Biotechnology Reports 8 (2015) 152–159

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Identification of oxidoreductases from the petroleum *Bacillus safensis* strain

Francine S.A. da Fonseca[a,b], Célio F.F. Angolini[a], Marco A. Zezzi Arruda[a], Cícero A.L. Junior[a], Clelton A. Santos[c], Antonio M. Saraiva[a,e], Eduardo Pilau[a], Anete P. Souza[c], Prianda R. Laborda[c], Patrícia F.L. de Oliveira[d], Valéria M. de Oliveira[d], Francisco de Assis M. Reis[a], Anita J. Marsaioli[a,*]

[a] *Chemistry Institute, University of Campinas, P.B 6154, 13083-970 Campinas, São Paulo, Brazil*
[b] *Institute of Agricultural Sciences, Federal University of Minas Gerais, 39404-547 Minas Gerais, Brazil*
[c] *Center for Molecular Biology and Genetic Engineering, University of Campinas, 13083-875 São Paulo, Brazil*
[d] *Microbial Resources Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas, CP 6171, CEP 13081-970, Campinas, SP, Brazil*
[e] *National Institute of Metrology, Quality and Technology – INMETRO, Xerém, Rio de Janeiro, Brazil*

ARTICLE INFO

Article history:
Received 7 July 2015
Received in revised form 25 August 2015
Accepted 2 September 2015
Available online 5 September 2015

Keywords:
Bacillus safensis
Catalase
Monooxygenase

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].

* Corresponding author at: Chemistry Institute, University of Campinas, PB 6154, 13083-970 Campinas, São Paulo, Brazil.
E-mail address: anita@iqm.unicamp.br (A.J. Marsaioli).

http://dx.doi.org/10.1016/j.btre.2015.09.001

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