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Optimization of lipase-catalyzed synthesis of β-sitostanol esters by response surface methodology

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ABSTRACT

The esters of β-sitostanol and fatty acids are known for their effect as cholesterol-lowering agents. In this work, the efficiency of three lipases as biocatalysts of the esterification of β-sitostanol and C16 and C18 fatty acids was compared. The sterol esterase of *Ophiostoma piceae* (OPEr) yielded the highest esterification rates and was selected for further optimization of the reaction. The effects of four parameters (temperature, enzymatic dosage, acyl donor concentration, and reaction time) on ester synthesis were investigated and the process conditions were optimized using response surface methodology (RSM). The best conditions for esterification for each fatty acid were predicted using a second-order model, and experimentally validated. Very high esterification efficiencies (86–97%) were observed using the predicted values for the four variables. This approach was shown to be suitable for optimizing the enzymatic production of β-sitostanol esters, which represents a green alternative to the chemical synthesis of these dietary complements.

1. Introduction

Clinical studies have shown a significant positive correlation between the incidence of cardiovascular disease (CVD) in humans and increased levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) (He et al., 2010). In this sense, plant sterols and their saturated forms (stanols) have been added to different food matrixes to serve as cholesterol lowering agents (Weingärtner et al., 2017). Due to their chemical and structural similarity to cholesterol, both phytosterols and phytostanols can compete with it, reducing its absorption (Ostlund, 2007). However, the information on the bioactivity of these compounds is controversial, especially for sterols. According to Musa-Veloso, Poon, Elliot and Chung (2011), free sterols produce only a slight reduction in serum cholesterol. Similarly, Weingärtner et al. (2017) reported no significant change in cholesterol levels in serum upon daily supplementation with 3 g of plant sterol esters. On the contrary, the effectiveness of β-sitostanol, the saturated β-sitosterol derivative, seems to be much higher, but this compound is virtually unabsorbable, as with other plant sterols (Miettinen, Puska, Gylling, Vanhanen & Vartiainen, 1995). However, the cholesterol-lowering effect of sitostanol may be increased when it is ingested as a fatty-acid ester, probably due to its higher bioavailability. Cater, Garcia-Garcia, Vega and Grundy (2005) showed that a daily intake of 2, 3, and 4 g of stanol esters reduces LDL-C by 12, 13, and 14%, respectively. These results agree with the data of Miettinen et al. (1995), which confirmed that substituting part of the daily fat intake by margarine enriched in sitostanol esters produced a decrease in serum TC and LDL-C in individuals with mild hypercholesterolemia.

On the other hand, sterol and stanol esters have semi-liquid consistency and properties comparable to those of edible fats and oils, which make them ideal to be added to fat-containing foods. Considering all these factors, the use of stanol esters as dietary complements may have an advantage over free stanols, free sterols, and sterol esters, to reduce hypercholesterolemia.

The most commonly used process to synthesize phytosterol esters is based in chemical esterification with alkali catalysts. This process is carried out at high temperature, which involves high-energy consumption, browning of products, low selectivity and generation of undesirable by-products (He et al., 2010). In this context, biocatalysis represents a green alternative for the synthesis of these compounds (He et al., 2017). Particularly in the food sector, the application of clean processes based on white biotechnology would be a key factor for

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producing such esters under mild conditions, as the only reaction products, and precluding the use of strong or toxic reagents, which is not possible for chemical catalysis (Feltes, Oliveira, Block & Ninow, 2012). Most research on this subject has been conducted using lipases as catalysts and fatty acids and phytosterols as substrates (Barba Cedillo et al., 2013; Miao et al., 2014; Panpipat, Xu, & Guo, 2013; Villeneuve et al., 2005; Vu, Shin, Lin & Lee, 2004; Weber, Weitkamp & Mukherjee, 2001). However, the reports on the enzymatic synthesis of phytostanyl esters are limited to the use of some commercial lipases (He et al., 2010), and the native (OPE) and recombinant (OPEr) versatile lipase/sterol esterase of *Ophiostoma piceae* (Molina-Gutiérrez, Hakalin, Rodríguez-Sanchez, Prieto & Martínez, 2017). Both OPE and OPEr have already been fully characterized (Barba Cedillo, Plou & Martínez, 2012; Barriuso, Vaquero, Prieto & Martinez, 2016; Calero-Rueda, Plou, Ballesteros, Martínez, & Martínez, 2002; Vaquero, Barriuso, Martínez & Prieto, 2016), and their efficiency for catalyzing the direct acylation of a mixture of soybean sterols and lauric acid was gathered in an application patent (Barba Cedillo et al., 2013). More recently, Molina-Gutiérrez et al. (2017) demonstrated that OPEr catalyzed the synthesis of β-sitostanyl esters more effectively than OPE and the commercial lipase from *Candida rugosa* (CRL).

The application of statistical tools, such as response surface methodology (RSM), can be a very interesting strategy to maximize the enzymatic production of these esters by optimizing operational factors. The use of this methodology involves the construction of quadratic models for the response variable as a function of each of the selected parameters. Since the interaction between variables can be determined by statistical techniques, RSM reduces the number of experiments or repetitions and improves the quality of the information obtained from the results, compared to the individual study of each variable.

This study is aimed at optimizing an enzymatic procedure for direct esterification of β-sitostanol with five fatty acids frequently found in plant biomass. The catalyst for this reaction was selected after evaluation of the efficiency of three enzymes: two commercial lipases from *Candida antarctica* (CalA and CalB), and OPEr. The effects of four variables (temperature, enzymatic dosage, acyl donor concentration, and reaction time) on the esterification rates will be examined using central composite design (CCD) as the experimental design for RSM. This strategy is compatible with the number of variables studied and allowed obtaining a predictive model. The best conditions for catalyzing β-sitostanol esterification with each one of the acyl donors studied were identified through a second-order model, and the predicted efficiencies were experimentally validated.

2. Material and methods

2.1. Chemicals and reagents

β-Sitostanol, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The enzymes NS 40020 (CalA) and Lipozyme® (CalB) were kindly provided by Novozymes. Other chemicals and solvents were of the purest available grade, provided by Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Yeast strain, culture conditions and crude OPEr production

Pichia pastoris GS115 strain containing the *ope* gene from the fungus *O. piceae* was cultivated for OPEr production as previously reported (Vaquero, Prieto, Barriuso & Martínez, 2015). Single colonies from the transformed *P. pastoris* plates were inoculated in 250 ml flasks with 20 ml YEPS medium. The flasks were incubated at 28 °C and 250 rpm. One-liter flasks with 100 ml of YEPS medium, inoculated with 3.5 ml of fresh cultures grown overnight in YEPS (OD_{600nm} 6–8), were incubated at 28 °C and 250 rpm. Methanol (0.5% p/v) was added daily for maintaining protein induction. When maximum activity was reached (generally after 4–6 days) the cells were harvested by centrifugation (10,000 rpm at 4 °C). Thereafter, supernatants were concentrated by sequential ultrafiltration in a Minissette filter (Pall-Filtron, Northborough, MA, USA) with a 10-kDa membrane and an YM3 Amicon device (Merck Millipore, Darmstadt, Germany). The obtained crude products were used without further purification.

2.3. Measurement of enzyme activity and protein content

The activity of the three catalysts (OPEr, CalA and CalB) was measured by monitoring *p*-nitrophenol release from hydrolysis of 1.5 mM *p*-nitrophenyl butyrate (*p*NPB) in 20 mM Tris-HCl pH 7.0 at room temperature, using a Shimadzu UV-160A spectrophotometer set at 410 nm. One unit of activity (1 U) is defined as the amount of crude enzymatic extract required to release 1 µmol of *p*-nitrophenol (ε is 15,200 M^{-1} cm^{-1}) per minute (Calero-Rueda et al., 2002). The BCA Protein Assay (Thermo Scientific) was used to determine protein concentration, using bovine serum albumin as standard.

2.4. Comparison of OPEr, CalA and CalB as catalysts of direct esterification of β-sitostanol with different acyl donors

As β-sitostanol is pretty insoluble, a diluted solution (7.5 mg ml^{-1}) in isooctane was first prepared, and the volume containing the final amount required for the reaction was dispensed into an empty vial. On the other hand, 60 mM solutions in isooctane of each acyl donor (palmitic, stearic, oleic, linoleic or linolenic acid) were prepared, and the appropriate volume dispensed in the reaction tube with β-sitostanol. Cholesten-3-one was always added to the reaction medium, as an internal standard for quantification of the residual β-sitostanol by gas chromatography. This compound has similar solubility to β-sitostanol and does not affect enzymes' activity. Isooctane was then added until a final volume of 500 µl. In order to achieve a homogeneous solution, the mix inside screw-capped glass vials was heated at 60 °C for 15 min and tempered before adding the catalyst dissolved in buffer (100 µl).

The standard reactions were performed at 28 °C, for a maximum period of 48 h, in isooctane:water biphasic systems. The reaction mixture contained equimolar amounts (10 mM) of the substrates in 500 µl of the organic solvent, and 6 U_{pNPB} ml^{-1} of the catalyst in 100 µl Tris-HCl buffer (20 mM, pH 7). The reactions were agitated in a Multi Bio RS-24 rotator set up at 100 rpm, with vertical rotation programmed at 10 s-cycles of 360° followed by of 30 s-cycles of 90°. Samples (10 µl) were withdrawn at regular intervals to follow the course of reactions. The effect of increasing temperature on synthesis of β-sitostanol esters was evaluated under the same experimental conditions at 40 °C.

2.5. Optimization of the synthesis of β-sitostanol esters by direct esterification

Central Composite Design (CCD) was selected as experimental RSM design for optimization of the enzymatic esterification of β-sitostanol with each acyl donor. Four independent variables were examined in different ranges (Table 1): temperature (20–36 °C), enzymatic dosage (3–15 U ml^{-1}), acyl donor concentration (4–60 mM) and reaction time (10–490 min). The experimental ranges −1 and +1 were chosen taking into account the results obtained previously (Molina-Gutiérrez et al., 2017). Four replicates of the center point (CP, coded as 0) were carried out, adding axial points in order to adjust the experimental results to a second-order model. The distance of the axial points from the central point is given by $\alpha = (2^K)^{1/4}$, where K is the number of variables, then $\alpha = 2$ for four variables. In general, in a CCD with two levels, the design is composed of 2^K factorial points plus $(2 \times K)$ axial points plus an arbitrary number of central points (Kim & Akoh, 2007). Then, each CCD was composed of 28 runs. The response variable was the synthesis efficiency for β-sitostanyl palmitate, β-sitostanyl stearate, β-sitostanyl oleate, β-sitostanyl linoleate or β-sitostanyl linolenate for palmitic,

Table 1
Variables and levels used in the Central Composite Design (CCD) for optimization of β-sitostanol ester synthesis catalyzed by OPEr.

Independent variable		Axial	Min	CP	Max	Axial
		−2	−1	0	+1	+2
Temperature (°C)	(X_1)	20	24	28	32	36
Enzymatic dosage (U ml^{-1})	(X_2)	3	6	9	12	15
Acyl donor (mM)	(X_3)	4	18	32	46	60
Time (min)	(X_4)	10	130	250	370	490

CP = center point; *Min* = minimum factorial level; *Max* = maximum factorial level.

stearic, oleic, linoleic or linolenic acid, respectively. The reactions were prepared and conducted as explained in Section 2.4, but the acyl donor concentration, temperature and reaction time of each run were set according to the CCD configuration. The statistical analysis of the results was performed using Design-Expert® software version 10 (Stat-Ease, Inc), to estimate the main effects. The optimum conditions to maximize the response variables were defined according to the model, and the predicted values were experimentally validated.

2.6. Gas-chromatography (GC) analysis

10 µl-aliquots from the organic phase of the above reactions were periodically withdrawn (the specific times are stated in each individual experiment) and diluted 5 times in isooctane into a clean autosampler vial. 1 µl of this diluted solution was injected in a 7890A gas chromatograph (Agilent, Palo Alto, CA). Injector and flame ionization detector were set up at 350 °C, and He (20 psi) was used as the carrier gas. The separation was carried out using a fused-silica capillary column SPB-1 (5 m × 250 µm × 0.25 µm, Supelco, Bellefonte, PA). The oven was maintained at 115 °C for 1 min and then a two-step temperature program was applied, with a 10 °C min^{-1} ramp rate to 170 °C, and a second ramp rate of 20 °C min^{-1} to reach a final temperature of 350 °C held for 4 min. The peak of β-sitostanol was identified from its retention time, compared to that of a commercial standard. A calibration curve of this compound was elaborated from the peak areas of the analyte and the internal standard. The esterification yields across the reaction times were calculated from the amount of residual free β-sitostanol.

3. Results and discussion

3.1. Direct esterification of β-sitostanol and fatty acids catalyzed by OPEr, CalA and CalB

The effectiveness of OPE and OPEr for direct esterification of a mixture of soybean sterols and oleic or lauric acid at 28 °C was previously described in a patent (Barba Cedillo et al., 2013). According to this report, these enzymes showed better performance than the commercial *C. rugosa* lipase (CRL). The effect of some reaction conditions, such as solvent, enzyme dose and molar excess of fatty acid, were also analyzed, reporting the best esterification yields in isooctane/water (< 10% water). The authors observed the highest acylation levels (85%) in 48 h using doses of OPE or OPEr over 3 U ml^{-1}, without increasing the yields with higher enzyme dosages. Recently, β-sitostanol esters have been synthesized at 28 °C *via* direct esterification or transesterification catalyzed by OPE and OPEr (Molina-Gutiérrez et al., 2017). In this report, the standard reactions of direct esterification were conducted in a biphasic isooctane:water system containing 10 mM β-sitostanol and lauric or oleic acid as acyl donors, reaching 90% esterification in 3 h with OPEr. The use of molar excesses of the free fatty acids did not improve the esterification rate, and the enzyme did not convert one of the acyl donors preferentially when both were simultaneously available. Then, these basal experimental conditions were selected to evaluate the ability of OPEr, CalA and CalB to esterify β-sitostanol with palmitic, stearic, oleic, linoleic or linolenic acids. These fatty acids are widely represented in the lipid fraction of plant wastes, which can be used as non–expensive sources of acyl donors for this reaction, contributing to their recycling and valorization. However, although these fatty acids have close structural resemblances, they differ in their chain-length and/or in the presence and number of double bonds, and these factors may condition the efficiency of the enzymatic conversion. Then, it is relevant to find their individual optimal reaction conditions for producing β-sitostanol esters.

The efficiency of the three enzymes for catalyzing direct acylation of β-sitostanol was first evaluated at 28 °C, and either saturated (palmitic acid, C16:0; stearic acid, C18:0), mono-unsaturated (oleic acid, C18:1), di-unsaturated (linoleic acid, C18:2) or tri-unsaturated (linolenic acid, C18:3) fatty acids were used as acyl donors.

The lipase CalB did not succeed in catalyzing direct esterification of β-sitostanol with any of the donors assayed. No products were detected at 24 h, and less of 15% esters were found in 48 h-reactions (14.2 ± 0.8, 12.0 ± 0.3, 13.5 ± 1.9, 2.8 ± 0.5, 12.6 ± 1.6% for palmitic, stearic, oleic, linoleic and linolenic acids, respectively). The low sterol esterase activity of this enzyme has already been reported (Panpipat et al., 2013; Villeneuve et al., 2005; Weber et al., 2001). On the other hand, both OPEr and CalA were good catalysts for this reaction, although their actions on these substrates were shown to be different.

Even after very short intervals, OPEr was highly efficient-catalyzing direct esterification of β-sitostanol at 28 °C with all fatty acids assayed (Fig. 1a). A high esterification rate was observed in the first 30 min, reaching around 80% conversion in 3 h. The reaction rates during the first 15 min demonstrated that palmitic acid was the best donor for OPEr and linoleic acid the worst. Considering that the conversion of all substrates did not follow analogous profiles, the performance of OPEr seemed to be significantly influenced by the chain length and/or number of double bonds of the acyl donor. He et al. (2010) reported similar findings, showing that the efficiency of the immobilized lipase CalB from *C. antarctica* (Novozym 435) was inversely correlated with the chain length of the acyl donor, probably due to steric effects derived from the size of the substrates. For that study, only saturated fatty acids from 12 to 18 C-atoms were evaluated as acyl donors. However, the current work involves the use of mono-, di-, and tri-unsaturated C18 fatty acids, and the number and position of the double bonds are probably additional factors affecting their individual spatial arrangement and thus their access to the active center of the enzyme. However, despite the differences observed at these short reaction times, from 3 h onwards the esterification efficiencies were similar for the five donors and the maximum conversion was achieved in 6 h with OPEr (Fig. 1b).

The synthesis of stanol esters catalyzed by CalA at 28 °C resulted in poor conversions at short reaction times (Fig. 1c), with much lower esterification rates than those observed when OPEr was the catalyst. The esters' yields in 3 h-reactions reached 70–80% for sitostanyl palmitate and stearate, 32–34% for sitostanyl oleate and linolenate, and around 18% for sitostanyl linoleate, showing again that this is the worst of the donors evaluated. As can be observed in Fig. 1d, the maximum efficiencies of esterification of β-sitostanol with palmitic or stearic acid were achieved at 6 h, while the synthesis efficiency of the oleic, linoleic or linolenic esters showed an increasing tendency across the 48 h. At the final reaction time, the esters' yields produced by both catalysts were comparable except for sitostanyl linoleate, whose production was significantly lower by using CalA. Therefore, at short reaction times, the lipase A from *C. antarctica* was influenced by the chain length of the fatty acids used for direct esterification of β-sitostanol, which agrees with the data reported by Panpipat et al. (2013). On the other hand, the synthesis rate was much faster for the saturated than for the unsaturated fatty acids. This was also observed in OPEr-catalyzed reactions although to a lower extent, suggesting a steric problem for the access of the unsaturated substrates to the active centers of both

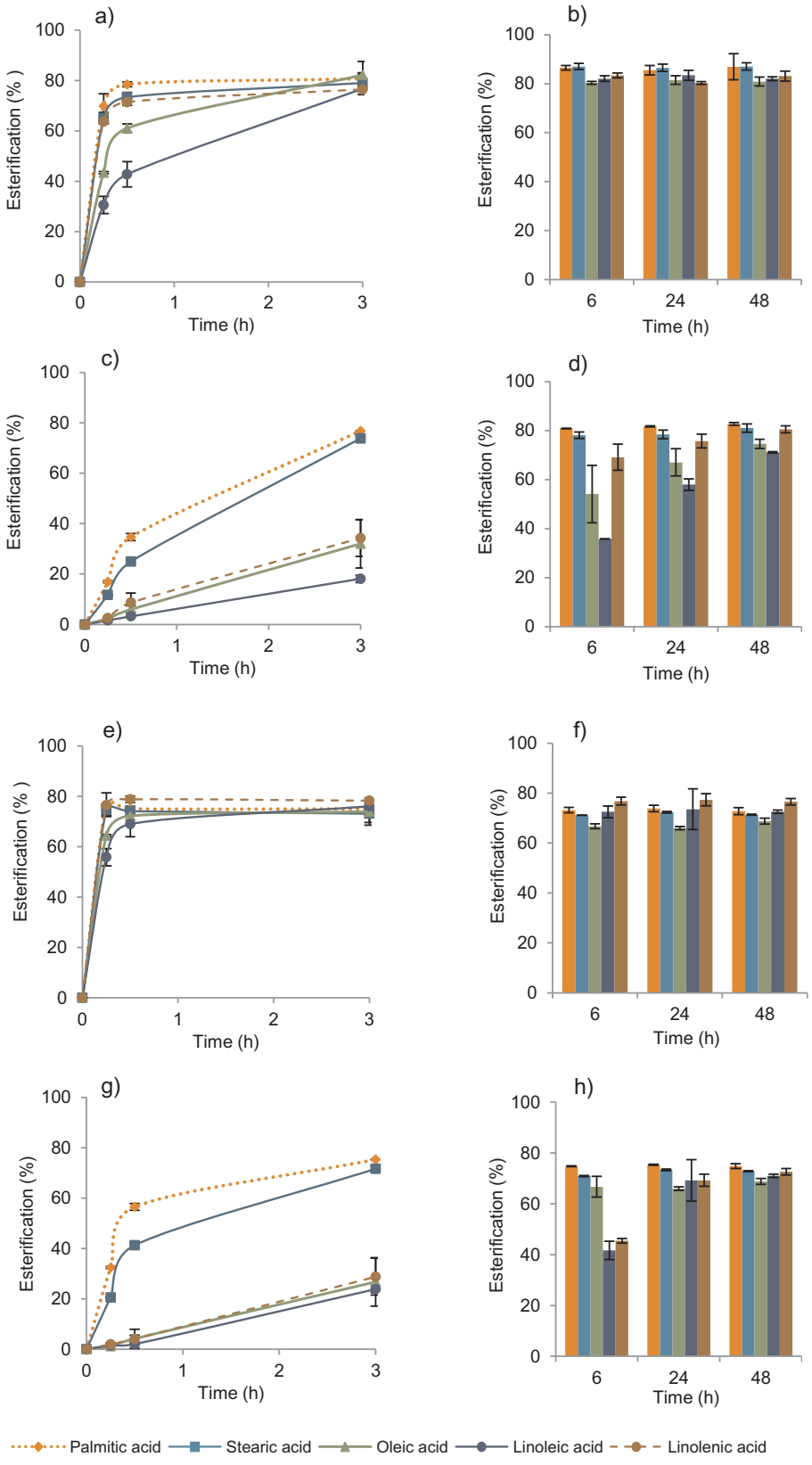
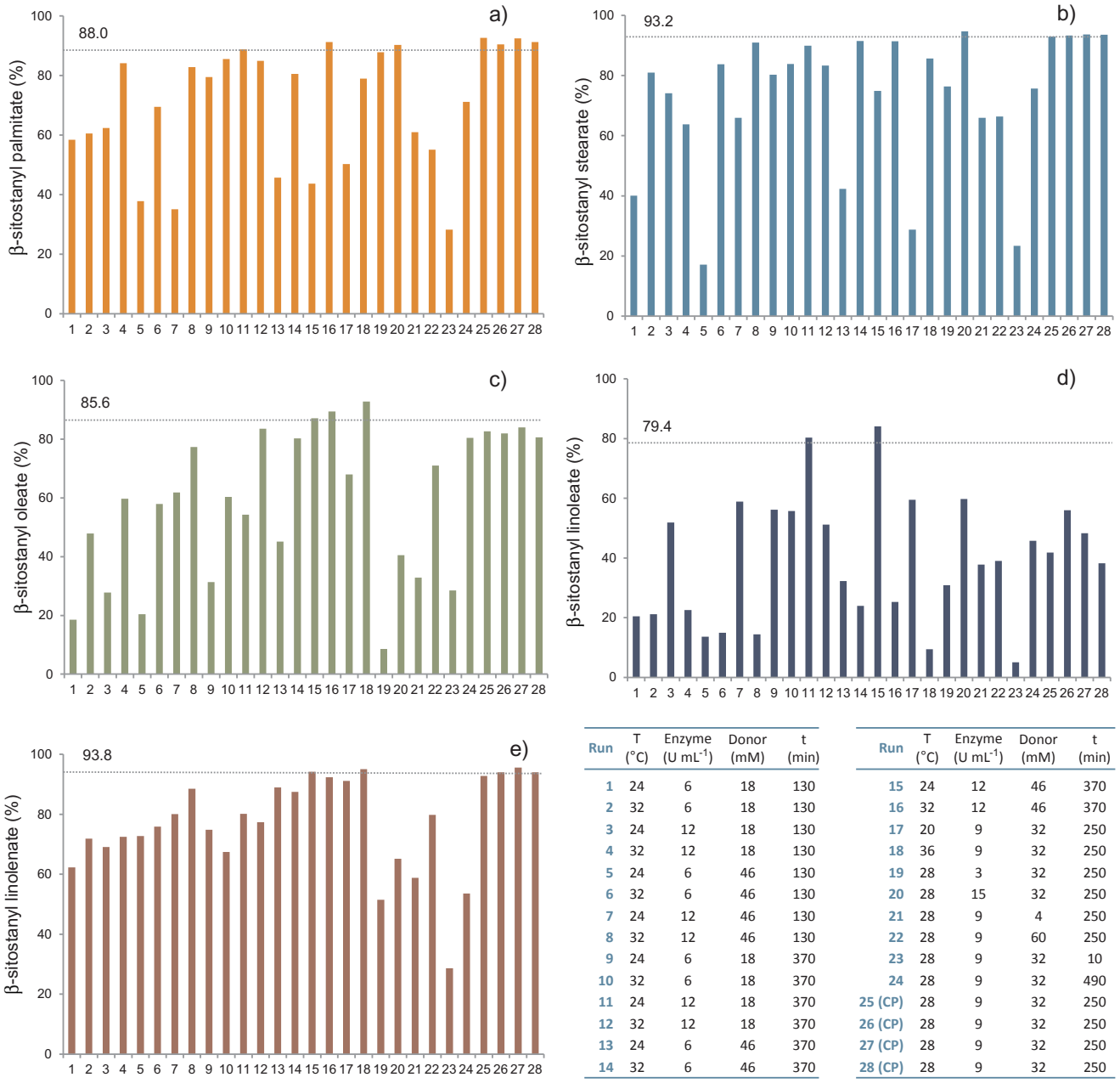


Fig. 1. Efficiency of catalysts in equimolar (10 mM) direct esterification of β-sitostanol and palmitic, stearic, oleic, linoleic or linolenic acids at 28 °C (a–d) and 40 °C (e–h). Detail of the progress of esterification during the first 3 h for OPEr (a and e) and CalA (c and g). Esterification yields at 6 h, 24 h and 48 h with OPEr (b and f) and CalA (d and h). All transformations were performed in isooctane/water biphasic systems, using 6 U of catalyst per ml of reaction and at 100 rpm.



Run	T (°C)	Enzyme (U mL^{-1})	Donor (mM)	t (min)
1	24	6	18	130
2	32	6	18	130
3	24	12	18	130
4	32	12	18	130
5	24	6	46	130
6	32	6	46	130
7	24	12	46	130
8	32	12	46	130
9	24	6	18	370
10	32	6	18	370
11	24	12	18	370
12	32	12	18	370
13	24	6	46	370
14	32	6	46	370

Run	T (°C)	Enzyme (U mL^{-1})	Donor (mM)	t (min)
15	24	12	46	370
16	32	12	46	370
17	20	9	32	250
18	36	9	32	250
19	28	3	32	250
20	28	15	32	250
21	28	9	4	250
22	28	9	60	250
23	28	9	32	10
24	28	9	32	490
25 (CP)	28	9	32	250
26 (CP)	28	9	32	250
27 (CP)	28	9	32	250
28 (CP)	28	9	32	250

Fig. 2. Efficiencies of β-sitostanyl palmitate (a), β-sitostanyl stearate (b), β-sitostanyl oleate (c), β-sitostanyl linoleate (d) and β-sitostanyl linolenate (e) synthesized with OPEr in each of the 28 CCD runs. The table shows the experimental matrix with the real levels for the four independent variables that are given on columns 2–5, while the first column shows the number of each run. *CP* = center point. The order in which the runs were carried out was randomized to avoid systematic errors. The dashed lines correspond to the confidence limit predicted for the best responses and all experimental efficiencies above the lines are within the maximum range expected.

enzymes. Then, the particular three-dimensional structure of the fatty acids assayed could be responsible for their different conversion efficiencies. As it is already known, while palmitic and stearic acids have lineal backbones of 16 and 18 carbon atoms, respectively, the oleic, linoleic and linolenic acid molecules present different curvatures due to the number and position of their double bonds. This three-dimensional arrangement may be the reason for the low efficiency of both enzymes for converting linoleic acid, which probably has difficulty accessing their catalytic center. Following the same line of thought, the CalA structure can provide a good explanation for its lower efficiency compared to OPEr. The CalA lid is seen to be the primary determinant of the acyl site. A narrow ~30-Å-long tunnel begins near the catalytic residues and snakes up through the helices of the lid structure, with a ~90° kink near its midpoint (Ericsson et al., 2008). In this sense, although OPEr also has an internal tunnel of similar length that connects the active site cleft to the outside at the opposite side, no outstanding curvature is present along the tunnel (Gutiérrez-Fernández et al., 2014), which may allow a more fluid passage of substrates and then a faster catalysis.

The three catalysts, OPEr, CalB and CalA, were also assayed for direct acylation of β-sitostanol and fatty acids at 40 °C. As expected, and due to the same reasons previously explained, CalB was not successful in catalyzing the synthesis of any of these esters at 40 °C, since no products were detected upon 48 h. On the other hand, both OPEr and CalA were able to esterify β-sitostanol with all donors assayed, although OPEr again showed much higher esterification rates. The data

presented in Fig. 1e indicate that 15 min of reaction with OPEr were sufficient to get the maximum efficiency (nearly 80%) in β-sitostanyl palmitate and stearate synthesis. The conversion rates observed for β-sytostanyl oleate and linolenate stabilized around the same values from 30 min onwards, while the esterification of β-sitostanol with linoleic acid took 3 h to achieve its maximum point. Based on this, it could be concluded that the effect of a higher temperature on the acylation of β-sitostanol and fatty acids was positive and significant for OPEr.

The effect of the temperature increase on catalysis with CalA was very similar, with higher synthesis rates at 40 °C than at 28 °C. In this case, the maximum esters' yields for sitostanyl palmitate and stearate were achieved at 3 h (Fig. 1g). On the other hand, the synthesis of sitostanyl oleate, linoleate and linolenate at 40 °C did not show an increasing tendency at the longest reaction times. The esters production with oleic acid stabilized after 6 h, while the maximum efficiencies for the linoleic and linolenic esters were detected at 24 h (Fig. 1h).

At the final reaction time, the esters' yields produced by OPEr and CalA at 40 °C were comparable except for sitostanyl linoleate, whose production was again significantly higher using the versatile lipase of *O. piceae* as the catalyst. Carrying out the reaction at 40 °C did not alter the catalytic preferences of the two enzymes related to the chain length and number and position of double bonds.

Finally, it should be noticed that no reversion of esterification to hydrolysis was observed in any of the conditions assayed (Fig. 1b, d, f and h). As other lipases, both OPEr and CalA are able to hydrolyze the newly formed β-sitostanol esters if the water content in the reaction exceeds a determined threshold. However, the initial water content in the reaction (15%) plus the water yielded from direct esterification was not high enough to cause the hydrolysis of the esterification products.

3.2. Experimental approach for central composite design (CCD)

Summarizing the above results, it can be concluded that OPEr exhibits a catalytic behavior far superior to those observed for the two lipases from *C. antarctica,* and also to those reported for the lipase from *C. rugosa*, and the native OPE (Molina-Gutiérrez et al., 2017). Reactions proceeded much faster when OPEr was the catalyst than when it was CalA (3–6 h *vs.* 6–48 h at 28 °C; and 15 min-3 h *vs.* 3–24 h at 40 °C). Therefore, OPEr was selected to tackle the optimization of the esterification process through RSM, specifically using CCD as the experimental design.

As demonstrated in the previous experiments, the chemical characteristics of the fatty acid used as an acyl donor has a decisive impact on the esterification yields, suggesting that the optimal reaction conditions may be different for different substrates. For this reason, a CCD was performed for each one of the fatty acids included in the current study in order to determine the effects (response) on esterification yields of four variables: temperature (*T*), enzymatic dosage (*ED*), acyl donor concentration (*AD*) and reaction time (*t*). With these data, the best conditions for esterification of β-sitostanol will be defined according to the substrate used as donor.

The plots in Fig. 2 show the efficiencies of the OPEr-catalyzed synthesis of β-sitostanyl palmitate, stearate, oleate, linoleate and linolenate under all 28 CCD runs, which are detailed in the table contained in this figure. As was clearly observed, the esterification efficiencies were greatly influenced by the variation of the reaction conditions. The enzymatic synthesis ranged from 28.2% to 92.6% for β-sitostanyl palmitate (Fig. 2a), from 17.1% to 94.7% for β-sitostanyl stearate (Fig. 2b), from 8.6% to 92.8% for β-sitostanyl oleate (Fig. 2c), from 5.1% to 84.0% for β-sitostanyl linoleate (Fig. 2d), and from 28.6% to 95.6% for β-sitostanyl linolenate (Fig. 2e). These results are graphically and numerically presented in Fig. 3. Each one of the charts shows the magnitude and statistical significance ($p \leq 0.10$) of the effect produced by a single independent variable on every esterification reaction. The plots in the two upper rows, corresponding to esterification with the saturated fatty acids, showed similar responses for the variables tested. In both cases, the increase in the acyl donor concentration from the level −1 to +1 caused a significant and negative response, while the other variables tested exhibited significantly positive impacts on esterification of β-sitostanol. Slight differences were observed at the statistical level: the parameter with the lowest significance for C16:0 was the enzymatic dosage ($p = 0.0966$), while for C18:0 it was the acyl donor concentration ($p = 0.0839$). The central row revealed that the four variables exerted a positive and statistically significant effect on the synthesis of β-sitostanyl oleate. Just down this row, the graphs showed the significant effect of all variables on the production of the linoleic acid ester. However, the influence of higher enzymatic dosage, or longer reaction time, were positive, while the increases in temperature or acyl donor concentration were detrimental. Finally, the last row in Fig. 3 displays the plots related to the synthesis of β-sitostanyl linolenate. In this case, the temperature-dependent effects were non-significant within the range evaluated, whereas the other variables played a significant role, and the fatty acid molarity was the most influential parameter.

The CCD responses confirmed that both the reaction time and the amount of catalyst produced significant positive effects on the esterification of β-sitostanol with all the donors evaluated. Concerning the first variable, the last column in Fig. 3 shows an enhancement of the reaction yields over time up to a maximum and, except for the linoleic ester, with a more or less sharp decrease in efficiency at longer reaction times. As already reported (Molina-Gutiérrez et al., 2017) this result suggests that successive synthesis and hydrolysis reactions can take place in the reaction tube depending on the total water content at different sampling times. As water and the ester are equimolar reaction products, the more efficient the reaction, the greater the amount of water released into the medium and the probability of reversion to hydrolysis. The increment of enzymatic dosage up to the maximum amount tested in this experimental design improved the esterification efficiency of β-sitostanol with either palmitic, stearic or linoleic acid. On the contrary, the use of catalyst doses over 12.5 U ml^{-1} hampered the synthesis of the oleic and linolenic acid esters. This decreased efficiency at the highest OPEr doses could be due to enzyme aggregation, taking into account that the recombinant sterol esterase from *O. piceae* is very hydrophobic. Numerous experimental and theoretical studies deal with the effect of steric exclusion by macromolecular crowding on the rates and equilibria of enzyme-catalyzed reactions. Such studies have included the effect of crowding on the transition between conformational states of proteins (Minton, 2005; Sasahara, McPhie & Minton, 2003; Tokuriki et al., 2004). It is generally agreed that the free energy in enzyme-substrate systems fluctuates significantly because of general protein motion (Raza, Fransson & Hult, 2001), which could be hampered at high enzyme dose.

Concerning the effects of temperature on the reaction yields, Fig. 3 illustrates well their diversity, as they depend on the fatty acid used as substrate. The synthesis of the esters of palmitic and stearic acid showed to have a maximum at a given temperature, with a slight decrease in the esterification yield once surpassed this value. However, the synthesis efficiency of the oleic ester showed a linear increasing tendency with the use of higher temperature, and that of the linolenic ester was virtually unaffected by the changes within the range evaluated. Interestingly, the synthesis of β-sitostanyl linoleate was more efficient at the minimum (20 °C) than at the maximum axial level (36 °C).

The molar ratio of substrates is also a key parameter that affects the enzymatic catalysis, and the presence of an excess of one of them can definitely deviate the equilibrium to the synthesis (He et al., 2010; Kim & Akoh, 2007; Miao et al., 2014; Teixeira, Santos & Crespo, 2011). In the present study the concentration of β-sitostanol was kept constant at 10 mM, while the fatty acid concentration varied from 4 mM to 60 mM according to each CCD run. The esterification rates decreased when the reaction mixture contained molar excesses of palmitic, stearic or linoleic acid. It suggests that OPEr is partially inhibited by high

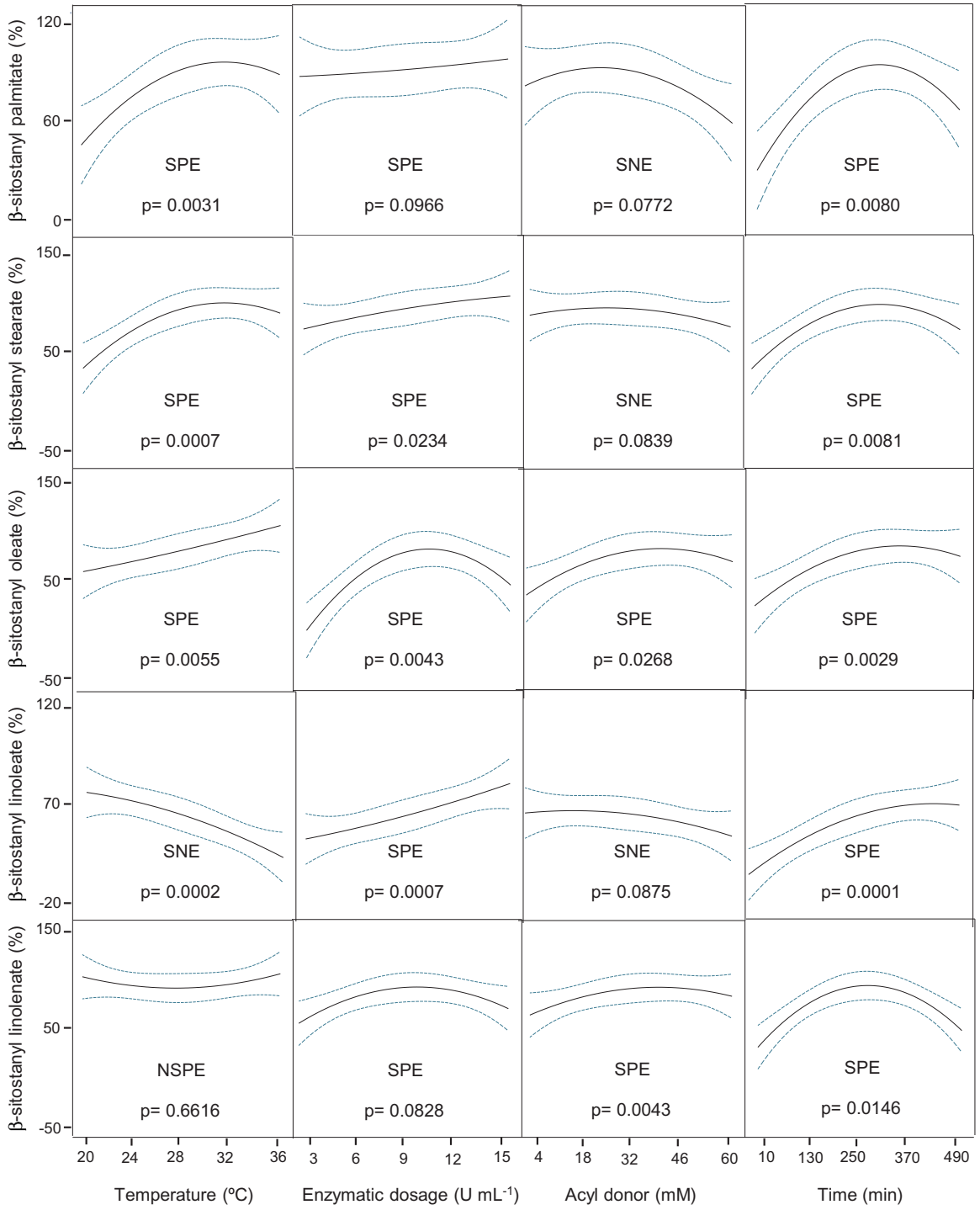


Fig. 3. Effect of temperature, enzymatic dosage of OPEr, acyl donor concentration and reaction time on synthesis of β-sitostanyl palmitate, β-sitostanyl stearate, β-sitostanyl oleate, β-sitostanyl linoleate and β-sitostanyl linolenate. *SPE:* Significant positive effect, *SNE:* Significant negative effect, *NSPE:* Non-significant positive effect.

concentrations of these substrates. On the other hand, this effect was not observed for oleic and linolenic acids, since increasing concentrations from the factorial level −1 to +1 had a slight positive effect on both esterification yields.

3.3. Process optimization: validation of the predicted conditions

Based on the CCD results it was possible to obtain second-order models, that were plotted as three-dimensional surfaces representing the responses (β-sitostanyl esters synthesis) as a function of two variables ($T \times ED$, $T \times t$, $T \times AD$, $ED \times AD$, $ED \times t$, $AD \times t$) for the experimental range considered. The whole set of response plots for each of the fatty acids is presented in the Supplementary Figs. S1–S5.

In four of the five cases, the response surfaces for the synthesis of β-sitostanyl esters showed a maximum point, indicating that their production was optimized within the ranges evaluated (Figs. S1–S3 and S5). The plots of the variables that produced the highest impact on the synthesis of these esters, as a function of the reaction time, are gathered in Fig. 4. Temperature was the most determinant factor for the enzymatic production of the β-sitostanyl esters of the two saturated fatty

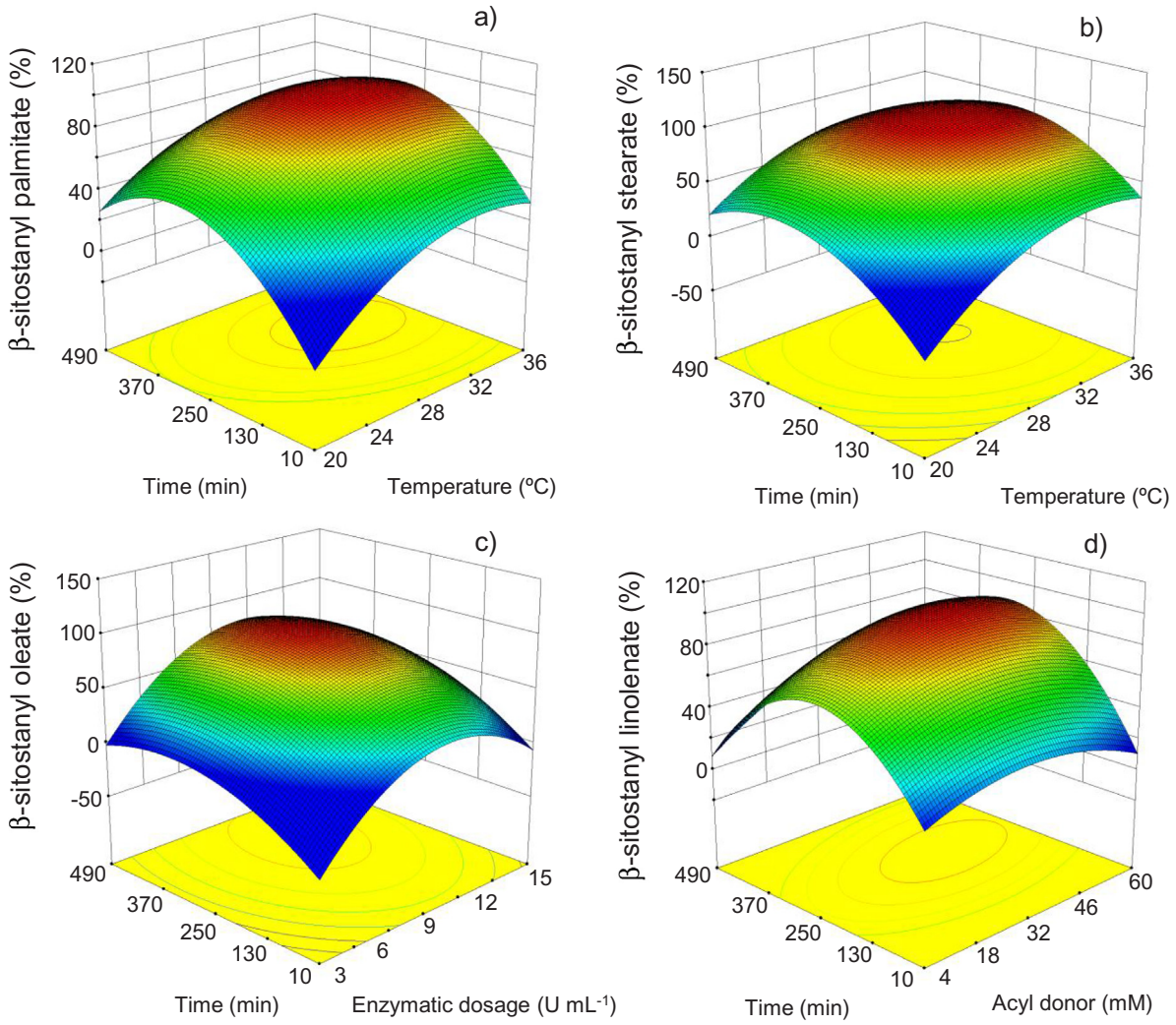


Fig. 4. Selection of the response surface plots of the variables that produced the highest impact on the OPEr-catalyzed synthesis of each ester, as a function of the reaction time: β-sitostanyl palmitate (a), β-sitostanyl stearate (b), β-sitostanyl oleate (c) and β-sitostanyl linolenate (d). The whole set of response surface plots from CCD are gathered in Figs. S1–S5.

acids, palmitate and stearate, while the enzymatic dosage affected more the synthesis of β-sitostanyl oleate and the acyl donor concentration had a stronger effect on esterification with linolenic acid.

The response surface curves for the synthesis of β-sitostanyl linoleate did not present an absolute maximum (Fig. S4). However, they show an increasing trend in the direction of a maximum point. This suggest that optimization of this reaction could be reached widening the ranges established in the current experimental design. According to our data, the use of extended reaction time, lower temperature and higher enzymatic dosage and C18:2 concentration would probably benefit the enzymatic production of the linoleic ester.

By considering the coded levels, equations for the mathematical models of β-sitostanol ester production were obtained as follows:

$$\begin{aligned}\beta\text{-sitostanyl palmitate (\%)} = {} & 20.41 + 10.21T + 2.51ED - 5.40AD + 8.77t \\ & - 0.08TED + 8.48TAD - 1.18Tt - 1.04EDAD \\ & - 1.30EDt + 0.17ADt - 5.81T2 + 0.27ED2 \\ & - 4.7AD2 - 10.54t^2 \end{aligned} \quad (1)$$

$$\begin{aligned}\beta\text{-sitostanyl stearate (\%)} = {} & 7.23 + 13.33T + 7.74ED - 2.77AD + 7.24t \\ & - 7.30TED + 8.48TAD - 1.72Tt - 3.22EDAD \\ & - 1.40EDt + 1.07ADt - 7.70T^2 + 0.72ED^2 \\ & - 2.72AD^2 - 10.25t^2 \end{aligned} \quad (2)$$

$$\begin{aligned}\beta\text{-sitostanyl oleate (\%)} = {} & 82.78 + 11.27T + 1.84ED + 8.14AD + 11.72t \\ & - 3.72TED - 1.14TAD - 1.72Tt + 1.35EDAD \\ & + 0.78EDt + 0.72ADt + 0.45T^2 - 13.51ED^2 \\ & - 4.84AD^2 - 7.27t^2 \end{aligned} \quad (3)$$

$$\begin{aligned}\beta\text{-sitostanyl linoleate (\%)} = {} & 45.08 - 11.02T + 7.23ED - 3.72AD + 11.52t \\ & - 7.24TED - 3.50TAD - 0.05Tt + 1.33EDAD \\ & - 0.05EDt - 4.23ADt - 1.54T^2 + 0.48ED^2 \\ & - 1.83AD^2 - 3.83t^2 \end{aligned} \quad (4)$$

$$\begin{aligned}\beta\text{-sitostanyl linolenate (\%)} = {} & 7.24 + 0.75T + 3.71ED + 4.75AD + 4.28t \\ & - 0.23TED + 0.14TAD - 2.31Tt - 0.85EDAD \\ & - 1.18EDt + 3.05ADt + 3.25T^2 - 7.20ED^2 \\ & - 4.34AD^2 - 13.48t^2 \end{aligned} \quad (5)$$

The statistical analysis indicated an appropriate adjustment of the empirical model to the experimental results, as confirmed by the high correlation coefficients obtained for β-sitostanyl palmitate, stearate, oleate, linoleate and linolenate (R^2 = 0.95, 0.94, 0.96, 0.94 and 0.86, respectively). The statistical insignificance for the lack of fit (p level > 0.10) and the low pure error values corresponding to 15%, or less, of the results' magnitude, confirmed that the model was suitable for prediction within the interval selected in the experimental design.

Table 2
Validation of optimized conditions for synthesis of β-sitostanol esters catalyzed by OPEr.

β-Sitostanol ester	Temperature (°C)	Enzymatic dosage (U ml^{-1})	Acyl donor (mM)	Time (min)	Limits		Predicted value (%)	Experimental result (%)
					−95%	+95%		
Palmitate	30.3	6.0	31.9	285.0	88.0	100	94.0	92.6 ± 1.3
Stearate	31.2	5.4	27.3	313.0	93.2	100	97.6	94.8 ± 0.5
Oleate	34.7	7.5	22.8	343.4	85.6	100	92.8	89.3 ± 0.7
Linoleate	21.2	12.3	23.7	297.7	79.4	100	90.7	84.0 ± 3.4
Linolenate	20.0	7.7	20.2	273.4	93.8	100	96.1	95.6 ± 0.8

Therefore, the use of mathematical models made it possible to predict the best conditions for the esterification of β-sitostanol with palmitic, stearic, oleic, linoleic or linolenic acids in the interval analyzed for each independent variable. The conditions related to a set of maximum efficiencies were visualized on the response surfaces, and their effects and statistical significance were considered. Finally, the lowest values of temperature, enzymatic dosage, acyl donor concentration and reaction time were selected to be experimentally validated. The predicted values for the synthesis efficiencies of β-sitostanol esters and the experimental data obtained in the validation of these conditions are presented in Table 2. Here, the confidence limits of −95% and +95% correspond to the interval of efficiencies that should be experimentally achieved using the best settings predicted by the model. As the experimental values are inside this range, we can state that the best conditions predicted for these reactions have been experimentally validated.

Plots in Fig. 2 display the experimental results from the 28 CCD runs performed for each fatty acid. In these plots, all responses above the dashed line are within the confidence limits and would represent an improvement to the process. Validation of the expected response under the mildest optimized conditions predicted by the model (Table 2) offers an efficient procedure for the green synthesis of these esters. Optimization led to the improvement of the esterification yields obtained with OPEr under the basal conditions initially established (< 85% in 6 h *vs.* 84–97% in < 6 h), together with the reduction of at least one of the process variables evaluated. Therefore, this approach allowed obtaining considerably high esterification efficiencies under mild conditions, and showed to be a suitable tool for optimizing the enzymatic production of different β-sitostanol esters.

4. Conclusion

Three biocatalysts were assayed to synthesize esters of β-sitostanol and long-chain fatty acids. Among them, OPEr demonstrated to be the most efficient against all the substrates evaluated and was selected for further optimization of these reactions using Central Composite Design as RSM strategy. A second-order model was obtained to predict conversion levels as a function of temperature, enzymatic dosage, acyl donor concentration and reaction time. The model provided information on potential optimal conditions for the enzymatic synthesis of β-sitostanol esters, confirming its suitability for maximizing the yield of each one of these reactions under the mildest experimental settings. Further research is on the way for the esterification of β-sitostanol with fatty acids from residual oleaginous materials applying the optimized conditions determined here, according to their main fatty acid component. In addition, works are in progress to scale up the production of OPEr and for its immobilization, as both aspects are of critical importance for a potential industrial application of this interesting catalyst.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2018.04.031.

References

Barba Cedillo, V., Prieto, A., Martínez, A. T., & Martínez, M. J. (2013). Procedimiento de acilación para la obtención de compuestos de interés alimenticio y/o farmacéutico utilizando esterol esterasas fúngicas. PCT/ES2012/070473 Patent WO2013001126 A1.

Barba Cedillo, V., Plou, F. J., & Martínez, M. J. (2012). Recombinant sterol esterase from *Ophiostoma piceae*: An improved biocatalyst expressed in *Pichia pastoris*. *Microbial Cell Factories, 11*, 73.

Barriuso, J., Vaquero, M. E., Prieto, A., & Martinez, M. J. (2016). Structural traits and catalytic versatility of the lipases from the *Candida rugosa*-like family: A review. *Biotechnology Advances, 34*, 874–885.

Calero-Rueda, O., Plou, F. J., Ballesteros, A., Martínez, A. T., & Martínez, M. J. (2002). Production, isolation and characterization of a sterol esterase from *Ophiostoma piceae*. *Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics, 1599*, 28–35.

Cater, N. B., Garcia-Garcia, A. B., Vega, G. L., & Grundy, S. M. (2005). Responsiveness of plasma lipids and lipoproteins to plant stanol esters. *American Journal of Cardiology, 96*, 23D–28D.

Ericsson, D., Kasrayan, A., Johansson, P., Bergfors, T., Sandström, A., Bäckvall, J.-E., & Mowbray, S. L. (2008). X-ray structure of *Candida antarctica* lipase A shows a novel lid structure and a likely mode of interfacial activation. *Journal of Molecular Biology, 376*, 109–119.

Feltes, M. M. C., Oliveira, D., Block, J. M., & Ninow, J. L. (2012). The production, benefits, and applications of monoacylglycerols and diacylglycerols of nutritional interest. *Food Bioprocess Technology, 6*, 17–35.

Gutiérrez-Fernández, J., Vaquero, M. E., Prieto, A., Barriuso, J., Martínez, M. J., & Hermoso, J. A. (2014). Crystal structures of *Ophiostoma piceae* sterol esterase: Structural insights into activation mechanism and product release. *Journal of Structural Biology, 187*, 215–222.

He, W., Jia, C., Ma, Y., Yang, Y., Zhang, X., Feng, B., & Yue, L. (2010). Lipase-catalyzed synthesis of phytostanyl esters in non-aqueous media. *Journal of Molecular Catalysis B: Enzymatic, 67*, 60–65.

He, Y., Li, J., Kodali, S., Balle, T., Chen, B., & Guo, Z. (2017). Liquid lipases for enzymatic concentration of n-3 polyunsaturated fatty acids in monoacylglycerols via ethanolysis: Catalytic specificity and parameterization. *Bioresource Technology, 224*, 445–456.

Kim, B. H., & Akoh, C. C. (2007). Modeling and optimization of lipase-catalyzed synthesis of phytosteryl esters of oleic acid by response surface methodology. *Food Chemistry, 102*, 336–342.

Miao, M., Liu, H., Jiang, B., Yang, C., Xia, X., & Zhang, T. (2014). Enzyme-catalysed synthesis of plant steryl laurate in non-aqueous media using salt hydrate pairs and its characterisation. *Journal of Functional Foods, 7*, 452–461.

Miettinen, T. A., Puska, P., Gylling, H., Vanhanen, H., & Vartiainen, E. (1995). Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *The New England Journal of Medicine, 333*, 1308–1312.

Minton, A. P. (2005). Models for excluded volume interaction between an unfolded protein and rigid macromolecular cosolutes: Macromolecular crowding and protein stability revisited. *Biophysical Journal, 88*, 971–985.

Molina-Gutiérrez, M., Hakalin, N. L. S., Rodríguez-Sanchez, L., Prieto, A., & Martínez, M. J. (2017). Green synthesis of β-sitostanol esters catalyzed by the versatile lipase/sterol esterase from *Ophiostoma piceae*. *Food Chemistry, 221*, 1458–1465.

Musa-Veloso, K., Poon, T. H., Elliot, J. A., & Chung, C. (2011). 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, Leukotrienes and Essential Fatty Acids, 85, 9–28.
Ostlund, R. E. (2007). Phytosterols, cholesterol absorption and healthy diets. *Lipids, 42*, 41–45.
Panpipat, W., Xu, X., & Guo, Z. (2013). Improved acylation of phytosterols catalyzed by *Candida antarctica* lipase A with superior catalytic activity. *Biochemical Engineering Journal, 70*, 55–62.
Raza, S., Fransson, L., & Hult, K. (2001). Enantioselectivity in *Candida antarctica* lipase B: A molecular dynamics study. *Protein Science, 10*, 329–338.
Sasahara, K., McPhie, P., & Minton, A. P. (2003). Effect of dextran on protein stability and conformation attributed to macromolecular crowding. *Journal of Molecular Biology, 326*, 1227–2123.
Teixeira, A. R. S., Santos, J. L. C., & Crespo, J. G. (2011). Production of steryl esters from vegetable oil deodorizer distillates by enzymatic esterification. *Industrial & Engineering Chemistry Research, 50*, 2865–2875.
Tokuriki, N., Kinjo, M., Negi, S., Hoshino, M., Goto, Y., Urabe, I., & Yomo, T. (2004). Protein folding by the effects of macromolecular crowding. *Protein Science, 13*, 125–133.
Vaquero, M. E., Barriuso, J., Martinez, M. J., & Prieto, A. (2016). Properties, structure, and applications of microbial sterol esterases. *Applied Microbiology and Biotechnology, 100*, 2047–2061.
Vaquero, M. E., Prieto, A., Barriuso, J., & Martínez, M. J. (2015). Expression and properties of three novel fungal lipases/sterol esterases predicted in silico: Comparison with other enzymes of the Candida rugosa like-family. *Applied Microbiology and Biotechnology, 99*, 10057–10067.
Villeneuve, P., Turon, F., Caro, Y., Escoffier, R., Baréa, B., Barouh, B., ... Pina, M. (2005). Lipase-catalyzed synthesis of canola phytosterols oleate esters as cholesterol lowering agents. *Enzyme and Microbial Technology, 37*, 150–155.
Vu, P. L., Shin, J.-A., Lim, C.-H., & Lee, K.-T. (2004). Lipase-catalyzed production of phytosteryl esters and their crystallization behavior in corn oil. *Food Research International, 37*, 175–180.
Weber, N., Weitkamp, P., & Mukherjee, K. D. (2001). Steryl and stanyl esters of fatty acids by solvent-free esterification and transesterification *in vacuo* using lipases from *Rhizomucor miehei, Candida antarctica*, and *Carica papaya*. *Journal of the Agricultural and Food Chemistry, 49*, 5210–5216.
Weingärtner, O., Bogeski, I., Kummerow, C., Schirmer, S. H., Husche, C., Vanmierlo, T., ... Laufs, U. (2017). Plant sterol ester diet supplementation increases serum plant sterols and markers of cholesterol synthesis, but has no effect on total cholesterol levels. *Journal of Steroid Biochemistry & Molecular Biology, 169*, 219–225.