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1 **Production of biosurfactant using the endemic bacterial** 2 **community of a PAHs contaminated soil, and its potential use** 3 **for PAHs remobilization**

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16 **Abstract**

17 Biosurfactants are surface-active agents produced by microorganisms whose use in soil
18 remediation processes is increasingly discussed as a more environmentally friendly
19 alternative than chemically produced surfactants. In this work, we report the production of a
20 biosurfactant by a bacterial community extracted from a polluted soil, mainly impacted by
21 PAHs, in order to use it in a soil-washing process coupled with bioremediation. Nutrient
22 balance was a critical parameter to optimize the production. Best conditions for biosurfactant
23 production were found to be 20 g/L of glucose, 2 g/L of NH₄NO₃, and 14.2 g/L of Na₂HPO₄,
24 corresponding to a C/N/P molar ratio equal to 13/1/2. Purification of the produced
25 biosurfactant by acidification and double extraction with dichloromethane as a solvent
26 allowed measuring the Critical Micellar Concentration (CMC) as equal to 42 mg/L. The
27 capacity of the purified biosurfactant to increase the apparent solubility of four reference
28 PAHs (naphthalene, phenanthrene, pyrene and benzo[a]pyrene) was completed. The
29 solubilisation ratios, in mg of PAH/g of biosurfactant for phenanthrene, pyrene and
30 benzo[a]pyrene are 0.214, 0.1204 and 0.0068, respectively. Identification of the bacteria
31 found in the colony producing the biosurfactant showed the presence of bacteria able to

32 produce biosurfactant (*Enterobacteriaceae*, *Pseudomonas*), as well as, others able to degrade
33 PAHs (*Microbacterium*, *Pseudomonas*, *Rhodanobacteraceae*).

34

35 **Keywords**

36 Bacterial biosurfactant, Polycyclic Aromatic Hydrocarbons, desorption, solubility, Critical
37 Micellar Concentration

38 **1 - Introduction**

39

40 Surface-active agents produced by microorganisms, including bacteria, are called
41 biosurfactants. These compounds are able to reduce the surface tension between liquids,
42 solids or gases. According to this property, biosurfactants can be used to increase the
43 solubility of hydrophobic compounds in water (Soberón-Chávez et al., 2005).

44 When present in soil, Polycyclic Aromatic Hydrocarbons (PAHs), have low accessibility,
45 availability and solubility due to their proper physical and chemical characteristics (especially
46 molecular weights) and to those of the soil (Haritash and Kaushik, 2009).

47 Biodegradation of PAHs is then limited due to their low bioavailability, defined as “the part of
48 the compound which is freely available to cross an organism membrane from the medium the
49 organism inhabits at a given point in time” (Riding et al., 2013). Due to their hydrophobicity,
50 in soil, an important part of the PAHs, isn’t bioavailable, but still bioaccessible (Riding et al.,
51 2013) namely “could become bioavailable if desorbed from the solid matrix”. However,
52 increasing the PAHs solubility could lead to an increase of the risk of PAHs dispersion in the
53 environment. Thus, a thorough control of the flow during their solubilisation process is
54 crucial in order to limit this risk.

55 Chemical surfactants are used in bioremediation to increase the apparent solubility of PAHs
56 in groundwater (Lamichhane et al., 2017; Lukić et al., 2016), but the bioavailability of PAHs
57 trapped in chemical surfactant micelles is not insured (Shao et al., 2017) and the toxicity of
58 the chemical surfactant itself could lead to a decrease in the biodegradation process. For
59 example, addition of SDS in high concentrations (100 and 500 mg/kg) increased the PAHs
60 concentration in water , but it decreased the biodegradation of PAHs due to the toxicity of the
61 SDS towards the bacterial strains (Deschênes et al., 1995; Lima et al., 2011).

62 To solve this problem, the use of biosurfactants for soil remediation has been increasingly
63 studied and is close to be implemented (Koch et al., 1991; Souza et al., 2014; Whang et al.,
64 2008). In comparison with chemically synthesized surfactants which are often toxic and
65 persistent in the environment, biosurfactants have no proven toxicity and are more
66 biodegradable (Mulligan, 2005; Rufino et al., 2014), which is more in line with their use for
67 environmental applications. Some studies (Lima et al., 2011; Varjani and Upasani, 2017a)
68 suggest that the biodegradation of biosurfactants happens after the consumption of other
69 carbon sources and should not affect the solubility of entrapped pollutants.

70 Remediation of soils polluted by hydrophobic compounds could lead to better achievements
71 if biosurfactants are used. This is due to the capacity of microorganisms to better degrade
72 pollutants trapped in micelles of biosurfactant. Using a bacterial culture able to produce
73 biosurfactant and to metabolize PAHs for bioremediation could then combine the two fold
74 effect of soil washing and biodegradation (Ali Khan et al., 2017; Chebbi et al., 2017; Gabet,
75 2004; Hassanshahian, 2014; Lamichhane et al., 2017; Li et al., 2015; Portet-Koltalo et al.,
76 2013).

77 Numerous studies have investigated the optimization of the production of biosurfactants
78 from bacteria isolated from polluted sites to this purpose (Ali Khan et al., 2017; Chebbi et al.,
79 2017; Hassanshahian, 2014; Koch et al., 1991; Liang et al., 2017; Pereira et al., 2013; Soberón-
80 Chávez et al., 2005).

81 Soil and groundwater remediation of PAHs often focus on the remediation of the source zone.
82 The remediation of pollution plumes is economically limited by the cost of the remediation
83 processes, and technically by the difficulty to access the sorbed PAHs, due to their
84 hydrophobicity. The association of soil washing with biosurfactant, coupled with a
85 bioremediation process could be an innovative method for the remediation of sites impacted
86 by PAHs pollution plumes though the fate of solubilized PAHs needs a careful monitoring
87 that must control and promulgate actions to avoid their dispersion in the environment. The
88 use of biosurfactant will increase the bioavailability of the PAHs and so their biodegradability
89 which will limit their dispersion. Due to the biodegradability of the biosurfactant, the
90 solubilisation of the PAHs will be time limited and the risk of dispersion in the environment
91 will decrease quickly.

92 In this present work are reported i) the extraction and isolation from a polluted soil impacted
93 by PAHs of bacterial populations able to produce biosurfactants, and ii) the development of a
94 method for the production and recovery of the biosurfactants. Each isolated colony was
95 tested for the production of biosurfactant. After the selection of one colony, tests were
96 performed to optimize the production medium based on nutrient nature and concentrations.
97 Two types of carbon sources, soluble and insoluble, were compared. The effect of the C/N/P
98 ratios for the production of this biosurfactant has also been studied.

99 **2 - Materials and methods**

100

101 **2.1. Chemicals**

102

103 Anhydrous D-glucose (99% purity), K_2HPO_4 (97% purity), NH_4NO_3 (>99% purity)
104 $FeCl_3 \cdot 6H_2O$ (99% purity) and Na_2HPO_4 (>99% purity) were purchased from VWR (VWR
105 International SAS, France).

106 KH_2PO_4 (analytical standard), KNO_3 (99% purity), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (>98% purity), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
107 (>99% purity), NaCl (99.5% purity) dichloromethane (>99.8% purity), naphthalene (NAPH)
108 (99% purity), phenanthrene (PHE) (98% purity), pyrene (PYR) (97% purity) and
109 benzo[a]pyrene (BaP) (96% purity) were purchased from Sigma-Aldrich (Merck KGaA,
110 Darmstadt, Germany).

111

112 **2.2. Site of interest and soil sampling**

113

114 A former coke plant located in the Northeast of France, in the region of Lorraine, was
115 selected for collecting water and soil samples and for *in-situ* implementation of the developed
116 remediation process.

117 The site is impacted by pure PAHs, BTEX and other hydrocarbons (TPH) in the source zone
118 through a layer of 4 m depth. A pollution plume due to an aquifer passing through the site at
119 a depth of 4 to 8 m affects the surroundings of the site.

120 To extract bacteria able to resist to PAHs, several soil samples were performed by drilling in
121 the pollution plume area. Three samples were collected from three different depths, the first
122 one at 2 m, the second one from 2 to 4 m depth and the last one, in the saturated zone from 4
123 to 8 m. Each sample was homogenized and aliquots were sent to Synlab (Gennevilliers,
124 France) for the analysis of the main pollutants, hydrocarbons, phenols, BTEX and PAHs, in
125 water and soil. The results showed high concentrations of PAHs, both in water and in soil.

126 The total BTEX concentration in water was 8400 $\mu\text{g/L}$, total concentration of the 16 PAHs
127 (US EPA) was about 10000 $\mu\text{g/L}$ and the concentration of the total hydrocarbons (C10 to
128 C40) in water was 14000 $\mu\text{g/L}$. The corresponding concentrations in soils were 33, 2164 and
129 2486 mg/kg, respectively. A more detailed summary of these analyses can be found in
130 supplementary materials (SM1). Granulometric and chemical analyses of the soil sample are
131 detailed in supplementary materials, SM2 and SM3, respectively.

132

133 **2.3. Experimental design**

134

135 To achieve the production of biosurfactant and evaluate its potential use in bioremediation
136 processes, different steps were necessary. Figure 1 presents the successive steps of the study,
137 from the extraction of the soil bacteria to the purification and characterization of the
138 produced biosurfactant and the PAHs desorption assays, with an important focus on the
139 optimization of the biosurfactant production.

140

141 **2.3.1. Extraction of cultivable bacteria from contaminated soil**

142

143 Aliquots of 10g of the three contaminated soil samples were added to 40 mL solution
144 containing 0.016 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of $CaCl_2 \cdot 2H_2O$, 0.040 g of KH_2PO_4 , 0.040 g of
145 K_2HPO_4 , 0.040 g of NH_4NO_3 and 0.003 g of $FeCl_3 \cdot 6H_2O$ with a pH fixed to 7, in 50 mL flasks
146 (adapted from Mcgrath et al., 1997). These suspensions were agitated for 48 h (25 °C, 180
147 rpm). Then 4 mL were taken and added to 71 mL of the same minimum medium in 100 mL
148 flasks. The suspensions were stirred for 120 h (20 °C, 180 rpm). These media were then
149 spiked with pure PAHs in powder (Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) at
150 concentrations above their solubility) as sole carbon source, in order to select only PAHs
151 tolerant bacteria. Colonies were obtained after plating on solid minimal medium (Bois et al.,
152 2011), and were used for next experiments.

153

154 **2.3.2. Selection of bacteria able to produce biosurfactant**

155

156 Each of the obtained colonies was cultivated in fresh media and then tested for the
157 production of biosurfactant. A first experimental medium was prepared following the
158 literature, selecting the most widespread components on biosurfactant production media to
159 increase the probability of having a positive result (Ali Khan et al., 2017; Davis et al., 1999;
160 Henkel et al., 2012; Liu et al., 2014; Mulligan et al., 1989; Ochsner et al., 1996; Pereira et al.,
161 2013; Reis et al., 2010; Robert et al., 1989; Rocha e Silva et al., 2014). This first medium was
162 composed of 1 mL of colza oil, 50 mL of water solution containing 0.1 g of NH_4NO_3 , 0.25 g of
163 K_2HPO_4 , 0.25 g of Na_2HPO_4 and 0.25 g of NaCl in 100 mL flasks. The suspensions were
164 agitated for 7 days (25 °C, 180 rpm). After 7 d, the collapsing drop test (Jain et al., 1991) was
165 used to prove the production of biosurfactant. A drop of each suspension was placed on a
166 colza oil coated glass side. Visually, the presence of biosurfactant can be observed: the
167 collapsed drop indicates the presence of biosurfactant, when a stable drop indicates its
168 absence, or its presence at a very low concentration.

169 **2.3.3. Optimization of the biosurfactant production medium**

170

171 For the optimization of the biosurfactant production medium, two carbon sources
172 (colza oil and glucose), and four nutrients (NH_4NO_3 , Na_2HPO_4 , K_2HPO_4 , KNO_3), were
173 selected in order to highlight the effect of C, N, P and K concentrations in the biosurfactant
174 production. For each experiment, running time was 7 d, and the flasks were agitated at 180
175 rpm at 20 °C. In the principle of having a fast, cheap and easily reproducible biosurfactant
176 production, the two carbon sources selected were colza oil and glucose.

177 Several tens of experiments were carried out following the scheme presented in Figure 2.

178

179 The first step of assays was achieved with the purpose of having an equal molar concentration
180 of 0.05 mol/L of K, N and P in the medium where they were present. The two carbon sources,
181 glucose and colza oil, were tested at 20 g/L.

182 The Collapsing Drop Test was performed daily to assess the production of biosurfactant. The
183 positive result of the test and the speed of production were the two critical factors for
184 selecting the best carbon source to optimize the production of biosurfactant. The objective of
185 the second step was to evaluate the effect of the presence or absence of the three different
186 nutrients on the biosurfactant production by the bacterial community. The same protocol was
187 used to assess the production of biosurfactant.

188 After the first two steps of tests, following the tests used, only a medium with glucose as
189 carbon source and NH_4NO_3 and Na_2HPO_4 as nutrients was selected. Next experiments were
190 carried out with various concentrations of each component to optimize the production of
191 biosurfactant: glucose, NH_4NO_3 and Na_2HPO_4 concentration varied from 20 to 40 g/L, 1 to 4
192 g/L, 7.1 to 14.2 g/L, respectively. Table 1 presents the composition of the media (named from
193 A to I) where biosurfactant production was achieved. Each medium will be named after this
194 table in the following of the text.

195

196 **2.3.4. Upscaling of biosurfactant production in batch bioreactor**

197

198 Once the optimization of the medium used for biosurfactant production was achieved,
199 a scale-up process to increase the production volume started. The objective of this scale-up
200 process was to obtain a large quantity of biosurfactant for its characterization and to use it in
201 PAHs desorption experiments in dynamic sand and soil columns.

202 The production was carried out in bioreactors of 5 L and the impact of several physical and
203 chemical conditions on biosurfactant production was tested: (i) temperature (10, 15, 20 and
204 40°C), (ii) pH (from 5 to 8) and (iii) water oxygenation of the batch bioreactor (bulk solution
205 between below saturation and full saturation in O_2).

206 For each bioreactor, pH, redox potential (with Pt/Ag-AgCl probe), temperature, conductivity
207 and dissolved O_2 content in the fluid were continuously monitored using probes (Mettler

208 Toledo, Viroflay, France) immersed in the batch bioreactor and were recorded continuously
209 with a data acquisition device (Keithley Instruments, model 2700, Cleveland, OH, USA).

210

211 **2.4. Characterization of the biosurfactant**

212 **2.4.1. Extraction and purification of the biosurfactant**

213

214 To extract and purify the produced biosurfactant from batch bioreactors, the protocol
215 was adapted from previous ones (Varjani and Upasani, 2017b). Three litres of the liquefied
216 foam obtained from three different 5 L bioreactors, were centrifuged, and the supernatant
217 filtered at 0.22 μm (MF-Millipore™ 0.22 μm mixed cellulose esters membrane, Merck
218 Millipore), to separate the bacteria from the biosurfactant. The obtained filtrate was then
219 acidified to pH 2 with hydrochloric acid and stored at 4 °C for 48 h. The precipitate resulting
220 from the acidification was extracted by centrifugation and filtration at 0.22 μm , and then re-
221 dissolved in ultrapure water. The pH was finally stabilized at 7. The biosurfactant was then
222 extracted from water two times using dichloromethane in separating funnel.
223 Dichloromethane was then evaporated under a nitrogen flux during 1 h, and finally freeze-
224 dried (Christ Alpha 2-4 LSCPlus freeze-dryer), to eliminate the remaining water.

225

226 **2.4.2. Determination of the Critical Micellar Concentration (CMC)**

227

228 To evaluate the CMC, the curve of the surface tension (mN/m) between the solution of
229 purified biosurfactant and air, as a function of the concentration of biosurfactant in solution
230 was made (Mahalingam and Sampath, 2014; Patowary et al., 2017; Varjani and Upasani,
231 2017b). Surface tensions were measured using a KRUSS DSA-100 goniometer and the
232 method of the pending drop (using Young-Laplace fit directly from the Advance software

233 combined to the KRUSS DSA-100). Using the tangent intersection method on the two
234 different parts of the curve, CMC was graphically evaluated and reported.

235

236 **2.4.3. PAHs solubilisation assays**

237

238 The ability to solubilize PAHs by the produced biosurfactant was tested. Four PAHs:
239 NAPH (99% purity), PHE (98% purity), PYR (97% purity) and BaP (96% purity) were used as
240 representative of the 16 priority PAHs. The PAHs were introduced in flasks in pure acetone at
241 concentrations equal to 10 times their solubility in water at 25 °C. On each batch, 6.4 mg of
242 NAPH (solubility at 25 °C = 32 mg/L), 0.24 mg of PHE (solubility at 25 °C = 1.2 mg/L), 0.032
243 mg of PYR (solubility at 25 °C = 0.140 mg/L) and 0.0016 mg of BaP (solubility at 25 °C = 6
244 µg/L) were introduced. After evaporation of the acetone for 24 h (and formation of PAHs
245 crystals), the flasks were filled with 20 mL of different solutions of biosurfactant at different
246 concentrations (final concentrations of 1/2 CMC, 1 CMC, 5 CMC, 10 CMC and 20 CMC). After
247 24 h at 20 °C with agitation at 180 rpm, extractions by Solid Phase Extraction (SPE) were
248 performed to evaluate the solubilized quantity of PAHs (Ma et al., 2010). Extracts were
249 analysed by Gas Chromatography coupled with Mass Spectrometry for PAHs concentrations
250 (Shimadzu QP 2020). For the extraction, SPE columns from Supelclean Envichrom (ENVI -
251 Chrom P SPE Tube) were used. The extraction protocol was adapted for this specific use. The
252 columns were conditioned with 2 mL dichloromethane, followed by 2 mL MilliQ water. The
253 water samples were percolated, and the cartridges were then washed with 3 mL MilliQ water.
254 The cartridges were finally dried with vacuum aspiration for 2 h, and samples were eluted
255 with 5 mL dichloromethane before analyses. Due to the low quantity of biosurfactant
256 extracted and purified, the tests could not be performed in triplicate.

257

258 **2.5. Characterization of the biosurfactant-producing bacterial community**

259

260 Firstly, DNA extractions were performed on culture from bioreactor producing
261 biosurfactant. A volume of 10 mL of culture were centrifuged and the supernatant discarded.
262 DNA extractions were carried out on the pellet using Fast Prep DNA kit for soils (MP
263 Biomedicals) following the manufacturer's instructions. The total extracted DNA was then
264 quantified with a Quantus Fluorometer (Promega) and stored at -20 °C prior to further
265 analyses. To characterize the bacterial community structure and the diversity in the
266 bioreactor, Capillary Electrophoresis-Single Strand Conformational Polymorphism (CE-
267 SSCP) profiles were performed based on an amplicon of the gene coding for 16S rDNA, and
268 amplified fragments of the gene coding for 16S rDNA were cloned and sequenced to identify
269 bacteria from the community.

270 For CE-SSCP fingerprints, about 200 bp of the V3 region of bacterial 16S rRNA genes were
271 amplified from DNA extract with the forward primer w49 (5'-ACGGTCCAGACTCCTACGGG-
272 3'; *E. coli* position, 331) and the reverse primer w34 (5'-TTACCGCGGCTGCTGGCAC-3'; *E.*
273 *coli* position, 533). The 5' end was labelled with the fluorescent dye FAM, 25 cycles,
274 hybridization at 61 °C, and 30 s elongation at 72 °C were used. One µL of diluted (5- to 100-
275 fold in nuclease-free water) PCR product was added to a mixture of 18.8 µL of deionized
276 formamide and 0.2 µL of Genescan-600 LIZ internal standard (Applied Biosystems). To
277 obtain single-strand DNA, samples were heated denaturised for 5 min at 95 °C, and
278 immediately cooled on ice. CE-SSCP analyses were performed on an ABI Prism 310 genetic
279 analyser using a 47-cm length capillary, a non-denaturing 5.6 % CAP polymer (Applied
280 Biosystems) and the following electrophoresis conditions: run temperature 32 °C, sample
281 injection for 5 s at 15 kV, data collection for 35 min at 12 kV. SSCP data was analysed using
282 BioNumerics V7.5 (Applied Maths) and lining SSCP profiles up to the internal standard and
283 to a common baseline.

284 The bacterial 16S rRNA gene sequences were then amplified from the extracted DNA.
285 Universal bacterial primers 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1406R (5'-
286 ACGGGCGGTGTGTRC-3') were used with elongation at 55 °C and 30 cycles. Purified PCR

287 products (Macherey–Nagel) were used to build a gene library with the TOPO-TA Cloning Kit
288 and TOP 10 chemically competent cells (Invitrogen). Sequences were then analysed by
289 Sanger sequencing (Eurofins genomics, Germany). Consensus 16S rRNA gene sequences
290 were constructed and manually verified with the BioEdit program version 7.5.2 (Hall, 1999).
291 The phylogenetic assignment was based on sequence analysis by the Ribosomal Database
292 Project version 11.5 classifier tool (Wang et al., 2007) and by the NCBI Blast tool
293 (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Phylogenetic tree was built with the MegaX
294 software (version 10.0.1). All sequences (5 clones corresponding to 5 peaks on fingerprint
295 profiles) were posted on GenBank platform (NCBI) under accession numbers MN422137 to
296 MN422141.

297

298 **2.6. Chemical analysis methods**

299

300 To analyse the biosurfactant in cultures, a High Performance Liquid Chromatography
301 with a fluorescence detector (HPLC-FLD, Hitachi LaChrom Elite® L-2400) was used. The
302 media were analysed with a Reverse Phase column (5 mm, 25 cm × 4.6 mm) RP C-18
303 (Purospher®, Merck), and as mobile phase a mix of acidified water (pH =2.5) and acetonitrile
304 at a ratio of 25:75 v:v (Mubarak et al., 2015). Due to absence of standard for the
305 biosurfactant, only relative quantifications between the different experiments were possible
306 with peak area values.

307 For PAH analyses, a GC-MS with a 30 m column (RXi®-5Sil MS from RESTEK, length: 30 m,
308 thickness: 0.25 µm and diameter: 0.25 mm) was used. The oven temperature was set at 80 °C
309 at the beginning of the analysis, and then increased up to 300 °C in 25 min and maintained at
310 300°C for 10 min. Helium was used as the gas carrier, at a flow rate of 1.30 mL/min. For the
311 mass spectrometer, the ion source temperature was set at 240 °C and the detector voltage at
312 1.20 kV (adapted from Crampon, 2015).

313

314 **2.7. Statistical analysis method**

315

316 Statistical analysis of the significant differences between data have been made with XLSTAT
317 software, version 2019.3.2 (Addinsoft).

318

319 **3. Results and discussion**

320

321 **3.1. Optimization of the production of biosurfactant**

322

323 After plating the extraction medium, 15 different colonies were grown. These colonies
324 were re-inoculated in fresh medium in batches, filled with the first biosurfactant production
325 medium (1 mL of colza oil, 50 mL of water solution containing 0.1 g of NH_4NO_3 , 0.25 g of
326 K_2HPO_4 , 0.25 g of Na_2HPO_4 and 0.25 g of NaCl). Only one of the batches had a positive
327 response on the collapsing drop test. The three replicates of the batch showed a positive
328 result, reinforcing the reliability of the result. In addition, an emulsion was visible in the
329 batches between the two immiscible fluids, the aqueous medium and the colza oil. This batch
330 seems to be the only one inoculated by a colony containing bacteria able to produce
331 biosurfactant with these conditions. The colony (containing several bacterial species), coming
332 from the 2 to 4 m depth sample, was then selected as the most suitable colony for
333 development and used in the following tests.

334 The second step of the assays, with colza oil medium and glucose medium was monitored for
335 two weeks. After 7 days, in the medium containing 20 g/L glucose, 2 g/L NH_4NO_3 , and 7.1
336 g/L Na_2HPO_4 , a positive result was obtained on the collapsing drop test. It took 14 days to
337 obtain a biosurfactant production in the medium containing 20 g/L colza oil, 2 g/L NH_4NO_3 ,
338 7.1 g/L Na_2HPO_4 and 4.35 g/L K_2HPO_4 . No other medium allowed the production of

339 biosurfactant. The medium containing glucose allowed a faster production of biosurfactant.
340 Glucose being an easily degradable carbon source, its biodegradation is faster, thus
341 improving the bacterial growth and so the production of the biosurfactant. In the case of any
342 development of a biosurfactant production process, the speed of production is a crucial
343 parameter. Glucose was then selected for the next experiments.

344 Variations of the selected medium with a lack of glucose, of NH_4NO_3 or of Na_2HPO_4 were
345 tested, but did not show any production of biosurfactant. The presence of the two nutrients
346 with glucose is mandatory to produce biosurfactant for the selected bacterial community.

347 The purpose of the next step of assays was to optimize the ratio of each nutrient and glucose
348 to increase the production of biosurfactant. Each batch showed a production of biosurfactant,
349 with positive collapsing drop tests. Using HPLC-Fluo analysis on these media, it was found
350 that the medium producing more biosurfactant was medium A (Figure 3). These
351 concentrations matched with a C/N/P ratio equal to 13/1/2. It was deduced that a C/N ratio
352 higher than 10 was mandatory for the biosurfactant production. This result was consistent
353 with others biosurfactant production studies, which have proved the influence of the C/N
354 ratio on biosurfactant production (Karanth, 1989; Reis et al., 2010). Influence of phosphorus
355 had been studied (Mulligan et al., 1989; Wei et al., 2007) and differed in function of the
356 bacterial community and biosurfactant type. Tukey analysis of the significant differences
357 between the different conditions shows different groups of result. The first condition,
358 medium A with the higher peak area, is in a distinct group than the others, meaning that it is
359 significantly different from the other ($p < 0.05$, Tukey ANOVA test), supporting the fact that
360 this medium is the best for the biosurfactant production.

361 This medium was then selected as biosurfactant production medium for the next
362 experiments.

363

364 **3.2. Monitoring of the parameters for the optimisation of biosurfactant**
365 **production in bioreactor**

366

367 Firstly, an open to air bioreactor with only mechanical stirring for oxygenation of the
368 solution showed no evidence of biosurfactant production. The lack of dissolved oxygen in the
369 water was a limiting parameter for the bacterial community growth, as shown below (Figure
370 4), where O₂ concentrations were close to 0 ppm during most of the time of the experiment.
371 Consumption of the O₂ by the bacteria in the bioreactor could be an explanation to these low
372 O₂ concentrations, with a too low input by natural dissolution of the O₂ present in the air.
373 Two assays to inject directly O₂ into the bioreactor did not allow the production of
374 biosurfactant with a surface tension remaining constant at *ca* 72 mN/m during the whole
375 experiment (Figure 6). Temperature – due to a thermal control –, pH and conductivity values
376 remained stable during the test and redox potential increased with the injection of O₂. The
377 lack of oxygen could have led to the non-consumption of the glucose, explaining the
378 stability of the physical parameters. These results seems to indicate that the bacteria
379 composing the studied community are aerobic bacteria.

380

381 For next experiments, oxygen was supplied by direct air injection in the bioreactors, what
382 generates a biosurfactant production after two days which was achieved and attested by
383 collapsing drop test and measurement of the surface tension (Jain et al., 1991).

384 During the production of biosurfactant, the consumption of glucose by bacteria led to the
385 production of carboxylic acids (probably pyruvic acid and its conjugate base, pyruvate)
386 (Schuit and Schuit, 2015), which decreased the pH of the solution (Figure 5). When pH was
387 lower than 7, the production of biosurfactant was stopped and the foam on the bioreactor
388 disappeared. Measurements of the surface tension and collapsing drop tests made at pH
389 inferior to 6.5 proved that the biosurfactant was no longer active. The acidification of the
390 medium could lead to the partial precipitation of the biosurfactant and then its

391 disappearance at the interface between water and air, preventing its role in the decrease of
392 the surface tension. It's also possible that the nature of the biosurfactant is affected by the pH
393 as it can happen to the amphoteric surfactant and so causing the destabilization of the
394 biosurfactant micelles (Johansson and Somasundaran, 2007).

395 As shown by Figure 6, surface tension stayed stable during the first day of experiment, during
396 the decrease of the pH. When pH was adjusted to 7, surface tension values began to decrease,
397 and foam production started again. Measurements of the surface tension between the
398 bioreactor medium and air at different pH in the bulk, showed that when pH was lower than
399 7, the biosurfactant was no longer active and the surface tension was close to 72 mN/m, close
400 to the reference value between pure water and air.

401 Other biosurfactants, like lipopeptides, can be produced at large pH range, from 4.5 to 10.5
402 (Makkar and Cameotra, 1997). However, at acidic pH, rhamnolipids and surfactin lower their
403 efficiency on emulsifying diesel (Whang et al., 2008). For other biosurfactants, a pH value
404 greater than 7 seems important for their production (Lotfabad et al., 2009; Zinjarde and
405 Pant, 2002) or stability (Mukherjee, 2007).

406 An explanation could be the change of the molecular aggregates morphologies of the
407 biosurfactant (Helvac et al., 2004) at acidic pH or the slowdown of the cells growth, leading
408 to a decrease in the production (Lotfabad et al., 2009).

409 To limit this phenomenon, some drops of a NaOH 2 M solution were regularly injected into
410 the bioreactor to keep the pH value higher than 7, explaining the curve shape (Figure 5).

411 Conductivity and temperature – due to a thermal control – remained stable during the test.
412 O₂ concentration decreased quickly at the beginning of the experiment due to its
413 consumption by bacteria. After the first drop, O₂ concentration increased and remained
414 stable at a concentration of approximately 9 ppm enabling enough oxygen for biosurfactant
415 production. After an initial decrease, due to the O₂ concentration drop, redox potential
416 increased during the experiment due to oxidation of the glucose by the bacteria. If a decrease

417 in redox potential can be observed when 2 M NaOH was injected into the bioreactor, it
418 always stays positive, meaning that the producing medium is in oxidative conditions, which is
419 in line with the idea of oxidizing the PAHs by the bacteria (Haritash and Kaushik, 2009).

420

421 Four different temperatures of production were tested, 10, 15, 20 and 40 °C. No production
422 of biosurfactant occurred at 10 °C and 40 °C and the surface tension remained at a value
423 around 72 mN/m during the whole experiment. Considering the 15 °C bioreactor, it took
424 more than 2 wk to observe a decrease in the surface tension and the decrease was lower than
425 the one in the 20 °C bioreactor. The bioreactors at 20°C showed a higher production of
426 biosurfactant; these results illustrate the low-mesophilic nature of the bacterial strains able
427 to produce biosurfactant. Finally, the upscaling of the biosurfactant production was a success.
428 The continuous monitoring of the physical parameters allowed determining that the O₂
429 saturation of the medium, the pH of 7 and a temperature of 20 °C were the most important
430 operating parameters to succeed the biosurfactant production. This preference for a
431 temperature of 20 °C may be due to the conditions of extraction and culture of the bacterial
432 community. To maximize the number of extracted bacterial species, it was decided to make
433 an extraction medium at a temperature of 20 °C (Dobrić and Bååth, 2018). Next media of
434 culture were all carried out at 20 °C. These tests could lead to the selection of only mesophilic
435 bacteria, explaining that later 20 °C is the optimum temperature for the biosurfactant
436 production, which could be due to a highest bacterial growth.

437 Enough biosurfactant could be produced to go further by purifying and characterizing it. In
438 parallel, the characterization of the producing community was achieved.

439

440 **3.3. Characterization of the biosurfactant**

441

442 **3.3.1. Characterization of the biosurfactant properties**

443 From the 3 L of foam and medium extracted used for the purification assays of the
444 biosurfactant, 80 mg of purified biosurfactant were obtained after lyophilisation of the
445 extracted product.

446 The surface tension as a function of the biosurfactant concentration can be obtained by
447 measuring surface tension between different solutions of purified biosurfactant and air. This
448 is achieved in order to determine its critical micellar concentration (CMC).

449 Two different behaviours, represented by the two different lines in figure 7, were recorded
450 with the increase of concentration. The surface tension decreased linearly with the increase in
451 concentration, due to the increasing concentration of biosurfactant molecule at the surface of
452 the drop medium. Then the surface tension remained stable with the increase of the
453 concentration, due to the saturation of biosurfactant at the surface of the drop. The junction
454 of the two lines is the point where the concentration of surfactant in solution is sufficient to
455 start to produce naturally micelles of surfactant; it corresponds to the CMC.

456 The estimated CMC of the purified biosurfactant is 42 mg/L, for a maximum reduction of the
457 surface tension from 72 mN/m to 40 mN/m (Figure 7).

458 This value of CMC at pH 7 and 20 °C is in the same range of value compared to the CMC of
459 other biosurfactants, like rhamnolipids – CMC = 10-400 mg/L – or surfactin – CMC = 20
460 mg/L – (Lang and Wullbrandt, 1999; Liu et al., 2014). It is also lower than the CMC of
461 chemically produced surfactants, like sodium dodecyl sulfate (SDS) – CMC = 2307 mg/L –,
462 Octylphenol ethylene oxide condensate (marketed as Triton X-100™) – CMC = 150 mg/L – or
463 Polyoxyethylene lauryl ether (marketed as Brij-35™) – CMC = 108 mg/L –. These three
464 surfactants are commonly used in soil washing processes (Befkadu and Chen, 2018). This
465 quite low value of CMC could be advantageous in a soil washing process perspective, with
466 micelle formation at lower concentrations, it will lead to a better efficiency for PAHs
467 desorption and a better control of the soil remediation method (Mao et al., 2015; Mulligan et
468 al., 2001). Moreover, another advantage is that the biosurfactant will not be regarded itself as
469 a pollutant and so it will not require recovery operations after the total removal of PAHs.

470 However, the precise monitoring of the PAHs concentration in the groundwater is necessary
471 until the end in order to control and promulgate actions to prevent the pollutant dispersion
472 in the groundwater due to the biosurfactant action.

473

474 **3.3.2 PAHs solubilisation assays**

475 The evolution of the concentration in water of each of the 4 PAHS was studied as a function
476 of biosurfactant concentration. For PHE, PYR and BaP, an increase in PAH concentration can
477 be observed with the increase of the biosurfactant production, attesting the efficiency of the
478 biosurfactant to solubilize PAHs in water (Figure 8). NAPH concentration is decreasing
479 slightly with the increase of biosurfactant concentration. This phenomenon could be
480 explained by the fact that the maximum solubility of naphthalene in water at 20 °C, which is
481 32 mg/L, was not reached. The method of PAH deposit, by solubilisation in acetone (Sawada
482 et al., 2004), injection in the dry batches and evaporation of the acetone, could have led to
483 the partial evaporation of NAPH and then a final concentration below its solubility value. The
484 chemical formula of the produced biosurfactant could not be determined, therefore molar
485 mass, it was not possible to calculate the Molar Solubilisation Ratios (MSR) (Ansari et al.,
486 2013). Then comparison with other bio-sourced and chemical surfactant of its ability to
487 solubilize PAHs was not possible. However, the solubilisation capacity of the biosurfactant
488 was evaluated thanks to the slope of the lines and presented as mg solubilized PAH per g
489 biosurfactant in solution. The solubilisation ratios of PHE, PYR and BaP are respectively
490 0.214 mg/g, 0.1204 mg/g and 0.0068 mg/g.

491 These results show the ability of the produced biosurfactant to increase the solubilisation of
492 PAHs. Concerning soil remediation, this ability is appealing and enables opportunity to
493 combine the desorption effect of the biosurfactant with a biodegradation process. The
494 analysis of the producing bacterial community could support this possibility by bringing to
495 light the potential ability of the bacteria to degrade PAHs.

496

497 **3.4. Identification of the bacterial community**

498

499 DNA extractions and analyses were performed on a sample from bioreactor in which
500 biosurfactant production was attested and optimal. First, CE-SSCP fingerprint (Figure 9a)
501 highlighted a relatively low biodiversity in the bacterial community, with five major peaks
502 corresponding to five dominant bacterial species in the culture. These species could be
503 determined by cloning and sequencing the 16S rRNA gene, and closer affiliations could be
504 determined as listed in a phylogenetic tree (Figure 9b). Clones 1 and 5, corresponding to
505 peaks 1 and 5 in the fingerprint profile, were found to be part of the *Pseudomonas* genus.

506

507 As *Pseudomonas* 16S rRNA genes are close one from each other, it was difficult here to
508 clearly link clones 1 and 5 to a particular *Pseudomonas* specie, nevertheless some of the
509 species close from our clones are described as able to produce biosurfactants (Abouseoud et
510 al., 2008; Mellor et al., 2011) and to degrade PAHs, generally low molecular ones (Isaac et al.,
511 2015). Some members of the *Enterobacteriaceae* family, especially *Klebsiela grimonti* who
512 was found to be close from clone 2, were described as able to produce biosurfactants (Jain et
513 al., 2013). The presence of these species in the consortium could then be interesting for
514 bioremediation perspectives.

515 Clone 3 was found to be close to members of the *Microbacterium* genus, especially from
516 *Microbacterium maritypicum* and *Microbacterium oxydans*. *Microbacterium maritypicum*
517 is not known to produce biosurfactants, but was described as able to degrade BaP (Mansouri
518 et al., 2017). Combined with the ability of some strains in our culture to produce a
519 biosurfactant able to increase the solubilisation (and so the bioavailability) of PAHs, the
520 presence of bacteria able to degrade High Molecular Weight (HMW) PAHs in the consortium
521 is very interesting. Other members of *microbacterium* genus were described as able to

522 degrade benzo[a]pyrene and pyrene (Qin et al., 2017). The presence of such strains in the
523 bacterial community clearly let us think that its use in soil for PAHs remediation could be
524 very interesting. The degradation of HMW PAHs such as BaP by some members of the
525 community could also lead to the production of degradation by-products, easier to
526 metabolize for other bacteria known to degrade Low Molecular Weight (LMW) PAHs, such as
527 *pseudomonas* strains (clones 1 and 5) or *Enterobacteriaceae* family members (clone 2).

528 Members of the *Rhodanobacteraceae* family (close to clone 4) have also been described as
529 able to mineralize BaP (Kanaly et al., 2002), as shown by the production of $^{14}\text{CO}_2$ from ^{14}C
530 labelled BaP on a high-boiling-point diesel fuel distillate (HBD). BaP solubilisation into the
531 culture suspension was however an essential step for rapid mineralization. In the case of our
532 consortium, the solubilisation could occur thanks to biosurfactant producing bacteria,
533 making the degradation of BaP possible.

534 Finally, the community grown in our study seems to cumulate the ability of different strains
535 that could lead to an efficient PAHs biodegradation, with biosurfactant producing strains that
536 could increase PAHs bioavailability (*Enterobacteriaceae*, *Pseudomonas*), and PAHs
537 degradation bacteria (*Microbacterium*, some *Pseudomonas*, *Rhodanobacteraceae*). The
538 more recalcitrant PAHs, such as benzo[a]pyrene, can be metabolized by some bacteria close
539 from members of our consortium, creating more easily degradable by products. LMW PAHs
540 such as NAPH or PHE can be degraded more easily by bacteria, including strains from our
541 consortium. Even if assays of remobilization and degradation in real matrices contaminated
542 with PAHs have to be performed to clearly conclude on the efficiency of the community for
543 bioremediation, all parameters seem to be very promising.

544

545 **4. Conclusions**

546

547 In this study, the extraction and isolation of a bacterial community able to produce
548 biosurfactant from a contaminated soil was achieved. The nutritional and operating
549 conditions were optimized to maximize biosurfactant production. Four main factors were
550 identified for the biosurfactant production: (i) a C/N ratio higher than 10, with a maximum
551 production with C/N ratio = 13/1, (ii) the presence of phosphorus in the culture medium,
552 with a maximum production with a C/N/P ratio = 13/1/2, (iii) a constant presence of
553 dissolved oxygen in the medium, and (iv) a pH superior to 7. Purification of the produced
554 biosurfactant allowed its characterization, with a CMC = 42 mg/L, and the determination of
555 the Molar Solubility Ratio for 4 PAHs with this biosurfactant, highlighting its capacity for
556 PAHs remobilization. Determination of the bacterial community has been achieved and
557 showed the presence of bacteria (*Enterobacteriaceae* family, *Microbacterium* genus) (i) able
558 to produce biosurfactant, which were responsible for the production observed in our
559 experiments, and (ii) bacteria able to metabolize both LMW and HMW PAHs. Despite the
560 proved effect of the studied biosurfactant in the PAHs desorption, due to the long and
561 expensive process of purification of biosurfactant, and the relatively small quantity obtained,
562 the use of purified biosurfactant in a real scale soil washing process seems difficult. However,
563 the fast and relatively not expensive production of biosurfactant in batch bioreactor, and its
564 low CMC value make possible the use of non-purified biosurfactant rather than the purified
565 product. When using raw bioreactor solution, the presence of biosurfactant combined with
566 bacteria, which are able to degrade both LMW and HMW PAHs, could lead to a combined
567 effect of desorption and biodegradation and so a reduction of the remediation cost, by only
568 using easily available nutrients to both help the bacterial growth and the biosurfactant
569 production. Furthermore, the biosurfactant is certainly biodegradable and non-toxic, which
570 facilitates the post-treatment acceptance on completion of the works, with or without
571 obligation of monitoring.

572 Due to these benefits, it seems that the consortium used in our study, and its produced
573 biosurfactant, is a good candidate for bioremediation tests, and future studies will determine
574 its possible use for increasing PAHs bioavailability and biodegradation in contaminated soils.

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576

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590

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592

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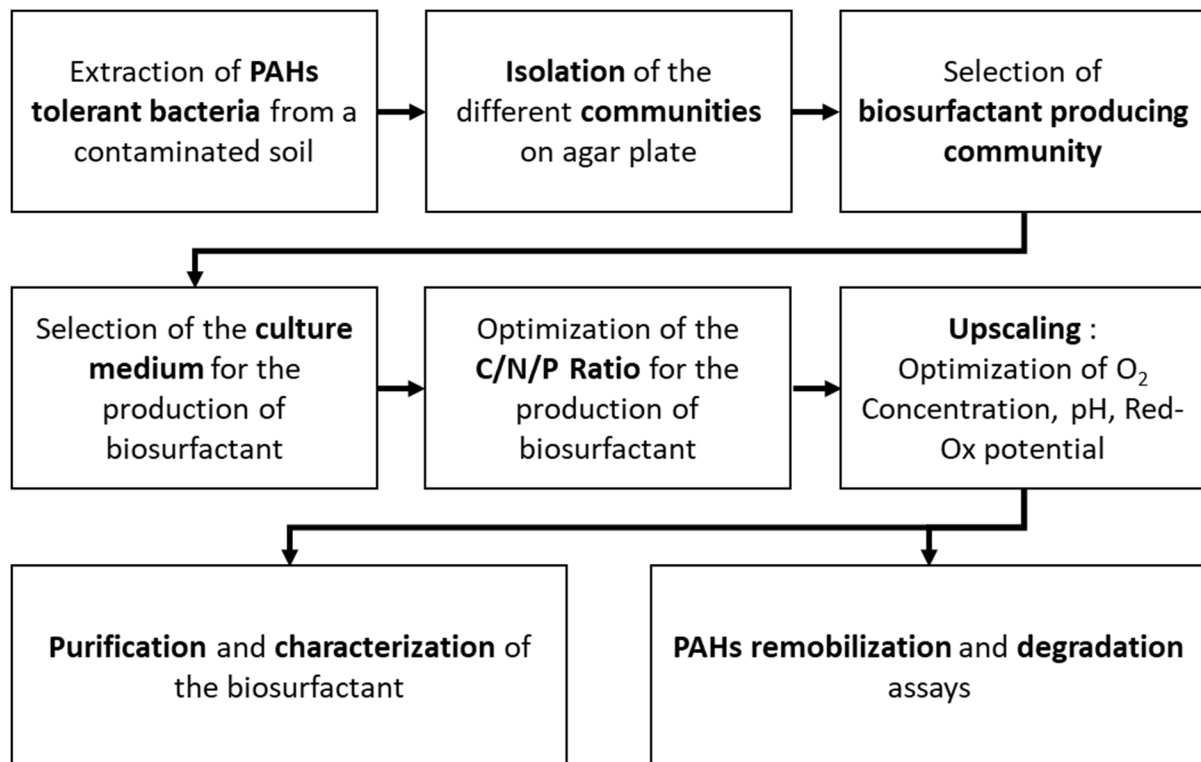


Figure 1: Experimental scheme showing all the successive steps from extraction of PAHs tolerant bacterial strains from contaminated soil to PAHs remobilization and degradation assays.

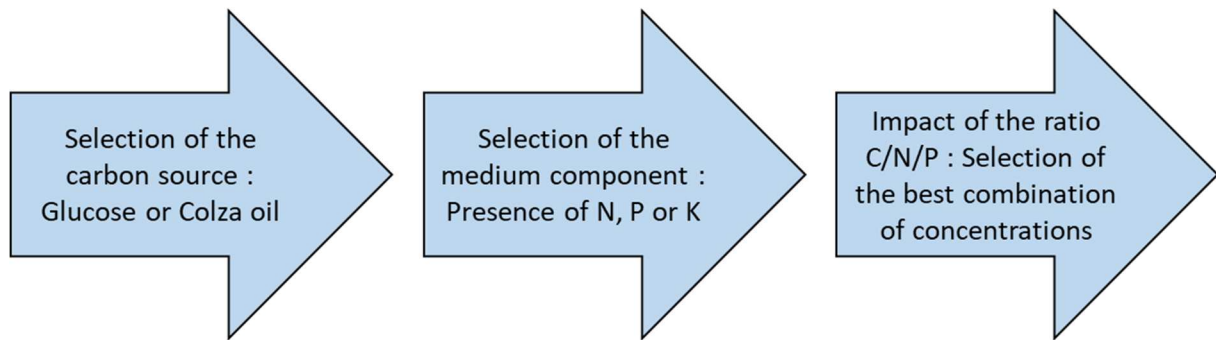


Figure 2: Experimental scheme showing the three successive steps from the selection of carbon source nutrients to the selection of the most optimized combination of concentrations in N, P and K as nutrients for the selected population of bacteria, tolerant to PAHs and producing biosurfactants.

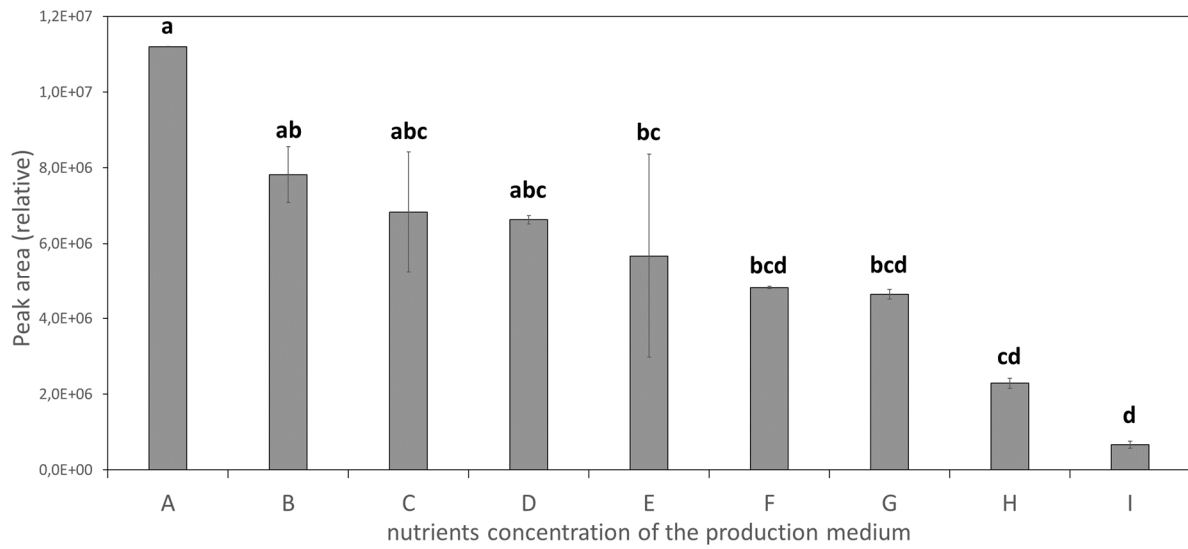


Figure 3: Relative biosurfactant concentration as shown by HPLC-FLD peak areas in the 9 media tested, where biosurfactant production was achieved, groups a to d represent significant differences between the conditions, analyzed by Tukey test with multiple pairwise comparisons from an ANOVA analysis of the results

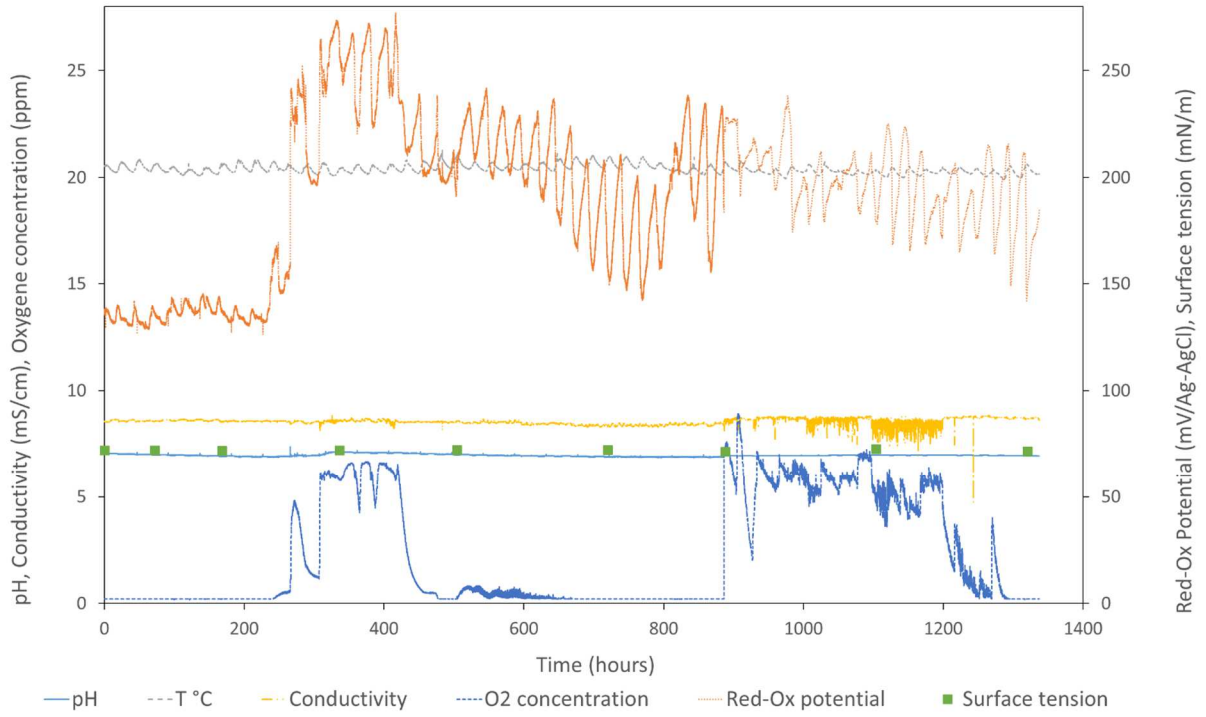


Figure 4: Monitoring of the main physical and chemical parameters of the solution (pH, redox potential, conductivity, dissolved oxygen concentration and surface tension between air and solution), on the first bioreactor

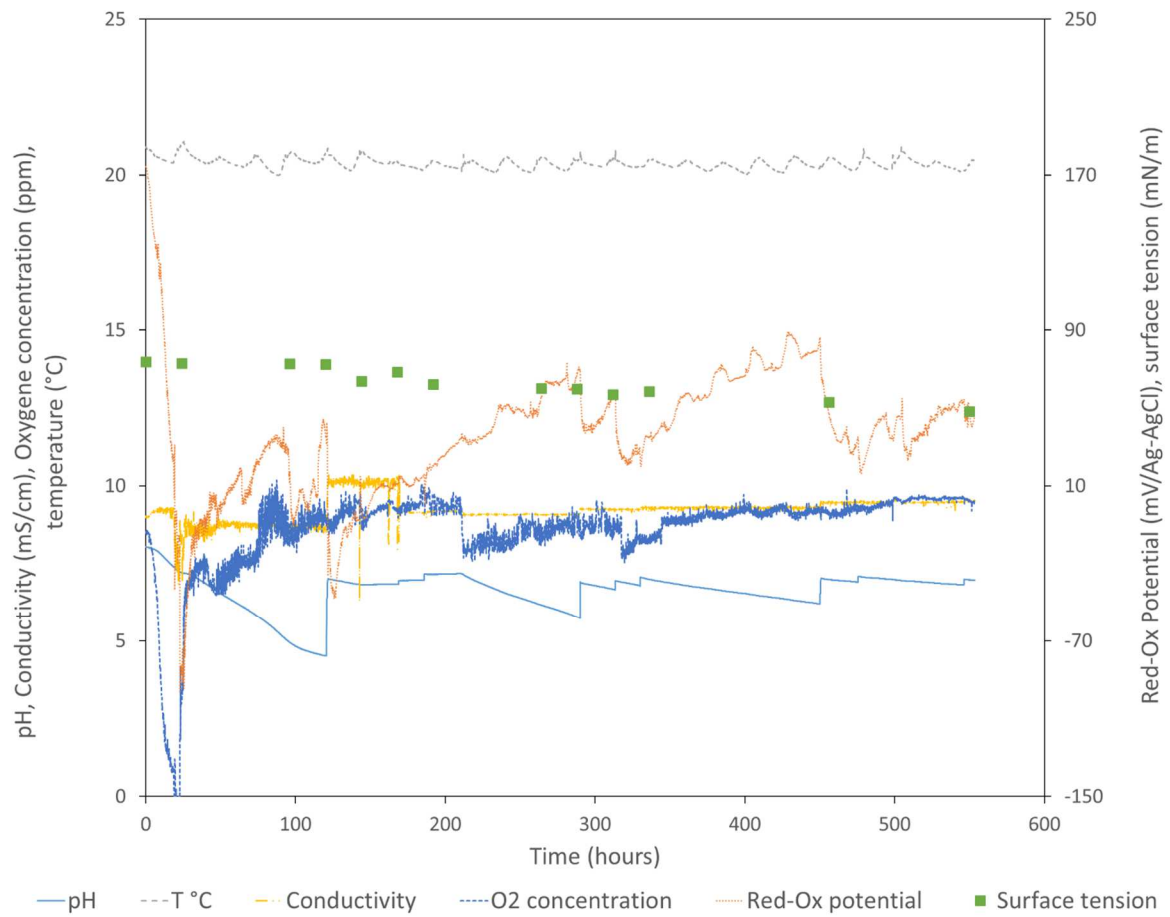


Figure 5: Monitoring of the main physical and chemical parameters of the solution (pH, redox potential, conductivity, dissolved oxygen concentration and surface tension between solution and air), on the second bioreactor.

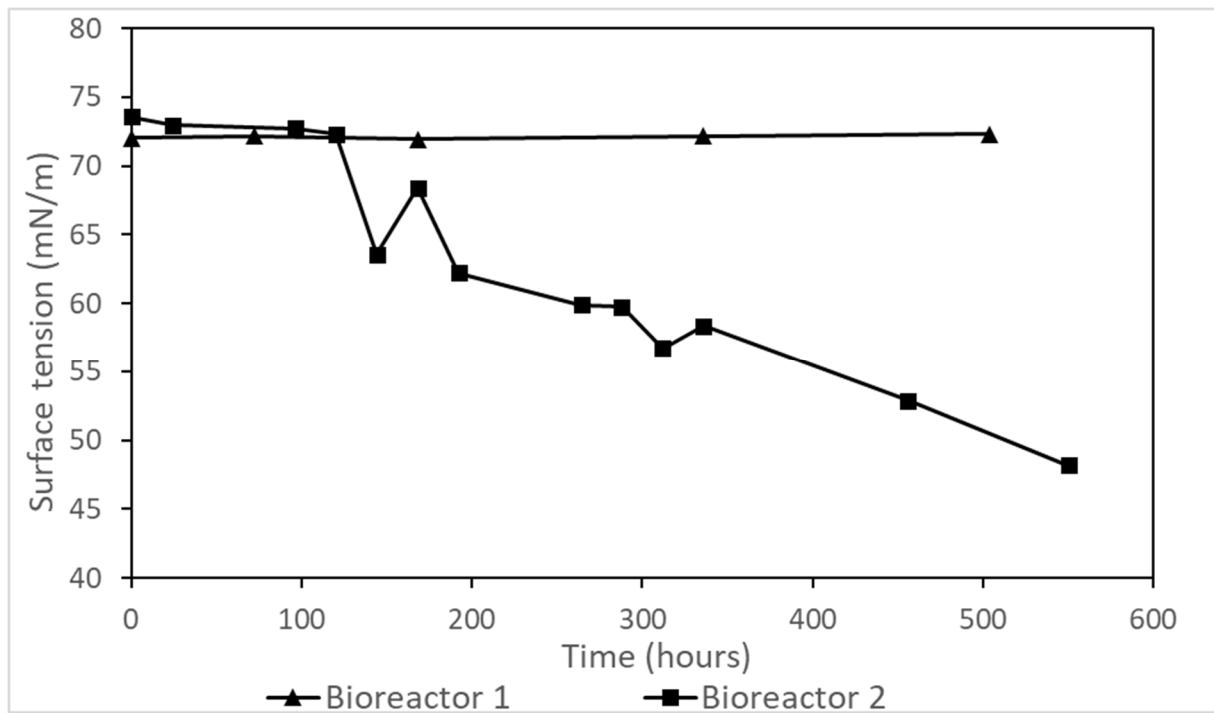


Figure 6: Evolution of the surface tension between solution and air in the solution of the two bioreactors

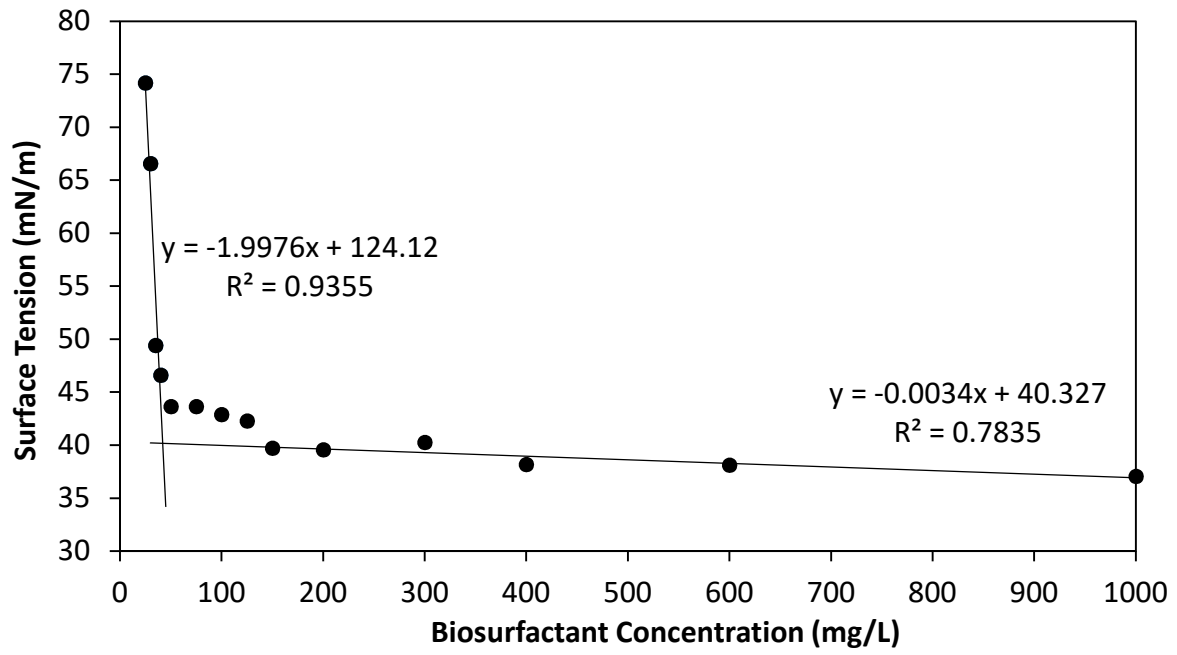


Figure 7: Surface tension between different purified biosurfactant solutions and air as a function of the purified biosurfactant concentration (in mg/L). The estimated CMC of the biosurfactant is equal to 42 mg/L, for a surface tension of 40 mN/m.

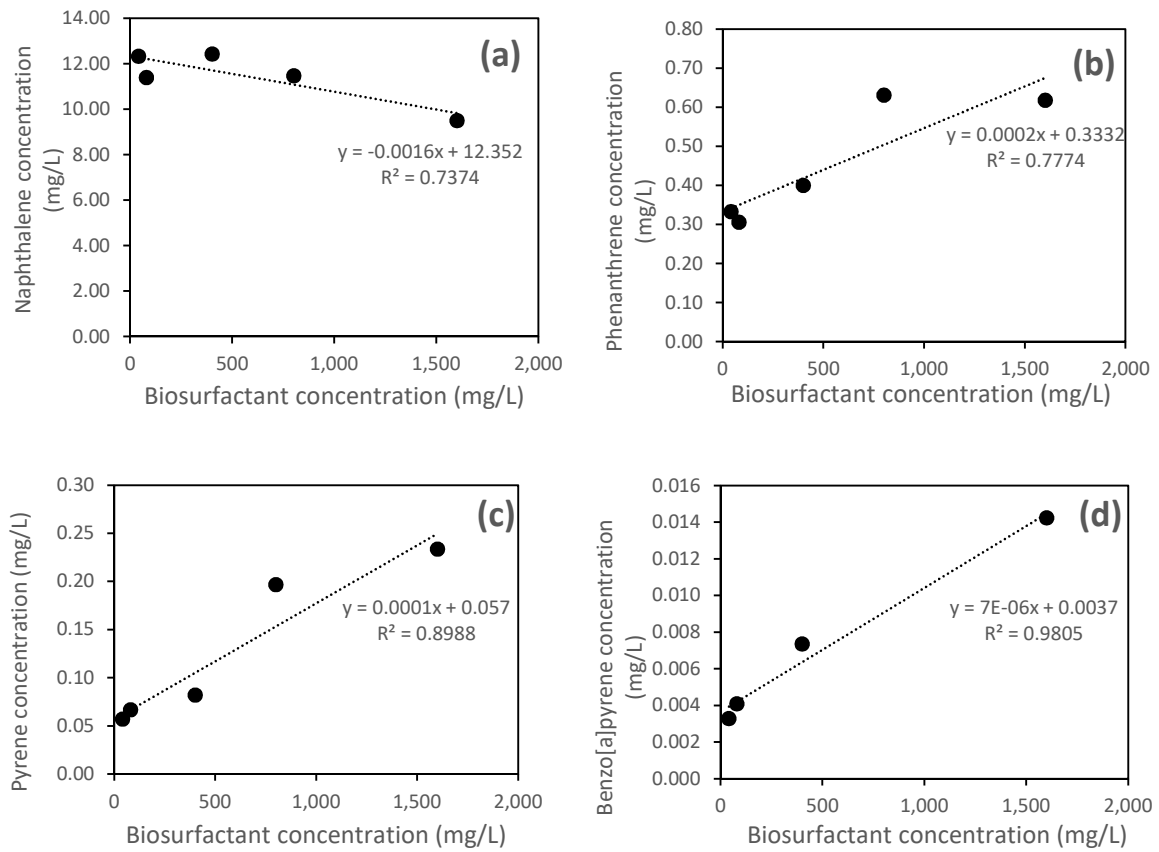


Figure 8: Molar solubilisation Ratio (MSR) in water of the four studied PAHs, (a) naphthalene, (b) phenanthrene, (c) pyrene, (d) benzo[a]pyrene, as a function of the biosurfactant concentration

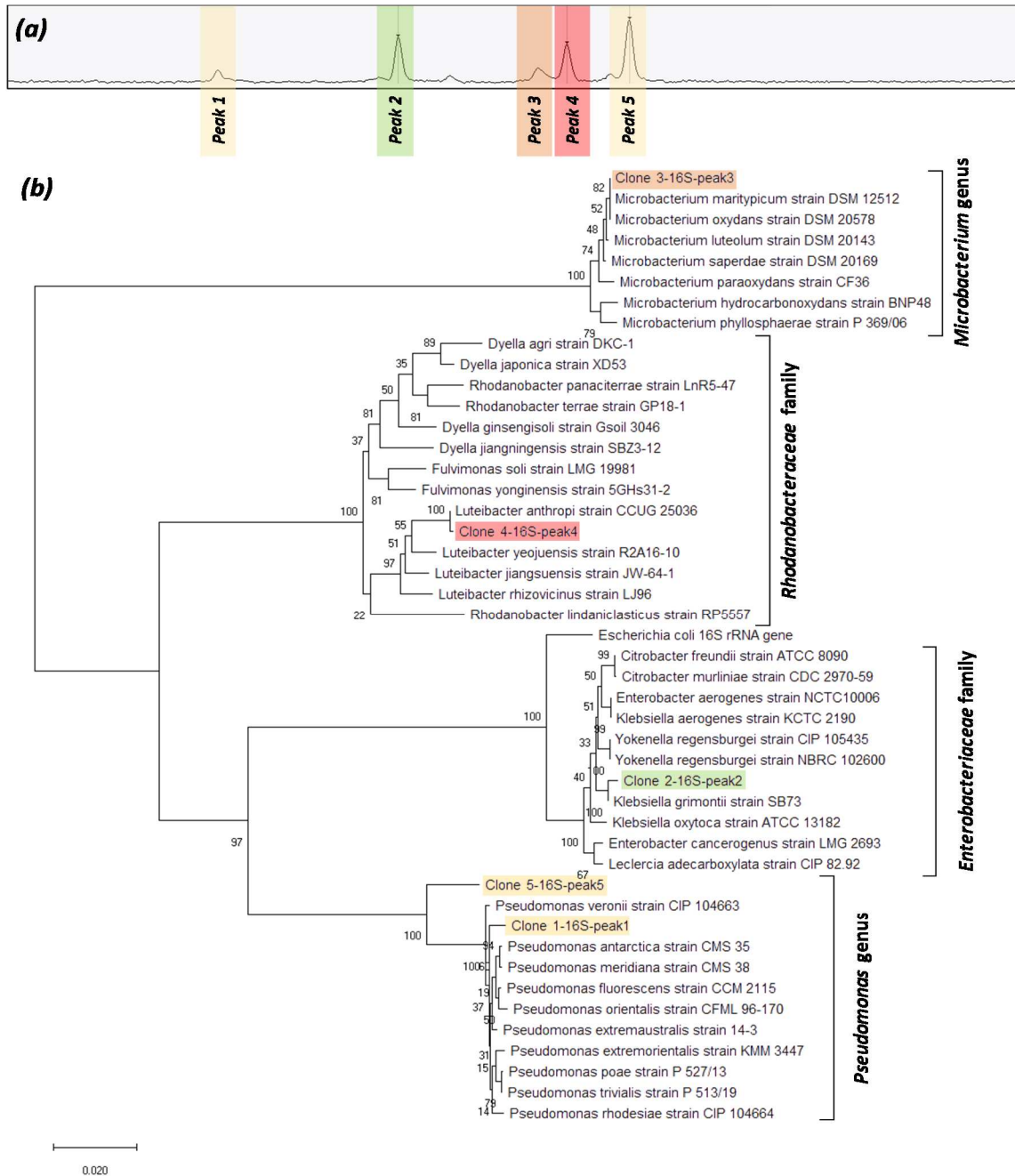


Figure 9 : (a) CE-SSCP fingerprint analysis showing the biodiversity of the bacterial community and (b) Evolutionary relationships of taxa inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.62696946 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the Jukes-Cantor method [3]. The analysis involved 46 nucleotide sequences, with a total of 1341 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

Table 1: Composition of the different media tested for the biosurfactant production

Medium name	A	B	C	D	E	F	G	H	I
Glucose concentration (g/L)	20	20	40	20	40	20	40	40	20
NH ₄ NO ₃ concentration (g/L)	2	4	2	2	4	4	4	2	1
Na ₂ HPO ₄ concentration (g/L)	14.2	14.2	14.2	7.1	7.1	7.1	14.2	7.1	7.1
C/N/P ratio	13/1/2	13/2/2	26/1/2	13/1/1	26/2/1	13/2/1	13/1/1	26/1/1	26/1/2

Graphical abstract

