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Enzymatic potential of heterotrophic bacteria from a neutral copper mine drainage

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ABSTRACT

Copper mine drainages are restricted environments that have been overlooked as sources of new biocatalysts for bioremediation and organic syntheses. Therefore, this study aimed to determine the enzymatic activities (esterase, epoxide hydrolase and monooxygenase) of 56 heterotrophic bacteria isolated from a neutral copper mine drainage (Sossego Mine, Canaã dos Carajás, Brazil). Hydrolase and monooxygenase activities were detected in 75% and 20% of the evaluated bacteria, respectively. Bacterial strains with good oxidative performance were also evaluated for biotransformation of organic sulfides. Fourteen strains with good enzymatic activity were identified by 16S rRNA gene sequencing, revealing the presence of three genera: *Bacillus*, *Pseudomonas* and *Stenotrophomonas*. The bacterial strains *B. megaterium* (SO5-4 and SO6-2) and *Pseudomonas* sp. (SO5-9) efficiently oxidized three different organic sulfides to their corresponding sulfoxides. In conclusion, this study revealed that neutral copper mine drainages are a promising source of biocatalysts for ester hydrolysis and sulfide oxidation/bioremediation. Furthermore, this is a novel biotechnological overview of the heterotrophic bacteria from a copper mine drainage, and this report may support further microbiological monitoring of this type of mine environment.

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Introduction

Brazil is the world's 15th largest producer of copper concentrate.[1] The mining company Vale S.A. is responsible for the majority of copper production, having reached 275,000 mt of copper concentrate in the first nine months of 2013,[2] and operates at the Sossego Mine (Canaã dos Carajás, PA, since 2004) and at the Salobo Mine (Marabá, PA, since 2012). The process used for copper ore concentration involves crushing and subsequent semi-autogenous grinding (SAG), followed by

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comminution in a ball mill. The material is classified according to the particle size and then passes through a flotation process. The generated waste (a slurry) is deposited in a dam approximately 5200 m in length.

The aqueous wastes generated from mining activities are known as mine drainages and are described as restricted environments that often have a high metal content and low organic matter concentration. In addition, water drainage from mine wastes and abandoned mines are often acidic as a result of the extended exposure of sulfidic minerals to water and oxygen. However, the pH of copper mine drainage may drastically differ according to the waste chemical composition, the time of exposure and the microbial community.[3]

Most microbial studies focus on the mineral-oxidizing prokaryotes from acid mine drainages (AMD), which usually consist of acidophilic bacteria and archaea. These microorganisms are able to oxidize ferrous iron and/or reduced forms of sulfur and accelerate the oxidative dissolution of sulfidic minerals, which may be applied to metal ore processing and concentrates in a biotechnological process known as biomining and bioleaching.[4–6]

However, the microbial community from copper mine drainages (acidic or neutral) has been overlooked as a source of new biocatalysts for bioremediation and the synthesis of organic compounds. To the best of our knowledge, there are no reports about the use of heterotrophic microorganisms isolated from these restricted environments as biocatalysts applied for bioremediation of organic pollutants or for synthetic organic chemistry.

From a biotechnological point of view, esterases, epoxide hydrolases and monooxygenases are some of the best-studied and most-applied enzymes. These biocatalysts are commonly used for the enantioselective production of value-added compounds, which are important to pharmaceutical and fine chemical industries. In this context, our research group has been working on the detection of enzymatic activity using fluorogenic probes and organic substrates of interest. Our focus has been on new biocatalysts from the Amazon and Atlantic rainforest,[7] petroleum oil and formation water,[8] human skin,[9] and from Brazilian Culture Collections.[10,11]

The present work aimed to investigate hydrolases and monooxygenases in heterotrophic bacteria isolated from a neutral copper mine drainage, applying fluorescence-based high-throughput screening (HTS) assays and multibioreactions to monitor organic sulfide oxidations. Therefore, this study represents a new biotechnological application of poorly investigated, heterotrophic microbiota.

Materials and methods

General methods

Reagents were purchased from Sigma–Aldrich (Steinheim, Germany). Solvents were distilled from technical solvents. Fluorogenic probes and products for HTS assays (**2–16**) were synthesized by our group.[12] A free sample of 2-methyl-4-propyl-1,3-oxathiane (**19**) from Givaudan (Jaguaré, SP, Brazil) was provided by Natura perfumery and cosmetic industry (Cajamar, SP, Brazil). Ethyl phenyl sulfide (**18**) and oxidation products of substrates **18**, **19** and **20** (sulfoxides **18a**, **19a** and **20a**, respectively) were synthesized as described by Porto et al. (2002).[13] All chemical reactions were monitored by silica gel TLC (aluminum foil, 60 F_{254} Merck), and visualization was obtained using UV or by spraying with *p*-anisaldehyde/sulfuric acid followed by heating at approximately 120 °C. Flash column chromatography was performed using Merck (Whitehouse Station, NJ, USA) silica gel 60 (0.04–0.063 mm, 230–400 mesh). NMR spectra were recorded on a Varian Inova 500 (Palo Alto, CA, USA) for ^{1}H (499.88 MHz) and ^{13}C (125.69 MHz) measurements. Chemical shifts (δ) are given in ppm, and coupling constants (*J*) are given in Hertz.

Enzymatic reactions were monitored by GC–MS using an Agilent 7890 gas chromatograph (Santa Clara, CA, USA) coupled with a Hewlett Packard 5975C-MSD (70 eV) spectrometer equipped with a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d. × 0.25 μm film thickness). GC–MS analyses were conducted using a 1 mL min^{-1} He flow, split mode (20:1) and the following temperature program: initial temperature 50 °C, increasing at 10 °C min^{-1} to 200 °C and at 20 °C min^{-1} to 300 °C, remaining constant for 5 min. Enantiomer discrimination was performed on an Agilent 6850 gas chromatograph (Santa Clara, CA, USA) coupled with a flame ionization detector (GC-FID) using a fused silica capillary column Lipodex-E (Macherey-Nagel Inc., Bethlehem, PA, USA) with chiral phase Octakis-(2,6-di-O-pentyl-3-O-butyryl)-γ-cyclodextrin (28 m × 0.25 mm i.d. × 0.25 μm film thickness). The GC-FID analyses were conducted using a 1 mL min^{-1} H_2 flow, split mode (10:1) and the following temperature program: initial temperature 50 °C, increasing at 10 °C min^{-1} to 100 °C and at 5 °C min^{-1} to 180 °C, remaining constant for 5 min.

Sample collection and isolation of heterotrophic bacteria

Samples from the Sossego copper mine drainage, Canaã dos Carajás, State of Pará, Brazil, were collected in August 2009. The samples were named SO5, SO6 and SO7; collected in sterile disposable flasks; and stored at 4 °C. The Sossego mining operation began in 2004. Thus, these are 5-year-old copper mine drainage samples.

The bacteria were isolated according to Eaton and Franson (2005).[14] Each sample (1 g) was homogenized with sterile water (9 mL) and serially diluted. One milliliter of each dilution was transferred to a Petri dish and homogenized with 12 mL of Plate Count Agar (PCA). The plates were incubated for 48 h at 35 °C. Colonies exhibiting a different morphology and color were re-isolated in trypticase soy agar (TSA). The bacteria were stored at −70 °C in LB medium containing glycerol.[15]

Fluorescence-based high-throughput screening assays

Bacteria were inoculated into LB solid medium[15] and incubated for 16 h at 37 °C. After growth, cell suspensions (0.2 mg mL^{-1}) were prepared in borate buffer (20 mmol L^{-1} pH 7.4).

Assays were performed in flat-bottom polypropylene 96-well microtiter plates, which were incubated at 28 °C and 200 rpm. Fluorescence intensities were measured using a plate reader spectrophotometer (FlashScan 530 Analytic Jena) at 460 nm (excitation wavelength: 390 nm).

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