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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 m³ 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⁻¹ 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⁻¹ 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: Na₂). 10 µL SDS (10 %) and 2.5 µL
130 Proteinase K (20 mg.ml⁻¹) 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⁻¹) 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 σ 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 1×10^8
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 NaSO₄ instead of anhydrous MgSO₄ 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 NaSO₄, 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 NaSO₄, 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- μ 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 μ 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>	<i>gyrB</i> 16	recA 48	<i>B. vietnamiensis</i>
BAP2y	benzo(a)pyrene	<i>B. vietnamiensis</i>	<i>gyrB</i> 16	recA 48	<i>B. vietnamiensis</i>
NAP1	naphthalene	<i>B. multivorans</i>	<i>gyrB</i> 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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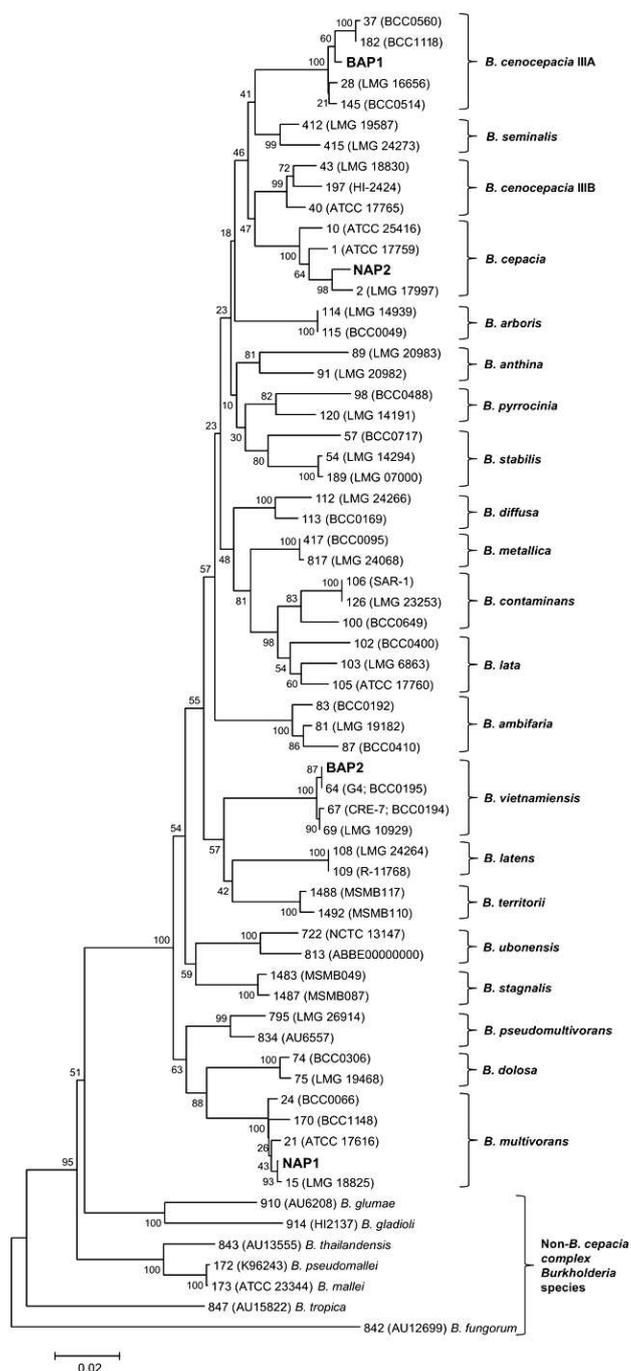
644 Table 2. Evaluation of biosurfactant production in liquid media after 14 days of incubation in minimal
645 media with benzo(a)pyrene as a carbon source.

	Surface tension		E_{24}	
	Average (mN/m)	Reduction related to the control (%)	Cell	Cell-free
Negative control ¹	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 ¹without bacterial inoculum. * Statistically significant according to One-Way ANOVA (PAST 3.0)

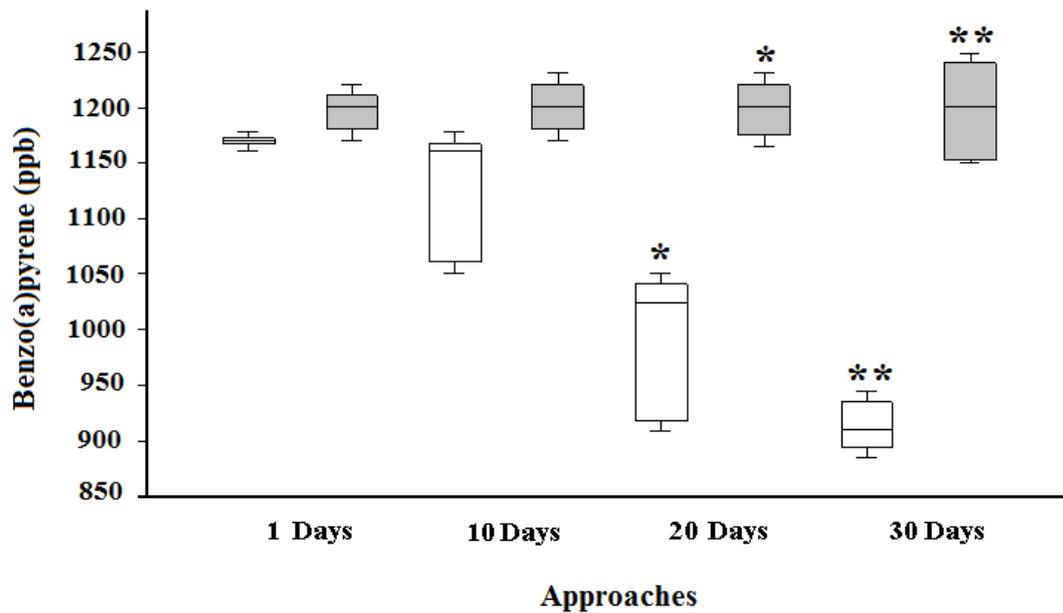
647 followed Tukey test, with a confidence interval of 95%. ** Statistically significant according to

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.