ozonation with a packed bed bioreactor that contained biochar immobilized with Providencia stuartii.[39] As a pre-treatment, the biological method removed dye with an efficiency of 92%. The complete decolorization of RR120 by *A. faecalis* subsp. *phenolicus*-immobilized cells in a batch continuous reactor were used in this study. Bacillus megaterium KY848339.1 was capable of decolorizing the acid red 337 azo dye at an optimal pH 7 and 91% efficiency at a primary concentration of 500 mg/l in less than a day. Dye biodegradation is influenced by effluent pH. Bacillus sp. removes the azo dyes Navy blue and methyl red at different pH values (5–8).[40] The degradation efficiency of Bacillus sp. strain CH12, when various carbon sources were added, was greater than that of the carbon-free culture (27–51%), with decreasing efficiency of 95% to 100%. In contrast to inorganic

sources, which produced the maximum degradation efficiency, the inclusion of peptone and yeast extract as a source of nitrogen produced the highest degradation efficiency (>90%) in the same study.[41] Parma and Shukla investigated how various dye concentrations affected the way Staphylococcus hominis subsp. DSM 20328 degraded the C.I. Reactive Blue 4, an anthraquinone-based dye. They studied an increase in the dye's quantity followed by a reduction in the degradation efficiency of dyes.[42] Arsenic, nickel, copper, and zinc salts were tolerated by the moderately halotolerant strain of Klebsiella at concentrations of 1.25 mM, 1.5 mM, 3 mM, and 5 mM, respectively, because they degraded Congo red and Acid Orange 743. In this study, the *A. faecalis* subsp. *phenolicus* strain decolorized RR120 when heavy metals were present (1% Ni, Pd, Cu, Cd, and Zn).

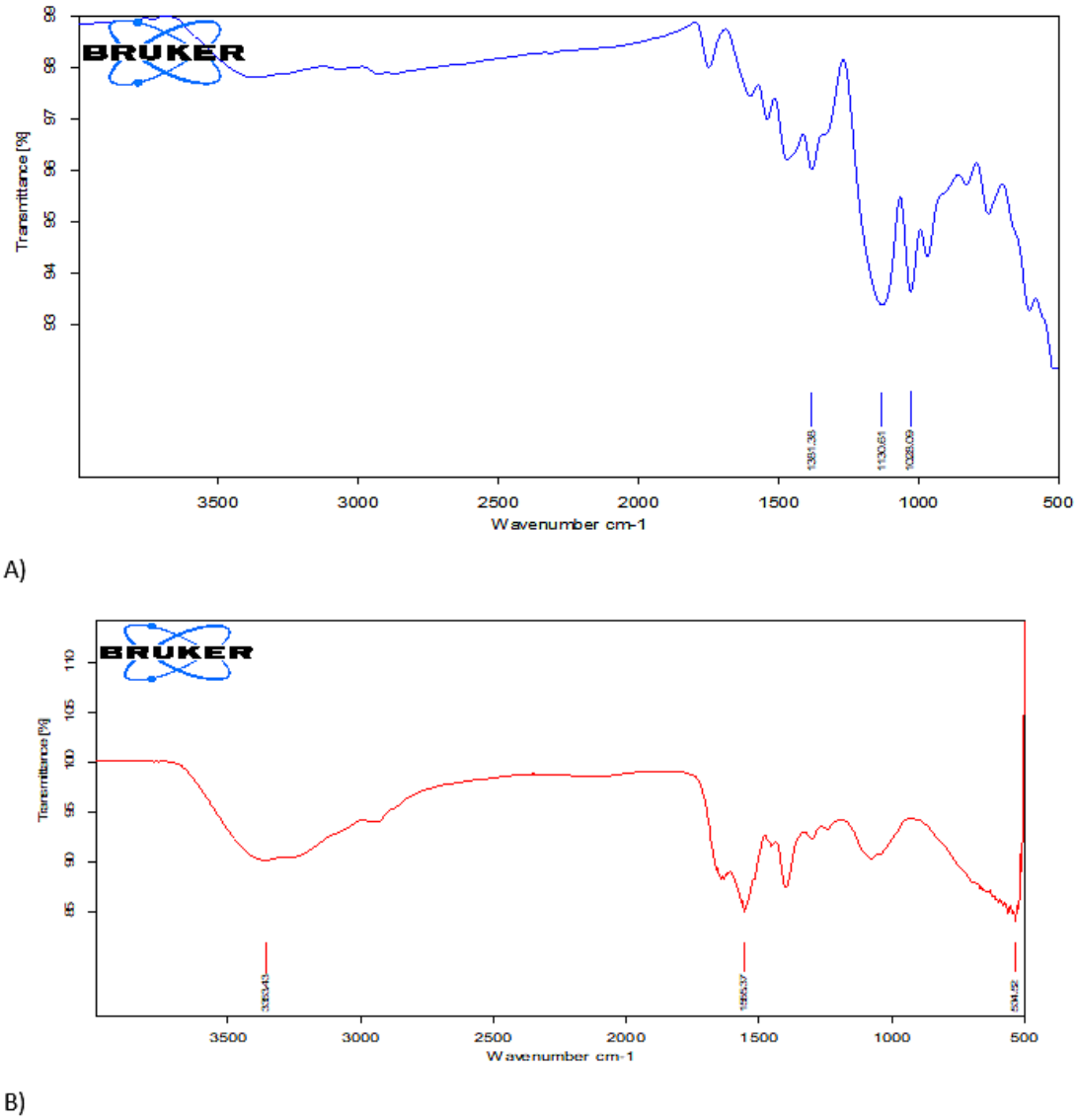


Fig. 8: FTIR analysis graph of (A) RR120 dye and (B) RR120 dye degraded metabolites

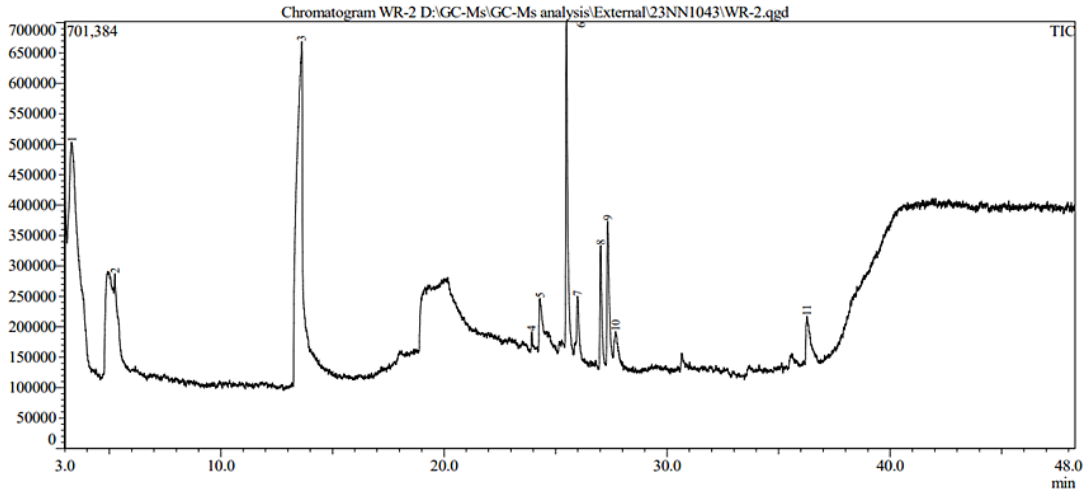


Fig. 9: GC-MS analysis graph of degraded RR120 metabolites

Table 5: GCMS identified compounds and degraded metabolites of RR120 dye

Compound	Molecular formula	R.Time (min.)	Molecular Weight (g/mol)	Peak Area %	Peak % Height
Pentanoic acid	$C_6H_{12}O_2$	3.315	116	12.18	8.08
Pentanoic acid	$C_6H_{12}O_2$	5.304	116	0.68	1.62
2-Piperidinone	C_5H_9NO	13.611	99	48.86	25.61
Methyl tetradecanoate	$C_{15}H_{30}O_2$	23.920	242	0.83	3.38
1,4-diazabicyclo[4.3.0] nonan-2,5-dione	$C_8H_{12}N_2O_2$	24.286	168	2.47	3.84
Cyclo(L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$	25.492	196	12.63	22.97
Cyclo(L-prolyl-L-valine)	$C_{10}H_{16}N_2O_2$	25.985	196	2.01	4.25
Hexadecanoic acid	$C_{17}H_{34}O_2$	26.804	270	0.42	1.54
Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	27.028	210	4.22	7.88
Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	27.342	210	7.13	11.05
Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{11}H_{18}N_2O_2$	27.691	210	1.63	2.15
Heptadecanoic acid	$C_{19}H_{38}O_2$	30.504	298	0.24	0.87
1-Isobutylsulphanylmethyl-2,8,9-trioxa -5-aza-1-sila-bicyclo[3.3.3]undecane $$ Isobuty	$C_{11}H_{23}NO_3SSi$	30.670	277	0.88	1.77
Pyrrolo[1,2-a]pyrazine-1,4-dione	$C_{14}H_{16}N_2O_2$	36.248	244	5.55	4.43
Ethyl homovanillate	$C_{14}H_{22}O_4$	47.211	282	0.27	0.58

Conclusion

Reactive red 120 dye was decoloured in simulated BHM using heavy metal-tolerant and EPS-producing *A. faecalis* subsp. *phenolicus* strains under various physicochemical and operational conditions. Comparing Taguchi's method of parameter design to full factorial analysis, the latter requires fewer experiments and obtains comparable results. The dye concentration, pH, carbon-nitrogen sources, and temperature all affected decolorization. A temperature of 35°C, pH of 7, dye concentration of 0.1 mg/l, carbon source of fructose, and nitrogen source of peptone, a duration of 12 hours, static conditions, and 0.3 ml of inoculums were the most

suitable parameters for Reactive red 120 dye removal. The immobilized beads were found to be stable for 12 hours in repeated batch cultivation for RR120 degradation when *A. faecalis* subsp. *phenolicus* was used continuously for this purpose. The results of this investigation demonstrated the great decolorization efficiency of this *A. faecalis* subsp. *phenolicus* bacteria strain. This strain decolored 76.51% of AB 113, 96.8% of orange II, 98.47% of congo red, 98.3% of RR120, 97.92% of phenol red individual dyes, and 94.72% of the dye mixture at the 12-hour. Aliphatic primary amines, nitro compounds halo compounds were detected by FTIR analysis in the isolated metabolites that were produced during dye decolorization. GC-MS analysis was used to identify low molecular weight substances. The significant removal of the color rate of the strain of bacteria in natural environments suggests that it has the potential to be both economically and environmentally beneficial when used in the microbial treatment of dye-containing effluents.

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Conflict of Interest
The authors do not have any conflict of interest.

Data Availability Statement
All dataset incorporated in the manuscript are produced by the authors

Ethics Statement
This research did not involve human participants, animal subjects, or any material that requires ethical approval

Authors' Contribution
Kusumlata; writing—original data preparation, Rajat Pratap Singh; review, Ashish Kumar; Data analysis, review, editing and visualization.

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