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Identification of oxidoreductases from the petroleum *Bacillus safensis* strain

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ABSTRACT

A gram-positive bacterium, denominated CFA-06, was isolated from Brazilian petroleum in the Campos Basin and is responsible for the degradation of aromatic compounds and petroleum aromatic fractions. The CFA-06 strain was identified as *Bacillus safensis* using the 16S rRNA and gyrase B sequence. Enzymatic assays revealed the presence of two oxidoreductases: a catalase and a new oxidoreductase. The oxidoreductases were enzymatically digested and analyzed via ESI-LTQ-Orbitrap mass spectrometry. The mass data revealed a novel oxidoreductase (named BsPMO) containing 224 amino acids and 89% homology with a hypothetic protein from *B. safensis* (CFA-06) and a catalase (named BsCat) with 491 amino acids and 60% similarity with the catalase from *Bacillus pumilus* (SAFR-032). The new protein BsPMO contains iron atom(s) and shows catalytic activity toward a monooxygenase fluorogenic probe in the presence of cofactors (NADH, NADPH and NAD). This study enhances our knowledge of the biodegradation process of petroleum by *B. safensis*.

1. Introduction

Bacillus is a gram-positive genus of rod-shaped bacteria that are obligate aerobes or facultative anaerobes and include more than 60 species. Under stress, the cells produce oval endospores and can remain dormant for extended periods [1,2]. This defence mechanism is associated with a temporary change in gene expression, causing a phenotypic modification of some cells and protecting the genetic material [3]. *Bacillus* spores are exceptionally resistant to heat, UV radiation and chemical agents (as peroxides and hypochlorite) impacting public health by surviving in relatively sterile environments, such as hospital and spacecraft assembly rooms (*Bacillus licheniformis* and *Bacillus pumilus*) [4].

A new *Bacillus* species was isolated from the spacecraft assembly facility at NASA and compared with *B. pumilus.* The new species possesses a unique gyrase B gene sequence and is thus named *Bacillus safensis,* in reference to the SAF Spacecraft Assembly Facility, the location of its first isolation and identification (FO-36b^T). This gram-positive, mesophilic, aerobic and chemotrophic species produces characteristic oxidoreductases (oxidase and catalase) and hydrolases (esterase and β-galactosidase) [5–7]. A microbial consortium containing *B. safensis* strains found in a wastewater electroplating process showed high tolerance to free cyanide (F-CN) [8]. Two additional strains of *B. safensis*—MS11 and JUCHE1—were isolated from the Mongolia desert soil and from milk serum, respectively [9,10]. A thermostable hydrolase (β-galactosidase) was isolated and characterised from *B. safensis* JUCHE 1, and its production process, via fermentation, was tested using distinct carbon sources [11,12]. *B. safensis* DVL-43, isolated from a Haryana soil sample (India), produces a new hydrolase (lipase), which is stable in organic solvents and is readily applicable for the synthesis of methyl laurate from lauric acid [13]. Other strains show potential for lipase production [14,15].

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Oxidoreductases, the second largest class of enzymes applied in biotechnology, are responsible for strategic redox reactions used for functional group interconversion [16–18]. These biocatalysts are cleaner and greener alternatives to traditional methodologies, reducing the use of solvents and toxic reagents [19,20]. They occur in natural and engineered microorganisms and can be used free, immobilized, or in whole cells. Each approach has advantages and limitations; free enzyme processes are usually regio-and enantioselective, but they generally require the addition of cofactors [21–23].

The genome from a *B. safensis* strain, harvested from the rhizosphere of a cumin plant (*Cuminum cyminum*) from the Radhanpar saline desert (Gujarat, India), has been described [24]. In addition, a *B. safensis* CFA-06 strain was recently isolated from highly degraded petroleum from the Pintassilgo Oil Field, Potiguar Basin in Rio Grande do Norte, Brazil, and its genome was sequenced [25]. The enzymatic profile of the *B. safensis* CFA-06 revealed, among other enzymes, oxidoreductase activity. Given the importance of this enzyme class in the petroleum degradation processes, the objective of this study was to identify oxidoreductases present in the strain CFA-06 of *B. safensis* isolated from biodegraded petroleum from Brazil.

2. Material and methods

2.1. Microorganism isolation, cultivation and monooxygenase screening

Biodegraded oil from the Potiguar Basin, Pintassilgo Field in Rio Grande do Norte, Brazil, was inoculated into various cultivation media (agar nutrient, trypticase soy agar, marine agar and glucose-yeast extract-malt extract) from Oxoid Ltd., Basingstoke, Hampshire, England, and the cultures were grown for 3 days at 28 °C. The isolated colonies were classified by color, texture, and type and identified via the 16S rRNA genomic method. The cells were inoculated in liquid cultivation media (500 mL) and stirred in an orbital shaker at 200 rpm for 3 days at 4 °C. The cells were harvested by centrifugation at 6000 rpm, and the pellets were used in high-throughput enzymatic screening (HTS).

The HTS assays were performed in 96-well microliter plates, using the following fluorogenic substrates [26]: 7-(2-oxopropoxy)-2H-chromen-2-one **(1)**, 7-(2-oxocyclohexyloxy)-2H-chromen-2-one **(3)**, 7-(2-oxocyclopentyloxy)-2H-chromen-2-one **(5)** and 7-(3-oxobutan-2-yloxy)-2H-chromen-2-one **(7)**. The reaction products (2-oxo-2H-chromen-7-yloxy) methyl acetate **(2)**, 7-(7-oxooxepan-2-yloxy)-2H-chromen-2-one **(4)**, 7-(tetrahydro-6-oxo-2H-pyran-2-yloxy)-2H-chromen-2-one **(6)**, 1-(2-oxo-2H-chromen-7-yloxy) ethyl acetate **(8)** and 7-(1-hydroxy-3-oxobutoxy)-2H-chromen-2-one **(9)** were used as the positive controls. The assay conditions were as follows: CFA 06 cells in a borate buffer (100 μL, 0.2 mg mL^{-1}), BSA (80 μL, 5.0 mg mL^{-1}), substrate (10 μL, 2 mmol L^{-1}) and borate buffer (10 μL 20 mmol L^{-1}, pH 8.8). The positive controls were: CFA 06 cell suspension (100 μL, 0.2 mg mL^{-1}), BSA (80 μL, 5.0 mg mL^{-1}), product of the enzymatic reaction (10 μL, 2 mmol L^{-1}), and borate buffer (10 μL 20 mmol L^{-1}, pH 8.8). The negative controls were: BSA (80 μL, 5.0 mg mL^{-1}), substrate (10 μL, 2 mmol L^{-1}) and borate buffer (110 μL, 20 mmol L^{-1}, pH 8.8). The microbial control was established with CFA-06 cells (100 μL, 0.2 mg mL^{-1}), and BSA (80 μL, 5.0 mg mL^{-1}) in a borate buffer (20 μL, 20 mmol L^{-1}, pH 8.8) [27].

2.2. Multibioreactions

The biodegradation potential of *B. safensis* CFA-06 was assessed using a multibioreaction protocol [28]. The evaluated substrates were phenanthrene **(11)** and 4-cholesten-3-one **(14)**. CFA-06 was inoculated (10 mg) in glucose–yeast extract–malt extract (GYM) and then incubated for three days at 28 °C, in an orbital shaker at 150 rpm. The cells were harvested by centrifugation (5000 rpm, 20 min, 18 °C). Two grams of cells were resuspended in 40 mL of Zinder solution [29], with 0.5 mL of vitamins solution, 0.5 mL of sodium bicarbonate aqueous solution (10% w/w) and 10 mg of substrate phenanthrene **(11)**, 4-colesten-3-one **(14)**. The resulting suspension was left in an orbital shaker at 28 °C and monitored weekly over 28 days. The reactions were extracted with 20 mL of ethyl acetate (2 × 10 mL), and the organic layer was dried over anhydrous $MgSO_4$. After derivatisation with diazomethane, the samples (1 mg mL^{-1}) were transferred to vials containing non-adecane solution (0.03 mg mL^{-1}) as the internal standard and were monitored by GC–MS using a Agilent 6890 gas chromatograph (Santa Clara, CA, USA) coupled to a Hewlett Packard 5975-MSD (70 eV) spectrometer equipped with a fused silica capillary column (HP-5MS, 30 m × 25.0 mm × 0.25 μm film thickness). CG–MS analyses were conducted using a 1 mL min^{-1} He flow, operating in split mode (20:1), and the temperature program started at 60 °C, increasing at 10 °C min^{-1} to 290 °C.

2.3. Identification of CFA-06

B. safensis CFA-06 was cultivated on agar plates, and the genomic DNA of the pure culture was isolated using a previously described protocol [30]. The PCR amplification of 16S rDNA gene fragments was performed using the primers 27F [31] and 1401R [32], which were complementary to the conserved regions of the 16S rRNA gene of the Bacteria domain. The 50 μL reaction mixtures contained 50–100 ng of genomic DNA, 2 U of *Taq* DNA polymerase (Invitrogen), 1× *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (GE Healthcare) and 0.4 μM each primer. The PCR amplification program, performed in an Eppendorf thermal cycler, consisted of the following: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and 1 cycle of final extension at 72 °C for 7 min. Primers *gyr* B UP-1 and UP-2r were used to amplify the DNA gyrase subunit B gene of the bacterial isolate [33]. The 25-μL-reaction mixtures contained 50 ng of genomic DNA, 2 U of *Taq* DNA polymerase (Invitrogen), 1× *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (GE Healthcare) and 0.4 μM of each primer. The PCR amplification program, performed in an Eppendorf thermal cycler, consisted of the following: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and 1 cycle of final extension at 72 °C for 7 min. The PCR amplification of the 16S rRNA and *gyr*B gene fragments was confirmed using 1% agarose gel stained with SYBR Safe (Invitrogen).

The PCR products were purified further using mini-columns (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and sequenced with an ABI 3500 XL automated sequencer (Applied Biosystem) according to the manufacturer's instructions. The primers used during sequencing were 10F, 1100R [31] and 782R [34] for the 16S rRNA gene and UP-1 and UP-2r [33] for the gyrase gene.

The partial gene sequences (16S rRNA or *gyr*B) obtained with each primer was assembled into a contig using the phred/Phrap/CONSED program [35,36]. Positive identification was achieved by comparing the contiguous 16S rRNA or gyrase sequences obtained with the sequence data from the reference and type strains available in the public databases of GenBank (2014) and RDP (Ribosomal Database Project–Release 10). The sequences were aligned using the CLUSTAL X program [37] and analysed using the MEGA software v.4 [38]. The evolutionary distances were derived from sequence-pair dissimilarities that were calculated as implemented in MEGA while using Kimura's DNA substitution model [39]. The phylogenetic reconstruction was performed using

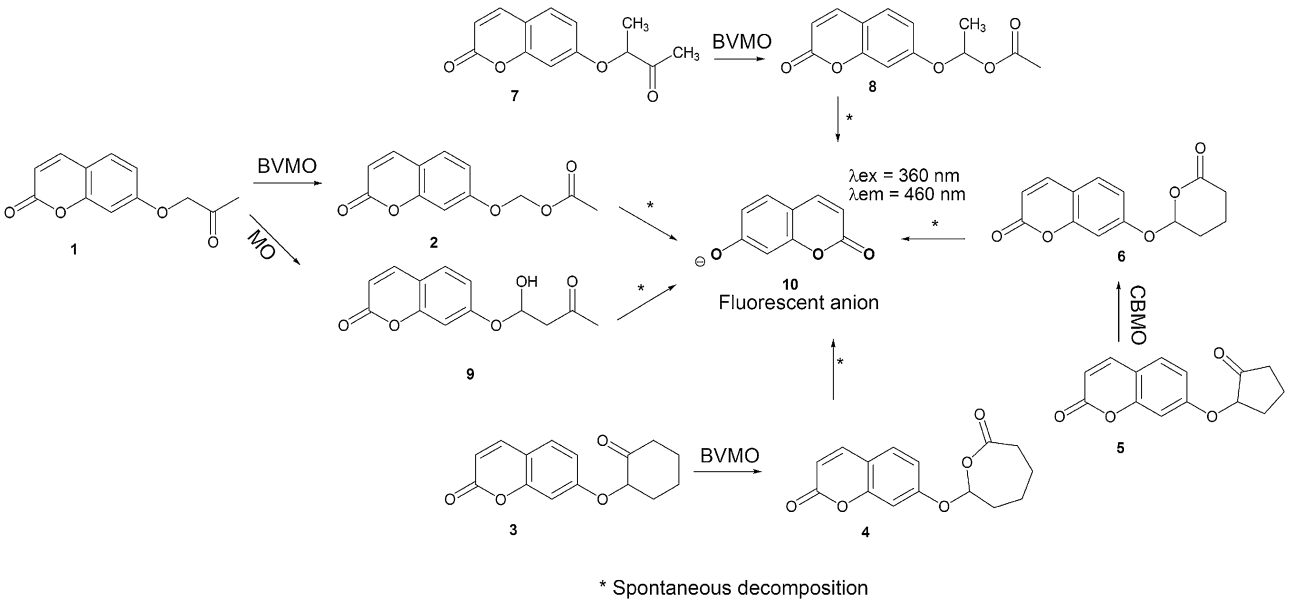

Fig. 1. Enzymatic oxidation of probes 1, 3, 5, and 7 and release of the fluorescent umbelliferyl anion (10).

the neighbour-joining (NJ) algorithm [40], with bootstrap values calculated from 1000 replicate runs.

2.4. Purification of B. safensis soluble proteins

Purification of the soluble proteins from two *B. safensis* cell cultures grown in GYM liquid media in the presence and absence of phenanthrene (200 mg) was performed to verify the influence of this substrate in the enzyme production. For both cultures, the cells were harvested at 4 °C (15 min at 5000 rpm). Pellets from 25 mL of the cultivation medium were resuspended in 32 mL of either sodium phosphate (50 mmol L^{-1}, pH 7.6 for the cation exchange) or Tris–HCl (50 mmol L^{-1}, pH 7.6 anion exchange). Protease inhibitor PMSF (0.320 mL, 1 mmol L^{-1}), β-mercaptoethanol (0.128 mL, 2 mmol L^{-1}), EDTA (0.064 mL, 1 mmol L^{-1}) and DTT (1 mmol L^{-1}) were added to the buffer solutions. The resulting solutions were sonicated (Cole Parmer 4710, EUA) using 8 cycles of 60 s (60% duty cycle) with 2 min between each cycle.

The cell extracts were centrifuged twice at 12,500 rpm and 4 °C for 40 min. The solution was purified with an ÄKTA-FPLC using a 1 mL HiTrap SP HP column (GE Healthcare). Buffer A (10 mL, sodium phosphate 50 mmol L^{-1} pH 7.6) was used to equilibrate the column. The flow-through fraction was eluted with buffer A (10 mL), and the remaining fractions (1 mL) were eluted with buffer A and increasing amounts of buffer B (0–100%, sodium phosphate 50 mmol L^{-1}pH 7.6 and NaCl 1 mol L^{-1}). The anionic exchange column (HiTrap Q HP of 1 mL, GE Healthcare) was eluted with buffered solutions of Tris–HCl (50 mmol L^{-1}, pH 7.6 buffer A) and Tris–HCl (50 mmol L^{-1}, pH 7.6 with NaCl 1 mol L^{-1}, buffer B). The resin was equilibrated with 10 mL of buffer A, and the soluble extract was applied to the FPLC with the superloop. The flow-through fraction was eluted with 10 mL of buffer A, and the remaining 1 mL fractions were eluted with buffer A and increasing amounts of buffer B. Each fraction (50 μL) was subjected to a 12% polyacrilamide gel (SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis) for 2 h (150 V), and then the gel was stained with a Coomassie Blue solution as previously described [41].

2.5. Enzymatic screening of FPLC fractions

After purification, the enzymatic activities of all fractions were immediately assayed with fluorogenic probes using 96-well microplates: substrate **1** (10 μL, 2 mmol L^{-1}), NADH (10 μL, 4 mmol L^{-1}), NADPH (10 μL, 4 mmol L^{-1}), NAD (10 μL, 4 mmol L^{-1}), BSA (110 μL, 5 mg mL^{-1}) and fraction (50 μL). The positive control was as follows: product of the substrate **2** (10 μL, 2 mmol L^{-1}), NADH (10 μL, 4 mmol L^{-1}), NADPH (10 μL, 4 mmol L^{-1}), NAD (10 μL, 4 mmol L^{-1}), BSA (110 μL, 5 mg mL^{-1}) and fraction (50 μL). The control fraction was as follows: substrate **1** (10 μL, 2 mmol L^{-1}), BSA (160 μL, 5 mg mL^{-1}) NADH (10 μL, 4 mmol L^{-1}), NADPH (10 μL, 4 mmol L^{-1}) and NAD (10 μL, 4 mmol L^{-1}). The fluorescent intensities of the released umbelliferyl anion **(10)** were measured in a plate reader spectrometer (Flashscan 530 Analitic Jena). Substrate conversion (%) into product was calculated by comparing fluorescence intensities of the reaction assay and positive control, considering the latter as 100%. Negative controls were used to monitor the spontaneous probe oxidation.

The presence of catalase was investigated in the purified fractions (ion exchange) and in whole *B. safensis* cells by adding hydrogen peroxide (200 μL, 30% v/v) [42].

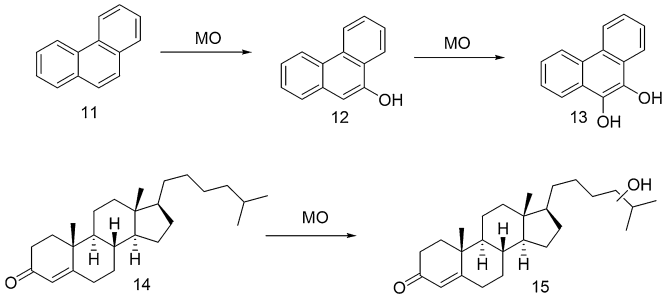

Fig. 2. Enzymatic assay of the strain (CFA-06) on the phenanthrene substrates (11) and the 4-colesten-3-nona (14). After 21 days of reaction the intermediate of the route of biodegradation (12, 13 and 15) were detected using GC–MS.

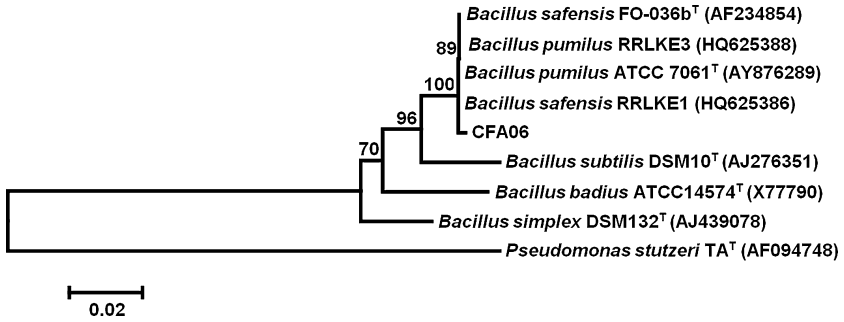

Fig. 3. Phylogenetic analysis based on the partial 16S rRNA sequences (~1000 pb) obtained from the isolate CFA-06 and the related species. Bootstrap values (1000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after the species names. *Pseudomonas stutzeri* was used as the outgroup.

2.6. Metal analyses

FPLC fractions presenting enzymatic activity detected with the fluorogenic substrate (**1**) were subjected to metal content analysis via inductively coupled plasma mass spectrometry (ICP-MS) on an Elan DRC-E mass spectrometer (PerkinElmer, Norwalk, CT, USA) equipped with a collision/reaction cell. Each fraction (100 μL) was diluted to 10 mL with aqueous HNO_3 (1% v/v), as shown in Supplemental Table S1 [43]. The elution buffer used in the chromatographic analysis (FPLC) was used as control in the analysis, along with other non-active fractions.

2.7. Determination of the exact mass protein

ESI-QTOF–MS was used to determine the exact mass of BsPMO. The protein samples were diluted to 10 μmol L^{-1} in H_2O/MeCN 1:1 with 0.1% of formic acid. The samples were introduced with a syringe pump at 10 μL min^{-1}. The parameters were as follows: capillary voltage 3 kV, cone voltage 30 V, source temperature 100 °C, desolvation temperature 200 °C, flow gas 30 L h^{-1}, flow gas of desolvation of 900 L h^{-1}, and trap collision and transfer energies of 6 and 4 V, respectively (QTOF-MS mode). A solution of phosphoric acid was used to calibrate the mass range from *m/z* 90 to 2000 [44,45].

2.8. Protein identification

The excised protein bands were digested as previously described [46]. The mixtures of peptides were analysed using an HPLC-LTQ Orbitrap XL (Thermo Fischer Scientific) with a 150 × 0.075 mm column packed with C_{18} (*Reprosil*) at 80 μL min^{-1} over 90 min with 0–95% acetonitrile/water gradient; both solvents contained 0.1% formic acid. The concentrations of the samples were 1.0 μg mL^{-1} in methanol:water (4:1) and 0.1% of formic acid. The analyses were performed using nanoelectrospray, the spray voltage was 1.5 kV, the capillary temperature 200 °C and the capillary voltage 35 V. The MS1 spectra were acquired using an Orbitrap analyser (400–1800 *m/z*) at a resolution of 60,000 (FWHM at *m/z* 445.1200). For each spectra, the 5 most intense ions were submitted to CID fragmentation (minimum signal required of 1000.0; isolation width of 2.00; normalized collision energy of 35.0; activation Q of 0.250 and activation time of 30 s) followed by MS2 acquisition on the linear trap analyzer [47].

2.9. Data analysis by PEAKS 6.0 software

The tandem mass spectra were extracted from the RAW files and compared to those in the NCBI and SwissProt databanks using PEAKS version 6.0 build 20120620 (Bioinformatics Solutions Inc., Canada) [48]. The raw files were initially subjected to data refining for precursor mass correction, peak centroiding, charge deconvolution and deisotoping. For further analysis, we used accuracy tolerances of 10.0 ppm for the precursor ions and 0.5 Da for the fragment-ions. The enzymatic digestion was semi-tryptic, with a maximum of two missed enzymatic cleavages per protein. All data were subjected to an initial *De Novo* search, allowing variable modifications for cysteine (+57.02 Da—carbamidomethylation), methionine, histidine and tryptophan (+15.99 Da—oxidation), with a maximum of two modifications per peptide allowed [49].

The next step was a search using the PEAKS DB tool, setting the mass of the precursor as monoisotopic and allowing variable modification of cysteine (+57.02 Da) with up to two modifications

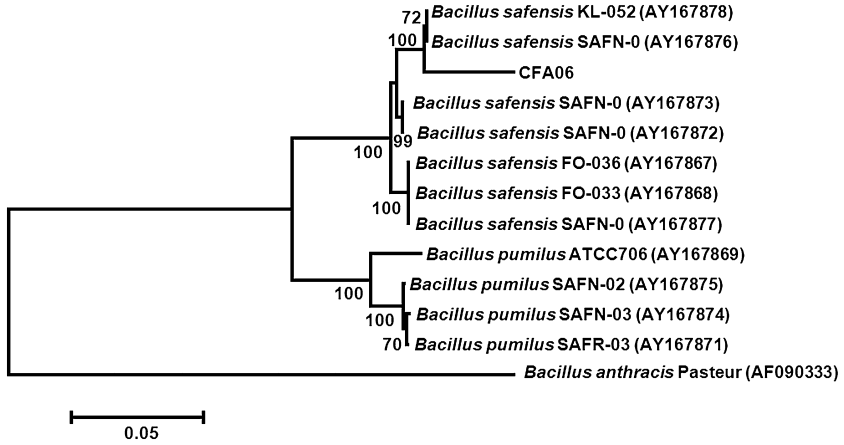

Fig. 4. Phylogenetic analysis based on partial gyrase gene sequences obtained from the isolate CFA-06 and *Bacillus safensis*/*Bacillus pumilus* strains. Bootstrap values (1000 replicate runs, shown as %) greater than 70% are listed. The GenBank accession numbers are listed after the species names. Bacillus anthracis was used as the outgroup.

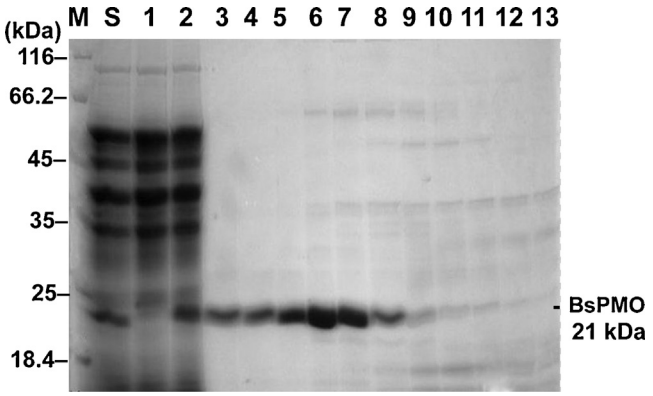

Fig. 5. SDS-PAGE containing the first channel of the comb molecular weight marker (protein ladder broad range) followed by the soluble extract (S) and the flow through collected fractions (1–13) of cation exchange.

per peptide. All searches were performed against the taxon bacteria (*B. safensis and B. pumilus*) in the NCBI non-redundant public database (downloaded on May 01, 2014). The last search was performed using the SPIDER tool; this coverage search was dedicated to finding novel peptide sequences that are not present in the protein database. Finally, the data from all of the searches were consolidated, and only results whose estimated false discovery rate (*FDR*) was less than or equal to 1% were considered reliable.

3. Results and discussion

3.1. Microorganisms isolation, cultivation and monooxygenase screening

B. safensis CFA-06 was isolated from biodegraded petroleum. This strain was subjected to high-throughput enzymatic screening using four distinct substrates for monooxygenases detection. Oxidation of probes **1**, **3**, **5** and **7** produces compounds **2**, **4**, **6**, **8** and **9**, respectively, which spontaneously release the fluorescent umbelliferyl anion (**10**). Reaction conversions using probes **3** and **7** did not reach the minimum acceptable conversion of 5% within the 96 h period. The fluorescent signals were most intense with fluorogenic probes **1** (11% conversion) and **5** (7% conversion), indicating the presence of monooxygenases (Fig. 1). Therefore, the performance of *B. safensis* strain enabled better characterization of its monooxygenase activities.

These enzymes can act by incorporating one (monooxygenases) or two (dioxygenases) oxygen atoms into different organic substrates. However, the transformation of **1** and **5** into **2** and **6**, respectively, is related to the presence of a Baeyer–Villiger monooxygenase (BVMO), and the transformation of **1** into **9** signalled the presence of cytochrome P450 and peroxidases activities. Thus, this test revealed that CFA-06 strain produced oxidoreductases that could oxidise two different fluorogenic substrates (**1** and **5**).

B. safensis CFA-06 oxidoreductase activity was confirmed by multibioreaction an assays using substrates **11** and **14** (Fig. 2). Biodegradation of these compounds can be initiated by the action of monooxygenases (mono- or di-oxygenases), which promotes the oxidation of the substrate forming diols, followed by the ring cleavage and formation of catechol [50].

Analyses using the BLAST algorithm revealed that the highest similar sequences in the databases were represented by *B. safensis* and *B. pumilus* strains, with sequence identity ranging from 99 to 100% and *e*-values of 0.0. A phylogenetic reconstruction based on the 16S rRNA gene allowed the recovery of the CFA-06 isolate in a tight cluster that was supported by a high bootstrap value (100%) with *B. pumilus* and *B. safensis* strains, including the type strains of these species (Fig. 3).

Because of the conserved nature of the 16S rRNA gene for the *B. safensis*/*B. pumilus* group, the *gyrB* gene was used as an alternative phylogenetic marker [6] when identifying the CFA-06 isolate at the species level. A phylogenetic reconstruction based on the gyrase gene revealed that the CFA-06 isolate grouped with *B. safensis* (100% bootstrap value) (Fig. 4), defining the identification of the isolate at the species level. These two species are closely related, and laborious techniques were used to differentiate them. Because of the industrial importance of these two species, the mass spectrometry technique (MALDI-TOF–MS) has been used to reveal biomarkers to facilitate their identification [51].

To characterize this monooxygenase, cell lysates from *B. safensis* cultivated in the presence and absence of phenanthrene (Supplemental Fig. S1) were purified by fast protein liquid chromatography (FPLC) with ion-exchange resins (anionic and cationic). The fractions and cell lysates were monitored by SDS-PAGE, as shown in Fig. 5.

The screening assays of the FPLC fractions (anion and cation exchange columns) and cell lysates were performed in 96-well plates using fluorogenic substrate **1** and three cofactors (NADH, NADPH and NAD). The choice of **1** was based on the best conversion yields obtained during the whole-cell enzymatic screening assays.

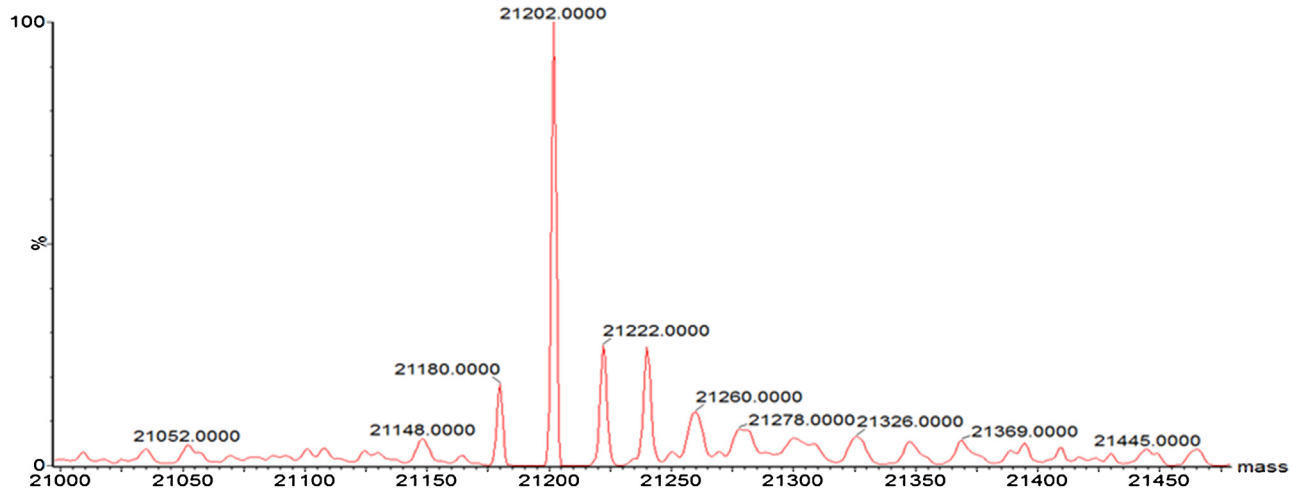

Fig. 6. Deconvoluted mass spectrum of fraction 6 BsPMO (ACN:H_2O (1:1), 0.1% HCOOH).

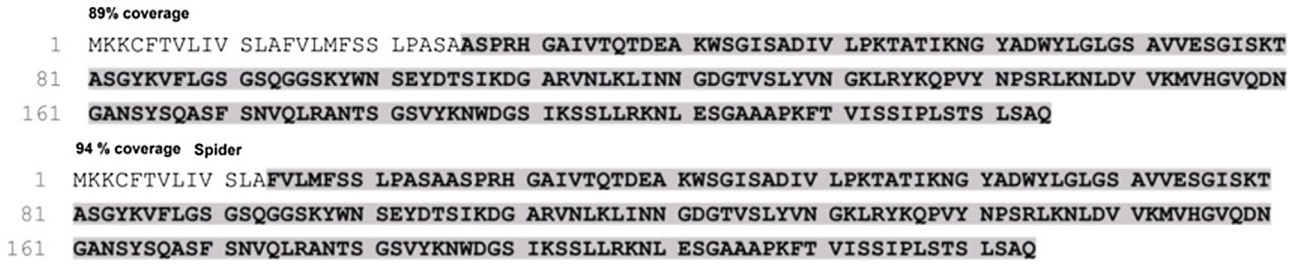

Fig. 7. Amino acid sequence of hypothetical protein CFA-06 (*B. safensis*), with mutations derived from the "*De Novo*" sequence analysis performed using the SPIDER algorithm from PEAKS 6.0.

Cofactors were added because the protein might have eluted without cofactors. No activity was detected in the anion exchange fractions; however, some activity was detected in the cell lysate and fractions **3–9** from the cation exchange purification. This result was obtained in the presence and absence of phenanthrene. These fractions revealed the presence of a predominant protein (SDS-PAGE, Fig. 5), with a molecular mass between 18 and 21 kDa. This protein was named BsPMO, and its molecular weight is 21 kDa, according to ESI(+)-QTOF/MS (Fig. 6).

Cation exchange fractions 3–9 were analysed by mass spectrometry using an inductively coupled plasma source (ICP-MS Elan DRC-e, PerkinElmer), revealing the presence of iron. These fractions were also analyzed in the presence of carbon monoxide and sodium dithionite, revealing that BsPMO protein was not a cytochrome P450. This test is specific for this class of enzymes and is considered positive only when the absorption band occurs at 450 nm due to the formation of an irreversible complex (Fe–CO). However, the P450 activity cannot be completely excluded since the protein may be inactive under the experimental conditions.

Additionally, the presence of peroxidases in the purified fractions of *B. safensis* lysate was investigated using H_2O_2 as a substrate. The release of oxygen was detected only in the flow-through fractions and the soluble extract. The addition of hydrogen peroxide directly on the culture plate also releases oxygen, which confirms the existence of a catalase (BsCat) in *B. safensis*.

Catalases have a significant role in oxidative stress processes in various species of *Bacillus* [52–54]. The catalases belong to a group of peroxidases that use hydrogen peroxide as an electron acceptor. The oxygen transfer reactions catalyzed by peroxidases are among the most relevant oxidative transformations [55,56]. These reactions are compared with the P450 monooxygenase-type reactions because of the versatility and similarity of the mechanisms, but have the advantage of being self-sufficient regarding cofactors [57].

3.2. Protein identification

To identify the primary sequence of BsPMO (gel fractions 3–9, Fig. 5), we used the "bottom-up" methodology with trypsin. The peptides generated were separated using liquid chromatography and analyzed using a mass spectrometry LTQ-Orbitrap-XL (Thermo Scientific, Germany). The equipment was operated using data-independent acquisition mode switching between MS and LTQ-Orbitrap-MS/MS, allowing isolation of the most intense peptide ions and sequencing using collision-induced dissociation (CID) as the fragmentation technique [58].

The fragmentation spectra for the BsPMO peptides were compared with those in the NCBI database (National Center for Biotechnology Information) and SwissProt using PEAKS 6.0. The primary sequence of BsPMO showed 89% coverage with a putative protein of unknown function based on the *B. safensis* (CFA-06) database. Analysis of the primary sequence of BsPMO has enabled predictions (Protparam program) [59] that in some cases have been confirmed by experimental data. The theoretical isoelectric

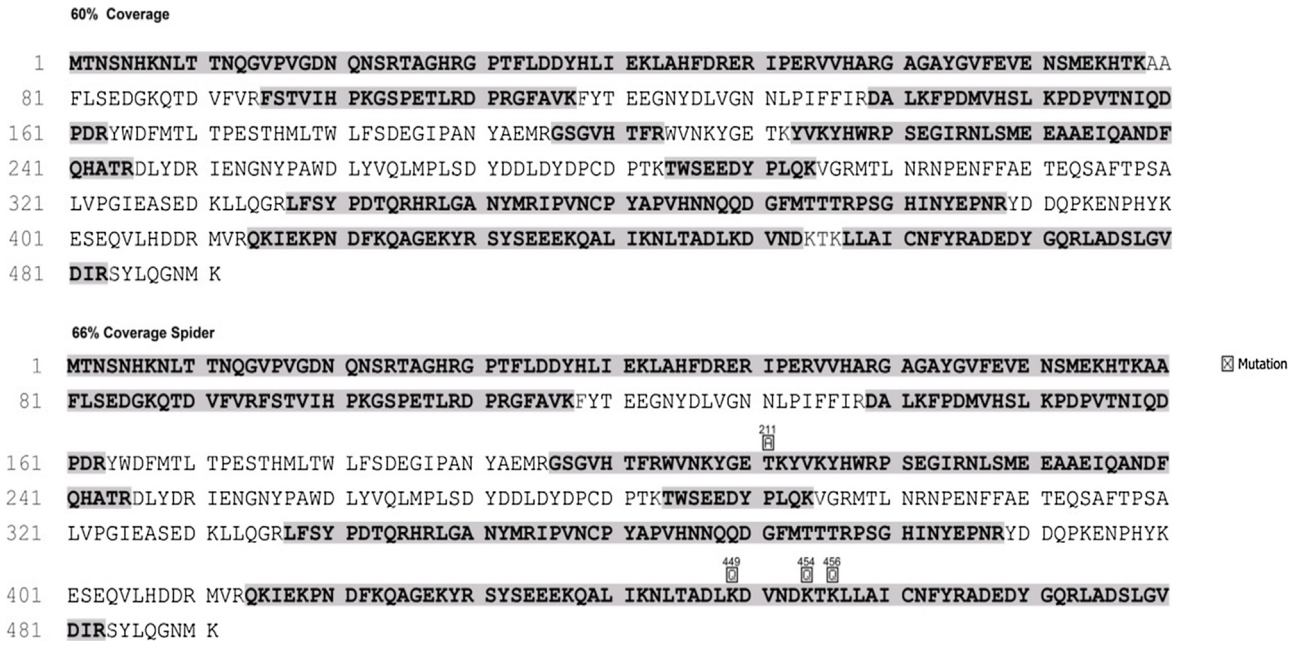

Fig. 8. Amino acid sequence of BsCat, with mutations derived from the "*De Novo*" sequence analysis performed using the SPIDER algorithm from PEAKS 6.0.

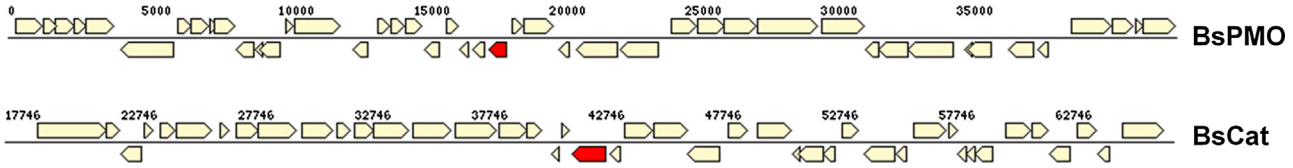

Fig. 9. BsPMO and BsCat genomic localisation. (A) The BsPMO locus was identified in contig 20, between nucleotide positions 17714 and 18388 in the anti-sense direction. (B) The BsCat locus was identified in contig 46, between nucleotides 42007 and 43482 in the anti-sense direction. Only a fragment of each contig is shown. The charts were obtained by means of the IMG/ER tool.

point (9.96) of the BsPMO is relevant to the type of separation. The Signal P 4.1 software [60] indicated the presence of a signal peptide in the primary sequence of BsPMO. This sequence was cleaved between amino acids 25 and 26 (between two alanines, which is not a trypsin site), suggesting that this protein is produced, cut and transported by extracellular environment. This evidence was demonstrated by mass spectrometry, which observed the absence of 1–25 peptide (Fig. 7) in all acquired data, therefore most of the produced protein has its signal peptide cleaved after the secretion process**.** Several species of the genus *Bacillus* are known as "cell factories", based on the amount and stability of the enzyme produced and secreted into the extracellular environment [61].

To improve the amino acid coverage in *B. safensis* BsPMO, sequencing was also performed using the PEAKS 6.0 program. This program constructed a theoretical mass spectrum with amino acid sequences from the mass spectra (MS/MS), containing the peptide fragments generated by CID [62,63]. The program calculated the best combination, predicting the theoretical sequence. In addition, the program enabled mutations in the amino acid sequence, replacing residues with the same functionalities on the side chain (SPIDER) [64,65]. Consequently, 94% coverage was achieved (Fig. 7; Supplemental Table S2).

The BsCat detected in fraction 3 eluted with other 14–116 kDa proteins. Therefore, bands containing the proteins were excised from the gel and trypsinized. The peptides were separated using liquid chromatography and analyzed using a mass spectrometry LTQ-Orbitrap-XL (Thermo Scientific, Germany). A search in the NCBI database (National Center for Biotechnology Information) and SwissProt using PEAKS 6.0 software identified a catalase containing 491 amino acid residues that was 60% similarity with the catalase KatX2 from *B. pumilus* (SAFR-032). BsCat sequencing was also performed using PEAKS 6.0 (Fig. 8; Supplemental Table S3).

Nucleotide sequences of both BsPMO and BsCat are available at GenBank (accession no. JNBO00000000) and IMG Database (project ID Gi23929) and approximate genomic localizations are presented in Fig. 9.

In conclusion, a new *B. safensis* (CFA-06) strain isolated from petroleum degrades aromatic compounds and expresses two distinct oxidoreductases (BsPMO and BsCat). These two enzymes were isolated and identified. BsPMO is a novel enzyme with a signal peptide that allows excretion into the extracellular environment and has iron but no haem group covalently linked to its structure. The second enzyme, BsCat, has 60% similarity with the catalase of the *B. pumilus* and is different from other enzymes previously described.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.09.001.

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