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Regio- and stereoselective biocatalytic hydration of fatty acids from waste cooking oils en route to hydroxy fatty acids and bio-based polyesters

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ABSTRACT

The development of biorefinery approaches is of great relevance for the sustainable production of valuable compounds. In accordance with circular economy principles, waste cooking oils (WCOs) are renewable resources and biorefinery feedstocks, which contribute to a reduced impact on the environment. Frequently, this waste is wrongly disposed of into municipal sewage systems, thereby creating problems for the environment and increasing treatment costs in wastewater treatment plants. In this study, regenerated WCOs, which were intended for the production of biofuels, were transformed through a chemo-enzymatic approach to produce hydroxy fatty acids, which were further used in polycondensation reaction for polyester production. *Escherichia coli* whole cell biocatalyst containing the recombinantly produced *Elizabethkingia meningoseptica* Oleate hydratase (Em_OhyA) was used for the biocatalytic hydration of crude WCOs-derived unsaturated free fatty acids for the production of hydroxy fatty acids. Further hydrogenation reaction and methylation of the crude mixture allowed the production of (*R*)− 10-hydroxystearic acid methyl ester that was further purified with a high purity (> 90%), at gram scale. The purified (*R*)− 10-hydroxystearic acid methyl ester was polymerized through a polycondensation reaction to produce the corresponding polyester. This work highlights the potential of waste products to obtain bio-based hydroxy fatty acids and polyesters through a biorefinery approach.

1. Introduction

With the increasing interest in the reuse of waste products for the production of valuable compounds through the creation of the so-called "industrial symbiosis"[1,2], green chemistry and "white biotechnology" are crucial for manufacturing bio-based compounds ranging from fine chemicals, biofuels and polymers using renewable resources following a circular bioeconomy approach [3].

Waste cooking oils (WCOs) are a great example of waste mixture to be used as a raw material for industrial production. WCOs are considered renewable resources as they contain a mixture of triglycerides with a composition that resembles the variety of oils used by local communities. Due to the cooking procedure, triglycerides and their unsaturated fatty acids can undergo hydrolysis and conformational changes [4]. WCOs are preliminarily treated with the so-called "regeneration process" to reduce both the acidity of the mixture, which is given by the presence of free fatty acids (FFA), and to reduce the amount of water, possibly present in the mixture for the production of biofuels [5,6].

Our society strongly relies on polymeric compounds, as they can find applications in different fields ranging from packaging, cosmetics, automotive and medicine. In 2018, only ca. 2% out of the 360 million tons of produced polymers derived from renewable resources [7].

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Bio-based polymers represent an important section of bioeconomy [8]. However, these polymers are currently produced mainly from edible agricultural feedstocks, affecting food chains, if not planned accordingly. Aliphatic polymers such as polyolefins have been considered of utmost importance for many uses. Nevertheless, this class of polymers shows lack in biodegradation, with a negative impact on the environment. The presence of heteroatoms on the aliphatic chain can increase the polymer biodegradability through different pathways, such as oxidation and hydrolysis. Aliphatic polyesters can find different industrial applications while showing high biodegradability in different environments [9]. Polyhydroxyalkanoates (PHA) and polylactic acid (PLA) are the most studied aliphatic polymers due to their biodegradability and their scalable production [10–12]. These two polymeric compounds contain building blocks which are formed by hydroxy acids that are biologically or synthetically polymerized for the production of final polymers, respectively. Polymerization through self-condensation of hydroxy acids has the main advantage to remove the need to control the stoichiometry of the reaction. Moreover, self-condensation of hydroxy acids can enhance the target of specific molecular weight, through a good control of temperature, high performance catalysts, and vacuum. Although self-condensation has many benefits, this procedure remains underexploited when compared to ring-opening polymerization (ROP) and step-growth polymerization [13–16]. To enhance the activity of catalyst and the removal of by-products, high temperatures and high vacuum are usually used. Metallic acid catalysts and lipases have been thoroughly studied for the production of polyesters [17].

Hydroxy acids belong to the broad class of synthons of the difunctional compounds that are widely distributed in nature and they are produced by plants and microorganisms through different metabolic pathways. Their chemical synthesis requires harsh conditions and involve numerous steps of protecting chemistry. For this reasons, either the extraction from biological sources or the biological synthesis is of utmost interest.

Naturally, longer chain difunctional monomers can be found in suberin and cutin as polyesters, in combination with other compounds [18]. Plant triglycerides are other natural sources of modified and unmodified fatty acids. Castor oil triglycerides, for instance, contain 90% of the hydroxy fatty acid ricinoleic acid, which is globally used for the production of industrially relevant polyesters and polyamides, such as poly-12-hydroxystearic acid and Nylon 11, respectively [19]. However, the high ricin content poses an important obstacle for castor bean cultivation [20]. Moreover, fertile soil should be mainly used for the production of food products, without disrupting this value chain.

By using microbial whole cells or isolated enzymes, selective modification of specific chemical structures can be obtained for the production of industrially-relevant compounds [21] by using waste materials and reducing the need of ad-hoc crops for the production of hydroxy acids and synthons [22,23].

Fatty acid hydratases (FAHs) are lyase enzymes that are able to perform the addition of a water molecule into unsaturated fatty acids for the stereo- and regioselective production of hydroxy fatty acids [24]. Oleate hydratase (OhyA, EC. 3.4.2.53) is an FAD-containing enzyme family that is able to specifically add a water molecule into the Δ9 double bond of unsaturated fatty acids [25]. Due to the need of cofactor addition, with a consecutive increase in production costs, *Escherichia coli* whole-cell biocatalyst expressing OhyA from different organisms has been widely studied. The whole-cell system containing the OhyA from *Stenotrophomonas maltophila* was shown to have a threefold higher conversion rate than the wild-type strain [26]. Another member of the OhyA is the *Elizabethkingia meningoseptica* OhyA (Em_OhyA) (PDB ID: 4UIR) [27], which has been thoroughly studied for the biocatalytic transformation of different fatty acids into hydroxy fatty acids and fatty alcohols from natural and non-natural substrates [28–33].

In this study, we report the selective cofactor-independent enzymatic transformation of WCO-derived free fatty acids into (*R*)− 10-hydroxystearic acid (10-HSA) employing as whole-cell biocatalyst a recombinant *Escherichia coli* expressing the Em_OhyA and its chemical conversion into methyl ester 10-HSA (10-HSAME) and further polycondensation for the synthesis of the polymer poly-10-hydroxystearic acid.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this work were of analytical grade. Regenerated WCOs was kindly provided from Greenoil s.r.l. (Italy). Buffer components, IPTG, MSTFA, Kanamycin sulphate, Ethyl acetate (EtOAc), ethanol (EtOH), H_2SO_4 and organic solvents were purchased at the higher commercial quality and used without further purification (Sigma-Aldrich, USA).

2.2. Preparation of the whole-cell biocatalyst

Oleate hydratase from *Elizabethkingia meningoseptica* was produced through recombinant expression in *E. coli* BL21(DE3) cells carrying the plasmid pET28a(+)_*emohyA*, as previously reported [34]. Briefly, *E. coli* cells were grown overnight in Lysogeny Broth (LB) medium supplemented with 40 μg mL^{-1} kanamycin at 37 °C. The precultures were used to inoculate larger cultures grown at 37 °C and 200 rpm. The Em_OhyA expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} of 0.7–0.8 for 20 h at 20 °C and 150 rpm. Cultures (500 mL in 2-L unbaffled flasks) were harvested by centrifugation at 4000 rpm at 4 °C for 20 min. The harvested cells were washed with reaction buffer (50 mM NaCitrate pH 6, 50 mL) and centrifuged again at 4000 rpm at 4 °C for 20 min and stored at − 20 °C until further analysis.

2.3. Protein analysis

E. coli cell lysate was prepared using CelLytic B (Sigma-Aldrich, USA) following the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysate was performed according to Laemmli using 4% (v v^{-1}) stacking gels and 12% (v v^{-1}) separating gels and run at 150 V. Proteins were stained with Coomassie method.

2.4. Preparation of FAME from WCOs and GC-MS analysis

The fatty acid composition of WCOs was analyzed by GC-MS by converting them into fatty acid methyl esters (FAME). Briefly, a solution of 6.75% (w v^{-1}) WCOs in hexane was mixed with the internal standard (IS) heptadecanoic acid (final concentration 0.018 mM). Hexane was evaporated under N_2 flow and 1 mL methanol with 0.5% (v v^{-1}) H_2SO_4 was added and incubated at 100 °C for 2 h. After cooling to room temperature, 1 mL 0.9% (w v^{-1}) NaCl was added to the reaction that was then extracted with 3 mL hexane. An aliquot of 1 mL of organic phase was then transferred into a 2-mL vial and dried under N_2. The mixture was then resuspended into 100 μL hexane and transferred into a GC vial for analysis.

FAME separation and analysis was carried out by GC-MS using a Waters Micromass Quattro Micro GC triple quadrupole mass spectrometer (Waters, USA), coupled to an Agilent 6890 N gas chromatograph (Agilent Technologies, USA). Samples (1 μL) were injected in splitless mode into the gas chromatograph and then separated with a fused silica DB-5MS + DG capillary column (30 m x 0.25 mm×0.25 μm, Agilent Technologies, USA). High purity helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injector temperature was set to 250 °C. The initial column temperature was kept at 50 °C for 1 min; first temperature ramp 50 °C min^{-1} to 170 °C; second temperature ramp at 3 °C min^{-1} to 260 °C; final temperature ramp at 50 °C min^{-1} to 290 °C and kept at the final temperature for 5 min. The interphase and ion source

temperatures were 280 and 240 °C, respectively. FAME were detected with electron ionization (70 eV) in scan mode (50–600 *m/z*) and selected ion monitoring mode at 55, 67, 74, and 79 *m/z* (for quantitative analysis). Data acquisition, and processing were performed using the Masslynx 4.0 (Waters, USA) and AMDIS 2.7 software, based on the masses, fragmentation patterns, and retention times, in comparison with Supelco 37 Component FAME Mix as reference standards, NIST 02 and Golm databases. Absolute amounts of FAME were quantified by integration of the peaks using the Openchrom software and normalizing data by the area of the peak of the internal standard.

2.5. Preparation of Free fatty acid mixture

For triglyceride hydrolysis, 15 g of WCOs (**1**) were reacted with 30 mL 20% KOH in a 7:1 water:ethanol solution for 1 h under stirring at 80 °C with reflux. The ethanol contained in the optimized reaction is known to improve the solubility of the potassium salts of released fatty acids. The reaction was cooled at room temperature and 15 mL of 20% H_2SO_4 solution were added. The reaction was transferred into a separating funnel and the product mixture was extracted with EtOAc. The organic phase was collected and dried over anhydrous sodium sulphate and concentrated to obtain a mixture containing free fatty acids (FFA). Obtained crude samples were analyzed by ^{1}H NMR to confirm the removal of glycerol (Fig. 2B) with a total product amount of 14.7 g.

2.6. Enzyme activity assay and biocatalytic hydration reaction

Hydratase activity assay of *E. coli* whole cell biocatalyst was performed as previously described with little modifications [27]. Briefly, 10 mg WCW of *E. coli* whole cell biocatalyst were added into 1 mL of 50 mM sodium citrate buffer pH 6 containing 2 mM oleic acid and 2% ethanol. The reaction was then extracted with 500 μL EtOAc and analyzed by GC-MS as previously described.

Biocatalytic transformation was carried out in 2-L unbaffled flasks containing 500 mL 50 mM sodium citrate pH 6 with 10 g L^{-1} WCW whole-cell biocatalyst containing the recombinantly produced Em_OhyA and 0.4 mM Tween 20. The reaction was started by the addition of 1% *v* v^{-1} FFA mixture. The reaction was incubated at 37 °C and 200 rpm for 24 h. The reaction was then mixed with 200 mL ethyl acetate and centrifuged at 4000 rpm for 20 min to separate the organic phase containing free fatty acids, such as **2a-3a**, and (*R*)− 10-hydroxy free fatty acids **2b** and **3b**. The organic mixture was collected and dried over anhydrous sodium sulphate and then dried to obtain a mixture containing (*R*)− 10-hydroxy fatty acids. Aliquots of the obtained samples were dissolved in 30 μL pyridine (Sigma-Aldrich, USA) and 30 μL MSTFA (Sigma-Aldrich, USA) and incubated at 80 °C for 40 min. Solutions were transferred into GC vials and analyzed by GC-MS using a Waters Micromass Quattro Micro GC triple quadrupole mass spectrometer, directly coupled to an Agilent 6890 N gas chromatograph. Samples (1 μL) were injected in splitless mode into the gas chromatograph and then separated with a fused silica DB-5MS + DG capillary column (30 m x 0.25 mm×0.25 μm, Agilent Technologies, USA). High purity helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injector temperature was set to 240 °C. The initial column temperature was kept at 120 °C for 1 min; first temperature ramp 10 °C min^{-1} to 265 °C; second temperature ramp at 25 °C min^{-1} to 290 °C kept for 1 min. The interphase and ion source temperatures were 280 and 240 °C, respectively. Data were acquired in Full Scan mode in the range of 80–550 *m/z*. The solvent was delayed for 8 min. Obtained crude samples (3.9 g) were analyzed by ^{1}H NMR, hydration percentage of fatty acids, calculated by comparing the integral of the signal at 3.60 ppm (C*H*-OH) with that of 0.88 ppm (CH_3) was 80% (Fig. 2 C).

2.7. Hydrogenation reaction

In order to reduce C-C double bonds that were present in the product of the biocatalytic hydration reaction, which contained products **2a**, **3a**, and **3b**, hydrogenation reaction was carried out with 20% *w* v^{-1} (*R*)− 10-hydroxy free fatty acids-containing mixture in ethanol, containing 10% *w* w^{-1} platinum oxide (Adam's catalyst) (Sigma-Aldrich, USA). The reaction was carried out in a stirred pressure autoclave at 1 bar hydrogen. The autoclave was sealed and flushed with hydrogen gas three times, then heated to 50 °C for 3 h under stirring. After the reaction mixture was cooled, it was filtered through Celite to remove the catalyst. The column was washed with ethyl acetate for the complete elution of the product. The organic mixture was first dried over anhydrous sodium sulphate and then concentrated to obtain a mixture of saturated products. Obtained crude samples (ca. 5.1 g) were analyzed by ^{1}H NMR to confirm the complete hydrogenation of C-C double bonds (Fig. 2D).

2.8. Synthesis of (R)− 10-hydroxystearic acid methyl ester

The mixture resulting from the hydrogenation reaction was then used to produce fatty acid methyl esters, such as **4c**, and (*R*)− 10-hydroxy fatty acid methyl esters, such as **2c**, through esterification reaction using 8% *w* v^{-1} of the hydrogenation reaction product in methanol containing a final concentration of 0.5 M HCl. The reaction was performed at 80 °C for 24 h under stirring. The reaction was then evaporated through rotavapor to remove the excess of methanol. The resulting methyl esters were then extracted by EtOAc and separated on a separating funnel. The organic phase was then dried over sodium sulphate and concentrated to have a mixture of methyl esters. Obtained crude samples (ca. 4.1 g) were analyzed by ^{1}H NMR to confirm the complete esterification of carboxylic acids (Fig. 2E).

The (*R*)− 10-hydroxystearic acid methyl ester (10-HSAME, **2c**) was then purified by silica gel column chromatography using as eluent a mixture of hexane:EtOAc:dichloromethane (8:1:2). The elution was tested on TLC by eluting with the eluent and stained with ceric ammonium molybdate. The fractions containing the compound **2c** were then collected and pooled. The solution was then concentrated to obtain a white solid powder of 1.2 g.

^{1}H NMR ($CDCl_3$, 600 MHz) δ 0.91 (3 H, t, $J = 6.3$ Hz, CH_3), 1.19–1.35 (22 H, m, 11 CH_2), 1.39–1.48 (4 H, m), 1.60–1.67 (2 H, m), 2.33 (2 H, t, $J = 7.5$ Hz, -CH_2-CO_2Me), 3.61 (1 H, br s, C*H*-OH), 3.69 (3 H, s, -CO_2CH_3). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 14.1, 22.6, 24.9, 25.5_8, 25.6_3, 29.0_8, 29.1_5, 29.2, 29.4, 29.5_7, 29.5_9, 29.7, 31.8, 34.1, 37.4, 37.5, 51.4, 72.0, 174.3. HRMS (ESI) *m/z* calculated for $[C_{19}H_{38}O_3 + Na]^+$: 337.2713; found: 337.2723.

2.9. NMR-analysis and determination of enantiomeric excess (e.e.) of (R)− 10-hydroxystearic acid methyl ester

All experiments were carried in oven-dried glassware. ^{1}H NMR spectra were recorded on a Bruker Advance spectrometer at 600 MHz. ^{19}F NMR spectra were recorded on a Bruker 500 spectrometer at 500 MHz^{-1}.

The esterification of **2c** was carried out with racemic α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher's acid chloride) and the optically pure (*R*)-(−)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride. The reaction was performed as previously described [35]. Briefly, in a 4-mL glass vial, **2c** (20 mg, 64 μmol) and dry pyridine (16 μL, 20 μmol) were dissolved in 1 mL anhydrous CH_2Cl_2. Racemic or optically pure Mosher's acid chloride (23 μL, 120 μmol) was added to the mixture. The reaction was capped and stirred at room temperature and monitored by TLC with a mobile phase composed of hexane and ethyl acetate in a ratio 4:1. After the reaction was complete (2–4 h), the formed ester was extracted in a separating funnel with water and ethyl ether. The organic fraction was collected and the aqueous fraction was extracted two more times. The organic fractions were collected and pooled. The pooled organic fractions were dried and resuspended on $CDCl_3$ for H^1 and ^{19}F NMR analyses. Methyl (*R*) and (*S*)− 10-[(3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl)oxy]octadecenoate ^{19}F NMR

($CDCl_3$, 282 MHz) δ − 71.68 (s), − 71.72 (s); (*R*)− 10-[(3,3,3-trifluoro-2-methoxy-2-phenylpropanoyl)oxy]octadecenoate ^{19}F NMR ($CDCl_3$, 282 MHz) δ − 71.69 (s).

2.10. Polymerization reaction

All glassware was dried at 150 °C for 24 h and additionally dried with a heating gun at 600 °C under reduced pressure. The polycondensation reaction was performed following a three-stage melt polycondensation method at 180 °C as previously described with little modification [36]. Briefly, the ester **2c** (1.91 mmol, 600 mg) and the catalyst dibutyl tin oxide (0.011 mmol, 2.85 mg) were added to a three-neck round--bottomed flask and mixed by magnetic stirring and three vacuum-argon cycles were performed. The system was then sparged with argon for 15 min. The system was heated to the set temperature, while continuously flushing with argon. The vessel containing the reaction was equipped with a magnetic stirrer and septa. Reactions were incubated for a total time of 24 h.

2.11. Polymer analysis

^1H (400 MHz) NMR spectra were recorded with a Bruker Avance AM 400 spectrometer (USA). The signal of the deuterated solvent, $CDCl_3$ (δ = 7.26 ppm), was used as reference. For SEC, a TOSOH EcoSEC HLC-8320GPC system (Japan), equipped with an EcoSEC RI detector and three PSS PFG 5 mm columns (microguard, 100 Å, and 300 Å; USA), was used. Poly(ethylene glycol) (PEG) standards were used for calibration and toluene was used as an internal standard. DSC was performed by using a Mettler Toledo DSC 820 module. Samples (5–10 mg) were prepared in 100 mL aluminum crucibles. The samples were subjected to heating from 30° to 170°C (or 160 °C), then cooled to − 60 °C (or −80 °C), and then heated again to 170 °C (or 160 °C) at a heating/cooling rate of 10 °C min^{-1} under a flow of nitrogen (50 mL min^{-1}). The data obtained from the second heating step was used for analyses. For TGA, a Mettler Toledo TGA/DSC1 instrument was used. Samples (5–7 mg) were prepared in 70 mL alumina crucibles and heated from 40° to 700°C at a heating rate of 10 °C min^{-1} under a flow of nitrogen (50 mL min^{-1}).

3. Results and discussion

3.1. Production of reagents for biocatalysis reaction

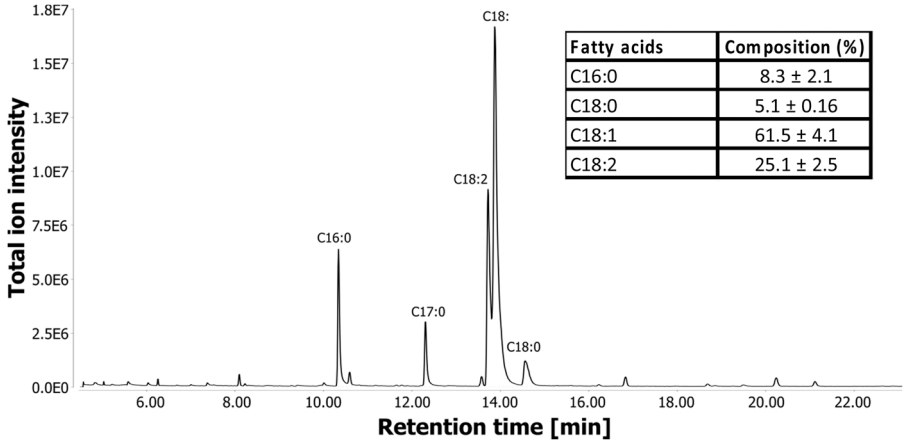
The starting material of our study was WCOs collected in the Italian region of Apulia and preliminarily regenerated. The composition of regenerated WCOs was analyzed by GC-MS after producing fatty acid methyl esters (FAMEs) and reported in Fig. 1. The studied WCOs had a liquid appearance due to the high concentration of unsaturated fatty acids that were also identified by GC-MS analysis. The GC-MS profile (Fig. 1) showed that triglycerides (**1**) in the regenerated WCOs contained mainly C18 unsaturated fatty acids. In particular, oleic acid (C18:1) (**2a**, Fig. 2A) was the unsaturated fatty acid with the highest concentration of 61.5%, while linoleic acid (C18:2) (**3a**, Fig. 2A)) was present at approximately 25.1%. On the other hand, saturated fatty acids were present at a concentration of approximately 13.4%, with mainly palmitic acid (C16:0) and stearic acid (C18:0) (**4a,** Fig. 2A) (Fig. 1).

^1H NMR spectrometry analysis showed a similar composition of triglycerides present in sunflower oil, especially in the alkene protons (δ = 5.3 ppm) and the methylene protons (δ = 3.2 ppm), showing the presence of single and multiple C═C double bonds (Fig. 2 A) [37,38].

In order to prepare the optimized substrate for the biocatalytic hydration step, triglycerides were hydrolyzed using a saponification reaction with an alcoholic solution of KOH for the release of FFA. Fatty acid potassium salts (soap 59salts) were acidified to pH 1 with H_2SO_4. FFA from triglyceride hydrolysis were separated from the reaction by liquid-liquid extraction, with a calculated product yield of 95% (Fig. 2B). The degree of completion of the hydrolysis reaction was 100%, as calculated by ^1H NMR spectrometry analysis (Fig. 2B). Although, different chemical [39,40] and enzymatic [28,41] methods can lead to the production of FFA, such as the use of lipases, which are thoroughly studied for the production of one-pot multienzyme transformation reactions [28,42], saponification reaction followed by acidification was the method of choice for its rapidity and the use of readily available compounds. It was previously shown that the carboxyl group of FFA is highly important for the binding of the substrate in the active site of the enzyme Em_OhyA for the hydration of C═C double bond [29].

3.2. Biocatalytic production of (R)− 10-Hydroxy fatty acids

The biocatalytic hydration of the mixture containing *cis*-unsaturated fatty acids at position Δ9, such as oleic acid (**2a**) and linoleic acid (**3a**), was performed with 20 U (2 g dry cell weight L^{-1}) of *E. coli* whole-cell biocatalyst containing the recombinantly produced *Elizabethkingia meningoseptica* Oleate hydratase (Em_OhyA). The enzyme was recombinantly expressed and the SDS-PAGE analysis of the cell-free extract showed a band at approximately 73 kDa (Fig. S1). After a 24-h reaction containing 1% w/v of the whole-cell biocatalyst, the chemical structure of the compounds present in the extracted reaction mixture was studied by ^1H NMR spectroscopy (Fig. 2 C) and GC-MS (Fig. 3) analyses, which confirmed the production of (*R*)− 10-hydroxy fatty acids **2b** and **3b**. ^1H



Fatty acids	Composition (%)
C16:0	8.3 ± 2.1
C18:0	5.1 ± 0.16
C18:1	61.5 ± 4.1
C18:2	25.1 ± 2.5

Fig. 1. GC-MS analysis of fatty acid methyl esters (FAME) present in WCOs. Fatty acid composition in % of Waste Cooking Oils analyzed by GC-MS using heptadecanoic acid (C17:0) as internal standard. Methyl esters of fatty acids are as follow: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Data are mean values of five different measurements with standard error.

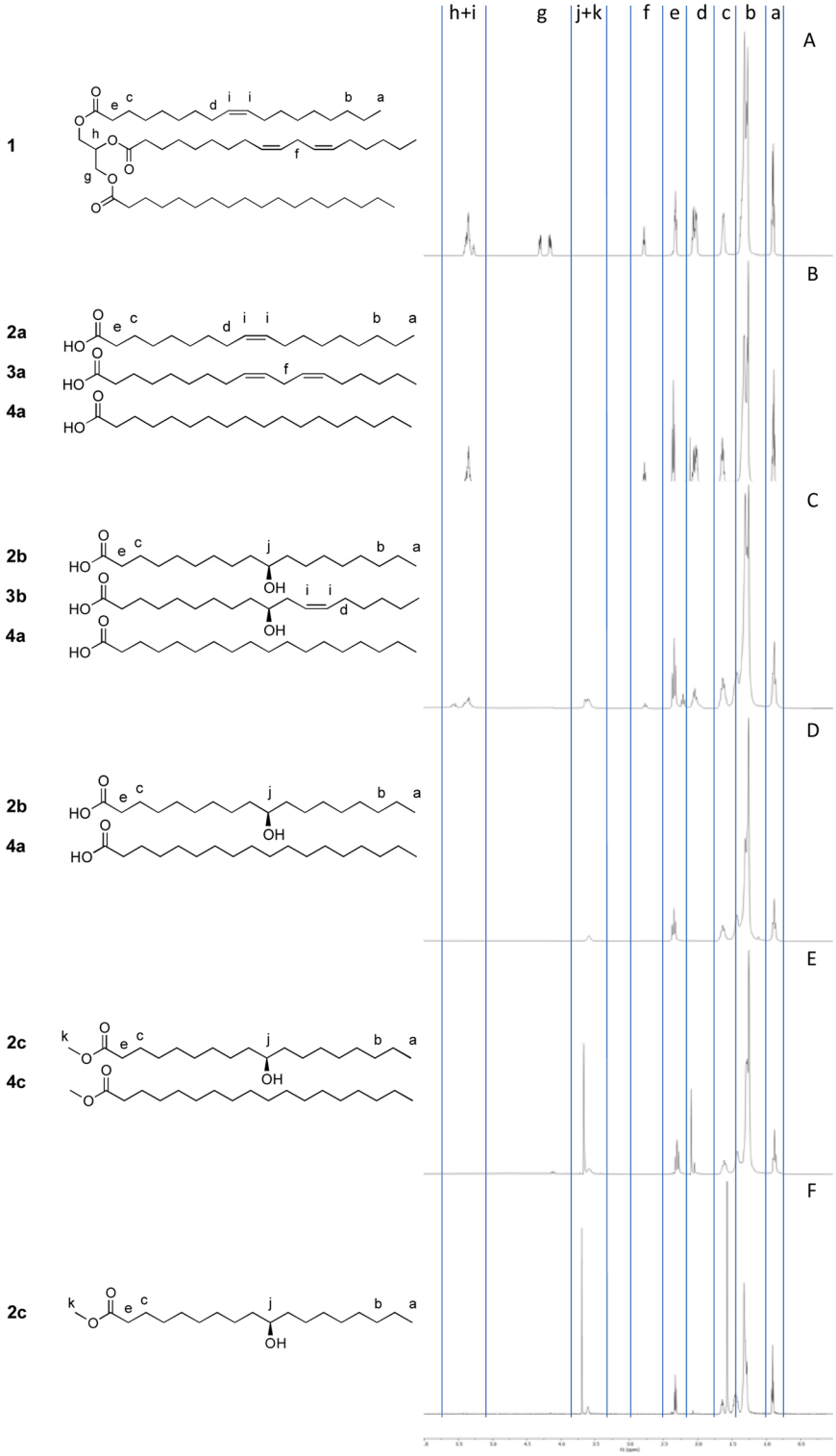


Fig. 2. ^{1}H NMR spectra of reagents and products of the chemo-enzymatic transformation of WCOs into (*R*)− 10-hydroxy fatty acid methyl ester. (**A**) Triglyceride (**1**); (**B**) mixture containing FFA oleic acid (**2a**), linoleic acid (**3a**) and saturated fatty acids represented by stearic acid (**4a**); (**C**) product mixture from the biocatalytic hydration reaction containing (*R*)− 10-hydroxystearic acid (**2b**) and (*R*)− 10-hydroxyoctadec-12-enoic acid (**3b**) and saturated fatty acids represented by **4a**; (**D**) product mixture from hydration reaction containing **2b** and saturated fatty acids, such as **4a**; (**E**) product mixture from esterification reaction containing the (*R*)− 10-hydroxystearic acid methyl ester (**2c**) and saturated fatty acid methyl esters represented by stearic acid methyl ester (**4c**); (**F**) purified **2c**.

NMR spectrum of the mixture showed the biocatalytic conversion with an overall increase of 80% on newly formed hydroxyl group by the analysis of hydroxyl proton ($\delta = 3.6$ ppm) (Fig. 2 C). The analysis of the mixture revealed a smaller amount of double bonds both for mono and polyunsaturated fatty acids compared to the reagents as shown by the reduction of the double bond protons ($\delta = 5.3$ ppm) and by the reduction of the methylene protons ($\delta = 3.2$ ppm), respectively (Fig. 2 C). The GC-MS analysis of the mixture showed that the hydration reaction was specifically active on the Δ9 double bond of unsaturated fatty acids by the presence of the characteristic ion 331 *m/z*, as previously reported [29]. Moreover, the ion 215 *m/z* for the peak at 14.89 min showed the presence of **2b,** whereas the ion 213 *m/z* for the peak at 14.78 min showed the presence of **3b** (Fig. 3).

Whole-cell biocatalysts can play an important role on enzymatic conversions. In fact, whole-cell biocatalysts can act as cofactor reservoir reducing the need for external cofactors, thus reducing overall production costs [43,44]. In this case, the enzyme Em_OhyA was previously shown to require the cofactor FAD, due to its dual role for the correct

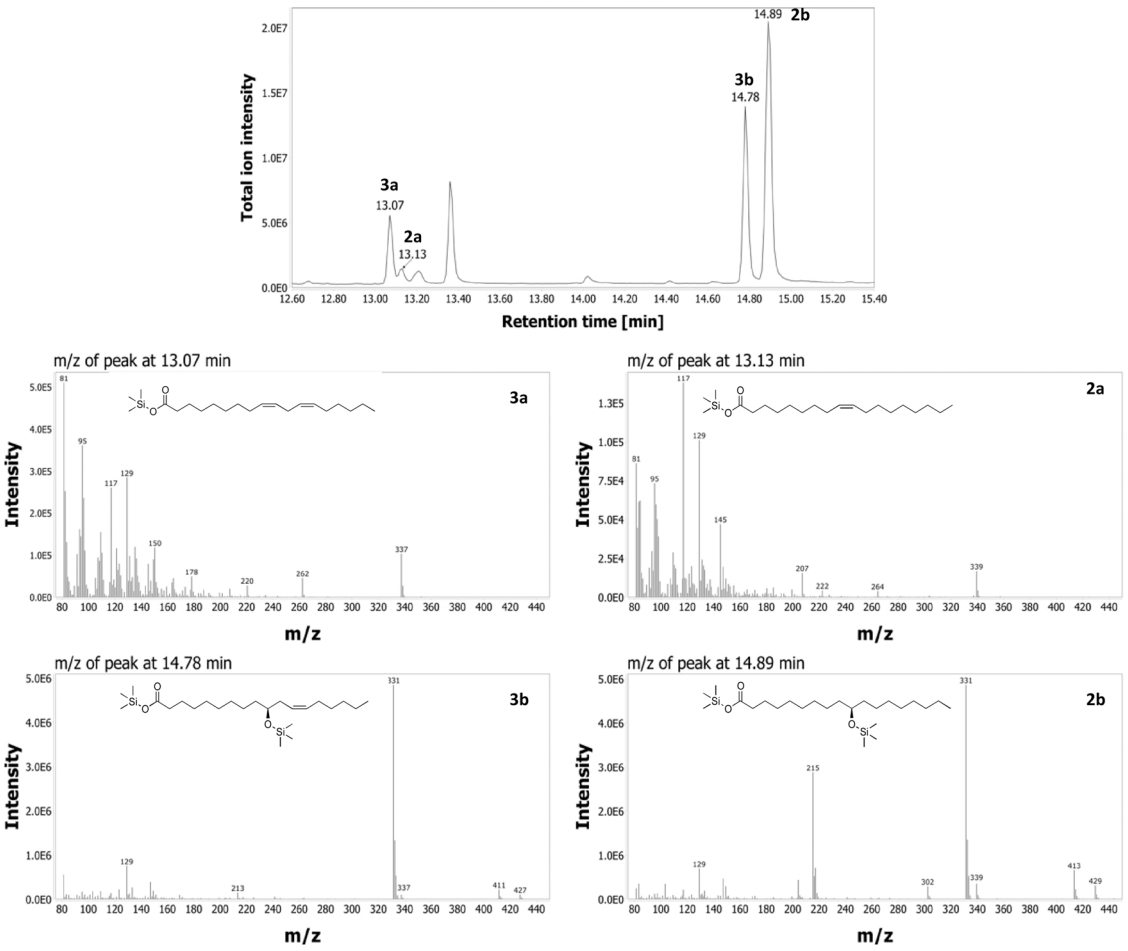


Fig. 3. Bioconversion of **2a** and **3a** derived from **1** of WCOs catalyzed by *E. coli* whole cells overexpressing Em_OhyA. GC-MS chromatogram showing retention time of the TMS-derivatives of the components of the reaction mixture (*top*), linoleic acid (**3a**): 13.07 min; oleic acid (**2a**): 13.13 min; (*R*)− 10-hydroxyoctadec-12-enoic acid (**3b**): 14.78 min; (*R*)− 10-hydroxyoctadecanoic acid (**2b**): 14.89 min. Unequivocal identification of the TMS-derivatives of free fatty acids and their hydroxylated homologues was supported by analysis of mass spectra (*bottom*).

organization of the active site and the stabilization of the partial positive charge in the transition state [27]. Indeed, it is shown that the whole-cell biocatalyst containing the recombinant enzyme Em_OhyA is able to perform the conversion of unsaturated fatty acids containing a C═C double bond at position Δ9 into (*R*)− 10-hydroxy fatty acids [29,34,45]. Control experiments confirmed that *E. coli* whole cells containing an empty vector at the studied conditions was not able to perform the biocatalytic hydration of *cis*-unsaturated fatty acids into (*R*)− 10-hydroxy fatty acids [29], demonstrating the Em_OhyA-dependent hydration reaction (Fig. S2).

However, a disadvantage of whole-cell biocatalysts can reside on the hindrance in the use of hydrophobic substrates, due to the reduced cell membrane mass transfer. In order to solve this problem, it was found that the use of a detergent was necessary to improve the permeability of the membrane and improve the mass transfer of substrates and products, as previously demonstrated for other biocatalytic reactions [46]. The detergent of choice must not contain moieties that could also be present in the substrate. For this reason, Tween 20 was chosen for this activity due to the presence of esterified lauric acid instead of Tween 80 containing esterified oleic acid. Other biological approaches were previously reported to reduce the low permeability of FFA to the cell membrane by the overexpression of transporters, such as the fatty acid transporter FadL in *E. coli* [47], or by the formation of caveolae-like structure [48], which led to the increase of reaction rate for biocatalytic reactions of hydrophobic substrates.

3.3. Chemical hydrogenation of hydroxy fatty acid mixture

The extracted product mixture was then reacted in an autoclave at 1 bar with H_2 in the presence of PtO_2 to produce saturated (*R*)− 10-hydroxy fatty acids and saturated fatty acids, where the residual C═C double bonds were reacted with H_2 in the presence of the catalyst PtO_2. The reaction was complete after 3 h, as identified by ^{1}H NMR. The catalyst was removed by celite and the product mixture was extracted. The extracted product had a yield of 90%. ^{1}H NMR spectroscopy analysis was used to confirm the complete reduction of double bonds into single bonds (Fig. 2D), the signals for double bond protons ($\delta = 5.3$ ppm) and methylene protons adjacent to two double bonds ($\delta = 3.2$ ppm) disappeared. The hydrogenation reaction led to the transformation of **3b** and **2–3a** into **2b** and **4a**, respectively. This reaction increased the yield of the product 10-HSA (**2b**) and saturated fatty acids, such as stearic acid (**4a**), in the mixture.

3.4. WCOs-based polyester by polycondensation

To synthesize bio-based polyesters, the mixture containing the (*R*)− 10-hydroxystearic acid **2b** was subjected to esterification to obtain methyl esters. Methyl esters were produced for two reasons. On the one hand, methyl esters are better separated on column chromatography in the purification step. On the other hand, they are known to be more reactive in polycondensation reactions, through transesterification [49]. The mixture was then converted into fatty acid methyl esters (FAME)

and hydroxy fatty acid methyl esters (H-FAME), the latter was represented by 10-HSAME (**2c**). The mixture was then extracted with ethyl acetate. The product formation had a yield of 90%. ^{1}H NMR spectroscopy analysis showed a complete methylation of the components of the mixture with the appearance of the methyl ester protons ($\delta = 3.7$ ppm) (Fig. 2E). Column chromatography was carried out to purify the monomer **2c** in high purity for the polycondensation reaction. After evaporation of the solvent, the product was collected with a yield of 30% and a purity of 95%. The remaining fractions were collected and identified as mixture of fatty acid methyl esters (FAME), which are thoroughly studied for the production of biofuels [6]. However, further functionalization with the aid of cytochrome P450 could enhance the production of different multifunctional compounds, such as ω-hydroxy saturated fatty acids, which could find further applications also for polyester production [50]. The low product yield during column chromatography was due to the presence of **2c** in other fractions which were discarded to allow high purity of the product for further polymerization. ^{1}H NMR spectrometry analysis showed the high purity of the methyl ester ($\delta = 3.6$ ppm) (Fig. 2F). Purified products **2c** and the saponified product **2b** were analyzed by ^{1}H NMR and ^{13}C NMR (Fig. S3 – S6).

As previously reported for the hydroxy fatty acid **2b** [27], the product **2c** was obtained with an enantiomeric excess (e.e.) of ≥ 98%, as confirmed by ^{19}F NMR spectroscopy analyses of Mosher esters (Fig. S7). The absolute configuration was determined by its optical rotation. The absolute configuration of **2c** was ascertained by negative sign of optical rotation [29].

Having control over the stereochemistry of the monomers allows to specifically tailor the physical properties of the polymer. This is due to the resulting differences in tacticity, thus the stereospecific arrangement of the "side chains" which has a direct impact on material properties, such as the degree of crystallinity, solubility, thermal properties etc. Having a random orientation of side chains usually results in a less stiff material with a lower degree of crystallization and a lower glass transition temperatures compared to the syndio and isotactic chains.

To showcase the possibility to polymerize the product 10-HSA, the purified monomer **2c** was homopolymerized to form the polymer poly-10-hydroxystearic acid (Poly10HSA). The obtained polymer had an amber color and was easily solubilized in dichloromethane (DCM), which is one of the most commonly industrially used solvent for solution processing of polymers. ^{1}H NMR spectroscopy analysis was used to investigate the chemical structure and to confirm the synthesis of the expected polyester.

^{1}H NMR spectroscopy analysis showed that the polymerization reaction had a 90% conversion as demonstrated by the reduction of hydroxyl group proton and the formation of ester bond of the Poly10HSA ($\delta = 4.7$ ppm) (Fig. 4A).

The molecular weight (M_n) was measured by size exclusion chromatography (SEC) giving a value of 3118 g mol^{-1} which corresponds to a polymer of 11 units (Fig. S8). However, the polydispersity index calculated by SEC showed a high polydispersity of 24. Low molecular weight could be due to the unoptimized polymerization protocol and to the small scale of the polymer synthesis, which influences the presence of impurities. These impurities can affect the stoichiometric balance and cause an upper limit to the degree of polymerization which causes an increase in the polydispersity index. Additionally, mass transfer limitations due to insufficient mixing or high viscosities can cause further broadening of molecular weight distribution. To investigate the physiochemical properties of the produced polymer differential scanning calorimetry (DSC) were performed on the produced polyester. DSC analysis showed that the polymer had a melting temperature (T_m) of − 36 °C (Fig. 4B). A glass transition temperature (T_g) peak was not visible probably due to the temperature limit of the instrument. This has been previously shown for aliphatic polyesters where the T_g could be lower than − 60 °C [51]. As previously shown, low molecular weight polymers could be used in different applications where high adhesion, low viscosity or high solubility is needed, such as dispersants and thickeners, and also used as prepolymers for the preparation of high molecular weight polymers for different applications[55]. Moreover, broad molecular weight distribution are generally appreciated in systems that need a high elongation, high yield strength, low toughness, low brittleness/hardness and a good adhesion.

4. Conclusions

In this paper, we showed the ability of *E. coli* whole-cell biocatalyst containing the recombinant enzyme Em_OhyA to perform the regio- and stereoselective hydration of unsaturated FFA derived from regenerated WCOs into (*R*)− 10-hydroxy fatty acids. The whole-cell biocatalyst was able to produce a product mixture with 80% content of newly formed hydroxyl group. The product mixture containing (*R*)− 10-hydroxy fatty acids was produced on a preparative scale with further hydrogenation and esterification. The esterified product (*R*)− 10-hydroxystearic acid methyl ester was purified by column chromatography with a 30% product yield. The product was further polymerized through polycondensation to prepare the polyester poly-10-hydroxystearic acid with a melting temperature T_m of − 36 °C.

The use of waste products as raw materials for the production of valuable compounds can contribute to boost the ecological transition in the polymer industry and to create sustainable second generation biorefinery. Further improvements in process engineering with lipase aided release of FFA in a multienzyme biocatalytic reaction, the optimization of the polymerization reaction and downstream processing can increase product yields can reduce the requirement of organic solvents for purification of intermediates, improving the overall process sustainability.

Ethical Statement

This article does not contain any studies with human participants or animals performed by any of the authors.

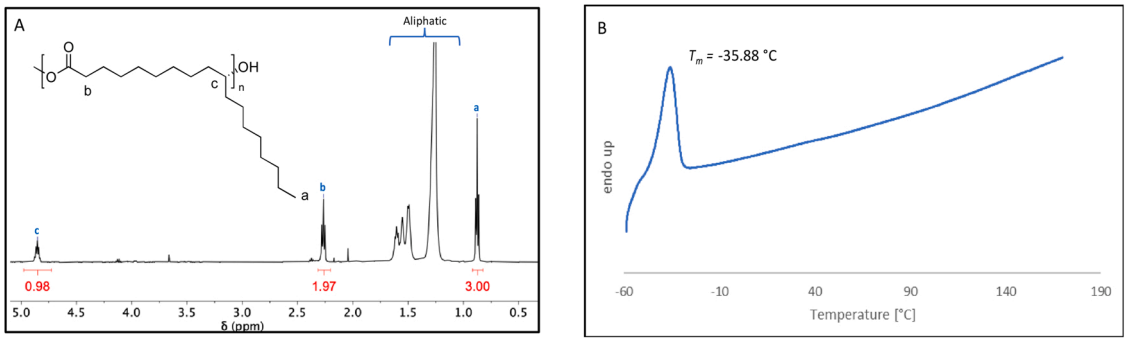


Fig. 4. Overview of the material properties of the synthesized Poly10HSA. (**A**) ^{1}H NMR spectrum of polyester Poly10HSA. (**B**) Differential scanning calorimetry thermogram of the polyester Poly10HSA.

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CRediT authorship contribution statement

A. B. conceived, designed and supervised the project together with I. P., A. S. and P.-O. S. performed the polymer analysis, V. C., P. V., F. P. performed NMR analysis and catalytic experiments with metal catalysts, R. G. and G. A. performed data analysis and GC-MS analysis. B. H. and S. C. participated in the discussion of reaction designs and results. The manuscript was drafted by A. B. and A. S. with inputs from all authors.

Author agreement

All authors have seen and approved the final manuscript before its submission. We declare that this manuscript is original, has not been published previously and is not currently under consideration for publication elsewhere.

Declaration of Competing Interest

All contributing authors declare that they have no conflict of interest.

Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.enzmictec.2022.110164.

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