Two-Step Biocatalytic Route to Biobased Functional Polyesters from ω-Carboxy Fatty Acids and Diols

Yixin Yang, Wenhua Lu, Xiaoyan Zhang, Wenchun Xie, Minmin Cai, and Richard A. Gross*

NSF I/UCRC for Biocatalysis and Bioprocessing of Macromolecules, Department of Chemical and Biological Sciences, Polytechnic Institute of NYU, Six Metrotech Center, Brooklyn, New York 11201

Received September 29, 2009; Revised Manuscript Received November 25, 2009

Biobased ω-carboxy fatty acid monomers 1,18-*cis*-9-octadecenedioic, 1,22-*cis*-9-docosenedioic, and 1,18-*cis*-9,10-epoxy-octadecanedioic acids were synthesized in high conversion yields from oleic, erucic and epoxy stearic acids by whole-cell biotransformations catalyzed by *C. tropicalis* ATCC20962. Maximum volumetric yields in shake-flasks were 17.3, 14.2, and 19.1 g/L after 48 h conversion for oleic acid and 72 h conversions for erucic and epoxy stearic acids, respectively. Studies in fermentor with better control of pH and glucose feeding revealed that conversion of oleic acid to 1,18-*cis*-9-octadecenedioic acid by *C. tropicalis* ATCC20962 occurred with productivities up to 0.5 g/L/h. The conversion of ω-carboxy fatty acid monomers to polyesters was then studied using immobilized *Candida antarctica* Lipase B (N435) as catalyst. Polycondensations with diols were performed in bulk as well as in diphenyl ether. The retension of functionality from fatty acid, to ω-carboxy fatty acid monomer and to corresponding polyesters resulted in polymers with with unsaturated and epoxidized repeat units and M_w values ranging from 25000 to 57000 g/mol. These functional groups along chains disrupted crystallization giving materials that are low melting (23−40 °C). In contrast, saturated polyesters prepared from 1,18-octadecanedioic acid and 1,8-octanediol have correspondingly higher melting transitions (88 °C). TGA results indicated that all synthesized polyesters showed high thermal stabilities. Thus, the preparation of functional monomers from *C. tropicalis* ω-oxidation of fatty acids provides a wide range of new monomer building blocks to construct functional polymers.

Introduction

Aliphatic α,ω-dicarboxylic acids (α,ω-diacids) are widely used as raw materials for the manufacture of engineered plastics, perfumes, fragrances, lubricants, and adhesives.[1] The majority of currently used α,ω-diacids are produced by chemical means from nonrenewable petrochemical feedstocks. For examples, adipic acid is manufactured by a two-step process involving stoichiometric nitric acid oxidation of a cyclohexanol−cyclohenanone mixture, which is aerobically generated from cyclohexane using a homogeneous cobalt-based catalyst.[2] Dodecanedioic acid is manufactured by a nickel-catalyzed cyclic trimerization of butadiene, followed by hydrogenation to cyclododecane, air oxidation to a mixture of cyclododecanone and cyclododecanol, and, finally, nitric acid oxidation to dodecanedioic acid.[3] Also, chemical routes to α,ω-diacids can be tedious and result in unwanted byproducts. Furthermore, chemical routes are unavailable to synthesize α,ω-diacids with carbon numbers greater than 13.[4]

Nature uses long-chain unsaturated and epoxidized dicarboxylic acids (mainly 9,10-epoxy octadecanedioic, 1,18-*cis*-9-octadecenedioic, and 9,10-dihydroxy octadecanedioic acids), as well as ω-hydroxyl carboxylic acids (mainly 9,10-epoxy-18-hydroxy octadecanoic and 9,10,18-trihydroxy octadecanoic acids) as building blocks to synthesize important plant polyesters such as suberin and cutin.[5] These and related monomers would be useful to design and synthesize unique functional polyesters that would also biodegrade. However, they are difficult to synthesize by chemical methods and are currently unavailable commercially.

* To whom correspondence should be addressed. Telephone: 718-260-3024. Fax: 718-260-3075. E-mail: rgross@poly.edu.

It is well-known that many microorganisms can convert *n*-alkanes and fatty acids to their corresponding α,ω-diacids, including *Candida tropicalis*,[4,6,7] *Candida cloaca*,[8] *Cryptococcus neoforman*,[1] and *Corynebacterium* sp.[9] *Candida tropicalis* and similar yeasts produce α,ω-diacids through an ω-oxidation pathway. The terminal methyl group is first hydroxylated by a cytochrome P450 monooxygenase that is further transformed via the action of fatty alcohol oxygenase and aldehyde dehydrogenase to form diacids.[10] Of particular interest with regards to this paper is the report of *C. tropicalis* ATCC20962, which was engineered to block its β-oxidation pathway.[4] Yi et al.[11] used a mutant of *C. tropicalis* to convert oleic acid to 1,18-*cis*-9-octadecenedioic acid. Picataggio et al.[4] used engineered *C. tropicalis* ATCC20962 and its P450 monooxygenase (P450alkl) and NADPH-oxidoreductase (CPR) amplified strain AR40 to convert methyl esters of saturated (C14 to C18) and unsaturated (oleic, erucic) fatty acids to their corresponding α,ω-diacids. Bioconversion of C12 to C22 *n*-alkanes and fatty acids to α,ω-diacids by strain AR40 was successfully performed without undesired modification of substrates or products via the β-oxidation pathway. Fabritius et al.[12,13] reported that a mutant of *C. tropicalis* M25 converts oleic acid[12] and linoleic acid[13] to their corresponding 3-hydroxydicarboxylic acids. Unfortunately, bioconversions using of *C. tropicalis* described in Yi et al.[11] and Fabritius et al.[12,13] resulted in low yields and product mixtures, including α,ω-diacids with different carbon-chain lengths or having variable contents of hydroxylation along chains. Furthermore, comparison of *C. tropicalis* ATCC20962-catalyzed conversion rates of unsaturated acids differing in chain lengths to their corresponding α,ω-diacids has not been studied. Moreover, whereas the in vitro ω-hydroxylation of 9,10-epoxystearic acid, catalyzed by cytochrome P450s in plants such

10.1021/bm901112m
Published on Web 12/10/2009

as CYP86A1, CYP86A8, CYP94A1, and CYP94A5, has been studied,[14] corresponding conversions of epoxy-containing fatty acids to their corresponding epoxidized α,ω-diacids by *C. tropicalis* has not been reported. Resulting α,ω-diacid monomers with an internal double bond, epoxy moiety, or other naturally occurring functionality in fatty acids can provide important building blocks for polymer synthesis. Also, conversions of unsaturated and epoxy functionalized α,ω-diacids to corresponding polyesters would be best performed by lipase catalysis for reasons discussed below.

Normally, polyesters are synthesized by ester interchange reactions or by direct esterification of diacid/diol or hydroxyacids.[15] These reactions often require harsh reaction conditions and metal catalysts, which are not compatible with retention of functional group structure during polymer synthesis. An alternative approach to traditional chemical polymerization methods is the use of cell-free, enzyme-catalyzed polycondensation reactions.[16] For example, lipase-catalyzed polymerizations of monomers containing a double bond or an epoxy group to synthesize polyesters have been reported by several authors.[17] In one example described by Olsen and Sheares,[17b] *trans-β*-hydromuconic acid (HMA) was copolymerized (90 °C, in-bulk, 48 h) with 1,8-octanediol using immobilized *Candida antarctica* Lipase B (CALB) to give polyesters with M_n 10500 g/mol ($M_w/M_n = 2.0$) whose double bonds remained intact.[17b]

A natural bifunctional fatty acid of interest as a monomer for polymer synthesis is ricinoleic acid [(*Z*,*R*)-12-hydroxy-9-octadecenoic acid], derived from castor oil. However, esterification of secondary hydroxyl groups by lipase-catalysis is generally slow.[18,19] Furthermore, to use ricinoleic acid as a bifunctional monomer to prepare high molecular weight products, ricinoleic acid must be rigorously purified to remove impurities that terminate chain growth.

Others have pursued naturally derived functional fatty acid monomers that, unlike ricinoleic acid, can more efficiently be converted by enzyme-catalysis to polyesters. Veld et al.[20] investigated aleuritic acid that has two secondary and one primary (ω-position) hydroxyl groups and is derived from ambrettolide that naturally occurs in musk abrette seed oil and is a valuable perfume base due to its desirable odor. Aleuritic acid was first converted to its isopropyl ester and then polymerized (90 °C, 550 m bar, 21 h) in a mixture of dry toluene and dry 2,4-dimethyl-3-pentanol. Poly(aleuriteate) (M_n = 5600 g/mol, PDI = 3.2) was isolated in moderate yield (43%) after precipitation. Interestingly, the polymerization was highly selective for monomer primary hydroxyl groups with no observable secondary hydroxyl esterification based on NMR studies. Olsson et al.[21] isolated *cis*-9,10-epoxy-18-hydroxyoctadecanoic acid from suberin, found in the outer bark of birch. This naturally derived monomer was converted to an epoxyfuctionalized polyester by N435 catalysis. Problems associated with deriving functional fatty acid monomers from specialty seed oils such as abrette is their low abundance. Furthermore, fatty acids from major seed oil sources lack of ω-hydroxyl or ω-carboxyl groups. Also, it is difficult to purify components of suberin and cutin that consist of severe mixtures of these useful building blocks. For these reasons, *C. tropocalis* ATCC20962 appears as a promising biocatalyst to provide a wide range of bifunctional fatty acid building blocks. In this paper, we report a new two-pot two-step route, using whole-cell bio-oxidation followed by cell-free lipase catalysis, to convert readily renewable fatty acids to functional polyesters. First, fermentations of *C. tropicalis* ATCC20962 were used to convert oleic, erucic and epoxystearic acids to their corresponding diacid functional monomers 1,18-*cis*-9-octadecenedioic acid (ω-carboxyl OA), 1,22- *cis*-9-docosenedioic acid (ω-carboxyl EA), and 1,18-*cis*-9,10-epoxy-octadecanedioic acid (ω-carboxyl epoxy SA). Effects of fatty acid chain length and presence of unsaturation versus epoxy groups at C18 carbons on the *C. tropicalis* ATCC20962-catalyzed conversion of these fatty acid substrates to diacids was studied. Diacid monomers were purified and characterized by gas chromatography (GC)-mass spectrometry (MS) and nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR). Conversion of unsaturated and epoxidized ω-carboxyl fatty acids to polyesters was performed by condensation copolymerizations with a diol using an immobilized CALB (N435) as catalyst. Comparison of CALB-catalyzed copolymerizations in-bulk, and in diphenyl ether, was performed and chain growth was monitored as a function of time. Effects of changes in ω-carboxyl fatty acid building blocks on thermal properties of resulting polyesters were also investigated.

Experimental Section

Materials. Oleic acid (technically, ~90% pure), erucic acid (~99% pure), 1,3-propanediol, 1,8-octanediol, 1,16-hexadecanediol, antifoam 204, and Glucose (HK) Assay Kit were purchased from Sigma-Aldrich (St. Louis, U.S.A.). The diols were obtained in the highest available purity and were used as received. 1,18-Octadecanedioic acid (~95% pure) and thin layer chromatography (TLC) aluminum sheets were purchased from TCI (Portland, U.S.A.) and Merck (Darmstadt, Germany), respectively. Novozym 435 (abbreviated as N435, specified activity 10000 PLU/g) was a gift from Novozymes (Bagsvaerd, Denmark) and consists of CALB physically adsorbed within the macroporous resin Lewatit VPOC 1600 (poly[methyl methacrylateco-butyl methacrylate], supplied by Bayer). All other chemicals not listed above were of analytical grade from Sigma-Aldrich (St. Louis, U.S.A.). *Candida tropicalis* ATCC20962 was purchased from The American Culture Collection (ATCC).

Microorganism. *Candida tropicalis* ATCC20962, used in this study, was obtained with its β-oxidation pathway blocked. This was accomplished by Picataggio et. al[4] who disrupted the *POX 4* and *POX 5* genes encoding its acyl-coenzyme A oxidase.

Lipase-Mediated Epoxidation of Oleic Acid. The method used was based on a previous literature report.[22] The reaction was performed in a 50-mL round-bottom flask containing 0.5 M oleic acid in 20 mL toluene and 300 mg immobilized *C. antarctica* lipase (N435). Hydrogen peroxide (30%, w/w) was added stepwise at 0.5 mL every hour during the first 4 h. The reaction mixture was stirred at 600 rpm and reaction temperature was maintained at 50 °C. After 8 h, TLC showed only a single band indicating complete conversion of oleic acid to 9,10-epoxy stearic acid. The reaction was terminated by removal of N435 by filtration and solvent removal was performed under vacuum with a rotary evaporator. The obtained product was directly used for the biotransformation without further purification.

9,10-epoxy stearic acid: ^{1}H NMR (8 mg in 0.7 mL $CDCl_3$; ppm) 2.92 (2H, bs, -C*H*-, *cis*-epoxide), 2.35 (2H, t, −CH_2-CO-), 1.2−1.8 (26H, bm, -CH_2-), 0.89 (3H, t, -CH_3).

Fermentation Procedure. *C. tropicalis* ATCC20962 was precultured in 30 mL YPD medium consisting of (g/L) yeast extract, 10; peptone, 20; glucose, 20; and shaken at 250 rpm, 30 °C for 20 h in a 500 mL flask. The cells were inoculated at 10% (v/v) to 30 mL conversion medium consisting of (g/L) peptone, 3; yeast extract, 6; yeast nitrogen base, 6.7; acetic acid, 3; K_2HPO_4, 7.2; KH_2PO_4 9.3; glucose, 20 in 500 mL flask. After 12 h of cultivation at 250 rpm and 30 °C, the biotransformation phase was begun by addition of oleic acid, erucic acid or 9,10-epoxystearic acid. The initial concentration of fatty acids was 20 g/L and pH was adjusted to 7.5 by addition of 2 mol/L NaOH solution. During the biotransformation, glucose (500 g/L) was fed (2.5% per day) as cosubstrates and the pH was maintained at 7.5−8.0 by addition of NaOH solution.

The biotransformation of oleic acid was also conducted in a 3-L Bioflo3000 fermentor (New Brunswick Scientific Co., U.S.A.) in fed-batch culture. The conversion medium above was used except for addition of 0.05% antifoam 204 (Sigma) and 0.5% oleic acid. The culture was grown at 30 °C at 900 rpm, with aeration rate of 1.5 vvm. After 12 h fermentations (growth phase), the biotransformation phase was started by feeding oleic acid at 2 mL/L. Glucose (500 g/L) as cosubstrate was fed continuously at 1.2 g/L/h. During the biotransformation phase, the pH was maintained at 7.6 automatically by addition of 4 mol/L NaOH solution and dissolved oxygen was controlled at about 60%. To avoid excessive foaming, antifoam 204 was added as needed. Every 12 h, a 5 mL aliquot from fermentation media was withdrawn to determine cell growth, glucose concentration, depletion of the fatty acid, and diacid production.

Extraction and Purification of Fermentation Products. Fermentation broths of oleic acid and erucic acid were acidified to pH 1.0 with HCl and extracted twice with diethyl ether. To avoid epoxy ring-opening during acidification, the fermentation broth of 9,10-epoxy stearic acid was slowly acidified to pH 3.0 with 5 N HCl and then extracted with diethyl ether. Diethyl ether was removed by rotoevaporation that gave a light yellow solid for all products from oleic acid, erucic acid, and 9,10-epoxy stearic acid. The crude product (4 g), dissolved in diethyl ether, was separated into components with a Biotage SP1 Flash Chromatography system (Biotage Inc., A Dynax Corp. Company, U.S.A.). Separations were performed using a Biotage SI 40+M column (150 × 40 mm ID; 100 g KP-Sil silica; 40−63 μm particle size; flow rate: 25−50 mL/min). The mobile phase consisted of *n*-hexane/diethyl ether mixtures that were run in gradient mode with compositions ranging from 30 to 80% diethyl ether in 10 column volumes (CVs) at 30 mL/min flow rate. Fractions were monitored at 220 and 280 nm by UV detection. Unreacted fatty acid substrate eluted first and then α,ω-diacid eluted in the second peak. Fractions in the second peak showing a single band on TLC plate were pooled and concentrated by rotoevaporation that gave a white powder. The purity of ω-carboxyl OA, ω-carboxyl EA, and ω-carboxyl epoxy SA used in polymerizations was >99% (GC-MS).

ω-Carboxyl OA. Yield: 95% (from crude mixture of diethyl ether extracted products); mp 66−68 °C. ^{1}H NMR (9.8 mg in 0.7 mL $CDCl_3$; ppm): 5.3−5.4 (2H, m, =C*H*-CH_2-), 2.35 (4H, t, -CH_2-CO-), 1.9−2.1 (4H, m, =CH-CH_2-), 1.6 (4H, m, -CH_2-CH_2-CO-), 1.2−1.4 (16H, m, -CH_2-). ^{13}C NMR (63.5 mg in 0.7 mL $CDCl_3$; ppm): 180.7 (C=O), 129.8 (=CH−), 34.1, 29.6, 29.1, 29.0, 28.9, 27.1, 24.6. IR (KBr; cm^{-1}): 1691 (s, C=O), 725 (m, C=C, *cis*-form). MS for dimethyl ester of ω-carboxyl OA, *m*/*z* (rel int, %): 340 (5) [M^+], 308 (49) [M^+ − CH_3OH], 276 (90) [M^+ − 2CH_3OH], 248 (18) [M^+ − 2CH_3OH − CO], 234 (7) [M^+ − 2CH_3OH − CO − CH_2], 165 (19) [M^+ − CH_3OH − $CH_3OOC(CH_2)_6$], 151 (27), 109 (37), 95 (31), 81 (100), 69 (48) [$C_5H_9^+$], 55 (81) [$C_4H_7^+$], 41 (63) [$C_3H_5^+$].

ω-Carboxyl EA. Yield: 70% (from crude mixture of diethyl ether extracted products); mp 71−73 °C. ^{1}H NMR (10.3 mg in 0.7 mL $CDCl_3$; ppm): 5.3−5.4 (2H, m, =C*H*-CH_2-), 2.33 (4H, t, -CH_2-CO-), 1.9−2.1 (4H, m, =CH-CH_2-), 1.6 (4H, m, -CH_2-CH_2-CO-), 1.2−1.4 (24H, m, -CH_2-). ^{13}C NMR (63.5 mg in 0.7 mL $CDCl_3$; ppm): 180.4, 180.5 (C=O), 130.0, 129.7 (=CH−), 34.1, 29.7, 29.6, 29.57, 29.53, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.2, 27.1, 24.6. IR (KBr; cm^{-1}): 1691 (s, C=O), 725 (m, C=C, *cis*-form). MS for dimethyl ester of ω-carboxyl EA, *m*/*z* (rel int, %): 396 (0.7) [M^+], 364 (8) [M^+ − CH_3OH], 332 (13) [M^+ − 2CH_3OH], 304 (4) [M^+ − 2CH_3OH − CO], 290 (2) [M^+ − 2CH_3OH − CO − CH_2], 165 (8), 151 (13), 109 (32), 95 (56), 81 (68), 69 (69) [$C_5H_9^+$], 55 (100) [$C_4H_7^+$], 41 (39) [$C_3H_5^+$].

ω-Carboxyl Epoxy SA. Yield: 92% (from crude mixture of diethyl ether extracted products); mp 85−86 °C. ^{1}H NMR (10.0 mg in 0.7 mL $CDCl_3$) (ppm): 2.92 (2H, bs, -C*H*-, *cis*-epoxide), 2.35 (4H, t, -CH_2-CO-), 1.2−1.8 (24H, bm, -CH_2-). ^{13}C NMR (50.6 mg in 0.7 mL $CDCl_3$; ppm): 180.1 (C=O), 57.3 (−CH−, *cis*-epoxide), 34.0, 29.2, 29.1, 28.9, 27.6, 26.4, 24.5. MS for TMSi-ether dimethyl ester derivative of ω-carboxyl epoxy SA, *m*/*z* (rel int, %): 429 (2) [M^+ − CH_3O], 413 (2) [M^+ − CH_3OH − CH_3], 274 (9), 259 (100) [M^+ − $(CH_3)_2COO(CH_2)_7CHO$], 201 (5) [$(CH_3)_2COO(CH_2)_7CHO^+$], 169 (4) [$CH_3COO(CH_2)_7C^+$], 155 (44) [$CHO(CH_2)CO^+$], 137 (12), 109 (18), 73 (53) [$TMSi^+$], 55 (11) [$C_4H_7^+$], 31 (9) [CH_3O^+].

Novozym 435-Catalyzed Condensation Polymerizations between ω-Carboxy Fatty Acids and Diols. Reactions were performed in a parallel synthesizer (AdvantageTM 2050, Argonaut) both in-bulk and in diphenyl ether at 90 °C. Unsaturated and epoxidized α,ω-diacids (1.0 mmol) and diol (1.0 mmol) were transferred into reactor tubes of the parallel synthesizer and, then, 10%-by-wt N435 (i.e., 1%-by-wt CALB) relative to monomer was added. For solution polymerizations, 1 mL diphenyl ether was added. Control reactions without addition of N435 were also performed in parallel. Vacuum (10 mmHg) was applied after 2 h. To follow the progress of polymerizations, aliquots were withdrawn at 2, 6, 12, 24, 36, and 48 h. Reactions were terminated by addition of cooled chloroform and N435 was removed by filtration. Filtrates were concentrated at 50 °C under vacuum to remove chloroform and then directly (i.e., without fractionation by precipitation) analyzed by gel permeation chromatography (GPC) and ^{1}H and ^{13}C NMR. Alternatively, the concentrated filtrate solutions were added into magnetically stirred cold methanol. Precipitated polymers were filtered, were washed three times with cold methanol, and were dried in vacuo overnight at 50 °C.

Polyester from ω-Carboxyl OA and 1,3-Propanediol (Poly(ω-carboxyl OA-co-PD)). Yield: 80%. ^{1}H NMR (10.7 mg in 0.7 mL $CDCl_3$; ppm): 5.2−5.35 (2H, m, =C*H*-CH_2-), 4.05 (4H, m, -(CO)-O-CH_2-), 2.23 (4H, t, -CH_2-CO-), 1.8−2.0 (6H, m, =CH-CH_2-, -(CO)-O-CH_2-CH_2-), 1.45−1.6 (4H, m, -CH_2-CH_2-CO-), 1.1−1.35 (16H, m, -CH_2-). ^{13}C NMR (76.8 mg in 0.7 mL $CDCl_3$; ppm): 173.7 (C=O), 129.8 (=CH−), 60.8 (-CH_2O-), 34.2, 29.7, 29.2, 29.1, 28.1, 27.2, 24.9.

Polyester from ω-Carboxyl OA and 1,8-Octanediol (Poly(ω-carboxyl OA-co-OD)). Yield: 79%. ^{1}H NMR (10 mg in 0.7 mL $CDCl_3$; ppm): 5.3−5.4 (2H, m, =C*H*-CH_2-), 4.05 (4H, m, -(CO)-O-CH_2-), 2.35 (4H, t, -CH_2-CO-), 1.9−2.1 (4H, m, =CH-CH_2-), 1.5−1.7 (8H, m, -CH_2-CH_2-(CO)-O-CH_2-CH_2-), 1.2−1.4 (24H, m, -CH_2-). ^{13}C NMR (80 mg in 0.7 mL $CDCl_3$; ppm): 173.9 (C=O), 129.8 (=CH−), 64.3 (-CH_2O-), 34.3, 29.6, 29.2, 29.1, 28.6, 27.1, 25.8, 24.9.

Polyester from ω-Carboxyl OA and 1,16-Hexadecanediol (Poly(ω-carboxyl OA-co-HD)). Yield: 89%. ^{1}H NMR (8.3 mg in 0.7 mL $CDCl_3$) (ppm): 5.3−5.4 (2H, m, =C*H*-CH_2-), 4.05 (4H, m, -(CO)-O-CH_2-), 2.30 (4H, t, -CH_2-CO-), 1.9−2.1 (4H, m, =CH-CH_2-), 1.5−1.7 (8H, m, -CH_2-CH_2-(CO)-O−CH_2-CH_2-), 1.1−1.4 (40H, m, -CH_2-). ^{13}C NMR (81.9 mg in 0.7 mL $CDCl_3$; ppm): 173.7 (C=O), 129.7 (=CH−), 64.3 (-CH_2O-), 34.3, 29.6, 29.5, 29.4, 29.2, 29.1, 29.0, 28.9, 28.6, 27.1, 25.8, 24.9.

Polyester from ω-Carboxyl EA and 1,8-Octanediol (Poly(ω-carboxyl EA-co-OD)). Yield: 79%. (a) ^{1}H NMR (7.5 mg in 0.7 mL $CDCl_3$; ppm): 5.3−5.4 (2H, m, =C*H*-CH_2-), 4.05 (4H, m, -(CO)-O-CH_2-), 2.33 (4H, t, -CH_2-CO-), 2.0 (4H, m, =CH-CH_2-), 1.5−1.7 (8H, m, -CH_2-CH_2-(CO)-O-CH_2-CH_2-), 1.2−1.4 (32H, m, -CH_2-). ^{13}C NMR ($CDCl_3$; 75 mg in 0.7 mL ppm): 173.9, 173.8 (C=O), 129.9, 129.7 (=CH−), 64.2 (-CH_2O-), 34.3, 29.7, 29.6, 29.54, 29.51, 29.4, 29.3, 29.2, 29.1, 29.06, 28.6, 27.2, 27.1, 25.8, 24.9.

Polyester from ω-Carboxyl Epoxy SA and 1,8-Octanediol (Poly(ω-carboxyl epoxy SA-co-OD)). Yield: 60%. ^{1}H NMR (10.0 mg in 0.7 mL $CDCl_3$; ppm): 4.05 (4H, m, -(CO)-O-CH_2-), 2.9 (2H, bs, -C*H*-, *cis*-epoxide), 2.29 (4H, t, -CH_2-CO-), 1.2−1.7 (36H, m, -CH_2-). ^{13}C NMR (82.7 mg in 0.7 mL $CDCl_3$; ppm): 173.8 (C=O), 64.2 (-CH_2O-), 57.1 (-CH-, *cis*-epoxide), 34.2, 29.3, 29.1, 29.0, 28.9, 27.7, 26.5, 25.7, 24.8.

Unlike the above copolymerizations, reaction of 1,18-octadecanedioic acid (ω-carboxyl SA) with 1,8-octanediol was performed in a 250 mL flask with addition of toluene using an azeotrope at 90 °C. 1,18-Octadecanedioic acid (20 mmol) and 1,8-octanediol (20 mmol) were transferred into a flask with 100 mL toluene and 10%-by-wt N435. Vacuum (200−250 mmHg) was applied after 2 h. To follow the progress of polymerizations, aliquots were withdrawn at 2, 6, 12, 24, 36, and 48 h. As above, an aliquot that was not precipitated was used

to determine molecular weight averages and polydispersity of products by GPC. The final time point of this reaction was worked-up as above to terminate the reaction and remove N435. Subsequently, the filtrate was slowly added with stirring to methanol to precipitate the polyester. The precipitated polymer was washed with methanol three times and then dried under vacuum (50 °C, 10 mmHg). The resulting precipitated product was then analyzed by ^{1}H and ^{13}C NMR and also used for thermal property analysis.

Polyester from ω-Carboxyl SA and 1,8-Octanediol (Poly(ω-carboxyl SA-co-OD)). Yield: 86%. ^{1}H NMR (7.9 mg in 0.7 mL $CDCl_3$; ppm): 4.05 (4H, m, -(CO)-O-CH_2-), 2.33 (4H, t, -CH_2-CO-), 2.0 (4H, m, =CH-CH_2-), 1.5−1.7 (8H, m, -CH_2-CH_2-(CO)-O-CH_2-CH_2-), 1.2−1.4 (32H, m, -CH_2-). ^{13}C NMR (62.5 mg in 0.7 mL $CDCl_3$; ppm): 173.9 (C=O), 64.2 (-CH_2O-), 34.3, 29.62, 29.57, 29.5, 29.4, 29.2, 29.1, 29.0, 28.6, 25.8, 25.0.

Analytical Methods. Cell growth was determined by measuring absorbance at 600 nm. A standard curve of absorbance versus cell dry weight was constructed and subsequently used to convert cell growth measurements from absorbance to cell dry weight values. Glucose was measured by the enzymatic method using Glucose (HK) Assay Kit (Sigma).

Thin layer chromatography analysis was done on aluminum sheets coated with silica gel 60. Samples were spotted on the sheet using a 5 μL micro glass pipet and developed with a mobile phase system comprising toluene and acetic acid (9:1, v/v). After developing, sheets were sprayed with primuline solution (50 mg primuline dissolved in 800 mL acetone and 200 mL distilled water). Fatty acid derivatives were detected after primuline spray under UV light.

Instrumental Methods. *Chemical Characterization of Fermentation Products.* Gas chromatography/mass spectrometry (GC/MS) analysis was performed at 70 eV using a ThermoFinnigan TraceGC Ultra gas chromatograph coupled with a Trace DSQ mass spectrometer. Purified products were esterified with BF_3 in methanol (10%, w/w) at 70 °C for 20 min.[23] Silylation of ω-carboxyl epoxy SA methyl ester was performed with HMDS/TMCS/pyridine at 70 °C for 10 min.[24] GC/MS experiments were performed with injector, ion source and interface temperatures of 200, 250, and 280 °C, respectively. Samples in hexane (1 μL) were injected in PTV split mode and run on a capillary column (Varian CP8944 VF-5MS, 0.25 mm × 0.25 μm × 30 m). The oven temperature was programmed at 120 °C for 1 min increasing to 260 at 20 °C/min, and then to 280 at 4.0 °C/min.

Oxidative cleavage was used to determine the position of double bonds. Purified products were treated by acidic $KMnO_4$ solution (2%) at 60 °C for 20 min. After methylation of oxidized products (see above), samples were analyzed by GC/MS.

Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker DPX300 NMR spectrometer. The chemical shifts (ppm) reported were referenced relative to internal tetramethylsilane (TMS, 0.00 ppm) or to the solvent resonance at the appropriate frequency.

Infrared spectroscopy (Avatar 360 FT-IR spectrometer) was used to characterize products and provide information on double bond configuration. Melting point measurements were performed using an electrothermal melting point apparatus.

The concentration of diacids during biotransformation was measured by liquid chromatography/mass spectrometry (LC/MS) with purified products as standards. The solvent delivery system was a Waters Alliance 2795 Separation Module (Milford, MA, U.S.A.) coupled with a Waters 2996 photodiode array detector and Waters ZQ detector with an electron spray ionization mode. The separation was carried on a reversed-phase column with dimensions 150 × 4.6 mm and particle size of 5 μm. The mobile phase used for separation contained 10% H_2O, 5% acetonitrile, 5% formic acid solution (1% in water), and 80% methanol.

Gel Permeation Chromatography (GPC). Molecular weights of polyesters were determined by GPC using a Waters HPLC system equipped with model 510 pump, Waters model 717 autosampler, model 410 refractive index detector, Viscotek model T-50/T-60 viscosity detector, and 500, 10^3, 10^4, and 10^5 Å Ultrastyragel columns in series. Sample concentrations and injection volumes were 0.2% (w/v) and 30 μL, respectively. Tetrahydrofuran was used as eluent at a flow rate of 1 mL/min. Trisec GPC software version 3 was used for calculations. Molecular weights were determined on the basis of conventional calibration curve generated by narrow molecular weight polystyrene standards obtained from Sigma.

Thermogravimetric Analysis (TGA). TGA measurements were performed with a TA Instruments TGA2950 analyzer under nitrogen atmosphere with about 10 mg samples at a heating rate of 10 °C/min from 25 to 700 °C.

Differential Scanning Calorimetry (DSC). DSC scans were recorded using a TA Instruments DSC-2920 analyzer. Polymer samples were heated and cooled under a nitrogen flow rate of 50 mL/min and a heating rate of 10 °C/min. Samples were first heated to 120 °C and then quenched to −70 °C with liquid nitrogen thereby erasing their thermal history. Data reported herein on thermal transitions were obtained from second heating scans, recorded immediately after samples were quenched to −70 °C by reheating from −70 to 120 °C at a heating rate of 10 °C/min.

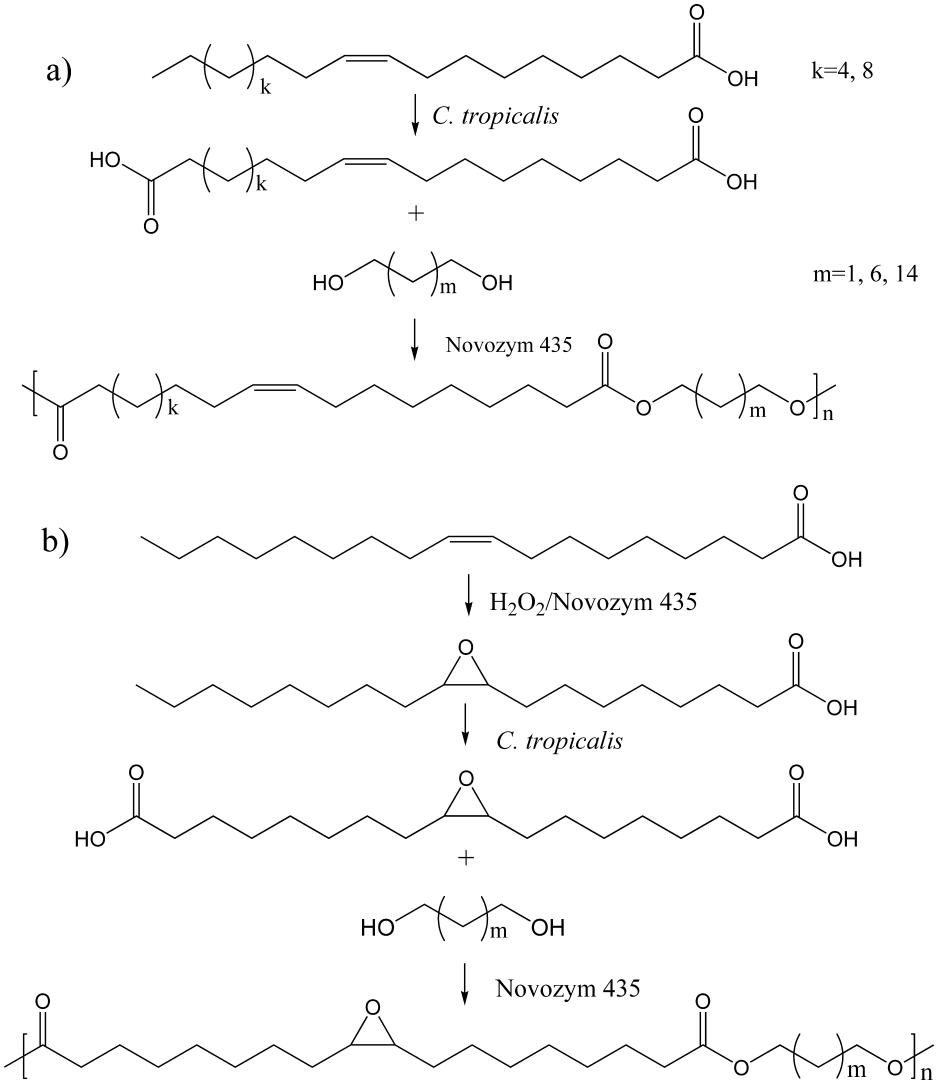
Results and Discussion

The following describes a unique two-pot two-step route by which whole-cell bio-oxidation followed by cell-free lipase catalysis is used to convert a series of unsaturated and epoxy fatty acid substrates to functional polyesters (Scheme 1). The first step investigates a fermentative reaction, catalyzed by *C. tropicalis* ATCC20962, to convert oleic, erucic and epoxystearic acids to their corresponding diacid functional monomers 1,18-*cis*-9-octadecenedioic acid (ω-carboxyl OA), 1,22- *cis*-9-docosenedioic acid (ω-carboxyl EA), and 1,18-*cis*-9,10-epoxyoctadecanedioic acid (ω-carboxyl epoxy SA). A key question addressed here is to what extent changes in chain length and functionality (double bond vs epoxy groups) of fatty acids affects their conversion to ω-carboxy diacids by *C. tropicalis* ATCC20962.

Biotransformation of Oleic Acid, Erucic Acid, and 9,10-Epoxy Stearic Acid by *C. tropicalis* ATCC20962. Synthesis of 9,10-epoxy stearic acid was performed by a chemoenzymatic method by which the peracid chemical catalysis of epoxidation was formed by a lipase-catalyzed esterification of oleic acid with hydrogen peroxide.[25] Epoxy stearic acid was obtained in quantitative yield and was directly used for biotransformation after removing toluene under vacuum with a rotary evaporator. The successful conversion of the double bond to an epoxy group is evident from ^{1}H NMR spectra that show the complete disappearance of signals corresponding to oleic acid olefin protons *a* of double bond at 5.3−5.4 ppm and the appearance of ^{1}H NMR signals for the product at 2.90 ppm due to methine protons *A* of the epoxy functionality (Figure 1). The resulting epoxy SA was then used as a substrate for fermentative bio-oxidation reactions.

Biotransformations of oleic acid, erucic acid, and 9,10-epoxy stearic acid, catalyzed by *C. tropicalis* ATCC20962, were conducted in 500 mL shake-flasks (30 mL conversion media) with initial fatty acid concentrations of 20 g/L. Figure 2A shows the consumption of fatty acid carbon sources and formation of unsaturated and epoxidized dicarboxylic acids as a function of biotransformation time determined by LC-MS. The highest conversion rate was obtained using oleic acid as substrate, reaching 14.1 and 17.3 g/L (77.6 mol %) ω-carboxyl OA in 24 and 48 h, respectively. In contrast, after 72 h, the maximum concentration of ω-carboxyl EA and ω-carboxyl epoxy SA reached to 14.2 g/L (64.8 mol %) and 19.1 g/L (86.2 mol %), respectively. Slower conversions of these two substrates may

Scheme 1. Lipase-Catalyzed Polycondensation of Unsaturated (a) and Epoxidized (b) Dicarboxylic Acids with Diols[a]



[a] Dicarboxylic acids were obtained from bioconversions of oleic acid, erucic acid, and 9,10-epoxy stearic acid catalyzed by *C. tropicalis* ATCC20962.

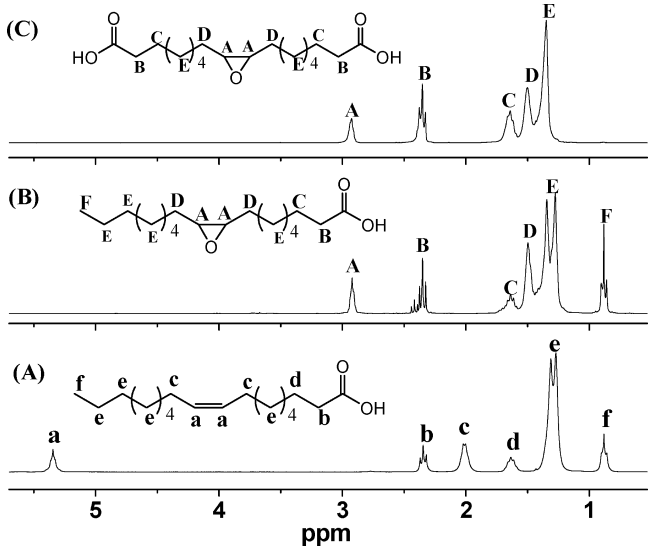


Figure 1. ^{1}H NMR (300 MHz) spectra recorded in chloroform-*d* of oleic acid (A), 9,10-epoxy stearic acid (B), and 1,18-*cis*-9,10-epoxy-octadecanedioic acid (C).

in part be due to their lower solubility in fermentation media. Nevertheless, at 72 h, the conversion of erucic acid to ω-carboxyl EA continues to increase, indicating that extending the fermentation time would result in still higher ω-carboxyl EA yield.

Scale-up biotransformation of oleic acid, catalyzed by *C. tropicalis* ATCC20962, was performed by fed-batch culture in a 3 L fermentor. Previous studies by Gmunder et al.[26] showed that high glucose concentrations in fermentation media for *C. tropicalis* inhibits cytochrome P450/reductase expression and activity. Hence, after the 12 h growth phase, high glucose concentrations in fermentation media during the biotransformation phase was avoided by slowly feeding glucose at 1.2 g/L/h. Concurrently, oleic acid was fed at 2.0 mL/h. Resulting cell growth, glucose consumption and production of ω-carboxyl OA are shown in Figure 2B. Maximum ω-carboxyl OA volumetric concentration of 31 g/L was obtained after 60 h. By conducting ω-carboxyl OA synthesis in a fermentor instead of in-shake flask cultures, the productivity increased from 0.36 to 0.5 g/L/h. This is due to improved control of fermentation parameters such as pH, DO, and low level of glucose concentration during the conversion phase that is maintained by continuous feeding in the fermentor.

In previous studies, oleic acid bioconversion by *C. tropicalis* mutants resulted in poor conversion efficiency and low yield of ω-carboxyl OA. Yi et al.[11] reported that, conversion of oleic

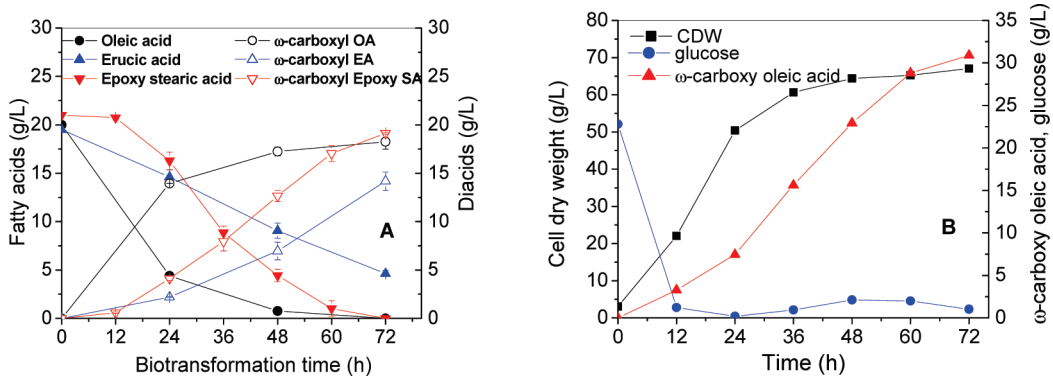


Figure 2. Fermentation profiles for *C. tropicalis* ATCC20962-catalyzed conversions of oleic acid, erucic acid, and 9,10-epoxy stearic acid to α,ω-dicarboxylic acids in shake-flask experiments (A) and (B) *C. tropicalis* ATCC20962-catalyzed conversion of oleic acid to ω-carboxyl oleic acid in a 3-L fermentor.

acid using *C. tropicalis* mutant S_{76} gave ω-carboxyl OA in up to ~4 g/L after a 72 h fermentation in shake flask. Furthermore, increased fermentation time resulted in decreased product yield. This is consistent with the fact that the shorter chain-length unsaturated diacids with 6−16 carbon atoms were also detected in media due to degradation of ω-carboxyl OA through the β-oxidation pathway.[27] Fabritius et al.[12] reported that, for conversion of oleic acid using mutants of *C. tropicalis* DSM3152 and M25 in fed-batch culture, the maximum concentration of ω-carboxyl OA reached values of 21.5 g/L at 86.5 h and 8.1 g/L at 192 h, respectively. Similarly, the decrease in ω-carboxyl OA production was observed presumably due to metabolism of product via the β-oxidation pathway. Indeed, 3-hydroxy-1,18-*cis*-9-octadecenedioic acid was found in large amounts along with ω-carboxyl OA in media. Picataggio et al.[4] compared oleic acid and erucic acid bioconversion using the P450 monooxygenase (P450alkl) and NADPH-oxidoreductase (CPR) amplified strain AR40 derived from *C. tropicalis* ATCC20962. They found that both ω-carboxyl OA and ω-carboxyl EA was obtained in high yield and productivity. However, the effect of chain length and functionality (double bond versus epoxy groups) of fatty acids on their conversion to ω-carboxyl fatty acids by *C. tropicalis* ATCC20962 has not been reported. Furthermore, to our knowledge, this is the first time that ω-carboxyl epoxy SA has been efficiently synthesized from its corresponding fatty acid by a whole-cell biotransformation.

Structural analysis of ω-carboxyl fatty acids was performed by multiple methods.

Molecular weights of resulting ω-carboxyl OA, ω-carboxyl EA and ω-carboxyl epoxy SA, determined by LC-MS, are 312, 368, and 328, respectively, in agreement with those calculated for each product. Methyl ester and silylated (only for ω-carboxyl epoxy SA) derivatives of purified ω-carboxyl products were analyzed by GC-MS. Indeed, for derivatized ω-carboxyl OA, ω-carboxyl EA and ω-carboxyl epoxy SA, single peaks with retention times of 10.54, 13.97, and 13.17 min, respectively, were detected. The mass fragment spectrum for each compound is listed in the Experimental Section and displayed in Figure 3. The mass fragment spectrum for ω-carboxyl OA dimethyl ester (Figure 3A) shows a molecular ion $[M^+]$ at *m/z* 340 and fragment ions at *m/z* 308 $[M^+ - CH_3OH]$ and 276 $[M^+ - 2CH_3OH]$ due to scission of one and two methanol groups, respectively. Fragment ions at *m/z* 248 $[M^+ - 2CH_3OH - CO]$, *m/z* 234 $[248 - CH_2]^+$, and *m/z* 69 $[C_5H_9^+]$ were also detected. The mass fragment spectrum was in agreement with that reported previously by Yi et al.,[11] obtained by GC-MS analysis of Δ^9-*cis*-1,18-octadecenedioic acid dimethyl ester. For the dimethyl ester of ω-carboxyl EA, its mass fragment spectrum shown in Figure 3B is also in accord with that expected. The molecular ion $[M^+]$ at *m/z* 396 and typical fragment ions at *m/z* 364 $[M^+ - CH_3OH]$ and 332 $[M^+ - 2CH_3OH]$, due to scission of one and two methanol groups, respectively, were observed as well as *m/z* 304 $[M^+ - 2CH_3OH - CO]$ and *m/z* 69 $[C_5H_9^+]$. The mass fragment spectrum of methylated and silyated ω-carboxyl epoxy SA is displayed in Figure 3C. As above, the observed spectrum is consistent with that expected for the corresponding product structure. Fragment ions at *m/z* 429 $[M^+ - CH_3O]$, 259 $[M^+ - (CH_3)_2COO(CH_2)_7CHO]$, 201 $[(CH_3)_2COO(CH_2)_7CHO^+]$, 155 $[259 - TMS - CH_3O]^+$, 169 $[201 - CH_3OH]^+$, 137 $[169 - CH_3OH]^+$, 109 $[137 - CO]^+$, 73 $[TMSi^+]$, and 31 $[CH_3O^+]$ were all detected.

To determine whether the double bond position remained intact or was isomerized to another position along the fatty acid, purified ω-carboxyl OA and ω-carboxyl EA were treated by acidic $KMnO_4$ solution. After methylation of oxidative products, the samples were analyzed by GC-MS. The mass spectrum with retention time 6.48 min in GC was identical to that of authentic dimethyl ester of azelaic acid (see Supporting Information, Figure S-1). Thus, as in the OA and EA used as feedstocks in fermentations, the position of double bonds in both compounds is between C-9 and C-10.

Structures of purified products were further confirmed by ^{1}H NMR, ^{13}C NMR and IR (see deposited spectra in Supporting Information, Figures S-2, S-3, and S-4, respectively). Indeed, spectra obtained are consistent with that of proposed product structures. ^{1}H NMR spectra of ω-carboxyl OA and ω-carboxyl EA have signals at 2.35 ppm with two overlapping triplets due to protons for two methylene groups adjacent to carboxylic acid moieties. Furthermore, signals at 5.35 ppm, due to olefin protons (−CH═CH−), are observed and integration ratios are as expected. Also, the characteristic signal corresponding to the terminal methyl group of fatty acid starting materials OA and EA at 0.88 ppm is not observed. As above, comparison of ^{1}H NMR spectra of 9,10-epoxy SA and ω-carboxyl epoxy SA are consistent with formation of the ω-oxidized product (Figure 1). Unique to these spectra is the signal at 2.90 ppm corresponding to methine protons *A* and the ^{13}C signal at 57.3 ppm corresponding to carbons of *cis*-epoxy groups.[23] Observation of epoxy proton signals in the product shows this group remains intact during the biotransformation. IR spectra of ω-carboxyl OA and ω-carboxyl EA show the characteristic vibrational absorption band at 725 cm^{-1} located in the fingerprint region corresponding to the presence of *cis*-configuration double bonds.[11]

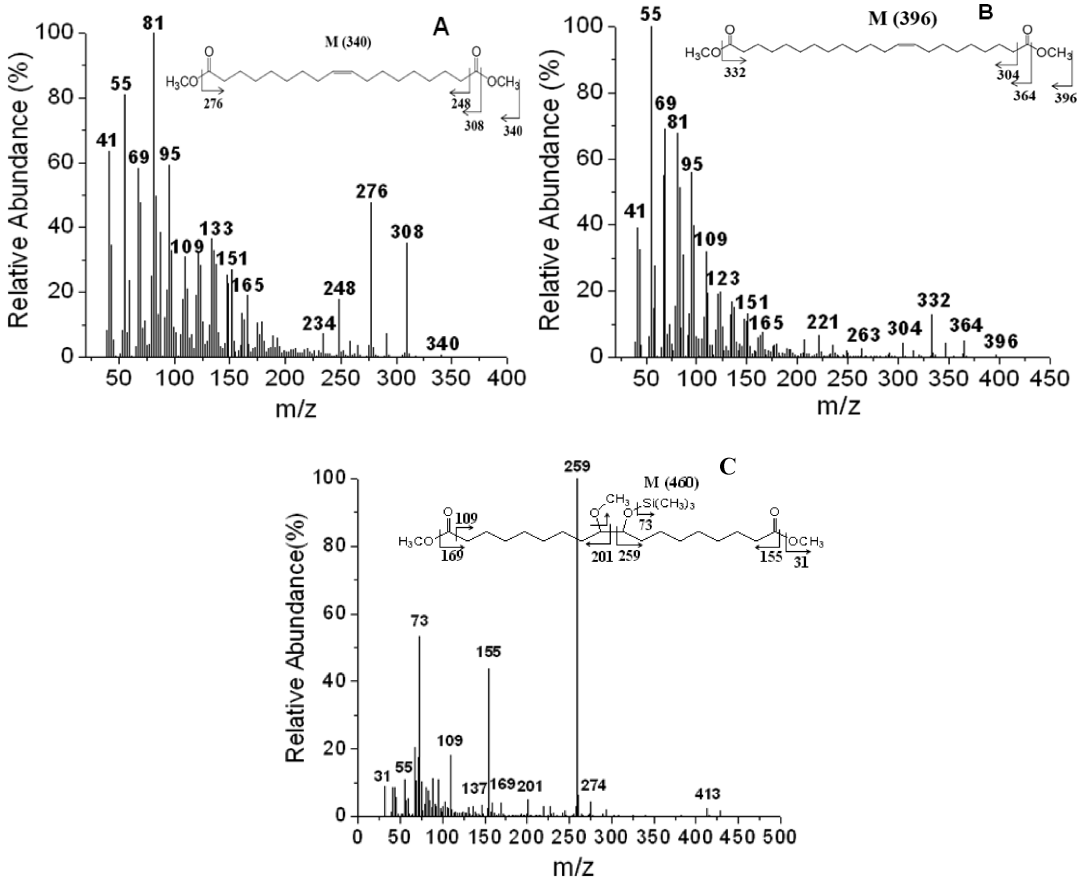


Figure 3. Mass spectral analysis of oleic acid, erucic acid, and 9,10-epoxy stearic acid metabolites produced by *C. tropicalis* ATCC20962. The mass spectra were obtained from GC peaks appearing at the retention times of A) 10.54 min (1,18- *cis*-9-octadecenedioic acid dimethyl ester derivative; B), 13.97 min (1,22- *cis*-9-docosenedioic acid dimethyl ester derivative; C), and 13.17 min (1,18-*cis*-9, 10-epoxy-octadecanedioic acid, TMS ether dimethyl ester derivative.

Novozym 435-Catalyzed Copolymerization of Unsaturated and Epoxidized α,ω-Dicarboxylic Acids with Diols. After successful preparation by whole-cell bio-oxidations of ω-carboxyl OA, ω-carboxyl EA, and ω-carboxyl epoxy SA, the second step of this newly developed two-pot, two-step route to unsaturated and epoxidized fatty acid-based polyesters was undertaken. That is, cell-free immobilized lipase catalysis was used to copolymerize α,ω-diacids with a series of diols. Of interest was to what extent do changes in structures of fatty acid derived α,ω-diacids and α,ω-alkylene diols affect lipase-catalyzed conversions of monomers to polyesters.

N435, consisting of CALB immobilized on a macroporous polyacrylate resin, was used as the lipase catalyst for polycondensations of ω-carboxy fatty acids and diols. Previous work by Mahapatro et al.[16g] demonstrated that, N435-catalyzed copolycondensations in-bulk, between adipic acid and 1,8-octanediol (OD), could be conducted at temperatures up to at least 90 °C without negatively impacting the outcome of reactions. Hence, to (i) melt unsaturated and epoxy diacids, (ii) attain monophasic liquid media for bulk copolymerizations, and (iii) minimize diffusion limitations under such conditions, all polymerizations conducted herein were performed at 90 °C.

The time course of changes in weight average molecular weight (M_w) and polydispersity (M_w/M_n) for N435-catalyzed in-bulk polycondensations of 1,8-octanediol with ω-carboxy OA to prepare poly(ω-carboxyl OA-*co*-OD) are displayed in Figure 4. Poly(ω-carboxyl OA-*co*-OD) M_w increased from about 15000 to 37000 g/mol from 2 to 24 h. Beyond 24 h, further increases in molecular weight occurred slowly. By 48 h, M_w and M_w/M_n values of poly(ω-carboxyl OA-*co*-OD) reached 44000 g/mol and 2.1, respectively. A decrease in M_w/M_n was observed from about 2.5 to 2.1 at 12−36 and 48 h, respectively. This decrease in polydispersity is explained by that, when reaction times are prolonged and high viscosity reaction media are formed, more rapid diffusion of lower molecular weight chains to propagating chain ends depletes the population of low-molecular weight species.[28] When this reaction was performed in diphenyl ether at 90 °C, poly(ω-carboxyl OA-*co*-OD) reached an M_w of 57000 g/mol in 36 h with M_w/M_n of 2.0. The attainment of higher molecular weights in solution is explained by higher diffusivity of reactants as well as easier removal of water from reaction media. However, given the (i) difficulty of removing diphenyl ether from reaction products (discussed further below), (ii) overall benefits in process efficiency by running bulk polymerizations, and (iii) ability to form high molecular weight polyesters without solvent, the focus of polymerization studies below is on use of bulk polymerization conditions.

While using the same ω-carboxyl diacid, the effect of varying diol chain length on unsaturated polyester molecular weight was investigated. Figure 4A shows that increasing the diol chain length from 1,8-octanediol to 1,16-hexadecanediol (HD) gave a similar trend in M_w increase as a function of time. The most notable difference in the molecular weight behavior during poly(ω-carboxyl OA-*co*-OD) and poly(ω-carboxyl OA-*co*-HD) synthesis is that, for extended reaction times, M_w/M_n decreased for the former but increased for the later. Increase in M_w/M_n is indicative of an increase in events of transesterification that appears to be more prevalent when using HD as comonomer.

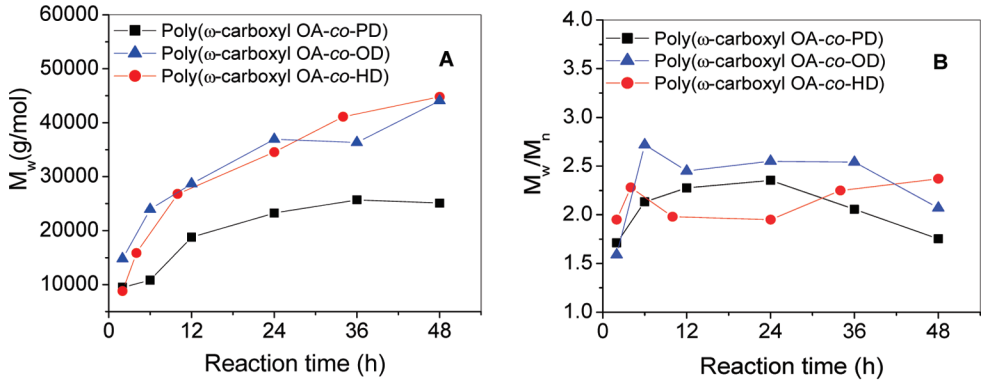


Figure 4. Molecular weight (M_w (A) and polydispersity index (M_w/M_n (B) as a function of reaction time for N435-catalyzed polymerization of ω-carboxyl OA with 1,3-propanediol, 1,8-octanediol, and 1,16-hexadecandediol. Reactions were performed in bulk at 90 °C.

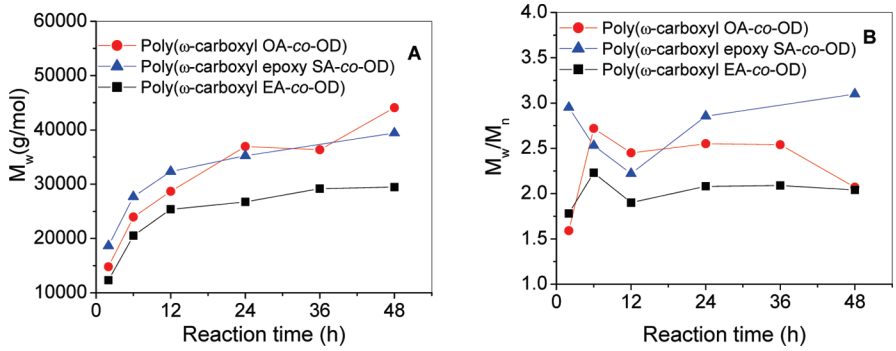


Figure 5. Molecular weight (M_w, A) and polydispersity index (M_w/M_n, B) as a function of reaction time for N435-catalyzed polymerization of 1,8-octanediol with ω-carboxyl OA, ω-carboxyl EA, and ω-carboxyl epoxy SA. Reactions were performed in bulk at 90 °C.

The rapid chain growth found herein for N435-catalyzed polymerizations of ω-carboxyl OA with longer chain diols OD and HD was similarly observed for N435-catalyzed bulk polymerizations of 12- and 16-carbon ω-hydroxyacids that, performed at ambient pressure, gave polyesters in 4 h with M_n up to 23000 g/mol (nonfractionated).[16g] By using 1,3-propanediol (PD) in place of OD and HD as comonomer for copolymerizations with ω-carboxyl OA, M_w increased more slowly with time. While M_w for poly(ω-carboxyl OA-*co*-OD) increased from 16000 to 27000 and 35000 g/mol at 6, 12, and 24 h, respectively, M_w for poly(ω-carboxyOA-*co*-PD) increased from 11000 to 19000 and 23000 g/mol at 6, 12, and 24 h, respectively. However, similarly to poly(ω-carboxyl OA-*co*-OD), M_w/M_n of poly(ω-carboxyl OA-*co*-PD) decreased with extended reaction time due, as described above, to higher diffusivity of low molecular weight fragments to condense, thereby narrowing the polydispersity. The slow reactivity of PD relative to OD and HD is interesting, given that PD is much closer in structure to glycerol, the acyl acceptor associated with lipase activity in nature, than is OD or HD. Indeed, the relatively low reactivity of shorter chain α,ω-alkylene glycols during N435-catalyzed polymerizations was observed by others. Uyama et al.[29] reported that reactions between short chain diols (1,2-ethane diol and 1,4-butanediol) and succinic anhydride gave only oligomeric products, whereas polymerizations between glutaric anhydride and 1,14-dihydroxytetradecane at 60 °C gave polyesters with M_n 7600 g/mol and M_w/M_n of 2.1. The difficulty in obtaining polyesters by N435-catalyzed polymerization using diol monomers with four or fewer carbons and nonactivated diacid comonomers was discussed by Azim et al.[30] Similarly, Mahapatro et al.[16f] reported that, for bulk N435-catalyzed copolymerizations of adipic acid with 1,4-butanediol, 1,6-hexanediol, and 1,8-octanediol at 70 °C, chain growth occurred most rapidly with 1,8-octanediol and slowest with 1,4-butanediol.

Figure 5 shows the time course of M_w and M_w/M_n for N435-catalyzed polymerization of 1,8-octanediol with ω-carboxyl EA (C22 unsaturated diacid), ω-carboxyl epoxy SA (C18 epoxy diacid), and ω-carboxyl OA, respectively. Poly(ω-carboxyl EA-*co*-OD) M_w rapidly increased from 12000 to 21000 and 25000 g/mol in 2, 6, and 12 h, respectively. Similarly, M_w of poly(ω-carboxyl epoxy SA-*co*-OD) rapidly increased from 19000 to 28000 and 32000 g/mol in 2, 6, and 12 h, respectively. Typical of diffusion limited reactions, further extending the reaction times beyond 12 h resulted in slow increases in M_w so that, by 48 h, copolymers of ω-carboxyl EA and ω-carboxyl epoxy SA reached M_w values of 29000 and 39000 g/mol, respectively. Increase in the diacid chain length by 4-methylene units for EA relative to OA and epoxy SA did not result in enhanced M_w increase (see Figure 5A). Variations in M_w/M_n between these three copolymerizations are difficult to explain and may be due to experimental error.

For all of the above-described polymerizations control experiments were performed without addition of N435. GPC analysis of reaction products under control conditions showed that no polymers (M_w < 1000 g/mol) were formed.

To ensure that conversion of ω-carboxyl fatty acids to polyesters occurred without disturbing double bond and epoxy moieties, ^{1}H and ^{13}C NMR spectra of corresponding polyesters were recorded. Chemical shifts of polyesters are listed in the Experimental Section and ^{1}H NMR spectra of poly(ω-carboxyl OA-*co*-OD), poly(ω-carboxyl EA-*co*-OD), and poly (ω-carboxyl epoxy SA-*co*-OD) are deposited in Supporting Information (Figures S-5 and S-6). For copolyesters with unsaturated and

Table 1. Thermal Properties of Saturated, Unsaturated, and Epoxidized Polyesters Catalyzed by Novozym 435[a]

polyester	M_w[b] (g/mol)	M_w/M_n[b]	T_d[c] (°C)	T_m[d] (°C)	ΔH_m[d] (J/g)
poly(ω-carboxyl OA-*co*-OD)	44000	2.1	388	23/36	11.7/21.1
poly(ω-carboxyl OA-*co*-PD)	25000	2.1	384	16/22	1.4/14.4
poly(ω-carboxyl OA-*co*-HD)	45000	2.4	375	35/57	8.3/83.4
poly(ω-carboxyl EA-*co*-OD)	29000	2.1	385	35/40	30.6/1.1
poly(ω-carboxyl epoxy SA-*co*-OD)	39000	3.1	381	33	52.9
poly(ω-carboxyl SA-*co*-OD)	76000	2.0	383	77/88	14.8/90.3

[a] Polycondensations of unsaturated and epoxidized diacids with diols were performed in bulk containing 1.0 mmol unsaturated diacid and 1.0 mmol 1,8-octanediol at 90 °C for 48 h. Polycondensation of 1,18-octadecanedioic acid with 1,8-octanediol was performed in toluene in a round-bottom flask. [b] Data from GPC were measured using THF as eluent. Samples without precipitation for unsaturated and epoxidized polyesters were used for measurements. [c] Data from TGA under nitrogen atmosphere at a heating rate of 10 °C/min from 25 to 700 °C. [d] Data from DSC from the second heating scan run at 10 °C/min.

epoxy moieties, ^{1}H NMR signals at 5.35 and 2.90 ppm due to vinyl and epoxy protons were found and integration intensities are consistent with these groups remaining intact. Low intensity signals at 3.65 ppm correspond to protons of -CH_2-OH end groups. Retention of double bond and epoxy group structure during conversion of monomers to polyesters is an important advantage of lipase-catalyzed condensation reactions. In contrast, chemical-catalyzed condensation methods conducted at high temperatures (>150 °C) with acid, base, or organo-metallic reagents cause the formation of undesired side products by reactions at unsaturated and epoxy groups.[17d]

Thermal Properties Analysis of Saturated, Unsaturated, and Epoxy Functionalized Polyesters. Thermal properties of polyesters were analyzed by TGA and DSC. For comparison with unsaturated and epoxy functionalized polyesters described above, it was deemed useful to prepare the corresponding nonfunctionalized polyester from 1,18-octadecanedioic (ω-carboxy stearic acid, ω-carboxy SA). To that end, N435-catalyzed synthesis of poly(ω-carboxyl SA-*co*-OD) was undertaken. Due to the high melting point of ω-carboxyl SA (122−123 °C),[31] bulk polycondensations at 90 °C with OD were unsuccessful. From previous work in our laboratory where polymers of similar structure were synthesized by N435 catalysis in diphenyl ether, difficulties were encountered when trying to remove residual diphenyl ether from products. To circumvent these problems, poly(ω-carboxyl SA-*co*-OD) synthesis was conducted in toluene under vacuum so that the reaction was maintained at 90 °C and water was removed azeotropically (see Experimental Section). At 48 h, GPC analysis showed that a high molecular weight polyester had formed with M_w 76000 g/mol and M_w/M_n 2.0. ^{1}H NMR analysis of this product was consistent with that expected (see Supporting Information, Figure S-5). Subsequently, poly(ω-carboxyl SA-*co*-OD) was isolated by precipitation, solvent was removed under vacuum at 50 °C, and the resulting product was used for thermal analysis.

Table 1 lists results from DSC and TGA analyses of saturated, unsaturated and epoxidized polyesters and their corresponding molecular weight averages. Comparison of TGA scans shows they are all alike in appearance and have temperatures at which 10% of weight loss was observed (T_d) that are similar in value ranging from 375 to 388 °C. Hence, the presence of epoxy and unsaturated values as well as the use of diols differing in structure led to polyesters of similarly high thermal stability.

Differential scanning calorimetry (DSC) traces of unsaturated poly(ω-carboxyl OA-*co*-OD) and poly(ω-carboxyl EA-*co*-OD) both show two peak melting transitions (T_m values) at 23/36 and 35/40 °C, respectively. Although the 40 °C transition has a low ΔH_m, poly(ω-carboxyl EA-*co*-OD) lacks the melting transition at 23 °C and the T_m at 35 °C has a ΔH_m of 30 J/g. Higher melting transitions and ΔH_m values of poly(ω-carboxyl EA-*co*-OD) relative to poly(ω-carboxyl OA-*co*-OD) is consistent with the longer chain length of ω-carboxyl EA relative to ω-carboxyl OA. An increase in the alkylene diol chain length of ω-carboxyl OA copolymers from 3 to 8 and 16 carbons results in corresponding increases in T_m values (Table 1). Large differences in melting transitions for poly(ω-carboxyl OA-*co*-HD) and poly(ω-carboxyl SA-*co*-OD) are intriguing. Poly(ω-carboxyl OA-*co*-HD) has only one point of *cis*-unsaturation that provides a kink along a repeat unit consisting of a C18 unsaturated diacid and C16 saturated diol. That one kink, along a 34-carbon AA-BB repeat unit structure, results in a predominant T_m transition at 57 °C. In contrast, poly(ω-carboxyl SA-*co*-OD), with an AA-BB repeat unit structure consisting of only 26 carbons, but that is fully saturated, has a predominant melting transition that is 31 °C higher (88 °C). This attests to the powerful affects of *cis*-unsaturation that nature uses on a regular basis to manipulate the fluidity and, therefore, biological properties of lipid bilayers in living systems.[32] Comparison of thermal transitions for poly(ω-carboxyl OA-*co*-OD) and poly(ω-carboxyl epoxy SA-*co*-OD) shows the relative affects of *cis*-unsaturation and epoxidation on disruption of crystalline order in nonbranched aliphatic polyesters containing ω-carboxy fatty acid repeat units. These two polyesters, with *cis*-unsaturation and epoxy moieties, have similar predominant melting transitions at 36 and 33 °C, respectively. However, ΔH_m of the latter is much higher (21 vs 53 J/g), while the former also has a substantial lower melting transition at 23 °C. Hence, it is concluded that *cis*-unsaturation relative to epoxidation has a greater affect on disrupting crystalline organization of nonbranched long-chain aliphatic polyesters.

Conclusion

This work reported a unique two-pot, two-step route, using whole-cell bio-oxidation followed by cell-free lipase catalysis to convert readily renewable fatty acids to functional polyesters. Whole-cell biotransformation was found to be an effective way to synthesize ω-carboxyl fatty acids with internal functional groups from renewable feedstocks. ω-Carboxyl OA, ω-carboxyl EA, and ω-carboxyl epoxy SA were produced using fermentations of *C. tropicalis* ATCC20962 in good yield and with intact double bond or epoxy groups. This biochemical route to oxidation of terminal fatty acid methyl groups to carboxyl moieties fills a gap in the expansive repertoire of organic chemical methods.

Immobilized CALB (N435) is an efficient catalyst for the polycondensation of unsaturated and epoxidized ω-carboxyl fatty acids with diols to give double bond- or epoxy-functionalized polyesters with high molecular weight. The effect of changes in ω-carboxyl fatty acid building blocks and length of diols on thermal properties of the resulting polyesters was studied. Linear unsaturated and epoxidized polyesters had high

molecular weights of 25000−57000 g/mol with low melting points (23−40 °C), whereas saturated polyesters from ω-carboxyl SA with 1,8-octanediol had a higher melting transition (77−88 °C). Increasing the chain length of diols resulted in higher molecular weights and melting point of unsaturated polyesters from ω-carboxyl OA. The results of this work pave the way for a new family of fatty acid derived polyesters from a wide-range of ω-carboxyl fatty acid monomers. Unlike polyesters from saturated diacids, polyesters with epoxy and unsaturated moieties as described herein can be modified, derivatized, or cross-linked at these sites, which will prove valuable in developing curable coatings as well as for decoration of these polymers with bioactive moieties for medical applications.

Acknowledgment. We thank the NSF and Industrial members of the NSF-Industry/University Cooperative Research Center (NSF I/UCRC) for Biocatalysis and Bioprocessing of Macromolecules at Polytechnic Institute of NYU for their financial support, intellectual input, and encouragement during the course of this research.

Supporting Information Available. Supplementary data of mass spectrum of oxidative products from ω-carboxyl OA and ω-carboxyl EA, ^{1}H and ^{13}C NMR spectra of ω-carboxyl OA, ω-carboxyl EA, and ω-carboxyl epoxy SA, IR spectra of ω-carboxyl OA and ω-carboxyl EA, and ^{1}H NMR spectra of polyesters. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Chan, E. C.; Kuo, J. *Enzyme Microb. Technol.* **1997**, *20*, 585–589.
(2) Sato, K.; Aoki, M.; Noyori, R. *Science* **1998**, *281*, 1646–1647.
(3) Debuan, F.; Hänssle, P. *J. Synth. Lubr.* **1985**, *1*, 254–267.
(4) Picataggio, S.; Rohrer, T.; Deanda, K.; Lanning, D.; Reynolds, R; Mielenz, J.; Eirich, L. D. *Bio/technology (N.Y.)* **1992**, *10*, 894–898.
(5) Kolattukudy, P. E. *Science* **1980**, *208*, 990–1000.
(6) Yi, Z. H.; Rehm, H. J. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *15*, 175–179.
(7) Hill, F. F.; Venn, I.; Lukas, K. L. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 168–174.
(8) Green, K. D.; Turner, M. K.; Woodley, J. M. *Enzyme Microb. Technol.* **2000**, *27*, 205–211.
(9) Broadway, N. M.; Dickinson, M.; Ratledge, C. *J. Gen. Microbiol.* **1993**, *139*, 1337–1349.
(10) Eschenfeldt, W. H.; Yeyan, Z. H.; Samaha, H.; Stols, L.; Eirich, D. C.; Ronald, W.; Donnelly, M. I. *Appl. Environ. Microbiol.* **2003**, *69*, 5992–5999.
(11) Yi, Z. H.; Rehm, H. J. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 327–331.
(12) Fabritius, D.; Schafer, H. J.; Steinbuchel, A. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 342–348.
(13) Fabritius, D.; Schafer, H. J.; Steinbuchel, A. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 83–87.
(14) Kandel, S.; Sauveplane, V.; Olry, A.; Diss, L.; Benveniste, I.; Pinot, F. *Phytochem. Rev.* **2006**, *5*, 359–372.
(15) Odian, G. *Principles of Polymerization*; John Wiley & Sons: New York, 2004; p 94.
(16) (a) Magolin, A. L.; Creene, J. Y.; Klibanov, A. M. *Tetrahedron. Lett.* **1987**, *28*, 1607–1612. (b) Chaudhary, A. K.; Lopez, J.; Beckman, E. J.; Russell, A. J. *Biotechnol. Prog.* **1997**, *13*, 318–325. (c) Linko, Y. Y.; Wang, Z. L.; Seppala, J. *Enzymol. Microb. Technol.* **1995**, *17*, 506–511. (d) Linko, Y. Y.; Lamsa, M.; Wu, X.; Uosukanum, E.; Seppala, J.; Linko, P. *J. Biotechnol.* **1998**, *66*, 41–50. (e) Uyama, H.; Inada, K.; Kobayashi, S. *Polym. J.* **2000**, *32*, 440–443. (f) Mahapatro, A.; Kalra, B.; Kumar, A.; Gross, R. A. *Biomacromolecules* **2003**, *4*, 544–551. (g) Mahapatro, A.; Kumar, A.; Kalra, B.; Gross, R. A. *Macromolecules* **2004**, *37*, 35–40. (h) Ebata, H.; Toshima, K.; Matsumura, S. *Macromol. Biosci.* **2007**, *7*, 798–803.
(17) (a) Wallace, J. S.; Morrow, C. J. *J. Polym. Sci., Polym. Chem. Ed.* **1989**, *27*, 2553. (b) Olson, D. A.; Sheares, V. V. *Macromolecules* **2006**, *39*, 2808–2814. (c) Uyama, H.; Kuwabara, M.; Tsujimoto, T.; Kobayashi, S. *Biomacromolecules* **2003**, *4*, 211–215. (d) Warwel, S.; Demes, C.; Steinke, G. *J. Polym. Sci., Part A: Polym. Chem.* **2001**, *39*, 1601–1609.
(18) Kelly, A. R.; Hayes, D. G. *J. Appl. Polym. Sci.* **2006**, *101*, 1646–1656.
(19) Ebata, H.; Toshima, K.; Matsumura, S. *Macromol. Biosci.* **2007**, *7*, 798–803.
(20) Veld, M. A. J.; Palmans, A. R. A.; Meijer, E. W. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 5968–5978.
(21) Olsson, A.; Lindstrom, M.; Lversen, T. *Biomacromolecules* **2007**, *8*, 757–760.
(22) Warwel, S.; Ruesch gen klaas, M. *J. Mol. Catal. B: Enzym.* **1995**, *1*, 29–35.
(23) Dawidowicz, E. A.; Thompson, T. E. *J. Lipid. Res.* **1971**, *12*, 636.
(24) Blau, K.; Halket, J. *Handbook of Derivatives for Chromatography*, 2nd ed.; John Wiley & Sons: New York, 1993.
(25) Cecilia, O.; Samuel, C.; Dietlind, A.; Bo, M.; Rajni, H. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 864–870.
(26) Gmunder, F. K.; Kappeli, O.; Fiechter, A. *Appl. Microbiol. Biotechnol.* **1981**, *12*, 135–142.
(27) Yi, Z. H.; Rehm, H. J. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 520–526.
(28) Jiang, Z. Z.; Liu, C.; Xie, W. H.; Gross, R. A. *Macromolecules* **2007**, *40*, 7934–7943.
(29) Uyama, H.; Wada, S.; Fukui, T.; Kobayashi, S. *Biochem. Eng. J.* **2003**, *16*, 145–152.
(30) Azim, H.; Dekhterman, A.; Jiang, Z. Z.; Gross, R. A. *Biomacromolecules* **2006**, *7*, 3093–3097.
(31) Tulloch, A. P.; Spencer, J. F. T.; Gorin, P. A. J. *Can. J. Chem.* **1962**, *40*, 1326–1338.
(32) Rawicz, W.; Olbrich, K. C.; McIntosh, T.; Needham, D.; Evans, E. *Biophys. J.* **2000**, *79*, 328–339.

BM901112M