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Enzymatic kinetic resolution of aliphatic *sec*-alcohols by LipG9, a metagenomic lipase

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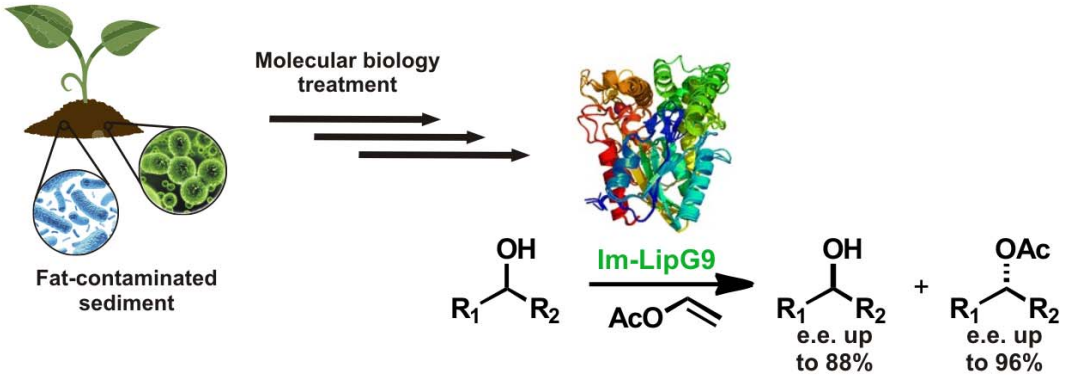
Graphical abstract (was substituted by this)



Highlights

- Metagenomics is a useful technique for finding new biocatalysts
- LipG9 was successful employed in enzymatic kinetic resolution of aliphatic alcohols
- LipG9 showed activity and selectivity comparable to commercially available enzymes for the explored reactions

ABSTRACT

Bioprospection for new enantioselective enzymes for application in organic synthesis is a prominent area of investigation in biocatalysis. In this context, here we present the evaluation of an immobilized lipase isolated from a metagenomic library (LipG9) for the enzymatic kinetic resolution (EKR) of aliphatic *sec*-alcohols, which are still challenging substrates, since low enantioselectivity values are usually observed for these resolutions. LipG9 was successfully employed in EKR of aliphatic alcohols, which were resolved with satisfactory conversions (19-59%) and enantiomeric excesses for alcohols (26-88%) and esters (30-96%) by

transesterification reactions, demonstrating that its performance is equal to or better than commercially available enzymes for the same reaction.

Keywords

Lipases, metagenomic, aliphatic alcohols, kinetic resolution

1. Introduction

Biocatalysis focused on organic synthesis is already a well-established practice in academy and industry [1-8]. For every synthetic transformation employing a conventional organic method, it is possible to propose an enzymatic approach as an alternative. In a synthetic route, the coupling between chemical and enzymatic (chemoenzymatic) processes is practicable [9,10]. The use of enzymes in organic synthesis implies using biodegradable catalysts that are isolated from renewable sources (plants, animals and microorganisms), since these are important properties for biotechnological applications. Among the enzymes that have been successfully used in biocatalysis are the lipases (glycerol ester hydrolases EC 3.1.1.3), which, *in vivo* can hydrolyze acyl esters in aqueous-lipid interfaces [11,12]. The great success of lipases in organic synthesis has several reasons, including no requirement for coenzymes; applications for a very broad range of synthetic substrates; and activity and stability in non-aqueous media such as organic solvents [13], ionic liquids [14,15] and supercritical fluids [16]. Furthermore, lipases usually exhibit high level of regio-, chemo- and enantioselectivity, making them valuable reagents for preparation of optically active compounds [17-22].

Kinetic resolution of racemates is the most widely used lipase-mediated reaction for preparation of optically active compounds [23]. Considering that the structural variability of synthetic substrates is much higher than that of the available commercial enzymes, bioprospection for new lipases has become a prominent area of investigation in biocatalysis. The traditional methods to isolate lipases rely on laboratory cultivation of microorganisms. This approach has a major limitation, given that only a small portion of such microorganisms (about 1%, in terms of prokaryotic genomes) can be cultivated under artificial conditions [24-26]. Therefore, the great majority of microorganisms (and consequently their enzymes) remain inaccessible and unknown. Besides this, many microorganisms can be harmful to health (e.g., pathogenic fungi and bacteria), so their manipulation requires special containment conditions.

In this context, a microorganism-independent culture technique that overcomes these limitations is a metagenomic approach [27,28]. In recent years, several enzymes have been isolated from metagenomic libraries, such as esterases [29-31], epoxy-hydrolases [32], celulases [33], proteases [34], nitrilases [35], beta-glucosidases [36], decarboxylases [37], oxirredutases [38], amidases e peptidases [39], deoxyribonucleases [40] and lipases [41-44]. Although several lipases [41-44] have been isolated from metagenomic libraries, few reports describe their application in organic synthesis focusing on the preparation of optically active compounds. To the best of our knowledge, there are few enzymatic kinetic resolutions [49-52] and syntheses of esters and biodiesel [42,49] mediated by metagenomic lipases.

In this paper, we report the evaluation of an immobilized metagenomic lipase, LipG9, in EKR of aliphatic alcohols. LipG9 is a 55 kDa protein, which was isolated from a metagenomic library constructed from a fat-contaminated sediment collected from a wastewater treatment plant [42,48]. The immobilized form, Im-LipG9, is thermostable (up to 60 °C) and active in different organic solvents, and showed maximum specific hydrolytic activity of 66 U mg^{-1} of protein employing triolein as substrate in *n*-heptane. A previous EKR assay [48] demonstrated

that LipG9 has excellent enantioselectivity (E > 200) in the transesterification reaction of 1-phenylethanol and the hydrolysis of 1-phenylethyl acetate, proving to be a promising biocatalyst for organic synthesis.

To increase the range of synthetic substrates, we evaluated Im-LipG9 in EKR of aliphatic *sec*-alcohols, a versatile class of organic compounds useful as synthetic intermediates in asymmetric synthesis for the preparation of anti-Alzheimer drugs [49], agrochemicals [50] and liquid crystals [51]. In spite of their applicability in organic synthesis, the preparation of enantioenriched aliphatic alcohols by biocatalysis remains a challenge since low values of enantioselectivity are usually obtained in EKR of this class of substrates [52].

2. Experimental data

2.1 Materials

Unless otherwise stated, commercially available materials were used without further purification. All solvents were analytical grade. Analytical thin-layer chromatography (TLC) analyses were performed by using aluminum-backed silica plates coated with a 0.25 mm layer of silica gel 60 F254 (Merck, Darmstadt, HE, Germany), visualized with an ultraviolet light (λ = 254 nm), followed by exposure to a vanillin solution and mild heating. Standard chromatographic purification methods were followed using 35-70 mm (220-440 mesh) silica gel (Sigma-Aldrich, MO, USA). ^{1}H (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded with a Bruker DPX 200 spectrometer (Bruker, Massachusetts, USA). The chemical shifts of ^{1}H and ^{13}C NMR were assigned to internal $CDCl_3$ (δ_H = 7.26 ppm), tetramethylsilane (δ_{TMS} = 0.00 ppm) or $CDCl_3$ (δ_C = 77.0 ppm). ^{1}H NMR spectra were recorded at 200 MHz frequency and the data are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sext = sextet, m = multiplet, brs = broad singlet), coupling constant (*J*) in Hertz and relative intensity. ^{13}C NMR spectra were recorded at 50 MHz frequency and data are reported as chemical shift in ppm (δ). Infrared spectra were recorded from KBr discs by FTIR with Bomem MB100 spectrometer (ABB Bomem, Zurich, Switzerland) with internal reference. Maximum absorption (ν_{max}) is reported in wavenumbers (cm^{-1}). Enantioselectivity parameters (ee, enantiomeric excess and E, enantioselectivity coefficient) were determined according to Chen [53] et al. by chiral GC analyses, which were performed with a GC-17A chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a hydrogen flame ionization detector and a CP Chirasil-DEX CB chiral column (25 m × 0.25 mm diameter, 0.25 μm film thickness). One μL of the samples was injected with a split ratio of 1:50, using N_2 as the carrier gas. The injector and detector were set at 220 °C. The temperature programming for all compounds was: 40 °C (1 min) to 100 °C – gradient rate: 2 °C min^{-1}. Aliphatic alcohols **1a-6a** did not show resolution in the chiral chromatography column used, requiring derivatization to respective propionates (**1c-6c**). The absolute configurations of compounds were attributed by comparison with the data available in the literature [52-56]. The retention times (t_R/min) were as follows for acetates (**1b-6b**) and propionates (**1c-6c**): (*R*)-**1b**: 9.91; (*S*)-**1b**: 8.38; (*R*)-**1c**: 13.38; (*S*)-**1c**: 12.25 (*R*)-**2b**: 15.19; (*S*)-**2b**: 12.95; (*R*)-**2c**: 18.91; (*S*)-**2c**: 17.63; (*R*)-**3b**: 20.79; (*S*)-**3b**: 18.94; (*R*)-**3c**: 25.10; (*S*)-**3c**: 23.99; (*R*)-**4b**: 27.12; (*S*)-**4b**: 25.46; (*R*)-**4c**: 31.50; (*S*)-**4c**: 30.63; (*R*)-**5b**: 11.61; (*S*)-**5b**: 10.38; (*R*)-**5c**: 15.32; (*S*)-**5c**: 14.60; (*R*)-**6b**: 13.53; (*S*)-**6b**: 12.77; (*R*)-**6c**: 17.80; (*S*)-**6c**: 17.28.

2.2 General procedure for synthesis of alcohols 1a-6a

To a solution of appropriated commercial ketone (50 mmol) in methanol (50 mL) at 0 °C, $NaBH_4$ (1.93 g, 51 mmol) was added in portions. In the sequence, the reaction was carried out under vigorous stirring at room temperature for 2h – 6h. Then the methanol was removed by simple distillation, distilled water was added to the white residue and the pH was adjusted to pH 6.0 with aqueous HCl solution 1 mol L^{-1}. The organic phase was extracted with dichloromethane (3 x 20 mL), dried over anhydrous magnesium sulphate, filtered off and concentrated by simple distillation, leading to racemic alcohols **1a-6a**.

Pentan-2-ol (**1a**): Colorless liquid, bp 119 °C, yield 85%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.91 – 1.11 (m, 3H), 1.28 (d, *J* = 6.2 Hz, 3H), 1.39 – 1.53 (m, 4H), 1.80 (brs, 1H), 3.73 – 3.80 (m, 1H). ^{13}C NMR (50 MHz, CDCl3), δ (ppm): 13.9, 18.8, 23.4, 41.5, 67.7; IR (cm^{-1}): 3341, 2959, 2922, 1467 and 1363.

Hexan-2-ol (**2a**): Colorless liquid, bp 138 °C, yield 95%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.84 – 0.99 (m, 3H), 1.18 (d, *J* = 6.2 Hz, 3H), 1.24 – 1.52 (m, 5H), 1.89 (brs, 1H), 3.79 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 22.6, 23.3, 27.9, 38.9, 68.0; IR (cm^{-1}): 3348, 2966, 2922, 2856, 1467, 1371 and 1113.

Heptan-2-ol (**3a**): Colorless liquid, bp 159 °C, yield 91%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89 – 1.39 (m, 14H), 1.73 (brs, 1H), 3.78 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 22.6, 23.4, 25.4, 31.8, 39.3, 68.1; IR (cm^{-1}): 3328, 2966, 2922, 2856, 1467 and 1371.

Octan-2-ol (**4a**): Colorless liquid, bp 174 °C, yield 89%. ^{1}H NMR (200 MHz, CDCl3, TMS), δ (ppm): 0.89 (s, 3H), 1.20 (d, *J* = 6.2 Hz, 3H), 1.22 (brs, 1H), 1.23 – 1.49 (m, 10H), 3.78 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 14.0, 22.6, 23.4, 25.7, 29.3, 31.8, 39.3, 68.1; IR (cm^{-1}): 3355, 2973, 2929, 2863, 1467, 1378 and 1327.

4-Methyl-pentan-2-ol (**5a**): Colorless liquid, bp 132 °C, yield 94%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (d, *J* = 6.6 Hz, 6H), 1.20 (d, *J* = 3.2 Hz, 3H), 1.33 – 1.51 (m, 1H), 1.57 (brs, 1H), 1.63 – 1.86 (m, 1H), 3.79 – 3.98 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 22.3, 23.1, 23.9, 24.8, 48.6, 66.1; IR (cm^{-1}): 3319, 2959, 2871, 1467, 1392, and 1327.

Hexan-3-ol (**6a**): Colorless liquid, bp 135 °C, yield 92%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (t, *J* = 7.2 Hz, 3H), 0.93 (t, *J* = 6.9 Hz, 3H), 1.31 – 1.56 (m, 6H), 1.74 (brs, 1H), 3.44 – 3.61 (m, 1H). 13C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.8, 14.1, 18.8, 30.1, 39.1, 73.0; IR (cm^{-1}): 3348, 2966, 2944, 2871, 1459 and 1319.

2.3 General procedure for synthesis of acetates 1b-6b

To a solution of appropriated alcohol **1a-6a** (25 mmol) in pyridine (15 mL), acetic anhydride (5.1 g, 50 mmol) was added. After stirring overnight at room temperature, ethyl acetate was added (20 mL) and the mixture was repeatedly washed with aqueous saturated solution of $CuSO_4$ until complete removal of pyridine. The organic phase was then isolated, dried over anhydrous magnesium sulfate, filtered off and concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel (*n*-hexane: ethyl acetate 9:1) to give acetates **1b-6b** in 58-84% yields.

2-Pentyl acetate (**1b**): Colorless oil, bp 134 °C, yield 58%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.92 (t, *J* = 7.1 Hz, 3H), 1.21 (d, *J* = 6.2 Hz, 3H), 1.25 – 1.68 (m, 10H), 2.04 (s, 3H), 4.83 – 4.99 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.8, 18.5, 19.8, 21.2, 30.0, 70.7, 170.7; IR (cm^{-1}): 2959, 1738, 1467, 1378 and 1238.

2-Hexyl acetate (**2b**): Pale yellow oil, bp 154 °C, yield 64%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.86 – 0.93 (m, 3H), 1.20 (d, *J* = 6.2 Hz, 3H), 1.25 – 1.33 (m, 4H), 1.42 – 1.63 (m, 2H), 2.03 (s, 3H), 4.89 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 19.8, 21.2, 22.4, 27.5, 35.6, 71.0, 170.7. IR (cm^{-1}): 2956, 1738, 1378, 1245 and 1018.

2-Heptyl acetate (**3b**): Yellow oil, bp 172 °C, yield 78%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.88 (t, *J* = 6.2 Hz, 3H), 1.20 (d, *J* = 6.2 Hz, 3H), 1.20 – 1.60 (m, 8H), 2.02 (s, 3H), 4.81 – 4.97 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 19.8, 21.3, 22.5, 25.0, 31.6, 35.8, 70.9, 170.6; IR (cm^{-1}): 2930, 1738, 1459, 1378, 1246 and 1121.

2-Octyl acetate (**4b**): Yellow oil, bp 195 °C, yield 84%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89 (t, *J* = 6.4 Hz, 3H), 1.21 (d, *J* = 6.2 Hz, 3H), 1.25 – 1.68 (m, 10H), 2.04 (s, 3H), 4.82 – 4.98 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 13.9, 19.8, 21.2, 22.5, 25.3, 29.0, 31.6, 71.0, 170.6; IR (cm^{-1}): 2929, 1738, 1467, 1371 and 1238.

4-Methyl-2-pentyl acetate (**5b**): Pale yellow oil, bp 147 °C, yield 81%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.88 – 0.91 (m, 6H), 1.19 (d, *J* = 6.2 Hz, 3H), 1.46 – 1.72 (m, 3H), 2.01 (s, 3H), 4.88 – 5.10 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 20.3, 21.2, 22.2, 22.7, 24.6, 45.0, 69.3, 170.6; IR (cm^{-1}): 2958, 1739, 1454, 1370 and 1232.

3-Hexyl acetate (**6b**): Pale yellow oil, bp 150 °C, yield 64%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.85 – 0.97 (m, 7H), 1.26 – 1.64 (m, 5H), 2.06 (s, 3H), 4.78 – 4.90 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.5, 13.9, 18.5, 21.1, 26.9, 35.7, 75.2, 170.9; IR (cm^{-1}): 2966, 1738, 1238, 907 and 738.

2.4 General procedure for synthesis of propionates 1c-6c

To solution of appropriated alcohol **1a-6a** (1 mmol) in dichloromethane (2 mL), propanoic anhydride (0.25 mL, 2 mmol) and *N*,*N*-dimethylaminopyridine (10 mg) were added. After vigorous stirring at room temperature for 10 minutes, the mixture was neutralized with portions of aqueous saturated solution of $NaHCO_3$. The organic phase was isolated, dried over anhydrous magnesium sulfate, filtered off and concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel (eluent: dichloromethane) to give propionates **1c-6c** in 73-87% yields.

2-Pentyl propionate (**1c**): Colorless liquid, yield 76%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.91 (t, *J* = 6.6 Hz, 3H) 1.13 (t, *J* = 7.5 Hz, 3H), 1.20 (d, *J* = 6.2 Hz, 3H), 1.25 – 1.68 (m, 4H), 2.29 (q, *J* = 7.5 Hz, 2H), 4.92 (sext, *J* = 6,2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.2, 13.8, 18.6, 19.9, 27.9, 38.1, 70.5 and 174.1. IV (cm^{-1}): 1194, 1459, 1731, 2871 and 2966.

2-Hexyl propionante (**2c**): Colorless oil, yield 81%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89 (t, *J* = 6.6 Hz , 3H), 1.13 (t, *J* = 7.5 Hz, 3H), 1.19 (d, *J* = 6.2 Hz,

3H), 1.23 – 1.66 (m, 7H), 2.29 (q, *J* = 7.5 Hz, 2H), 4.90 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.2, 13.9, 19.9, 22.5, 27.5, 27.9, 35.6, 70.7 and 174.1. IV (cm^{-1}): 1194, 1467, 1731, 2856, 2929 and 2966.

2-Heptyl propionate (**3c**): Yellow oil, yield 78%. ^{1}H NMR (200 MHz, CDCl3, TMS), δ (ppm): 0.89 (t, *J* = 6.3 Hz, 3H), 1.14 (t, *J* = 7.5 Hz, 3H), 1.21 (d, 6.2, 3H), 1.24 – 1.73 (m, 8H), 2.30 (q, 7.5, 2H), 4.91 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.1, 13.9, 19.9, 22.5, 24.9, 27.9, 31.6, 35.8, 70.8 and 174.1. IV (cm^{-1}): 1121, 1194, 1459, 1731, 2853 and 2930.

2-Octyl propionate (**4c**): Yellow oil, yield 87%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89 (t, *J* = 6.3 Hz, 3H), 1.14 (t, *J* = 7.5 Hz, 2H), 1.21 (d, *J* = 6.2 Hz, 3H), 1.24 – 1.70 (m, 10H), 2.30 (q, *J* = 7.5 Hz, 2H) 4.91 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.1, 13.9, 19.9, 22.5, 25.3, 27.9, 29.0, 31.7, 35.9, 70.7 and 174.1. IV (cm^{-1}): 1187, 1459, 1731, 2856 and 2936.

4-Methyl-2-pentyl propionate (**5c**): Colorless oil, yield 82%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.89 (d, *J* = 4.5 Hz, 6H), 1.13 (t, 7.5, 3H), 1.19 (d, 6.2, 3H), 1.25-1.57 (m, 3H), 2.29 (q, *J* = 7.5 Hz, 2H), 5.00 (sext, *J* = 6.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 9.1, 20.4, 22.3, 22.8, 24.7, 45.1, 69.1 and 174.0. IV (cm^{-1}): 1194, 1467, 1731, 2863 and 2951.

3-Hexyl propionate (**6c**): Colorless oil, yield 73%. ^{1}H NMR (200 MHz, $CDCl_3$, TMS), δ (ppm): 0.87 (t, *J* = 7.6 Hz, 3H), 0.90 (t, *J* =7.3 Hz, 3H), 1.14 (t, *J* = 7.6, 3H), 1.32 – 1.67 (m, 6H), 2.31 (q, *J* = 7.6, 2H), 4.83 (quint, *J* = 6.2, 1H). ^{13}C RMN (50 MHz, $CDCl_3$), δ (ppm): 9.2, 9.4, 13.8, 18.5, 26.9, 27.8, 35.7, 74.9 and 174.2. IV (cm^{-1}): 1194, 1459, 1731, 2871 and 2966.

2.5 General procedure for enzymatic kinetic resolution by transesterification reactions

To a solution of appropriated alcohol **1a-6a** (0.1 mmol) in *n*-hexane (2 mL), vinyl acetate (34 mg, 0.4 mmol) and 50 mg (corresponding to 11 U of activity) of immobilized enzyme (LipG9) were added and the was reaction carried out at controlled temperature and 150 rpm. The progress of the reaction was monitored by GC chiral analysis.

2.6 Lipase production and immobilization

The expression of LipG9 was done according to Martini [42] et al. The purified LipG9 was immobilized by physical adsorption on the polypropylene Accurel MP 1000, according to Alnoch [48] et al.

2.7 Lipase activity assay

The hydrolytic activity of Im-LipG9 was determined from spectrophotometric quantification of oleic acid resulting from the hydrolysis of triolein in *n*-heptane. In a 125 mL Erlenmeyer flask, triolein (1.4 mmol, 1.23 g), and *n*-heptane (4.9 mL), distilled water (2% v/v) and LipG9 (20 mg) were added. The mixture was incubated in an orbital shaker at 200 rpm and 40 °C. The progress of the hydrolysis reaction was monitored by determination of fatty acids using the Lowry-Tinsley method [57]. One unit of activity

(U) corresponded to the production of 1 μmol of fatty acid per minute, under the assay conditions.

3. Results and discussion

3.1 Syntheses of substrates

The applicability of a novel Im-LipG9 in organic synthesis was first evaluated by its performance in transesterification reactions of aliphatic alcohols (**1a-6a**). Enzymatic kinetic resolution (EKR) is already a well-established synthetic method especially for secondary aromatic alcohols. Nonetheless, EKR of secondary aliphatic alcohols is more difficult and low enantiomeric excesses are frequently observed [52]. Since Im-LipG9 was previously evaluated in EKR of 1-phenylethanol with high performance [48], we decided to expand its application to aliphatic alcohols. Chemical reduction of commercial ketones **1-6** with sodium borohydride led to aliphatic alcohols **1a-6a** in 85-95% yields, which were subsequently used as starting materials to produce the respective esters **1b-6b** employing chemical acetylation with acetic anhydride (Ac_2O) in 58-84% yields (Scheme 1).

3.2 Enzymatic kinetic resolution of racemic alcohols in lipase-catalysed transesterification

The initial exploratory transesterification assays were carried out employing 50 mg of Im-LipG9, which corresponds to 11 U of activity in triolein hydrolysis reaction, *n*-hexanes as solvent, and vinyl acetate as acyl donor at 35 °C [48] (Table 1). The initial screening already demonstrated the potential applicability of Im-LipG9 in organic synthesis, since its performance was superior to the majority of those results reported in literature. Im-LipG9 mediated EKR of (*R*,*S*)-pentan-2-ol (**1a**) with high conversion (49%) and enantiomeric excess for (*S*)-**1a** (81%) and (*R*)-**1b** (88%, Table 1, entry 1) after 24 hours, resulting in an enantiomeric ratio (E) equal to 39. These results are better than those described in the literature: *Pseudomonas cepacia* (Amano PS) lipase-catalysed EKR of **1a** reaction led to (*S*)-**1a** with just 20% ee, while commercially available lipases from porcine pancreatic (PPL), *Penicillium* sp., *Pseudomonas* sp. and *Mucor* sp. led to racemic ester **1b** [54]. In view of these results, we decided to expand the scope of substrates by varying the R_2 position with different alkyl chains, in order to investigate Im-LipG9 in EKR of a series of more challenging aliphatic alcohols **2a-6a**, (Table 1, entries 2-6).

Our results confirmed the difficulty of applying EKR to those compounds and reinforce the relevance of bioprospecting for new enzymes or reaction conditions for that class of alcohols. When EKR reactions of alcohols **2a-6a** were compared with EKR of **1a** in Im-LipG9-mediated transesterification reactions, the influence of alkyl chain size (at R_2 position) was noticeable. When (*R*,*S*)-hexan-2-ol (**2a**) was used as substrate, despite high conversion (54%), ee of ester (*R*)-**2b** decreased from 88 to 74% and the enantiomeric ratio also decreased (from 39 to 19). However, in the literature, EKR of **2a** catalyzed by the commercial lipase from *P. cepacia* (Amano Pharmaceutical Co. Ltd.) also presented low enantiomeric ratio (E = 13 in *n*-hexane and E = 4 in 2,2-

dimethylbutane as solvent) [55]. On the other hand, for the EKR of **2a** mediated by *P. fluorescens* lipase (Amano AK) immobilized in sol-gel, the enantiomeric ratio was high (E>200) but the conversion was only 24% after 24 hours [56].

The negative influence of a long alkyl chain at R_2 position on the enantiomeric ratio can also be seen for EKR of aliphatic racemic alcohols **3a** and **4a**. (*R,S*)-heptan-2-ol (**3a**) and (*R,S*)-octan-2-ol (**4a**) in LipG9-mediated reactions presented 52% and 53% conversions, respectively, after 24 hours (Table 1, entries 3 and 4), but the enantiomeric ratios were low (E = 4 and E = 3, respectively) for both compounds. These results indicate a possible unfavorable fitting of a bigger aliphatic chain (*n*-pentyl and *n*-hexyl for alcohols **3a** and **4a**, respectively) attached to the chirality center at the catalytic site of Im-LipG9, which shows the dependence between the structure of the substrate and the enantioselectivity of lipase.

Transesterification reactions of **3a** and **4a** catalyzed by commercial lipase from *P. fluorescens* (Amano Enzyme Inc.) immobilized in sol-gel also presented low enantiomeric ratio (E = 11 and E = 4, respectively) [56], demonstrating the agreement of our results with those in the literature. Furthermore, for EKR of (*R,S*)-heptan-2-ol (**3a**) catalyzed by lipases from porcine pancreas (PPL, Sigma Chemicals) and *Candida antarctica* (CAL, Fluka 62299), the conversions were only 2% and 6% after 24 hours, respectively, and low enantiomeric ratios were also observed (E = 13 and E = 2.2). For EKR of **3a** catalyzed by *Candida cylindracea* lipase (CCL, Fluka 62316) and pig liver esterase (immobilized on Eupergit C, PLE, Fluka 46064), conversions were 56% and 24% after 24 hours, respectively, but these commercial enzymes were not enantioselective for these substrates (E = 1.2 for both lipases). The lipases from *Penicillium roqueforti* (PRL, Fluka 62308) and *Aspergillus oryzae* (AOL, Fluka 62285) almost did not catalyze the EKR of **3a** (conversions were 1% and 0.1%, respectively) [57].

The contribution of the structural design to the low stereoselectivity parameters could be verified in EKR of the (*R,S*)-4-methyl-pentan-2-ol (**5a**), an alcohol with a branched chain at the R_2 position. EKR of **5a** mediated by Im-LipG9 led to (*R*)-**5b** with 96% ee and E=63 (Table 1, entry 5). The comparison between EKR of **2a** and **5a** reinforce the hypothesis of the structural influence of the substrate on Im-LipG9 selectivity. Both alcohols contain a butyl moiety at the R_2 position, but for an *n*-butyl chain (alcohol **2a**), poor stereoselectivity parameters were observed. On the other hand, in the EKR of **5a**, which contains a branched *sec*-butyl chain at R_2, higher stereoselectivity parameters were obtained. The EKR of **5a** catalyzed by the commercial immobilized lipase from *Candida antarctica B* (CALB, Novozymes) presented high enantiomeric ratio (E>200) and conversion (c = 50 %) [52].

For Im-LipG9-mediated transesterification reaction of (*R,S*)-hexan-3-ol (**6a**), although high conversion was obtained (59%, Table 1, entry 6), both enantiomeric ratio and ee were low, which can be explained by the small difference in the steric bulk of the groups R_1 and R_2 (ethyl and *n*-propyl, respectively) attached to the stereogenic center in **6a**. We did not find any reports about the EKR of alcohol **6a**.

3.3 Effect of reaction medium on LipG9-catalyzed transesterification

In an attempt to improve the enantioselectivity of Im-LipG9, the influence of acyl donors, organic solvents and temperature in the EKR of alcohol **1a** was investigated. The EKR employing isopropenyl acetate and ethyl acetate as acylating agents presented lower conversion rates (36% and 8%, respectively) and enantiomeric ratios (E = 22 and E = 3, respectively) than EKR employing vinyl acetate (c = 49%, E = 39). In addition to hexane, four other organic solvents were tested in transesterification reactions, as follows: *n*-heptane, toluene, methyl *t*-butyl ether (M*t*BE) and acetonitrile (ACN). As shown in Figure 1, the transesterification reactions occurred in all solvents, but heptane (c = 60%, E = 13) and hexane (c = 58%, E = 17) were the best ones. For the transesterification reactions of **1a** mediated by LipG9 employing ACN, M*t*BE and toluene as organic solvents, in addition to lower conversions (c = 50%, 35% and 6%, respectively), the enantiomeric ratios also decreased (E = 8, 8 and 12, respectively). The change of reaction temperature from 35 °C to either 25 °C or 45 °C also promoted a decrease in the enantiomeric ratio (E = 20 and E = 17, respectively), but no significant change was observed in conversions. Taking into account these results, no significant improvement of Im-LipG9 enantioselectivity was obtained by changing the acyl donor, the organic solvent or the temperature. The initial reaction conditions (vinyl acetate, hexane and 35 ^{o}C) were the best ones for EKR of compound **1a**.

3.4 Hydrolysis reaction

Im-LipG9 was evaluated in the hydrolysis reaction of racemic esters **1b-6b** (Scheme 1). For the initial exploratory hydrolysis assay, distillated water was employing as nucheophile and *n*-hexane as solvent, at 35 °C. However, this reaction did not occur. Despite this unsuccessful attempt, other reaction conditions were evaluated for enzymatic hydrolysis. Phosphate buffer solution (0.1 mol L^{-1}, pH = 7) and toluene as solvent and a higher temperature (70 °C) were tested. Nevertheless, no hydrolysis product was detected. These results indicate that Im-LipG9 presents a high degree of chemoselectivity, since EKR of the alcohols **1a-6a** was successfully performed by transesterification reaction, while no reaction was observed for the hydrolysis of the corresponding esters.

4. Conclusions

Metagenomic lipase LipG9 is a promising biocatalyst, since it showed enantioselectivity equal to or better than commercially available lipases for the EKR of aliphatic *sec*-alcohols by transesterification reactions. LipG9 was successfully employed in EKR of aliphatic alcohols, which were resolved with satisfactory conversions (19-59%) and enantiomeric excesses for alcohols (26-88%) and esters (30-96%) by transesterification reactions. Changing the reaction parameters (acyl donor, organic solvent or temperature) did not cause significant improvement of Im-LipG9 enantioselectivity, so the initial reaction conditions (vinyl acetate, hexane and 35 ^{o}C) were the best ones for EKR catalyzed by Im-LipG9. Hydrolysis of aliphatic esters **1b-6b** did not occur, indicating that these compounds are not good substrates for hydrolysis reactions mediated by Im-LipG9. However, given the results obtained, Im-LipG9 presented chemoselectivity to aliphatic compounds, since transesterification reactions occurred while hydrolysis did not. There is still room for improvements in the performance of LipG9, considering that this native lipase was only purified, immobilized

and directly evaluated in EKR of aliphatic alcohols without others chemical or biological interventions. The need for new enzymes or the engineering of the reaction media in EKR of aliphatic alcohols should be fostered, since the synthetic challenge presented by this class of alcohols is evident and the influence of structural aspects of the substrate on the conversions and the enantiomeric ratio is dramatic. The results presented here for the immobilized metagenomic lipase LipG9 show that metagenomics is a useful technique for finding new biocatalysts with application in enzymatic kinetic resolutions.

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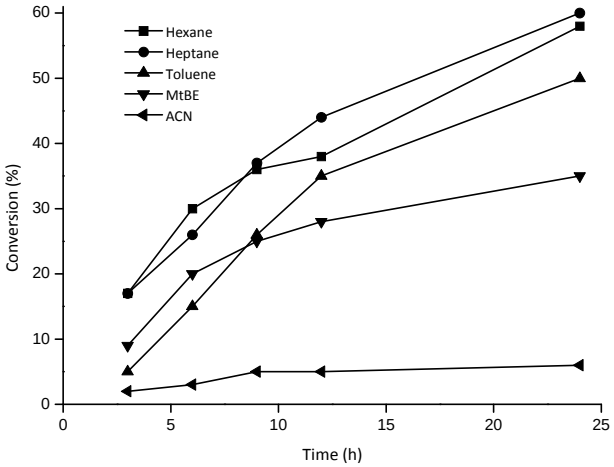
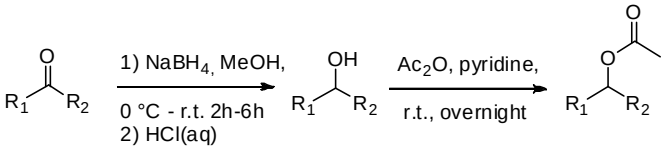


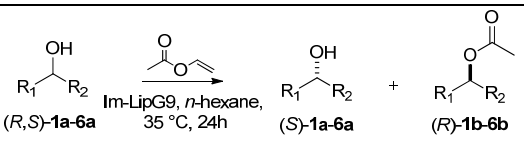
Figure 1 Effect of organic solvent on the conversion of transesterification of (*R*,*S*)-pentan-2-ol (**1a**) mediated by LipG9. Reaction conditions: substrates (0.1 mmol), vinyl acetate (0.4 mmol), organic solvents (2 mL), immobilized LipG9 (Im-LipG9) (11 U), 45 °C and 24 hours.



R_1 = Me, R_2 = *n*-Pr; **1**	**1a** (85%)	**1b** (58%)
R_1 = Me, R_2 = *n*-Bu, **2**	**2a** (95%)	**2b** (84%)
R_1 = Me, R_2 = *n*-Pent, **3**	**3a** (91%)	**3b** (78%)
R_1 = Me, R_2 = *n*-Hex, **4**	**4a** (89%)	**4b** (83%)
R_1 = Me, R_2 = *i*-Bu, **5**	**5a** (94%)	**5b** (81%)
R_1 = Et, R_2 = *n*-Pr, **6**	**6a** (92%)	**6b** (64%)

Scheme 1 Syntheses of aliphatic *sec*-alcohols **1a-6a** and esters **1b-6b**

Table 1 Enzymatic kinetic resolution of alcohols **1a-6a**



Entry	Alcohol	*c* [a] (%)	ee (%)[b]		E[c]
			alcohol	ester	
1	**1a**	49	81	88	39
2	**2a**	54	88	74	19
3	**3a**	52	47	46	4
4	**4a**	53	36	30	3
5	**5a**	19	26	96	63
6	**6a**	59	32	52	4

Reaction conditions: substrates (0.1 mmol), vinyl acetate (0.4 mmol), n-hexane (2 mL), immobilized LipG9 (11 U), 35 °C and 24 hours.
[a]Conversion (c). [b]Enantiomeric excess (ee): determinate GC analysis using chiral column Chiral-Dex-CB; [c] Enantiomeric ratio (E).