Enzyme and Microbial Technology 112 (2018) 29–34

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Characterization of two styrene monooxygenases from marine microbes

Wei Pu[a,b,c], Can Cui[a,b,c], Chao Guo[a,b], Zhong-Liu Wu[a,b,*]

[a] Key Laboratory of Environmental and Applied Microbiology, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China
[b] Environmental Microbiology Key Laboratory of Sichuan Province, Chengdu 610041, China
[c] University of the Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Keywords:
Monooxygenase
Oxide
Whole-cell biocatalyst
Epoxidation
Asymmetric synthesis

ABSTRACT

Styrene monooxygenases (SMOs) are highly stereoselective enzymes that catalyze the formation of chiral epoxides as versatile building blocks. To expand the enzyme toolbox, two bacterial SMOs were identified from the genome of marine microbes *Paraglaciecola agarilytica* NO2 and *Marinobacterium litorale* DSM 23545, and heterologously expressed in *Escherichia coli* in soluble form. Both of the resulting whole-cell biocatalysts exhibited maximal activity at 30 °C and pH 8.0. They catalyzed the sulfoxidation reactions, and the epoxidation of both conjugated and unconjugated styrene derivatives with up to > 99%ee. *Mls*SMO displayed higher activity toward most substrates tested. Compared to an established SMO from *Pseudomonas* species (*Ps*SMO), *Mls*SMO achieved 3.0-, 3.4- and 2.6-fold conversions for substrates styrene, cinnamyl alcohol and 4-vinyl-2, 3-dihydrobenzofuran, respectively.

1. Introduction

Enantiopure epoxides bear a highly reactive oxirane function that can be opened by various nucleophiles or undergo elimination, reduction or rearrangements to a multitude of chiral intermediates, which makes them extremely versatile building blocks in organic synthesis and pharmaceutical industry [1–4]. Although chemo-catalyzed asymmetric epoxidations have been extensively developed in the last few decades, biocatalytic epoxidation remains a strong candidate for preparing enantiopure epoxides in an eco-friendly fashion [5–9]. Particularly, classical chemical methods often lack high stereoselectivity for nonfunctional terminal alkenes, such as styrene, while biocatalytic epoxidation can provide the corresponding epoxides with excellent enantiomeric excess.

Styrene monooxygenase (SMO) is a flavin-dependent monooxygenase that catalyzes the epoxidation of styrene to (*S*)-styrene oxide in the upper catabolic pathway of styrene degradation [10,11]. SMOs adopt oxygen as the oxidant, and often display absolute stereoselectivity to afford the (*S*)-styrene oxide with > 99%ee under mild reaction conditions. Their potential as a tool in organic synthesis has been demonstrated by the pilot-scale production of (*S*)-styrene oxide [12], the expanded substrate scope that includes a variety of conjugated and unconjugated styrene derivatives, heterocyclic analogues and aliphatic alkenes [13–16], and a series of cascade reactions based on the asymmetric epoxidation coupled with other enzymatic transformations [17–20].

Most SMOs reported so far are two component flavin-dependent monooxygenase composed of a FAD-dependent styrene epoxidase (StyA) and a NADH dependent flavin reductase (StyB), encoded by *styA* and *styB* genes [10]. They all originate from *Pseudomonas* [21–26] or *Rhodococcus* species [27,28] except one from metagenome [29]. A self-sufficient one-component SMO has been reported as well, which was isolated from *Rhodococcus opacus* 1CP [30]. In general, database analysis has revealed that SMOs represent only a small proportion of flavoprotein monooxygenases [29,31], and up to now the efforts devoted to the searching for new SMOs remain limited, which has hindered the extensive application of such a group enzymes of superior stereoselectivity and unique substrate preference that complements to classic chemical epoxidations.

The marine microbes are known to play many important roles in the ecosystem, and also have provided abundant resources for bioactive small molecules and proteins [32–34]. However, to the best of our knowledge, no SMO from such a source has been investigated. Here we report the functional characterization of two SMOs from marine microbes. The whole-cell systems expressing those two SMOs exhibited maximal activity on pH 8.0. They displayed similar substrate preference as the SMO from *Pseudomonas* species (*Ps*SMO), but one of them could yield significantly increased conversion toward styrene and derivatives with excellent stereoselectivity.

* Corresponding author at: Chengdu Institute of Biology, Chinese Academy of Sciences, 9 South Renmin Road, 4th Section, Chengdu, Sichuan 610041, China.
E-mail address: wuzhl@cib.ac.cn (Z.-L. Wu).

https://doi.org/10.1016/j.enzmictec.2018.02.001
Received 14 December 2017; Received in revised form 29 January 2018; Accepted 1 February 2018
Available online 07 February 2018

2. Materials and methods

2.1. Chemicals

The substrates styrene (**1a**), cinnamyl alcohol (**2a**), thioanisole (**5a**), 4-vinyl-2, 3-dihydrobenzofuran (**6a**) were purchased from Alfa-Aesar (Tianjin, China) or Acros Organics (Geel, Belgium). The substrate 1-phenylprop-2-en-1-ol (**3a**) and 2-allylphenol (**4a**) were prepared from the corresponding aldehydes and Grignard reagent [14,35]. Racemic and (*S*)-styrene epoxides were from Sigma-Aldrich (St. Louis, MO, USA). Other racemic epoxides were prepared from the corresponding substrates using meta-chloroperoxybenzoic acid as the oxidant [36]. Methyl phenyl sulfone was purchased from Energy Chemical (Shanghai, China). Oligonucleotides were synthesized by Invitrogen (Shanghai, China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Kanamycin and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Amresco (Solon, OH, USA).

2.2. Construction of expression vectors

The DNA fragment encoding *Ps*SMO (GenBank accession no. GU593979.1) was previously isolated from *Pseudomonas* sp. LQ26, and annotated as *styAB2* [26]. It was constructed into pET28a (+) vector (Novagen, Madison, WI) for expression [26,37]. The amino acid sequence and the native DNA sequence of *Pa*SMO and *Ml*SMO were obtained from NCBI database (GenBank accession no.: NZ_BAEK01000054.1 and NZ_AUAZ01000037.1, respectively) using a genome mining approach that adopted the amino acid sequence of StyA from *Pseudomonas* sp. LQ26 as the reference sequence. The source strains were *Paraglaciecola agarilytica* NO2 (previously *Glaciecola agarilytica*) [38] and *Marinobacterium litorale* DSM 23545, respectively. Both DNA sequences were optimized for *E. coli* BL21 (DE3) expression using Optimum-Gene technology and synthesized by GenScript Biotech Co. (Nanjing, China). The DNA fragments were each digested with *Bam*H I and *Sac* I, and ligated into pET28a (+). The constructed plasmids were verified by DNA sequencing at Invitrogen Biotechnologies (Shanghai, China), and designated as pET-*Pa*SMO and pET-*Ml*SMO, respectively. The resulting plasmids were each introduced into *E. coli* BL21 (DE3) for expression.

2.3. Expression of SMOs in E. coli

Single colonies were grown overnight at 37 °C in Luria-Bertani (LB) media containing kanamycin (50 μg/mL) and D-glucose (5 g/L). Two milliliter of the overnight culture was then inoculated into 200 mL of Terrific Broth (TB medium) containing kanamycin (50 μg/mL) and D-glucose (5 g/L) in a 500 mL flask. The cultures were incubated at 37 °C for 3 h before the addition of 0.1 mM IPTG, and the incubation was continued at 20 °C for another 20 h with gyratory shaking at 200 rpm on an INFORS Multitron shaker. The cells were harvested by centrifugation, washed twice with potassium phosphate buffer (0.1 M, pH 7.0) and stored at 4 °C. Bacterial soluble fractions were prepared by high pressure homogenizer (ATS-AH100B, ATS Engineering Inc., Canada) followed by centrifugation at 4 °C, and the crude cell extracts were analyzed using SDS-PAGE.

2.4. Whole-cell biotransformation and product analysis

Harvested cells with a dry cell weight (DCW) of 0.2 g were re-suspended in 5 mL potassium phosphate buffer (pH 7.5, 100 mM) containing 5 mg substrate, and incubated at 30 °C for 1 h with gyratory shaking at 200 rpm. For the substrate styrene, a biphasic system [23] containing 10% (v/v) cyclohexane was applied instead. All the reactions were carried out in duplicate. The reactions were terminated by extraction with an equal volume of ethyl acetate for twice. The combined organic phases were dried with anhydrous sodium sulfate, concentrated under vacuum, and subjected to GC or HPLC analysis.

To determine the temperature optimum, the reactions were performed in 5 mL potassium phosphate buffer (pH 6.5, 100 mM) containing 10% (v/v) cyclohexane and 5 mg styrene for 4 h at temperatures varying from 20 to 45 °C. To determine the pH optimum, the reactions were performed in 5 mL potassium phosphate buffer (100 mM) or Tris-HCl buffer (50 mM) containing 10% (v/v) cyclohexane and 7.5 mg styrene for 2 h at 30 °C with pH values ranging from 5.5 to 9.0.

Enantiomeric excesses or conversions were determined using chiral HPLC on a Shimadzu LC 20-AD (Shimadzu, Japan) connected to a photodiode array detector at 30 °C using a Daicel Chiralpak IC column (for **1a**, *n*-hexane/2-propanol 98:2, 0.6 mL/min, $t_R(R)$ 10.2 min, $t_R(S)$ 11.5 min; for **2a**, *n*-hexane/2-propanol 95:5, 0.5 mL/min, $t_R(R)$ 49.5 min, $t_R(S)$ 41.8 min; for **6a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, $t_R(R)$ 13.5 min, $t_R(S)$ 20.1 min), or Chiralpak AS-H column (for **3a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, t_{R1} 21.3 min, t_{R2} 25.3 min, t_{R3} 26.8 min, t_{R4} 30.3 min; for **4a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, $t_R(S)$ 17.4 min, $t_R(R)$ 19.0 min), or Chiralpak OD-H column (for **5a**, *n*-hexane/2-propanol 97:3, 0.5 mL/min, $t_R(R)$ 26.6 min, $t_R(S)$ 34.8 min).

3. Results and discussion

3.1. Sequence analysis of SMOs

The two subunits of both *Ml*SMO and *Pa*SMO are hypothesized monooxygenases (StyA) and flavin reductases (StyB) revealed by genome sequencing projects. The sequence of *Ml*SMO is mined from the genome of *Marinobacterium litorale* DSM 23545, a mesophile bacteria isolated from surface seawater, and that of *Pa*SMO is from the genome of *Paraglaciecola agarilytica* NO2, an agar-digesting marine bacterium isolated from marine sediment [39]. As far as we know, neither strain has been reported to degrade styrene or related compounds.

StyAs of *Ml*SMO and *Pa*SMO showed 65.8% identity to each other, and had similar identities to other two-component SMOs from *Pseudomonas* sp. LQ26 (69.2% and 65.5%, respectively) [26], *Rhodococcus* sp. ST-10 (57.9% and 55.6%, respectively) [27], or metagenome (50.0% and 48.8%, respectively) [29]. StyBs of *Ml*SMO and *Pa*SMO showed 43.3% identity to each other, and had similar identities to those of *Pseudomonas* sp. LQ26 (60.8% and 44.7%, respectively) [26] and *Rhodococcus* sp. ST-10 (41.6% and 33.2%, respectively) [27].

The amino acid sequences of *Ml*SMO and *Pa*SMO SMOs were aligned with other SMOs of known function, which showed the presence of conserved sequence motifs typical for a mechanistic involvement of flavins and nicotinamide adenine dinucleotides (Fig. 1a). Both SMOs have the functionally conserved residue Thr47 that is important to catalytic activity in StyA [40,41]. The FAD-binding fingerprints GXGXXG, GG and DX6G were found in both *Ml*SMO and *Pa*SMO (Fig. 1a). The GXGXXG sequence (residues 9–14) is a fingerprint sequence among FAD- and NAD(P)H-dependent oxidoreductases and play an important role in FAD-binding proteins in general [42–44]. The second FAD binding motif contains the GDX6P sequence (*Ml*SMO residues 294–302; *Pa*SMO residues 292–300), where the highly conserved Asp residue connects with the O3′ of the ribose moiety of FAD [45]. The DG sequence is highly conserved among all flavoprotein hydroxylases [43], but only the Gly is conserved in StyA (*Ml*SMO residue 171; *Pa*SMO residue 169).

3.2. Heterologous expression

The heterologous expression of both *Pa*SMO and *Ml*SMO were performed at 20 °C to achieve soluble proteins in *E. coli* BL21 (DE3) cells. SDS-PAGE analysis indicated that both SMOs showed a major band at around 45 kDa, which was in accordance with the size of the open reading frame of StyA (Fig. 2).

StyB was estimated to be around 19 kDa. Its over-expression was not

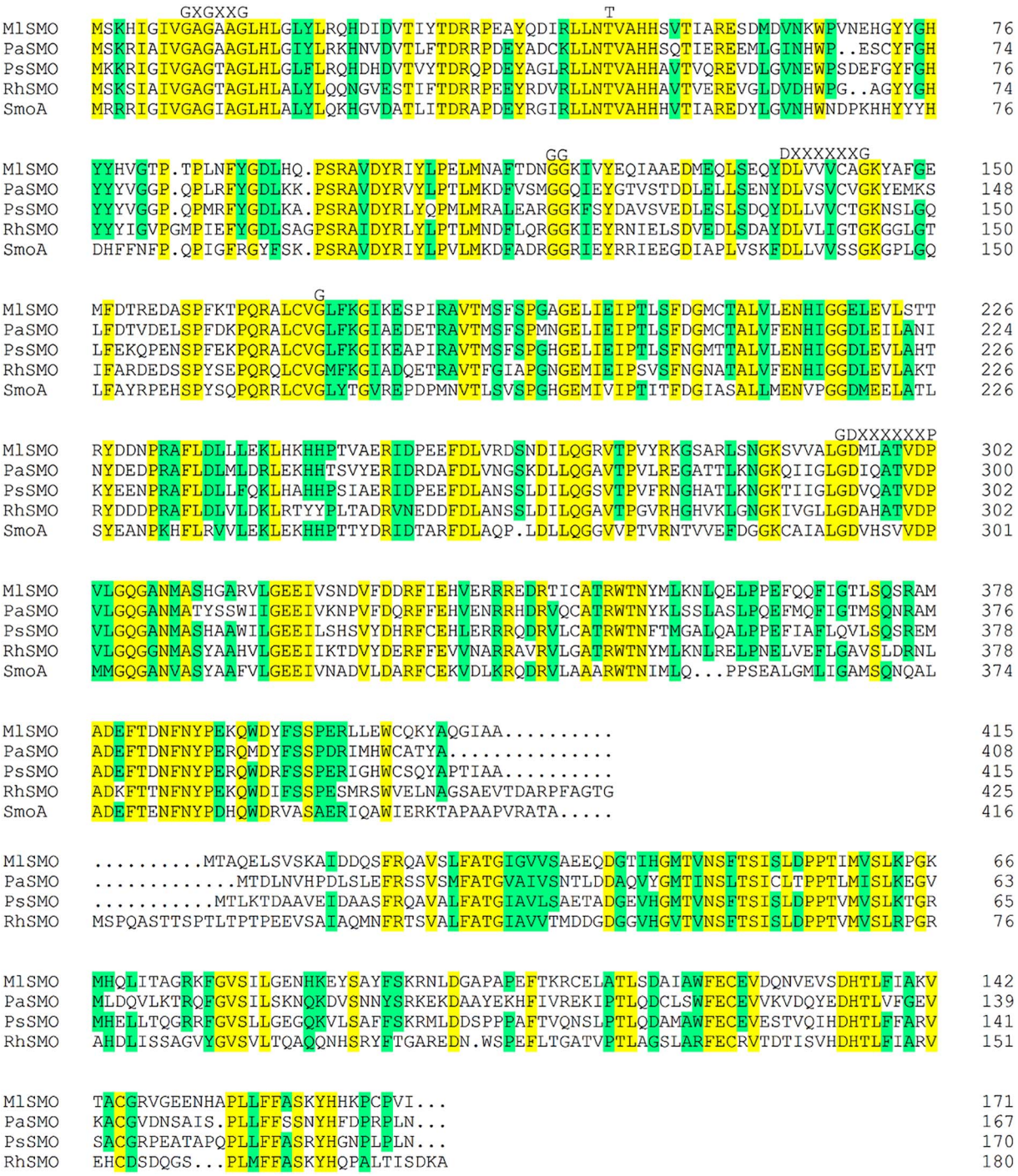

Fig. 1. Multiple sequence alignment of the two subunits of SMOs, StyA (a) and StyB (b). Shown are *Ml*SMO (*Marinobacterium litorale* DSM 23545, WP_027855270 and WP_051299263), *Pa*SMO (*Paraglaciecola agarilytica* NO2, WP_008305084 and WP_008305080), *Ps*SMO (*Pseudomonas* sp. LQ26, ADE62390 and ADE62391), *Rh*SMO (*Rhodococcus* sp. ST-10, AB594506) and SmoA (metagenome, ABV24041). Motifs that are conserved in the flavoprotein hydroxylase family are indicated.

detected on SDS-PAGE (Fig. 2). However, the formation of blue dye was observed during the cultivation, which was formed by the oxidative-coupling of the *1H*-indole that was produced by *E. coli*. This reaction is well known to be catalyzed with oxygenases such as cytochrome P450 and SMO, and the products are commonly a mixture of indigo and indirubin [46–48]. The control sample expressing the vector without the incorporation of *styAB* did not result in dye formation. On the other hand, the dye formation could be suppressed by adding D-glucose during cultivation, which was in accordance with a previous report [49]. The results indicated that the functional expression of both *Pa*SMO and *Ml*SMO were successfully achieved.

3.3. Effect of reaction temperature and pH

The biotransformation reactions catalyzed with *E. coli* whole-cells expressing *Ml*SMO or *Pa*SMO were performed in a bi-phase system containing 10% (v/v) cyclohexane using styrene as substrate [50]. The temperature dependence of activities was measured from 20 to 45 °C (Fig. 3). Both *Ml*SMO and *Pa*SMO displayed the maximal activity at 30 °C, but the maximal activity of *Ml*SMO was 2.0-fold of that of *Pa*SMO. *Pa*SMO appeared to be more sensitive to reaction temperature than *Ml*SMO, and retained 50% of the maximal activity at 40 °C, while *Ml*SMO retained > 80% of the maximal activity at the temperature

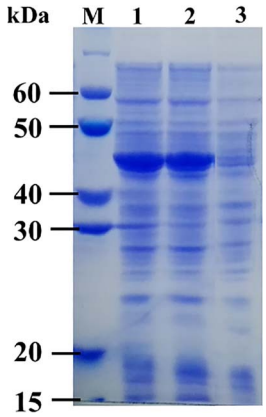

Fig. 2. SDS-PAGE analysis of recombinant SMOs. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. M: molecular marker, lane 1–3: cell-free extracts of the transformants containing pET-*Mls*MO, pET-*Pa*SMO and pET28a (+), respectively.

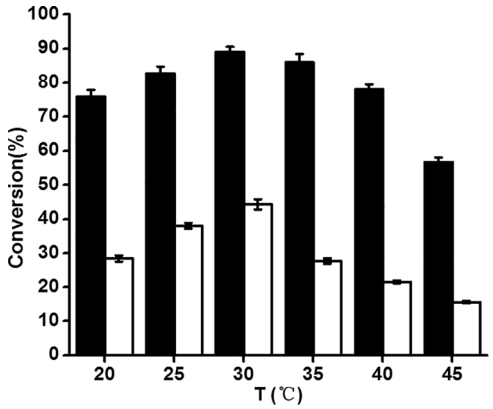

Fig. 3. Effect of reaction temperature on the activity of whole-cells expressing *Mls*MO (black bar) and *Pa*SMO (white bar).

range of 20–40 °C.

Both *Mls*MO and *Pa*SMO were active at a wide range of pH, and displayed the maximal activity at pH 8.0 in potassium phosphate buffer (Fig. 4). The activity of *Mls*MO was 2.5-fold of the *Pa*SMO at optimum pH 8.0 in potassium phosphate buffer. The components of the buffer had significant impact on the enzymatic activity. When Tris-HCl buffer of pH 8.0 was applied instead of potassium phosphate buffer, the activities of *Mls*MO and *Pa*SMO decreased by 29.4% and 18.1%, respectively.

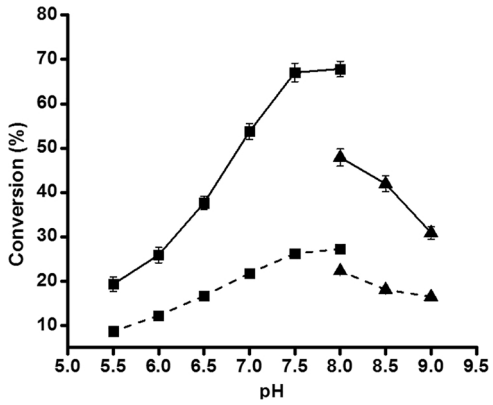

Fig. 4. Effect of pH on the activity of whole-cells expressing *Mls*MO (solid line) and *Pa*SMO (dotted line). Potassium phosphate (■) and Tris–HCl (▲) were applied as reaction buffers.

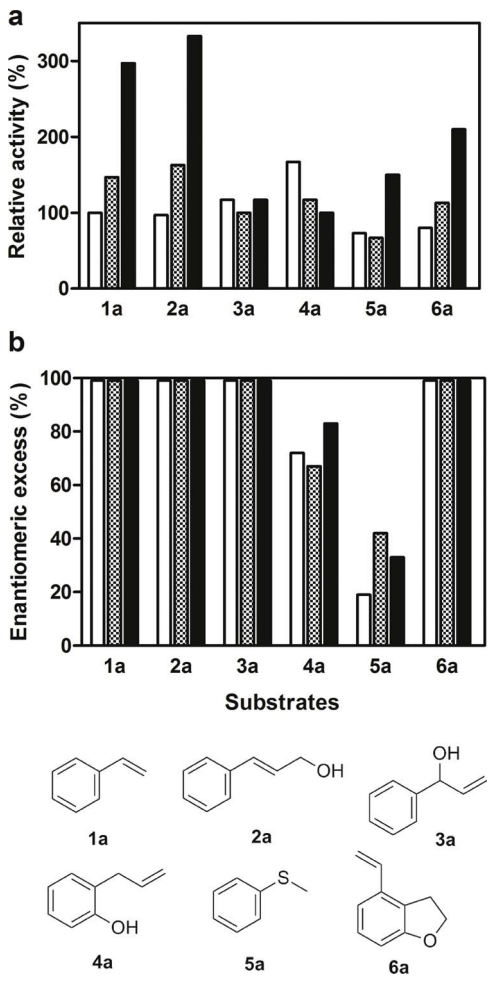

Fig. 5. Biotransformation of alkenes and sulfide using whole-cells expressing *Ps*SMO (white bar), *Pa*SMO (dotted bar) and *Mls*MO (black bar). Shown are conversions (a) and enantiomeric excesses (b) for substrates styrene (**1a**), cinnamyl alcohol (**2a**), 1-phenylprop-2-en-1-ol (**3a**), 2-allylphenol (**4a**), thioanisole (**5a**), and 4-vinyl-2,3-dihydrobenzofuran (**6a**). Activities were normalized as percentages of the activities of *Ps*SMO toward substrate **1a**. The absolute configuration of products is *S* for substrates **1a**-**4a** and **6a**, and *R* for **5a**.

In general, *Mls*MO consistently displayed higher activity than *Pa*SMO under the same reaction conditions, and the enantiomeric excess of the product (*S*)-styrene oxide was > 99%ee for all the assays.

3.4. Whole-cell biotransformation of various alkenes

The activity and stereoselectivity of *Mls*MO and *Pa*SMO were investigated using the whole-cell biotransformation system with six substrates (Fig. 5). Among them, substrates **1a**, **2a** and **6a** are conjugated alkenes. The chiral epoxide derived from **6a** is a key intermediate of Tasimelteon [51], which is a drug that was approved by the US FDA in January 2014 for the treatment of non-24-h sleep-wake disorder. It is currently produced via a two-step procedure that involves Sharpless asymmetric dihydroxylation followed by cyclization [52]. Substrates **3a** and **4a** are unconjugated alkenes, and substrate **5a** is a sulfide (Fig. 5). As a comparison, the same system expressing the SMO from *Pseudomonas* sp. LQ26 (*Ps*SMO) [26] was assayed side-by-side.

All three SMOs displayed similar substrate preference. They catalyzed the asymmetric epoxidation of both conjugated and unconjugated aromatic alkenes, as well the oxidation of sulfide, but failed to show activity toward aliphatic alkenes, such as 1-heptene, which was previously reported to be catalyzed with *Rh*SMO [13].

In general, *Mls*MO appeared to prefer conjugated substrates, while

*Ps*SMO showed the highest activity toward the unconjugated substrate **4a** (Fig. 5a). For the epoxidation of substrates **1a**, **2a** and **6a**, both *Ml*SMO and *Pa*SMO displayed increased activities compared to the previously established *Ps*SMO [26], and *Ml*SMO showed the highest whole-cell activity among the three SMOs. Compared to *Ps*SMO, *Ml*SMO achieved 3.0-, 3.4- and 2.6-fold conversions for substrates **1a**, **2a** and **6a**, respectively (Fig. 5a). For the sulfoxidation reaction of **5a**, *Ml*SMO also showed the highest activity.

The stereoselectivity of all three SMOs were excellent for substrates **1a**, **2a, 3a** and **6a**, delivering the corresponding (*S*)-oxide with > 99% ee (Fig. 5b). However, for substrates **4a**, the enantiomeric excess of the epoxide product was only medium (67–83%ee), while the sulfoxidation reaction of **5a** resulted in poor selectivity (19–42%ee) (Fig. 5b).

4. Conclusions

We have identified two bacterial two-component SMOs, *Pa*SMO and *Ml*SMO, from the genomes of marine microbes, and functionally expressed each of the SMOs in *E. coli*. The resulting whole-cell biocatalysts catalyzed the asymmetric epoxidation of styrene and analogues, as well the oxidation of sulfide. The two SMOs, particularly *Ml*SMO, displayed significantly higher activity, compared to a previous established SMO from *Pseudomonas* species, toward conjugated alkenes, such as styrene, cinnamyl alcohol and 4-vinyl-2,3-dihydrobenzofuran, the Tasimelteon intermediate, while remaining excellent stereoselectivity, implicating great potential for bio-epoxidation reactions.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (21572220, 21372216 and 21708038) and the Open Fund of Key Laboratory of Environmental and Applied Microbiology (KLCAS-2016-08 and KLCAS-2015-01) of the Chinese Academy of Sciences.

References

[1] R.L. Davis, J. Stiller, T. Naicker, H. Jiang, K.A. Jørgensen, Asymmetric organocatalytic epoxidations: reactions, scope, mechanisms, and applications, Angew. Chem. Int. Ed. 53 (2014) 7406–7426.
[2] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Kesseler, R. Sturmer, T. Zelinski, Industrial methods for the production of optically active intermediates, Angew. Chem. Int. Ed. 43 (2004) 788–824.
[3] V. Farina, J.T. Reeves, C.H. Senanayake, J.H.J. Song, Asymmetric synthesis of active pharmaceutical ingredients, Chem. Rev. 106 (2006) 2734–2793.
[4] K.B. Sharpless, Searching for new reactivity (Nobel lecture), Angew. Chem. Int. Ed. 41 (2002) 2024–2032.
[5] H. Lin, J.Y. Liu, H.B. Wang, A.A.Q. Ahmed, Z.L. Wu, Biocatalysis as an alternative for the production of chiral epoxides: a comparative review, J. Mol. Catal. B: Enzym. 72 (2011) 77–89.
[6] M.M. Hussain, P.J. Walsh, Tandem reactions for streamlining synthesis: enantio- and diastereoselective one-pot generation of functionalized epoxy alcohols, Acc. Chem. Res. 41 (2008) 883–893.
[7] D.L. Chang, J. Zhang, B. Witholt, Z. Li, Chemical and enzymatic synthetic methods for asymmetric oxidation of the C-C double bond, Biocatal. Biotransform. 22 (2004) 113–130.
[8] M.-Y. Xu H. Lin, Y. Liu, Biocatalytic epoxidation for green synthesis, in: R.N. Patel (Ed.), Green Biocatalysis, John Wiley & Sons, 2016, pp. 351–372.
[9] G. Sello, T. Fumagalli, F. Orsini, Recent developments in epoxide preparation, Curr. Org. Synth. 3 (2006) 457–476.
[10] K. Otto, K. Hofstetter, M. Rothlisberger, B. Witholt, A. Schmid, Biochemical characterization of StyAB from *Pseudomonas* sp. strain VLB120 as a two-component flavin-diffusible monooxygenase, J. Bacteriol. 186 (2004) 5292–5302.
[11] A. Mooney, P.G. Ward, K.E. O'Connor, Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications, Appl. Microbiol. Biotechnol. 72 (2006) 1–10.
[12] S. Panke, M. Held, M.G. Wubbolts, B. Witholt, A. Schmid, Pilot-scale production of (S)-styrene oxide from styrene by recombinant *Escherichia coli* synthesizing styrene monooxygenase, Biotechnol. Bioeng. 80 (2002) 33–41.
[13] H. Toda, T. Ohuchi, R. Imae, N. Itoh, Microbial production of aliphatic (S)-epoxyalkanes by using *Rhodococcus* sp strain ST-10 styrene monooxygenase expressed in organic-solvent-tolerant *Kocuria rhizophila* DC2201, Appl. Environ. Microbiol. 81 (2015) 1919–1925.
[14] H. Lin, Y. Liu, Z.-L. Wu, Highly diastereo- and enantio-selective epoxidation of secondary allylic alcohols catalyzed by styrene monooxygenase, Chem. Commun. 47 (2011) 2610–2612.
[15] A. Schmid, K. Hofstetter, H.J. Feiten, F. Hollmann, B. Witholt, Integrated biocatalytic synthesis on gram scale: the highly enantio selective preparation of chiral oxiranes with styrene monooxygenase, Adv. Synth. Catal. 343 (2001) 732–737.
[16] H. Toda, R. Imae, N. Itoh, Bioproduction of chiral epoxyalkanes using styrene monooxygenase from *Rhodococcus* sp ST-10 (RhSMO), Adv. Synth. Catal. 356 (2014) 3443–3450.
[17] S. Wu, Y. Zhou, T. Wang, H.P. Too, D.I. Wang, Z. Li, Highly regio- and enantioselective multiple oxy- and amino-functionalizations of alkenes by modular cascade biocatalysis, Nat. Commun. 7 (2016) 11917.
[18] Y.-C. Liu, Z.-L. Wu, Switchable asymmetric bio-epoxidation of alpha,beta-unsaturated ketones, Chem. Commun. 52 (2016) 1158–1161.
[19] Y.C. Liu, C. Guo, Y. Liu, H.B. Wang, Z.L. Wu, Enzymatic cascades for the stereocomplementary epimerisation of in situ generated epoxy alcohols, Org. Biomol. Chem. 15 (2017) 2562–2568.
[20] Y. Zhou, S. Wu, Z. Li, Cascade biocatalysis for sustainable asymmetric synthesis: from biobased l-phenylalanine to high-value chiral chemicals, Angew. Chem. Int. Ed. Engl. 55 (2016) 11647–11650.
[21] S. Hartmans, M. van der Werf, J. de Bont, Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase, Appl. Environ. Microbiol. 56 (1990) 1347–1351.
[22] F. Beltrametti, A.M. Marconi, G. Bestetti, C. Colombo, E. Galli, M. Ruzzi, E. Zennaro, Sequencing and functional analysis of styrene catabolism genes from *Pseudomonas fluorescens* ST, Appl. Environ. Microbiol. 63 (1997) 2232–2239.
[23] S. Panke, B. Witholt, A. Schmid, M.G. Wubbolts, Towards a biocatalyst for (S)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120, Appl. Environ. Microbiol. 64 (1998) 2032–2043.
[24] A. Velasco, S. Alonso, J.L. Garcia, J. Perera, E. Diaz, Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2, J. Bacteriol. 180 (1998) 1063–1071.
[25] N.D. O'Leary, K.E. O'Connor, W. Duetz, A.D. Dobson, Transcriptional regulation of styrene degradation in Pseudomonas putida CA-3, Microbiology 147 (2001) 973–979.
[26] H. Lin, J. Qiao, Y. Liu, Z.-L. Wu, Styrene monooxygenase from *Pseudomonas* sp. LQ26 catalyzes the asymmetric epoxidation of both conjugated and unconjugated alkenes, J. Mol. Catal. B: Enzym. 67 (2010) 236–241.
[27] H. Toda, R. Imae, T. Komio, N. Itoh, Expression and characterization of styrene monooxygenases of *Rhodococcus* sp ST-5 and ST-10 for synthesizing enantiopure (S)-epoxides, Appl. Microbiol. Biotechnol. 96 (2012) 407–418.
[28] A. Riedel, T. Heine, A. Westphal, C. Conrad, P. Rathsack, W. van Berkel, D. Tischler, Catalytic and hydrodynamic properties of styrene monooxygenases from *Rhodococcus opacus* 1CP are modulated by cofactor binding, AMB Express 5 (2015) 30.
[29] E.W. van Hellemond, D.B. Janssen, M.W. Fraaije, Discovery of a novel styrene monooxygenase originating from the metagenome, Appl. Environ. Microbiol. 73 (2007) 5832–5839.
[30] D. Tischler, J.A.D. Groning, S.R. Kaschabek, M. Schlomann, One-component styrene monooxygenases: an evolutionary view on a rare class of flavoproteins, Appl. Biochem. Biotechnol. 167 (2012) 931–944.
[31] W.J.H. van Berkel, N.M. Kamerbeek, M.W. Fraaije, Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts, J. Biotechnol. 124 (2006) 670–689.
[32] J.W. Blunt, B.R. Copp, R.A. Keyzers, M.H.G. Munro, M.R. Prinsep, Marine natural products, Nat. Prod. Rep. 29 (2012) 144–222.
[33] J. Kennedy, J.R. Marchesi, A.D.W. Dobson, Marine metagenomics: strategies for the discovery of novel enzymes with biotechnological applications from marine environments, Microb. Cell Fact. 7 (2008) 8.
[34] A. Trincone, Marine biocatalysts: enzymatic features and applications, Mar. Drugs 9 (2011) 478–499.
[35] D. Gryko, Z. Urbanczyk-Lipkowska, J. Jurczak, Diastereoselective addition of vinylmagnesium halides to variously N-mono- and N,N-diprotected L-alaninals, Tetrahedron: Asymmetry 8 (1997) 4059–4067.
[36] H. Lin, Y. Liu, Z.-L. Wu, Asymmetric epoxidation of styrene derivatives by styrene monooxygenase from *Pseudomonas* sp LQ26: effects of alpha- and beta-substituents, Tetrahedron: Asymmetry 22 (2011) 134–137.
[37] Y. Liu, Y.-C. Liu, Z.-L. Wu, Asymmetric bio-epoxidation catalyzed with the styrene monooxygenase from *Pseudomonas* sp. LQ26, Bioresour. Bioprocess. 3 (2016) 1–7.
[38] S. Shivaji, G.S. Reddy, Phylogenetic analyses of the genus Glaciecola, Int. J. Syst. Evol. Microbiol. 64 (2014) 3264–3275.
[39] Q.L. Qin, B.B. Xie, Y. Yu, Y.L. Shu, J.C. Rong, Y.J. Zhang, D.L. Zhao, X.L. Chen, X.Y. Zhang, B. Chen, B.C. Zhou, Y.Z. Zhang, Comparative genomics of the marine bacterial genus Glaciecola reveals the high degree of genomic diversity and genomic characteristic for cold adaptation, Environ. Microbiol. 16 (2014) 1642–1653.
[40] A.A. Qaed, H. Lin, D.F. Tang, Z.L. Wu, Rational design of styrene monooxygenase mutants with altered substrate preference, Biotechnol. Lett. 33 (2011) 611–616.
[41] K.A. Feenstra, K. Hofstetter, R. Bosch, A. Schmid, J.N.M. Commandeur, N.P.E. Vermeulen, Enantioselective substrate binding in a monooxygenase protein model by molecular dynamics and docking, Biophys. J. 91 (2006) 3206–3216.
[42] U.E. Ukaegbu, A. Kantz, M. Beaton, G.T. Gassner, A.C. Rosenzweig, Structure and ligand binding properties of the epoxidase component of styrene monooxygenase, Biochemistry 49 (2010) 1678–1688.
[43] M.H.M. Eppink, H.A. Schreuder, W.J.H. VanBerkel, Identification of a novel conserved sequence motif in flavoprotein hydroxylases with a putative dual function in FAD/NAD(P)H binding, Protein Sci. 6 (1997) 2454–2458.
[44] G. Kleiger, D. Eisenberg, GXXXG and GXXXA motifs stabilize FAD and NAD(P)-

binding Rossmann folds through C-alpha-H center dot center dot center dot O hydrogen bonds and van der Waals interactions, J. Mol. Biol. 323 (2002) 69–76.
[45] G. Eggink, H. Engel, G. Vriend, P. Terpstra, B. Witholt, Rubredoxin reductase of *Pseudomonas oleovorans* – structural relationship to other flavoprotein oxidoreductases based on one nad and 2 fad fingerprints, J. Mol. Biol. 212 (1990) 135–142.
[46] E.M. Gillam, A.M. Aguinaldo, L.M. Notley, D. Kim, R.G. Mundkowski, A.A. Volkov, F.H. Arnold, P. Soucek, J.J. DeVoss, F.P. Guengerich, Formation of indigo by recombinant mammalian cytochrome P450, Biochem. Biophys. Res. Commun. 265 (1999) 469–472.
[47] Z.L.L. Wu, P. Aryal, O. Lozach, L. Meijer, E.P. Guengerich, Biosynthesis of new indigoid inhibitors of protein kinases using recombinant cytochrome P450 2A6, Chem. Biodivers. 2 (2005) 51–65.
[48] T. Heine, K. Tucker, N. Okonkwo, B. Assefa, C. Conrad, A. Scholtissek, M. Schlomann, G. Gassner, D. Tischler, Engineering styrene monooxygenase for biocatalysis: reductase-epoxidase fusion proteins, Appl. Biochem. Biotechnol. 181 (2017) 1590–1610.
[49] S. Panke, M.G. Wubbolts, A. Schmid, B. Witholt, Production of enantiopure styrene oxide by recombinant *Escherichia coli* synthesizing a two-component styrene monooxygenase, Biotechnol. Bioeng. 69 (2000) 91–100.
[50] S. Panke, V. de Lorenzo, A. Kaiser, B. Witholt, M.G. Wubbolts, Engineering of a stable whole-cell biocatalyst capable of (S)-styrene oxide formation for continuous two-liquid-phase applications, Appl. Environ. Microbiol. 65 (1999) 5619–5623.
[51] R.N. Patel, Synthesis of chiral pharmaceutical intermediates by biocatalysis, Coord. Chem. Rev. 252 (2008) 659–701.
[52] J.S. Prasad, T. Vu, M.J. Totleben, G.A. Crispino, D.J. Kacsur, S. Swaminathan, J.E. Thornton, A. Fritz, A.K. Singh, Development of Jacobsen asymmetric epoxidation and sharpless asymmetric dihydroxylation methods for the large-scale preparation of a chiral dihydrobenzofuran epoxide, Org. Process Res. Dev. 7 (2003) 821–827.