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Sabra Hemidouche, Lidia Favier, Abdeltif Amrane, Patrick Dabert, Sophie Le  
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1 **Successful biodegradation of a refractory pharmaceutical compound by an indigenous**  
2 **phenol tolerant *Pseudomonas aeruginosa* strain**

3 **Sabra Hemidouche<sup>a,b</sup>, Lidia Favier<sup>c</sup>, Abdeltif Amrane<sup>c</sup>, Patrick Dabert<sup>d</sup>, Sophie Le Roux<sup>d</sup>, Zahra Sadaoui<sup>a</sup>**  
4

5 <sup>a</sup>Laboratoire de Génie de la Réaction, Faculté de Génie Mécanique et de Génie des Procédés, Université des  
6 Sciences et de la Technologie Houari – Boumediene, BP n°32 el Alia Bab -Ezzouar 16111, Alger, Algeria

7 <sup>b</sup>Centre de Recherche Scientifique et Technique en Analyses Physico-Chimiques, BP 384, Siège ex-Pasna Zone  
8 Industrielle, Bou-Ismaïl CP 42004, Tipaza, Algeria

9 <sup>c</sup>Ecole Nationale Supérieure de Chimie de Rennes, CNRS, UMR 6226, 11 Allée de Beaulieu, CS 50837, 35708  
10 Rennes Cedex 7, France

11 <sup>d</sup>IRSTEA -UR Gestion Environnementale et traitement biologique des déchets(GERE), 17, avenue de Cucillé -  
12 CS 64427, 35 044 Rennes, Cedex, France

13 **\*Corresponding Authors:**

14  
15 Dr. Lidia Favier, Associate Professor  
16 Ecole Nationale Supérieure de Chimie de Rennes, CNRS, UMR 6226, 11 Allée de Beaulieu CS 50837, 35708  
17 Rennes Cedex 7, France.  
18 Tel.: +33 2 23 23 81 55; Fax: +33 2 23 23 81 20  
19 E-mail: [lidia.favier@ensc-rennes.fr](mailto:lidia.favier@ensc-rennes.fr)  
20

21 Prof. Abdeltif Amrane  
22 Ecole Nationale Supérieure de Chimie de Rennes, Université de Rennes 1, CNRS, UMR 6226, 11 Allée de  
23 Beaulieu CS 50837, 35708 Rennes Cedex 7, France.  
24 Tél: +33 2 23 23 81 55; Fax: +33 2 23 23 81 20  
25 E-mail: [abdeltif.amrane@univ-rennes1.fr](mailto:abdeltif.amrane@univ-rennes1.fr)  
26

27 Prof. Zahra Sadaoui  
28 Laboratoire de Génie de la Réaction, Faculté de Génie Mécanique et de Génie des Procédés, Université des  
29 Sciences et de la Technologie Houari – Boumediene, BP n°32 el Alia Bab -Ezzouar 16111 Alger, Algérie  
30 E-mail: [sadaouizahra@yahoo.fr](mailto:sadaouizahra@yahoo.fr)

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  $\text{ngL}^{-1}$  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  $240 \text{ ngL}^{-1}$   
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  $2 \text{ mgL}^{-1}$ . 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<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> tryptone, 5 gL<sup>-1</sup> meat extract and 5 gL<sup>-1</sup> 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<sup>-1</sup>): K<sub>2</sub>HPO<sub>4</sub>, 4.25; KH<sub>2</sub>PO<sub>4</sub>, 1.70; NH<sub>4</sub>Cl, 2.10;  
115 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.41; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.06; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01; CaCl<sub>2</sub>·2H<sub>2</sub>O, 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<sup>-1</sup>). For each experiment, 10 mL of inoculum (OD<sub>600</sub>=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<sup>-1</sup> of glucose and 1 mgL<sup>-1</sup> 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<sup>-1</sup>, and gradually increasing CLA concentrations from 0.25  
166 to 2 mgL<sup>-1</sup>, 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)<sub>2</sub> and ZnSO<sub>4</sub>·7H<sub>2</sub>O. 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)<sub>2</sub> (0.3 M) and 0.25 mL ZnSO<sub>4</sub>·7H<sub>2</sub>O (5% w/w). The supernatant obtained after 10 min of  
188 centrifugation at 5000 rpm was filtered through a membrane filter of Polyester (Chromfil<sup>®</sup> 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<sup>-1</sup>. 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<sup>-1</sup>. 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<sup>-1</sup>.  
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<sup>-1</sup>): 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  $\mu\text{L}^{-1}$ ), 2.5  $\mu\text{L}$  of 10 $\times$ PCR buffer, 2  $\mu\text{L}$  of dNTP (2.5 mM each), 1.5  $\mu\text{L}$  of forward primer (10  $\mu\text{M}$ ) and 1.5  $\mu\text{L}$  of  
239 reverse primer (10  $\mu\text{M}$ ), 0.75  $\mu\text{L}$  of  $\text{MgCl}_2$  (50 mM), 1  $\mu\text{L}$  of DNA template and sterile water to 25  $\mu\text{L}$  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  $\text{mgL}^{-1}$ ). 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  $\text{mgL}^{-1}$  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  $\text{mgL}^{-1}$  and more than 70h at 250  $\text{mg L}^{-1}$ .

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  $\text{OD}_{600}$  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 sulfametoxazole 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  $\text{mgL}^{-1}$ . 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 clofibrac 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, clofibrac 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  $1\text{mgL}^{-1}$  of CLA in BMM supplemented with  $2\text{gL}^{-1}$   
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. Fakhrudin 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 ( $\text{OD}_{600}$  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.

354 2009) found that the presence of acetate ( $100 \text{ mgL}^{-1}$ ) as an additional carbon source increases the CLA removal.  
355 In their previous work, Fakhruddin and Quilty (2005) observed the degradation of 2-chlorophenol by  
356 *Pseudomonas putida* CP1 in the culture media supplemented with glucose. Likewise, *Streptomyces* bacteria  
357 isolated from soils cometabolise CLA and carbamazepine in the presence of glucose as additional source of  
358 carbon and energy (Popa et al. 2014, 2015 and 2016).  
359 Moreover, our results clearly showed that, the CLA removal proceed until a certain value and then did not  
360 continue. One explanation is that, the pollutant elimination is not limited by the glucose consumption because it  
361 continues after its depletion from the culture medium but, by an unknown nutrient (oxygen or another) present in  
362 the culture media or by the accumulation of an inhibitory metabolite which affects the CLA degradation. Similar  
363 results have been previously reported by Salgado et al. (2012) for the same molecule.  
364 The obtained data show that *Pseudomonas aeruginosa* RZS9 is able to remove the target molecule in the  
365 presence of glucose. However, the CLA removal yield obtained with unacclimated culture still remains low  
366 confirming the high resistance to microbial attack of this molecule and suggests that adaptation of the strain to  
367 pollutant may be required to have a significant elimination of the target molecule. Several studies have reported  
368 that the capacity of bacteria to remove toxic compounds can be enhanced by acclimation (Evangelista et al.  
369 2010; Ferro Orozco et al. 2013; Sahinkaya and Dilek 2005). Acclimation is considered as the key issue to  
370 achieve the degradation of recalcitrant compounds. Indeed, microorganisms which are repeatedly exposed to a  
371 pollutant may develop new capabilities to degrade toxic molecules. Thus in this study, the impact of the  
372 acclimation on the removal of the target molecule was also investigated.

373

### 374 **3.3 Investigation of acclimation strategy of *Pseudomonas aeruginosa* RZS9 to CLA**

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

388 In order to examine the impact of the acclimation process in the presence of a primary carbon source (glucose)  
389 two acclimation strategies were performed as described in the section 2.3.2. As stated before, the acclimation  
390 strategy used here was based on enrichment or multiple transfer approach by using inoculum of different  
391 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<sup>-1</sup> of  
399 CLA and 1.25 gL<sup>-1</sup> 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<sup>-1</sup> of CLA and 1.25 gL<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> of CLA and at 1.25 gL<sup>-1</sup> of glucose for an incubation time of 72h. It was observed that the  
422 OD<sub>600</sub> 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 rhodochromus* an elimination yield of 90% for a solution of CLA of 100 mgL<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup>.

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 serves 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 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<sup>-1</sup> CLA and 1.25 mg L<sup>-1</sup> glucose. Different  
490 initial glucose concentrations ranging from 0.25 to 10 gL<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup>. 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<sup>-1</sup>, 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<sup>-1</sup> 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

531

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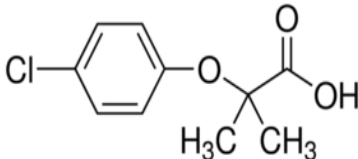
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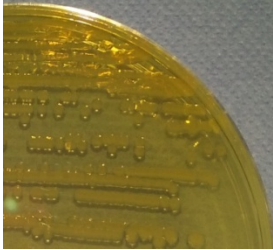

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**Table 1** Physicochemical properties of clofibric acid (CLA).

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

<sup>a</sup> Zhang et al. 2012.<sup>b</sup> Zhang et al. 2014.

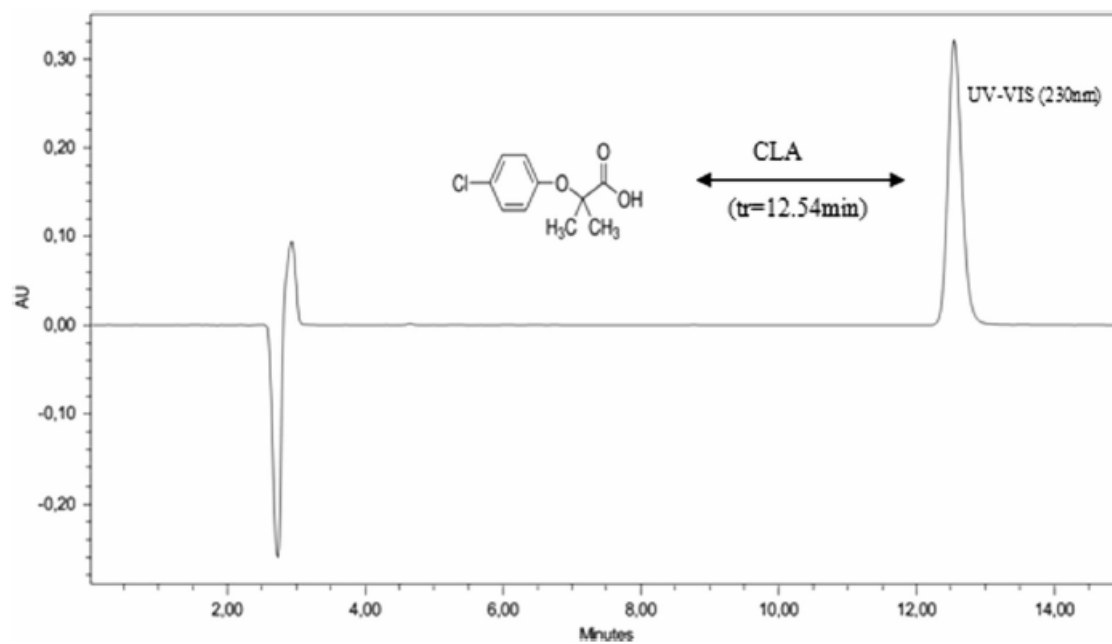
**Table 2** Morphological and biochemical characteristics of isolated strain.

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

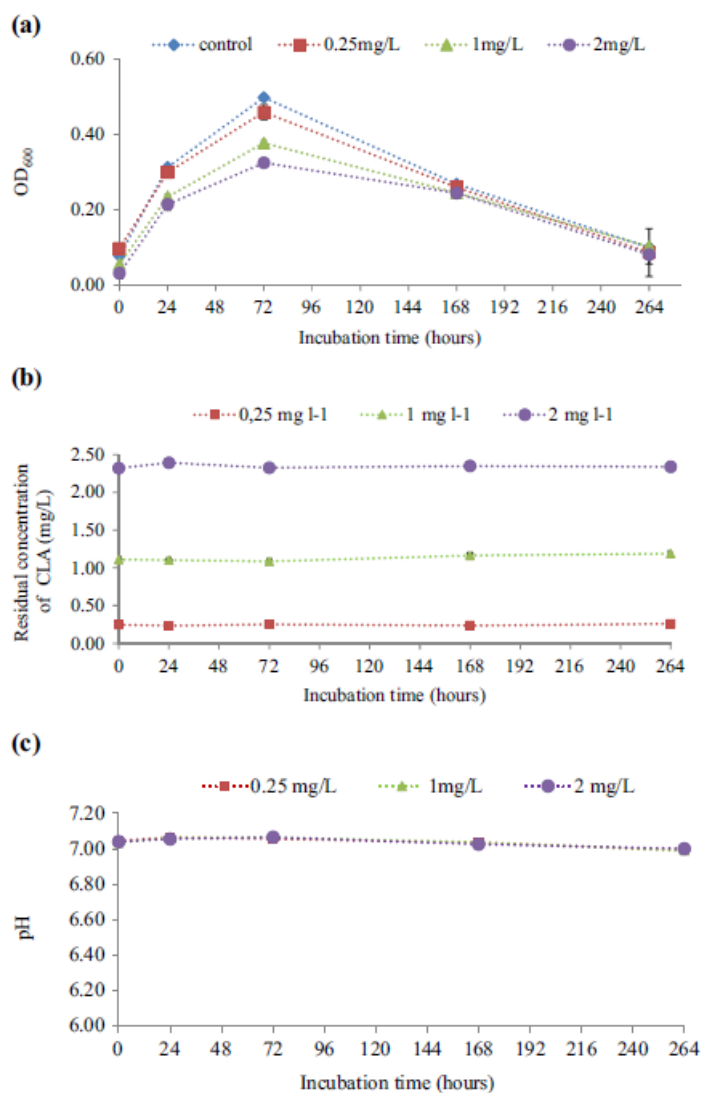
<sup>a</sup> Positive result.

<sup>b</sup> Negative result.

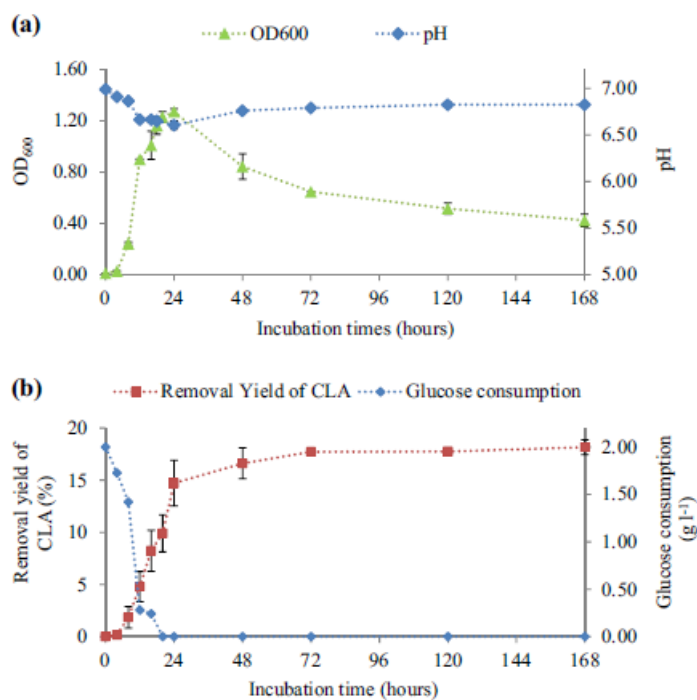
**Figure captions**



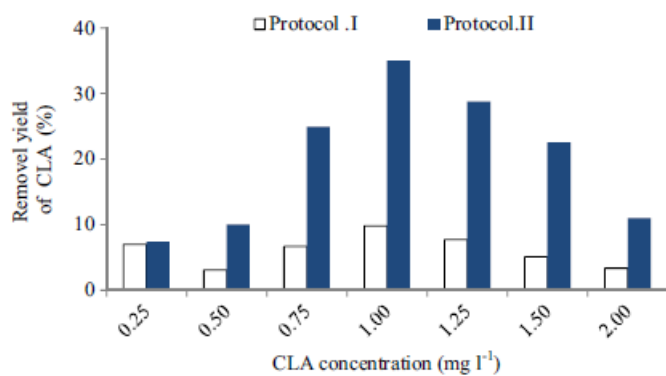
**Fig. 1** Chromatogram obtained by HPLC-UV analysis for standard solution of CLA.



**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.

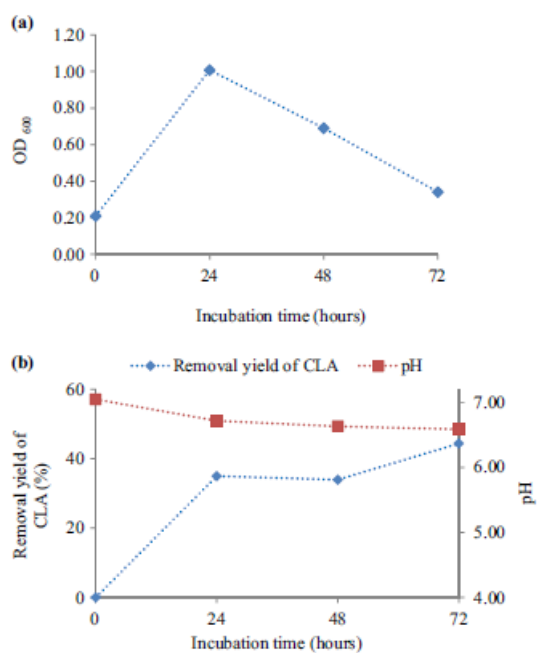


**Fig. 3** Time-course variation in OD<sub>600</sub> values, pH, CLA removal yield and glucose consumption for the biodegradation test carried out with glucose as carbon co-substrate and non-adapted cells. (a) cell growth and pH profiles; (b) removal yield of CLA and glucose consumption; [CLA]<sub>0</sub> = 1 mg L<sup>-1</sup>; [glucose]<sub>0</sub> = 2 g L<sup>-1</sup>; 37 °C; 150 rpm ; pH=7±0.1.

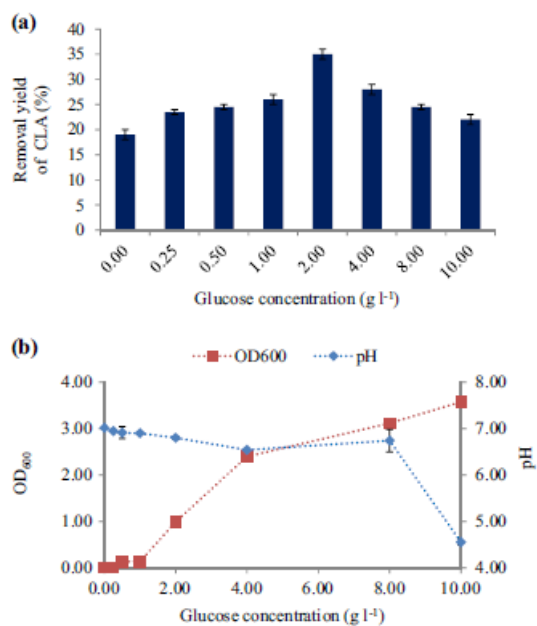


**Fig. 4** Removal yields of CLA at the end of exponential phase (24h) as a function of initial CLA concentration during the consecutive batch assays performed for the both acclimation procedures. [glucose]<sub>0</sub> = 2, 1.75, 1.5, 1.25, 1, 0.5, 0.25 g L<sup>-1</sup>; 37°C; 150rpm; pH=7±0.1.





**Fig. 5** Time course of the acclimation experiments during the batch assay performed at  $[CLA]_0 = 1 \text{ mg L}^{-1}$ ;  $[\text{glucose}]_0 = 1.25 \text{ g L}^{-1}$ ;  $37^\circ \text{ C}$ ; 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  $\text{g L}^{-1}$ ;  $37^\circ \text{ C}$ ; 150 rpm;  $\text{pH} = 7 \pm 0.1$ .