Research Paper

Operational stability of immobilised lipase/acyltransferase during interesterification of fat blends

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The lipase/acyltransferase from *Candida parapsilosis* is an unusual enzyme that preferably catalyses alcoholysis over hydrolysis in biphasic aqueous/organic media. The aim of this study was to evaluate the operational stability of an immobilised form of this enzyme during the interesterification of fat blends containing *n*-3 polyunsaturated fatty acids, in solvent-free media, at 60 °C, carried out continuously and batchwise. When the interesterification was performed in a continuous fluidised-bed reactor, an operational half-life of 9 h was estimated. The biocatalyst was also reused in consecutive 23-h batches, in a total of four batches, either using fresh medium with no water addition or adding water to rehydrate the biocatalyst. When no water and extra water was added to the reaction medium, the obtained half-lives were 10 and 18 h, respectively. Thus, the loss of activity may be explained by a progressive dehydration occurring along the reaction rather than by product or substrate inhibition effects. The interesterification activity was accompanied by changes in the acylglycerol profile. An increase in compounds of low equivalent carbon number (ECN) and in triacylglycerols (TAG) of ECN 42 and 44 was observed. This increase was accompanied by the consumption of TAG of ECN 46, 48 and 50.

Keywords: Batch operational stability / *Candida parapsilosis* lipase/acyltransferase / Continuous operational stability / Interesterification / *n*-3 Polyunsaturated fatty acids

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1 Introduction

In the food industry, the interesterification of fats mainly aims at the production of fats with specific melting properties and crystallisation behaviour, comparable to those obtained by hydrogenation, but free of *trans* fatty acids. Conversely to the consumption of hydrogenated fats containing *trans* fatty acids, no significant effects of interesterified fats on human blood lipid parameters have been detected. Interesterified fats are used for the manufacture of high-quality margarines and spreads with no oiling-off or sandy mouth feel [1].

The interesterification process involves the rearrangement of the fatty acids on the glycerol backbone of triacylglycerols (TAG) in the presence of chemical or enzymatic catalysts. Microbial lipases have been used as catalysts for enzymatic interesterification of oils and fats. The natural function of lipases is to catalyse the hydrolysis of acylglycerols and other fatty acid esters, but this reaction is easily reversible and such enzymes are also effective catalysts for esterification and interesterification reactions [2].

Chemical interesterification is usually faster than when biocatalysts are used, but it leads to full randomisation of acyl chains within TAG molecules. Due to the high 1,3-regioselectivity of some lipases, a control of the position of acyl

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chains on the glycerol backbone can be achieved, thus improving the nutritional properties of the products. Moreover, lipase-catalysed interesterification is conducted under mild conditions (normal pressure and temperatures below 70 °C) that are favourable to the chemical stability of polyunsaturated fatty acyl chains. The control of the conversion degree for producing optimal products with the desired physical properties is also easier than when inorganic non-selective catalysts are used [3–5]. The conversion degree is critical for industrial applications because it is related to the properties of the interesterified fat blends [6].

Nowadays, the cost of commercial non-immobilised and immobilised lipases and their relatively low operational stability have been considered as the major constraints to their use for the production of commodity fats in the food industry. In addition, the mechanism of the interesterification reaction involves the hydrolysis of the ester bond followed by re-esterification. This may lead to the accumulation of high levels of free fatty acids (FFA) in the reaction medium, principally in the presence of water [3–5, 7–10].

Several studies have been carried out to investigate the possibility to implement lipase-catalysed interesterification reactions in continuous reactors to lower the cost of the enzymatic process and make it competitive with the current chemical processes [2, 4, 9, 11–15]. The operational stability of immobilised lipases was shown to depend on several parameters such as the biocatalyst itself, the water content of the fat and the presence of oxidation products, related to the degree of refining of these fats [3, 9, 14]. Also, the bioreactor configuration and operation mode will affect the operational stability of the biocatalyst. Continuous packed-bed reactors (PBR) may be preferably used when product inhibition, substrate activation and/or reaction reversibility occur, while continuous well-stirred-tank reactors (CSTR) are more adequate for processes involving substrate inhibition or product activation. The kinetic behaviour of the continuous fluidised-bed reactors (FBR) normally lies between that of the PBR and the CSTR (M. Chaplin at http://www.lsbu.ac.uk/bi ology/enztech/cstr.html, 2004). Fluidised-bed bioreactors perform consistently better than the equivalent PBR. The higher conversion in the FBR is probably because, in PBR, part of the activity of the fixed bed is not available to contact the substrate, due to (i) preferential flow patterns through the bed and/or (ii) occlusion of surface area due to particle-particle or particle-wall contact [16].

The framework of this study is the search for biocatalysts with eventual novel properties as an alternative to the commercial immobilised lipases used in the majority of interesterification studies. In this context, *Candida parapsilosis* lipase/acyltransferase, which preferentially catalyses alcoholysis over hydrolysis when in aqueous or in biphasic aqueous/organic media [17–22], was previously tested for the interesterification of fat blends containing *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), in solvent-free media [23]. In that study, the immobilised enzyme presented, at a water activity (a_w) of 0.97, an interesterification activity similar to that exhibited by commercial immobilised lipases at a_w values lower than 0.5.

In the present study, batch and continuous operational stabilities of the immobilised *C. parapsilosis* lipase/acyltransferase in the interesterification of fat blends containing *n*-3 PUFA, in solvent-free media, were evaluated. The interesterification was carried out at laboratory scale in a continuous FBR. The biocatalyst was also reused in consecutive 23-h batches, in a total of four batches, with or without rehydratation of the biocatalyst by the addition of water to the fresh medium. The interesterification activity was indirectly followed by the decrease of the amount of crystallised fat [solid fat content (SFC)], at storage, processing and consumption temperatures (10, 20, 30 and 35 °C). The modification in acylglycerol profile and the accumulation of oxidation products and FFA, throughout the enzymatic interesterification, were also assessed.

2 Materials and methods

2.1 Materials

The refined, bleached and deodorised fats used in the experiments, palm stearin (PS) and palm kernel oil (PK), were donated by FIMA/VG, Produtos Alimentares, Portugal. The commercial concentrate of TAG rich in *n*-3 PUFA, "EPAX 4510TG" (45% EPA and 10% DHA), was a gift from EPAX AS, Lysaker, Norway.

The lipase/acyltransferase from *C. parapsilosis* was produced by overexpression of the corresponding gene in *Pichia pastoris* according to Brunel *et al.* [24] and immobilised on Accurel MP 1000 (Membrana GmbH, Obernburg, Germany) as previously described [23]. The hydranal® Coulomat AG-H, for water content assay, was from Riedel-de-Häen, Germany. Molecular sieves 4A and acetonitrile for HPLC of gradient grade were from Sigma-Aldrich, Germany. HPLC-grade acetone was from Fisher Scientific, UK. The other reagents used were of *p.a.* grade and obtained from various sources.

2.2 Methods

2.2.1 Preliminary batch interesterification reactions

Preliminary studies were carried out in a batch reactor (i) to investigate the maximum $SFC_{35\,°C}$ reduction that could be achieved by the interesterification catalysed by *C. parapsilosis* lipase/acyltransferase, (ii) to select the blend formulation for subsequent studies, and (iii) to investigate mass transfer limitations.

Reactions were carried out in thermostatted cylindrical glass reactors (100 mL) closed with rubber stoppers, under magnetic stirring at 60 °C for 30 h. The biocatalyst was used

at its original water activity (a_w = 0.97 at 30 °C; water content of 46.0%, wet basis) and its load was fixed at 5 wt-%. Two blend formulations were tested in order to choose the best one for the continuous FBR operation. Blend I was a mixture of PS (55 wt-%), PK (35 wt-%) and "EPAX 4510TG" (10 wt-%). Blend II consisted of PS (45 wt-%), PK (45 wt-%) and "EPAX 4510TG" (10 wt-%).

The choice of these formulations was based on the following aspects. In a previous work [23], the interesterification activity of the biocatalyst used showed to be higher when lower PS concentrations were used. Also, due to the high melting point of this fat (slip melting point higher than 44 °C), blends with a high content of PS are not adequate for use in continuous reactors because fat solidification in tubing can easily occur even at 60 °C. Conversely, from an industrial point of view, blends rich in PS are preferred due to its low cost. Thus, PS contents of 45 and 55% were tested.

During the time course of the reaction, 5-mL samples were taken and paper-filtered (Whatman 4) in an oven at approximately 70 °C. All samples were stored at –18 °C for subsequent analysis (*cf.* 2.2.5).

In order to evaluate possible mass transfer limitations, batch interesterification was carried out using blend II, under the same conditions but without agitation of the reaction medium.

For each reaction time, t, the percentage of $SFC_{35\,°C}$ reduction was calculated as follows:

$$SFC_{35°C}reduction = \left(\frac{SFC_0 - SFC_t}{SFC_0}\right) * 100 \quad (1)$$

where SFC_0 and SFC_t are the $SFC_{35\,°C}$ of the initial blend and after a time t of interesterification.

Initial interesterification rates were calculated by linear regression on the initial data points (time, % of $SFC_{35\,°C}$ reduction) and were expressed as percentage of $SFC_{35\,°C}$ reduction per hour.

2.2.2 Continuous interesterification experiments

The performance of *C. parapsilosis* lipase/acyltransferase for the interesterification was tested in a continuous FBR operating at 60 °C during 300 h. The FBR was a jacketed glass column (internal diameter of 2 cm; total height of 20 cm). The temperature in the reactor was maintained at 60 °C by circulating water in the jacket. The substrate used was fat blend II, with an initial $SFC_{35\,°C}$ value of 15.3. This blend was continuously pumped from a reservoir at 60 °C through silicone tubing at a flow rate of 0.67 mL/min to the bottom end of the bioreactor. To avoid solidification of the fat inside the tubing, the bioreactor had to be covered with a coiled insulation strap containing a thermostatted electrical resistance. An amount of 10 g biocatalyst was used, which corresponded to an apparent volume of 18.9 cm^3. The volume of the expanded bed was 28.3 cm^3, corresponding to a residence time of 14 min and a biocatalyst concentration of 53.6 wt-%. Samples were taken along the operation time, as previously described (*cf.* 2.2.1.).

2.2.3 Batch operational stability test

Batch operational stability was followed in consecutive batches of 23 h duration each, using the blend II formulation. Interesterification was carried out as previously described (*cf.* 2.2.1). After each 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 four batches were performed using the same biocatalyst sample. Samples were taken during the 23-h reaction time of each batch.

In parallel, similar four consecutive batches were carried out using fresh medium with water addition, in order to rehydrate the biocatalyst. The water content of the reaction medium was monitored for each batch (*cf.* 2.2.5.). The amounts of water added to the reaction medium were calculated in order to reach an average value of about 0.2 wt-% water. Water addition was performed after 7.5 h in the second batch and 0 h in batches 3 and 4, when a noticeable decrease in the water content of the reaction medium was detected and not following a previously established protocol.

2.2.4 Lipase deactivation kinetics

The $SFC_{35\,°C}$ values (*cf.* 2.2.5.) were used to assess the residual activity of the biocatalyst. Each experimental data point, at time t, was converted to the fraction of the original activity, *i.e.* its residual activity.

In continuous experiments, the residual activity, a, was defined as the ratio between the observed $SFC_{35\,°C}$ reduction at time t and the initial $SFC_{35\,°C}$ reduction. The initial reduction was assumed to be that obtained after 1 h of operation, *i.e.* when a pseudo-steady state was reached.

With respect to batch operational stability tests, the activity of each batch corresponds to the $SFC_{35\,°C}$ reduction observed after 23 h of reaction time. The first batch was used as the reference (100% activity). The residual activity (a_n, %) of the biocatalyst at the end of each batch n (n = 1, . . ., 4) was thus estimated as follows:

$$a_n = \left(\frac{SFC_{35°C-Batch\_1}}{SFC_{35°C-Batch\_n}}\right) * 100 \quad (2)$$

For each batch n, the relative activity was determined as the residual activity of the biocatalyst in the batch with water addition (a_{Wn}) *vs.* that in the corresponding batch without adding water (a_n), as follows:

$$Relative\ Activity = \frac{a_{Wn}}{a_n} \quad (3)$$

For the biocatalyst used in FBR, the following first-order deactivation kinetics model was fitted to the deactivation profiles:

$$a = A * e^{-k_d t} \quad (4)$$

where k_d is the deactivation rate constant (h^{-1}), t is the operation time (h), and A is a constant.

When the biocatalyst was used in consecutive batches, the following equation was used in analogy to Eq. (4):

$$a = A * e^{-k_d n} \quad (5)$$

where k_d is the deactivation rate constant expressed in (batch number)$^{-1}$, n is the 23-h batch number, and A is a constant.

The operational half-life time of the biocatalyst, *i.e.* the operation time needed to reduce its original activity to 50%, was estimated by the models fitted to the deactivation profiles.

The fit of kinetic models to experimental data was carried out using the solver add-in from Excel for Windows, version 8.0 SR2, by minimising 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^{-6}; 5% of tolerance and 10^{-4} convergence.

2.2.5 Analytical methods

2.2.5.1 SFC assay

The interesterification of fat blends was indirectly evaluated by the assay for the amount of the solid fraction at different temperatures, known as SFC, by nuclear magnetic resonance (NMR). The SFC values at 10, 20 and 30 °C ($SFC_{10\,°C}$, $SFC_{20\,°C}$, $SFC_{30\,°C}$) are related to the rheological behaviour of fats at storage, packaging and utilisation of bakery margarines, respectively. The SFC at 35 °C ($SFC_{35\,°C}$) is particularly important for table margarines, since it is related to the extent of melting in the mouth. The $SFC_{35\,°C}$ values of the interesterified fats must be smaller than their original counterparts, and as low as possible to prevent a sandy and coarse texture of the margarine.

The SFC values of the blends were assayed in a pulsed NMR spectrophotometer (Bruker Minispec mq20 NMR analyser, Germany). For NMR analyses, 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 prior to the SFC measurement [25].

2.2.5.2 Free fatty acids assay

The FFA content was assayed by titration with a 0.1 N sodium hydroxide aqueous solution. Its percentage (wt/wt) was calculated on the basis of the molecular weight of oleic acid.

2.2.5.3 Oxidation products assay

Thermal oxidation of the fat was indirectly evaluated by UV absorbance at 232 nm (Abs_{232nm}, related to the presence of initial products of oxidation, *i.e.* conjugated hydroperoxides) and at 270 nm (Abs_{270nm}, related to final oxidation products, *i.e.* FFA, aldehydes and ketones) of 1% (wt/vol) fat blends in *iso*-octane.

2.2.5.4 Assay for acylglycerol profile

The fatty acid composition of each fat and the acylglycerol profiles of the blends used, before and after interesterification catalysed by *C. parapsilosis* lipase/acyltransferase, have been described previously [10, 23].

The changes in acylglycerol profile due to the interesterification reaction were evaluated by non-aqueous reverse-phase high-performance liquid chromatography (RP-HPLC), using a Merck Hitachi (Germany) chromatograph equipped with an RP column (100 Superspher 100-RP-18; 250 × 4 mm i.d., 5 µm particle size) and a refractive index detector. Analysis and tentative peak identification according to their equivalent carbon number (ECN) were performed as previously described [23, 26]. Each chromatogram showed up to 25 peaks, at different approximate retention times (rt, min) with the following ECN values: peak 1: ECN ≤28, rt = 3.2; peak 2: ECN ≤28, rt = 3.8; peak 3: ECN = 28, rt = 4.3; peak 4: ECN = 28, rt = 4.7; peak 5: ECN = 30, rt = 5.6; peak 6: ECN = 32, rt = 6.0; peak 7: ECN = 34, rt = 6.8; peak 8: ECN = 36, rt = 7.8; peak 9: ECN = 38, rt = 9.1; peak 10: ECN = 40, rt = 10.2; peak 11: ECN = 40, rt = 10.7; peak 12: ECN = 42, rt = 12.1; peak 13: ECN = 42, rt = 12.7; peak 14: ECN = 44, rt = 13.7; peak 15: ECN = 44, rt = 14.4; peak 16: ECN = 44, rt = 15.2; peak 17: ECN = 46, rt = 16.0; peak 18: ECN = 46, rt = 16.9; peak 19: ECN = 48, rt = 18.6; peak 20: ECN = 48, rt = 19.6; peak 21: ECN = 48, rt = 20.7; peak 22: ECN = 48, rt = 22.0; peak 23: ECN = 50, rt = 23.7; peak 24: ECN = 50, rt = 25.2; peak 25: ECN = 50, rt = 26.9.

2.2.5.5 Water content assay

Solutions in *n*-hexane (10%, wt/vol) previously dried by molecular sieves 4A were prepared for each fat sample [10]. Aliquots of 0.5 mL were withdrawn from these organic solutions using a 0.5-mL glass chromatography syringe, and the water content was measured, in triplicate, with a Metrohm 684 Karl Fischer Coulometer.

3 Results and discussion

3.1 Preliminary batch interesterification reactions

The results obtained in 30-h batch interesterification reactions, performed with blends I and II under agitation or without agitation of the reaction medium, are presented in Fig. 1.

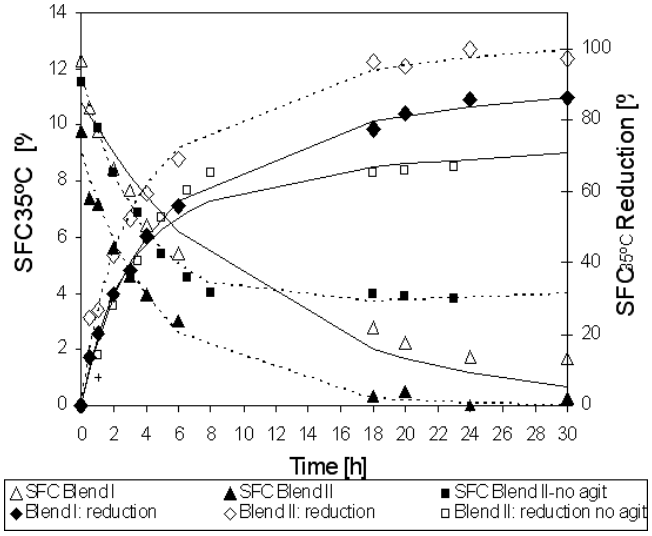

Figure 1. Time course of the SFC at 35 °C ($SFC_{35\,°C}$) and the corresponding percentages of reduction, obtained in the batch interesterification of fat blends I and II at 60 °C during 30 h, under magnetic stirring or with no agitation, catalysed by *C. parapsilosis* lipase/acyltransferase.

Quasi-equilibrium was attained after 20 and 18 h of reaction, corresponding to 82 and 97% $SFC_{35\,°C}$ reduction, respectively, when blends I and II were used under completely mixed conditions.

For reaction times similar to chemical interesterification times (less than 3 h), $SFC_{35\,°C}$ reductions of about 38 and 52% were obtained for blends I and II, respectively. These results are analogous to those previously obtained by lipase-catalysed interesterification of similar fat blends in solvent-free media [8, 10, 27].

When the interesterification of blend II was carried out without agitation of the reaction medium, the $SFC_{35\,°C}$ reduction values were always lower than those obtained with the same blend under agitation. In addition, the quasi-equilibrium was reached after about 8 h of reaction and only 65% $SFC_{35\,°C}$ reduction was achieved. However, the initial interesterification rate (10.4% SFC decrease/h) was similar to that observed for the interesterification of blend I under agitation (10.9% SFC decrease/h) and lower than the value obtained with blend II, under agitation (13.6% SFC decrease/h). The presence of mass transfer limitations in the experiments with no medium agitation explains the differences observed in initial rates and also in the conversion value at quasi-equilibrium situation.

Blend I is richer in PS and poorer in PK than blend II. The differences observed between blends I and II may thus be explained by a higher affinity of the enzyme for PK (lauric fat) or a lower affinity for PS, as previously reported [23, 28]. Also, a higher viscosity of blend I, due to the higher content of PS, may promote mass transfer limitations comparable to those observed in non-agitated media.

Blend II was chosen for subsequent experiments, regarding the best results obtained with this blend. Also, using a lower PS content, fat solidification problems inside the tubing of the continuous bioreactor are minimised.

3.2 Continuous interesterification experiments

The FBR was operated continuously at 60 °C for 300 h. The evolution of the SFC values at different temperatures of the interesterified fat blends during the continuous operation of the reactor is shown in Fig. 2. When the steady state was reached (after 1 h of operation), a reduction in all the SFC values was observed upon interesterification. However, a progressive decrease in SFC reduction was observed during the first 26 h of operation, from an initial $SFC_{35\,°C}$ reduction value of 46.7% down to 2.5% after 26 h of operation.

In order to describe the apparent deactivation kinetics, each experimental data point, at time t, was converted to the fraction of the original activity, *i.e.* its residual activity (*cf.* 2.2.4.).

The inactivation profile of *C. parapsilosis* lipase/acyltransferase in an FBR could be well described by the following first-order deactivation exponential model:

$$a = 105.8 * e^{(-0.076t)} \quad (6)$$

An operational half-live of 9 h was estimated from this equation.

During the continuous operation of the bioreactor, FFA, water content and the presence of first and final oxidation products in the outlet stream were assayed (Fig. 3).

FFA may come from the first step of interesterification or from the competing hydrolysis reaction [7, 29]. At the beginning of the operation, high levels of FFA were observed (ca. 7 wt-%) in the interesterified fat. A sharp decrease in this value was then observed and after 5 and 19 h of running, 5.7 and 1.9% FFA, respectively, were present in the outlet stream. From 26 h of operation time onwards, the average FFA content of the outlet fat stream was only 0.55% (standard deviation = 0.07, 10 samples). This decrease was accompanied by a similar trend for the water content in the outlet stream, as previously reported [30]. The immobilised *C. parapsilosis* enzyme showed an optimum interesterification activity at a water activity (a_w) of 0.97 [23]. Thus, the observed inactivation may be explained by a dehydration of the biocatalyst due to the removal of water by the fat stream. Inactivation by reaction substrates or products, namely FFA, may also occur.

In spite of the high temperature used (60 °C), no significant increase in first or final oxidation products was observed (Abs_{232nm} = 4.42, standard deviation = 0.34; Abs_{270nm} = 0.64, standard deviation = 0.09; average values of 18 samples). This indicates that under the reaction conditions used, the thermal oxidation of the fats can be neglected, despite the considerable amounts of *n*-3 PUFA in the blends.

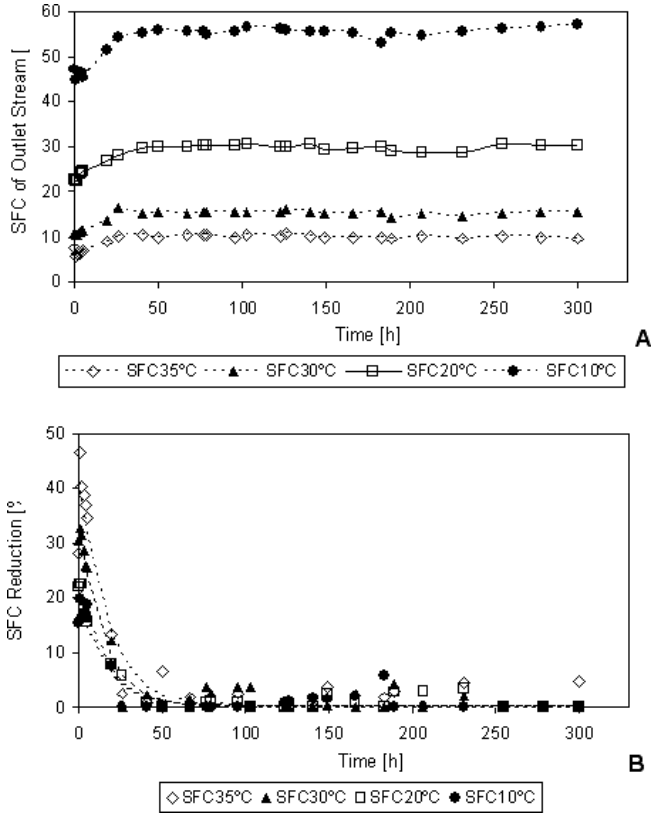

Figure 2. SFC at 10, 20, 30 and 35 °C ($SFC_{10\,°C}$, $SFC_{20\,°C}$, $SFC_{30\,°C}$ and $SFC_{35\,°C}$) (A) and the corresponding percentages of reduction (B) of the fat blends obtained by continuous interesterification at 60 °C, catalysed by *C. parapsilosis* lipase/acyltransferase in a fluidised-bed bioreactor.

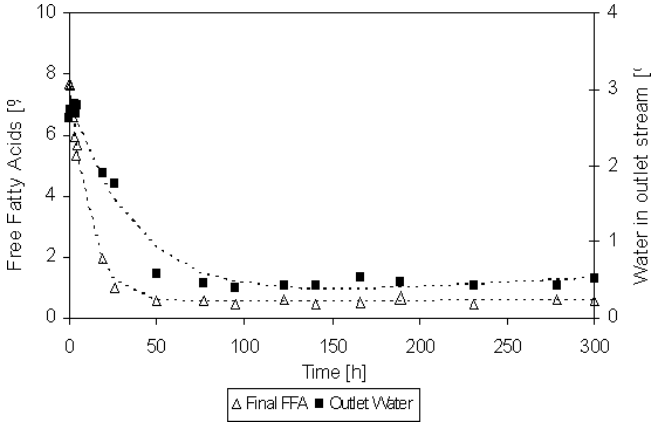

Figure 3. FFA values of the interesterified fat blends obtained during 300 h of continuous operation of an FBR at 60 °C.

As previously reported [23], remarkable differences between the acylglycerol profiles of the inlet and outlet fat blends taken after the first hours of continuous FBR operation were observed: A decrease in the peaks of higher ECN (≥48) was accompanied by an increase in the peaks of medium (36≤ ECN <48) and low ECN (≤34). The observed increase in TAG of medium ECN values confirms the interesterification activity of the *C. parapsilosis* lipase/acyltransferase. The increase in the peaks with low ECN values may correspond to diacylglycerols (DAG) and/or to new TAG species formation [23]. The latter are TAG containing long-chain PUFA originally present in the "EPAX 4510TG" concentrate.

The evolution of the main peaks in the acylglycerol profile of the outlet stream blends obtained during the first 26 h of operation (when interesterification activity was detected) are presented in Fig. 4. In this figure, the inlet stream blend corresponds to the sample at time 0 and the sample at 1 h corresponds to the first sample taken under steady-state conditions.

Peak 2 (ECN ≤28) is mainly formed by DAG [23], the concentration of which showed a maximum at 1 h of reaction time, decreasing thereafter. This profile may be explained by the promotion of the first hydrolytic step of the interesterification reaction during the first hours of bioreactor operation, due to water availability in the support. When water is removed, a decrease in this peak is observed. Also, an increase in peak 6 (ECN of 32) and peak 7 (ECN 34) areas was observed during the operation time. For both peaks, a 70% increase was measured after 1 h, and a maximum of 180% increase occurred thereafter, which was maintained during the first 26 h of operation.

Conversely to peaks 2, 6 and 7, after 1 h of operation, a decrease in about 63% of the area of peak 8 (ECN 36) and 66% of peak 9 (ECN 38) was observed. From this time on, during the 26 h of run time, the biocatalyst maintained its ability to catalyse the conversion of the compounds associated with these peaks.

With respect to peaks of ECN 40 (peaks 10 and 11), the area of peak 10 increased about 1.4 times after 1 h of operation. This value was maintained during the first 26 h. Conversely, 74% of peak 11 was consumed after 1 h and the consumption of this peak was observed during the first 26 h of operation.

Different profiles were observed for peaks of ECN 48 (peaks 19–22): about 55% decrease in the area of peak 19 was observed during the 26 h of continuous operation; 19% reduction in peak 21 was observed at 1 h of operation, but the ability of the biocatalyst to consume this peak decreased thereafter. For peaks 20 and 22, no significant variation was observed during the 26 h of operation time.

3.3 Batch operational stability

A very low operational activity of the biocatalyst was observed in the continuous fluidised-bed bioreactor. Among other factors, the operational stability of a biocatalyst depends on the bioreactor type and the operation mode used. In this context, the operational stability in consecutive runs carried out in completely mixed-batch reactors was addressed. Also, in

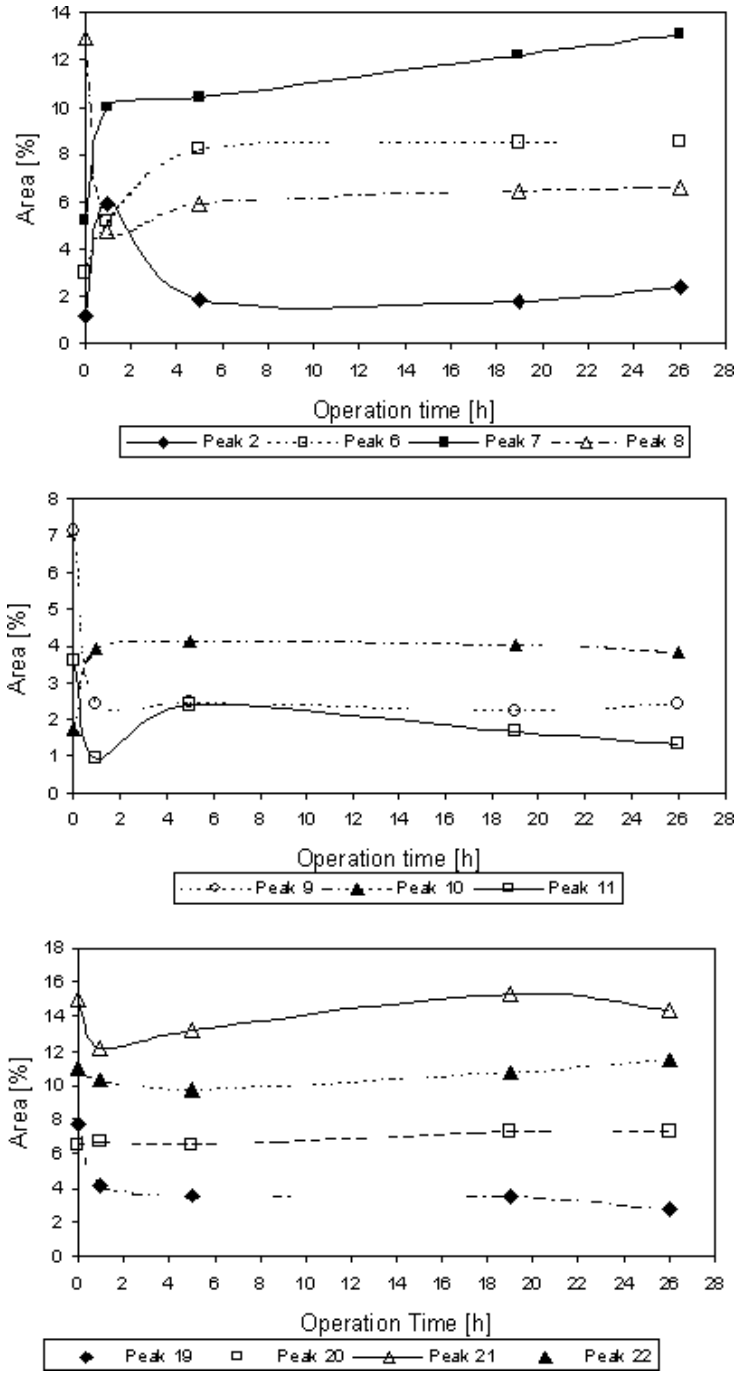

Figure 4. Evolution of the main peaks in the acylglycerol profiles of interesterified fat blends obtained during 26 h of continuous operation of an FBR. See "Assay for acylglycerol profile" (2.2.5.4) for peak identification.

order to investigate if the observed inactivation was due to dehydration of the immobilised biocatalyst, a set of consecutive batches with fed-batch water addition was carried out (*cf.* 2.2.3.).

$SFC_{35\,°C}$ values were used to calculate the activity of the biocatalyst at the end of each batch, either with or without the addition of water (*cf.* 2.2.4.). The activity of the biocatalyst in the first batch was assumed to be 100% and used as reference. For both sets of experiments, the activity decay of the biocatalyst followed a first-order exponential deactivation kinetics (*cf.* 2.2.4.). When no water was added to the reaction medium, the following model equation was fit:

$$a = 470 * e^{(-1.55n)} \quad (7)$$

Therefore, when the biocatalyst was used with no water addition, its operational half-life was 0.45 batches, *i.e.* 10 h, a value

close to that estimated for the FBR. When water was added to the reaction medium, the inactivation of the biocatalyst was slower, with an estimated half-life time of 18 h (0.88 batches of 23 h of duration each). The fitted equation was:

$$a = 239 * e^{(-0.88n)} \quad (8)$$

In fact, a beneficial effect of adding water to the reaction medium was observed during the four consecutive batches, suggesting that the inactivation of the biocatalyst may result from its dehydration.

The relative activity, as a function of batch number n, can be described by the following exponential function ($r^2 = 0.956$):

$$Relative\ Activity = 0.63e^{0.481n} \quad (9)$$

The water and FFA contents of the reaction media during the four consecutive batches, either without or with water addition, are presented in Fig. 5. Comparing these two sets of experiments, no significant differences in the water content of the reaction media could be noticed, in spite of the differences observed in enzyme activity.

Also, the profiles of the FFA content of the reaction medium, during the four consecutive batches, were similar for both sets of experiments (Fig. 5). These results suggest that the added water was not consumed in the hydrolysis reaction and was primarily used to stabilise the active conformation of the enzyme.

With respect to first and final oxidation products, no significant variation was observed during these experiments (data not shown). Again, the thermal oxidation of *n*-3 PUFA in 23-h batch interesterification at 60 °C can be neglected.

Concerning the modification of the acylglycerol profile during the consecutive batches, Fig. 6 shows the relative amounts of each peak of the chromatogram in the initial blend samples, in the first interesterification batch using the fresh biocatalyst (sample A1) and after the second utilisation of the same biocatalyst, without (sample A2) or with water addition to the reaction medium (sample B2).

In the samples obtained at the end of the first batch with no water addition, the largest differences in the acylglycerol profiles were (i) the production of acylglycerols of ECN equal or lower than 30 (peaks 2–5) and of ECN 44 (peaks 15 and 16) and (ii) the consumption of peak 8 (ECN 36) and acylglycerols of ECN 48 (peaks 20–22). In this set of experiments, the acylglycerol profiles of interesterified blends from batches 2–4 were not very different from that of the original blend (data from batches 3 and 4 are not shown). Conversely, when water was added to the reaction medium, the acylglycerol profile of the second batch (sample B2) showed pronounced differences from that of the initial blend sample (Fig. 6).

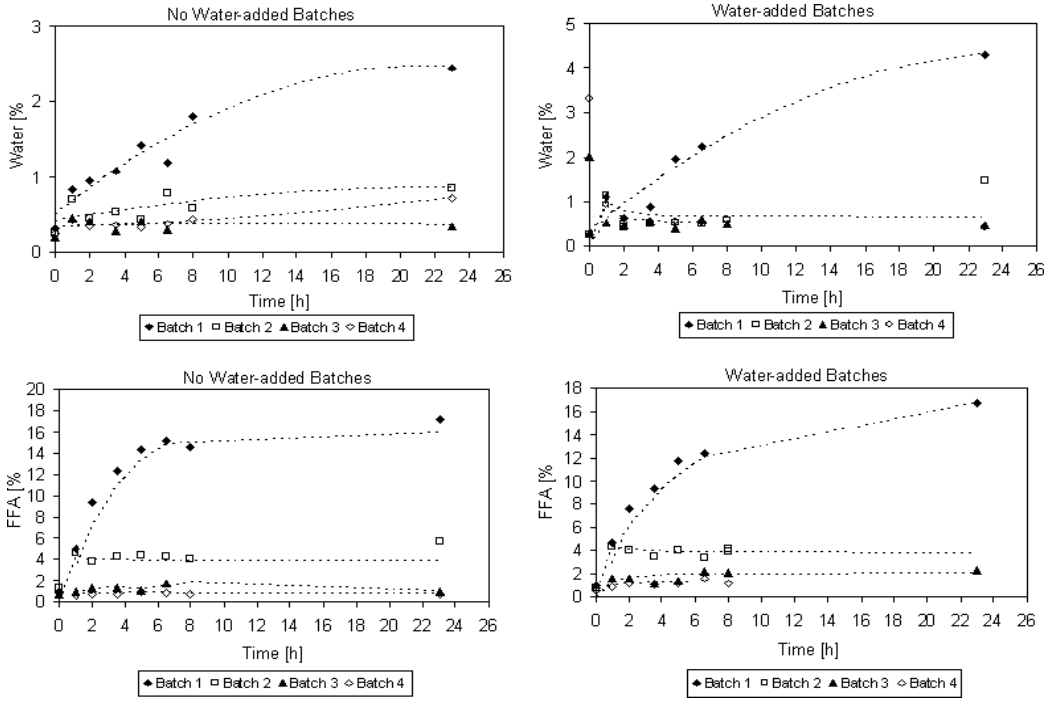

Figure 5. Water and FFA contents of reaction media during the four consecutive batches without water addition or with water added to the reaction medium.

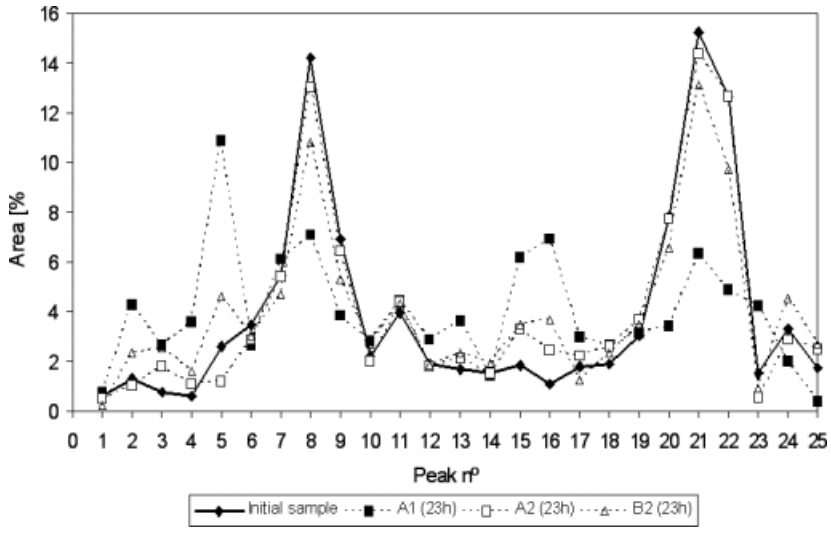

Figure 6. Acylglycerol profiles of initial blend II and at the end of batch 1 (A1) and batches 2, without (A2) or with (B2) water addition to the reaction medium. See "Assay for acylglycerol profile" (2.2.5.4) for peak identification.

4 Conclusions

The immobilised *C. parapsilosis* lipase/acyltransferase exhibited a low operational stability during the interesterification of fat blends at 60 °C in a medium consisting of melted fat and immobilised catalyst, both in continuous operation carried out in an FBR (apparent half-life of 9 h) and when used in four consecutive 23-h batches (apparent half-life of 10 h). However, the fed-batch addition of water during the consecutive batches allowed the increase of the immobilised biocatalyst operational stability up to 18 h. The loss of activity may thus be attributed to a progressive dehydration occurring during the bioreaction, rather than to product and/or substrate inhibition effects.

When compared to commercially available lipases, the biocatalyst assayed in this study has the great advantage of presenting its maximum interesterification activity under high water activity values. In practice, highly hydrated preparations are usually obtained as a result of enzyme immobilisation. In the case under study, no drying of the immobilised preparation is required. On the contrary, a large activity decrease is observed when water is removed from the immobilised preparation. It should therefore be possible to considerably improve the operational stability of this biocatalyst by maintaining the immobilised preparation highly hydrated. The rehydration of the immobilised lipase between consecutive batches, up to a water activity of about 0.97, should thus be a feasible strategy. In the continuous FBR case, water should be added to the fresh medium to ensure the hydration of the enzyme present in the bed. The hydration of the enzyme during the operation, which is easier and cheaper to perform than dehydration, is crucial for the maintenance of its activity and for a high operational stability. The optimisation of immobilisation matrices may also contribute to attain these goals.

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Conflict of interest statement

The authors have declared no conflict of interest.

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