

1 **Indigenous *Bacillus paramycoides* and *Alcaligenes faecalis*: potential solution**
2 **for the bioremediation of wastewaters**

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Abstract

24 Farmers near towns and cities are using wide range of untreated wastewaters for crop irrigation in
25 Pakistan due to severe freshwater shortage. The present study aimed to treat different types of wastewater
26 including domestic, hospital, textile, pharmaceutical and mixed wastewaters using indigenous bacterial
27 isolates to remove contaminants and render these wastewaters safer for irrigation. 37 bacterial strains were
28 isolated from the 5 wastewater samples collected from different sites in Lahore, Pakistan. Under optimum
29 growth conditions, the isolates D6, D7 and P1 showed maximum decolourisation potential of 96, 96, 93 %,
30 respectively against hospital wastewater. GCMS analysis of the untreated hospital wastewater confirmed
31 the presence of pharmaceutic pollutants i.e. Phenol, Salicylic acid, Caffeine, Naproxen, Octadecene and
32 Diazepam. These organic compounds were biodegraded into derivate Ticlopidine in the case of isolate D6,
33 derivatives Tetradecene and Griseofulvin in the case of isolate D7, and derivatives Lidocaine and Butalbital
34 in the case of isolate P1. 16S rDNA sequencing was used to identify these isolates. Isolates D6 and D7
35 showed 100 and 99.86 % homology to *Bacillus paramycoides*, a novel strain from *Bacillus cereus* group
36 (Liu et al., 2017). Isolate P1 showed 97.47 % homology to *Alcaligenes faecalis*. These strains therefore
37 could represent a low-cost and low-tech alternative to bioremediate complex wastewaters prior to irrigation
38 to support the achievement of the Sustainable Development Goal 6 - clean water and sanitation in Pakistan.

39 **Keywords:** *Bacillus paramycoides*, *Alcaligenes faecalis*, wastewaters, biotreatment,
40 bioremediation.

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45 **1. Introduction**

46 The Planet Earth contains only less than 1% of freshwater (Gleick, 2014). The increasing
47 population, urbanization, human activities and unjustifiable usage of freshwater are the foremost
48 reasons of causing its further shortage (Khosro et al., 2015). The South Asian region, mainly
49 Pakistan, has the worst condition in this scenario (Roberts, 2017; Wagan and Khoso, 2013).
50 Despite having world's largest glaciers, researchers have proclaimed that the country is on its way
51 to become the most water-stressed country in the region by year 2040 (WRI, 2015). The country's
52 agricultural, domestic and industrial sectors have too scored high on the World's Resource
53 Institute's water stress index. Its per capita annual water availability is just 1017 m³ now (IMF,
54 2015) which is scarily closer to the scarcity threshold level (1000 m³). Being an agricultural
55 country, this scarcity of freshwater resources has driven local farmers in Pakistan to reuse untreated
56 wastewater for irrigation of crops (Mahmood and Malik, 2014). These wastewaters contain many
57 harmful chemicals and heavy metals which accumulate in crops (Afonne and Ifediba, 2020; Topal
58 et al., 2020; Zhang et al., 2020; Zoqi and Doosti, 2020) and up the food chain making them
59 hazardous for consumption.

60 Pakistan Water Sector Strategy (PWSS, 2002) reported that the total quantity of wastewater
61 produced in Pakistan is 962335 million gal per annum including 674009 million gal from domestic
62 and 288326 million gal from industrial use. The domestic and industrial wastewater is either
63 discharged directly to a sewer system, a natural drain or water body, a nearby field or an internal
64 septic tank in Pakistan (Murtaza and Zia, 2012). Generally, this wastewater is not treated and none
65 of the cities have any biological treatment process except Islamabad and Karachi (EPMS, 2002),
66 and even these cities treat only a small proportion (<8%) of their wastewater before disposal
67 (Bashir, 2012; Steenbergen and Oliemans, 2002). These wastewaters contain considerable amount

68 of dyes, suspended solids, heavy metals, additives, detergents, surfactants, carcinogenic amines
69 and formaldehyde (Azizullah et al., 2011). They also contain organic and inorganic particles and
70 compounds, macro-solids, gases, emulsions, toxins, microplastics (Gatidou et al., 2019),
71 pharmaceuticals like endocrine disrupting compounds, hormones, antibiotics, anesthetics,
72 perfluorinated compounds (Arvaniti and Stasinakis, 2015), siloxanes (Bletsou et al., 2013), drugs
73 of abuse (Gatidou et al., 2016) and various biological pathogens (Andersson et al., 2016). These
74 untreated or insufficiently effluents treated wastewaters pose a serious environmental threat
75 (Salgot et al., 2006). The complex nature of these effluents and lack of centralized wastewater
76 treatment infrastructure make the treatment difficult in Pakistan. One area, that is a considerable
77 challenge is the removal of colour contamination.

78 The dyes, impurities and chemicals released from the textile industries impart colour to
79 wastewater drains and cause colour contamination, thus diminishing the water quality (Carmen
80 and Daniela, 2012). Various physicochemical methods have been used worldwide to remove
81 colour and impurities from wastewater, i.e. adsorption (Patel and Vashi, 2010), ion exchange
82 (Karcher et al., 2002), membrane filtration (Marcucci et al., 2001), ozonation (Ince and Tezcanli,
83 2001), photooxidation (Hai et al., 2007) and reverse osmosis (Suksaroj et al., 2005). Pakistan being
84 a developing economy has not adopted any of these methods on a large scale as these methods are
85 prohibitively expensive and require large complex infrastructure (Verma et al., 2012). Only one
86 full scale domestic wastewater treatment plant was set up on the conventional activated sludge
87 process in Islamabad, Pakistan but it is not maintained well by the plant operators (Fatima and
88 Khan, 2012). Decentralised biological treatment methods could offer a potential low-cost and low-
89 tech solution for communities in developing countries such as Pakistan.

90 Biological processes play a major role in the removal of pollutants. Due to ubiquitous nature

91 of bacteria, they can be used as invaluable tools for the biological treatment of different types of
92 wastewater, i.e. domestic, hospital, pharmaceutical and textile industrial wastewaters. The
93 bioremediation potential of bacterial isolates is an economically viable method and environment
94 friendly thus presents a good alternative to other engineered process (Dwivedi and Tomar, 2018).
95 Biological treatment takes advantage of the catabolic versatility of microorganisms including
96 bacteria to degrade or convert toxic compounds to non-toxic compounds (Díaz, 2008). One
97 strategy – to use native or indigenous isolates from wastewater to degrade, detoxify and decolour
98 specific wastewater has been the source of intensive research. Many authors have isolated
99 microorganisms from industrial textile wastewaters and then demonstrated their ability to
100 decolourise specific classes of dyes in the laboratory (e.g. Zhang et al., 2010; Meerbergen et al.,
101 2018; Alalewi and Jiang, 2012; Buthelezi et al., 2012; Mahmood et al., 2011). Shukor et al (2009)
102 demonstrated isolates from hospital wastewater were capable of degrading acrylamide compounds.
103 Others have used similar strategies to demonstrate the removal of heavy metals (Helmy et al.,
104 2018; Afzal et al., 2017; Das and Kumari, 2016).

105 Researchers have established the identity of many of these isolates from different
106 wastewaters and their ability to specific chemical compounds, e.g. *Bacillus cereus* isolated from
107 domestic wastewater for degrading acrylamide (Shukor et al., 2009) and hydrocarbons (Kostka et
108 al., 2011), *B. subtilis* isolated from pharmaceutical wastewater for removing antibiotic cephalixin
109 and heavy metals (Adel et al., 2015), *Aeromonas hydrophila* isolated from industrial wastewater
110 for degrading Triarylmethane dyes (Ogugbue and Sawidis, 2011), *Alcaligenes faecalis* spp.
111 isolated from petrochemical industrial wastewater for degrading phenol (Manafi et al., 2011),
112 *Rhodococcus pyridinivorans* isolated from gold mine wastewater for degrading cyanomethane
113 (Sulistinah et al., 2019), *Dracaena sanderiana* isolated from plastic industry wastewater for

114 degrading bisphenol A (Suyamud et al., 2020) or *Sphingomonas trueperi* isolated from wastewater
115 sludge for the degradation of allethrin (Bhatt et al., 2020). However, these biotreatment studies do
116 not represent the complex environment of mixed wastewater. Moreover, isolates are generally
117 tested against specific compounds in simplistic lab conditions and thus, the potential to degrade
118 these compounds in complex raw wastewater is largely unknown. This therefore is not sufficient
119 for the real-world situation in countries like Pakistan where wastewaters from household, hospitals
120 and wide range of industries is combined.

121 The present research aimed to i) characterise the pollutants and metals in a variety of
122 complex raw wastewaters in Pakistan, ii) isolate novel decolourising isolates from the raw
123 wastewater, iii) determine the decolourisation and degradation potential of these isolates in raw
124 hospital wastewater and finally iv) to identify the isolates with the maximum potential for
125 decolourisation and degradation of organic compounds.

126 **2. Materials and methods**

127 *2.1 Collection of wastewaters*

128 Four wastewater (domestic, hospital, textile and pharmaceutical) samples (50 L each) from
129 the points of discharge of drainage sites in Lahore, Pakistan were collected in sterile bottles
130 according to the standard protocols (APHA, 2005). The geographical coordinates of Lahore city
131 are 31° 34' 55.3620" north and 74° 19' 45.7536" east at an altitude of 217 m (712 ft). Mixed
132 wastewater (50 L) was also collected from a collective drainage site of the different wastewaters.
133 All five samples were collected in October, 2018. The temperature of the wastewaters and
134 environment were measured on-site with the help of digital thermometer (HUBDIC).

135 *2.2 Characterisation of the wastewaters*

136 The wastewaters were analysed immediately after the collection for the characterisation to

137 ensure the bacterial viability and to avoid any self-degradation of organic compounds. Following
138 physicochemical parameters were investigated according to standard protocols (APHA, 2005; Ali
139 et al., 2009) *i.e.* colour, smell, temperature, pH, electrical conductivity (EC), total suspended solids
140 (TSS), total dissolved solids (TDS), chemical oxygen demand (COD), biological oxygen demand
141 (BOD_5), salinity (ppt) and turbidity (NTU). The concentrations of heavy metals, *i.e.* Arsenic,
142 Cadmium, Chromium, Lead and Nickel, were estimated through Atomic Absorption
143 Spectrophotometer (AA 7000 F with Autosampler and Hydride Vapour Generator, Shimadzu,
144 Japan). The same physicochemical parameters were investigated in treated wastewaters and were
145 compared with untreated wastewaters. Biodegradability index (BI) is the ratio of BOD_5 : COD. It
146 is a parameter for evaluating the potential biodegradability of a biological treatment in wastewater
147 (Padoley et al., 2012). The values of BI for all decolourised wastewaters were compared with the
148 values of BOD_5 : COD of the original wastewaters to access the level of biodegradability. The
149 biodegradation of wastewaters with lesser BOD_5 / COD value is not possible to biodegrade as it
150 contains extremely toxic contaminants. If the BOD_5 / COD value would be lower than 0.3, then
151 the biodegradation will not proceed, thus it cannot be treated biologically, because the wastewater
152 generated from these activities inhibits the metabolic activity of bacteria due to their toxicity.

153 *2.3 Isolation and screening of bacteria*

154 The isolation of bacterial strains from each of the five types of wastewaters was carried out
155 through serial dilution method (Verma et al., 2001). The isolates from each wastewater's inocula
156 were incubated on sterile nutrient agar medium (0.8% Nutrient broth and 2% Agar) plates in static
157 incubator at 37°C for 24 hours and were then purified by streaking on nutrient agar medium plates.
158 Streaking was done thrice in zig zag manner. The purified cultures were shifted to prepared slants
159 of Luria-Bertani medium (LB) with Agar in test tubes and were preserved in a refrigerator (4 °C)

160 (Mahmood et al., 2011). The bacterial slants were maintained every two weeks on freshly prepared
161 agar slants to circumvent the susceptibility of the isolates (ISO 11133, 2014).

162 The domestic wastewater was chosen as the preliminary testing sample for screening. The
163 isolated bacterial strains were inoculated (10 %) and incubated at 37 °C for 24 hours in domestic
164 wastewater (100 mL) for initial screening. The percentage decolourisation was measured using
165 UV/VIS (AE-S80) spectrophotometer at 545 nm (Nanthakumar et al., 2013). The bacterial isolates
166 showing more than 50% decolourisation were then tested and inoculated using the same
167 methodology against each type of wastewater separately (D5, D6, D7, D8, H6, T4, T5, T6, P1, M5
168 and M8). The bacterial isolates showing maximum decolourisation (more than 90%) against all
169 wastewaters tested were further selected for testing optimal conditions (See supplementary data)
170 for colour contamination removal in complex wastewaters.

171 *2.4 Testing decolourisation potential of isolated bacteria*

172 The parameters incubation time, temperature and inoculum concentration were selected for
173 the estimation of optimal growth conditions of three bacterial isolates for testing their
174 decolourisation potential. For incubation time, the conical flasks (250 mL) containing 100 mL of
175 domestic wastewater each were inoculated with the screened isolates (10 % inoculum) in shaking
176 incubator at 120 rpm (PMI Labortechnik GMBH, WIS-20R) (Taran et al., 2007). The flasks were
177 incubated for 24, 48, 72 and 96 hours at 37 °C. For testing the optimal temperature, the inoculum
178 (10%) of screened isolates was added to domestic wastewater (100 mL) in conical flasks (250 mL)
179 for 24 h. Flasks were incubated at 30, 37, 44, 51 and 58°C in a shaking incubator (PMI
180 Labortechnik GMBH, WIS-20R). For the inoculum concentration, a loop full of bacterial colony
181 from a plate was added in distilled water (100 mL). The optical density (OD) was adjusted to 1 at
182 545 nm wavelength using UV/VIS spectrophotometer (A&E Labmed, AE-S80) in order to

183 maintain equal number of bacterial cells to each inoculum. The inoculum concentrations tested
184 were 5, 10, 15, 20, 25 and 30 % (Getha et al., 1998). The bacterial cell count per mL of each
185 screened isolate was also done through haemocytometer slide bridge (Neubaur improved HBG,
186 Marinefield, Germany). On optimum inoculum concentration (10 %), optimum incubation time
187 (48 h) and optimum temperature (37 and 51 °C), the decolourisation tests was conducted.

188 The three bacterial isolates were inoculated (10 %) separately in five types of wastewaters
189 (100 mL each) present in conical flasks (250 mL) for 48 hours at 37 and 51 °C (Jadhav et al.,
190 2010). The percentage decolourisation was calculated using Equation (1) (Cheriaa et al., 2012) at
191 545 nm using UV/VIS (AE-S80) spectrophotometer:

$$192 \quad \text{Decolourisation percentage (\%)} = \frac{(A_0 - A)}{A_0} \times 100 \quad (1)$$

193 Where A_0 = Initial absorbance, A = Absorbance of medium after decolourisation at the λ_{max} (nm).

194 The decolourisation experiments were performed in triplicates.

195 *2.4.1 Organic compounds degradation*

196 Bioremediation potential of the hospital wastewater sample that showed maximum
197 decolourisation percental and biodegradability index (Section 2.2 and 2.4) was further analysed
198 for organic compounds degradation. The hospital wastewater sample was analyzed by gas
199 chromatography mass spectrometry (GCMS) technique using an Agilent Gas Chromatograph (GC,
200 AgiTech-7260) and Mass Spectrometer (MS, Maspec-6595). In total, four samples (10 mL each)
201 were prepared for the analysis, *i.e.* one uninoculated hospital wastewater sample (control) and
202 three inoculated (*i.e.* decolourised) hospital wastewater samples. The inoculated three samples
203 were centrifuged (8000g for 15 min) to remove the biomass and the supernatants were shifted in
204 polypropylene falcon tubes (15 mL). All the samples were acidified to pH 1–2 with concentrated
205 HCl and then thoroughly extracted with three volumes of ethyl acetate. The organic layer was

206 collected, dewatered over anhydrous Na₂SO₄ and filtered through Whatman filter paper (no. 54).

207 All the GC separations were accomplished using a 20 m×0.3 mm (as internal diameter) fused-

208 silica capillary column with a 0.45 μm coated 6% phenylmethyl silicone film in the instrument.

209 The aliquot of the sample (5 μL) was injected in split-less mode (0.5 min) at 290°C. The

210 oven temperature was set as follows: initial temperature (45°C), raised to 58-92°C/min and then

211 12-210°C/min, 10-285°C/min and 6-320°C/min with a hold time of 5 min. The pressure control

212 was adjusted for a constant electronic flow of helium as the carrier gas (mL/min). Mass

213 Spectrometer was adjusted as follows: 120°C analyzer, 210°C source, 280°C interface and electron

214 ionization at 80 eV. The data was collected from 50-450 atomic mass unit (amu). The retention

215 time (±0.1 min), quantification ions, confirmation ions (156.18 and 184.25 m/z) and internal

216 standards (Acenaphthene and Phenanthrene) of each sample were set at optimal levels (Spiking

217 level = 0.05 μg/g; recovery = 98.9 and 93.47 %; coefficient of variation (CV) = 4.22 and 7.39 %)

218 and run in accordance with the system sequence. The base-peak ion was employed for quantitation

219 and two qualifier ions were used for confirmation. The compound concentrations were compared

220 with internal standard quantitation (LoQ = 0.05 mg/kg) and calibration curves. In order to identify

221 the low molecular weight compounds derived from bacterial treatment, the mass spectra were

222 compared with National Institute of Standard and Technology (NIST) database library software

223 available in the instrument and by comparing the retention time with those of authentic compounds

224 available. Quantification of these compounds was conducted by relating the ratio of the peak area

225 of the compound of interest over the peak area of the internal standard (Acenaphthene and

226 Phenanthrene) to the calibration curve of standard solution.

227 *2.5 Metal tolerance limits*

228 100 mg/L solutions of following ten metal salts were prepared in deionized water, *i.e.*

229 PbNO₃, CoCl₂, CaCl₂, ZnSO₄, MnSO₄, MgSO₄, FeSO₄, K₂Cr₂O₇, Na₂MoO₄ and CuSO₄ (Sigma
230 Aldrich, Uk). The three bacterial isolates were streaked on the prepared metal salt-nutrient agar
231 plates and kept in static incubator (at 37°C) for 24 h. At 100 mg/L concentration, the metal salt
232 plates with more than 65% bacterial growth were selected. The solutions of these metal salts
233 (CaCl₂, MgSO₄, K₂Cr₂O₇, Na₂MoO₄ and PbNO₃) were then prepared in 50, 100, 150, 200, 250
234 and 300 mg/L concentrations. The bacterial growth of three isolates on these concentrations was
235 assessed.

236 *2.6 Identification of the bacterial isolates*

237 The three bacterial isolates showing > 90% decolourisation potential above were selected
238 for identification through 16S rDNA sequencing (Mignard and Flandrois, 2006). Neat DNA (0.5
239 mL) was sent to the Macrogen sequencing company in South Korea for sequencing analysis.
240 Polymerase chain reaction (PCR) was carried on the three isolates using the following forward and
241 reverse primer set (See supplementary data): 27F (AGA GTT TGA TCM TGG CTC AG) and
242 1492R (TAC GGY TAC CTT GTT ACG ACT T) (Muyzer et al., 1993). 20 ng of genomic DNA
243 template was taken in a 30 µL reaction mixture using *EF-Taq* (SolGent, Korea) as follows: *Taq*
244 polymerase activation for 2 min at 95°C, 35 cycles for 1 min at 95°C, 1 min each at 55°C and 72°C
245 were performed finishing with 10 min step at 72°C. Amplification products were purified with a
246 multiscreen filter plate (Millipore Corporation, Bedford, Ma, USA). The sequencing reaction was
247 performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. DNA samples
248 containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster
249 City, CA). The mixture was incubated for 5 min at 95°C, followed by 5 min on ice and then
250 analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

251 The forward and reverse sequence chromatograms (abi files) were initially viewed in

252 FinchTV version 1.5.0 and then interrogated using MacVector version 17.5.4. Raw sequences were
253 examined in MacVector and ambiguous bases were edited by comparing the individual
254 electrograms per strain. Low quality ends were trimmed. The forward and reverse reads were
255 imported into BioEdit version 7.2. A consensus sequence per strain was subsequently assembled
256 using the contig assembler program (CAP; Huang, 1992) using the forward read and reverse
257 complement of the reverse read. The full sequence information and raw chromatogram details are
258 presented in the Supplementary Information. BLAST analysis was carried out on the assembled
259 sequences. The sequences of the three isolates were deposited in GenBank with accession
260 numbers. Phylogenetic analysis of the strains was carried out using the top 20 BLAST hits for each
261 isolate. This was achieved by aligning the sequences using Muscle version 3.8.425 (Edgar, 2004)
262 and a phylogenetic tree assembled in Geneious Prime using Tamura-Nei genetic distance method
263 and Neighbor-Joining tree building method. This tree was then imported in newick file format and
264 edited in Evolview (Zhang et al., 2012).

265 **3. Results and discussion**

266 *3.1 Characterisation of the wastewaters*

267 The apparent colours of domestic, hospital, textile, pharmaceutical and mixed wastewaters
268 were light grey, light yellow, greenish grey, light brown and blackish, respectively. The true colour
269 values for the wastewaters were 101, 188, 221, 103 and 311 PCU, respectively. The smell of the
270 domestic, textile and mixed wastewaters was pungent, while hospital and pharmaceutical
271 wastewaters had fishy smell. The values of most of the physicochemical parameters were beyond
272 the level of National Environment Quality Standards (NEQS, 2000). Like the pH values of textile
273 and pharmaceutical wastewater were 8.7 and 10.4, respectively, before treatment keeping in mind
274 that the NEQS range for pH is 6.6-8.5. The pH values of domestic, hospital and mixed wastewaters

275 were within the NEQs range before treatment. This indicated that these wastewaters had their pH
276 corrected before being discharged. Similarly, the values of total suspended solids of domestic,
277 hospital, textile, pharmaceutical and mixed wastewaters were 1920, 2300, 2150, 2120 and 2670
278 mg/L. These values were too beyond the range of NEQS standard (i.e. < 500 mg/L). The values
279 for the turbidity should be less than 5 NTU as per NEQS range. While the turbidity values for
280 domestic, hospital, textile, pharmaceutical and mixed wastewaters were 38, 51, 76, 61 and 123
281 NTU (Nephelometric Turbidity Units), respectively.

282 The wastewaters became colourless and odourless after the biotreatment (Figure 1 a,b). The
283 true colour values for domestic, hospital, textile, pharmaceutical and mixed wastewaters were
284 reduced to 28, 55, 61, 38 and 64 PCU, respectively. Results showed that the values of analyses of
285 various physicochemical parameters were within the levels of National Environment Quality
286 Standards (NEQS, 2000) after treatment (Table 1). Like, the pH of textile and pharmaceutical
287 wastewaters after the treatment were reduced to 7.5 and 8.2, respectively (pH range: 6.6-8.5). The
288 reduction in pH after the decolourisation of textile wastewater has been reported previously by
289 Ogugbue and Sawidis (2011b). Similarly, the values of total suspended solids (TSS) were reduced
290 to 363, 483, 425, 398 and 491 mg/L after the biotreatment (TSS range: < 500 mg/L). The turbidity
291 values were reduced after the biotreatment to 4, 5, 5, 3 and 4 which were within the range of NEQS
292 turbidity value (i.e. ≤ 5 NTU).

293 The values of BOD₅ for domestic, hospital, textile, pharmaceutical and mixed wastewaters
294 were 39, 78, 14, 68 and 40 mg/L, respectively. This was out of the range of NEQs which is 80 –
295 250 mg/L. The values of COD for these wastewaters were 76, 260, 17, 133 and 99 mg/L,
296 respectively. The value of COD in all wastewaters were below the range (150 – 400 mg/L) except
297 hospital wastewater. The BOD₅ / COD ratio for these wastewaters were 0.51, 0.3, 0.82, 0.51 and

298 0.4, respectively. One important thing to notice is that if the value of BOD_5 / COD is in between
299 0.3 and 0.6, then wastewater is required to treat it biologically, because the process would be
300 relatively slow, as the acclimatization of the microorganisms that help in the degradation process
301 takes time (Abdalla and Hammam, 2014). All of our wastewater samples lie in the same range
302 between 0.3 and 0.6. However, the lowest value of this ratio recorded was of hospital wastewater
303 that showed it was the most contaminated wastewater than all other types.

304 The values of BOD_5 after the biotreatment of domestic, hospital, textile, pharmaceutical and
305 mixed wastewaters were 176, 246, 174, 223 and 169 mg/L, respectively that were within the range
306 of NEQs (80 – 250 mg/L). The values of COD for these wastewaters after biotreatment were 212,
307 396, 153, 269 and 235 mg/L, respectively that were too within the range (150 – 400 mg/L). The
308 BOD_5 / COD ratio for these wastewaters were 0.83, 0.62, 1.14, 0.83 and 0.72, respectively. As per
309 previously reported work, the value of BOD_5 / COD ration > 0.6 confirms the biotreatment of
310 wastewater (Abdalla and Hammam, 2014). All the values of biodegradability index in our
311 wastewater samples were more than 0.6. Even the value of most contaminated hospital wastewater
312 was also 0.62 that showed significant biodegradability index.

313 Heavy metal chromium was detected in the hospital (1.8 mg/L), pharmaceutical (1.7 mg/L)
314 and mixed (0.9 mg/L) wastewaters which was exceeding the NEQs limit (< 0.05 mg/L). Lead was
315 only present in the hospital wastewater (0.17 mg/L). Nickel was present in domestic (0.08 mg/L),
316 hospital (1.76 mg/L), textile (0.19 mg/L), pharmaceutical (1 mg/L) and mixed (0.5 mg/L)
317 wastewaters (Table 1). The hospital wastewater seemed to have more heavy metals than all other
318 types of wastewaters under study. After treatment, the chromium became absent in hospital
319 wastewater. Its amount was reduced to the NEQ limit (< 0.05 mg/L) in pharmaceutical (0.05
320 mg/L) and mixed (0.019 mg/L) wastewaters after biotreatment (Table 1). Lead which was only

321 present in the hospital wastewater was not detected after biotreatment. The values of Nickel were
322 reduced to 0.07, 0.25, 0.08, 0.5 and 0.22 after the biotreatment of domestic, hospital, textile,
323 pharmaceutical and mixed wastewaters, respectively. Our results agree well with previous work.
324 For example, Abo-Amer et al. (2015) and Naik et al., (2012) have reported the removal of heavy
325 metals from sewage and electroplating wastewaters, respectively. Also, Ali et al. (2009) have
326 reported reduction in colour, temperature, pH, EC, BOD₅, COD, TSS, TDS and heavy metals ions
327 present in textile wastewaters after the bioremediation by isolated bacteria.

328 *3.2 Isolation and screening of bacteria*

329 In total, 37 bacterial strains were isolated from domestic, hospital, textile, pharmaceutical
330 and mixed wastewaters. Eight bacteria were isolated from the domestic wastewater (D1-D8), nine
331 bacteria were isolated from the hospital wastewater (H1-H9), six from the textile wastewater (T1-
332 T6), six from the pharmaceutical wastewater (P1-P6) and eight were isolated from the mixed
333 wastewater (M1-M8). The isolations of bacteria have been reported from domestic (Jin et al.,
334 2015), hospital (Yamina et al., 2014), textile (Alalewi and Jiang, 2012) and pharmaceutical
335 (Madukasi et al., 2010) wastewaters. Meerbergen et al. (2018) isolated the bacterial isolates from
336 textile wastewater to decolourise azo dyes. Similarly, four bacterial strains were isolated from
337 marine and tannery saline wastewater samples that were proven to be salt-tolerant and carried out
338 successful bioremediation (Sivaprakasam et al. 2008). Shomar et al. (2020) researched on the
339 significance of using the isolated (viable) bacteria for wastewater treatments.

340 Eleven bacteria, isolated from domestic, hospital, textile, pharmaceutical and mixed
341 wastewaters, had the potential to decolourise the preliminary tested domestic wastewater in
342 comparison with other bacterial isolates under study. The percentage decolourisations of these
343 bacterial strains isolated from domestic (D5, D6, D7 and D8), hospital (H6), textile (T4, T5, and

344 T6), pharmaceutical (P1) and mixed wastewaters (M5 and M8) were > 50% (Figure 2). After final
345 screening, three bacterial strains showed more than 70% decolourisation potential against all
346 wastewaters *i.e.* D6, D7 and P1 (Figure 3). The isolate D6 exhibited 71, 93, 70, 83 and 73 %
347 decolourisation of domestic, hospital, textile, pharmaceutical and mixed wastewaters, respectively.
348 The isolate D7 showed 74, 91, 70, 83 and 73 % decolourisation of domestic, hospital, textile,
349 pharmaceutical and mixed wastewaters, respectively. The isolate P1 showed 82, 92, 71, 77 and 75
350 % decolourisation of domestic, hospital, textile, pharmaceutical and mixed wastewaters,
351 respectively. Chen et al. (2003) reported varied decolourisation capabilities (14 – 90 %) of six
352 bacterial strains isolated from textile wastewater for azo, anthraquinone and indigoid dye groups.
353 Meerbergen et al. (2018) reported > 80 % decolourisation potential of five bacterial strains isolated
354 from domestic wastewater treatment plant to decolourise azo dyes. However, most of the work has
355 been done on synthetic components of textile wastewaters (e.g. azo dyes) while our work has
356 provided a complex combination and is more representative of the real-world scenario in Pakistan.

357 *3.3 Testing decolourisation potential of isolated bacteria*

358 The strain D6 exhibited 87, 96, 80, 93 and 83 % decolourisation of domestic, hospital,
359 textile, pharmaceutical and mixed wastewaters, respectively. The strain D7 showed 84, 96, 88, 89
360 and 83 % decolourisation of domestic, hospital, textile, pharmaceutical and mixed wastewaters,
361 respectively. The strain P1 showed 89, 93, 81, 87 and 85 % decolourisation of domestic, hospital,
362 textile, pharmaceutical and mixed wastewaters, respectively (Figure 4). The high decolourisation
363 potential of 95-98 % have been reported previously in textile wastewater (Deng et al. 2008).
364 Similarly, Saha et al. (2017), Modi et al. (2010), Kanagaraj et al. (2012) and Liao et al. (2013)
365 have also worked on the decolourisation potential of bacterial isolates for textile wastewater.
366 However, to our knowledge, this study has proven significant regarding the decolourisation and

367 bioremediation potential of these strains for pharmaceutical industrial, hospital, domestic and
368 mixed wastewaters that are frequently discharged in Pakistan.

369 *3.3.1 Organic compounds degradation*

370 Considering the maximum decolourisation potential, fluctuating physicochemical values
371 and significant biodegradability index value against bacterial isolates D6, D7 and P1, the untreated
372 and decolourised samples of hospital wastewater were analyzed for degradation of organic
373 compounds. GCMS analysis of untreated hospital wastewater confirmed the presence of six
374 pharmaceutical pollutants in the effluent. These pollutants belonged to following different major
375 groups: aromatic, metabolite, stimulant, NSAID, organic and sedative (Table 2). The pollutants
376 belonging to these groups (with concentrations) were Phenol (0.876 ppm), Salicylic acid (0.048
377 ppm), Caffeine (0.007 ppm), Naproxen (0.023 ppm), Octadecene (0.185 ppm) and Diazepam
378 (0.014 ppm). The retention time (min) for these pollutants were 26.72, 6.51, 7.96, 9.16, 28.65 and
379 38.06 minutes, respectively. The confirmation (m/z) ion for these pollutants were 58.15, 147.64,
380 266.82, 412.07, 581.46 and 685.39 m/z, respectively (See supplementary data). Nair et al., (2008)
381 have described the hazardous nature of phenolic pollutants even at relatively low concentration.
382 Accumulation of phenol creates toxicity both for flora and fauna. Rodil et al., (2012) have reported
383 that salicylic acid is one of the emerging most concentrated pollutant (exceeding the 1 µg/L) which
384 is very hard to remove from the wastewaters even after biotreatment. Motuzas et al. (2017) have
385 reported caffeine as an environmentally emerging micro-pollutant. The presence of non-steroidal
386 anti-inflammatory drug (NSAID) like naproxen in the environment is an emerging problem due to
387 their potential influence on human health and biocenosis or microbial communities
388 (Wojcieszynska et al., 2014). Octadecene was found as an organic priority pollutant in Potato crop
389 (concentration = 0.06 mg/kg; retention time = 21.12 minutes) that was irrigated with ground water

390 having pesticides and herbicides residues (Gushit et al., 2013). Rosal et al. (2010) reported
391 diazepam as an emerging pollutant in urban wastewater with an average concentration of 3 ng/L
392 (that equals LOQ).

393 In the hospital wastewater sample treated with bacterial isolate D6, all other four pollutants
394 were completely biodegraded except salicylic acid and caffeine which were now present at very
395 low concentrations (0.007 and 0.004 ppm) that showed their partial degradation leading to
396 reduction in its concentration from its concentrations in untreated sample 0.048 and 0.007 ppm,
397 respectively. However, a new intermediate compound Triclopidine belonging to Fibrinolytic group
398 was found with 0.011 ppm concentration, 31.95 minutes retention time and 534.12 m/z
399 confirmation ion. Previous researches have supported our ecofriendly biodegradation in this
400 treated sample as Ticlopidine helps in prevention of stroke even better than Aspirin (Grotta et al.,
401 1992). It is also helpful in coronary stenting and as antiplatelet agent during coronary interventions
402 to cure the patients with acute myocardial infarction (AMI) (Cherian et al., 1998).

403 In the hospital wastewater sample treated with bacterial isolate D7, all other four pollutants
404 were completely biodegraded except naproxen and octadecene which were now present at very
405 low concentrations (0.006 and 0.019 ppm) that showed their partial degradation leading to
406 reduction in its concentration from its concentrations in untreated sample 0.023 and 0.185 ppm,
407 respectively. However, two new intermediate compounds Tetradecene and Griseofulvin belonging
408 to organic and antibacterial groups were found present with 0.035 and 0.028 ppm concentrations,
409 7.08 and 46.18 minutes retention times and 190.86 and 692.95 m/z confirmation ions. The
410 formation of these essentially important compounds has been supported by previous researches.
411 For example, Roth et al. (1959) reported the Griseofulvin as an antifungal and antibiotic. It is very
412 interesting that a bacterial strain has helped in the formation of an antibiotic through degradation

413 of organic pollutants. Similarly, Tetradecene is a very important compound used in making
414 polyalphaolefins (PAO) at a very low viscosity and excellent cold temperatures (Goze et al., 2007).

415 In the hospital wastewater sample treated with bacterial isolate P1, all other four pollutants
416 were completely biodegraded except phenol and salicylic acid (0.381 and 0.015 ppm). However,
417 two new intermediate compounds Lidocaine and Butalbital belonging to anesthetic and barbiturate
418 groups were found present with 0.122 and 0.054 ppm concentrations, 20.26 and 30.88 minutes
419 retention times and 368.27 and 625.51 m/z confirmation ions. Previously reported work has
420 supported this biodegradation as an ecofriendly one. For example, Lidocaine is said to possess
421 analgesic (Hollmann et al., 2000; Hollmann et al., 2005), antihyperalgesic (Nagy et al., 1996) and
422 anti-inflammatory (Sugimoto et al., 2003) properties. It is also known to accelerate the return of
423 bowel function after surgery (Marret et al., 2008). It is helpful for post-operative pain and acute
424 rehabilitation after laparoscopic nephrectomy (Tauzin-Fin et al., 2014). Additionally, Butalbital is
425 an analgesic usually prescribed for the treatment of migraine and tension-type headaches
426 (Silberstein and McCrory, 2001). The maternal periconceptional use of butalbital also supports in
427 healing congenital heart defects (Browne et al., 2013). However, its overuse causes headache and
428 discontinuation syndromes (Devine et al., 2005).

429 *3.4 Metal tolerance limits*

430 At 100 mg/L concentration of metal salts of PbNO₃, MgSO₄, MnSO₄, ZnSO₄, K₂Cr₂O₇,
431 CaCl₂, Na₂MoO₄, CuSO₄, CoCl₂ and FeSO₄, the isolate D6 exhibited growth of 25, 70, 50, 5, 35,
432 80, 45, 20, 5 and 50 %, respectively. It showed maximum growth of 80 % against CaCl₂. The
433 isolate D7 indicated growth of 65, 35, 25, 12, 20, 35, 45, 3, 0 and 45%, respectively. It showed
434 maximum growth of 65 % against PbNO₃. The isolate P1 showed growth of 95, 65, 40, 15, 60, 75,

435 95, 20, 0 and 35 %, respectively. It showed maximum growth of 95 % against both PbNO₃ and
436 Na₂MoO₄ (Figure 5).

437 For isolate D6, CaCl₂ and MgSO₄ metal salts were selected that showed overall maximum
438 growth of 78 and 70 % at 300mg/L concentrations, respectively. For isolate D7, PbNO₃ metal salt
439 was selected that showed maximum growth of 82 % at 300mg/L concentration. For isolate P1,
440 PbNO₃, Na₂MoO₄, CaCl₂, MgSO₄ and K₂Cr₂O₇ metal salts were selected that showed maximum
441 growth of 65, 90, 73, 73 and 75 % at 300mg/L concentration of these metal salts, respectively
442 (Figure 6 a,b,c). On one hand, this has confirmed that all three strains have the potential to tolerate
443 these metals efficiently along with remediating the organic compounds from wastewaters even in
444 co-existence with heavy metals. On the other hand, it also supported our results (Section 3.1) that
445 these isolates have potential to adsorb the heavy metals to remove them from wastewaters. The
446 high metals concentration is really a big challenge for wastewater treatments as it leads to the
447 inhibition of the microbial populations etc. These strains were resistant to high metal
448 concentrations and thus tolerated the harsh environments of these complex wastewaters.

449 *3.5 Identification of the bacterial isolates*

450 BLAST analysis indicated that strain D6 was a *Bacillus* species with 100% homology to
451 *Bacillus paramycooides* (Table 3). Phylogenetic analysis reveals that it closely resembled *Bacillus*
452 *pseudomycooides* (Figure 7) but formed a separate outgroup, indicating that the isolated species was
453 phylogenetically distinct from the BLAST reference sequences. It was one of the nine novel
454 species of the *Bacillus cereus* group reported by Liu et al. (2017). BLAST analysis indicated that
455 strain D7 was also a *Bacillus* species with 99.86% homology to *Bacillus paramycooides* (Table 4).
456 Phylogenetic analysis reveals that it closely resembled *Bacillus pseudomycooides* (Figure 8) but
457 formed a separate outgroup, indicating that the isolated species was phylogenetically distinct from

458 the BLAST sequences. Thus, D6 and D7 isolates share a high similarity. BLAST analysis indicated
459 that strain P1 was an *Alcaligenes* species with 97.47% homology to *Alcaligenes faecalis* (Table
460 5). Phylogenetic analysis reveals that it closely resembled *Paenalcaligenes suuwonensis* and
461 *Paenalcaligenes hominis* (Figure 9) but formed a separate outgroup, indicating that the isolated
462 species was phylogenetically distinct from the BLAST sequences. The nucleotide sequences of
463 these isolates D6, D7 and P1 have been submitted to GenBank under accession number [GenBank:
464 MT477810], [GenBank: MT477812], and [GenBank: MT477813], respectively. The three isolates
465 were then phylogenetically compared with each other and the top BLAST hit sequences. This
466 result indicated that these isolates were more closely related to each other than the blast sequences.
467 D6 and D7 clustered together demonstrating that these isolates were highly similar. The closest
468 cluster was identified as *Paenalcaligenes suuwonensis* and *Paenalcaligenes hominis* (Figure 10).

469 Authors have previously found *Bacillus* species such as *Bacillus paramycooides* to be part of
470 plant growth-promoting rhizobacteria (Osman and Yin, 2018) and associated with bioremediation
471 of toxic effluents containing cyanide (Wu et al., 2014), alkylphenols (Chang et al., 2020) and
472 hydrocarbons (Kostka et al., 2011). Similarly, *Alcaligenes faecalis* was also noted as a biocontrol
473 agent by Yokoyama et al. (2013). It must be noted that both species are potential human pathogens
474 (Bottone, 2010; Kaliaperumal et al., 2006). The potential pathogenicity of these isolates would
475 warrant further investigation prior to any bioamendment strategies.

476 It is very important to highlight that the optimal incubation time for *B. paramycooides* has
477 not been reported previously. For *A. faecalis* JBW4, isolated from activated sludge, the optimal
478 incubation time was 5 days (Kong et al., 2013). In present study, these two strains have proven to
479 be very efficient in terms of requiring less time of incubation (48 hours) with more decolourisation
480 potential. The optimal temperature for growth of *Bacillus* spp. is reported between 30 – 37 °C

481 (Gilbert et al., 2009). *Alcaligenes faecalis* was previously reported to be grown at 37 °C (Schroll
482 et al., 2001). Syed et al. (2015) have found heavy metal resistance and their degradation by *B.*
483 *cereus* strains. *A. faecalis* was found to be heavy metal resistant bacteria isolated from sewage
484 wastewater and responsible for the synthesis of silver nanoparticles (Abo-Amer et al., 2015). The
485 capability of *A. faecalis* to degrade phenol as a carbon source has been previously reported
486 (Rehfuss and Urban 2005). This supports our results showing biodegradation of phenol into other
487 non-toxic low molecular organic compounds. *A. faecalis* has been proven to be efficient to
488 bioremediate ϵ -Caprolactam too from nylon-6 produced wastewater plant (Baxi and Shah 2002).
489 But to the best of the authors knowledge, *B. paramycoides* have never been reported for any type
490 of wastewater bioremediation. The antibiotic degradation potential of different isolated bacterial
491 species from pharmaceutical wastewaters (Tahrani et al., 2015) and the biodegradation of
492 acrylamide by *Enterobacter aerogenes* isolated from domestic wastewater (Buranasilp and
493 Charoenpanich, 2011) has only been reported previously. Majorly, they looked at the individual
494 wastewaters while our work has investigated a complex combination and is more representative
495 of the real-world scenario in Pakistan.

496 **4. Environmental implications**

497 Our work suggests that *B. paramycoides* D6, *B. paramycoides* D7 and *A. faecalis* are
498 capable to bioremediate domestic, hospital, textile, pharmaceutical and mixed wastewaters under
499 optimal conditions. These optimal conditions for temperature (37 and 51 °C) are achievable in
500 Pakistan's arid climate (in temperate zone) and the incubation time is achieved in 48 h only. The
501 utilization of these bacterial strains has several advantages as compared to the conventional
502 methods such as physicochemical approaches for the removal of contaminants. Bacterial treatment
503 with these strains is a cost-effective and low-tech method as the strains are isolated from the same

504 wastewater needed to be treated (Phugare et al., 2011). Further, these bacterial strains have been
505 found here to be efficient for the biotreatment of a wide range of wastewaters, *i.e.* domestic,
506 hospital, textile, pharmaceutical and mixed wastewaters. The strains degraded pharmaceutic
507 pollutants into ecofriendly derivatives and showed high decolourisation potential. Thus, this work
508 suggests that the biological treatment of wastewaters using *B. paramycoides* and *A. faecalis* can
509 be an eco-friendly and efficient method which may help developing countries such as Pakistan to
510 meet the Sustainable Development Goal of Clean Water and Sanitation (SDG-6). Future work may
511 require to focus on scaling-up this methodology at commercial level and to form a consortium of
512 these strains for achieving much higher efficiency.

513 **5. Conclusion**

514 Bacterial strains *B. paramycoides* D6, *B. paramycoides* D7 and *A. faecalis* have been proven
515 to be efficient in terms of possessing bioremediation potential against different wastewaters, *i.e.*
516 domestic, hospital, textile, pharmaceutical and mixed wastewaters. These bacterial isolates
517 significantly biodegrade the pollutants from the wastewaters into non-toxic organic compounds
518 within 48 hours of incubation, 10 % of inoculum and 37 and 51°C temperatures, respectively.
519 Under these optimal growth conditions, the strains *B. paramycoides* D6, *B. paramycoides* D7 and
520 *A. faecalis* showed maximum decolourisation potential of 96, 96, 93 %, respectively against
521 hospital wastewater. GCMS analysis confirmed the biodegradation of pharmaceutic pollutants, *i.e.*
522 Phenol, Salicylic acid, Caffeine, Naproxen, Octadecene and Diazepam, present in the hospital
523 wastewater into Ticlopidine in the case of *B. paramycoides* D6, Tetradecene and Griseofulvin in
524 the case of *B. paramycoides* D7 and Lidocaine and Butalbital in the case of *A. faecalis*. At 300
525 mg/L concentration, *B. paramycoides* D6, showed overall maximum growth of 78 and 70 % for
526 CaCl₂ and MgSO₄, respectively; *B. paramycoides* D7 showed maximum growth of 82 % for

527 PbNO₃; *Alcaligenes faecalis* showed maximum growth of 65, 90, 73, 73 and 75 % for PbNO₃,
528 Na₂MoO₄, CaCl₂, MgSO₄ and K₂Cr₂O₇, respectively. Our work recommends that the development
529 of a consortium from these strains may prove more efficient source of bioremediation of
530 wastewaters.

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


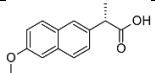

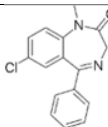


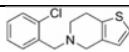
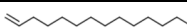
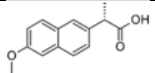
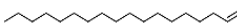
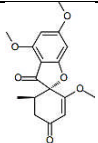


901 **Table 1: Physiochemical characterisation of untreated and treated wastewaters in**
 902 **comparison to NEQS**

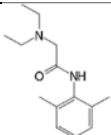
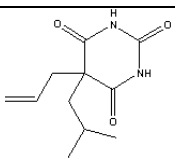


Parameters	NEQS	Wastewaters										
		DWW		HWW		TWW		PWW		MWW		
		Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	
Colour (PCU)	-	Light grey	Colourless	Light yellow	Colourless	Greenish grey	Colourless	Light brown	Colourless	Blackish	Colourless	
	-	101	28	188	55	221	61	103	38	311	64	
Smell	Acceptable / Bearable	Pungent	No smell	Fishy	No smell	Pungent	No smell	Fishy	No smell	Pungent	No smell	
Temperature (°C)	≤3°C	-	25	4	25	4	22	4	28	4	21	4
	-	-	29	26	30	26	33	26	33	26	31	26
pH	6.6-8.5	7.8	6.9	7.4	6.7	8.7	7.5	10.4	8.2	8.4	7.4	
EC (µs/cm)	-	413	214	444	267	861	574	350	193	775	435	
TSS (mg/L)	<500 mg/L	1920	363	2300	483	2150	425	2120	398	2670	491	
TDS (mg/L)	1000	296	213	296	220	608	398	105	87	541	323	
COD (mg/L)	150-400	76	212	260	396	17	153	133	269	99	235	
BOD ₅ (mg/L)	80-250	39	176	78	246	14	174	68	223	40	169	
BOD ₅ : COD	>0.6	0.51	0.83	0.3	0.62	0.82	1.14	0.51	0.83	0.4	0.72	
Salinity (ppt)	-	0.2	0.1	0.2	0.1	0.5	0.3	0.3	0.2	0.4	0.3	
Turbidity (NTU)	5	38	4	51	5	76	5	61	3	123	4	
Arsenic (As)	0.05 mg/L	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	
Cadmium (Cd)	0.01 mg/L	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	
Chromium (Cr)	0.05 mg/L	Nd	Nd	1.8	Nd	Nd	Nd	1.7	0.05	0.9	0.02	
Lead (Pb)	0.05 mg/L	Nd	Nd	0.17	Nd	Nd	Nd	Nd	Nd	Nd	Nd	
Nickel (Ni)	0.02 mg/L	0.08	0.07	1.8	0.25	0.18	0.08	1.0	0.5	0.5	0.22	

903 *NEQS = National Environment Quality Standards
 904 *Nd = Not Detectable
 905 *DWW = Domestic Wastewater
 906 *HWW = Hospital Wastewater
 907 *TWW = Textile Wastewater
 908 *PWW = Pharmaceutical Wastewater
 909 *MWW = Mixed Wastewater

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Table 2: Analysis of untreated and treated hospital wastewater through GCMS

Wastewater samples	Pollutants	Major Group/Class	Chemical structure	Retention time (min)	Confirmation ion (m/z)	Conc. (ppm)
Untreated HWW (Control)						
	Phenol	Aromatic		26.72	58.15	0.876
	Salicylic acid	Metabolite		6.51	147.64	0.048
	Caffeine	Stimulant		7.96	266.82	0.007
	Naproxen	NSAID		9.16	412.07	0.023
	Octadecene	Organic		28.65	581.46	0.185
	Diazepam	Sedative		38.06	685.39	0.014
Treated HWW (D6)						
	Salicylic acid	Metabolite		6.51	147.64	0.007
	Caffeine	Stimulant		7.96	266.82	0.004
	Ticlopidine (Derivative)	Fibrinolytic		31.95	534.12	0.011
Treated HWW (D7)						
	Tetradecene (Derivative)	Organic		7.08	190.86	0.035
	Naproxen	NSAID		9.16	412.07	0.006
	Octadecene	Organic		28.65	581.46	0.019
	Griseofulvin (Derivative)	Antibacterial		46.18	692.95	0.028
Treated HWW (P1)						
	Phenol	Aromatic		26.72	58.15	0.381
	Salicylic acid	Metabolite		6.51	147.64	0.015

Lidocaine (Derivative)	Anesthetic		20.26	368.28	0.122
Butalbital (Derivative)	Barbiturate		30.88	625.51	0.054
Internal standards					
Acenaphthene	<i>Na</i>		24.27	156.18	<i>Na</i>
Phenanthrene	<i>Na</i>		29.74	184.25	<i>Na</i>

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Table 3. Top 10 BLAST hits for isolate D6

Hit Number	Description	Max Score	Total Score	Query Cover	Percentage Identity	Accession number
1.	<i>Bacillus paramycoides</i> strain MCCC 1A04098 16S ribosomal RNA, partial sequence	2743	2743	100%	100.00%	NR_157734.1
2.	<i>Bacillus tropicus</i> strain MCCC 1A01406 16S ribosomal RNA, partial sequence	2737	2737	100%	99.93%	NR_157736.1
3.	<i>Bacillus nitratireducens</i> strain MCCC 1A00732 16S ribosomal RNA, partial sequence	2737	2737	100%	99.93%	NR_157732.1
4.	<i>Bacillus luti</i> strain MCCC 1A00359 16S ribosomal RNA, partial sequence	2737	2737	100%	99.93%	NR_157730.1
5.	<i>Bacillus albus</i> strain MCCC 1A02146 16S ribosomal RNA, partial sequence	2737	2737	100%	99.93%	NR_157729.1
6.	<i>Bacillus cereus</i> strain CCM 2010 16S ribosomal RNA, partial sequence	2732	2732	100%	99.87%	NR_115714.1
7.	<i>Bacillus cereus</i> ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	2732	2732	100%	99.87%	NR_074540.1
8.	<i>Bacillus proteolyticus</i> strain MCCC 1A00365 16S ribosomal RNA, partial sequence	2726	2726	100%	99.80%	NR_157735.1
9.	<i>Bacillus cereus</i> strain IAM 12605 16S ribosomal RNA, partial sequence	2721	2721	99%	99.86%	NR_115526.1
10.	<i>Bacillus wiedmannii</i> strain FSL W8-0169 16S ribosomal RNA, partial sequence	2721	2721	100%	99.73%	NR_152692.1

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Table 4. Top 10 BLAST hits for isolate D7

Hit Number	Description	Max Score	Total Score	Query Cover	Percentage Identity	Accession number
1.	<i>Bacillus paramycoides</i> strain MCCC 1A04098 16S ribosomal RNA, partial sequence	2715	2715	100%	99.86%	NR_157734.1
2.	<i>Bacillus tropicus</i> strain MCCC 1A01406 16S ribosomal RNA, partial sequence	2710	2710	100%	99.80%	NR_157736.1
3.	<i>Bacillus nitratireducens</i> strain MCCC 1A00732 16S ribosomal RNA, partial sequence	2710	2710	100%	99.80%	NR_157732.1
4.	<i>Bacillus luti</i> strain MCCC 1A00359 16S ribosomal RNA, partial sequence	2710	2710	100%	99.80%	NR_157730.1
5.	<i>Bacillus albus</i> strain MCCC 1A02146 16S ribosomal RNA, partial sequence	2710	2710	100%	99.80%	NR_157729.1
6.	<i>Bacillus cereus</i> strain CCM 2010 16S ribosomal RNA, partial sequence	2704	2704	100%	99.73%	NR_115714.1
7.	<i>Bacillus cereus</i> ATCC 14579 16S ribosomal RNA (<i>rrnA</i>), partial sequence	2704	2704	100%	99.73%	NR_074540.1
8.	<i>Bacillus cereus</i> strain IAM 12605 16S ribosomal RNA, partial sequence	2702	2702	99%	99.86%	NR_115526.1
9.	<i>Bacillus cereus</i> strain NBRC 15305 16S ribosomal RNA, partial sequence	2702	2702	99%	99.86%	NR_112630.1
10.	<i>Bacillus cereus</i> strain JCM 2152 16S ribosomal RNA, partial sequence	2702	2702	99%	99.86%	NR_113266.1

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Table 5. P1 Isolate Top 10 Blast hits

Hit Number	Description	Max Score	Total Score	Query Cover	Percentage Identity	Accession number
1.	<i>Alcaligenes faecalis</i> strain NBRC 13111 16S ribosomal RNA, partial sequence	2473	2473	99%	97.47%	NR_113606.1
2.	<i>Alcaligenes aquatilis</i> strain LMG 22996 16S ribosomal RNA, partial sequence	2455	2455	99%	97.20%	NR_104977.1
3.	<i>Alcaligenes faecalis</i> strain IAM 12369 16S ribosomal RNA, partial sequence	2429	2429	99%	96.99%	NR_043445.1
4.	<i>Alcaligenes endophyticus</i> strain AER10 16S ribosomal RNA, partial sequence	2388	2388	100%	96.39%	NR_156855.1
5.	<i>Alcaligenes faecalis</i> subsp. <i>parafaecalis</i> strain G 16S ribosomal RNA, partial sequence	2364	2364	96%	97.17%	NR_025357.1
6.	<i>Alcaligenes pakistanensis</i> strain NCCP-650 16S ribosomal RNA, partial sequence	2320	2320	96%	96.67%	NR_145932.1
7.	<i>Alcaligenes faecalis</i> subsp. <i>phenolicus</i> strain J 16S ribosomal RNA, partial sequence	2303	2303	99%	95.25%	NR_042830.1
8.	<i>Paenalcaligenes suwonensis</i> strain ABC02-12 16S ribosomal RNA, partial sequence	2204	2204	97%	94.76%	NR_133804.1
9.	<i>Parapusillimonas granuli</i> strain Ch07 16S ribosomal RNA, partial sequence	2200	2200	99%	94.36%	NR_115804.1
10.	<i>Pusillimonas ginsengisoli</i> strain DCY25 16S ribosomal RNA, partial sequence	2200	2200	100%	94.07%	NR_116103.1

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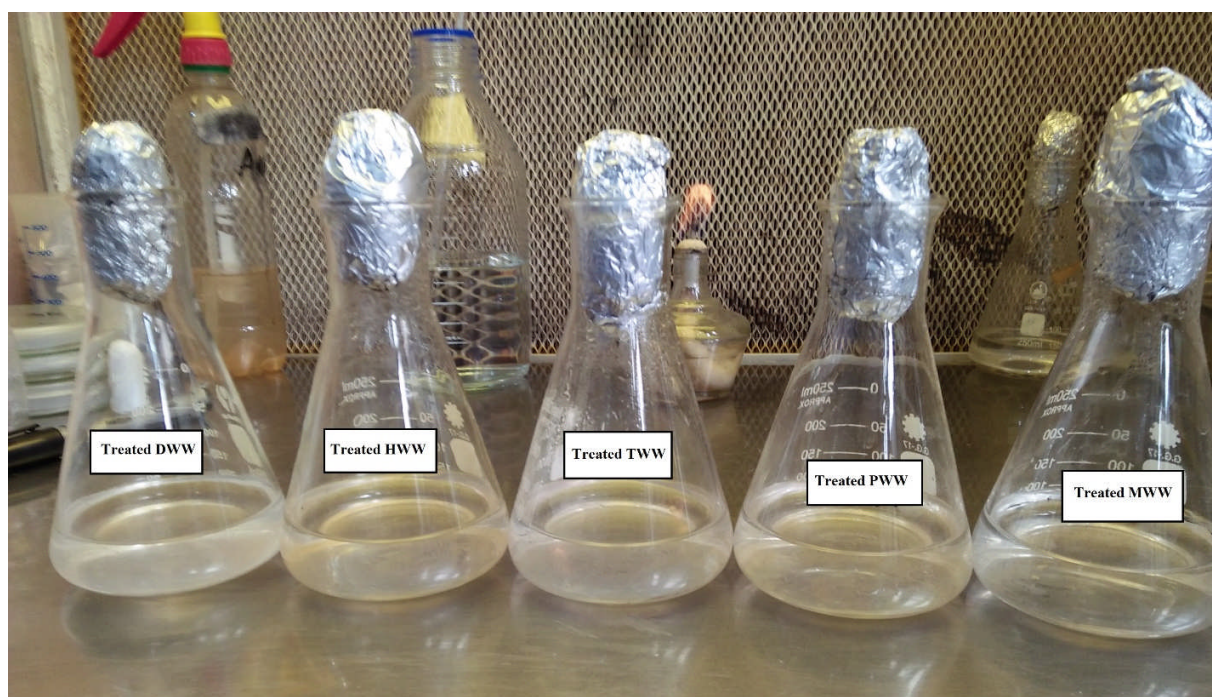
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(a) Untreated wastewaters



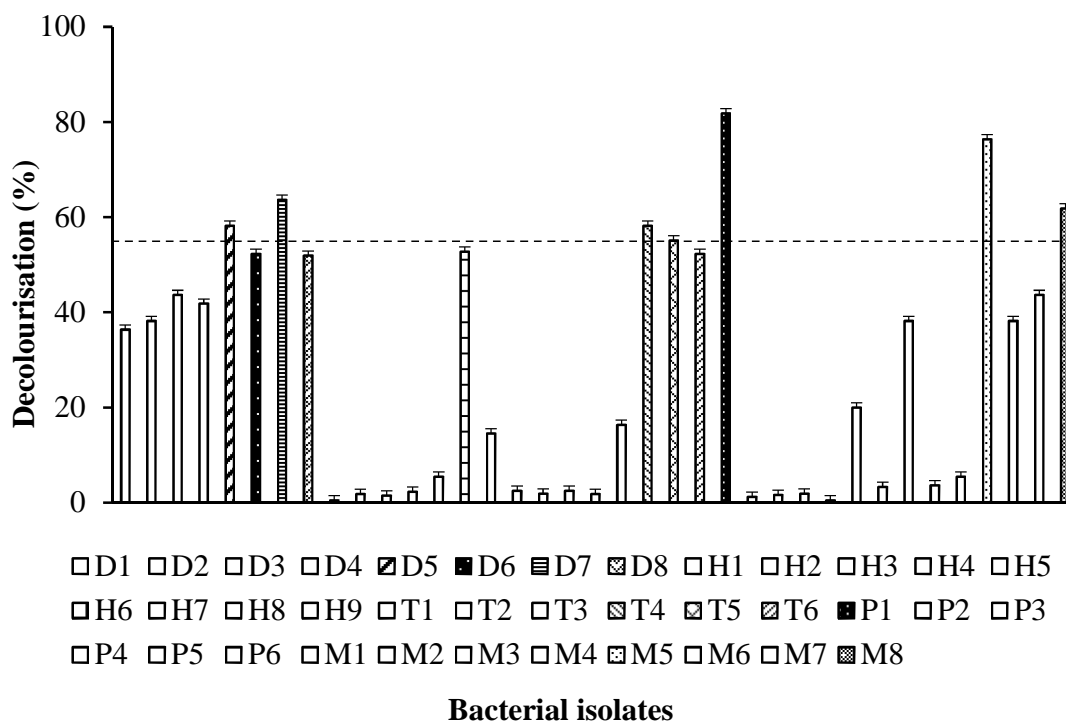
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(b) Treated wastewaters

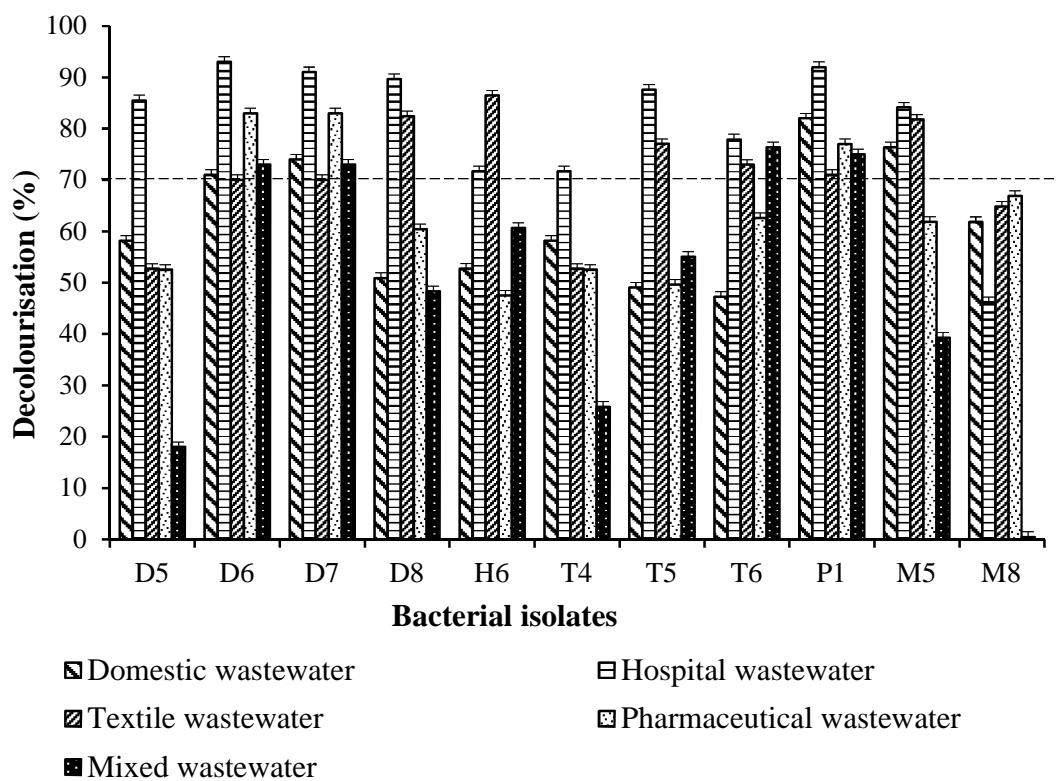
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Figure 1: Comparison of wastewaters before and after decolourisation



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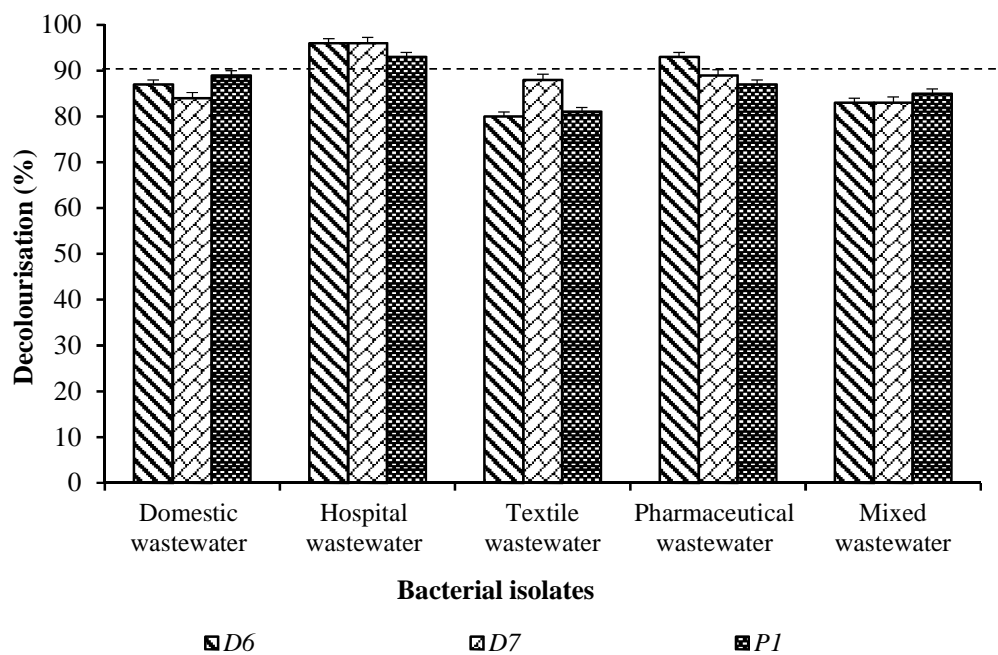
944 **Figure 2. Initial screening from 37 bacterial isolates (>50%); D1-D8 in domestic**
 945 **wastewater, H1-H9 in hospital wastewater, T1-T6 in textile wastewater, P1-P6 in**
 946 **pharmaceutical wastewater and M1-M8 in mixed wastewater**



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948 **Figure 3. Final screening from 11 bacterial isolates (>70%) against domestic,**
949 **hospital, textile, pharmaceutical and mixed wastewaters**

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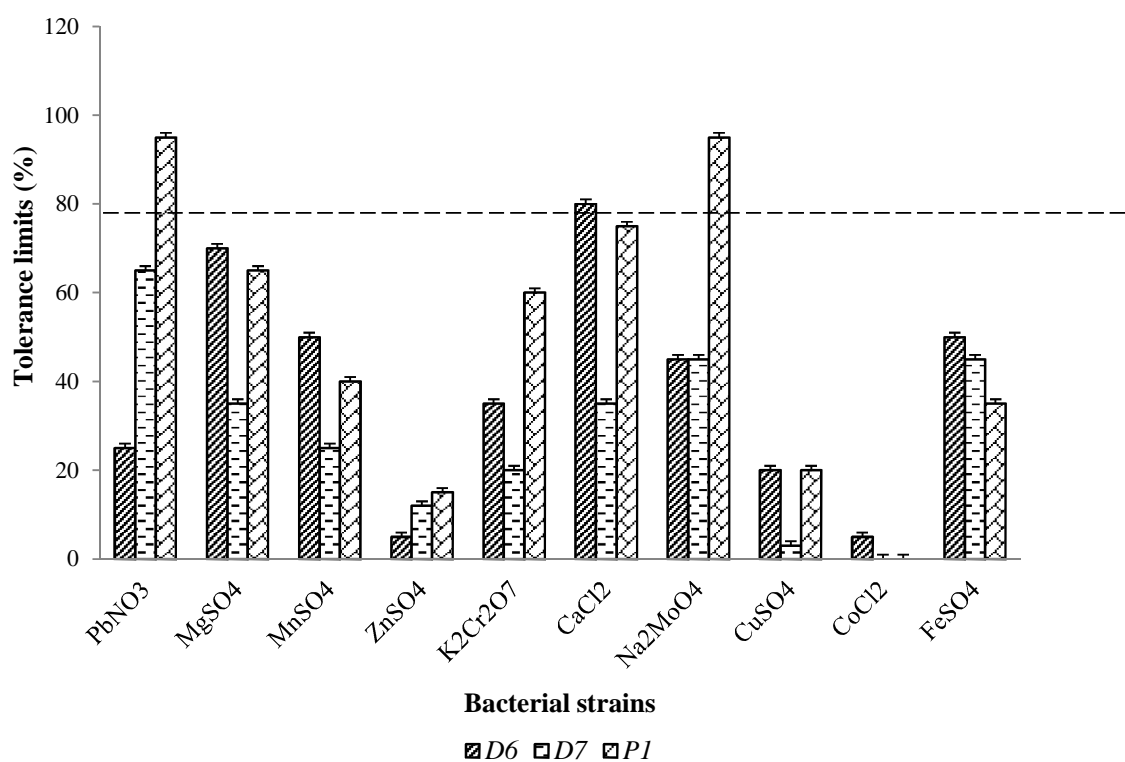
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952 **Figure 4. Decolourisation of domestic, hospital, textile, pharmaceutical and mixed**

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wastewaters against D6, D7 and P1

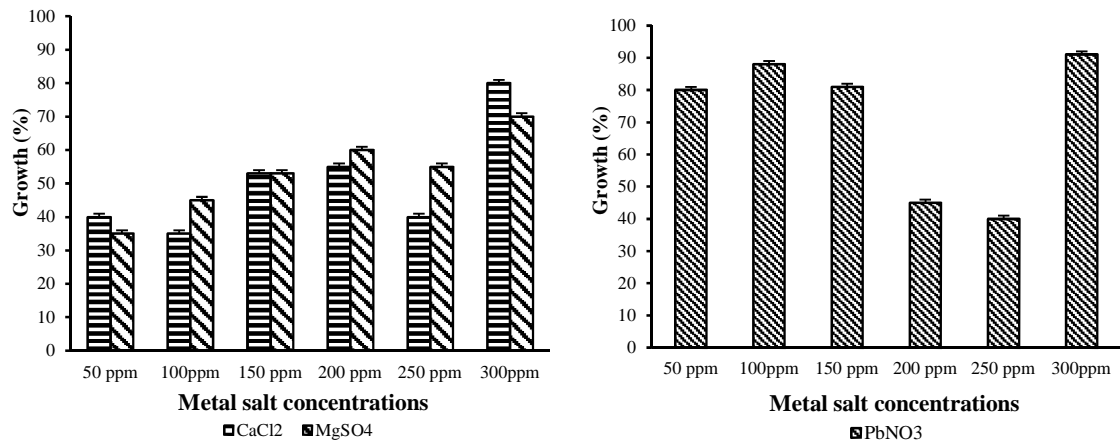
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Figure 5. Tolerance limits of bacterial isolates against metal salts (>65%)

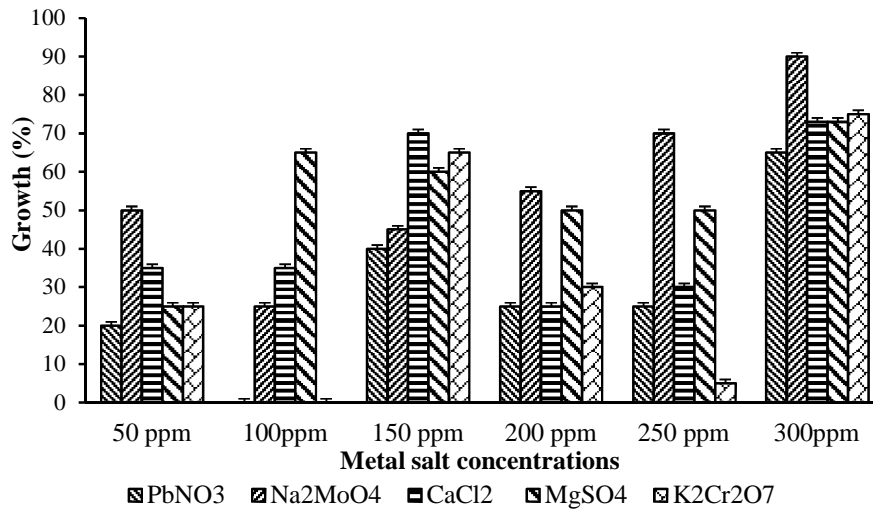


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(a)

(b)



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(c)

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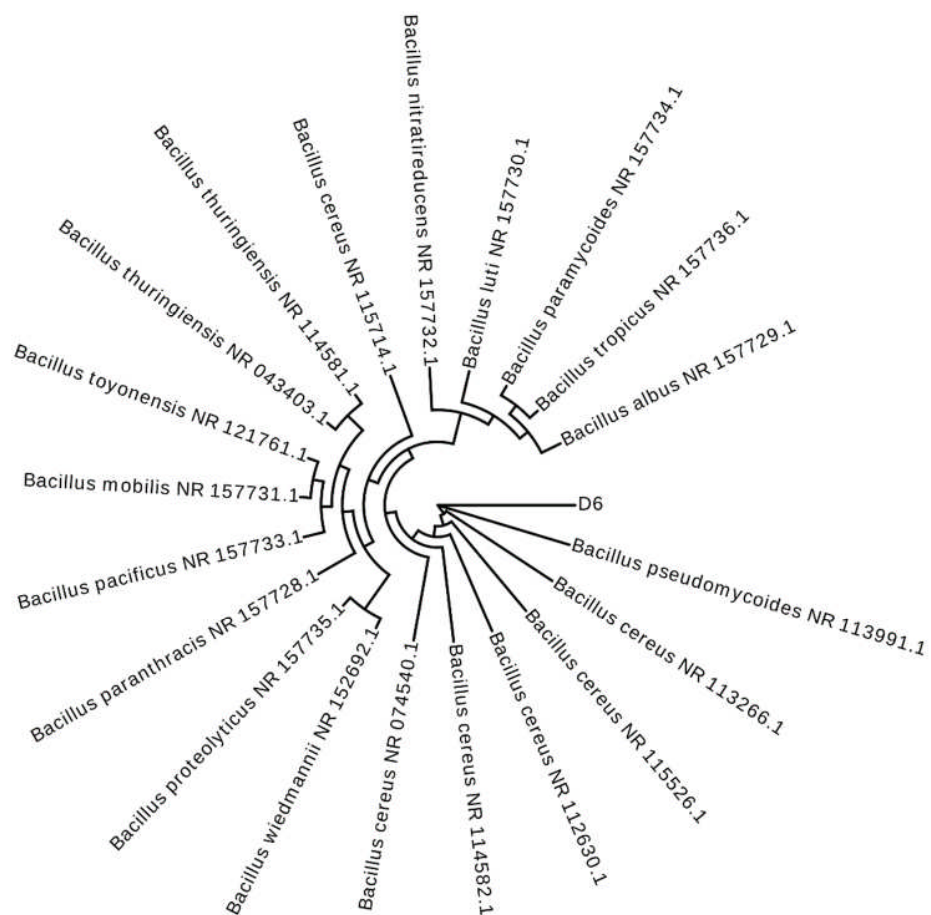
Figure 6. Maximum tolerance limits of bacterial isolates to metals salts solutions at 50, 100, 150, 200, 250 and 300 ppm concentrations (a) D6, (b) D7, (c) P1

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Figure 7. Phylogenetic distance between D6 isolate and top 20 BLAST sequences

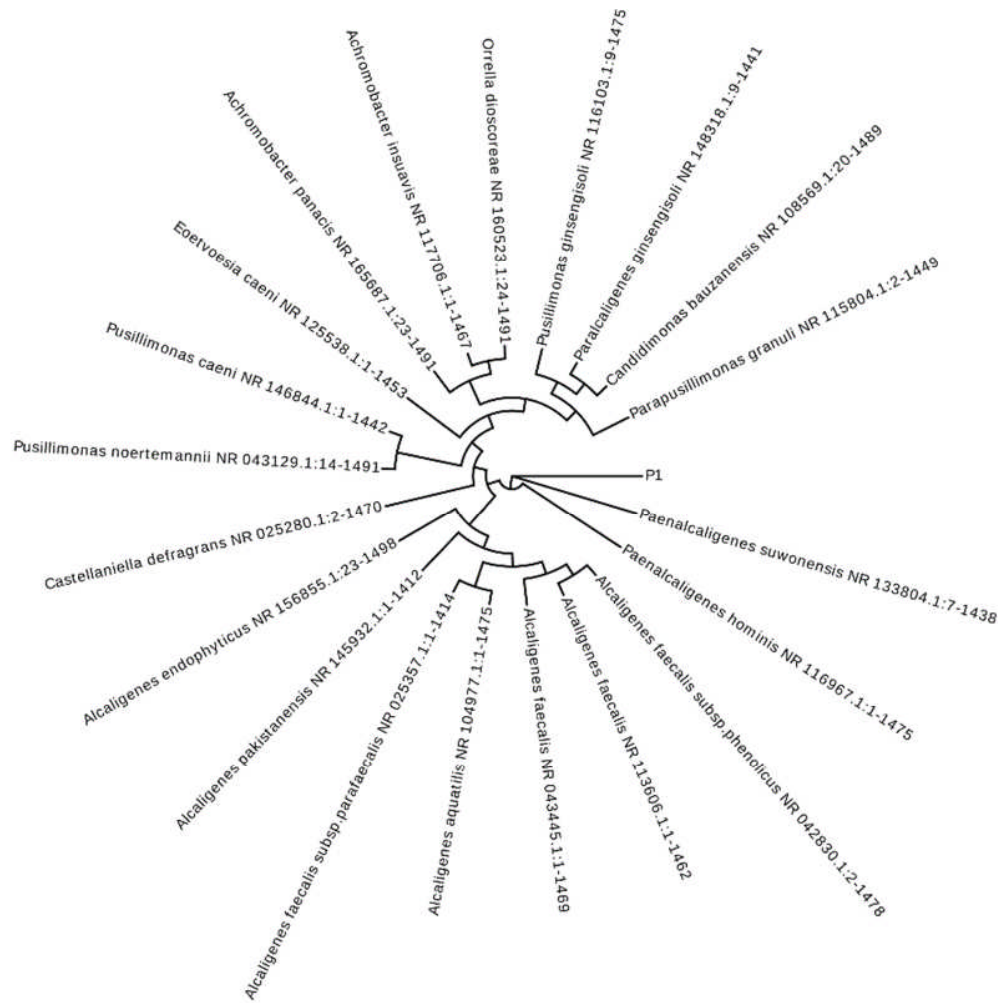


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Figure 8. Phylogenetic distance between D7 isolate and top 20 BLAST sequences

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Figure 9. Phylogenetic distance between P1 isolate and top 20 BLAST sequences

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