Cocoa olein glycerolysis with lipase *Candida antarctica* in a solvent free system

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SUMMARY: In this paper we present the valorization of cocoa olein obtained from the acid fat-splitting of soapstocks. The aim is to develop a solvent free process (enzymatically catalyzed) to maximize the production of a final product with high content of monoglycerides (MAG) and diglycerides (DAG). The effect of the enzyme dose, glycerol content, reaction times as well as the modification of the raw material and pressure were studied. The yield of the reaction increased up to 90-95% when using a vacuum of 2-3 mbar at 65 °C, enough to evaporate the water which is generated as a by-product, an enzyme dose of 1% and molar ratio oil:glycerol of 1:2. The highest yield in terms of MAG and DAG production was obtained by starting from a raw material which was rich in free acidity (FFA), rendering oil with 33.4 and 44.2% MAG and DAG, respectively. Short reaction times (6-8 h) were observed compared to previously reported results (24 h).

KEYWORDS: *By-products; Diglyceride; Enzymatic Glycerolysis; Lipase CALB; Monoglyceride; Olein*

RESUMEN: *Glicerólisis de oleínas de cacao con lipasa* **Candida antarctica** *en un sistema libre de solventes.* En el presente trabajo se pretende valorizar la oleína vegetal de cacao procedente de la ruptura ácida de las pastas de refinación química. El objetivo es poner a punto un proceso de glicerólisis enzimática en un sistema libre de solventes maximizando la producción de monoglicéridos (MAG) y diglicéridos (DAG). Se ha estudiado el efecto de la dosis de enzima, el contenido de glicerol y el tiempo de reacción, la modificación de la presión de reacción y la composición de la materia prima. Se concluye que el rendimiento de la reacción aumenta hasta el 90-95% cuando se aplica un vacío de 2-3 mbar a 65 °C suficiente para evaporar el agua que se va generando como producto, una dosis de enzima del 1% y una relación molar aceite:glicerol 1:2. El mayor rendimiento en cuanto a la producción de MAG y DAG se ha conseguido partiendo de una materia prima rica en acidez libre (FFA), obteniéndose un aceite con un 33.4 y 44.2% de MAG y DAG, respectivamente. Se observa que los tiempos de reacción son cortos (6-8h) comparados con los descritos en la bibliografía encontrada (24h).

PALABRAS CLAVE: *Diglicéridos; Glicerólisis enzimática; Lipasa CALB; Monoglicéridos; Oleína; Subproductos oleaginosos*

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1. INTRODUCTION

Oleins are obtained as a by-product in the process of the fat splitting of soapstocks, but they have low market value because they are a mixture of free fatty acids and TAG. At least 50% of it is constituted by free fatty acids and 40% by TAG, with the remaining 10% being MAG, DAG, unsaponifiable lipids and, to a lesser extent, methyl and ethyl esters.

Oleins obtained from the fat-splitting of soapstocks have various uses, including the manufacture of animal feed (fat additive) as well as other multiple technical uses by employing olein as such or via some of their derivatives obtained by chemical transformation. The most widespread use is the manufacture of biodiesel (Ribeiro *et al.*, 2011; Narvaez Rincón *et al.*, 2004; Pereda Marín *et al.*, 2003), for which the olein is subjected to a process of chemical esterification with methanol catalyzed by acids (Otadi *et al.*, 2011) and subsequently to a process of chemical transesterification catalyzed by a strong base. As a result, free fatty acids (FFA), as well as MAG, DAG and TAG can be transformed into methyl esters (FAMEs).

MAG and DAG are biodegradable compounds classified as GRAS (Generally Recognized As Safe) by the FDA (Food Drug Administration). These molecules are used as anionic surfactants, emulsifiers and stabilizers (E471) (Camino Feltes *et al.*, 2013) for the food industry (bakery products, dairy products, and other products that may contain milk or flour) as well as for the textile industry and the plastic and biolubricant industry (formulations of oils for use in different types of machinery). They have proven to be so versatile that MAG has even shown to have antimicrobial activity (Ramesh *et al.*, 2017). Their production, using both chemical and enzymatic reactions, can reach 90% yields when carried out in organic solvents (hexane, toluene, chloroform, acetone, etc). The disadvantage of using this type of solvents is that, in addition to denaturing the enzymes, they are toxic, carcinogenic, flammable and not biodegradable. In addition, a further purification step for the final product is required to remove these chemicals.

The glycerolysis reaction from oleins produces oils which are rich in MAG and DAG. This process can be carried out chemically or enzymatically, with the industry favoring the chemical approach (Satriana *et al.*, 2016). Such a process is carried out with a basic catalyst (NaOH, KOH or Ca(OH)$_2$), at high temperatures (190-250 °C), for 3-5 h with constant agitation. The final product resulting from the reaction under these conditions usually has the following composition: MAG 30-60%, DAG 35-50% and TAG 1-20%, with certain residual acidity. Thus, in order to achieve a concentration of over 80% MAG and DAG in the final product, a further process of molecular distillation is needed (Ramesh *et al.*,

2017; Solaesa *et al.*, 2016). Consequently, the current industrially implemented processes of chemical glycerolysis tend to entail costly energy demands and have additional drawbacks, such as raw material degradation and darkening of the desired product due to the use of very aggressive catalysts. When a minor degradation of raw material is required, the use of Ca(OH)$_2$ as a catalyst is preferred. However, although smaller, the use of this catalyst cannot prevent the degradation of a significant part of the unsaturated fatty acids (Solaesa *et al.*, 2016; Tiankui *et al.*, 2005), with the subsequent reduction in the potential health benefits of the oil.

Enzymatic glycerolysis has several advantages over chemical glycerolysis (Fregolente *et al.*, 2008; Csanadi *et al.*, 2009; Kapoor *et al.*, 2012; Camino Feltes *et al.*, 2012). The main ones are lower working temperatures (50-80 °C) and a reaction pH close to neutrality (pH 5.0-6.0), both having a direct impact on the quality improvement of the obtained final products. Also, energetically speaking, the process is a less costly one that can be easily scaled up and implemented at industrial scale, generating fast economic benefits and returns. However, it also has disadvantages, with the main one being the high costs associated with the enzymatic catalyst. To give some estimation of costs, increasing the enzyme load from 0.5 to 1% doubles the process costs.

Nonetheless, enzymatic catalyst is a highly viable process leading to a final product with MAG concentrations similar to or even higher than those obtained by the chemical route with the added benefit of a less degraded product (Solaesa *et al.*, 2016; Tiankui *et al.*, 2005).

Lipases (E.C. 3.1.1.3) belong to the family of hydrolases, since they catalyze the hydrolysis of TAG at the lipid-water interface. In addition to their physiological roles, lipases catalyze the hydrolysis or enantio- and regio-selective synthesis of a wide variety of natural substrates, as well as carrying out esterification, interesterification and transesterification reactions in non-aqueous media. This versatility explains why this enzymatic group has a great biotechnological application in the oil industry (Fariha *et al.*, 2005; Godfrey T, 2005; Rivera-Pérez *et al.*, 2007), and in the production of drugs, agrochemicals, etc. The main lipases of industrial interest are produced by fungi (*Candida cylidracae, Aspergillus niger, Candida rugosa, Candida antarctica, Mucor miehei, Staphylococcus aureus, Rhizopus arrhizus,* etc.); whereas others are extracted from bacteria. Some are non-specific enzymes, while others act specifically on a very precise fragment of the TAG. There is also another group of lipases that can work at slightly higher temperatures (60-75 °C) namely, thermostable lipases. CALB is a non-specific enzyme capable of attacking the three positions (sn-1, 2 and 3) of the TAG molecule to produce glycerol and free fatty acids (Grazia *et al.*, 2015). Regardless of the

different features of this great family of enzymes, the great advantage of the use of lipases (for low temperature fat processing processes) lies in their ability to significantly reduce the formation of *trans* fats, which are formed in high amounts in homologous chemical processes (Rivera-Pérez *et al.*, 2007).

For all the reasons mentioned above, the objective of this research is to develop an enzymatic technology in a solvent-free system (Noureddini *et al.*, 1998; Valerio *et al.*, 2010; Vazquez *et al.*, 2016; Wiphum *et al.*, 2005; Zhao *et al.*, 2013; Ziobrowski *et al.*, 2009), with yields close to 80% (joint content of MAG and DAG).

The reaction of any Gycerolysis can be seen in Eqs. 1-3.

$$TAG + Glycerol \longrightarrow DAG + MAG \quad \text{Eq.1}$$
$$DAG + Glycerol \longrightarrow 2MAG \quad \text{Eq.2}$$
$$\mathbf{TAG + 2Glycerol \longrightarrow 3MAG} \quad \text{Eq.3}$$

Finally, it is also worth mentioning that since oleins contain a high concentration of FFA (free acids concentration can represent up to 50-70% of the olein), the typical reactions of glycerolysis (Eqs. 1 to 3) compete with the reaction between glycerol and FFA (direct esterification), resulting in the glycerolysis of oleins in a set of competing reactions (Eqs. 4 to 7). A secondary reaction of direct esterification (Eq. 5) reduces the concentration of MAG but unfortunately cannot be avoided, as they happen simultaneously. Both types of syntheses lead to a balance which shows a greater shift to the right as the temperature and reaction time increase (Chemical Glycerolysis).

$$FFA + Glycerol \longrightarrow MAG + H_2O \quad \text{Eq.4}$$
$$MAG + FFA \longrightarrow DAG + H_2O \quad \text{Eq.5}$$
$$DAG + FFA \longrightarrow TAG + H_2O \quad \text{Eq.6}$$
$$\mathbf{3FFA + Glycerol \longrightarrow TAG + 3H_2O} \quad \text{Eq.7}$$

Thus, when the raw material has a high acidity value, such as oleins, the composition in the final product of the glycerolysis reaction is higher in TAG (up to 15-25% of total) compared to products obtained from other sources.

In this research we have studied the enzymatic glycerolysis reaction of cocoa olein in a solvent-free medium. We will study the effect of the enzyme concentration (dose), glycerol concentration, reaction time and vacuum effect in the final composition of the product (FFA and MAG, DAG and TAG contents).

2. MATERIALS AND METHODS

2.1. Materials

The raw material used in the experiments was an olein from cocoa (*Theobroma cocoa*) soapstocks, obtained from OLEOFAT TRADER S.L. (Tudela – Navarra -Spain) by acid fat-splitting. Cocoa soapstocks were provided by Moner Cocoa S.A. (La Selva del Camp – Tarragona – Spain). The enzymes Lipozyme CALB and Eversa transform 2.0 were purchased from Novozymes (Bagsværd -Copenhagen). Glycerol (99.9%) was purchased from Labbox, NaOH, KOH and the solvents used for analysis (ethanol, *n*-hexane, diethyl ether, methanol and acetone) were from Scharlab. The HMDS+TMCS+Pyridine 3:1:9 (Sylon HTP) mix used to quantify MAG, DAG and TAG and the internal standards such as 1,2,4-butanetriol and tricaprin were purchased from Supelco (Sigma Chemical Co., St Louis, MO). The standards for the calibration curves in the GC analysis, 1-Monolarin, Diolein, Triolein and glycerol, were purchased from Larodan and the 30 mg/ml FAME mix (28 components) was purchased from Restek.

2.2 Methods

2.2.1. *Enzymatic glycerolysis*

The enzymatic glycerolysis reaction was performed in a batch system (Figure 1; Table 1). Raw material (200 gr cocoa olein) was placed in the reactor and 0.8% NaOH (oil weight) was added in order to reach a pH of 5.5 (the olein had a pH of 3.88) (Table 2). After this glycerol was added up to a concentration of 7.0-46.0% (w/w), with molar ratios

Experimental Procedure

NaOH   Enzyme
Oil   Glycerol

Procedure
Enzyme 0,5-2,0 %
Time 24 h
Stirring 800 rpm
Temperature 50-80 ºC
Vacuum 5-10 mbar
Ratio FFA:glycerol 1:2

Enzymatic Inactivation
80-90 ºC
30 min

Light phase
FFA, MAG, DAG and TAG
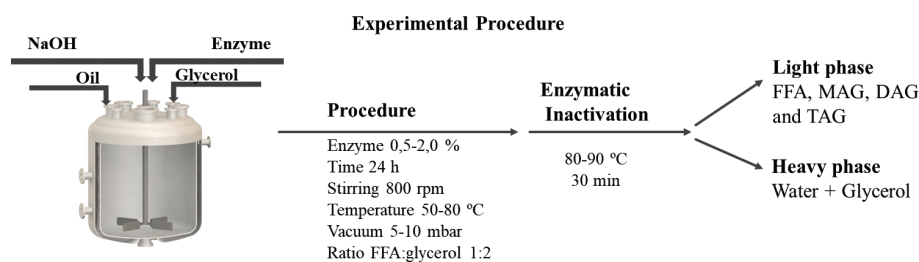
Heavy phase
Water + Glycerol

FIGURE 1.   Enzymatic Glycerolysis procedure: reaction conditions, raw material, reagents and final products of the reaction.

TABLE 1.   Experimental conditions of Enzymatic reactions.

Enzymatic reaction	Enzyme	Temperature (ºC)	Time (h)	pH	Stirring (rpm)	Dose (%)	Vacuum (mbar)	Glycerin (% w/w)	Water (%)
Glycerolysis	Lipozyme CALB	65	24	5.5-6.0	800	0.2-2.0	1-2	7-35	1-3
Hydrolysis	Eversa transform 2.0	55	24	5.5-6.0	400	0.5-2.0	No	0	50

TABLE 2.   Composition of cocoa olein, raw material used in this study. [1]MAG (monoglycerides); DAG (diglycerides) and TAG (triglycerides).

Acidity (%)	Unsaponifiable (%)	pH	Water (%)	MAG[1] (%)	DAG[1] (%)	TAG[1] (%)	Melting point (ºC)
46.3	1.66	3.88	1.13	2.70	7.70	38.90	36.0
Fatty acid profile (%)		**C16:0**	**C18:0**	**C18:1 (*cis*-9)**	**C18:2 (*cis*-9, 12)**	**C18:3 (*cis*-9, 12, 15)**	**C22:0**
		30.94	26.21	40.32	0.82	0.80	0.89

oil:glycerol between 1:0.5 and 1:2, depending on the composition of the desired final product.

Thus, when high doses of MAG are desired a higher dose of glycerol is needed (molar ratio oil:glycerol up to 1:1); whereas in a reaction intended for the production of TAG the molar ratio of oil:glycerol must be increased (working with a stoichiometric defect of glycerol). Finally, the enzyme Lipozyme CALB was added (0.5-3.5% of oil weight). CALB is a non-specific enzyme capable of attacking the three positions (sn-1, 2 and 3) of the TAG molecule to produce glycerol and free fatty acids.

The glycerolysis reaction was carried out at 65 ºC, for 24 h, at 800 rpm and with controlled vacuum (2-3 mbar) using a vacuum pump. The enzyme was thermally denatured at 85 ºC for 30 min, the final product was decanted after 1-2 h to separate the heavy phase, which contained the water and glycerol produced during the glycerolysis reaction. The acidity as well as the MAG, DAG and TAG profile were analyzed in the upper phase with acid base titration and a GC-FID-on-column system, respectively. In this work, all the reactions were performed in duplicate.

2.2.2. Enzymatic hydrolysis

The enzymatic hydrolysis reaction was performed based on previous works in a batch system (Goswami *et al.*, 2012). The conditions are shown in Table 1. Raw material (cocoa olein) was placed in the reactor (2000 g) then 0.8% NaOH (oil weight) was added to reach a pH of 5.5, since e olein has a pH of 3.88 (Table 2). Then water was added (50% v/v) followed by the enzyme Eversa transform 2.0 (1% of oil weight). Temperature and stirring were set at 55 ºC and 400 rpm for 24h for the hydrolysis reaction. The enzyme was thermally denatured at 85 ºC for 30 min, and the final product was decanted after 1-2 h to separate the heavy phase,

which contained water and the glycerol produced during the hydrolysis reaction. Acidity was analyzed in the upper phase with acid base titration.

2.2.3. Chromatographic analyses: MAG, DAG and TAG profile

The chromatographic analyses were carried out using an Agilent 6890N gas chromatograph, equipped with on-column injector and a flame ionization detector (FID). A DB-5HT capillary column (15 m x 0.32 mm i.d.) was used for the GC separation. 1 μl of sample was injected using hydrogen as carrier gas with linear velocity of 1 ml/min and flame ionization detector at 380 ºC. The column oven temperature program was 50 ºC (1 min), 15 ºC/min up to 180 ºC, 7 ºC/min up to 230 ºC, 30 ºC/min up to 380 ºC (held for 15 min); on-column injector with direct injection mode and temperature at 70 ºC (held for1 min), 20 ºC/min up to 380 ºC (held for 10.31 min). The standard mixtures and samples (100 μl to 10 mg/ml) were silylated with 100 μl MSTFA, and after 20 min, 8 ml *n*-heptane were added. The retention time of the first internal standard (1,2,3-butanetriol) was used to identify glycerin and the retention time of the second internal standard (tricaprin) was used to identify the mono-glycerides, diglycerides and triglycerides. Glycerin, monoolein, diolein and triolein were used to calculate the linear calibration curves.

2.2.4. Acid base titration

The acidity of the oil was determined using an acid-base titration according to ISO 660:2009 standard and using an automatic titrator (HI901 from Hanna Instruments). Oil (0.3-1.0 g) was dissolved in 100 ml of diethyl ether:ethanol 50:50 (v/v) and the sample was titrated with an ethanolic solution of KOH 0.1 N.

2.2.5. Melting point

The melting point of the oils was determined following the open capillary tube method, which was immersed in water under stirring and heating according to AOCS method Cc 3-25 (2004).

2.2.6. Determination of fatty acid composition

Fatty acid composition was analyzed by gas chromatography (GC) after methylation of the fatty acids to fatty acid methyl esters (FAMEs) using a chromatograph Varian 430 model equipped with a flame ionization detector (FID) and a Supelco SP2380 60 m x 0.25 mm x 0.2 μm capillary column. About 50 μl of oil were methylated by adding 150 μl of KOH 2N-methanol in a glass tube. The mixture was stirred for 30 seconds and 1 ml of hexane was added. The process conditions were: N_2 as a carrier gas with 0.9 ml/min flow, FID at 250 °C, injector (1:100 split ratio) at 250 °C and injection volume of 1 μl. The column temperature was programmed to 2 min at 60 °C followed by an increase to 240 °C at 8 °C/min. The peaks of individual fatty acids were identified by comparing the retention times of known mixtures of fatty acid standards (FAME mix (28 components) injected in similar conditions. The results were expressed as percentages in relation to the total fatty acids.

2.2.7. Determination of unsaponifiable matter

To determine the degree of unsaponifiable matter, a method of extraction with diethyl ether was used (ISO 3596:2002). Oil (5 g) was saponified with 50 ml of KOH 1M (65 °C, 300-400 rpm, 1h) and subsequently washed with 100 ml of distilled water and 100 ml of diethyl ether. The organic phase was washed with distilled water (3 x 40 ml), KOH 0.5M (3 x 40 ml) and once again distilled water (3 x 40 ml), after which the diethyl ether was evaporated on a rotary evaporator at 50 °C. The quantity of unsaponifiable matter was calculated by weight.

3. RESULTS AND DISCUSSION

3.1. Raw material: cocoa olein

Cocoa olein results from the acid fat-splitting with H_2SO_4 of cocoa soapstocks. Likewise, cocoa soapstocks are obtained when cocoa oil is refined using a chemical neutralization with NaOH in order to reduce the acidity in the cocoa oil. Cocoa soapstocks are light brown, they smell like chocolate and they are solid at room temperature, with a pH over 12.0. The composition and free fatty acid profile of the cocoa olein were determined (Table 2).

3.2. Effect of the enzyme concentration in the glycerolysis reaction

Enzymes require a minimum amount of water to maintain their structure and flexibility. Moreover, water content is necessary because these enzymes act at the oil-water interface. Since the raw material contained 1.1% of water (Table 2), which was considered sufficient for the reaction to run successfully based on the working specifications set for lipases (Table 1), further amounts of water were not added to the reaction mixtures.

In this experiment, an average performance of 58.6% ± 0.6 was found for all experimental conditions with enzyme concentrations ranging from 0.5 to 3.5%, which suggested that the yield of the enzymatic glycerolysis did not seem to depend on the dose of the enzyme (Figure 2). Based on these results, in the following set of experiments the enzyme dose was kept between 0.5-1.0% so as to reduce the costs of industrial processing. The amount of water measured in the final products was 2.7% ± 0.3.

Even in the presence of small amounts of water in the glycerolysis reaction medium, unwanted hydrolysis reactions must be considered (Solaesa *et al.*, 2016). Taking into account that enzymatic glycerolysis produces up to 3 equivalents of water (equations 4 to 7), working at low pressure would favor the formation of MAG, DAG and TAG as water would be removed from the reaction medium as vapor.

In good agreement with this, when the enzymatic glycerolysis reaction was carried out under vacuum (2-3 mbar, enzyme concentration between 0.5-1.0% (oil weight)), the efficiency of the reaction increased by up to > 90% (Table 3) showing the beneficial effect of vacuum in the glycerolysis reaction. As for the enzyme dosage, increasing from 0.5 to 1.0% improved the reaction yield a further 5% (from 90.3 to 95.1%) generating an oil with an acidity below 2.5%. Humidity was determined in the final reaction product at less than 0.1%.
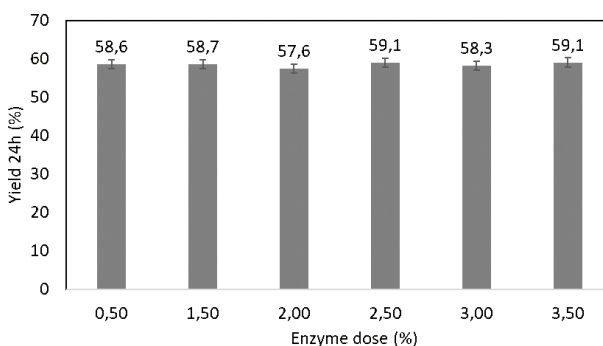

FIGURE 2. Effect of the enzyme dose in the yield of enzymatic glycerolysis reaction. Reaction condition: 65 °C, 24 h, 800 rpm, pH 5.5 and molar ratio glycerin/oil of 2:1. Each point represents the mean value of two replicates +/- SD.

3.3. Effect of glycerol in enzymatic glycerolysis

As can be observed in Figure 3, the yield of the enzymatic glycerolysis depended on the quantity of glycerol added, and ranged from 79 (using 16.7% of glycerol) to 89% (using 45.9% of glycerol). In all conditions studied the acidity value was lower than 10%.

When vacuum was applied (2-3 mbar), the reaction progressed favorably and acidity values below 7.0% were reached in all cases, while yields higher than 85% were obtained (Table 4). Humidity in the very final product was determined to be less than 0.1%, reaching a similar acidity value (less than 7) in this group of experiments. As previously discussed in section 2.2, the MAG, DAG and TAG profiles were conditioned by the amount of glycerol used in the reaction medium (Table 4).

TABLE 3. Effect of using vaccum on acidity reduction in the enzymatic glycerolysis reaction with different enzyme doses. Reaction condition: 65 ºC, 24 h, 800 rpm, pH 5.5 and molar ratio glycerin:oil 2:1. Each point represents the mean value of two replicates +/− SD.

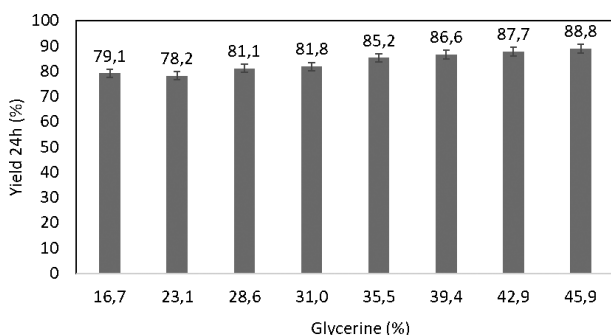| Enzyme dose (%)	Acidity 24h (%)	Yield (%)
0.5	4.5 ± 0.1	90.3 ± 0.1
1.0	2.3 ± 0.2	95.1 ± 0.4

FIGURE 3. Effect of the glycerin concentration on the yield of the enzymatic glycerolysis reaction. Reaction condition: 65 ºC, 24 h, 800 rpm, pH 5.5 and enzyme dose of Lipozyme CALB of 1%. Each point represents the mean value of two replicates +/− SD.

In the selected working conditions for this group of experiments glycerol was in stoichiometric defect with respect to FFA. Not surprisingly, in these working conditions MAG formation was favored, reaching the highest conversion at 9.2% glycerol (molar ratio 0.6:1; glycerol:FFA). In this set of experiments (molar ratios glycerol:oil of 0.5:1 (7.0%), 0.6:1 (9.2%) y 0.75:1 (11.2%)), TAG concentration was higher for lower concentrations of glycerol, which suggested that the content of MAG and DAG increased as TAG content decreased.

3.4. Time period of the enzymatic glycerolysis reaction

Samples were taken at 2, 4, 6 and 24 h, following the procedure described by Choong (Choong *et al.*, 2018). Time periods were studied for three different reactions: the first one with a molar ratio of oil:glycerol 1:2 (Figure 4A), the second with a molar ratio of oil:glycerol 1:1 (Figure 4B) and the last one using hydrolyzed cocoa olein as raw material (Figure 4C). The reaction conditions are described in detail in section 2.2. The composition of the hydrolyzed olein was 87.2% FFA, 0% MAG, 3.1% DAG, 4.7% TAG and pH 5.0. The enzymatic hydrolysis reaction had a yield of up to 80%.

As previously stated, increasing the amount of glycerol in the reaction medium favored the formation of MAG and DAG (Table 5). When the raw material used for the enzymatic glycerolysis reaction was a material with high acidity and low TAG content (such as hydrolyzed cocoa olein) a final product with a high quantity of MAG and DAG (77%) was obtained.

It is important to highlight that although the enzymatic glycerolysis reaction is usually a long process (24 h), when cocoa olein was used as raw material the reaction was completed in only 6-8 h, regardless of the composition of the raw material (cocoa olein or hydrolyzed cocoa olein). This is noteworthy since previous studies on enzymatic glycerolysis reported longer reaction times (20-72 h) (Table 6).
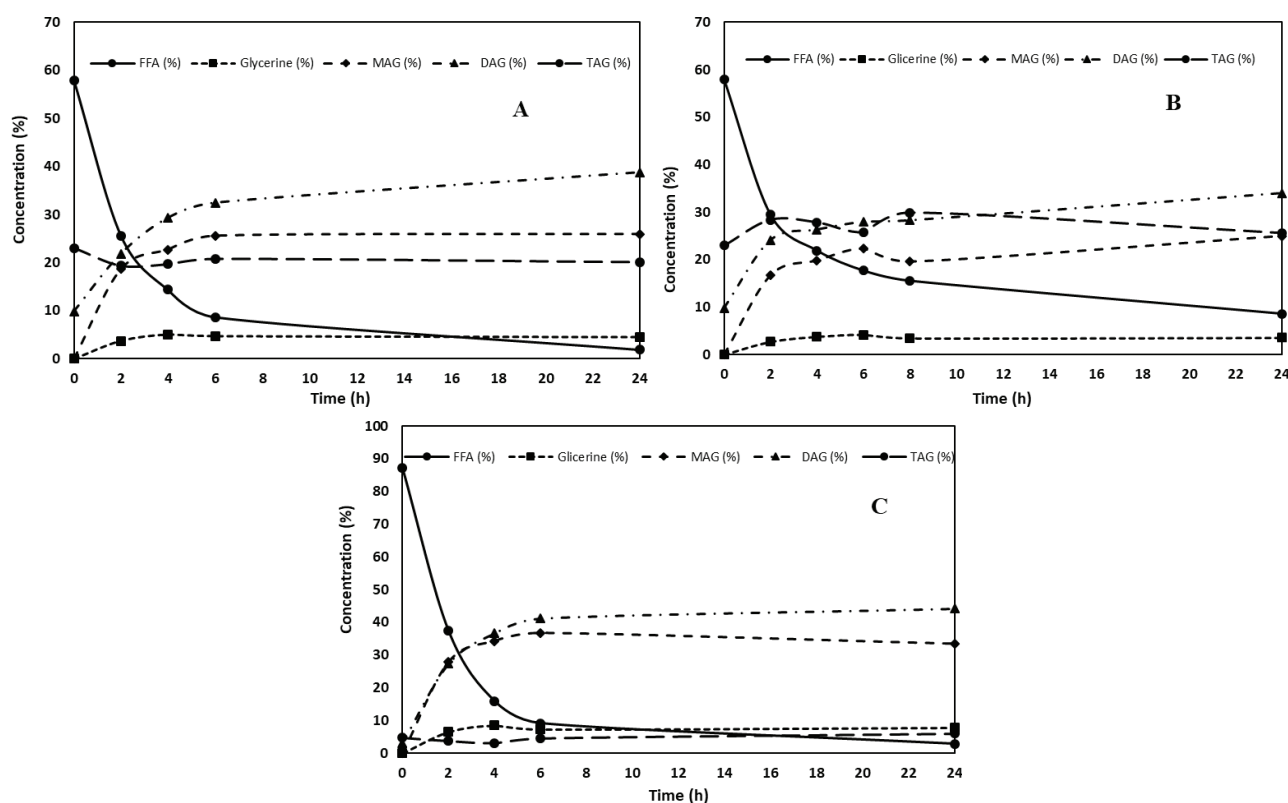
TABLE 4. Effect of glycerin/oil molar ratio on product formation in the enzymatic glycerolysis reaction. Reaction condition: 65 ºC, 24 h, 800 rpm, 2-3 mbar, pH 5.5 and enzyme dose of 0.5%. [1]FFA (free fatty acid); MAG (monoglycerides); DAG (diglycerides) and TAG (triglycerides). Each point represents the mean value of two replicates +/− SD.

Sample	Glycerin (%)	Water 24h (%)	FFA[1] (%)	MAG[1] (%)	DAG[1] (%)	TAG[1] (%)	Yield (%)
Cocoa olein	0.0	1.13 ± 0.05	46.3 ± 2.3	2.7 ± 0.2	7.7 ± 0.4	38.9 ± 1.9	-
Final product	7.0	0.05 ± 0.01	7.0 ± 0.4	21.4 ± 1.0	34.1 ± 1.7	31.4 ± 1.6	84.9 ± 0.9
	9.2	0.08 ± 0.01	4.7 ± 0.3	42.2 ± 2.1	21.5 ± 1.1	29.2 ± 1.5	89.8 ± 0.1
	11.2	0.03 ± 0.01	2.1 ± 0.1	32.2 ± 1.6	34.1 ± 1.7	24.5 ± 1.2	95.6 ± 0.1

FIGURE 4. Time period of the enzymatic glycerolysis reaction. Reaction condition: 65 ºC, 24 h, 800 rpm, pH 5.5, 2-3 mbar, enzyme dose of Lipozyme CALB of 1% and a molar ratio oil:glycerin of 1:2 (A); a molar ratio oil:glycerin of 1:1 (B) and a different raw material, the product from enzymatic hydrolysis of cocoa olein and a molar ratio oil:glycerin of 1:2 (C). FFA (free fatty acid); MAG (monoglycerides); DAG (diglycerides) and TAG (triglycerides). Each point represents the mean value of two replicates.

TABLE 5. Final product composition from the enzymatic glycerolysis reaction. Reaction condition: 65 ºC, 24 h, 800 rpm, 2-3 mbar, pH 5.5 and enzyme dose of 0.5%. [1]FFA (free fatty acid); MAG (monoglycerides); DAG (diglycerides) and TAG (triglycerides). Each point represents the mean value of two replicates +/− SD.

Raw material	Molar Ratio Oil:Glycerin	FFA[1] (%)	Glycerin (%)	MAG[1] (%)	DAG[1] (%)	TAG[1] (%)
Cocoa Olein	1:1	8.6 ± 0.4	3.5 ± 0.2	24.9 ± 1.2	34.0 ± 1.7	25.6 ± 1.0
Cocoa Olein	1:2	1.9 ± 0.1	4.4 ± 0.3	25.9 ± 1.5	38.8 ± 1.5	20.1 ± 0.8
Hydrolyzed Cocoa Olein	1:2	2.9 ± 0.2	7.7 ± 0.4	33.4 ± 2.0	44.2 ± 1.2	5.9 ± 0.4

4. CONCLUSIONS

The free lipase B of *Candida antarctica* was used in the glycerolysis of cocoa olein and produced an oil enriched in MAG and DAG in a solvent-free system. It was found that the maximum yield was reached when the reaction was carried out at low pressure (2-3 mbar) and at 65 °C, with an enzyme dose of 1% and a molar oil:glycerol ratio of 1:2. In addition, FFA enrichment of cocoa olein, used as raw material, yielded an improvement in the MAG/DAG ratio which went from MAG/DAG = 26/39% to MAG/DAG = 33/44%. Finally, we also observed a remarkable reduction in reaction time compared to the typical enzymatic processes described for this kind of reactions (Table 6).

The combination of shorter reaction times with decreasing prices of enzymes could pave the way for the scale up and industrialization of these processes for the valorization of by-products such as sunflower, soybean, olive, rapeseed and cocoa oleins from soapstocks.

TABLE 6. Comparison of different glycerolysis reactions over oils using enzymes; IM (Immobilized Enzyme)

Lipase	Raw material	T (°C)	T (h)	MAG/DAG (%)	Molar ratio Gly/TAG	Reference
Candida antarctica	Cocoa olein	65	24	26 / 39	2:1	This study
Candida antarctica	Hydrolyzed cocoa olein	65	24	33 / 44	2:1	This study
Candida Antarctica B	Palm stearin olein	50	20	12 / 25	1.5:1	Subroto E et al., (2019)
Candida antarctica	Olive oil	40	3	26 / 30	2:1	Singh A.K et al., (2012)
Candida antarctica IM	Soybean oil	70	24	32 / 48	4:1	Fregolente P et al., (2010)
Candida antarctica IM	Fish oil	65	24	13 / 60	1:3	Baeza-Jiménez R. et al., (2013)
Candida antarctica B	Fish oil	60	24	20 / 42	0.9:1	Torres. et al., (2002)
Burkholderia cepacia	Anchovy oil	45.8	4	24.8 / 51.9	3:1	Palacios et al., (2019)
Pseudomonas sp.	Palm olein	45	24	22 / 45	2.5:1	Kaewthong W. et al.,(2003)
Mucor miehei	Rapeseed oil	75	20	17.4 / 36.8	6:1	Inger Elfman-Börjesson et al., (1999)
Chromobacterium	Sunflower oil	40	72	62 (Total)	3:1	F.A. Zaher et al., (1998)
Thermomyces lanuginose IM	Sunflower oil	40	17	5 / 50	1:1	Yang et al., (2005)
Candida antarctica IM	Coconut oil (*)	50	4	62.5 / 30.4	4:1	Baophing Zha et al., (2014)

(*) Surfactant sodium (bis-2-ethyl-hexyl) sulfosuccinate (AOT) 16%.

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