



Enantioselective degradation and unidirectional chiral inversion of 2-phenylbutyric acid, an intermediate from linear alkylbenzene, by *Xanthobacter flavus* PA1

Yishan Liu^a, Ping Han^a, Xiao-yan Li^b, Kaimin Shih^b, Ji-Dong Gu^{a,c,*}

^a School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

^b Department of Civil Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong, China

^c The Swire Institute of Marine Science, The University of Hong Kong, Shek O, Cape d'Aguilar, Hong Kong, China

ARTICLE INFO

Article history:

Received 15 February 2011

Received in revised form 10 June 2011

Accepted 29 June 2011

Available online 5 July 2011

Keywords:

Enantioselective biodegradation

Chiral inversion

Linear alkylbenzene sulfonates

2-Phenylbutyric acid

Xanthobacter flavus

ABSTRACT

Microbial degradation of the chiral 2-phenylbutyric acid (2-PBA), a metabolite of surfactant linear alkylbenzene sulfonates (LAS), was investigated using both racemic and enantiomer-pure compounds together with quantitative stereoselective analyses. A pure culture of bacteria, identified as *Xanthobacter flavus* strain PA1 isolated from the mangrove sediment of Hong Kong Mai Po Nature Reserve, was able to utilize the racemic 2-PBA as well as the single enantiomers as the sole source of carbon and energy. In the presence of the racemic compounds, *X. flavus* PA1 degraded both (*R*) and (*S*) forms of enantiomers to completion in a sequential manner in which the (*S*) enantiomer disappeared much faster than the (*R*) enantiomer. When the single pure enantiomer was supplied as the sole substrate, a unidirectional chiral inversion involving (*S*) enantiomer to (*R*) enantiomer was evident. No major difference was observed in the degradation intermediates with either of the individual enantiomers when used as the growth substrate. Two major degradation intermediates were detected and identified as 3-hydroxy-2-phenylbutanoic acid and 4-methyl-3-phenyloxetan-2-one, using a combination of liquid chromatography–mass spectrometry (LC–MS), and ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy. The biochemical degradation pathway follows an initial oxidation of the alkyl side chain before aromatic ring cleavage. This study reveals new evidence for enantiomeric inversion catalyzed by pure culture of environmental bacteria and emphasizes the significant differences between the two enantiomers in their environmental fates.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Many organic pollutants discharged into the environment are chiral compounds containing stereo isomers. The environmental fates of chiral pollutants can be stereoselective, and the biological degradation, uptake, accumulation and toxicological effect of enantiomers may also be quite different [1–4]. With respect to stereochemistry, research on biodegradation of chiral pollutants can provide a better understanding of environmental safety of these chemicals to humans, animals and the environment [5–10]. In most cases, microorganisms enantioselectively degrade the chiral compounds: a specific strain might degrade only one enantiomer [11,12] or it might sequentially degrade both enantiomers [13–15]. Enantiomer-specific enzymes [16–20] were reported to be involved in the degradation or uptake process.

Under selective conditions, enantiomerization/racemization, the chiral conversion of one enantiomer into its antipode, may occur in the environment. Chiral phenoxy propionic acids mecoprop [(*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid] and dichlorprop [(*RS*)-2-(2,4-chlorophenoxy)propionic acid] [21–23] were reported to proceed a biochemically mediated enantiomerization in soil and lakes in both directions, resulting in the formation of the *S* enantiomers from the *R* enantiomers and *vice versa*. Enantiomerization may influence efficacy and side effects of the enantiopure products, especially in optical active drugs development [24] and their environmental fate.

Both phenyl and sulfophenyl carboxylates are degradation metabolites of the most widely used surfactant-linear alkylbenzene sulfonates (LAS) and their occurrences have been reported in fresh water [25], marine waters [26,27], riverine and coastal sediments [28,29] and sewage sludge [30]. Although the degradation of LAS has been studied extensively [31–37], the fates of their degradation intermediate metabolites are still unclear. Furthermore, due to the presence of an asymmetrically substituted C-atom in the carboxyl alkyl moiety, most of the phenyl carboxylates are chiral

* Corresponding author at: School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China. Tel.: +852 2299 0605; fax: +852 2559 9114.
E-mail address: jdgu@hkucc.hku.hk (J.-D. Gu).

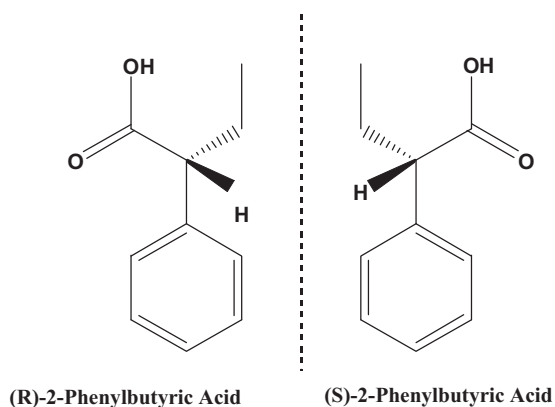


Fig. 1. Chemical structures of (S)-(+)-2-phenylbutyric acid and (R)-(-)-2-phenylbutyric acid.

and that makes their degradative mechanisms more complicated. Sariaslani et al. described two different degradation biochemical pathways of 3-phenylbutyric acid by *Pseudomonas* sp. [38] through an initial oxidation of the benzene ring and of the side chain. However, enantiomer-specific analytical tools were not applied in their research to further delineate the enantioselectivity in the degradation processes.

With respect to stereochemistry, *Rhodococcus rhodochrous* PB1 [39] isolated from soil enantioselectively metabolizes 3-phenylbutyric acid: only (R) enantiomer supports growth of strain PB1, whereas the (S) enantiomer is only co-metabolized to a dihydroxylated intermediate (S)-3-(2,3-dihydroxyphenyl)butyric acid. A meta-cleavage pathway of (R) enantiomer is also suggested to form 2-hydroxy-6-oxo-2,4-nonadiene-1,9-dicarboxylic acid from dioxygenation product 3(2,3-dihydroxyphenyl)propionic acid. A *Delftia acidovorans* SPB1 [40], isolated from activated sludge, is able to utilize the enantiomers of 2-(4-sulfophenyl) butyrate sequentially. The (R) enantiomer is degraded first and, only when it is exhausted, the (S) enantiomer starts to degrade. Metabolism of the two enantiomers converges at 4-sulfocatechol and subsequently undergoes through ortho-cleavage. Enantioselectivity is also reported by Schleheck et al. [33] in that the (S) enantiomer of RS-4-C6-SPC[RS-4-(4-sulfophenyl)hexanoate] is utilized faster than (R) enantiomer by *D. acidovorans* SPH-1. Although enantioselectivity is reported in these degradation processes of phenyl and sulfophenyl carboxylates, more mechanism information including detailed degradation pathway, specific enzyme systems involved and chiral stability remain to be further explored.

In this study, we investigated the degradation of chiral 2-phenylbutyric acid (2-PBA) (Fig. 1) by *Xanthobacter flavus* PA1 using enantiomer-specific analytical techniques. We described the enantioselectivity and two major degradation intermediates produced during the biodegradation process. A unidirectional chiral inversion from (S) enantiomer to (R) enantiomer of 2-PBA catalyzed by pure culture of environmental bacteria was reported for the first time.

2. Materials and methods

2.1. Enrichment culture and isolation

The initial aerobic enrichment culture was established in mineral salts medium (MSM) by adding mangrove sediments taken from the Mai Po Nature Reserve of Hong Kong as inoculant, with 2-PBA (starting concentration 0.62 mM [100 mg/l]) as the sole source of carbon and energy. The positive enrich-

ment cultures were further transferred and incubated at least 4 times in freshly made MSM before isolation of bacteria. Individual colonies were picked and streaked on selective agar plates supplemented with 2-PBA (1.85 mM [300 mg/l]) to isolate pure species of bacteria. After confirmation on their degradation capability, strain PA1 capable of degrading 2-PBA was identified using 16S rDNA sequence as a *X. flavus* and was used in subsequent experiments.

2.2. Mineral salts medium

The MSM used for growth of strain PA1 consisted of a buffer solution (adjusted to pH 7.0) containing KH_2PO_4 (0.8 g/l), K_2HPO_4 (0.2 g/l), $(\text{NH}_4)_2\text{SO}_4$ (1.0 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/l). The following trace elements were supplied from a stock solution to the final concentrations (mg/l): CaCl_2 5, FeSO_4 10, NiSO_4 32, $\text{Na}_2\text{O}_7 \cdot \text{H}_2\text{O}$ 7.2, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$ 14.4, ZnCl_2 23, $\text{CoCl}_2 \cdot \text{H}_2\text{O}$ 21, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 10 and $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 30. The medium was also supplemented with 10 mg of yeast extract (Difco Lab., Detroit, Michigan) per liter. The carbon sources in the R, S, or racemic form of 2-PBA were added to MSM before autoclaving because neither loss nor transformation of these chemicals was observed after autoclaving at 121 °C for 30 min.

2.3. Complex culture medium

The non-selective complex culture medium consisting of 20 g bacteriological peptone (Lab M, Bury, England) per liter was used for preservation of the cultures and preparation for growth experiments.

2.4. Chemicals

Analytical standards of racemic 2-phenylbutyric acid (98%) and (S)-(+)-2-phenylbutyric acid (99%) were purchased from International Laboratory (San Bruno, CA, USA). (R)-(-)-2-phenylbutyric acid (99%) was purchased from Sigma-Aldrich (Steinheim, Germany). All solvents used as the chromatographic eluent in the experiments were of chromatography pure quality and purchased from Tedia (Fairfield, OH, USA). Other chemicals were from Sigma-Aldrich or Acros Organics (Fair Lawn, NJ, USA).

2.5. Growth experiments

The bacterial cultures used for inoculation in the experiments were pre-incubated in complex culture medium in a 300 ml Erlenmyer flask maintained in an incubator shaker (New Brunswick Scientific Co., Inc., NJ, USA) at 30 °C and 150 rpm. In order to remove the residual organic carbon in the bacterial cultures prior to the growth experiments, the bacterial cultures were harvested by centrifugation (10 min, 13,000 rpm) at the middle exponential growth phase, washed, and re-suspended in fresh MSM. The inoculation started with 2% (v/v) of the purified bacterial culture into the newly prepared MSM. Degradation experiments were carried out in 150 ml culture medium in 300 ml flasks kept in an incubator at 30 °C and 150 rpm. The racemic, R- or S-2-PBA was added into respective MSM as the sole source of carbon and energy for growth, with an initial concentration of 2.45, 1.23, 1.23 mM (400, 200, 200 mg/l). The growth experiments were all performed in triplicate, and control experiments were carried out under identical operating conditions as mentioned above with autoclaved cells (inoculant). When large amount of cultures were required for intermediate isolation, the growth experiments were carried out in 500 ml culture medium in 11 Erlenmyer flasks in an incubator maintained at 30 °C and 150 rpm.

2.6. Biomass measurement

During growth experiments, the culture medium was taken at regular time intervals. The microbial biomass was determined by optical density measurements at 580 nm using an UV-1201 spectrophotometer (Shimadzu, Japan).

2.7. Sample storage and preparation

After biomass measurement, the culture samples taken from growth experiments were stored at -20°C for further chemical analysis. Prior to HPLC analysis, the samples were thawed, centrifuged (10 min, 13,000 rpm), and filtered through 0.2- μm -pore-size cellulose ester membrane filter (Advantec MFS, Inc., Dublin, CA, USA) to remove bacteria cells and undesirable particulates.

2.8. Purification and isolation of metabolites

When large quantities of degradation intermediates were needed for chemical identification, culture samples at appropriate time of incubation were centrifuged (20 min, 13,000 rpm) and filtered through 0.2- μm -pore-size mixed cellulose ester membrane to remove the bacteria cells. Acetone was then used for protein precipitation and removal of the undesirable soluble proteins in the filtrate. The aliquot sample was mixed with 2 volumes of acetone and kept at -20°C overnight. Centrifugation (20 min, 13,000 rpm) and filtration were performed again to remove the precipitated proteins. The filtrate was acidified with HCl to pH 2 and extracted three times with 0.5 volume of ethyl acetate. The extracts were combined and concentrated by removing the solvent under reduced pressure. Then the two major intermediates were separated and concentrated using HPLC with a semi-preparative Alltima C18 column (250 mm \times 10 mm, Alltech Associates, Inc., Deerfield, IL, USA). The solvent in each of the fractions was removed under reduced pressure, and the residues obtained were dissolved in methanol for LC-MS. Collected fractions were concentrated and evaporated to dryness under vacuum for NMR analyses.

2.9. Analytical procedures

2.9.1. HPLC

High-pressure liquid chromatography (HPLC) analysis was performed on an Agilent 1100 system with a G1311A QuatPump and a diode array G1315B DAD detector (Agilent Technologies, Santa Clara, CA). (*R*)- and (*S*)-2-PBA (retention times, 15.0 and 15.3 min, respectively) were separated on a Chiralcel OD-RH column (150 mm \times 4.6 mm, Daicel Chemical Industry, Ltd., Osaka, Japan) and detected at a wavelength of 210 nm. The system was operated with an eluent consisting of 50% CH_3CN and 50% $\text{NaClO}_4\text{-HClO}_4$ solution (200 mM; pH 2.0) at a flow rate of 1.2 ml/min. When there was no need for chiral separation, the system was performed on a Eclipse XDB-C8 column (150 mm \times 4.6 mm, Agilent, Santa Clara, CA) with a 40% CH_3OH and 60% NaH_2PO_4 (50 mM; pH 3.0) solution as the eluent at a flow rate of 1.0 ml/min. A semipreparative Alltima C18 column (250 mm \times 10 mm, Alltech Associates, Inc., Deerfield, IL, USA) was used for separation and purification of the major intermediates. The mobile phase consisted of 40% CH_3OH and 60% NaH_2PO_4 (50 mM; pH 3.0) solution at a flow rate of 2.0 ml/min.

2.9.2. LC-MS

Liquid chromatography-mass spectrometry (LC-MS) analysis of degradation metabolites was performed on an Agilent quadrupole mass selective detectors (G1946B system) outfitted with Agilent 1100 system with a G1312 Binary pump and a diode array G1315B

DAD detector (Agilent Technologies, Santa Clara, CA). The system was performed on an ODS column (150 mm \times 2.1 mm, Shimadzu, Japan) with CH_3CN and 0.05% formic acid solution as the eluent at a flow rate of 1.0 ml/min. The mobile phase program was with 10% CH_3CN increasing to 90% in 15 min. Mass analysis was carried out using positive ion mode electro spray ionization (ESI).

2.9.3. NMR spectroscopy

Vacuum-dried fraction samples derived from growth experiments were dissolved in 400 μL of deuteriochloroform (CDCl_3) with tetramethylsilane (TMS) as internal standard and placed in 15 cm length and 4 mm diameter NMR tubes for NMR analyses. The NMR spectrum was obtained on a Bruker Avance DPX 500 Fourier Transform Spectrometer, operating at a proton frequency of 500 MHz for ^1H and 125 MHz for ^{13}C . Proton NMR spectra were recorded with a sweep-width of 7 kHz, 32 K data points per sample at a temperature of 300 K. In addition, two-dimensional NOESY experiments were recorded with a spectral width of 3980 Hz, 512 steps in f_1 -dimension, and a repetition delay of 6.4 s.

3. Results and discussion

3.1. Growth characteristics of *X. flavus* PA1 with racemic 2-phenylbutyric acid

X. flavus strain PA1, could utilize both enantiomers as the sole carbon and energy source in an enantioselective manner. When racemic compound 2-PBA was supplied as the growth substrate, degradation of both enantiomers to completion in an enantioselective manner by *X. flavus* PA1 was observed over 350 h of incubation (Fig. 2A). At the beginning of the incubation, equal amounts of both enantiomers were supplemented and bacteria started to grow after a lag phase of about 50 h. Accompanied with an apparent decrease of (*S*) enantiomer, the growth of *X. flavus* PA1 was observed to proceed through two kinetically distinguishable phases. The first growth phase started from the end of the lag phase and lasted for about 50 h, at a cell growth rate of 0.0356 h^{-1} . In the second growth phase, strain PA1 grew apparently slower at a growth rate of 0.0038 h^{-1} . The decline of (*S*) enantiomer was much faster than the (*R*) enantiomer. A notable phenomenon was observed in that the amount of (*R*) enantiomer increased gradually to reach a maximum of 130% after 125 h of incubation, and then decreased rapidly until disappeared completely.

3.2. Evidence for chiral inversion in growth experiments with single enantiomer

A unidirectional enantiomeric inversion was observed in that only the (*S*) enantiomer could be converted into its antipode before mineralization. When the single (*S*) enantiomer was provided to strain PA1 as the sole source of carbon and energy for growth (Fig. 2B), a decline of (*S*) enantiomer was observed with the formation and subsequent degradation of (*R*) enantiomer in the culture media. Both enantiomers were degraded to completion after 200 h of incubation, the concentration of (*R*) enantiomer reached a maximum of 0.55 mM (90 mg/l) in the culture medium after 135 h. On the contrary, when only the single (*R*) enantiomer was provided as the growth substrate (Fig. 2C), the (*S*) enantiomer could not be detected during the entire incubation period. These results clearly indicated that the enantiomeric inversion catalyzed by strain PA1 was a unidirectional one in which only the (*S*) enantiomer of 2PBA was converted to its antipode. Representative HPLC chromatograms (Fig. S1) from the batch experiments with the single (*S*) enantiomer after 0, 116 and 155 h of incubation, show an initially pure composition of (*S*) enantiomer and then the formation of (*R*) enantiomer from (*S*) enantiomer in the cultures. Furthermore, the

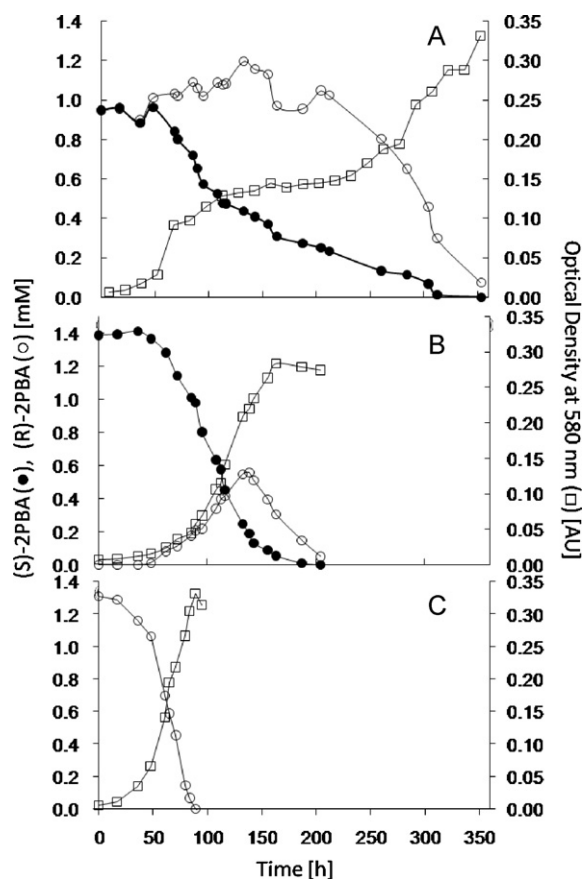


Fig. 2. Substrate utilization experiments with *X. flavus* PA1 incubated with racemic 2-phenylbutyric acid, (S)-2-phenylbutyric acid or (R)-2-phenylbutyric acid. (A) Growth on the racemic mixtures, (B) growth on the pure (S) enantiomer, and (C) growth on the pure (R) enantiomer.

concentrations of both enantiomers remain constant in the control experiments during the entire experiment period (data not shown), indicating that the degradation and chiral inversion processes were biochemically mediated.

The chiral inversion may be bidirectional or unidirectional. The mechanisms and racemases, the enzymes that catalyze the inversion of stereochemistry in biological molecules, are summarized [41,42]. It is noted that, one drug derivative 2-arylpropionic acid (APA) (so-called profens), with structure similar to 2-PBA, is known to undergo metabolic chiral inversion *in vivo*. Within the APA derivatives, ibuprofen is the most studied and has been shown to undergo unidirectional chiral inversion (from *R* to *S*) in different animal species and humans [43–45]. The enzymatic mechanism [46–49] of chiral inversion of ibuprofen contains three steps involving the formation of the acyl-CoA thioester by stereoselective activation of *R* enantiomer in the presence of acyl-CoA synthetase and enzymatic epimerization of the *R*-thioester to the *S*-(+)-thioester followed by the formation of *S*-(+)-enantiomer by hydrolysis of *S*-(+)-thioester.

3.3. Kinetics considerations

The rate equations for a process involving both degradation and enantiomerization (chiral inversion) assuming first-order kinetics are [21]:

$$\frac{d[R]}{dt} = -k_R[R] - k_{RS}[R] + k_{SR}[S] \quad (1)$$

$$\frac{d[S]}{dt} = -k_S[S] - k_{SR}[S] + k_{RS}[R] \quad (2)$$

whereby $[R]$ and $[S]$ are the concentrations, k_R and k_S are the rates of decomposition, and k_{RS} (*R* to *S*) and k_{SR} (*S* to *R*) are the rates of chiral inversion of the *R* and *S* enantiomers, respectively. Enantiomer fraction (EF), which is considered superior to the enantiomer ratio (ER) [50], is used as the standard descriptor of enantiomeric signatures as follows:

$$EF_S = \frac{[S]}{[R] + [S]} \quad (3)$$

The EF values can only range from 0 to 1.0 with EF = 0.5 representing a racemic mixture.

Due to the unidirectional chiral inversion from (*S*) enantiomer of 2PBA to (*R*) enantiomer by the strain PA1, $k_{RS} = 0$ (inversion from *R* to *S* can be neglected). During the incubation processes with racemic mixtures as well as (*S*) enantiomer of 2PBA, there was a continuous formation of (*R*) from (*S*) enantiomer. Thus, the rate equations can be described as

$$\frac{d[R]}{dt} = -k_R[R] + k_{SR}[S] \quad (4)$$

$$\frac{d[S]}{dt} = -k_S[S] - k_{SR}[S] \quad (5)$$

In the growth experiment with racemic 2PBA, the net (overall) dissipation rate (k_{net} , d^{-1}) of (*S*) enantiomer can be described as the sum of decomposition and enantiomeric inversion ($k_{net} = k_S + k_{SR}$). Following a first-order kinetic reaction, the net dissipation rate of (*S*) enantiomer was derived from “ $\ln(C_0/C)$ vs. t ” plots (neglecting the lag phase) and calculated as $0.009 h^{-1}$, and corresponding half-life for degradation of (*S*) enantiomer was 77 h. The kinetics in dissipation of (*R*) enantiomer was more complicated, and the net dissipation rate of (*R*) enantiomer was concerned to concentrations of both enantiomers. In an early stage of incubation, the concentrations of the two enantiomers were equal; and in the late stage, the concentration of (*S*) enantiomer was much lower than (*R*) enantiomer. Thus, the dissipation of (*R*) enantiomer was assumed to follow a pseudo-first-order kinetic reaction, and proceeded a two-phase kinetic with an initially slow formation rate ($-0.0007 h^{-1}$) followed by a more rapid dissipation rate ($0.0154 h^{-1}$). The concentration of (*R*) enantiomer increased to reach a maximum of 130% after 125 h of incubation, indicating that in the early stage of incubation the inversion rate from (*S*) enantiomer to (*R*) enantiomer was higher than the decomposition rate of (*R*) enantiomer. The EF_S values decreased gradually from 0.5 to 0, indicating the preferential dissipation of (*S*) enantiomer by strain PA1 in the presence of racemic 2PBA.

For the incubation process with single (*R*) enantiomer, chiral inversion can be neglected and the equation will be

$$\frac{d[R]}{dt} = -k_R[R] \quad (6)$$

The degradation rate of *R* enantiomer was calculated (neglecting the lag phase about 20 h) as $0.0426 h^{-1}$ and corresponding half-life was 16.3 h.

When the strain PA1 was incubated with single (*S*) enantiomer ($EF_S = 1$), the overall dissipation rate of (*S*) enantiomer was calculated (neglecting the lag phase about 50 h) as $0.032 h^{-1}$ and corresponding half-life was 21.7 h. This time, interestingly, the concentration curves for the *R* and *S* enantiomers intersected (Fig. 2B) after 120 h of incubation ($EF_S = 0.5$), indicating reversed enantiomeric composition with higher ratio of the *R* enantiomer. The kinetics in dissipation of (*R*) enantiomer also can be divided into two kinetic phases with rates as -0.026 and $0.0277 h^{-1}$.

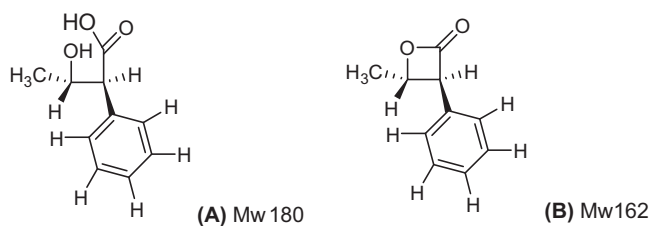


Fig. 3. Chemical structures of the two major degradation intermediates (A) 3-hydroxy-2-phenylbutanoic acid and (B) 4-methyl-3-phenyloxetan-2-one.

3.4. Identification of intermediates produced during growth of *X. flavus* PA1

The formation and further degradation of the two major degradation intermediates were detected using HPLC on the culture aliquot samples and no difference was observed on intermediates produced between the two enantiomers. After purification and isolation steps by semipreparative HPLC, as mentioned above, two major degradation intermediates were identified using a series of LC–MS and NMR analyses.

Combining LC–MS spectra and ^1H NMR, ^{13}C NMR, COESY and HSQC spectroscopy (Supplement material), we concluded the two degradation intermediates have the following chemical structures: 3-hydroxy-2-phenylbutanoic acid (A) and 4-methyl-3-phenyloxetan-2-one (B) (Fig. 3). These two major degradation intermediates produced during degradation process indicate an initial oxidation of the alkyl side chain before aromatic ring cleavage.

The biochemical degradation pathway of racemic 2-PBA by strain PA1 is proposed (Fig. 4).

LC–MS spectra included M+1-H₂O (163), M+H (181), M+Na (203), M+2Na-1 (225) except the core structure part ions, such as 117, 135. In contrast, the spectra (Supplement material) did not contain the 181 peak, but M+H (163), M+18+Na (203), M+2Na-1 (225) ions were found. Compared with their similarity in NMR spectra, the degradation intermediate B is a further dehydrated product of intermediate A. The chemical shifts of peak and peak assignment from NMR analysis are summarized and shown in Table 1.

For the stereochemistry of these two degradation metabolites, NOESY analysis was also conducted (Fig. 5). The NOE effect between protons a and c is stronger than that between a and b from the NOESY spectra while the NOE effect between protons b and h is stronger than that between c and h. Thus, we conclude that proton a and b of the two degradation metabolites should have a *trans* relationship combining the NMR results presented above.

Our data showed that, the initial metabolic attack of 2-PBA is at the β -C atom of the carboxyl alkyl side chain through hydroxylation, not at the aromatic ring. This may be due to the steric hindrance caused by the spatial structure of the chiral carboxyl alkyl moiety attached on the benzene ring. It might be too hard to directly attack through oxidation at the aromatic ring for further ring fission. This view is in agreement with results from the previous studies on degradation of phenyl and sulfophenyl carboxylates, 3-phenylbutyric acid and 2-(4-sulophenyl)butyrate [39,40]. When *R. rhodochrous* PB1 was incubated with 3-phenylbutyric acid [39]: the (*R*) enantiomer was initially demethylated at the chiral center of the carboxyl alkyl side chain to decrease the steric hindrance

Table 1

A summary of chemical shifts for the two degradation intermediates detected in transformation of 2-phenylbutyric acid by *Xanthobacter flavus* PA1.

Intermediate A									
^1H chemical shifts (ppm vs. TMS)									
a	b	c	d	e	f	g	h		
3.56(d, $J=6.9$ Hz)	4.41–4.38(m)	1.23(d, $J=6.3$ Hz)	7.39–7.37(m)	7.37–7.35(m)	7.34–7.31(m)	7.37–7.35(m)	7.39–7.37(m)		
^{13}C chemical shifts (ppm vs. TMS)									
2	3	4	5	6	7	8	9	10	11
176.7	58.4	68.4	20.5	134.5	129.3	128.9	128.1	128.9	129.3
Intermediate B									
^1H chemical shifts (ppm vs. TMS)									
a	b	c	d	e	f	g	h		
3.55(d, $J=9.2$ Hz)	4.36(qd)	1.23(d, $J=6.3$ Hz)	7.30–7.27(m)	7.35–7.31(m)	7.37–7.34(m)	7.35–7.31(m)	7.30–7.27(m)		
^{13}C chemical shifts (ppm vs. TMS)									
2	3	4	5	6	7	8	9	10	11
177.0	59.8	69.6	20.5	135.7	128.9	128.4	128.0	128.4	128.9

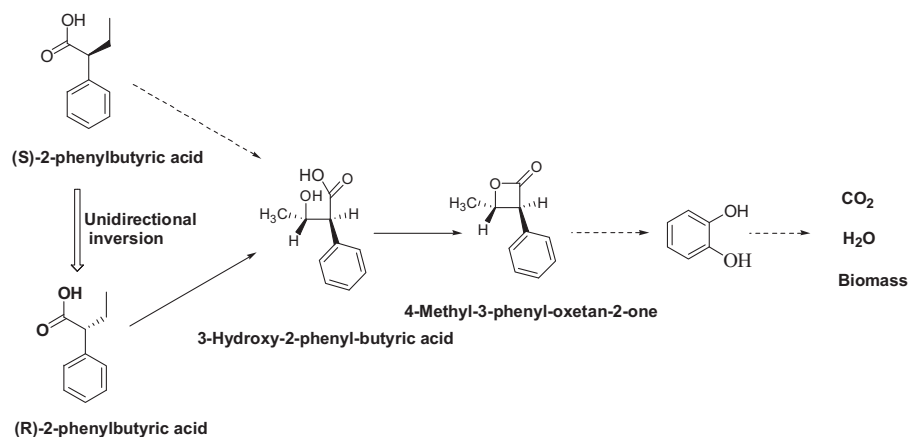


Fig. 4. Biochemical pathway proposed for the metabolism of (R)- and (S)-2-phenylbutyric acid by *X. flavus* PA1. A unidirectional chiral inversion from (S) to (R) enantiomer was observed; together with further metabolically transfer to 3-hydroxy-2-phenylbutanoic acid and 4-methyl-3-phenyloxetan-2-one. Side chain cleavage is supposed to occur before the oxidation and opening of aromatic ring.

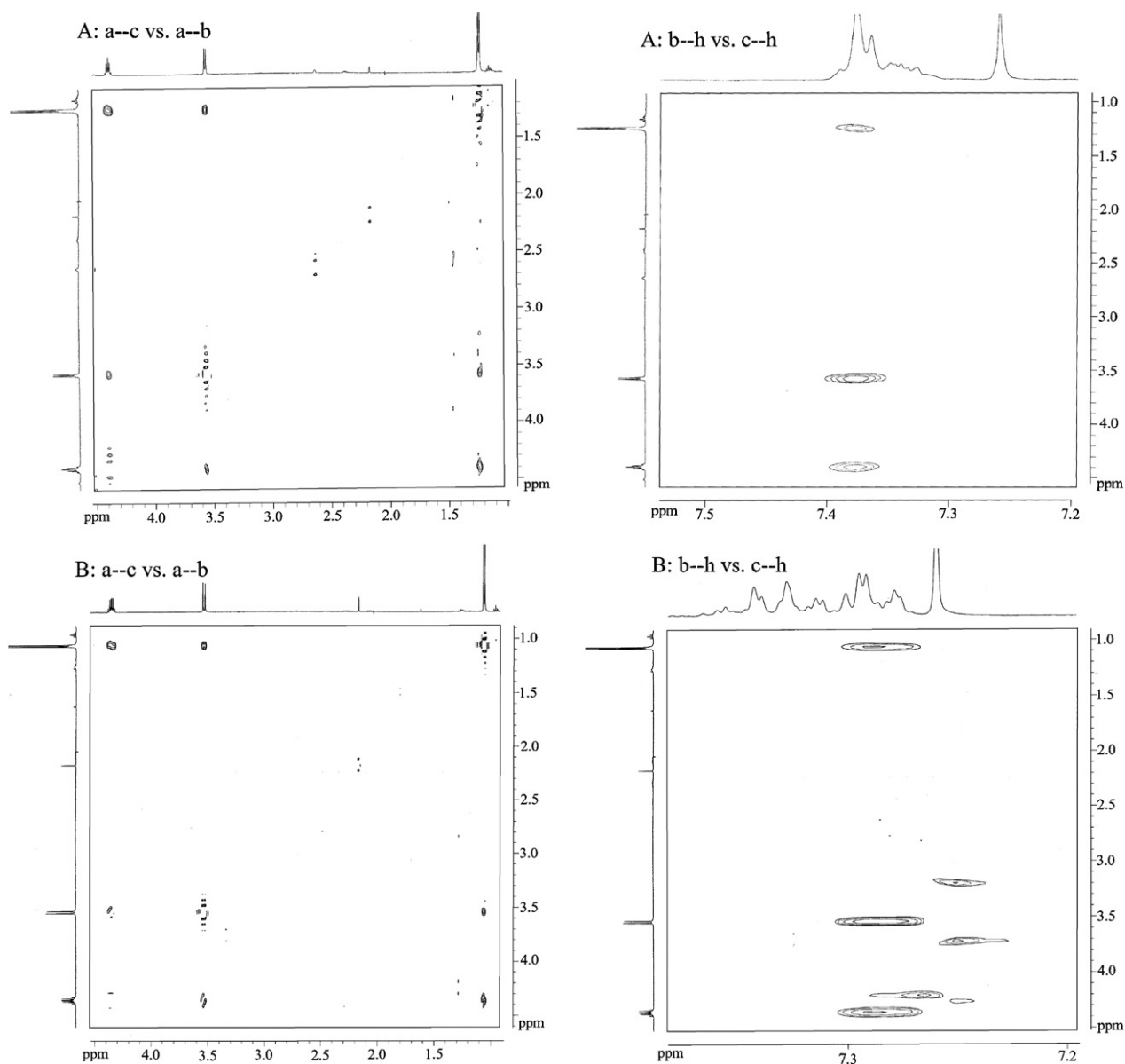


Fig. 5. NOESY spectra of the two degradation intermediates detected in this study.

and further metabolized via *meta* ring cleavage; whereas the (*S*) enantiomer is only cometabolically oxidized to (*S*)-3-(2,3-dihydroxyphenyl)butyric acid without ring cleavage. In another study [40] with *D. acidovorans* SPB on 2-(4-sulfophenyl)butyrate, metabolism of the two enantiomers converges at 4-sulfocatechol indicating the initial side chain cleavage, but no further information is available prior to the detection of this degradation intermediate.

3.5. Proposed mechanisms of enantioselective metabolism of 2-PBA by *X. flavus*

Due to the absence of intermediates which have hydroxyl groups on the aromatic ring together with alkyl side chain, we propose that the major metabolic pathway may follow an initial hydroxylation at the β -C of carboxyl alkyl side chain and further dehydration to a tetratomic ring, and then followed by side chain cleavage and further oxidation at the aromatic ring for ring fission. The *trans* position relationship between the two protons of degradation intermediates may be due to the specific attack direction and chiral structures of the corresponding enzymes.

The mechanisms and enzymes involved in the enantioselective metabolism of 2-PBA by *X. flavus* PA1 are still unclear. Some enantiomer-specific enzymes including racemase catalyzing chiral inversions may be involved, causing the unidirectional enantiomeric inversion from *S*-(+)-2-PBA to *R*-(-)-2-PBA. During the incubation process with only the single *S*-(+)-2-PBA as the sole source of carbon and energy for growth, the decomposition and enantiomeric inversion were observed concurrently. Although the (*S*) enantiomer could be degraded to completion, there is no evidence to ascertain whether the strain PA1 could decompose *S*-(+)-2-PBA directly, or it could only selectively decompose the *R*-(-)-2-PBA which was inverted from the (*S*) enantiomer. In other words, relevant degradative enzymes for alkyl side chain might also be enantioselective.

4. Conclusions

Nowadays, more and more new optical active products, manufactured by agrochemical and pharmaceutical industry, are introduced into market aiming to increase efficacy and specificity, and reduce toxicity. These chemical may be dispersed and accumulated in the environment. However, little information is available concerning stereochemistry in the study of environmental fates of diverse chiral compounds. In this study, we described the complete microbial degradation of the chiral 2-phenylbutyric acid, a metabolite of surfactant LAS, in an enantioselective manner by *X. flavus* PA1. Furthermore, a unidirectional enantiomeric inversion catalyzed by pure culture of environmental bacteria has been established for the first time. Our study emphasizes the importance of stereospecificity in the microbial metabolism and environmental fate of chiral compounds. Further work on biochemical enzymes involved for these phenyl carboxylates concerning stereochemistry will be carried out to advance our understanding on these mechanisms.

Acknowledgements

This research project was supported in part by grants from Faculty of Science, Faculty of Engineering and The University of Hong Kong on Sustainable Water. We would like to thank Xiang Li and Baofu Zheng for their help with NMR spectrometry and stimulating discussions on enantiomer-specific analyses, Li Pan for 16S rRNA gene sequencing, and Jessie Lai for laboratory assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.06.088.

References

- [1] C.S. Wong, Environmental fate processes and biochemical transformations of chiral emerging organic pollutants, *Anal. Bioanal. Chem.* 386 (2006) 544–558.
- [2] W. Liu, J. Gan, D. Schlenk, W.A. Jury, Enantioselectivity in environmental safety of current chiral insecticides, *Proc. Natl. Acad. Sci.* 102 (2005) 701–706.
- [3] T.A. Müller, H.-P.E. Kohler, Chirality of pollutants—effects on metabolism and fate, *Appl. Microbiol. Biotechnol.* 64 (2004) 300–316.
- [4] L. Wang, S. Zhou, K. Lin, M. Zhao, W. Liu, J. Gan, Enantioselective estrogenicity of *o,p'*-dichlorodiphenyltrichloroethane in the MCF-7 human breast carcinoma cell line, *Environ. Toxicol. Chem.* 28 (2009) 1–8.
- [5] S. Qjin, R. Budd, S. Bondarenko, W. Liu, J. Gan, Enantioselective degradation and chiral stability of pyrethroids in soil and sediment, *J. Agric. Food Chem.* 54 (2006) 5040–5045.
- [6] S.A. Law, T.F. Bidleman, M.J. Martin, M.V. Ruby, Evidence of enantioselective degradation of α -hexachlorocyclohexane in groundwater, *Environ. Sci. Technol.* 38 (2004) 1633–1638.
- [7] H.-R. Buser, M.D. Müller, T. Poiger, M.E. Balmer, Environmental behavior of the chiral acetamide pesticide metalaxyl: enantioselective degradation and chiral stability in soil, *Environ. Sci. Technol.* 36 (2002) 221–226.
- [8] D.L. Lewis, A.W. Garrison, K.E. Wommack, A. Whittemore, P. Steudler, J. Melillo, Influence of environmental changes on degradation of chiral pollutants in soils, *Nature* 401 (1999) 898–901.
- [9] C. Zipper, T. Fleischmann, H.-P.E. Kohler, Aerobic biodegradation of chiral phenoxyalkanoic acid derivatives during incubations with activated sludge, *FEMS Microbiol. Ecol.* 29 (1999) 197–204.
- [10] M.K. Ramezani, D.P. Oliver, R.S. Kookana, W. Lao, G. Gill, C. Preston, Faster degradation of herbicidally-active enantiomer of imidazolinones in soils, *Chemosphere* 79 (2010) 1040–1045.
- [11] V.A. Tett, A.J. Willetts, H.M. Lappin-Scott, Enantioselective degradation of the herbicide mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid] by mixed and pure bacterial cultures, *FEMS Microbiol. Ecol.* 14 (1994) 191–199.
- [12] V.A. Tett, A.J. Willetts, H.M. Lappin-Scott, Biodegradation of the chlorophenoxy herbicide (*R*)-(+)-mecoprop by *Alcaligenes denitrificans*, *Biodegradation* 8 (1997) 43–52.
- [13] C. Zipper, M. Bunk, A.J.B. Zehnder, H.-P.E. Kohler, Enantioselective uptake and degradation of the chiral herbicide dichlorprop [(*RS*)-2-(2,4-dichlorophenoxy)propanoic acid] by *Sphingomonas herbicidovorans* MH, *J. Bacteriol.* 180 (1998) 3368–3374.
- [14] C. Zipper, K. Nickel, W. Angst, H.-P.E. Kohler, Complete microbial degradation of both enantiomers of the chiral herbicide mecoprop [(*RS*)-2-(4-chloro-2-methylphenoxy)propionic acid] in an enantioselective manner by *Sphingomonas herbicidovorans* sp. nov., *Appl. Environ. Microbiol.* 62 (1996) 4318–4322.
- [15] L.-A. Garbe, M. Moreno-Horn, R. Tressl, H. Görisch, Preferential attack of the (*S*)-configured ether-linked carbons in bis-(1-chloro-2-propyl) ether by *Rhodococcus* sp. strain DTB, *FEMS Microbiol. Ecol.* 55 (2006) 113–121.
- [16] T.A. Müller, T. Fleischmann, J.R. van der Meer, H.-P.E. Kohler, Purification and characterization of two enantioselective α -ketoglutarate-dependent dioxygenases, RdpA and SdpA, from *Sphingomonas herbicidovorans* MH, *Appl. Environ. Microbiol.* 72 (2006) 4853–4861.
- [17] K.M. Schleinitz, S. Kleinstüber, T. Vallaes, W. Babel, Localization and characterization of two novel genes encoding stereospecific dioxygenases catalyzing 2-(2,4-dichlorophenoxy)propionate cleavage in *Delftia acidovorans* MC1, *Appl. Environ. Microbiol.* 70 (2004) 5357–5365.
- [18] T.A. Müller, S.M. Byrde, C. Werlen, J.R. van der Meer, H.-P.E. Kohler, Genetic analysis of phenoxyalkanoic acid degradation in *Sphingomonas herbicidovorans* MH, *Appl. Environ. Microbiol.* 70 (2004) 6066–6075.
- [19] K. Nickel, M.J.-F. Suter, H.-P.E. Kohler, Involvement of two α -ketoglutarate-dependent dioxygenases in enantioselective degradation of (*R*)- and (*S*)-mecoprop by *Sphingomonas herbicidovorans* MH, *J. Bacteriol.* 179 (1997) 6674–6679.
- [20] M. Pogorevc, K. Faber, Purification and characterization of an inverting stereospecific and enantioselective sec-alkylsulfatase from the gram-positive bacterium *Rhodococcus ruber* DSM 44541, *Appl. Environ. Microbiol.* 69 (2003) 2810–2815.
- [21] M.D. Müller, H.-R. Buser, Conversion reactions of various phenoxyalkanoic acid herbicides in soil. 1. Enantiomerization and enantioselective degradation of the chiral 2-phenoxypropionic acid herbicides, *Environ. Sci. Technol.* 31 (1997) 1953–1959.
- [22] H.-R. Buser, M.D. Müller, Conversion reactions of various phenoxyalkanoic acid herbicides in soil. 2. Elucidation of the enantiomerization process of chiral phenoxy acids from incubation in a D₂O/soil system, *Environ. Sci. Technol.* 31 (1997) 1960–1967.
- [23] H.-R. Buser, M.D. Müller, Occurrence and transformation reactions of chiral and achiral phenoxyalkanoic acid herbicides in lakes and rivers in Switzerland, *Environ. Sci. Technol.* 32 (1998) 626–633.
- [24] I. Agranat, H. Caner, J. Caldwell, Putting chirality to work: the strategy of chiral switches, *Nat. Rev. Drug Discov.* 1 (2002) 753–768.

- [25] P. Eichhorn, T.P. Knepper, F. Ventura, A. Diaz, The behavior of polar aromatic sulfonates during drinking water production: a case study on sulfophenyl carboxylates in two European waterworks, *Water Res.* 36 (2002) 2179–2186.
- [26] V.M. León, A. Gómez-Parra, E. González-Mazo, Biodegradation of linear alkylbenzene sulfonates and their degradation intermediates in seawater, *Environ. Sci. Technol.* 38 (2004) 2359–2367.
- [27] E. González-Mazo, M. Honing, D. Barceló, A. Gómez-Parra, Monitoring long-chain intermediate products from the degradation of linear alkylbenzene sulfonates in the marine environment by solid-phase extraction followed by liquid chromatography/ion spray mass spectrometry, *Environ. Sci. Technol.* 31 (1997) 504–510.
- [28] P.A. Lara-Martin, A. Gomez-Parra, T.K. Kochling, J.L. Sanz, R. Amils, E. Gonzalez-Mazo, Anaerobic degradation of linear alkylbenzene sulfonates in coastal marine sediments, *Environ. Sci. Technol.* 41 (2007) 3573–3579.
- [29] V.M. León, E. González-Mazo, A. Gómez-Parra, Handling of marine and estuarine samples for the determination of linear alkylbenzene sulfonates and sulfophenylcarboxylic acids, *J. Chromatogr. A* 889 (2000) 211–219.
- [30] Y. Huang, A. Latorre, D. Barceló, J. García, P. Aguirre, R. Mujeriego, J.M. Bayona, Factors affecting linear alkylbenzene sulfonates removal in subsurface flow constructed wetlands, *Environ. Sci. Technol.* 38 (2004) 2657–2663.
- [31] D. Schleheck, W. Dong, K. Denger, E. Heinze, A.M. Cook, An α -proteobacterium converts linear alkylbenzenesulfonate surfactants into sulfophenylcarboxylates and linear alkyldiphenyletherdisulfonate surfactants into sulfodiphenylethercarboxylates, *Appl. Environ. Microbiol.* 66 (2000) 1911–1916.
- [32] P.A. Lara-Martin, A. Gomez-Parra, T. Kochling, J.L. Sanz, E. Gonzalez-Mazo, Monitoring the primary biodegradation of linear alkylbenzene sulfonates and their coproducts in anoxic sediments using liquid chromatography–mass spectrometry, *Environ. Sci. Technol.* 41 (2007) 3580–3586.
- [33] D. Schleheck, T.P. Knepper, K. Fischer, A.M. Cook, Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria, *Appl. Environ. Microbiol.* 70 (2004) 4053–4063.
- [34] J.-C. Sigoillot, M.-H. Nguyen, Complete oxidation of linear alkylbenzene sulfonate by bacterial communities selected from coastal seawater, *Appl. Environ. Microbiol.* 58 (1992) 1308–1312.
- [35] D. Schleheck, F. von Netzer, T. Fleischmann, D. Rentsch, T. Huhn, A.M. Cook, H.P. Kohler, The missing link in linear alkylbenzenesulfonate surfactant degradation: 4-sulfoacetophenone as a transient intermediate in the degradation of 3-(4-sulfophenyl)butyrate by *Comamonas testosteroni* KF-1, *Appl. Environ. Microbiol.* 76 (2010) 196–202.
- [36] P.A. Lara-Martin, A. Gomez-Parra, J.L. Sanz, E. Gonzalez-Mazo, Anaerobic degradation pathway of linear alkylbenzene sulfonates (LAS) in sulfate-reducing marine sediments, *Environ. Sci. Technol.* 44 (2010) 1670–1676.
- [37] L.L. de Oliveira, R.B. Costa, D.Y. Okada, D.V. Vich, I.C. Duarte, E.L. Silva, M.B. Varesche, Anaerobic degradation of linear alkylbenzene sulfonate (LAS) in fluidized bed reactor by microbial consortia in different support materials, *Bioresource Technol.* 101 (2010) 5112–5122.
- [38] F.S. Sariaslani, J.L. Sudmeier, D.D. Focht, Degradation of 3-phenylbutyric acid by *Pseudomonas* sp., *J. Bacteriol.* 152 (1982) 411–421.
- [39] S. Simoni, S. Klinke, C. Zipper, W. Angst, H.-P.E. Kohler, Enantioselective metabolism of chiral 3-phenylbutyric acid, an intermediate of linear alkylbenzene degradation, by *Rhodococcus rhodochrous* PB1, *Appl. Environ. Microbiol.* 62 (1996) 749–755.
- [40] S. Schulz, W. Dong, U. Groth, A.M. Cook, Enantiomeric degradation of 2-(4-sulfophenyl)butyrate via 4-sulfocatechol in *Delftia acidovorans* SPB1, *Appl. Environ. Microbiol.* 66 (2000) 1905–1910.
- [41] M.E. Tanner, Understanding nature's strategies for enzyme-catalyzed racemization and epimerization, *Acc. Chem. Res.* 35 (2002) 237–246.
- [42] I. Ali, V.K. Gupta, H.Y. Aboul-Enein, P. Singh, B. Sharma, Role of racemization in optically active drugs development, *Chirality* 19 (2007) 453–463.
- [43] A. Reddy, M. Hashim, Z. Wang, L. Penn, C.J. Stankovic, D. Burdette, N. Surendran, H. Cai, A novel method for assessing inhibition of ibuprofen chiral inversion and its application in drug discovery, *Int. J. Pharm.* 335 (2007) 63–69.
- [44] C.-S. Chen, W.-R. Shieh, P.-H. Lu, S. Harriman, C.-Y. Chen, Metabolic stereoisomeric inversion of ibuprofen in mammals, *Biochim. Biophys. Acta: Protein Struct. Mol. Enzymol.* 1078 (1991) 411–417.
- [45] E.J.D. Lee, K. Williams, R. Day, G. Graham, D. Champion, Stereoselective disposition of ibuprofen enantiomers in man, *Brit. J. Clin. Pharmacol.* 58 (2004) S759–S764.
- [46] T.S. Tracy, D.P. Wirthwein, S.D. Hall, Metabolic inversion of (R)-ibuprofen. Formation of ibuprofenyl-coenzyme A, *Drug Metab. Dispos.* 21 (1993) 114–120.
- [47] Q. Xiaotao, S.D. Hall, Modulation of enantioselective metabolism and inversion of ibuprofen by xenobiotics in isolated rat hepatocytes, *J. Pharmacol. Exp. Ther.* 266 (1993) 845–851.
- [48] C. Reichel, R. Brugger, H. Bang, G. Geisslinger, K. Brune, Molecular cloning and expression of a 2-arylpropionyl-coenzyme A epimerase: a key enzyme in the inversion metabolism of ibuprofen, *Mol. Pharmacol.* 51 (1997) 576–582.
- [49] P. Bhaumik, W. Schmitz, A. Hassinen, J.K. Hiltunen, E. Conzelmann, R.K. Wierenga, The catalysis of the 1,1-proton transfer by α -methyl-acyl-CoA racemase is coupled to a movement of the fatty acyl moiety over a hydrophobic, methionine-rich surface, *J. Mol. Biol.* 367 (2007) 1145–1161.
- [50] T. Harner, K. Wiberg, R. Norstrom, Enantiomer fractions are preferred to enantiomer ratios for describing chiral signatures in environmental analysis, *Environ. Sci. Technol.* 34 (2000) 218–220.