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Production of structured lipids by acidolysis with immobilized *Rhizomucor miehei* lipases: Selection of suitable reaction conditions

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ABSTRACT

Enzymatic modification of vegetable oils can be used to produce plastic fat or structured lipids (SL) avoiding the generation of *trans* fatty acids. Biocatalysts previously prepared by immobilization of *Rhizomucor miehei* lipases onto alkylated chitosan microspheres were used to synthesize SL by acidolysis reaction of sunflower oil and palmitic–stearic acids mixture. Effects of some reaction parameters, substrate molar ratio (SR), amount of biocatalyst (*E*) and amount of solvent (*H*), over selected response variables were analyzed by applying an incomplete factorial design of three factors with three levels (Box–Behnken). Responses were selected to evaluate not only the development of desired TAG but also the yield and quality of obtained products, through by-products and undesired trisaturated lipids quantification. The analysis of variable effects showed that *E* was the most significant factor on each analyzed response. Multiresponse optimization with restrictions from practical considerations revealed that maximum saturated fatty acids composition in glycerides of the SL (38.6%) is obtained when SR = 3, *E* = 0.34 g and *H* = 3.2 ml are used. Under these conditions, a change in the composition of the desired TAG group (monounsaturated TAG) from 2.5% in the original oil to 33.9% in the final product was achieved. On the other hand, time and temperature behavior studies showed that generation of trisaturated triacylglycerols and by-products were favored mainly by increasing temperature. After 12 h of reaction, overall hydrolysis and esterification reactions rates were comparable. However, TAG distribution continued changing.

1. Introduction

Nowadays, fats and oils modification is one of the main areas in food processing industry that demands novel economical and green technologies. In this respect, modification of lipids by enzymatic catalysis has become an attractive option. Lipase mediated modifications are specific and can be carried out at moderate reaction conditions with fewer side products. More specifically, the selectivity and specificity of lipases make it possible to obtain structured lipids (SL), which are tailor-made lipids with desired characteristics, like certain physical or chemical properties and/or nutritional benefits. Important structured triacylglycerols, like cocoa butter substitutes, low calorie fats, PUFA-enriched oils, and oleic acid enriched oils, have been synthetized with such technologies [1].

Likewise, processes to obtain SL can be employed as alternative methods to hydrogenation, used to convert vegetable liquid oil into semi-solid fats without the formation of the undesired *trans* fatty acids. It has been shown that *trans* fats can extend shelf life and flavor stability of products, but it can increase the risk of cardiovascular disease by raising the low – density lipoprotein cholesterol (LDL) and decreasing the high – density lipoprotein cholesterol (HDL) [2]. Among other processes, semi-solid fats can be synthesized by acidolysis reactions catalyzed by lipases, where it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol through the hydrolysis and reesterification reaction steps. In particular, by the use of *sn*-1,3-specific lipase as *Rhizomucor miehei*, free fatty acids (FFA) which are present in a reaction medium could be incorporated in *sn*-1 and *sn*-3 positions of triglycerides while original fatty acids are ideally kept in *sn*-2 position. Following this route for the synthesis of SL, oils and saturated FFA have been used in order to obtain semi-solid fats which are beneficial for human nutrition due to the fact that they preserve unsaturated or polyunsaturated long-chain fatty acids in *sn*-2 position [3–6].

Additionally, undesired reactions could take place because of the presence of diacylglycerols (DAG) produced in the hydrolysis

Abbreviations: AP, Adeq precision; BP, by-products; *D*, desirability function; DAG, diacylglycerols; *E*, amount of biocatalyst; FAME, fatty acid methyl esters; FA, fatty acid; FFA, free fatty acids; *H*, amount of solvent (hexane); LOF, lack of fit; MAG, monoacylglycerols; P + St, palmitic and stearic acids; PUFA, polyunsaturated fatty acids; R^2, *R*-squared; R^2adj, adjusted *R*-squared; R^2pred, predicted *R*-squared; S, saturated fatty acid; SL, structured lipids; SO, refined sunflower oil; SPFA, palmitic–stearic acids blend; SR, substrate molar ratio; SSS, trisaturated triacylglycerols; SUS, monounsaturated triacylglycerols; TAG, triacylglycerol; U, unsaturated fatty acid.

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step. Acyl migration of the long-chain acyl groups, from position *sn*-2 to *sn*-1 or *sn*-3 caused by a certain amount of DAG in the reaction mixture, has been reported in acidolysis, decreasing the yield and the purity of the desired TAG [7,8].

The potential of enzymatic processes to obtain SL has been subject of numerous researches, in which commercial biocatalysts were mainly used [5,9–12]. Likewise, the high cost and the easy attrition of the commercially available immobilized lipases restrict its use in large scale processes. As a possible solution, in a previous work we prepared 8 biological catalysts suitable to produce SL using modified chitosan microspheres as support for immobilization of R. miehei lipases [13]. It is worth mentioning, that the development of this support was based on the well-known properties of chitosan [14] and the need of changing its hydrophilic nature to a hydrophobic one in order to stabilize the open conformation of lipases and promotes their hyperactivation after their immobilization [15]. The obtained biocatalysts were used in acidolysis of sunflower oil and palmitic and stearic FFA at specific conditions. The most active biocatalyst achieved a change in the composition of palmitic and stearic acid from a value of 9.6% in the original oil to 49.1% in the final SL, representing an almost 3-fold enzyme hyperactivation. Additionally, they proved to be mechanically resistant even after several hours of use [13]. Even so, in order to scale this procedure, a wider knowledge of process variables is necessary. In this contribution, the influence of specific parameters of the reaction – the amount of biocatalyst (*E*), the substrate mole ratio (SR) and the amount of solvent (*H*) – has been studied on selected response variables. These were: % P + St, palmitic and stearic acids composition respect to total fatty acids in glycerides, % SUS, monounsaturated triglycerides (desired TAG) respect to total triglycerides, % BP, by-products (mono and diglycerides) respect to total glycerides and % SSS, trisaturated triglycerides respect to total triglycerides, as indicative of the possible acyl migration. In order to evaluate the effect of experimental factors on the responses, with a minimum number of trials, an incomplete factorial design of three factors with three levels (Box–Behnken) was applied. A fitting model for each response was obtained and a multi-response optimization was made to obtain optimal working conditions. Independent supplementary experiments were carried out at the optimum condition in order to determine the validity of fitting models in the explored domain. Additionally, the time-course of acidolysis reaction assayed in the optimum condition at two different reaction temperatures (50–60 °C) was also studied.

To sum up, this contribution intends to achieve a full understanding of the effect of main reaction parameters when a novel biocatalyst, specially prepared for modifying lipids, is used. Likewise, it wants to find the most suitable reaction conditions in order to produce the greatest change in the sunflower oil composition based on practical considerations.

2. Materials and methods

2.1. Materials

Chitosan of low molecular weight with a degree of 75–85% of deacetylation and 12.6% of moisture was obtained from Sigma–Aldrich. Refined sunflower oil (SO) was purchased from a local grocery store and it was used as received (peroxide value (PV): 1.94 mequiv./kg). *R. miehei* lipase (>20,000 U/g, Novozymes) from *Aspergillus oryzae* conditioned in the form of liquid, dodecyl aldehyde (92%, Aldrich), sodium cyanoborohydride $NaCNBH_3$ (>95%, Fluka), palmitic–stearic acids blend (SPFA) with purity grade 49–54% and 40–51% respectively (Fluka) were purchased from Sigma–Aldrich (Germany). Fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). Other standards (1,2,3-trioctadecanoyl-glycerol, 1,2,3-trihexadecenoyl-glycerol, 1,2,3-trioctadecadienoyl-glycerol, 1,2,3-trioctadecenoyl-glycerol, 1,2,3-trihexadecanoyl-glycerol, 1,2,3-tridecanoyl-glycerol, 1,2-distearoyl-3-palmitoyl-rac-glycerol, 1,3-dipalmitoyl-2-oleoylglycerol, 1,3-dioleoyl-2-palmitoyl-glycerol, 1,2-dilinoleyl-3-palmitoyl-rac-glycerol, 1,2-dioleoyl-3-stearoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, 1,2-distearoyl-3-oleoyl-rac-glycerol, 1,3-dipalmitoyl-rac-glycerol, 1-monopalmitoyl-rac-glycerol, octadecenoic acid, tetradecane) were of more than 98% purity and were obtained from Sigma Chemical Co. (St. Louis, USA). Pyridine was from J.T. Baker (Philipsburg, USA) and N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Fluka (Buchs, Switzerland). All the other reagents, gases and solvents were of analytical or chromatographic grade.

2.2. Preparation of biocatalyst

Modified chitosan microspheres were prepared following the procedure described in our previous work [13], using dodecyl aldehyde in monomolar ratio 1:1 NH_2/aldehyde and 1.5 h of contact times with aldehyde. In the same way, lipase immobilization on obtained support was carried out as reported in the aforementioned work.

2.3. Lipase-catalyzed acidolysis

Enzymatic acidolysis reactions were carried out with refined sunflower oil and a mixture of free palmitic and stearic acids (SPFA). Acidolysis was performed as follows: 110 mg of SO and the correspondent amount of SPFA, needed to obtain the desired molar ratio of substrates, were dissolved in the corresponding amount of hexane and later mixed and heated at 60 °C. The reaction began when immobilized lipase was added. Reactions were performed in a screw-capped test tube in a water bath with temperature controller and magnetic agitation at 250 rpm. After 12 h (unless another time is specified), reactions were stopped removing enzymes by filtering. The solvent from the reaction mixtures was removed by evaporation under a nitrogen atmosphere and then, the reaction mixtures were maintained at −20 °C until analysis.

2.4. Experimental design

In order to evaluate the influence of chosen reaction parameters on response variables, an incomplete design of three-level and three-factor factorial with three central points (Box–Behnken design) was used, in which experimental points have been especially selected to allow an efficient estimation of coefficients in quadratic models. The three selected factors were: the amount of biocatalyst (*E*), the substrate mole ratio (SR) and the amount of solvent, hexane (*H*). They were decided according to similar works [6,16,17] in which these variables had significant effects. The setting of the factors was determined in accordance with previous studies, in addition to practical considerations. The amount of hexane in the reaction medium was set so that both, the substrates-solvent diluted systems, commonly used in continuous operations, and free solvent green systems could be simulated. Consequently, the ranges chosen were: *E* (0.21–0.39 g), SR (3–6 mol SPFA/mol SO) and *H* (0.0–3.2 ml).

Matrix design was performed using the DESIGN EXPERT 7.0 software. Used factors and levels, together with analyzed responses, are shown in Table 1. The experiment order was completely randomized.

Likewise the mentioned software was used to find fitting models in which the coefficients of the postulated models were calculated