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Production of lipase SMG1 and its application in synthesizing diacylglyecrol

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ABSTRACT

Exploration and evaluation of lipases from different sources will always be required by the swiftly developing industries for modification of oils and fats. Lipase SMG1 from *Malassezia globosa* was expressed in recombinant *Pichia pastoris*, and its catalytic activity in the hydrolysis and synthesis of partial glycerides was evaluated. Lipase SMG1 could not hydrolyze soybean oil; however, enhanced the hydrolysis of soybean oil by combining with Palatase 20000L, suggesting it showed strict specificity on mono- and diacylglycerol. Hydrolysates of soybean oil were esterified with glycerol to produce diacylglycerol, and the optimized results for the esterification reaction were with an fatty acids/glycerol molar ratio of 1:4, lipase SMG1 at a concentration of 120 U/g (U/w, with respect to total reactants), initial water content 1% (w/w, with respect to total reactants) and 30 °C, which yielded 62.03% of diacylglycerol in reaction mixture (80.5% in acylglyceride) after 12 h of reaction. The results showed that lipase SMG1 is a prospective enzyme which could be used in the oils and fats industry.

1. Introduction

Diacylglycerols (DAGs) are esters of glycerol in which two of the hydroxyl groups are esterified with fatty acids. Compared with traditional cooking oils (rich in triacylglycerols, TAGs), intake of DAGs can reduce body weight and visceral fat accumulation in rats and humans [1]. Consumption of DAG oil may also produce less postprandial elevation in plasma TAG levels in humans and lower fasting serum TAG concentrations in animals and humans [2]. Studies in animals and humans suggest that DAG, especially 1,3-DAG, have a number of beneficial effects on lipid metabolism [3]. The beneficial functions of DAG are probably attributable to differences in DAG and TAG absorption and metabolism. So the synthesis of DAG becomes more significant because of the minor content of DAG in natural form.

DAG can be produced chemically or enzymatically through esterification, glycerolysis and partial hydrolysis processes. The enzymatic approach provides an alternative process due to its mild performance conditions, the regioselectivity of the lipases and the low environmental impact [4]. Various methods have been reported for the production of DAG using the enzymatic approach such as the glycerolysis of triglycerides [5] and ethyl esters [6], the partial hydrolysis of oils [7], the re-esterification of monoacylglycerol (MAG) and fatty acids [8], and the esterification of fatty acids and glycerol in organic solvents [9] and in a solvent-free system [10]. However, much more attention has been focused on the development of reaction process, and less work has been carried out to evaluate the potential of lipases from different resources in the production of DAG. Among published enzymatic approaches for DAG preparation, lipase RM IM (*Rhizomucor miehei*), Novozym 435 (*Candida Antarctica*) and lipozyme TL IM (*Thermomyces lanuginous*) are mainly selected to catalyze the reaction. So, further exploration and evaluation of lipases from different sources will always be required by the swiftly developing industries for modification of oils and fats.

Lip1 from *Malassezia globosa*, has specific property which shows positive activity to diolein, but negative activity to triolein [11]. It may be a potential enzyme which could be used in oils and fats industry. In this work, lipase SMG1 (Lip1) was expressed in recombinant *pichia pastoris*, and its potential application in the production of DAG was evaluated in detail. Lipase SMG1 catalyzed esterification of glycerol and soybean oil hydrolysates were adopted to produce DAG, and conditions for the reaction process were investigated.

2. Materials and methods

2.1. Materials and reagents

Soybean oil was supplied by Kerry Oils & Grains Ltd. (Shenzhen, China). Glycerol (99.5%) contained 0.2% water was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). The

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liquid lipase from *M. miehei* (Palatase 20000L, 8050 U/g) was kindly donated by Novozymes A/S, Bagsvaerd, Denmark. *n*-Hexane and 2-propanol were of HPLC grade from Kermel Chemical Reagent Co., Ltd. (Tianjin). Acylglycerol standards (triolein, diolein, and monoolein) and oleic acid were obtained from Sigma (Shanghai, China).

2.2. Crude *M. globosa* Lip1 production by recombinant pichia pastoris

Lipase SMG1 gene (GenBank accession number XM_001732152.1) was artificially synthesized by Sangon Biotech, Inc. (Shanghai, China) and cloned into the *Kpn*I and *Not* I restriction sites of the pUC57 plasmid. To subclone *smg1* gene into *P. pastoris* expression vector pGAPZαA (Invitrogen), the *smg1* gene fragment was released from pUC57 vector by digestion of *Kpn*I and *Not* I and inserted into the same site of pGAPZαA to generate pGAPZαA-*smg1* vector. The *P. pastoris* X-33 transformants harbouring the pGAPZαA-*smg1* constructive expression vector was used to produce lipase SMG1. Strains were stored in 50% glycerol solution (v/v) at −80 °C.

Seed medium consisting of yeast extract/peptone/dextrose (YPD) medium was inoculated and incubated at 30 °C in a 250 rpm rotary shaker for 24 h. After the cultivation, 50 μL of the seed culture was inoculated in 50 mL liquid medium of YPD in a 300 mL glass flask and this culture was grown under the same conditions above. The YPD medium contained (per liter): 1% (m/v) yeast extract, 2% bactopeptone (m/v) and 2% glucose (m/v). After 72 h of cell culture, the supernatant was clarified by centrifugation (1500 rpm, 4 min, 4 °C), and was filtered through a 0.45 mm cellulose acetate filter and then concentrated by ultrafiltration through a 5 kDa relative molecular mass membrane (Vivaflow 200, Sartorius, Germany). The concentrated supernatant was collected and lyophilized. The lyophilized lipase SMG1 powder was kept at 4 °C.

2.3. Lipase activity assay

Lipase assay was performed with DAG oil (purchased from the Kao Corporation of Japan) emulsion. One unit of lipase (U) is the amount of enzyme which releases 1 μmol of titratable fatty acids per minute under the described conditions. Substrate solution: DAG oil and 2% polyvinyl alcohol solution were emulsified at a volume ratio of 1:3 at 10,000 rpm for 10 min. Analysis conditions: 4 mL of DAG oil emulsion, 5 mL of 0.05 M KH_2PO_4/Na_2HPO_4 buffer (pH 6.0) buffer and 1 mL of enzyme solution were mixed and incubated at 25 °C for 15 min. The reaction was terminated with the addition of 95% ethanol (15 mL) after incubation, and the liberated fatty acids were titrated with 0.05 M NaOH. Blanks were measured with a heat-inactivated enzyme sample, for which an enzyme stock solution was kept at 100 °C for 15 min. After cooling to ambient temperature, the solution was used as described for the active enzyme sample. Lipase activity of Palatase 20000L was determined according to the olive emulsion method [12]. The substrate solution was the emulsion of olive oil and polyvinyl alcohol.

2.4. Effect of temperature on the lipase activity of lipase SMG1

The effect of temperature on the enzyme activity was investigated. Lipase SMG1 was kept under each temperature condition (20–60 °C) for 2 h before activity assay. The activity of the lipase was measured by the method mentioned above.

2.5. Preparation of soybean oil hydrolysates

50 g of soybean oil and 5 g of distilled water were added into a 250 mL Florence flask, and stirred magnetically (IKA RH Basic 2 IKAMAG Magnetic Stirrers) at 25 °C, 200 rpm. 100 U/g (lipase with respect to the soybean oil) of Palatase 20000L and lipase SMG1 were employed as biocatalysts. About 0.1 g of the reaction mixture was withdrawn periodically and centrifuged at 10,000 × *g* for 5 min. The upper layer was transferred into another centrifugation tube and was mixed with anhydrous sodium sulfate and 1 mL of *n*-hexane and 2-propanol (15:1, v/v) by swirling. The mixture was underwent centrifugation at 10,000 × *g* for 1 min, and the supernatants was removed for HPLC analysis.

Reaction mixture was separated after 36 h of hydrolysis of soybean oil, and it was extracted with 100 mL of *n*-hexane, then the *n*-hexane layer was dried over anhydrous sodium sulfate. Soybean oil hydrolysates were obtained by removing hexane with a rotary evaporator at 40 °C under vacuum.

2.6. Lipase SMG1-catalyzed esterification of soybean oil hydrolysates and glycerol to produce DAG

2.8 g of extracted hydrolysates of soybean oil together with different amount of glycerol and lipase were added into 25 mL stoppered conical flask for esterification on magnetic stirrer at a stirring rate of 200 rpm. The reaction conditions of enzyme load (30, 60, 90, 120 and 150 U/g), temperature (25, 30, 35 and 40 °C), initial water content (0, 1%, 2%, 3% and 4%, w/w with respect to total reactants) and substrate molar ratio of glycerol to fatty acids (1:1, 2:1, 3:1, 4:1 and 5:1) were varied for optimization. The reactions were allowed to proceed for 24 h. 20 μL of the reaction mixture was withdrawn at periodic intervals and mixed with 1 mL of *n*-hexane and 2-propanol (15:1, v/v). The mixture was centrifuged at 10,000 × *g* for 5 min to remove the glycerol then the upper layer was filtered through a 0.45 μm nylon membrane to remove the enzyme. 10 μL of supernatants was drawn for HPLC analysis.

2.7. Composition analysis by HPLC

Reaction products were analyzed by Normal Phase HPLC (NP-HPLC, refractive index detector) to separate and quantify the acylglycerols and fatty acids. A phenomenex Luna column (Phenomenex Corporation, 4.6 mm i.d. × 250 mm, 5 μm particle size) was used. The mobile phase was *n*-hexane and 2-propanol (15:1, v/v). The flow rate was 1 mL/min. Peaks in HPLC were evaluated by comparison of their retention times with those of known standards. Peak percentages and areas were calculated using Waters 2695 integration software. Analysis was carried out in triplicate, and the values were of the average of triplicate measurements.

3. Results and discussion

3.1. Preparation of crude lipase SMG1

A recent genome sequence analysis of *M. globosa* showed that the *M. globosa* genome encodes abundant hydrolase genes (e.g. lipases, phospholipases, proteases and sphingomyelinases) [13]. Until now, biochemical properties of Lip1 and Lip2 from *M. globosa* were reported, and Lip1 showed lipase activity [11], Lip2 was verified as an esterase [14]. In this work, Lip1 (named as lipase SMG1 in this study) were overexpressed in recombinant *P. pastoris*. The recombinant lipase SMG1 was secreted into culture media, and then concentrated according to the method described in the materials and methods section. The SDS-PAGE electrophoresis (Fig. 1) showed the band of lipase SMG1 in the crude lipase SMG1 solutions after 72 h cell culture. In contrast to the transformant harbouring plasmid without lipase gene, the recombinant *P. pastoris* cells have a good expression of lipase SMG1. 99.5% of lipase SMG1 yield was obtained when the culture medium was concentrated by ultrafiltration through 5-kDa membrane. The molecular weight was

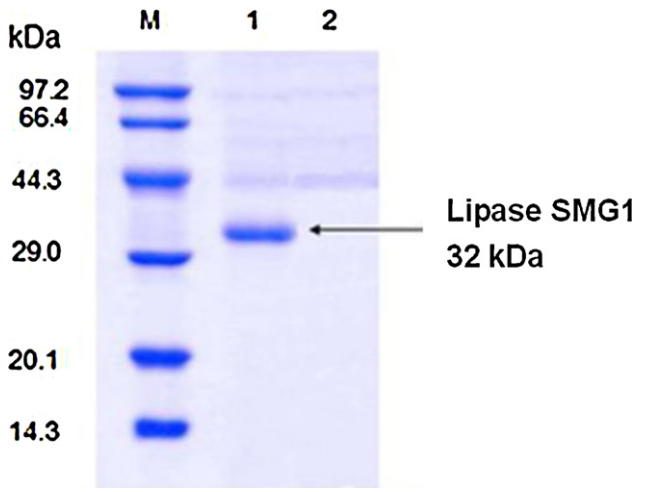

Fig. 1. SDS-PAGE analysis for crude lipase SMG1. Lane M, molecular mass standards indicated in kDa; lane 1, concentrated culture medium of lipase SMG1; lane 2, concentrated culture medium of transformant harbouring pGAP-ZαA without lipase gene.

estimated to be about 32 kDa according to the standard protein markers. The activity of lyophilized lipase SMG1 was 7000 U/g.

3.2. Preparation of soybean oil hydrolysates

Hydrolysis of soybean oil, which was catalyzed by lipase SMG1, Palatase 20000L, and the combination of these two lipases, has been performed. Hydrolysis of soybean oil could not be initiated by lipase SMG1 (data unshown) that was because lipase SMG1 can only be used in hydrolyzing partial glycerides but not for triglycerides, which is agreement with the reports [11]. A relatively low degree of hydrolysis of soybean oil was found with the reaction catalyzed by Palatase 20000L (Fig. 2A). 46.54% of fatty acids (FA) were gained after 36 h, and the hydrolysates also contained 30.22% TAG, 22.57% DAG and 0.66% MAG (w/w). That the hydrolysis degree was low by Palatase 20000L might because the accumulation of partial glycerides such as MAG and DAG limited the hydrolytic reaction. Therefore, the combined use of Palatase 20000L and lipase SMG1 in hydrolysis of soybean oil for synthesis of fatty acids was evaluated (Fig. 2B). After a total reaction time of 36 h, hydrolysates of soybean oil contained 86.12% FFA, 11.68% TAG, 2.21% DAG and less than 0.1% MAG (w/w) were achieved. The results further proved that lipase SMG1 showed good hydrolytic activity to DAG and MAG, and negative activity on TAG, and it was also suggested that the combination of lipases with different specificities is a potentially useful strategy for oils/fats modification. The separated soybean oil hydrolysates were used in the succeeding esterification for synthesizing DAG.

3.3. Lipase SMG1-catalyzed synthesis of DAG by esterification of soybean oil hydrolysates and glycerol

Different reaction conditions on DAG production were investigated with the catalysis of lipase SMG1. The factors to be discussed include lipase load, reaction temperature, molar ratio of glycerol to fatty acids, water content and reaction time.

3.4. Effect of enzyme load

Effect of enzyme load on esterification reactions was evaluated under the conditions of 1:1 molar ratio of glycerol to fatty acids, 2% initial water content and 25 °C. It can be observed from Fig. 3 that higher DAG contents were achieved with the 120 U/g and 150 U/g enzyme loadings, and fast initial reaction rates were

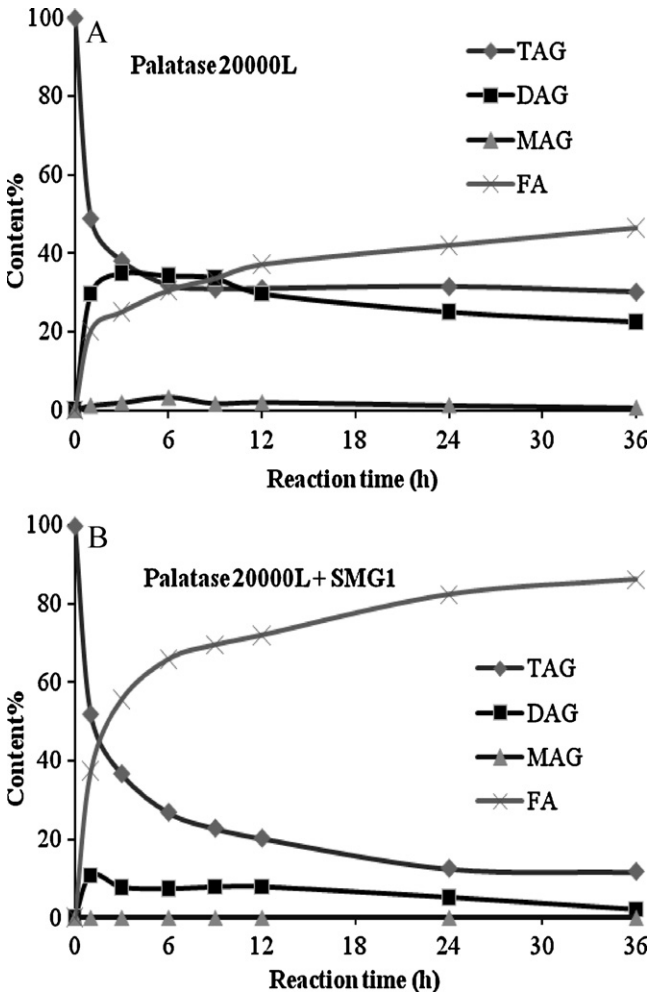

Fig. 2. (A) Palatase 20000L catalyzed hydrolysis of soybean oil. (B) Hydrolysis of soybean oil catalyzed by the combined enzymes of Palatase 20000L and SMG1. Reaction conditions were as follows: soybean oil, 50 g; distilled water, 5 g; temperature, 25 °C; reaction time, 36 h; enzyme loading of each lipase, 100 U/g (with respect to the soybean oil).

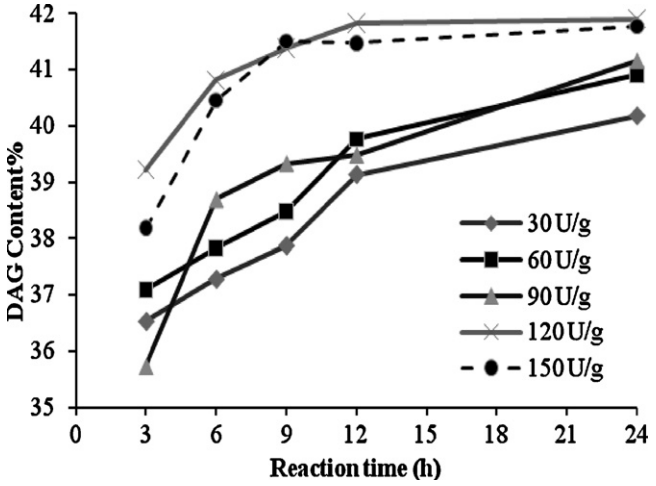

Fig. 3. Effect of enzyme load on the esterification. Reaction conditions were as follows: substrate molar ratio (glycerol:fatty acids), 1:1: temperature, 25 °C; reaction time, 24 h; water content, 2 wt%.

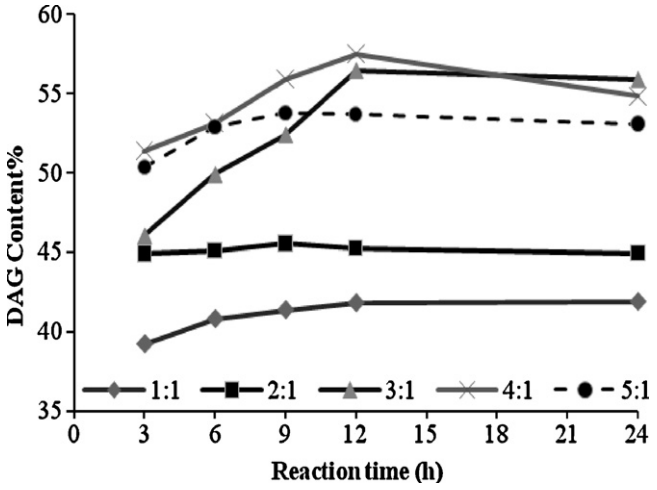

Fig. 4. Effect of substrate molar ratio of glycerol to fatty acids on the esterification. Reaction conditions were as follows: enzyme loading, 120 U/g (with respect to total mixture); temperature, 25 °C; reaction time, 24 h; water content, 2 wt%.

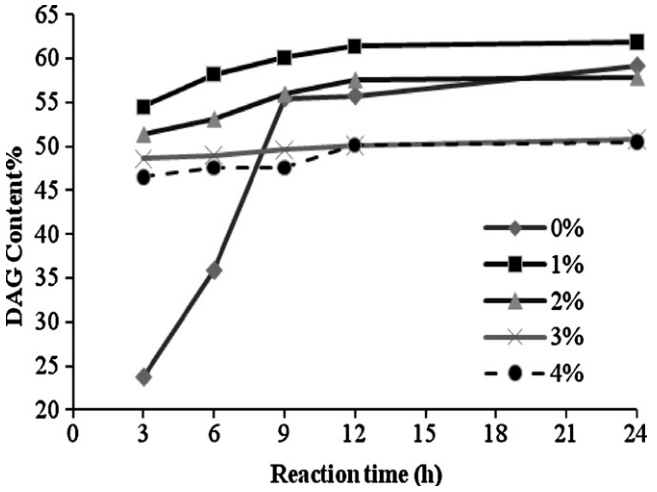

Fig. 5. Effect of initial water content on esterification. Reaction conditions were as follows: substrate molar ratio (glycerol:fatty acids), 4:1; enzyme loading, 120 U/g (with respect to total mixture); temperature, 25 °C; reaction time, 24 h.

also found under these conditions. In general, a larger amount of lipase load arouses esterification to achieve equilibrium faster. As the results shown in Fig. 3, an increase in enzyme concentration from 30 U/g to 150 U/g led to a considerable enhancement in the production of DAG, especially at the beginning stage. There was no improvement found when the enzyme loading was increased from 120 U/g (41.90% of DAG content) to 150 U/g (41.77% of DAG content) probably because part of lipase molecule became inaccessible to the substrate. This also met the agreement with previous reports that high enzyme concentrations might lead to the formation of aggregates, thus making the enzyme-active sites unavailable to the substrates [15,16]. Therefore, the enzyme load of 120 U of every gram of reaction substrates was used for the subsequent experiments. And after 12 h the 1,3-DAG and 1,2-DAG content in the reaction product was 30.26% and 11.56%, respectively.

3.4.1. *Effect of the molar ratio of glycerol to fatty acids*

The amount of fatty acids, 86.12% (w/w) in hydrolysates of soybean oil, was calculated to prepare the esterification substrates at different molar ratios. The effect of molar ratio of glycerol to fatty acids on the esterification was accomplished at 25 °C with the enzyme and initial water at fixed concentrations of 120 U/g and 2% (w/w with respect to total reactants). It was found from the experimental results (Fig. 4) that excess of glycerol would accelerate the reaction rate. The content of DAG in the reaction products was increased dramatically after 3 h of esterification when the proportion of glycerol was increased from 1:1 to 4:1. The maximum DAG content (57.51%, w/w in the product mixture) was obtained after 12 h with the molar ratio of glycerol to fatty acid 4:1. The initial reaction rate and DAG content were decreased when the molar ratio of glycerol to fatty acids increased further from 4:1 to 5:1. It might be because the concentration of FFA was diluted by the excess glycerol, thus the reduction of FFA was declined. The 1,3-DAG and 1,2-DAG content was 38.60% and 18.91% after 12 h of reaction time at the molar ratio of 4:1. The molar ratio of glycerol to fatty acids was selected at 4:1 for the subsequent experiments.

3.5. *Effect of water content*

Lipase-catalyzed reactions are reversible and governed by the water content of the reaction mixture. An appropriate amount of water is needed to maintain enzyme activity. However, the degree of synthesis would be reduced if the water content is too high in the reaction mixtures. In the esterification reactions, the role of the water content is more important because water is one of the reaction products. Therefore, the effect of initial water content on the DAG production and on the composition of the products mixture was studied.

Hydrolysates from soybean oil (contained 86.12% fatty acids) were esterified at 25 °C with addition of different amounts of water (Fig. 5). The degree of the DAG synthesis was very low in the first 3 h of esterification when there was no additional water added, but it was increased dramatically between the 3 h and 9 h. This is because that the enzyme could be activated fully even by a small amount of water generated during esterification. In another words, the enzyme could be activated fully even by a small amount of water generated during esterfication [17]. Addition of 1% (w/w, with respect to total reactants) of water accelerated the DAG synthesis from 23.7% of DAG content to 54.8% after 3 h of esterification. Addition of 2% and more water had no improved positive effect on the DAG synthesis (Fig. 6). The results showed that lipase SMG1 requires a small amount of water for full expression of its activity and 1% of initial water of the reaction substrate in weight would be the most suitable amount for addition. The 1,3-DAG and 1,2-DAG content was 42.46% and 18.91% after 12 h of reaction time at the molar ratio of 4:1 with 1% of initial water.

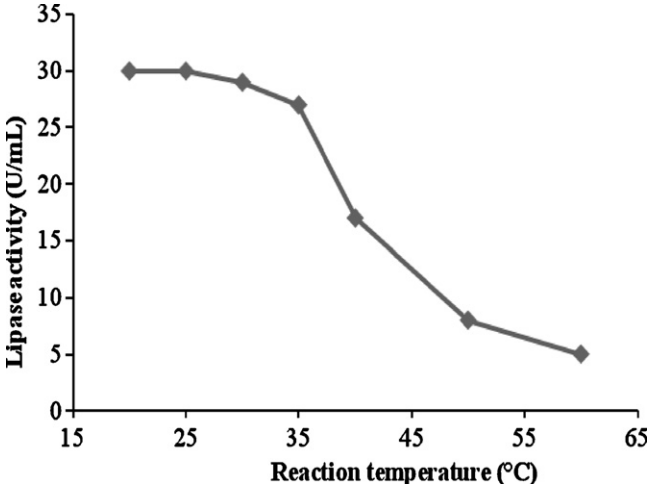

Fig. 6. Effect of temperature on the activity of lipase SMG1.

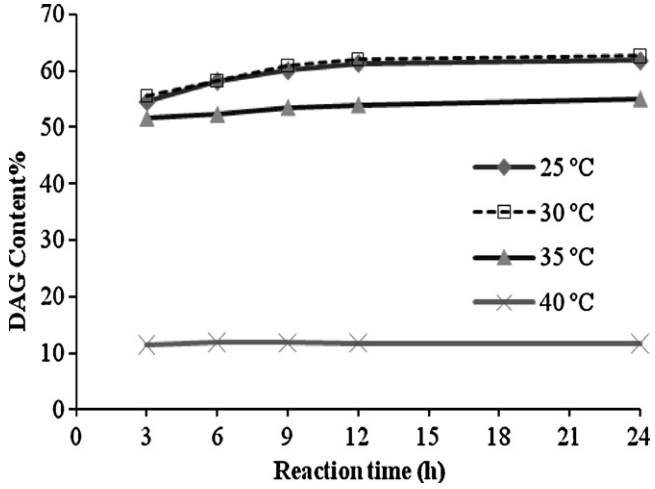

Fig. 7. Influence of the temperature on the DAG content in the reaction mixture. Reaction conditions were as follows: substrate molar ratio (glycerol:fatty acids), 4:1; enzyme loading, 120 U/g; reaction time, 24 h; water content, 1 wt%.

3.6. Effect of temperature

The effect of temperature on the activity of lipase SMG1 was firstly investigated as the results shown in Fig. 6. The recombinant lipase SMG1 had relatively high activities between 20 °C and 30 °C. The lipase activity was reduced dramatically when the temperature further increased.

To evaluate the effect of temperature (25, 30, 35 and 40 °C) on the produced DAG content, the other reaction conditions were fixed at a molar ratio of glycerol to fatty acids 4:1, enzyme loading 120 U/g, and 1% of initial water content. Almost the same amount of DAG was synthesized after 12 h and 24 h under the reaction temperature of 25 °C and 30 °C as shown in Fig. 7. The content of DAG decreased from 62.03% and 62.65% to 53.94% and 55.01% after 12 h and 24 h, respectively when the temperature was increased from 30 °C to 35 °C. The lowest DAG production was observed at 40 °C as the lipase was deactivated because of protein denaturation by high temperature. The optimum temperature of lipase SMG1 was 25 °C and 30 °C, and it was inactivated swiftly when the temperature was 40 °C in this reaction system. The result was consistent with the effect of temperature on the lipase activity as discussed above.

In the publication on the DAG production by esterification, lipase RM IM and Novozyme 435 are successfully used enzymes. The DAG content in the reaction products could be up to 51–84% by esterification of fatty acids and glycerol [9,15,18]. The experimental results in this study (62.03% DAG content in the products) indicated that lipase SMG1 is another potential enzyme for DAG production.

4. Conclusion

Lipase SMG1 showed specific activity on hydrolysis or synthesis of partial acylglycerols, but non-specific selectivity on the production of 1,3-DAG and 1,2-DAG was observed. The optimum conditions for esterification of glycerol and soybean oil hydrolysates using lipase SMG1 were: lipase SMG1 at a concentration of 120 U/g (U/w with respect to total reactants), fatty acids/glycerol molar ratio of 1:4, initial water content 1% (w/w, with respect to total reactants) and 30 °C for 12 h. There was 62.03% DAG (80.5% in acylglycrides) in the esterification products after 12 h under those optimum conditions, of which 1,3-DAG and 1,2-DAG was 42.25% and 19.78%, respectively.

In conclusion, lipase SMG1 is a prospective enzyme which will be used in the oil and fats industry, and it is highly significant to develop the working on molecular modification of lipase SMG1 to screen the mutants with high activity, and then much more detailed research on its properties as biocatalyst.

Acknowledgements

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