Sustainable and green synthesis of stanol esters from oil wastes

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ABSTRACT. The recombinant lipase of *Ophiostoma piceae* (OPeR) is characterized for 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 stanols transesterification. An 85% conversion was achieved in 2 h from the second cycle onwards, 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 at the basis of the use of phytosterols to reduce cholesterol levels, as they compete with cholesterol absorption in the gut in 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 done by chemical methods using acid or basic catalysts, and the development of competitive enzymatic technologies would be welcome. A small group of lipases and sterol esterases have shown their ability to catalyze the esterification/transesterification of these hydrophobic and bulky substrates in 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 re-use, and reducing costs. Within the food, cosmetics and pharmaceutical sectors, where the final product delivered to the consumer must be free of residual enzyme,¹³ only processes based on the use of immobilized enzymes, attached 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 in the synthesis of stanol esters by direct esterification or transesterification, and under extremely mild conditions, than CRL,⁴ the 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.

MATERIALS AND METHODS

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

Strains, culture conditions, and preparation of enzyme crudes. *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 an 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 μ mol of *p*-nitrophenol ($\epsilon_{410} = 15,200 \text{ M}^{-1} \text{ cm}^{-1}$) 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), and 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 NaIO₄ 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 NaBH₄ (1 h) and washed with 100 mM and 20 mM Tris-HCl pH 7, and the final catalyst was named AMNPs-CH-OPeR.

The activity provided, the immobilized activity (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 substrate. The immobilization parameters were calculated according to Sheldon and van Pelt:¹³

Yield (%) = (immobilized activity/starting activity) x 100

Efficiency (%) = (observed activity/immobilized activity) x 100

Activity recovery (%) = (observed activity/starting activity) x 100

Enzymatic synthesis of stanol esters by solvent-free transesterification. A 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 GC quantification), 20% of 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 acted also 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 not commercial standards of the synthesized esters were available, the esterification yields were calculated from the amount of residual free stanols according to the formula:

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

Sequential synthesis of acyl donors and stanol esters catalyzed by AMNP-CH-OPeR.

500 μ L of wastes of cooking olive oil was 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 others from the FAMES. Then, it was used as 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 μ m film thickness). To analyze the commercial sample of β -

130 sitostanol, the temperature program was: 180 °C to 205 °C (ramp rate 3 °C/min), then
131 to 220 °C (10 °C/min) maintaining this temperature for 5 min, and finally from 220 °C to
132 400 °C, keeping the final temperature for 5 min. Commercial methyl oleate and the
133 mixture of FAMES enzymatically synthesized in the laboratory were analyzed using a
134 program that started at 80 °C (4 min) and then raised to 300 °C (20 °C/min). The
135 compounds detected were identified using commercial standards, analyzed under
136 identical conditions to the samples or through their mass spectra using the NIST 2011
137 library and the ChemStation software (Agilent, Santa Clara, CA, USA).

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

145 Human HeLa cells from cervix carcinoma were continuously maintained in DMEM
146 enriched with high glucose and pyruvate. Non-small cell lung carcinoma A549 cells were
147 cultured in RPMI-1640 with L-glutamine at 37 °C in 5% CO₂. Both media were
148 supplemented with 10% fetal bovine serum, 10 U/mL penicillin, and 10 µg/mL
149 streptomycin. Cells were seeded in a 96-well plate at a density of 120,000 cells/mL for
150 HeLa and 160,000 cells/mL for A549 cells. Then, 80 µL were added per well and cultured
151 in 5% CO₂ at 37 °C for 24 h. Cells were then exposed to 20 µL serial dilutions of the four
152 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 in a MTT assay²² with some modifications Briefly, 20 μ L 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 in an automated Multiscan microplate reader (Spectra Max Plus 384, Molecular Devices, San Jose, CA, USA). Controls containing medium without cells were used as blanks and colchicine was the positive control ($\frac{1}{2}$ 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 plot of compound concentration against 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, USA).

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, 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's conformation and catalytic properties might change depending on the area implicated in the attachment, we have prepared and tested four versions of OPeR

immobilized on functionalized magnetic nanoparticles, whose characteristics are summarized in Table 1. The immobilization 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 positive to maintain 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 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 obtained according to 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. Schiff's bases are formed *via* reductive amination,⁴² probably by multiple points, and the oxidized carbohydrate chains act as slightly flexible linkers. This has a positive impact in

maintaining the enzyme conformation since the protein moiety is not attached in a rigid conformation, there are not 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), obtaining esterification yields 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 the biocatalysts tested transesterified both stanols in a similar way, yielding the corresponding oleic esters (Figure 1), which confirmed that the wide substrate specificity of OPer¹⁷ 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 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 to this behavior, the increase of the activity detected after recycling could be due to not only to the gradual accommodation of the enzyme to 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 papers 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 35-55 °C for 6-24 h.^{46–49} The recyclability, if evaluated, varied between 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 discarded 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 negative control and colchicine, a drug that depolymerizes microtubules and blocks the mitotic spindle, affecting cellular viability,⁵² as positive control.

According to the analysis of IC_{50} (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 in the other treatments are comparable to these. Hence, microscopic inspection of the cells supported the IC_{50} results. There are not observable differences between untreated and treated cells, while the micrographs of the colchicine control showed the massive presence of non-adherent cells.

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 character of fatty acid esters makes them an interesting choice for this and other processes devoted to the synthesis of added-value commodities.⁵³ However, the industrial production of FAMES relies in 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 synthesis of FAMES, but the product must be cost-competitive. Several lipases are able of producing FAMES, and OPeR has shown to do it sustainably, using triglycerides from cooking oil wastes in a solventless

reaction medium at ambient temperature.⁴² Thus, in the search of a process compliant with the principles of green chemistry, we explored the possibility of coupling triglycerides' 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 in the synthesis of FAMES⁴² 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 in 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 the 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 the 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 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 onwards (Figure 4c). This observation confirms 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 acyl donor, probably due to a positive effect of other components of the FAMEs 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 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 CRL⁴⁶ produced 74-96.3% sterol or stanol esters using cosolvents, and temperatures between 40-55 °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 435⁵⁴ produced 96% esters at 85 °C and using SC-CO₂, 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

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 biocatalyst, and stanols, wasted cooking oil and methanol as substrates. The whole procedure involves a cascade of two consecutive reactions, oil methanolysis and stanols transesterification, and the biocatalyst can be recycled for further reactions without activity loss. These results point to the suitability of this novel strategy to synthesize stanol esters by a green and sustainable procedure from non-expensive wastes as substrates, according to the current concept of circular economy.

313 **Abbreviations**

314 AMNP: magnetic nanoparticles functionalized with reactive amino groups in their
315 surface

316 AMNPs-GA-OPeR: OPeR covalently immobilized on AMNPs treated with glutaraldehyde
317 to leave reactive aldehyde surface groups

318 AMNPs-CH-OPeR: OPeR covalently immobilized, through aldehyde groups generated by
319 oxidation of its glycidic chains, on AMNPs

320 APTS: 3-aminopropyl)triethoxysilane

321 FAMES: fatty acid methyl esters

322 GC: gas chromatography

323 GC/MS: gas chromatography/mass spectrometry

324 mCLEAs-OPeR: magnetic cross-linked enzyme aggregates

325 MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

326 *p*NPB: *p*-nitrophenyl butyrate

327 OPeR: versatile lipase of *Ophiostoma piceae*

328 SDS: sodium dodecyl sulphate

329 SiMAG-Octyl-OPeR: OPeR non-covalently immobilized by hydrophobic interaction on
330 magnetic nanoparticles with octyl groups in their surface

331

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REFERENCES

- (1) Ostlund, R. Phytosterols, Cholesterol Absorption and Healthy Diets. *Lipids* **2007**, 42 (1), 41–45.
- (2) Musa-Veloso, K.; Poon, T. H.; Elliot, J. A.; Chung, C. A Comparison of the LDL-Cholesterol Lowering Efficacy of Plant Stanols and Plant Sterols over a Continuous Dose Range: Results of a Meta-Analysis of Randomized, Placebo-Controlled Trials. *Prostaglandins Leukot. Essent. Fat. Acids* **2011**, 85 (1), 9–28.
- (3) He, W.-S.; Zhu, H.; Chen, Z.-Y. Plant Sterols: Chemical and Enzymatic Structural Modifications and Effects on Their Cholesterol-Lowering Activity. *J. Agric. Food Chem.* **2018**, 66 (12), 3047–3062.
- (4) Molina-Gutiérrez, M.; Hakalin, N. L. S.; Rodríguez-Sánchez, L.; Prieto, A.; Martínez, M. J. Green Synthesis of β -Sitostanol Esters Catalyzed by the Versatile Lipase/Sterol Esterase from *Ophiostoma piceae*. *Food Chem.* **2017**, 221, 1458–1465.
- (5) He, W. Sen; Li, L.; Zhao, J.; Xu, H.; Rui, J.; Cui, D.; Li, H.; Zhang, H.; Liu, X. Candida

358 Sp. 99-125 Lipase-Catalyzed Synthesis of Ergosterol Linolenate and Its
 359 Characterization. *Food Chem.* **2019**, *280*, 286–293.

360 (6) Negishi, S.; Hidaka, I.; Takahashi, I.; Kunita, S. Transesterification of Phytosterol
 361 and Edible Oil by Lipase Powder at High Temperature. *JAOCs, J. Am. Oil Chem.*
 362 *Soc.* **2003**, *80* (9), 905–907.

363 (7) Torrelo, G.; Torres, C. F.; Reglero, G. Enzymatic Strategies for Solvent-Free
 364 Production of Short and Medium Chain Phytosteryl Esters. *Eur. J. Lipid Sci.*
 365 *Technol.* **2012**, *114* (6), 670–676.

366 (8) Villeneuve, P.; Turon, F.; Caro, Y.; Escoffier, R.; Baréa, B.; Barouh, B.; Lago, R.;
 367 Piombo, G.; Pina, M. Lipase-Catalyzed Synthesis of Canola Phytosterols Oleate
 368 Esters as Cholesterol Lowering Agents. *Enzyme Microb. Technol.* **2005**, *37* (1),
 369 150–155.

370 (9) Vu, P.-L.; Shin, J.-A.; Lim, C.-H.; Lee, K.-T. Lipase-Catalyzed Production of
 371 Phytosteryl Esters and Their Crystallization Behavior in Corn Oil. *Food Res. Int.*
 372 **2004**, *37* (2), 175–180.

373 (10) Weber, N.; Weitkamp, P.; Mukherjee, K. D. Steryl and Stanyl Esters of Fatty Acids
 374 by Solvent-Free Esterification and Transesterification in Vacuo Using Lipases from
 375 *Rhizomucor miehei*, *Candida antarctica*, and *Carica papaya*. *J. Agric. Food Chem.*
 376 **2001**, *49* (11), 5210–5216.

377 (11) Dong, Z.; Liu, Z.; Shi, J.; Tang, H.; Xiang, X.; Huang, F.; Zheng, M. Carbon
 378 Nanoparticle-Stabilized Pickering Emulsion as a Sustainable and High-
 379 Performance Interfacial Catalysis Platform for Enzymatic
 380 Esterification/Transesterification. *ACS Sustain. Chem. Eng.* **2019**, *7* (8), 7619–

7629.

- (12) Xiao, Y.; Zheng, M.; Liu, Z.; Shi, J.; Huang, F.; Luo, X. Constructing a Continuous Flow Bioreactor Based on a Hierarchically Porous Cellulose Monolith for Ultrafast and Nonstop Enzymatic Esterification/Transesterification. *ACS Sustain. Chem. Eng.* **2019**, 7 (2), 2056–2063.
- (13) Sheldon, R. A.; van Pelt, S. Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chem. Soc. Rev.* **2013**, 42 (15), 6223–6235.
- (14) Ansorge-Schumacher, M. B.; Thum, O. Immobilised Lipases in the Cosmetics Industry. *Chem. Soc. Rev.* **2013**, 42 (15), 6475–6490.
- (15) Turcheniuk, K.; Tarasevych, A.; Kukhar, V.; Boukherroub, R.; Szunerits, S. Recent Advances in Surface Chemistry Strategies for the Fabrication of Functional Iron Oxide Based Magnetic Nanoparticles. *Nanoscale* **2013**, 5 (22), 10729–10752.
- (16) Xu, J.; Sun, J.; Wang, Y.; Sheng, J.; Wang, F.; Sun, M. Application of Iron Magnetic Nanoparticles in Protein Immobilization. *Molecules* **2014**, 19 (8), 11465–11486.
- (17) Barriuso, J.; Vaquero, M. E.; Prieto, A.; Martínez, M. J. Structural Traits and Catalytic Versatility of the Lipases from the *Candida rugosa*-like Family: A Review. *Biotechnol. Adv.* **2016**, 34 (5), 874–885.
- (18) Hakalin, N. L. S.; Molina-Gutiérrez, M.; Prieto, A.; Martínez, M. J. Optimization of Lipase-Catalyzed Synthesis of β -Sitostanol Esters by Response Surface Methodology. *Food Chem.* **2018**, 261, 139–148.
- (19) Barriuso, J.; Prieto, A.; Martínez, M. J. Fungal Genomes Mining to Discover Novel Sterol Esterases and Lipases as Catalysts. *BMC Genomics* **2013**, 14 (1).
- (20) Vaquero, M. E.; Prieto, A.; Barriuso, J.; Martínez, M. J. Expression and Properties

- 404 of Three Novel Fungal Lipases/Sterol Esterases Predicted in Silico: Comparison
405 with Other Enzymes of the *Candida rugosa*-like Family. *Appl. Microbiol.*
406 *Biotechnol.* **2015**, *99* (23), 10057–10067.
- 407 (21) Molina-Gutiérrez, M.; Hakalin, N.; Rodríguez-Sánchez, L.; Alcaraz, L.; López, F.;
408 Martínez, M.; Prieto, A. Effect of the Immobilization Strategy on the Efficiency and
409 Recyclability of the Versatile Lipase from *Ophiostoma piceae*. *Molecules* **2019**, *24*
410 (7), 1313.
- 411 (22) Mosmann, T. Rapid Colorimetric Assay for Cellular Growth and Survival:
412 Application to Proliferation and Cytotoxicity Assays. *J. Immunol. Methods* **1983**,
413 *65* (1–2), 55–63.
- 414 (23) Shuai, W.; Das, R. K.; Naghdi, M.; Brar, S. K.; Verma, M. A Review on the Important
415 Aspects of Lipase Immobilization on Nanomaterials. *Biotechnol. Appl. Biochem.*
416 **2017**, *64* (4), 496–508.
- 417 (24) Cao, L. *Carrier-bound Immobilized Enzymes. Principles, Applications and Design*;
418 Wiley-VCH: Weinheim, Germany, 2005.
- 419 (25) Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Rodrigues, R. C.;
420 Fernandez-Lafuente, R. Glutaraldehyde in Bio-Catalysts Design: A Useful
421 Crosslinker and a Versatile Tool in Enzyme Immobilization. *RSC Adv.* **2014**, *4* (4),
422 1583–1600.
- 423 (26) Betancor, L.; López-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Mateo, G. D.-O.
424 C.; Fernández-Lafuente, R.; Guisán, J. M. Different Mechanisms of Protein
425 Immobilization on Glutaraldehyde Activated Supports: Effect of Support
426 Activation and Immobilization Conditions. *Enzyme Microb. Technol.* **2006**, *39* (4),

- 427 877–882.
- 428 (27) Kim, M.; Park, J. M.; Um, H. J.; Lee, D. H.; Lee, K. H.; Kobayashi, F.; Iwasaka, Y.;
 429 Hong, C. S.; Min, J.; Kim, Y. H. Immobilization of Cross-Linked Lipase Aggregates
 430 onto Magnetic Beads for Enzymatic Degradation of Polycaprolactone. *J. Basic*
 431 *Microbiol.* **2010**, *50* (3), 218–226.
- 432 (28) Zhang, Y.; Ge, J.; Liu, Z. Enhanced Activity of Immobilized or Chemically Modified
 433 Enzymes. *ACS Catal.* **2015**, *5* (8), 4503–4513.
- 434 (29) Cruz-Izquierdo, Á.; Picó, E. A.; López, C.; Serra, J. L.; Llama, M. J. Magnetic Cross-
 435 Linked Enzyme Aggregates (MCLEAs) of *Candida antarctica* Lipase: An Efficient
 436 and Stable Biocatalyst for Biodiesel Synthesis. *PLoS One* **2014**, *9* (12), e115202.
- 437 (30) Barbosa, O.; Torres, R.; Ortiz, C.; Fernandez-Lafuente, R. Versatility of
 438 Glutaraldehyde to Immobilize Lipases: Effect of the Immobilization Protocol on
 439 the Properties of Lipase B from *Candida Antarctica*. *Process Biochem.* **2012**, *47*
 440 (8), 1220–1227.
- 441 (31) Cui, Y.; Li, Y.; Yang, Y.; Liu, X.; Lei, L.; Zhou, L.; Pan, F. Facile Synthesis of Amino-
 442 Silane Modified Superparamagnetic Fe₃O₄ Nanoparticles and Application for
 443 Lipase Immobilization. *J. Biotechnol.* **2010**, *150* (1), 171–174.
- 444 (32) Rodrigues De Melo, R.; Alnoch, R. C.; Ferreira Lopes Vilela, A.; Maltempi De Souza,
 445 E.; Krieger, N.; Ruller, R.; Sato, H. H.; Mateo, C. New Heterofunctional Supports
 446 Based on Glutaraldehyde-Activation: A Tool for Enzyme Immobilization at Neutral
 447 PH. *Molecules* **2017**, *22* (7).
- 448 (33) Xie, W.; Ma, N. Immobilized Lipase on Fe₃O₄ Nanoparticles as Biocatalyst for
 449 Biodiesel Production. *Energy & Fuels* **2009**, *23* (3), 1347–1353.

- 450 (34) Netto, C. G. C. M.; Toma, H. E.; Andrade, L. H. Superparamagnetic Nanoparticles
451 as Versatile Carriers and Supporting Materials for Enzymes. *Journal of Molecular*
452 *Catalysis B: Enzymatic*. Elsevier January 2013, pp 71–92.
- 453 (35) Liu, Y.; Guo, C.; Liu, C.-Z. Novel Magnetic Cross-Linked Lipase Aggregates for
454 Improving the Resolution of (R, S)-2-Octanol. *Chirality* **2015**, 27 (3), 199–204.
- 455 (36) Gricajeva, A.; Kazlauskas, S.; Kalėdienė, L.; Bendikienė, V. Analysis of *Aspergillus*
456 *Sp.* Lipase Immobilization for the Application in Organic Synthesis. *Int. J. Biol.*
457 *Macromol.* **2018**, 108, 1165–1175.
- 458 (37) Arana-Peña, S.; Lokha, Y.; Fernández-Lafuente, R. Immobilization on Octyl-
459 Agarose Beads and Some Catalytic Features of Commercial Preparations of Lipase
460 a from *Candida antarctica* (Novocor ADL): Comparison with Immobilized Lipase B
461 from *Candida antarctica*. *Biotechnol. Prog.* **2019**, 35 (1).
- 462 (38) Fernandez-Lopez, L.; Pedrero, S. G.; Lopez-Carrobles, N.; Gorines, B. C.; Virgen-
463 Ortíz, J. J.; Fernandez-Lafuente, R. Effect of Protein Load on Stability of
464 Immobilized Enzymes. *Enzyme Microb. Technol.* **2017**, 98, 18–25.
- 465 (39) Rodrigues, R. C.; Virgen-Ortíz, J. J.; dos Santos, J. C. S.; Berenguer-Murcia, Á.;
466 Alcantara, A. R.; Barbosa, O.; Ortiz, C.; Fernandez-Lafuente, R. Immobilization of
467 Lipases on Hydrophobic Supports: Immobilization Mechanism, Advantages,
468 Problems, and Solutions. *Biotechnology Advances*. Elsevier April 2019, pp 746–
469 770.
- 470 (40) Tacias-Pascacio, V. G.; Virgen-Ortíz, J. J.; Jiménez-Pérez, M.; Yates, M.;
471 Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Evaluation
472 of Different Lipase Biocatalysts in the Production of Biodiesel from Used Cooking

- 473 Oil: Critical Role of the Immobilization Support. *Fuel* **2017**, *200*, 1–10.
- 474 (41) Zaak, H.; Siar, E. H.; Kornecki, J. F.; Fernandez-Lopez, L.; Pedrero, S. G.; Virgen-
 475 Ortíz, J. J.; Fernandez-Lafuente, R. Effect of Immobilization Rate and Enzyme
 476 Crowding on Enzyme Stability under Different Conditions. The Case of Lipase from
 477 *Thermomyces Lanuginosus* Immobilized on Octyl Agarose Beads. *Process*
 478 *Biochem.* **2017**, *56*, 117–123.
- 479 (42) Prieto, A. M.; Molina-Gutiérrez, M.; Martínez, Á. T.; Martínez, M. J. Synthesis of
 480 Biodiesel Catalysed by an Enzymatic Crude Immobilised on Magnetic Particles,
 481 2018.
- 482 (43) Knezevic, Z.; Milosavic, N.; Bezbradica, D.; Jakovljevic, Z.; Prodanovic, R.
 483 Immobilization of Lipase from *Candida Rugosa* on Eupergit® C Supports by
 484 Covalent Attachment. *Biochem. Eng. J.* **2006**, *30* (3), 269–278.
- 485 (44) Chaibakhsh, N.; Basri, M.; Rahman, M. B. A.; Adnani, A.; Salleh, A. B. Lipase-
 486 Catalyzed Synthesis of Ergosterol Ester. *Biocatal. Agric. Biotechnol.* **2012**, *1* (1),
 487 51–56.
- 488 (45) Hammann, S.; Lehnert, K.; Vetter, W. Esterified Sterols and Their Contribution to
 489 the Total Sterols in Edible Mushrooms. *J. Food Compos. Anal.* **2016**, *54*, 48–54.
- 490 (46) Zheng, M.-M.; Dong, L.; Lu, Y.; Guo, P.-M.; Deng, Q.-C.; Li, W.-L.; Feng, Y.-Q.;
 491 Huang, F.-H. Immobilization of *Candida rugosa* Lipase on Magnetic Poly(Allyl
 492 Glycidyl Ether-Co-Ethylene Glycol Dimethacrylate) Polymer Microsphere for
 493 Synthesis of Phytosterol Esters of Unsaturated Fatty Acids. *J. Mol. Catal. B Enzym.*
 494 **2012**, *74* (1–2), 16–23.
- 495 (47) Zheng, M.-M.; Lu, Y.; Huang, F.-H.; Wang, L.; Guo, P.-M.; Feng, Y.-Q.; Deng, Q.-C.

496 Lipase Immobilization on Hyper-Cross-Linked Polymer-Coated Silica for
 497 Biocatalytic Synthesis of Phytosterol Esters with Controllable Fatty Acid
 498 Composition. *J. Agric. Food Chem.* **2013**, 61 (1), 231–237.

499 (48) Zheng, M. M.; Lu, Y.; Dong, L.; Guo, P. M.; Deng, Q. C.; Li, W. L.; Feng, Y. Q.; Huang,
 500 F. H. Immobilization of *Candida rugosa* Lipase on Hydrophobic/Strong Cation-
 501 Exchange Functional Silica Particles for Biocatalytic Synthesis of Phytosterol
 502 Esters. *Bioresour. Technol.* **2012**, 115, 141–146.

503 (49) Shang, C. Y.; Li, W. X.; Zhang, R. F. Immobilization of *Candida rugosa* Lipase on ZnO
 504 Nanowires/Macroporous Silica Composites for Biocatalytic Synthesis of
 505 Phytosterol Esters. *Mater. Res. Bull.* **2015**, 68, 336–342.

506 (50) He, W.-S.; Cui, D.-D.; Zhang, Y.-L.; Liu, Y.; Yin, J.; Chen, G.; Jia, C.-S.; Feng, B. Highly
 507 Efficient Synthesis of Phytosterol Linolenate Catalyzed by *Candida rugosa* Lipase
 508 through Transesterification. *Food Sci. Technol. Res.* **2017**, 23 (4), 525–533.

509 (51) Miao, M.; Liu, H.; Jiang, B.; Yang, C.; Xia, X.; Zhang, T. Enzyme-Catalysed Synthesis
 510 of Plant Steryl Laurate in Non-Aqueous Media Using Salt Hydrate Pairs and Its
 511 Characterisation. *J. Funct. Foods* **2014**, 7 (0), 452–461.

512 (52) Buey, R. M.; Barasoain, I.; Jackson, E.; Meyer, A.; Giannakakou, P.; Paterson, I.;
 513 Mooberry, S.; Andreu, J. M.; Díaz, J. F. Microtubule Interactions with Chemically
 514 Diverse Stabilizing Agents: Thermodynamics of Binding to the Paclitaxel Site
 515 Predicts Cytotoxicity. *Chem. Biol.* **2005**, 12 (12), 1269–1279.

516 (53) Lee, S.; Yuk, J. S.; Park, H.; Kim, Y. W.; Shin, J. Multiblock Thermoplastic Elastomers
 517 Derived from Biodiesel, Poly(Propylene Glycol), and L-Lactide. *ACS Sustain. Chem.*
 518 *Eng.* **2017**, 5 (9), 8148–8160.

- 519 (54) Hu, L.; Llibin, S.; Li, J.; Qi, L.; Zhang, X.; Yu, D.; Walid, E.; Jiang, L. Lipase-Catalyzed
520 Transesterification of Soybean Oil and Phytosterol in Supercritical CO₂. *Bioprocess*
521 *Biosyst. Eng.* **2015**, 38 (12), 2343–2347.
- 522 (55) Sengupta, A.; Pal, M.; Silroy, S.; Ghosh, M. Comparative Study of Sterol Ester
523 Synthesis Using *Thermomyces lanuginosus* Lipase in Stirred Tank and Packed-Bed
524 Bioreactors. *JAOCS, J. Am. Oil Chem. Soc.* **2010**, 87 (9), 1019–1025.
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Figure captions

Figure 1. Scheme of the reaction of transesterification of 80 mM β -sitostanol and methyl oleate catalyzed by immobilized OPEr. Methyl oleate acts as solvent and as donor of oleic acid. Once completed the reaction, the catalyst is retained in the reaction vessel in seconds, with the help of a Nd magnet. The reaction mixture can be recovered, free of protein, for further purification of the products. The GC chromatograms of samples analyzed at 0 h and 15 h are shown at the bottom of the figure. The signal has been magnified from minute 8.5 onwards (x10) to improve visualization of the peaks of free stanols and stanol esters.

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 of stanols with commercial methyl oleate (99%). Reactions were performed at 25 °C for 15 h. b) Detail of the transesterification yields of the four nanobiocatalysts after 2 h of reaction in the fourth and fifth cycles. R1-R5: reaction cycles 1 to 5.

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.

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 (x 5)

549 from minute 7 onwards to improve visualization of the peaks of free stanols and stanol
550 esters. c) Recycling of the catalyst: % of stanol esters produced in 2 h of
551 transesterification across five successive reaction cycles. R1-R5: reaction cycles 1 to 5.
552
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554 Tables

555 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
	AMNP-GA-OPEr	65 ± 8	334 ± 5
Covalent	mCLEAS-OPEr	99 ± 2	769 ± 58
	AMNP-CH-OPEr	96 ± 3	709 ± 19

556 ^aImmob. yield (%) = (immobilized activity/starting activity) x 100

557 ^bThe specific activity of the different preparations with the versatile lipase attached to
 558 magnetic nanoparticles was determined using *p*NPB as substrate.

559

560 Table 2. IC50 values of nanobiocatalysts extract and colchicine as standard control.

Extract/compound	IC50 HeLa	IC50 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

561 ^aPositive control
562

1. Lipase immobilization

Magnetite nanoparticles

+

Lipase
OPeR

2. Green synthesis of stanol esters

+ Methanol

25 °C

Recovery of the
catalyst with a magnet

+ FAMES
mixture

+ Stanols

25 °C

Stanol
esters

Fig. 1

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567

568 **Fig. 2**

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573 **Fig. 3**

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577 **Fig. 4**