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1

1 **New benzo(a)pyrene-degrading strains of the *Burkholderia cepacia* complex prospected**  
2 **from Activated Sludge in a Petrochemical Wastewater Treatment Plant**

3

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31 coordinator's own funds.

## 32 **Abstract**

33 The prospection of bacteria that are resistant to polyaromatic hydrocarbons (PAH) of  
34 activated sludge from a Petrochemical Wastewater Treatment Plant (WWTP) allows  
35 investigating potential biodegraders of PAH. For this purpose, sludge samples were cultured  
36 with benzo(a)pyrene and/or naphthalene as carbon sources. The recovered isolates were  
37 characterized by biochemical methods and identified based on the analysis of the sequence of  
38 three genes: 16S, *recA* and *gyrB*. The isolated strains were shown to be capable of producing  
39 surfactants, which are important for compound degradation. The ability to reduce  
40 benzo(a)pyrene in vitro was tested by gas chromatography. After twenty days of experiment,  
41 the consortium that was enriched with 1 mg/L of benzo(a)pyrene was able to reduce 30% of  
42 the compound when compared to a control without bacteria. The four isolated strains that  
43 significantly reduced benzo(a)pyrene belong to the *Burkholderia cepacia* complex and were  
44 identified within the consortium as the species *B. cenocepacia* IIIa, *B. vietnamiensis*, *B.*  
45 *cepacia* and *B. multivorans*. This finding demonstrates the biotechnological potential of the  
46 *B. cepacia* complex strains for use in wastewater treatment and bioremediation. Previous  
47 studies on hydrocarbon-degrading strains focused mainly on contaminated soil or marine  
48 areas. In this work, the strains were prospected from activated sludge in a WWTP and  
49 showed the potential of indigenous samples to be used in both improving treatment systems  
50 and bioremediation of areas contaminated with petrochemical waste.

51

52 **Keywords:** Indigenous microbiota. PAH degradation. Surfactant production. Bioremediation.  
53 Polyaromatic Hydrocarbons. Bioprospection.

## 54 **Introduction**

55           Scientific and industrial communities have already celebrated the 106th anniversary  
56 of one of the most important applications of biotechnology, Activated Sludge, which is used  
57 to purify sewage in Wastewater Treatment Plants (WWTPs) worldwide (Daims et al. 2006;  
58 Jenkins et al. 2014; Valentín-Vargas et al. 2012). Activated Sludge is a powerful tool for the  
59 treatment of sewage from different matrices. The complex community of microorganisms  
60 that make up activated sludge is associated to and varies according to the type of wastewater  
61 that is treated (Greene et al. 2002; Shchegolkova et al. 2016; Winkler et al. 2013; Ye and  
62 Zhang 2013; Yi et al. 2012). However, while it is known that understanding microbial  
63 communities is essential to managing biotechnology and obtaining better microbial services,  
64 there is still a long way to go before we can fully understand these communities and their  
65 interactions and apply the activated sludge technique to its full potential (Rittmann 2006).

66           In petrochemical wastewater, Polycyclic Aromatic Hydrocarbons (PAHs) are the  
67 main pollutants and a special challenge for wastewater treatment plants, as many of these  
68 compounds have high toxicity, stability and end up accumulating in the environment (Ghosal  
69 et al. 2016; Haritash and Kaushik 2009; Hernandez-Raquet et al. 2013; Kipopoulou et al.  
70 1999; Viesser et al. 2020). Due to these characteristics, these compounds are not always  
71 totally degraded through treatment with activated sludge and new processes are needed to  
72 deal with the accumulation of these compounds, which are important environmental  
73 liabilities.

74           Thus, the prospection of bacteria from contaminated sites or effluent treatment  
75 systems becomes an important tool to be used in bioaugmentation and bioremediation  
76 techniques. Since these microorganisms are adapted to environments that are contaminated  
77 with toxic and/or stable compounds, they end up having the capacity to degrade specific  
78 pollutants with a higher success rate (Ławniczak et al. 2020; Cerqueira et al. 2012; Ma et al.  
79 2009; Moreno-Forero et al. 2016; Rodrigues et al. 2015). Through classic taxonomy methods  
80 and metagenomic techniques, we can better understand the structure of microbial  
81 communities in these sites, as well as monitor the impacts generated by the management of  
82 bioremediation processes, which over time can reveal a whole new set of microorganisms  
83 that are often neglected because they are considered unculturable (Roy et al, 2018; Woźniak-  
84 Karczewska et al., 2019; Greene et al. 2002; Ju et al. 2014; Winkler et al. 2013; Ye and  
85 Zhang 2013). The combination of next-generation sequencing techniques with advances in

86 knowledge of culture-enrichment methods, using certain nutrients and sufficient time for  
87 growth (Pham and Kim 2012; Stewart 2012; Vartoukian et al. 2010), allows us to recover  
88 “non-culturable” degrading microorganisms, such as PCB- or PAH-degrading bacteria  
89 (Cerqueira et al. 2011, 2012; Leigh et al. 2006).

90 Many already described genera of bacteria can degrade low-molecular-weight PAHs  
91 (up to three aromatic rings) such as naphthalene. However, high-weight PAHs (with four or  
92 more aromatic rings), such as benzo(a)pyrene, are more worrisome because they are  
93 structurally stable and, consequently, more recalcitrant to microbial attack (Juhasz and Naidu  
94 2000; Tonini et al. 2010). Thus, prospecting bacteria and knowing more widely their PAH-  
95 degradation metabolism become the focus for current research to improve the efficiency of  
96 treatment in WWTPs and bioremediation of sites that are contaminated with these  
97 compounds (Pinhati et al. 2014; Seo et al. 2009; Van Hamme et al. 2003; Withey et al. 2005).  
98 With this objective, we prospect and characterize bacteria with the capacity to degrade  
99 naphthalene and benzo(a)pyrene (highly stable compound) from activated sludge in a  
100 Petrochemical WWTP to evaluate their degradation potential for possible use in  
101 bioremediation techniques of contaminated areas and improvement of operational services in  
102 effluent-treatment stations.

## 103 **Materials and Methods**

### 104 **Wastewater Treatment Plant**

105 This study was performed in a WWTP dedicated to the treatment of waste from  
106 Brazil’s Third Petrochemical Plant, City of Triunfo, Rio Grande do Sul, Brazil (29° 51’  
107 35.02” S, 51° 20’ 50.17” W). The WWTP has been operating since 1982, with two  
108 bioreactors of Conventional Activated Sludge (CAS) with a volume of 13,000 m<sup>3</sup> each and  
109 interchanged operation, from which all the samples were obtained.

### 110 **Isolation of strains**

111 Activated sludge samples were collected from the CAS bioreactor into sterile 50-ml  
112 tubes. Bacteria were cultured by the enrichment methodology in minimum mineral media  
113 (MM1) following Cerqueira et al. (2011). One per cent of activated sludge was added to 50  
114 ml of MM1 enriched with 10 mg l<sup>-1</sup> of either benzo(a)pyrene (a model within high molecular  
115 weight compounds, with 5 aromatic rings) or naphthalene (2 rings, low molecular weight  
116 compound, chosen for being a more easily degradable carbon source) and incubated at 30 °C

117 and 180 rpm. Negative and positive controls were made either without any carbon source or  
118 with 10 mg l<sup>-1</sup> of glucose. Every fifth day, an aliquot of 1 ml of the growth was transferred to  
119 fresh 50 ml of MM1 and incubated under the same conditions. After five transfers, the growth  
120 was harvested by centrifugation and serially diluted in solid media with the same composition  
121 as the liquid enrichments except for the activated sludge. Pools of bacteria were enriched  
122 from these cultures and examined as follows.

### 123 **Genomic DNA isolation**

124 Bacterial genomic DNA isolation was performed according to Sambrook and Russell  
125 (2001), with modifications. Briefly, either a pool or a single colony was transferred to a  
126 microtube and mixed for 5 min with 750 µL of Lysis Buffer I (0.32 M sucrose, 10 mM Tris-  
127 HCl, 5 mM MgCl, 1 % Triton X-100). The mix was centrifuged for 10 min at 10,000 g, the  
128 aqueous phase was discarded, and the pellet was resuspended in 100 µL of Lysis Buffer II  
129 (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA: Na<sub>2</sub>). 10 µL SDS (10 %) and 2.5 µL  
130 Proteinase K (20 mg.ml<sup>-1</sup>) were added and the mix was incubated at 37 °C for 15 min and 60  
131 °C for 60 min. Afterwards, 67.5 µL NaCl (5 M) were added and the mixture was centrifuged  
132 at 10,000 g for 20 min. The aqueous phase was transferred to new clean tubes and DNA was  
133 precipitated with 2 volumes of isopropanol at -20 °C for 60 min. The solution was  
134 centrifuged at 10,000 g for 30 min, the pellet was washed in 70% ethanol once, dried at 30  
135 °C, resuspended in 30 µL of ultrapure water and treated with 1 µL RNase A (10 mg.ml<sup>-1</sup>) for  
136 1 hour at 37 °C.

### 137 **Denaturant Gradient Gel Electrophoresis**

138 Before selecting isolates for further identification, the pool of colonies was analysed  
139 via Denaturant Gradient Gel Electrophoresis (DGGE). The amplification targeted the  $\sigma$  factor  
140 *rpoB* gene, which appears to be present in only one copy per bacteria and has shown a good  
141 discrimination power to be used in pattern analysis. The primers were *rpoB*1698f, containing  
142 a CG clamp, and *rpoB*2041r. All primers in this study are described in the supplementary  
143 table S4. The technique was performed according to Dahllöf et al. (2000). This analysis was  
144 used to infer the taxon diversity in the pool.

### 145 **Morphological and Biochemical characterization of the strains.**

146 Morphological examination of the isolated colonies was done with an optical  
147 microscope (Zeiss AXIO LabA1) after Gram staining. Four morphologically distinct colonies

148 were selected from benzo(a)pyrene and three from naphthalene growths, and biochemical  
149 tests were performed using Bactray III kit (Laborclin, Brazil) according to manufacturer  
150 instructions. Colour and odour were evaluated, as well as the following biochemical tests:  
151 oxidase, cetrimide, acetamide, malonate, citrate, maltose, esculin, urea and indol.

## 152 **Molecular identification of strains**

153 The 16S rRNA gene was target using the primers 27F (DeLong 1992) and LPW205  
154 (Woo 2002), with the addition of a cytosine (C) at position 5' (in bold). The reaction was  
155 prepared in 25 µL using Dream Taq PCR Master Mix (Thermo Fischer Scientific) and PCR  
156 conditions were: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1  
157 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 5 min. Also,  
158 partial Multilocus Sequence Typing (MLST) was performed using the primers: *recA*-F and  
159 *recA*-R; *gyrB*-F and *gyrB*-R (Spilker *et al.* 2009). Reactions were prepared in a total volume  
160 of 25 µL using Dream Taq PCR Master Mix (Thermo Fischer Scientific) and cycling of the  
161 PCR followed Spilker *et al.* (2009). The PCR products were visualized on 1% agarose gel  
162 and were subsequently enzymatically purified using FastAP (Thermosensitive Alkaline  
163 Phosphatase, Thermo Scientific, Delaware, USA) and EXO I (Exonuclease I, Thermo  
164 Scientific, Delaware, USA). The fragments were subsequently cloned into pGEM®-T  
165 Vectors (Promega, Madison, USA) and subjected to blue-white screening. Successful cloning  
166 was tested by PCR using the vector primers T7 and SP6 (Promega, Madison, USA). The  
167 cloned products were then sequenced in both directions at Macrogen (Macrogen Inc., Seoul,  
168 Korea). Quality of sequences was evaluated and an alignment of both directions to form a  
169 consensus was performed using Staden Package 2.0 (available at  
170 <http://staden.sourceforge.net/>). The BlastN tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was  
171 used to provisionally identify the isolates based on the similarity of 16S, *recA* and *gyrB*  
172 fragments of our samples to those from GenBank database. Also, partial sequences of *recA*  
173 and *gyrB* genes were used to identify alleles in the *Burkholderia cepacia* complex MLST  
174 database (<http://pubmlst.org/bcc/>) by aligning our sequences with the available allele  
175 sequences from the database in ClustalW and MEGA 6 (Tamura *et al.* 2013) and, when  
176 necessary, corrected using BioEdit 5.0.9 (Hall, 1999). Nucleotide sequences were deposited  
177 to GenBank and had the accession numbers KU169245 – KU169256 assigned to them.

## 178 **Identification by phylogenetic analysis**

179 A phylogenetic reconstruction using partial sequences of *recA* and *gyrB* genes from  
180 the *Burkholderia* isolates was used to identify them to the species level as follows. The  
181 individual *recA* and *gyrB* gene sequences were aligned using the ClustalW tool in MEGA 6  
182 (Tamura et al. 2013) against the current allele's diversity in the *B. cepacia* complex MLST  
183 database (403 *recA* and 687 *gyrB*, respectively). The sequences were trimmed to match the  
184 MLST alleles (451 bases for *gyrB* and 393 for *recA*) and phylogenetically analysed using the  
185 Neighbor-joining method (Saitou and Nei, 1987) and Kimura'2-parameter model (Kimura  
186 1980) in MEGA 6 (with 1000 bootstrap phylogeny testing). Reference sequences  
187 neighbouring the WWTP isolates were selected from these single-gene phylogenies and  
188 downloaded as a concatenated, aligned sequence and set together with reference alleles to  
189 generate a dataset comprising 53 species of the *B. cepacia* complex and seven *Burkholderia*  
190 species from outside the complex. The trimmed *gyrB* and *recA* alleles from the WWTP  
191 isolates were concatenated, combined, and re-aligned with the reference sequence dataset. A  
192 final Neighbor-joining tree based on the concatenated *gyrB* and *recA* sequences was  
193 constructed in MEGA 6 as described above to identify each WWTP isolate to the species  
194 level.

### 195 **Biosurfactant production**

196 Biosurfactants are compounds that act by decreasing the surface tension between the  
197 hydrocarbon molecule and the medium, allowing the bacterial cell to incorporate the  
198 pollutant into its metabolism. For this reason, the production of biosurfactants was measured  
199 through two different approaches (Patowary et al. 2017). For the surface tension, the cells  
200 were removed by centrifugation at 10,000 g during 10 min and a digital surface tension meter  
201 (Gibertini, Milan, Italy) was used according to Cerqueira et al. (2011) The results were  
202 analysed using the One-Way ANOVA followed by the Tukey test with a 95% confidence  
203 level. Kerosene emulsification (E24) was determined with and without cells following Bento  
204 et al. (2005). An aliquot of 4 ml of mineral medium and cultured cells with benzo(a)pyrene as  
205 a carbon source was mixed for 2 minutes with an equal volume of kerosene and left resting  
206 for 24 hours before measurement to determine the ratio of emulsified height to total height.  
207 The results were analysed using the Mann-Whitney U test and the Kruskal-Wallis test with a  
208 confidence level of 95 % in PAST software version 3.25 (Hammer *et al.*, 2001).

209

### 210 **Quantification of Benzo(a)pyrene degradation test**



211 After identification of the strains, we evaluated their potential to degrade  
212 benzo(a)pyrene *in vitro* and compared it to *Burkholderia vietnamiensis* G4, a model strain for  
213 hydrocarbon degradation (L.A. O’Sullivan and Mahenthiralingam 2005). Around  $1 \times 10^8$   
214 cells from four strains of *Burkholderia*, previously isolated and identified, were mixed in a  
215 consortium and distributed into 20 ml aliquots of MM1 media containing 1 mg/L of  
216 benzo(a)pyrene, incubated at 35 °C and 180 rpm for 29 days. Samples were extracted on the  
217 1st, 10th, 20th and 30th day after incubation to monitor benzo(a)pyrene concentration  
218 decrease in 4 independent approaches. The extractions were made using a modified  
219 QuEChERS method, with anhydrous NaSO<sub>4</sub> instead of anhydrous MgSO<sub>4</sub> in both extractions  
220 and clean up stages (Prestes et al. 2009). The protocol was made in triplicates. For each  
221 sample, a 20-ml aliquot of acetonitrile was added and the mixture was shaken vigorously for  
222 1.5 min, followed by addition of QuEChERS extraction kit, which contained 8 g of  
223 anhydrous NaSO<sub>4</sub>, 2 g of NaCl and 2 g of sodium citrate. After 1 min of shaking and 5 min of  
224 centrifugation at 4000 g, 12 ml of the upper layer were added to the clean-up kit, containing  
225 1800 mg of anhydrous NaSO<sub>4</sub>, 300 mg of PSA, 300 mg of C18, and then shaken for 1 min  
226 and centrifuged for 5 min at 4000 g. An upper layer of 8 ml was filtered through a 20- $\mu$ m  
227 filter. After evaporation, samples were resuspended with 1 ml of dichloromethane containing  
228 chrysene as an internal standard at the concentration of 0.5 mg/L to validate the  
229 chromatographic method. Samples were then quantified in gas chromatography with mass  
230 spectrometry (GC-MS). We performed a paired t-test to evaluate differences between initial  
231 and final benzo(a)pyrene content of the samples with the bacteria consortium (tested group)  
232 against the bacteria-free control using PAST software version 3.25 (Hammer *et al.*, 2001).

## 233 **Results and Discussion**

### 234 **Bacterial isolation and preliminary characterization**

235 After 25 days of culturing (including five transfers) in liquid medium, only the  
236 positive control containing glucose presented turbidity, and no turbidity was observed in  
237 benzo(a)pyrene or naphthalene liquid media. Nevertheless, 4 days after plating 100 and 200  
238  $\mu$ L aliquots from each growth in solid media with the same composition, all plates showed  
239 extensive growth (more than 300 colonies per plate) with more than one evident  
240 morphological type. Pools of bacteria were recovered from both carbon sources and DNA  
241 was extracted and used in DGGE analysis to estimate taxon diversity. For both pools, only 2  
242 DNA fragments were visualized in gel, indicating a low variety of taxa in the samples (data

243 not shown). This result was used to reduce the number of selected colonies for further  
244 identification. Four different morphological types were chosen from benzo(a)pyrene (named  
245 BAP1, BAP1a, BAP2x, and BAP2y) and three were chosen from naphthalene (named NAP1,  
246 NAP2 and NAP3). The results of biochemical and morphological essays using the Bactray 3  
247 kit were inconclusive but suggested *Pseudomonas*-related taxa (Supporting information -  
248 Table A1).

### 249 **Molecular identification of strains**

250 When compared to the GenBank database, all 16S-fragment sequences had more than  
251 98% of similarity with the genus *Burkholderia*: However, it was not possible to reach the  
252 species level using this tool, as the amplified region often showed 100% similarity with more  
253 than one species within this genus (Supporting information - Table A2).

254 To solve this problem, fragments from genes *recA* and *gyrB* were also amplified and  
255 sequenced (NCBI accession numbers of the sequences isolated in this study are in  
256 Supplementary Table S3). Using MLST *Burkholderia cepacia* complex database, it was  
257 possible to identify the specific status of our strains from those in the database, as shown in  
258 Table 1. According to the results, the isolates were identified as belonging to four different  
259 strains and renamed *Burkholderia* sp. BAP1 (identical alleles to BAP1a); *Burkholderia*  
260 *vietnamiensis* BAP2 (BAP2x and BAP2y alleles were identical); *Burkholderia multivorans*  
261 NAP1 and *Burkholderia* sp. NAP2 (identical alleles to NAP3).

262 To solve the species identification of strains BAP1 and NAP2, which had novel  
263 MLST alleles (Supporting information - Table A3); a phylogenetic tree encompassing the  
264 current species-diversity of the *B. cepacia* complex was built using the concatenated *gyrB*  
265 and *recA* sequences (Figure 1). The *B. vietnamiensis* BAP2 strain was placed within the *B.*  
266 *vietnamiensis* species cluster, corroborating the individual allele analysis. In addition, as  
267 expected, the *B. multivorans* NAP1 strain clustered within the *B. multivorans* group. The two  
268 unresolved isolates were placed as follows: NAP2 was identified as *B. cepacia* and BAP1  
269 clustered with isolates of *B. cenocepacia* that belonged to the IIIA phylogenetic lineage  
270 (Vandamme et al. 2003) (Figure 1).

### 271 **Biosurfactant production by the strains**

272 Biosurfactants are biologically produced by several bacterial genera such as  
273 *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Clostridium*, among others, and vary according to the

274 substrate in which the microorganisms are inserted. The advantage of producing  
275 biosurfactants using bacteria is that they increase the solubilization of compounds that are  
276 present in the medium, enabling the use of a wide variety of compounds as a source of energy  
277 and carbon (Jimoh and Lin 2019). Both the reduction of surface tension and the  
278 emulsification of kerosene varied among the strains, but it has been demonstrated that they all  
279 have surfactant properties through one or both test methods (Table 2). Several studies  
280 demonstrate that surfactant producing bacteria can be used in bioremediation of soils and  
281 other matrices contaminated with PAHs and other pollutants such as DDT (Cecotti et al.  
282 2018; Ebadi et al. 2017; Ławniczak et al. 2020; Wang et al. 2018). Thus, the production of  
283 biosurfactants by *Burkholderia* strains indicates not only their capacity to degrade these  
284 compounds but also the capacity of this group to survive in highly impacted environments.  
285 This shows great potential for using these strains in bioremediation techniques of  
286 contaminated areas, enabling improvements in the treatment of petrochemical effluents.

#### 287 ***In vitro* reduction of Benzo(a)pyrene**

288 To investigate the benzo(a)pyrene-degradation ability by the previously isolated and  
289 identified *Burkholderia* strains, we used gas chromatography with mass spectrometry (GC-  
290 MS). The time course studies for the degradation showed a constant increase in the level of  
291 degradation of this PAH by bacteria when compared to the control (Figure 2). Although the  
292 box plot demonstrated a reduction in the amount of benzo(a)pyrene in the first experiments (1  
293 and 10 days), only with 20 and 30 days the differences were significant ( $P < 0.05$ ) (Figure 2).  
294 In the first 20 days, we found a significant differences of benzo(a)pyrene concentration when  
295 comparing the test group against the control group ( $t = -3.3019$ ,  $P = 0.0029$  in 20 days, and  $t$   
296  $= -9.2181$ ,  $P = 0,0007$  in 30 days). In the 30-day inoculation experiment, it was possible to  
297 observe a 23.7% decrease of benzo(a)pyrene concentration compared to the control group.

298 This degradation rate found in the study was similar when compared with studies  
299 using the genus *Burkholderia* for the degradation of several pollutants, as well as several  
300 studies that use benzo(a)pyrene as a model of PAH for degradation tests. Aziz et al. (2018)  
301 found a benzo(a)pyrene degradation rate of 26% and 20% by the bacteria *Ochrobactrum*  
302 *anthropi* and *Stenotrophomonas acidaminiphila*, respectively. Wang et al. (2021) tested the  
303 degradation of benzo(a)pyrene through bacterial communities whose main genera were  
304 *Nocardioides*, *Micromonospora*, *Sacarothrix*, *Lysobacter*, *Methylium*, *Burkholderia* and  
305 *Phenylobacterium*, and obtained a degradation rate of 29.5% and 25.3%. Morya et al. (2020)

306 conducted a review of studies that used species of *Burkholderia* for the degradation of  
307 aromatic compounds. The authors present *Burkholderia fungorum* as able to degrade three  
308 aromatic compounds, viz., phenanthrene, pyrene and fluoranthene, with a decrease of 100%,  
309 98% and 99%, respectively, and a bacterial consortium of *Burkholderia* sp. which obtained  
310 33.4% in the degradation of pyrene and benzo(a)pyrene. Nzila and Musa (2020), in their  
311 review article, presented a relationship between benzo(a)pyrene and some bacteria that can  
312 degrade it alone or in a consortium. Some of the mentioned genera were *Beijerinckia*,  
313 *Pseudomonas*, *Mycobacterium*, *Flavobacterium*, *Sphingomonas*, *Burkholderia*, *Bacillus*,  
314 *Stenotrophomonas* and *Ochrobactrum*, among others.

315         The use of an enriched culturing method allowed us to isolate strains using PAHs as  
316 the sole source of carbon (Fulekar 2017). Fast and simple approaches are especially useful to  
317 recognize the traits related to the degradation abilities of these microorganisms. One common  
318 technique for this preliminary identification of degrading ability is the production of  
319 biosurfactants (Xiao et al. 2012) as these compounds act as a solubilizing agent in surfactant-  
320 enhanced remediation processes (Bordoloi and Konwar 2009; D'aes et al. 2009; Lamichhane  
321 et al. 2017; Wattanaphon et al. 2008). This characteristic is why several researchers are trying  
322 to isolate bacteria with this capacity (Ben Belgacem et al. 2015; Wattanaphon et al. 2008).  
323 Here, we highlight that being able to find indigenous sludge bacteria with this ability is  
324 already a good indication that we can use them in further applications (Embar et al. 2006;  
325 Ray et al. 2021). Furthermore, not only have we found this important indicator, but also our  
326 strains, when in a consortium, significantly reduced benzo(a)pyrene *in vitro*, confirming what  
327 was predicted with their surfactant production. These characteristics were reinforced when  
328 we identified our strains as belonging to the genus *Burkholderia* since this group is well-  
329 known for its degradation abilities (L.A. O'Sullivan and Mahenthiralingam 2005). Among  
330 several bacterial groups that can be used for bioremediation, the *Burkholderia cepacia*  
331 complex is a group of many phenotypically similar species (Depoorter et al. 2016, 2020;  
332 Eshwar Mahenthiralingam et al. 2005). Formerly known as *Pseudomonas*, they have only  
333 been transferred to the genus *Burkholderia* in 1992 (Beukes et al. 2017). Despite first known  
334 for their pathogenic characteristics, they usually have beneficial interactions with plants, are  
335 considered ecologically versatile, and have the potential for bioremediation since their  
336 significant metabolic capacity enables them to degrade a variety of common pollutants  
337 (Eshwar Mahenthiralingam et al. 2005). *Burkholderia vietnamiensis* G4 was first isolated in  
338 1986, when it was found to degrade trichloroethylene (Nelson et al. 1986, 1987). Nowadays it

339 is considered to have major biotechnological potential and is also recognized by its ability to  
340 degrade benzene, *o*-cresol, *p*-cresol, phenol, toluene, chloroform, naphthalene and  
341 benzo(a)pyrene (Cauduro et al. 2020; Morya et al. 2020; Nzila et al. 2018; L.A. O'Sullivan  
342 and Mahenthiralingam 2005; Louise A. O'Sullivan et al. 2007).

343 Interestingly, WWTP activated sludge contains and can be enriched specifically for *B.*  
344 *cepacia* complex strains by growth in the presence of benzo(a)pyrene and naphthalene, as this  
345 was the only genus found in all strains isolated in this work. While *B. cepacia* and *B.*  
346 *vietnamiensis* isolates may be easily cultured from a variety of environmental niches,  
347 including polluted soils and the rhizosphere (Mahenthiralingam et al. 2008), environmental  
348 sources of *B. multivorans* and specifically the IIIa strains of *B. cenocepacia* are poorly  
349 understood. The WWTP isolate *B. cenocepacia* BAP1 recovered herein is a very rare IIIA  
350 strain with an authenticated environmental source. It is also striking that all the WWTP  
351 strains isolated after this enrichment were members of the *B. cepacia* complex (Figure 1),  
352 suggesting that this closely related group of species have evolved a great capacity to survive  
353 and grow in the presence of PAHs.

## 354 **Conclusions**

355 Four new bacterial strains, viz., *B. cenocepacia* IIIA, *B. vietnamiensis*, *B. cepacia* and  
356 *B. multivorans*, were prospected in a activated sludge of a WWTP dedicated to the treatment  
357 of waste from a Petrochemical Plant and characterized by biochemical and molecular  
358 methods. All species belong to the *Burkholderia cepacia* complex, a group known for its  
359 ability to survive in several environments and widely used in bioremediation techniques in  
360 several impacted areas. All strains were able to produce surfactants and degrade  
361 benzo(a)pyrene, with a decrease of 23.7% of the compound over 30 days. These  
362 characteristics are important and indicate the biotechnological potential of the group for use  
363 in bioremediation.

364 The bioprospecting of new bacteria contributes to the understanding and improvement  
365 of bioremediation processes, as observed in this study. However, most studies on  
366 hydrocarbon-degrading bacteria are concentrated on contaminated soils and marine waters. In  
367 our study, activated sludge from a wastewater treatment plant was explored, demonstrating  
368 the potential of indigenous samples for improvements in the treatment of oil residues. The  
369 challenge now is to convert this knowledge into better "microbial services", using these

370 microorganisms in real applications, such as in wastewater treatment, bioaugmentation and  
371 bioremediation of contaminated soils.

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385

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641 Table 1. Identification of isolates using partial MLST by analysis of the *gyrB* and *recA* genes.

Isolate	Carbon source	Preliminary		Final	
		Species identification	Allele <i>gyrB</i>	Allele <i>recA</i>	Species identification
BAP1	benzo(a)pyrene	<i>Burkholderia</i> sp.	new	recA 14	<i>B. cenocepacia</i>
BAP1a	benzo(a)pyrene	<i>Burkholderia</i> sp.	new	recA 14	<i>B. cenocepacia</i>
BAP2x	benzo(a)pyrene	<i>B. vietnamiensis</i>	gyrB 16	recA 48	<i>B. vietnamiensis</i>
BAP2y	benzo(a)pyrene	<i>B. vietnamiensis</i>	gyrB 16	recA 48	<i>B. vietnamiensis</i>
NAP1	naphthalene	<i>B. multivorans</i>	gyrB 475	recA 7	<i>B. multivorans</i>
NAP2	naphthalene	<i>Burkholderia</i> sp.	new	new	<i>B. cepacia</i>
NAP3	naphthalene	<i>Burkholderia</i> sp.	new	new	<i>B. cepacia</i>

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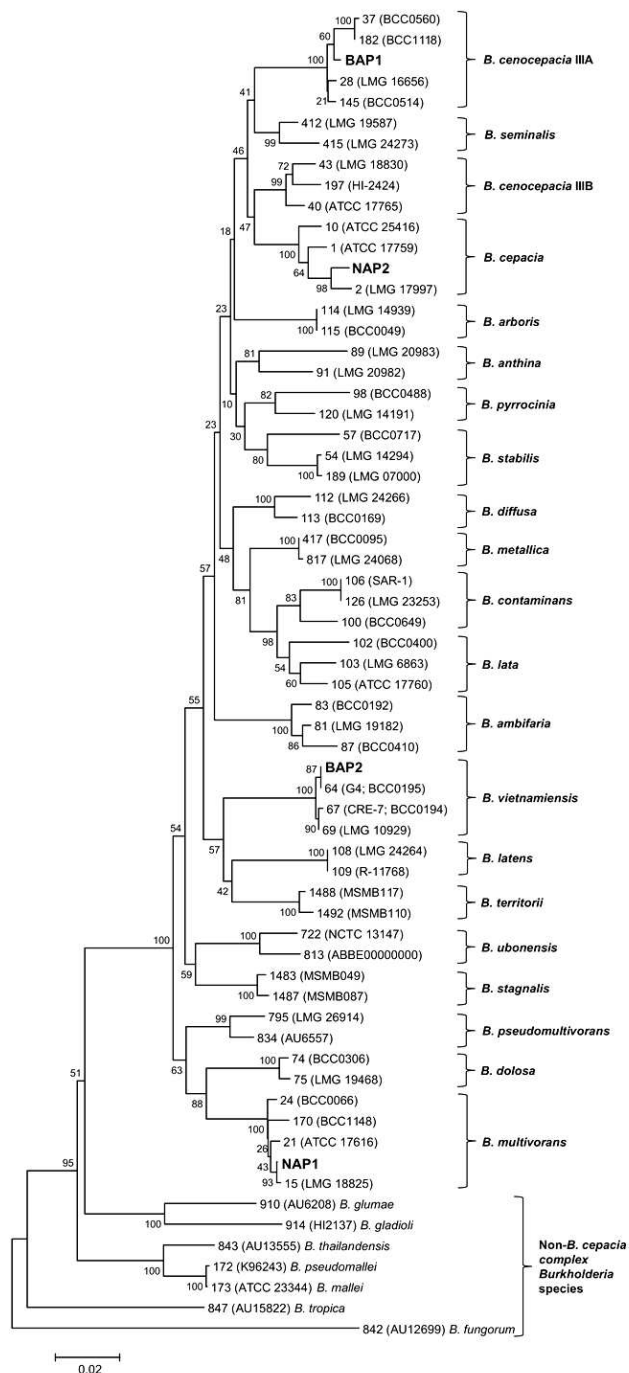
644 Table 2. Evaluation of biosurfactant production in liquid media after 14 days of incubation in minimal  
645 media with benzo(a)pyrene as a carbon source.

	Surface tension		$E_{24}$	
	Average (mN/m)	Reduction related to the control (%)	Cell	Cell-free
Negative control <sup>1</sup>	73.07		NE	NE
<i>B. cenocepacia</i> BAP1	67.65	7.41	**5.71 ± 1.05	**3.45 ± 1.31
<i>B. vietnamiensis</i> BAP2	68.18	*12.00	**1.76 ± 0.41	NE
<i>B. multivorans</i> NAP1	69.30	5.16	**3.77 ± 1.33	1.58 ± 1.83
<i>B. cepacia</i> NAP2	67.05	*8.23	**5.21 ± 0.96	**2.89 ± 1.46
<i>B. vietnamiensis</i> G4	65.37	*10.54	1.73 ± 2.01	1.58 ± 1.83

646 <sup>1</sup>without bacterial inoculum. \* Statistically significant according to One-Way ANOVA (PAST 3.0)

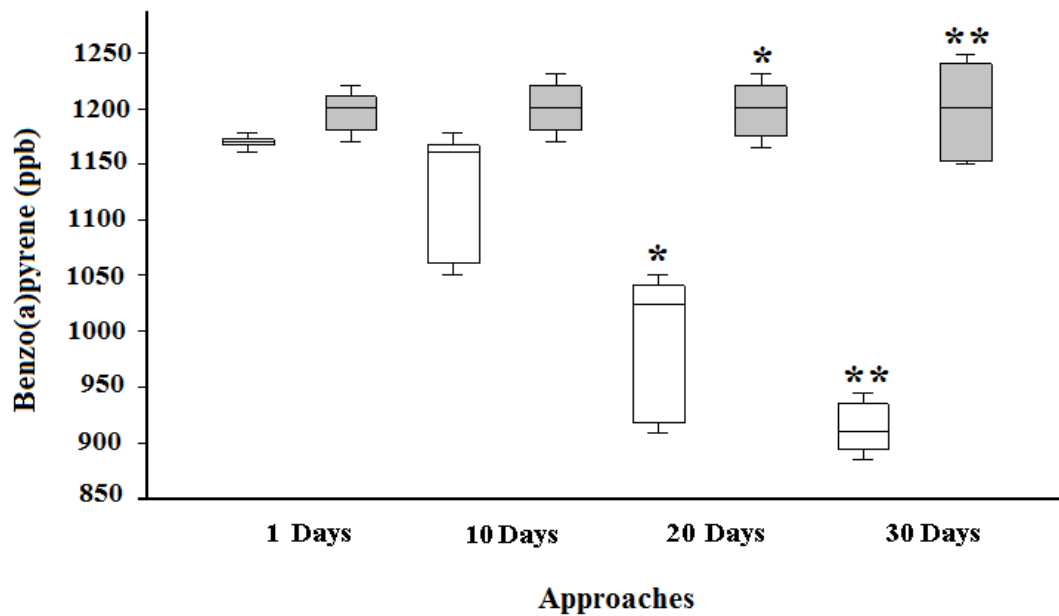
647 followed Tukey test, with a confidence interval of 95%. \*\* Statistically significant according to

648 Mann-Whitney. NE = not emulsified.



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650 **Fig. 1** Phylogenetic analysis of concatenated *gyrB* and *recA* genes for the identification of  
 651 *Burkholderia* species. The species identity of the WWTP isolates BAP1, BAP2, NAP1 and NAP2 was  
 652 determined after phylogenetic analysis, resulting in the Neighbor-joining tree shown above using  
 653 MEGA 6. The scale of genetic distance and phylogeny testing of each node (based on 1000  
 654 bootstraps) are indicated. The WWTP isolate sequences are shown in bold and the species designation  
 655 is based on *Burkholderia* sequences obtained from GenBank.



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657 **Fig. 2** Box plot representing median and interquartile values of benzo(a)pyrene (ppm) from control  
658 sample (grey box) and a consortium of the *Burkholderia* strains in 4 independent experiments (1, 10,  
659 20 and 30 days). We compared differences among the quantities of benzo(a)pyrene in gas  
660 chromatography with mass spectrometry (GC-MS) using the paired t-test in the PAST software  
661 version 2.17 (Hammer *et al.*, 2001). Significance level: \* P < 0.05 and \*\* P < 0.005.



**Response to reviewers' and editor comments:**

Reviewer comments: The manuscript titled "New benzo(a)pyrene-degrading Burkholderia cepacia complex strains prospected from Activated Sludge in a Petrochemical Wastewater Treatment Plant" has been examined. However, there are some grammatical mistakes and typos across the whole manuscript. The manuscript needs a revision and the authors should carefully address my comments below:

We thank the reviewers enormously for their excellent contributions. The answers to the questions asked by the reviewers are detailed below. Also, we are forwarding a new version of the manuscript incorporating all the reviewers' suggestions.

**1. In Abstract Line 6, "the sequence analysis of three genes", which three genes?**

**Reply:** We added the name of the genes in the new version (lines 6 and 7).

**2. In Abstract Line 6-9, "All the strains ... without bacteria", firstly, what was the initial concentration of benzo(a)pyrene. Moreover, this sentence was too long, and I suggest that the author should revise it into two sentences.**

**Reply:** We agree with the reviewer. We rewrote the sentence and incorporated the missing information into the new version of the manuscript.

**3. I suggest that the Introduction should be revised. The logicity should be improved, and the significance of the study should be clarified clearly.**

**Reply:** We agree with the reviewers and are submitting a remodeled introduction. In addition, we have pointed out the objectives of the study more clearly (lines 67 – 71).

**4. I do not understand why did the author investigated the production of biosurfactant?**

**Reply:** We agree with the reviewers that we do not make clear the intent of such an investigation. In the new version of the manuscript, between lines 168 - 170 (material and methods) and 244 - 258 (results and discussion), we explain the importance of this approach to the study. It is important to highlight that biosurfactants are compounds that act by reducing the surface tension between the hydrocarbon molecule and the medium, allowing the bacterial cell to incorporate the pollutant into its metabolism. Therefore, species of bacteria that produce biosurfactants use a wide range of compounds more easily as a source of energy and carbon. It has been shown that such surfactant-producing microorganisms would be candidates for use in bioremediation programmes in soil, e.g., contaminated with PAHs and other pollutants such as DDT. Considering that our objective was to characterize bacteria with the ability to degrade these compounds, we believe that the demonstration of such activity would be a further indication of the potential of such bacterial strains.

**5. In Line 211-213, the author only described the results without deep discussion. For example, what are the advantages for producing biosurfactant by the test Burkholderia cepacia complex?**

**Reply:** We agreed and had a discussion (lines 244-258) as suggested by the reviewer. In this, we presented the state of the art regarding the importance of biosurfactant production by certain microorganisms and justified the choice for this approach in this study.

**6. In Line 222-223, "In the 30 ... with the control group", I suggest that the benzo(a)pyrene reduction rate should be calculated and compared with those of other bacteria.**

**Reply:** We thank the reviewer for their suggestion and inform that a new paragraph (lines 271 - 287) was incorporated in the discussion where we made the recommended comparisons.

**7. I suggest that some important results of this study should be clarified clearly in Conclusions.**

**Reply:** We agree with the reviewer and incorporated a paragraph in the study's conclusions (lines 329-335).

**8. Keywords must be relevant for database search, and different that those already appearing in the title.**

**Reply:** We agree with the reviewer and new keywords have been incorporated into the new version of the manuscript.