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Short Communication

Expanding the toolbox for enantioselective sulfide oxidations: *Streptomyces* strains as biocatalysts

Maria Laura Mascotti [a], Martin Alejandro Palazzolo [b], Elizabeth Lewkowicz [b], Marcela Kurina-Sanz [a,*]

[a] INTEQUI-CONICET, Facultad de Química Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, CP 5700 San Luis, Argentina
[b] Laboratorio de Biocatálisis y Biotransformaciones, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, CP 1876 Bernal- Buenos Aires, Argentina

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ABSTRACT

Sulfide oxidase activity of *Streptomyces* genus is reported here for the first time. Three *Streptomyces* strains were selected as new tools to access to enantioenriched *R*- and *S*-dialkyl sulfoxides. The bacterial screening was carried out using cyclohexyl methyl sulfide as model substrate. Both sulfoxide antipodes were obtained in a one-pot, time-dependent biotransformation employing *Streptomyces phaeochromogenes* NCIMB 11741 as biocatalyst. *Streptomyces flavogriseus* ATCC 33331produced mainly the *S*-enantiomer, while *Streptomyces hiroshimensis* ATCC 27429 yielded enantiopure *R*-cyclohexyl methyl sulfoxide.

1. Introduction

Enantiomerically pure sulfoxides are valuable organic compounds which are extensively used as building blocks in the production of bioactive compounds (Wojaczynska and Wojaczynski, 2010). Among the approaches to obtain optically active sulfoxides, the oxidation of prochiral sulfides has become a very useful strategy (Holland, 2001). Particularly, enzymatic oxidation is one of the most direct and versatile strategies to achieve this goal. A wide variety of sulfide-oxidizing enzymes has been described in recent years, with special focus on Baeyer–Villiger monooxygenases (Rioz-Martínez et al., 2010; Mascotti et al., 2013), peroxidases (Colonna et al., 1990), flavin monooxigenases (Rioz-Martinez et al., 2011) and dioxygenases (Boyd et al., 2004). Although their use as biocatalysts in large-scale processes is hampered because of the low stability of the enzymes under reaction conditions and the necessity of expensive cofactors, the utilization of microbial whole-cells might overcome these issues. Hence, several microorganisms have been successfully employed for the asymmetric oxidation of aromatic sulfides, including fungi, bacteria and yeasts (Adam et al., 2005; Pinedo-Rivilla et al., 2007; Mascotti et al., 2012; Elkin et al., 2013). Nevertheless, the discovery of novel wild-type whole-cell biocatalysts capable of performing the oxidation of dialkyl sulfides with high enantioselectivity is still a challenge. In the context of our research topic, we have conducted a bacterial screening using cyclohexyl methyl sulfide as model substrate in order to find novel biocatalysts capable of oxidizing dialkyl sulfides with high stereoselectivity. Subsequently we studied the biocatalytic performance of the selected microorganisms which showed promising sulfide oxidase activity.

2. Materials and methods

2.1. Chemicals and microorganisms

Cyclohexyl methyl sulfide was purchased from Alfa Aesar. *rac*-Cyclohexyl methyl sulfoxide and cyclohexyl methyl sulfone were prepared by chemical oxidation of the corresponding sulfide and exhibited physical and spectral properties in agreement with those previously reported (Holland et al., 1994).

Culture media components were obtained from Merck, Difco and Britania, and solvents from Merck, Sintorgan and Biopack. Microorganisms were kindly supplied by the Colección Española de Cultivos Tipo (CECT), Universidad de Valencia (Spain).

2.2. Growth conditions

Strains were cultured in liquid media, in an orbital shaker (200 rpm) for 48 h at optimal temperature and medium according to the American Type Culture Collection (ATCC), as follows: *Streptomyces* (28 °C) in *Streptomyces* medium, *Pseudomonas* (26 °C), *Arthrobacter* (28 °C), *Aeromonas hydrophila* (30 °C) in nutrient broth II, *Brevibacterium* (30 °C) in *Corynebacterium* medium; *Citrobacter koseri* (37 °C); *Xanthomonas traslucens* (26 °C); *Bacillus*

* Corresponding author. Tel.: +54 266 4423789 114; fax: +54 2664 439909.
E-mail address: marcelakurina@gmail.com (M. Kurina-Sanz).

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cereus (30 °C) and *Bacillus stearothermophilus* (55 °C) in nutrient broth I; *Bacillus thermoglucosidasius* (55 °C) in *Bacillus thermoglucosidasius* medium, *Alicyclobacillus acidocaldarius* (55 °C) in *Alicyclobacillus acidocaldarius* medium. The saturated culture broths (stationary phase, $OD_{600}=3.9$) were centrifuged (3,500 rpm, 20 min). Cell pellets were washed with 0.1 M sodium phosphate buffer pH 6.0. After centrifugation (10,000 rpm, 3 min) pellets were directly used as wet whole-cell biocatalysts.

2.3. General procedure for biotransformation

Assays consisted on 2 mL final volume of 0.1 M sodium phosphate buffer pH 6.0, 20 mM cyclohexyl methyl sulfide and 75 $mg.mL^{-1}$ of wet weight biocatalyst (corresponding to 8 mg of dry weight for *S. hiroshimensis* ATCC 27429) in 8 mL flasks. Mixtures were stirred at 28 °C for 48 h. Samples were taken at 0, 18, 24 and 48 h for screening assays, and at 0, 6, 12, 20, 24, 30, 36 and 48 h for time-course experiments. Samples were centrifuged (10,000 rpm, 3 min) and supernatants were extracted with ethyl acetate and analyzed by GC-FID. Screening assays and time-course experiments were repeated three times.

When sulfoxide overoxidation was assessed, racemic cyclohexyl methyl sulfoxide (20 mM) was employed as substrate. For the evaluation of cosolvents, cyclohexyl methyl sulfide (20 mM) was dissolved in 2% ($v.v^{-1}$) of IPA or DMSO. Biotransformations were carried out in 10 mL final volume.

2.4. Analytical methods

GC-FID analyses were performed on a Perkin Elmer-Clarus 500 instrument. Conversion was determined using a 007 methyl 5% phenyl silicone column. Temperature setting: 100 °C hold 2 min, 2 °C min^{-1} to 140 °C, hold 2 min. Injector: 200 °C; carrier gas N_2: 25 cm seg^{-1}; FID: 300 °C. Retention times: cyclohexyl methyl sulfide 5.29 min, cyclohexyl methyl sulfoxide 16.20 min and cyclohexyl methyl sulfone 19.15 min. Optical purity was determined with a β-DEX-column. Temperature setting: 140 °C, 0.5 °C min^{-1} to 150 °C, hold 10 min, 0.5 °C $min^{-1}$to 160 °C, hold 10 min. Injector: 200 °C, carrier gas N_2: 25 cm seg^{-1}; FID: 300 °C. Retention times: *S*-sulfoxide 50.1 min, *R*-sulfoxide 50.9 min. Absolute configuration of enantiomers was assigned using analytical pure samples.

3. Results and discussion

The bacteria genera screened were chosen on the basis of their known ability to catalyze several oxidative biotransformations (Adam et al., 2004; Médici et al., 2011; Linares-Pastén et al., 2012). Therefore, strains of *Streptomyces*, *Pseudomonas*, *Citrobacter*, *Arthrobacter*, *Bacillus*, *Alicyclobacillus*, *Aeromonas*, *Brevibacterium* and *Xanthomonas* were initially tested as biocatalysts to perform the oxidation of the model sulfide. Out of the thirty one screened microorganisms, *Pseudomonas putida*, *Pseudomonas stutzeri* and *Citrobacter koseri* showed poor conversion of the substrate, whereas *Bacillus cereus*, *Streptomyces badius*, *S. hiroshimensis*, *S. phaerocromogenes* and *S. flavogriseus* produced significant amounts (> 25%) of the sulfoxide (Table 1).

In a second stage, the stereoselectivity of the microorganisms showing positive sulfide oxidase activity was analyzed. *Bacillus cereus* and *Streptomyces badius* gave a racemic mixture. The three *Streptomyces* strains, namely *S. hiroshimensis* ATCC 27429, *S. flavogriseus* ATCC 33331 and *S. phaeochromogenes* NCIMB 11741, performed the sulfoxidation reaction with different enantioselectivities (Fig. 1). It should be highlighted that *S. hiroshimensis* and *S. flavogriseus* oxidized the substrate to the *R*- and *S*-sulfoxide,

Table 1
Sulfide oxidase activity screening.

Strain	*Conversion*
Aeromonas hydrophila ATCC 7966	–
Alicyclobacillus acidocaldarius ATCC 27009	–
*Arthrobacter oxydans*ATCC15359	–
Arthrobacter oxydans ATCC 14358	–
Bacillus cereus ATCC 9634	++
Bacillus stearothermophilus ATCC 12980	–
Bacillus thermoglucosidasius ATCC 43742	–
Brevibacterium helvolum ATCC 19239	–
Brevibacterium linens ATCC 9172	–
Brevibacterium linens ATCC 9175	–
Citrobacter koseri ATCC 27156	+
Pseudomonas putida ATCC 12633	+
Pseudomonas syringae ATCC 10862	–
Pseudomonas stuzeri ATCC 17588	+
Streptomyces sp. ATCC 11238	–
Streptomyces sp. ATCC 27448	–
Streptomyces badius ATCC 39117	++
Streptomyces baldaccii ATCC 23615	–
Streptomyces hiroshimensis ATCC 27429	+++
Streptomyces blastmyceticus ATCC 19731	–
Streptomyces cattleya ATCC 35852	–
*Streptomyces flavogriseus*ATCC 33331	+++
Streptomyces fradiae ATCC 21096	–
Streptomyces griseostramineus ATCC 19768	–
*Streptomyces griseus*ATCC10137	–
Streptomyces halstedii ATCC 10897	–
Streptomyces mobaraensis ATCC 27441	–
Streptomyces netropsis DSMZ 10846	–
Streptomyces phaeochromogenes NCIMB 11741	+++
Streptomyces setonii ATCC 39116	–
Xanthomonas traslucens ATCC 19319	–

Conversion is expressed as –, +, ++, +++, corresponding to ranges < 5%, 5–25%, 25–50% and > 50%, respectively. Samples were analyzed by GC-FID.

respectively, with no overoxidation to the sulfone. Notably, the substrate concentration tolerance was found to be higher than the already reported for whole-cell sulfoxidation procedures (Kelly et al., 1996). It is known that *Streptomyces* catalyzes aromatic hydroxylations (Gopishetty et al., 2007), *O*- and *N*-dealkylations (Niraula et al., 2011), amidations of carbonyl moieties (Bright et al., 2011), alcohol oxidations (Liu et al., 2006), glycosylations (Marvalin and Azerad, 2011) and hydrolysis of epoxides (Zocher et al., 2000) and esters (Molinari et al., 2005). Hence, the sulfide-oxidase activity of this genusis described herein for the first time.

To assess the biocatalytic performance of the selected microorganisms, time-course experiments were performed. It was found that *S. phaeochromogenes* showed a complex biotransformation profile since the stereoselectivity was inverted throughout the process (Fig. 2-A). The substrate was initially oxidized to the *S*-sulfoxide with an excellent enantioselectivity ($ee > 99\%$). Later, the *ee* noticeably dropped ($ee = 31\%$), revealing an evident oxidation to the *R*-isomer. After this point, the reaction progressed favoring the *R*-enantiomer accumulation. Finally, at 48 h an *ee* of 55% was achieved with a good conversion ($c = 55\%$). This fact might be explained by the occurrence of at least two simultaneously-acting enzymes that reached their highest rates at different times. Moreover, these may be expressed at different moments or reach their highest activity under diverse metabolic conditions. Since the formation of the sulfone was evidenced, a subsequent biotransformation employing *rac*-cyclohexyl methyl sulfoxide as substrate was performed to determine the contribution of this reaction to the sulfoxide optical purity (Table 2). Only a small amount of the racemic substrate was oxidized to the sulfone and there was no change in the enantiomeric ratio of both stereoisomers in the remaining substrate. As a consequence, it was confirmed that this specific reaction did not contribute to the final *ee* of the *R*-sulfoxide.

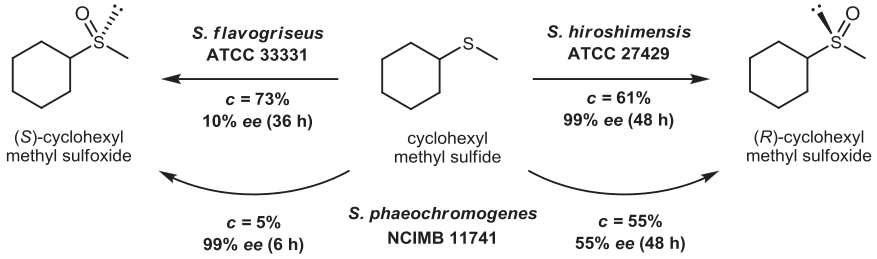

Fig. 1. Biotransformation of cyclohexyl methyl sulfide catalyzed by the selected *Streptomyces* strains.

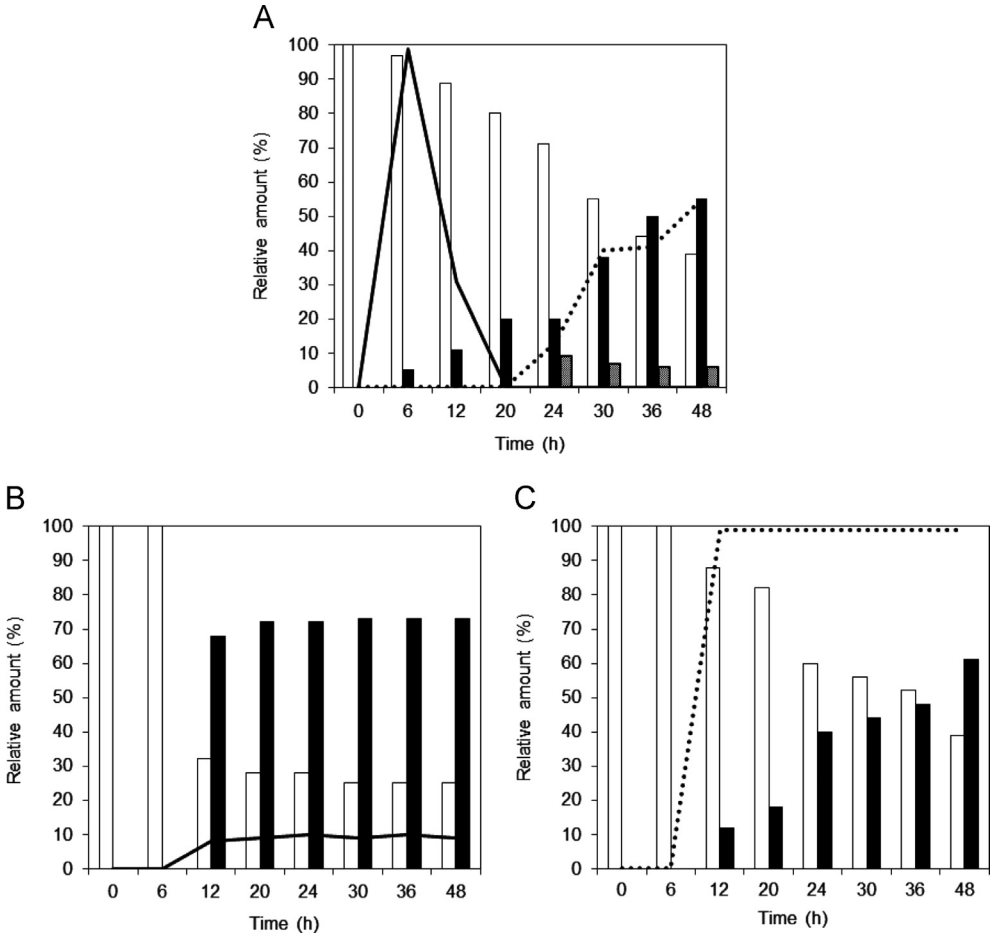

Fig. 2. Time-course biotransformation profiles. Graphs A, B and C correspond to *S. phaeochromogenes* NCIMB 11741, *S. flavogriseus* ATCC 33331 and *S. hiroshimensis* ATCC 27429, respectively. (White bars: sulfide, black bars: sulfoxide, grey bars: sulfone, continuous lines: *S*-sulfoxide *ee*, dashed lines: *R*-sulfoxide *ee*).

Table 2
Biotransformation of *rac*-cyclohexyl methyl sulfoxide by *S. phaeochromogenes* NCIMB 11741.

Time (*h*)	*Sulfoxide* (%)	*Sulfone* (%)	*Sulfide* (%)	*Configuration and ee* (%)
0	100	0	0	*Racemic mixture*
24	90	10	0	*Racemic mixture*
48	90	10	0	*Racemic mixture*

When *S. flavogriseus* was used as biocatalyst, the maximal sulfide conversion (c=73%) was achieved in 20 h. Although the optical purity of the product was poor, this strain was the only one which showed a constant *S* enantiopreference (Fig. 2-B).

Finally, the assays carried out with *S. hiroshimensis* led to the production of enantiopure *R*-sulfoxide ($ee > 99\%$) with good conversion (c=61%) (Fig. 2-C). Considering that many of the processes described for the asymmetric oxidation of dialkyl sulfides deal with engineered bacteria or mutant enzymes (Kayser, 2009; Rioz-Martinez et al., 2011), this result should be highlighted due to an enantioselective transformation is successfully achieved by using a wild-type ready-available microorganism.

It is known that the use of organic solvents might improve the biotransformation of xenobiotic substrates in aqueous media (Zheng et al., 2012). In the attempt of improving the substrate conversion, further experiments with the addition of IPA and DMSO, on the basis of their recognition as green solvents (Sheldon, 2011), were run using *S. hiroshimensis* as biocatalyst. These procedures did not improve the results obtained under standard conditions, since lower conversions were observed. Moreover, the addition of IPA resulted in a drop of the *ee* (Table 3). In order to explain this, we assumed an evident, but unexpected, inactivation of the enzyme/s involved in the transformation. It has been reported that DMSO can decrease or inhibit the enzymatic activity, as in the case of peroxidases and catalases

Table 3
Cosolvent effect in the oxidation of cyclohexyl methyl sulfide catalyzed by *S. hiroshimensis* ATCC 27429.

Cosolvent	*Conversion* (%)	*Configuration and ee* (%)
DMSO	18	(*R*) > 99
IPA	30	(*R*) 66
None*	60	(*R*) > 99

Biotransformations were conducted with the addition of 2% ($v.v^{-1}$) DMSO or IPA during 48 h. Whole reaction volumes were extracted and analyzed by GC-FID.

* Standard conditions.

(Rammler, 1967). Besides, some BVMOs may exhibit lower biotransformation yields and enantioselectivities when IPA is added (de Gonzalo et al., 2006). This solvent may also trigger cofactor-regenerating systems, thus activating other enzymes which might affect the overall *ee*.

4. Conclusions

The ability of the *Streptomyces* genus of performing sulfooxidations has been demonstrated for the first time. Three wild-type *Streptomyces* strains capable of oxidizing cyclohexyl methyl sulfide in an enantiocomplementary fashion as whole-cell biocatalysts were discovered. These biocatalytic tools allowed the access to the desire optically-enriched sulfoxide by simple, mild and green processes, since moderate to good conversions and excellent stereo- and chemoselectivities were achieved. The further optimization of the reaction parameters may contribute to improve the productivity and envision large-scale applications.

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