Marine microorganisms as source of stereoselective esterases and ketoreductases: kinetic resolution of a prostaglandin intermediate

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Abstract

A screening among bacterial strains isolated from water-brine interface of the deep hypersaline anoxic basins (DHABs) of the Eastern Mediterranean was carried out for the biocatalytical resolution of racemic propyl ester of *anti*-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (*R,S*)-**1**, a key intermediate for the synthesis of D-cloprostenol. *B. hornekiae* 15A gave highly stereoselective reduction of (*R*,*S*)-**1**, whereas *H. aquamarina* 9B enantioselectively hydrolysed (*R*,*S*)-**1**; in both cases, enantiomerically pure unreacted (*R*)-**1** could be easily recovered and purified at molar conversion below 57-58%, showing the potential of DHABs extremophile microbiome and marine-derived enzymes in stereoselective biocatalysis.

Keywords: esterase, ketoreductase, deep-sea hypersaline anoxic basins (DHABs), marine enzymes, stereoselective, biocatalysis, prostaglandin

1. Introduction

The biocatalytic preparation of optically pure chiral building blocks for the fine chemistry as well as pharmaceutical sector is a valid alternative to conventional chemical methods (Patel 2008). In this context, the recruitment of new robust enzymes able to catalyse stereoselective reactions is highly demanded. Microorganisms from extreme environments are thoroughly adapted for surviving and growing under harsh conditions due to the development of various structural and physiological adjustments (Burton *et al.* 2002). Therefore, enzymes produced by marine microorganisms often show higher stability in operational conditions (i.e. relatively high ionic strength, organic solvents) and different types of functional properties when compared with enzymes isolated by conventional sources (Ferrer *et al.* 2005a; Ferrer *et al.* 2007; Trincone 2011; Dionisi *et al.* 2012). Recently, the use of seawater as reaction media has been suggested as an interesting alternative for future bioprocesses (Domínguez de María, 2013) and marine enzymes may represent ideal biocatalysts for such applications. Deep-sea hypersaline anoxic basins (DHABs) of the Eastern Mediterranean are extreme environments characterized by high salinity and corresponding density, high hydrostatic pressure, absence of light, anoxia, and the presence of a sharp chemocline between brines and the above seawater. Moreover, DHABs contain brines with different composition, and hence each of them constitutes an independent environment, which hosts a peculiar and stratified prokaryotic community, highly adapted to the extreme geosetting (Van der Wielen *et al.* 2005; Daffonchio *et al.,*2006; Yakimov *et al.* 2007, Borin *et al.* 2009). These habitats resulted, therefore, in the selection of unusual microorganisms with peculiar enzyme endowment, e.g. DHAB esterases proved exceptional stability in polar solvents, besides high activities, specificities, enantioselectivities (Ferrer *et al.* 2005b; Wei *et al.* 2013). Thus, DHABs seem a suitable habitat for mining enzymes potentially useful for industrial biotransformations.

anti-2-Oxotricyclo[2.2.1.0]heptane-7-carboxylic acid is a useful chiral intermediate for prostaglandin synthesis (i.e. D-cloprostenol) and can be easily synthesised from norbornadiene as a racemic mixture, but its synthetic utility is limited to the (7*R*)-enantiomer (Stibor *et al.* 1985). The resolution of *anti*-2-oxotricyclo[2.2.1.0]heptan-(*R,S*)-7-carboxylic acid can be achieved by precipitation with chiral amines, although the process is characterised by low yields (Grieco *et al.*,1980). Alternatively, resolution can also be achieved by enzymatic hydrolysis, starting from the corresponding ester and using esterases or lipases (Kingery-Wood and Johnson 1996; Holla *et al.* 1996; Romano A. *et al.* 2005), or by reduction of the carbonyl function using ketoreductases from yeasts (Romano A. *et al.* 2005).

The main goal of this work was to select DHAB microorganisms producing enzymes to be applied to the resolution of the propyl ester of *anti*-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid.

2. Materials and Methods

Chemicals were of reagent grade and purchased form Fluka, Milano, Italy.

2.1 Bacteria isolation, genotypic characterisation and identification

The bacteria tested in the present work were isolated on 246 DSM medium from seawater-brine interface samples collected from the following Eastern Mediterranean DHABs: Bannock (34° 17.949' N, 20° 00.985' E), Discovery (35°17' N, 21°41' E), L'Atalante (35°18.27' N, 21°23.46' E) and Urania (35°13.023' N, 21°30.682' E). Bannock basin is situated on the southern limit of the Mediterranean Ridge (De Lange *et al.*, 1990), while Discovery, L'Atalante and Urania basins are located on the north-eastern limit of the ridge (MEDRIFF consortium, 1996). Details on the geochemical setting and the extreme conditions occurring at the DHABs were previously described (Van der Wielen *et al.* 2005). DNA extraction was performed on each isolated strain. Genotypic characterisation and identification were carried out as previously described (Mapelli *et al.* 2013). Nucleotide sequences were edited in Chromas Lite 2.01 (http://www.technelysium.com.au) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial 16S rRNA gene sequences obtained from the bacterial isolates have been deposited in the EMBL database under accession numbers HG799612– HG799644.

2.2 Analytical methods

Merck Silica Gel 60 F_{254} plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200-400 mesh). ^{1}H NMR spectra were recorded on a Varian Mercury 300 MHz spectrometer. Rotary power determinations were carried out using a Jasco P-1010 spectropolarimeter, coupled with a Haake N3-B thermostat. The enantiomeric composition of **1** and **3** was determined by gas chromatography using a chiral capillary column (diameter 0.25 mm, length 25 m, thickness 0.25 mm, DMePeBeta-CDX- PS086, MEGA, Legnano, Italy) at 130 °C. (*R*)-**1**: retention time 14.4 min; (*S*)-**1**: retention time 15.7 min; (*R*)-**3**: retention time 20.1; (*S*)-**3**: retention time 21.3 min. The enantiomeric composition of **2** was determined by HPLC using a chiral column (Chiralcel OD, 4.6-250 mm, Daicel Chemical Industries Ltd., Tokio, Japan) mobile phase: n-hexane/2-propanol/HCOOH 90:10:1, flow 0.5 mL/min, temperature 28 °C, detection UV 280 nm. (*S*)-**2**: retention time 9.1 min; (*R*)-**2**: retention time 10.3 min. The absolute configuration was determined by comparison with optically pure compounds obtained as previously

described (Romano A. *et al*., 2005).

2.3 Synthesis of anti-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid propyl ester [(R,S)-1]

Anti-2-oxotricyclo[2.2.1.0]heptan-(*R,S*)-7-carboxylic acid (5.0 g, 32.86 mmol) was dissolved in dry CH_2Cl_2 (60 mL) and dimethylaminopyridine (DMAP, 401 mg, 3.29 mmol) was added. The mixture was stirred for 10 minutes at 25°C and then *n*-propanol (2.0 ml) was added. The reaction mixture was brought to 4 °C and dicyclohexylcarbodiimide (DCC, 6.78 g, 32.86 mmol) was added; the reaction mixture was then stirred for 5 h at 25°C. The solid was removed by filtration and the filtrate was evaporated under vacuum. The crude residue was purified by flash chromatography (*n*-hexane/ethyl acetate 1:1) to give 5.51 g (28.36 mmol, 86% yield) of (*R,S*)-**1.**

2.4 Microorganisms, media and culture conditions

Strains were routinely maintained on marine broth agar slants and cultured in liquid marine broth (Difco) by incubation for 24 h at 30°C on a reciprocal shaker (120 spm).
Strains were also cultured in 2.0 L Erlenemeyer flasks containing 200 mL of CYSP broth (Romano D. *et al.* 2005) (casitone 15 g /L, yeast extract 5 g/L, soytone 3 g/L, peptone 2 g/L, $MgSO_4 \cdot 7H_2O$ 15 mg/L, $FeCl_3$ 115 mg/L, $MnCl_2$ 20 mg/L, pH 7.0) with or without NaCl (30 g/L) and incubated for 24 h at different temperatures on a reciprocal shaker (120 spm). The dry weights were determined after centrifugation of 100 mL of cultures: cells were washed with distilled water and dried at 110 °C for 24 h.
Growth optimum was assessed growing the bacterial strains on CYSP medium supplemented by increasing NaCl concentration (ranging from 0 to 9%) in 96 wells microplate. The cultures were incubated and growth was monitored by microplate reader (TECAN Infinite Pro200). Data elaboration was carried out using Excel software. For each strain, the NaCl percentage at which the growth curve showed the higher slope value was identified as the optimum NaCl concentration.

2.5 Biotransformations

Biotransformations with whole growing cultures were performed by adding neat substrate directly to flasks containing the growing cultures after 24 h and by incubating the flasks at 30°C on a reciprocal shaker (120 spm). Biotransformations with resting cells were carried out by harvesting cells from liquid cultures by centrifugation and by suspended in aqueous buffers (10 mL) until reaching the desired OD (600 nm).

Extracellular activity was checked by using the supernatant obtained from liquid cultures after centrifugation. Reactions were started by addition of the substrate and, in the selected cases, addition of glucose; incubation was performed under reciprocal shaking (120 spm). A sample of the biotransformation mixture (200 μL) was acidified with 0.1 N HCl until pH 1 and extracted with EtOAc (200 μL). The organic extracts were dried over anhydrous Na_2SO_4 and analysed by TLC. Two TLC eluents were used: *n*-hexane/ethyl acetate 1:1 (R_f of **1** = 0.46, R_f of **2** = < 0.1, R_f of **3** = 0.26) and ethyl acetate/acetic acid 98:2 (R_f of **1** = 0.93, R_f of **2** = 0.24, R_f of **3** = 0.74).

Optimization studies with *Bacillus hornekiae* 15A were carried out by suspending the desired amount of cells in different phosphate buffers (0.1 M, pH range 5.8-7.8) containing glucose (50 g/L); neat substrate and NaCl were added to reach the desired concentration; the suspensions obtained were magnetically stirred at different temperatures. Optimization studies with *Halomonas aquamarina* 9B were carried out by suspending the desired amount of cells in different phosphate buffers (0.1 M, pH range 5.8-7.8) and adding neat substrate and NaCl to reach the desired concentration; the suspensions obtained were magnetically stirred at different temperatures. Each experiment was carried out in triplicate.

*2.6 Preparative reduction of (*R,S*)-**1** with Bacillus hornekiae 15A*

Resting cells of *Bacillus hornekiae* 15A (DO_{600nm}=20.0) were suspended in 500 mL of phosphate buffer (0.1 M, pH 6.5) containing 25.0 g of glucose and the mixture stirred for 45 min at 30°C; (*R,S*)-**1** (700 mg) was then added and the reaction outcome was followed by HPLC. After 8 h, the enantiomeric excess of the substrate was 100% with 54-56% molar conversion and the reaction was stopped by adding 0.1 N HCl until pH 1. The mixture was centrifuged and the aqueous phase was extracted twice with ethyl acetate (300 mL), the organic extracts were collected, dried over anhydrous Na_2SO_4 and evaporated to give 695 mg of crude residue. The crude residue was dissolved in MeOH (25 mL) containing acetic acid (2.0 mL) and solid (carboxymethyl)trimethylammonium chloride hydrazide (Girard T reagent, 1.40 g) was added. The mixture was refluxed for 15h, then cooled at 20 °C and NaOH (20% aqueous solution) was added until pH 7.0. Methanol was distilled under vacuum and the remaining solution was extracted with CH_2Cl_2 (25 ml). The organic extracts were washed with brine, dried over Na_2SO_4 and evaporated giving 285 mg of enantiomerically pure (*R*)-**1** (41%). $[\alpha]_D^{25}$= +23.2 (*c* = 1.0 MeOH); $[\alpha]_D^{25}$ lit. = +23.1 (*c* = 1.0 MeOH) (Romano A. et al. 2005). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.98-1.02 (t, *J* = 4.2 Hz, 3H), δ 1.45-1.51 (dt, *J* = 2.1

Hz, 1H), δ 1.63-1.74 (m, 2H), δ 1.84-1.90 (dt, *J* = 6.2 Hz, 1H), δ 1.95-2.01 (dt, *J* = 6.2 Hz, 1H), δ 2.20-2.27 (m, 1H), δ 2.38-2.45 (m, 1H), δ 3.01 (bt, 1H), δ 4.05 (t, *J* = 4.2 Hz, 2H).

*2.7 Preparative hydrolysis of (*R,S*)-**1** with Halomonas aquamarina 9B*

Resting cells of *Halomonas aquamarina 9B* (OD_{600nm}=20.0) were suspended in 500 mL of phosphate buffer (0.1 M, pH 7.2) and (*R,S*)-**1** (600 mg) was added; the biotransformation was carried out at 2°C under magnetic stirring and the reaction followed by HPLC. After 7 h, the enantiomeric excess of the substrate was 100% with 55-57% molar conversion and the reaction was stopped by adding 0.1 N NaOH until pH > 12. The mixture was centrifuged and the aqueous phase extracted twice with EtOAc (300 mL); the organic extracts were collected, washed twice with water, dried over Na_2SO_4 and evaporated giving 260 mg of crude residue. The crude residue was purified by flash chromatography (*n*-hexane/ethyl acetate 1:1) to give 239 mg of (*R*)-**1** (40%). $[\alpha]_D^{25}$= +23.1 (c = 1.0 MeOH).

3. Results and Discussion

3.1 Screening for biocatalytical activity

In this work 33 halotolerant bacterial strains have been selected from a wide collection of isolates obtained from the seawater-brine interface of different deep hypersaline anoxic basins (DHABs) of the Eastern Mediterranean Sea, namely Urania, Bannock, Discovery and L'Atalante basins. According to the taxonomic identification, performed through 16S rRNA sequence analysis, the bacterial collection comprised isolates belonging to ten different genera of the phylogenetic groups gamma-Proteobacteria and Firmicutes, including, respectively, 11 and 22 isolates. Four bacterial genera, *Bacillus*, *Halomonas*, *Oceanobacillus* and *Virgibacillus* were the most represented, comprising 19 species (Table 1). Subspecies diversity was analysed by the application of ITS-fingerprinting (Mapelli *et al.* 2013), which identified 23 different ITS haplotypes. Strains belonging to the same haplotype were in all cases belonging to the same species and were isolated from the same DHABs. Only for one species, *H. aquamarina*, strains with the same ITS haplotype were isolated from different DHABs, L'Atalante and Urania (Table 1). All the isolated bacteria have been tested for the ability to biotransform the propyl ester of 1,2 *anti*-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (**1**).

Table 1. Taxonomic identification, haplotype diversity and growth optimum (% NaCl) of the tested bacterial strains.

Strain code	DHABs	Closest described species (Acc. N°)	Phylum/Class	% identity	ITS haplotype	Growth optimum (NaCl %)
3B	Bannock	*Oceanobacillus profundus* (HQ595230)	Firmicutes	99	19	0-3-6
5Bt	Bannock	*Oceanobacillus profundus* (HQ595230)	Firmicutes	99	19	n.d.
6B	Bannock	*Oceanobacillus profundus* (HQ595230)	Firmicutes	99	19	n.d.
7B	Bannock	*Oceanobacillus profundus* (HQ595230)	Firmicutes	99	19	n.d.
17B	Bannock	*Bacillus selenatarsenatis* (JF506004)	Firmicutes	99	23	6
19B	Bannock	*Bacillus selenatarsenatis* (JF506004)	Firmicutes	99	23	n.d.
8B	Bannock	*Halomonas meridiana* (EU441001)	Gammaproteobacteria	99	20	n.d.
9B	Bannock	*Halomonas aquamarina* (EU440965)	Gammaproteobacteria	99	21	3
15B	Bannock	*Halomonas meridiana* (EU441001)	Gammaproteobacteria	99	22	n.d.
11D	Discovery	*Bacillus firmus* (DQ089748)	Firmicutes	99	13	n.d.
12D	Discovery	*Bacillus firmus* (DQ089748)	Firmicutes	99	13	3
13D	Discovery	*Halobacillus trueperi* (HM179214)	Firmicutes	99	14	3
15D	Discovery	*Bacillus selenatarsenatis* (JF506004)	Firmicutes	99	15	n.d.
20D	Discovery	*Bacillus lehensis* (NR_036940)	Firmicutes	99	17	n.d.
21D	Discovery	*Virgibacillus pantothenticus* (JN791392)	Firmicutes	100	18	6-9
18D	Discovery	*Halomonas cupida* (AB681327)	Gammaproteobacteria	99	16	3
13A	L'Atalante	*Virgibacillus halodenitrificans* (AB697714)	Firmicutes	97	9	3-6

Strain code	DHABs	Closest described species (Acc. N°)	Phylum/Class	% identity	ITS haplotype	Growth optimum (NaCl %)
14A	L'Atalante	*Virgibacillus halodenitrificans* (AB697714)	Firmicutes	97	9	3-6
15A	L'Atalante	*Bacillus horneckiae* (JN002383)	Firmicutes	99	10	0-3
16A	L'Atalante	*Virgibacillus proomii* (FN397532)	Firmicutes	99	11	n.d.
26A	L'Atalante	*Virgibacillus pantothenticus* (JN791392)	Firmicutes	99	12	3-6
9A	L'Atalante	*Halomonas aquamarina* (EU430083)	Gammaproteobacteria	99	5	n.d.
9U	Urania	*Exiguobacterium acetylicum* (JN852813)	Firmicutes	99	4	n.d.
11Ub	Urania	*Bacillus thioparans* (NR_043762)	Firmicutes	99	6	n.d.
16U	Urania	*Bacillus thioparans* (NR_043762)	Firmicutes	99	6	n.d.
16Ub	Urania	*Bacillus thioparans* (NR_043762)	Firmicutes	99	6	n.d.
23U	Urania	*Virgibacillus salinus* (FM205010)	Firmicutes	99	7	n.d.
1U	Urania	*Idiomarina loihiensis* (AY505529)	Gammaproteobacteria	99	1	3-6
2U	Urania	*Idiomarina loihiensis* (NR_025119)	Gammaproteobacteria	99	1	n.d.
3U	Urania	*Enterobacter aerogenes* (JQ682634)	Gammaproteobacteria	99	2	n.d.
5U	Urania	*Pseudoalteromonas ganghwensis* (DQ011614)	Gammaproteobacteria	100	3	n.d.
13U	Urania	*Halomonas aquamarina* (AB681582)	Gammaproteobacteria	99	5	3
24U	Urania	*Salinisphaera shabanensis* (JF281734)	Gammaproteobacteria	99	8	n.d.

Acc. N°: accession number; n.d. not determined

The first screening was performed by growing the strains in liquid cultures using marine broth and by using the whole cultures for the biotransformation of the propyl ester of 1,2 *anti*-2-oxotricyclo[2.2.1.0]heptan-*(R,S)*-7-carboxylic acid (**1**). Kinetic resolution of (*R,S*)-**1** can be achieved by ester hydrolysis with enantioselective formation of *anti*-2-oxotricyclo[2.2.1.0]heptan-*(S)*-7-carboxylic acid (*S*-**2**) or by carbonyl reduction with formation of the propyl ester of *anti*-2-hydroxytricyclo[2.2.1.0]heptan-*(S)*-7-carboxylic acid (*S*-**3**). The biotransformations were performed using whole cultures grown in Marine broth at 30°C and firstly analysed by TLC; 33 strains were able to give detectable transformation of the substrate and these biotransformations were also analysed by chiral GC for determining stereochemical composition. Interestingly, a homogeneous stereopreference was observed with prevalent formation of *S*-**2** by activity of microbial esterases and prevalent formation of *S*-**3** upon ketoreductase activity (Figure 1). Therefore, in all the cases, the synthetically useful enantiomer *R*-**1** was mostly left unreacted. Interestingly, no traces of the products deriving from carbonyl reduction of **2** or from ester hydrolysis of **3** were observed in all the tested biotransformations, showing high substrate specificity of the involved enzymes.

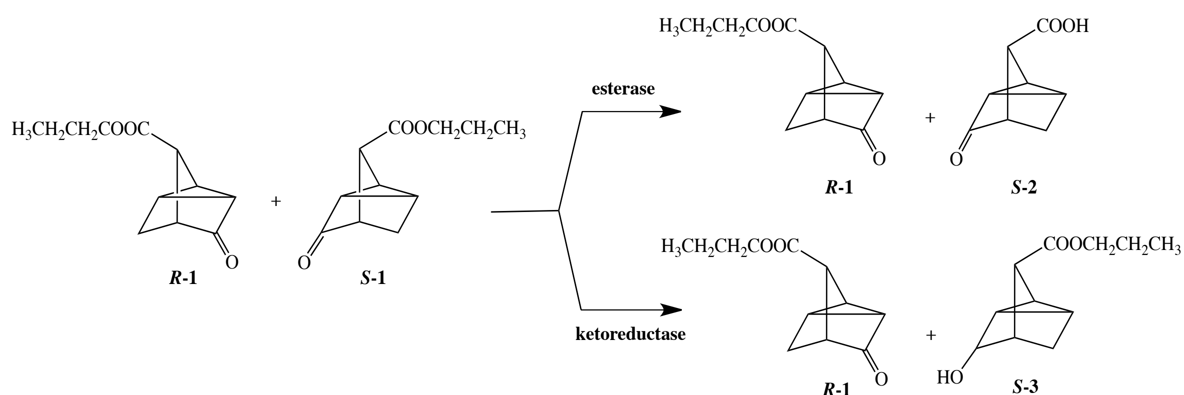

Figure 1. Biocatalytical approaches for the resolution of racemic propylic ester of *anti*-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (*R,S*)-**1**.

Table 2 reports the results and the stereochemical outcome obtained with cells growing in 96-well microplates (2 ml biotransformation volume) in marine broth containing glucose.

Table 2. Biotransformation of **1** (2 mg/mL) with whole growing cultures (2 mL) of DHAB microorganisms. Conversions (%) into **2** and **3** and enantiomeric excesses (ee %) were determined by chiral GC analysis. The results are given as the mean values of two separate experiments.

		1		2		3		
entry	**Strain**	(%)	e.e. (%)	(%)	e.e. (%)	(%)	e.e. (%)	Time (h)
1	*Bacillus firmus* 11D	10	92	5	-	85	25	96
2	*Bacillus firmus* 12D	16	100	13	31	70	30	120
3	*Bacillus hornekiae* 15A	< 5	-	5	-	90	54	96
4	*Bacillus lehensis* 20D	15	100	< 5	-	82	22	96
5	*Bacillus selenatarsenatis* 15D	5	-	5	-	88	11	120
6	*Bacillus selenatarsenatis* 17B	< 5	-	7	47	90	50	24
7	*Bacillus selenatarsenatis* 19B	< 5	-	< 5	-	85	21	96
8	*Bacillus thioparans* 11Ub	< 5	-	< 5	-	92	8	48
9	*Bacillus thioparans* 16U	10	90	< 5	-	86	20	120
10	*Bacillus thioparans* 16Ub	5	-	5	-	85	11	96
11	*Enterobacter aerogenes* 3U	8	96	12	6	80	13	96
12	*Exiguobacterium acetylicum* 9U	10	88	< 5	-	85	< 5	48
13	*Halomonas aquamarina* 9A	9	84	9	19	82	14	120
14	*Halomonas aquamarina* 9B	0	-	30	74	70	53	48
15	*Halomonas aquamarina* 13U	17	< 5	25	35	58	33	120
16	*Halomonas cupida* 18D	0	-	27	85	73	55	48
17	*Halomonas meridiana* 8B	10	95	< 5	-	88	21	72
18	*Halomonas meridiana* 15B	5	-	5	-	90	5	96
19	*Halomonas trueperi* 13D	0	-	15	53	85	54	120
20	*Idiomarina loihiensis* 1U	< 5	-	75	50	25	40	96
21	*Idiomarina loihiensis* 2U	5	73	25	25	70	24	96
22	*Oceanobacillus profundus* 3B	10	75	20	50	70	41	120
23	*Oceanobacillus profundus* 5Bt	23	80	10	20	68	42	120
24	*Oceanobacillus profundus* 6B	25	80	15	22	65	47	120
25	*Oceanobacillus profundus* 7B	11	10	25	23	64	35	120
26	*Pseudoalteromonas gangwensis* 5U	5	-	0	-	95	< 5	96

The primary screening showed that only one strain (*Idiomarina loihiensis* 1U, entry 20) showed prevalent esterase activity, giving (*S*)-**2** with 75% conversion and 50% ee, whereas all the remaining strains exhibited a predominance of ketoreductase activity, furnishing (*S*)-**3** as a major product.

Microorganisms were selected according to different enzymatic behaviour towards **1**. Namely, a first group containing strains showing enantioselective (ee ≥ 50%) ketoreductase activity (8 strains: entries 3, 6, 14, 16, 19, 28, 30, 31) and a second group with strains showing enantioselective esterase activity (6 strains: entries 14, 16, 19, 20, 22, 28). Interestingly, four strains showed both enantioselective esterase and ketoreductase activity.

Generally, the growth observed with marine broth used in the first screening was quite poor; thus, the selected microorganisms were also grown in a richer medium (CYSP) with or without NaCl (3-9%) for evaluating the effect of the presence of salt during the growth on the enzymatic activities. CYSP liquid medium was found to be very effective in favouring bacterial growth and esterase expression (Romano *et al.*, 2005). Only three out of the 10 selected strains were able to grow in the absence of NaCl. The majority of the bacterial strains showed the growth optimum at NaCl concentration equal to 3% or comprised between 3 and 6% (Table 1) with the exception of strains 17B and 21D, showing the optimum growth respectively at 6 and 6-9% NaCl.

3.2 Optimization of ketroreductase activity

Microorganisms selected for their enantioselective ketoreductase activity were grown in CYSP and 3% NaCl and used as resting cells (in phosphate buffer pH 7.0, 0.1 M) for the biotransformation of **1** in the presence of glucose. The presence of glucose is known to favour ketoreductase activity over esterase in many microbial biotransformations, as a consequence of redox cofactor regeneration, while esterase activity is mostly observed in the absence of co-substrates (Gandolfi *et al.* 2009, Rimoldi *et al*. 2011). Resting cells were used with and without 3% NaCl; the strains able to efficiently grow at higher concentrations of the salt were also tested at 6-9% NaCl (Table 3).

Table 3. Biotransformation of **1** (5mM) with resting cells of marine microorganisms grown in CYSP medium with 3% salt. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 7, 10 mL) in the presence of 5% glucose. Conversions (%) into **2** and **3** and

enantiomeric excesses (ee %) were determined by chiral GC analysis; enantiomeric ratio (E) was given only for the biotransformation leading to one product. The results are the average of three replicates.

			1		**2**		**3**			
Entry	Strain	NaCl (%)	(%)	e.e.(%)	(%)	e.e.(%)	(%)	e.e.(%)	E	Time (h)
1	*B. hornekiae* 15A	0	48	95	0	-	53	84	42	2
2	*B. hornekiae* 15A	3	51	79	0	-	49	81	22	5
3	*B. selenatarsenatis* 17B	0	40	100	0	-	60	65	32	8
4	*B. selenatarsenatis* 17B	3	50	79	0	-	50	81	22	5
5	*B. selenatarsenatis* 17B	6	81	7	0	-	19	33	2.1	5
6	*H. aquamarina* 9B	0	48	90	17	90	36	52	-	2
7	*H. aquamarina* 9B	3	66	43	20	93	14	27	-	2
8	*H. cupida* 18D	0	45	25	10	60	45	50	-	1
9	*H. cupida* 18D	3	40	23	14	97	46	35	-	2
10	*H. trueperi* 13D	0	57	50	0	-	43	60	6.5	5
11	*H. trueperi* 13D	3	44	38	0	-	56	30	2.6	5
12	*V. halodenitrificans* 13A	0	79	20	0	-	21	75	8.5	2
13	*V. halodenitrificans* 13A	3	84	13	0	-	16	67	5.7	5
14	*V. pantothenticus* 21D	0	58	95	0	-	58	70	20	2
15	*V. pantothenticus* 21D	3	59	61	0	-	41	86	24	5
16	*V. pantothenticus* 21D	6	46	90	0	-	54	76	22	5
17	*V. pantothenticus* 21D	9	50	77	0	-	50	80	20	5
18	*V. pantothenticus* 26A	0	60	57	0	-	40	91	37	2
19	*V. pantothenticus* 26A	3	76	45	0	-	34	87	22	2

The use of resting cells generally allowed for higher rates, conversion and enantioselectivity (Table 3). The presence of salt generally decreased reaction rate. *Bacillus hornekiae* 15A, *B. selenarsenatis* 17B, *Halomonas trueperi* 13D, *Virgibacillus halodenitrificans* 13A, *Virgibacillus pantothenticus* 21D and *Virgibacillus pantothenticus* 26A only gave reduction of the substrate giving (*S*)-**3** with medium/high enantioselectivity. *Bacillus hornekiae* 15A gave the highest enantiomeric ratio (E=42) and high rates, reaching 50% conversion in 2 hours, when used in the absence of salt (entry 1).

Bacillus hornekiae 15A was selected for an optimization study due to its highly enantioselective performances. Optimization was carried out using the Multisimplex® experimental design, which simultaneously evaluates different parameters of the biotransformation and was previously shown to be suited for fast optimization of different biotransformations, also on preparative scale (Romano *et al.* 2005; Romano *et al*. 2006, Romano *et al.* 2011). The experimentally investigated response parameters were product yields (mM) and enantiomeric ratio (E), while the control variables were temperature, pH, [substrate], [cells], [NaCl]. The best conditions for the biotransformation of (*R,S*)-**1** were 0.1 mM phosphate buffer at pH 6.5, 30°C, 1.4 g/L substrate concentration in the presence of 20 $g_{dry\ weight}$/L of cells; the kinetic resolution of (*R,S*)-**1** by reduction using cell-bound ketoreductase of *Bacillus hornekiae* 15A is reported in Figure 2a. Under the optimised conditions, the overall resolution occurred with an enantiomeric ratio (E) of 50-55 and, notably, no acid **2** was observed, allowing for the recovery of optically pure (*R*)-**1**.

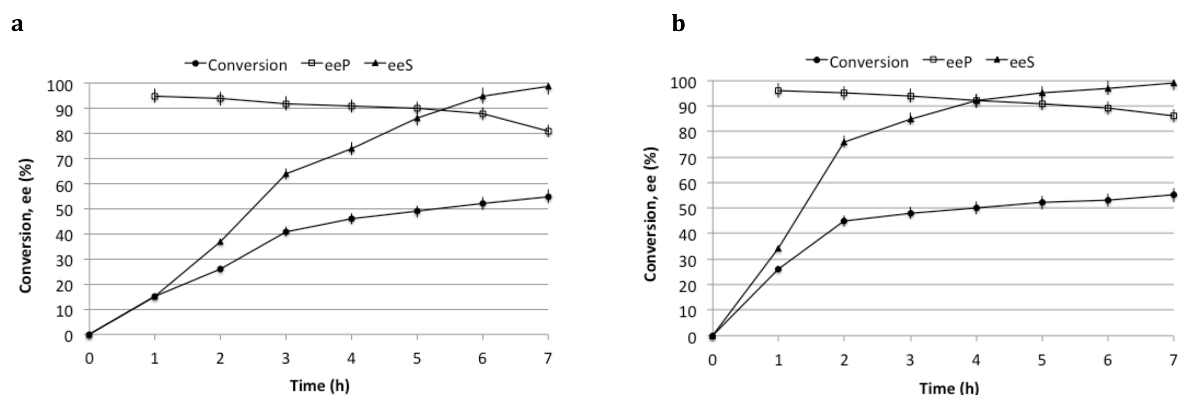

Figure 2. Experimental data of the kinetic resolution of (*R,S*)-**1** under optimised conditions. **a**: reduction of (*R,S*)-**1** (1.4 g/l) with *Bacillus hornekiae* 15A to alcohol **3**; **b**: hydrolysis of (*R,S*)-**1** (1.2 g/L) with *Halomonas aquamarina* 9B to acid **2**. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 6.5-7.2, 10 mL) and stirred at 30°C. Results are given as mean values of three replicates.

The bioreduction of (*R,S*)-**1** was also carried out on semi-preparative scale (500 mL biotransformation volume) showing similar results; the crude extract, recovered after centrifugation of the biomass, extraction with ethyl acetate and solvent evaporation, was derivatized with Girard T reagent for easy separation of the alcohol produced. The enantiomerically pure ester was hydrolysed and used for next steps aimed at the production of D-cloprostenol (Romano *et al.* 2005).

3.3 Optimization of esterase activity

Hydrolysis of (*R,S*)-**1** was also studied using the six strains which had previously displayed enantioselective activity as growing cells. Hydrolytic activity observed in whole cultures can be due to extracellular or cell-bound activity (Gandolfi *et al.* 2000; Guglielmetti *et al.* 2008). Thus, extracellular and cell-bound activities were measured independently, showing that esterase activity was mostly cell-bound, whereas conversions lower than 5% for all the tested strains were observed using the supernatants.

Resting cells of the six strains were employed for the biotransformation of (*R,S*)-**1** in the absence of glucose for decreasing ketoreductase activity and favouring esterase activity (Table 4).

Table 4. Biotransformation of **1** (5mM) with resting cells of marine microorganisms grown in CYSP medium with 3% salt. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 7, 10 mL). Conversions (%) into **2** and **3** and enantiomeric excesses (ee %) were determined by chiral GC analysis. The results are the average of three replicates.

			1		**2**		**3**		
Entry	Strain	NaCl (%)	(%)	e.e.(%)	(%)	e.e.(%)	(%)	e.e.(%)	Time (h)
1	*H. aquamarina* 9B	0	45	94	41	93	14	52	5
2	*H. aquamarina* 9B	3	50	94	45	94	5	53	5
3	*H. cupida* 18D	0	0	-	16	> 97	84	40	8
4	*H. cupida* 18D	3	12	> 97	34	94	54	84	8
5	*H. trueperi* 13D	0	39	83	0	-	61	< 5	12
6	*H. trueperi* 13D	3	58	50	5	> 97	37	53	12
7	*I. loihiensis* 1U	0	17	38	24	58	59	60	24
8	*I. loihiensis* 1U	3	16	25	50	25	34	69	24
9	*O. profundus* 3B	0	65	7	5	7	31	7	24
10	*O. profundus* 3B	3	96	< 5	< 5	-	0	-	24
11	*V. halodenitrificans* 13A	0	80	< 5	5	6	16	< 5	24
12	*V. halodenitrificans* 13A	3	92	9	6	55	0	-	24

Generally, the biotransformations carried out with the same strains but in the absence of glucose furnished a higher amount of acid **2**; *H. trueperi* 13D, *O. profundus* 3B and *V. halodenitrificans* 13A showed low overall esterase activity. *Halomonas aquamarina* 9B predominantly gave the acid **2** with high enantioselectivity; the presence of salt (entry 2) tended to decrease the competing ketoreductase activity, giving only traces of the corresponding alcohol. The kinetic resolution of (*R,S*)-**1** by enantioselective hydrolysis catalysed by cell-bound esterase of *Halomonas aquamarina* 9B was ameliorated using the Multisimplex® approach, showing (under optimised conditions) the time-course reported in Figure 2B.

The biotransformation was carried out on semi-preparative scale (500 mL biotransformation volume) with resolution of the racemic substrate (E= 60-70) and obtainment of optically pure *R*-**1** at 54-56% molar conversion. Unreacted optically pure *R*-**1** was easily separated from the product **2** by selective extraction with ethyl acetate, after centrifugation of the biomass and alkalinization of the aqueous solution up to pH 10. These results are positively compared with previous reports about the use of commercial enzymes for the kinetic resolution of the racemic methyl ester of **2**: the average recovery of optically pure (*S*)-**2** using *Candida antarctica* Lipase A was 30% achieved after 2 days (Kingery-Wood and Johnson 1996), while the use of the thermostable lipase SP 526 from *Candida antarctica* afforded the desired (*R*)-enantiomer as (remaining) ester with ≥ 99% enantiomeric excess in 82% yield (Holla *et al.* 1996).

4. Conclusions

Newly isolated marine microorganisms were screened for the kinetic resolution of racemic (*R,S*)-**1**, a key intermediate for prostaglandin synthesis (Romano *et al.* 2005). Strains showing enantioselective ketoreductase and esterase activity in marine broth were grown using a richer medium containing 3% NaCl, resulting in higher and sometimes more stereoselective activity. *B. hornekiae* 15A gave highly stereoselective reduction of (*R,S*)-**1**, while *H. aquamarina* 9B enantioselectively hydrolysed (*R,S*)-**1**; in both cases, enantiomerically pure unreacted substrate could be easily recovered and purified at molar conversion below 57-58%. Although wild-type whole cells were used, good results have been obtained, showing the potential of DHABs extremophile microbiome and marine-derived enzymes in biocatalysis.

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Conflicts of Interest

The authors declare no conflict of interest.

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Captions

Figure 1. Biocatalytical approaches for the resolution of racemic propylic ester of *anti*-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (*R,S*)-**1**.

Figure 2. Experimental data of the kinetic resolution of (*R,S*)-**1** under optimised conditions. **a**: reduction of (*R,S*)-**1** (1.4 g/l) with *Bacillus hornekiae* 15A to alcohol **3**; **b**: hydrolysis of (*R,S*)-**1** (1.2 g/L) with *Halomonas aquamarina* 9B to acid **2**. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 6.5-7.2, 10 mL) and stirred at 30°C. Results are given as mean values of three replicates.

Table 1. Taxonomic identification, haplotype diversity and growth optimum (% NaCl) of the tested bacterial strains.

Table 2. Biotransformation of **1** (5mM) with whole growing cultures (2 mL) of DHAB microorganisms. Conversions (%) into **2** and **3** and enantiomeric excesses (ee %) were determined by chiral GC analysis.

Table 3. Biotransformation of **1** (5mM) with resting cells of marine microorganisms grown in CYSP medium with 3% salt. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 7, 10 mL) in the presence of 5% glucose. Conversions (%) into **2** and **3** and enantiomeric excesses (ee %) were determined by chiral GC analysis. Each experiment was carried out in triplicate.

Table 4. Biotransformation of **1** (5mM) with resting cells of marine microorganisms grown in CYSP medium with 3% salt. Biotransformations were carried out with freshly prepared cells suspended in phosphate buffer (0.1 M, pH 7, 10 mL). Conversions (%) into **2** and **3** and enantiomeric excesses (ee %) were determined by chiral GC analysis. Each experiment was carried out in triplicate.