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Monoglycerides and Diglycerides Synthesis in a Solvent-Free System by Lipase-Catalyzed Glycerolysis

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Abstract Five lipases were screened (*Thermomyces lanuginosus* free and immobilized forms, *Candida antarctica* B, *Candida rugosa*, *Aspergillus niger*, and *Rhizomucor miehei*) to study their ability to produce monoglycerides (MG) and diglycerides (DG) through enzymatic glycerolysis of soybean oil. Lipase from *C. antarctica* was further studied to verify the enzyme load (wt% of oil mass), the molar ratio glycerol/oil, and the water content (wt% of glycerol) on the glycerolysis reaction. The best DG and MG productions were in the range 45–48% and 28–30% (*w*/*w*, based on the total oil), respectively. Using immobilized lipases, the amount of free fatty acids (FFA) produced was about 5%. However, the amount of FFA produced when using free lipases, with 3.5% extra water in the system, is equivalent to the MG yield, about 23%. The extra water content provides a competition between hydrolysis and glycerolysis reactions, increasing the FFA production.

Keywords Monoglycerides · Diglycerides · Lipase-catalyzed glycerolysis · Soybean oil

Introduction

Monoglycerides (MG) and diglycerides (DG) are widely used as emulsifiers in foods, cosmetics, and pharmaceutical products [1–3]. Often, mixtures of MG and DG are used in these applications because they are cheap and give proper performance [4]. Besides these applications, a great number of studies on DG in human dietary have been carried out. The use of DG replacing triglyceride (TG) in food has been studied to prevent lipid accumulation in abdominal tissue and, therefore, prevent some diseases related to obesity. Researchers recently showed that long-term ingestion of dietary oil containing mainly DG

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reduces body fat accumulation in humans as compared to TG oil with a similar fatty acid composition [5–7]. Industrially, the production of MG and DG consists on the interesterification of TG with glycerol (GL), in the presence of inorganic catalysts at high temperatures (220–260 °C). This reaction is known as glycerolysis and produces approximately 50% MG content mixture. Due to the high temperatures used, undesirable sub-products are formed [8, 9]. Currently, food industries present interest on the production of more healthful products due to the market requirements, like products free of sub-products of polymerization reactions and of fats in the *trans* configuration. The product produced by this strategy has several drawbacks, e.g., low yield, dark color, and burnt taste [10]. Thus, the substitution of the chemical synthesis of MG and DG for the enzymatic route is presented as an attractive alternative because, in this process, low temperatures and near neutral pH are used to prevent the formation of undesirable products [11]. An efficient alternative to produce high yield of MG is the use of solvent or a mixture of solvents to improve the miscibility of substrates. However, the optimization of the solvent media is complex considering the interaction between solvents and enzymes and the safety requirements for food applications [12].

Lipase-catalyzed glycerolysis of oils and fats using solvent-free system at atmospheric pressure and lower temperature have attracted interest in both academic and industrial fields. Because of the possibility of further separation of the reaction products by vacuum distillation, lower energy requirements, selectivity of the enzymes [13], and possibility of recoverability and recyclability of immobilized enzymes [14], it is believed to be a practical alternative method for MG and DG production.

In this work, MG and DG are produced through lipase-catalyzed glycerolysis of soybean oil in a batch reactor using *Candida antarctica* B, *Thermomyces lanuginosus*, *Rhizomucor miehei*, *Candida rugosa*, and *Aspergillus niger* lipases, in a solvent-free system.

Materials and Methods

Materials

Commercial refined soybean oil was used. Glycerol (99%, 0.5% of water content) was supplied by Synth (Diadema, Brazil). *C. antarctica* B (CA-IM, immobilized lipase), *T. lanuginosus* (TL-IM and TL-L, immobilized and free lipases, respectively), *A. niger* (AN-L, free lipase), and *R. miehei* (RM-IM, immobilized lipase) were generously supplied by Novozymes A/S (Bagsvaerd, Denmark). *C. rugosa* (CR-IM, immobilized lipase) was supplied by Sigma Chemicals Co. Lipase from *R. miehei* is immobilized by adsorption on a macroporous anion exchange resin phenolic type. This is a thermostable 1,3-specific lipase preparation that can be used at temperatures between 30 and 70 °C. Novozyme 435 B is the lipase from *C. antarctica* immobilized on a macroporous acrylic resin. It is also a thermostable lipase preparation with a maximum activity in the range 70–80 °C. The immobilization matrix of TL IM lipase is silicon dioxide.

Glycerolysis Reaction

The enzymatic glycerolysis reaction was carried out in batch system. The reaction mixture consisted of glycerol and soybean oil in different molar ratios (glycerol/oil), immobilized or free lipase (the amount of lipase was based on the weight of oil), and an extra 3.5% of water (based on the glycerol amount). A water thermostatic bath was used to control the temperature, which ranged from 40 to 70 °C. The reaction was stirred using a magnetic stirrer at 300 rpm. The reaction was stopped after 24 h by heating the reaction until 90 °C for 15 min, as described by Tuter et al. [15]. Samples of the reaction mixture were filtrated to remove the lipases before analysis.

Determination of Lipase Hydrolytic Activity

The method is based on the speed at which the enzyme hydrolyzes tributyrin at pH 7.0. The butyric acid that is formed is titrated with sodium hydroxide and the consumption of the latter recorded as a function of time. One unit (lipase activity unit) is the amount of enzyme that releases 1 µmol titratable butyric acid per minute of reaction at 37 °C.

Analysis of Glycerides by HPSEC

The determination of the composition of the acylglycerols, FFA and GL, were performed using a gel-permeation chromatography (GPC), also called high-performance size-exclusion chromatography (HPSEC) [16]. The chromatographic system consists of an isocratic pump, model 515 HPLC Pump (Waters, Milford, MA, USA), a differential refractometer detector model 2410 (Waters), and an oven for columns maintained at 40 °C by a temperature control module (Waters). Two HPSEC columns Styragel HR 0.5 and HR 1 (Waters) were connected in series. These columns are packed with styrenedivinylbenzene co-polymer. The mobile phase used was high-performance liquid chromatography-grade tetrahydrofuran from Tedia Inc. (Fairfield, OH, USA), and the flow rate was 1 ml/min. The typical pressure at this flow rate was 450 PSI (3,102 kPa). All the standards were obtained from Supelco, Inc. (Bellefonte, PA, USA). The products of reaction, DG, MG, and FFA, as well as TG and GL are separated because of the differences of molar weight.

Results

Screening of Lipases for the Enzymatic Glycerolysis of Soybean Oil

To select the most suitable lipase for the production of DG and MG through enzymatic glycerolysis reactions, different lipases were examined under the same experimental conditions. The reaction products are presented as mass fraction (%) of TG, DG, MG, and FFA. The mass percentage of TG, DG, MG, and FFA after 24 h of reaction for each lipase is presented in Fig. 1. In all cases, lipases led to the high production of DG. As can be seen, RM-IM lipase does not present a satisfactory performance to produce DG and MG, as it presented lower lipase activity (Table 1). Furthermore, according to Bornscheuer and Kazlauskas [17] and Mukherjee [18], this lipase present more selectivity for short chain fatty acids (10−6 carbons), which are not significantly present in soybean oil. The fatty acids compositions of the starting material was 22.8% of C18:1, 43.4% of C18:2, and 16.8% of C18:3. Under the reaction conditions, CR-IM lipase also did not obtain satisfactory results on the production of DG and MG. Figure 1 shows that glycerolysis catalyzed by CA-IM lipase presented the highest yield of DG and MG. According to Ferreira-Dias et al. [11], the aquaphilicity (Aq) of the supports might be used as an indicator of their affinity for water and, this way, evaluate possible differences in hydrophilicity between the supports used in the immobilized lipases. The support used to immobilize CA-IM lipase presents lower hydrophilicity than the support used as immobilization matrix for

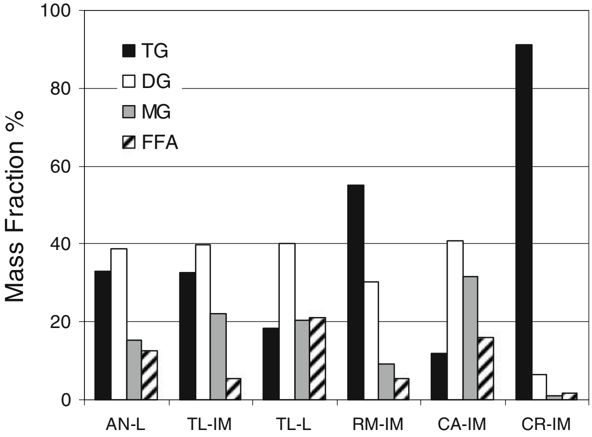

Fig. 1 Glycerolysis of soybean using various lipases. Conditions: T=40°C, substrate molar ratio glycerol/oil=4, 10% enzyme load (wt% of oil mass), 3.5% water content (wt% of glycerol mass), 24 h of reaction

RM-IM lipase [11]. The observed results may be explained by a different affinity of glycerol (hydrophilic compound) for each immobilization matrices. According to Ferreira-Dias et al. [11], the support of the RM-IM presents Aq about six times higher than that of CA-IM, leading to higher glycerol concentrations in the microenvironment; the formation of a glycerol layer around the lipase particles restraining the contact between the lipase and the hydrophobic substrates (TG and DG) may occur [11]. Besides the strong activity, the high performance in the production of MG and DG obtained by CA-IM also can be explained because its support presents low Aq, decreasing the unavailable caused by GL in the interface lipase-oil.

TL-L and AN-L resulted in the production of high amount of FFA (considered byproducts), even with lesser MG production, compared to other lipases as CA-IM and TL-IM. In this case, an extra 3.5% of water content was added to the system, which already contained a concentration similar to water from free lipase (lipase in aqueous solution). Therefore, with the mixture containing an excess of water, a competition between glycerolysis and hydrolysis occurs. In this case, the hydrolysis can predominate in relation to glycerolysis reaction, producing high amount of FFA (principal characteristic of hydrolysis reaction).

Effect of Water Content

Enzymes require a minimum amount of water to maintain their structure and flexibility. Water content is necessary because these enzymes act at the oil–water interface, allowing

Table 1 Initial lipases hydrolytic activity in the tributyrin substrate.

Lipases	Commercial name	Lipase activity (U/mg)
RM-IM	Lipozyme IM	39.1
TL-IM	Lipozyme TL IM	145
TL-L	Lipozyme TL 100L	246
AN-L	Palatase 20000L	230
CA-IM	Novozyme 435	1,056
CR-IM	Sigma—typeVII	800

the formation of an acyl–enzyme complex. As can be seen in Fig. 2(a), the conversion of TG for the reaction that contains 3.5% of water is 48.15% higher than the reaction without extra water in the same conditions [Fig. 2(b)]. The reaction illustrated in Fig. 2(b) occurred because of the minimum of 0.5% water present on glycerol. The production of DG and MG are, respectively, 23% and 58% lower when the enzymatic glycerolysis is carried out in the absence of extra water content.

According to Table 2, free lipases in the absence of extra water content produce around 50–57% lesser FFA because of the decrease in the competition between glycerolysis and hydrolysis reaction. Similarly, the mass fractions of DG and MG for both free lipases increase, 15% and 13% of DG and MG for TL-L and 11% and 8% of DG and MG for AN-L, respectively, prevailing glycerolysis reaction. Reaction employing TL-L in the absence of extra water can be compared with glycerolysis reaction using TL-IM lipase in relation to the compositions of DG and MG. However, the use of free lipases presents disadvantages, such as impossibility of recycle to the system.

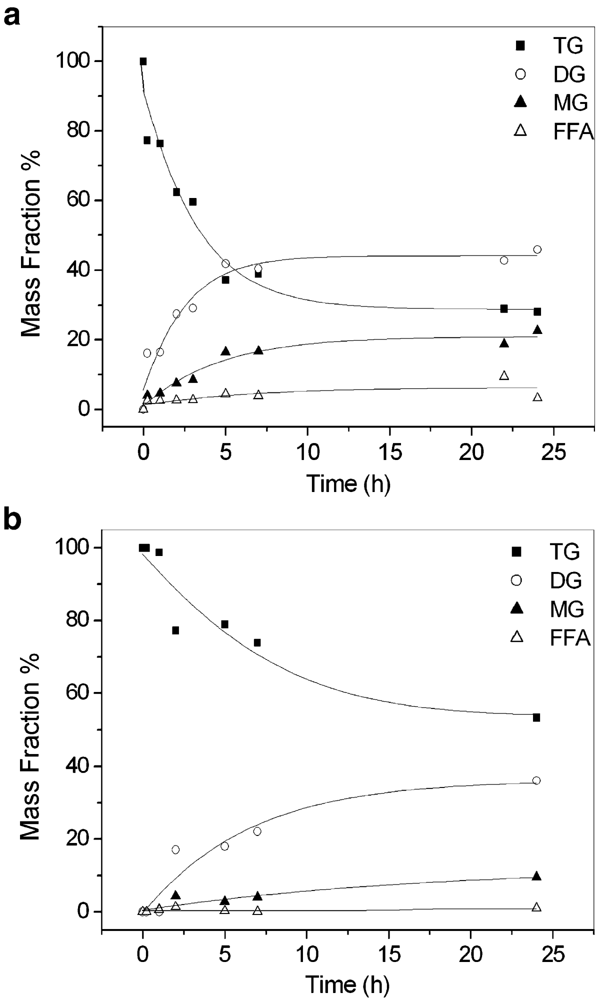

Fig. 2 Time course of the enzymatic glycerolysis reaction using 10% of TL-IM lipase (oil mass) at 40 °C, substrate molar ratio glycerol/oil=8, **a** 3.5% water content (wt% of glycerol mass), **b** absence of extra water content

Table 2 Effect of water content in the glycerol on DG, MG, and FFA productions for TL-L and AN-L lipases.

Mass fraction (%)					
Lipases		TG	DG	MG	FFA
TL-L	(a)	19.47	47.00	22.87	10.65
	(b)	18.46	40.20	20.37	20.96
AN-L	(a)	31.14	44.88	16.66	7.31
	(b)	33.13	38.89	15.30	12.66
TL-IM	(b)	29.43	44.59	21.41	4.56

Absence extra water (a); extra 3.5% water (b) (wt% of glycerol mass). T=50 °C, molar ratio glycerol/oil=8, 10% enzyme load (wt% of oil mass), 24 h of reaction. Standard error=±0.72.

Effect of Enzyme Load

The effect of the amount of lipase was investigated using immobilized lipase from *C. antarctica* B, as it presented higher performance to produce DG and MG. Figure 3(a) and (b) shows the kinetics of the enzymatic glycerolysis of soybean oil in a solvent-free system,

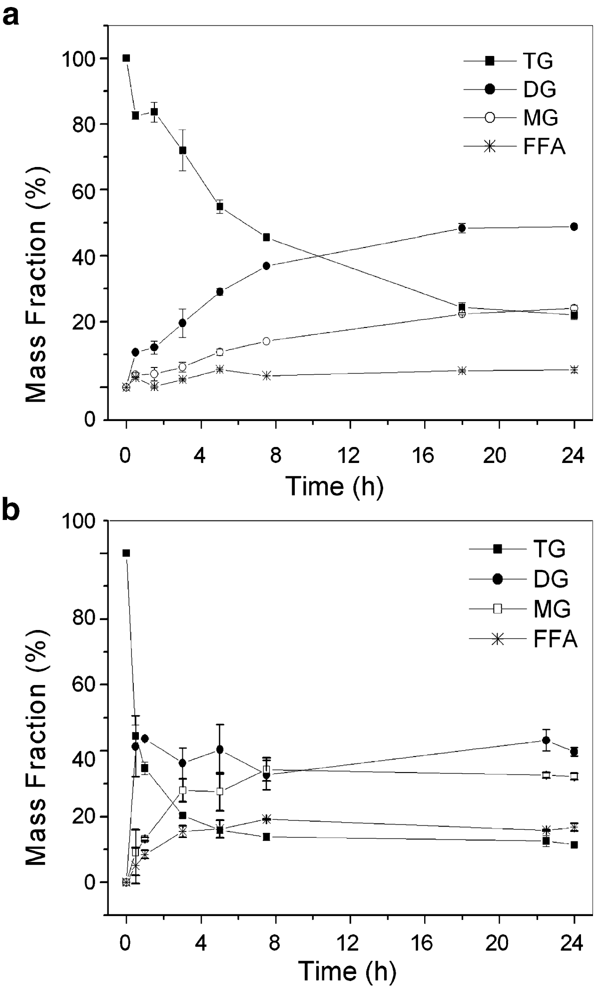

Fig. 3 Time course of the enzymatic glycerolysis reaction using CA-IM (wt% of oil mass) at 50 °C, substrate molar ratio glycerol/oil=8, **a** 2% CA-IM, **b** 10% CA-IM

using 2% [Fig. 3(a)] and 10% [Fig. 3(b)] of CA-IM at 70 °C. As can be seen, in adding 2% of CA-IM, the reaction reaches the equilibrium slower. The compositions of DG and MG are 48.76% and 24.02%, respectively, and the production of byproducts is minimized, around 5.2% of FFA after 24 h. The same behavior of the reaction is verified when 5% of CA-IM is used in the same conditions. The production of DG and MG are similar, 48.28% and 24.64%, respectively, and the mass fraction of FFA is 5.83% after 24 h. In contrast, the reaction equilibrium is reached quickly when 10% of CA-IM is used; however, the production of FFA is higher, around 12.5%, after 24 h. The yield of MG is 27% higher than reactions with 2% and 5% of lipase; however, the excess of lipase and the increase in the production of FFA are strong disadvantages.

Conclusions

The lipase CA-IM was the most efficient in producing DG and MG in a solvent-free system through the catalyzed lipase glycerolysis of soybean oil. The maximum production reached, after 24 h of reaction, for DG and MG was 48% and 32%, respectively. CA-IM presented higher hydrolysis activity and, also, its immobilization matrix presents low hydrophilicity, which provided a good interaction lipase–oil. Water content influences directly on the production of MG, as the lipase acts at the oil–water interface. However, excess of water content leads to the competition between glycerolysis and hydrolysis reaction. The addition of extra water in glycerolysis reactions using free lipases produces high amount of FFA, once the system with excess water is favorable, for the lipases conduct hydrolysis rather than the glycerolysis reaction. The glycerolysis reaction reaches the equilibrium quickly when high amount of lipases (10%) is used; however, lower quantities of CA-IM (2%) catalyzes the reaction slower and producing lower quantity of by-products (FFA). It implies the reduction of lipase load to catalyze the production of DG and MG, decreasing the cost of enzymes in an enzymatic process.

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References

1. Kristensen, J. B., Xu, X., & Mu, H. (2005). *Journal of the American Oil Chemists' Society*, *82*, 329–334.
2. Kaewthong, M., Sirisansaneeyakul, S., Prasertsan, P., & H-Kittikun, A. (2005). *Process Biochemistry*, *40*, 1525–1530.
3. Chang, C., & Bodmeier, R. (1998). *International Journal of Pharmaceutics*, *173*, 51–60.
4. Fureby, A. M., Tian, L., Adlercreutz, P., & Mattiasson, B. (1997). *Enzyme and Microbial Technology*, *20*, 198–206.
5. Taguchi, H., Nagao, T., Watanabe, H., Onizawa, K., Matsuo, N., & Tokimitsu, I. (2001). *Lipids*, *36*, 379–382.
6. Maki, C. K., Davidson, M., Tsushima, R., Matsuo, N., Tokimitsu, I., Umporowicz, D. N., et al. (2002). *American Journal of Clinical Nutrition*, *76*, 1230–1236.
7. Meng, X., Zou, D., Shi, Z., Duan, Z., & Mao, Z. (2006). *Lipids*, *39*, 37–41.
8. Sonntag, N. O. V. (1982). *Journal of the American Oil Chemists' Society*, *59*, A795–A802.
9. Fregolente, L. V., Batistella, C. B., Maciel, R., & Wolf-Maciel, M. R. (2005). *Journal of the American Oil Chemists' Society*, *82*(9), 673–678.
10. Bornscheuer, U. T. (1995). *Enzyme and Microbial Technology*, *17*, 578–586.
11. Ferreira-Dias, S., Correia, A. C., Baptista, F. O., & Fonseca, M. M. R. (2001). *Journal of Molecular Catalysis. B, Enzymatic*, *11*, 699–711.
12. Yang, Y., Vali, S. R., & Ju, Y. (2003). *Journal of the Chinese Institute of Chemical Engineers*, *34*, 617–623.

13. Noureddini, H., & Harmeier, S. E. (1998). *Journal of the American Oil Chemists' Society*, *75*, 1359–1365.
14. Guo, Z., & Xu, X. (2006). *Green Chemistry*, *8*, 54–62.
15. Tuter, M., Aksoy, H. A., Ustun, G., Riva, S., Secundo, F., & Ipekler, S. (2003). *Journal of the American Oil Chemists' Society*, *80*, 237–241.
16. Schoenfelder, W. (2003). *European Journal of Lipid Science and Technology*, *105*, 45–48.
17. Bornscheuer, U. T., Kazlauskas R. J. (1999). *Hydrolases in organic synthesis: Regio- and stereoselective biotransformations*. Weinheim: Wiley-VCH, pp. 164–167.
18. Mukherjee, K. D. (1998). Lipid biotechnology. In C. C. Akoh, & D. B. Min (Eds.)*Food lipids* (pp. 589–639). New York: Marcel Dekker.