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# Interesterification of fat blends using a fermented solid with lipolytic activity

Kátia Rasera[a,d], Natália M. Osório[a,b], David Alexander Mitchell[d], Nadia Krieger[c], Suzana Ferreira-Dias[a,*]

[a] Instituto Superior de Agronomia, CEER-Biosystems Engineering, Technical University of Lisbon, Tapada da Ajuda, 1349-017 Lisbon, Portugal
[b] Instituto Piaget, ISEIT de Almada, 2800-005 Almada, Portugal
[c] Departamento de Química, Universidade Federal do Paraná, Cx.P. 19081, Centro Politécnico, Curitiba 81531-980, Brazil
[d] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Cx.P. 19046, Centro Politécnico, Curitiba 81531-980, Brazil

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ABSTRACT

Enzymatic processes to produce interesterified fats and oils with desirable properties to incorporate in margarines and shortenings are currently quite expensive. In the current work, fermented solids, produced by growing *Rhizopus oryzae* and *Rhizopus microsporus* on a mixture of sugarcane bagasse and sunflower seed meal, were able to catalyze the interesterification of a mixture of palm stearin, palm kernel oil and a concentrate of triacylglycerols enriched with ω-3 polyunsaturated fatty acids ("EPAX 4510TG"). The best conditions for the interesterification reaction with the fermented solid produced by *R. oryzae*, found by response surface methodology, were a temperature of 65 °C, a palm stearin content of 38% and an EPAX content of 15%. Under these conditions, after 24 h, the product had a solid fat content at 35 °C ($SFC_{35\,°C}$) of 2.3%, making it suitable for the production of margarines and shortenings. If the reaction time can be decreased, this process has good potential to lower the costs of the enzymatic interesterification process, since the fermented solids are reasonably cheap to produce and their use avoids the recovery and immobilization steps that are required for lipases produced by submerged fermentation.



## 1. Introduction

The physical, nutritional and sensory properties of margarines are attained by appropriate processing of selected blends of fats and oils [1]. Until the 1990s, hardstocks for margarines and shortenings were obtained through hydrogenation of polyunsaturated oils [1–3]. Although this process is relatively inexpensive, it destroys essential polyunsaturated fatty acids (PUFAs) and creates non-natural isomers of saturated fats [3]. Further, the partial hydrogenation of PUFAs results in the formation of undesirable *trans* fatty acids [1]. Nowadays, interesterification, in which fatty acids are exchanged between triacylglycerols (TAGs), is increasingly being used [1,4].

Interesterification of blends of palm stearin (PS), palm kernel oil (PK) and TAGs rich in omega-3 polyunsaturated fatty acids (ω-3 PUFAs) can produce healthy shortenings or margarines that have adequate physical properties, while being free of *trans* fatty acids. Palm stearin (PS) is relatively inexpensive, but its high content of saturated fatty acids and high melting point (44–56 °C) mean that it does not confer the required plasticity on the end-product [5]. Palm kernel oil (PK) has 40–50% of its fatty acid content constituted by lauric acid, which provides for easy digestion and confers plasticity on the final product [6,7]. The incorporation of ω-3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5$n-3$) and docosahexaenoic acid (DHA, 22:6$n-3$) brings benefits to human health, as these compounds help not only in the prevention and treatment of heart diseases, but also in the regulation of blood pressure and inflammatory conditions [8,9]. Additionally, DHA is important for the development of the brain and nervous system of infants and in the proper functioning of neuronal cells in adults [10,11].

Interesterification can be achieved through chemical or enzymatic processes. Chemical interesterification often involves the use of metal alkali catalysts. Disadvantages of chemical interesterification include the random interchange of acyl groups, contamination of the final product with residual catalyst, and the formation of soaps (sodium salts of fatty acids, monoacylglycerols and diacylglycerols) [12]. Conversely, interesterification with lipases (triacylglycerol acyl-hydrolases, E.C. 3.1.1.3) enables regioselective exchange of fatty acids and avoids side-reactions. When *sn*-1,3 selective lipases are used, the presence of a PUFA at the original *sn*-2 position in acylglycerols of vegetable oils is maintained, with nutritional benefits.

Over the last decade, lipase-catalyzed interesterification processes have been developed for the production of margarine and

* Corresponding author. Tel.: +351 21 3653540; fax: +351 21 3653200.
*E-mail address:* suzanafdias@mail.telepac.pt (S. Ferreira-Dias).

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**Nomenclature**

*List of symbols*

| | |
|---|---|
| $Abs_{232\,nm}$ | UV absorbance at 232 nm |
| $Abs_{270\,nm}$ | UV absorbance at 270 nm |
| $a_w$ | thermodynamic activity of water |
| CCRD | central composite rotatable design |
| DHA | docosahexaenoic acid |
| EPA | eicosapentaenoic acid |
| EPAX 4510TG | concentrate of triacylglycerols enriched with ω-3 PUFA (44% of EPA and 15% of DHA) |
| FFA | free fatty acids |
| FS | fermented solid |
| PS | palm stearin |
| PK | palm kernel oil |
| PUFAs | polyunsaturated fatty acids |
| $R^2$ | determination coefficient (quadratic correlation coefficient) |
| $SFC_{35\,°C}$ | solid fat content measured at 35 °C by nuclear magnetic resonance |
| SSF | solid state fermentation |
| TAG | triacylglycerol |

shortenings [2,3,13–21] and some processes have already been implemented at industrial scale [4,22]. However, they are quite expensive since the enzyme itself is costly and the required amount of enzyme is relatively high [16–19,23–25]. Costs can be reduced by immobilizing the enzyme, such that it can be recovered and used in subsequent batches or in continuous bioreactors [18,19,21], but this is only possible if the enzyme is highly stable.

The present study represents a search for a cheap biocatalyst as an alternative to the high-cost commercial immobilized lipases used in the majority of interesterification studies. It is inspired by the work of Salum et al. [26], who produced the lipase of *Burkholderia cepacia* by solid-state fermentation (SSF) and added the lyophilized fermented solid (FS) directly to a solvent-free reaction medium containing triacylglycerols and ethanol, in order to produce biodiesel esters. This strategy avoids the need for recovery of the enzyme and immobilizes it without the need for expensive supports or immobilization procedures [27]. The specific aim of the work is, therefore, to investigate whether fermented solids, produced by growing *Rhizopus oryzae* and *Rhizopus microsporus* on a mixture of sugarcane bagasse and sunflower seed meal, are capable of catalyzing the interesterification of a mixture of palm stearin, palm kernel oil and a concentrate of triacylglycerols enriched with ω-3 PUFA ("EPAX 4510TG"), in a solvent-free reaction medium. Species of *Rhizopus* were chosen for this biocatalytic process since several members of this genus are generally recognized as safe (GRAS) for use in the production of foods [28]. The success of the interesterification was characterized in terms of the solid fat content at 35 °C ($SFC_{35\,°C}$), which represents the amount of crystallized fraction. The $SFC_{35\,°C}$ of table margarines must be as low as possible, in order to ensure essentially complete melting in the mouth and to prevent a coarse and sandy texture [29,30].

## 2. Materials and methods

### 2.1. Materials

Refined, bleached and deodorized palm stearin (PS) and palm kernel oil (PK) were donated by Unilever Jerónimo Martins, Lda, Portugal. "EPAX 4510TG", a concentrate of TAG enriched with ω-3 PUFA, containing about 62% of ω-3 PUFA (including 44% of EPA and 15% of DHA), was a gift from EPAX AS, Lysaker, Norway. All other reagents were of analytical grade.

### 2.2. Solid-state fermentation

*R. microsporus* CPQBA 312-07 DRM, originally isolated from soil samples from Guadalajara, Mexico, and *R. oryzae* ATCC 34612, were used. Spore suspensions were prepared by growing the strains on potato dextrose agar at 30 °C for five days and harvesting the spores with a Tween 80 solution (0.01%, w/v). The spore concentration was determined using a Neubauer chamber.

The fermented solid was obtained by fermentation of a mixture of sugarcane bagasse and sunflower seed meal (1:3, w/w on a dry basis) following the procedure described by Alberton et al. [31]. The sugarcane bagasse (donated by Usina de Álcool Melhoramentos, Jussara, Paraná, Brazil) was used as it came from the industry, without any kind of pre-treatment. It was oven-dried at 60 °C and contained particles between 0.8 mm and 1.5 mm. Sunflower seeds (purchased at a local market) were milled and then sieved to obtain particles between 0.85 and 1.4 mm.

The solid-state fermentations were done in 250 mL Erlenmeyer flasks, each containing 10 g of dry substrate. Phosphate buffer solution (0.1 M, pH 7.0) was added to attain 75% moisture (w/w, wet basis), determined in an infrared moisture balance (Gehaka IV 2000, São Paulo, SP, Brazil). Flasks were plugged with cotton wool and autoclaved at 121 °C for 20 min. After cooling, the substrates were inoculated with 1 mL of spore suspension and incubated at either 30 °C (for *R. microsporus*) or 25 °C (for *R. oryzae*) for 24 h.

### 2.3. Assay for lipolytic activity

The fermented solid containing lipolytic activity was dried by lyophilization for 12 h at −45 °C in a Jouan (Paris, France) LP3 lyophilizer. An amount of 0.300 g of dried solid was added directly to 20 mL of an emulsion prepared with triolein (62 mM), gum arabic (3%, w/v), 2 mM $CaCl_2$, 2.5 mM Tris–HCl buffer and 150 mM NaCl [32], maintained at 37 °C in a pH-Stat 718 (Metrohm – Herisau, Switzerland). The release of fatty acids was followed for 10 min, with the pH being maintained at 7.0 through the addition of 0.05 M NaOH solution [31]. For both fungi this method gave a hydrolytic activity of 40 ± 2 U/g of dried fermented solid, where a unit of activity (U) is defined as the release of 1 μmol of fatty acid per minute, under the assay conditions.

### 2.4. Interesterification reactions

Interesterification reactions were performed in a magnetically stirred cylindrical batch reactor with temperature control. Each reaction medium consisted of a ternary blend of PS, PK and "EPAX 4510TG", in the stated proportions. After the reaction medium melted completely, a load of 6.5% (w/v) fermented solid was added. For analysis, samples of 5 mL were taken, the fermented solids were removed by filtration through Whatman #1 filter paper at 70 °C and the filtrate was stored at −18 °C.

### 2.5. Modeling the enzymatic interesterification

Interesterification experiments were carried out following a central composite rotatable design (CCRD), as a function of 3 factors (independent variables), in a total of 17 experiments (8 factorial points, 6 axial points and 3 central points) [33]. The 3 independent variables were PS concentration, "EPAX 4510TG" concentration and temperature. Table 1 shows the values of the three variables that correspond to each coded level. A load of 0.300 g of fermented solid, at its original water activity (0.7 for *R. microsporus* and 0.5 for *R. oryzae*), was used in 10 mL of reaction medium. After 24 h, samples were taken and filtered and stored as described above.

**Table 1**
Variables and levels for the central composite rotatable design.

| Variables | Symbol | Coded variable levels | | | | |
|---|---|---|---|---|---|---|
| | | −1.68 | −1 | 0 | +1 | +1.68 |
| Temperature (°C) | *T* | 56 | 60 | 65 | 70 | 73 |
| Concentration of palm stearin (%) | PS | 38 | 45 | 55 | 75 | 71 |
| Concentration of EPAX 4510TG (%) | EPAX | 06 | 10 | 15 | 20 | 23 |

### 2.6. Analytical methods

The water activity ($a_w$) of the fermented solids was measured at 30 °C in a ROTRONIC HYGROSKOP DT humidity sensor (DMS-100H).

As in the margarine industry, the interesterification of fat blends was indirectly followed by using a pulsed NMR spectrometer (Minispec P-20i, IBM) to determine the solid fat content at 35 °C ($SFC_{35\,°C}$) [16,34,35]. Samples were first melted at 60 °C, maintained at this temperature for about 10 min, kept at 0 °C for 60 min and finally maintained for 30 min at 35 °C prior to the SFC measurement [36]. Considering that the blends have significantly different initial $SFC_{35\,°C}$ values, due to their different compositions, it is convenient also to express the results in terms of the percentage reduction (%Red) of $SFC_{35\,°C}$, calculated as:

$$SFC_{35\,°C}(\%\text{Red}) = \frac{SFC_{35\,°C}(I) - SFC_{35\,°C}(F)}{SFC_{35\,°C}(I)} \times 100\% \quad (1)$$

where "*I*" and "*F*" represent the $SFC_{35\,°C}$ values at 0 and 24 h, respectively.

The free fatty acid (FFA) content was determined by titration with a 0.1 M NaOH solution. FFA percentage (w/w) was calculated on the basis of the molecular weight of oleic acid [6,37].

Thermal oxidation of the fat was indirectly evaluated by UV absorbance at 232 nm ($Abs_{232\,nm}$) and at 270 nm ($Abs_{270\,nm}$) of a 1% (w/v) fat blend in *iso*-octane [6,37].

### 2.7. Statistical analysis

The results of the CCRD experiments were analyzed using "Statistica™", version 7 (Statsoft, USA). The linear and quadratic effects of each of the 3 independent variables (factors), as well as their linear interactions, on interesterification reaction and hydrolysis were calculated. Their significance was evaluated by analysis of variance. The use of 5 levels for each factor (Table 1) enables the fitting of second order polynomials to the experimental data points and, therefore, the fitting of curved surfaces. First and second order coefficients were generated by regression analysis. The goodness of fit of the statistical models was evaluated by the coefficient of determination ($R^2$). High values of $R^2$ obtained suggest a good fit of the model to the experimental data points [10,33].

## 3. Results

### 3.1. Time course of interesterification

The substrates used in the interesterification reactions were chosen based on the works of Osório et al. [19–21] and Nascimento et al. [17], who used reaction media composed of PS, PK and "EPAX 4510TG", and commercial lipases or a non-commercial lipase/acyltransferase as biocatalyst, either in batch or in continuous mode. Preliminary interesterification experiments were carried out at 65 °C, using PS (55%), "EPAX 4510TG" (15%) and PK (30%), in order to establish the time of reaction and to select the microorganism to be used in the experimental design. The fermented solids (FS) from both *R. oryzae* and *R. microsporus* catalyzed interesterification, reducing the $SFC_{35\,°C}$ from an initial value of around 15% to a final value of 8–9% within 24 h (Fig. 1).

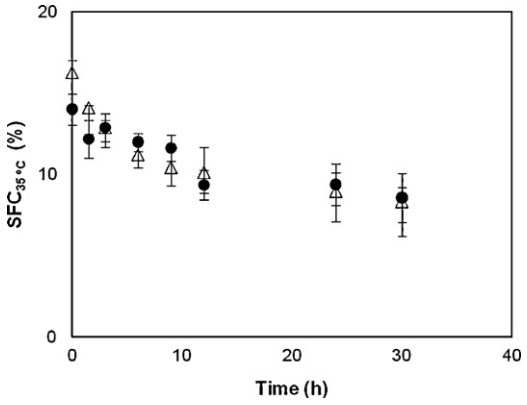


**Fig. 1.** Time course of the interesterification reaction of fat blends catalyzed by the fermented solids produced with (●) *R. microsporus* and (Δ) *R. oryzae*. Conditions: PS (55%), EPAX 4510TG (15%) and PK (30%), at 65 °C, with 6.5% (w/v) of fermented solid. Error bars represent the standard error of the mean.

The presence of FFA, a product of the hydrolysis reaction, can lead to a decrease in the yield of interesterified products and the formation of rancid flavors if these free fatty acids are oxidized [16,17,20]. With both fermented solids, the FFA content increased rapidly over the first 12 h and then increased more slowly, reaching 9% FFA for the *R. microsporus* FS and 7% FFA for the *R. oryzae* FS at 24 h (Fig. 2). The higher FFA content obtained for the *R. microsporus* FS may be due to the fact that it had a higher $a_w$ (0.7) than did the *R. oryzae* FS (0.5), since higher $a_w$ values are known to favor the production of FFA [16,20].

### 3.2. Application of response surface methodology

A CCRD was carried out in order to find conditions under which a product with an acceptably low $SFC_{35\,°C}$ value could be obtained. It was carried out using the *R. oryzae* FS since in the preliminary studies it generated a slightly lower final FFA level. Table 2 shows the experimental matrix, the $SFC_{35\,°C}$ values of reaction media

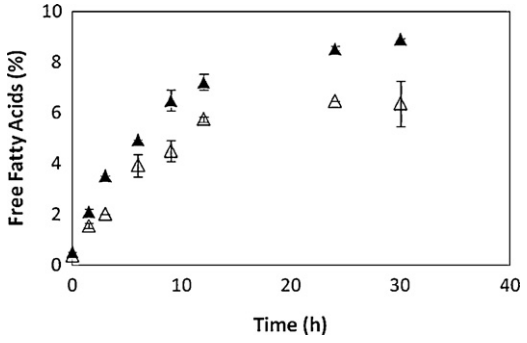


**Fig. 2.** Time course of the production of free fatty acids (FFA, %, w/w) during the interesterification reaction of fat blends catalyzed by the fermented solids with lipolytic activity produced by (▲) *Rhizopus microsporus* and (Δ) *Rhizopus oryzae*. Conditions: PS (55%), EPAX 4510TG (15%) and PK (30%), 65 °C, 6.5% (w/v) of the fermented solid. Error bars represent the standard error of the mean.

**Table 2**
CCRD – Uncoded values and the experimental and predicted results using fermented solids of *R. oryzae*.

| Experiment | $T$ (°C) | PS (%) | EPAX (%) | $SFC_{35\,°C}$ (%) | | Observed $SFC_{35\,°C}$ (% Red) | Predicted $SFC_{35\,°C}$ (% Red) ($\hat{Y}$) | FFA (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | Initial | Final | | ($\hat{Y}$) | |
| 1 | 60 | 45 | 10 | 16.8 | 6.0 | 64.2 | 67 | 7.1 |
| 2 | 60 | 45 | 20 | 17.7 | 5.9 | 66.8 | 68 | 7.1 |
| 3 | 60 | 65 | 10 | 29.6 | 17.7 | 40.2 | 37 | 5.6 |
| 4 | 60 | 65 | 20 | 30.4 | 16.9 | 44.6 | 44 | 6.2 |
| 5 | 70 | 45 | 10 | 13.4 | 6.0 | 55.2 | 58 | 5.9 |
| 6 | 70 | 45 | 20 | 14.1 | 7.7 | 45.8 | 51 | 4.3 |
| 7 | 70 | 65 | 10 | 25.0 | 16.1 | 35.6 | 36 | 6.4 |
| 8 | 70 | 65 | 20 | 27.4 | 17.5 | 36.3 | 36 | 6.8 |
| 9 | 56 | 55 | 15 | 18.9 | 9.5 | 49.7 | 50 | 6.3 |
| 10 | 73 | 55 | 15 | 18.5 | 11.2 | 39.5 | 35 | 5.9 |
| 11 | 65 | 38 | 15 | 12.3 | 2.3 | 81.3 | 74 | 7.4 |
| 12 | 65 | 71 | 15 | 24.9 | 16.6 | 33.3 | 36 | 5.5 |
| 13 | 65 | 55 | 06 | 19.0 | 9.0 | 52.6 | 51 | 6.6 |
| 14 | 65 | 55 | 23 | 24.6 | 11.3 | 54.1 | 51 | 6.3 |
| 15 | 65 | 55 | 15 | 23.0 | 12.2 | 47.2 | 49 | 4.9 |
| 16 | 65 | 55 | 15 | 18.4 | 8.6 | 53.1 | 49 | 6.2 |
| 17 | 65 | 55 | 15 | 22.3 | 11.6 | 47.9 | 49 | 5.9 |

before and after interesterification and the FFA content of each final interesterified fat blend. The FFA content of initial blends varied from 0.28 to 0.55%.

The percentage $SFC_{35\,°C}$ reduction of the interesterified fat blends catalyzed by the *R. oryzae* FS can be described by the following second-order polynomial model:

$$\hat{Y} = -72.85 + 10.61T - 6.63\mathrm{PS} + 2.68\mathrm{EPAX} - 0.09T^2 + 0.02\mathrm{PS}^2 + 0.02\mathrm{EPAX}^2 + 0.04(T)(\mathrm{PS}) - 0.07(T)(\mathrm{EPAX}) + 0.03(\mathrm{PS})(\mathrm{EPAX}) \quad (2)$$

where $T$, PS and EPAX represent the decoded values of the independent variables (see Table 1). Table 2 also shows the value of $SFC_{35\,°C}$ (%Red) predicted by this fitted statistical model.

Table 3 shows the analysis of variance of the estimated linear, quadratic and interaction coefficients of Eq. (2). Only the linear terms for $T$ and PS were significant ($p \leq 0.05$). The value of $R^2$ for the regression was 0.94, indicating that 94% of the variation in the response can be attributed to the independent variables and only 6% cannot be explained by the model. The model proposed for interesterification reactions was significant, with an $F$-value (11.15) greater than the tabulated one (3.29). Further, the lack-of-fit of the regression was not significant at the 5% level ($p = 0.29$), which indicates that the equation can be used to predict the effect of any combination of the studied variables.

Fig. 3 shows the response surface for $SFC_{35\,°C}$ (%Red) values, given by the model of Eq. (2). This is a 4-dimensional surface, but it can be illustrated by two three-dimensional surfaces, where only two of the three initial factors vary while keeping the remaining one constant at its value for the central point. Thus, the response surface is represented as a function of the temperature and PS concentration, with the EPAX 4510TG concentration fixed at 15% (Fig. 3A), and as a function of PS and EPAX concentrations, maintaining the temperature at 65 °C (Fig. 3B). In this figure, the highest value of $SFC_{35\,°C}$ reduction (%Red), of 81%, was obtained with a PS content of 38% and a temperature of 65 °C, with no local maximum in the experimental region.

None of the variables had a significant effect on the FFA content of the blends after 24 h reaction. At the beginning of the reaction, the fat blends had low acidities (from 0.28 to 0.55% FFA), whereas the final acidities varied from 4.3 to 7.4% FFA. Also, none of the experiments showed remarkable differences between the initial and final contents of oxidation products (Table 4).

## 4. Discussion

This study shows that fermented solids with lipolytic activity, produced using *R. oryzae*, can be used to catalyze the interesterification of palm stearin with palm kernel oil and a concentrate of triacylglycerols enriched with ω-3 PUFA (EPAX 4510TG). Although fermented solids containing lipolytic activity have previously been used to catalyze the resolution of racemates [38] and esterification and transesterification reactions to produce biodiesel esters [26,27], this is the first time that a fermented solid has been used to catalyze an interesterification reaction. The importance of this result is that it opens the possibility of reducing the costs of the enzymatic interesterification process used for the production of margarines and shortenings. This process should be acceptable to regulatory agencies since *R. orzyae* is recognized as GRAS by the FDA [39]. Further, after filtration of the final interesterified product to remove the fermented solids, any components that might have been extracted from the sugarcane bagasse or sunflower seed meal would be unlikely to cause problems as these are natural materials.

One of the key factors in any biocatalytic process is to reduce the cost of the enzyme preparation itself, which can contribute up to 35% of the total process cost, even if the enzyme is highly stable [40,41]. Tufvesson et al. [40] recently estimated the cost of immobilizing commercial lipases (produced by submerged fermentation) on hydrophilic and hydrophobic supports. The cost of the final immobilized product was estimated at 300–550 euros per kilogram, depending on the size of the production batch. Our solid-state fermentation process offers two potential cost advantages over the use of lipases produced in submerged fermentation. Firstly, the costs of production of lipases by solid-state fermentation are estimated to be only 30% of those in submerged fermentation, especially if solid agro-industries residues are used as substrates [42]. Secondly, the direct use of the dried fermented solid means that downstream processing steps such as enzyme recovery and immobilization are avoided [27]. It should be possible to decrease the costs of the fermented solid even further, since lyophilization is an expensive method of drying. The use of a simple warm-air drying should be investigated, as air drying at 55 °C has been successful in the production of dried fermented solids produced using the same substrate mixture and *R. microsporus* CPQBA 312-07 DRM [31]. Of course, in order for the low cost of the fermented solid to bring real cost advantages to the process, it will be necessary to demonstrate that it maintains its catalytic activity over long periods.

With the CCRD, it was possible to identify conditions in which a product with a low value of $SFC_{35\,°C}$ was obtained: with a

**Table 3**
Statistical analysis of the observed data for the $SFC_{35\,^{\circ}C}$ (% Red) using *R. oryzae* fermented solid.

| Source | Estimated coefficients | Sum of squares | Degrees of freedom | Mean squares | Calculated *F* | *p*-Value |
|---|---|---|---|---|---|---|
| Mean | −72.85 | 2260.42 | 9 | 2260.42 | 11.15 | 0.00001** |
| *T* | 10.61 | 264.72 | 1 | 264.72 | 11.50 | 0.036780* |
| PS | −6.63 | 1791.40 | 1 | 1791.40 | 173.90 | 0.00570** |
| EPAX | 2.68 | 0.033 | 1 | 0.033 | 0.0032 | 0.95983 |
| $T^2$ | −0.09 | 66.93 | 1 | 66.93 | 6.49 | 0.12556 |
| $PS^2$ | 0.02 | 47.27 | 1 | 47.27 | 4.59 | 0.16544 |
| $EPAX^2$ | 0.02 | 4.86 | 1 | 4.86 | 0.47 | 0.56304 |
| $T \times PS$ | 0.04 | 36.76 | 1 | 36.76 | 3.57 | 0.19945 |
| $T \times EPAX$ | −0.07 | 30.77 | 1 | 30.77 | 2.98 | 0.22606 |
| $PS \times EPAX$ | 0.03 | 17.67 | 1 | 17.67 | 1.71 | 0.32050 |
| Residuals | | 161.09 | 7 | 23.01 | | |
| Lack of fit | | 140.49 | 5 | 28.09 | 2.72 | 0.28973 |
| Pure error | | 20.60 | 2 | 10.30 | | |
| Total | | 2471.26 | 16 | | | |

$R^2 = 0.94$; $F_{5,7,0,05} = 3.29$. *T*: temperature; PS: palm stearin; EPAX: EPAX 4510TG.
* Significant at 5% level.
** Significant at 1% level.

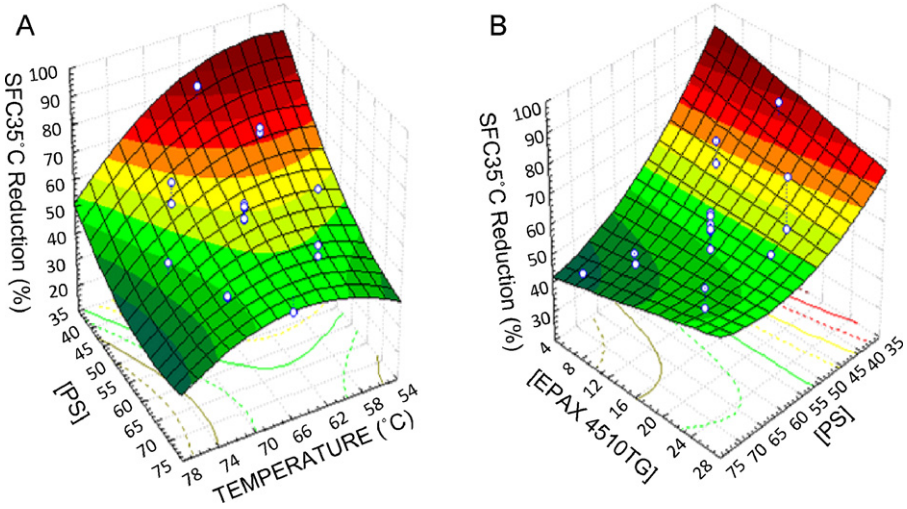


**Fig. 3.** Response surface fitted to the $SFC_{35\,^{\circ}C}$ (%Red) values of interesterified fat blends obtained using fermented solids of *R. oryzae* as a function of (A) PS concentration and temperature, with the EPAX value fixed at 15%, and of (B) PS and EPAX concentrations, with the temperature fixed at 65 °C.

temperature of 65 °C, a palm stearin content of 38% and an EPAX content of 15%, the final product had a $SFC_{35\,^{\circ}C}$ of 2.3%. Since in this case, the initial $SFC_{35\,^{\circ}C}$ was 12.3%, this corresponds to a $SFC_{35\,^{\circ}C}$ (%Red) of 81.3% (Table 2). The final SFC value is comparable with previous results obtained in various systems with different original oil blends and different lipase sources (Table 5). However, this interesterified product takes around 24 h to reach the final $SFC_{35\,^{\circ}C}$ value, compared to values as low as 30 min reported for Lipozyme TL IM [1]. Using an original blend of oils not too different from that used in the current work, Osório et al. [16] obtained an $SFC_{35\,^{\circ}C}$ value of 3% in 120 min using Novozym 435 as the catalyst. We need to obtain significant improvements in reaction rate in order to attain a similar reaction time. For example, it would be possible to increase the lipolytic activity by adding more solids. Following our work in the synthesis of biodiesel esters in a co-solvent-free system [26], it might be possible to do this by implementing this reaction in a packed-bed column reactor with continuous recirculation.

Improvements in the fermentation process itself to increase the level of lipolytic activity in the fermented solids would help in providing better reaction rates. For example, if we were able to obtain lipolytic activity levels as high as 1500 U $gDS^{-1}$, such as have been reported by Diaz et al. [43], it should be possible to decrease the interesterification reaction time significantly. It is worthy to notice that the results obtained in the present study are comparable to some others obtained with commercial lipases, in which 24–48 h interesterification reactions are reported [44,45].

**Table 4**
Oxidation products for *R. oryzae* reactions with different temperatures and substrate concentrations.

| Temperature (°C) | PS (%) | EPAX (%) | Primary oxidation products ($A_{232}$) | | Secondary oxidation products ($A_{270}$) | |
|---|---|---|---|---|---|---|
| | | | Initial | Final | Initial | Final |
| 56.59 | 55 | 15 | 4.20 | 4.36 | 1.53 | 1.74 |
| 73.40 | 55 | 15 | 4.14 | 4.58 | 1.81 | 1.81 |
| 65 | 38.18 | 15 | 0.50 | 0.48 | 0.22 | 0.14 |
| 65 | 71.82 | 15 | 3.99 | 4.44 | 2.02 | 2.01 |
| 65 | 55 | 6.6 | 3.89 | 4.28 | 2.50 | 2.41 |
| 65 | 55 | 23.4 | 5.13 | 5.29 | 3.96 | 4.69 |
| 65 | 55 | 15 | 4.72 | 4.74 | 1.62 | 1.84 |

**Table 5**
Studies of lipase-catalyzed interesterification.

| Authors | Substrates | Lipase[a] | Lipase concentration | Temperature | Results | Time |
|---|---|---|---|---|---|---|
| Silva et al. [46] | Lard, soybean oil | Lipozyme TL IM | 5% (w/w) | 60 °C | $SFC_{35\,^{\circ}C\ final}$ = 0% | 6 h |
| Paula et al. [45] | Soybean oil, milkfat | *Rhizopus oryzae* lipase (L036P, Biocatalysts) | 970 U/g | 45 °C | <2% FFA<br>High interesterification degree (76% consistency reduction) | 48 h |
| Li et al. [44] | Soybean oil, sunflower oil | Lipozyme TL IM | 10% (w/w) | 70 °C | $SFC_{35\,^{\circ}C\ final}$ = 7%<br>4–6% FFA | 24 h |
| Houmoller et al. [1] | Palm stearin, coconut oil, rapeseed oil | Lipozyme TL IM | 1% (w/w) | 70 °C | $SFC_{35\,^{\circ}C\ final}$ = 6.65%<br>0.2–2.2% FFA | 30 min |
| Laia et al. [34] | Palm stearin, palm kernel olein | Lipozyme IM 60 | 1% (w/w) | 60 °C | $SFC_{30\,^{\circ}C\ final}$: commercial margarine = 5%;<br>Experimental margarine = 14% | 6 h |
| Zainal and Yusoff [2] | Palm stearin and palm kernel olein | Lipozyme IM 60 | 1% (w/w) | 100 °C | $SFC_{35\,^{\circ}C\ final}$ = 0–4.1%<br>2.0–2.9% FFA | 24 h |
| Ming et al. [47] | Palm stearin and sunflower oil | Lipozyme IM 60, *Pseudomonas sp.* Lipase | 1% (w/w) | 60 °C | $SFC_{35\,^{\circ}C\ final}$ = 0–4.5% | 6–8 h |
| This work | Palm stearin, palm kernel oil, EPAX 4510TG | *Rhizopus oryzae* fermented solid | 6.5% (w/w) | 56 °C–73 °C | $SFC_{35\,^{\circ}C}$ = 2.3%<br>4.3% FFA | 24 h |

[a] Enzymes used were immobilized. The reactions were performed in media without solvent.

As with the work of Osório et al. [16,18–20], who studied the interesterification of similar blends of oils using commercial lipases and a non-commercial lipase/acyltransferase, FFA levels were undesirably high in the present work. Specifically, the experiment that gave the lowest final $SFC_{35\,^{\circ}C}$ of 2.9% also generated a FFA level of 7.4%. In fact, an increase in FFA levels during lipase-catalyzed interesterification of binary or ternary fat blends in solvent-free media is a general phenomenon [2,16,17,46–49]. The control of the water activity of the reaction medium may help to reduce FFA levels [16,17]. In addition, when interesterification reactions are implemented in continuous fluidized or packed-bed reactors, FFA levels decrease to about 1% [18,19]. Even if it is not possible to reduce FFA levels significantly, it should be noted that the free fatty acids are removed in the deodorization process that is routinely applied at the end of chemical or enzymatic interesterification processes [4]. In this process, free fatty acids (and other volatiles) are removed using superheated steam at 200–270 °C under low pressure [50].

## 5. Conclusions

It was shown that a fermented solid containing lipolytic activity, produced through the solid-state fermentation of a mixture of sugarcane bagasse and sunflower seed meal by species of *Rhizopus*, can be used to catalyze the interesterification of palm stearin, palm kernel oil and a concentrate of triacylglycerols enriched with ω-3 PUFA (EPAX 4510TG), producing interesterified fat blends with low $SFC_{35\,^{\circ}C}$. The use of fermented solid has good potential to reduce the costs of enzymatic interesterification for the production of margarines and shortenings, although further research is required to reduce the reaction time.

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## References

[1] L.P. Houmoller, D. Kristensen, H. Rosager, Talanta 71 (2007) 868–873.
[2] Z. Zainal, M.S.A. Yusoff, J. Am. Oil Chem. Soc. 76 (1999) 1003–1008.
[3] I. Karabulut, S. Turan, J. Food Comp. Anal. 19 (2006) 55–58.
[4] H.C. Holm, D. Cowan, Eur. J. Lipid Sci. Technol. 110 (2008) 679–691.
[5] L.O. Minga, H.M. Ghazali, C.C. Let, Food Chem. 64 (1999) 83–88.
[6] N.O.V. Sonntag, in: D. Swern (Ed.), Bailey's Industrial Oil and Fat Products, 4 ed., Wiley-Interscience, New York, 1982, pp. 97–173.
[7] A.R. Norizzah, C.L. Chong, C.S. Cheow, O. Zaliha, Food Chem. 86 (2004) 229–235.
[8] J.M. Kremer, Arthritis Rheum. 33 (1990) 810–820.
[9] A.P. Simopoulos, in: A.P. Simopoulos (Ed.), World Rev. Nutr. Diet, Basel, Switzerland, 1998, pp. 1–11.
[10] P.D. Haaland, Experimental Design in Biotechnology, Marcel Dekker Inc., New York, 1989.
[11] E.E. Zeijdner, A.C. Houwellingen, A.D.M. Kester, G. Hornstra, Prostaglandins Leukot. Essent. Fatty Acids 56 (1997) 395–401.
[12] M.D. Erickson, in: D.R. Erickson (Ed.), Practical Handbook of Soybean Processing and Utilization, AOCS Press and United Soybean Board, Champaign, 1995, pp. 277–296.
[13] X. Xu, S. Balchen, C.E. Hoy, J. Adler-Nissen, J. Am. Oil Chem. Soc. 75 (1998) 301–308.
[14] H. Mu, X. Xu, C.E. Hoy, J. Am. Oil Chem. Soc. 75 (1998) 1187–1193.
[15] V. Seriburi, C.C. Akoh, J. Am. Oil Chem. Soc. 75 (1998) 511–516.
[16] N.M. Osório, S. Ferreira-Dias, J.H. Gusmão, M.M.R. da Fonseca, J. Mol. Catal. B: Enzym. 11 (2001) 677–686.
[17] A.C. Nascimento, C.S.R. Tecelão, J.H. Gusmão, M.M.R. da Fonseca, S. Ferreira-Dias, Eur. J. Lipid Sci. Technol. 106 (2004) 599–612.
[18] N.M. Osório, J.H. Gusmão, M.M.R. da Fonseca, S. Ferreira-Dias, Eur. J. Lipid Sci. Technol. 107 (2005) 455–463.
[19] N.M. Osório, M.M.R. da Fonseca, S. Ferreira-Dias, Eur. J. Lipid Sci. Technol. 108 (2006) 545–553.
[20] N.M. Osório, E. Dubreucq, M.M.R. da Fonseca, S. Ferreira-Dias, Eur. J. Lipid Sci. Technol. 111 (2009) 120–134.
[21] N.M. Osório, E. Dubreucq, M.M.R. da Fonseca, S. Ferreira-Dias, Eur. J. Lipid Sci. Technol. 111 (2009) 358–367.
[22] U. Schörken, P. Kempers, Eur. J. Lipid Sci. Technol. 111 (2009) 627–645.
[23] S.F.A. Halim, A.H. Kamaruddin, W.J.N. Fernando, Bioresour. Technol. 100 (2009) 706–710.
[24] D. Royon, M. Daz, G. Ellenrieder, S. Locatelli, Bioresour. Technol. 98 (2007) 648–653.
[25] W. Du, Y.Y. Xu, D.H. Liu, J. Zeng, J. Mol. Catal. B: Enzym. 30 (2004) 125–129.

[26] T.F.C. Salum, P. Villeneuve, B. Barea, C.I. Yamamoto, L.C. Côcco, D.A. Mitchell, N. Krieger, Process Biochem. 45 (2010) 1348–1354.
[27] M.L.M. Fernandes, E.B. Saad, J.A. Meira, L.P. Ramos, D.A. Mitchell, N. Krieger, J. Mol. Catal. B: Enzym. 44 (2007) 8–13.
[28] M. Raimbault, C.R. Toro, in: S. Roussos, B.K. Lonsane, M. Raimbault, G. Viniegra-Gonzalez (Eds.), Advances in Solid State Fermentation, Springer, Dordrecht, 1997, pp. 169–181.
[29] B.S. Ghotra, S.D. Dyal, S.C. Narine, Food Res. Int. 35 (2002) 1015–1048.
[30] H.M.D.N. Lida, A.R. Ali, J. Am. Oil Chem. Soc. 75 (1998) 1625–1631.
[31] D. Alberton, D.A. Mitchell, J. Cordova, P. Peralta-Zamora, N. Krieger, Food Technol. Biotechnol. 48 (2010) 28–35.
[32] A. Tiss, F. Carrière, R. Verger, Anal. Biochem. 294 (2001) 36–43.
[33] M.C. Gacula Jr., J. Singh, Food Science and Technology: A Series of Monographs, Academic Press, New York, 1984, pp. 214–273.
[34] O.M. Laia, H.M. Ghazalia, F. Cho, C.L. Chong, Food Chem. 71 (2000) 173–179.
[35] L. Faur, in: A. Karleskind, J.P. Wolff (Eds.), Oils and Fats Manual, Association Française pour l'Étude des Corps Gras, London, Paris & New York, 1996, pp. 923–995.
[36] H.J. Fiebig, J. Lüttke, Eur. J. Lipid Sci. Technol. 105 (2003) 377–380.
[37] J.L. Perrin, in: A. Karleskind, J.P. Wolff (Eds.), Oils & Fats Manual, second ed., Intercept Ltd., Andover, 1996, pp. 1025–1042.
[38] V. Nagy, E.R. Toke, L.C. Keong, G. Szazker, D. Ibrahim, I.C. Omar, G. Szakács, L. Poppe, J. Mol. Catal. B: Enzym. 39 (2006) 141–148.
[39] U. Bakir, S. Yavascaoglu, F. Guvenc, A. Ersayin, Enzyme Microb. Technol. 29 (2001) 328–334.
[40] P. Tufvesson, U. Tornvall, J. Carvalho, A.J. Karlsson, R. Hatti-Kaul, J. Mol. Catal. B: Enzym. 68 (2011) 200–205.
[41] D. Adlercreutz, P. Tufvesson, A. Annerling, R. Hatti-Kaul, Ind. Biotechnol. 6 (2010) 204–211.
[42] L. Castilho, C.M.S. Polato, E.A. Baruque, G.L. Sant'Anna Jr., D.M.G. Freire, Biochem. Eng. J. 4 (2000) 239–247.
[43] J.C.M. Diaz, J.A. Rodriguez, S. Roussos, J. Cordova, A. Abousalham, F. Carriere, J. Baratti, Enzyme Microb. Technol. 39 (2006) 1042–1050.
[44] D. Li, P. Adhikari, J.A. Shin, J.H. Lee, Y.J. Kim, X.M. Zhu, J.N. Hu, J. Jin, C.C. Akoh, K.T. Lee, LWT – Food Sci. Technol. 43 (2010) 458–464.
[45] A.V. Paula, G.F.M. Nunes, L. Freitas, H.F. de Castro, J.C. Santos, J. Mol. Catal. B: Enzym. 65 (2010) 117–121.
[46] R.C. Silva, L.N. Cotting, T.P. Poltronieri, V.M. Balcão, D.B. de Almeida, L.A.G. Goncalves, R. Grimaldi, L.A. Gioielli, LWT – Food Sci. Technol. 42 (2009) 1275–1282.
[47] L.O. Ming, H.M. Ghazali, C.C. Let, Food Chem. 64 (1999) 83–88.
[48] H. Zhang, X.B. Xu, J. Nilsson, H.L. Mu, J. Adler-Nissen, C.E. Hoy, J. Am. Oil Chem. Soc. 78 (2001) 57–64.
[49] H.M. Ghazali, S. Hamidah, Y.B. Che Man, J. Am. Oil Chem. Soc. 72 (1995) 633–639.
[50] J. Čmolík, J. Pokorný, Eur. J. Lipid Sci. Technol. 102 (2000) 472–486.