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Environmental Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tent20

CHARACTERIZATION OF A PHENOL-DEGRADING BACTERIUM ISOLATED FROM AN INDUSTRIAL EFFLUENT AND ITS POTENTIAL APPLICATION FOR BIOREMEDIATION

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Available online: 11 Jun 2012

To cite this article: Cintia E. Paisio, Melina A. Talano, Paola S. González, Eloisa Pajuelo-Domínguez & Elizabeth Agostini (2012): CHARACTERIZATION OF A PHENOL-DEGRADING BACTERIUM ISOLATED FROM AN INDUSTRIAL EFFLUENT AND ITS POTENTIAL APPLICATION FOR BIOREMEDIATION, Environmental Technology, DOI:10.1080/09593330.2012.701238

To link to this article: http://dx.doi.org/10.1080/09593330.2012.701238

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CHARACTERIZATION OF A PHENOL-DEGRADING BACTERIUM ISOLATED FROM AN INDUSTRIAL EFFLUENT AND ITS POTENTIAL APPLICATION FOR BIOREMEDIATION

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23 Abstract

24

25 The use of native microorganisms is a useful strategy for phenol bioremediation. In the
26 present work, a bacterial strain, named RTE1.4, was isolated from effluents of a chemical
27 industry. The strain was able to grow at high concentrations of phenol and its derivatives,
28 such as guaiacol, 2,4-dichlorophenol and pentachlorophenol, as well as in a medium
29 containing industrial effluents. This bacterium was identified as *Acinetobacter* sp. using
30 morphological, physiological, biochemical and 16S rRNA gene analysis. *Acinetobacter* sp.
31 RTE1.4 degraded phenol (200 to 600 mg/L) at wide pH range and temperature (5-9 and 25-
32 37 °C, respectively) demonstrating high adaptation ability to different conditions. The strain
33 would metabolize phenol by the *ortho*-pathway since catechol 1,2-dioxigenase activity was
34 detected. When bacteria were grown in medium containing phenol, an altered whole-cell
35 protein pattern was observed by SDS-PAGE, with the lack of some low-molecular mass
36 polypeptides and an increase in the relative abundance of high-molecular mass proteins after
37 treatment. Considering that the use of native strains in bioremediation studies shows several
38 ecological advantages and that the studied bacterium showed high tolerance and
39 biodegradation capabilities, *Acinetobacter* sp. RTE1.4 could be an appropriate
40 microorganism to improve bioremediation and biotreatment of areas polluted with phenol
41 and/or some of its derivatives. Moreover, the establishment of the optimal growth conditions
42 (pH, temperature, concentration of the pollutant) would provide base line data for bulk
43 production of the strain and its use in bioremediation processes.

44

45

46

47 Keywords: phenol removal, optimal growth conditions, protein patterns, catechol 1,2-
48 dioxigenase, *ortho* pathway

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51 ## 1. INTRODUCTION

52

53 Phenol is a toxic substance frequently found in aquatic environments due to
54 contamination derived from many industrial processes, such as oil refineries, chemical
55 industries, explosives and dye manufacturing, wood preservation, pulp and paper production,
56 and coke ovens.

57 Phenol is toxic to several aquatic organisms including anuran amphibians, fishes and
58 crustaceans [1,2]. It is also toxic to humans, inducing carcinogenicity and causing
59 reproductive and developmental toxicity, neurotoxicity, and acute toxicity. Accidental
60 exposure to high phenol concentrations may cause severe local effects, systemic
61 intoxications and even death [3]. Consequently, it is reasonable to assume that living
62 organisms may be at risk when they are exposed to water contaminated with phenol. In this
63 context, due to its high toxicity, phenol has been listed as a priority pollutant by different
64 regulatory agencies such as the US Environmental Protection Agency [4] and the Agency for
65 Toxic Substances and Disease Registry [5]. Therefore, phenol remediation is important for a
66 safe environment.

67 Current chemical procedures for phenol removal from fluid streams involve expensive
68 treatments such as distillation, liquid–liquid extraction with different solvents, adsorption,
69 membrane pervaporation and membrane–solvent extraction [6]. However, in the past few
70 decades, with advances in biotechnology, bioremediation has become one of the most
71 rapidly developing fields of environmental restoration, utilizing microorganisms to reduce the
72 concentration and toxicity of various chemical pollutants. This approach represents a good
73 alternative compared with physico-chemical strategies, which have high costs and produce
74 other toxic end products [7]. Thus, the role of biotechnology in bioremediation is to efficiently

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75 apply existing resources available in nature to clean up contaminated environments. In this
76 sense, scientists are making efforts to find adequate microorganisms, with high metabolic
77 activities that would be able to efficiently remove pollutants. Bioremediation must address
78 multiphasic and heterogenous environments, including clean-up of ground water, soils,
79 lagoons, sludges, and process-waste streams, where there has been either accidental or
80 intentional release of pollutants that pose a risk to human, animal or ecosystem health.
81 Because of this complexity, successful bioremediation is dependent on having proper
82 microorganisms in the right place with an adequate control of environmental factors for
83 degradation to occur [8]. Among the several factors that directly impact on bioremediation are
84 energy sources (electron donors), electron acceptors, nutrients, pH, temperature, and
85 inhibitory substrates or metabolites. Even with some obstacles, bioremediation has been
86 successfully applied for efficient clean-up of environments contaminated with inorganic or
87 organic xenobiotics. Regarding organic compounds, phenol and its derivatives has a high
88 toxicity, as it was pointed out before, hence, there is an increasing interest in isolating and
89 identifying microorganisms with phenol metabolizing capacity. Bioremediation of phenolic
90 compounds can be carried out by many microorganisms including bacteria and fungi, which
91 have been notified as phenol degrading agents and they are often able to completely
92 degrade this pollutant. Among them, bacteria from different genera, such as *Pseudomonas,*
93 *Vibrio, Serratia, Bacillus and Achromobacter* are able to metabolize phenol [9]. Some of
94 these microorganisms have the ability to completely mineralize phenol through the action of
95 several enzymes. In fact, phenol can be metabolized by two major pathways known as *ortho*
96 and *meta* cleavage [10]. Phenol hydroxilase represents the first enzyme in the metabolic
97 pathway of phenol degradation [11]. This enzyme catalyzes the oxidation of phenol to form
98 catechol. In the next step, two enzymes can be induced, catechol 1,2- or 2,3-dioxigenase,
99 which belong to the *ortho* and *meta* ring fission pathways, respectively [10,11]. This ability to
100 use phenol as carbon and energy source is exploited to bioremediate soil, water or effluents

101 contaminated with this compound. However, phenolic compounds can frequently inhibit
102 microbial growth. Thus, the optimization of degradation conditions (pH, temperature,
103 concentration of pollutants) is necessary for bio-oxidative processes in industrial and
104 environmental applications.

105 Phenol degrading microorganisms are frequently isolated from phenol-polluted
106 environments. However, to our knowledge, the isolation of native strains from the proximity of
107 Ctalamochita River (Río Tercero, Argentina) and its use for phenol bioremediation has not
108 been documented. This river receives effluents from domestic activities and mainly from
109 several chemical and petrochemical industries and represents a dangerous polluted area.
110 So, this site was selected to isolate microbes which, after a deep study, could be returned to
111 the same place for bioremediation/bioaugmentation purposes and even more for the
112 treatment of other polluted areas.

113 Thus, the aims of the present study were to (a) isolate and characterize, indigenous
114 bacteria capable to tolerate and degrade phenol, from industrial effluents near Ctalamochita
115 River; (b) investigate phenol degradation as well as establish optimal degradation conditions;
116 (c) study the kinetics of biodegradation, the possible degrading pathways and changes in
117 total protein profiles, in the presence of the pollutant.

118

119 ## 2. MATERIALS AND METHODS

120

121 ### 2.1 Sample collection

122

123 Bacterial strains were obtained from effluents of chemical industries located near Río
124 Tercero city (Córdoba Province), Argentina. Effluent samples were collected in sterile bottles
125 from the end of a channel discharging wastewater into Ctalamochita River.

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127 2.2 Isolation and selection of phenol-degrading strains

128

129 For selection of phenol-degrading strains, appropriately diluted water samples (10^{-3})
130 were spread on agar plates with TY medium [(g/L): 5 tryptone; 3 yeast extract; 0.65 $CaCl_2$; 13
131 agar] supplemented with 100 mg/L phenol (Merck). Cultivation was carried out for 48 h at
132 28±2 °C. Morphologically different colonies obtained from the plates were transferred to agar
133 mineral medium (MM) plates with phenol (100 mg/L). Subsequently, isolated colonies
134 exhibiting visible growth and different morphology were selected and stored for further
135 studies. MM used in these assays contained (g/L): 2.8 Na_2PO_4H; 9 KPO_4H_2; 2.5 NaCl; 1
136 NH_4Cl; 13 agar. Phenol was used as sole source of carbon and energy.
137 An isolated colony, named RTE1.4 until its identification, with capability to fast growth in
138 MM agar containing phenol was selected and identified for further characterization and
139 phenol degradation studies.

140

141 2.3 Screening of tolerance for phenol and its derivatives

142

143 Phenol tolerance as well as tolerance to different phenolic compounds such as 2,4-
144 dichlorophenol (2,4-DCP), guaiacol and pentachlorophenol (PCP) was evaluated. For that,
145 the isolated bacterium was spread in MM agar supplemented with phenol (100 to 1000
146 mg/L), 2,4-DCP (100-1000 mg/L), guaiacol (50-1000 mg/L) and PCP (20-400 mg/L). The
147 maximum tolerated concentration (MTC) was established as the highest concentration of the
148 contaminant at which bacteria could grow, after 7 d at 28±2 °C.

149

150 2.4 Bacterial tolerance in a cork processing wastewater

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152 RTE1.4 strain was tested for its ability to grow in the presence of an effluent derived
153 from the cork industrial process. This effluent usually contains some cork wood extracts, such
154 as phenolic acids, tannic fraction, 2,4,6-trichloroanisol and PCP [12]. Exhausted cork boiling
155 wastewater (after being used for 20-30 cycles) was collected from cork transformation
156 industry. Solid MM was supplemented with 10, 25, 50 and 100% of this liquid effluent. Plates
157 were spread with the strain and incubated at 28±2 °C for 72 h. The bacterial growth in these
158 plates was evaluated.

159

160 2.5 Characterization and identification of bacterial strain

161

162 RTE1.4 strain was subjected to several morphological, physiological and biochemical
163 tests for its characterization and identification.
164 The isolate was characterized by Gram staining kit (Britania) using an Axiolab (Zeiss)
165 microscope. To determine oxidase activity, isolated single colonies were picked from the
166 plate and were gently scratched on oxidase disks (Britania). The change to purple colour of
167 the disc was indicative of a positive test. On one single colony, suspended in 0.9% NaCl, 3%
168 H_2O_2 was dropped and the appearance of effervescence caused by free oxygen release
169 demonstrated the presence of catalase enzyme.
170 A commercially available kit for bacterial biochemical analysis was used in this study
171 (API 20 NE system, BioMerieux® SA). The protocols followed the manufacturer's instructions.
172 The kit monitors 8 conventional and 12 assimilation tests. Results of the biochemical test
173 were analyzed with API web program, which allows identification of the studied
174 microorganism.
175 A genotypic study was used to confirm the identification of RTE1.4 strain. For that, a
176 PCR analysis for 16S rRNA gene was performed with a pair of forward (5' -
177 CCAGCAGCCGCGGTAATACG -3') and reverse (5' -TACCAGGGTATCTAATCC- 3') primers

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178 and the PCR product was sequenced. All the procedures were carried out by "MacroGen"
179 company (Korea). The sequence was compared using BLAST program [13,
180 http://www.ncbi.nlm.lih.gov] and RDP [14, http://www.rdp.cme.msu.edu] and the results were
181 deposited in GenBank, under the accession number JF304543.

182

183 2.6 Phylogenetic analysis

184

185 A phylogenetic tree was constructed based on the 16S rRNA gene sequences. First,
186 multiple alignments of the different 16S rRNA gene sequences downloaded from GenBank
187 were performed using CLUSTAL-X software. The evolutionary distances were calculated by
188 the Tamura-Nei model. Phylogenetic tree was constructed by Neighbor-Joining method by
189 MEGA4 software [15] and a bootstrap analysis of up to 1000 iterations were carried out.

190

191 2.7 Phenol biodegradation experiments

192

193 Biodegradation studies of RTE1.4 strain were carried out at different pH, temperature
194 and initial phenol concentrations. For all the experimental designs, the bacterial strain was
195 grown in TY liquid medium until late exponential phase. Then, this culture was used to
196 inoculate Erlenmeyer flasks (to give an initial optical density (OD) of 0.05) containing 30 mL
197 of liquid MM with phenol as sole carbon source. The flasks were placed in a shaker at 100
198 rpm at controlled temperature. Samples were taken at different intervals depending on each
199 experiment and analyzed for bacterial biomass and phenol consumption, until phenol was
200 completely removed. All experiments were carried out by triplicate set for checking the
201 consistency of data.
202 To establish optimum growth and phenol biodegradation conditions, bacterium was
203 incubated in liquid MM plus 200 mg/L phenol under different temperatures (from 25 to 37 °C)

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204 and pH (from 5 to 11). Assays were performed for two days and then, residual phenol
205 concentration was determined as described in section 2.8.

206 Finally, the potential of RTE1.4 for phenol degradation and the effect of the initial
207 concentration on its degradation ability were assayed under optimal pH and temperature
208 previously determined, with phenol concentrations from 200 to 700 mg/L.

209 All experiments were carried out by triplicate and repeated three times.

210 2.8 Phenol determination

211

212 In all experiments, phenol concentration was estimated by a colorimetric assay [16]
213 based on its rapid condensation with 4-aminoantypirine, followed by oxidation with potassium
214 ferricyanide under alkaline conditions to give a red colour product, which was measured at
215 510 nm wavelength using Beckman DU640 UV-Vis spectrophotometer. Phenol
216 concentrations were calculated using a calibration curve, which was carried out with known
217 concentrations of pure phenol.

218

219 2.9 Elucidation of phenol degradation pathway

220

221 RTE1.4 strain was grown in liquid MM containing 600 mg/L of phenol until it reached
222 50% of phenol removal. This culture was centrifuged at 12,000*g* at 4 °C for 10 min. To
223 prepare crude extracts, cell pellets were washed twice with 0.9% NaCl and resuspended in
224 Tris-HCl buffer (pH 9) obtaining 0.1 g/mL of wet cell biomass. These cells were disrupted at 4
225 °C for 6 cycles of 0.5 min each, with a gap of 0.5 min between the cycles, using a
226 Sonic&Materials VibraTMCells sonicator. Unbroken cells and cell debris were removed by
227 centrifugation at 24,000*g* at 4 °C for 30 min. The supernatant was used as enzyme crude
228 source for all the assays. The enzyme activity was spectrophotometrically determined in
229 potassium phosphate buffer (0.1 M, pH 7.5) adding 15 µl of the crude extract and 7.5 µl of

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230 catechol (10 mM) as substrate. Catechol 1,2-dioxigenase and catechol 2,3-dioxigenase
231 activities were estimated by measuring the reaction products cis,cis-muconic acid at 260 nm
232 and 2-hydroxy muconic semialdehyde at 375 nm, respectively, as described by Pradhan and
233 Ingle [17]. One unit (U) of enzyme activity was defined as the amount which catalyzed the
234 formation of 1 μmol of product/min at 25 °C. Specific activity was calculated as Units per mg
235 of protein. Proteins were determined by Bradford method with bovine serum albumin as a
236 standard [18].

237

238 2.10 Analysis of changes in protein profiles induced by phenol

239

240 Cultures grown in liquid MM with different initial phenol concentrations (200, 400 and
241 600 mg/L) and a control culture, grown with 500 mg/L of L-lactate as a carbon source, were
242 collected, in exponential growth phase, by centrifugation at 10,000*g* for 10 min at 4 °C. Then,
243 the pellet was washed twice with 0.9% NaCl and centrifuged again in the same conditions.
244 The pellets were resuspended in Tris-HCl buffer (pH 7.5) and disrupted by sonication on ice
245 using 6 cycles of 0.5 min each, with a gap of 0.5 min between the cycles. After sonication,
246 suspensions were centrifuged at 20,000*g* for 20 min at 4 °C, and the supernatants were
247 collected. Protein concentrations were measured as was described before.

248 The protein patterns of cell extracts were analyzed by polyacrilamide gel
249 electrophoresis (PAGE) under denaturizing conditions according to Laemmli [19], using 25 μg
250 of protein per lane. The acrylamide/bis-acrylamide concentration was 4% and 12% (W/V) in
251 the stacking and resolving gels, respectively. The gel was stained with Coomasie Brilliant
252 Blue R-250. To determine molecular mass values, a Kaleidoscope Prestained Standard (Bio-
253 Rad) was used.

254

255 2.11 Statistical analysis

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256

257 Statistical analysis was performed using STATISTICA 7.1 software package. All the
258 data were analyzed using ANOVA. In all cases $p \leq 0.05$ was statistically significant. Dunnett
259 test was used for comparing several treatment groups with a control.

260

261

262

263

264 3. RESULTS

265

266 3.1 Isolation of bacterial strains and phenol tolerance

267

268 Four morphologically different microbial isolates were able to grow on TY agar medium
269 plates supplemented with 100 mg/L of phenol. These strains were tested for their capability
270 to grow in the presence of phenol (100 mg/L) as sole carbon source, in MM agar plates. One
271 of them, RTE1.4, was selected for further studies because it showed a fast and visible growth
272 in this medium, whereas the other three isolates were not able to grow in this condition.
273 When this isolate was spread in plates containing MM agar with different phenol
274 concentrations (100 to 1000 mg/L), RTE1.4 was able to tolerate concentrations as high as
275 1000 mg/L after 48 h at 28±2 °C.

276

277 3.2 Tolerance assay using phenol derivatives and an industrial wastewater

278

279 Tolerance of the isolated strain to various phenolic derivatives was also analyzed. After
280 7 days, the MTC for RTE1.4 were 1000 mg/L for guaiacol, 250 mg/L for 2,4-DCP and 200
281 mg/L for PCP.

282 In addition, growth of RTE1.4 in the presence of cork processing wastewater, containing
283 20 mg/L of total phenolic compounds was evaluated. After 72 h of incubation, this strain
284 proved to be tolerant to this wastewater and showed ability to grow even in plates containing
285 pure effluent.

286

287 3.3 Characterization and identification of bacterial strain

288

289 RTE1.4 strain was characterized based on morphological, physiological and
290 biochemical assays. On MM agar colonies were 1-2 mm in diameter and white-yellow. After
291 single colony isolation the strain was characterized as Gram negative, oxidase-negative and
292 catalase-positive, bacilli. Table 1 shows full details of biochemical and physiological
293 characteristics of RTE1.4 strain. Based on the results of the API 20 NE biochemical test, this
294 microorganism was identified as *Acinetobacter lwoffi* with a 99% of identity.

295 By PCR amplification, 1388 bp 16S rRNA gene fragment of RTE1.4 strain was obtained
296 and submitted for sequencing. The BLAST and RDP search indicated that RTE1.4 was
297 closely related to species of *Acinetobacter* genus.

298

299 3.4 Phylogenetic analysis

300

301 Figure 1 shows the phylogenetic relationship of the RTE1.4 strain with other
302 *Acinetobacter* strains, based on 16S rRNA gene sequences. As can be seen, the 16S rRNA
303 gene sequence of RTE1.4 strain showed closest relation to three strains of *Acinetobacter sp*
304 and two strains of *A. tandoii*, forming a cluster in which the homology between these species
305 was high (97-99%). RTE1.4 also showed high homology with several *Acinetobacter* species
306 (such as *A. schlinderi*, *A. lwoffii*, *A. baumanii*, among other) but these bacterial species were

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307 included in separate clusters. Taking into account these results, we consider that RTE1.4
308 strain is a typical member of *Acinetobacter* genus.
309
310 3.5 Effect of pH, temperature and initial phenol concentration on biodegradation
311
312 Factors affecting phenol degradation capability of *Acinetobacter* sp. RTE1.4 strain such
313 as pH, temperature and initial phenol concentration were investigated.
314 The strain was able to degrade phenol (200 mg/L) in all the tested pH (5 to 11) at 28±2
315 °C and the highest phenol degradation (100%) was achieved at the neutral condition (Figure
316 2). At pH 5, 6, 8 and 9, degradation was still high (above 50%). However, at pH 10 and 11, a
317 very low phenol degradation was observed and only 10 mg/L of the pollutant were removed.
318 Similarly, optimal temperature for phenol removal was determined incubating bacterial
319 strain at different temperatures (25-40 °C), at pH 7, with 200 mg/L of the pollutant and
320 monitoring residual phenol concentration (Figure 3). The optimal temperature registered was
321 28 °C, reaching 100% of phenol removal after 48 h and showing statistically significant
322 differences respect to the other assayed temperatures. In addition, at 25 °C phenol
323 degradation was around 80%. Nevertheless, when incubation was carried out at 33 or 37 °C,
324 low phenol degradation was observed (40%), while at 40 °C the removal was as low as 5%.
325 According to these results, the optimal conditions for phenol removal by *Acinetobacter* sp.
326 RTE1.4 strain were pH 7 and 28 °C, and they were selected for further experiments.
327 As it is shown in Figure 4a, initial phenol concentrations were varied from 200 to 700
328 mg/L to analyze and compare the degree of biodegradation and the time required for
329 complete degradation whereas Figure 4b shows growth curves at different phenol
330 concentrations. The time required for phenol degradation significantly increased as a function
331 of initial phenol concentration in the culture medium. Total degradation of 200 mg/L phenol
332 occurred after 48 h and a short *lag* phase was observed (6 h, data not shown). The

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333 degradation time was prolonged to 7 and 11 d, for 400 and 600 mg/L of phenol, respectively,
334 whereas only 28% of phenol degradation was achieved for 700 mg/L after 13 d. In this case,
335 the strain showed a 6 d *lag* phase to grow and consume phenol.

336 Thus, the strain was able to tolerate and degrade high phenol concentrations, removing
337 completely 200 to 600 mg/L of this contaminant at 28 °C and pH 7, which were determined
338 as optimal conditions.

339

340 3.6 Elucidation of phenol degradation pathway

341

342 The catechol 1,2-dioxigenase and catechol 2,3-dioxigenase activities were examined in
343 cell extracts obtained from *Acinetobacter* sp. RTE1.4. The increase in absorbance at 260 nm
344 was observed in the supernatant of cell suspensions containing catechol as substrate,
345 indicating formation of cis,cis-muconic acid. Specific activity of catechol 1,2-dioxigenase was
346 0.058 U/mg protein whereas 2,3-dioxigenase activity was not detected. These results
347 indicated that *Acinetobacter* sp. RTE1.4 metabolized phenol via *ortho*-pathway while the
348 *meta*-cleavage pathway was discarded.

349

350 3.7 Analysis of changes in protein profiles induced by phenol

351

352 The SDS-PAGE showed several polypeptides which ranged from 17 to 200 kDa.
353 Growth in the presence of phenol altered the whole-cell protein patterns compared with cell
354 growing in a preferential carbon source such as L-lactate. Some protein bands disappeared,
355 other appeared and some of them varied their intensity (Figure 5). For instance, the control
356 pattern showed a small protein of 18.6 kDa, which was absent in phenol treated cells.
357 Moreover, this pattern presented a lower number of bands. Despite the concentration used,
358 phenol treated cells showed similar protein patterns. The most relevant differences were

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359 observed in high molecular mass proteins (120 and 145 kDa), which did not appear in control
360 cells. Besides, 600 mg/L of phenol produced a protein pattern with many differences
361 compared with 200 and 400 mg/L, such as less intensity in bands of 28.2 and 145 kDa and
362 more intensity in a band of 20 kDa.

363

364 **4. DISCUSSION**

365

366 During the past few decades the incidence and threat of phenol pollution has resulted in
367 extensive research. In this context, the use of native microbial communities constitutes a
368 useful strategy for phenol bioremediation. In the present study, four microorganisms with
369 ability to grow *in vitro* in liquid MM supplemented with phenol, have been isolated from an
370 industrial effluent, which is discharged into Ctalamochita River. One of them (RTE1.4) was
371 selected by its high phenol tolerance.

372 In industrial processes, a number of other toxic compounds besides phenol are formed,
373 giving multicomponent composition to wastewaters. Therefore, the strains used for
374 decontamination should not only be tolerant and efficient for phenol removal but they should
375 also be tolerant to the remainder of the pollutants. In this sense, bacteria have adaptively
376 evolved by developing catabolic pathways to utilize a variety of compounds that are available
377 in the natural environment [20]. Many bacteria have the metabolic potential to degrade
378 several aromatic compounds. Although the metabolic pathways are very diverse, they are
379 channeled into a limited number of key intermediates, such as catechol and substituted
380 catechols [21]. Exploring the tolerance of *Acinetobacter* sp. RTE1.4 to different phenol
381 compounds such as guaiacol, 2,4-DCP and PCP as well as to effluents from the cork
382 industry, we found that this strain tolerated them at high concentrations and also showed
383 tolerance to pure effluent. Taking into account that the above mentioned wastewaters
384 contained phenolic compounds (20 mg/L), the results obtained are consistent with bacterial

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385 ability to degrade several phenolic compounds. Considering that polyphenols in cork
386 processing wastewater may form chemical complexes which may have an inhibitory effect on
387 the microbial growth [22], the obtained results are promissory. Therefore, the tolerance
388 showed by the isolated strain could indicate that it could be used to remediate complex
389 effluents, prior to discharge into the environment.

390 Using different approaches such as 16S rRNA gene sequencing and phylogenetic
391 analysis, this strain was identified as *Acinetobacter* sp. In addition, through the use of
392 biochemical tests, this microorganism was identified as *Acinetobacter lwoffi* with a 99% of
393 identity. However, for the identification of species from a specific genus, sequencing of
394 different genes should be performed. These experiments are currently undergone in our
395 laboratory. Thus, until now, we tentatively classified this strain as *Acinetobacter* sp RTE1.4.

396 In the present work, several factors which could affect phenol degradation capability of
397 the bacterial strain, such as pH, temperature and initial phenol concentration were
398 investigated. When *Acinetobacter* sp. RTE1.4 was exposed to the lowest phenol
399 concentration assayed (200 mg/L), a short *lag* phase was observed (6 h) and complete
400 degradation of phenol was reached after 48 h, whereas for 600 mg/L of the contaminant, the
401 strain showed a 4-d-*lag* phase to grow and 11 d to complete phenol consumption. This
402 prolonged *lag* phase could be attributed to the toxic effect of phenol on cells. Pradhan and
403 Ingle [17] showed that *Serratia plymuthica* strain GC had 4-d-*lag* phase when it was grown in
404 the presence of high phenol concentrations (1050 mg/L). Similar events were shown by
405 Wang et al. [23], which also remarked that phenol toxicity, at high concentrations, could
406 inhibit the growth of free cells resulting in a lower removal efficiency of the pollutant.
407 Additional support was given by Geng and Lim [24], which indicated that high phenol
408 concentration treatments include not only inhibition of bacterial growth but also the up-
409 regulation of oxidative stress proteins, heat shock proteins, and a membrane protein, the
410 ABC type sugar transporter. Based on these findings, it is clear that phenol consumption is

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411 strongly dependent on the initial phenol concentration due to toxic effects induced by the
412 pollutant. Despite of phenol toxicity, *Acinetobacter* sp. RTE1.4 was able to tolerate and
413 degrade high phenol concentrations, removing completely 200 to 600 mg/L of this
414 contaminant at 28 °C and pH 7, which were determined as optimal conditions. However,
415 *Acinetobacter* sp. RTE1.4 could also degrade phenol at wide pH range and temperature
416 conditions (5-9 and 25-37 °C, respectively) demonstrating high adaptation ability to different
417 conditions. This is a suitable feature for microorganisms selection to be used for the
418 biotreatment of effluents, because they could have periodic fluctuations in the inlet substrate
419 concentration, influent flow rate, aeration rate, pH and temperature. Some problems in the
420 bioremediation process are frequently associated with such fluctuations since these factors
421 play an important role in the establishment and maintenance of the microbial communities
422 which can remove the organic constituents [25].

423 As it is well known, the aerobic metabolism of phenol is initiated by two different
424 pathways, either the *ortho*- or *meta*-pathway. The final products of both pathways are
425 molecules that can enter the tricarboxylic acid cycle. In this study, the enzymatic
426 determinations showed that *Acinetobacter* sp. RTE1.4 only exhibited catechol 1,2-
427 dioxigenase activity, which indicated that this strain would metabolize phenol by the *ortho*-
428 pathway. This result is in agreement with those described by Zaki [26], which showed that
429 high phenol hydroxylase and catechol 1,2-dioxigenase activities were detected in
430 *Acinetobacter* isolates.

431 One approach to understand the ability of bacteria to tolerate xenobiotic stress has
432 been the identification of stress-induced changes of individual proteins under the assumption
433 that stress adaptation results from alterations in gene expression. When we analyzed SDS-
434 PAGE profiles, many changes were detected after phenol treatment. A large number of
435 proteins were observed in the presence of this contaminant in all the assayed concentrations,
436 compared to control cells growing in L-lactate medium. Therefore, both qualitative and

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437 quantitative differences were detected, mainly the lack of some low-molecular mass
438 polypeptides and an increase in the relative abundance of high-molecular mass proteins after
439 phenol treatment. In addition, protein profiles derived from cells treated with 200 and 400
440 mg/L of phenol, showed slight differences, mainly in the intensity of bands, compared with
441 those grown with 600 mg/L of phenol. Similarly, Soussi et al. [27] and Unni and Rao [28]
442 described the increase of some high-molecular mass proteins in *Mesorhizobium ciceri* and
443 *Rhizobium sp* strains, respectively under other stress conditions. They concluded that these
444 proteins along with other factors may be responsible for growth and hence survival under
445 stress conditions. It must be noted that, the results presented here were a preliminary study
446 of proteome analysis. Thus, an exhaustive study would be necessary to provide a more
447 comprehensive approach related to particular proteins associated with tolerance and
448 degradation of this harmful chemical. However, the presence of several proteins induced in
449 response to phenol stress may suggest that they could have an important role in
450 homeostasis and maintenance of vital cellular functions under this adverse condition.

451 One of the best ways to minimize the ecological risks and regulatory problems
452 associated with the introduction of non-native biota, including genetically modified species,
453 into ecosystems is the use of native species for bioremediation. The addition of specifically
454 selected bacteria from polluted areas have ecological advantages, such as already
455 adaptation of these strains to the environmental conditions of a given site and the reduction
456 of competition pressure. Taking into account these aspects, *Acinetobacter* sp. RTE1.4 could
457 be an appropriate strain for bioremediation of industrial zones because it belongs to a
458 polluted environment and has not been genetically engineered.

459

460 5. CONCLUSION

461

462 Bioremediation uses the resources available in nature to clean up contamination and
463 usually involves lower costs and additional advantages compared to chemical treatment
464 processes. In this work, a bacterial strain was isolated from effluents of a chemical industry
465 and identified as *Acinetobacter* sp. RTE 1.4 using morphological, physiological, biochemical
466 and 16S rRNA gene analysis. The strain was able to tolerate phenol and phenol derivatives,
467 such as guaiacol, 2,4-dichlorophenol and pentachlorophenol and to grow in a medium
468 containing industrial effluents. The strain was also able to efficiently metabolize high phenol
469 concentrations by the *ortho*-pathway, at wide pH range and temperature (pH 5-9 and 25-37
470 °C, respectively). Protein patterns of *Acinetobacter* sp. RTE 1.4 changed in response to
471 phenol stress, showing mainly the induction of several proteins.
472 The biochemical and enzymatic findings, as well as phenol compounds tolerance and
473 removal capability of this native bacterium, would be helpful and provide base line data for
474 further studies associated with its application for effluent bioremediation or bioaugmentation
475 of areas polluted with phenolic compounds.
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481 **6. ACKNOWLEDGEMENTS**
482
483 P.S.G, M.T and E.A. are members of the research career from Consejo Nacional de
484 Investigaciones Científicas y Técnicas (CONICET) (Argentina). C.E.P. has a fellowship from
485 CONICET-MinCyT Córdoba. We wish to thank PPI (SECyT- UNRC), CONICET, MinCyT
486 Córdoba and PICT (FONCyT) for financial support. The authors thank Iliana Martinez, for
487 language correction of the manuscript.

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559

560 Table 1. Physiological and biochemical characteristics of RTE1.4 strain (+, positive
561 reaction; -, negative reaction).
562
563

Characteristics	**RTE1.4 strain**

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Cell morphology	Bacilli
Gram reaction	-
Cell size (µm)	1-3
Nitrates reduction	+
Indole production	-
D-glucose fermentation	-
Enzyme production	
Oxidase	-
Catalase	+
Arginine dihydrolase	-
Urease	-
β-glucosidase	-
Protease (Hydrolisis of gelatin)	-
β-galactosidase	-
Sugar assimilation	
Glucose	-
Arabinose	-
Mannose	-
Mannitol	-
N-acetyl-glucosamine	-
Maltose	-
Potassium gluconate	-
Acid assimilation	
Capric acid	+
Adipic acid	-
Malic acid	+
Trisodium citrate	-
Phenylacetic acid	+

564

565

566

567

568 **FIGURE CAPTIONS**

569

570 **Figure 1.** Phylogenetic tree based on 16S rRNA gene sequence analysis (1000
571 bootstrap for the confidence level) showing the relationship between RTE1.4 strain and
572 representative species of *Acinetobacter* genus. The bacterial species are indicated in the

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573 figure by their accession number between parentheses followed by the taxonomical
574 identification. The scale bars represents 5 substitutions per 100 nucleotides.
575
576 **Figure 2.** Phenol (200 mg/L) degradation by *Acinetobacter* sp. RTE1.4 strain at different
577 pH and 28±2 °C, after 48 h of incubation. Values are mean of three independent experiments
578 and asterisk (*) represents significant differences respect to pH 7, according to Dunnett´s test
579 ($p < 0.05$).
580
581 **Figure 3.** Effect of temperature on degradation of 200 mg/L of phenol in liquid MM at pH
582 7 and after 48 h of incubation. Values are mean of three independent experiments and
583 asterisk (*) represents significant differences respect to 38 °C, according to Dunnett´s test (p
584 < 0.05).
585
586 **Figure 4.** Effect of initial phenol concentrations on: biodegradation (a) and growth (b) of
587 *Acinetobacter* sp. RTE1.4.
588
589 **Figure 5.** SDS-PAGE of total proteins from *Acinetobacter* sp. RTE1.4 grown in: control
590 medium (L-lactate) (Lane 2), and in culture medium containing phenol at different
591 concentrations: 200 mg/L (Lane 3); 400 mg/L (Lane 4); 600 mg/L (Lane 5). Lane 1: Molecular
592 mass marker. Each lane was loaded with the same protein amount (25 μg).
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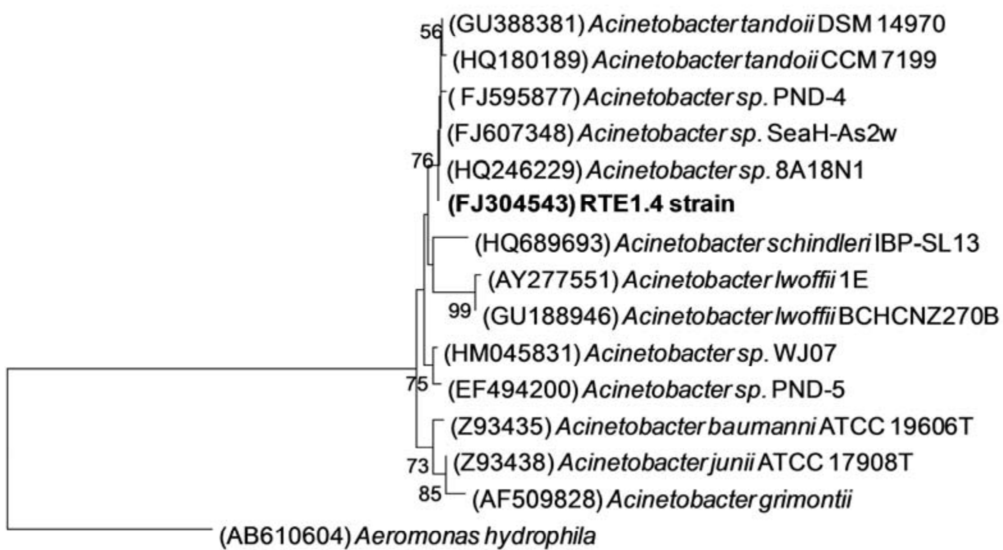

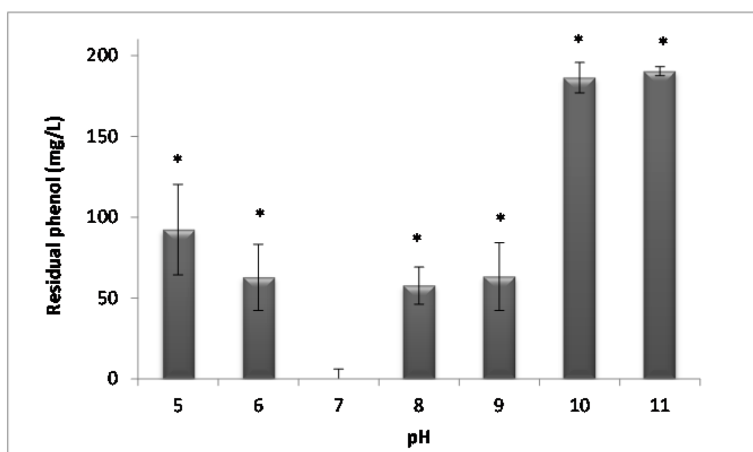

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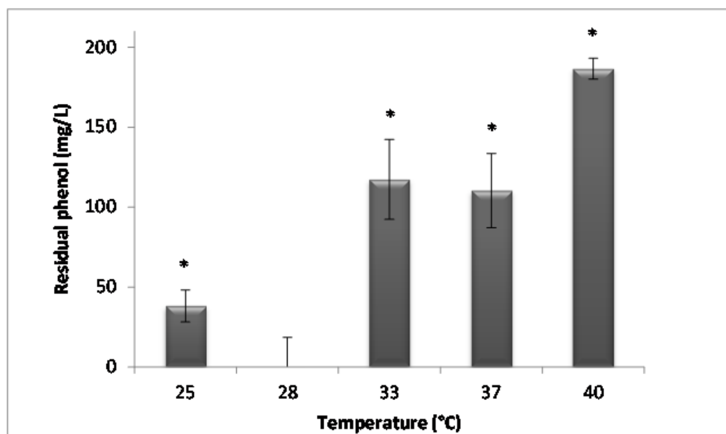

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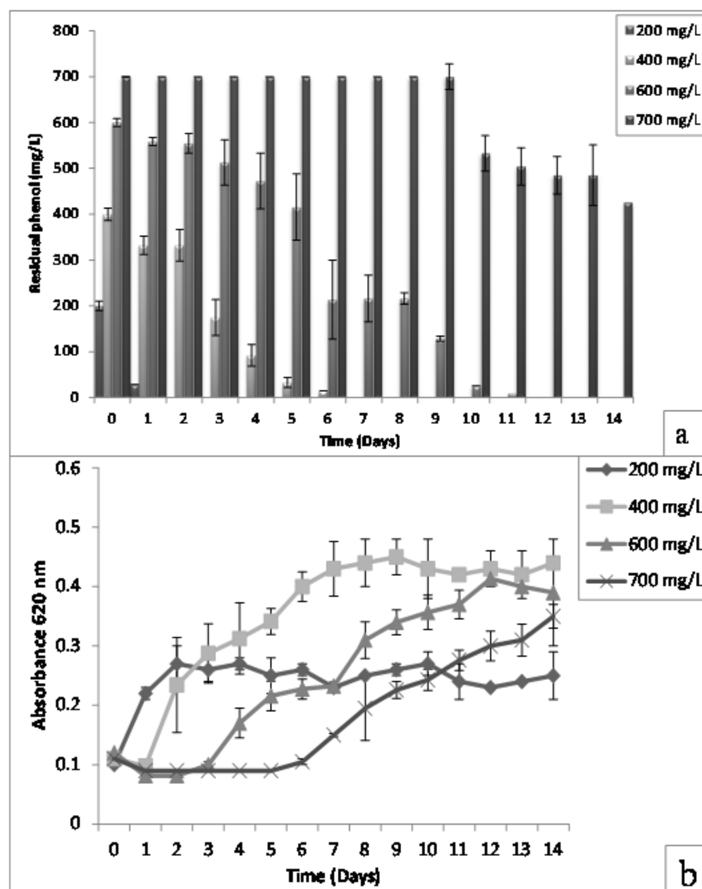

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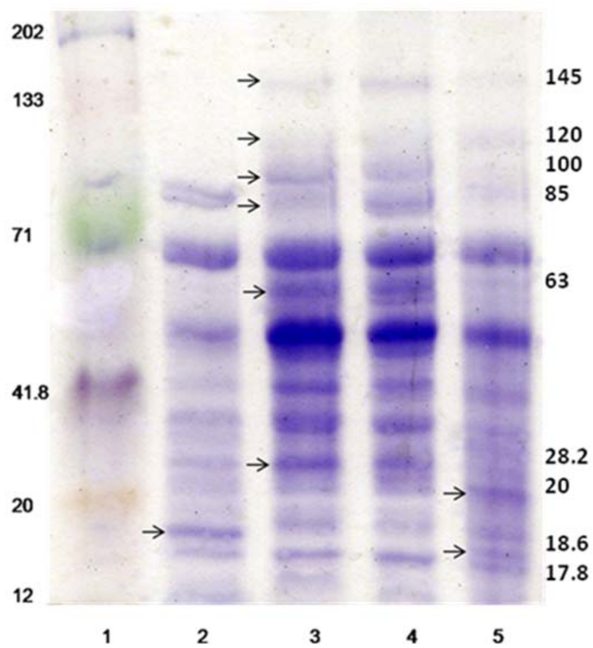

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