Food Bioscience 58 (2024) 103769

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Enzymatic synthesis of structured lipids from sacha inchi (*Plukenetia volubilis*) oil with capric acid via acidolysis reaction in stirred tank and packed bed mini reactors

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ARTICLE INFO

Keywords:
Sacha inchi
Lipase
Oil
Acidolysis
Immobilization
Functional triglycerides

ABSTRACT

The present study describes an efficient and sustainable synthesis method for producing functional triglycerides (FTs) by conducting an acidolysis reaction of sacha inchi (*Plukenetia volubilis*) oil with capric acid (C10), catalyzed by *Rhizopus oryzae* (RO) lipase immobilized in low-cost corn cob powder, to obtain medium-large-medium chain (MLM) triglycerides. Initially, acidolysis reactions were performed between sacha inchi oil, C10, and the immobilized enzyme in a stirred tank at various temperatures (35 °C, 45 °C, 55 °C, and 59 °C), different molar ratios (1:2, 1:3, 1:4 oil:fatty acid), and various hydrolytic enzyme activities (83.6, 124, and 217 IU/mg). Subsequently, to optimize the degree of incorporation, the best condition from the stirred tank reactions (45 °C, 1:3 M ratio, and 217 U of hydrolytic activity) was selected, considering the highest nutritional quality of the lipid in terms of the migration of the sn*2* position, and was tested in a packed bed reactor with a spatial time of 112 min, residence time of 107 min, and a flow rate of 0.5 mL/min. The highest incorporation degree achieved in packed bed reactor was 36% at 45 °C. Afterward, it was developed for the best condition an integrative platform for the recycling of the RO lipase immobilized in low-cost corn cob powder (up to four successive cycles without any make up). Subsequent assays on the functional triglycerides revealed atherogenic and thrombogenicity lipid index values of 0.04 ± 0.10 and 4.15 ± 0.05, respectively, which were lower than those of pure sacha inchi oil, indicating the potential to enhance the nutritional quality of the oil through C10 incorporation.

1. Introduction

Food technology has focused on modifying oils and fats to exploit the properties of different composites that are important for maintaining human health. One of the most studied techniques has been the modification of common lipids to obtain specific nutritional applications and to satisfy the growing demand for healthier foods that improve the benefits to human health (Xu et al., 2023). Functional triglycerides (FTs), also known as structured lipids, low-calorie lipids, or triglycerides (TG) of medium-large-medium chain (MLM) type, are produced through chemical or enzymatic reactions (Bassan et al., 2018; Moreria, Santos, Gambero, & Macedo, 2017; Sivakanthan et al., 2019). These reactions aim to modify the fatty acid composition in triglycerides, where 1, 3-regio-selective lipases are used to remove fatty acids (FAs) from the sn*1* and sn*3* positions (hydrolysis) and then incorporate medium-chain fatty acids (MCFAs) into the same positions (esterification). This process results in the formation of MLM triglycerides, which have various health benefits (Iwasaki & Yamane, 2000).

Regio-selective lipases, such as those produced by the filamentous fungi *Rhizopus oryzae* (RO), are used in these reactions either free or immobilized. Immobilization techniques allow for their reuse in several cycles and improve their stability, which is essential for sustainable

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https://doi.org/10.1016/j.fbio.2024.103769
Received 17 December 2023; Received in revised form 16 February 2024; Accepted 17 February 2024
Available online 19 February 2024

bioreactor use. While stirred tank bioreactors have been reported to standardize conditions in such processes, the use of fixed bed reactors in the biosynthesis of structured lipids has not been extensively reported (Remonatto et al., 2022). Fixed bed reactors enable the scaling up of processes and the resolution of several drawbacks related to the degradation of the enzyme derivative (Guisan, 2006; Sheldon & van Pelt, 2013). This technology holds great promise to produce functional triglycerides (FTs), which are an alternative for individuals who need to consume healthy food. Moreover, it has been demonstrated that FTs are suitable for controlling the progressive development of obesity and weight gain, making them ideal for people with metabolism problems (Morales-Medina et al., 2017) or fat absorption issues (Martínez-Galán et al., 2021). FTs have a very high nutritional value because, after the modification, the essential fatty acids can be fully utilized for various biological functions in metabolism. Furthermore, medium-chain fatty acids (MCFAs) are efficiently hydrolyzed by lipases in lipid metabolism (Zhang et al., 2016).

The sources to synthesize FTs are recognized as common or alternative oils, mainly from seeds such as the *Plukenetia volubilis* L seed (Bassan et al., 2018; Ferreira-Dias et al., 2022; Martínez-Galán et al., 2021; P. Nunes et al., 2010; Zam, 2015), commonly known as the sacha inchi seed. This seed belongs to the Euphorbiaceae family and is distributed in some countries of South America, including Colombia, Peru, Ecuador, and Bolivia (Cárdenas et al., 2021). Due to Colombia's climatic diversity and the plant's adaptability, the sacha inchi oil is readily accessible in the region.

Sacha Inchi oil is renowned for its high nutritional value, containing approximately 85% of essential fatty acids, with 34% being linoleic acid and 52% linolenic acid (Torres Sánchez et al., 2021). This high content of both fatty acids, particularly linolenic acid, promotes a balanced ratio between omega-3 and omega-6, countering the high consumption of omega-3 in food. Additionally, the transesterification reaction enhances the nutritional quality by reducing long-chain saturated fatty acids (LCFAs). The unique composition of sacha inchi oil makes it a versatile and beneficial addition to the human diet. Moreover, the nutritional quality of functional triglycerides (FTs) from sacha inchi oil can be represented not only by the ratios usually used (PUFAs/SFAs) but also by healthy lipid indexes that consider the role of fatty acids (FAs) on health, such as the Nutritive Value Index (NVI), which evaluates the nutritional value of FAs, the Atherogenic index (AI), the Thrombogenicity index (TI), and hypocholesterolemic FAs (DFA). Conversely, undesirable effects on hypercholesterolemia (OFA) can also be evaluated using the Nutritive Value Index (NVI) (Akinbule et al., 2022; Chen and Liu, 2020; Paszczyk et al., 2019).

In this study, the objective was to synthesize medium-large-medium (MLM) type triglycerides from sacha inchi oil using enzymatic modification through acidolysis reactions by a regio-selective sn-1,3 RO lipase. To achieve this, an experimental strategy was implemented to assess the impact of temperature, molar ratio, and the hydrolytic activity of the RO enzyme on the incorporation of capric acid in sacha inchi oil within a stirred tank reactor, with the aim of achieving the optimal incorporation degree. Subsequently, the most favorable condition was chosen to evaluate the effect of packed bed reactor on capric acid incorporation followed by the assessment of enzyme RO recycling on packed bed reactor and the evaluation of healthy lipid indexes to estimate the nutritional quality of developed structured oil. Hence, this work not only contributes to the development of alternative lipid products with improved nutritional properties but also offers a more sustainable approach to oil modification using immobilized biocatalysts. These advancements have the potential to benefit both human health and the environment, making this research an important contribution to the field of food technology.

2. Materials and methods

2.1. Materials

Sacha inchi oil (*Plukenetia volubilis*) was acquired from a commercial establishment in Medellín, Colombia. The capric acid standard and fatty acids methyl esters (FAMEs) standard mixture (37-component FAME Mix, Supelco) were provided by Sigma-Aldrich (Darmstadt, Germany). The corn cob powder used as an organic support for the enzymatic immobilization was donated by RASUL in Brazil. The uncommercial free enzyme was generously donated by the company PROZYN™ in São Paulo (Brazil) and was immobilized on a corn cob. All other chemicals and solvents used for extraction and analysis were of high purity and analytical grade.

2.2. Characterization of sacha inchi oil

The fatty acid composition was determined using gas chromatography, following the American Oil Chemists' Society Method Ce 2–66. Additionally, the acid index value (Ca 5a-40 method), peroxide index value (Cd 8b-90 method), and iodine value (Cd 1c-85) were determined in accordance with the official methods of the American Oil Chemists' Society (AOCS, 2017). The tests were conducted using a gas chromatograph (Shimadzu®, model GC-2014) equipped with a Split injector, flame ionization detector (FID), and a column SH-Stabilwax-DA with a length-to-inner diameter ratio of 30 m × 0.25 μm. The injector and detector were held at 250 °C, employing a Split injection mode (1:10), and nitrogen was used as the carrier gas at a flow rate of 0.55 mL/min. The initial column temperature was set at 60 °C, then ramped up to 210 °C at a heating rate of 20 °C/min, where it remained for 7 min. Subsequently, the temperature was increased to 250 °C (20 °C/min) and held at that temperature for 14 min.

2.3. Enzymatic immobilization and hydrolytic activity

The activation of the support was carried out using the methodology previously described by Bassan et al. (N. Bassan et al., 2018) and Martínez-Galán et al. (Martínez-Galán et al., 2021). Enzymatic immobilization was conducted as follows: 1 g of activated support was added to 10 mL of 0.5 mol/L sodium phosphate buffer (pH 7.0), containing an enzymatic solution (25 mg/mL). The suspension was agitated for 24 h at 25 °C in a roll bed. Note that, the immobilization of enzyme was indeed carried out via covalent attachment using glutaraldehyde chemistry, which is a widely recognized method in the field of enzyme immobilization. This process involves a two-step mechanism: initially, the protein adsorbs onto the support material, followed by a subsequent covalent reaction. In our experimental procedure, the support material, which contains amine groups, was activated using glutaraldehyde. Specifically, we utilized epoxy or aldehyde with ethylenediamine (EDA) as the activated support. This activation step ensures the presence of reactive sites for the covalent bonding of the lipase enzyme (Martínez-Galán et al., 2021).

The protein quantification was measured by the Bradford method (Bradford, 1976) on the supernatant before starting the contact time and at the end to calculate the immobilization percentage of the enzyme on the support. Hydrolytic activity was assessed before and after the immobilization process on the free enzyme, on the recovered supernatant immediately after immobilization, and on the immobilized enzyme by using an olive oil emulsion (1:1, w/v) with a modified triton-like emulsifier (Paula et al., 2015). Note that, in this study, RO refers to *Rhizopus oryzae* lipase. The numerical suffixes 1, 2, and 3 signify different instances of immobilization, each involving a separate batch of enzyme from the same supplier (PROZYN™). RO-1 represents the first immobilization process where *Rhizopus oryzae* lipase was immobilized onto the corn cob material. Similarly, RO-2 and RO-3 denote the second and third immobilization instances, respectively. Parameters of

immobilization: immobilization yield from protein quantification (YIP), immobilization yield from activity quantification (YIA), efficiency (E), recover activity (RA) were calculated using Equations (1)–(3).

$$\text{Yield}\ (\%) = (\text{immobilized activity} / \text{starting activity})\ \text{x}\ 100\% \quad \text{(Eq. 1)}$$

$$\text{Efficiency}\ (\%) = (\text{observed activity} / \text{immobilized activity})\ \text{x}\ 100\% \quad \text{(Eq. 2)}$$

$$\text{Activity recovery}\ (\%) = (\text{observed activity} / \text{starting activity})\ \text{x}\ 100\% \quad \text{(Eq. 3)}$$

2.4. Acidolysis reaction on stirred tank reactor operated in batch mode

The stirred tank mini reactor (50 mL), and the entire medium was set at 30 g to ensure proper homogenization between the oil, acid, and biocatalyst. Magnetic stirring was performed at a consistent speed using the same stirring plate (500 rpm) for all reactions. The enzyme loading was 10% (w/w) of the entire medium (3 g), and the molar ratio was 1:3 (oil: fatty acid). The temperature was maintained at 45 °C using a thermostatic bath. The acidolysis reaction was conducted in batch mode for 24 h, with 10% enzyme loading of the entire medium and constant stirring (500 rpm). Ten experiments were carried out to assess different reaction conditions, including varying temperature, molar ratio, and the hydrolytic activity of the biocatalyst. After each reaction, the peroxide value, hydrolytic activity of the immobilized enzyme, and fatty acid profile were analyzed.

2.5. Acidolysis reaction on packed bed reactor in batch mode

The packed bed reactor, with a volume of 60.61 mL and a flow rate of 0.5 mL/min controlled by a peristaltic pump, was characterized through a tracer test. A non-random mixture was created using a fat-soluble colorant (red colorant) and food-grade tracer solution to determine the axial dispersion, following the methodology described by De Paula et al. (De Paula et al., 2018). The tracer test was conducted using a Gilson brand peristaltic pump (SL-64 SOLAB), which was initially stabilized under conditions of temperature, flow, and height. A tracer pulse was then introduced using a syringe containing 2 mL of a concentrated solution. Samples were taken every 10 min for 2 h. The dimensions of the reactor, spatial and residence time were calculated using Equations (4)–(7):

$$\text{System volume} = \text{VR} + \text{VPB} + \text{VU} + \text{VH} + \text{VH} - \text{VE} \quad \text{(Eq. 4)}$$

Where: VR: volume of the reactor (mL); VPB: volume of the packed bed (mL); VU: volume of the tube (mL); VH: volume of the hose (mL); VE: volume of the enzyme (mL)

$$t = V/Q \quad \text{(Eq. 5)}$$

Where: t: space time V: system volume (mL), Q: flow (mL/minute)

$$\text{Useful volume} = \text{VPB} - \text{VE} \quad \text{(Eq. 6)}$$

Where: VPB is the volume of the packed bed (mL) and VE is the volume of the enzyme (mL)

$$d = w/v \quad \text{(Eq. 7)}$$

Where: d = corn cob powder density w = weight (g) v = volume (mL).

These equations were used to calculate the system volume, space time, useful volume, and corn cob powder density for the packed bed reactor.

Subsequently to the tracer test, we evaluated the acidolysis reaction in a packed bed reactor (60.61 mL) containing immobilized RO lipase as a packed bed. The reactor was operated in batch mode with recirculation of the mixture for 120 h at 45 °C. The determined flow was 0.5 mL/min. To perform a tracer test, a tracer solution was introduced into the reactor. Samples were periodically collected over time, neutralized, and methylated immediately afterward. The fatty acid composition was then determined by gas chromatography to verify the incorporation degree.

2.6. Neutralization and methylation of triglycerides

After the acidolysis reaction, 3 g of the FTs samples was weighed. Subsequently, 60 mL of hexane and 20 mL of a 0.8 M KOH hydro-alcoholic solution (30% ethanol) were added to remove the free fatty acids produced during the reaction. The mixture was allowed to stand for 30 min until two phases were observed, after which the organic phase was collected and subjected to a rotary evaporator to extract any remaining hexane residues (AOCS, 2017; Wang et al., 2012). For the methylation process, 0.05 g of FTs after the neutralization process was weighed with 2 mL of sodium hydroxide and 2 M methanolic, heated at 80 °C and placed under magnetic stirring for 15 min. Subsequently, 2 mL of H_2SO_4 1 M methanolic were added with the same conditions of the first solution. After 15 min, 1 mL of NaCl saturated methanolic solution and 2 mL of hexane were added. The mixture was kept until two phases were observed, then the organic phase was collected. This step was repeated three times (ISO 12966-2, 2011) and fatty acid esters were collected for further analysis.

2.7. Determination of fatty acid composition and incorporation degree of FTs

FAMEs were analyzed by gas chromatography according to the American Oil Chemists' Society Method as reported for composition oil described in the section *'Characterization of sacha inchi oil'*, carried out using a standard mixture of fatty acids methyl esters standards (37-component FAME Mix, Supelco). The results are presented as the relative amount of each fatty

Acid (% of FA/100 g of sample).

The percentage of fatty acid incorporation degree was calculated according to **equation (8)**:

$$\%\ ID = (MFA / MT)\ * 100 \quad \text{(Eq. 8)}$$

Where: MFA is the number of moles of the C:10 fatty acid, MT is the total number of moles of fatty acids in the triglycerides.

2.8. Fatty acid analysis at the sn-2 position of the modified triglycerides

For the *sn-2* fatty acid composition, 100 mg of the sample was weighed. Then, 1 mL of HCl tris buffer (0.1 M, pH 8), 250 mL of bile salts (cofactor), and 0.1 mL of calcium chloride (cofactor) were added. After 10 min, 100 mg of porcine pancreatic lipase (LPP) was added to initiate hydrolysis. The mixture was incubated at 40 °C in a water bath for 30 min with stirring (500 rpm). Subsequently, 1 mL of HCl and 3 mL of ethyl ether were added to stop the reaction. Immediately after, the mixture was centrifuged for 10 min at 2500 rpm. The organic phase was then collected and placed on a silica-gel layer chromatographic (TLC) plate. The TLC plate was positioned in a chromatographic chamber for almost 2 h. In the chamber, the mobile phase (hexane, ether, and acetic acid in a 70:30:1 v:v:v ratio) was added. Following this, the TLC plate was removed and sprayed with 2.7 dichlorofluorescein (2%). The *sn-2* monoacylglycerol band was then collected, methylated, and the fatty acid composition of the *sn-2* position was determined by gas chromatography (Jennings et al., 2000).

2.9. Assessment of enzyme RO recycling on packed bed reactor

To explore the potential for reusing RO in corn cob powder, we prepared immobilized RO lipase by combining 0.3 g (10%) of enzymatic powder per gram of support (total 3 g). Subsequently, 0.8 g (dry mass) of the resulting biocatalyst was incubated with a ratio of 1:3 (SIO: C10), allowing the reaction to proceed for a maximum of 120 h at 45 °C. After

each batch, the biocatalyst was carefully extracted from the reaction medium and thoroughly washed with cold hexane to eliminate any substrate and product retained in its microenvironment. The cleaned biocatalyst was then introduced into a fresh medium, and reactions were regularly monitored by assessing the conversion percentage.

2.10. Health lipid indices

The fatty acid profile was used to calculate the main health lipid indices for sacha inchi oil and FTs to evaluate whether the properties would be beneficial in structured lipids. The indices were calculated using Equations (9)–(13) (Chen and Liu, 2020; Ulbricht & Southgate, 1991; Ulbricht & Southgate, 1991; Janiszewski et al., 2016; Janiszewski et al., 2016):

$$\text{Nutritive Value Index : NVI} = (C18:0 + C18:1)/C16:0 \quad \text{(Eq. 9)}$$

$$\text{Atherogenic index : AI} = (C14:0 + C16:0 + C18:0) \Big/ \sum \text{UFA} \quad \text{(Eq. 10)}$$

$$\text{Thrombogenicity index : TI} = (C14:0 + C16:0 + C18:0) \Big/ \left[\left(0,5 * \sum \text{MUFA}\right) + \left(0,5 * \sum \text{PUFA n} - 6\right) + \left(3 * \sum \text{PUFA n} - 3\right) + \left(\sum \text{PUFA n} - 3 \Big/ \sum \text{n} - 6\right) \right] \quad \text{(Eq. 11)}$$

$$\text{Dietary FA having undesirable effect on hypercholesteremic : OFA} = (C14:0 + C16:0) \quad \text{(Eq. 12)}$$

$$\text{Dietary FA having a desirable effect on hypercholesteremic : DFA} = \left(\sum \text{MUFA} + \sum \text{PUFA} + C18:0\right) \quad \text{(Eq. 13)}$$

2.11. Statistical analysis

The obtained results were presented as the mean ± standard deviations. Three independent variables (temperature, molar ratio, and hydrolytic activity) were correlated with the incorporation degree. Data were expressed as mean ± standard deviation and analyzed by ANOVA using SPSS software. A significance level of $p < 0.05$ was considered statistically significant, and Tukey's test, Duncan's multiple range tests, and multiple comparison tests (LSD) were applied. Correlation analysis was calculated by Kendall's correlation method in R statistical framework using the cor. test function from the stats package corrplot to investigate the relationship between factors. Graphics (ggplot2 packages for boxplots and scatter plot) were generated in RStudio.

3. Results and discussion

3.1. Feedstock (sacha inchi oil) physical-chemical characterization

The sacha inchi oil employed in acidolysis reactions underwent prior characterization, focusing on its peroxide, acidity, and iodine levels. Note that, the physicochemical characterization of an oil is crucial for assessing its nutritional quality and understanding its potential benefits for consumers or as an ingredient in various food products. In this study, the characterization of the oil revealed that the peroxide index (3.03 ± 0.27 mEq O_2/kg of oil) fell within the acceptable range specified by CODEX STAN (CODEX, 1999) (10 mEq O_2/kg of oil). However, the acidity index (0.78 ± 0.05 mg KOH/g of oil) slightly exceeded the norm (0.6 mg KOH/g of oil), potentially indicating influences from the oil production process, transport, or storage conditions (Trung Nghiem et al., 2023, p. 2682). The iodine value (219.86 ± 0.8 mg I_2/0.5 g oil), associated with unsaturated fatty acids (Haro et al., 2016), reflects a high level of unsaturation in the oil, particularly in oleic, linoleic, and linolenic acids. The peroxide value result suggests that the sacha inchi oil is suitable for conducting reactions, meeting the requirements for food applications (Table S1).

Sacha inchi oil was also characterized as for its compositional profile in fatty acids using gas chromatography. Average concentrations, as well as their respective standard deviations, are presented in Table 1.

Table 1 provides a comprehensive profile of the fatty acid composition, revealing that approximately 85% of the sample consists of unsaturated fatty acids, including essential fatty acids. These findings align with similar reports in the literature (Benítez et al., 2018; Torres Sánchez et al., 2021; Wang et al., 2012). Notably, the sacha inchi oil exhibits a substantial presence of linolenic acid (C18:3; 52.47%) closely followed by linoleic acid (C18:2; 32%), a pattern consistent with findings in previous studies (Benítez et al., 2018; Paucar-Menacho et al., 2015; Torres Sánchez et al., 2021). The combined content of linoleic, linolenic, and oleic acids (C18:1; 7.92%) imparts notable nutritional benefits to sacha inchi oil, contributing to human health. These fatty acids have been associated with preventing cardiovascular diseases (Chen and Liu, 2020), aiding in the control of obesity, and proving advantageous for individuals with metabolism or fat absorption challenges (Martínez-Galán et al., 2021).

3.2. RO enzyme immobilization

The preparation of the biocatalyst is a pivotal step in this investigation. The use of immobilized enzymes has demonstrated the capability to augment enzyme activity per reactor volume in industrial processes, leading to expedited reaction rates and reduced time for product formation. Consequently, we scrutinized the specific type of RO enzyme employed in the immobilization method to achieve a biocatalyst with enhanced catalytic activity. The parameters for immobilization were systematically executed for each immobilization process on the support. Furthermore, we conducted the immobilization process three RO lipase batches, which was immobilized through covalent linkage to glutaraldehyde-corn cob powder support, specifically targeting the amino group of the terminal lysine of the enzyme (Table 2).

Typically, immobilization progress is tracked through protein quantification (Pinto et al., 2014). The immobilization yield (YIP), calculated based on this parameter, exhibited values close to 100%. However, in the pursuit of a more nuanced understanding of enzymatic behavior, the yield was also calculated by activity tracking (YIA), revealing values lower than 50%. The observed discrepancies in YIA values may stem from differences in both the support material utilized and the batches of RO enzyme employed. The organic support chosen for immobilization is recognized for its porous structure, as noted in previous research (Bassan et al., 2016). This porous nature could potentially impede the entry of the enzyme, leading to some fraction of the enzyme remaining adsorbed to the support, resembling adsorption immobilization mechanisms elucidated by Ismail and Baek (Ismail & Baek, 2020). Consequently, this could elucidate the higher YIP percentages calculated through protein quantification, a phenomenon documented in several

Table 1
Fatty acid profile of sacha inchi oil characterized using gas chromatography, values are expressed in terms of mean (% of FA/100 g of sample) ± standard deviation.

Fatty acids	(%)
C10	–
C16	4.14 ± 0.00
C18	2.69 ± 0.30
C18:1	7.92 ± 0.13
C18:2	32.79 ± 0.00
C18:3	52.47 ± 0.20

Table 2
Parameters of immobilization: immobilization yield from protein quantification (YIP), immobilization yield from activity quantification (YIA), efficiency (E), recover activity (RA). Values of hydrolytic activity are expressed in terms of mean + standard deviation.

Enzyme	YIP (protein) (%)	YIA (activity) (%)	E (%)	RA (%)	Hydrolytic activity ($IU/mg_{biocatalyst}$)
RO- 1	83.21	12.66	20.73	23.74	83.6 ± 1.38
RO- 2	75.00	29.13	48.82	68.89	124 ± 1.19
RO- 3	71.86	40.88	59.12	90.42	217 ± 2.21

studies (Pinto et al., 2014). Another crucial factor to highlight is the careful selection of enzyme batches and ensuring their viability, as these factors can significantly influence both the efficiency and activity of the immobilized enzyme. This underscores the importance of validating YIP values through enzymatic activity measurements, particularly since monitoring often relies on residual protein quantification, which might yield false positive signals during product reactions. Various parameters derived from enzymatic activity, such as hydrolytic activity, efficiency of immobilization (E), and recovery activity (RA), were computed to evaluate the effectiveness of the immobilization process, as detailed in Table 2.

Significantly, enzyme RO-3 exhibited notably higher values in both efficiency (59%) and recovery activity (90%) compared to RO-1 and RO-2. The recovery activity (RA) percentage holds particular significance as it reflects the overall success of immobilization by indicating the activity retained in the support, available for catalytic actions (Sheldon & van Pelt, 2013). The calculated parameters collectively suggest that RO-3 achieved the most effective immobilization. This observation could potentially elucidate a positive correlation between the hydrolytic activity and the degree of C:10 incorporation, as discussed in the subsequent section (Bassan et al., 2018; Martínez-Galán et al., 2021). Furthermore, the notably lower RA values of RO-1 and RO-2 in comparison to RO-3 could account for the decreased C:10 incorporation degree observed in reactions catalyzed by these enzymes, which exhibited a decrease of 15% (discussed in the next section).

3.3. Acidolysis reaction on stirred tank reactor

A stirred tank reactor was first utilized to conduct four acidolysis reactions on sacha inchi oil at different temperatures (35, 45, and 59 °C) for 24 h. Three reactions were performed with molar ratios of fatty acid to oil at 1:4, 1:3, and 1:2, aligning with the stoichiometric values for the acidolysis of TAGs when *sn*-1,3 regioselective lipases are employed. Additionally, three reactions were carried out with different enzymatic activities of the enzymes set at 83.6, 124, and 217 IU/mg (Fig. 1).

Fig. 1-A indicate a significant increase in incorporation degree, approximately 30%, when the temperature reaches 59 °C, aligning with findings of several published studies (Bassan et al., 2018, 2019; Martínez-Galán et al., 2021; Simões et al., 2021). This is attributed to the positive correlation between temperature and incorporation degree, promoting the acyl migration of triglyceride bonds and enhancing the

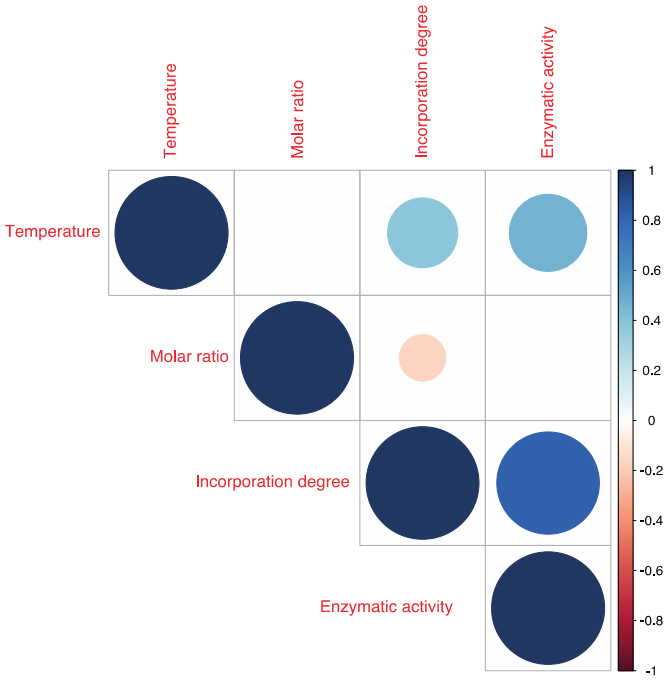

Fig. 2. Correlation matrix between variables involved on structuration of FTs. Effect of the hydrolytic activity into the incorporation degree of experimental reactions activity. Each cell shows the correlation between two variables. The color-code shows the values according to the Kendall correlation statistics. The values between (0–1) show positive correlation, the values between (0- (−1)) show negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

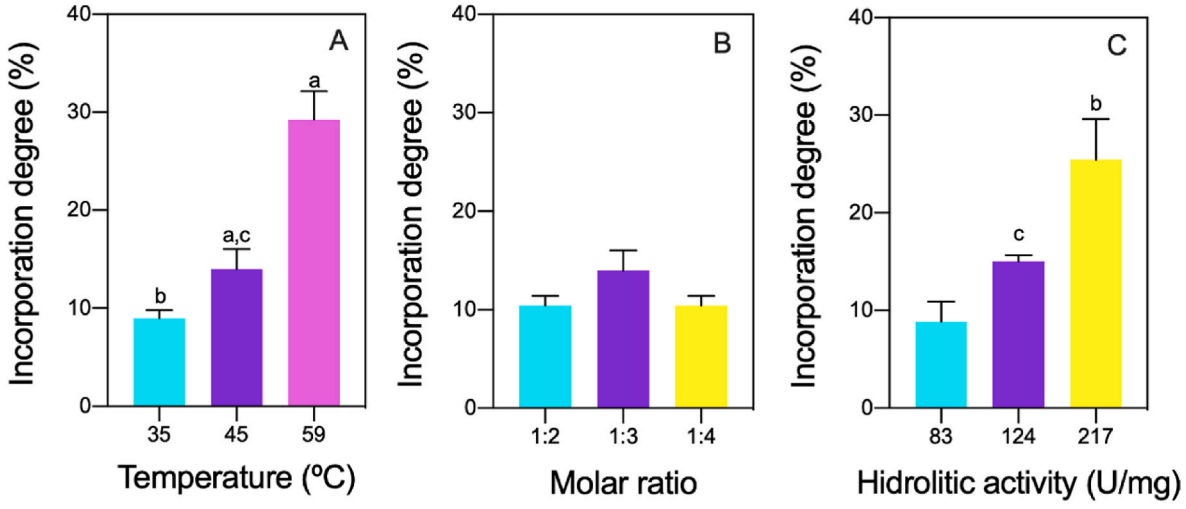

Fig. 1. Effect of temperature, molar ratio, and hydrolytic activity on incorporation of capric acid into sacha inchi oil. **A:** reactions with different temperatures; **B:** reactions with different molar ratios and **C:** reactions with different enzymatic activity. Each reaction was performed for 24 h at 10% load enzymatic. Different letters mean significant difference ($p < 0.05$).

acidolysis reaction (Bassan et al., 2016; Cao et al., 2013; Kim et al., 2010; Simões et al., 2021), as also depicted in the correlational analysis (Fig. 2). These results suggest that increasing the temperature close to 60 °C would significantly enhance the incorporation degree. However, note that higher temperatures reduce substrate viscosity, favoring the acidolysis reaction, but extreme temperatures can lead to enzyme inactivation, oxidation processes, or a decline in nutritional quality (Zou et al., 2020). Additionally, the reaction carried out at 45 °C demonstrated the second-highest degree of incorporation, potentially related to the optimal hydrolytic activity of the immobilized enzyme (Martínez-Galán et al., 2021), favoring hydrolysis in acidolysis reactions, as described earlier (Zou et al., 2020). The reaction at 35 °C exhibited a low C10 incorporation degree, consistent with expectations under these temperature ranges.

Furthermore, increasing the acyl donor is known to positively affect fatty acid incorporation. However, in these reactions, substrate saturation may hinder the enzyme by excess or reduce product formation due to its absence. This explains the observed decrease in incorporation degree in the 1:2 and 1:4 ratios, as reported by Yue, Ben, Wang, Li, and Yu (2019), while the 1:3 ratio exhibited the highest incorporation degree (Fig. 1-B), suggesting an equilibrium in esterification reactions and indicating it as ideal for acidolysis reactions, as reported in different studies (Bassan et al., 2018; Korma, Zou, Ali, Abed, & Wang, 2018; Zou et al., 2020).

The effect of hydrolytic activity is illustrated in Fig. 1-C, showing a significant increase in the C10 incorporation degree due to a strong positive correlation between these variables, emphasizing the influence of hydrolytic activity on acidolysis reactions, as previous reported (Brígida et al., 2008; Palla et al., 2012; Pinto et al., 2014). Temperature, hydrolytic activity, and molar ratio as variables collectively influence acidolysis reactions for C10 incorporation degree (Fig. 2), and correlation analysis indicates a very strong positive correlation between hydrolytic activity and temperature variables with the incorporation degree (Abed et al., 2017). It is also observed that there is a kind of negative correlation between molar ratio and the degree of incorporation, as previous explained.

Increasing incorporation degrees while preserving the quality of structured lipids is crucial. It is expected that the composition of *sn*-2 fatty acids does not change significantly, even though the reaction is catalyzed by a 1,3 regio-selective enzyme. Nevertheless, migration might occur due to external factors such as temperature, although to a lesser extent than with nonspecific chemical catalysts (Pacheco et al., 2015). Acyl migration is a thermodynamic process regulated by the Arrhenius equation (Kim & Akoh, 2005; Kim et al., 2010), resulting in an enhanced acyl migration at the sn-2 position under elevated temperatures, as evidenced in RO- 59 (16%) and corroborated by findings in various studies (Chopra et al., 2008; Kim & Akoh, 2005). Additionally, it has been noted that acyl migration follows an exponential pattern, as reported by P. A. Nunes et al. (2011). Nonetheless, it is notable that acyl migration on RO- 45 (5%) is lower than RO-5 9 (16%) and other studies with similar temperature conditions (Bassan et al., 2018; Chopra et al., 2008; Nunes et al., 2011), which is interesting for the stability of the reaction to maintain the nutritional properties of the modified oil.

The subsequent phase of the acidolysis reaction in the stirred tank reactor involved assessing the sn-2 fatty acid composition. This evaluation was conducted after performing the reactions at two different temperatures, referred to as R-45 for 45 °C and R-59 °C for 59, respectively. Both reactions were carried out at a molar ratio of 1:3 (oil: acid) for 24 h, aiming to ascertain the impact of temperature on acyl migration. The fatty acid profile values are detailed in Table 3, with C18:2 and C18:3 continuing to represent the majority FAs in the modified oil.

In conclusion, stirred tank reactors have found extensive application in the synthesis of FTs (Bassan et al., 2019; Martínez-Galán et al., 2021; Simões et al., 2021; Turan et al., 2006), primarily due to their ability to establish the initial conditions of reactions (Turan et al., 2006). This is particularly relevant in our case, where we are working with sacha inchi oil as a raw material for the first time. These reactors are preferred for their ease of use, requiring small sample quantities, simple equipment, and straightforward operation. Nevertheless, when scaled up to industrial levels, production may be hampered by unproductive time accumulation. This challenge can be addressed by employing a packed bed reactor, offering the potential to enhance production efficiency (Utama et al., 2020).

Table 3
Fatty acid profile at *sn*-2 position of sacha inchi and modified oil, values are presented as percentage of FA expressed in terms of mean (% of FA/100 g of sample) ± standard deviation in sacha inchi oil (SIO), reaction at 45 °C (R-45) and reaction at 59 °C (R-59).

Fatty acid	SIO	R- 45	R- 59
C10	–	5.14 ± 0.40	16.01 ± 0.12
C16	3.10 ± 0.50	2.93 ± 0.03	2.46 ± 0.04
C18	1.37 ± 0.00	1.88 ± 0.10	1.31 ± 0.50
C18:1	0.00 ± 0.30	0.00 ± 0.24	0.00 ± 0.20
C18:2	52.16 ± 0.10	47.42 ± 0.00	38.65 ± 0.00
C18:3	43.33 ± 0.20	42.58 ± 0.30	41.53 ± 0.00

3.4. Acidolysis reaction on packed bed reactor

One of the prominent challenges associated with stirred tank reactors is the stirring rate, which has the potential to compromise immobilized enzymes and impact the homogeneity of the substrate-medium (Remonatto et al., 2022; Sharma & Kanwar, 2014), subsequently influencing incorporation degrees that may affect the overall reaction. In addressing these concerns, the packed bed reactor emerges as a viable alternative, mitigating issues related to stirring rate problems with immobilized enzymes and concurrently extending reaction times. Packed bed reactors are widely employed in heterogeneous liquid-solid catalysis (Hita et al., 2009). First, to comprehend the hydrodynamics of the packed bed reactor, we conducted a characterization using a tracer test to ascertain the presence of preferential paths. The reactor dimensions were measured to calculate the reactor volumes (see Table 2 from Supplementary Material).

One specific condition was chosen from the stirred tank reactor—specifically, operating at 45 °C with a molar ratio of 1:3 (oil:fatty acid) and employing RO-3 enzyme —as the optimal condition based on the considerations previous discussed. This condition, encompassing factors like *sn-2* migration, was then extrapolated to the packed bed reactor. The packed bed reactor was characterized through a tracer test, revealing a residence time of 107 min, closely aligned with the calculated space time of 112 min. The minimal 3.7% variance suggests that preferential paths are not significantly influential (Ferreira-Dias et al., 2022), and the immobilized enzyme packaging was deemed suitable for the reaction.

First, the acidolysis reaction was conducted for 24 h to assess the impact of time on the incorporation degree compared to stirred tank reactor. Samples were collected at intervals, revealing a positive correlation between time and incorporation degree (Fig. 3), consistent with findings from previous studies (Abed et al., 2017; Aragon et al., 2013; Simões et al., 2021; Utama et al., 2020).

The ultimate incorporation degree reached approximately 20%. However, the 24-h duration appears insufficient for a thorough optimization of reaction conditions to achieve maximum incorporation degrees. Notably, the percentages of C18:2 and C18:3 remained elevated following the acidolysis reaction in both reactors. To better comprehend the impact of time on the structuration of FTs, the acidolysis reaction time was extended to 120 h only on the packed bed reactor at a temperature of 45 °C. Samples were collected over time, revealing that prolonged reaction time significantly increased the incorporation degree of C10 (Fig. 4). After 120 h in FTPB, the incorporation degree reached 36%.

As depicted in Fig. 4, the acidolysis reaction was conducted over a

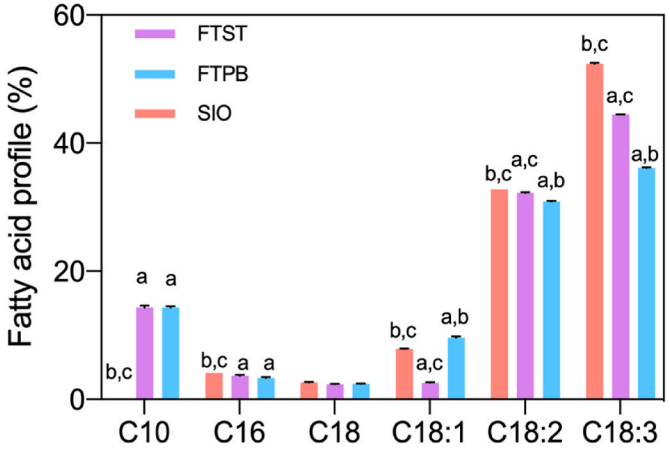

Fig. 3. Fatty acid profile of sacha inchi oil, and the FTs in stirred tank and packed bed reactor operating in batch mode. Sacha inchi oil (SIO), FTs synthesized on stirred tank reactor (FTST), FTs synthesized on packet bed reactor (FTPB). Values are expressed in terms of mean ± standard deviation. Values with different letters represent significant difference ($p < 0.05$).

120-h period to assess the influence of time on the C10 incorporation degree. Samples were systematically collected during this duration, revealing a positive correlation between time and incorporation degree, consistent with findings previous reported (Abed et al., 2017; Aragon et al., 2013; Simões et al., 2021; Utama et al., 2020). The ultimate incorporation degree reached 36%, indicating a notable improvement of 20% compared to the 24-h mark. This underscores the packed bed reactor's efficacy in optimizing reaction conditions and enhancing incorporation degrees of fatty acids. The sustained elevation of C18:2 and C18:3 percentages further emphasize the reactor's potential for sustainable production of FTs. The observed advantages make the packed bed reactor a promising and sustainable approach for the efficient incorporation of fatty acids in FT synthesis.

3.5. Recycling of RO lipase immobilized in corn cob powder

In this work, an innovative platform for the enzymatic synthesis of structured lipids from SIO with C10 via acidolysis reaction in packed bed mini reactors was proposed envisaging a future implementation at industrial scale following circular and sustainable precepts was proposed. Five incorporation cycles were evaluated as proof-of-concept, namely, a first incorporation using fresh immobilized enzyme and three subsequent cycles reusing recycled immobilized enzyme (Fig. 5).

As illustrated in Fig. 5, the RO lipase immobilized in corn cob powder exhibits remarkable recyclability, being successfully reused up to four times without the need for any 'make-up,' while maintaining the incorporation degree at a steady 10%. However, after completing three recycling cycles, the incorporation degree slightly decreased to 5%, marking a reduction of approximately 30% compared to the freshly immobilized RO lipase. The findings suggest that RO lipase immobilized in corn cob powder holds promise as an intriguing alternative for structuring fluidized beds in a packed bed reactor. Nonetheless, to establish a circular and sustainable industrial platform based on RO for the incorporation of fatty acids into TAG, further studies are crucial. These studies should encompass several aspects, including the optimization of operational conditions, exploration of potential catalyst modifications, and a comprehensive assessment of the economic and environmental feasibility of the proposed approach.

3.6. Health lipid indices for FTs application in human health

Various dietary factors play a pivotal role in the development of coronary heart diseases (CHD). Fatty acids, in particular, have been

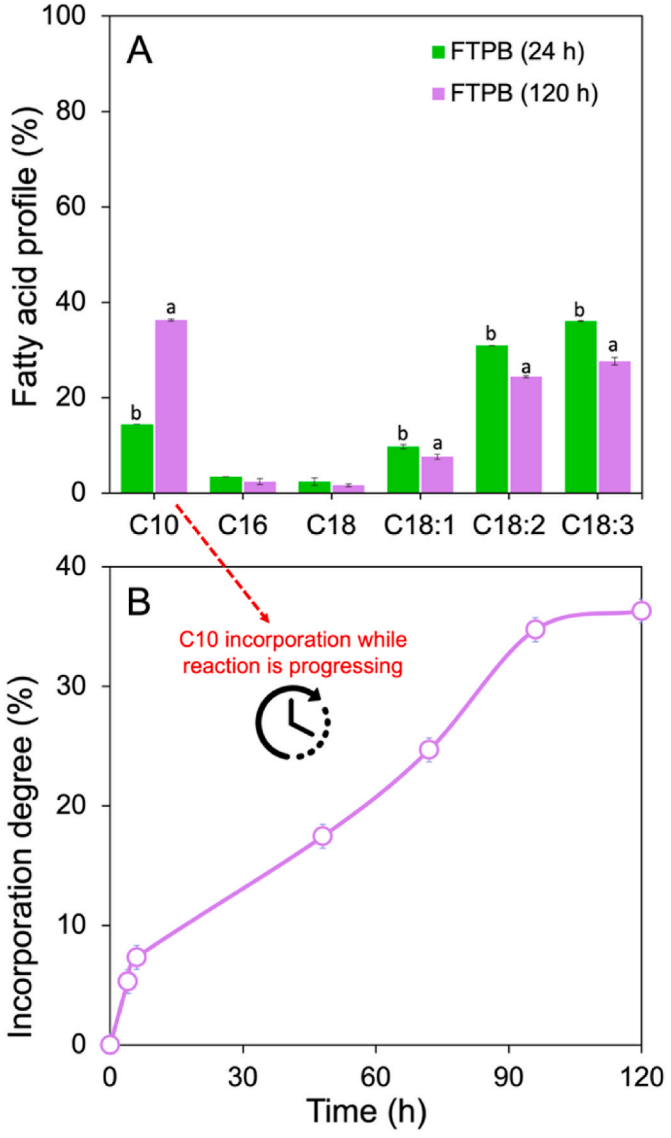

Fig. 4. **A-** Effect of time (24 and 120 h) on the structuration of FTs in a packed bed reactor (FTPB) operated in batch mode. **B-** The incorporation degree, representing the percentage of C10 incorporation as the reaction progresses calculated at various time intervals (h). Values with different letters represent significant difference ($p < 0.05$).

widely acknowledged as significant contributors either promoting or protecting against CHD development (Wereńska et al., 2021). Saturated fatty acids (SFA) are associated with cholesterol elevation and arterial obstruction, while unsaturated fatty acids (USFA) are recognized for their beneficial effects in preventing CHD. Although there is a widely reported recommended minimum value for the PUFA/SFA ratio (0.45), we disagree with using this parameter as the primary indicator for the health index of lipids, especially in lipid modification processes. Incorporating a medium-chain fatty acid (MCFA) would be expected to increase the SFA proportion. However, it has been noted that long-chain saturated fatty acids (LCSFA), primarily myristic, palmitic, and stearic acids are heavily implicated in CHD diseases (Wereńska et al., 2021). Therefore, we have opted to determine the health lipid value using indices that consider stronger relationships between the types of fatty acids (Fig. 6).

As depicted in Fig. 6, the NVI values were very close between the tested conditions, demonstrating that the nutritional value remains constant after the oil modification. Conversely, we anticipated that TI

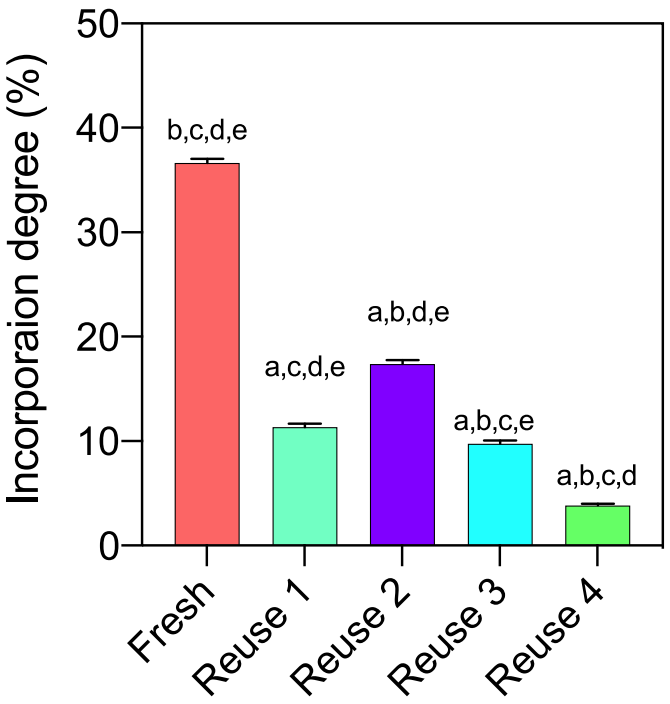

Fig. 5. Structuration of FTs in a packed bed reactor (FTPB) operated in batch mode using fresh and recycled RO lipase immobilized in corn cob powder. Values with different letters represent significant difference ($p < 0.05$).

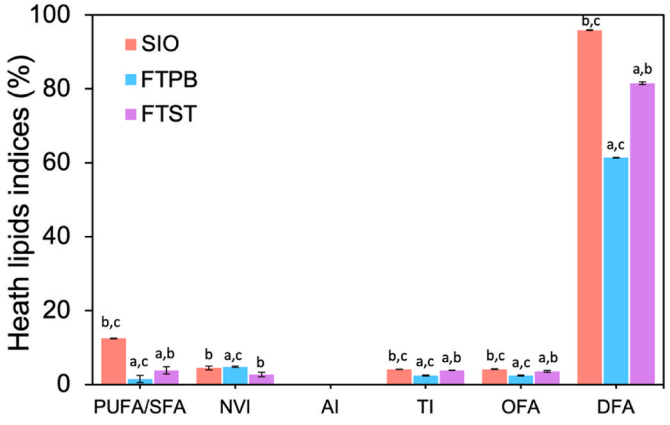

Fig. 6. Heath lipids indices of sacha inchi oil (SIO), FTs synthesized on stirred tank reactor (FTST) at 24 h and FTs synthesized on packet bed reactor (FTPB) at 120 h. Note that values are expressed in terms of mean ± standard deviation. Tukey test was used for multiple comparisons and different letters mean significant difference ($p < 0.05$).

indices would be lower in the SIO than in the FTPB. This suggests that FTs may have a potential protective function against coronary heart diseases (CHD) (Hooper et al., 2016). Regarding AI, we observed that all values were consistently close to 0, lower than those reported for other conventional oils (Filip et al., 2011; Ratusz et al., 2018). OFA and DFA are indices that elucidate the effects of fatty acid types on hypercholesterolemia. Notably, the OFA index is lower in FTST and FTPB compared to SIO, with the minimum value reported in FTPB. This suggests that achieving a 36% incorporation degree of medium-chain fatty acids (MCFA) in the oil could positively impact the nutritional value of lipids. Values for DFA were smaller than the original oil, as expected due to the reduction in unsaturated fatty acids resulting from the incorporation of capric acid. However, the values remained above 60, indicating good lipid quality, validating the potential benefits of the modified SIO in promoting heart health.

4. Conclusion

The successful production of functional triglycerides (FTs) enriched with capric acid (C10) was achieved through the immobilized lipase *Rhizopus oryzae* (RO) on corn cob powder using Sacha Inchi oil (SIO) as a viable alternative raw material. The robust hydrolytic activity of the RO-3 enzyme, with a recovery activity of around 90%, underscores the positive correlation with the observed incorporation degree. The immobilization enzyme on corn cob powder emerged as an attractive alternative support, with optimal reaction conditions identified at a temperature of 45 °C, a molar ratio of 1:3, and an enzymatic load of 10% of the medium. These conditions, designed to minimize acyl migration resulted in the highest incorporation degree of 36% using packed bed reactor. Despite this, the health lipid indices confirm that the FTs maintain good lipid quality, indicating the potential cardiovascular benefits of the modified SIO. The pursuit of optimal conditions is crucial for the integration of enzymatic production systems of structured lipids MLM type in the food industry, In the same sense, we recommend future studies that include the identification of molecular species of TAGs in order to confirm the success of the incorporation of MLM TAGs in FTs.

CRediT authorship contribution statement

Angie Vanessa Caicedo-Paz: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cassamo Ussemane Mussagy:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Conceptualization. **Victoria Mesa:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Rodney Helder Miotti Junior:** Validation, Methodology. **Rodrigo Valenzuela:** Valenzuela, Writing – review & editing, Visualization, Validation, Conceptualization. **Ariela Veloso de Paula:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Conceptualization. **Julián Paul Martinez-Galan:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

AVCP was financially supported by Ministry of Science, Technology, and Innovation of Colombia (Minciencias), Postsecondary Education Agency of the Mayor's Office of Medellin (Colombia), Internationalization fund under the modality of international counterparts. University of Antioquia. CUM was supported by grants from the VINCI-DI de l PUCV (grants no. 039.338/2023 and 039.369/2023).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.103769.

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