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RESEARCH ARTICLE

Sulphoxidation reactions catalysed by the Baeyer-Villiger monooxygenase OTEMO from *Pseudomonas putida* ATCC 17453

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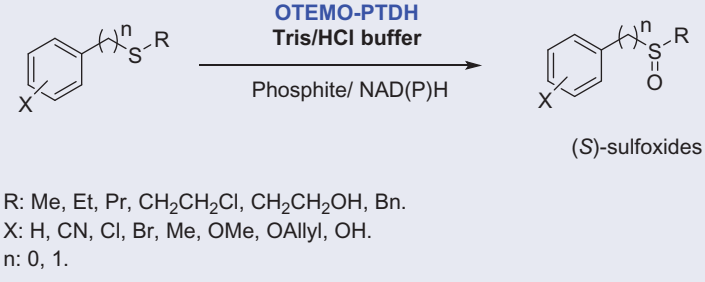
ABSTRACT

The Baeyer-Villiger monooxygenase OTEMO from *Pseudomonas putida* ATCC 17453 has been employed as biocatalyst in the asymmetric synthesis of a set of optically active sulphoxides. Several alkyl aryl sulphides are oxidized by this biocatalyst leading to the (*S*)-sulphoxides. Especially for those substrates containing electron-donating groups in the aromatic ring or in the alkyl moiety, good to high enantiopurities can be obtained. OTEMO is also able to perform the kinetic resolution of racemic sulphoxides, but with low enantioselectivities. Finally, parameters that can affect its biocatalytic properties, such as pH, temperature, organic cosolvents and substrate concentration, have been tested to get a better insight into the biocatalytic potential of this hitherto poorly explored oxidative biocatalyst.

GRAPHICAL ABSTRACT



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Biocatalysis; Baeyer-Villiger monooxygenases; sulphoxidations; kinetic resolution

1. Introduction

Optically active sulphoxides are very valuable building blocks in organic synthesis, as the chiral sulphinyl group is present in several APIs as well as other high value compounds (Bentley 2005). Due to their unique properties, sulphoxides have also been employed as chiral auxiliaries and ligands in asymmetric synthesis and enantioselective catalysis (Jia 2019; Salom-Roig and Bauder 2020). Among the different methodologies for the preparation of these compounds (Wojaczynska and Wojaczynski 2020), one of the most employed one is the selective oxidation of prochiral sulphides. The search for novel green oxidative methodologies (Domínguez de María 2021), including those for the preparation of chiral sulphoxides, has experienced a great development in the last years (Dong 2018). Biocatalytic procedures present some advantages when compared with more classical approaches that involve metal catalysts, such as the use of mild and environmentally friendly reaction conditions as well as non-hazardous reagents (Winkler 2021; Alcántara 2022). Different types of oxidoreductases have been successfully applied for the synthesis of chiral sulphoxides (Mączka et al. 2018), including monooxygenases (Rioz-Martínez 2010), dioxygenases (Boyd 2012), peroxidases (Gao 2015) and peroxygenases (Bassanini 2017). Among the monooxygenases, Baeyer-Villiger monooxygenases (BVMOs) have been widely applied in sulphoxidation procedures since the seminal works using cyclohexanone monooxygenase (Colonna 1996; Reetz 2004). BVMOs have been shown to be excellent

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biocatalysts in the preparation of valuable compounds such as omeprazole (Xu 2020), ceralasertib (Goundry 2017) and modafinil (Ang 2012) at industrial scale. These flavin-dependent enzymes require NADPH for their activity and are efficient in using dioxygen as oxidant. Except for regio- and enantioselective sulphoxidations, BVMOs are also able to catalyse oxygenations of other heteroatom-containing compounds (nitrogen, selenium and phosphorous) as well as the Baeyer-Villiger oxidation, usually with exquisite chemo-, regio- and/or enantioselelectivities (Fürst 2019; de Gonzalo & Alcántara 2021). A large number of BVMOs has been described in recent literature, but the substrate acceptance profiles have often been poorly explored (Fürst 2019).

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA 1,2-monooxygenase (OTEMO) from *Pseudomonas putida* ATCC 17453 is a type I NADPH-dependent FAD-containing BVMO (van Berkel 2006). This enzyme catalyses the lactonization of key intermediate in camphor metabolism, being described for the first time in 1993 (Grogan 1993). The biocatalyst has been recently cloned and overexpressed in *Escherichia coli* (Leisch 2012). A preliminary study on the biocatalytic performance of OTEMO revealed that it can be employed for the selective synthesis of chiral lactones starting from 4-substituted cyclohexanones, while it is also able to selectively oxidize bicyclic ketones. In 2017, OTEMO was employed as biocatalyst for the desymmetrisation of different cyclic and bicyclic ketones, including 3-vinylcyclobutanone, which led to enantiopure (*R*)-Taniguchi lactone, a valuable building block for the synthesis of natural product analogues (Rudroff 2017). In the present manuscript, the synthetic repertoire of this biocatalyst has been expanded by analysing the application of OTEMO in sulphoxidations of different prochiral sulphides.

2. Experimental section

2.1. Materials and methods

Purified PTDH-fused OTEMO was obtained from GECCO-Biotech (www.gecco-biotech.com). The PTDH-BVMO fusion enzyme was produced using the developed pCRE2 expression vector. This results in OTEMO being fused N-terminally to a robust phosphite dehydrogenase variant from *Pseudomonas stutzeri* (Torres Pazmiño, 2009). The two enzymes are fused via a flexible 6-amino acid linker (SRSAAG). Sodium phosphite dibasic pentahydrate, starting sulphides **1a**, **3a**, **15a**, **18a** and racemic sulphoxide (±)-**1 b** were products from Sigma-Aldrich. NADPH and starting compounds **5a**, **6a**, **9a** and **10a w**ere obtained from Alfa Aesar. Racemic sulphoxide (±)-**17 b** was purchased from Acros Organics. Compounds **2a**, **4a**, **7a**, **8a**, **11-13a** and **17a** were products from TCI Europe. The rest of racemic sulphoxides were prepared by oxidation of the corresponding sulphides employing hydrogen peroxide in methanol. Unless otherwise stated, analytical grade solvents and commercially available reagents were used without further purification.

GC/MS analyses were performed with a GC Hewlett Packard 7890 Series II equipped with a Hewlett Packard 5973 chromatograph MS (Agilent Technologies) using a HP-5MS cross-linked methyl siloxane column (30 m × 0.25 mm × 0.25 μm, 1.0 bar N_2). To monitor levels of conversion, substrates and products were quantified by using calibration curves. HPLC analyses were carried out with a Thermo-Fischer UltiMate chromatograph equipped with a Thermo UltiMate detector. To determine the level of conversion of the esomeprazole sulphide (**8a**) oxidation, a calibration curve using HPLC was employed. Absolute configuration of the chiral sulphoxides was established by comparing with the data described in bibliography (de Gonzalo 2005; Rioz-Martínez 2011).

2.2. Synthesis of 4-allyloxyphenyl methyl sulphide (16a)

To a solution of sulphide **18a** (500 mg, 3.6 mmol) in acetone (15 mL), allyl bromide (0.75 mL, 8.9 mmol) was added. After some minutes stirring at room temperature K_2CO_3 (937 mg, 7.2 mmol) was slowly added and the reaction was followed by TLC using *n*-hexane:EtOAc (1:2) as eluent. Once completed, water (15 mL) was poured, and the crude was extracted with CH_2Cl_2 (3 × 20 mL). Organic layers were dried onto Na_2SO_4 and solvent was evaporated to obtain **16a** (460 mg, 77% yield) as a colourless oil that was employed without any further purification. ^{1}H-NMR (300.13 MHz, $CDCl_3$) δ 7.19–7.08 (m, 2H), 6.80–6.67 (m, 2H), 5.94 (ddd, $J = 17.8$, 10.5 and 6.8 Hz, 1H),7.14–7.11 (m, 1H), 5.35 (dd, $J = 17.8$ and 2.8 Hz, 1H), 5.20 (dd, $J = 10.5$ and 2.8 Hz, 1H), 4.43 (d, $J = 6.8$ Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 157.2 (C_{ar}), 140.2 (CH), 133.1 (C_{ar}), 130.1 ($2CH_{ar}$), 117.7 (CH_2), 115.5 (CH_{ar}), 67.0 (CH_2), 18.0 (CH_3). HRMS: *m/z* calculated for $[C_9H_{10}OS]^+$: 166.0452; found: 166.0459.

2.3. General procedure for the OTEMO-catalysed sulphoxidation of sulphides 1–18a

Unless otherwise stated, prochiral sulphides **1**–**18a** (5–10 mM) were dissolved in 1.0 mL Tris/HCl 50 mM

(pH 8.0) containing DMSO (10 μL), NADPH (0.2 mM), sodium phosphite (10 mM) and OTEMO (2.0 μM). Reactions were stirred at the temperatures selected and 220 rpm for the times established. Once finished, the reactions were extracted with EtOAc (2 × 0.5 mL), dried onto Na_2SO_4 and the samples were directly analysed by GC/MS and HPLC to determine the level of conversion, the percentage of sulphones **1**–**18c**, as well as the enantiomeric excesses of sulphoxides (*S*)-**1**–**3b**, (*R*)-**4b** and (*S*)-**5-18b**.

2.4. General method for the OTEMO-catalysed kinetic resolution of racemic sulphoxides

To a solution of the racemic sulphoxides (±)-**1,2 b**, (±)-**12 b**, (±)-**15 b** or (±)-**17 b** (10 mM) in 1.0 mL Tris/HCl 50 mM pH 9.0 containing 10 μL DMSO, NADPH (0.2 mM), sodium phosphite (10 mM) and OTEMO (2.0 μM) were added. Reactions were stirred at 30 °C and 220 rpm for 20 h. Reactions were then extracted with EtOAc (2 × 0.5 mL), dried onto Na_2SO_4 and the samples were directly analysed by GC/MS and HPLC to determine the level of conversion as well as the enantiomeric excesses of sulphoxides (*S*)-**1,2b**, (*S*)-**12b**, (*S*)-**15b** or (*S*)-**17b**.

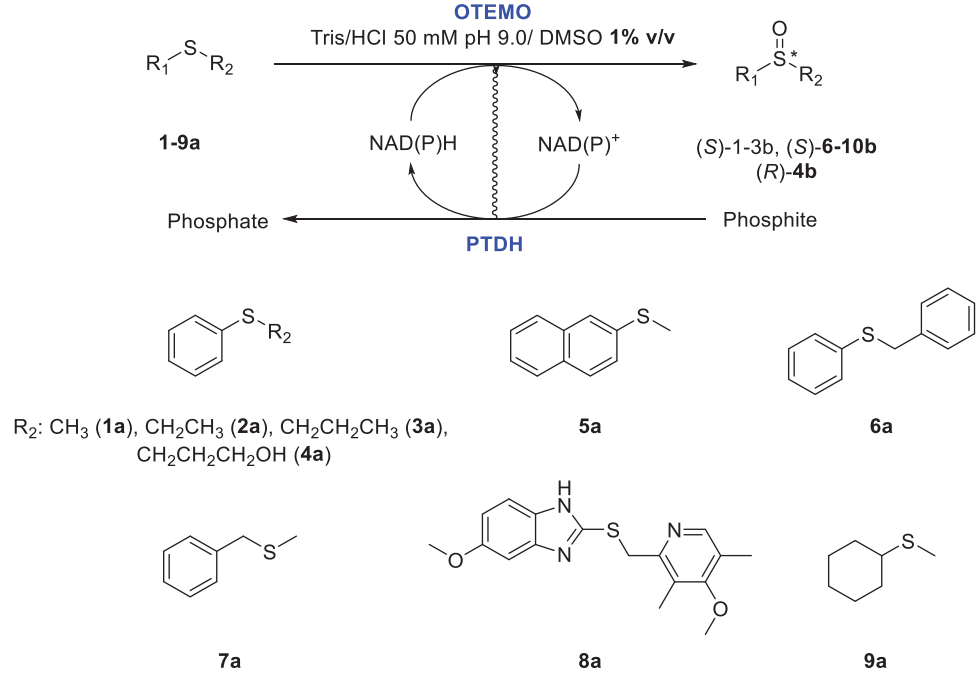
3. Results and discussion

Initial experiments were devoted to determining the substrate specificity of OTEMO concerning sulphides (Scheme 1). The biocatalyst was employed as a fusion PTDH-OTEMO protein in which the PTDH (phosphite dehydrogenase from *P. stutzeri*) supports cofactor regeneration at the expense of phosphite as cheap cosubstrate. Sulphoxidations were carried out incubating 10 mM prochiral starting sulphides with 2.0 μM OTEMO in buffer (Tris/HCl pH 9.0), which has previously shown to be the optimal buffer for the enzymatic activity (Leisch, 2012). Gratifyingly, 8 out of 9 tested sulphides were found to be converted (Table 1) at this biocatalyst concentration, as when employing 1.0 μM much lower conversions were afforded. All the produced sulphoxides were obtained with *S* configuration, except for compound **4 b**, for which the (*R*)-enantiomer was recovered due to a change in the

Table 1. Enzymatic sulphoxidation of prochiral sulphides **1–10a** catalysed by OTEMO.[a]

Entry	Sulphide	*t* (h)	Conv (%)[b]	% Sulphone[b]	*ee* (%)[c]	Config.
1	**1a**	16	80	10	63	*S*
2	**2a**	14	74	19	77	*S*
3	**3a**	14	73	17	70	*S*
4	**4a**	16	45	≤3	80	*R*[d]
5	**5a**	24	21	12	28	*S*
6	**6a**	40	13	≤3	23	*S*
7	**7a**	20	71	≤3	93	*S*
8[e]	**8a**	48	≤3	≤3	n.d.	n.d.
9	**9a**	24	48	≤3	39	*S*

[a]For reaction details, see Experimental Section.
[b]Determined by GC/MS.
[c]Determined by HPLC.
[d]Absolute configuration is reversed due to a change in the substituent priority according to the sequence rules.
[e]Sulphide concentration was 5 mM.



Scheme 1. OTEMO-catalysed sulphoxidation of prochiral sulphides **1**–**9a**.

substituent priority according to the CIP priority rules. Thioanisole (**1a**) was oxidized to (*S*)-methyl phenyl sulphoxide (**1 b**) with 80% conversion after 20 h and a moderate selectivity (*ee* = 63%, entry 1). It must be mentioned that some amount of the sulphone overoxidation product (**1c**) was observed (10%), indicating that this biocatalyst is also able to catalyse the (over)-oxidation of the sulphoxide moiety. Sulphoxidation of ethyl phenyl sulphide afforded (*S*)-**2b** with higher enantiopurity than the methyl derivative (entry 2), but a 19% of sulphone **2c** was recovered after 14 h. A substrate presenting a longer alkyl chain, as the *n*-propyl group (**3a**), was oxidized by OTEMO with a similar result to **2a**, as shown in entry 3. Phenyl propyl sulphoxide was recovered with 70% *ee* and 73% conversion, with 17% of sulphone **3c**. Oxidation of 2-(phenylthiol) ethanol (**4a**) afforded the (*R*)-sulphoxide with 45% conversion and good optical purity after 16 h (80% *ee*), with no sulphone **4c** formation. Bulkier sulphides were also accepted by OTEMO. Thus, methyl naphthyl sulphide (**5a**) was converted into (*S*)-**5b** with low conversion and enantiopurity (entry 5), whereas 13% of (*S*)-benzyl phenyl sulphoxide (**6 b**) was recovered after 40 h with *ee* = 27%, but a low conversion (entry 6). When the sulphur moiety was located further away from the aromatic ring, as for benzyl methyl sulphide, OTEMO showed a good performance. Thus, (*S*)-**7b** was achieved with an excellent enantiopurity (93% *ee*) and good conversion (71%), as shown in entry 8. OTEMO is not able to oxidize omeprazole sulphide (**8a**, 5 mM). After 40 h at 30 °C, no omeprazole formation was observed. An aliphatic substrate, cyclohexyl methyl sulphide (**9a**), was converted into (*S*)-**9b** with moderate results (entry 9). After 24 h, (*S*)-cyclohexyl methyl sulphoxide was recovered in 48% conversion and 39% *ee*.

It was observed that for some sulphides, the overoxidation sulphone product was obtained in amounts higher than 10% of the reaction conversion, thus reducing the amount of chiral sulphoxide obtained. Such overoxidation was studied in more detail with ethyl phenyl sulphide (**2a**), studying the time course of its OTEMO-catalysed sulphoxidation (Figure 1). Until 4 h, the reaction proceeds with the formation of 52% of the corresponding sulphoxide and a relatively low sulphone percentage (6% of **2c**). During the four following hours, the increase in the amount of sulphoxide is low, achieving a maximum value in the formation of (*S*)-**2b** (57.5%) whereas much more sulphone **2c** is obtained. This is especially clear after 14 h, where the main process catalysed by OTEMO is the oxidation of sulphoxide to the sulphone. After

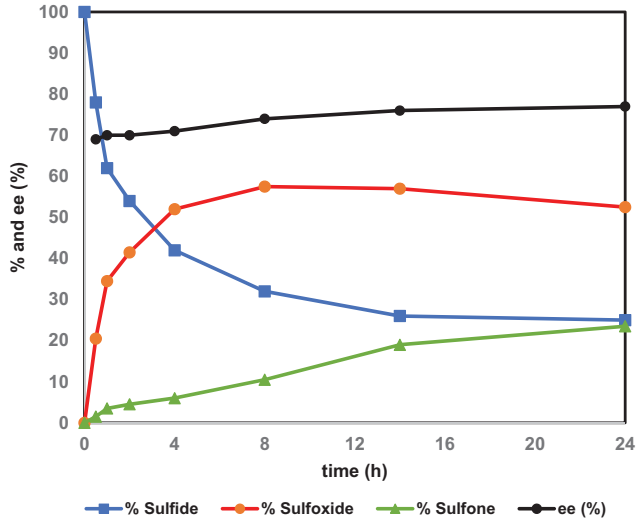


Figure 1. Time course in the biocatalysed oxidation of ethyl phenyl sulphide (**2a**) in presence of OTEMO.

Table 2. OTEMO-catalysed sulphoxidation of substituted methyl phenyl sulphides **10–18a**.[a]

Entry	Sulphide	X	Time (h)	Conv. (%)[b]	% Sulphone[b]	*ee* (%)[c]	Config.
1	**10a**	*p*-CN	16	37	5	47	*S*
2	**11a**	*m*-Cl	16	34	14	30	*S*
3	**12a**	*p*-Cl	16	40	15	53	*S*
4	**13a**	*o*-Cl	16	63	18	35	*S*
5	**14a**	*p*-Br	16	49	25	60	*S*
6	**15a**	*p*-CH_3	20	87	≤3	85	*S*
7	**16a**	*p*-OAllyl	20	70	≤3	91	*S*
8	**17a**	*p*-OCH_3	20	83	≤3	93	*S*
9	**18a**	*p*-OH	20	59	≤3	85	*S*

[a]For reaction details, see Experimental Section.
[b]Determined by GC/MS.
[c]Determined by HPLC.

24 h, only 52% of (*S*)-**2b** is recovered whereas a 29% of sulphone **2c** was observed. Regarding the enantiopurity of the chiral sulphoxide, it increased during the whole reaction time, even when the formation of the sulphone is the main process, indicating that OTEMO preferentially oxidizes the (*R*)-enantiomer of the sulphoxide thus increasing the enantiomeric excess of (*S*)-**2b**.

The effect of different substituents at the aromatic ring of thioanisole derivatives was also studied, as shown in Table 2. As a general trend, higher enantiomeric excesses were obtained in presence of electron-donating groups when compared with electron-withdrawing substituents. A correlation between the enantiomeric excesses of the (*S*)-sulphoxides and the Hammet parameter (σ) (Hansch 1991) was observed, as indicated by the negative slope observed in Figure 2. Thus, treatment of sulphide **10a**, presenting a cyano group, afforded the corresponding (*S*)-sulphoxide with moderate enantiopurity and 37% conversion after 16 h. OTEMO-catalysed sulphoxidation

of thioanisole derivative presenting halogen atoms at the aromatic rings afforded the corresponding (*S*)-sulphoxides with moderate conversions (34–63%) and enantiomeric excesses (30–60%). For the latter sulphides, some sulphone formation was observed (entries 2–5). For instance, the oxidation of **14a** leads to 25% of *p*-bromophenyl methyl sulphone (**14c**) after 24 h. There is no clear effect of the position of the substituents in the aromatic ring, as can be observed for the chlorine derivatives. A relatively high enantiopurity was achieved for the *para* position (entry 3, **12 b**), whereas the highest conversion was achieved with the *ortho*-derivative (*S*)-**13b**. (*S*)-*p*-Tolyl methyl sulphoxide (**15 b**) was achieved with high conversion (87% after 20 h) and good enantioselectivity (85% *ee*, entry 6). *O*-alkylated thioanisole derivatives seemed to be optimal compounds for OTEMO. Both methyl *p*-allyloxy (**16 b**) and *p*-methoxy (**17 b**) phenyl sulphoxides were recovered with good conversions and excellent enantiopurities (91% and 93% *ee*, respectively). *p*-Hydroxyphenyl methyl sulphide was also a good substrate for this biocatalyst in terms of enantioselectivity. Thus, (*S*)-**18b** was obtained with 85% *ee* and moderate conversion (59%), as shown in entry 9.

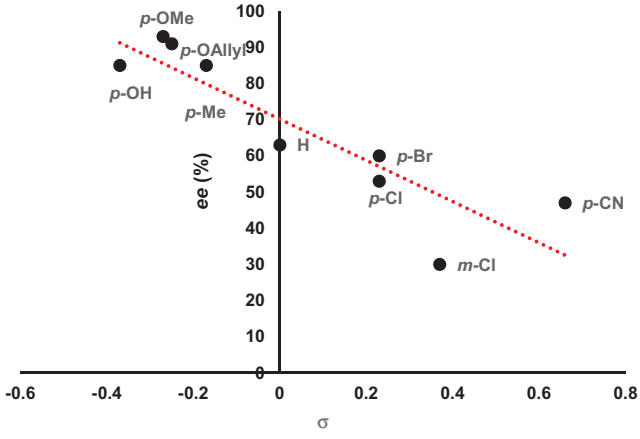


Figure 2. Plot between the enantiomeric excess of sulphoxides (*S*)-**10**–**18b** versus the Hammet parameter (σ) in the OTEMO-catalysed sulphoxidation of thioanisole derivatives catalysed by OTEMO.

3.1. Otemo-biocatalysed oxidation of racemic sulphoxides

As it was observed that OTEMO can catalyse the oxidation of sulphoxides to the corresponding sulphones, this process was studied in more detail by starting from racemic sulphoxides, analysing their kinetic resolution (Scheme 2). As shown in Table 3, a set of alkyl aryl sulphoxides presenting different groups at the aromatic moiety were oxidized in presence of OTEMO, leading in all cases to high conversions after 20 h, while recovering the unreacted *S* enantiomer of the corresponding sulphoxide with moderate enantiomeric excesses. These results indicate that the kinetic resolutions of sulphoxides by OTEMO occur with low enantioselectivities (*E* values around 3–5) (Chen, 1982). This shows that the selectivity achieved in the synthesis of the chiral sulphoxides was mainly due to the enantioselective oxidation of the prochiral sulphides.

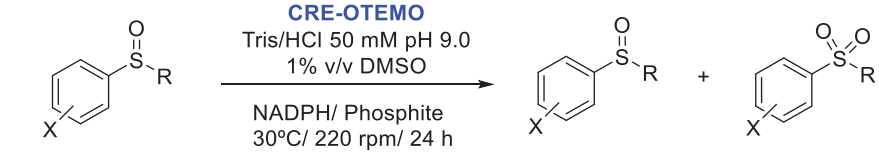
3.2. Parameters that affect to OTEMO biocatalytic properties

Water is the solvent in which most of the BVMO-catalysed transformations are performed. Yet, some of these flavoenzymes have shown to work in presence of organic or neoteric solvents, even with improved activities and/or selectivities in certain cases and with the possibility of increasing the process performance

Table 3. Kinetic resolution of racemic sulphoxides catalysed by OTEMO.[a]

Entry	Sulphoxide	*t* (h)	Conv (%)[b]	*ee* (%)[c]	Config.	*E*[d]
1	(±)-**1b**	20	64	54	*S*	3
2	(±)-**2b**	20	58	43	*S*	4
3	(±)-**12b**	20	66	32	*S*	3
4	(±)-**15b**	20	62	41	*S*	3
5	(±)-**17b**	20	53	51	*S*	5

[a]Reactions were carried out at 30 °C and pH 9.0 for 24 h. For other details, see Experimental Section.
[b]Determined by GC/MS.
[c]Determined by HPLC.
[d]Enantiomeric ratio (*E*) is calculated from the conversion and the enantiomeric excess of the remaining sulphoxide; $E=\ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ees)]$.



R: CH_3, CH_2CH_3
X: H, *p*-Cl, *p*-CH_3, *p*-OCH_3

Scheme 2. Kinetic resolution of racemic substituted alkyl phenyl sulphoxides catalysed by OTEMO.

by employing higher substrate concentrations (de Gonzalo 2017). Due to the results obtained in buffer alone with 1% v/v DMSO, thioanisole (**1a**) was selected as model substrate to check the effect of organic solvents presenting different physicochemical properties. Studies were initially developed with a low cosolvent concentration (5% v/v), to analyse their influence on OTEMO-biocatalysed sulphoxidations. As shown in the supplementary information (Table S1), for almost all the solvents there is a negative effect on the performance of OTEMO, as lower conversions and/or enantiopurities were obtained. Only when employing 5% v/v diethyl ether, (*S*)-**1b** was obtained with a similar enantiomeric excess (*ee* = 60%) and conversion (80%) when compared with buffer alone. The presence of this solvent has the advantage that no sulphone formation was observed. The use of dibutyl ether also led to (*S*)-**1b** with 62% *ee*, but unfortunately a very low conversion (*c* = 14%) was observed in presence of this ether.

As previously shown, the optimal pH for OTEMO activity in the Baeyer-Villiger oxidation of ketones was 9.0. To have a better view on the effect of this parameter on the enzyme when performing sulphoxidations, the synthesis of (*S*)-**1b** was carried out at pH values ranging from 7.0 to 9.0. It was found that altering the pH did not significantly influence the conversions (entries 1–3, Table 4). For all the pH values tested, the chiral sulphoxide was obtained with similar enantiopurities, while the conversions were around 80% and the sulphone percentage was close to 10%.

Temperature can have an important effect on biocatalysts' performance. For this reason, OTEMO-catalysed sulphoxidations of sulphides presenting different structures were tested at 45 °C (Table 4, entries 4–8). As it was found that the increase of the temperature has a negative impact on OTEMO performance, as for all the substrates, both lower conversions and enantioselectivities are achieved when comparing with the reactions carried out at 30 °C. For all the compounds tested (**1**–**2a**, **8a**, **12a** and **17a**), no sulphone formation was observed at this temperature, with the (*S*)-sulphoxides being the only oxidation product, but as mentioned, with poorer enantiomeric excesses.

Finally, the effect of the substrate concentration on OTEMO biocatalytic properties has been analysed. As a model substrate, methyl 4-methoxyphenyl sulphide (**17a**) was chosen, due to its good results in the sulphoxidation reaction. In order to compare the results obtained at different reaction times, the reaction rate was defined as the mmoles of **17a** oxidized per litre and per hour (mmol L^{-1} h^{-1}). As can be seen in

Table 4. Effect of temperature and pH on OTEMO biocatalytic properties.[a]

Entry	Sulphide	Time (h)	pH	*T* (°C)	Conv. (%)[b]	% Sulphone[b]	*ee* (%)[c]
1	**1a**	16	7.0	30	80	9	61
2	**1a**	16	8.0	30	78	12	63
3	**1a**	16	9.0	30	80	10	63
4	**1a**	16	9.0	45	57	≤3	33
5	**2a**	14	9.0	45	36	≤3	51
6	**7a**	20	9.0	45	34	≤3	61
8	**12a**	16	9.0	45	9	≤3	23
9	**17a**	20	9.0	45	30	≤3	66

[a]For reaction details, see Experimental Section.
[b]Determined by GC/MS.
[c]Determined by HPLC.

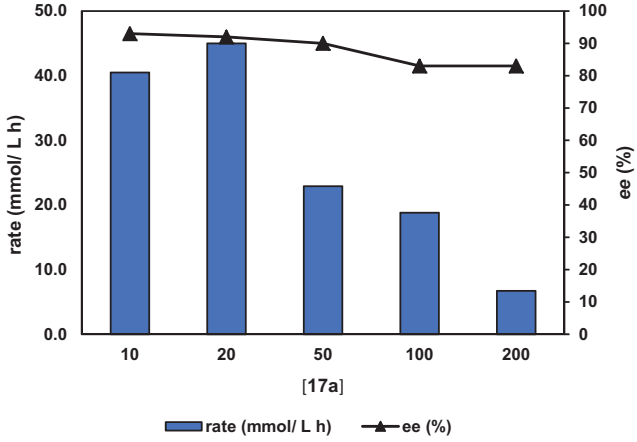


Figure 3. Effect of substrate **17a** concentration on the activity and selectivity of OTEMO-catalysed sulphoxidation.

Figure 3, similar results were obtained when employing low substrate concentrations (10–20 mM), with a maximum reaction rate at 20 mM of **17a** (45.0 mmol L^{-1} h^{-1}). Higher amounts of the starting material led to an important decrease in efficiency. When employing 200 mM [**17a**], OTEMO is still active, but displays a very low reaction rate (close to 7.0 mmol L^{-1} h^{-1}). Regarding the effect on the enantiopurity, there is no effect on this when using 10–50 mM substrate concentrations, but the use of 100–200 mM afforded (*S*)-**17b** with slightly lower enantiomeric excesses (83% *ee* vs. 93% *ee* at 10 mM), as shown in Figure 3.

4. Conclusions

OTEMO, a BVMO from *P. putida* ATCC 17453 overexpressed in *E. coli*, was shown to be a promising biocatalyst for the preparation of optically active sulphoxides. This biocatalyst can catalyse the biooxidation of several aryl alkyl sulphides to the corresponding (*S*)-sulphoxides with different results depending on the substrates structures. The best results in terms of enantioselectivity were obtained for those alkyl aryl

sulphides containing electron-donating groups, in the aromatic ring or in the alkyl chain, and for benzyl methyl sulphide. The analysis of the different reaction parameters that can affect to the biocatalyst properties revealed that OTEMO is sensitive to the presence of organic solvents, temperature, and the use of substrate concentrations higher than 20 mM.

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Disclosure statement

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