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1 **Successful biodegradation of a refractory pharmaceutical compound by an indigenous**
2 **phenol tolerant *Pseudomonas aeruginosa* strain**

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31 **Abstract**

32 This study provides an alternative solution for the bioremediation of a recalcitrant pharmaceutical
33 micropollutant. Clofibric acid (CLA) was chosen as target molecule, because of its environmental persistence
34 and resistance to wastewater treatment technologies. The aim of this study was to investigate the potential of a
35 phenol resistant *Pseudomonas aeruginosa* strain isolated from the activated sludge to degrade CLA.

36 In order to evaluate the effect of acclimation process with glucose as carbon co-substrate, two protocols were
37 carried out, in which the transfer of the inoculum is carried out either in the exponential growth phase or in the
38 decline phase. The results showed a removal efficiency of CLA of 35% when cells in the decline phase were
39 used for inoculation. In contrast, a very low removal yield (10%) was achieved when cells harvested in the
40 exponential phase were used as inoculum.

41 This work is the first one reporting on the capability of this bacterium to remove this drug. The obtained data
42 showed that the isolated strain is able to degrade target molecule and might be a promising agent for the
43 elimination of this refractory compound.

44 **Keywords:** pharmaceutically active compounds; clofibric acid; biodegradation; *Pseudomonas aeruginosa* RZS9,
45 acclimation.

46 1. Introduction

47

48 During the last decade, the occurrence of pharmaceutically active compounds (PhACs) into the aquatic
49 environment have become an increasingly issue for environmental regulators and pharmaceutical industry
50 (Cardoso et al. 2014; Favier et al. 2015; Zuccato et al. 2010). Several investigations have shown that substances
51 of pharmaceutical origin are not efficiently removed during the wastewater treatment process, and also not
52 biodegraded in the environment (Salgado et al. 2012; Semrany et al. 2012; Tauxe-Wuersch et al. 2005). Clofibric
53 acid (CLA) is the main metabolite and the pharmacologically active component of the lipid regulators clofibrate,
54 etofibrate, and etofyllinclofibrate (Cruz-Morató et al. 2013). These substances are used as antilipaemic agents
55 (Kosjek et al. 2009). Their concentration levels in surface water and effluent from sewage treatment plants
56 (STPs) have been shown to lie in the ngL^{-1} to $\mu\text{g L}^{-1}$ range (Sacher et al. 2001; Salgado et al. 2010). Recent
57 investigations report that CLA concentration in natural surface water at the highest levels approaches 240 ngL^{-1}
58 (Luo et al. 2014). The environmental concern related to CLA arises from its environmental persistence, with an
59 estimated environmental residence time of 21 years (Doll et al. 2003; Khetan and Collins 2007). Several
60 techniques have been investigated for the CLA removal including ozonation, photocatalysis, advanced oxidation
61 processes and biological treatment (Nakada et al. 2007). Physicochemical methods lead to the best CLA
62 degradation yields (>90%), but the inherent drawbacks due to the tendency of the formation of secondary toxic
63 by-products and the cost related to these kind of advanced technologies are significant and they pose
64 maintenance problems (Jones et al. 2007), which make them economically unfeasible for many municipalities.
65 Microbial degradation with specialized cultures is considered as an attractive alternative because of economic
66 reasons and low possibility of hazardous byproducts formation (Sahinkaya and Dilek 2007). Up to now, only
67 Zwiener and Frimmel (2003) have studied the biodegradation of three PhACs (clofibric acid, ibuprofen, and
68 diclofenac) in a pilot sewage plant (PSP) and biofilm reactors (BFR) under aerobic and anaerobic conditions.
69 The obtained results clearly showed that the both systems are inefficient for the elimination of clofibric acid and
70 diclofenac under aerobic conditions with only approximately 5% of degradation. More recently, Salgado et al.
71 (2012) evaluated the elimination of CLA in an aerobic sequencing batch reactor (SBR) with mixed microbial
72 cultures after acclimation over a period of 20 months. The maximum removal yield achieved was 51% for an
73 initial CLA concentration of 2 mgL^{-1} . Furthermore, Evangelista et al. (2010) studied the biodegradation of three
74 chlorophenoxy acids using axenic cultures of bacteria under cometabolic conditions. Nevertheless, none of the
75 tested microorganisms was able to degrade clofibric acid suggesting that the recalcitrance of clofibric acid is due
76 to the additional methyl group adjacent to the ether bond (2010).

77 Some studies reported that cultures acclimated to a specific type of xenobiotics compounds such as
78 chlorophenols with an aromatic ring have also the ability to degrade other compounds with similar structure
79 (Murcia et al. 2012; Sahinkaya and Dilek 2007).

80 However, literature data on the biodegradation of CLA with short-term experiments by pure cultures of bacteria
81 belonging to the genus of *Pseudomonas* remains very scarce.

82 In this framework, the present study evaluates the ability of a phenol resistant *Pseudomonas aeruginosa* strain to
83 remove CLA in liquid system. For this purpose, an acclimation study of the strain to the micropollutant was
84 carried out in the presence of carbon co-substrate (D-glucose). The effect of glucose concentration on the CLA
85 biodegradation was also examined.

86 2 Materials and methods

87 2.1 Microorganisms

88 The strain *Pseudomonas aeruginosa*, used in this work was isolated from an activated sludge collected in the
89 aeration tank of the Municipal Sewage Treatment Plant of Beni Messous (Algeria). 500 mL of sludge was
90 introduced in an Erlenmeyer flask and was aerated for a period of 24h. The aeration process was then stopped for
91 30 min in order to separate the sludge from the liquid phase. 250 mL of the supernatant was replaced by a
92 solution volume of phenol in order to have a final concentration of 500 mgL⁻¹ and then aeration was started
93 again. After 24h of incubation time, 89% of phenol biodegradation was noted. Appropriate serial dilutions of
94 resistant strain suspension were then prepared with NaCl (0.9%, w/w) before plating onto nutrient agar (NA) and
95 incubated for 24h at 37°C. Colonies showing good growth were selected and transferred into a fresh nutrient
96 agar. This procedure was repeated three times in order to ensure the cultures purity. The stock culture was stored
97 at -20°C in 80% glycerol.

98 2.2 Chemicals and growth media

99 CLA (>99% of purity) was purchased from Acros Organics (New Jersey, USA). Table 1 shows the physico-
100 chemical characteristics of the target compound. The solvents of HPLC-grade, acetonitrile, formic acid (99.9%)
101 were provided by Fisher Scientific (Geel, Belgium) and D-glucose anhydrous was bought from Merck
102 (Darmstadt, Germany).

103 **Table 1**

104 Mili-Q ultrapure (resistivity of 18MΩ cm at 25 °C) obtained from a Milipore system was used for the
105 preparation of all aqueous solutions. All other chemicals and nutrient media were purchased from Acros
106 Organics (New Jersey, USA). For the batch degradation experiments and acclimation studies, a stock solution of
107 CLA was prepared at 50 mgL⁻¹ in ultrapure water and stored at 4°C until use.

108 Inoculum preparation was carried out in nutrient broth medium (N.B.) in order to promote increased biomass
109 growth. The used medium contained: 10 gL⁻¹ tryptone, 5 gL⁻¹ meat extract and 5 gL⁻¹ sodium chloride. 5 g of the
110 N.B. powder were weighed and slowly dissolved by magnetic stirring until complete dissolution in 250 mL of
111 ultrapure water into an Erlenmeyer flask of 500 mL. The flask was then covered by a cotton cap and aluminum
112 foil and autoclaved at 121 °C for 15 minutes.

113 For the biodegradation and acclimation experiments a basal mineral medium (BMM) was employed. It contains
114 the following compounds with concentrations expressed in (gL⁻¹): K₂HPO₄, 4.25; KH₂PO₄, 1.70; NH₄Cl, 2.10;
115 MgSO₄·7H₂O, 0.41; MnSO₄·H₂O, 0.06; FeSO₄·7H₂O, 0.01; CaCl₂·2H₂O, 0.03. The medium pH was adjusted to
116 7.3 with NaOH (2.5 M) prior to sterilization. Finally, CLA was added in the BMM and then autoclaved at 121°C
117 for 20 min, as it was determined that the target molecule is not heat labile.

118 The carbon co-substrate (D-glucose) was dissolved in ultrapure water and was sterilized separately to avoid
119 Maillard reactions and hence a color change of the medium during the autoclaving process, and then added
120 aseptically to the sterilized medium to yield the appropriate final concentration according to the requirements for
121 the biodegradation tests or acclimation procedure.

122 2.3 Growth experiments

123 2.3.1 Preparation of the inoculum

124 Between experiments, the bacterial isolate was preserved by nutrient agar plating at 4°C and then subcultured.
125 Before each experiment, the strain was cultured for 48h in nutrient broth (Biokar Diagnostics, Beauvais) at 150
126 rpm and at 37°C. The obtained culture suspension was then centrifuged at 5000 rpm for 10 min (Jouan C412,
127 Saint-Herblain, France), washed twice with sterile ultrapure water in order to remove organic substances
128 adsorbed on biomass and was finally re-suspended in the same volume in minimal basal media (BMM). The
129 concentration of the final cell suspension was adjusted to a suitable optical density (1.2) at 600 nm. This
130 procedure was repeated each time for inoculum preparation.

131

132 2.3.2 Biodegradation and biosorption experiments

133 All biodegradation tests were performed in duplicate and were conducted in batch mode. For this purpose
134 Erlenmeyer flasks with a volume of one liter stoppered with cotton plugs were employed. For the assays with
135 CLA as the sole carbon source the drug was added in 250 mL of basal minimal medium in appropriate
136 proportions to give the desired pollutant concentration and then sterilized. Different initial concentrations of
137 CLA were examined (ranging from 0.25 to 2 mgL⁻¹). For each experiment, 10 mL of inoculum (OD₆₀₀=1.2) was
138 added. The flasks were maintained at 37°C, pH = 7±0.1 and at constant stirring rate (150 rpm). Furthermore, two
139 controls were done in parallel with CLA without microorganism and with biomass without drug in order to study
140 the bacterial growth in the absence of the target molecule and the possible abiotic degradation of CLA.

141 In order to investigate the effect of glucose on the degradation of CLA, *Pseudomonas aeruginosa* cells were
142 grown in basal mineral medium with 2 gL⁻¹ of glucose and 1 mgL⁻¹ of CLA. This test was carried out under the
143 same conditions as the biodegradation test with CLA and without co-substrate.

144 In addition, biosorption experiments were carried out under similar conditions to those described for
145 biodegradation tests in order to evaluate the significance of the adsorption process by the bacterial biomass on
146 the total removal of the target molecule. They were performed with inactivated cells (autoclaved 121°C for 20
147 min). The samples were collected from the flask at several time intervals (0.5, 1, 1.5, 2, and 24h) and the CLA
148 content in the supernatant was analyzed by HPLC-UV.

149 The presence of CLA adsorbed onto the biomass was also highlighted by the use of the modified method of
150 ultrasonic solvent extraction described by Salgado et al. (2012). For this method, samples were taken at the end
151 of the biodegradation experiments (168 h) and centrifuged as described before (section 2.3.1). Briefly, 2 x 4 mL
152 of acetonitrile was added to the centrifuged biomass sample, which was subjected to sonication treatment to
153 suspend the biomass. The CLA extraction process was performed in an ice bath with four bursts of 30 s
154 (amplitude 20%) with 59 s intervals using a Vibra Cell sonifier (Bioblock Scientific, Illkirch, France). After
155 sonication the samples were centrifuged again at 5000 rpm for 10 min. The obtained supernatants were filtered
156 using 0.2 µm pore size syringe filters in HPLC vials and then were analyzed in HPLC-UV.

157

158

159 2.3.3 Acclimation process

160 In order to obtain acclimated cultures, two different protocols were tested in this work. They were performed by
161 consecutive batch assays (multiple transfer or enrichment) in the presence of carbon co-substrate. In order to
162 select the most efficient acclimation strategy, the degradation of CLA by *Pseudomonas aeruginosa* was
163 investigated.

164 **Protocol I:** The cultures were acclimated to CLA by sequential transfers, in a series of shake flasks (1L),
165 decreasing glucose concentrations from 2 to 0.25 gL⁻¹, and gradually increasing CLA concentrations from 0.25
166 to 2 mgL⁻¹, over a period of one week. Every 24h (end of exponential phase), bacterial biomass was harvested by
167 centrifugation (5000 rpm for 10 min) and washed twice with ultrapure water. The pellet was then re-suspended
168 in fresh BMM medium. 10 mL of cells suspension were used to inoculate in a new flask containing fresh BMM
169 medium (different concentrations of glucose and CLA).

170 **Protocol II:** Similar to Protocol I, but the acclimation period was longer (one month). All cultures were
171 regularly transferred at intervals of 72h (decline phase) to fresh medium, under the same conditions as protocol I.
172 It should be noted that the both strategies were conducted at a fix reaction time (cycle duration 24h or 72h) in
173 order to use different quality of inoculum and as a consequence to evaluate the effect of this parameter for the
174 acclimation. Moreover, the both procedures are independent of the degree of pollutant removal. For each assay,
175 samples were taken at different times to determine the residual CLA concentration and the optical density. The
176 acclimation process was monitored by calculating the CLA removal yield.

177

178 2.4 Analytical methods

179 Samples were aseptically taken at regular intervals and investigated for cell density, medium pH, CLA removal
180 and glucose consumption. 4 mL of sample from each flask was taken for the determination of all cited parameters.
181 Medium pH was measured using a small pH electrode (CyberScan, Thermo Scientific, France) suitable for small
182 volume samples. Cell growth was monitored by measuring the absorbance at 600 nm using UV-Vis single beam
183 spectrophotometer (Thermospectronic Helios, Cambridge, UK).

184 To prevent the fouling of the chromatographic column, the samples require, before injection, a very simple
185 pretreatment based on deproteinization. This treatment was performed with Ba(OH)₂ and ZnSO₄·7H₂O. Both
186 compounds cause the precipitation of proteins present in the samples. 2 mL of sample was treated by addition of
187 0.25 mL of Ba(OH)₂ (0.3 M) and 0.25 mL ZnSO₄·7H₂O (5% w/w). The supernatant obtained after 10 min of
188 centrifugation at 5000 rpm was filtered through a membrane filter of Polyester (Chromfil[®] Xtra, Macherey-
189 Nagel, Germany, porosity 0.2 µm) and used for CLA and glucose analysis.

190

191 The residual CLA concentration was determined using a Waters 2695 Alliance HPLC system equipped with a
192 UV-detector (Waters 2489 dual λ absorbance detector) operating at a wavelength of 230 nm. Chromatographic
193 separation was achieved on a C18 column (250 x 4.6 mm, 5 µm, Waters, Ireland) and at 30°C. The mobile phase
194 consisted of a mixture of acetonitrile-ultrapure water (40:60, v/v) and 0.1% formic acid at a flow rate of 1.0 mL
195 min⁻¹. The injection volume was 50 µL. The retention time of CLA under these analytical conditions was 12.54
196 min and the instrumental quantification limit (LOQ) for this drug was < 150 µgL⁻¹. The chromatogram of a standard
197 CLA solution is shown in Fig.1.

198

199

Figure 1

200

201 Glucose consumption during the biodegradation experiments was verified by ion chromatography using the
202 Dionex DX 600 system (Dionex Corporation, Sunnyvale, Canada) equipped with a pulsed amperometric detector
203 with a gold working electrode, a GP50 gradient pump, and an AS40 automated sampler (Dionex Corporation,
204 Sunnyvale, CA). A guard column (4x50 mm) was placed in-line prior to the analytical column in order to protect
205 it for fouling. Analyte separation was achieved with a CarboPac PA1 Analytical (4x250 mm) column. The
206 column was eluted by a mixture of ultrapure water - NaOH (200mM) (90:10, v/v) at a flow rate of 1.0 mL min⁻¹.
207 The volume of the injected sample was 20µL. Under these analytical conditions the retention time of glucose
208 was 7.81 min. Data acquisition was achieved with Chromeleon software, v.6.80 (Dionex Corporation,
209 Sunnyvale, CA).

210 All measurements were carried out in duplicate and mean values were used for the data analysis and
211 calculations.

212

213 3 Results and discussion

214 3.1 Strain identification

215 For preliminary identification at species level, bacterial isolate was examined by using standard biochemical and
216 morphological tests (macroscopic and microscopic) provided in the Bergey's Manual of Determinative
217 Bacteriology (Holt et al. 1994). For macroscopic evaluation nutrient agar from Biokar diagnostics, containing (g
218 L⁻¹): tryptone, 10; meat extract, 5; NaCl, 5; bacteriological agar, 15; pH 7.1, was used as a basal medium to study
219 the morphological characteristics of pure culture. Bacterial colonies in this medium were circular with a diameter
220 1-3mm, with fluorescent yellow green color, smooth shape and mucous consistency. Moreover, the obtained
221 bacterial colonies could be easily scraped off from the solid agar media incubated at 37 °C. Biochemical tests
222 and microscopic evaluation revealed that, the bacterial isolate is Gram negative, motile by polar flagella, positive
223 for oxidase, catalase, citrate, arginine dihydrolase (ADH) and glucose in MEVAG medium (medium for the
224 study of the carbohydrates attack way) through oxidative way. However, they are negative for indole, methyl red
225 and Voges proskauer tests (glucose fermentation ability). Indeed, the selected strain is not able to use glucose
226 under anaerobic condition. The results of strain identification are detailed in Table 2.

227

228

Table 2

229

230 According to Holt et al. (1994) all these morphological and biochemical characteristics allow us to identify the
231 studied strain as a species of the *Pseudomonas* genus.

232 In order to complete the identification of the bacterial isolate, 16S RNA gene of pure bacterial culture was
233 amplified and sequenced. As previously reported, such approach is considered as a valuable technique in
234 taxonomic analysis of bacteria. DNA was extracted using NucleoSpin®Microbial DNA kit. The 16S rDNA gene
235 was amplified by PCR using forward and reverse primers 9F (5'-GAGTTTGATCMTGGCTCAG-3') (Gordon et
236 al. 1997) and 1509R (5'- GNTACCTTGTTACGACTT-3') (Weisburg et al. 1991), based on the *Escherichia coli*
237 numbering system and the PCR product was sequenced. PCR mixture contained 0.5 µL of Taq polymerase (5U

238 μL^{-1}), 2.5 μL of 10 \times PCR buffer, 2 μL of dNTP (2.5 mM each), 1.5 μL of forward primer (10 μM) and 1.5 μL of
239 reverse primer (10 μM), 0.75 μL of MgCl_2 (50 mM), 1 μL of DNA template and sterile water to 25 μL reaction
240 volume. PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 2
241 min, followed by 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final
242 extension at 72°C for 10 min. DNA sequencing of the PCR fragments was carried out by the Biogen Ouest
243 facility (Nantes, France). Obtained 16S rRNA gene sequence was compared to GenBank entries and identified
244 via BLAST algorithm. The analysis showed that the 16S rRNA of the isolated strain shared 100% similarity with
245 the 16S rRNA gene of the *Pseudomonas aeruginosa* strain RZS9, deposited in GenBank with the accession
246 number KP866815.

247

248 **3.2 Biodegradation studies of CLA without strain acclimation**

249 **3.2.1 Study without co-substrate**

250 In order to evaluate the capacity of bacterial isolate to grow in the presence of CLA as sole carbon and energy
251 source, series of growth experiments were conducted in BMM medium at different CLA concentrations (ranging
252 from 0.25 to 2 mgL^{-1}). Time-course variations in biomass (expressed as optical density), pH and CLA residual
253 concentration were followed for the unacclimated cultures. The corresponding results are given in Fig.2 (a, b,
254 c). It was seen from the results that similar growth profiles are obtained for all individual experiments and the rise
255 of the initial concentration of CLA from 0.25 to 2 mgL^{-1} led to only a slow decrease of the bacterial growth
256 ($\text{OD}_{600} = 0.5$ to 0.3) (Fig.2a). No lag phase was observed at the beginning of the strain growth. *Pseudomonas*
257 *aeruginosa* RZS9 directly entered in growth phase from an incubation time between 0 and 72h while, from 72h
258 to 264 h an important decrease in the cell growth was observed. The absence of the lag phase in the bacterial
259 growth can be due to the low pollutant concentrations used in this work. This observation agrees with the study
260 of Murcia et al. (2012) with *Pseudomonas putida* where no lag time was achieved with the low concentrations in
261 4-chlorophenol, whereas bacterial required a lag phase of 40h before exhibiting the growth at a pollutant
262 concentration of 200 mgL^{-1} and more than 70h at 250 mg L^{-1} .

263 Moreover, Fig 2a shows that bacterial cells survived in the presence of the pollutant. However, the maximum
264 cell density achieved at the end of the growth phase remains quite low (up to 0.5 OD_{600} units). The time required
265 to reach the maximum biomass concentration was similar (72h) for all the tested conditions. These observations
266 are in agreement with those reported previously by Larcher and Yargeau (Larcher and Yargeau (2011) for
267 *Pseudomonas aeruginosa* (PA01) and *Pseudomonas putida* (123633) exposed to sulfamethoxazole as the sole
268 carbon source.

269 In addition, it should be noted that a control experiment without pollutant was also conducted (as previously
270 reported in section 2.3.3) and the growth profile was quite identical to the one of the experiment done at initial
271 CLA concentration of 0.25 mgL^{-1} . Based on the successful cells growth in the presence of drug it was expected
272 to observe a pollutant removal under the considered experimental conditions. However, no degradation of the
273 target molecule was observed with unacclimated biomass during 264h of incubation time at any of the tested
274 initial pollutant concentrations (Fig.2b). These results were in agreement with those reported for clofibric acid
275 and carbamazepine when exposed to cultures of *Streptomyces* spp (Popa et al. 2014; Popa et al. 2016). Larcher

276 and Yargeau (2011) observed a very low removal yield (0-6.6%) for sulfamethoxazole degradation experiments
277 with individual bacterial species. Moreover, Gauthier et al. (2010) found that *Rhodococcus rhodochrous* and
278 *Aspergillus niger* are not able to degrade carbamazepine and sulfamethoxazole when these molecules are the sole
279 carbon sources.

280 The results obtained in our work confirmed that the isolated strain is unable to degrade the target molecule at all
281 the investigated concentrations in monosubstrate system and the recalcitrance of CLA. Similar observations were
282 reported by Evangelista et al. (2010). It can also be noted that no volatile loss of CLA was observed during the
283 abiotic test (without microorganisms) (result not shown).

284

285 **Fig. 2(a), (b), (c)**

286

287 In addition, the pH of the medium remained almost constant during the biodegradation tests. This result indicates
288 the absence of degradation metabolites usually formed during biodegradation processes which cause pH
289 modification of the culture medium. Moreover, it confirms the data obtained on the removal of the target
290 molecule.

291 The obtained results showed that during the degradation experiments, *Pseudomonas aeruginosa* RZS9 cells
292 survive in the presence of pollutant but, no pollutant removal was detected in the presence of the target molecule.
293 This suggests that CLA may not be used as the sole carbon or energy source to maintain the cells growth and to
294 induce the enzymes needed for its assimilation. The bacterial growth observed under the investigated culture
295 conditions could be probably attributed to the use of the carbon stored intracellularly during the preculture period
296 conducted in nutrient broth allowing the cells maintenance and bacterial growth during the first 72h of
297 cultivation.

298 To overcome the toxic effect of the pollutant, especially at high pollutant concentration more energy is required.
299 In this context, the presence of a growth substrate or another utilizable compound is obligatory required to
300 maintain the biomass growth and to induce the corresponding enzymes and/or necessary for biodegradation. This
301 process is well-known as co-metabolism (Tran et al. 2013). Therefore, it is of great interest to investigate if the
302 addition of a carbon co-substrate reduce the toxicity of the target organic compound and enhance bacterial
303 growth and the removal process.

304

305 **3.2.2 Study with co- substrate**

306 It was reported that in mono-substrate system, clofibric acid was resistant to microbial degradation [18]. As
307 previously stated, different studies proved that microorganisms are able to degrade xenobiotics only under
308 cometabolic conditions (Domaradzka et al. 2015; Fisher et al. 2010; Gauthier et al. 2010). Indeed, the addition of
309 a co-substrate, which is a readily available source of carbon and energy, increases biomass production.
310 Moreover, the presence of the growth substrate may also induce enzymes of xenobiotic decomposition pathways
311 and in this way increases its degradation rate (Quintana et al. 2005; Grenni et al. 2013). For example, Fakhruddin
312 and Quilty (2005) showed that the degradation of 2-chlorophenol was enhanced in the presence of glucose (up to
313 1%). Similar results have been found for the degradation of another recalcitrant molecule, benzo[a]pyrene, by

314 *Pseudomonas saccharophilia* and *Stenotrophomonas maltophilia* (Chen and Aitken 1999; Juhasz and Naidu
315 2000).

316 So for that reason, the effect of the addition of a growth substrate on the *Pseudomonas aeruginosa* RZS9 growth
317 and CLA removal was investigated in this work. Study was carried out using glucose, as carbon co-substrate. To
318 investigate its effect, an additional batch experiment test with 1mgL^{-1} of CLA in BMM supplemented with 2gL^{-1}
319 of glucose was carried out. The operating conditions of this test were the same to those used for the
320 biodegradation experiments without co-substrate. The corresponding results are given in Fig.3 (a, b).

321

322

Fig. 3 (a), (b)

323

324 These figures clearly illustrate the significant effect of the addition of glucose on bacterial growth, pH and CLA
325 removal. Indeed, the carbon co-substrate addition led to an absence of the lag phase and to an increase in the
326 optical density. The measured value is higher than the one determined in the presence of CLA as sole source of
327 carbon and energy. Fakhruddin and Quilty (2005) have attributed this effect to the production of large quantities
328 of cofactors such as NADH and NADPH resulting with oxidation of sugars.

329 The maximum growth (OD_{600} up to 1.3) was reached after 24 hours of culture(Fig.3a) with practically total
330 consumption of glucose (Fig.3b) while, in the absence of glucose 72h were needed (Fig.3a). This value is more
331 than the double that the one achieved with CLA as the sole source of carbon suggesting that glucose stimulate
332 the bacterial cells growth. Regarding the measured pH, it was observed that, during this period the pH of the
333 culture medium decrease to 6.61. According to Loh and Wang (1997) and Salgado et al. (2012), this can be
334 attributed to the formation of glucose degradation metabolites (acetic acid and lactic acid) leading to the
335 acidification of the culture medium. As shown in Fig.3b, CLA removal reached almost 20% after 72h of
336 incubation, and remained constant beyond this time. It is noteworthy that CLA removal occurred simultaneously
337 with glucose consumption during this time and was accompanied by an increase in biomass. Moreover, the
338 increase of pollutant removal observed in the presence of glucose can be due to an increased production of
339 cofactors such as NADPH which could be the required cofactors for the key enzymes involved in the CLA
340 metabolism.

341 It should be pointed out that, in separate sets of tests, abiotic elimination of pollutant (using the same conditions
342 but without biomass) as well as, the adsorption of CLA to the biomass (data not shown) were investigated.
343 Moreover, as previously stated (section 2.3.3), to check for possible adsorption of CLA onto the biomass at the
344 end of the experiment, a supplementary adsorption test was performed at the end of the culture, 168h (data not
345 shown). The obtained results suggest that, there is no evidence for abiotic degradation as well as for adsorption
346 of drug to the cells surface (biosorption) confirming that the CLA removal observed in Fig. 3b was only
347 attributed to the biodegradation process. This result was also confirmed by chromatograms obtained by HPLC-
348 UV analysis (data not shown) and is consistent to literature data. Indeed, several studies reported that the CLA
349 sorption to sludge, suspended matter and sediments is not considered to be an important contribution to the
350 elimination of this molecule from waste and surface waters (Kent et al. 2006; Kosjek et al. 2009). Moreover, in
351 their work, Kosjek et al. (2009) excluded the possibility of abiotic degradation of CLA.

352 The addition of a more readily available carbon sources exerted a stimulatory effect on the cellular activity and
353 as consequence, on the cells growth and the removal yield of CLA. Similarly to our study, Tran et al. (Tran et al.

2009) found that the presence of acetate (100 mgL^{-1}) as an additional carbon source increases the CLA removal. In their previous work, Fakhruddin and Quilty (2005) observed the degradation of 2-chlorophenol by *Pseudomonas putida* CP1 in the culture media supplemented with glucose. Likewise, *Streptomyces* bacteria isolated from soils cometabolise CLA and carbamazepine in the presence of glucose as additional source of carbon and energy (Popa et al. 2014, 2015 and 2016). Moreover, our results clearly showed that, the CLA removal proceed until a certain value and then did not continue. One explanation is that, the pollutant elimination is not limited by the glucose consumption because it continues after its depletion from the culture medium but, by an unknown nutrient (oxygen or another) present in the culture media or by the accumulation of an inhibitory metabolite which affects the CLA degradation. Similar results have been previously reported by Salgado et al. (2012) for the same molecule. The obtained data show that *Pseudomonas aeruginosa* RZS9 is able to remove the target molecule in the presence of glucose. However, the CLA removal yield obtained with unacclimated culture still remains low confirming the high resistance to microbial attack of this molecule and suggests that adaptation of the strain to pollutant may be required to have a significant elimination of the target molecule. Several studies have reported that the capacity of bacteria to remove toxic compounds can be enhanced by acclimation (Evangelista et al. 2010; Ferro Orozco et al. 2013; Sahinkaya and Dilek 2005). Acclimation is considered as the key issue to achieve the degradation of recalcitrant compounds. Indeed, microorganisms which are repeatedly exposed to a pollutant may develop new capabilities to degrade toxic molecules. Thus in this study, the impact of the acclimation on the removal of the target molecule was also investigated.

373

3.3 Investigation of acclimation strategy of *Pseudomonas aeruginosa* RZS9 to CLA

Acclimation of an indigenous microbial species or population is essential to increase the potential of the elimination yield of an organic pollutant. Some studies indicated that the degradation of a persistent xenobiotic compound can be achieved only after going through an acclimation process (Chong 2009; Huang et al. 2014; Mangat and Elefsiniotis 1999). Indeed, during the acclimation process the microbial population can acquire new metabolic pathways for xenobiotic degradation (Chong and Lin 2007). In aerobic microbial communities the acclimation periods generally range from some hours to several days while for anaerobic communities this period is typically more longer (from two weeks to six months or longer) (Ye and Shen 2004). Other works pointed out that, this process depends on type and concentration of the xenobiotic compound, the type of considered microorganisms, the type of culture (pure or mixed), the presence of readily biodegradable compounds (Chong et al. 2008; Ye and Shen 2004), the level of inoculation and the physiological state of the inoculated cells, as well as the strategy under which acclimation was performed. Moreover, it has been mentioned that the degree of adaptation of a microorganism or a mixed culture varies depending on the used acclimation procedure.

In order to examine the impact of the acclimation process in the presence of a primary carbon source (glucose) two acclimation strategies were performed as described in the section 2.3.2. As stated before, the acclimation strategy used here was based on enrichment or multiple transfer approach by using inoculum of different qualities. Another possibility is to conduct this procedure in a single flask. CLA removal and optical density

392 were monitored periodically for evaluation acclimation efficiency purposes. The removal yields of CLA at the
393 end of the exponential phase (24 h) during acclimation are displayed in Fig. 4 for both investigated protocols.

394

Fig. 4

395 It can be observed that after acclimation in the presence of glucose as a primary carbon substrate the bacterial
396 strain acquired the capability to degrade CLA. Moreover, the increase in the pollutant concentration had an
397 impact on the level of degradation. Indeed, for both acclimation strategies the removal efficiency of
398 *Pseudomonas aeruginosa* RZS9 increased with the initial pollutant concentration up to a threshold value (1 mgL⁻¹ of
399 CLA and 1.25 gL⁻¹ of glucose). In the Protocol I, namely cells harvested in the exponential phase, after 24h of
400 incubation, only 10% of CLA was degraded. However, in the same conditions, 35% of CLA biodegradation was
401 observed following the Protocol II, namely cells harvested in the decline phase, after 72h of incubation. These
402 results suggested that, Protocol II appeared more efficient to the increase in pollutant concentration and its
403 degradation than Protocol I. Protocol II is therefore more sensitive to the increase in pollutant concentration than
404 the Protocol I. In fact, for the Protocol II, each step of the acclimation phase lasted 72h and hence, the existing
405 glucose in the medium would be totally consumed at this stage of bacterial growth and consequently, only CLA
406 remained in the acclimation medium; the cells would be therefore, better-adapted and more capable for pollutant
407 degradation comparatively to the Protocol I. In other words, the adaptive pressure in the Protocol II was higher
408 than during the Protocol I which leads to a lower degradation efficiency.

409 Pinto et al. (Pinto et al. 2012) reported a similar behavior for *P. brevicompactum* isolated from contaminated soil
410 for the removal of difenoconazole. Awasthi et al. (Awasthi et al. 2009) also found that consortium adapted to
411 mixed 2-ABTS/glucose substrates demonstrated rapid glucose removal with concomitant degradation of 2-
412 aminobenzenesulfonate.

413 Beyond the optimal concentrations (1 mgL⁻¹ of CLA and 1.25 gL⁻¹ of glucose), the removal yield decreased with
414 the increase of initial CLA concentrations for the both considered protocols. This result could be attributed to the
415 inhibitory and toxic effects caused by high pollutant concentrations (Fig.4). Similarly, Yamanaka et al. (2007)
416 reported a toxic effect caused by high bisphenol A (BPA) concentrations. They found that in the range of 2-25
417 mg L⁻¹ BPA was effectively degraded by *Bacillus pumilus* but higher pollutant concentrations caused the
418 inhibition of the degrading activity of the acclimated cells. Moreover, Zhang et al. (2007) reported that the
419 degrading activity of *Achromobacter xylosoxidans* was affected by increased levels of toxicity for BPA.

420 Figure 5a depicts the biomass concentration profiles during the acclimation Procedure II for the batch test
421 operated at 1 mgL⁻¹ of CLA and at 1.25 gL⁻¹ of glucose for an incubation time of 72h. It was observed that the
422 OD₆₀₀ value increased up to 1.0 during the first 24h of incubation after that, a marked decreased of this
423 parameter (up to 0.30) was reached after 72h of culture reflecting the consumption of the more available carbon
424 source (glucose) by the bacterial strain. Moreover, a concomitant decrease of the pH was observed during the first 24h
425 of culture, probably due to the formation of biodegradation metabolites and then, the measured pH values
426 remained without significant changes until the end of the batch assay (Fig.5b).

427

428

Fig.5 (a), (b)

429

430 In addition, during this incubation time, a progressive augmentation of CLA removal yield can be observed until
431 reaching a value of 46% after 72h of incubation (Fig.5b). This value is higher than the one obtained under the
432 same conditions but with unacclimated biomass, suggesting the positive role of the used acclimation strategy for
433 the removal of this refractory pharmaceutical molecule. However, the complete removal of CLA was not obtained.
434 It can be also observed that the removal of the target molecule continues also after the complete depletion of
435 glucose from the culture media. As the culture time proceeds and with the presence of the target molecule, the
436 acclimated biomass acquired new degradation capabilities due to a biochemical adjustment at intracellular level.
437 While the degradation of an ordinary substrate is generally reflected by the microorganism growth, the
438 degradation of a toxic molecule involves a capability factor within the biomass which has not always reflected in
439 biomass growth. Only when a capability is gained, the utilization of the xenobiotic compound as substrate
440 started, resulting in the growth of the adapted microorganism (Chong and Lin 2007). Thus, our results proved
441 that during the acclimation process the *Pseudomonas aeruginosa* RZS9 biomass acquired new biodegradation
442 abilities and can promote the pollutant removal even if the carbon co-substrate was completely consumed and
443 the total biomass started to decrease. Indeed, under these conditions it is supposed that the resting active biomass
444 can continue the degradation process. At this culture time, two types of cells are present in the culture media: the
445 active acclimated biomass (degraders) which acquired the capability to degrade the CLA during the acclimation
446 process and can use the pollutant as sole source of carbon and the non-degraders of the xenobiotic compound
447 due to the cells death during the endogenous decay process. A pure evaluation of the active biomass and the
448 calculation of the acclimation effectiveness in relation with this parameter can lead to a better understanding of
449 the acclimation phenomenon considered in this work. Similar phenomena were reported previously by Chong
450 and Lin (2007), when a mixed culture (such as activated sludge) was exposed to a xenobiotic compound (e.g.
451 BPA).

452 To the best of our knowledge, the results obtained in our work with acclimated biomass are very promising
453 because the achieved CLA removal efficiency was higher than those reported previously. Evangelista et al.[18]
454 found for *Rhodococcus rhodochrous* an elimination yield of 90% for a solution of CLA of 100 mgL⁻¹ but after a
455 long acclimation period of 20 days. Winkler et al. (2001) reported that CLA was not removed at an initial
456 concentration of 90 µgL⁻¹ over a time period of 400h and only 27% of elimination was achieved in a biofilm
457 reactor feed with river water spiked at 11 µgL⁻¹ after a culture time of 95h. In another work, Tran et al. (2009)
458 obtained a maximum elimination yield of 25% with enriched nitrifier culture for the same molecule, and an
459 initial concentration of 200 mgL⁻¹.

460 According to literature data, the molecular structure of clofibric acid is involved in its recalcitrant nature. Indeed,
461 the significant role of this parameter in the recalcitrance of xenobiotic compounds is well known (Evangelista et
462 al. 2010). It is acknowledged that, chlorinated substitutions or structural characteristics such as ring substitutions
463 but also the nature of aliphatic side chain of compounds influence their persistence in the environment, affecting
464 their susceptibility to biological degradation. The target molecule considered in this study contains a
465 chlorophenolic group, but its resistance to biodegradation is in fact due to the steric hindrance from a single extra
466 methyl group (Evangelista et al. 2010).

467 Results obtained in this work demonstrate, that the both acclimation protocols investigated here had the ability to
468 acclimate *Pseudomonas aeruginosa* RZS9 biomass to degrade CLA but the biomass obtained from the Protocol

469 II had higher pollutant elimination efficiency. For this reason, further optimization experiments were designed
470 with acclimated biomass obtained under this acclimation strategy.

471

472 **3.3 Bioremediation of CLA by acclimated biomass: effect of the initial concentration of carbon co-** 473 **substrate**

474

475 As stated above, in many cases the xenobiotic molecule cannot serve as the sole carbon and energy sources to
476 maintain biomass and induce expression of relevant enzymes and/or cofactors for the assimilation by
477 microorganisms. Many works suggested that the main biodegradation pathway of such compounds is probably
478 attributed to a microbial cometabolic activity based on the use of a growth substrate or to another utilizable
479 compound. It requires the obligatory presence of a growth substrate which helps and maintains the biomass
480 growth and produces the relevant enzymes/cofactors involved in the cometabolic biodegradation of the pollutant
481 (Tran et al. 2013). Moreover, it should be noted the degradation efficiency of a pollutant as well as the formation
482 of by-products is influenced by the different growth substrates added. The degradation of organic pollutants
483 refractory to biological decomposition was frequently observed or enhanced in the presence of easily assimilated
484 carbon substrates (Gren et al. 2010). For all these reasons the biodegradation of CLA by acclimated biomass was
485 also investigated under cometabolic conditions. For comparison purposes glucose was used as a simple source of
486 carbon. Tests were carried out for different initial glucose concentrations in order to determine the optimum
487 conditions allowing an efficient removal of the target molecule.

488 The experiments were performed in batch reactors, using as inoculum, biomass that was previously acclimated to
489 CLA following the Protocol II (cells in the decline phase) with 1 mgL⁻¹ CLA and 1.25 mg L⁻¹ glucose. Different
490 initial glucose concentrations ranging from 0.25 to 10 gL⁻¹ were tested. Fig 6a shows the CLA removal
491 efficiencies determined after 72h of incubation. It can be observed that the CLA degradation activity was
492 modified by the addition of glucose as carbon co-substrate. For the investigated conditions an optimal value of 2
493 gL⁻¹ was found for the glucose concentration (Fig.6a). Indeed, the CLA removal yield increased from 19 to 35%
494 with a rise of the glucose concentration from 0 to 2 gL⁻¹. Similarly, Popa et al. (2014) and Popa Ungureanu et al.
495 (2016) showed that the addition of glucose improved the carbamazepine and CLA degradation by *Streptomyces*
496 spp. Liu et al. (Liu et al. 2013) also demonstrated that the addition of different utilizable carbon substrates (such
497 as sucrose and succinate) enhance the elimination of an insecticide (imidacloprid) by the bacterial strain
498 *Stenotrophomonas maltophilia*. Moreover, obtained results suggest once again, that the addition of readily
499 available carbon and energy sources facilitate the cells growth (Fig. 6b), but also can act as an inducing agent for
500 the production of relevant enzymes and provide cofactors (i.e. NADPH or NADH) needed for the degradation of
501 the target pollutant.

502 Beyond a glucose concentration of 2 gL⁻¹, the biodegradation yield decreased and was only 22%, which was
503 nearly to the value obtained for the control test (without glucose) carried out with acclimated biomass (19%)
504 after 72h of incubation time (Fig 6a). This result also confirms the positive effect of the bacterial strain
505 acclimation and demonstrates that acclimated *Pseudomonas aeruginosa* RZS9 strain is able to grow in the
506 presence of the pollutant as the sole carbon and energy source. It can also be noted the decrease of the pH of the
507 culture medium from 7.3 to 4.5 (Fig.6b). This effect will be attributed to a catabolite repression by glucose,
508 which has been reported by some researchers (Loh and Wang 1997; Satsangee and Ghosh 1990).

509 Their findings suggest that the presence of high glucose concentrations can inhibit the use of the target substrate.
510 Moreover, Wang and Loh (1999) and Fakhruddin and Quilty (2005) reported that high concentrations of glucose
511 caused significant drop in pH and the inhibition of the assimilation of other substrates present in the culture
512 medium.

513

514

Fig.6 (a), (b)

515 4 Conclusions

516 This is the first study on the degradation of clofibric acid by *Pseudomonas aeruginosa* RZS9, a phenol resistant
517 strain isolated from the activated sludge in a sewage treatment plant from Algeria. This work demonstrates that
518 the cometabolic conditions influence the removal efficiency of the CLA with a simultaneous increase of biomass
519 growth. However, under these conditions the strain exhibits a low biodegradation yield; the reached maximum
520 elimination yield was only of 20% after 72h of incubation. Information concerning the acclimation process of the
521 isolated strain to CLA is also given; the acclimation strategy has a significant impact on the removal of the target
522 molecule. Indeed, the acclimation protocol based on the use of cells harvested during the decline phase produced
523 biomass with higher activity. Optimal CLA removal (up to 35%) was obtained with acclimated biomass in
524 the presence of 2 gL⁻¹ of glucose. In addition, the presence of high glucose concentrations negatively impacted
525 the consumption of CLA by the acclimated biomass. In conclusion, the results obtained demonstrated the ability
526 of the selected strain for the removal of the target molecule. To complete this work, research is ongoing dealing
527 with the identification of the degradation metabolites and their toxicity. Moreover, further research is needed to
528 optimize the growth culture conditions of the acclimated strain in order to increase its degradation efficiency.

529

530

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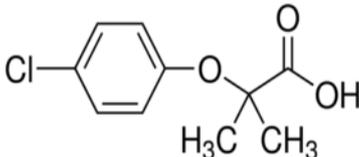
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Table 1 Physicochemical properties of clofibric acid (CLA).

Chemical Structure	
CAS Number	<u>882-09-7</u>
Formula	C ₁₀ H ₁₁ ClO ₃
Therapeutic class	lipidregulator
Appearance	white solid
Molecular weight	214.65 g mol ⁻¹
Water solubility	573 mg l ⁻¹ (25 °C) ^b
pK _a	3 ^a
Log k _{ow}	2.57 ^a
Log D _{ow}	-0.42 ^a
Henry'slaw constant	2.19 10 ⁻⁸ Pa m ³ mol ⁻¹ (25 °C) ^b

^a Zhang et al. 2012.^b Zhang et al. 2014.

Table 2 Morphological and biochemical characteristics of isolated strain.

Test	Observation	
Morphological tests	Bacterial culture 	pigmentation Fluorescent yellow- green
		shape smooth
		consistency mucous
	Nutrient agar Gram's strain 	Gram-negative
	Mobility	+ ^a
Biochemical tests	Catalase	+
	Oxydase	+
	Citrate assimilation	+
	Arginine dihydrolase (ADH)	+
	Glucose (MEVAG medium)	+
	Indole	- ^b
	Methylred	-
	Vogesproskauer	-

^a Positive result.

^b Negative result.

Figure captions

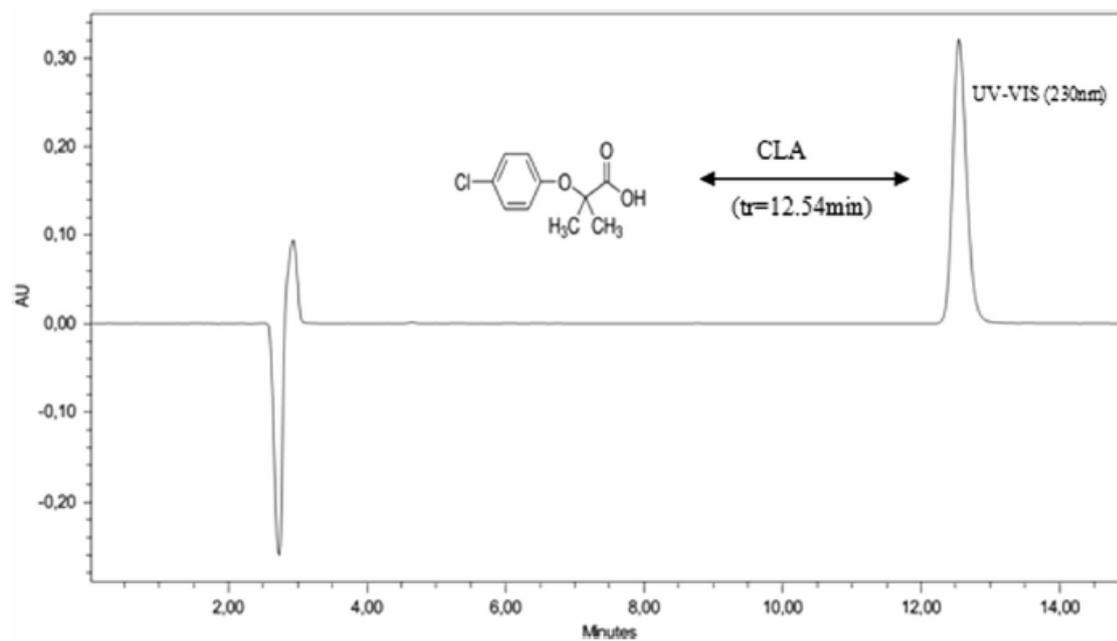


Fig. 1 Chromatogram obtained by HPLC-UV analysis for standard solution of CLA.

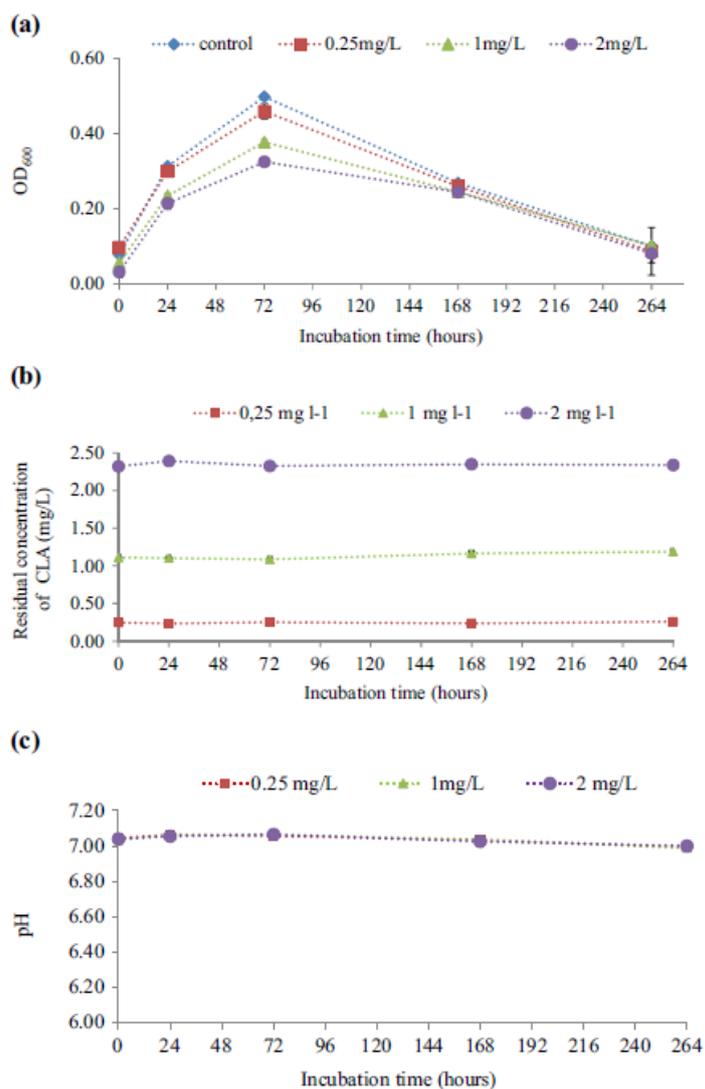


Fig. 2 Profiles of cells growth, pH and residual concentration of CLA for the biodegradation experiments without co-substrate and non-adapted cells conducted at different initial pollutant concentrations; 37°C, 150 rpm and an initial pH=7±0.1. (a) *Pseudomonas aeruginosa* RZS9 growth curves (expressed as optical density at 600 nm); (b) residual concentration of CLA; (c) pH profiles.

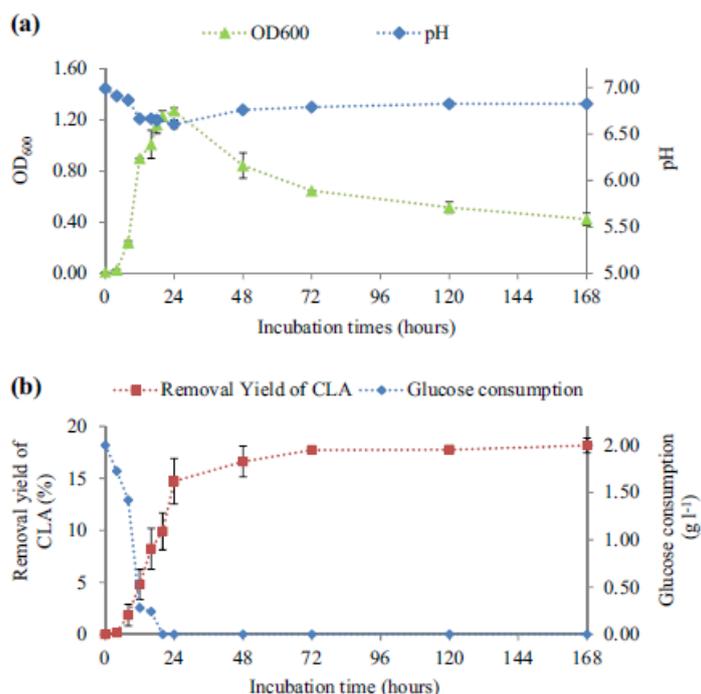


Fig. 3 Time-course variation in OD₆₀₀ values, pH, CLA removal yield and glucose consumption for the biodegradation test carried out with glucose as carbon co-substrate and non-adapted cells. (a) cell growth and pH profiles; (b) removal yield of CLA and glucose consumption; [CLA]₀ = 1 mg L⁻¹; [glucose]₀ = 2 g L⁻¹; 37 °C; 150 rpm ; pH=7±0.1.

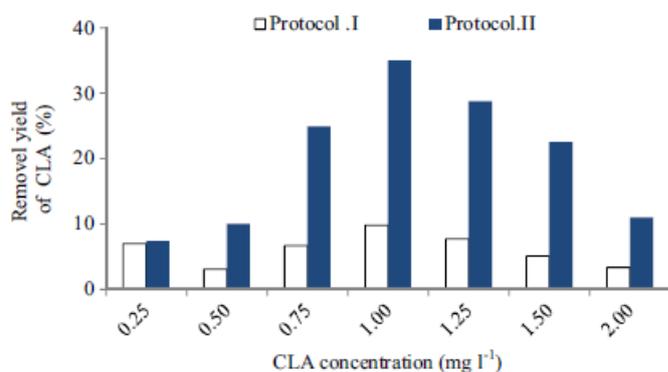


Fig. 4 Removal yields of CLA at the end of exponential phase (24h) as a function of initial CLA concentration during the consecutive batch assays performed for the both acclimation procedures. [glucose]₀ = 2, 1.75, 1.5, 1.25, 1, 0.5, 0.25 g L⁻¹; 37°C; 150rpm; pH=7±0.1.

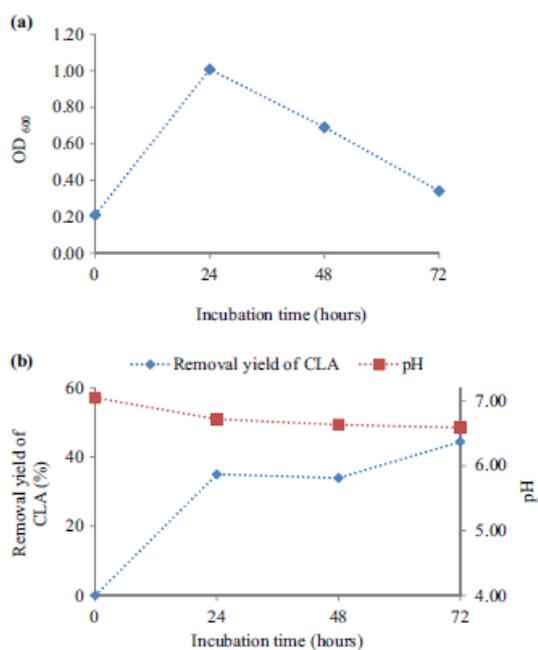


Fig. 5 Time course of the acclimation experiments during the batch assay performed at $[CLA]_0 = 1 \text{ mg L}^{-1}$; $[\text{glucose}]_0 = 1.25 \text{ g L}^{-1}$; 37° C ; 150 rpm; $\text{pH} = 7 \pm 0.1$ for cells acclimation to CLA following the protocol II. (a) growth of *Pseudomonas aeruginosa* RZS9 strain; (b) removal yield of CLA and pH profiles.

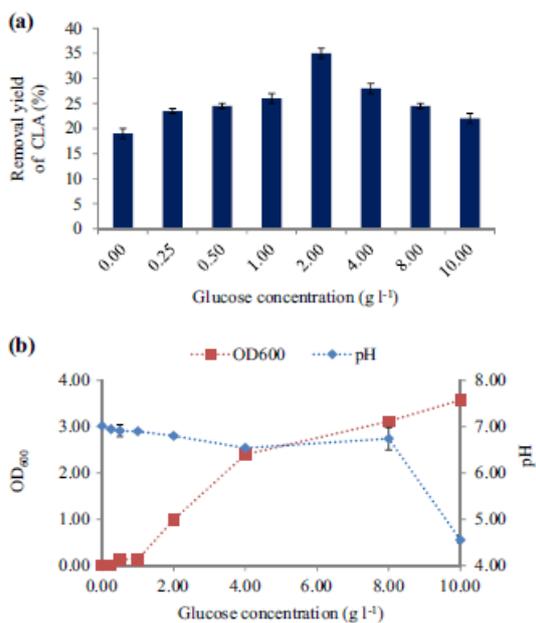


Fig. 6 Effect of different initial glucose concentrations, after 72h of incubation time. (a) removal yield of CLA; (b) cell growth and pH profiles. $[CLA]_0 = 1 \text{ mg L}^{-1}$; $[\text{glucose}]_0$, ranging from 0 to 10 g L⁻¹; 37° C ; 150rpm; $\text{pH} = 7 \pm 0.1$.