Advanced Synthesis & Catalysis

Very Important Publication

DOI: 10.1002/adsc.201400893

Biotransformation of Linoleic Acid into Hydroxy Fatty Acids and Carboxylic Acids Using a Linoleate Double Bond Hydratase as Key Enzyme

Hye-Jin Oh,[+a] Sae-Um Kim,[+b] Ji-Won Song,[b] Jung-Hoo Lee,[b] Woo-Ri Kang,[a] Ye-Seul Jo,[a] Kyoung-Rok Kim,[a] Uwe T. Bornscheuer,[c] Deok-Kun Oh,[a,*] and Jin-Byung Park[b,*]

[a] Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea
E-mail: deokkun@konkuk.ac.kr
[b] Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, Republic of Korea
Fax: (+82)-2-3277-4213; phone: (+82)-2-3277-4509; e-mail: jbpark06@ewha.ac.kr
[c] Institute of Biochemistry, Department of Biotechnology & Enzyme Catalysis, Greifswald University, 17487 Greifswald. Germany
[+] H.-J. Oh and S.-U. Kim equally contributed to this work.

Received: September 10, 2014; Revised: November 21, 2014; Published online: ■■ ■■, 0000

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201400893.

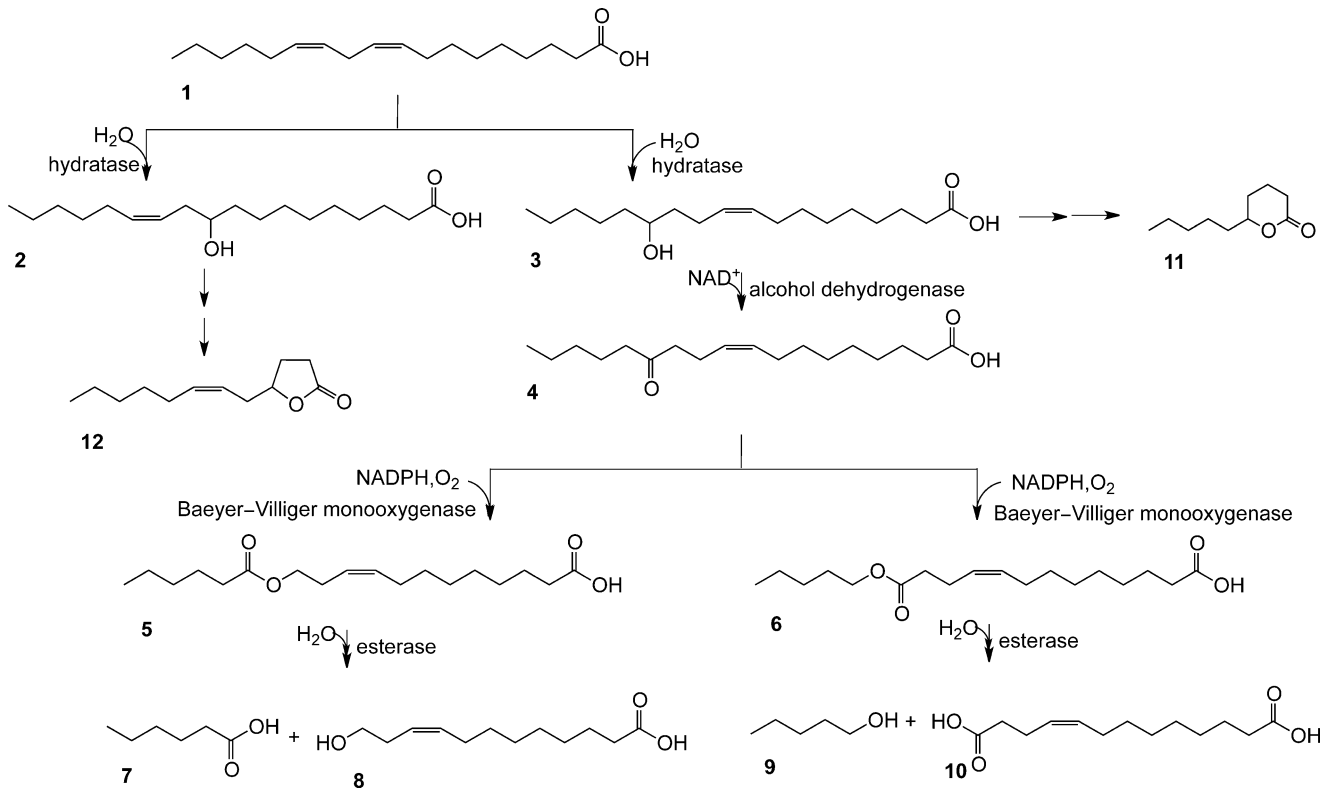
Abstract: Hydroxy fatty acids are used as starting materials for the production of secondary metabolites and signalling molecules as well as in the manufacture of industrial fine chemicals. However, these compounds are usually difficult to produce from renewable biomass by chemical means. In this study, linoleate double bond hydratases of *Lactobacillus acidophilus* NBRC 13951 were cloned for the first time. These enzymes were highly specific for the hydration of the C-9 or the C-12 double bond of unsaturated fatty acids (e.g., linoleic acid). Thereby, the enzymes allowed the selective production of hydroxy fatty acids such as 13-hydroxy-*cis*-9-octadecenoic acid and 10-hydroxy-*cis*-12-octadecenoic acid from linoleic acid. In addition, the hydroxy fatty acids were further converted into industrially relevant carboxylic acids (e.g., 12-hydroxy-*cis*-9-dodecenoic acid, α,ω-tridec-9-enedioic acid) and lactones (i.e., δ-decalactone, γ-dodecelactone) *via* whole-cell biocatalysis using an enzyme cascade. This study thus contributes to the preparation of hydroxy fatty acids, unsaturated carboxylic acids, and lactones from renewable unsaturated fatty acids.

Keywords: carboxylic acids; enzyme catalysis; hydration; hydroxy fatty acids; lactones

Introduction

Hydroxy fatty acids, which contain one or more hydroxy groups in the carbon skeleton of fatty acids, are found as components of cerebrosides, triacylglycerols, waxes, and other essential lipids in living organisms. Hydroxy fatty acids are also used as starting materials for the production of diverse secondary metabolites and signalling molecules.[1,2] For instance, 10-hydroxyoctadecenoic acid, 9- and 13-hydroxyoctadecadienoic acids, and leukotriene B4 are metabolized to lactones through the β-oxidation pathway. In the chemical industry, hydroxy fatty acids are widely used to manufacture flavors, resins, waxes, nylons, plastics, lubricants, polymers, and are used as additives in coatings and paintings.[3–6]

A variety of hydroxy fatty acids were reported to be produced by various microbes including lactic acid bacteria and ruminal microorganisms. 10-Hydroxystearic acid was produced from oleic acid by myosin cross-reactive antigen proteins from *Elizabethkingia meningoseptica*,[7] *Streptococcus pyogenes*,[8] and *Bifidobacterium breve*.[9] Linoleic acid was converted to 10-hydroxy-12-octadecenoic acid by *Lactobacillus acidophilus* AKU1137,[10] *L. plantarum,*[11] and *Streptococcus bovis.*[12] Linoleic acid was also transformed to 13-hydroxy-9-octadecenoic acid and/or 10,13-dihydroxyoctadecanoic acid by *L. acidophilus, Pediococ-*



Scheme 1. Linoleic acid biotransformation pathway. Linoleic acid (**1**) is converted into 13-hydroxy-*cis*-9-octadecenoic acid (**3**) by a linoleate C-12 double hydratase, which is further converted into *n*-hexanoic acid (**7**) and 12-hydroxydodec-9-enoic acid (**8**) or *n*-pentanol (**9**) and α,ω-tridec-9-enedioic acid (**10**) by the multistep enzyme reactions. 13-Hydroxy-*cis*-9-octadecenoic acid (**3**) and 10-hydroxy-*cis*-12-octadecenoic acid (**2**) could be also transformed into δ-decalactone (**11**) and γ-dodecelactone (**12**) *via* whole-cell biocatalysis.

cus pentosaceus,[13] *Pediococcus* sp. AKU 1080,[14] and ruminal strain *S. bovis* JB1.[15]

Most of the hydroxy fatty acids produced by microbes are considered to be synthesized by fatty acid double bond hydratases (e.g., oleate hydratase), which catalyze the regiospecific, irreversible addition of a hydrogen atom and a hydroxy group from water to the carbon-carbon *cis*-double bond of unsaturated fatty acids.[2,7] Until now, a number of C-9 double bond hydratases (e.g., myosin cross-reactive antigen proteins from *E. meningoseptica*,[7] *S. pyogenes*,[8] *B. breve*,[9] *L. acidophilus*,[16] oleate hydratases from *S. maltophilia,*[17] *Macrococcus caseolyticus*[18]) were cloned and characterized in detail, whereas C-12 double bond hydratases remained unidentified.

In this study, we cloned linoleate double bond hydratases of *L. acidophilus* NBRC 13951, which was known to produce 13-hydroxyoctadec-9-enoic acid (**3**) from linoleic acid,[13] for the first time. The enzymes were very specific to hydration of the C-9 or the C-12 double bond of linoleic acid and thereby allowed the selective production of 10-hydroxy-*cis*-12-octadecenoic acid (**2**) or 13-hydroxy-*cis*-9-octadecenoic acid (**3**) from linoleic acid. These hydroxy fatty acids were further transformed to δ-decalactone (**11**) and γ-dodecelactone (**12**) *via* whole-cell biocatalysis using wild-type yeast cells. 13-Hydroxy-*cis*-9-octadecenoic acid was also converted to 12-hydroxy-*cis*-9-dodecenoic acid (**8**) and *n*-hexanoic acid (**7**) *via* enzymatic cascade reactions (Scheme 1), which was based on our previous study.[19]

Results and Discussion

Cloning, Expression, and Purification of Putative Fatty Acid Hydratases

Screening of a novel putative linoleate double bond hydratase was based on known bacterial genome sequences. The amino acid sequences of the fatty acid C-9 double bond hydratases (see the Supporting Information, Table S2 for details) were used as probes to identify putative linoleate double bond hydratases. It was found that 70 genomes appeared to harbor at least two different genes encoding putative linoleate double bond hydratases out of 1,851 bacterial genomes examined (Supporting Information, Table S3). Notably, 9 of the 70 genomes belonged to *L. acidophilus* strains including the strain *L. acidophilus*

NBRC 13951, which was known to produce 13-hydroxyoctadec-9-enoic acid from linoleic acid[13] (Supporting Information, Table S4 and Figure S1). Next, the primers were designed to clone the putative linoleate double bond hydratase genes from *L. acidophilus* NBRC 13951 (Supporting Information, Table S5). This led to the identification of two genes (accession numbers KJ560553, KJ560554) from the genomic DNA of *L. acidophilus* NBRC 13951. These genes were cloned followed by expression of the putative hydratases in *Escherichia coli* ER2566. The resulting soluble enzymes were purified by His-Trap affinity chromatography (Supporting Information, Figure S2) and gave single bands in SDS-PAGE at the molecular weight of approximately 68 kDa. The amino acid sequences of the putative linoleate hydratases (AHW98239.1, AHW98240.1) from *L. acidophilus* NBRC 13951 were 57% identical to each other and similar to oleate hydratase of *Lysinibacillus fusiformis* KCTC 3454 (identity: *ca.* 60%) (Supporting Information, Table S6 and Figure S1).

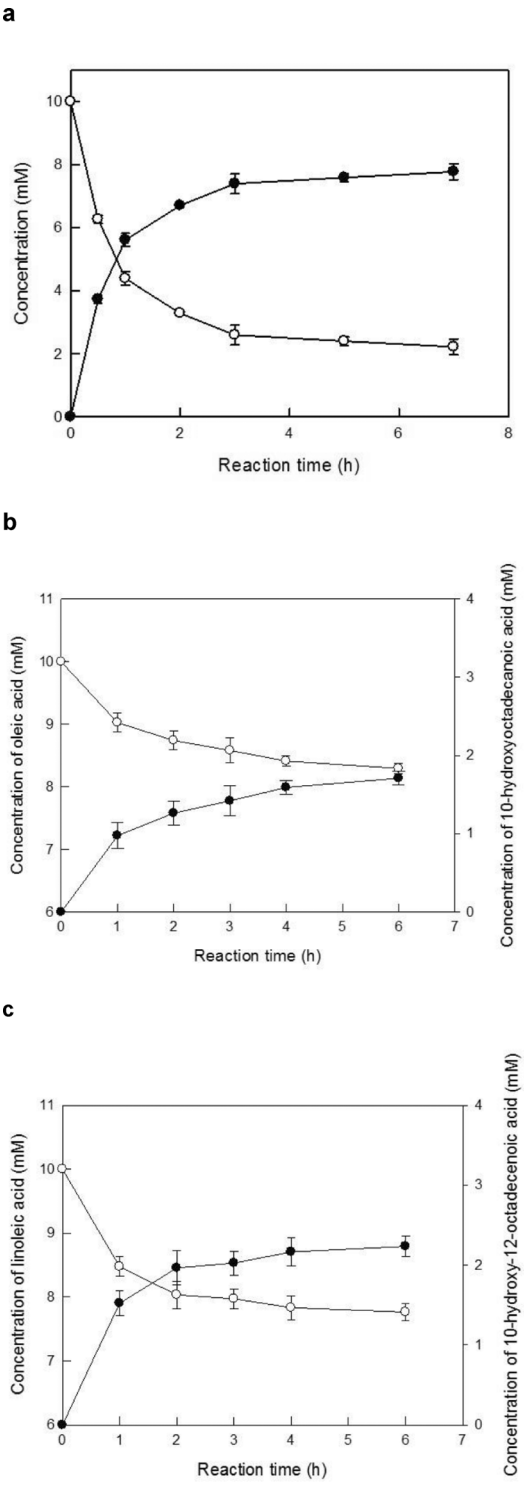


Figure 1. Time course of the biotransformations. (**a**) Linoleic acid (**1**) was converted into 13-hydroxyoctadec-9-enoic acid (**3**) by the linoleate C-12 double bond hydratase (AHW98239.1) isolated from *L. acidophilus* NBRC 13951. (**b**) Oleic acid and (**c**) linoleic acid were converted into 10-hydroxyoctadecanoic acid and 10-hydroxy-*cis*-12-octadecenoic acid (**2**), respectively, by the linoleate C-9 double bond hydratase (AHW98240.1) isolated from *L. acidiphilus* NBRC 13951. The biotransformations were carried out in 50 mM citrate-phosphate buffer (pH 7.0) containing 10 mM of individual unsaturated fatty acid and 2% (v/v) ethanol. The experiments were carried out in triplicate and the error bars indicate standard deviation. The symbols indicate concentration of linoleic acid (**a**, **c**), oleic acid (**b**) (○) and 13-hydroxyoctadec-9-enoic acid (**a**), 10-hydroxyoctadecanoic acid (**b**), 10-hydroxy-*cis*-12-octadecenoic acid (**c**) (●).

Catalytic Activity of the Putative Fatty Acid Hydratases

The substrate specificity of the purified enzymes AHW98239.1 and AHW98240.1 for fatty acids was investigated using oleic acid (*cis*-9-octadecenoic acid) and linoleic acid (*cis*-9,12-octadecadienoic acid). Addition of the purified enzyme AHW98239.1 into 50 mM citrate-phosphate buffer (pH 7.0) containing 10 mM linoleic acid resulted in accumulation of a single product in the medium (Figure 1a), whereas no product was observed from the oleic acid biotransformation. The resulting product was isolated and identified *via* GC/MS analysis (Supporting Information, Figures S3 and S4). It turned out to be 13-hydroxy-*cis*-9-octadecenoic acid (**3**). The concentration of 13-hydroxy-*cis*-9-octadecenoic acid in the reaction medium increased up to 8 mM within 3 h. The maximal specific enzyme reaction rate reached 127 U g protein^{-1} at $t < 0.5$ h. The enzymatic conversion, which was calculated based on the substrate depletion and the product concentration determined by GC/MS, reached 96%. The isolated yield, measured based on amount of the products isolated and amount of the substrates added into the reaction medium, was *ca.* 60% having a purity of 96% (Supporting Information,

Table 1. Products accessible through biocatalysis.[a]

Starting material	Final products	Product yield [%][b]
linoleic acid (**1**)	13-hydroxyoctadeca-9-enoic acid (**3**)	96 ± 2 (61%, 96%)[c]
	10-hydroxyoctadeca-12-enoic acid (**2**)	94 ± 4
13-hydroxyoctadeca-9-enoic acid (**3**)	12-hydroxydodeca-9-enoic acid (**8**)	91 ± 4 (54%, 71%)[c]
	δ-decalactone (**11**)	73 ± 4[d] (60%, 97%)[c]
10-hydroxyoctadeca-12-enoic acid (**2**)	γ-dodecelactone (**12**)	86 ± 3 (50%, 90%)[c]

[a] Linoleic acid was converted into 13-hydroxyoctadeca-9-enoic acid (**3**) and 10-hydroxy-*cis*-12-octadecenoic acid (**2**) by the linoleate C-12 and C-9 double bond hydratases, respectively, isolated from *L. acidophilus* NBRC 13951. Biotransformation of 13-hydroxyoctadec-9-enoic acid (**3**) into 12-hydroxydodeca-9-enoic acid (**8**) was carried out by the recombinant *E. coli* BL21(DE3) pACYC-ADH, pET-BmoF1, pCOLA-PFE1. Transformation of 13-hydroxyoctadeca-9-enoic acid (**3**) and 10-hydroxyoctadeca-12-enoic acid (**2**) into the corresponding lactones were conducted *via* whole-cell reactions using *Waltomyces lipofer* KCTC 17657 or *Candida boidinii* KCTC 17776, respectively.

[b] The product yield was calculated based on the substrate depletion and the product concentration, which was determined by GC/MS. All experiments were carried out in triplicate.

[c] The numbers in parenthesis indicate the isolated yield and purity, respectively. The isolated yields were calculated based on amount of the products isolated and amount of the substrates added into the reaction medium.

[d] The product yield was calculated based on the substrate depletion and the product concentration measured at t = 18 h in Figure 3a.

Figure S4 and Table 1). The high regioselectivity of the enzyme toward a double bond between C-12 and C-13 of linoleic acid indicated that the enzyme AHW98239.1 could be a linoleate C-12 double bond hydratase. Bioconversion of **1** to **3** has been also examined by using the wild-type strain of *Pediococcus* sp. AKU 1080.[14] However, significant amounts of by-products such as 10-hydroxy-*cis*-12-octadecenoic acid and 10,13-dihydroxyoctadecanoic acid were formed during the biotransformation of linoleic acid. The product yield was less than 20%. This indicated that our enzyme process is quite efficient in terms of selectivity of the biotransformation.

The other enzyme AHW98240.1 was able to selectively catalyze hydration of a double bond between C-9 and C-10 of oleic acid and linoleic acid under identical reaction conditions as given in Figure 1a (Figure 1b and c). 10-Hydroxystearic acid and 10-hydroxy-*cis*-12-octadecenoic acid (**2**) were the major products from oleic and linoleic acid, respectively (see Supporting Information, Figures S5 and S6 for product identification). The maximal specific enzyme reaction rate of **1** to **2** reached 14 U g protein^{-1} at t < 1 h. The biotransformation rate was greater with linoleic acid than oleic acid, indicating that the enzyme AHW98240.1 might be a linoleate C-9 double bond hydratase. Most fatty acid C-9 double bond hydratases reported so far including myosin cross-reactive antigen (MCRA) showed greater activity with oleic acid compared to linoleic acid.[2] The higher activity of AHW98240.1 with linoleic acid is one of the most striking characteristics of the enzyme.

The biotransformation of linoleic acid with both AHW98239.1 and AHW98240.1 resulted in the accumulation of 10,13-dihydroxyoctadecanoic acid (Supporting Information, **S4** in Figure S8) as a product in the reaction medium (Supporting Information, Figure S7). This result indicates that 10,13-dihydroxyoctadecanoic acid, which was observed in the fermentation products of lactic acid bacteria[13,14] and ruminal bacteria,[15] could be produced by a combined reaction of a linoleate C-9 double bond hydratase and a linoleate C-12 double bond hydratase (Supporting Information, Figure S8).

Biotransformation of 13-Hydroxy-*cis*-9-octadecenoic acid (3) into Carboxylic Acids

Hydroxy fatty acids are used as starting materials for the production of fine chemicals.[1–6] Here, we investigated the biotransformation of 13-hydroxy-*cis*-9-octadecenoic acid (**3**) into a C-12 carboxylic acid [i.e., 12-hydroxy-*cis*-9-dodecenoic acid (**8**)], which can be used for the production of polyester and/or polyamide monomers such as 1,12-dodecanedioic acid and 12-aminododecanoic acid.[20] The Baeyer–Villiger monooxygenase (BVMO) enzymes, which determine the chemical structure of the final products due to their regioselectivity, were first examined. The BVMOs that are active with long chain aliphatic ketones[21–23] were chosen as candidates. When the recombinant *E. coli* BL21(DE3) pACYC-ADH, pET-BmoF1, pCOLA-PFE1 expressing an alcohol dehydrogenase (ADH) from *Micrococcus luteus* NCTC2665, a BVMO from *Pseudomonas fluorescens* DSM 50106[23] and an esterase from *P. fluorescens* SIK WI, was added to the reaction medium containing 13-hydroxy-*cis*-9-octadecenoic acid (**3**), the compound was selectively converted into 12-hydroxy-*cis*-9-dodecenoic acid (**8**) and *n*-hexanoic acid (**7**) *via* 13-oxo-*cis*-9-octadecenoic acid (**4**) and ester (**5**) (Scheme 1) (Fig-

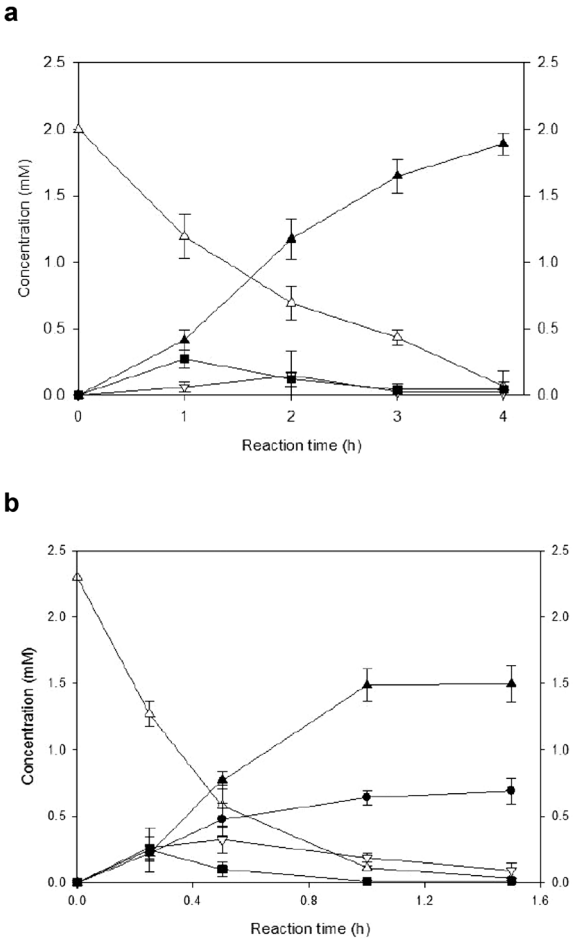


Figure 2. Time course of the biotransformation of 13-hydroxyoctadec-9-enoic acid (**2**). 13-Hydroxyoctadec-9-enoic acid (**2**) was converted into carboxylic acids by (**a**) the recombinant *E. coli* BL21(DE3) pACYC-ADH, pET-BmoF1, pCOLA-PFE1 and (**b**) the recombinant *E. coli* BL21 pACYC-ADH-BVMO, pCOLA-PFE1 (Scheme 1). The biotransformation was initiated by adding 2 mM of 13-hydroxyoctadec-9-enoic acid into 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 g L^{-1} Tween 80 and 7.2 g dry cells L^{-1} of the recombinant *E. coli* BL21(DE3) cells. The experiments were carried out in triplicate and the error bars indicate standard deviation. The symbols indicate concentration of 13-hydroxyoctadec-9-enoic acid (**2**) (△), 13-ketooctadec-9-enoic acid (**3**) (▽), the ester (**4**, **5**) (■), and 12-hydroxydodec-9-enoic acid (**7**) (▲), α,ω-tridec-9-enedioic acid (**9**) (●).

ure 2a and Supporting Information, Figures S9 and S10). Notably, the final product 12-hydroxy-*cis*-9-dodecenoic acid (**8**) was produced to a concentration of 1.8 mM from 2.0 mM of the substrate 13-hydroxy-*cis*-9-octadecenoic acid. The maximal specific reaction rate of the recombinant cells reached 1.4 U g dry cells^{-1} at $t < 2$ h. The target product (**8**) was also produced from linoleic acid by serial reactions of recombinant *E. coli* expressing the linoleate C-12 double bond hydratase and recombinant *E. coli* BL21(DE3) pACYC-ADH, pET-BmoF1, pCOLA-PFE1 in one-pot (Supporting Information, Figure S11). Overall, 12-hydroxy-*cis*-9-dodecenoic acid (**8**), which was reported to stimulate growth of the main root and lateral roots of plant seedlings and to act as antifungal metabolites of the wild rice *Oryza officinalis*,[24] could be synthesized from renewable biomass with a high yield for the first time.

On the other hand, addition of the recombinant *E. coli* BL21(DE3) pACYC-ADH-BVMO, pCOLA-PFE1, expressing an ADH from *M. luteus* NCTC2665, a BVMO from *Pseudomonas putida* KT2440[22] and an esterase from *P. fluorescens* SIK WI resulted in the production of not only 12-hydroxy-*cis*-9-dodecenoic acid (**8**) and *n*-hexanoic acid (**7**), but also α,ω-tridec-9-enedioic acid (**10**) and *n*-pentanol (**9**) (Figure 2b and Supporting Information, Figure S12). It was assumed that the ester compound (**6**) was produced *via* the formation of the abnormal ester, which is driven by migration of the less-substituted carbon center during BVMO catalysis.[25] The BVMO from *Pseudomonas veronii* MEK700[21] was poorly active with 13-ketooctadec-9-enoic acid (**4**). Overall, 12-hydroxy-*cis*-9-dodecenoic acid (**8**) and α,ω-tridec-9-enedioic acid (**10**) were produced *via* linoleate *cis*-C-12 hydratase- and BVMO-based biocatalysis from linoleic acid (Table 1).

Biosynthesis of δ-Decalactone (11) and γ-Dodecelactone (12)

13-Hydroxy-*cis*-9-octadecenoic acid (**3**) was then used for the production of δ-decalactone (**11**), which was reported to be suitable for use in flavor compositions.[26] This was accomplished by whole-cell biotransformation. A variety of yeasts were examined with the substrate. It turned out that the yeast *Waltomyces lipofer* KCTC 17657 was one of the best strains to produce the target compound. When the *W. lipofer* cells were added into 50 mM citrate-phosphate buffer (pH 7.0) containing 8 mM 13-hydroxy-*cis*-9-octadecenoic acid (**3**) and 0.05% (w/v) Tween 80, δ-decalactone (**11**) was produced to a concentration of 8 mM in the medium at $t = 15$ h (Figure 3a and Supporting Information, Figure S13). The maximal specific reaction rate of the yeast cells increased up to 1.1 U g dry cells^{-1} at $t < 12$ h. The isolated yield of δ-decalactone reached 60% with a purity of 97% (Table 1 and Supporting Information, Figure S14). This is to the best of our knowledge the first report for the synthesis of δ-decalactone (**11**) from renewable biomass.

Biosynthesis of γ-dodecelactone (**12**) from 10-hydroxy-*cis*-12-octadecenoic acid (**2**) (Scheme 1 and Supporting Information, Figure S15), which had been produced from linoleic acid by the linoleate C-9 double bond-hydratase, was also carried out with various yeasts. *Candida boidinii* KCTC 17776 showed the

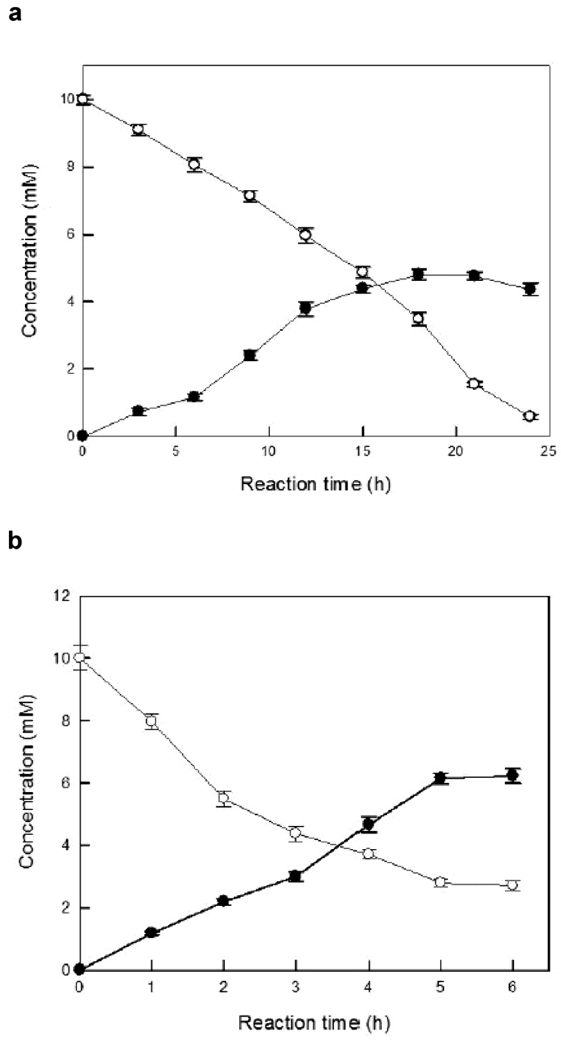


Figure 3. Time course of the transformation of hydroxy fatty acids into lactones. (**a**) 13-Hydroxy-*cis*-9-octadecenoic acid (**3**) was converted into δ-decalactone (**11**) by *Waltomyces lipofer* KCTC 17657. (**b**) 10-Hydroxy-*cis*-12-octadecenoic acid (**2**) was transformed into γ-dodecelactone (**12**) (Scheme 1 and Supporting Information, Figure S15) by *Candida boidinii* KCTC 17776. See the production of lactones from hydroxy fatty acids in the Experimental Section for details. The experiments were carried out in triplicate and the error bars indicate standard deviation. The symbols indicate concentration of 13-hydroxy-*cis*-9-octadecenoic acid (**a**), 10-hydroxy-*cis*-12-octadecenoic acid (**b**) (○) and δ-decalactone (**a**), γ-dodecelactone (**b**) (●).

highest productivity among the strains tested. γ-Dodecelactone was produced to 6.5 mM within 5 h biotransformation in the set-up identical to that for the production of δ-decalactone (Figure 3b). The productivity during biotransformation reached over 4 U g dry cells^{-1} and 1.2 mM h^{-1}. The isolated yield of γ-dodecelactone reached 50% with a purity of 90% (Table 1 and Supporting Information, Figure S16). γ-Dodecelactone was also produced from linoleic acid by serial reactions of the linoleate C-9 double bond hydratase and *C. boidinii* KCTC 17776 in one-pot (Supporting Information, Figure S17). Overall, γ-dodecelactone was synthesized from linoleic acid with a yield of *ca.* 43%.

Conclusions

The bacterial linoleate double bond hydratases from *L. acidophilus*, which are highly specific for the hydration of the C-9 or the C-12 double bond of unsaturated fatty acids, were cloned for the first time. The recombinant enzymes were successfully used for the production of hydroxy fatty acids such as 13-hydroxy-*cis*-9-octadecenoic acid (**3**) and 10-hydroxy-*cis*-12-octadecenoic acid (**2**) from linoleic acid. Furthermore, the industrially relevant carboxylic acids [e.g., 12-hydroxy-*cis*-9-dodecenoic acid (**8**), α,ω-tridec-9-enedioic acid (**10**)] and lactones [i.e., δ-decalactone (**11**), γ-dodecelactone (**12**)] could be produced from the hydroxy fatty acids via the whole-cell biocatalysis.

Experimental Section

Cultivation of Microbial Strains

Lactobacillus acidophilus NBRC 13951 (Supporting Information, Table S1), which was used to clone the putative hydratase genes, was cultivated in de Man, Rogosa and Sharpe (MRS) medium as described elsewhere.[10] Recombinant *Escherichia coli* ER2566 harboring the linoleate hydratase genes from *L. acidophilus* was grown at 37 °C with vigorous shaking in Luria–Bertani (LB) medium supplemented with 50 μg mL^{-1} of ampicillin. Expression of the recombinant hydratase genes was induced by adding 0.1 mM of isopropyl β-D-thiogalactopyranoside (IPTG) and/or 2 g L^{-1} rhamnose at an optical density (OD_{600}) of 0.5. Afterwards, the culture was further incubated at 16 °C for 16 h. Recombinant *E. coli* BL21(DE3), used for the biotransformation of hydroxy fatty acids, was cultivated in Riesenberg medium, which was supplemented with 10 g L^{-1} glucose and the appropriate antibiotics for plasmid maintenance (Supporting Information, Table S1). The Riesenberg medium consisted of 4 g L^{-1} $(NH_4)_2HPO_4$, 13.5 g L^{-1} KH_2PO_4, 1.7 g L^{-1} citric acid, 1.4 g L^{-1} $MgSO_4$, and 10 mL L^{-1} trace metal solution [10 g L^{-1} $FeSO_4$, 2.25 g L^{-1} $ZnSO_4$, 1.0 g L^{-1} $CuSO_4$, 0.5 g L^{-1} $MnSO_4$, 0.23 g L^{-1} $Na_2B_4O_7$, 2.0 g L^{-1} $CaCl_2$, and 0.1 g L^{-1} $(NH_4)_6Mo_7O_{24}$]. *Waltomyces lipofer* KCTC 17657 and *Candida boidinii* KCTC 17776 used for the production of lactones were cultivated in yeast malt (YM) broth as described previously.[27]

Screening of Linoleate Hydratase Genes

The fatty acid double bond hydratase genes originating from *Elizabethkingia meningoseptica*, *Macrococcus caseolyticus*, *Streptococcus pyogenes* NZ131, and *L. acidophilus* NCFM were chosen as probes to detect the putative linoleate hy-

dratase genes in public databases (Supporting Information, Table S2). A total 2,875 of bacterial oleate hydratase homologues were found in InterPro (http://www.ebi.ac.uk/interpro/entry/IPR010354/taxonomy) and 1,018 homologous in 897 bacterial species were found in Pfam (http://pfam.xfam.org/family/PF06100.6#tabview=tab7) registered as PF01600 (67 kDa myosin cross-reactive antigen). After removing overlapping sequences, the genomes that harbored at least two different genes encoding putative linoleate double bond hydratases were selected using the organism-specific genome search option in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Supporting Information, Table S3). The genes encoding protein sequences too short (smaller than 400 amino acids in length) or fragmented were excluded for functional candidates at this study because existing functional hydratases were reported to consist of 564 to 732 amino acids in length.[7]

Cloning of Linoleate Hydratase Genes

Among the 70 genomes containing at least two different genes encoding putative linoleate double bond hydratases, the genome of *L. acidophilus* NBRC 13951, which is known to produce 13-hydroxyoctadec-9-enoic acid from linoleic acid[13] (Supporting Information, Table S4 and Figure S1), was used for gene cloning. The two genes (LACIP7613_00033 and LACIP7613_01498) encoding putative linoleate hydratases were amplified by polymerase chain reaction (PCR) with the primers shown in the Supporting Information, Table S5. The PCR products were designed to include the *Nde*I and *Xho*I restriction sites. Each PCR product was subcloned into the pET-15b plasmid (Novagen). The resulting plasmid was used to transform *E. coli* ER2566 as an expression host.

Purification of Linoleate Hydratases

After cultivation of recombinant *E. coli* ER2566 harboring the putative linoleate hydratase genes, the cell mass was harvested, washed, and resuspended into 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, and 0.1 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The resuspended cells were disrupted using an ultrasonicator (Fisher Scientific) with the samples kept on ice. The cell debris was removed by centrifugation at 13,000 *g* for 20 min at 4 °C, and the supernatant was filtered through a 0.45 μm filter. The filtrate was applied to a His-Trap HP affinity chromatography column (GE Healthcare) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. The column was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient of 10 to 250 mM imidazole at a flow rate of 1 mL min^{-1}. The active fractions were collected and dialyzed against 50 mM citrate-phosphate buffer (pH 7.0). After dialysis, the solution was used as the purified enzyme. The purification step was carried out in a cold room at 4 °C using a fast protein liquid chromatography system (Bio-Rad Laboratories).

Enzymatic Hydration of Unsaturated Fatty Acids

Enzymatic biotransformations of oleic acid and linoleic acid into hydroxy fatty acids [e.g., 13-hydroxy-*cis*-9-octadecenoic acid (**2**), 10-hydroxy-*cis*-12-octadecenoic acid, 10-hydroxyoctadecanoic acid] was performed in 50 mM citrate-phosphate buffer (pH 7.0) containing 10 mM of individual unsaturated fatty acid and 2% (v/v) ethanol. In a 15-mL polypropylene tube with a screw cap, 3 mL of reaction mixture were incubated at 35 °C for 6 h with agitation at 200 rpm. For biotransformation of linoleic acid into 13-hydroxy-*cis*-9-octadecenoic acid, 1.5 mg mL^{-1} of purified hydratase was applied. To produce 10-hydroxyoctadecanoic acid and 10-hydroxy-*cis*-12-octadecenoic acid from oleic acid and linoleic acid, 2 mg mL^{-1} of purified hydratase was used. One unit (U) of the hydratase activity is defined as μmol of 10-hydroxy-12-octadecenoic acid or 13-hydroxy-9-octadecenoic acid produced per min at 25 °C by enzymes.

Biotransformations of Hydroxy Fatty Acids into Carboxylic Acids

Biotransformations of the hydroxy fatty acids with recombinant *E. coli* cells were carried out on the basis of our earlier work.[19,27] Briefly, the recombinant cells were cultivated in Riesenberg medium at 30 °C, and expression of the target genes was induced with 0.1 mM IPTG and/or 2.0 g L^{-1} rhamnose at an OD_{600} of 0.5. Thereafter, the cultivation temperature was reduced to 20 °C to facilitate the soluble expression of the target genes. When the added glucose was consumed, the recombinant cells were harvested by centrifugation at 3,500 *g* for 15 min at 4 °C and used as biocatalysts. The biotransformation was initiated by adding 2 mM of a hydroxy fatty acid to 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 g L^{-1} Tween 80 and 7.2 g dry cells L^{-1} of the recombinant *E. coli* BL21(DE3) that expressed the ADH from *Micrococcus luteus*, the BVMO from *Pseudomonas putida* KT2440 or *Pseudomonas fluorescens* DSM 50106, and the esterase from *P. fluorescens* SIK WI. The biotransformations were conducted in a shaking incubator (35 °C and 200 rpm). One unit (U) of whole cell activity was defined as μmol of the product produced per min at 35 °C by whole cells.

Production of Lactones from Hydroxy Fatty Acids

Lactones were produced from 13-hydroxy-*cis*-9-octadecenoic acid or 10-hydroxy-*cis*-12-octadecenoic acid on the basis of previous studies.[28] A variety of yeasts (e.g., *W. lipofer*, *C. boidinii*, *Yarrowia lipolytica*, and *Candida tropicalis*) were cultivated and used for biotransformation of the hydroxylated fatty acids into lactones. A single colony was inoculated into 15 mL of YM medium and cultivated at 28 °C with 200 rpm of agitation for 12 h. The culture was transferred into a 2-L baffled flask containing 500 mL of production medium consisting of 5.0 g L^{-1} glucose, 2.5 g L^{-1} ammonium chloride, 0.1 g L^{-1} yeast extract, 2.1 g L^{-1} KH_2PO_4, 4.51 g L^{-1} K_2HPO_4, 0.1 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 9.14 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 0.5 mg L^{-1} $ZnCl_2$, and 1.56 mg L^{-1} $CuSO_4 \cdot 5H_2O$. Lactone production was induced by supplementation of 5 g L^{-1} oleic acid, and the cells were further cultivated at 28 °C with agitation at 200 rpm for 12 h. The cells were harvested by centrifugation at 13,000 *g* for 20 min at 4 °C and washed twice with 50 mM citrate/phosphate buffer (pH 5.5) to prepare concentrated cell suspensions for the production of lactones from hydroxy fatty acids.

δ-Decalactone (**11**) was produced in 10 mL reaction medium [i.e., 50 mM citrate-phosphate buffer (pH 7.0)] in

a 100-mL baffled flask containing 10 mM 13-hydroxy-9-octadecenoic acid, 5 g L^{-1} whole cells (as dry cell weight), and 0.05% (w/v) Tween 80 at 28 °C for 15 h with 200 rpm. Afterwards, the reaction solution was acidified at 100 °C for 10 min with 10% (v/v) HCl for lactonization.[27] γ-Dodecelactone (**12**) was produced in 10 mL of reaction medium [i.e., 50 mM citrate-phosphate buffer (pH 5.5)] in a 100-mL baffled flask containing 10 mM 10-hydroxy-9-octadecenoic acid, 5 g L^{-1} cells (as dry cell weight), 3.4 g L^{-1} yeast nitrogen base, and 0.05% (w/v) Tween 80 at 25 °C with agitation at 200 rpm. For lactonization, the reaction solution was acidified at 100 °C for 10 min with 10% HCl.

Product Analysis by GC/MS

Concentrations of remaining fatty acids and accumulating carboxylic acids in the medium [i.e., oleic acid, linoleic acid, 13-hydroxy-*cis*-9-octadecenoic acid (**3**), 10-hydroxyoctadecanoic acid, 10-hydroxy-*cis*-12-octadecenoic acid (**2**), 10,13-dihydroxyoctadecanoic acid, *n*-hexanoic acid (**7**), *n*-pentanol (**9**), 12-hydroxy-*cis*-9-dodecenoic acid (**8**), α,ω-tridec-9-enedioic acid (**10**), and α,ω-tridec-6,9-dienedioic acid] were determined as described previously.[20] The reaction medium was mixed with an equal volume of ethyl acetate containing 2 or 5 g L^{-1} palmitic acid as an internal standard. The organic phase was harvested after vigorous vortexing and then derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (TMS). The TMS derivatives were analyzed using a Thermo Ultra Trace GC system connected to an ion trap mass detector (Thermo ITQ1100 GC-ion Trap MS; Thermo Scientific). The derivatives were separated on a non-polar capillary column (30 m length, 0.25 μm film thickness, HP-5MS; Agilent, Santa Clara, CA, USA). A linear temperature gradient was programmed as follows; 90 °C, 15 °C min^{-1} to 200 °C; 200 °C, 5 °C min^{-1} to 250 °C or 90 °C, 15 °C min^{-1} to 200 °C; 200 °C, and 5 °C min^{-1} to 280 °C. The inject port temperature was 230 °C. Mass spectra were obtained by electron impact ionization at 70 eV. Scan spectra were obtained within the range of 100–600 m/z. Selected ion monitoring (SIM) was used for the detection and fragmentation analysis of the reaction products.

Product Analysis by GC

The obtained hydroxy fatty acids were derivatized by 3:1 mixture of pyridine and TMS. The TMS-derivative hydroxy fatty acids, lactones, and unsaturated fatty acids were analyzed by GC (Agilent 6890N) equipped with a flame ionization detector and a SPB-1 capillary column (15 m × 0.32 mm inside diameter, 0.25 μm thickness; Supelco) using palmitic acid as an internal standard.[15,27] The column temperature increased from 150 to 210 °C at 4 °C min^{-1} and then 30 °C min^{-1} until 280 °C, and was maintained at 280 °C for 5 min. The injector and detector temperatures were held at 260 and 250 °C, respectively.

Identification of the Biotransformation Products

The biotransformation products were identified *via* GC/MS analysis. 13-Hydroxy-*cis*-9-octadecenoic acid (**3**), 10-hydroxyoctadecanoic acid, 10-hydroxy-*cis*-12-octadecenoic acid (**2**), 10,13-dihydroxyoctadecanoic acid, 12-hydroxy-*cis*-9-dodecenoic acid (**8**), α,ω-tridec-9-enedioic acid (**10**), δ-decalactone (**11**), and γ-dodecelactone (**12**) were identified *via* GC/MS analysis and comparison to authentic reference compounds (Supporting Information, Figures S3, S5, S6, S7, S9, S12, S13, and S15).

Purification of the Biotransformation Products

Purification of hydroxy fatty acids: The hydroxy fatty acids (e.g., 13-hydroxy-*cis*-9-octadecenoic acid) were isolated from the enzyme reaction medium by solvent extraction and column chromatography as described previously.[15,29] In brief, the biotransformation products were isolated *via* extraction using two volumes of ethyl acetate two times. After the organic layer was separated from the aqueous phase, the organic extracts were dried with Na_2SO_4 and concentrated *via* evaporation under vacuum. The concentrate was purified *via* silicic acid column chromatography with methanol gradient (0–5 %, v/v) in CH_3Cl (50 mL). Impurities and remaining substrate were washed off with CH_3Cl and 1% methanol in CH_3Cl (seethe Supporting Information, Figure S4 for purification of 13-hydroxy-*cis*-9-octadecenoic acid).

Purification of unsaturated carboxylic acids: The medium chain unsaturated carboxylic acids [e.g., 12-hydroxydodec-9-enoic acid (**8**)] were isolated from the whole-cell reaction medium, as described previously.[19] In brief, the biotransformation products were extracted with ethyl acetate three times. The extract was dried over $MgSO_4$ and concentrated *via* evaporation under vacuum. The impurities (e.g., *n*-alkanoic acids coproduced during biotransformations, long chain fatty acids originating from the starting materials) were removed by washing with hexane (see the Supporting Information, Figure S10 for purification of 12-hydroxydodec-9-enoic acid (**8**)).

Purification of lactones: γ-Dodecelactone and δ-decalactone were isolated from whole cell biotransformation medium, as described previously.[27] In short, the biotransformation products were isolated *via* extraction with a half volume of mineral oil (Sigma–Aldrich). The organic layer was separated and boiled at 180 °C in an oil bath. The lactones were evaporated and collected in a receiving flask (see the Supporting Information, Figures S14 and S16 for the purification).

Acknowledgements

This study was supported by the Marine Biomaterials Research Center grant from Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Korea, and the Bio-industry Technology Development Program funded by the Ministry for Food and Agriculture, Republic of Korea (No. 112002-3).

References

[1] Y. C. Joo, D. K. Oh, *Biotechnol. Adv.* **2012**, *30*, 1524.
[2] K. R. Kim, D. K. Oh, *Biotechnol. Adv.* **2013**, *31*, 1473.
[3] S. C. Liu, C. Li, X. C. Fang, Z. A. Cao, *Enz. Microb. Technol.* **2004**, *34*, 73.
[4] L. S. Liu, X. Fang, *Petrochem. Technol.* **2002**, *31*, 558.

[5] S. Huf, S. Krugener, T. Hirth, S. Rupp, S. Zibek, *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 548.
[6] W. H. Lu, J. E. Ness, W. C. Xie, X. Y. Zhang, J. Minshull, R. A. Gross, *J. Am. Chem. Soc.* **2010**, *132*, 15451.
[7] L. E. Bevers, M. W. H. Pinkse, P. Verhaert, W. R. Hagen, *J. Bacteriol.* **2009**, *191*, 5010.
[8] A. Volkov, A. Liavonchanka, O. Kamneva, T. Fiedler, C. Goebel, B. Kreikemeyer, I. Feussner, *J. Biol. Chem.* **2010**, *285*, 10353.
[9] E. Rosberg-Cody, A. Liavonchanka, C. Gobel, R. P. Ross, O. O'Sullivan, G. F. Fitzgerald, I. Feussner, C. Stanton, *BMC Biochem.* **2011**, *12*, 12.
[10] J. Ogawa, K. Matsumura, S. Kishino, Y. Omura, S. Shimizu, *Appl. Environ. Microbiol.* **2001**, *67*, 1246.
[11] Y. Yamada, H. Uemura, H. Nakaya, K. Sakata, T. Takatori, M. Nagao, H. Iwase, K. Iwadate, *Biochem. Biophys. Res. Commun.* **1996**, *226*, 391.
[12] J. A. Hudson, B. Morvan, K. N. Joblin, *FEMS Microbiol. Lett.* **1998**, *169*, 277.
[13] N. Kishimoto, I. Yamamoto, K. Toraishi, S. Yoshioka, K. Saito, H. Masuda, T. Fujita, *Lipids* **2003**, *38*, 1269.
[14] M. Takeuchi, S. Kishino, K. Tanabe, A. Hirata, S.-B. Park, S. Shimizu, J. Ogawa, *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 386.
[15] J. Hudson, C. MacKenzie, K. Joblin, *Appl. Microbiol. Biotechnol.* **1995**, *44*, 1.
[16] S. J. O'Flaherty, T. R. Klaenhammer, *Microbiology-Sgm* **2010**, *156*, 3360.
[17] Y.-C. Joo, E.-S. Seo, Y.-S. Kim, K.-R. Kim, J.-B. Park, D.-K. Oh, *J. Biotechnol.* **2012**, *158*, 17.
[18] Y.-C. Joo, K.-W. Jeong, S.-J. Yeom, Y.-S. Kim, Y. Kim, D.-K. Oh, *Biochimie* **2012**, *94*, 907.
[19] J. W. Song, E. Y. Jeon, D. H. Song, H. Y. Jang, U. T. Bornscheuer, D. K. Oh, J. B. Park, *Angew. Chem.* **2013**, *125*, 2594; *Angew. Chem. Int. Ed.* **2013**, *52*, 2534.
[20] J. W. Song, J.-H. Lee, U. T. Bornscheuer, J. B. Park, *Adv. Synth. Catal.* **2014**, *356*, 1782–1788.
[21] A. Volker, A. Kirschner, U. T. Bornscheuer, J. Altenbuchner, *Appl. Microbiol. Biotechnol.* **2008**, *77*, 1251.
[22] J. Rehdorf, A. Kirschner, U. T. Bornscheuer, *Biotechnol. Lett.* **2007**, *29*, 1393.
[23] A. Kirschner, J. Altenbuchner, U. T. Bornscheuer, *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1065.
[24] Y. Suzuki, O. Kurita, Y. Kono, H. Hyakutake, A. Sakurai, *Biochem. Biotechnol. Biochem.* **1995**, *59*, 2049.
[25] J. Rehdorf, M. D. Mihovilovic, U. T. Bornscheuer, *Angew. Chem.* **2010**, *122*, 4609; *Angew. Chem. Int. Ed.* **2010**, *49*, 4506.
[26] S. Gocho, K. Rumi, K. Tsuyoshi, U.S. Patent 5,763,233, **1997**.
[27] H.-Y. Jang, E.-Y. Jeon, A.-H. Baek, S.-M. Lee, J.-B. Park, *Process Biochem.* **2014**, *49*, 617.
[28] J.-U. An, D.-K. Oh, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8265.
[29] E.-Y. Jeon, J.-H. Lee, K.-M. Yang, Y.-C. Joo, D.-K. Oh, J.-B. Park, *Process Biochem.* **2012**, *47*, 941.