Biochemical, molecular and antibiotic resistance profile of multi-potential toluene metabolizing bacteria isolated from tannery effluents

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Abstract:

The focus of present study was to isolate and characterize bacteria which can be effectively used for toluene, a highly recalcitrant pollutant, bioremediation. For isolation of bacteria from the tannery effluents selective enrichment and serial dilution methods were employed. The isolated bacteria were subjected to growth curve analysis, estimation of toluene removal efficiencies, biochemical tests, antibiotic sensitivity assays and molecular characterization based upon 16S rRNA gene. The rRNA genes sequences were analyzed through BLAST to determine similarity index of isolates with bacterial database sequences. To trace the evolutionary history, phylogenetic trees were constructed using MEGA version 7. Total twenty toluene metabolizing bacteria (IUBT1-2, 4-12, 16, 19, 21, 23-26, 28 and 30) were isolated and characterized. Their rRNA gene sequences have been submitted to Genbank. Fifteen of the twenty isolates showed homology to

28 *Brevibacillus agri* strain NBRC 15538, four found similar to *Bacillus paralicheniformis*
 29 strain KJ-16 and one homologous to *Burkholderia lata* strain 383. All bacterial isolates
 30 were resistant to chloramphenicol but sensitive to teicoplanin and linezolid. However,
 31 few (i. e.; IUBT9 and 26) were sensitive to oxacillin. Biochemical characterization
 32 indicated all bacteria positive for alkaline phosphatases (100%). While many were found
 33 positive for p-nitrophenyl N-acetyl β , D-glucosaminidase (35%), hydroxyproline β -
 34 naphthylaminopeptidase (15%), esculinase (65%), mannitol (75%), sorbitol (95%) and
 35 inulin (90%) fermentation. Biochemical profile suggests the use of isolated bacteria for
 36 future exploitation in several fields like bioremediation of toluene, ethanol production,
 37 biomass hydrolysis, biosensors, biofertilizers, as a marker for milk pasteurization in dairy
 38 industries and evaluation of soil quality.

39 **Importance**

40 Toluene is a highly toxic environmental pollutant. We have isolated bacteria which can
 41 be effectively used for the removal of toluene from environmental resources. Moreover,
 42 these bacteria are capable to produce many valuable enzymes which can be used in many
 43 industrial processes for the production of a wide range of products. Further study may
 44 help to exploit these bacterial for the benefit of humanity.

45

46 **Key words:** toluene metabolizing bacteria, bioremediation, toluene removal efficiency,
 47 *Brevibacillus agri*, *Bacillus paralicheniformis*, *Burkholderia lata*, rRNA profiling

48

49 **Introduction**

50 Toluene (methylbenzene) is an omnipresent pollutant and is a cause of concern due to its
 51 resistance to chemical, photolytic and biological degradation, lipophilic nature,
 52 bioaccumulation, long-range transport and wide range adverse effects on environment,
 53 wild life, biota and human health (1). Being lipophilic, it gets accumulated in lipid bilayer
 54 of cell membrane and alters the structure of living cells (2). Toluene is a man-made
 55 hydrocarbon and its major sources in urban air environment are industrial effluents and
 56 motor vehicle emissions (3). It is used as solvent and is essential component of printing
 57 inks, adhesives, rubber, disinfectants, leather tanners, petrol, paints, lacquers and cleaning
 58 agents. Moreover, toluene is added to gasoline fuels to boost octane number (4).

59 Breathing in indoor or ambient air around toluene sources is the cause of toluene
 60 exposure and may lead to various health issues. Low level exposure (200 ppm) promotes
 61 headache, fatigue, slowed reflexes and paresthesia while exposure to moderate levels of
 62 toluene (600 ppm) elevate feelings of confusion and high levels (800 ppm) of toluene
 63 lead to euphoria (5). The other chronic consequences of toluene exposure include
 64 teratogenic effects, neurotoxic and immunotoxic effects, dementia and toluene
 65 leukoencephalopathy (6-8).

66 Due to associated health risks, it is essential to mitigate toluene. Various traditional
 67 techniques like soil vapor extraction, incineration, chemical oxidation, ultraviolet
 68 oxidation and nanotech remedial technology etc. have been used for this purpose (9-12).
 69 However, conventional approaches due to associated limitations like partial toluene
 70 degradation, high operating and maintenance cost, maximum disturbance to land,

71 production of more hazardous substances, transport of contaminated material and
72 increased exposure to toluene for the workers are not preferentially used.

73 The bioremediation has become a preferable technique for toluene detoxification. The
74 process of bioremediation involves scavenging of environmental (water or soil) organic
75 pollutants by naturally occurring or deliberately introduced cultured microbes which are
76 capable to consume and metabolize the contaminants and thus neutralize the environment
77 (13). The bioremedial approach is; far less disruptive and invasive, can be improved by
78 using genetically engineered microorganisms, avoids further pollution, more accessible
79 and cheaper alternative (14). Utilization of bacteria to eradicate environmental pollution
80 is beneficial due to their rapid growth rate, broad metabolic capabilities and diverse
81 enzymes which are active in aerobic as well as anaerobic environments. Moreover
82 bacteria can survive under wide range of temperature and salinity conditions.

83 Variety of toluene metabolizing bacterial species isolated from different sources has been
84 reported (Table 1).

85 Being a highly reduced molecule, toluene needs to be oxidized before its assimilation.
86 For this purpose, bacteria have adopted diverse degradative pathways selectively
87 operating under aerobic or anaerobic conditions (Table 2).

88 Although a number of toluene metabolizing bacteria have been isolated and studied, there
89 is need to find more highly efficient and environment friendly bacteria. Under different
90 habitats, bacteria express different biodegradative enzymes and pathways (15). Moreover,
91 utilization of bacterial consortia with broad spectrum of enzymes will be proven more
92 effective for toluene remediation (16).

93 The industries which manufacture and use toluene are the primary sources of toluene
 94 pollution in the environment. Toluene is a commonly used adhesive in leather industry.
 95 Hence, leather tanning and processing industries and manufacturing units of footwears,
 96 leather bags, leather furniture and other miscellaneous leather goods are the major
 97 sources of toluene exposure. Knowing thing in present study, tannery effluent was
 98 selected as a source sample for the isolation of toluene metabolizing bacteria. It was
 99 hypothesized that we might find some novel bacteria capable to degrade toluene in a
 100 more effective and versatile manner in tannery industry effluents. The accomplishment of
 101 this project might provide an eco-friendly way out to reclaim toluene contaminated
 102 environmental resources.

103 **Materials and methods:**

104 **Medium and growth conditions:**

105 For bacterial isolation, standard culture enrichment technique was employed by involving
 106 use of carbon deficient minimal salt media (17). The carbon deficient minimal salt
 107 medium ($4\text{ g L}^{-1} \text{ KH}_2\text{PO}_4$; $4\text{ g L}^{-1} \text{ Na}_2\text{HPO}_4$; $2\text{ g L}^{-1} \text{ NH}_4\text{Cl}$; $0.2\text{ g L}^{-1} \text{ MgCl}_2$; 0.001 g L^{-1}
 108 CaCl_2 ; $0.001\text{ g L}^{-1} \text{ FeCl}_3$) was prepared and autoclaved at $121\text{ }^\circ\text{C}$ for 20 min. Prior to
 109 inoculation, the medium was supplemented with 1% (v/v) toluene as the only carbon
 110 source. The pH of medium was maintained at 6.8. Isolated bacteria were grown
 111 aerobically on this toluene supplemented media at $50\text{ }^\circ\text{C}$.

112 **Morphology:**

113 Morphological aspects of isolates like gram staining and bacterial cell shape were
 114 documented. The colony forming units (CFUs) were also calculated for all these isolates.

115 **Growth rate:**

116 The growth rate was evaluated indirectly by assessment of turbidity as optical density
117 (OD). The optical density was measured at 600 nm and at different time intervals using
118 UV- visible spectrophotometer. The optical density was plotted versus time to obtain
119 growth curves and determine growth behavior of each isolate.

120 **Toluene removal assay:**

121 For determining the toluene removal rate of bacterial isolates, initially the bacterial
122 culture was centrifuged. The supernatant containing the residual toluene of the medium
123 (3ml) was extracted using 3ml n-hexane. Followed by this, the n- hexane containing
124 toluene was separated using a separating funnel. The optical density of toluene was
125 measured against a blank at 260 nm wavelength (Supplementary Data Figure 1). Medium
126 containing toluene without bacterial culture was used as blank. Toluene degradation
127 efficiency has been calculated by following formula;

128
$$\text{Toluene degradation efficiency (\%)} = [T_i - T_f / T_i] \times 100$$

129 Whereas; T_i = initial absorbance of toluene

130 T_f = final absorbance of toluene

131 **Morphological and Biochemical characterization:**

132 The isolated bacteria were characterized by gram staining, colony morphology (18) on
133 nutrient agar medium and morphological characteristics. For biochemical identification
134 of bacteria, a qualitative micromethod of Remel RapID STR System (19) was used. In
135 this method, toluene metabolizing bacteria were subjected to different biochemical tests.

136 i. e.; arginine dehydrolase test, esculinase test, mannitol fermentation test, sorbitol
137 fermentation test, raffinose fermentation test, inulin fermentation test, ρ -nitrophenyl- α ,D-
138 galactosidase test, Tyrosine β -naphthylamidase test, ρ -Nitrophenyl- α ,D-glucosidase test,
139 ρ -Nitrophenyl-n-acetyl- β ,D-glucosaminidase test, Lysine β -naphthylamidase test,
140 alkaline phosphatase test and pyrrolidonyl peptidase test.

141 **Antibiotic susceptibility test:**

142 Antibiotic test was performed for all the isolated bacteria by using disc diffusion method
143 (20). Antibiotics tested were teicoplanin (30 μ g), linezolid (30 μ g), linezolid (10 μ g),
144 oxacillin (1 μ g) and chloramphenicol (30 μ g).

145 **Molecular characterization by 16S rRNA gene sequence analysis:**

146 For molecular characterization of bacterial isolates their genomic DNA was extracted by
147 organic method (21). The 16S rDNA gene was amplified by polymerase chain reaction
148 (PCR) using the rDNA specific primers F1 and R1 (Supplementary Data Table 1). The
149 region of 16S rRNA gene targeted by these primers is shown (Supplementary data Figure
150 2). In 50 μ l of PCR reaction mixture, 50 ng of template DNA, 5 μ l of 10X PCR reaction
151 buffer (Mg^{++} free), 5 μ l of $MgCl_2$, 1 μ l of 10mM dNTPs, 2 μ l of 10 pM forward primer, 2
152 μ l of 10 pM reverse primer, 0.25 μ l of Taq DNA polymerase and 29.75 μ l of nuclease
153 free water was used. PCR amplification conditions were as follows; initial denaturation at
154 95 °C for 5 min, , 38 cycles consisting of denaturation at 94 °C for 40 seconds, annealing
155 at 58 °C for 40 seconds and extension at 72 °C for 30 seconds. The final extension of
156 PCR products was carried out at 72 °C for 10 min. After PCR amplification, the PCR
157 products were resolved on agarose gel. The PCR products were purified by Monarch

158 DNA Gel Extraction Kit and sequenced bidirectionally using sequencer based on
159 principle of Sangers Chain Termination. For this purpose, samples were sent to
160 MacroGen, Korea. FASTA sequences obtained from MacroGen were subjected to
161 microbial databases similarity searches through NCBI (National Center for
162 Biotechnology Information) BLAST analysis
163 (<http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi>). Sequences of highest similarity obtained
164 through BLAST analysis were recorded. Clustal omega multiple sequence alignment
165 software (22) was used to align the sequences. The sequences were refined by removing
166 the gaps and only ungapped aligned regions were analyzed by MEGA7 software to
167 perform phylogenetic analysis of the identified bacteria. Phylogenetic trees were
168 constructed to show the evolutionary relationship among the identified bacteria. Different
169 parameters used for phylogenetic tree construction included neighbor joining statistical
170 method, maximum composite likelihood substitution method and Bootstrap analysis with
171 1000 replicates.

172 Sequences of twenty isolates have been submitted to NCBI and accession numbers
173 assigned are; IUBT1 (MH014864), IUBT2 (MG241284), IUBT4 (MH014871), IUBT5
174 (MH014859), IUBT6 (MH266116), IUBT7 (MH201270), IUBT8 (MG190354), IUBT9
175 (MG190357), IUBT10 (MG263995), IUBT11 (MH266117), IUBT12 (MG263996),
176 IUBT16 (MG190391), IUBT19 (MH014861), IUBT21 (MH014862), IUBT23
177 (MG263997), IUBT24 (MG190870), IUBT25 (MH266118), IUBT26 (MG263998),
178 IUBT28 (MH014868) and IUBT30 (MH014869).

179 **Results**

180 **Morphology:**

181 All the bacteria were found to be gram positive and rod shaped. Their Colony Forming
182 Units (CFUs) have been calculated and are given (Supplementary Data Table 2). The
183 bacteria IUBT1, IUBT2, IUBT4 and IUBT5 were isolated from the plate inoculated with
184 original culture without any dilution.

185 **Molecular characterization of toluene metabolizing bacteria:**

186 Ribotyping of toluene metabolizing bacteria revealed three groups among twenty isolates
187 (Figure 1). Group 1 comprised of single isolate IUBT1, homologous to *Burkholderia lata*
188 strain 383. Group 2 included fifteen bacteria homologous to *Brevibacillus agri* strain
189 NBRC 15538 while group 3 contained isolates IUBT4, IUBT24, IUBT28 and IUBT30
190 showing homology to *Bacillus paralicheniformis* strain KJ-16. All sequences were
191 deposited to Genbank database (www.ncbi.nlm.nih.gov/genbank) and assigned accession
192 numbers.

193 **Phylogenetic analysis of bacterial isolates:**

194 To study the evolutionary relationships between the isolated toluene metabolizing
195 bacteria phylogenetic trees were constructed. Bacterium IUBT1 from group 1, was
196 assigned to genus *Burkholderia*. Study of its evolutionary relationship revealed its
197 common ancestry with *Burkholderia lata strain 383* (Accession number NR 102890) as
198 both originated from the same node (Figure 2). The similarity percentage between two
199 strains was 99%.

Four bacterial isolates IUBT4, IUBT24, IUBT28 and IUBT30 were assigned to genus *Bacillus*. All shared common ancestor, belonged to same clade and shared close similarity with *Bacillus paralicheniformis* strain KJ-16 (Figure 3).

Among the 20 isolates in this study, 15 were assigned to genus *Brevibacillus* in Group 2. The phylogenetic study revealed that IUBT7 is distantly related with rest of the isolates and same is the case of IUBT9. Both of these are not sharing clade with any other isolate. IUBT12 and 19 are sharing the same clade. IUBT21 and IUBT23 are related through strong bootstrap value of 99. IUBT5 and IUBT11 are also closely related because they are converging at a common ancestor. IUBT6 is distantly related with IUBT26 through bootstrap value of 22. IUBT2 is related with *Bacillus glycinifermentans* through strong bootstrap value of 100. IUBT8 and IUBT10 are more related with each other as compared to IUBT16 and IUBT25. IUBT25 is sharing the same lineage with *Brevibacillus gelatini* strain through bootstrap value of 48 (Figure 4).

Biochemical characterization:

The isolated bacteria were screened for fourteen biochemical tests. All of these were negative for arginine dehydrolase (ARG) test, raffinose fermentation (RAF) test, ρ -nitrophenyl- α , D-galactoside (GAL) test, tyrosine β -naphthylamide (TYR) test, ρ -nitrophenyl- α , D-glucoside (GLU) test and lysine β -naphthylamide (LYS) test. The positive response was observed for the esculinase (ESC) test, mannitol fermentation test (MNL), sorbitol fermentation test (SBL), inulin fermentation test (INU), hydroxyproline β -naphthylamide (HPR) test, ρ -nitrophenyl-n-acetyl- β -D-glucosaminide (NAG) test, ρ -nitrophenyl phosphate (PO_4) test and pyrrolidine β -naphthylamide (PYR) test. Thirteen

222 bacteria were esculinase producers and can be exploited for improved ethanol production
 223 through increased saccharification of cellulose. All except two (IUBT8 and 16) were
 224 capable of fermenting inulin and fifteen (IUBT2, 4, 5-7, 9, 11-12, 19, 21, 2-26 and 30)
 225 were mannitol fermenting bacteria. All the isolates were positive for sorbitol fermentation
 226 except IUBT16. Seven were n-acetyl glucosaminidase producers and possess potential for
 227 future applications like single cell protein (SCP) production, for conversion of chitinous waste
 228 into biofertilizers and as an additive in food industry. Only two (IUBT5 and 19) were
 229 hydroxyproline β -naphthylamidases producers and can be utilized in pharmaceutical
 230 industries. All isolates were positive for PO_4 test and thus can be employed in
 231 diagnostics immunoassays, in biosensors for environment monitoring, as biofertilizers, in
 232 soil quality evaluation, in limnological studies and dairy industries. Only one bacterium
 233 IUBT5 was positive for PYR test and can be utilized in protein sequencing to free the
 234 amino terminal amino acid being blocked by pyrrolidonyl group. This analysis helped us
 235 to find out unique characteristics of Group 2 and Group 3 members. Biochemical
 236 behavior of IUBT10, IUBT8, IUBT19, IUBT5 and IUBT16 was different from each
 237 other and from ten other *Brevibacilli* of Group 2. So, group 2 members were further
 238 categorized into two major groups. The isolates IUBT2, IUBT6, IUBT7 and IUBT26
 239 were similar biochemically so placed in the group G2B. These bacteria were sorbitol and
 240 inulin fermenting and esculinase, p-nitrophenyl-n-acetyl- β -D-glucosaminidase and p-
 241 nitrophenyl phosphatase producers. The IUBT9, IUBT11, IUBT12, IUBT21, IUBT23
 242 and IUBT25 were placed in group G2C as they all were mannitol, sorbitol and inulin
 243 fermenting and esculinase and p-nitrophenyl phosphatase producers. All the four isolates
 244 from Group III were found biochemically different. Hence, biochemical analysis
 245 confirmed their distinctiveness (Supplementary Data Table 3).

246 **Growth study:**

247 To sort out the unique bacteria among those having same molecular and biochemical
248 profile their growth behavior was studied. Assuming that same bacteria must possess
249 same growth pattern, the isolates IUBT2, IUBT26, IUBT11 and IUBT12 were found
250 unique in their growth behavior (Supplementary data Table 4). Results helped to identify
251 three groups of bacteria G2B2 consisting of IUBT6 and IUBT7, G2C3 containing IUBT9
252 and IUBT21 and G2C4 including IUBT23 and IUBT25 (Supplementary Data Figure 3).
253 The members of each group exhibited similar growth pattern hence, they might be the
254 same bacteria.

255 **Toluene removal efficiency:**

256 Toluene removal efficiency assay served as fourth layer of screening and helped to
257 differentiate among bacteria exhibiting similarity in molecular, biochemical and growth
258 behaviors. The toluene removal potentials for IUBT6, IUBT7, IUBT23 and IUBT25 were
259 found to be $83\% \pm 1.41$, $93\% \pm 4.95$, $28\% \pm 4.24$ and $45\% \pm 10.6$ respectively
260 (Supplementary data Figure 4). The IUBT9 and IUBT21 had toluene removal potentials:
261 $83\% \pm 2.12$ and $83\% \pm 0.70$, respectively. However, IUBT9 and IUBT2 might be the
262 same bacteria due to similarity in their molecular aspects, biochemical behavior, growth
263 pattern and toluene removal efficiencies.

264 **Antibiotic profile:**

265 All of the twenty isolates were tested for their antibiotic susceptibility (Supplementary
266 Data Table 5). All of the isolates were sensitive to teicoplanin and linezolid. Among these
267 isolates, 100% were resistant to chloramphenicol and 90% to oxacillin. Only two of

these, IUBT9 and IUBT26 showed sensitivity to oxacillin and 100% showed sensitivity to teicoplanin and linezolid. The zone of inhibition was measured for the antibiotics to which bacteria exhibited susceptibility. The maximum zone of inhibition was recorded 39 mm against the antibiotic linezolid (10µg) in case of IUBT19. In case of teicoplanin, linezolid (30µg) and oxacillin, maximum zones of inhibition were 35 mm (IUBT6), 37 mm (IUBT16, 26 and 30) and 30 mm (IUBT26), respectively. A comparison of zone of inhibitions for different antibiotics is given (Supplementary Data Figure 5).

Discussion:

Grown on minimal salt medium (MSM) supplemented with 1% toluene, morphological, biochemical and molecular analyses of all isolates (n=20) revealed their capability to metabolize toluene as the only source of carbon.

Morphology

Morphologically these strains were found to be rod shaped and gram positive bacteria. Our results are consistent with earlier studies because most of the toluene metabolizing bacteria reported in literature are gram positive: *Bacterium* Ex-DG74 (17), *Mycobacterium* sp. IBB_{pol} (23), *Mycobacterium* strains T103 and T104 (24). However, contrary to our findings previous studies have reported few toluene metabolizing gram negative bacteria (23).

Molecular characterization

Traditional bacterial identification based on phenotypic characteristics is not a precise method in comparison with genotypic tools. Similarly through the 16S rRNA gene profiling the poorly described, phenotypically anomalous, rarely isolated strains and even

uncultured strains can be identified (25). In present study, the molecular characterization of isolates showed homology with three different genera and species. i. e.; *Brevibacillus agri*, *Burkholderia lata* and *Bacillus licheniformis*. The *Burkholderia* and *Bacillus licheniformis*, are known to possess toluene degradation ability (26-28). Although a thermophilic bacterium (growth temp. 45 °C) *Brevibacillus agri* strain 13 has been reported to grow and tolerate high concentrations of toluene (5% and 20%, v/v) but it was not capable of utilizing toluene as sole carbon source (29). Hence, to the best of our knowledge, this is the first study reporting isolation of toluene metabolizing *Brevibacillus agri* strain. Similarity score of less than 97% could be considered as an indicative of a new species within a known genus. Moreover, isolate IUBT7 with 89% similarity could be considered as an archetype of new species within the genus *Brevibacillus*.

301 **Biochemical characterization**

To better comprehend the ongoing metabolic activities isolates were subjected to biochemical analysis. Although previous studies have performed biochemical characterization of toluene metabolizing bacteria through standard biochemical tests (30, 31). Esculinase, hydroxyproline β -naphthylamidase and alkaline phosphatase activities of toluene degrading bacteria, have not been yet studied.

Esculinase (ESC) test is an assay used for the detection of beta-glucosidase enzyme, also known as esculinase. It hydrolyzes esculin into esculetin and dextrose. Followed by this esculetin and ferric chloride of the medium react and black brown color is produced. Thirteen isolates (IUBT1-2, 6-7, 9-12, 16, 21, 23, 25-26) showed positive result for esculinase. Few gram positive strains like *Enterococcus faecium*, *Enterococcus faecalis*

312 and *Streptococcus bovis* are known to be positive for esculinase test. This test aids in
 313 differentiating among few families of *Enterobacteriaceae*. Esculinase or β - glucosidase
 314 enzyme due to its hydrolytic activity can be effectively used for biofuel production,
 315 synthesis of antitumor agent (aglycone moiety), for removing bitterness from citrus fruit
 316 juices, cooked soybean syrup, unripe olive and for detoxification of cassava. By reverse
 317 hydrolysis, this enzyme can also be used for synthesis of o-alkyl glucoside which can be
 318 used in food industry, extraction of organic dyes, in cosmetics and pesticides formulation
 319 (32). In this study, thirteen isolates (IUBT1-2, 6-7, 9-12, 16, 21, 23 and 25) have been
 320 found positive for this test. As esculinase enzyme has wide range of applications, hence
 321 the isolates found positive hold multipurpose potential.

322 Mannitol, sorbitol, raffinose and inulin fermentation tests were performed to detect the
 323 capability of bacteria to utilize the carbohydrate substrates like mannitol, sorbitol,
 324 raffinose and inulin respectively. Bacteria usually transform mannitol, raffinose and
 325 inulin into acidic products which reduce the pH of medium, change the color of indicator
 326 added to the medium. Our 75% isolates were positive for mannitol test, 95% were
 327 positive for sorbitol test, 90% were positive for inulin test while none of the isolates
 328 showed positive result for raffinose test. In literature, toluene metabolizing bacteria have
 329 been reported to exhibit mannitol and sorbitol fermenting potential (31). However, this is
 330 the first study reporting on inulin and raffinose fermenting capability of toluene
 331 metabolizing bacteria. Bacterial inulases have tremendous industrial applications and can
 332 be utilized for the production of fructose syrup, gluconic acid, inulooligosaccharides,
 333 lactic acid, mannitol, ethanol and 2,3- butanediol (33). Hence, the isolates found to
 334 possess inulases can be exploited for these purposes.

335 Hydroxyproline β -naphthylamide (HPR) test detects the presence of hydroxyproline β -
 336 naphthylamidase enzyme which catalyzes the hydrolysis of hydroxyproline β -
 337 naphthylamide and β -naphthylamine. In present study, three bacterial isolates were found
 338 positive for this test.

339 The p -Nitrophenyl-n-acetyl- β -D-glucosaminide (NAG) test is a biochemical assay to
 340 detect p -Nitrophenyl-n-acetyl- β -D-glucosaminidase (NAGase) in bacteria. This enzyme
 341 hydrolyzes p -nitrophenyl substituted glycoside and releases p -Nitrophenol. In present
 342 study, seven isolates showed positive result for this test. Few toluene metabolizing and
 343 glucoaminidase producing strains have been reported (31). A strain *Burkholderia*
 344 *pseudomallei* is also known for the production of this enzyme (34). This enzyme can be
 345 employed for analyzing complex sugar chains in glycopeptides and glycoproteins,
 346 synthesis of variety of biologically important compounds and as biocontrol agents etc
 347 (35).

348 The p -nitrophenyl phosphate (PO_4) test confirms the presence of phosphatase enzyme
 349 which expedites the breakdown of p -nitrophenyl phosphate to yellowish p -nitrophenol.
 350 All the bacterial isolates of our study analyzed for this test showed positive result. This
 351 enzyme has diverse applications such as in immunoassays, biomedical industry due to its
 352 resistance to denaturation, biosensors for environmental monitoring, as a marker for
 353 adequate milk pasteurization, bioremediation agent to mineralize organophosphates and
 354 evaluation of heavy metals precipitation from industrial effluents (36)

355 Pyrrolidine beta-naphthylamide (PYR) test facilitates the detection of enzyme L-
 356 pyrroglutamyl amino-peptidase found in *Streptococcus pyogenes* which hydrolyzes the

pyrrolidine β - naphthylamide yielding β - naphthylamide. In present study, only one bacterium showed positive result for this test. This enzyme has potential applications in protein sequencing to release the amino acid blocked by pyrrolidonyl group at N-terminus and bacterial diagnosis (37).

The isolates of present study being the source of variety of enzymes can be destined for utilization in scientific and industrial entities. They can be employed in areas like clinical diagnosis, food industry, dairy technology, agroecosystem, molecular biology, genetic engineering and environmental remediation of pollutants.

Growth study:

Majority of the isolates (n=14) were slow growing and found to exhibit logarithmic growth until 48 hours (IUBT3, 19 and 24), 51 hours (IUBT1, 26 and 28), 68 hours (IUBT5, 8-9, 12, 14 and 21) and even until 74 hours (IUBT30). Consistent with our study, four slow growing toluene metabolizing bacteria, exhibiting slow growth rate with exponential growth phase until 90 hours, have been reported (38). The isolates IUBT2, IUBT4, IUBT5, IUBT6 and IUBT8 showed 0.4-0.5 optical density (OD) at 600 nm during the log phase which is consistent with the optimum optical density (OD) of toluene metabolizing bacteria reported in literature (38). The maximum OD at 600 nm reported for toluene metabolizing bacteria so far is 0.8 (17). While in present study IUBT16 and IUBT25 showed OD of 0.9 and IUBT9, IUBT12, IUBT21, IUBT23, IUBT24 and IUBT28 showed OD of 1.0. Hence, present work reports the maximum biomass growth of toluene metabolizing bacteria.

Toluene removal efficiency:

379 Toluene removal efficiencies of bacteria were determined by toluene removal assay. The
 380 isolates having highest toluene removal efficiency (Figure 2) were IUBT16 ($93\% \pm 1.41$),
 381 IUBT1 ($90\% \pm 2.83$) and IUBT19 ($90\% \pm 0$), IUBT2 ($89\% \pm 1.41$), and IUBT4 ($89\% \pm$
 382 0.71). The observed toluene removal efficiencies are consistent with those reported in
 383 earlier literature .i.e.; 92.4% for bacterium J2, 84.8% for bacterium J6 (39). However, the
 384 highest toluene metabolizing efficiencies (99 and 98%) have been reported so far for
 385 *Rhodococcus erythropolis* and *Alcaligenes xylosoxidans* respectively
 386 (40, 41). The isolates with minimal toluene removal were found to be IUBT23 ($28\% \pm$
 387 4.24) and IUBT26 ($50\% \pm 9.19$). However, in literature minimum toluene removal
 388 efficiency has been reported to be 43% and 49% in rhizosphere bacterial community (42).

389 **Antibiotic profiling:**

390 All of the twenty bacteria reported can be used for toluene remediation. But there may be
 391 some associated potential risks due to their virulent nature. Although this study has not
 392 detected virulence status of isolates, the antibiotic resistance profiling may give us a clue
 393 about their user friendly nature. Environment friendly bacteria should not possess
 394 antibiotic resistance. Moreover, the exploitation of these bacteria to remediate toluene
 395 can be decided on the bases of their sensitivity as well as resistance to the drugs.
 396 Application of a whole bacterial cell to deal unfavorable toluene burden can only be
 397 suggested, if it is drug sensitive. Hence, it was crucial to test the isolated bacteria for
 398 antibiotic resistance. All of the bacteria were gram positive so we selected the antibiotics
 399 which are most commonly used for treatment of infections caused by gram positive
 400 bacteria .i.e; teicoplanin, linezolid, oxacillin and chloramphenicol. All of the bacteria in
 401 our study have shown resistance to some of the antibiotics so the whole cell remediation

402 will not be recommended. Rather their enzymes and genes associated with toluene
403 degrading pathways can be beneficially used and three approaches should be followed in
404 this regard.

405 (a) If the required enzymes are extracellular then nanoparticles can be synthesized by
406 using supernatant and can be destined for remediation.

407 (b) If intracellular enzymes are associated with toluene catabolism then these
408 enzymes and other proteins can be purified from cell lysate for synthesis of
409 nanoparticles.

410 (c) Associated genes can also be identified and can be cloned into any environmental
411 friendly bacteria for bioremediation.

412 **Conclusion:**

413 Twenty thermophilic bacteria have been isolated which are capable of toluene
414 catabolism. Biochemical analysis has revealed other catabolic attributes possessed by
415 these bacteria which can be exploited for different industrial applications. There is still
416 need to elucidate the pathways associated with toluene degradation. Study of genes
417 encoding enzymes, factors as well as conditions contributing to maximum toluene
418 breakdown would help to exploit their potential in an environmentally favorable way. By
419 combining knowledge about these attributes of bacteria and bioremediation approach,
420 toluene degrading potential of these bacteria can be harnessed to mitigate toluene
421 pollution in the environment.

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Table 1: Toluene metabolizing bacteria

Bacterial species	Source	Reference
<i>Bacillus cereus</i> THH39	Oil polluted soils	(43)
<i>Magnetospirillum</i> sp. strain 15-1	Rhizospheres of constructed wetlands.	(44)
<i>Bacterium</i> Ex-DG74	Wastewater	(17)
<i>Pseudomonas aeruginosa</i> UKMP-14T and <i>Bacillus cereus</i> UKMP-6G	oil contaminated soil	(45)
<i>Acinetobacter</i> <i>genospecies</i> Tol 5	gasoline contaminated sediment at a gas station	(46)
<i>Pseudomonas aeruginosa</i> AT18	crude oil contaminated soil	(47)

Table 2: Pathways for aerobic oxidation of toluene

Pathway	Bacteria	Reference
Dioxygenase mediated pathway	<i>Thauera</i> species strain DNT-1	(48)
Toluene-2-monooxygenase pathway (TOM)	<i>Burkholderia cepacia</i> G4	(49)
Toluene-3-monooxygenase pathway (TBU)	<i>Ralstonia pickettii</i> PKO1	(50)
Toluene-4-monooxygenase pathway (TMO)	<i>Pseudomonas mendocina</i> KR1	(51)
Ortho cleavage pathway	<i>Pseudomonas mendocina</i> KR1	(27)
Meta cleavage pathway via catechol	<i>Burkholderia cepacia</i> G4	(26)
Tod pathway	<i>Pseudomonas putida</i> F1	(52)

Figure 1: Flowsheet for twenty toluene metabolizing bacteria: The molecular, biochemical, growth pattern and toluene removal analysis data showed eighteen bacteria IUBT1, IUBT2, IUBT4, IUBT5, IUBT6, IUBT7, IUBT8, IUBT9, IUBT10, IUBT11, IUBT12, IUBT16, IUBT23, IUBT24, IUBT25, IUBT26, IUBT28, IUBT30 were unique. And two bacteria IUBT9 and IUBT21 might be the same on the basis of similarity in their toluene removal efficiencies.

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634 **Figure 2:** Neighbour joining tree based on 16S rRNA gene sequence for IUBT1 and its
635 closest relatives. Numbers with the tree nodes are depicting percentage bootstrap support
636 for 1000 replicates.

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652 **Figure 3:** Neighbour joining tree illustrating the evolutionary relationship between

653 IUBT4, IUBT24, IUBT28, IUBT30 and other related strains.

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662 **Figure 4:**

663 Neighbour joining tree illustrating phylogenetic relationship of isolates assigned to genus

664 *Brevibacillus* and other related strains.

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