A Biocatalytic Cascade Reaction to Access a Valuable Long-Chain ω-Hydroxy Fatty Acid

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Environmentally-friendly processes for the manufacturing of valuable industrial compounds like ω-hydroxy fatty acids (ω-OHFAs) are highly desirable. Herein, we present such an approach by establishing a two-step enzymatic cascade reaction for the production of 2,15,16-trihydroxy hexadecanoic acid (THA). Starting with the easily accessible natural compound ustilagic acid (UA) that is secreted by the corn smut fungus *Ustilago maydis*, the recombinantly expressed esterase BS2 from *Bacillus subtilis* and the commercial β-glucosidase from almonds were applied yielding 86% product. Both hydrolases do not require expensive cofactors, making the process economically attractive. Additionally, no harmful solvents are required, so that the product THA can be labelled natural to be used in food and cosmetic products.

Introduction

As a result of increasing global environmental awareness, the demand for sustainable resources in the food, cosmetic, pharmaceutical and chemical industry is rising. This makes it inevitable to exploit new sources for natural active compounds and make them accessible through environmentally-friendly processes. With fats and oils being the most important feedstock,[1,2] especially hydroxy fatty acids (OHFAs), which are challenging and expensive to synthesize chemically, are in the center of attention as they offer a wide field of application.[3]

Compared to non-hydroxylated fatty acids, they are characterized by a higher stability, viscosity and a higher miscibility with solvents.[4,5] Because of these positive features, they are attractive precursors in industrial processes being utilized in the production of flavorings,[6] emulsifiers,[7,8] lubricants,[9,10] adhesives,[11,12] cosmetics,[13] and pharmaceuticals.[14] For example, OHFAs can be found in the medical area, making use of their antibacterial,[15,16] antimycotic[17–19] and anticancerogenic[20] properties.

The common approach for the enzymatic production of ω-OHFAs relies on the hydroxylation of simple saturated and unsaturated fatty acids. By utilizing a combination of different fatty acid-hydroxylation enzymes, like P450s or hydratases, not only mono- but also di- and trihydroxy fatty acids could be produced, provided that enzymes with the desired selectivity are available.[3] For instance, starting from long-chain unsaturated fatty acids, hydrating and oxidative enzymes were applied in a complex biocatalytic cascade in combination with an esterase to yield C_9–C_{13} ω-OHFAs.[21] Moreover, the production of C_{12}-C_{18} ω-OHFAs by engineered *C. tropicalis* – being classified as a risk group 2 organism, restricting the usability in the industry – starting from vegetable oil fatty acids was described,[22] as well as the microbial conversion of glucose to C_{12} and C_{14} ω-OHFAs.[23]

All these approaches have in common that not only the availability of the required starting material from renewable sources is crucial but also that a variety of perfectly coordinated enzymes with high activities and regioselectivities is necessary to yield ω-OHFAs that, e.g., are hydroxylated at various positions. Other constraints are the need of expensive cofactors and cofactor regeneration systems, as well as elaborate purification procedures due to by-product formation, especially in the microbial approaches that, additionally, are limited in the acceptance of substrates and intermediates. However, the isolation of complex ω-OHFAs from natural sources like animals, plants or microorganisms – offering a great diversity of glycolipids, triacylglycerols, or waxes with OHFAs as major components[24] – represents an easy alternative with reduced effort.

Such a naturally occurring source for an interesting ω-OHFA is ustilagic acid (UA). This natural substance is a mixture of acetylated cellobiose lipids that is not only esterified with a medium-chain hydroxy fatty acid (C_6 or C_8)[25] but is also *O*-glycosidically linked to a long-chain ω-hydroxy fatty acid,[26] predominantly 2,15,16-trihydroxy hexadecanoic acid (THA)[27] (Scheme 1).

This mixture of cellobiose lipids is secreted by the Basidiomycota *Pseudozyma fusiformata*,[28] *Pseudozyma gramnicola*[29] and *Ustilago maydis*.[30] The latter is a corn smut

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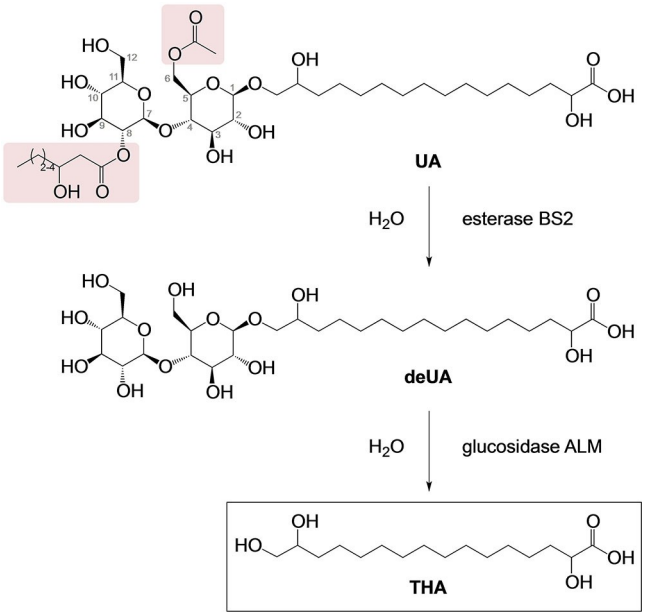

Scheme 1. Schematic depiction of the two-step biocatalytic cascade reaction to yield 2,15,16-trihydroxy hexadecanoic acid (THA). After BS2-catalyzed deacylation of the ustilagic acid (UA) at positions 6 and 8 (highlighted in red) to obtain the deacylated ustilagic acid (deUA), the THA is released by use of the glucosidase ALM.

fungus that produces the UA under nitrogen deficiency and offers the advantage of being effortlessly cultivatable yielding up to 15 $g l^{-1}$ UA in 2–3 days.[31]

Thus, this easily accessible compound serves as natural precursor for the 2,15,16-trihydroxy hexadecanoic acid (THA) which itself has industrial relevance, e.g. in the production of macrocyclic musks as fragrance component.[32,33] Due to the limited availability and lack of a natural production route of the THA, its full potential for several applications could not be exploited yet.

Hence, we present here the first approach for the environmentally-friendly synthesis of the industrially attractive THA applying a two-step enzymatic cascade reaction with independency from expensive cofactors. The use of isolated (commercial) enzymes can be slightly more costly, but unlike whole cell systems it results in a reduced by-product formation and ensures the harmlessness for food and cosmetic products.

Results and Discussion

Searching for suitable biocatalysts

As the THA is linked to the cellobiose unit of the substrate ustilagic acid via its terminal hydroxy group, a glycosidase is necessary to obtain free THA. However, the acyl substitutions at positions 6 and 8 of the cellobiose moiety (Scheme 1) were shown to inhibit the activity of β-glucosidases and hence no THA was released.[34] Thus, it had to be screened for an esterase that would hydrolyze the ester bonds of the sterically demanding ustilagic acid first to yield deacylated ustilagic acid (deUA).

Among 31 tested recombinant and commercial esterases, only a few (e.g. *Bacillus stearothermophilus* Esterase, BsteE[35,36]) showed activity regarding the deacylation of the medium-chain OHFAs 3-hydroxyoctanoic and 3-hydroxyhexanoic acid at position 8, resulting in UA that is still acetylated in position 6 (acUA). The mass of the acUA was verified via TLC-MS (m/z 669,0 $[M–H]^-$). As 3-hydroxy fatty acids were shown to be building blocks for industrially valuable polyesters with several applications,[37,38] a possible utilization for these formed by-products is indicated. Only the esterase BS2 from *Bacillus subtilis* DSM402 – which was recombinantly expressed in *E. coli* BL21 (DE3) as described previously[39] – catalyzed both deacylation at position 8 and deacetylation at position 6 of UA, producing deUA (m/z 626,7 $[M\text{-}2H]^{2-}$).

The deacylated deUA was now readily accepted by six commercial glycosidases (β-glucosidase from almonds, cellulase from *Aspergillus niger*, clara-diastase, β-glucuronidase from *Helix pomatia* type HP-2, naringinase from *Penicillium decumbens* and glucosidase from *Aspergillus niger*) that showed full conversion to THA (Figures S5b and S7). As the glucosidase ALM also converted acUA to THA, it can be concluded that either the acylation of the cellobiose moiety by its own or the combination of both acylation at position 8 and acetylation in position 6 hinder the glycosidases sterically.

As the esterase BS2 was the only enzyme showing complete deacylation under equal conditions (Figures S5a and S6), it was selected to conduct further experiments. Regarding the glycosidases, the β-glucosidase from almonds (ALM) was chosen because of its good stability under various reaction conditions[40,41] and its commercial availability.

Determination of pH and temperature optima of the biocatalysts

The esterase BS2 had been investigated before concerning its temperature and pH optimum, showing the highest stability at 40 °C and the highest activity between pH 8 and 9.[39]

The commercially available β-glucosidase from almonds (ALM) was characterized regarding its pH and temperature optimum utilizing the chromogenic surrogate *p*-nitrophenyl β-D-cellobioside (pNPC). By monitoring the release of *p*-nitrophenol spectrophotometrically, the activity under different conditions was determined. To ensure measurement over a wide pH range, the pH-independent isosbestic point of pNP at 348 nm[42] was used for detection, instead of the characteristic absorption maximum only present in alkaline solution. ALM generally showed high activity over a broad pH range, as depicted in the literature,[40,41] retaining at least 80% of its activity between pH 4 and 6 (Figure 1). The optimal conditions were investigated to be 40 °C in a sodium acetate buffer at pH 5.5.

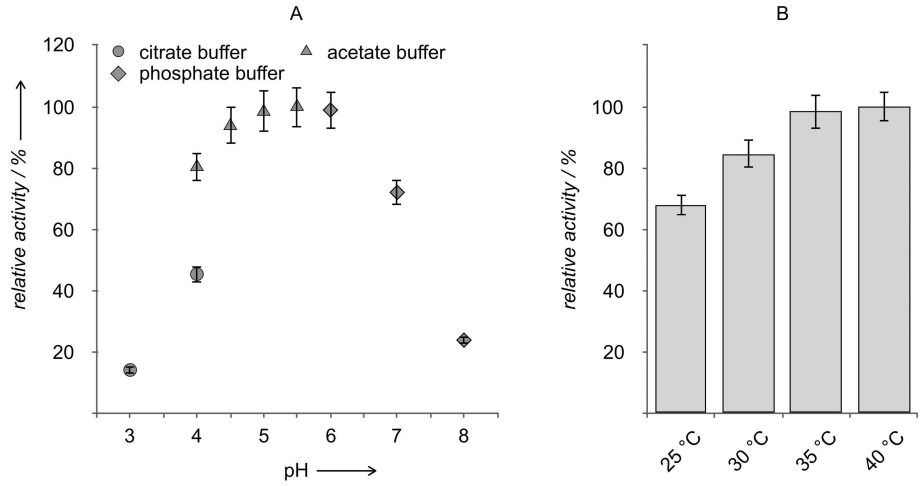

Figure 1. Determination of optimal pH and temperature for the β-glucosidase from almonds (ALM). The relative activity of ALM (0.2 mg ml^{-1}) normalized to maximum activity towards the model substrate pNPC (1 mM) is shown (A) in different buffer systems (40 mM, pH 3–8) at 30 °C and (B) under different temperatures (25–40 °C) in 40 mM sodium acetate buffer pH 5.5. All measurements were performed in triplicate.

Small-scale cascade reactions

Hereinafter, small-scale biocatalytic cascade reactions were conducted in 1.8 ml reaction vessels in order to optimize reaction conditions for obtaining the THA. BS2 (230 U ml^{-1} towards pNPA at pH 8 and 40 °C) and ALM (625 mU ml^{-1} towards pNPC at pH 5.5 and 40 °C) were combined simultaneously under different pH conditions (pH 6 to 8) at 40 °C with the substrate UA (5 mg ml^{-1}). Full conversion was observed at pH 8 after 72 h (Figures 2 and S8). At pH 6 and 7, however, the conversion of the substrate UA to the intermediate deUA was not as effective: At pH 7 only 86% and at pH 6 even only a fifth of the conversion of the biocatalysis at pH 8 was achieved according to HPLC analysis. This can be explained by the insufficient buffer capacity (25 mM) that was not able to compensate for the released fatty acids by BS2. Thus, the pH in the reactions with buffers of pH 6 and 7 may have dropped faster to a level where BS2 lost activity. Another reason for the ineffective reaction at lower pH is that the temperature-dependent solubility of UA – generally being low in water – is superior in alkaline milieu.[33,43] Moreover, it shows that BS2 has a narrower acceptance of pH conditions compared to ALM and thus is limiting concerning the selection of reaction conditions.

Furthermore, reduction of the concentration of biocatalysts was investigated under optimal conditions of 40 °C and pH 8 to minimize costs. If the substrate load was doubled to 10 mg ml^{-1}, BS2 and ALM concentrations of 500 µg ml^{-1} (corresponding to 23 U ml^{-1} BS2 towards pNPA at pH 8 and 63 mU ml^{-1} ALM towards pNPC at pH 5.5, both at 40 °C) were sufficient to achieve high conversion within 72 h.

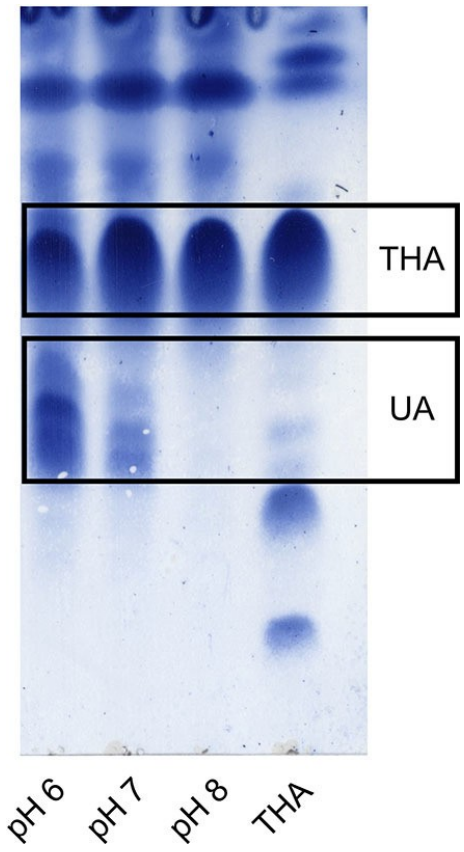

Figure 2. TLC analysis of the small-scale hydrolysis of UA (5 mg ml^{-1}) to THA catalyzed by adding esterase BS2 (5 mg ml^{-1}) and β-glucosidase ALM (5 mg ml^{-1}) simultaneously. The reaction product after 72 h under different conditions (pH 6 to 8) in 25 mM sodium phosphate buffer at 40 °C is shown. Mobile phase: chloroform:MeOH:H_2O (65:25:4, v/v). Spots were verified via TLC-MS.

Establishment of a preparative-scale two-step biotransformation

In small scale it could be shown that full conversion of UA to THA can be achieved by utilizing the enzymes BS2 and ALM simultaneously at pH 8. In contrast, the biotransformation in a larger scale of 2.5 g UA was conducted sequentially, not only to allow for an enhanced end product purity by implementing a purification step between the two reactions, but also to exploit the enzymes' full activity under their individual pH optimum and thus to perform the reactions even more efficient. Indeed, ALM is still active at pH 8 – the optimum of BS2 – but the activity is lower by the factor 4 compared to its optimum at pH 5.5 (Figure 1). To assure a constant pH value with respect to the release of middle-chain hydroxy fatty acids and acetate by the esterase activity, the reaction was coupled to a pH meter and an automatic burette that was equipped with 200 mM NaOH. The reaction of UA (10 g l^{-1}) with BS2 (400 mg l^{-1}, 46 U mg^{-1} towards pNPA at 40 °C and pH 8) at 40 °C in 200 mM sodium phosphate buffer pH 8 under pH control was monitored via TLC and stopped after 96 h. A high conversion could be achieved despite UAs relatively low solubility in water, as more UA was dissolved in the course of reaction – also as an effect of the increase in volume by the addition of overall 55 ml NaOH. Any cosolvent was omitted to perform the reaction more sustainable and to prevent limiting the enzymes activity.

The intermediate deUA was then precipitated by acidifying and cooling down the reaction to 4 °C resulting in a decreased solubility. The precipitate was extracted with EtOAc to remove the released middle-chain fatty acids in order to facilitate end product purification. After resuspending the precipitate in 50 mM sodium acetate buffer with pH 5.5, the glycosidase ALM (400 mg l^{-1}) was added to release the *O*-glycosidically linked THA. This reaction could be conducted without pH control as no acidic functionalities were released. The deglycosylation proceeded much faster than the ester hydrolysis: 86% conversion was already reached after 2.5 h (Figure S12). Downstream processing was performed by acidic precipitation of the THA at 4 °C – the remaining cellobiose exhibits a high solubility in water –, followed by filtration. This resulted in an overall isolated yield of 66% in $>$80% purity. Finally, the structure of THA was verified via NMR spectroscopy (Figure S1).

Conclusion

This work demonstrated a novel method to obtain a valuable ω-OHFA for industrial applications, the 2,15,16-trihydroxy hexadecanoic acid, in 86% conversion with an isolated yield of 66% and good purity using the naturally occurring compound ustilagic acid as starting material. The biocatalysts BS2 and ALM applied in this two-step cascade reaction are advantageous in terms of their stability and in particular their cofactor-independency, resulting in a low-cost process that, on top of that, omits the use of harmful solvents and harsh reaction conditions. This natural process paves the way for an application in food and cosmetic industry. Furthermore, the formed by-products (3-OHFAs) may be applied in the production of polyesters and the cellobiose could function as an energy source for microbial growth resulting in a minimal waste approach.

To further enhance the effectiveness of the demonstrated system an immobilization of the participating enzymes could be considered. This could not only enhance the enzymes' pH tolerance but would also facilitate downstream processing and implementation for the reuse of the enzymes.

Experimental Section

General

The substrate, UA, was received from AnalytiCon Discovery GmbH (Potsdam, DE). β-glucosidase from almonds (ALM, G0395) was obtained from Sigma-Aldrich (St. Louis, US). For all tested biocatalysts, see Supporting Information. All other reagents were purchased from Sigma–Aldrich (St. Louis, US) and Carl-Roth (Karlsruhe, DE), if not stated otherwise. A mixture of chloroform, methanol and water (65:25:4, v/v) was used as mobile phase in TLC analysis with 0.2 mm ALUGRAM® SIL G/UV_{254}, 4×8 cm (Macherey-Nagel GmbH & Co. KG, DE) as stationary phase. Staining of TLC plates was achieved by using Cerium phosphomolybdic acid. TLC-MS measurements were performed using an expression^{L} Compact Mass Spectrometer with ESI ionization source (Advion, US) coupled to *Plate Express* (Advion, US). The ^{1}H NMR spectrum was recorded using a 500 MHz Bruker instrument at 500 MHz with TMS as internal standard. HPLC analysis was performed with a Merck Hitachi system equipped with a reversed phase column (Superspher® RP-select B 4 μm, 125×4 mm) and a Sedere Sedex 75 ELSD (3.5 bar, nebulizer temperature 35 °C). At a flow rate of 1 ml min^{-1} and 23 °C column temperature the following gradient was applied using mobile phase A (water with 5 mM ammonium formate and 0.1% formic acid), B (methanol/acetonitrile 1:1 v/v with 5 mM ammonium formate and 0.1% formic acid) and C (isopropanol): linear gradient from 15% A and 85% B to 100% B in 15 min, hold at 100% B for 2.5 min, linear gradient to 50% B and 50% C in 0.5 min, hold for 2 min, linear gradient to 85% A and 15% B in 0.1 min, hold for 3.9 min.

Identification of suitable biocatalysts

Screening for a suitable biocatalyst was conducted in 1.8 ml glass vials with 5 mg ml^{-1} esterase or glucosidase and 5 mg ml^{-1} UA or deUA in a suitable buffer system (25 mM sodium phosphate buffer pH 7–8 or acetate buffer pH 4–5.5) at 40 °C. After 72 h a 100 μl sample was acidified with 20 μl 2 M HCl and extracted twice with 200 μl EtOAc. Reaction success was monitored via TLC analysis.

Esterase production and activity assay

The esterase BS2 from *Bacillus subtilis* DSM402 (GenBank: AQZ92317.1) was expressed using the L-rhamnose inducible vector pGaston in *E. coli* BL21 (DE3) as described previously.[39] In brief, the culture was grown in LB media supplemented with 100 μg ml^{-1} ampicillin until an OD_{600} of 0.5 was reached. Esterase production was induced with 0.2% w/v rhamnose. After incubation at 37 °C and 200 rpm for 4 h, cells were harvested by centrifugation at 4500×g and 4 °C for 15 min. Subsequently, the cells were washed twice with 50 mM sodium phosphate buffer pH 7.5 and disrupted via sonification (10 min, 50% power, 50% pulse) with SONOPULS

HD 2070 (BANDELIN electronic GmbH & Co. KG, DE). The crude extract, received after centrifugation at 10,000×g and 4 °C for 20 min, was lyophilized and used for further experiments. The activity of lyophilisate (50 μg ml^{-1}) was determined spectrophotometrically via hydrolysis of *p*-nitrophenyl acetate (pNPA, 1 mM in DMSO) at 40 °C in 45 mM sodium phosphate buffer pH 8. The *p*-nitrophenol released by BS2 was quantified at 410 nm using the Infinite M200 PRO microplate reader (Tecan Group, CH). One unit of activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under assay conditions.

Glucosidase activity assay and determination of pH and temperature optima

Glucosidase activity of ALM (0,2 mg ml^{-1}) at different temperatures (25–40 °C) and in various buffer systems (40 mM, pH 3–8) was determined spectrophotometrically in triplicates by hydrolysis of pNPC (1 mM). The released *p*-nitrophenol was quantified at 348 nm using the Infinite M200 PRO microplate reader (Tecan Group, CH). One unit of activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute under assay conditions.

Small-scale cascade reactions

Small-scale cascade reactions to hydrolyze UA (5–10 mg ml^{-1}) were performed at 40 °C and 650 rpm in 1.8 ml glass vials containing both BS2 and ALM in concentrations of 500 μg ml^{-1} to 5 mg ml^{-1}. The reaction media consisted of 25 mM sodium phosphate buffer pH 6 to 8. After 72 h a 100 μl sample of the reaction was extracted twice with 200 μl EtOAc after acidification with 20 μl 2 M HCl and analyzed via TLC as described above.

Preparative scale two-step biotransformation

2.5 g UA were dissolved in 250 ml 200 mM sodium phosphate buffer pH 8 at 40 °C. Thereupon, 400 mg l^{-1} BS2 (46 U mg^{-1} at 40 °C referred to *p*-nitrophenyl acetate) were added and pH was maintained constant by titration of NaOH (200 mM stock solution) via an automatic burette controlled by a pH meter (pH 211, Hanna Instruments Deutschland GmbH, DE). The reaction was stopped after 96 h by precipitation of the reactants with 6 ml concentrated HCl at 4 °C. The dry precipitate was extracted three times with 30 ml EtOAc to remove the short- and middle-chain fatty acids. The residual substance was extracted three times with 30 ml methanol to remove the enzyme residue. The dried extract was then resuspended in 50 mM sodium acetate buffer pH 5.5 and heated to 40 °C, followed by addition of 400 mg l^{-1} ALM. After one-week reaction time 1 ml concentrated HCl was added and the reaction was cooled down to 4 °C. To separate the precipitated end product from the enzyme, it was extracted six times with 15 ml MeOH and lyophilized. The reaction progress was monitored by HPLC analysis and the final product 2,15,16-trihydroxy-hexadecanoic acid (636 mg, 2.1 mmol) was obtained in 66% isolated yield and verified via NMR spectroscopy. ^{1}H NMR (500 MHz, CD_3OD): $\delta = 4.11$ (dd, $J =$ 8.4 Hz, $J = 4.0$ Hz, 1H; CH), 3.59 (m, 1H, CH), 3.49 (dd, $J = 11.5$ Hz, $J =$ 4.6 Hz, 2H; CH_2), 3.43 (dd, $J = 11.5$ Hz, $J = 6.9$ Hz, 2H; CH_2), 1.75 (m, 2H; CH_2), 1.65 (m, 2H; CH_2), 1.29–1.53 ppm (m, 22H; CH_2); MS (ESI): *m/z* (%): 303,2 (100) $[M–H]^-$.

Abbreviations

acUA, acetylated ustilagic acid; **ALM**, β-glucosidase from almonds; **BS2**, esterase from *Bacillus subtilis* DSM402; **BsteE**, esterase from *Bacillus stearothermophilus*; **deUA**, deacylated ustilagic acid; **OHFA**, hydroxy fatty acid; **pNPC**, *p*-nitrophenyl acetate; **pNPC**, *p*-nitrophenyl β-D-cellobioside; **THA**, 2-15-16-trihydroxy hexadecanoic acid; **UA**, ustilagic acid.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · cascade reaction · ω-hydroxy fatty acid · natural products · ustilagic acid

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