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Olive oil glycerolysis with the immobilized lipase *Candida antarctica* in a solvent free system

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RESUMEN

Glicerolisis de aceites de oliva mediante lipasa inmovilizada *Candida antarctica* en sistema libre de solventes.

En el presente trabajo se ha estudiado la glicerolisis sin disolvente de aceites de oliva para la producción de monoglicéridos (MG) y diglicéridos (DG), con la lipasa inmovilizada *Candida antarctica*. Los experimentos fueron realizados por lotes, variando distintos parámetros del proceso. Los resultados mostraron que los rendimientos de MG y DG dependen de las condiciones de operación como el tiempo, la temperatura, la relación molar glycerol/aceite, la concentración de la enzima y del contenido en agua del glicerol. El tiempo óptimo de la operación fue de 3h para un rendimiento máximo en peso del 26% de MG, y del 30% de la producción en peso de DG. La velocidad de reacción inicial ha sido estudiada variando los diferentes parámetros del proceso durante 1h. La velocidad de reacción aumenta a la temperatura de 30 °C, con una relación molar 2:1 glicerina/aceite, un contenido de agua en glicerol de 3,5% (w/w) y una carga de enzima de 0.015g. Se presentan datos comparativos de redimientos de MG y DG para diferentes aceites y combinaciones de enzima.

PALABRAS CLAVE: Aceite de oliva – Digliceride – Glicerina – Glicerolisis – Lipasa – Lipasa B Candida Antarctica – Monoglicéride.

SUMMARY

Olive oil glycerolysis with an immobilized lipase *Candida antarctica* in a solvent free system.

In the present work, the solvent free lipase glycerolysis of olive oil for the production of monoglyceride (MG) and diglyceride (DG) with an immobilized Lipase B *Candida antarctica* was studied. The experiments were performed in batch mode by varying different process parameters. The Results showed that the MG and DG yields were dependent on operating conditions such as time, temperature, glycerol/oil molar ratio, enzyme concentration and the water content in glycerol. The optimum operating time for maximum MG, 26 wt% and DG, 30 wt% production was 3h. The initial reaction rate was studied by varying different process parameters for 1h. The initial reaction rate increased at 30 °C temperature, 2:1 glycerol/oil molar ratio, 3.5% (w/w) water content in glycerol and 0.015g of enzyme loading. Comparative data for MG and DG yields for different oils and enzyme combinations were presented.

KEY-WORDS: Glycerolysis – Glycerol – Lipase – Lipase B Candida Antarctica – Monoglyceride – Olive oil.

1. INTRODUCTION

The lipids monoglyceride (MG) and diglyceride (DG) are products from the degradation of triglycerides (TG). MG and DG are readily biodegradable and generally recognized as safe (GRAS) (Blasi *et al.*, 2007). MG and DG are widely used as non-ionic surfactants and emulsifiers (Zaher *et al.*, 1998; Ghamgui *et al.*, 2006) and stabilizers (E.E.C. code: E471) (Jackson and King, 1997) in the food industry. MG, or its mixtures with DG, accounts for about 75% of the worldwide emulsifier production (Damstrup, 2006a). MG is also used in textile processing, the production of plastics and the formulation of oil for different types of machinery (Pawongrat *et al.*, 2007; Ferreira-Dias *et al.*, 2001). DG oil has beneficial effects on the prevention and management of obesity compared to TG, the main component of edible oils. One report suggests that the consumption of DG may reduce the accumulation of visceral abdominal fat (Kasamatsu *et al.*, 2005; Nagao *et al.*, 2000). MG, or its mixtures with DG, accounts for about 75% of the worldwide emulsifier production (Damstrup *et al.*, 2006a). Olive oil is a readily available source of long-chain monounsaturated fatty acids, especially, oleic acid (C18:1 about 78%), linoleic acid (C18:2 about 16%), palmitic acid (C16:0 about 12%) and stearic acid (C18:0 about 5%). Highly monounsaturated fatty acid concentration improves the consistency and stability of olive oil. Olive oil also produces a consideral amount of MG and DG in glycerolysis compared to conventional fats and oils (Firestone, 2005). A literature review supports that glycerolysis activity in olive oil by lipase is high (Ferreira-Dias and Fonseca. 1995).

Commercial MG and DG production is by chemical glycerolysis of oils/fats with glycerol at high temperatures of 220-260 °C, in the presence of inorganic alkaline catalysts (e.g., KOH and NaOH) in an inert atmosphere. Due to the high reaction temperature the reaction is energy intensive and dark-colored, low yield product formation has been observed (Damstrup, 2006b; Kaewthong *et al.*, 2005). More importantly, unsaturated fatty acids, which have beneficial health and functional properties, may be destroyed during the high

temperature process (Ferreira-Dias *et al.*, 2001; Damstrup *et al.*, 2006a). The chemical glycerolys produces a mixture containing 35-60% MG, 35-50% DG, 1-20% TG and 1-10% free fatty acid (FFA) and undesirable by-products like alkali metal salts (Langone *et al.*, 2002; Noureddini *et al.*, 2004).

Lipase-catalyzed glycerolysis reactions are believed to be a potential alternative method to the chemical process as they are a gentler technology with a much lower temperature required (below 80 °C) (Berger and Schneider, 1992; Kaewthong and H-Kittikun, 2004). The low temperature improves product quality and results in the production of new heat-sensitive MG and DG with polyunsaturated fatty acids (PUFA), which is difficult with the existing chemical glycerolysis process. Thus, MG and DG from enzymatic catalyzed glycerolysis involves industrially viable ingredients with the improved functionality of the fatty acid (FA) profile (Damstrup *et al.*, 2006b).

The lipase-catalyzed glycerolysis of oils/fats using a solvent-free system at atmospheric pressure and lower temperature is of interest to both researcher and industry. Because of the possibility of easy separation of products by vacuum distillation, lower energy requirement and possible reusability of immobilized enzymes (Guoand Xu, 2006), it is believed to be a practical alternative method for MG and DG production. Many reports exist on work about the use of lipases as catalysts for the glycerolysis processes. MG and DG production have been reported from *Pseudomonas fluorescens* lipase in the glycerolysis of beef tallow and palm oil (McNeill and Yamane, 1991; McNeill *et al.*, 1991) and soybean oil using *Candida antarctica* Lipase B in a solvent-free system (Fregolente *et al.*, 2010). Other authors have investigated the effects of lipase from *Humicola lanuginose* on the solvent-free glycerolysis of palm and palm kernel oils and compared the fatty acids profiles of MG, DG, and TG fractions of their glycerolysis products with those of original oils (Tüter and Aksoy, 2000). Few reports are also available on olive oil glycerolysis using *Pseudomonas* sp. lipase (Cao *et al.*, 1996) in a solvent-free system and different surfactants in olive oil with an immobilized lipase *Candida antarctica* (Valério *et al.*, 2010). There is limited information in the literature on the study of commercially immobilized lipase B *Candida antarctica* catalyzed glycerolysis of olive oil in a solvent-free system for the production of MG and DG.

The chemical reaction for any oil including olive oil glycerolysis can be seen in Eqs. (1-3):

$$TG + Gly \leftrightarrow DG + MG \quad (1)$$

$$DG + Gly \leftrightarrow 2MG \quad (2)$$

Thus, the overall, simplified equation is:

$$TG + 2Gly \leftrightarrow 3MG \quad (3)$$

The objective of this work was to investigate the production of MG and DG with the enzymatic glycerolysis of olive oil in a solvent-free system. The experiments were performed in batch mode by varying different process parameters namely the molar ratio of glycerol and olive oil, water content in glycerol, enzyme loading, reaction temperature and time. The initial reaction rate was also studied by varying different process parameters.

2. MATERIALS AND METHODS

2.1. Materials

The substrates used in the experiments were the commercially immobilized lipase B *Candida antarctica* (immobilized on immobead 150, recombined from *Aspergillus oryzae*); glycerol (99.9%) and olive oil (Sigma Chemical Co., St. Louis, MO). Distilled water (Millipore, India) was mixed with glycerol. The standards for high-performance liquid chromatography (HPLC) analysis such as Tripalmitate, Dipalmitin, DL-α Palmitin, Glyceryl trioleate 1,2-Diolein, 1,3-Diolein and 1-Oleoyl-*rac*-glycerol were purchased from Sigma Chemical Co., St. Louis, MO. The solvents used for analysis e.g. acetone, acetonitrile, n-hexane, isopropanol and acetic acid of HPLC grade were purchased from Merck, Mumbai, India.

2.2. Methods

2.2.1. Experimental procedure

The enzymatic glycerolysis reaction was performed in a batch system. The lipase B *Candida antarctica* (0.01g) was placed in a conical flask. The reaction mixture consisted of glycerol and olive oil with molar ratios of 1:1, 3:2, 2:1, 5:2, 3:1 and 7:2. The (w/w)% water in glycerol used for the experiments was 0, 1.5, 3.5, 5, 8 and 10. The reaction mixture was stirred in a water bath using a magnetic stirrer at 600 rpm. The incubation time varied from 1, 2, 3, 4, 5, 6 and 7 h at 30 °C for MG and DG production. The water thermostatic bath (Remi, India) was used to control the temperature in the range of 30 to 70 °C. Samples of the reaction mixture were filtrated through a syringe filled with water-repellent cotton to remove the lipase before analysis at room temperature. All experiments were replicated at least three times.

2.2.2. Analytical method

Quantitative analyses of the MG, DG, and TG were conducted using an HPLC system from the Agilent 1100 Series, with a refractive index detector. The following instrumentation and conditions were used: Zorbax C18 column (4.6 m × 250 mm, 5 μm), flow rate of 1.0 mL/min, column temperature of 35 °C; detector temperature 45 °C; the mobile phase n-hexane and isopropyl alcohol (4:5 v/v). n-hexane and isopropyl alcohol were used as a sample dissolving solvents with injection volume of 25 μL. Standards for MG, DG, and TG were used to establish the calibration chart. All of the integration results were corrected for mole percentages of the individual components in these calibration charts.

3. RESULTS AND DISCUSSION

The triglyceride compositions of the olive oil obtained by the repeated glycerolysis were analyzed by HPLC. Thirteen peaks (1 to 13) were obtained by glycerolysis of olive oil (Fig. 1). The retention times of MG, DG, and TG were 9-13, 19-22, and 24-27 min, respectively. The wider peaks of TG revealed that these lipid classes consist of a mixture of different components. On the other hand, the sharper peaks of MG and DG represented the narrower range of product spread within MG and DG. The fatty acid compositions of these peaks were analyzed and the structures identified as follows. Peak 1: unknown substance; peak 2: 2-Ln, 2-monolinolenin; peak 3: 2-P, 2-monopalmitolein; peak 4: 1-O, 1-monoolein; peak 5: 1,3-LL, 1,3-dilinolein; peak 6: Dipalmitin; peak 7: 1,3-LLn, 1,3-linoleoyl-linolenoyl-glycerol; peak 8: 1,3-diolein; peak 9: POL palmitoyl-oleoyl-linoleoyl-glycerol; peak 10: OLLn,oleoyl-linoleoyl-linolenoyl-glycerol; peak 11: LLL, trilinolein, OLnLn dilinolenoyl-oleoyl-glycerol; peak 12: OLL dilinoleoyl-oleoyl-glycerol, OOLn dioleoyl-linoleoyl-glycerol; peak 13: OOO triolein and POO palmitoyl-dioleoyl-glycerol.

3.1. Effect of time

The effects of reaction time on the production of MG and DG were investigated with an enzyme concentration of 0.01g, water content in glycerol of 3.5%(w/w) and a glycerol to olive oil molar ratio of 2:1 at 30 °C. Figure 2 shows that the initial reaction rate was very rapid with a maximum production of MG of 26 (wt%) and DG of 30 (wt%) in 3h at 30 °C and continued at a slower rate for the remaining time period. The measurements indicated that MG and DG production started as soon as the enzyme was introduced into the system but reduced enzyme activity could be the cause of a decrease in MG and DG production after 3h. Theoretically, MG and DG yields increased with the stoichiometric excess of glycerol in the reaction mixture, however, the yield of MG and DG were depended on favored equilibrium under particular operating conditions.

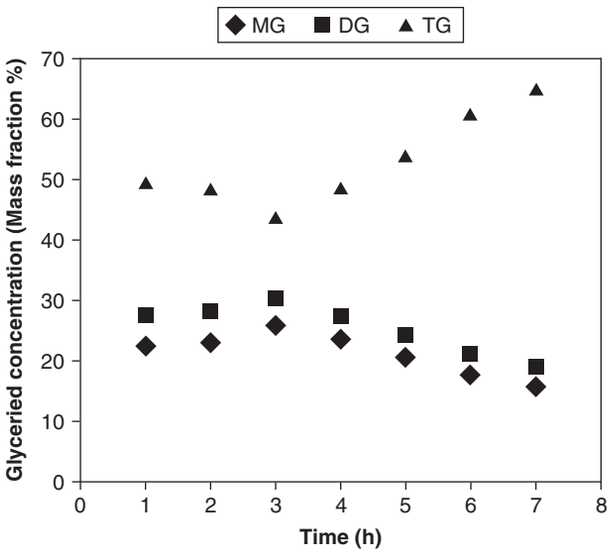

Figure 2
Effect of time on olive oil glycerolysis. Reaction condition: 2: l molar ratio glycerol/oil, 0.01 g lipase/1g oil and water content in glycerol of 3.5% (w/w). The reaction was carried out at 30 °C. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

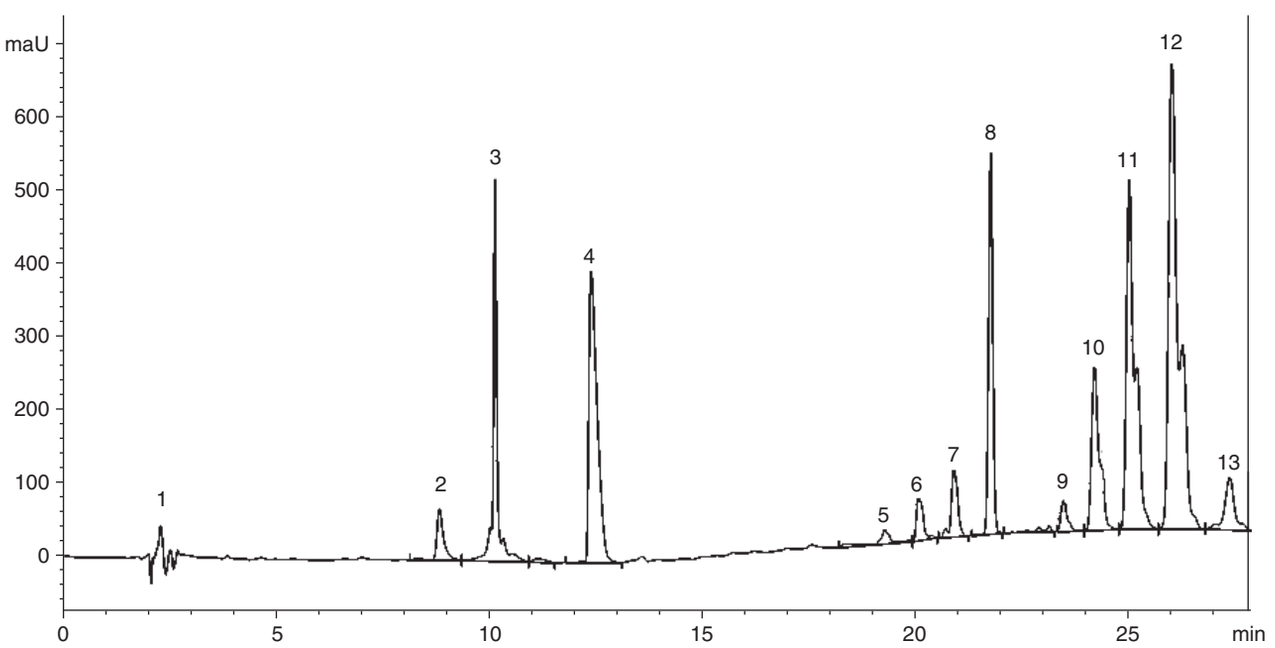

Figure 1
Typical chromatograms obtained by HPLC upon separation of MG, DG and TG mixture after olive oil glycerolysis. Notation of compounds (1) unknown substance; (2) 2-Ln 2-monolinolenin; (3) 2-P 2-monopalmitolein; (4) 1-O1-monoolein; (5) 1,3-LL 1,3-dilinolein; (6) Dipalmitin; (7) 1,3-LLn 1,3-linoleoyl-linolenoyl-glycerol; (8) 1,3-diolein; (9) POL palmitoyl-oleoyl-linoleoyl-glycerol; (10) OLLn oleoyl-linoleoyl-linolenoyl-glycerol; (11) LLL trilinolein, OLnLn dilinolenoyl-oleoyl-glycerol; (12) OLL dilinoleoyl-oleoyl-glycerol, OOLn dioleoyl-linoleoyl-glycerol; (13) OOO triolein, POO palmitoyl-dioleoyl-glycerol.

Valério *et al.* (2009) studied the effect of time on the enzymatic glycerolysis of olive oil by *Candida antarctica* lipase with Triton X-100 and showed that optimum yields of 18 (wt%) MG and 16 (wt%) DG in 3h at 70°C. Zhong *et al.* (2009) used tert-butanol and isopropanol as solvents and showed that the optimum time for the maximum yield of MG and DG for the glycerolysis of soybean oil using *Thermomyces lanuginosus* was 4h.

3.2. Effect of temperature

Temperature played a very important role in the initial rate of the enzymatic glycerolysis reaction. Glycerol showed low miscibility with oils. At high temperatures, the viscosity could be reduced and the substrate solubility or its diffusion could be increased. However, if the temperature was set too high, lipase denaturation possibly occurs. Therefore, the temperature was selected as 1h. The effect of temperature on the initial rate of the glycerolysis reaction was performed with an enzyme concentration of 0.01g, water content in glycerol of 3.5% (w/w) and a glycerol to olive oil molar ratio of 2:1 for 1h. Temperatures in the range of 10-70°C were tested at 10°C increments for 1h. The reaction products as a function of temperature for the glycerolysis of olive oil with lipase B *Candida antarctica* are presented in Figure 3. Figure 3 showed that the maximum MG and DG were produced at 30°C in 1h. The product formation reaches a maximum and starts to decrease as temperature was increased further. This was coherent with the expected loss in enzyme activity at elevated temperatures (above 30°C) due to enzyme denaturation. Temperature dependency in the formation of products from the glycerolysis reaction was also in accordance with the glycerolysis activity. Yang *et al.* (2005) evaluated the effects of temperature on the enzymatic glycerolysis of sunflower oil in a solvent-free system catalyzed by Novozym 435 (10 wt%), a glycerol to oil molar ratio of 4.5:1, without surfactant, at 40 and 70°C. These authors observed that after 24h of reaction, the conversion of MG reached 16 wt% at 40°C. Ferreira-Dias and da Fonseca (1995) reported that the highest yield of MG (70 wt%) in a glycerolysis reaction of olive oil catalyzed by the *Candida rugosa* lipase with n-hexane was reached in 48h at 30°C. Stevenson *et al.* (1993) showed the maximum MG yield (35wt%) for the glycerolysis of tallow with *Mucor meihei* lipase at 50°C. The glycerolysis of palm oil catalyzed by lipase from *Humicola lanuginosa* at 40°C produced with a 43 (wt%) MG content (McNeill and Yamane, 1991).

3.3. Effect of the molar ratio of glycerol to olive oil

To evaluate the effects of the molar ratios of glycerol to olive oil on the initial rate of glycerolysis reactions, experiments were carried out at 30°C with an enzyme concentration of 0.01g per 1g olive

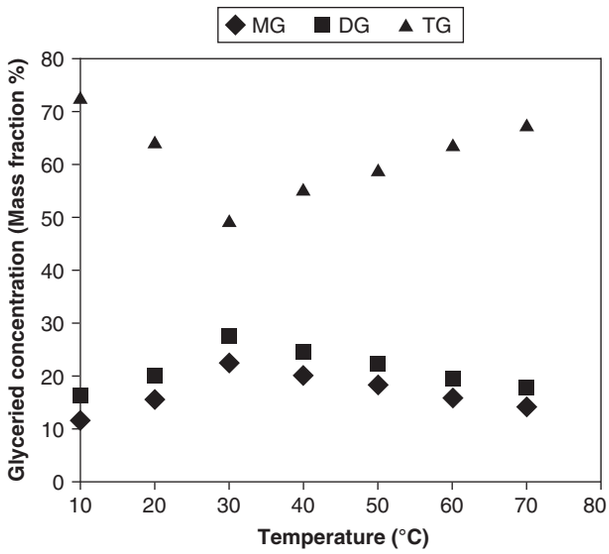

Figure 3
Effect of temperature on glycerolysis of olive oil. Reaction condition: 2: l molar ratio glycerol/oil, 0.01 g lipase /1g oil and water content in glycerol of 3.5% (w/w). The reaction was carried out for 1h. (◆)Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

oil and water content in glycerol of 3.5% (w/w) for 1h. The results for the glycerol to olive oil molar ratio 1:1, 3:2, 2:1, 5:2, 3:1 and 7:2 are presented in Figure 4. The molar ratio of glycerol to olive oil affected the yields of MG and DG. It was observed that with increasing glycerol concentration, the yields of MG and DG production were increased. At a constant water concentration in glycerol, increasing glycerol proportion also increased the water content in the reaction. Hence, hydrolysis might have occurred. A decrease in the reaction yield above glycerol to olive oil 2:1 was unexpected but could be due to the decreased substrate or

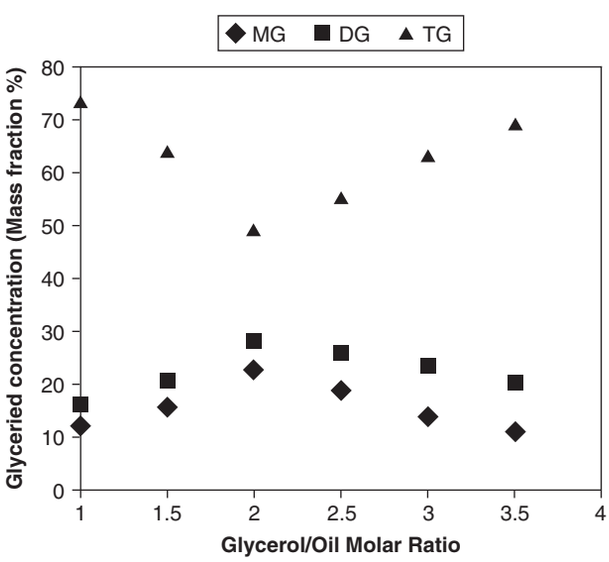

Figure 4
Effect of glycerol/oil molar ratio on product formation in the glycerolysis of olive oil. Reaction condition: 0.01 g lipase/1 g oil and water content in glycerol of 3.5% (w/w). The reaction was carried out at 30°C for 1h. (◆) Monoglycerides, (■) Diglycerides, (▲) Triglycerides.

product diffusion due to increased viscosity as the glycerol content increases. Therefore, the molar ratio of glycerol to oil at 2:1 showed the highest yield of MG and DG. Coteron *et al.* (1998) studied the effect of glycerol to oil molar ratio (3:1 and 6:1) on the enzymatic glycerolysis of olive oil without solvents at 75 °C and observed that after 4h, both reacting systems reached steady levels of MG and DG concentrations (about 23 and 42 wt %) and after 7h, the excess of glycerol had no effect on the product content. Yang *et al.* (2005) investigated the effect of the glycerol to oil molar ratio in the range of 4.5:1-46:1 for sunflower oil glycerolysis at 70 °C, 8% of Novozym 435, and 5h of batch time, in a surfactant and solvent-free system. The authors obtained 17% MG with a glycerol to oil molar ratio of 9:1. Tuter and Aksoy (2000) showed the molar ratio of glycerol to palm kernel oil by *Humicola lanuginose* lipase at 2:1 was the optimum for the production of MG (31 wt %) and DG (42 wt %).

3.4. Effect of enzyme loading

To evaluate the effect of the enzyme concentration on the initial rate of the glycerolysis reaction, experiments were carried out at 30°C with a glycerol to olive oil molar ratio of 2:1 and a water content in glycerol of 3.5% (w/w) for 1h. The reaction results for enzyme concentrations 0.005, 0.01, 0.015, 0.02, 0.025 and 0.03 g are represented in Figure 5. Lipases require a certain amount of water to function properly. As the enzyme concentration increased from 0.005 to 0.015%, considerable enhancement in the production of MG and DG at 1h of reaction was observed. Maximum glycerolysis activity occurred at a ratio of 0.015 g enzyme/g of oil due to increased MG and DG production at that point. Increasing the enzyme concentration from 0.015 to 0.03 g had no significant effect on the yield of MG and DG, probably due to poor mixing of the reaction, substrate deficiencies or an insufficient amount of water in the reaction. The active sites of the enzyme molecules present in large excess were not exposed to the substrates possibly due to protein aggregation (Ghamgui *et al.*, 2006). Pawongrat *et al.* (2007) studied the effect of enzyme loading on the MG and DG production from tuna oil with IM-AK lipase enzyme in tert-butyl methyl ether. The authors observed that MG and DG productions were 25 and 42 wt %, after 24h at 45 °C with 0.3 g of enzyme loading.

3.5. Effect of water content in glycerol

A trace amount of water was necessary to maintain enzyme structure, flexibility and stability. Lipase-catalyzed glycerolysis involved both hydrolysis and esterification reactions. In the first step, water was a reactant. In the second step, water was a product. Thus a suitable water content was necessary to maximize the reaction in both steps. If the amount of water was not enough for the enzyme, the reaction yield reduced. In contrast, excessive water causes acyl migration, leading to a decrease in MG and DG yields (Wongsakul *et al.*, 2003). To evaluate the effect of the water content in glycerol on the initial rate of the glycerolysis reaction, experiments were carried out at 30 °C with a glycerol to olive oil molar ratio of 2:1 for 1h. The results for water concentrations 0, 1.5, 3.5, 5.0, 8.0 and 10.0% (w/w) are shown in Figure 6. The 3.5% (w/w) water content in glycerol showed 23 and

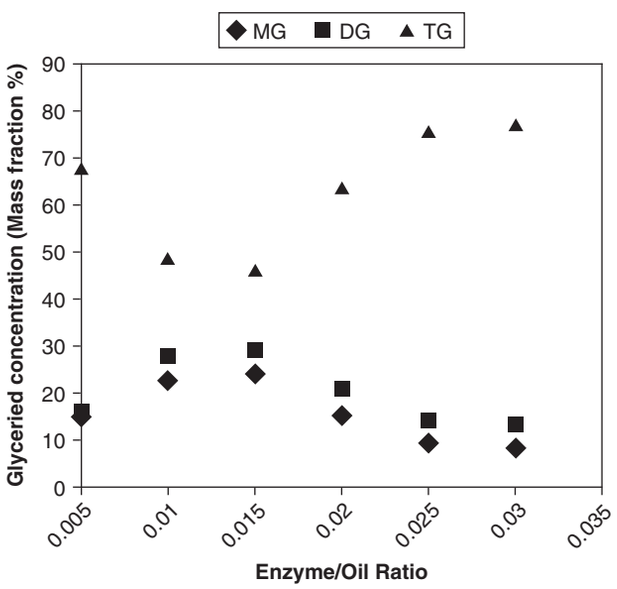

Figure 5
Effect of Enzyme/oil ratio on product formation in the glycerolysis of olive oil. Reaction condition: glycerol/oil of 2: l and water content in glycerol of 3.5% (w/w). The reaction was carried out at 30 °C for 1h. (◆) Monoglycerides; (■) Diglycerides; (▲) Triglycerides.

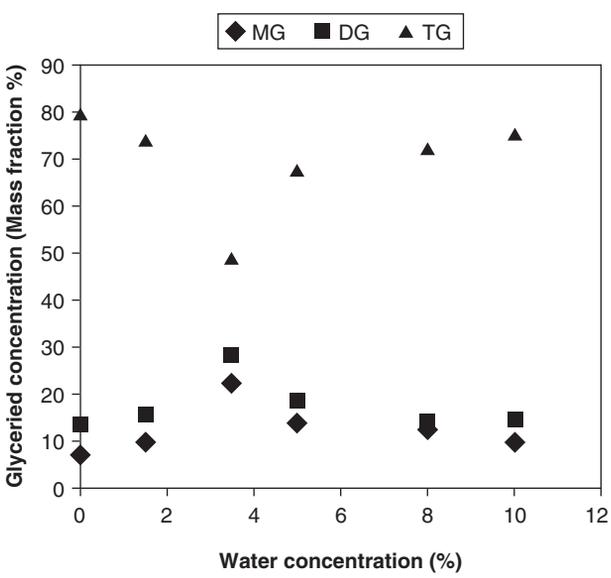

Figure 6
Effect of water content in glycerol on continuous MG and DG production by glycerolysis of olive oil. Reaction condition: 0.01 g lipase/1 g oil and glycerol/oil of 2: l. The reaction was carried out at 30 °C for 1h. (◆) Monaglycerides; (■) Diglycerides; (▲) Triglycerides.

Table 1
Comparison of maximum MG and DG yield by immobilized lipase-catalyzed glycerolysis reactions

Source of Lipase	TG	Solvent / surfactant	Temp. (°C)	Time (h)	Yield MG and DG (wt%)	Gly/TG (mol/mol)	References
Candida antarctica	Olive oil	No	40	3	26, 30	2:1	This study
Candida antarctica	Olive oil	Tween 65 & Triton X-100	70	2	43, 37	6:1	Valério *et al.* (2010)
Candida rugosa	Olive oil	n-hexane	30	48	70, –	4.5:1	Ferreira-Dias and Fonseca (1995)
Candida antarctica	Soybean oil	No	70	24	22, 47	8:1	Fregolente *et al.* (2010)
Thermomyces lanuginosus	Soybean oil	tert-butanol&isopropanol	45	4	72, –	3.5:1	Zhong *et al.* (2009)
Candida antarctica	Camellia oil	tert-butyl alcohol	60	8	82, 16	6:1	Zeng *et al.* (2010)
Candida antarctica	Sunflower oil	tert-butanol&tert-pentanol	50	1-2	68, 82	4:1	Damstrup *et al.* (2005)
Pseudomonas cepacia	Sunflower oil	No	35	25	25, –	3:1	Ota *et al.* (1997)
Pseudomonas sp.	Palm olein	No	45	24	21, 32	2.7:1	Kaewthong *et al.* (2005)
Pseudomonas sp.	Palm olein	Acetone & isooctane	45	24	56, –	8:1	Kaewthong and H-Kittikun (2004)
Mucor miehei	Rapeseed oil	Isooctane	55	20	28, 36	2:1	Elfman-Borjesson, and Harrod. (1999)
Pseudomonas fluorescens	Beef tallow	No	48–50	25	30, –	2:1	McNeill *et al.* (1991)
Mucor meihei	Mutton tallow	No	50	24	35, –	2:1	Stevenson *et al.* (1993)
Pseudomonas cepacia	Butter oil	No	40-45	10	22, –	2:1	Garcia *et al.* (1996)

28wt% yield of MG and DG. Further increase in water content above 3.5% (w/w) led to a reduction in MG and DG yields which was probably caused by hydrolysis due to the high content of water. These results were in agreement with other reports available in the literature. For example, Fregolente *et al.* (2008) showed that maximum MG (23 wt%) and DG (47 wt%) were achieved at 3.5% (w/w) water concentrations in glycerol during the glycerolysis of corn oil by immobilized *Thermomyces lanuginosus* lipase. Yamane *et al.* (1986) concluded that 4% (w/w) or less water concentration in glycerol maximized MG and DG formation during the lipase-catalyzed glycerolysis of corn oil. Yamane *et al.* (1994) found that the FFA content at equilibrium depended on the water concentration in the glycerol phase.

Comparative data of MG and DG synthesis by immobilized lipase-catalyzed glycerolysis reactions with the reported work for different oil and enzyme combinations are presented in Table 1. The comparative data presented is for experiments with and without solvent and surfactants.

4. CONCLUSIONS

The lipase B *Candida antarctica* is capable of producing MG and DG through the lipase catalyzed glycerolysis of olive oil in a solvent-free system. The optimum operating time for maximum MG, 26 wt% and DG, 30 wt% production is 3h. The initial reaction rate increased at 30 °C with a 2:1 glycerol/oil molar ratio and 3.5% (w/w) water content in glycerol and 0.015 g of enzyme loading. This implies that low lipase concentration is favorable for the production of MG and DG and in turn it reduces the cost of enzymed in an enzymatic process.

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