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Interesterification of fat blends rich in ω-3 polyunsaturated fatty acids catalysed by immobilized *Thermomyces lanuginosa* lipase under high pressure

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Abstract

The interesterification of natural fats will improve certain physical and nutraceutical properties by modification of their acylglycerol profile.

In this study, the effect of high pressure in the interesterification kinetics of fat blends, in solvent-free medium, catalysed by a commercial immobilized lipase from *Thermomyces lanuginosa* was evaluated. Reaction media were ternary blends of palm stearin, palm kernel oil and a concentrate of triacylglycerols (TAG) rich in ω-3 polyunsaturated fatty acids. Reactions were carried out at 60 °C, at 0.1, 50, 100 and 150 MPa. The interesterification was followed (i) by the decrease in "Solid Fat Content" values of the blend at 35 °C ($SFC_{35\,^\circ C}$) and (ii) by the changes in the acylglycerol profile.

The biocatalyst presented interesterification activity at least up to 150 MPa. Results obtained at 0.1 MPa, with no agitation of the reaction medium, were found to be similar to those obtained under high-pressure conditions. The observed decrease in $SFC_{35\,^\circ C}$ values was accompanied by important changes in the acylglycerol profile. An increase in compounds of low equivalent carbon number (ECN) and in TAG of ECN 44 and 46 were observed. This increase was accompanied by a consumption of TAG of ECN 48 and 50 for all pressure values, and also of trilaurin (ECN = 36) at normal pressure. High pressures seem to affect lipase selectivity towards lauric acid.

Batch operational stability tests showed a linear inactivation profile for each pressure. Half-lives of about 15, 6 and 4 h were estimated for the biocatalyst under 0.1, 50 and 150 MPa, respectively.

Keywords: High pressure; Immobilized lipase; Interesterification; ω-3 polyunsaturated fatty acids; Operational stability

1. Introduction

The functional properties of fats are determined by their fatty acid composition and also by the distribution pattern of fatty acid residues in triacylglycerols (TAG). Interesterification (ester interchange also called transesterification) is a route to improve certain physical, sensory and nutraceutical properties of natural fats. These changes result from the rearrangement of the acyl residues on the acylglycerol backbone, leading to a modification of the acylglycerol profile. By interesterification among TAG, it is possible to modify crystallisation and melting properties of fats, without modifying the fatty acid composition. This is of much interest for applications in margarine, confectionary and bakery industries, as well as for pharmaceutical and cosmetics purposes.

Industrial interesterification of fats is at present carried out using metal alkylates or alkali metals as catalysts, at temperatures ranging from 50 to 120 °C, for less than 2 h. The interchange of acyl groups proceeds at random. Also, final products may remain contaminated by residual catalyst and the formation of considerable amounts of side

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Nomenclature

DAG diacylglycerol(s);
DHA docosahexaenoic acid (22:6 (ω-3));
ECN equivalent carbon number;
EPA eicosapentaenoic acid (20:5 (ω-3));
EPAX 4510TG concentrate of TAG rich in ω-3 PUFA, EPAX AS, Lysaker, Norway;
FFA free fatty acid(s);
NMR nuclear magnetic resonance;
PK palm kernel oil;
PS palm stearin;
PUFA polyunsaturated fatty acid(s);
$SFC_{35\,^{\circ}C}$ solid fat content at 35 °C;
TAG triacylglycerol(s);
ω-3 PUFA ω-3 polyunsaturated fatty acid(s).

products, with a subsequent decrease in yield, may occur [1].

The extent of interesterification is usually indirectly assessed by the measurement of the solid fat (crystallised) content (SFC) of the blend, after and prior to interesterification.

The SFC of a fat blend defines the plastic or workability range of such fat and is an important empirical parameter. Thus, the knowledge of SFC of a fat helps to decide about the suitability of a fat for further processing. For example, the SFC values at 10, 20, 30 and 35 °C are related with the rheological behaviour of fats at storage, packaging, bakery in winter and summer, and consumption temperatures, respectively. The SFC at 35 °C ($SFC_{35\,^{\circ}C}$) is particularly important for table margarines, since it is related to the extent of melting in the mouth. The $SFC_{35\,^{\circ}C}$ values of the interesterified fats should be lower than their original counterparts, and as low as possible, to prevent a sandy and coarse texture of the margarine.

The assessment of the SFC follows a standard protocol related to the measurement, by nuclear magnetic resonance (NMR), of the extent of fat crystallisation [2]. The determination of the melting point of a blend is of limited value, since significant differences in the melting behaviour of a certain blend, evaluated by SFC at different temperatures, correspond to small differences in melting temperature [1]. Also, the alteration in the physical properties has not yet been fully correlated with the modifications in the acylglycerol profile which occurred during interesterification.

The growing consumer demands for natural and healthy foods have become a challenge for the food industry. In this context, the current trend includes the replacement of chemical catalysts by biocatalysts, recognized as natural, and the search for new formulations with nutraceutical properties. New products enriched in ω-3 polyunsaturated fatty acids (ω-3 PUFA), especially in eicosapentaenoic acid (EPA, 20:5 (ω-3)) and docosahexaenoic acid (DHA, 22:6 (ω-3)) have a great potential due to their known benefits in human health [3]. Since the majority of diets do not include adequate amounts of fish rich in ω-3 PUFA, the incorporation of these fatty acids in food products more readily available for consumption, such as vegetable oils and margarines, may be an option.

In the field of oils and fats, the replacement of inorganic catalysts by lipases (acylglycerol acylhydrolases, EC 3.1.1.3) has been attempted in the last years, due to the benefits of the enzymatic route relatively to chemical processes [4,5]. Lipase-catalysed interesterification of fat blends has been carried out, either in the presence of organic solvents [6–8] or in solvent-free media [9–19]. These reactions were always performed at normal pressure since high pressures have been ascribed to lipase inactivation [20].

In fact, high pressure has been successfully applied in the processes of pasteurization and sterilization in the food and pharmaceutical industries. The use of pressure as a parameter for changing reaction rates and equilibria in bioprocesses has been studied to a lesser extent [21,22]. The enzyme instability at high-hydrostatic pressures is one of the main reasons to restrain its use in biocatalytic processes.

It was found that pressure could either activate or inhibit enzymatic activities, depending on the proteins and conditions. The kinetics and equilibrium of enzyme reactions may differ significantly when carried out under high pressure and in some cases the selectivity and stability of the biocatalyst may be influenced [23]. Pressure can modify the catalytic behaviour of enzymes by changing the rate-limiting step or modulating the selectivity of the enzyme [24,25].

The following studies are presented to illustrate the effect of high-pressure conditions in biocatalysis:

The hydrolysis of naringin, a flavonoid responsible for the bitterness in grape fruit juices, catalysed either by free or immobilized naringinase in calcium alginate beads, was carried out under high-pressure conditions [26,27]. At 160 MPa, an increase in activity and operational stability was observed for both systems.

When the Aspergillus niger fructosyl-transferase was used for the sucrose conversion under high-hydrostatic pressure, the inhibition of the main transfer reaction without affecting sucrose hydrolysis, was observed. These pressure effects were shown to be reversible up to 200 MPa [25].

The Candida rugosa lipase was also tested under high-pressure conditions (up to 10 MPa) as catalyst for the transesterification of (±)-menthol with propionic acid anhydride in chloroform and water. A significant decrease in enantioselectivity E of this lipase was observed [22].

In addition to the performance of enzymatic reactions under high-pressure conditions, high-pressure hydrostatic treatments have been carried out to modify the original stability and selectivity of enzymes [28,29].

The effect of treatments at high-hydrostatic pressures of soluble Rhizomucor miehei lipase in aqueous solution on its residual stability, assayed by the hydrolytic activity at normal pressure, was also investigated [28,29]. For this lipase, a protective effect of pressure treatment ranging from 50 to 350 MPa, against thermal inactivation (50–60 °C) was observed [28]. When higher pressures treatments (300–500 MPa) were performed, a hyperbaric irreversible deactivation, described by

a series-type inactivation model, was observed. However, the specific activity of the Rhizomucor miehei lipase was enhanced after pressure treatment [29]. All these results stress the importance of investigate the influence of high pressure on enzymatic systems.

The aim of this study was to investigate the effect of high pressure (50–150 MPa) in the kinetics of interesterification of fat blends, in solvent-free medium, catalysed by an immobilized lipase from *Thermomyces lanuginosa* ("Lipozyme™ TL IM", Novozymes, Denmark). This biocatalyst has been successfully used in research studies on the interesterification of fats, at normal pressure [5,8,12,14,16,18,19]. Reactions were carried out at 60 °C, under pressures of 0.1 MPa (atmospheric pressure), 50, 100 and 150 MPa. In addition, batch operational stability tests were performed in order to investigate the possibility of reusing the biocatalyst at high-pressure conditions. In parallel, similar experiments were carried out under atmospheric pressure.

Reaction media were blends of palm stearin (PS), palm kernel (PK) oil and a concentrate of triacylglycerols rich in ω-3 polyunsaturated fatty acids (EPAX 4510TG, EPAX AS, Norway). Palm stearin, the high-melting fraction (≥44 °C) obtained from the fractionation of palm oil, contains a high level of saturated fatty acids (mainly palmitic acid). Due to its low price, it is commonly used in the margarine industry. However, PS tends to crystallise slowly and has a low ability to impart the required body and plasticity to the desired final products. By blending PS with other fats and/or oils, such as palm kernel oil, a lauric oil with the capability of improving the melting and crystallisation properties of the blend, better functional properties, namely mouthfeel and handling can be obtained [30]. The incorporation of an oil rich in ω-3 polyunsaturated fatty acids enhances spreadability of margarines at refrigeration temperatures and adds known benefits for human health.

The interesterification was monitored (i) indirectly, by the "Solid Fat Content" values of the blends at 35 °C ($SFC_{35\,°C}$) assayed by NMR and (ii) by the changes in the acylglycerol profile.

2. Materials and methods

2.1. Materials

Palm stearin and palm kernel oil were donated by FIMA/VG, Produtos Alimentares, Portugal; a commercial concentrate of triacylglycerols, rich in ω-3 PUFA, "EPAX 4510TG" (45% EPA and 10% DHA), was a gift from EPAX AS, Lysaker, Norway. Fatty acid composition of these fats is similar to that previously published [16]. The free fatty acid content (FFA, %) of PS, PK, and "EPAX 4510TG" were 0.58, 0.28, and 0.28, and their Peroxide values were 10, 10, and 5 meq/kg, respectively.

The commercial immobilized thermostable 1,3-selective lipase from *T. lanuginosa*, in food-grade granulated silica, "Lipozyme™ TL IM", was a gift from Novozymes A/S, Bagsvaerd, Denmark.

Acetone HPLC grade, from Fisher Scientific, UK, and acetonitrile for HPLC, gradient grade, from Sigma-Aldrich, Germany, were used. Tripalmitin, tristearin, and triolein standards were from Sigma-Aldrich, Germany. All the other reagents used were analytical grade obtained from various sources.

2.2. Methods

2.2.1. Lipase-catalysed interesterification at normal pressure

Interesterification reactions were performed in thermostated cylindrical batch reactors, at 60 °C and at normal pressure (0.1 MPa). Parallel experiments were run (i) under magnetic stirring and (ii) with no agitation, to simulate the same conditions as in the experiments under high pressure.

The reaction media consisted of two different ternary blends: blend I (55% PS, 35% PK and 10% "EPAX 4510TG") and blend II (45% PS, 45% PK and 10% "EPAX 4510TG"). A load of 6% (w/w) of the immobilized lipase was used. Prior to and at the end of each experiment, 5-mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70 °C. All samples were stored at −4 °C for subsequent analysis.

All the experiments and analysis were in duplicate and average results were reported.

2.2.2. Lipase-catalysed interesterification at high pressure

The interesterification reactions were carried out in a stainless-steel vessel immersed in a thermostatic water bath [27], at 60 °C, under 50, 100 and 150 MPa.

Pressure and temperature measurements were maintained constant within, respectively, ±2 MPa and ±0.1 °C. The pressurization fluid was hydraulic oil (EnerpacHF95 Y) and the required pressure was achieved with a 400 MPa manual pump (Enerpac, model P228) and controlled using a pressure gauge (Budenberg Gauge Co. Limited).

The high-pressure enzymatic reactions were carried out in 3 cylindrical glass cells with a volume of 9 mL and an internal diameter of 2.1 cm, each. The three cells were mounted along a stainless-steel central vertical bar. The biocatalyst was added only to two of the cells; the third cell was used as a blank reactor. The pressure was increased steadily in 1–3 min, depending on the pressure required, and maintained for different periods of time. Each reaction time (20, 40 and 60 min) corresponded to a different batch. Subsequently, the pressure was released within 1 min; the reaction media was immediately filtered at approximately 70 °C to remove the immobilized biocatalyst. Each data point was obtained in duplicate kinetic runs. All samples were stored at −4 °C for subsequent analysis.

Reaction media composition, temperature and biocatalyst load were the same used in normal pressure experiments.

2.2.3. Batch operational stability test

The interesterification of blend II was carried out for 20 min, at 60 °C, under 0.1, 50 and 150 MPa, catalysed by 6% Lipozyme TL IM, as previously described (cf. Sections 2.2.1 and 2.2.2). After the first batch, the biocatalyst was removed from the reaction medium by paper filtration and reused in the next batch with fresh medium, under the same reaction conditions. A total of 4 batches were performed using the same biocatalyst sample.

The operational stability of the immobilized lipase was assayed as described for interesterification experiments (cf. Section 2.2.5). The $SFC_{35\,^{\circ}C}$ values of the blends upon each batch were used to calculate the relative activity (A, %) of the biocatalyst at the end of that batch:

$$A(\%) = \left[\frac{SFC_{35\,^{\circ}C} \text{ of the blend of Batch 1 at 0.1 MPa}}{SFC_{35\,^{\circ}C} \text{ of the blend of Batch } n \text{ under } X \text{ MPa}}\right] 100$$

where n is the batch number ($n = 1,\ldots,4$) at a given pressure X (0.1, 50 or 150 MPa). Thus, the activity of the biocatalyst upon the first batch at 0.1 MPa, was assumed to be 100% and was used as the reference value also for the experiments at 50 and 150 MPa.

The operational half-life time of the biocatalyst, i.e., the operation time needed to reduce its original activity (as measured in the first batch) to 50%, was estimated by the models fitted to the deactivation profiles.

The fit of kinetic models to experimental data was carried out using "solver" add-in from Excel for Windows, version 8.0 SR2, by minimizing the residual sum-of-squares between the experimental data points and those estimated by the respective model and considering the following options: Newton method; 100 iterations, precision of 10^{-5}; 5% of tolerance and 0.001 convergence.

2.2.4. Assessment of the activity recovery of the biocatalyst previously used under high-pressure conditions in consecutive batches

After the 4 consecutive batches at 50 and 150 MPa, the recovered biocatalyst was used in the interesterification of blend II, at normal pressure and 60 °C, for 24 h, in order to evaluate the effect of high pressures on the residual activity of the biocatalyst at 0.1 MPa. In parallel, similar experiments were carried out with fresh biocatalyst, under magnetic stirring, in order to achieve the maximum conversion. The residual activity was evaluated by the decrease in $SFC_{35\,^{\circ}C}$ of the initial fat blend sample (cf. Section 2.2.5) and compared with the results obtained with the fresh biocatalyst sample.

2.2.5. Analytical methods

2.2.5.1. Solid fat content assay. The interesterification reaction was indirectly evaluated by the decrease in the extent of fat crystallisation at 35 °C ($SFC_{35\,^{\circ}C}$) assayed by NMR in a pulsed NMR spectrophotometer (Minispec P-20i, IBM). For NMR analysis, samples were melted at 60 °C, maintained at this temperature for about 10 min, then kept at 0 °C for 60 min, and, finally, maintained for 30 min at the test temperature (35 °C) prior to the $SFC_{35\,^{\circ}C}$ measurement [2].

2.2.5.2. Assay for acylglycerol profile. The changes in acylglycerol profile, occurring by interesterification reaction, were evaluated by non-aqueous reverse-phase high performance liquid chromatography (HPLC) using a Merck HITACHI (Germany) chromatograph equipped with a reverse-phase column (100 Superspher 100-RP-18; 250 mm × 4 mm i.d., 5 μm particles size) and a refractive index detector. The following conditions were used: mobile phase of acetone/acetonitrile (63.5:36.5, v/v), at a flow rate of 0.8 mL/min; oven temperature of 40 °C; injection of 10 μL of a solution of the fat sample (2%, w/v) in acetone HPLC grade.

For each blend type, up to 25 peaks, corresponding to the various groups of acylglycerols separated as a function of their "equivalent carbon number" (ECN), were obtained in the chromatograms. ECN value is calculated by the following equation [31]:

$$ECN = C - 2n$$

where, C is the number of carbon atoms in fatty acids chains of the acylglycerols and n, the number of double bonds in these fatty acid residues.

The presence of a wide variety of TAG, some of them with very similar equivalent carbon numbers (ECN), make their identification (based on relative retention times) extremely difficult and uncertain. In addition, the problem is further confounded by the lack of authentic standards for these TAG [30].

Therefore, a tentative identification of the various peaks separated under the HPLC conditions followed in this study (Table 1), was attempted (i) using a limited number of TAG standards (tripalmitin, tristearin and triolein), by (ii) comparison with chromatograms obtained with similar fats and previously reported [6,9,12,30] and (iii) based on the fatty acid composition of the individual fats used in each blend.

Palm stearin mainly contains high ECN peaks (Table 1: peaks 17–25; ECN 46–50) due to high amounts of palmitic (c.a. 60%) and oleic (c.a. 25%) acids, while palm kernel oil is characterized by the presence of lower ECN triacylglycerols (ECN lower than 46) due to the presence of high amounts of medium-chain saturated fatty acids (c.a. 44% lauric and 15% myristic acids) and also about 10% of palmitic acid. The concentrate EPAX 4510TG has the lowest ECN species (peaks 1–7) due to the presence of long-chain polyunsaturated fatty acids: 63% of total ω-3 PUFA from a total of 67% PUFA content and 21% of mono-unsaturated fatty acids [16]. Peak identification for the EPAX 4510TG was not possible due to the lack of standards and of available information about TAG composition of this oil. Thus, the contents of Table 1 are merely tentative and based on EPAX 4510TG original composition.

3. Results and discussion

3.1. Time-course experiments

The interesterification of blends I and II, catalysed by Lipozyme TL IM, during 24 h under atmospheric pressure, at 60 °C, when the reaction medium was stirred or not stirred, is presented in Fig. 1. The slope of the initial linear part of the curves, showing the decrease in $SFC_{35\,^{\circ}C}$ values along reaction time, was used as a measure of the initial reaction rate.

The presence of mass transfer limitations in the experiments with no medium agitation explains the differences observed in initial rates and also in the time to attain a quasi-equilibrium situation. In fact, the initial rates obtained in agitated media (blend I = 6.27; blend II = 8.05% SFC decrease/h) were about 5 and 2.5-fold the values observed when only diffusion-controlled reaction

Table 1
Tentative identification of the individual peaks observed in the chromatograms, as a function of their equivalent carbon number (ECN) and fatty acid composition

Peak no.	Retention time (min)	ECN	Initial samples	Final samples
1	3.192	?	EPAX + DAG	EPAX + DAG
2	3.642	?	EPAX + DAG	EPAX + DAG
3	4.025	28	LaCaCa + DAG	LaCaCa + DAG + EDCa
4	4.742	28	CCCa + DAG	CCCa + DAG
5	5.492	30	CCC + DAG + EPAX (EEE, DDD, EED, DDE)	CCC + DAG + EPAX (EEE, DDD, EED, DDE) EEC
6	6.042	32	LaCC/CaLaLa	DAG + LaCC + CaLaLa + LnDC
7	6.675	34	LaLaC/CLaM	DAG + LaLaC + LDC + LnLaE
8	7.883	36	LaLaLa	DAG + LaLaLa + DOC + ELLa
9	9.208	38	LaLaM	LaLaM + EOLa + PLaD + SDC + MMD
10	10.333	40	LaLaO	LaLaO + LaES
11	10.867	40	LaMM	LaMM + PEM
12	12.267	42	LaOM	LaOM
13	12.925	42	LaPM	LaPM
14	13.842	44	LaOO	LaOO
15	14.600	44	LaOP	LaOP
16	15.475	44	LaPP/MOM	LaPP/MOM
17	16.250	46	MOO/OOL	MOO/OOL
18	17.125	46	MOP/PLP	MOP/PLP
19	18.883	48	OOO	OOO
20	19.975	48	POO	POO
21	21.075	48	POP	POP
22	22.550	48	PPP	PPP
23	24.250	50	OOS	OOS
24	25.783	50	POS	POS
25	27.533	50	PPS	PPS

DAG, diacylglycerols; EPAX, non-identified peaks from "EPAX 4510TG"; Fatty acids in TAG: C, capric (C10:0); Ca, caprylic (C8:0); D, DHA (C22:6); E, EPA (C20:5); L, linoleic (C18:2); La, lauric (C12:0); Ln, linolenic (C18:3); M, myristic (C14:0); O, oleic (C18:1); P, palmitic (C16:0); S, stearic (C18:0).

occurred (blend I = 1.22; blend II = 3.26% SFC decrease/h), for the systems with blend I and II, respectively. The differences observed between systems I and II may be ascribed to a higher amount of palm stearin in blend I (55%, w/w) which is a high-melting point and viscous fat, when compared with the composition of blend II (45% palm stearin).

The time-course of interesterification of both blends I and II, at 0.1 MPa, with or without agitation, and also under high-pressure conditions, assayed by the decrease in $SFC_{35\,^{\circ}C}$, is presented in Fig. 2. Experiments showed that, with both blends tested, the enzymatic preparation presents interesterification activity at least up to 150 MPa, i.e., at a pressure 1500 times higher than the normal pressure. The highest interesterification activity was observed, for both blends, in the experiments at atmospheric pressure, under agitation.

Under pressures of 50, 100 and 150 MPa, a quasi-equilibrium state was observed after 20 min reaction time. After longer reaction times, the $SFC_{35\,^{\circ}C}$ presented a constant value when blend I was tested. In the experiments with blend II, the results obtained at 50 and 100 MPa were similar; under 150 MPa, slightly higher $SFC_{35\,^{\circ}C}$ values of the interesterified blends were observed, suggesting a lower activity of the biocatalyst.

The results obtained at 0.1 MPa with no stirring were close to those obtained under high-pressure conditions. With blend II, after 20 min reaction time, the decrease in $SFC_{35\,^{\circ}C}$ values was even higher under 50–150 MPa than under normal pressure with no agitation. The lower enzymatic activity observed at high pressures (50–150 MPa) may also be due to a diffusion-controlled reaction, and may not only result from a pressure-induced inactivation. In blend II experiments under atmospheric pressure, a continuous decrease in $SFC_{35\,^{\circ}C}$ values was observed, both with and without stirring, with values of 3.90 and 9.00, respectively, at 60 min reaction time.

The observed decrease in $SFC_{35\,^{\circ}C}$ values was accompanied by noticeable changes in the acylglycerol profiles. Fig. 3 shows the modifications occurred in acylglycerol profile of blend II upon 20 min interesterification at 60 °C, under 0.1, 50, 100 and 150 MPa, catalysed by Lipozyme TL IM. The profiles of the initial blend and of the interesterified blend obtained under normal pressure conditions (in agitated medium) are always presented together with the acylgycerol profile of the interesterified fat blend obtained at each high pressure tested, in order to facilitate the comparisons between experiments.

Under all pressure conditions tested, a decrease in peak 21 (POP; ECN = 48) was observed. Under normal pressure, a remarkable decrease (34%) in trilaurin (peak no. 8; ECN = 36) was observed, conversely to that occurred under high-pressure conditions. In addition, the consumption of the triacylglycerol POS (peak 24; ECN 50) at 0.1, 50 and 100 MPa, and also of PPS (peak 25; ECN 50), under 0.1 MPa was detected. The consumption of these species was accompanied by a considerable increase in peaks 15–17, corresponding to TAG of ECN 44 and 46, and also in peaks of lower ECN (peaks 2 and 5).

When the reaction occurred at atmospheric pressure, an increase in the amounts of peaks 10 (LaLaO), 11 (LaMM) and 13 (LaPM) was also observed. It is worthy to notice that the increase in pressure seems to affect the ability of the lipase to attack molecular species containing lauric acid. Thus, a

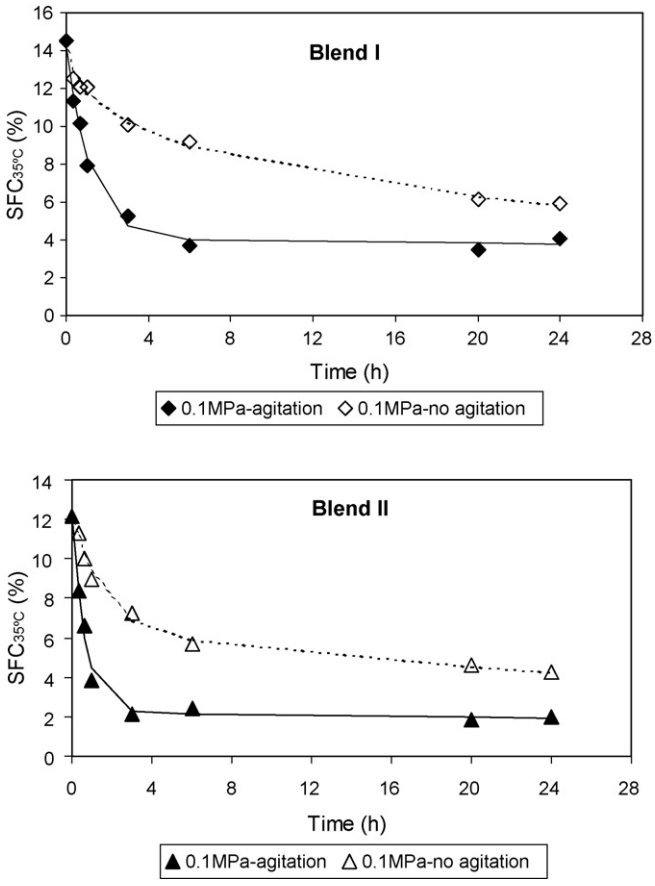

Fig. 1. Time-course of interesterification of blends I and II, catalysed by Lipozyme TL IM, at 0.1 MPa (normal pressure) when the medium was agitated or not, monitored by the decrease in $SFC_{35\,^{\circ}C}$.

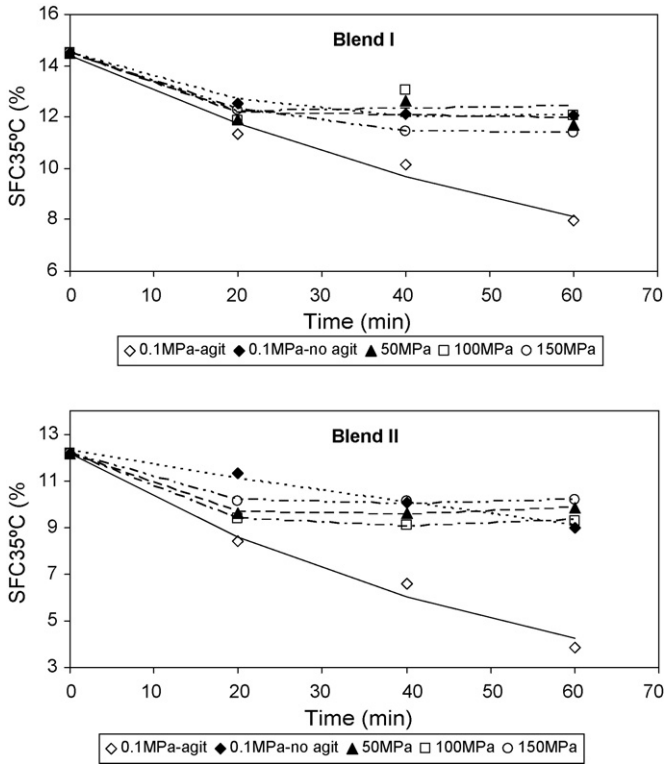

Fig. 2. The effect of pressure in lipase-catalysed interesterification of blends I and II, monitored by the decrease in $SFC_{35\,^{\circ}C}$.

modification in lipase selectivity towards TAG species may occur, although it may not be clearly reflected in a great variation of SFC value of the blend.

In fact, the most pronounced modifications in TAG profile were observed by interesterification under 0.1 MPa. The increase in pressure was accompanied by a decrease in the modification of the acylglycerol profile as a result of a decrease in lipase activity, as shown by the $SFC_{35\,^{\circ}C}$ values of the various fat blends.

A high affinity of Lipozyme TL IM towards lauroyl residues was also observed in the production of structured TAG containing conjugated linoleic acid at sn2 position and lauric acid at external positions, in solvent-free media at normal pressure [32]. On the contrary, when the same lipase preparation was used as catalyst for the interesterification between two TAG (mono-acid saturated or unsaturated TAG), in *n*-hexane at 0.1 MPa, no selectivity towards any fatty acid or TAG was detected [8].

Probably, diacylglycerols (DAG) are among the new compounds of low equivalent carbon number produced. In fact, the increase of DAG and FFA levels, by lipase-catalysed interesterification of binary or ternary fat blends in solvent-free media, under normal pressure, was previously reported [9,12,13,15–18,33]. This has been ascribed to the mechanism of the lipase-catalysed interesterification reaction. This reaction involves the hydrolysis of the ester bond followed by re-esterification, which may lead to the accumulation of considerable levels of free fatty acids and partial acylglycerols (mono and diacylglycerols) in the reaction medium, principally in the presence of water [34].

3.2. Batch operational stability test

In order to investigate if high-pressure conditions promote biocatalyst inactivation, 4 consecutive batches of 20 min duration each were carried out at 0.1 MPa (under agitation), 50 and 150 MPa. These experiments were performed with blend II, since the most pronounced differences among the experiments under different pressure conditions were observed with this blend.

The $SFC_{35\,^{\circ}C}$ values of the fat blends after each batch are shown in Fig. 4. Lower $SFC_{35\,^{\circ}C}$ values were achieved at normal pressure for all the 4 batches. Similar $SFC_{35\,^{\circ}C}$ values were observed upon the first batch at 50 and 150 MPa. From this batch on, a linear increase in $SFC_{35\,^{\circ}C}$ values was observed with batch number, with a greater slope when 150 MPa was used (0.533 against 0.31 for 50 MPa). This indicates a decrease in interesterification activity along the four consecutive batches.

The $SFC_{35\,^{\circ}C}$ values were used to calculate the relative activity of the biocatalyst at the end of each batch. The activity of the biocatalyst upon the first batch at 0.1 MPa, was assumed to be 100% and used as the reference value also for the experiments under 50 and 150 MPa (Fig. 4). A linear activity decay of the biocatalyst was observed. At atmospheric pressure, the inactivation of the biocatalyst is slower with an estimated half-life

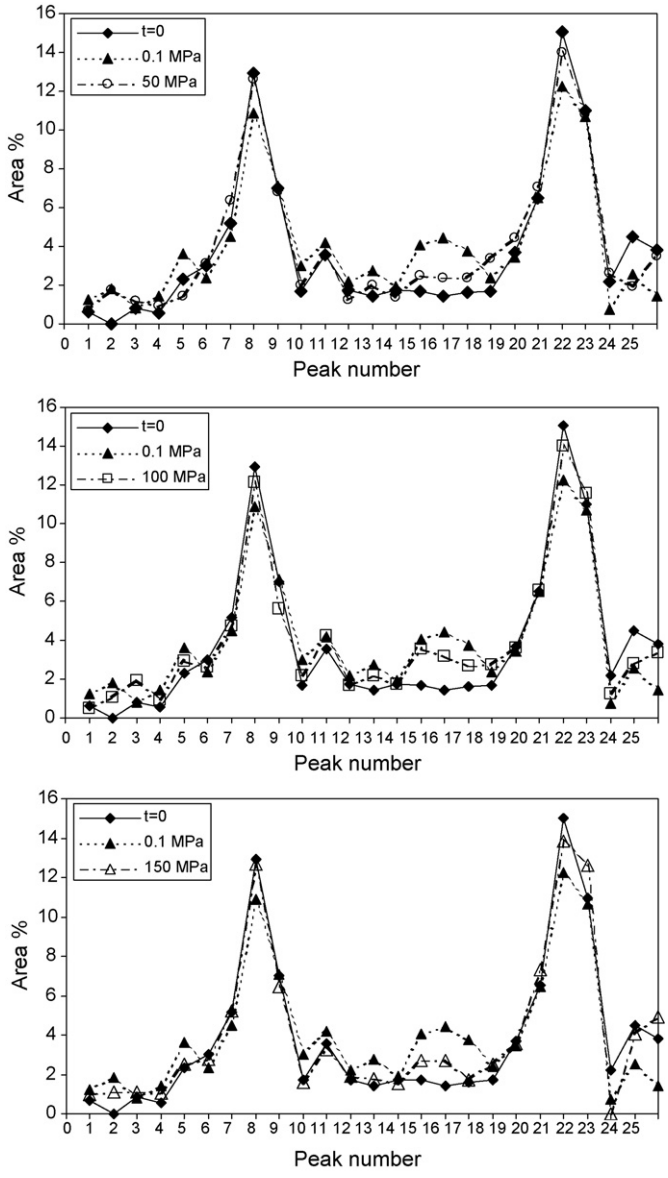

Fig. 3. Modification in acylglycerol profile of blend II upon 20 min interesterification at 60 °C, under 0.1, 50, 100 and 150 MPa, catalysed by Lipozyme TL IM.

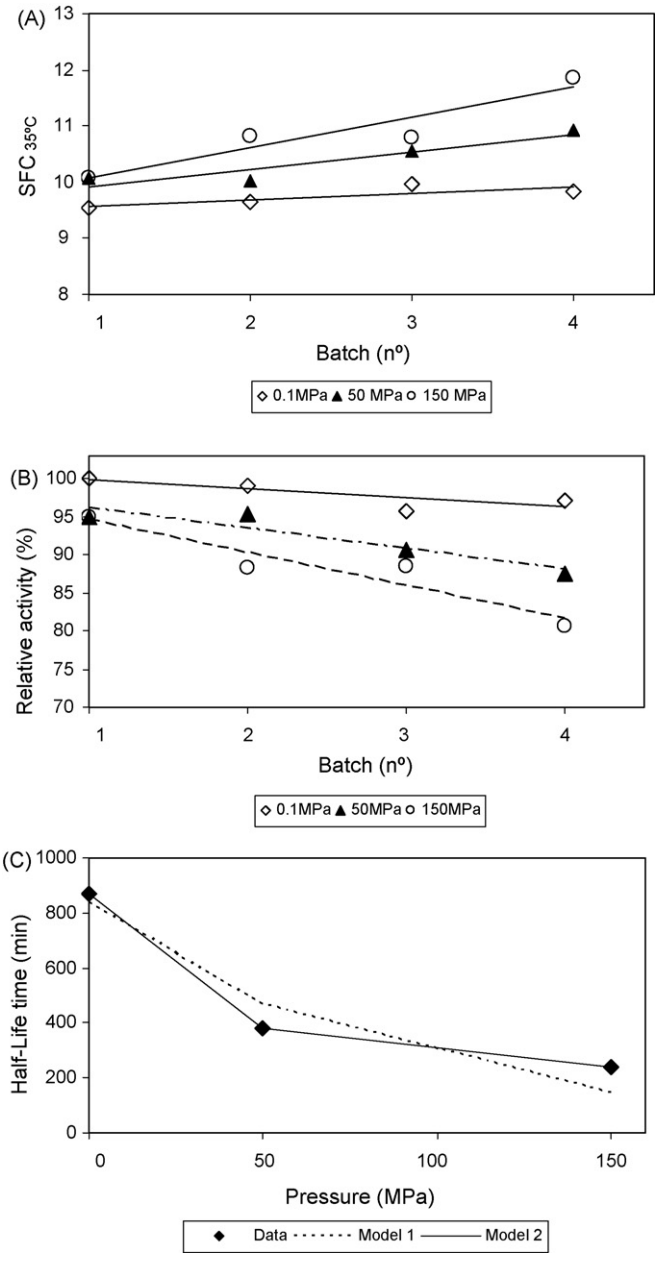

Fig. 4. Batch operational stability tests: (A) the $SFC_{35\,°C}$ values of blend II, upon each of the 4 consecutive batches of 20 min duration, at 60 °C, and 0.1 MPa (with agitation of the reaction media), 50 and 150 MPa, and (B) respective relative activities; (C) the dependence of half-life time of Lipozyme TL IM with pressure conditions, in consecutive batches and the fit of single first-order (model 1) and two component first-order decay models (model 2) (see text for details).

time ($t_{1/2}$) of 870 min (equivalent to 43.5 batches). When the same biocatalyst was used at 50 or 150 MPa, its half-life time was reduced to 378 min (19 batches) and 240 min (12 batches), respectively. In fact, a considerable decrease in the operational stability was observed when the biocatalyst operated under high-pressure conditions, probably due to a deep modification in the conformation of the lipase. Comparing with $t_{1/2}$ of the biocatalyst at normal pressure, the most pronounced decrease in $t_{1/2}$ was observed for the biocatalyst at 50 MPa (a decrease of 492 min). The increase in pressure, from 50 to 150 MPa, only conducted to a further decrease in $t_{1/2}$ of 138 min. It seems that from a certain pressure value, the enzyme structure will become rigid enough to be less sensitive to pressure effects.

The half-life ($t_{1/2}$; min) of Lipozyme TL IM may be described by the following two component first-order decay model, as a function of pressure, P (MPa):

$$t_{1/2} = a\,e^{(-k_1 \cdot P)} + b\,e^{(-k_2 \cdot P)}$$

where parameters k_1 and k_2 are the deactivation constants (MPa^{-1}) of the rapid and of the slow breakdown, respectively. The fit of this model to the experimental data gave the following model equation:

$$t_{1/2} = 463.95e^{(-0.0513.P)} + 408.95e^{(-0.0036.P)}$$

The good fit of this model to the experimental data, as well as the lack of fit of the single first-order exponential model ($t_{1/2} = a\,e^{(-k_1 \cdot P)}$), are illustrated in Fig. 4.

A considerably higher operational stability, with a half-life of 77 h, was observed for Lipozyme TL IM when used in a continuous packed-bed bioreactor for the interesterification of a similar fat blend, in solvent-free media, at normal pressure [18]. This value was more than 5 times the estimated value for the consecutive batches, carried out in the present study also under normal pressure. In fact, tubular reactors are particularly suitable when product inhibition occurs, while stirred tank reactors are more adequate for substrate inhibited situations [35].

The lower operational stability of Lipozyme TL IM when used in consecutive batches may be explained by the inhibitory effect of FFA, produced both by interesterification and by the competing hydrolytic reaction.

Concerning the modification of acylglycerol profile along the consecutive batches, Fig. 5 shows the relative amounts of each peak of the chromatogram in the initial blend samples and upon interesterification using the fresh biocatalyst (batch 1) and the same biocatalyst upon 3 reutilizations (batch 4), at 0.1, 50 and 150 MPa. In experiments at atmospheric pressure, the acylglycerol profiles of interesterified blends from batches 1 to 4 are similar and different from that of the original blend. Conversely, for the high-pressure interesterification experiments, as pressure and batch number increase, the acylglycerol profile of the final samples are more alike to that of the initial blend samples. In the samples obtained at 150 MPa, the largest differences in the acylglycerol profiles seem to occur in TAG of 42–46 ECN (peaks 13–18). Thus, the acylglycerol profile appears to be an indicator of the activity decay of the biocatalyst. However, it is not still possible to predict the SFC or any other physical fat property from the knowledge of its composition in acylglycerols.

3.3. Assessment of the activity recovery of the biocatalyst previously used under high-pressure conditions in consecutive batches

In order to evaluate the residual interesterification activity presented by the biocatalyst samples, after being used in the 4 consecutive batches of 20 min duration each, at 50 and 150 MPa, the recovered biocatalyst was used in the interesterification of blend II, at 60 °C and normal pressure, for 24 h (cf. Section 2.2.4). In parallel, similar experiments were carried out also with fresh biocatalyst, under magnetic stirring.

The reduction of $SFC_{35\,°C}$ values of interesterified blends were used to measure lipase activity. The percentage of $SFC_{35\,°C}$ reduction upon 24 h interesterification at normal pressure was considered as 100% activity. The residual activity was calculated with respect to this value.

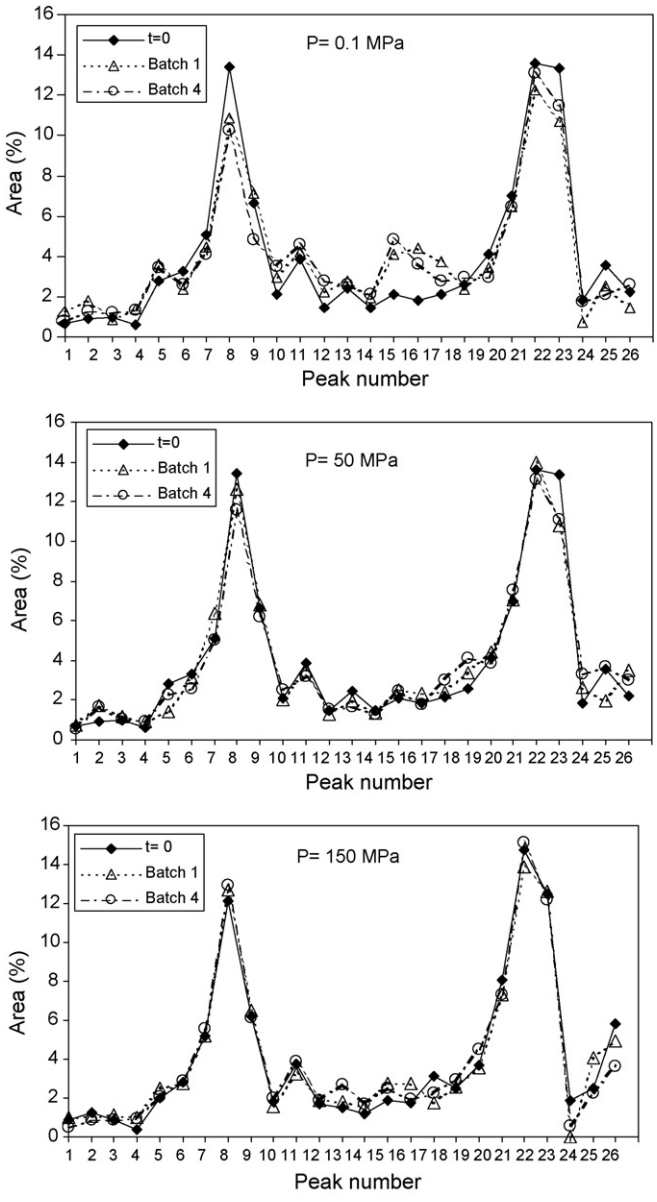

Fig. 5. Modification in acylglycerol profile of blend II upon the first and the fourth consecutive batch interesterification at 60 °C, under 0.1, 50 and 150 MPa, catalysed by Lipozyme TL IM.

The obtained results are presented in Table 2. As observed, both preparations previously used under high-pressure conditions exhibit a residual activity when reused under normal pressure. A high-$SFC_{35\,°C}$ reduction (76.6%) was achieved with

Table 2
Residual activity of the recovered biocatalyst samples used in 4 consecutive batches at 50 and 150 MPa, in the interesterification of blend II, at normal pressure, under magnetic stirring, at 60 °C and for 24 h

Previous pressure (MPa)	$SFC_{35\,°C}$ (initial blend)	$SFC_{35\,°C}$ (final blend)	Reduction of $SFC_{35\,°C}$ (%)	Activity (%)
Fresh biocatalyst	12.19	1.95	84	100
50	11.66	2.73	76.6	91.2
150	11.66	9.08	22.1	26.3

the biocatalyst sample previously used at 50 MPa, indicating a high activity recovery (91.2%). This value is similar to the relative activity exhibited by the same biocatalyst sample during the third reutilization (batch 4) at 50 MPa (Fig. 4).

With the Lipozyme TL IM sample previously submitted to 150 MPa along the 4 consecutive batches, only 22% reduction of $SFC_{35\,^{\circ}C}$, corresponding to 26.3% activity recovery, was obtained. This value is considerably lower than the relative activity (80%) showed by this lipase sample in batch 4 at 150 MPa (Fig. 4).

4. Conclusions

The obtained results are rather innovative and promising since they show the feasibility of using immobilized lipases as catalysts for interesterification reactions in solvent-free media, under conditions traditionally related with lipase inactivation. High pressures may be used to modify the original selectivity of the lipase towards the desirable direction.

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