

**1 Functional characterization of bacterial isolates from dye decolorizing**  
**2 consortia and a step-up metabolic engineering based on NADH-**  
**3 regeneration**

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## 18 **Abstract**

19 Azo dye decolorizing acclimatized decolorizing consortia are enriched microbial sources  
 20 of potential azoreductase-efficient bioremediation strains. Here, we characterized eight  
 21 selected consortial members for their azo decolorization and azoreductase profiling. These  
 22 efficient dye decolorizing bacterial isolates were affiliated to two major phyla viz.  
 23 *Firmicute* (genus-*Enterococcus*) and *Proteobacteria* ( $\gamma$ -group). Redox-mediators such as  
 24 AQDS and AQS were found to significantly increase decolorization except for menadione,  
 25 and IR functional group signatures highlighted the azo bond reduction and degraded  
 26 metabolites profiles of each strain. Among isolates, *Enterococcus* sp. L2 was found to be  
 27 the most effective strain as it could reduce >90mg/L Reactive violet 5R (RV5R) dye in 3h  
 28 of incubation. Furthermore, strain L2 possesses profound high NADH and NADPH-  
 29 dependent azoreductase activity which also corroborated with its superior azo  
 30 decolorization. As per physicochemical parameters, strain L2 showed an optimum  
 31 decolorization at pH 8, 40 °C and up to 2% w/v salinity. To channelize reducing  
 32 equivalence (NADH) to further enhance the dye decolorization in NADH-azoreductase  
 33 efficient *Enterococcus* sp. L2, we augmented an NADH co-factor regeneration system.  
 34 Using pMGS100, a Gram-positive expression vector a constitutive heterologous expression  
 35 of *Mycobacterium vaccae* encoded NAD<sup>+</sup>-dependent formate dehydrogenase enhanced  
 36 NADH pool which led to a significant 3.2 fold increased dye decolorization in  
 37 *Enterococcus* sp. L2 harboring pMGS100 *fdh* along with a positive effect on growth.  
 38 Ultimately, an augmentation of formate utilization step could further accelerate azo dye  
 39 decolorization by fulfilling the co-factor (NADH) requirement of azoreductase along with  
 40 a growth advantage in the non-model azoreductase-efficient environmentally important  
 41 strain L2.

## 42 **Keywords**

43 Azoreductase; Azo dye decolorization; Heterologous expression; NAD<sup>+</sup>-dependent  
44 formate dehydrogenase; NADH-regeneration.

## 45 **1. Introduction**

46 Azo dyes are one of the largest classes of dyeing chemicals that account for >70% of the  
47 global industrial dye requirement of around 9 million tons (Rawat et al., 2018; Sarvajith et  
48 al., 2018; Guo et al., 2020; Routoula and Patwardhan, 2020). Due to their genotoxic and  
49 carcinogenic capability, the annual disposal of ~4,500,000 tons of azo dyes and their  
50 metabolites are an environmental and financial task (Rawat et al., 2016). For the  
51 developing economy, azo compounds and their metabolism, in various biological systems  
52 is a top-order agenda of the environment protection and conservation agency which are  
53 mainly applied in the textile industry. As per estimates, ~12% of textile-industry associated  
54 synthetic azo dyes utilized annually are discharged to wastewater resources. Due to  
55 inadequate effluent treatment, these account for ~20% of the total dye pollution to the  
56 environment (Saratale et al., 2011). In the last few decades, multiple biological treatments  
57 have been attempted using various individual strains and artificial or acclimatized  
58 consortia which can bioremediate azo dyes efficiently (Rathod and Archana, 2013; Patel et  
59 al., 2016; Rathod et al., 2017; Sreedharan and Bhaskara Rao, 2019; Guo et al., 2021;  
60 Samuchiwal et al., 2021), and hunt of new effective strains and their further optimizations  
61 remains a challenging task till now.

62 Azo (-N=N-) bond reduction or decolorization has been the bottle-neck step of the  
63 dye degradation pathway, and each strain possesses its unique mode of azo dye  
64 decolorization (Sreedharan and Bhaskara Rao, 2019). Multiple mechanisms such as  
65 enzymatic, non-specific redox mediator based or direct reduction by reduced metabolites

66 including quinones that can extracellularly reduce azo dye (Stolz, 2001; Chengalroyen and  
67 Dabbs, 2013; Rathod et al., 2017) Hong and Gu, 2010).

68       Enzymatically azoreductase, mono-di oxygenase, peroxidase, laccase, and flavin  
69 reductase are the main set of enzymes catalyzing azo decolorization (Chen, 2006; Pandey  
70 et al., 2021), out of them azoreductases are recognized critical catalytical component of  
71 xenobiotic metabolism and found to be omnipresent in the various biological system  
72 (Bafana and Chakrabarti, 2008) (Misal and Gawai, 2018). Azoreductases are also known to  
73 possess flavin-dependent quinone reductase activity (Deller et al., 2008; Leelakriangsak,  
74 2013; Suzuki, 2019; Rathod et al., 2022). To catalyze azo cleavage, azoreductases derive  
75 reduction potentials from either NADH, NADPH or FADH<sub>2</sub> (Morrison et al., 2012; Punj  
76 and John, 2009). Out of all oxidoreductases, ~80% of enzymes require NADH as a co-  
77 factor compared to only up to 10% requiring NADPH (Wu et al., 2013). Therefore, the  
78 availability of intracellular NADH to an efficient azoreductase activity remains a  
79 competitive metabolic challenge (Rathod et al., 2017). Further, the augmentation of the  
80 NADPH-regeneration system is highly complex, and their metabolic requirement of  
81 fastidious metabolites (Oeggel et al., 2018), makes it unfavorable for bioremediation  
82 application. Previously, we have reported a single gene amended efficient NADH-  
83 regeneration system which has been reported to increase azo dye reduction in model  
84 bacterial systems. However, their compatibility in non-conventional and environmentally  
85 important azo dye reducers has not been studied.

86       This study aims to characterize azo dye decolorization and azoreductase profiles of  
87 unique bacterial strains isolated from our lab enriched acclimatized consortia Rathod and  
88 Archana (2013). Among these isolates, we selected the most efficient *Enterococcus* sp. L2  
89 possessing a native NADH-dependent azoreductase activity to further improvise its azo

dye decolorization profile. To achieve this, we heterologously overexpress *Mycobacterium vaccae* encoded NAD<sup>+</sup>-dependent formate dehydrogenase in strain L2 to replenish the intracellular NADH pool which is required for efficient azoreductase catalysis. Ultimately, we could accomplish a significant enhancement in azo dye reduction in a non-model and bioremediation points of view important strain.

## 2. Materials and methods

### 2.1. Azo dye decolorization studies

Reactive Violet 5R (RV5R) was used as the model azo dye which consists of a mono azo group linking benzene and a naphthalene ring (Jain et al., 2012; Rathod et al., 2017). RV5R was procured from Meghmani Dyes And Intermediates Ltd, GIDC Vatva, Ahmedabad, India. Methyl red was obtained from HiMedia Laboratories, India. The Bushnell Haas Medium (BHM) [MgSO<sub>4</sub>, 0.2 g/L; K<sub>2</sub>HPO<sub>4</sub>, 1.0 g/L; CaCl<sub>2</sub>, 0.02 g/L; FeCl<sub>3</sub>, 0.05 g/L; NH<sub>4</sub>NO<sub>3</sub>, 1.0 g/L] and various media components used in this study were from HiMedia Laboratories, India. Considering the consortium source of isolate the following media were used, 1) BHM with 0.5% w/v glucose and 0.5% w/v yeast extract as medium A used for isolate ME1; BHM with 0.5% v/v glycerol and 0.5% w/v yeast extract used as medium B for isolate A3; 2% w/v peptone, 0.15% w/v K<sub>2</sub>HPO<sub>4</sub>, 0.15% w/v MgSO<sub>4</sub>, 1% (v/v) glycerol used as medium C for isolate E2 and K1, and 4) 1.5% w/v Tryptone, 0.5% w/v soya peptone, 0.5% w/v NaCl used as medium D for isolate C1, G1, L1 and L2. Filter-sterilized solution of RV5R and MR dyes was added to obtain its 100 mg/L final concentration in media. To evaluate the NAD<sup>+</sup>-dependent formate dehydrogenase over-expression studies medium with chloramphenicol 10 µg/ml was used. Dye decolorization at different intervals was monitored by withdrawing

114 aliquots and followed by centrifugation at 14,000 g for 10 min to isolate the bacterial  
115 cell mass. By measuring the absorbance of the supernatant at maximum wavelength for  
116 the Reactive Violet 5R ( $\lambda_{\max} = 558\text{nm}$ ) and Methyl red ( $\lambda_{\max} = 420\text{ nm}$ ) using Spectronic  
117 20D+ (Thermo Scientific) the decolorization percentage was calculated using below  
118 equation,

119 Decolorization (%) = [O.D. at time ( $t_0$ ) - O.D. at time ( $t_1$ )] / O.D. at time ( $t_0$ ) \* 100. Dye  
120 decolorization experiments were done in triplicates.

## 121 ***2.2. Identification of bacterial isolates by 16S rRNA gene sequencing***

122 For genomic DNA isolation, freshly grown cells were harvested from 2 mL of the  
123 culture suspension by centrifugation at 14,000 g, 4°C for 10 min. Cell pellet was  
124 resuspended in TE25S buffer followed by lysis and purification steps as per standard  
125 molecular biology protocols (Sambrook and Russel, 2001). Finally, DNA was dissolved  
126 in 50  $\mu\text{l}$  TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Using eubacterial universal  
127 primers 27F and 1107R, 16S rRNA gene was amplified using PCR (Chaturvedi and  
128 Archana, 2012). The PCR product was sequenced using reverse primer (1107R),  
129 generating optimum sequence length for the identification (Pillai and Archana, 2008).  
130 The sequence data were analyzed using RDP database. MEGA 4.0 was used to  
131 construct the phylogenetic tree. Additionally, bacterial identifications were further  
132 confirmed using biochemical tests specified by Bergey's manual (Staley J.R., 2001).  
133 Growth analysis was performed by withdrawing cell suspension aliquots at time  
134 intervals. To avoid any spectral interference of the residual dye, harvested cells were  
135 washed with PBS and growth was measured by taking O.D. at 600nm. Growth  
136 experiments were done in triplicates.

### 137      ***2.3.Nucleotide sequence accession numbers***

138      All the isolates' 16S ribosomal RNA sequence GenBank accession number are JQ745287-  
139      94.

### 140      ***2.4.Azoreductase assay***

141      Azoreductase assay was performed by quantifying the reduction in optical density at  $\lambda_{\max}$   
142      of the Reactive violet 5R dye with a Shimadzu UV-visible spectrophotometer at room  
143      temperature. The reaction mixture (1.0 ml) contained 25mM potassium phosphate buffer  
144      (pH 7.1), 25  $\mu$ M azo dye, 0.1mM NADH, 10  $\mu$ M FMN, and a appropriate amount of  
145      enzyme. The reaction was initiated by the NADH and quick mixing. One unit (U) of  
146      enzyme activity was defined as the amount of enzyme needed to decolorize 1  $\mu$ mole of azo  
147      dye/min/mg of total protein (Chen et al., 2004). Protein concentration was quantified using  
148      the Bradford assay (Pierce) and bovine serum albumin (BSA) was used as the standard.

### 149      ***2.5.Functional group identification of RV5R degradation products by Fourier*** 150      ***Transformed Infrared spectroscopy (FTIR)***

151      Decolorization or degradation products of azo dye by isolates was studied by FTIR  
152      analysis. Endpoint metabolites were extracted by an equal volume of ethyl acetate and  
153      dried in SpeedVac (Thermo Electron Corporation, Waltham, MA). FTIR Analysis was  
154      done by mixing with HPLC grade potassium bromide (KBr) in the ratio of 5:95 and  
155      analyzed at mid-IR region (400–4000  $\text{cm}^{-1}$ ) by FTIR using Spectrum GX (PerkinElmer,  
156      USA).

### 157      ***2.6.Heterologous expression of NAD<sup>+</sup>-dependent formate dehydrogenase in*** 158      ***Enterococcus sp. L2***

159 *Enterococcus* sp. L2 being a Gram-positive isolate, we used pMGS100 *pbacA* including its  
160 ribosome-binding site-driven constitutive expression system. The plasmid pMGS100*fdh*  
161 was constructed by cloning of coding region (ORF) of *mycfdh* amplified using primers  
162 MGS100*fdh*F (5' ATG GCA AAG GTC CTG TGC GTT CTT TAC G 3') and *Mycfdh*R (5'  
163 TAT AGG TAC CTT CGG ATC CTC AGA CCG CCTT CTT GA 3') into NruI site of  
164 pMGS100. Clones with correct orientation of *fdh* with constitutive promoter of bacitracin  
165 resistance gene (*pbacA*) were screened by BamHI digestion and pcr conformation. *In vitro*  
166 handling of DNA molecules for cloning was done utilizing standard protocols (Sambrook  
167 and Russel, 2001). The pMGS100 *fdh* was transferred to *Enterococcus* sp. L2 using  
168 protoplast electroporation describe by (Dunny et al., 1991).

## 169 **2.7.SDS-PAGE analysis**

170 Cells were harvested and heat-lysed using a boiling water bath for 15 min. A  
171 resolving gel (12%) and separating gel were used for SDS-PAGE and as a molecular  
172 weight standard protein marker (97, 66, 43, 29, 20, 14kDa) (Merck, India) was used.  
173 SDS-PAGE gels were run in 5X Tris-glycine buffer at 70 V for initial 15-20 min and  
174 then, at 100 V up to 2h. After electrophoresis, proteins on gels were visualized by  
175 staining with 0.25% Coomassie brilliant blue R250 and de-stained according to  
176 Sambrook and Russel (2001).

177

## 178 **2.8.NAD<sup>+</sup>-dependent formate dehydrogenase assay**

179 To assay NAD<sup>+</sup>-dependent formate dehydrogenase (Fdh) activity, whole-cell  
180 lysate was prepared as mentioned for azoreductase activity in sodium phosphate buffer  
181 at pH 7.5 along with 0.1M β mercaptoethanol according to Rathod et al. 2017. Using  
182 molar extinction coefficient of NADH as 6220 M<sup>-1</sup> cm<sup>-1</sup> enzyme units were calculated.



183 One unit (U) of Fdh is defined as the enzyme needed to oxidize 1  $\mu$ mole formate per  
184 minute. Using Bradford method, total protein concentration in cell extracts was  
185 measured and bovine serum albumin was utilized as standard.

## 186 **2.9. Intracellular reducing equivalent estimation**

187 Cultures were grown overnight medium containing chloramphenicol 10  $\mu$ g/ ml. To a re-  
188 inoculated freshly grown culture, at the mid-log phase (0.4 O.D.) 1mM IPTG was added  
189 which was induced for 6 h along with amendment of 300mM Na-formate. This induced  
190 cell culture was centrifuged at 5000 g for 10 min and the resulting pellet was washed  
191 twice with 0.01M sodium phosphate buffer (pH 7.5). Decanted pellet was resuspended  
192 in 1mL 0.01M sodium phosphate buffer (pH 7.5) and sonicated for 3 min by using  
193 Sonics VibraCell™, USA. After centrifugation at 14,000 g, 4°C for 10 min cell debris  
194 were removed and supernatant as cell lysate was used to estimate the reducing  
195 equivalents. Using nanophotometer (Implen, GmbH) [NADH] at 340 nm and [Protein  
196 concentration] at 280nm was measured, and [NADH]/[Protein] as 340/280 nm ratio was  
197 determined.

## 198 **2.10. Statistical analysis**

199 The significant differences among the different treatments were analyzed by one-way  
200 analysis of variance (ANOVA) with a pairwise multiple comparison procedure (Fishers  
201 LSD). T-test has been performed between treatments of redox mediators to control as  
202 well as over-expressing *fdh* transformant to vector control. Sigma Stat 3.5 was used for  
203 the statistical analysis.

## 204 **3. Results and Discussion**

### 205 **3.1. Azo dye decolorization kinetics of isolates and 16S-rRNA gene-based** 206 **identification**

207 In our previous study by Rathod and Archana (2013), we have reported the enrichment of  
208 twelve acclimatized Reactive violet 5R decolorizing effective consortia from diverse  
209 environmental pools. The study also reported a total of 28 isolates from these consortia.  
210 These consortia harbored several heterogeneous, active, and profound azo dye decolorizing  
211 members which have the potential for efficient azo dye bioremediation. Based on the  
212 efficient decolorization properties, out of 28, eight isolates were selected for further  
213 taxonomic identification and characterization.

214 These potential isolates were analyzed for decolorization of complex model dye Reactive  
215 violet 5R (RV5R) and Methyl red (mono azo with two benzene rings, MR) in their native  
216 growth media of respective consortium. Isolates L2 and ME1 were found to decolorize  
217 RV5R more than 90% in 3 h (Figure 1a). Isolate C1 and G1 took up to 30h to decolorize  
218 up to 90%. Decolorization of methyl red (mono azo, benzene rings containing dye) was  
219 studied for these isolates, resulted 99% decolorization of MR by isolates L2 and ME1 by  
220 6h and isolate C1 decolorized 98% of MR decolorization by C1 isolate in 12 h (Figure 1b),  
221 whereas the rest of the isolates took 18h to decolorize MR more than 90%. The chemical  
222 structures of the model dyes used in this study are depicted in Figure. 1c, d.

223 Table 1 shows the phylogenetic affiliation of the eight isolates based on 16S rRNA gene  
224 sequence. Biochemical key identification results are given in Tables S1-4. The Gram  
225 positive were identified as *Enterococcus spp.*, whereas six of the gram negatives were  
226 found to belong to  $\alpha$ -*Proteobacteria*, out of which two belonged to *Providencia* and  
227 *Klebsiella spp.*, whereas remaining two were similar to *Acinetobacter* and *Citrobacter*  
228 genera. Using 16S rRNA gene sequence similarities, best matches were selected along with  
229 their 16S rRNA sequences from Ribosome data project (<http://rdp.cme.msu.edu/>) for  
230 building phylogenetic tree (Figure 1e). The optimal tree with the sum of branch length was

0.37043799. Further, we correlated the identified member strains with previously reported taxonomic neighbors with dye bioremediation features and their mode of azo dye reduction. *Klebsiella* spp. have been known for micro-aerophilic -aerobic sequential decolorization/degradation process of various textile azo dyes (Franciscon et al., 2009). *Klebsiella* spp. obtained in these studies showed 99% phylogenetic similarity with the reported *Klebsiella* strains showing heavy metal resistance, heavy metals are widely used for the chemical stability of the azo dye and found to be major co-contaminant in the effluents of dye manufacture and application industries. Interestingly, isolate *Klebsiella* sp. E2 showed phylogenetic similarity with copper resistant *Klebsiella pneumoniae* strain SW (accession no. AB641122) and *Klebsiella* sp. K1 with nickel resistant *Klebsiella pneumoniae* strain ZB (accession no. KC243315) (Table. 1). Azo-reducing bacteria such as *Shewanella*, *Citrobacter*, *Acinetobacter*, *Pseudomonas* have shown to reduce azo dyes with molecular H<sub>2</sub>, electron donors which includes short-chain fatty acids and redox mediators that are known to profoundly involved in dye decolorization (Hong et al., 2008; Cui et al., 2020). Thus, obtained isolates specifically *Enterococcus* sp. L2 from the current study should be further investigated for their best potentials.

### 3.2. Azoreductase profiling of isolates

Among different enzymes catalyzing dye decolorization step, a significant role is contributed by azoreductase in different microbial systems (Liu et al., 2009; Punj and John, 2009; Chen et al., 2010; Husain and Husain, 2012). Azoreductase activity was detected from the isolates, and *Enterococcus* L2 and ME1 had highest NADH- and NADPH-dependent azoreductase activities compared to the rest of the isolates (Table 2). *Enterococcus* L2 and ME1 showed NADH-azoreductase specific activity of  $18.73 \pm 1.91$  and  $8.89 \pm 1.23$ , whereas NADPH-azoreductase specific activities were  $29.87 \pm 2.14$  and

15.48  $\pm$  0.57, respectively. Liu et al., (2007) characterized the *azoA* gene from *Enterococcus faecalis* as broad substrate aerobic FMN dependent NADH- azoreductase homodimer of 23kDa subunits. Furthermore, Macwana et al., (2010) characterized *acpD* gene product AzoEf1 from *Enterococcus faecium* as utilized both NADH and NADPH for the reduction of azo dyes. Although, *Enterococcus* sp. L2 has both NADH- and NADPH-dependent azoreductase activities, strengthening the NADH-azoreductase catalysis in strain L2 will be advantageous and physiologically feasible modification as mentioned earlier to optimize azo dye decolorization.

### 3.3. Enhancement of RV5R decolorization by preferred redox mediators

Redox mediators involvement in bacterial -N=N- bond reductive cleavage under anaerobic condition have been reported (dos Santos et al., 2003; Van der Zee and Cervantes, 2009; Li et al., 2021), however, their preferences in microbial system and their participation in aerobic conditions for the dye decolorization remains vaguely defined. Flavin enzyme cofactors, such as flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN) and riboflavin, along with other quinone compounds, such as Anthraquinone-2, 6-disulfonate (AQDS), Anthraquinone-2-sulfonate (AQS), and lawsone, are known redox mediators. Most of azoreductases which plays direct role in azo dye decolorization also belong to flavin dependent quinone reductase family, thus physiologically have the ability to accept quinones as substrates (Liu et al., 2008; Rathod et al., 2022). Different concentrations 1.0, 1.5 and 2.0% of Menadione, AQS, AQDS and 1% Lawsone were checked to see the effect of the redox mediators on RV5R decolorization. In the presence of 2.0 mM Menadione the bacterial isolates *Klebsiella* spp. K1 and E2 and *Acinetobacter* sp. L1 had removed approximately double the amount of the dye i.e. 91.82%, 87.89% and

74.46% respectively than the control within 15 h (Figure 2a). In case of increase in the menadione concentration from >1.0 mM enhance decolorization of the RV5R, except *Enterococcus spp.* *Providencia spp.* showed range specific the positive decolorization effect for menadione which corroborated results by Rau et al., (2002) using menadione. We predict that menadione being electrophilic quinone in nature; imposed oxidative stress on *Citrobacter sp.* A3, *Enterococcus sp.* L2 and *Enterococcus sp.* ME1 which at high concentrations might have led to negative effect decolorization. Significant results were obtained in presence of AQS and AQDS showing ~10 to 20% increase in decolorization at optimum concentration. It was also found that isolates also decrease decolorization beyond optimum concentration of quinones (Figure2b, c). In case of *Klebsiella* strains effect of most of the redox mediators were found to be highly significant, although only *Klebsiella sp.* K1 shown 1.6 fold increases in RV5R decolorization in 1% lawsone (Figure 2d) which is corroborated with the results by Olivo-Alanis et al. (2018). The enhancement mechanism of redox mediators have been elucidated by Zee and Villaverde, (2005), as redox mediators (RMs) accelerate the reaction rate by coupling the microbial oxidation of primary electron donors via shuttling electrons to the acceptor azo dyes (Van der Zee et al., 2003). Yeast extract has demonstrated to improve azo dye decolorization as it can serve as a source of reducing equivalents and electron shuttle which can reduce azo dye (Imran et al., 2016). Hydroxyquinone was also checked for its effect on decolorization, which was found negative (data not shown). Ultimately, a strain-specific effect of RMs was observed on azo dye decolorization as these isolates were equipped by unique set of quinone reductase system which also includes many azoreductases.

301

### 302 **3.4.FTIR analysis of the dye decolorization/degradation end products**

Functional groups absorption peaks shifting or dis-appearance in treated samples to control dye sample demonstrates various steps or chemical modifications of the decolorization/degradation process (Jain et al., 2012; Patel et al., 2020). FTIR spectrum form decolorized end product was extracted and compared with the control (RV5R) (Figure S1a-i). Reactive Violet 5R FTIR spectra of as control showed signature peaks for multi-substituted benzene ring along with the peaks at 1,139, 1,185 and 1,547  $\text{cm}^{-1}$  which corresponds to two  $-\text{SO}_3\text{H}$  group, a symmetric  $\text{SO}_2$  and azo bone, respectively (Desai et al. 2009). Azo bond peak at 1547  $\text{cm}^{-1}$  was prime signature of a mono-azo reactive azo dye RV5R and loss of this peak in the decolorized extracts of various culture supernatants determined the cleavage of the azo bond (Table 3). FTIR analysis of extracted metabolites of degraded RV5R showed peaks 1630- 1680 $\text{cm}^{-1}$  of primary amines. The peak corresponding to  $-\text{CN}$  asymmetric stretching at 1048.48  $\text{cm}^{-1}$  and  $-\text{SO}_3\text{H}$  group 1139.89 and 1185.13  $\text{cm}^{-1}$  peak was also disappeared, in all the strains except in *Acinetobacter* sp. L1. Further suggesting that these isolates were capable of removing the sulfonate group from the dye structure and reducing its charge properties, which might enable them to pass through the membrane barrier. The asymmetrical stretching of C–H of alkane ( $-\text{CH}_3$ ) peak between 2,920-2930  $\text{cm}^{-1}$  were observed in degraded metabolites which is corroborated with the findings of asymmetrical C–H stretching in degradation of disperse dye Brown 3REL by *Bacillus* sp. VUS (Dawkar et al. 2008). Thus, these consortial isolates are expected to play a vital and active role in azo dye decolorization and effective bioremediation even as pure culture.

324

325 **3.5. Effect of physicochemical parameters such as pH, Temperature, and salinity on**  
326 **dye decolorization by *Enterococcus* sp. L2**

327 The *Enterococcus* sp. L2 was found to decolorize RV5 dye (100mg/L) at an optimum  
 328 medium pH of 7-8 and temperature 40°C under static conditions (Figure 3a, b). The  
 329 isolate showed complete decolorization between 35 to 40°C, however sharp decrease in  
 330 the decolorization was observed above and below this optimum range (Figure 3b).  
 331 Similarly, Sahasrabudhe et al. (2011) reported *Enterococcus* strain to decolorize Reactive  
 332 yellow at an optimum pH 5 and temperature for the decolorization at 37°C. Maximum  
 333 RV5R decolorization was found to be in the range of 0.5-2% NaCl (Figure 3c), which is  
 334 the survival and growth range of salinity for *Enterococcus* spp. (Fisher & Phillips, 2009).  
 335 Recently, similar halo-tolerant and thermophilic bacterial system have been reported for  
 336 the dye decolorization application (Guo et al., 2021). Interestingly, during *Enterococcus*  
 337 sp. L2 growth and azo dye decolorization, a significant pH drop was also observed (Figure  
 338 3d). Flahaut et. al (1996) reported “flash adaptation” in *E. faecalis*, which makes this  
 339 bacteria ideal for survival and growth under stress conditions under the bioremediation  
 340 category. Therefore, *Enterococcus* sp. L2 was selected for additional evaluations.

### 341       **3.6.       Augmentation of NADH-regeneration systems by heterologous** 342       **overexpression of NAD<sup>+</sup>-dependent formate dehydrogenase to further enhance** 343       **decolorization potential of *Enterococcus* sp. L2**

344 The selected isolate, *Enterococcus* sp. L2, was shown to possess NAD(P)H-  
 345 azoreductase activity, and these reducing equivalences are essential co-factors for  
 346 azoreductase. While NADH regeneration is physiologically more feasible compared to  
 347 NADPH<sub>2</sub> (Oeggli et al., 2018), therefore, we decide to further enhance the NADH to  
 348 support azoreductase catalysis. To replenish the NADH pool, NAD<sup>+</sup>-dependent formate  
 349 dehydrogenase was employed which oxidize formate to H<sub>2</sub>O and CO<sub>2</sub> while reducing  
 350 NAD<sup>+</sup> to NADH. Using pMGS100, a Gram-positive expression vector the

351 *Mycobacterium vaccae* encoded NAD<sup>+</sup>-dependent formate dehydrogenase was  
 352 heterologously overexpressed by a constitutive *bacA* promoter (Figure 4a). A pMGS100  
 353 *fdh* construct was confirmed by BamH1 digestion and a PCR amplification (Figure 4b,  
 354 c). *Enterococcus* sp. L2 harboring pMGS100*fdh* showed the expected overexpressed  
 355 protein band of 44kDa (Figure 4d). *Enterococcus* sp. L2 *fdh* transformant showed  
 356 specific activity of 12.56 U/mg with a fold increase of 6.05 compared to its vector  
 357 control (Figure 4e). The absorbance ratio of A<sub>340/280nm</sub> was used as a measure of  
 358 intracellular NADH concentration relative to the total protein concentration. In medium  
 359 amended with 300mM Na-formate the average absorbance for A<sub>340/280nm</sub> ratio for vector  
 360 control and *fdh* transformant were 0.395 ± 0.009 and 0.455 ± 0.012, respectively. This  
 361 determined a 1.15 fold NADH increase in *fdh* transformant. Additionally, *Enterococcus*  
 362 spp. are known to accumulate formate (Leblanc, 2006), and they do not possess  
 363 formate-hydrogen lyase enzymes or native NAD<sup>+</sup>-dependent formate dehydrogenase  
 364 activity, therefore, a significant incorporation of final formate oxidation linked to co-  
 365 factor reductive regeneration. Ultimately, *Enterococcus* sp. L2 *fdh* transformant showed  
 366 73.45% decolorization compared to only 22.97% RV5R decolorization by control in 6h,  
 367 demonstrating a 3.2 fold increase (Figure 4f). This augmentation also led to a significant  
 368 physiological advantage with positive effect on growth when cell grown with or without  
 369 supplement of 300mM formate amendment as shown in figure 4g, h. This could be  
 370 attributed to modified enterococcal system which is now able to utilize formate for the  
 371 regeneration of NADH when formate was added externally. *Enterococcus* spp. possess  
 372 pyruvate formate lyase which also naturally produces formate as they could not further  
 373 utilize it (Leblanc, 2006; Ramsey et al., 2014). Natural accumulation of formate as  
 374 terminal product of C-metabolism supports the implemented formate dehydrogenase



375 driven NADH-regeneration in *fdh* transformant even when no external formate is added.

376 It is noteworthy that *fdh*-based NADH-regeneration system augmentation in

377 *Enterococcus* sp. L2 could boost its azo dye decolorization and growth.

378 3.7. Potential of NADH-regeneration system in xenobiotic remediation

#### 379 4. Conclusion

380 Among azo dye decolorizing bacterial isolates from acclimatized consortia, *Enterococcus*

381 sp. L2 was recognized as the most efficient azo dye decolorizer by reducing >90mg/L

382 Reactive violet 5R (RV5R) dye in 3h. A strain-specific preference for redox mediators was

383 demonstrated. A low-cost redox mediator, crude lawsone powder (1%) extract of *Lawsonia*

384 *inermis* showed positive effect on *Klebsiella* sp. K1's dye decolorization only. At optimum

385 concentration, AQDS was found to be most preferred redox mediator enhancing dye

386 decolorization in all isolates. It is noteworthy that strain L2 is a NAD(P)H-dependent

387 azoreductase efficient system. Further, strain L2 showed an optimum decolorization at pH

388 8, 40 °C and up to 2% w/v salinity that were supporting physiochemical features for

389 utilizing strain L2 for biological treatment. NADH-regeneration augmentation in

390 *Enterococcus* sp. L2 by overexpressing NAD<sup>+</sup>-dependent formate dehydrogenase could

391 enhance NADH pool leading to a significant 3.2 fold increased dye decolorization with a

392 positive effect on growth. Ultimately, this study highlighted salient azo dye decolorization

393 traits of strain L2 and its possibility of further optimization by an augmentation of NADH-

394 regeneration system in the non-model azoreductase-efficient environmentally important

395 strain.

396

#### 397 Acknowledgment

398 Authors acknowledge funding support by the Department of Biotechnology (DBT),  
399 Ministry of Science and Technology, New Delhi, India (Project No. BT/PR-6555-  
400 BCE/08/424/2005) to GA. JR is thankful to DBT-India for project Junior and Senior  
401 Research Fellowships and University Grants Commission, New Delhi, India for  
402 Research Fellowship in Sciences for Meritorious Students (UGC-RFSMS).

403

#### 404 **References:**

- 405 Bafana, A., Chakrabarti, T., 2008. Lateral gene transfer in phylogeny of azoreductase  
406 enzyme. *Comput. Biol. Chem.* 32, 191-197.
- 407 Chen, H., 2006. Recent advances in azo dye degrading enzyme research. *current pt*,  
408 101-111.
- 409 Chengalroyen, M.D., Dabbs, E.R., 2013. The microbial degradation of azo dyes:  
410 minireview. *World J. Microbiol. Biotechnol.* 29, 389-399.
- 411 Cui, D., Zhang, M., Wang, J., Wang, H., Zhao, M., 2020. Effect of quinoid redox  
412 mediators during azo dye decolorization by anaerobic sludge: Considering the  
413 catalyzing mechanism and the methane production. *Ecotoxicol. Environ. Saf.*  
414 202, 110859.
- 415 Deller, S., Macheroux, P., Sollner, S., 2008. Flavin-dependent quinone reductases. *Cell.*  
416 *Mol. Life Sci.* 65, 141.
- 417 dos Santos, a.B., Cervantes, F.J., Yaya-Beas, R.E., van Lier, J.B., 2003. Effect of redox  
418 mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine  
419 group in a thermophilic anaerobic EGSB reactor. *Enzyme Microb. Technol.* 33,  
420 942-951.

421 Guo, G., Li, X., Tian, F., Liu, T., Yang, F., Ding, K., Liu, C., Chen, J., Wang, C., 2020.  
422 Azo dye decolorization by a halotolerant consortium under microaerophilic  
423 conditions. *Chemosphere* 244, 125510.

424 Guo, G., Liu, C., Hao, J., Tian, F., Ding, K., Zhang, C., Yang, F., Liu, T., Xu, J., Guan,  
425 Z., 2021. Development and characterization of a halo-thermophilic bacterial  
426 consortium for decolorization of azo dye. *Chemosphere* 272, 129916.

427 Hong, Y., Guo, J., Sun, G., 2008. Characteristics and phylogenetic analysis of the  
428 facultative anaerobic dissimilatory azoreducing bacteria from activated sludge.  
429 *Int. Biodeterior. Biodegradation* 61, 313-318.

430 Jain, K., Shah, V., Chapla, D., Madamwar, D., 2012. Decolorization and degradation of  
431 azo dye - Reactive Violet 5R by an acclimatized indigenous bacterial mixed  
432 cultures-SB4 isolated from anthropogenic dye contaminated soil. *J. Hazard.*  
433 *Mater.* 213-214, 378-386.

434 Leblanc, D.J., 2006. *Enterococcus*. in: Dworkin, M., Falkow, S., Rosenberg, E.,  
435 Schleifer, K.-H., Stackebrandt, E. (Eds.). *The Prokaryotes: Volume 4: Bacteria:*  
436 *Firmicutes, Cyanobacteria*. Springer US, New York, NY, pp. 175-204.

437 Leelakriangsak, M., 2013. Molecular approaches for bacterial azoreductases.  
438 *Songklanakarin J. Sci. Technol.* 35.

439 Li, T., Song, H.-L., Xu, H., Yang, X.-L., Chen, Q.-L., 2021. Biological detoxification  
440 and decolorization enhancement of azo dye by introducing natural electron  
441 mediators in MFCs. *J. Hazard. Mater.*, 125864.

442 Misal, S.A., Gawai, K.R., 2018. Azoreductase: a key player of xenobiotic metabolism.  
443 *Bioresour. Bioprocess.* 5, 17.

444 Oegg, R., Neumann, T., Gätgens, J., Romano, D., Noack, S., Rother, D., 2018. Citrate  
445 as cost-efficient NADPH regenerating agent. *Front. Bioeng. Biotechnol.* 6.

446 Olivo-Alanis, D., Garcia-Reyes, R.B., Alvarez, L.H., Garcia-Gonzalez, A., 2018.  
447 Mechanism of anaerobic bio-reduction of azo dye assisted with lawsone-  
448 immobilized activated carbon. *J. Hazard. Mater.* 347, 423-430.

449 Pandey, A.K., Gaur, V.K., Udayan, A., Varjani, S., Kim, S.-H., Wong, J.W., 2021.  
450 Biocatalytic remediation of industrial pollutants for environmental  
451 sustainability: Research needs and opportunities. *Chemosphere* 272, 129936.

452 Patel, D.K., Tipre, D.R., Dave, S.R., 2016. Selection and development of efficient  
453 consortia for decolorization of metal complex dyes. *Toxicol. Environ. Chem.*  
454 2248, 1-13.

455 Patel, V.R., Khan, R., Bhatt, N., 2020. Cost-effective in-situ remediation technologies  
456 for complete mineralization of dyes contaminated soils. *Chemosphere* 243,  
457 125253.

458 Ramsey, M., Hartke, A., Huycke, M., 2014. The physiology and metabolism of  
459 enterococci. *Enterococci: From Commensals to Leading Causes of Drug*  
460 *Resistant Infection* [Internet].

461 Rathod, J., Archana, G., 2013. Molecular fingerprinting of bacterial communities in  
462 enriched azo dye (Reactive Violet 5R) decolorising native acclimatised bacterial  
463 consortia. *Bioresour. Technol.* 142, 436-444.

464 Rathod, J., Dhebar, S., Archana, G., 2017. Efficient approach to enhance whole cell azo  
465 dye decolorization by heterologous overexpression of *Enterococcus* sp. L2  
466 azoreductase (azoA) and *Mycobacterium vaccae* formate dehydrogenase (fdh) in  
467 different bacterial systems. *Int. Biodeterior. Biodegradation* 124, 91-100.

468 Rathod, J., Pandey, S., Mahadik, K., Archana, G., 2022. Homologous overexpression of  
469 azoreductase (azoA) in *Enterococcus* sp. L2 moderated growth and azo dye  
470 decolorization while gaining an oxidative and heavy metal stress resistance: A  
471 trade-off. Environ. Technol. Innov. 102531.

472 Rawat, D., Mishra, V., Sharma, R.S., 2016. Detoxification of azo dyes in the context of  
473 environmental processes. Chemosphere 155, 591-605.

474 Rawat, D., Sharma, R.S., Karmakar, S., Arora, L.S., Mishra, V., 2018. Ecotoxic  
475 potential of a presumably non-toxic azo dye. Ecotoxicol. Environ. Saf. 148, 528-  
476 537.

477 Routoula, E., Patwardhan, S.V., 2020. Degradation of anthraquinone dyes from  
478 effluents: A review focusing on enzymatic dye degradation with industrial  
479 potential. Environ. Sci. Technol. 54, 647-664.

480 Samuchiwal, S., Gola, D., Malik, A., 2021. Decolourization of textile effluent using  
481 native microbial consortium enriched from textile industry effluent. J. Hazard.  
482 Mater. 402, 123835.

483 Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P., 2011. Bacterial  
484 decolorization and degradation of azo dyes: A review. J. Taiwan Inst. Chem.  
485 Eng. 42, 138-157.

486 Sarvajith, M., Reddy, G.K.K., Nanchaiah, Y.V., 2018. Textile dye biodecolourization  
487 and ammonium removal over nitrite in aerobic granular sludge sequencing batch  
488 reactors. J. Hazard. Mater. 342, 536-543.

489 Sreedharan, V., Bhaskara Rao, K.V., 2019. Biodegradation of Textile Azo Dyes. in:  
490 Gothandam, K.M., Ranjan, S., Dasgupta, N., Lichtfouse, E. (Eds.). Nanoscience

491 and biotechnology for environmental applications. Springer International  
 492 Publishing, Cham, pp. 115-139.

493 Stolz, a., 2001. Basic and applied aspects in the microbial degradation of azo dyes.  
 494 Appl. Microbiol. Biotechnol. 56, 69-80.

495 Suzuki, H., 2019. Remarkable diversification of bacterial azoreductases: primary  
 496 sequences, structures, substrates, physiological roles, and biotechnological  
 497 applications. Appl. Microbiol. Biotechnol. 103, 3965-3978.

498 Van der Zee, F.P., Cervantes, F.J., 2009. Impact and application of electron shuttles on  
 499 the redox (bio)transformation of contaminants: a review. Biotechnol. Adv. 27,  
 500 256-277.

501 Wu, H., Tian, C., Song, X., Liu, C., Yang, D., Jiang, Z., 2013. Methods for the  
 502 regeneration of nicotinamide coenzymes. Green Chem. 15, 1773-1789.

503

## TABLES

**Table 1. 16s rRNA gene sequence based identification of isolates**

Source	Isolates	GenBank	Best match	Similarity	Taxonomic	Best match
consortium		accession	with accession	with best	group	organism
		number	number	match		source/ details <sup>b</sup>
				(number of		
				bases) <sup>a</sup>		
<b>Gly</b>	<b>A3</b>	JQ745287	<i>Citrobacter</i> sp. S7	99% (512)	$\gamma$ - <i>Proteobacteria</i>	Fecal contaminated soil
			(HF572839)			
<b>MITZ</b>	<b>C1</b>	JQ745288	<i>Providencia</i>	99% (777)	$\gamma$ - <i>Proteobacteria</i>	<i>Centella asiatica</i> -Associated Bacteria
			<i>vermicola</i> strain			
			AR_PSBH1			
			(HM582881)			
<b>PBC</b>	<b>E2</b>	JQ7452289	<i>Klebsiella</i>	99% (722)	$\gamma$ - <i>Proteobacteria</i>	Copper resistant isolate
			<i>pneumoniae</i>			

519				strain SW			
520				(AB641122)			
521	<b>MW</b>	<b>G1</b>	JQ7452290	<i>Providencia</i> sp.	100% (647)	$\gamma$ - <i>Proteobacteria</i>	Isolated from <i>Lucilia sericata</i> larva
522				Sal2			
523				(JN790944)			
524	<b>PBR</b>	<b>K1</b>	JQ7452291	<i>Klebsiella</i>	99% (694)	$\gamma$ - <i>Proteobacteria</i>	Nickel resistant isolate
525				<i>pneumoniae</i>			
526				strain ZB			
527				(KC243315)			
528	<b>Dalc</b>	<b>L1</b>	JQ7452292	<i>Acinetobacter</i>	100% (772)	$\gamma$ - <i>Proteobacteria</i>	No relevant details found
529				<i>baumannii</i>			
530				strain DSM 30007T			
531				(HE978267)			
532	<b>Dalc</b>	<b>L2</b>	JQ7452293	<i>Enterococcus</i>	99% (698)	<i>Firmicutes</i>	Probiotic strain
533							



534				<i>faecalis</i> strain				
535				symbioflor 1				
536				(HF558530)				
537	<b>ME</b>	<b>ME1</b>	JQ7452294	<i>Enterococcus</i>	99% (763)	<i>Firmicutes</i>	<i>Plutella xylostella</i> gut isolate	
538				<i>casseliflavus</i>				
539				strain PX-EC				
540				(KC150018)				

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541 <sup>a</sup> Numbers in parentheses correspond to the number of bases used for sequence identity.

542 <sup>b</sup> The ecological/ environmental niche from where the organism that best matches was obtained.

**Table 2. NADH and NADPH dependent azoreductase specific activity of bacterial isolates (Reactive violet 5R as substrate)**

Isolate	Azo reductase specific activity (μmole of dye reduced/min/mg of total protein)	
	NADH <sup>a</sup>	NADPH <sup>b</sup>
<i>Citrobacter</i> sp. A3	5.79 ± 0.76(33.4%)	11.55 ± 0.9(66.6%)
<i>Providencia</i> sp. C1	5.77 ± 0.96(27.3%)	15.37 ± 1.00(72.7%)
<i>Klebsiella</i> sp. E2	6.84 ± 1.04(31.5%)	14.88 ± 1.16(68.5%)
<i>Providencia</i> sp. G1	5.62 ± 0.49(27.3%)	14.93 ± 0.8(72.7%)
<i>Klebsiella</i> sp. K1	5.07 ± 0.77(24.3%)	15.79 ± 0.37(75.7%)
<i>Acinetobacter</i> sp. L1	5.34 ± 0.68(30.7%)	12.08 ± 1.35(69.3%)
<i>Enterococcus</i> sp. L2	18.73 ± 1.91(38.5%)	29.87 ± 2.14(61.5%)
<i>Enterococcus</i> sp. ME1	8.89 ± 1.23(36.5%)	15.48 ± 0.57(63.5%)

<sup>a, b</sup> Percentage in the parentheses correspond to the percent distribution based on the co-factor (NADH or NAD(P)H) driven azoreductase activity

**Table 3. Fourier transformed infrared spectroscopy (FTIR) analysis of extract end products from the decolorized supernatant from isolates.**

Sample	-N=N- at 1547 and 1434cm <sup>-1</sup>	Out of plane Aromatic C- H bends 675- 900cm <sup>-1</sup>	Naphthalen e ring at 1470cm <sup>-1</sup>	-CN asymmetri c stretching at 1048.48c m <sup>-1</sup>	1139.89, 1339.24, 1185.13c m <sup>-1</sup> -SO <sub>3</sub> H group	2920- 2930cm <sup>-1</sup> asymmetrica l stretching of C-H in CH <sub>3</sub>	1455- 1465cm <sup>-1</sup> C-C stretchin g in ring
Control	+	+	+	+	+	+	shifted
A3	-	-	-	-	-	+	-
C1	-	+	-	-	-	+++	+
E2	-	+	-	-	-	++	+
G1	-	+	-	-	-	++	+
K1	-	+	-	-	-	++	+
L1	-	+	-	+	-	++	+
L2	-	+	-	-	-	+	+
ME1	-	+	-	-	-	++	+

## List of Figures:

**Figure 1.** Azo dye decolorization and 16S rRNA gene based phylogenetic analysis of isolates. a) RV5R decolorization; b) Methyl red (MR) decolorization; c) Structure of Reactive violet 5R (RV5R); d) Structure of Methyl red (MR); e) Unrooted phylogenetic tree depicting taxonomic affiliations of the azo dye decolorizing bacterial isolates. Phylogenetic analyses were conducted in MEGA4. RV5R decolorizing isolates of the present study are indicated by dark circles. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (scale bar corresponds to 0.2 nucleotide substitution per site). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

**Figure 2.** Effect of redox mediators on Reactive violet 5R decolorization. a) Menadione (1, 1.5, 2 mM) b) Anthraquinone-2-sulfonate (AQS) (1, 1.5, 2 mM) c) Anthraquinone-2,6-disulfonate (AQDS) (1, 1.5, 2 mM) d) 1% crude Lawsone. Asterisk sign denotes the statistical significance at  $p < 0.05$  for the increase in dye decolorization amended with specific redox mediator at respective concentration compared to control of without amendment of electron mediator.

**Figure 3.** Effect of physicochemical factors on *Enterococcus* sp. L2 Reactive violet 5R decolorization. a) pH; b) Temperature; c) Salinity-NaCl (% w/v) and d) pH-reduction while *Enterococcus* sp. L2 decolorization.

**Figure 4.** Augmentation of NADH-regeneration systems by heterologous overexpression of NAD<sup>+</sup>-dependent formate dehydrogenase to further enhance decolorization potential of *Enterococcus* sp.

L2. a) pMGS100 *fdh* construct map; b) BamH1-digestion confirmation of the *fdh* overexpressing construct and c) PCR confirmation of the *fdh* overexpressing construct; d) Overexpression of 44 kDa protein of *Mycobacterium vaccae* NAD<sup>+</sup>-dependent formate dehydrogenase in *Enterococcus* sp. L2 (VC-vector control, *fdh*- *fdh*-transformant); e) NAD<sup>+</sup>-dependent formate dehydrogenase activity of *Enterococcus* sp. L2 *fdh* transformant and its vector control; f) Reactive violet 5R decolorization comparison between *Enterococcus* sp. L2 *fdh* transformant and its vector control at 6 h incubation; g-h) Growth comparison between *Enterococcus* sp. L2 *fdh* transformant and its vector control in medium with and without 300mM formate amendment. (Asterisk denotes statistical significance at  $p < 0.01$  of increase in Fdh activity and dye decolorization for *fdh* transformant of strain L2 compared to its vector control.)

Figure 1.

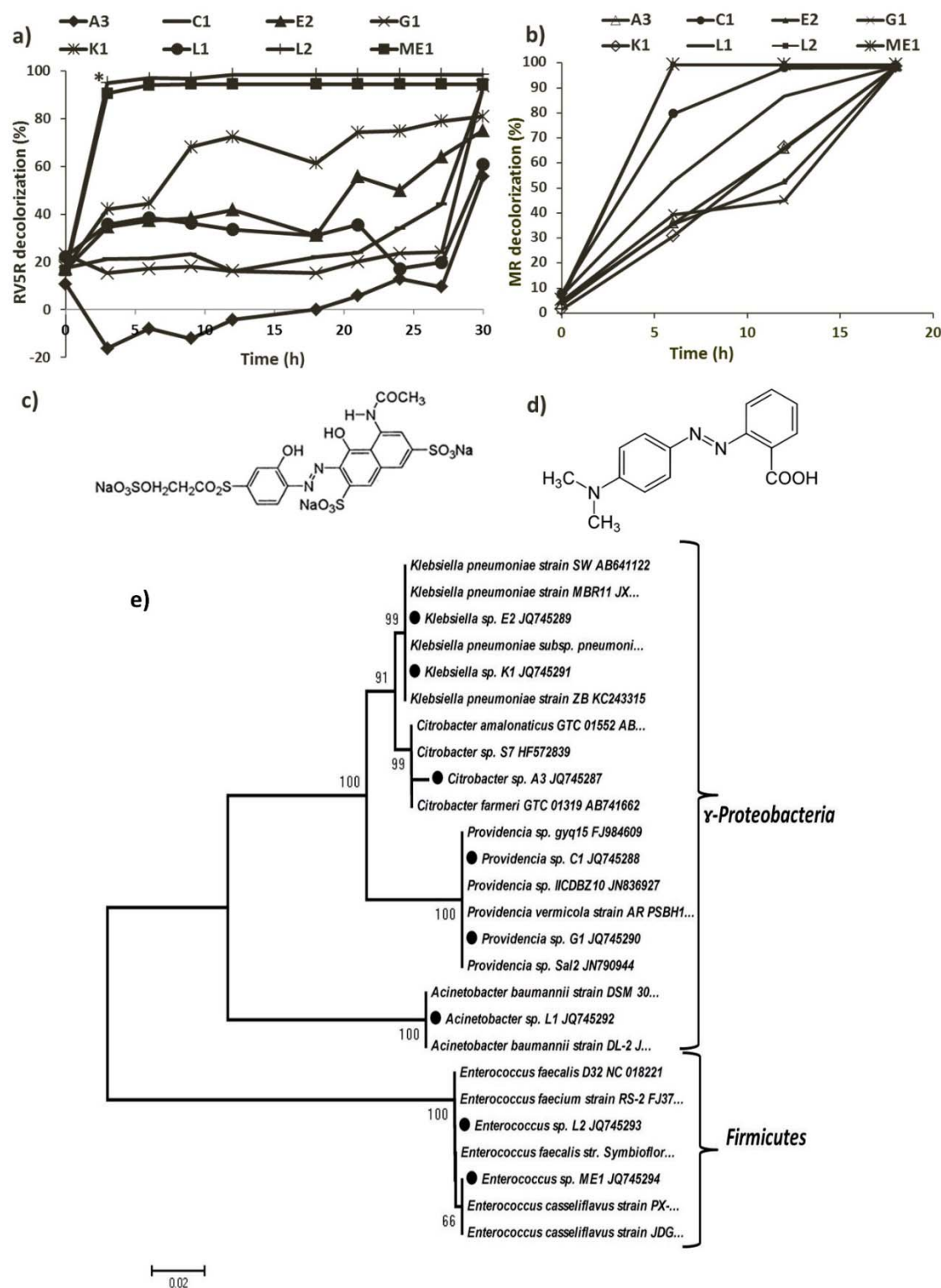


Figure 2.

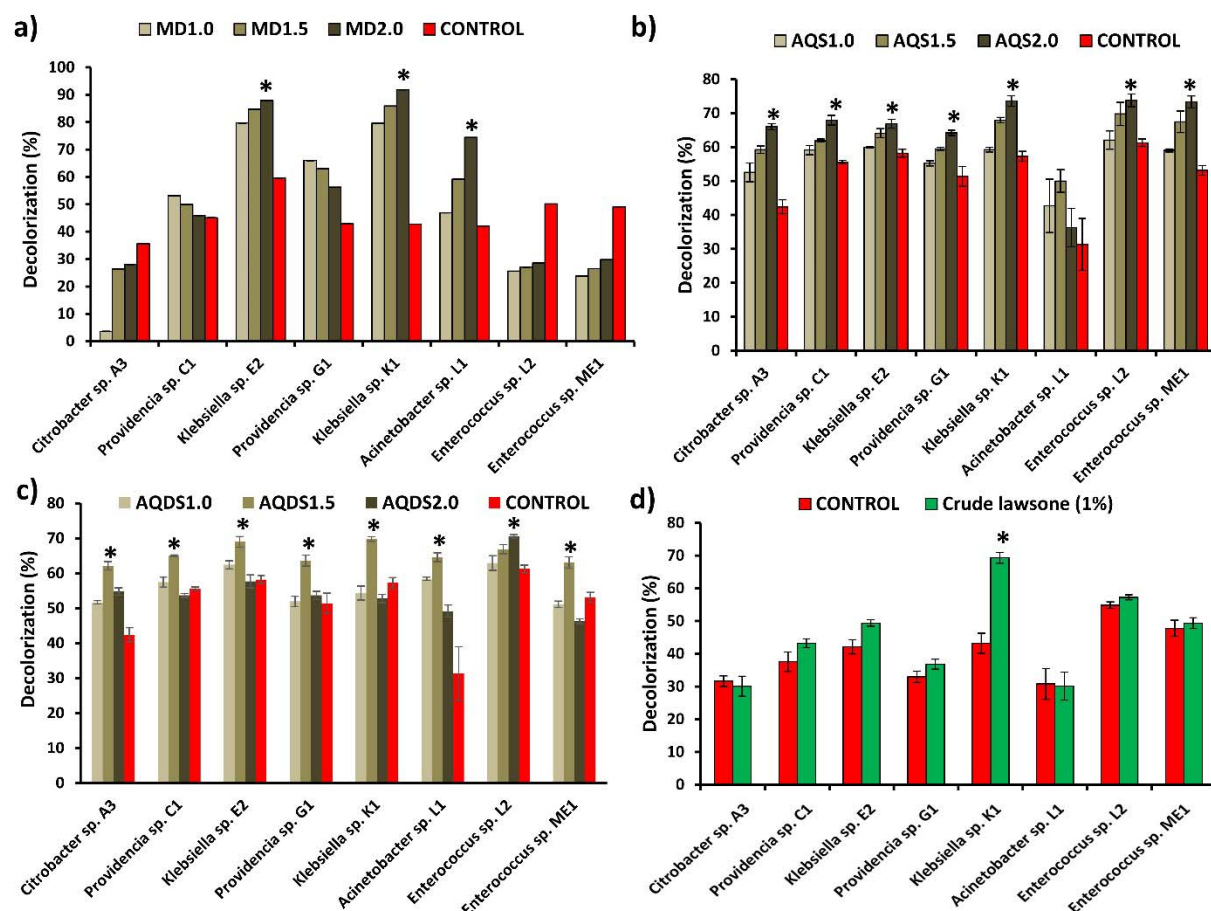


Figure 3.

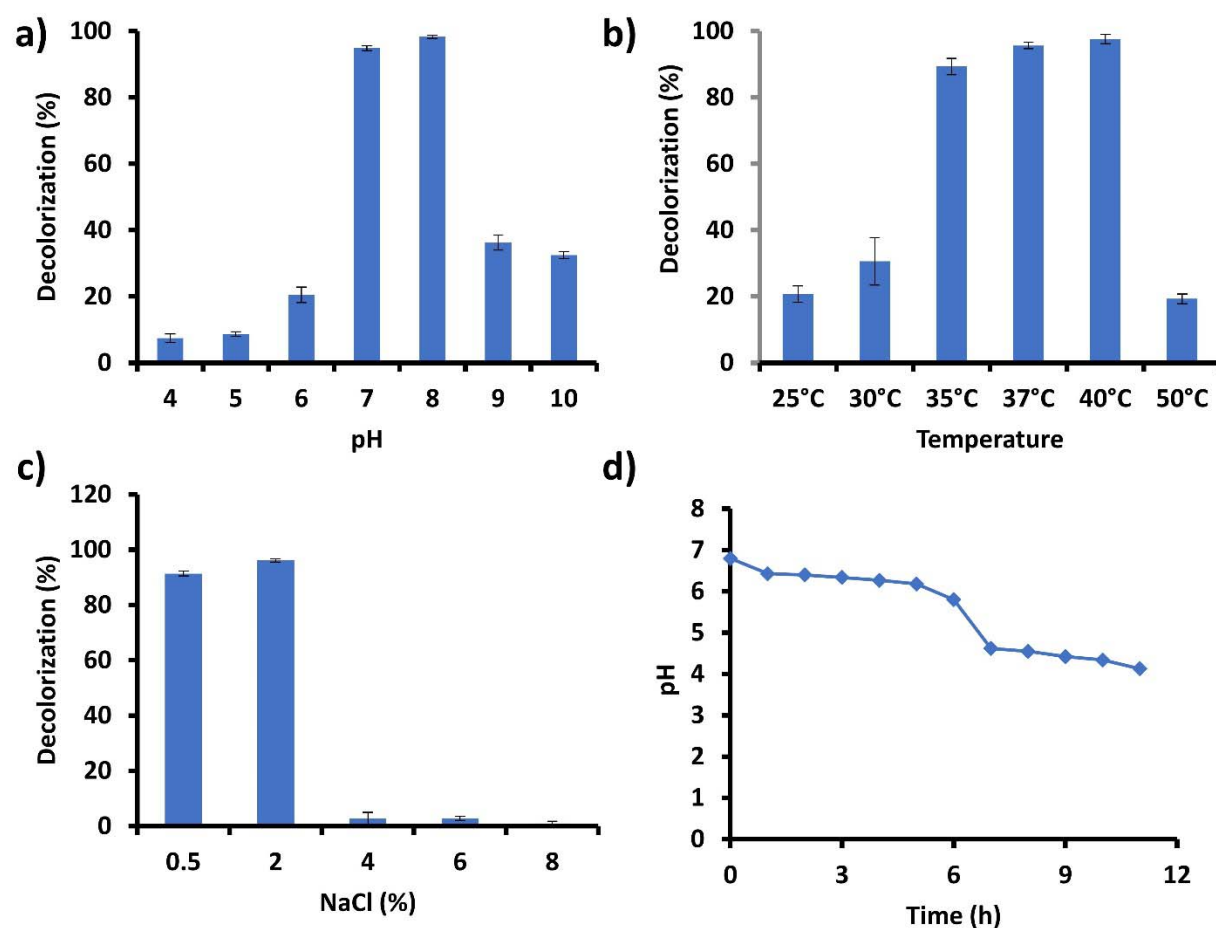




Figure 4.

