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# Use of hydrocarbons sludge as a substrate for the production of biosurfactants by *Pseudomonas aeruginosa* ATCC 27853

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33 **Keywords:** *Hydrocarbons sludge; Pollution; Refinery; Recycling; Enzymatic induction*  
34 *Growth; Biosurfactants.*

## 35 **Introduction**

36

37 Hydrocarbons sludge is an effluent provided from the crude oil refining industry resulting from  
38 various processes along the crude oil processing lines. They constitute a serious threat to the  
39 environment and need to be treated to avoid harming human health and the sustainability of the  
40 ecosystems. (Linda et al., 2016).

41 The study conducted by Pan et al., 2015 evaluates several essential state-of-the-art waste-to-  
42 energy (WTE) technologies including combustion, gasification and anaerobic digestion. Since  
43 strategies under appropriate policies were essential for the implementation of the WTE supply  
44 chain, a critical review on the challenging barriers and overcome strategies was performed.  
45 Several successful case studies around the world were illustrated based on their respective  
46 energy, environmental and economic benefits of the WTE supply chain for the district energy  
47 supply (DES) system. Qomarudin et al., 2010, described the problem of oil waste management  
48 as a consideration of the environmental impact of the oils contained in the waste. The  
49 production of hydrocarbons sludge is massive since oil production has increased in all  
50 producing countries as a result of its involvement in their economies, (Bhattacharyya et al.,  
51 2003); for instance, it can reach up to 2000 tons/day in Indonesia (Tse , 2003). This raise in  
52 petroleum production has increased the threat to the environment and has reached the sub-soil,  
53 (Qomarudin et al., 2010). The amount of hydrocarbons sludge continues to increase and its  
54 severity on the environment becomes worrying due to the density, and the viscosity of the oils  
55 contained. Generally, higher refining capacity is associated with a higher amount of oil sludge  
56 production (Wang et al., 2012; Guangji et al., 2013). The oil contained in the sludge crosses the  
57 earth's layer and slowly reaches the subsoil, which poses a direct risk of contamination of the  
58 subsoil and shallow groundwater.

59 In addition, the light hydrocarbons evaporated in the sludge are mixed with atmospheric dust,  
60 which causes air pollution during the harmattan season. This evaporation phenomenon  
61 provokes serious respiratory problems and eventually human disease and environmental  
62 damage.

63 Irreversible ecological disasters can be realized during releases or voluntary dumping. They  
64 present a significant toxicological risk even at low concentrations, in particular by their  
65 mutagenic and carcinogenic properties (Cravo-Laureau et Duran 2016).

66 In order to preserve the environment and eliminate these pollutants, it is necessary to develop  
67 reliable and effective methods of pollution control. The diagnosis of the treatment techniques  
68 used has shown major imperfections that can cause harm to human health, the ecosystem, and  
69 the environment. In this aim, Marin et al., 2005, studied the bioremediation of a refinery sludge  
70 containing hydrocarbons using agricultural techniques, to assess its ability to reduce the total  
71 content of hydrocarbons added to the soil with the sludge in a semi-arid climate and also to  
72 evaluate its effect on the microbial activity of the soil concerned.

73 Despite the multiple uses of physicochemical processes in the restoration of soil polluted by  
74 petroleum products, bioremediation remains the most effective, best controlled and least costly  
75 solution. Its principle is based on the complete mineralization of petroleum products which do  
76 not generate any toxic by-product. (Azubuike et al., 2016; Rajesh et al., 2017).

77 The addition of surfactants is a promising approach to increase the biodegradation of  
78 hydrocarbons. Nevertheless, these molecules of chemical origin present a risk to the  
79 environment due to their toxicity and their non-biodegradability (Geetha et al., 2018; Banat et  
80 al., 2000). Recently, several authors were interested in the production of biosurfactants due to  
81 the advantages presented by these biomolecules compared to their chemical counterparts, such  
82 as biodegradability, low toxicity, efficacy under extreme conditions (pH, temperature and  
83 salinity) (Makkar et al., 2011). Furthermore, as result of their emulsification and solubilization  
84 capacity, they have many potential applications covering a wide variety of fields such as  
85 medicine, the petroleum industry and the bioremediation of sites contaminated by hydrocarbons  
86 (Santos et al. ., 2017). Biosurfactants produced from *Pseudomonas aeruginosa* are constituted  
87 by the rhamnose fraction and the fatty fraction resulting from the growth on the hydrocarbon  
88 substrate, they are specific to a given carbon substrate for the growth of the producing strain.

89 In their study, Montagnolli et al., 2019 compared the effects of biosurfactants on the  
90 biodegradation of biodiesel and vegetable oils by two conceptually divergent methodologies.  
91 They found that biosurfactants caused an increase in oil absorption, increasing biodegradation  
92 performance. Also, they found that the biodiesel substrate stood out with the highest  
93 biodegradation rates. These results indicated that a rapid metabolic shift from the parent  
94 compound initially favored biodiesels in the uptake of organic carbon for a set of specialized  
95 microbial inoculum. The data has been successfully combined with mathematical models and  
96 statistical tools to describe and predict the actual environmental behavior of biodiesel and  
97 vegetable oils. The models confirmed and predicted the effectiveness of biodegradation with  
98 biosurfactants and allowed to estimate the time needed to satisfactorily remove contaminants.

99 The enzymes involved in the conversion of hydrocarbons in *P. aeruginosa* are of two types,  
100 namely rhamnolipase I and II with self-inductive capacity. However, in the literature, no  
101 author was interested in the efficiency of the inductive metabolism of *P. aeruginosa* in  
102 removing the oils contained in the residual sludge of the petroleum refining industries. The  
103 bacterial genus *Pseudomonas* is characterized by a multitude type of biosurfactants and  
104 synthesizing enzymes by induction in a medium in which phosphate and nitrogen are limiting.  
105 The enzymatic induction phenomenon of *P. aeruginosa* is at the origin of the synthesis of  
106 several rhamnolipids (Hanna et al., 2011; Ellen et al., 2014). Also, the metabolic process of *P.*  
107 *aeruginosa* constitutes a great opportunity for the discharge sites by the presence of  
108 biosurfactants which enrich the environment with biodegradable organic substances (Abhijit et  
109 al., 2014; Tanja et al., 2017).

110 The challenge of this study dealing with the treatment of hydrocarbons sludge is to highlight  
111 the importance of the inductive metabolism of *P. aeruginosa* in the elimination of hydrocarbons  
112 oils in sludge. For this purpose, the physicochemical behavior of the biosurfactants produced,  
113 allowing the extraction of the oils contained in the sludge by wetting, solubilization, and  
114 emulsification was examined. In addition, the conditions for the inductive growth of the strain  
115 leading to an efficient elimination and evacuation of sludge without risk of ecosystem  
116 degradation were investigated.

117

## 118 **Materials and methods**

119

120 The strain used in this work was *P. aeruginosa* ATCC 27853. It was kindly provided by the  
121 hospital of Medea (Algeria). The hydrocarbons sludge was furnished by the Niger-SORAZ  
122 industry. It was packaged in powder form after several treatments to destroy all traces of  
123 microorganisms. The culture medium was formulated according to the medium proposed by  
124 Milena et al., 2012. It was conditioned using an oven, and then wet heated in an autoclave.  
125 Biomass growth (OD at 600 nm) and hydrocarbon consumption by COD (transmittance at 420  
126 nm) monitoring were performed using a spectrophotometric method.

127

### 128 **Culture medium formulation**

129

130 To ensure the microbiological quality of the hydrocarbons sludge, double sterilization was  
131 undertaken. The first step consisted of humidifying the hydrocarbons sludge to reactivate all  
132 the microorganisms contained. The hydrocarbons sludge were then placed in an oven for one

133 hour at 180°C before being cooled to 70°C. In addition, 1 g of this suspension was dissolved in  
134 100 mL of distilled water. This solution was then tested on a solid agar medium for a period of  
135 48 to 72 h at 37°C. The second step consisted of sterilization with moist heat at 121°C for 15  
136 minutes followed by the same test to evaluate the sterility (Pacheco et al., 2010).

137 The culture medium formulated for the production of glycolipids biosurfactant was constituted  
138 by the following mixture: NaCl (1g/L); KNO<sub>3</sub> (4g/L); MgSO<sub>4</sub>·3H<sub>2</sub>O (0.2g/L); CaCl<sub>2</sub>·2H<sub>2</sub>O  
139 (0.05 g/L); FeCl<sub>3</sub>·7H<sub>2</sub>O (0.01 g/L); yeast extract (6 g/L); as well as KH<sub>2</sub>PO<sub>4</sub> monobasic  
140 phosphate buffer (4 g/L). 1 mL/L of the following trace elements solution was added to the  
141 culture medium: ZnSO<sub>4</sub> (50 mg); MnSO<sub>4</sub> (23 mg); FeSO<sub>4</sub>·5H<sub>2</sub>O (10 mg); CuSO<sub>4</sub> (10 mg);  
142 CaCl<sub>2</sub>·6H<sub>2</sub>O (36 mg); H<sub>3</sub>BO<sub>3</sub> (12 mg); KI (15 mg) and EDTA (80 mg). (Milena et al., 2012).

143 The hydrocarbons sludge powder 3% (w/v) was added as a source of carbon and energy to the  
144 medium (Linda et al., 2016). The solution was dissolved and magnetically stirred for 5 min for  
145 homogenization. The medium was adjusted to a pH = 6.0 ± 0.01 by the addition of sodium  
146 hydroxide solution (1N) or hydrochloric acid (1N). It was then sterilized for 15 minutes at  
147 121°C in an autoclave. After sterilization, the medium was cooled with tap water and the pH  
148 was then adjusted to pH = 7.0 ± 0.01 before inoculation, (Patil et al., 2014).

149

#### 150 Culture conditions

151

152 The culture was performed in batch mode at 37°C for 48 h with an agitation speed  $W = 250$   
153 rpm at pH = 7.00 ± 0.01. Germination was carried out in a sterilized solid selective medium  
154 (cetrimide agar) for 24 hours containing as carbon source a mixture of n-heptane and cetane to  
155 prevent a long latency phase. The microbial cells were then seeded for 48 h of culture with a  
156 regular sampling of 10 mL every 2 h on average for kinetic monitoring (Saimmai et al., 2012).

157

#### 158 Kinetic Monitoring

159

160 The kinetic study focused on biomass growth through the measurement of the optical density  
161 at 600 nm, which was examined versus the evolution of nitrogen, the consumption of  
162 hydrocarbons sludge and the productivity of biosurfactants. Samples of 10 mL were collected  
163 and centrifuged at  $W = 6000$  rpm for 10, 15 and 20 min with the removal of the residual solid  
164 mass ; this latter was diluted in 10 ml of water and measured at 600 nm. Then, 2 mL of cell-  
165 free medium was taken for the preparation of the emulsifying index. The emulsion samples  
166 were left for 24 h. The emulsifying index EI<sub>24</sub>% was given by the ratio between the height of

167 the emulsified layer on that of the whole column (Kumara et al., 2006; Aparna et al., 2011).  
168 The rest of the cell-free medium was precipitated after 24, 48, 72 and 96 h, with absolute ethanol  
169 (1:3 v/v), then centrifuged and maintained for 24 h at 4°C (Prabakaran et al., 2015).  
170 Biosurfactants were recovered and dried in a vacuum desiccator. The precipitated medium was  
171 measured by the COD-spectroscopic method at 420 nm. Samples of 2 mL were then taken,  
172 diluted and digested in a digester. The monitoring of the biomass growth was performed by  
173 optical density (OD) at 600 nm using a UV/VIS spectrophotometer (Senthil et Jayalakshmi,  
174 2013). The nitrogen concentration in the culture medium during the treatment was determined  
175 by the iron oxidation method in acidic medium, followed by a spectrophotometric  
176 determination of ferrous ion at 510 nm.

177

#### 178 Effect of the phosphate buffer

179

180 The effect of buffer was performed owing to its significant impact on culture; it stimulates  
181 growth, the associated production of biosurfactants and stabilizes the pH of the medium  
182 (Graziela et al., 2010). Experiments were spread on an experimental scale according to the  
183 lower and upper limits of buffer concentration and productivity. It allows to find a maximum  
184 production of biosurfactants and to determine the stimulating effect of the buffer on the growth  
185 of the strain. This assessment was done according to three key areas, namely low stimulation,  
186 optimal stimulation and repressive stimulation (Bassirou et al 2019).

187

#### 188 Characterization of the Biosurfactants

189

190 The detection of biosurfactants in the medium during the culture was based on the  
191 emulsification index and the method of Cooper and Goldenberg (1987). In addition, a test was  
192 carried out to demonstrate the rhamnose fraction of the biosurfactants produced based on the  
193 determination of hydrocarbon groups of the biosurfactants using the method of Dubois et al  
194 (Dubois et al., 1956). A volume  $V = 0.5$  mL of cell-free medium was collected and mixed with  
195 0.5 mL of phenol solution (5%) and 2.5 mL of sulfuric acid. The mixture was set for 15 minutes  
196 and then the absorbance was read at 490 nm.

197

#### 198 FTIR Characterization of crude biosurfactants

199

200 The infrared method was used to analyze the crude biosurfactants and to identify its various  
201 groups, characteristic of biomolecules (Prabakaran et al., 2015). The characterization method  
202 consisted of assaying KBr pellets of 0.23 mm containing a small amount of crude  
203 biosurfactants. The analysis was then carried out in a wavelength range from 400  $\text{cm}^{-1}$  to 4000  
204  $\text{cm}^{-1}$  with a resolution of 2  $\text{cm}^{-1}$ . The unit was controlled by software (IR/FP Solutions) which  
205 regulated the humidity and the ambient air (correction of atmospheric carbon dioxide).

206  
207 Physicochemical characterization of biosurfactants.

#### 208 209 *Surface tension and CMC*

210  
211 Equilibrium surface tension ( $\gamma$ ) was measured at a temperature of 25°C with a Krüss K9 digital  
212 tensiometer (Krüss, Helsinki, Finland) by the ring method of the aqueous solution at different  
213 concentrations of crude biosurfactant (Habib et al., 2013). The instrument was calibrated  
214 against ultrapure water ( $\gamma = 72 \text{ mN/m}$ ) to ensure accuracy over the entire range of surface  
215 tension. The ring was rinsed with ethanol than with water to avoid any particles or dust which  
216 may contaminate the latter (Milena et al., 2012). For the calibration of the instrument, the  
217 surface tension of distilled water was measured before each series of experiments. All assays  
218 were performed in triplicate along with distilled water as a control.

#### 219 220 *Effect of the salinity*

221  
222 The effect of salinity on aqueous solutions of crude biosurfactant was studied by adding NaCl  
223 in the range of 25 to 250 g / L with a step of 25 g / L. After addition, the samples were placed  
224 in the dark for 20 minutes. The objective was to assess the reactivity of the biosurfactant facing  
225 extreme chemical conditions. Thus, the monitoring was carried out by measurements of the  
226 surface tension after the addition of NaCl (Rajesh et al., 2014).

#### 227 228 229 *Effect of the pH*

230  
231 The stability of the biosurfactant versus pH variations was examined using a solution of 0.6 g/L  
232 of crude biosurfactant. The pH of the samples was adjusted within a range of 2 to 12 with a step  
233 of 1 for biosurfactant concentrations in the range of 1 to 600 mg/L (Habib et al., 2013). The



234 surface tension of the solutions was measured after adjusting the pH and after 30 minutes of  
235 contact time. This allowed to determine the optimal pH linked to the reactivity of the  
236 biosurfactant tested and also to evaluate the activity of the bioproducts regarding pH variations  
237 of the medium (Govindammal et Parthasarathi, 2013).

238

### 239 *Displacement and oil solubilization*

240

241 The oil displacement test is a method of determining the surface activity of the biosurfactant by  
242 measuring clear zone diameter. The method of displacement of the oil by solubilization was  
243 used to measure the stretching area diameter of the immiscible oil. The solubilization and  
244 displacement were provoked by the addition of an aqueous solution of crude biosurfactant at  
245 the oil-water interface (Rath et al., 2016).

246 The following procedure was considered to study the oil displacement, namely the expansion  
247 of the diameter of the oil droplet on a surface of distilled water: a Petri dish was filled with 50  
248 mL of distilled water, a volume of 20  $\mu\text{L}$  of oil was deposited on the surface of water, and then  
249 15  $\mu\text{L}$  of a solution of the crude biosurfactant at a concentration of 1 g/L was filled on the oil  
250 surface (Hamid et al., 2012).

251 Moreover, to investigate the solubility of the oil in an aqueous solution of crude biosurfactant,  
252 a Petri dish was filled with 25mL of a 1 g/L biosurfactant solution ; then, 15 $\mu\text{L}$  of oil were  
253 removed and deposited on the surface of the aqueous solution of crude biosurfactant. In both  
254 cases, the value of the diameter of the oil displacement was expressed in cm.

255

## 256 **Results and discussion**

257

258 The treatment efficiency characterizes the bioconversion and the efficiency of the treatment of  
259 hydrocarbon sludge to avoid damage to the environment and the ecosystem. It responds to the  
260 objective assigned to treatment, and therefore to the development of decontamination  
261 technologies in environments polluted by hydrocarbons. This allows to have a completely  
262 biodegradable and stable product, ES5D = 96.17% for an emulsification index  $EI_{24} = 71.80\%$ ,  
263 useful for various industries.

264 The results obtained include the regeneration of all the genetic material, the production of  
265 primary metabolites (enzymes, structural proteins, intra, and extracellular signaling systems,  
266 nucleic acids ...), since *Pseudomonas aeruginosa* synthesizes a complex called quorum whose  
267 maturity is essential to the growth and the production of secondary metabolites in fermentative

268 medium. Facing this consideration, the hydrocarbon and nitrogen substrates abruptly decreased  
269 between 0 and 3 h, leading to a treatment yield of 36.5% after 3 h. Also, a part of the substrate  
270 was used for the production of biosurfactants stimulated by the presence of hydrocarbon  
271 substrates.

272 The characteristic of the biosurfactants produced should highlight the importance of its  
273 biomolecules in the treatment of hydrocarbons sludge by their actions of wetting, solubilization,  
274 emulsification and extraction of the contained oils. The presence of biosurfactants in the  
275 medium promotes the contact between the microbial cells of *Pseudomonas aeruginosa* and the  
276 source of carbon and energy, namely the hydrocarbons contained in the sludge, to achieve  
277 optimal growth.

278

279 Determination of the phosphate buffer concentration

280

281 The determination of the phosphate buffer content, both as a growth stimulant and for pH  
282 stabilization was envisaged. The results regarding the evaluation of the importance of the  
283 phosphate buffer are summarized in Table 1, showing an optimum for 30 mmol/L.

284

285

286 **Table 1.** Comparative results of phosphate buffer values. Threshold and optimal  
287 concentrations (pH = 7, T = 37°C, W = 250 rpm).

288

289 In light of the results obtained and based on the tests carried out, the lowest critical value of  
290 productivity was 10mmol/L. It is important to note that the initial conductivity measurements  
291 are subject to the effect of the measured temperature and the volume of sodium hydroxide or  
292 hydrochloric acid used for pH adjustment after medium sterilization and cooling, (Cha et al.,  
293 2008; Priya et Usharani 2009). Nutrients assimilation allowed to decrease the conductivity of  
294 the medium to an extreme level, having a significant impact on the beginning of the growth and  
295 biosurfactant production. In addition, when the buffer concentration is low in the medium, the  
296 conductivity of nitrate ions dominates the overall conductivity, owing to the high conductivity  
297 of this anion.

298 Biosurfactants productivity showed a steady evolution until 40 hours, while there was an  
299 increase of almost 10% at 48 h (EI<sub>24</sub> = 57.2%) (Fig. 1); this result is in agreement with previous  
300 reports (Senthil et Jayalakshmi, 2013). This account for the inefficiency of the buffer, since the  
301 conductivity at the beginning of biosurfactants production (G = 11.20 mS/cm after 3 h of

302 culture) had not reached the threshold value of productivity observed with 30 mmol/L after 48  
303 h,  $G = 15.10$  mS/cm. This conductivity value was however observed after 48 h of culture, (Ould  
304 boudia, 2013). The increased production of biosurfactants obtained at 40 h can be related to a  
305 deficiency in nutrients and especially hydrocarbons substrate. Indeed, such nutrients limitation  
306 induced an increase in biosurfactants production to solubilize the remaining hydrocarbons  
307 substrate (20.12 mg/L for 10 mmol/L of phosphate buffer).

308 It can also be noted that the consumption of nitrogen (nitrate) with respect to hydrocarbons was  
309 0.20 g/g of carbon source. Nitrates consumption for 10 mmol/L buffer was nearly identical to  
310 the value observed for 30 mmol/L. The stimulating effect of the buffer was therefore observable  
311 in these conditions since this latter did not induce a significant difference regarding the lowering  
312 of the conductivity of the medium allowing maximal growth of the strain. Therefore, as shown  
313 through the emulsification index (Table 1), an increase in the production of biosurfactants was  
314 observed from 40 h of culture due to nutrients starvation (Wei et al., 2005; Whang et al., 2008).  
315 Productivity was significantly lower both in terms of biomass and product level, confirming the  
316 importance of the buffer for the growth of the strain.

317 **Fig. 1.** Evolution of the emulsification index versus time (pH = 7, T = 37°C, W = 250 rpm,  
318  $\text{KH}_2\text{PO}_4 = 10$  mmol/L,  $p \leq 0.05$ ).

319  
320 Whereas, the value of 50 mmol/L buffer showed to be critical in terms of repression of the strain  
321 by the salinity of the medium (Ilori et al., 2005), the emulsification index at this level showed  
322 a maximum at 5 h before declining abruptly up to 48 h. Medium conductivity did not decrease  
323 to a threshold limit allowing growth and biosurfactant production, owing to the lack of salt  
324 tolerance of the used strain; conductivity remained almost constant throughout culture (Table  
325 1). From this, it can be assumed that nitrate respiration most likely occurred throughout the  
326 culture.

327 As shown in Fig 2a and b, the best emulsification was obtained between 3 h and 7 h. Indeed, to  
328 produce biosurfactants without variations of the other medium components, the buffer  
329 concentration must satisfy the optimal physiological needs of the strain to allow an adequate  
330 response to the stimulus. Therefore, the concentration of 50 mmol/L exceeded the optimal needs  
331 of *Pseudomonas aeruginosa* ATCC 27853 in the considered medium and hence caused a  
332 repressive stimulation of the strain (Gorna et al., 2011; Al-Saleh et Akbar 2015).

333 In agreement with the work of Anna et Partcharathi (2014), owing to the excess of salinity  
334 due to the phosphate buffer, cell lysis was observed since the considered *Pseudomonas* strain

335 was not osmotolerant. As shown in Fig 2a and b, this effect can be compared to that of NaCl  
336 (Rikalović et al., 2012; Anna et Partcharathi, 2014).

337 The N/C ratio showed that 0.67 g of nitrate was needed to consume 1 g of carbon, namely three  
338 times the normal respiration observed for the value of 30 mmol/L. This shows that the  
339 environment was not optimal for high biosurfactants production, since the components of the  
340 environment were not well balanced (Dos-Santos et al., 2010; Senthil et Jayalakshmi 2013). In  
341 addition, an approximate lethality and inhibition of the emulsification index were estimated to  
342 be 27.3% and 19.9%, respectively. Lethality and inhibition were deduced from the ratios of the  
343 emulsification index values at 24 and 48 h for the former and 7 and 22 h for the latter (Bassirou  
344 et al., 2019), according to the following equations:

$$345 \text{Lethality} = \frac{EI_{24 \text{ from } 24 \text{ h to } 48 \text{ h}}}{EI_{24 \text{ max obtained}}} \times 100\% - 100\% \quad (1)$$

$$347 \text{Inhibition} = \frac{EI_{24 \text{ from } 7 \text{ h to } 48 \text{ h}}}{EI_{24 \text{ max obtained}}} \times 100\% - 100\% \quad (2)$$

348  
349 **Fig. 2.** Biomass (a) and Emulsification index versus time (b) (pH = 7, T = 37°C, W=250 rpm,  
350  $\text{KH}_2\text{PO}_4 = 50 \text{ mmol/L}$ ,  $p \leq 0.05$ ).

351  
352  
353 Culture conditions

354  
355 Fig 3 a, b, c and d shows growth in two-steps, illustrating enzymatic induction growth namely  
356 diauxic phenomena. Regarding the results represented in Fig. 3, the first growth phase, until 20  
357 h, corresponded to the consumption of the most readily treatment hydrocarbons contained in  
358 the sludge and then the less biodegradable hydrocarbons contained was assimilated until 48 h  
359 of culture (Bassirou et al., 2017).

360 The treatment efficiency can be estimated to be  $\theta = 96.8\%$  with a final hydrocarbons source  
361 concentration  $C = 250.1 \text{ mg/L}$  and a nitrogen to carbon ratio  $\text{N/C} = 20.85\%$ ; consequently, the  
362 biomass on substrate yield was  $Y_{X/S} = 52.7\%$  and the product on substrate yield was  $Y_{P/S} =$   
363  $20.3\%$  over 48 h of culture. The quantitative productivity showed two maxima (Fig 3d),  $P_1 =$

364 64.1 mg at 7 h and  $P_2 = 75.6$  mg at 28 h corresponding to each exponential phase of growth  
365 (Fig. 3a).

366 Based on the related literature, the comparison of the emulsification index obtained in 30  
367 mmol/L buffer and the concentration of biosurfactants produced ( $C = 1560$  mg/L) suggests that  
368 the diauxic growth in the present work occurred on a substrate approaching a lighter substrate  
369 as kerosene for the first phase and diesel for the second phase (Wei et Chu , 2002). Furthermore,  
370 the results of Fig. 3 demonstrated the presence of two hydrocarbons sources in the medium and  
371 growth by enzymatic induction or diauxic growth, which characterizes the metabolic pathway  
372 of the strain. The two peaks reflected the presence of two rhamnolipids, because of the  
373 specificity of biosurfactants to the carbon substrate and the productivity associated with growth.

374

375 **Fig. 3.** Evolution of Growth (a); hydrocarbons substrate consumption (b); nitrogen uptake (c)  
376 and biosurfactants product (d) versus time (pH=7, T=37°C, W=250 rpm,  $\text{KH}_2\text{PO}_4 = 30$   
377 mmol/L,  $p \leq 0.05$ ).

378

379 Following the Cooper and Goldenberg method, the emulsification kinetics showed the  
380 existence of two peaks, corroborating the growth of the strain and the evolution of substrates  
381 assimilation. As shown in Fig. 4, the values of the emulsification indexes  $\text{EI}_{24}$  found are of  
382 46.70% at 7 h (1) and 71.80% at 46 h (2). These two emulsification peaks correspond perfectly  
383 to inductive or diauxic growth

384

385 **Fig.4.** Emulsification index versus time (pH=7, T=37°C, W=250 rpm,  $\text{KH}_2\text{PO}_4 = 30$  mmol/L,  
386  $p \leq 0.05$ ).

387

388 The stability of the emulsion over five days allowed to test the biosurfactants according to the  
389 time and the environmental factors. According to Figure 5, the emulsions remained stable after  
390 five days. They showed ES5D stability of 81.63% for the first growth phase with an  $\text{EI}_{24}$  of  
391 46.70% and ES5D stability of 96.17% for the second growth phase with an  $\text{EI}_{24}$  of 71.80%.  
392 This stability of the formulated foaming is important for the treatment on-site of the sludge  
393 discharges without risk of degradation of the ecosystem due to the capacity of the biosurfactants  
394 to extract the oils contained in the sludge (Yan et al., 2012). The treatment yield was  
395 intrinsically dependent on the stability of the formulated foaming.

396

397 **Fig. 5.** Stability of the emulsions against the time after five days

(pH=7, T=37°C, W=250 rpm, KH<sub>2</sub>PO<sub>4</sub> = 30 mmol/L, p ≤ 0.05).

398  
399  
400

#### 401 Characterization of the biosurfactants

402

403 Biosurfactants were characterized by highlighting their presence in the cell-free medium. For  
404 this purpose, emulsions were prepared under Cooper and Goldenberg conditions. Consistency  
405 and stability can be related to the emulsification index after 24 h. Indeed, no disintegration of  
406 the emulsion was observed during the five days of monitoring at ambient temperature. The  
407 quality of the foam proves the importance of the molecular weight of the biosurfactants  
408 produced. The formation of flocs was very fast because of their high molecular weight. The  
409 rhamnose test, at 490 nm absorbance, was positive, showing their glycolipids structure and  
410 rhamnolipid type.

411

#### 412 *Infrared Characterization of the crude biosurfactants*

413

414 The obtained spectrum showed two regions with two peaks of maximum absorbance  
415 characteristics of the functional groups. The first of high intensity included two amplitudes. The  
416 first at 3420 cm<sup>-1</sup> (OH<sup>-</sup> band alcohol, typical of polysaccharides) and the second at 3277.17 cm<sup>-1</sup>  
417 (OH<sup>-</sup> band characteristic of carboxylic acid). This band constitutes a stretching vibration of  
418 the O-H groups, carboxylic acid dimmers, with large absorption of the band O-H between 3300  
419 and 2500 cm<sup>-1</sup>; while the second region had a peak of high intensity of narrowband which  
420 absorbed at 1105 cm<sup>-1</sup>. The characteristic band of carboxylic acids was easily distinguishable  
421 from that of alcohol by its lower and extended absorbance, covering the C-H bands, in contrast  
422 to that of alcohol between 3420 cm<sup>-1</sup> and 1105 cm<sup>-1</sup>. Besides these bands, the spectrum showed  
423 two bands of 2862.46 cm<sup>-1</sup> and 2926.11 cm<sup>-1</sup> (C-H bands characteristic of a CH<sub>2</sub>-CH<sub>3</sub>  
424 hydrocarbon chain).

425 Other bands can also be observed, at 1647.26 cm<sup>-1</sup> (characteristics of C=O ester groups) and at  
426 1454.38 cm<sup>-1</sup> (characteristics of C-H, asymmetric of CH<sub>3</sub>) (Amani et al., 2010; Pereira et al.,  
427 2013). Thus, the extended C-O band, which absorbs from 1105.25 to 1384.94 cm<sup>-1</sup>,  
428 characterized the presence of a bond between the carbon atoms and the hydroxyl groups of the  
429 chemical structure of rhamnose. According to Govindammal et Partcharathi (2013); Anna et  
430 Partcharathi (2014), the maximum absorption observed at 3466.24 cm<sup>-1</sup> and 3424.60 cm<sup>-1</sup> can  
431 be attributed to OH<sup>-</sup> groups and those at 881.50 cm<sup>-1</sup> and 698.25 cm<sup>-1</sup> are characteristic of CH<sub>2</sub>

432 groups ; all characteristics of the rhamnose cycle, showing that the obtained infrared spectrum  
433 was consistent with the glycolipids structure and the rhamnolipid type of the obtained  
434 biosurfactants (Fig. 6).

435

436 **Fig. 6.** Spectral IR characterization of biosurfactants produced by *Pseudomonas aeruginosa*  
437 ATTC 27853.

438

439 Glycolipids are composed of carbohydrates and long-chain aliphatic acids or hydroxyl aliphatic  
440 acids. An example of the most widely studied glycolipids is rhamnolipids produced by *P.*  
441 *aeruginosa* strains. Although there are several types of rhamnolipid species, all of them possess  
442 similar chemical structures. In general, the two major types of rhamnolipid species are 1-  
443 rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or mono-rhamnolipid (Rha-C<sub>10</sub>-C<sub>10</sub>) and  
444 1-rhamnosyl 1-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or di-rhamnolipid (Rha-  
445 Rha-C<sub>10</sub>- C<sub>10</sub>) (Prabakaran et al., 2015).

446 In comparison to the results obtained by Prabakaran et al., (2015) by FTIR analysis of  
447 biosurfactants products and Habib et al. (2013) related to the physicochemical characterization  
448 of mono-rhamnolipid, the biosurfactants product in the present study according to the FTIR  
449 result analysis are di and mono-rhamnolipids. According to the enzymatic induction  
450 phenomenon (diauxic growth) displayed in the fig. 3, the difference in the composition of  
451 rhamnolipid species might be due to the age of the culture, the strain of *P. aeruginosa*, substrate  
452 composition and the specific culture conditions (Habib et al., 2013). Although the bacterium  
453 produced predominantly a di-rhamnolipid, it also produced a mono-rhamnolipid in minor  
454 proportion.

455

456 Physicochemical characterization of the biosurfactants

457

458 *Surface tension and CMC*

459

460 The CMC is an index used to evaluate the surface-active activity that is the minimum  
461 biosurfactants concentration needed to achieve the lowest values of surface tension, from which  
462 the formation of micellar aggregates begins (Juliana et Crispin, 2013). Fig.7 exhibits the  
463 variation of the surface tension according to the concentration of biosurfactants in aqueous  
464 solutions. Indeed, the crude biosurfactants showed a lowering capacity of the surface tension  
465 of water from  $\gamma = 71.80$  mN/m at 25°C to  $\gamma = 40.52$  mN/m with a crude biosurfactants

466 concentration  $C = 600$  mg/L. The critical micellar concentration of the biosurfactant was CMC  
467  $= (21.0 \pm 0.1)$  mg/L for a series of samples between  $C = [0 - 150]$  mg/L as shown in Fig 7. It  
468 is significantly above sodium dodecyl sulfate (SDS), a synthetic surfactant commonly used in  
469 many industrial sectors, which has a CMC of 21.0 mg/L in the same conditions test (Juliana et  
470 Crispin, 2013).

471

472 **Fig. 7.** Evolution of the surface tension versus the biosurfactants concentration  
473 (pH = 7, T = 37°C, W=250 rpm,  $\text{KH}_2\text{PO}_4 = 30$  mmol/L,  $p \leq 0.05$ ).

474

#### 475 *Effect of the salinity*

476

477 The results of the stability effect related to the action of NaCl on the biosurfactants showed a  
478 strong reactivity in the medium below the CMC in terms of lowering the surface tension, even  
479 exceeding the behavior observed in the absence of salt. It was found that the addition of NaCl  
480 from 25 to 100 g/L, for biosurfactant concentrations ranging from 0 to 20 mg/L increased the  
481 efficiency of the biosurfactants to reduce the surface tension of the medium owing to the  
482 competition induced between the electrolytes.

483 However, beyond the CMC a slight increase in the surface tension was observed despite a very  
484 good reactivity of the crude biosurfactants. This increase should be most likely related not only  
485 to a competition effect, but even more to medium saturation by the electrolytes. The saturation  
486 concentration of NaCl was  $C = 150$  g/L for a concentration  $C = 37$  mg/L of biosurfactants. The  
487 maximum reduction of the surface tension was  $\gamma = 32.36$  mN/m. Several authors described  
488 similar behavior for biosurfactants produced by other microorganisms, *P. putida*, *Bacillus*  
489 *subtilis*, *P. aeruginosa*, *B. cereus* and *Rhodococcus erythropolis* (Morikawa et al., 2000; Xia et  
490 al., 2011).

491

#### 492 *Effect of the pH*

493

494 The stability's tests related to the pH indicated the ability of biosurfactants to react facing an  
495 increase of the ionic strength above and below the CMC, in terms of lowering the surface  
496 tension after pH adjustment. The results showed that pH = 7 was optimal for biosurfactant  
497 reactivity allowing a sufficient lowering of the surface tension (Fig.8).

498 The pH is an important factor, it can limit or enhance the effect of biosurfactants in the medium.

499 The effect of the pH is characterized by a modification of the surface charges of the



500 biosurfactants contained in the crude extract depending on the critical micellar concentration.  
501 It is at the origin of the solubilizing, wetting, foaming and emulsifying capacity of  
502 biosurfactants during culture. In an acidic medium, the surface tension decreased gradually until  
503 a value  $C = 18.75 \text{ mg/L}$ . The electrical competitiveness of the biosurfactants resulted from  
504 negative charges which allow the stability of molecules, particularly di-rhamnolipids, in  
505 agreement with the related literature (Amani et al., 2010; Xia et al., 2011)

506 From pH 4, the acidity of the medium caused a considerable decrease in surface tension by  
507 modifying the specific CMC due to mono-rhamnolipids. The CMC previously determined  
508 depended on the variation of the pH of the medium and decreased with acidic pH. In an alkaline  
509 medium, the decrease in surface tension was due to the presence of highly reactive mono-  
510 rhamnolipids in this pH range. This reactivity can be related to their negative repulsive charges  
511 which stabilize the emulsification of the medium in terms of the heterogeneous nature of the  
512 fatty acids of the mono-rhamnolipids produced by *P. aeruginosa* (Özdemir et al., 2004 ; Zhong  
513 et al., 2008).

514 However, for pH values greater than 7, most of the mono-rhamnolipid molecules are negatively  
515 charged, while they are neutral at pH 4. Thus, the determined CMC was characteristic of a  
516 mixture of mono and di-rhamnolipids produced simultaneously by the strain depending on the  
517 nature of the carbon source (Habib et al., 2013). For low ionic strengths ( $\text{NaCl} = 0\text{-}50 \text{ mM}$ ), the  
518 neutral mono-rhamnolipid had a CMC value less than the ionized form. This difference could  
519 be explained by the presence of electrostatic repulsions between the neutral and negatively  
520 charged mono-rhamnolipid molecules, which made their association in the micellar aggregates  
521 more difficult, compared to the neutral molecules present at pH 4.0.

522 Similar results were found using *P. aeruginosa*, *B. subtilis*, *R. erthropolis*, *P. putida* and *B.*  
523 *cereus* (Morikawa et al., 2000; Chen et al., 2010; Ikhwani et al., 2017).

524

525 **Fig. 8.** Evolution of the surface tension of the crude biosurfactant versus pH **(a)** and NaCl  
526 amount **(b)**

527  $(T = 37^\circ\text{C}, W=250 \text{ rpm}, \text{KH}_2\text{PO}_4 = 30 \text{ mmol/L}, p \leq 0.05)$ .

528

529 *Displacement and solubilization of oil*

530

531 The purpose of the displacement and the solubilization of oil is to highlight the ability of the  
532 biosurfactants to solubilize and expand the oil droplet deposited on a hydrophilic surface. A  
533 high capacity of the biosurfactants to solubilize and to move the oil after the contact of  $20 \mu\text{L}$

534 of a1 g/L biosurfactants solution with 15  $\mu$ L of oil deposited on 50 mL of distilled water was  
535 shown. This confirmed the high capacity of the biosurfactants in the solubilization of  
536 immiscible liquids, by lowering the interfacial tension between oil and water and by an  
537 enlargement of the oil diameter from 0 to 2.5 cm. This displacement occurs due to the decrease  
538 of the oil viscosity.

539 Hamid et al. (2012) and Rath et al. (2016) described qualitatively the activity of the  
540 biosurfactants by crossing the barrier of the oil droplet deposited on the surface of the water by  
541 the solution of biosurfactant which gradually expands the oil diameter. It is in agreement with  
542 the observed action of the biosurfactants produced by *Pseudomonas aeruginosa* ATCC 27853  
543 applied to the oil droplet deposited on the surface of the water.

544 Furthermore, the displacement of the oil on 25 mL of biosurfactants at a concentration of 1 g/L  
545 reached up to 5 - 8 cm ; it is in agreement with the related literature (Rath et al., 2016). Indeed,  
546 the oil was dissolved in the solution of biosurfactants by expanding its diameter. This result  
547 highlighted the solubilizing capacity of the produced biosurfactants and can be the subject of a  
548 more widespread application for the environmental safeguarding and protecting against the  
549 pollution of hydrocarbons sludge of refinery.

550

## 551 **Conclusion**

552

553 The results obtained showed the performance and the efficiency of the treatment of  
554 hydrocarbons sludge of *Pseudomonas aeruginosa* strain using the combined effect of  
555 biosurfactants and inductive metabolism. This observed phenomenon showed the ability of  
556 *Pseudomonas aeruginosa* to grow on complex hydrocarbons. The presence of biosurfactants  
557 had a positive effect on the mobility of hydrophobic components of soil, facilitating the leaching  
558 of hydrocarbons contained in the sludge. The mechanism of wetting, solubilization,  
559 emulsification and extraction by biosurfactants of *Pseudomonas aeruginosa* is an useful way to  
560 remove hydrocarbons oils. Ultimately, the application of the crude biosurfactants in the  
561 solubilization and the displacement of oil confirmed the promising properties of the  
562 rhamnolipids produced and hence, to prevent pollution and damage, it constitutes an  
563 opportunity of treatment of various hydrocarbons contained in crude oil and spilled into the  
564 ecosystem.

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#### 568 **Conflict of interest**

569

570 The authors declare that there is no conflict of interests.

571

#### 572 **Author Disclosure Statement**

573

574 No competing financial interests exist.

575

#### 576 **Abbreviation**

577

578 C: concentration (mg/L) ;

579 CMC: critical micellar concentration (mg/L);

580 COD: chemical oxygen demand

581 DES: district energy supply;

582 EI<sub>24</sub>: emulsification index (%);

583 FTIR: Fourier Transformer Infrared;

584 G: conductivity (mS/cm);

585 OD: optical density;

586 Rp: speed of the biosurfactants production (mg/h.L);

587 V: volume (mL);

588 W: speed of rotation (rpm);

589 WTE: waste-to-energy;

590 X: normalized biomass (g);

591 Y<sub>X/S</sub>: yield of biomass/substrate (%);

592 Y<sub>P/S</sub>: yield of product/substrate (%)

593  $\gamma$ : surface tension (mN/m);

594

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