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Communication

Relationships between substrate promiscuity and chiral selectivity of esterases from phylogenetically and environmentally diverse microorganisms

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Abstract: Substrate specificity and selectivity of a biocatalyst are determined by the protein sequence and structure of its active site. Finding versatile biocatalysts acting against multiple substrates while at the same time being chiral selective is of interest for the pharmaceutical and chemical industry. However, the relationships between these two properties in natural microbial enzymes remain underexplored. Here, we performed an experimental analysis of substrate promiscuity and chiral selectivity in a set of 145 purified esterases from phylogenetically and environmentally diverse microorganisms, which were assayed against 96 diverse esters, 20 of which were enantiomers. Our results revealed a negative correlation between substrate promiscuity and chiral selectivity in the evaluated enzymes. Esterases displaying prominent substrate promiscuity and large catalytic environments are characterized by low chiral selectivity, a feature that has limited commercial value. Although, low level of substrate promiscuity does not guarantee high chiral selectivity, the probability that esterases with smaller active sites possess chiral selectivity factors of interest for industry (>25) is significantly higher than for promiscuous enzymes. Together, the present study unambiguously demonstrates that promiscuous and selective esterases appear to be rare in nature and that substrate promiscuity can be used as an indicator of the chiral selectivity level of esterases, and vice versa.

Keywords: esterase; metagenomics; promiscuity; selectivity

46 **1. Introduction**

47 Presently, there is a great necessity of suitable biocatalysts with high process performance, as
48 greener alternatives to chemical synthesis [1,2]. It is expected that up to 40% of bulk chemical
49 synthesis processes could be substituted by enzymatic catalysis by 2020 [1]. Along with
50 requirements of a technical nature such as process development and optimization, it is however
51 widely recognized that the establishment of enzymatic processes is mainly a problem of finding,
52 optimizing or designing new and/or better performing enzymes. Nature is a rich reservoir from
53 where enzymes can be isolated [3,4], because they are continuously evolving as a consequence of
54 natural selection. Promiscuous enzymes are effective for converting multiple substrates, thus, they
55 are industrially relevant [4-6]. Enzymes need to also be robust and, preferably, chiral selective to
56 reduce raw material costs in the synthesis of pure chiral compounds [1,2,4]. That is, they need to be
57 able to cleave preferentially only one chiral ester when offered a racemic mixture. Is it possible to
58 find versatile enzymes displaying prominent substrate range and stringent chiral selectivity?
59 Evaluating this possibility was the starting point of the present study.
60 In this study we are interested in investigating as model enzymes serine ester hydrolases,
61 hereafter referred to as esterases, from the structural superfamily of α/β-hydrolases. The activity of
62 these esterases relies mainly on a catalytic triad usually formed by Ser, Asp/Glu and His [8]. This
63 enzyme class was selected for a number of reasons: it is widely distributed in the environment, it has
64 important physiological functions, it includes hydrolases that are among the most important
65 industrial biocatalysts, and extensive biochemical knowledge has been accumulated [4,5,7].
66 Just focusing on those from uncultivated microorganisms discovered through metagenomic
67 approaches, esterases with prominent chiral selectivity have been identified and their use in the
68 kinetic resolution of a number of esters reported. Recent examples include those preferably
69 hydrolyzing one of the chiral esters in racemic mixtures of ibuprofen esters [9,10], ketoprofen esters
70 [11-14], solketal esters [15], esters of phenylalkyl carboxylic acids,
71 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate and 3,7-dimethyl-1,6-octadien-3-yl acetate [16,17],
72 methyl 3-phenylglycidate [18], 1-phenylethyl acetate [19,20], ofloxacin butyl ester [21], 1-octin-3-ol,
73 3-chlor-1-phenyl-1-propanol, trimethylsilylbutinol, *cis*/*trans*-1,2-cyclohexanediol and
74 isopropylidenglycerol acetate [22], glycidyl butyrate [23], methyl-mandelate,
75 glycidyl-4-nitrobenzoate, methyl-3-bromo-2-methyl propionate, methyl lactate, menthyl acetate,
76 neomenthyl acetate, pantolactone, and methyl 3-hydroxybutyrate [22,24,25], 1-octin-3-ol,
77 3-chlor-1-phenyl-1-propanol, and trimethylsilylbutinol [22], methyl-3-hydroxy-2-methylpropionate
78 [26], and esters of secondary alcohols [27,28], to cite some. The advances in metagenomics
79 techniques and screening methods have allowed the discovery of these and other selective esterases
80 [29]. These studies exemplify that esterases with selective character occur naturally, and that their
81 chiral preference depend on structural factors in the proximity of the active-site. However, whether
82 the selective character of these esterases, and many others, is linked to a broad or a narrow substrate
83 spectrum has not been investigated, due to limited substrate sets employed.
84 Here we investigate the relationships between the level of substrate promiscuity and chiral
85 selectivity of a large set of 145 phylogenetically and environmentally diverse microbial esterases,
86 whose specific activity against 96 distinct esters that included 20 chiral esters have been recently
87 reported [5]. We provide unambiguous experimental evidences suggesting a negative association
88 between substrate specificity and chiral selectivity in native esterases.

89 **2. Results and Discussions**

90 *2.1. Relationships between substrate promiscuity and chiral selectivity*

91 We have recently described an extensive analysis of the substrate spectra of 145
92 phylogenetically and environmentally diverse microbial esterases [5]. Experimental data on
93 substrate conversion (i.e., units g^{-1} or U g^{-1}) followed for 24 h, at pH 8.0 and 30ºC was reported for 96
94 distinct esters. They included esters with variation in size of acyl and alcohol groups and with

growing residues (aromatic, aliphatic, branched and unbranched), halogenated esters, sugar esters, lactones, an alkyl di-ester, and 20 chiral esters (including (*R*) and (*S*) enantiomers of menthyl acetate, N-benzyl-proline ethyl ester, methyl mandelate, ethyl 4-chloro-3-hydroxybutyrate, methyl 3-hydroxybutyrate, methyl 3-hydroxyvalerate, neomenthyl acetate, methyl and ethyl lactate, and pantolactone). By meaning of the partitioning coefficient (log P value), which reflects electronic and steric effects and hydrophobic and hydrophilic characteristics, the 96 esters do show a broad chemical and structural variability [5]. This chemical variability characterized also the chiral esters tested (Figure 1).

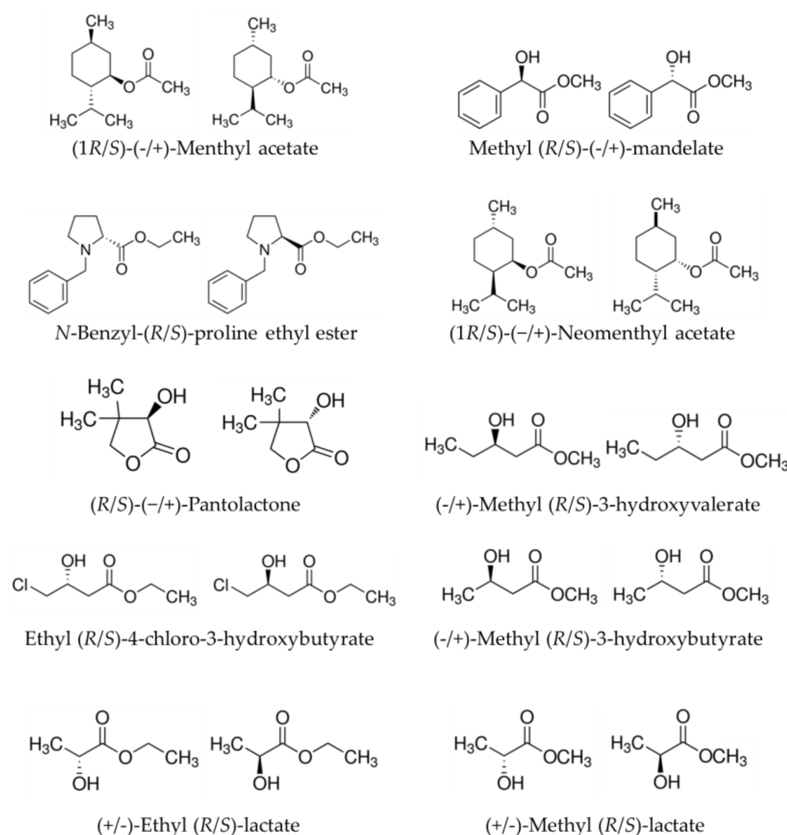


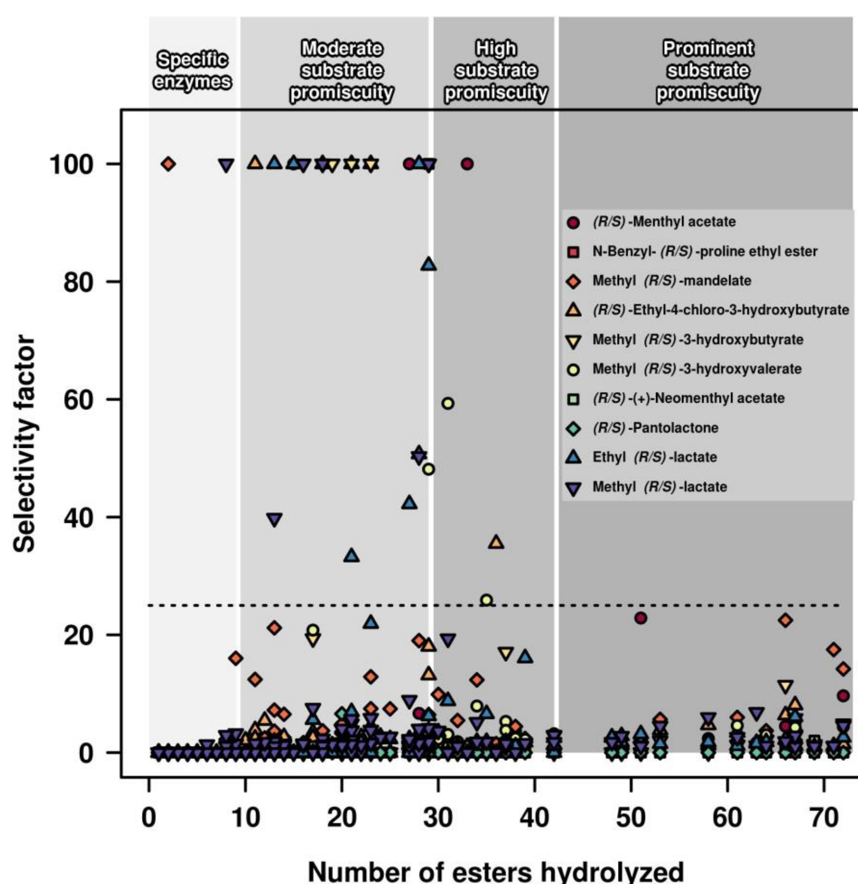
Figure 1 Representative chemical structures of 20 chiral esters used to evaluate chiral selectivity.

To find the relationships between substrate promiscuity and chiral selectivity we calculated the chiral selectivity factor for each of the 145 esterases and the 20 chiral esters tested (i.e. two enantiomers per pair) that were included in the 96-ester library (Figure 1). Selectivity factor was calculated as the ratio of specific activity (U g^{-1}) of the preferred over the non-preferred chiral ester when both esters were tested separately (see Materials and Methods). These calculations were extracted from datasets reported previously [5]. It should be mentioned that these apparent values may not correspond to true selectivity or enantiomeric factors calculated when the enzyme is confronted to a racemic mixture, because the rates of hydrolysis of the enantiomers were measured separately; nevertheless, recent studies have clearly demonstrated that apparent and true selectivity values closely match each other [15]. These values were plotted against the number of esters hydrolyzed by each of the esterases (Figure 2), previously reported for each of th esterases [5].

From the 145 esterases, 40 did not show appreciable activity under assay conditions for any of the chiral esters tested. From those being active against at least one of the chiral esters (105 in total), 80 esterases were characterized by selectivity factors below a threshold of 25. Although esterases with stringent selectivity are preferred, it is commonly considered that enzymes with selectivity factor of 25 or above begin to have commercial value [31]. On the other hand, we found 25 chiral selective esterases, as jugded by a selectivity factor above 25 (Figure 2; Figure 3). Ten of them

122 showed stringent selectivity, that is they were capable of hydrolyzing only one of the enantiomer
123 (Figure 3). Twelve of them were characterized by selectivity factors ranging from 25.9 to 59.3, and
124 three did show prominent selectivity factors ranging from 219 to 686 (Figure 2; Figure 3).

125 As shown in Figure 2, we found a negative association between the level of substrate
126 promiscuity, by meaning of the number of esters hydrolyzed, and the chiral selectivity factor. More
127 in details, according to criteria previously established [5] we considered an esterase specific if it
128 hydrolyses 9 esters or fewer, as moderate-to-highly promiscuous if it hydrolyses between 10 and 42
129 esters, and as prominently promiscuous if it hydrolyses 42 or more esters. None of the 25 hydrolases
130 which showed a selectivity factor ≥ 25 were prominently promiscuous. Rather, they were capable of
131 accepting 36 or fewer substrates. However, not all hydrolases converting 36 or fewer esters and
132 acting against chiral esters were selective according to the 25-selective factor threshold. Indeed, only
133 25 out of 85 in total (or 29%) were selective, with different selectivity factors and chiral preferences
134 (Figure 3). This is most likely due to the fact that the ability to selectively hydrolyze an enantiomer in
135 a racemate may depend on the topology of the catalytic environment [5].



136

137 **Figure 2** Chiral selectivity factor vs number of esters hydrolyzed per each of the 145 hydrolases
138 tested. Selectivity factor was calculated per each pair of enantiomers as the ratio of specific activity
139 (U g^{-1}) of the preferred over the non-preferred chiral ester when each of the chiral esters was tested
140 separately. Chiral esters are color coded. The value 100 was arbitrarily given to represent those
141 esterases capable of hydrolyzing, under our assay conditions, only one of the enantiomers (100%
142 selective) and those with selectivity factors higher than 100. These data are based on the data
143 reported previously [5], using conditions described in Materials and Methods. The level of
144 promiscuity, according to criteria previously established [5], is marked under a shadowed grey
145 background. The 25-selectivity factor threshold at which an esterase started to have commercial
146 value is indicated by a horizontal dashed gray line.

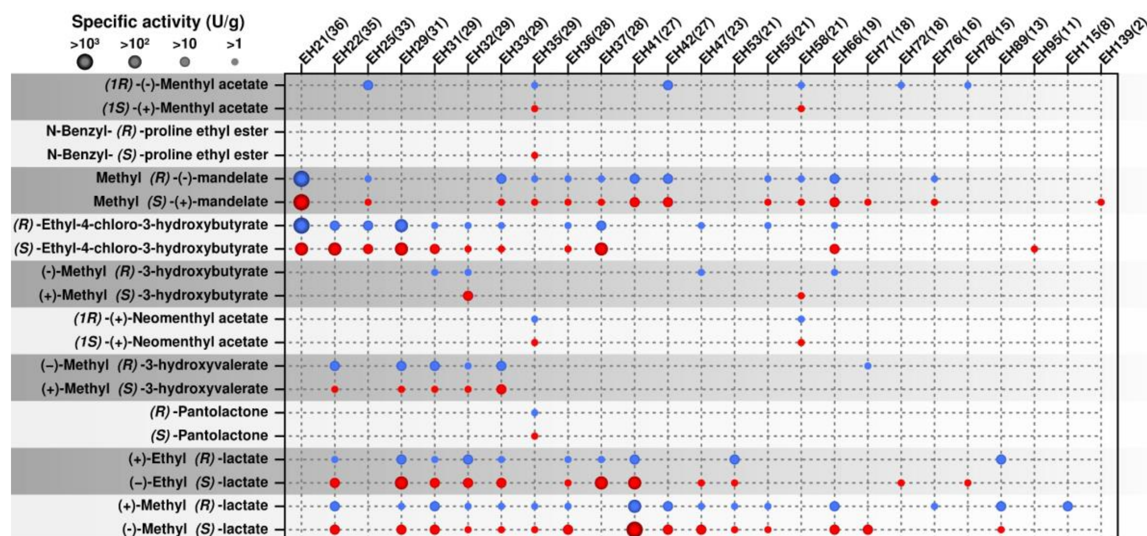


Figure 3 Chiral preferences of 25 hydrolases which were found to be selective for at least one chiral ester according to the 25-selective factor threshold. The figure illustrates the specific activity (U g^{-1}; represented by the size of the circles) of each esterase per each of the 20 chiral esters tested. The ID code for each esterase (for full description see ref. [5]) is shown on the top; the number of esters (out of 96 tested) hydrolyzed by each esterase is shown in brackets. The Figure was created with the R language console from data previously reported [5]. The list of the 20 chiral esters tested is shown on the left, with (*R*)-enantiomer in blue and (*S*)-enantiomer in red color. The protocol established and used to identify the esters hydrolyzed by each esterase is described in Materials and Methods.

2.2. Occurrence of multi selective esterases

Figure 3 summarizes the chiral preference of esterases fitting to the 25-selective factor threshold for each of the 20 chiral esters tested. As can be seen in Figure 3 esterases showed different level of promiscuity and preference for (*R*) or (*S*) methyl acetate, menthyl mandelate, methyl 3-hydroxybutyrate, N-benzyl-proline ethyl ester, ethyl-4-chloro-3-hydroxybutyrate, and (m)ethyl lactate. Esterases selective for (*R*) or (*S*)-pantolactone, N-benzyl-proline ethyl ester and neomenthyl acetate were the less abundant , suggesting these chiral esters are less preferred substrates. As shown in Figure 3, we also found that 5 out of 25 esterases fitting to the 25-selective threshold did show stringent selectivity or selectivity factor higher than 25 for several chiral esters differing in chemical and structural nature (Figure 3). They include one being selective for methyl 3-hydroxybutyrate (*R*-selective) and ethyl lactate (*S*-preference) (EH31), one for ethyl-4-chloro-3-hydroxybutyrate (*R*-selective), methyl 3-hydroxybutyrate (*R*-selective) and ethyl lactate (*S*-selective) (EH47), one for methyl mandelate (*S*-selective), methyl 3-hydroxyvalerate (*S*-selective) and methyl lactate (*S*-selective) (EH71), and two for menthyl acetate (*R*-selective) and methyl lactate (*S*-selective) (EH72 and EH78). The other 20 esterases did show the capacity to preferentially hydrolyze only one enantiomer (Figure 3). This suggests that multi selective esterases may have a lower abundancy.

3. Materials and Methods

3.1. Source of chemicals, enzymes and datasets

All chemicals for which activity data are reported were of the purest grade available and were purchased as reported [5]. The present study used datasets of hydrolytic activity (U g^{-1}) for 145 esterases assayed at 550 nm using 96-structurally diverse esters in 384-well plates. Reactions were followed for 24 h, at pH 8.0 and 30ºC. Datasets are available elsewhere [5].

179 *3.2. Selectivity factor calculation*

180 The chiral selectivity factor is defined as the ratio of the specific activity [30] for each
181 enantiomer, measured separately as described previously [5]. Briefly, reaction mixture contains 5
182 mM N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid buffer, pH 8.0, 4.5% (v/v) acetonitrile
183 or dimethyl sulfoxide, 0.45 mM Phenol Red (used as a pH indicator), a concentration of each of the
184 esters of 1.14 mg ml^{-1}, and 2 μg of proteins. Reactions were allowed to proceed kinetically at 30ºC
185 and hydrolytic activity (U g^{-1}) calculated followed for 24 h [5]. Selectivity factor was calculated
186 considering the preferred over the non-preferred chiral ester, whatever the preferred (*R*) or (*S*) ester.

187 **4. Conclusions**

188 Herein we show the value of the systematic investigation of enzyme activity to deepen our
189 understanding of the relationships between substrate promiscuity and chiral selectivity. By
190 comparing the number of esters that 145 diverse esterases hydrolyze as an indicator of the substrate
191 promiscuity level, and their selectivity factors as an indicator of enantio-selectivity, we found
192 unambiguous evidences that esterases with broad substrate spectra do commonly show low
193 selectivity for chiral molecules. In this study, the proportion of esterases with both prominent
194 promiscuity and selectivity approaches zero percent. By contrast, the proportion of esterases with
195 low to moderate promiscuity but prominent selectivity was as high as 29%. This suggests that the
196 substrate promiscuity may be used as an indicator of the selective character of esterases.
197 Promiscuous esterases acting against multiple substrates, while at the same time being
198 enantio-selective appear to be rare in nature, or at least in the habitats from where the esterases
199 herein described were isolated [5]. As these enzymes are of interest for application purposes [1-6,32],
200 protein engineering and rational design may be needed to obtain esterases being promiscuous and
201 selective for industrial applications. We anticipate that the possibility to transform a promiscuous
202 but not selective esterase into an efficient enantio-selective biocatalyst would require less
203 engineering effort because increasing the selectivity for an enantiomer may involve a reduced
204 number of contacts close to the active sites [for a recent example see ref. 33]. Opposite, increasing the
205 substrate spectra of a selective non-promiscuous esterase would require large rearrangement of the
206 catalytic environment which may, at the same time, result in significant reduction or even loss of
207 enantioselectivity. This is because non-promiscuous esterases are characterized by catalytic
208 environments that are highly exposed and have small volumes, while an esterase for being
209 promiscuous requires a large active site volume and lower relative solvent accessible surface area
210 [5], that are difficult to be designed through few mutations.

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226 G.E.K.B., A.B., R.S., I.H.S., O.V.G., M.M.Y., K-E.J. and A.F.Y. contributed to enzyme collection and data
227 analysis; M.F. drafted the manuscript which was revised by all co-authors.

228 **Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the
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231 **References**

232 1. Martínez-Martínez, M.; Bargiela, R.; Ferrer, M. Metagenomics and the search for industrial enzymes. In:
233 *Biotechnology of Microbial Enzymes, 1st Edition*; Brahmachari, G..; Demain, A.L.; Adrio, J.L. eds.; Academic
234 Press, Chennai, India, **2015**; pp. 167-184.
235 2. Martínez-Martínez.; M.; Bargiela.; R.; Coscolín.; C.; Navarro-Fernández.; J.; Golyshin.; P.N.; Ferrer.; M.
236 Functionalization and modification of hydrocarbon-like molecules guided by metagenomics: enzymes
237 most requested at the industrial scale for chemical synthesis as study cases. In *Consequences of Microbial*
238 *Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals;* Lee.; S.Y..; Ed.; Springer
239 International Publishing AG, Switzerland, **2016**; pp. 1-26.
240 3. Yarza, P.; Yilmaz, P.; Pruesse, E.; Glöckner, F.O.; Ludwig, W.; Schleifer, K.H.; Whitman, W.B.; Euzéby, J.;
241 Amann, R.; Rosselló-Móra, R. Uniting the classification of cultured and uncultured bacteria and archaea
242 using 16S rRNA gene sequences. *Nat. Rev. Microbiol*. **2014**, *12*, 635-645.
243 4. Ferrer, M.; Martínez-Martínez, M.; Bargiela, R.; Streit, W.R.; Golyshina, O.V.; Golyshin, P.N. Estimating
244 the success of enzyme bioprospecting through metagenomics: current status and future trends. *Microb.*
245 *Biotechnol.* **2016**, *9*, 22-34.
246 5. Martínez-Martínez.; M.; Coscolín.; C.; Santiago.; G.; Chow.; J.; Stogios. P.; Bargiela.; R.; Gertler.; C.;
247 Navarro-Fernández.; J.; Bollinger.; A.; Thies.; S.; Méndez-García.; C.; Popovic.; A.; Brown.; G.;
248 Chernikova.; T. N.; García-Moyano.; A.; Bjerga.; G. E. K.; Pérez-García.; P.; Hai.; T.; Del Pozo.; M. V.;
249 Stokke.; R.; Steen.; I. H.; Cui.; H.; Xu.; X.; Nocek.; B.; Alcaide.; M.; Distaso.; M.; Mesa.; V.; Peláez.; A. I.;
250 Sánchez.; J.; Buchholz.; P. C. F.; Pleiss.; J.; Fernández-Guerra.; A. F.; Glöckner.; F. O.; Golyshina.; O. V.;
251 Yakimov.; M. M.; Savchenko.; A.; Jaeger.; K-E.; Yakunin.; A. F.; Streit.; W. R.; Golyshin.; P. N.; Guallar.; V.;
252 Ferrer.; M. Determinants and prediction of esterase substrate promiscuity patterns. *ACS Chem. Biol*. **2017**,
253 DOI 10.1021/acschembio.7b00996 [Epub ahead of print].
254 6. Schmid, A..; Dordick, J. S..; Hauer, B..; Kiener, A..; Wubbolts, M.; Witholt, B. Industrial biocatalysis today
255 and tomorrow. *Nature* **2001**, *409*, 258-268.
256 7. Ferrer, M.; Bargiela, R.; Martínez-Martínez, M.; Mir, J.; Koch, R.; Golyshina, O.V.; Golyshin, P.N.
257 Biodiversity for Biocatalysis: a review for α/β-hydrolases of the esterase-lipase superfamily as case of
258 Study. *Biocatal. Biotransform.* **2015**, *33*, 235-249.
259 8. Aranda, J.; Cerqueira, N.M.; Fernandes, P.A.; Roca, M.; Tuñon, I.; Ramos, M.J. *Biochemistry* **2014**, *53*,
260 5820-5829.
261 9. Elend, C.; Schmeisser, C.; Hoebenreich, H.; Steele, H.L.; Streit, W.R. Isolation and characterization of a
262 metagenome-derived and cold-active lipase with high stereospecificity for (R)-ibuprofen esters. *J.*
263 *Biotechnol.* **2007**, *130*, 370-377.
264 10. Chow, J.; Kovacic, F.; Dall Antonia, Y.; Krauss, U.; Fersini, F.; Schmeisser, C.; Lauinger, B.; Bongen, P.;
265 Pietruszka, J.; Schmidt, M.; Menyes, I.; Bornscheuer, U.T.; Eckstein, M.; Thum, O.; Liese, A.;
266 Mueller-Dieckmann, J.; Jaeger, K.E.; Streit, W.R. The Metagenome-derived enzymes LipS and LipT
267 increase the diversity of known lipases. *PLoS One* **2012**, *7*, E47665.
268 11. Kim, Y.J.; Choi, G.S.; Kim, S.B.; Yoon, G.S.; Kim, Y.S.; Ryu, Y.W. Screening and characterization of a novel
269 esterase from a metagenomic library. *Protein Expr. Purif.* **2006**, *45*, 315-323.
270 12. Yoon, S.; Kim, S.; Ryu, Y.; Kim, T.D. Identification and characterization of a novel (S)-ketoprofen-specific
271 esterase. *Int. J. Biol. Macromol*. **2007**, *41*, 1-7.
272 13. Ngo, T.D.; Ryu, B.H.; Ju, H.; Jang, E.J.; Kim, K.K.; Kim, T.D. Crystallographic analysis and biochemical
273 applications of a novel penicillin-binding protein/beta-lactamase homologue from a metagenomic library.
274 *Acta Crystallogr. D Biol. Crystallogr.* **2014**, *70*, 2455-2466.
275 14. Kim, J.; Seok, S.H.; Hong, E.; Yoo, T.H.; Seo, M.D.; Ryu, Y. Crystal structure and characterization of
276 esterase Est25 mutants reveal improved enantioselectivity toward (S)-ketoprofen ethyl ester. *Appl.*
277 *Microbiol. Biotechnol.* **2017**, *101*, 2333-2342.
278 15. Ferrer, M.; Golyshina, O.V.; Chernikova, T.N.; Khachane, A.N.; Martins dos Santos, V.A.P.; Yakimov,
279 M.M.; Timmis, K.N.; Golyshin, P.N. Microbial enzymes mined from the Urania deep-sea hypersaline
280 anoxic basin. *Chem. Biol*. **2005**, *12*, 895-904.

281 16. Kourist, R.; Hari Krishna, S.; Patel, J.S.; Bartnek, F.; Hitchman, T.S.; Weiner, D.P.; Bornscheuer, U.T. Identification of a metagenome-derived esterase with high enantioselectivity in the kinetic resolution of arylaliphatic tertiary alcohols. *Org. Biomol. Chem*. **2007**, *5*, 3310-3313.
284 17. Fernández-Álvaro, E.; Kourist, R.; Winter, J.; Böttcher, D.; Liebeton, K.; Naumer, C.; Eck, J.; Leggewie, C.; Jaeger, K.E.; Streit, W.; Bornscheuer, U.T. Enantioselective kinetic resolution of phenylalkyl carboxylic acids using metagenome-derived esterases. *Microb. Biotechnol.* **2010**, *3*, 59-64.
287 18. Ouyang, L.M.; Liu, J.Y.; Qiao, M.; Xu, J.H. Isolation and biochemical characterization of two novel metagenome-derived esterases. *Appl. Biochem. Biotechnol*. **2013**, *169*, 15-28.
289 19. Martini, V.; Glogauer, A.; Muller-Santos, M.; Lulek, J.; de Souza, E.; Mitchell, D.; Pedrosa, F.; Krieger, N.First co-expression of a lipase and its specific foldase obtained by metagenomics. *Microb. Cell. Fact*. **2014**, *13*, 171.
292 20. Alnoch, R.C.; Martini, V.P.; Glogauer, A.; Costa, A.C.; Piovan, L.; Muller-Santos, M.; de Souza, E.M.; de Oliveira Pedrosa, F.; Mitchell, D.A.; Krieger, N. Immobilization and characterization of a new regioselective and enantioselective lipase obtained from a metagenomic library. *PLoS One* **2015**, *10*, e0114945.
296 21. Jeon, J.H.; Kim, J.T.; Kang, S.G.; Lee, J.H.; Kim, S.J. Characterization and its potential application of two esterases derived from the arctic sediment metagenome. *Mar. Biotechnol*. **2009**, *11*, 307-316.
298 22. Elend, C.; Schmeisser, C.; Leggewie, C.; Babiak, P.; Carballeira, J.D.; Steele, H.L.; Reymond, J.L.; Jaeger, K.E.; Streit, W.R. Isolation and biochemical characterization of two novel metagenome-derived esterases. *Appl. Environ. Microbiol*. **2006**, 72, 3637-3645.
301 23. Martínez-Martínez, M.; Alcaide, M.; Tchigvintsev, A.; Reva, O.; Polaina, J.; Bargiela, R.; Guazzaroni, M-E.; Chicote, Á.; Canet, A.; Valero, F.; Rico Eguizabal, E.; Guerrero, Mdel C.; Yakunin, A.F.; Ferrer, M. Biochemical diversity of carboxyl esterases and lipases from Lake Arreo (Spain): a metagenomic approach. *Appl. Environ. Microbiol*. **2013**, *79*, 3553-3562.
305 24. Alcaide, M.; Tchigvintsev, A.; Martinez-Martinez, M.; Popovic, A.; Reva, O.N.; Lafraya, A.; Bargiela, R.; Nechitaylo, T.Y.; Matesanz, R.; Cambon-Bonavita, M.A.; Jebbar, M.; Yakimov, M.M.; Savchenko, A.; Golyshina, O.V.; Yakunin, A.F.; Golyshin, P.N.; Ferrer, M. Identification and characterization of carboxyl esterases of gill chamber-associated microbiota in the deep-sea shrimp *Rimicaris exoculata* by using functional metagenomics. *Appl. Environ. Microbiol*. **2015**, *81*, 2125-2136.
310 25. Placido, A.; Hai, T.; Ferrer, M.; Chernikova, T.N.; Distaso, M.; Armstrong, D.; Yakunin, A.F.; Toshchakov, S.V.; Yakimov, M.M.; Kublanov, I.V.; Golyshina, O.V.; Pesole, G.; Ceci, L.R.; Golyshin, P.N. Diversity of hydrolases from hydrothermal vent sediments of the Levante Bay.; Vulcano Island (Aeolian archipelago) identified by activity-based metagenomics and biochemical characterization of new esterases and an arabinopyranosidase. *Appl. Microbiol. Biotechnol*. **2015**, *99*, 10031-10046.
315 26. Lee, H.W.; Jung, W.K.; Kim, Y.H.; Ryu, B.H.; Kim, T.D.; Kim, J.; Kim, H. Characterization of a novel alkaline family viii esterase with s-enantiomer preference from a compost metagenomic library. *J. Microbiol. Biotechnol.* **2016**, *26*, 315-325.
318 27. Gao, W.; Fan, H.; Chen, L.; Wang, H.; Wei, D. Efficient kinetic resolution of secondary alcohols using an organic solvent-tolerant esterase in non-aqueous medium. *Biotechnol. Lett*. **2016**, *38*, 1165-1171.
320 28. Kumar, R.; Banoth, L.; Banerjee, U.C.; Kaur, J. Enantiomeric separation of pharmaceutically important drug intermediates using a metagenomic lipase and optimization of its large scale production. *Int. J. Biol. Macromol.* **2017**, *95*, 995-1003.
323 29. Böttcher, D.; Zägel, P.; Schmidt, M.; Bornscheuer, U.T. A microtiter plate-based assay to screen for active and stereoselective hydrolytic enzymes in enzyme libraries. *Methods Mol. Biol.* **2017**, *1539*, 197-204.
325 30. Gawley, R.E. Do the terms "% ee" and "% de" make sense as expressions of stereoisomer composition or stereoselectivity? *J. Org. Chem*. **2006**, *71*, 2411-2416.
327 31. Reetz, M.T. Introduction to directed evolution. In *Directed evolution of selective enzymes: catalysts for organic chemistry and biotechnology;* Reetz, M.T. Ed.; Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, **2016**; pp. 1-16
330 32. Romano, D.; Bonomi, F.; de Mattos, M.C.; de Sousa Fonseca, T.; de Oliveira Mda, C.; Molinari, F. Esterases as stereoselective biocatalysts. *Biotechnol. Adv*. **2015**, 33, 547-565.
332 33. Wikmark, Y.; Svedendahl Humble, M.; Bäckvall, J.E. Combinatorial library based engineering of *Candida antarctica* lipase A for enantioselective transacylation of sec-alcohols in organic solvent. *Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 4284-4288.

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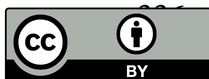

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