

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* (γ -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⁺-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⁺-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 FADH₂ (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⁺-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) [MgSO₄, 0.2 g/L; K₂HPO₄, 1.0 g/L; CaCl₂, 0.02 g/L; FeCl₃, 0.05 g/L; NH₄NO₃, 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 K₂HPO₄, 0.15% w/v MgSO₄, 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⁺-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 μl 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 λ_{\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 μ M azo dye, 0.1mM NADH, 10 μ 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 μ 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 cm^{-1}) by FTIR using Spectrum GX (PerkinElmer,
156 USA).

157 *2.6.Heterologous expression of NAD⁺-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⁺-dependent formate dehydrogenase assay**

179 To assay NAD⁺-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⁻¹ cm⁻¹ enzyme units were calculated.

183 One unit (U) of Fdh is defined as the enzyme needed to oxidize 1 μ 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 μ 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 α -*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 H₂, 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 ± 1.91 and 8.89 ± 1.23 , whereas NADPH-azoreductase specific activities were 29.87 ± 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 cm^{-1} which corresponds to two $-\text{SO}_3\text{H}$ group, a symmetric SO_2 and azo bone, respectively (Desai et al. 2009). Azo bond peak at 1547 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 cm^{-1} of primary amines. The peak corresponding to $-\text{CN}$ asymmetric stretching at 1048.48 cm^{-1} and $-\text{SO}_3\text{H}$ group 1139.89 and 1185.13 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 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⁺-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 NADPH₂ (Oeggli et al., 2018), therefore, we decide to further enhance the NADH to
 348 support azoreductase catalysis. To replenish the NADH pool, NAD⁺-dependent formate
 349 dehydrogenase was employed which oxidize formate to H₂O and CO₂ while reducing
 350 NAD⁺ to NADH. Using pMGS100, a Gram-positive expression vector the

351 *Mycobacterium vaccae* encoded NAD⁺-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_{340/280nm} 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_{340/280nm} 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⁺-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⁺-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

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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 ^b
				(number of		
				bases) ^a		
Gly	A3	JQ745287	<i>Citrobacter</i> sp. S7	99% (512)	γ - <i>Proteobacteria</i>	Fecal contaminated soil
			(HF572839)			
MITZ	C1	JQ745288	<i>Providencia</i>	99% (777)	γ - <i>Proteobacteria</i>	<i>Centella asiatica</i> -Associated Bacteria
			<i>vermicola</i> strain			
			AR_PSBH1			
			(HM582881)			
PBC	E2	JQ7452289	<i>Klebsiella</i>	99% (722)	γ - <i>Proteobacteria</i>	Copper resistant isolate
			<i>pneumoniae</i>			

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

534				<i>faecalis</i> strain				
535				symbioflor 1				
536				(HF558530)				
537	ME	ME1	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)				

541 ^a Numbers in parentheses correspond to the number of bases used for sequence identity.

542 ^b 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 ^a	NADPH ^b
<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%)

^{a, b} 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 ⁻¹	Out of plane Aromatic C-H bends 675- 900cm ⁻¹	Naphthalen e ring at 1470cm ⁻¹	-CN asymmetri c stretching at 1048.48c m ⁻¹	1139.89, 1339.24, 1185.13c m ⁻¹ -SO ₃ H group	2920- 2930cm ⁻¹ asymmetrica l stretching of C-H in CH ₃	1455- 1465cm ⁻¹ 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⁺-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⁺-dependent formate dehydrogenase in *Enterococcus* sp. L2 (VC-vector control, *fdh*- *fdh*-transformant); e) NAD⁺-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.

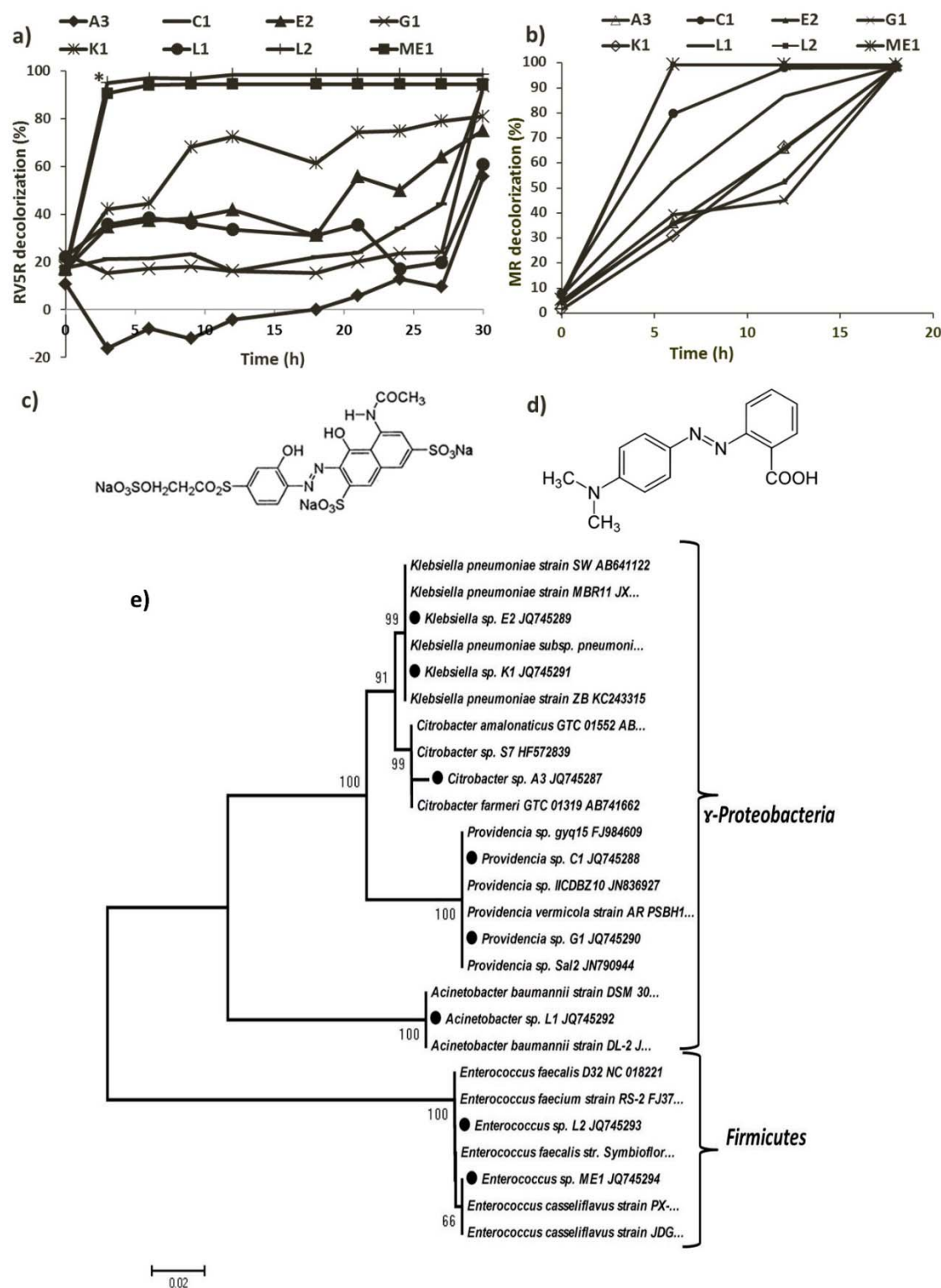


Figure 2.

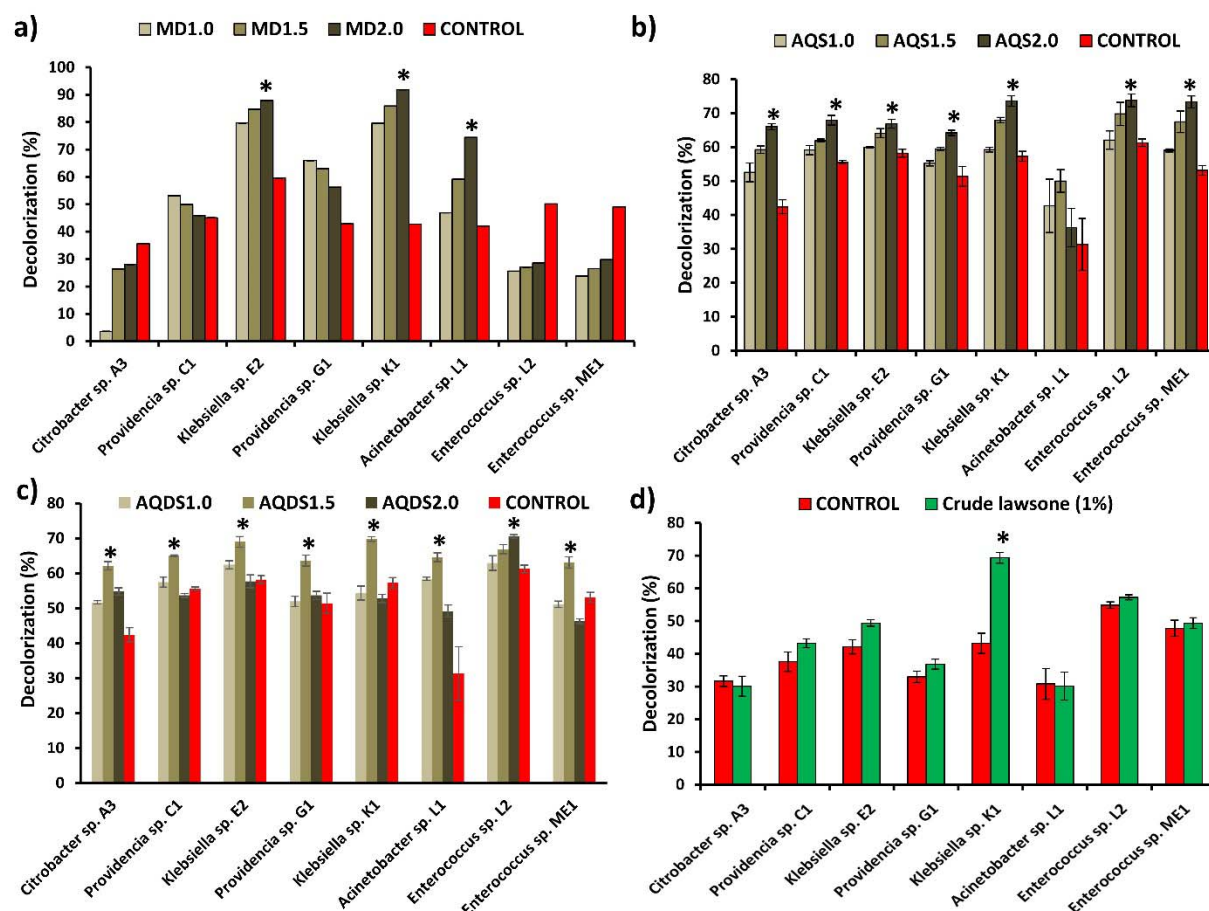


Figure 3.

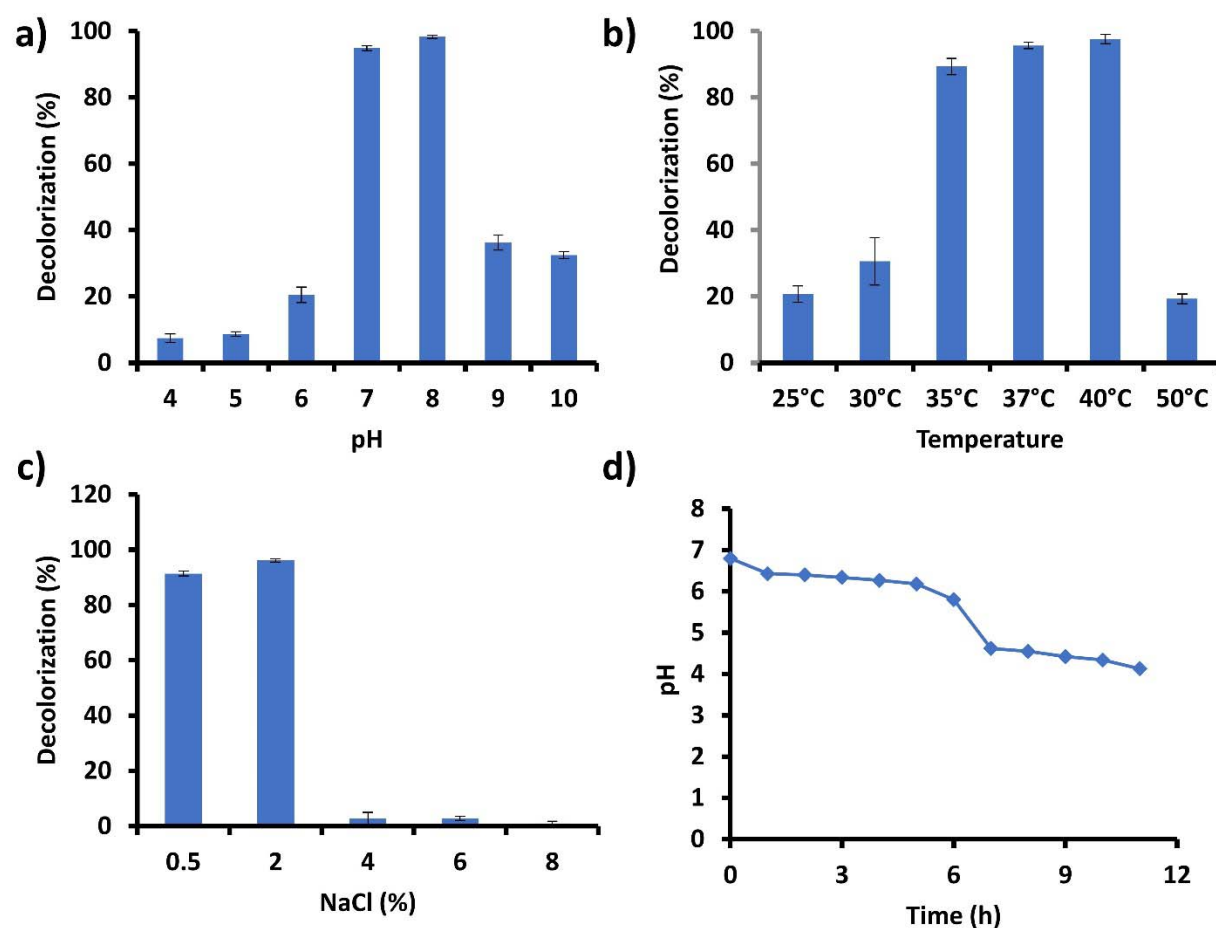


Figure 4.

