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

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Abstract

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

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

Introduction

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

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

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

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

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

Materials and Methods

Wastewater Treatment Plant

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

Isolation of strains

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

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

Genomic DNA isolation

Bacterial genomic DNA isolation was performed according to Sambrook and Russell (2001), with modifications. Briefly, either a pool or a single colony was transferred to a microtube and mixed for 5 min with 750 µL of Lysis Buffer I (0.32 M sucrose, 10 mM Tris-HCl, 5 mM MgCl, 1 % Triton X-100). The mix was centrifuged for 10 min at 10,000 g, the aqueous phase was discarded, and the pellet was resuspended in 100 µL of Lysis Buffer II (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA: Na2). 10 µL SDS (10 %) and 2.5 µL Proteinase K (20 mg.ml^{-1}) were added and the mix was incubated at 37 °C for 15 min and 60 °C for 60 min. Afterwards, 67.5 µL NaCl (5 M) were added and the mixture was centrifuged at 10,000 g for 20 min. The aqueous phase was transferred to new clean tubes and DNA was precipitated with 2 volumes of isopropanol at -20 °C for 60 min. The solution was centrifuged at 10,000 g for 30 min, the pellet was washed in 70% ethanol once, dried at 30 °C, resuspended in 30 µL of ultrapure water and treated with 1 µL RNAse A (10 mg.ml^{-1}) for 1 hour at 37 °C.

Denaturant Gradient Gel Electrophoresis

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

Morphological and Biochemical characterization of the strains.

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

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

Molecular identification of strains

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

Identification by phylogenetic analysis

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

Biosurfactant production

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

Quantification of Benzo(a)pyrene degradation test

After identification of the strains, we evaluated their potential to degrade benzo(a)pyrene *in vitro* and compared it to *Burkholderia vietnamiensis* G4, a model strain for hydrocarbon degradation (L.A. O'Sullivan and Mahenthiralingam 2005). Around 1 x 10^8 cells from four strains of *Burkholderia*, previously isolated and identified, were mixed in a consortium and distributed into 20 ml aliquots of MM1 media containing 1 mg/L of benzo(a)pyrene, incubated at 35 °C and 180 rpm for 29 days. Samples were extracted on the 1st, 10th, 20th and 30th day after incubation to monitor benzo(a)pyrene concentration decrease in 4 independent approaches. The extractions were made using a modified QuEChERS method, with anhydrous $NaSO_4$ instead of anhydrous $MgSO_4$ in both extractions and clean up stages (Prestes et al. 2009). The protocol was made in triplicates. For each sample, a 20-ml aliquot of acetonitrile was added and the mixture was shaken vigorously for 1.5 min, followed by addition of QuEChERS extraction kit, which contained 8 g of anhydrous $NaSO_4$, 2 g of NaCl and 2 g of sodium citrate. After 1 min of shaking and 5 min of centrifugation at 4000 g, 12 ml of the upper layer were added to the clean-up kit, containing 1800 mg of anhydrous $NaSO_4$, 300 mg of PSA, 300 mg of C18, and then shaken for 1 min and centrifuged for 5 min at 4000 g. An upper layer of 8 ml was filtered through a 20-µm filter. After evaporation, samples were resuspended with 1 ml of dichloromethane containing chrysene as an internal standard at the concentration of 0.5 mg/L to validate the chromatographic method. Samples were then quantified in gas chromatography with mass spectrometry (GC-MS). We performed a paired t-test to evaluate differences between initial and final benzo(a)pyrene content of the samples with the bacteria consortium (tested group) against the bacteria-free control using PAST software version 3.25 (Hammer *et al*., 2001).

Results and Discussion

Bacterial isolation and preliminary characterization

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

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

Molecular identification of strains

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

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

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

Biosurfactant production by the strains

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

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

In vitro reduction of Benzo(a)pyrene

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

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

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

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

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

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

Conclusions

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

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

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

Declarations

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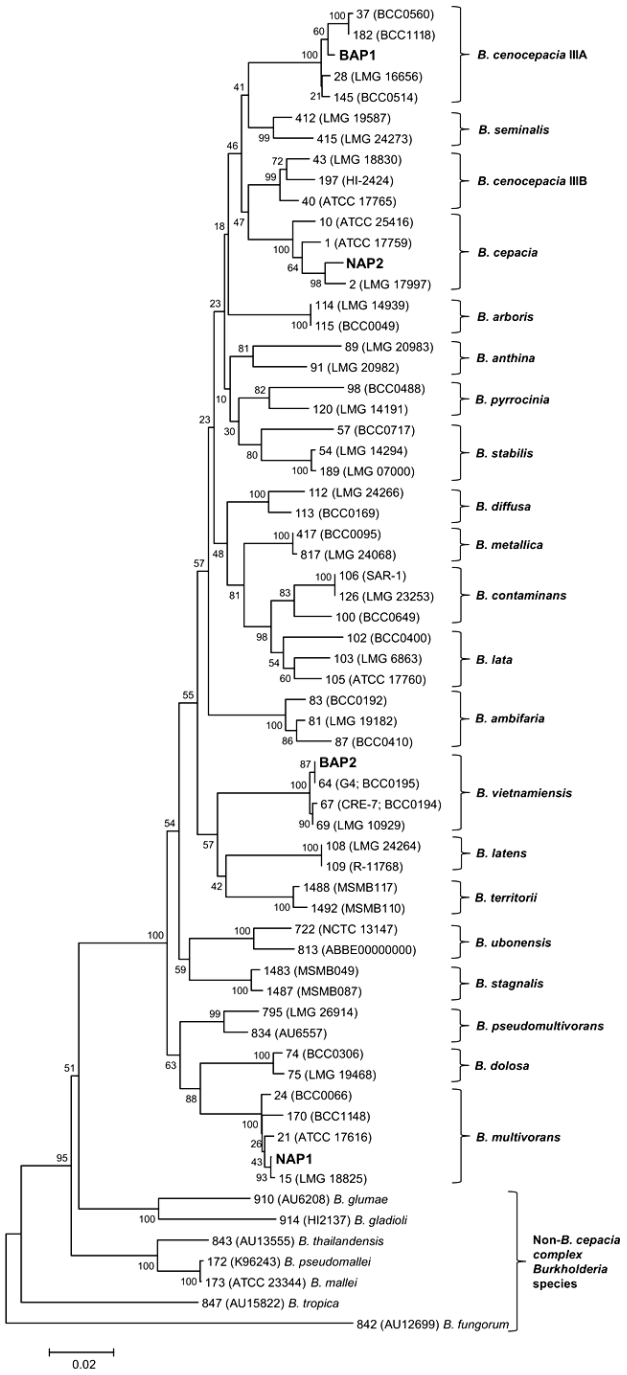
Table 1. Identification of isolates using partial MLST by analysis of the *gyrB* and *recA* genes.

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

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

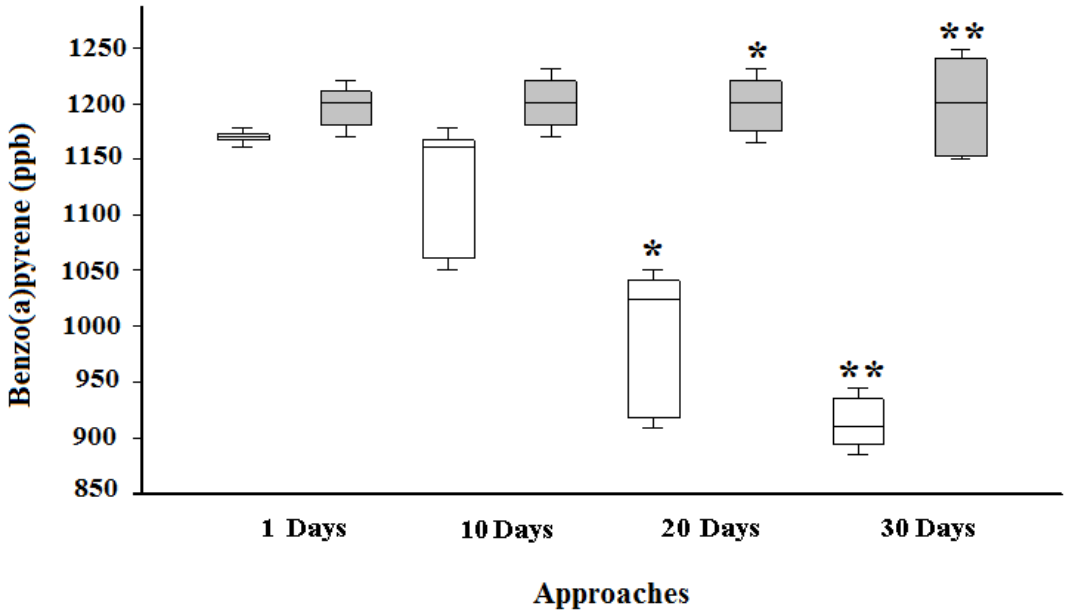
	Surface tension		E_{24}	
	Average (mN/m)	Reduction related to the control (%)	Cell	Cell-free
Negative control[1]	73.07		NE	NE
B. cenocepacia BAP1	67.65	7.41	**5.71 ± 1.05	**3.45 ± 1.31
B. vietnamiensis BAP2	68.18	*12.00	**1.76 ± 0.41	NE
B. multivorans NAP1	69.30	5.16	**3.77 ± 1.33	1.58 ± 1.83
B. cepacia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

[1]without bacterial inoculum. * Statistically significant according to One-Way ANOVA (PAST 3.0) followed Tukey test, with a confidence interval of 95%. ** Statistically significant according to Mann-Whitney. NE = not emulsified.



649

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.



656

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 logicality 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.