



## Environmental Microbiology

## Enzymatic potential of heterotrophic bacteria from a neutral copper mine drainage



Bruna Zucoloto da Costa<sup>a,1</sup>, Viviane Drumond Rodrigues<sup>b,1</sup>, Valéria Maia de Oliveira<sup>c</sup>,  
Laura Maria Mariscal Ottoboni<sup>b</sup>, Anita Jocelyne Marsaioli<sup>a,\*</sup>

<sup>a</sup> Universidade Estadual de Campinas, Instituto de Química, Campinas, SP, Brazil

<sup>b</sup> Universidade Estadual de Campinas, Centro de Biologia Molecular e Engenharia Genética, Campinas, SP, Brazil

<sup>c</sup> Universidade Estadual de Campinas, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Campinas, Brazil

## ARTICLE INFO

## Article history:

Received 25 March 2015

Accepted 22 February 2016

Available online 26 July 2016

Associate Editor: Cynthia Canêdo da Silva

## Keywords:

Copper mine drainage

High-throughput screening

Hydrolases

Monoxygenases

## 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.

© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Brazil is the world's 15th largest producer of copper concentrate.<sup>1</sup> 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,<sup>2</sup> 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

\* Corresponding author at: Chemistry Institute, University of Campinas (UNICAMP), POB 6154, 13084-971 Campinas, SP, Brazil.  
E-mail: [anita@iqm.unicamp.br](mailto:anita@iqm.unicamp.br) (A.J. Marsaioli).

<sup>1</sup> These authors contributed equally to this work.

<http://dx.doi.org/10.1016/j.bjm.2016.07.004>

1517-8382/© 2016 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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.<sup>3</sup>

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.<sup>4–6</sup>

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,<sup>7</sup> petroleum oil and formation water,<sup>8</sup> human skin,<sup>9</sup> and from Brazilian Culture Collections.<sup>10,11</sup>

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 multi-bioreactions 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.<sup>12</sup> A free sample of 2-methyl-4-propyl-1,3-oxathiane (19) from Givaudan (Jaguarié, 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).<sup>13</sup> All chemical reactions were monitored by silica gel TLC (aluminum foil, 60 F<sub>254</sub> 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 <sup>1</sup>H (499.88 MHz) and <sup>13</sup>C (125.69 MHz) measurements. Chemical shifts ( $\delta$ ) 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  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness). GC–MS analyses were conducted using a 1 mL min<sup>-1</sup> He flow, split mode (20:1) and the following temperature program: initial temperature 50 °C, increasing at 10 °C min<sup>-1</sup> to 200 °C and at 20 °C min<sup>-1</sup> 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)- $\gamma$ -cyclodextrin (28 m  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness). The GC-FID analyses were conducted using a 1 mL min<sup>-1</sup> H<sub>2</sub> flow, split mode (10:1) and the following temperature program: initial temperature 50 °C, increasing at 10 °C min<sup>-1</sup> to 100 °C and at 5 °C min<sup>-1</sup> to 180 °C, remaining constant for 5 min.

### Sample collection and isolation of heterotrophic bacteria

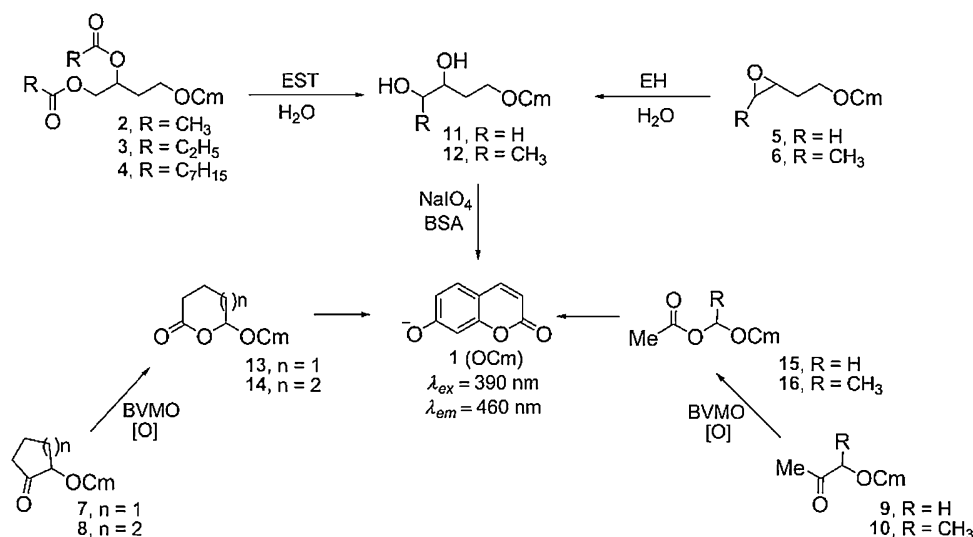
Samples from the Sossego copper mine drainage, Canaã dos Carajás, State of Pará, Brazil, were collected in August 2009. The samples were named SO5, SO6 and SO7; collected in sterile disposable flasks; and stored at 4 °C. The Sossego mining operation began in 2004. Thus, these are 5-year-old copper mine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

The bacteria were isolated according to Eaton and Fran-son (2005).<sup>14</sup> 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.<sup>15</sup>

### Fluorescence-based high-throughput screening assays

Bacteria were inoculated into LB solid medium<sup>15</sup> and incubated for 16 h at 37 °C. After growth, cell suspensions (0.2 mg mL<sup>-1</sup>) were prepared in borate buffer (20 mmol L<sup>-1</sup> 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).



**Scheme 1 – Fluorescence-based HTS assay for detection of esterases (EST, 2–4), epoxide hydrolases (EH, 5–6) and monooxygenases (BVMO, 7–10).**

Hydrolase screening was performed using fluorogenic probes 2–6 (Scheme 1, 100  $\mu\text{mol L}^{-1}$ ), NaIO<sub>4</sub> (2 mmol L<sup>-1</sup>), BSA (2 mg mL<sup>-1</sup>) and bacterial cell suspension (0.1 mg mL<sup>-1</sup>) and was monitored up to 24 h. Monooxygenases were investigated using probes 7–10 (Scheme 1, 100  $\mu\text{mol L}^{-1}$ ), BSA (2 mg mL<sup>-1</sup>) and bacterial cell suspension (0.1 mg mL<sup>-1</sup>) and were monitored up to 72 h. All enzymatic reactions were followed by positive controls, including hydrolyzed or oxidized fluorogenic products 11–16 instead of probes, and using negative controls developed in the absence of bacterial cells.

Substrate conversion (%) into product was calculated by comparing the fluorescence intensities of the reaction assay and positive control, considering the latter as 100%. Negative controls were used to monitor spontaneous probe hydrolysis or oxidation. Enzymatic activity was considered positive for conversions greater than 5%.

### Multibioreaction assays

Bacteria were grown in LB liquid medium for 16 h at 37 °C and 200 rpm. Cells were recovered by centrifugation at 4500  $\times g$  for 15 min. Wet bacterial cells (0.25 g) were mixed with Sørensen buffer<sup>16</sup> (5 mL, 100 mmol L<sup>-1</sup>, pH 7.0) containing 1.5  $\mu\text{L}$  of each substrate (18–21) in Erlenmeyer flasks (25 mL). The resulting solutions were incubated at 37 °C and 200 rpm. After 72 and 120 h, the reactions were interrupted by saturation with NaCl and extraction with ethyl acetate (2  $\times$  2 mL). The organic phases were combined, dried over anhydrous MgSO<sub>4</sub>, derivatized with diazomethane, and an internal standard was added (benzophenone; 0.05 mg mL<sup>-1</sup>). All samples were analyzed by GC–MS and chiral GC–FID. Substrate stability and volatility under reaction conditions were monitored by reaction controls in the absence of bacterial cells.

Substrate conversions (%) were calculated by comparing the chromatographic peak areas of the reactants, products and internal standards.

### Identification of bacteria

The bacterial strains displaying hydrolytic activity above 99% or monooxygenases capable of oxidizing organic sulfur compounds were identified by sequencing of 16S rRNA. Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega), following the manufacturer's instructions. PCR amplification and sequencing of partial 16S rRNA genes were conducted as described by Miqueletto et al. (2011).<sup>17</sup> The sequences obtained were compared with sequences available at GenBank (<http://www.ncbi.nlm.nih.gov>) and the Ribosomal Database Project (<http://www.cme.msu.edu/RDP/html/index.html>). Multiple alignments of 16S rRNA gene sequences were performed using ClustalW 1.6,<sup>18</sup> and evolutionary analyses were conducted in the Molecular Evolutionary Genetics Analysis (MEGA) 6.06 package,<sup>19</sup> using the DNA substitution model Kimura 2-parameter.<sup>20</sup> A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter=0.5288)).<sup>21</sup> The phylogenetic reconstruction was performed using the Maximum Likelihood method with 1000 bootstrapped replicates.

## Results

### Enzymatic activity detected by HTS

The 56 heterotrophic bacteria isolated from the three samples of a neutral copper mine drainage were evaluated by fluorescence-based HTS screening using probes 2–10 (Scheme 1).

Assays performed in the presence of probes 2 and 3 (Table 1), which were used to detect esterases catalyzing the hydrolysis of short chain esters (acetate and propionate, respectively), indicated that 27 bacteria were able to hydrolyze probe 2, five of which converted more than 99% of the substrate (S05-6, S05-13, S07-7, S07-10 and S07-15). Probe 3

**Table 1 – Esterase and monooxygenase activities of heterotrophic bacteria isolated from a neutral copper mine drainage detected by fluorescence-based HTS assays using probes 2–4<sup>a</sup> and 7–10.<sup>a</sup>**

Strain ID	Conversion (%)						
	2	3	4	7	8	9	10
SO5-1	–	29	–	5	7	–	–
SO5-2	–	–	–	9	–	12	–
SO5-3	45	–	–	–	–	–	–
SO5-4	–	–	–	–	16	–	–
SO5-5	–	–	–	–	14	7	–
SO5-6	>99	21	–	–	–	–	–
SO5-7	–	10	5	–	–	30	–
SO5-8	63	–	–	–	–	–	–
SO5-9	–	–	–	–	5	21	–
SO5-10	–	17	–	–	–	29	–
SO5-13	>99	11	–	–	–	–	–
SO5-14	76	10	–	–	–	–	–
SO5-16	64	–	–	–	–	–	–
SO5-17	70	–	–	–	–	–	–
SO5-18	90	8	–	8	–	–	–
SO5-19	–	18	–	–	–	–	–
SO5-21	76	–	–	–	–	–	–
SO6-2	–	56	–	–	29	–	–
SO6-3	–	19	–	–	–	–	–
SO6-4	–	15	–	–	–	–	–
SO6-5	84	6	–	–	–	–	–
SO6-6	33	–	–	–	–	–	–
SO6-7	89	–	–	–	–	–	–
SO6-8	69	–	–	–	–	–	–
SO6-9	57	–	–	–	–	–	–
SO6-12	14	>99	–	6	–	–	–
SO6-14	–	17	–	–	–	–	–
SO6-15	88	18	–	–	–	–	–
SO6-16	–	15	–	–	–	–	–
SO6-18	–	33	–	–	–	–	–
SO6-19	–	9	–	–	–	–	–
SO6-20	82	–	–	–	–	–	–
SO6-22	75	–	–	–	–	–	–
SO7-1	–	13	–	–	–	–	–
SO7-2	–	27	11	7	–	–	–
SO7-3	91	46	–	–	–	–	–
SO7-6	25	12	–	–	–	–	–
SO7-7	>99	–	–	–	–	–	–
SO7-8	19	11	–	–	–	–	–
SO7-9	–	13	–	–	–	–	–
SO7-10	>99	–	–	–	–	–	–
SO7-11	80	–	–	–	–	–	–
SO7-12	10	11	–	–	–	–	–
SO7-13	–	31	–	–	–	–	–
SO7-14	21	11	–	–	–	–	–
SO7-15	>99	–	–	–	–	–	–

<sup>a</sup> See Scheme 1.

was also hydrolyzed by 27 bacteria, and SO6-12 displayed outstanding performance, converting more than 99% of the substrate.

The results for short-chain ester biohydrolysis can be divided into four distinct groups: (a) 15 bacteria that exclusively catalyzed the hydrolysis of acetate esters (2), (b) 15 bacteria that exclusively hydrolyzed propionate esters (3), (c) 12 bacteria that were not able to discriminate between 2 and

**Table 2 – Chromatographic conversion (%) of multibioreaction assays for sulfide (18–21) oxidation,<sup>a</sup> catalyzed by bacteria from a neutral copper mine drainage.**

Strain ID	Conversion (%)			
	18	19	20	21
SO5-1	3	1	–	–
SO5-2	1	–	–	–
SO5-4	20	7	11	–
SO5-5	7	–	1	–
SO5-7	10	–	1	–
SO5-9	22	7	30	–
SO5-10	4	–	–	–
SO6-2	12	5	9	–

<sup>a</sup> See Scheme 2.

3, and (d) 14 bacteria with no esterase activity, as determined using the tested fluorogenic probes.

The heterotrophic bacteria evaluated in this work clearly prefer short-chain esters as only two strains (SO5-7 and SO7-2) were able to hydrolyze the medium-chain ester 4 (Table 1).

None of the strains were able to hydrolyze 5 and 6, which were specific for epoxide hydrolases, after a 24 h reaction period. Activity toward these probes was observed only after 72 h (data not shown); however, the conversions were below 10%, and these biocatalysts were not considered any further.

Monooxygenase screening using fluorogenic probes 7–10 indicated that among the 56 strains under evaluation, 11 were active (Table 1). Strains SO5-2, -18, SO6-12 and SO7-2 catalyzed the conversion of cyclic ketone 7 into 13. Strains SO5-1, -4, -5 and SO6-2 catalyzed the oxidation of 8 to lactone 14, and strains SO5-2, -5, -7, -9 and -10 oxidized ketone 9 to ester 15. None of the strains oxidized probe 10.

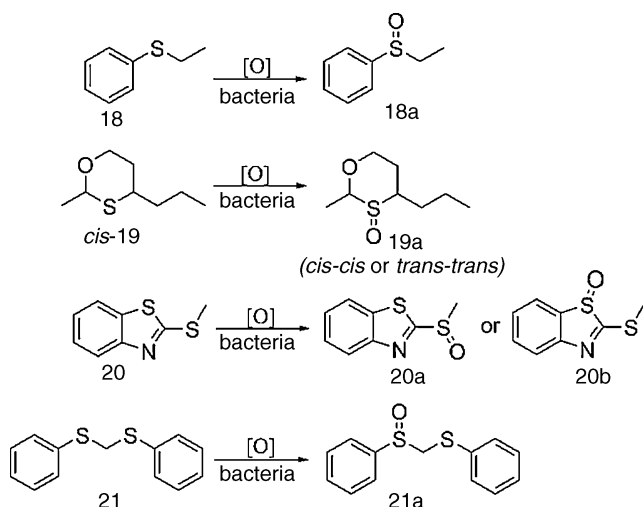
#### Biocatalytic sulfide oxidation by multibioreaction assays

Multibioreaction experiments (Table 2) revealed that bacteria isolated from a neutral copper mine drainage were readily able to oxidize organic sulfur compounds to their respective sulfoxides (Scheme 2). All tested strains were able to catalyze the oxidation of at least one of the substrates. Eight strains oxidized 18, and three strains oxidized 19 and 20; however, none of the strains oxidized 21. The best results were observed for strains SO5-4, SO5-9 and SO6-2, which were able to oxidize three of the sulfides tested (18, 19 and 20).

The enantioselectivities of the sulfide biooxidations catalyzed by SO5-4, SO5-9 and SO6-2 were assessed by chiral GC-FID, revealing low enantiomeric excesses (from 5 to 15%, data not shown). Nonetheless, oxidation of *cis*-19 was highly diastereoselective (over 99% de), providing only a racemic mixture of *trans-trans*-19a (2*S*,3*S*,4*S* and 2*R*,3*R*,4*R*, Scheme 3).

#### Isolation and identification of heterotrophic bacteria from mine environments

All of the evaluated copper mine drainage samples had a pH between 7 and 8. Thus, specific low pH culture media for isolation of acidophilic bacteria were not used. Heterotrophic bacteria (56 strains) were isolated from the Sossego copper



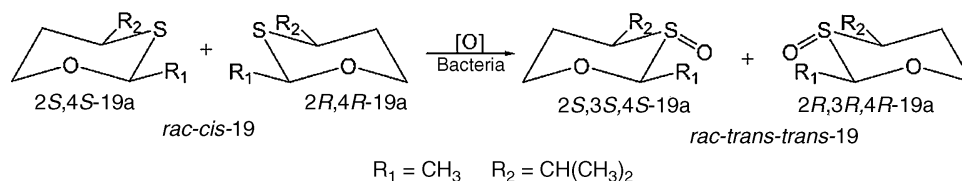
**Scheme 2 – Substrates (18–21) used for detection of organic sulfide biooxidation and their respective, expected products (18a–21a).**

**Table 3 – Identification of heterotrophic bacterial strains isolated from a neutral copper mine drainage by sequencing of the 16S rRNA gene.**

Strain ID	Sample	Identification	GenBank ID
SO5-1	SO5	<i>Stenotrophomonas maltophilia</i>	KC859435
SO5-2	SO5	<i>Pseudomonas</i> sp.	KC859436
SO5-4	SO5	<i>Bacillus megaterium</i>	KC859437
SO5-5	SO5	<i>Pseudomonas</i> sp.	KC859438
SO5-6	SO5	<i>Bacillus thuringiensis</i>	KC859439
SO5-7	SO5	<i>Bacillus</i> sp.	KC859440
SO5-9	SO5	<i>Pseudomonas</i> sp.	KC859441
SO5-10	SO5	<i>Pseudomonas</i> sp.	KC859442
SO5-13	SO5	<i>Bacillus thuringiensis</i>	KC859443
SO6-2	SO6	<i>Bacillus megaterium</i>	KC859444
SO6-12	SO6	<i>Bacillus megaterium</i>	KC859445
SO7-7	SO7	<i>Bacillus thuringiensis</i>	KC859446
SO7-10	SO7	<i>Bacillus cereus</i>	KC859447
SO7-15	SO7	<i>Bacillus cereus</i>	KC859448

mine drainage samples (18 strains were isolated from sample SO5, 23 from sample SO6 and 15 from sample SO7) using serial dilutions and PCA.

Fourteen strains with outstanding hydrolytic or oxidative activity (above 99% and 10%, respectively) were identified after partial sequencing of the 16S rRNA gene (Table 3, Fig. 1). Nine strains of *Bacillus* were identified as *B. thuringiensis*, *B. megaterium*, *B. cereus* and *Bacillus* sp. Other strains recovered were *Stenotrophomonas maltophilia* and *Pseudomonas* sp.



**Scheme 3 – Diastereoselective biooxidation of sulfide *rac-cis-19* catalyzed by bacteria SO5-4, SO5-9 and SO6-2.**

## Discussion

The 56 heterotrophic bacterial strains isolated from this neutral copper mine drainage were evaluated by fluorescence-based HTS enzymatic screening and displayed distinct enzymatic activity toward the selected probes. Esterases and monooxygenases were found in 75% and 20% of strains, respectively. Hydrolases and oxidoreductases are frequently employed in industrial biocatalyzed processes,<sup>22</sup> and the search for novel sources of these enzymes is essential for the development of new processes. The HTS results, therefore, indicate that the restricted environment of a copper mine drainage is a promising source of new biocatalysts.

The best oxidation results were obtained using strains isolated from sample SO5 (Table 1), which were further evaluated by multibioreaction assays assessing sulfide biooxidation.

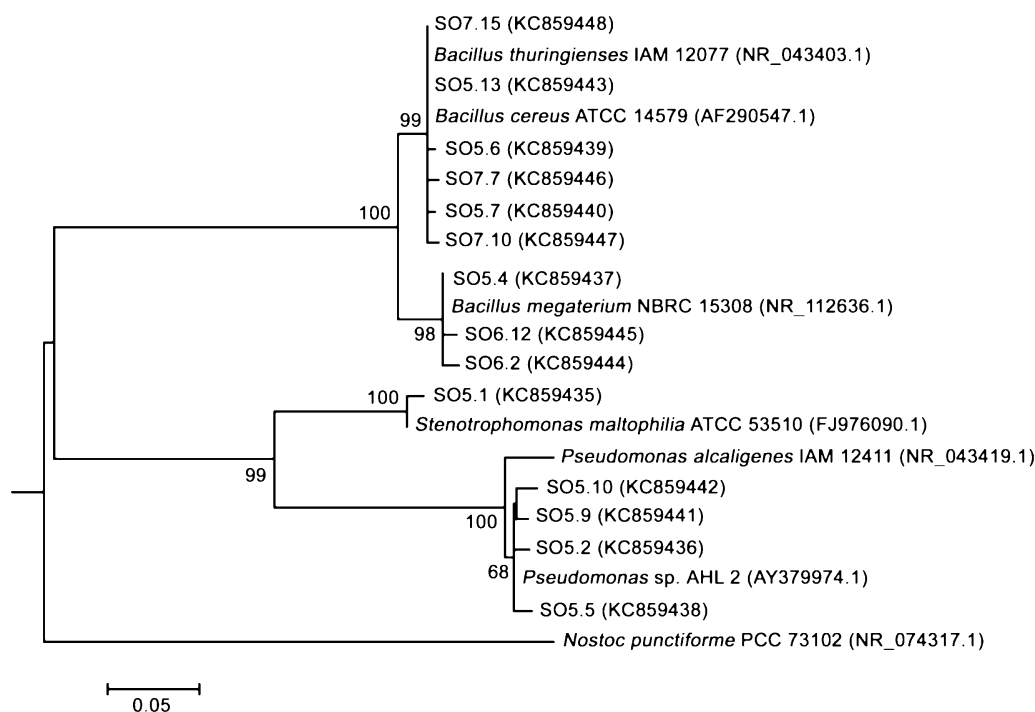
Reduced organic sulfur compounds, such as sulfides, were chosen as targets because of their presence in industrial aqueous wastes. These organic sulfides are often bad smelling and environmentally damaging. They are classified as air and water pollutants, and their bioremediation is an important ecological issue.<sup>23</sup>

Sulfur atom oxidation is the first bioremediation step, producing sulfoxides (monooxygenation) or sulfones (dioxygenation), which are less volatile and have a less unpleasant odor. Furthermore, sulfoxides are industrially relevant compounds that have potential as drugs and are usually applied as precursors of aromas and flavors and used in asymmetric organic syntheses.<sup>24</sup>

Compound 18 is usually employed to detect microbial sulfide oxidation and, in this study, was oxidized by all screened bacteria. Sulfide 19 is a common, fruity odor constituent and was oxidized to sulfoxide 19a by bacteria SO5-4, SO5-9 and SO6-2, which resulted in a change from its original odor to a new aroma valued by fragrance industries.<sup>25</sup> Furthermore, oxidation of *rac-cis-19* provided only the *rac-trans-trans-19a* (Scheme 3), with over 99% of diastereomeric excess toward *rac-cis-cis-19a*, which is chemically expected as both enantiomers have all substituents at equatorial positions. Oxidation of sulfide 20 was also accomplished by SO5-4, SO5-9 and SO6-2, providing sulfoxide *rac-20a* instead of *rac-20b* (Scheme 2) because the lone pair of the external sulfur atom of 20 is not part of the aromatic benzothiazole system.

These results are promising and indicate that biooxidation/bioremediation of organic sulfides can be performed by bacteria isolated from the restricted environment of neutral copper mine drainages.

Most isolated bacteria with outstanding enzymatic activities were identified as belonging to the *Bacillus* genus, which is consistent with the widespread distribution of *Bacillus* spp.,



**Fig. 1 – Phylogenetic analysis based on partial 16 rRNA gene sequences of the identified bacterial strains and related microorganisms. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model. The numbers at nodes indicate bootstrap percent values from the Maximum Likelihood analysis, based on 1000 resampled data sets. Only significant bootstrap values greater than 65% are shown near each node. Bars represent sequence divergence, and the sequence accession numbers are indicated in parenthesis. *Nostoc punctiforme* was used as the outgroup.**

including in mining environments.<sup>26–28</sup> *Bacillus* species have displayed broad catabolic abilities and are the major industrial hydrolase producers, responsible for approximately 50% of the total enzyme market production.<sup>29</sup>

The other genera recovered were *Stenotrophomonas* and *Pseudomonas*, identified, respectively, as *S. maltophilia* and *Pseudomonas* sp. Both *Stenotrophomonas* and *Pseudomonas* have been previously isolated from heavy metal-contaminated soils and other environmental mining samples.<sup>30–33</sup> Bacterial species from the *Stenotrophomonas* and *Pseudomonas* genera are well known for their metabolic diversity, multidrug resistance and ability to degrade a wide range of compounds, including pollutants.<sup>34–36</sup>

In this work, the *Bacillus* strains (SO5-6, SO5-13, SO6-12, SO7-7, SO7-10 and SO7-15) displayed good hydrolase activity (Tables 1 and 3). These strains, as well as the strains identified as *Pseudomonas*, presented a moderate level of activity for monooxygenases (Tables 1 and 3).

The best biocatalytic performance for sulfide oxidation was accomplished by *Pseudomonas* sp. (SO5-9), followed by two *Bacillus megaterium* strains (SO5-4 and SO6-2) (Tables 2 and 3). Mahmood et al. (2009)<sup>37</sup> isolated a strain of *Pseudomonas stutzeri* that was characterized as a chemolithoautotrophic sulfide-oxidizing and nitrite-reducing strain from the sludge of an anoxic sulfide-oxidizing reactor. Lee et al. (2012)<sup>38</sup> identified a *Pseudomonas* sp. strain isolated from a sludge reactor that could successfully convert sulfide to elementary sulfur and

thus demonstrated that sulfide oxidation could be applied to electricity generation from sulfide-containing industrial effluent. Therefore, our results indicate the mine strains' potential application as biocatalysts in organic syntheses and bioremediation, corroborating previous data that demonstrated the use of *Pseudomonas* and *Bacillus* in industry.

In conclusion, this study provides a novel overview of the heterotrophic bacteria from a copper mine drainage, which might be useful for future investigations of industrial processes, enzyme expression and bioremediation of contaminated environments. Furthermore, the obtained results will support further monitoring of the microbiological processes occurring in this type of restricted environment.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank Pablo dos Santos Pina for the samples from the Sossego mine. We also thank Companhia Ambiental do Estado de São Paulo (CETESB) for the isolation of the bacterial strains, especially Ana Paula G. Christ and Nancy de Castro Stoppe.

## REFERENCES

- ICSG – The International Copper Study Group. *The World Copper Factbook 2013*. Lisbon: ICSG; 2013.
- Blamey A. Brazil's Vale targets 289,000 mt nickel, 405,000 mt copper in 2014. Platts; 2013. Available at: <http://www.platts.com/latest-news/metals/london/brazils-vale-targets-289000-mt-nickel-405000-26507146>. Accessed 28.01.14.
- Johnson DB. Chemical and microbiological characteristics of mineral spoils and drainage waters at abandoned coal and metal mines. *Water Air Soil Pollut.* 2003;3:47–66.
- Faltesek L, Čepička I. Microbiology of diverse acidic and non-acidic microhabitats within a sulfidic ore mine. *Extremophiles.* 2012;16:911–922.
- Watling HR, Watkin ELJ, Ralph DE. The resilience and versatility of acidophiles that contribute to the bio-assisted extraction of metals from mineral sulphides. *Environ Technol.* 2010;31:915–933.
- Hallberg KB. New perspectives in acid mine drainage microbiology. *Hydrometallurgy.* 2010;104:448–453.
- Mantovani SM, Oliveira LG, Marsaioli AJ. Whole cell quick-E for epoxide hydrolase screening using fluorescent probes. *J Mol Catal B: Enzym.* 2008;52–53:173–177.
- Cruz GF, Angolini CFF, Oliveira LG, et al. Searching for monooxygenases and hydrolases in bacteria from an extreme environment. *Appl Microbiol Biotechnol.* 2010;87:319–329.
- Silva CP. *Enzymatic Potential of the Human Skin Microbiota and its Effects on Fragrance Ingredients*. University of Campinas; 2012 [Ph.D. thesis].
- Gonçalves RAC, Porto ALM, Pinheiro L, Cagnon JR, Manfio GP, Marsaioli AJ. Multibioreaction methodology for Baeyer-Villiger monooxygenase monitoring. *Food Technol Biotechnol.* 2004;42:355–361.
- Sicard R, Chen LS, Marsaioli AJ, Reymond J-L. A fluorescence-based assay for Baeyer-Villiger monooxygenases, hydroxylases and lactonases. *Adv Synth Catal.* 2005;245:1041–1050.
- Bicalho B, Chen LS, Grognum J, Reymond JL, Marsaioli AJ. Studies on whole cell fluorescence-based screening for epoxide hydrolases and Baeyer-Villiger monooxygenases. *J Braz Chem Soc.* 2004;15:911–916.
- Porto ALM, Cassiola F, Dias SLP, et al. *Aspergillus terreus* CCT 3320 immobilized on chrysolite or cellulose/TiO<sub>2</sub> for sulfide oxidation. *J Mol Catal B: Enzym.* 2002;19–20:327–334.
- Eaton AD, Franson MH. *Standard Methods for the Examination of Water and Wastewater*. 21st ed. Washington, DC: American Public Health Association; 2005.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
- Sørensen SPL. Enzymstudien. II. Mitteilung. Über die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. *Biochem Z.* 1909;21:131–304.
- Miqueletto PB, Andreote FD, Dias ACF, Ferreira JC, Dos Santos Neto EV, Oliveira VM. Cultivation-independent methods applied to the microbial prospection of oil and gas in soil from a sedimentary basin in Brazil. *AMB Express.* 2011;1:1–16.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994;22:4673–4680, <http://dx.doi.org/10.1093/nar/22.22.4673>.
- Tamura K, Stecher G, Peterson D, Filipksi A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol.* 2013;30:2725–2729.
- Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* 1980;16:111–120.
- Yang Z. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J Mol Evol.* 1994;39:306–314.
- Kirk O, Borchert TV, Fuglsang CC. Industrial enzyme applications. *Curr Opin Biotechnol.* 2002;13:345–351.
- Habibi MH, Vosoughian H. Photocatalytic degradation of some organic sulfides as environmental pollutants using titanium dioxide suspension. *J Photochem Photobiol A: Chem.* 2005;174:45–52.
- Bentley R. Role of sulfur chirality in the chemical processes of biology. *Chem Soc Rev.* 2005;34:609–624.
- Bentley R. The nose as a stereochemist. Enantiomers and odor. *Chem Rev.* 2006;106:4099–40112.
- Selenska-Pobell S, Flemming K, Tzvetkova T, Raff J, Schnorpfeil M, Geibler A. Bacterial communities in uranium mining waste piles and their interaction with heavy metals. In: Merkel BJ, Planer-Friedrich B, Wolkersdorfer, eds. *Uranium in the Aquatic Environment*. Berlin: Springer-Verlag; 2002.
- Freitas DB, Reis M, Lima-Bittencourt CI, et al. Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant waste. *BMC Res Notes.* 2008;1:92.
- Dhanjal S, Cameotra SS. Aerobic biogenesis of selenium nanospheres by *Bacillus cereus* isolated from coalmine soil. *Microb Cell Fact.* 2010;9:52–62.
- Schallmeyer M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol.* 2004;50:1–17.
- Freitas DB, Lima-Bittencourt CI, Reis MP, et al. Molecular characterization of early colonizer bacteria from wastes in a steel plant. *Lett Appl Microbiol.* 2008;47:241–249.
- Morel MA, Ubalde MC, Oliveira-Bravo S, Callejas C, Gill PR, Castro-Sowinski SC. Cellular and biochemical response to Cr (VI) in *Stenotrophomonas* sp. *FEMS Microbiol Lett.* 2009;291:162–168.
- Nangia Y, Wangoo N, Goyal N, Shekhawat G, Suri CR. A novel bacterial isolate *Stenotrophomonas maltophilia* as living factory for synthesis of gold nanoparticles. *Microb Cell Fact.* 2009;8:39–46.
- Choudhary S, Sar P. Uranium biomineralization by a metal resistant *Pseudomonas aeruginosa* strain isolated from contaminated mine waste. *J Hazard Mater.* 2011;186:336–343.
- Samanta SK, Singh OV, Jain RK. Polycyclic 420 aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotechnol.* 2002;20:243–248.
- Puchałka J, Oberhardt MA, Godinho M, et al. Genome-scale reconstruction and analysis of the *Pseudomonas putida* KT2440 metabolic network facilitates applications in biotechnology. *PLoS Comput Biol.* 2008;4:e1000210.
- Ryan RP, Monchy S, Cardinale M, et al. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol.* 2009;7:514–525.
- Mahmood Q, Zheng P, Hu B, et al. Isolation and characterization of *Pseudomonas stutzeri* QZ1 from an anoxic sulfide-oxidizing bioreactor. *Anaerobe.* 2009;15:108–115.
- Lee CY, Ho KL, Lee DJ, Su A, Chang JS. Electricity harvest from nitrate/sulfide containing wastewaters using microbial fuel cell with autotrophic denitrifier, *Pseudomonas* sp. C27. *Int J Hydrogen Energy.* 2012;37:15827–15832.