JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Sustainable and Green Synthesis of Stanol Esters from Oil Wastes

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Cite This: *J. Agric. Food Chem.* 2021, 69, 286−293

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ABSTRACT: The recombinant lipase of*Ophiostoma piceae* (OPEr) is characterized by its prominent sterol esterase activity. The protein was immobilized on magnetic nanoparticles, giving four enzyme variants that have been tested in solvent-free transesterification of methyl oleate and sitostanol. The yields of stanol esters reached 85%, and the catalysts can be reused. Stanol esters were also obtained in a two-step cascade reaction; a mixture of fatty acid methyl esters was enzymatically synthesized from cooking oil wastes and then used for stanol transesterification. An 85% conversion was achieved in 2 h from the second cycle onward, maintaining the activity over 5 cycles. The biocatalysts can be safely used since they don't release toxic compounds for HeLa and A549 cell lines. These procedures comply with the principles of green chemistry and contribute to the sustainable production of these nutraceuticals from secondary raw materials, like the lipid fraction from industrial or agricultural residues.

KEYWORDS: *biocatalysis, enzymatic transesterification, phytosterols, FAMEs, sustainability, wastes, OPEr.*

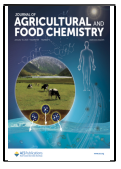
INTRODUCTION

Sterols are a family of compounds that perform relevant structural and physiological functions in eukaryotes as components of the cell membranes of animals, plants, and fungi. Chemically, sterols are lipids that have a bulky, hydrophobic ring with a β-3-hydroxyl group, which confers a slight polarity to the molecule, and a small aliphatic chain. This structural similarity is the basis of the use of phytosterols to reduce cholesterol levels, as they compete with cholesterol for absorption in the gut of animals and humans.[1] Stanols are the saturated forms of sterols, and appear to be more efficient cholesterol-lowering agents because they are more hydrophobic, have less micellar solubility, and therefore, their intestinal absorption is lower.[2,3] While the use of free sterols and stanols as dietary supplements is limited by their low solubility, their esters have properties comparable to edible fats and oils, which facilitates their incorporation into fatty foods, and are more bioavailable.[4]

The industrial production of these nutraceutical esters is usually accomplished by chemical methods using acidic or basic catalysts, and the development of competitive enzymatic technologies would be desirable. A small group of lipases and sterol esterases has shown their ability to catalyze the esterification/transesterification of these hydrophobic and bulky substrates under mild reaction conditions, without generating unwanted by-products.[3,5−12] The use of immobilized enzymes may help upscaling the process, improving the catalyst stability, enabling its recovery and reuse, and reducing costs. In the food, cosmetics, and pharmaceutical sectors, where the final product delivered to the consumer must be free of residual enzymes,[13] only processes based on the use of immobilized enzymes, attached to or embedded in an insoluble carrier, are gaining industrial importance.[14] Many types of scaffolds, from biopolymers to inorganic compounds, have been used to immobilize lipases,[3] and magnetic nanoparticles have become very popular carriers for their excellent properties. One of them is superparamagnetism, which allows easy and virtually immediate separation of the catalyst under external magnetic fields.[15,16]

The commercial lipase CRL from *Candida rugosa* is probably the best-known biocatalyst applied for the synthesis of phytosterol esters and has been tested under many experimental conditions.[3] However, the recombinant form of a versatile lipase produced naturally by the fungus *Ophiostoma piceae* (OPEr) shows outstanding activity against phytosterols and their esters.[17] We have demonstrated that OPEr was much more efficient for the synthesis of stanol esters by direct esterification or transesterification, and under extremely mild conditions, than CRL,[4] commercial lipases Cal A and Cal B from *Candida antarctica*,[18] and other recombinant lipases produced in our laboratory.[19,20] We recently immobilized and tested OPEr for direct esterification of volatile fatty acids with good results,[21] and an additional immobilized variant of OPEr has been prepared and tested in this work. Here, we address the synthesis of stanol esters in green reactions, at room temperature and without the addition of organic solvents, catalyzed by several immobilized forms of OPEr. We have also designed and tested a cascade reaction to produce these nutraceuticals using vegetable oil residues as fatty acid donors.

Received: October 15, 2020
Revised: December 7, 2020
Accepted: December 9, 2020
Published: December 29, 2020



MATERIALS AND METHODS

Materials. β-Sitostanol, *p*-nitrophenyl butyrate (*p*NPB), (3-aminopropyl)triethoxysilane 99% (APTS), methyl oleate, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), colchicine, sodium dodecyl sulfate (SDS), and dimethylformamide were from Sigma-Aldrich (St. Louis, MO). Other chemicals and solvents of the purest available grade were provided by Merck (Darmstadt, Germany).

Strains, Culture Conditions, and Preparation of Crudes Enzyme. *Pichia pastoris* GS115 strain containing the *ope* gene was maintained and cultivated to produce the versatile lipase OPEr, as previously reported.[20] The cultures were then centrifuged (27 666*g*, 4 °C) and fungal biomass discarded. Supernatants were concentrated by ultrafiltration in a YM3 Amicon device (Merck Millipore, Darmstadt, Germany) with a 50 kDa membrane. The crude extract obtained was used without further purification.

Evaluation of Enzyme Activity. The standard assay to determine the activity of the catalyst was carried out monitoring the release of *p*-nitrophenol from hydrolysis of 1.5 mM *p*NPB in 20 mM Tris−HCl pH 7.0 at room temperature at 410 nm using a Shimadzu UV-160A spectrophotometer. One unit of activity (1 U) is defined as the amount of enzyme used to release 1 μmol of *p*-nitrophenol (ε_{410} = 15 200 M^{-1} cm^{-1}) per minute under the defined conditions.

Immobilization of OPEr. Magnetic nanoparticles (IoLiTec, GmbH Heilbronn, Germany) were silanized and functionalized with NH_2 groups as described.[21] Concentrated extracts containing OPEr were immobilized on amino-functionalized magnetic nanoparticles (AMNP) by four procedures. The enzyme was non-covalently bound by hydrophobic interaction (SiMAG-Octyl-OPEr), covalently to glutaraldehyde-activated AMNPs (AMNPs-GA-OPEr), and as magnetic cross-linked enzyme aggregates (mCLEAs-OPEr), as previously reported.[21] The fourth immobilization procedure takes advantage of the fact that OPEr is glycosylated, and involved oxidation of the glycidic chains of OPEr with 10 mM $NaIO_4$ in 5 mM Tris−HCl pH 7 at 4 °C for 3 h. After treatment, the samples were dialyzed against 20 mM Tris−HCl, pH 7, in a 30 kDa cut off centrifuge filter (Amicon Ultra-15, Millipore). The enzyme solution was mixed with AMNPs in 100 mM Tris−HCl, pH 8, with 150 mM trimethyl amino borane, stirring at 100 rpm and 28 °C for 150 min. The AMNPs with the bound protein were captured with a magnet, treated with $NaBH_4$ (1 h), and washed with 100 and 20 mM Tris−HCl, pH 7, and the final catalyst was named AMNPs-CH-OPEr.

The activity provided, the immobilized activity (the difference between starting activity and residual activity measured in the supernatant after immobilization), and the activity of the immobilized catalysts (observed activity), were determined using *p*NPB as the substrate. The immobilization parameters were calculated according to Sheldon and van Pelt[13]

$$\text{yield (\%)} = (\text{immobilized activity}/\text{starting activity}) \times 100$$

$$\text{efficiency (\%)} = (\text{observed activity}/\text{immobilized activity}) \times 100$$

$$\text{activity recovery (\%)} = (\text{observed activity}/\text{starting activity}) \times 100$$

Enzymatic Synthesis of Stanol Esters by Solvent-Free Transesterification. An 80 mM solution of stanols in 99% methyl oleate was first prepared and warmed (50 °C) for a few minutes to ensure that stanols were completely dissolved before pipetting. Then, 500 μL of the solution were placed in a vial, adding cholest-4-en-3-one (internal standard for gas chromatography (GC) quantification), 20% water, and 35 U of OPEr, maintaining the reaction at 25 °C for up to 15 h with 100 rpm rotational mixing (Multi Bio RS-24, Biosan). No co-solvent was added since methyl oleate also acted as the reaction solvent. After each reaction cycle, the biocatalysts were washed with isooctane and 20 mM Tris−HCl buffer, pH 7, and reused under the same conditions. The experiments were performed in duplicate, monitoring the reaction by gas chromatography (GC) as previously reported.[4] Since no commercial standards of the synthesized esters were available, the esterification yields were calculated from the amount of residual free stanols according to the formula

$$\text{esterification (\%)} = 100 \times \frac{[\text{stanol}]_i - [\text{stanol}]_f}{[\text{stanol}]_i}$$

where $[\text{stanol}]_i$ and $[\text{stanol}]_f$ are the initial and final concentrations of stanols.

Sequential Synthesis of Acyl Donors and Stanol Esters Catalyzed by AMNP-CH-OPEr. Cooking olive oil wastes (500 μL) were used to obtain a mixture of fatty acid methyl esters (FAMEs) by solvent-free methanolysis (25 °C, 100 rpm, molar ratio oil/methanol 1:4) using AMNP-CH-OPEr (70 U) as the catalyst. Once the reaction was completed, the liquid part of the reaction mixture was analyzed by gas chromatography/mass spectrometry (GC/MS) to confirm the disappearance of the peaks from triglycerides and the appearance of those from the FAMEs. Then, it was used as the reaction solvent and source of acyl donors to synthesize stanol esters. Transesterification with AMNP-CH-OPEr was accomplished as indicated in the previous section, except for the acyl donor. The reaction mixture containing the stanol esters was analyzed by gas chromatography (GC) as reported.[4]

GC/MS Analysis. The analyses were performed in an Agilent instrument equipped with a 7890A GC, a 5975C quadrupolar mass detector, and a DB5-HT column (30 m, 0.25 mm internal diameter, 0.1 μm film thickness). To analyze the commercial sample of β-sitostanol, the temperature program was as follows: 180−205 °C (ramp rate 3 °C/min); then 220 °C (10 °C/min), this temperature was maintained for 5 min; and finally from 220 to 400 °C, maintaining the final temperature for 5 min. Commercial methyl oleate and a mixture of FAMEs enzymatically synthesized in the laboratory were analyzed using a program that started at a temperature of 80 °C (4 min), which was then increased to 300 °C (20 °C/min). The compounds detected were identified using commercial standards, analyzed under identical conditions to those for the samples or through their mass spectra using the NIST 2011 library and ChemStation software (Agilent, Santa Clara, CA).

***In Vitro* Cytotoxicity Assay.** The migration of potentially toxic compounds from the immobilized biocatalysts was also evaluated. Stock suspensions of each biocatalyst in sterile PBS, pH 7.4 (700 mg/mL for AMNP-CH-OPEr, mCLEAs-OPEr, and SiMAG-Octyl-OPEr, and 1250 mg/mL for AMNP-GA-OPEr) were maintained under agitation for 24 h. The supernatants were recovered and filtered (0.22 μm) before being added to the cell cultures in appropriate amounts. Cytotoxicity was tested by exposing human cell lines, HeLa and A549, to these supernatants.

Human HeLa cells from cervix carcinoma were continuously maintained in Dulbecco's modified Eagle's medium (DMEM) enriched with high glucose and pyruvate. Non-small cell lung carcinoma A549 cells were cultured in RPMI-1640 with L-glutamine at 37 °C in 5% CO_2. Both media were supplemented with 10% fetal bovine serum, 10 U/mL penicillin, and 10 μg/mL streptomycin. Cells were seeded in a 96-well plate at a density of 120 000 cells/mL for HeLa and 160 000 cells/mL for A549 cells. Then, 80 μL were added per well and cultured in 5% CO_2 at 37 °C for 24 h. The cells were then exposed to 20 μL serial dilutions of the four stock solutions of the biocatalysts for 48 h. The maximum concentrations tested corresponded to suspensions with 140 mg/mL of each nanobiocatalyst (250 mg/mL for AMNP-GA-OPEr). Viable cells were determined by MTT assay[22] with some modifications. Briefly, 20 μL of 2.5 mg/mL MTT was added to each well, incubated for 4 h at 37 °C, and then treated with 0.1 mL of a solution containing 10% SDS and 45% dimethylformamide, pH 5.5, and incubated overnight at 37 °C to solubilize the blue formazan precipitate. The absorbance at 595/690 nm was measured using an automated Multiscan microplate reader (Spectra Max Plus 384, Molecular Devices, San Jose, CA). Controls containing medium without cells were used as blanks, and colchicine was used as the positive control (1/2 dilutions 800−1.5 nM). The results were expressed as the dose that inhibits 50% growth after the incubation period (IC_{50}), estimating the values from a $\log_{10}$

https://dx.doi.org/10.1021/acs.jafc.0c06581
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plot of compound concentration against the percentage of viable cells. Data were processed using SigmaPlot 13.0. Micrographs were obtained using an AxioCam ERc5s digital camera in a phase-contrast inverted microscope Zeiss IM (Hicksville, NY).

RESULTS AND DISCUSSION

Immobilization of OPEr. Immobilization implies protein fixation to a non-soluble solid support. If the activity of the enzyme resists this process, then the immobilized biocatalyst is often more stable than the free enzyme and can be easily separated from the reaction medium to be reused.[23] However, there is no rational approach to predict which immobilization method will be best suited to produce a robust biocatalyst for a given application, as it depends on the enzyme, the application, and the support.[24] Since protein conformation and catalytic properties might change depending on the area implicated in the attachment, we prepared and tested four versions of OPEr immobilized on functionalized magnetic nanoparticles, whose characteristics are summarized in Table 1. The immobilization

Table 1. Summary of the Procedures Used for OPEr Immobilization

immobilization	catalyst	immob. yield (%)[a]	specific activity[b] (mU/mg carrier)
hydrophobicity	SiMAG-Octyl-OPEr	99 ± 1	843 ± 69
covalent	AMNP-GA-OPEr	65 ± 8	334 ± 5
	mCLEAS-OPEr	99 ± 2	769 ± 58
	AMNP-CH-OPEr	96 ± 3	709 ± 19

[a]Immob. yield (%) = (immobilized activity/starting activity) × 100. [b]The specific activity of different preparations with versatile lipase attached to magnetic nanoparticles was determined using *p*NPB as the substrate.

yields were close to 100% except for AMNP-GA-OPEr (65%), which could be due to the lower protein load accepted in this preparation and justifies its lower specific activity.

SiMAG-Octyl-OPEr is prepared by a quite simple method, in which the hydrophobic regions of the lipase are non-covalently attached to a hydrophobic carrier. The enzyme has few flexibility restrictions, but the catalyst may be lost during catalysis. The other three methods tested involve the formation of covalent linkages causing the irreversible binding of the lipase to the carrier. In each of these cases, the protein is attached through different structural regions, depending on the carrier functionalities and on the immobilization conditions.[21,25−27] In AMNP-GA-OPEr and mCLEAs-OPEr, the peptide chain is involved in the linkages, which probably causes protein rigidification. This can be useful for maintaining activity and stability[25,28] but may also hamper the accessibility of bulky substrates to the active center. SiMAG-Octyl-OPEr, mCLEAs-OPEr, and AMNP-GA-OPEr were successfully applied in the synthesis of esters of volatile fatty acids by direct esterification,[21] and other lipases have been immobilized by these procedures on the same or other carriers.[27,29−41]

The fourth preparation (AMNP-CH-OPEr) was accomplished through a less conventional strategy, avoiding the implication of the protein moiety of the lipase in its immobilization. Here, the covalent linkage is held between free amino groups of the carrier and aldehydes produced in the glycidic component of OPEr. Multiple Schiff's bases are formed *via* reductive amination,[42] and the oxidized carbohydrate chains act as slightly flexible linkers. This has a positive impact on maintaining the enzyme conformation since the protein moiety is not attached in a rigid conformation, there are no additional structural constraints, and the enzyme has more chances to adapt for the entry of large substrates. Nevertheless, this flexibility could make the enzyme more susceptible to inactivation.[43]

Transesterification of β-Sitostanol and Methyl Oleate. In a former work, Molina-Gutiérrez et al.[4] reported that β-sitostanol is fairly soluble at 25 °C in methyl oleate even at the highest concentration assayed (80 mM), affording esterification yields of around 90% in 2 h with soluble OPEr in the absence of co-solvents. Thus, this β-sitostanol concentration and temperature were chosen for the experiments performed in the current work. GC/MS analysis of the commercial substrate indicated that it contained 80% β-sitostanol and a second component that was identified as ergostanol based on its mass spectrum. All of the biocatalysts tested transesterified both stanols in a similar way, yielding the corresponding oleic esters (Figure 1), which confirmed that the wide substrate specificity

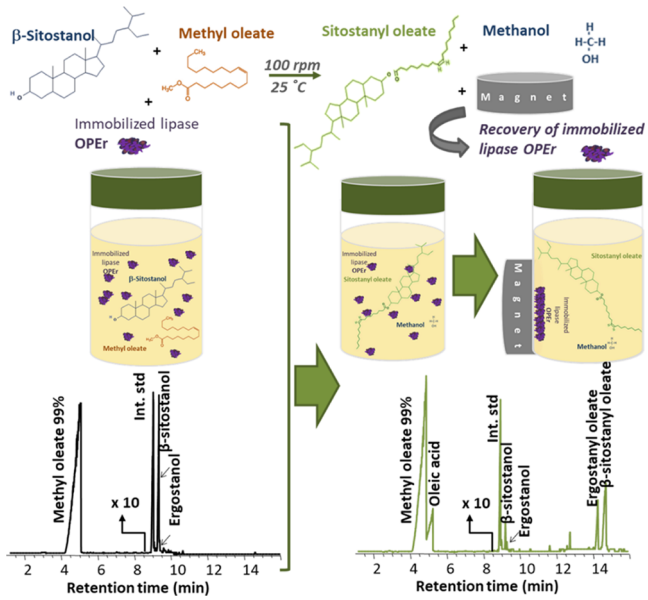

Figure 1. Scheme of the transesterification reaction of 80 mM β-sitostanol and methyl oleate catalyzed by immobilized OPEr. Methyl oleate acts as a solvent and as a donor of oleic acid. Once the reaction is complete, the catalyst is recovered from the reaction vessel in seconds, with the help of a Nd magnet. The reaction mixture can be recovered, free from protein, for further purification of the products. The GC chromatograms of samples analyzed at 0 and 15 h are shown at the bottom of the figure. The signal has been magnified from the 8.5th minute onward (×10) to improve the visualization of the peaks of free stanols and stanol esters.

of OPEr[17] is maintained upon immobilization. Several lipases have been used for ergosterol esterification,[5,44,45] but we have not found any report on esterification of ergostanol.

The results from the esterification and recycling of the catalysts assayed are presented in Figure 2. In the first reaction cycle (Figure 2a, R1), both the free OPEr and SiMAG-Octyl-OPEr produced 86 and 72% esters in 15 h, while the other catalysts gave esterification yields between 18 and 40%. However, an enhanced esterification efficiency was observed in the second (R2) and third (R3) cycles for all of them, with

https://dx.doi.org/10.1021/acs.jafc.0c06581
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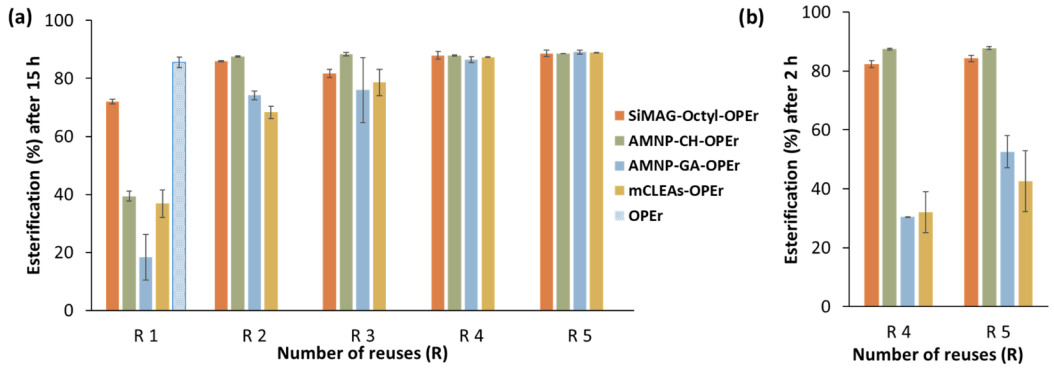

Figure 2. (a) Esterification yields of free OPEr (only one reaction cycle) and the OPEr-nanobiocatalysts, and recyclability of the immobilized enzyme in the solventless transesterification of 80 mM stanols with commercial methyl oleate (99%). Reactions were performed at 25 °C for 15 h. (b) Details of the transesterification yields of the four nanobiocatalysts after 2 h of reaction in the fourth and fifth cycles. R1−R5: reaction cycles 1−5.

stably high conversions in the fourth (R4) and fifth (R5) cycles. It is interesting to note that in 2 h reactions catalyzed by SiMAG-Octyl-OPEr and AMNP-CH-OPEr the esterification yield determined in cycles R4 and R5 was very close to the maximum values produced in 15 h in the previous cycles. As commented before, the degree of flexibility of OPEr is expected to be higher in these two preparations, allowing an easier and faster accommodation of the active center. Notably, in the fourth and fifth cycles, the catalytic activity of AMNP-CH-OPEr modestly overpassed that of the catalyst attached by hydrophobicity.

Although we cannot give a conclusive explanation for this behavior, the increase in the activity detected after recycling could not only be ascribed to the gradual accommodation of the enzyme under the reaction conditions but also to a beneficial effect of the presence of residual amounts of components from the previous reaction cycle.

Concerning the activity reported for other immobilized lipases, several studies deal with the enzymatic synthesis of sterol esters catalyzed by CRL immobilized on different scaffolds. In general, esterification and/or transesterification reactions were carried out in the presence of a co-solvent, at temperature ranges between 35 and 55 °C for 6−24 h.[46−49] The recyclability, if evaluated, varied from a complete activity loss after 5 cycles to nearly full maintenance of the catalytic efficiency after 12 cycles. Several non-covalently immobilized commercial lipases (Novozyme 435, Lipozyme RM IM, and Lipozyme TL IM) tested for this application displayed lower efficiency than CRL.[50,51]

Stanol esters are included in foods intended for human consumption, and thus the possible release of toxic compounds from the immobilized biocatalysts to the reaction medium must be prevented before considering the potential biotechnological application of this approach. To do so, we evaluated the effect of supernatants from concentrated suspensions of the biocatalysts on the viability of HeLa and A549 cells. Untreated cells were used as the negative control and colchicine, a drug that depolymerizes microtubules and blocks the mitotic spindle, affecting cellular viability,[52] as the positive control.

According to the analysis of IC_{50} values (Table 2), none of the extracts produced cytotoxic effects after 48 h of exposure, while low concentrations of colchicine strongly affected cellular viability.

As an example, Figure 3 shows the micrographs of the cells treated with the extract from AMNP-CH-OPEr at the maximum concentration evaluated, together with a positive and a negative control. The images obtained for other treatments are comparable to these. Hence, microscopic inspection of the cells supported the IC_{50} results. There are no observable differences between untreated and treated cells, while the micrographs of the colchicine control showed the presence of a massive number of non-adherent cells.

Table 2. IC_{50} Values of Nanobiocatalysts Extracts and Colchicine as the Standard Control

extract/compound	IC_{50} HeLa	IC_{50} A549	[biocatalyst] in the reactions
colchicine[a]	63.2 ± 9.4 nM	93.0 ± 5.7 nM	
AMNPs-CH-OPEr	>140 mg/mL	>140 mg/mL	76 mg/mL
AMNPs-GA-OPEr	>250 mg/mL	>250 mg/mL	204 mg/mL
SiMAG-Octyl-OPEr	>140 mg/mL	>140 mg/mL	84 mg/mL
mCLEAs-OPEr	>140 mg/mL	>140 mg/mL	96 mg/mL

[a]Positive control.

Cascade Reactions for Green and Sustainable Synthesis of Stanol Esters Using Oil Wastes as Secondary Raw Materials. The above results proved the suitability of the catalysts to produce stanol esters and confirmed methyl oleate as an excellent solvent and acyl donor. In fact, the renewable, nontoxic, and biodegradable nature of fatty acid esters makes them an interesting choice for this and other processes devoted to the synthesis of value-added commodities.[53] However, the industrial production of FAMEs relies on chemical transesterification that involves the use of toxic and polluting catalysts and reagents, high temperatures, and leaves a hardly recyclable waste. Cleaner biocatalytic manufacturing processes can be implemented for the synthesis of FAMEs, but the product must be cost-competitive. Several lipases can produce FAMEs, and OPEr has shown to do it sustainably, using triglycerides from cooking oil wastes in a solventless reaction medium at ambient temperatures.[42] Thus, in search of a process compliant with the principles of green chemistry, we explored the possibility of coupling triglyceride methanolysis with the synthesis of stanol esters, using the same OPEr biocatalyst in sequential reactions.

Among the four immobilized biocatalysts available, we selected AMNP-CH-OPEr for these cascade reactions because this preparation was the most efficient for the synthesis of

https://dx.doi.org/10.1021/acs.jafc.0c06581
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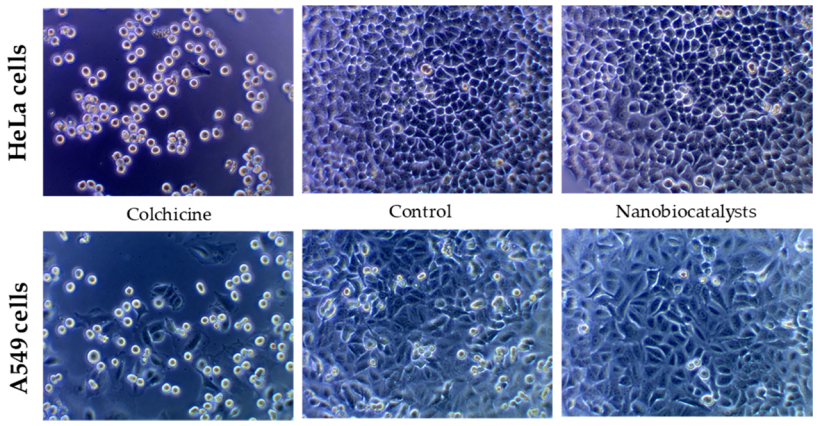

Figure 3. Micrographs of control and treated HeLa and A549 cells after 48 h incubation. Left: cells treated with colchicine at the maximum concentration assayed. Middle: untreated cells. Right: cells treated with the maximum concentration of AMNP-CH-OPEr extract.

FAMEs[42] and one of the best to synthesize stanol esters, according to the results presented in Figure 2. Indeed, the first reaction of the cascade, consisting of the methanolysis of triglycerides from oil residues, yielded a semi-solid residue of glycerol and a liquid fraction composed of 96.6% FAMEs, 1% diglycerides, and 2.4% free fatty acids. Methyl oleate was, by far, the main component, but up to four methyl esters were identified by GC/MS. This liquid fraction was mixed with stanols without further treatment or purification, and transesterification was catalyzed by the same biocatalyst without adding any other component.

Samples were analyzed after 2 and 15 h by GC, showing the peaks of oleic, palmitic, stearic, and linoleic stanol esters and a decrease in the content of FAMEs, diglycerides, and free stanols over time (Figure 4a,b). The reaction reached the maximum esterification (85%) after 15 h in the first cycle, but around 80% of stanol esters were synthesized in 2 h from the second cycle onward (Figure 4c). This observation confirmed that, more than losing activity, this OPEr catalyst gains efficiency when recycled, maintaining esterification yields of 80% in 2 h of reaction after five reuses (Figure 4c). In addition, the reaction proceeded faster and more efficiently than when commercial methyl oleate was used as an acyl donor, probably due to a positive effect of other components of the FAME mixture resulting from the previous enzymatic synthesis.

He et al.[3] reviewed the most recent publications on lipase-catalyzed synthesis of fatty acid esters of phytosterols. Few papers report on the synthesis of these compounds using an immobilized enzyme as catalysts and oils or mixtures of FAMEs as acyl donors and, in general, the experimental conditions applied are much less eco-friendly than those used in the current work. For example, non-covalently immobilized CRL[46] produced 74−96.3% sterol or stanol esters using co-solvents, and temperatures between 40 and 55 °C, but recyclability was not tested. Similarly, a novel approach in which transesterification was performed at 55 °C in a Pickering emulsion stabilized by mesoporous carbon nanospheres with immobilized CRL gave excellent transesterification yields and recyclability.[11] Transesterification of phytosterols with soybean oil catalyzed by Novozyme 435[54] produced 96% esters at 85 °C using SC-CO_2, and the use of Lipozyme TL IM in a packed bed continuous flow reactor operated at 60 °C yielded 70 and 57% phytosterol esters from linseed and fish oil in *n*-hexane, respectively.[55]

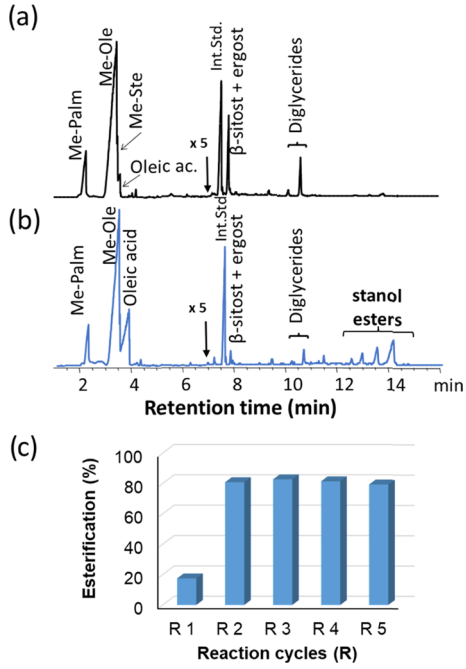

Figure 4. Synthesis of stanol esters catalyzed by AMNP-CH-OPEr in a two-step cascade reaction. GC chromatograms of the transesterification of FAMEs and stanols in samples taken at: (a) 0 h and (b) 15 h. In both chromatograms, the signal has been magnified (×5) from the 7th minute onward to improve the visualization of the peaks of free stanols and stanol esters. (c) Recycling of the catalyst: % of stanol esters produced in 2 h of transesterification across five successive reaction cycles. R1−R5: reaction cycles 1−5.

To summarize, this work has demonstrated that stanol esters can be easily synthesized at room temperature using a single immobilized variant of the lipase OPEr as the biocatalyst, and stanols, waste cooking oil, and methanol as substrates. The whole procedure involves a cascade of two consecutive reactions, oil methanolysis, and stanol transesterification, and the biocatalyst can be recycled for further reactions without activity loss. These results point to the suitability of this novel strategy for synthesizing stanol esters by a green and sustainable procedure from non-expensive wastes as substrates, according to the current concept of circular economy.

https://dx.doi.org/10.1021/acs.jafc.0c06581
J. Agric. Food Chem. 2021, 69, 286−293

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Funding

This work was supported by the Spanish projects GLYSUS RTI2018-093683-B-I00 from MICIU/AEI/FEDER, and RETOPROSOST-2-CM S2018/EMT-4459 from Comunidad de Madrid.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge Isabel Barasoaín for her help in designing the cytotoxicity experiments and Mercedes Sánchez from the Gas Chromatography Facility at CIB, for technical help. The authors would also like to thank the IBISBA1.0 project (H2020 730976) and the SusPlast-CSIC Interdisciplinary Platform for their support.

ABBREVIATIONS USED

AMNP, magnetic nanoparticles functionalized with reactive amino groups on their surface; AMNPs-GA-OPEr, OPEr covalently immobilized on AMNPs treated with glutaraldehyde to leave reactive aldehyde surface groups; AMNPs-CH-OPEr, OPEr covalently immobilized, through aldehyde groups generated by oxidation of its glycidic chains, on AMNPs; APTS, (3-aminopropyl)triethoxysilane; FAMEs, fatty acid methyl esters; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; mCLEAs-OPEr, magnetic cross-linked enzyme aggregates; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *p*NPB, *p*-nitrophenyl butyrate; OPEr, versatile lipase of *Ophiostoma piceae*; SDS, sodium dodecyl sulfate; SiMAG-Octyl-OPEr, OPEr non-covalently immobilized by hydrophobic interaction on magnetic nanoparticles with octyl groups on their surface

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