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# Highly enantioselective stereo-inverting *sec*-alkylsulfatase activity of hyperthermophilic *Archaea*†

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*rac-sec*-Alkyl sulfate esters **1a**–**8a** were resolved in low to excellent enantioselectivities with *E*-values up to >200 using whole cells of aerobically-grown hyperthermophilic sulfur-metabolizers, such as *Sulfolobus solfataricus* DSM 1617, *Sulfolobus shibatae* DSM 5389 and, most notably, *Sulfolobus acidocaldarius* DSM 639. Significantly enhanced selectivities were obtained using cells grown on sucrose-enriched Brock-medium. The stereochemical course of this biohydrolysis was shown to proceed with strict *inversion* of configuration, thus the preferred (*R*)-enantiomers were converted into the corresponding (*S*)-*sec*-alcohols to furnish a *homochiral* product mixture.

## Introduction

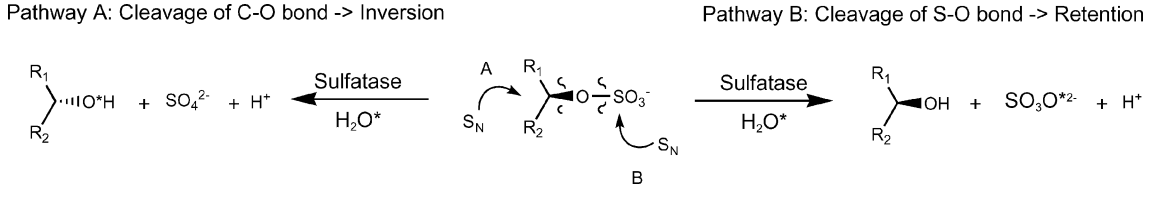
Driven by the demand to improve the economic balance of chemical processes for the synthesis of chiral compounds, the transformation of racemates into a single stereoisomeric product in quantitative yield has become a prime issue for contemporary asymmetric synthesis. Besides the widely employed dynamic kinetic resolution,[1] enantio-convergent processes – during which each enantiomer is transformed into the same stereoisomeric product *via* independent pathways, *i.e.* through *retention* and *inversion* of configuration – were shown to be a promising alternative.[2] Biocatalysts, which show this stereochemically complex potential to affect the stereochemistry of the substrate in a controlled fashion during catalysis are rather rare; they encompass haloalkane dehalogenases,[3] epoxide hydrolases[4] and sulfatases.[5,6]

Sulfatases catalyze the hydrolytic cleavage of the sulfate ester bond. In contrast to the majority of hydrolytic biotransformations catalyzed by lipases, esterases and proteases,[7] which do not alter the stereochemistry of the substrate during catalysis, the stereochemical course of sulfate ester hydrolysis can be controlled by the choice of the appropriate subtype of sulfatase enzyme (Scheme 1). On the one hand, arylsulfatases[8] generally act through *retention* of configuration at the sulfated carbon atom by cleavage of the S–O bond.[9] Their mechanism of action is well understood[10,11] and comprises the nucleophilic attack of an aldehyde hydrate (formed from a Cys- or Ser-residue by post-translational modification[12]) onto the sulfur atom, by liberating the corresponding alcohol by retaining its stereochemistry.[13] On the contrary, much less is known on *inverting* sulfatases,[6,14–16] which were shown to act on *sec*-alkyl sulfate esters by breaking the C–O bond of the sulfate ester, going in hand with *inversion* of configuration. As a result, a homochiral product mixture is obtained from racemic starting material. In order to remove the sulfate moiety from the remaining non-reacted sulfate ester enantiomer, a protocol for acid-catalyzed hydrolysis with strict retention of configuration has been recently developed.[17]

Based on vague hints on the stereospecific and enantioselective hydrolysis of alkyl sulfate esters,[14] we recently reported an inverting alkylsulfatase (termed 'RS2') from *Rhodococcus ruber* DSM 44541.[16,18] On the one hand, the enzyme displayed absolute *stereo*specificity by acting with strict inversion of configuration on simple *sec*-alkyl esters; on the other hand, its *enantio*selectivity was less than perfect: although 2-octyl sulfate (*rac*-**1a**) was resolved with an acceptable *E*-value of 21, no appreciable enantioselectivities were observed for 3- and 4-octyl sulfate ($E < 5$).[16] Attempts to enhance selectivities by enzyme inhibition (*e.g.* addition of $Fe^{3+}$)[19] were successful but (as usual in inhibition) led to a significant loss of catalytic activities. Furthermore, the substrate tolerance of sulfatase RS2 was rather narrow, as substrates bearing bulky aryl groups (*e.g. rac*-**4a**) were not accepted.[16]

Our search for novel (and more selective) inverting alkylsulfatases was led by the notion that organisms known to possess a rich *inorganic* sulfur metabolism (encompassing inorganic sulfur species of all oxidation states ranging from sulfide to sulfate[20]) might also act on sulfated *organic* species, such as alkyl sulfates.[21] Sulfur-based redox-chains were a prime energy-source for life before molecular oxygen was released into the atmosphere by cyanobacteria some 3.5 billion years ago.[22] Typically, sulfur-metabolizers belong to the kingdom of *Archaea* and were adapted to the harsh environment of the early biosphere, such as high growth temperatures (55–95 °C), low pH (0.0–4.0) and/or high salinity (0.4–3.5 M NaCl).[23] In their mode to gain energy they are extremely flexible and exhibit a great variety of pathways: obligate chemolithoautotrophs utilize only $CO_2$, hydrogen and different inorganic sulfur compounds, such as $S^{2-}$,

† Electronic supplementary information (ESI) available: general analytical and synthetic methods, the synthesis of substrates (*rac*-**1a**–**9a**) and reference materials [(*S*)-**3b**, (*S*)-**7d**, (*S*)-**8d**, (*S*)-**7e**, (*S*)-**8e**, (*S*)-**7f**, (*S*)-**8f**, (*R*)-**7b**, (*R*)-**8b**] and their spectroscopic data. See http://www.rsc.org/suppdata/ob/b5/b504883d/




**Scheme 1** Enzymatic stereo-divergent hydrolysis of sulfate esters catalyzed by sulfatases.

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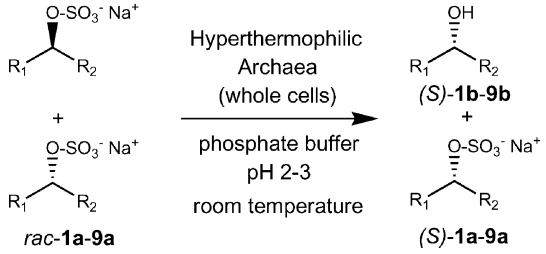
$S_2^{2-}$, $S_8^0$, $SO_3^{2-}$, $S_2O_3^{2-}$, $SO_4^{2-}$, or nitrate. On the other hand, chemo-organotrophs obtain their energy from the oxidation of organic compounds (*e.g.* organic acids, alcohols, sugars, amino acids or polymers, such as starch or chitin) using $S_8^0$, $SO_4^{2-}$ or molecular oxygen as the oxidant.[24]

## Results and discussion

In order to cover a broad range of the kingdom of *Archaea*,[25] a selection of extremophilic sulfur-metabolizers was chosen, and these were grown aerobically[26] and anaerobically[27] on the recommended media. Whole cells were tested for alkylsulfatase activity at pH 2–3, which corresponds to that of their natural habitats, using 2-octyl sulfate (*rac*-**1a**), 1-phenyl-2-propyl sulfate (*rac*-**6a**) and 6-methylhept-5-ene-2-yl sulfate ('sulcatyl sulfate', *rac*-**9a**) as substrates (Scheme 2). For all compounds, blank experiments were performed in the absence of biocatalyst to exclude any undesired spontaneous hydrolysis. From the beginning, a clear picture emerged as all of the eight anaerobically-grown strains[27] proved to be completely inactive on the above-mentioned substrates. On the contrary, all of the 11 aerobically-grown strains could hydrolyze *rac*-**1a** (Table 1, entries 1–11). Best reaction rates were obtained with three *Sulfolobus* spp.: *S. solfataricus* DSM 1617, *S. shibatae* DSM 5389 and *S. acidocaldarius* DSM 639 (entries 9–11). For the sterically more demanding substrate *rac*-**6a**, only the latter strains exhibited reasonable activities (entries 12–14). This trend was even more pronounced for the branched long-chain substrate *rac*-**9a**, which was only accepted at a slow rate by *Sulfolobus acidocaldarius* DSM 639 (entry 15), which clearly emerged as the 'champion' with respect to reaction rates and substrate tolerance.[28]




| compound | $R_1$ | $R_2$ |
|---|---|---|
| **1a**,**b** | $CH_3$ | *n*-$C_6H_{13}$ |
| **2a**,**b** | $C_2H_5$ | *n*-$C_5H_{11}$ |
| **3a**,**b** | *n*-$C_3H_7$ | *n*-$C_4H_9$ |
| **4a**,**b** | $CH_3$ | *n*-$C_5H_{11}$ |
| **5a**,**b** | $CH_3$ | *n*-$C_7H_{15}$ |
| **6a**,**b** | $CH_3$ | Ph-$CH_2$ |
| **7a**,**b** | $CH_3$ | *p*-F-$C_6H_4$-$CH_2$ |
| **8a**,**b** | $CH_3$ | *p*-Cl-$C_6H_4$-$CH_2$ |
| **9a**,**b** | $CH_3$ | $(CH_3)_2C{=}CH{-}(CH_2)_2$ |

**Scheme 2** Enantioselective microbial hydrolysis of *sec*-alkyl sulfate esters with inversion of configuration.

A first look into the enantioselectivities – expressed as *E*-values[29] – by measuring the enantiomeric excess of the formed *sec*-alcohol and the conversion using an internal standard was promising (Table 1). Although no measurable enantiopreference could be detected for the majority of strains showing low activity (entries 1–8), the three most active *Sulfolobus* spp. gave good to excellent enantioselectivities for *rac*-**1a**, with *Sulfolobus acidocaldarius* DSM 639 again being best (*E*-value 106). Low values were observed for *rac*-**6a** (*E*-values up to 2.8).

In comparison to the majority of bacteria and fungi most widely used for whole-cell biotransformations, the growth rates of *Archaea* on the usually recommended complex media are comparably low and values for optical densities did not exceed an OD of *ca.* 0.15 after a period of 35 d, which is unacceptable for preparative-scale experiments. In order to speed up the growth rates, experiments were undertaken with respect to medium optimisation for the most promising strain – *Sulfolobus acidocaldarius* DSM 639 – by supplementing the basal medium according to Brock *et al.*[30] with various 'simple' carbon sources. As shown in Fig. 1, a low cell density (OD 0.17) was obtained on the standard Brock-medium after 35 d. Enrichment of this medium by the addition of glucose at concentrations of 5, 10 and 20 g $L^{-1}$ did not lead to any significant improvement in growth rates. Similarly disappointing results were observed with glycerol (20 g $L^{-1}$) and mannitol (20 g $L^{-1}$). Finally, a three-fold improved growth rate was achieved by using sucrose (20 g $L^{-1}$) as a supplement, which led to an acceptable $OD_{40d}$ of 0.95.

With a greatly facilitated access to biomass in hand, enantioselectivities were re-checked for *Sulfolobus acidocaldarius* DSM 639 grown on sucrose-supplemented medium using substrates *rac*-**1a**–**9a** (Table 2).

We were suprised to see that *S. acidocaldarius* DSM 639 grown on sucrose-enriched medium showed significantly enhanced enantioselectivitites: excellent enantioselectivity was observed for 2-octyl sulfate (*rac*-**1a**, $E > 200$). The most plausible explanation for this phenomenon is the selective induction of an appropriate alkylsulfatase or the suppression of competing enzyme(s) by the sucrose-enriched medium. In comparison,

**Table 1** Screening for *sec*-alkylsulfatase activity in aerobically-grown hyperthermophilic *Archaea*[a]

| Entry | Substrate | Microorganism | Growth medium | Yield[b] (%) | Product/ee (%) | Enantioselectivity (*E*-value) |
|---|---|---|---|---|---|---|
| 1 | *rac*-**1a** | *Acidianus brierley* DSM 1651 | Brock + sucrose + $S_8^0$ | 7.4 | (*R*)-**1b**/2 | *ca.* 1 |
| 2 | *rac*-**1a** | *Sulfolobus metallicus* DSM 6482 | Brock + sucrose + $S_8^0$ | 8.0 | (*R*)-**1b**/2 | *ca.* 1 |
| 3 | *rac*-**1a** | *Sulfololobus hakoniensis* DSM 7519 | Brock + sucrose + $S_8^0$ | 8.4 | (*R*)-**1b**/2 | *ca.* 1 |
| 4 | *rac*-**1a** | *Sulfolobus acidocaldarius* IFO 15159 | Brock + sucrose + $S_8^0$ | 8.3 | (*S*)-**1b**/2 | *ca.* 1 |
| 5 | *rac*-**1a** | *Acidianus infernus* DSM 3191 | Brock + sucrose + $S_8^0$ | 25 | (*R*)-**1b**/3 | *ca.* 1 |
| 6 | *rac*-**1a** | *Acidianus ambivalens* DSM 3772 | Brock + sucrose + $S_8^0$ | 13 | (*R*)-**1b**/3 | *ca.* 1 |
| 7 | *rac*-**1a** | *Metallosphaera sedula* DSM 5348 | Brock + sucrose + $S_8^0$ | 9 | (*R*)-**1b**/3 | *ca.* 1 |
| 8 | *rac*-**1a** | *Sulfurisphaera ohwakuensis* DSM 12421 | Brock + sucrose + $S_8^0$ | 11 | (*R*)-**1b**/3 | *ca.* 1 |
| 9 | *rac*-**1a** | *Sulfolobus solfataricus* DSM 1617 | DSM #182 | 56 | (*S*)-**1b**/77 | 35 |
| 10 | *rac*-**1a** | *Sulfolobus shibatae* DSM 5389 | DSM #88 | 42 | (*S*)-**1b**/92 | 48 |
| 11 | *rac*-**1a** | *Sulfolobus acidocaldarius* DSM 639 | DSM #88 | 33 | (*S*)-**1b**/97 | 106 |
| 12 | *rac*-**6a** | *Sulfolobus solfataricus* DSM 1617 | DSM #182 | 20 | (*S*)-**6b**/35 | 2.3 |
| 13 | *rac*-**6a** | *Sulfolobus shibatae* DSM 5389 | DSM #88 | 20 | (*S*)-**6b**/43 | 2.8 |
| 14 | *rac*-**6a** | *Sulfolobus acidocaldarius* DSM 639 | DSM #88 | 16 | (*S*)-**6b**/25 | 1.8 |
| 15 | *rac*-**9a** | *Sulfolobus acidocaldarius* DSM 639 | DSM #88 | 4 | (*S*)-**9b**/n.d.[c] | n.d.[c] |

[a] Time = 5 d. [b] GC-analysis. [c] n.d. = not determined due to exceedingly low conversion.

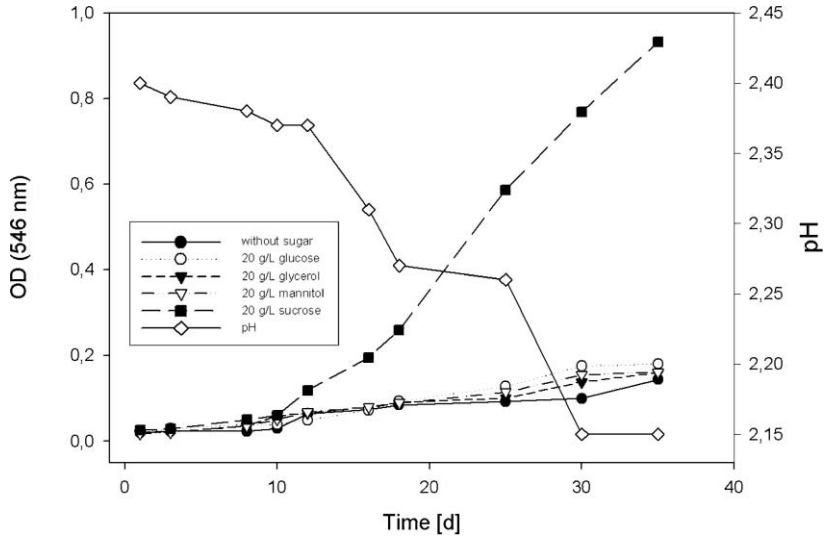



**Fig. 1** Medium optimisation for *Sulfolobus acidocaldarius* DSM 639 by variation of the carbon source.

**Table 2** Enantioselectivities of the microbial hydrolysis of *sec*-alkyl sulfate esters *rac*-**1a**–**9a** using *Sulfolobus acidocaldarius* DSM 639 aerobically grown on sucrose-supplemented Brock-medium[a]

| Substrate | Conversion (%) | Product/ee (%) | Enantioselectivity (*E*-value) |
|---|---|---|---|
| *rac*-**1a** | 32 | (*S*)-**1b**/>99 | >200 |
| *rac*-**2a** | 40 | (*S*)-**2b**/59 | 6 |
| *rac*-**3a** | 43 | (*S*)-**3b**/55 | 5 |
| *rac*-**4a** | 32 | (*S*)-**4b**/62 | 6 |
| *rac*-**5a** | 38 | (*S*)-**5b**/65 | 7 |
| *rac*-**6a** | 42 | (*S*)-**6b**/>99 | >200 |
| *rac*-**7a** | 10 | (*S*)-**7b**/16 | <2 |
| *rac*-**8a** | 11 | (*S*)-**8b**/16 | <2 |
| *rac*-**9a** | 7 | **9b**/n.d.[b] | n.d.[b] |
| (*R*)-**1a**[c] | 40 | (*S*)-**1b**/⩾97 | n.a.[d] |

[a] Time = 5 d. [b] n.d. = not determined due to low conversion. [c] ee ⩾ 97%. [d] n.a. = not applicable.

sulfatase RS2 from *Rhodococcus ruber* DSM 44541 showed only $E = 21$ for *rac*-**1a** in the absence of enzyme inhibitors.[16] When the sulfate ester moiety was gradually moved towards the center of the molecule (3-octyl-sulfate, *rac*-**2a**; 4-octyl sulfate, *rac*-**3a**), the enantioselectivities for *S. acidocaldarius* DSM 639 decreased due to the fact that the alkyl groups flanking the sulfate ester group became similar in size, thus making the chiral recognition process more difficult. We were particularly pleased to see that the sterically demanding substrate *rac*-**6a** (which was not accepted by sulfatase RS2 [16]) was not only well accepted, but also displayed excellent enantioselectivity.

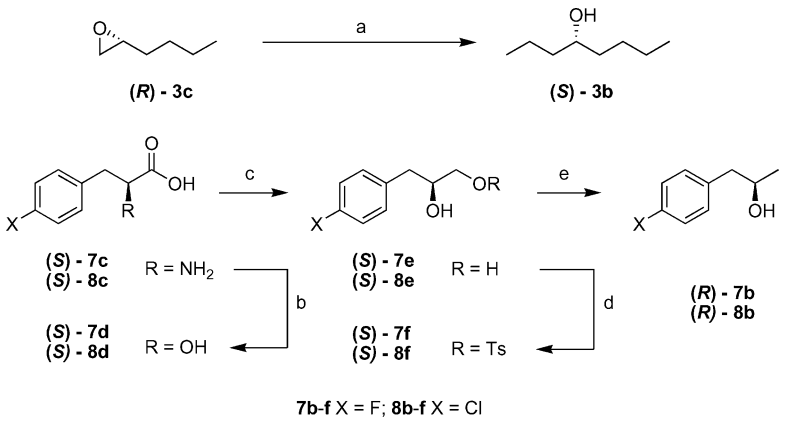
In order to prove the stereochemical course of the hydrolysis with respect to *retention* or *inversion* of configuration, (*R*)-2-octyl sulfate **1a** (ee ⩾ 97%) was hydrolysed with optimised *S. acidocaldarius* DSM 639 cells. As the sole product, (*S*)-2-octanol (ee ⩾ 97%) was detected indicating a highly desired complete inversion of configuration. The enantio-preference of *S. acidocaldarius* DSM 639 for substrates *rac*-**1a**–**8a** revealed a homogeneous picture, *i.e.* the (*R*)-enantiomers were preferentially hydrolysed to furnish the corresponding (*S*)-configurated *sec*-alcohols.

### Determination of absolute configuration

The absolute configuration of products **1b**–**8b** was determined by co-injection of commercially available or independently synthesized reference samples on GC using a chiral stationary phase. (*S*)-4-Octanol was obtained by coupling of ethylmagnesium bromide to (*R*)-(+)-1,2-epoxyhexane.[31] *p*-Halophenylpropanols (*R*)-**7b** and (*R*)-**8b** were synthesized by the following method (Scheme 3). Starting from amino acids (*S*)-**7c** and (*S*)-**8c**, various attempts were undertaken to synthesize (*R*)-**7b** and (*R*)-**8b** *via* the corresponding α-hydroxy acids (*S*)-**7d** and (*S*)-**8d** in a single-step reduction based on a protocol of Gevorgyan *et al*.[32] Thus, the α-hydroxyl group of (*S*)-**7d** and (*S*)-**8d** was protected as benzyl ether[33] and the carboxylic acid moiety was then subsequently reduced using $HSiEt_3$ in the presence of a catalytic amount of $B(C_6F_5)_3$. The reduction, however, failed. Alternatively, single-step reduction of the α-hydroxy acid methyl ester,[34] or the non-protected α-hydroxy acid and the α-hydroxy acid methyl ester were unsuccessful. In a different approach, dehalogenation of the *p*-halogen using Pd on carbon failed likewise.[35] Finally, reduction of (*S*)-**7d** and (*S*)-**8d** by $LiAlH_4$ gave diols (*S*)-**7e** and (*S*)-**8e**, which were transformed *via* the corresponding tosylates (*S*)-**7f** and (*S*)-**8f** into (*R*)-**7b** and (*R*)-**8b**.

## Conclusions

In summary, whole cells of hyperthermophilic sulfur-metabolizers, such as *Sulfolobus solfataricus* DSM 1617, *Sulfolobus shibatae* DSM 5389 and *Sulfolobus acidocaldarius* DSM 639, were identified as a convenient source of *sec*-alkylsulfatase activity with enhanced enantioselectivities and a wider substrate spectrum compared to *Rhodococcus* sulfatase RS2, when grown on a sucrose-supplemented Brock-medium. The full substrate-selectivity pattern of these novel biocatalysts is currently being studied.




**Scheme 3** Synthesis of reference material for (*S*)-**3b**, (*R*)-**7b** and (*R*)-**8b**. *Reagents and conditions*: a) EtMgBr, $Li_2CuCl_4$, THF, −78 °C to rt, 6 h; b) $NaNO_2$, $H_2SO_4$, 0 °C to rt, 18 h; c) $LiAlH_4$, $Et_2O$, reflux to rt, 17 h; d) *p*-TsCl, pyridine, 0 °C to rt, 24 h; e) $LiAlH_4$, $Et_2O$, 0 °C to rt, 3 h.

## Experimental

For general analytical and synthetic methods, the synthesis of substrates (*rac*-**1a**–**9a**) and reference materials [(*S*)-**3b**, (*S*)-**7d**, (*S*)-**8d**, (*S*)-**7e**, (*S*)-**8e**, (*S*)-**7f**, (*S*)-**8f**, (*R*)-**7b**, (*R*)-**8b**] and their spectroscopic data see ESI.†

### Biocatalytic procedures

**Bacterial strains.** All strains except *Sulfolobus acidocaldarius* IFO 15159, were obtained from the Deutsche Stammsammlung von Mikroorganismen, Braunschweig, Germany. *Sulfolobus acidocaldarius* IFO 15159 was obtained from the Institute of Fermentation, Osaka, Japan.

**Strain maintainance.** Cultures were frozen and stored in 50% glycerol solution at −80 °C.

**Anaerobic cultures.** Strains were grown according to the recommended DSMZ media in standing cultures (50 ml flasks flushed with $N_2$ and capped with a septum) at 80 or 90 °C depending on the optimal temperature conditions in a conventional thermostated drying oven.

**Aerobic cultures.** *Sulfolobales* strains were grown in Brock's basal salts mixture:[30] 1.3 g $(NH_4)_2SO_4$, 0.28 g $KH_2PO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CuCl_2 \cdot 2H_2O$, 0.03 mg $VOSO_4 \cdot 2H_2O$, 0.01 mg $CoSO_4 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$ and 4.5 μg $Na_2B_4O_7 \cdot 10H_2O$, per liter, supplemented with 1 g of yeast extract. Additional sugars (glucose, glycerol, mannitol, sucrose) were added in amounts of 20 g $L^{-1}$. The pH was adjusted to 3–3.5 with 50% aqueous $H_2SO_4$. All cells were grown aerobically in standing cultures at 70 °C in a conventional thermostated drying oven.

Cell growth was monitored spectrophotometrically by measurement of the optical density *via* the absorption at 546 and 600 nm. Cells were used for biotransformations when the optical density of the biomass reached an OD-value of 1.

### General screening procedure

*Rac*-2-octyl sulfate (50 g $L^{-1}$), *rac*-1-phenyl-2-propyl sulfate (50 g $L^{-1}$) and *rac*-6-methyl-5-hepten-2-yl sulfate (50 g $L^{-1}$) were selected as test substrates for a screening for new alkylsulfatase activity at different temperatures: rt (anaerobic); rt, 60, 85 and 95 °C, respectively (aerobic). 200 μL of the sulfate ester solution (10 mg sulfate, 200 μl Tris buffer, pH 7.5, 50 mM) was added to 1 mL of the culture and the mixture was shaken at 140 rpm at the given temperature for 3, 5 or 7 d. Every 3rd, 5th and 7th day a sample was withdrawn and analyzed as follows.

### Determination of conversion

An aliquot of 1 mL from the reaction mixture was extracted with 600 μL of ethyl acetate. After vigorous vortexing (30 s), centrifugation and drying over $Na_2SO_4$, 350 μL of the organic phase was mixed with 470 μL ethyl acetate and 70 μL of a stock solution of 2-dodecanol (1 : 10 in EtOAc) as internal standard and the conversion was determined by GC on column A.

### Determination of enantiomeric excess of product

For the determination of the enantiomeric excess, the alcohol formed during biohydrolysis of the sulfate ester was derivatized ($Ac_2O$, DMAP, EtOAc) to obtain the corresponding acetate ester in order to achieve enantioseparation on a chiral GC-column. An aliquot of the enzyme reaction mixture (300 μL) was extracted with 400 μL of ethyl acetate and dried over $Na_2SO_4$. After addition of 500 μL of acetic anhydride and cat. DMAP, the capped Eppendorff vial was shaken at rt for 30 min. Finally, the samples were extracted with 500 μL of water, the organic phase was dried over $Na_2SO_4$ and the samples were analyzed on column B. For GC-data see the ESI.†

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