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Solvent-free production of 1,3-diglyceride of CLA: Strategy consideration and protocol design

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Abstract

Enzymatic production of a homogeneous 1,3-diglyceride of polyunsaturated fatty acids (PUFAs) was carried out using Novozym 435 as biocatalyst and conjugated linoleic acid (CLA) as a model fatty acid. Three different operation modes, namely, magnetic stirring under vacuum, vacuum-driven N_2 bubbling and incubation with molecular sieves, were examined to find an efficient protocol for the enzymatic production. Studies on the effects of mass transfer showed that the occurrence of mass transfer limitation was strongly dependent on the operational modes. Vacuum-driven N_2 bubbling proved to be capable of eliminating mass transfer resistance, creating effective interaction for a multiple-phase reaction system and yielding an efficient water removal and a faster reaction rate. Hence, vacuum-driven N_2 stirring was considered as the best choice among the tested strategies for the production of pure 1,3-diglyceride of PUFAs with industrial interests, because it gave a higher yield of the desired product, higher productivity and lower impurity content due to its suppression of the acylmigration of 1,3-diglyceride to 1,2-diglyceride. The yield of 92–96% 1,3-dCLG could be obtained when 5 mmol of glycerol were incubated with 10–12 mmol CLA for about 3 h at 45–55 °C and a pressure less than 10 mbar, with enzyme loading of 40–70 g l^{-1}. Among the operational parameters, temperature and reaction time were found to have profound effects on the acylmigration and yield of 1,3-diglyceride. Moreover, the enzyme showed excellent operational stability in this protocol under the optimized conditions (little activity loss of enzyme was observed after 10 consecutive batch reactions), indicating the potential of this technology for industrial application.

Keywords: Conjugated linoleic acid; Enzymatic catalysis; *Candida antarctica* lipase B; Acylmigration; 1,3-Diglyceride

1. Introduction

Diglyceride (DG) finds a wide variety of use in foods, cosmetics and pharmaceuticals, depending on its purity. Mixtures of mono- and diglycerides are often used as food emulsifiers (Bornscheuer, Stamatis, Xenakis, Yamane, & Kolisis, 1994; Kristensen, Xu, & Mu, 2005; Plou, Barandiarán, Calvo, Ballesteros, & Pastor, 1996). As identified by recent studies of nutritional properties and dietary effects, diglycerides, of which 1,3-diglyceride is a major component, in contrast to triglyceride, is capable of reducing serum triglyceride concentration, resulting in a decrease of body weight and visceral fat mass (Maki et al., 2002; Meng, Zou, Shi, Duan, & Mao, 2004). A type of cooking oil of DG, consisting of about 70% 1,3- and 30% 1,2-isomers, claimed to be clinically beneficial, is now commercially available in Japan and the USA (ADM Kao LLC, www.enovaoil.com). Pure diglycerides also have great potential use as building blocks for synthesis of lipid derivatives (Fureby, Tian, Adlercreutz, & Mattiasson, 1997; Haftendorn & Ulbrich-Hofmann, 1995), or can be utilized as starting materials for drug formulation, such as 1,3-diglyceride conjugated chlorambucil for treatment of lymphoma (El Kihel, Bourass, Richomme, Petit, & Letourneux, 1996; Garzon-Aburbeh, Poupaert, Claesen, Dumont, & Atassi, 1983). However, large scale preparation of

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1,3-diglyceride, with high purity, still remains a problem with traditional chemical methods, especially for those with polyunsaturated fatty acids due to harsh reaction conditions (Sonntag, 1982; Watanabe et al., 2003). Enzymatic approach provides a promising alternative due to its mild performance conditions, the regioselectivity of lipases and less environmental impact (Berger, Laumen, & Schneider, 1992; Rosu, Yasui, Iwasaki, & Yamane, 1999).

Among those enzymatic approaches for diglyceride preparation, alcoholysis of triglycrides was proven to be efficient for the preparation of 1,2-diglycerides (Fureby et al., 1997). To obtain pure 1,3-diglyceride, many efforts have been made, which include in situ removal of diglyceride during the glycerolysis of triglyceride by temperature-programmed cooling crystallization (Yamane, Kang, Kawahara, & Koizumi, 1994), creating an artificial interface between glycerol and fatty acids (Waldinger & Schneider, 1996) and applying solvent engineering (Liao, Tsai, Chang, & Shieh, 2003). To resolve the compatibility of glycerol and fatty acid, Berger et al. (1992) and Waldinger and Schneider (1996) absorbed glycerol onto silica before reaction, to promote the interaction of glycerol and fatty acids and allow a high purity synthesis of 1,3-DG. Herein we present an alternative for high-yield and high-purity production of 1,3-diglyceride, aiming at developing a facile and efficient reaction system.

The attempt of this work is to establish a universal protocol for enzymatic production of high-purity 1,3-diglyceride containing polyunsaturated fatty acids from free fatty acid and glycerol. To this end, conjugated linoleic acid (CLA), a structurally representative polyunsaturated fatty acid with conjugated bonds, which is sensitive to oxygenation and polymerization (Minemoto et al., 2003; Timm-Heinrich, Skall Nielsen, Xu, & Jacobsen, 2004), is selected as a model fatty acid to demonstrate the designed protocol in this work. A thorough consideration and evaluation of the practical feasibility and industrial potential of the protocol, mass transfer and acylmigration concerns is given with comparison to other approaches. Effects of important parameters on the incorporation rate, the yield of 1,3-diglyceride and acylmigration were examined. Repeated use of Novozym 435 was also investigated, to test its durability under the optimized conditions and to envisage the industrial perspective of this method.

2. Materials and methods

2.1. Materials

Safflower oil for linoleic acid enrichment was from a local source and with a content of linoleic acid of 79.8% by GC analysis. Glycerol (purity 99.0%) was purchased from the local market and dried by activated 3Å molecular sieves before use. $BF_3 \cdot O(C_2H_5)_2$ was from J.T. Baker Chemical Co. (Germany). Conjugated linoleic acid methyl ester, used as a standard, was from Sigma Chemical Co. (St. Louis, MO). Methanol, acetonitrile and methylene chloride were of HPLC grade from Fisher Scientific (Fair Lawn, NJ). All other regents were of analytical grade and purified before use. Molecular sieves (3 Å) were from Dalian Institute of Chemical Physics (Dalian, China) and activated before use. Lipozyme RM IM (from *Rhizomucor miehei*, RML), Lipozyme TL IM (from *Thermomyces lanuginose*, TLL) and Novozym 435 (from *Candida antarctica* B, CAL) were provided by Novozymes A/S (Bagsvaerd, Denmark).

2.2. Preparation and analysis of CLA

Linoleic acid was enriched from safflower oil fatty acids according to the procedure reported earlier (Guo, Zhang, & Sun, 2003). GC analysis of linoleic acid revealed that the product was 99.01% pure. The alkali isomerization of linoleic acid was performed using the procedure developed by Chen, Lu, and Sih (1999). Conjugated linoleic acid was methylated by BF_3 in methanol at room temperature for 30 min, as described by Werner, Luedecke, and Schultz (1992). A gas chromatograph Agilent 6890N (Agilent Technologies, DE) equipped with a splitless/split injector, a flame-ionization detector and a DB-23 capillary column (0.25 μm film thickness, 60 m length, 0.25 mm I.D.), was used to detect fatty acid methylated ester. Both the injector and detector were set at 250 °C, and nitrogen was used as the carrier gas. The oven temperature was programmed from 50 to 200 °C at 20 °C min^{-1} and held at 200 °C for 15 min, and then to 250 °C at 20 °C min^{-1}, finally held at 250 °C for 15 min. GC analysis of the isomerized linoleic acid product showed that the total amount of conjugated linoleic acids was 96.9%, of which 9c, 11t- and 10t, 12c-18:2 comprised 48.6% and 37.8%, respectively.

2.3. Determination and evaluation of reaction protocols

As depicted in previous work, we have developed a vacuum-driven N_2 bubbling protocol for efficient preparation of 1,3-DG at high yield, using Lipozyme RM IM as a model enzyme, and Novozym 435 was screened as an optimal lipase from commercial sources in the designed protocol (Guo & Sun, 2004). In this study the esterification of CLA with glycerol, employing Novozym 435 as biocatalyst (typically 0.46 g glycerol and 2.8 g CLA with 0.2 g enzyme) in the vacuum-driven N_2 bubbling protocol was conducted and compared with the reaction performances by vacuum magnetic stirring and incubation with molecular sieves regarding their effects on acylmigration and yield of 1,3-diglyceride. The reaction was initiated by the addition of immobilized lipase and a 20 μl aliquot was withdrawn at different time intervals for HPLC analysis. All experiments were conducted in duplicate.

The particle size distribution of Novozym 435 was measured by a Masterizer 2000 particle size analyzer (Malvern Instrument, UK). The recovered Novozym 435 from the reaction mixture was washed with 70% ethanol and

measured to give a mean diameter of 0.572 mm. This value was used for mass transfer calculation.

2.4. HPLC analysis

HPLC analysis was performed according to a modified method described by Marcato and Cecchin (1996). The HPLC system was composed of an Agilent 1100 series solvent delivery system (Agilent Technologies, DE) equipped with an evaporative light-scattering detector (ELSD) (Alltech AsSociates Inc., IL), a Model 100 Column Heater (CBL Photoelectron Technology, Tianjin, China) and the Agilent Chemical Station (Agilent Technologies, DE). A Waters symmetry C_{18} reverse phase column (Waters Corporation, Milford, MA) (4.6 mm i.d. × 150 mm, 5 μm particle size) was used for the analysis. The chromatographic conditions were set as follows: flow-rate 1.0 ml min^{-1}; column temperature, 40 °C; detector temperature, 85 °C; carrier air flow-rate 2.0 ml min^{-1} for ELSD.

Based on the HPLC-ELSD analysis, area percentage on a glycerol-free basis was used as mass for the calculation of the conversion of fatty acid and yield of 1,3-diglyceride.

2.5. Optimization of individual parameters

Reaction time, temperature, substrate mole ratio, enzyme load and vacuum degree are considered as important factors for the solvent-free enzymatic synthesis. Of the five variables, each one was optimized when keeping the other four at constant values, and the changes of the composition of glycerides, conversion of CLA and acylmigration with the variable were examined. Acylmigration (Am) is defined as the esterified fatty acids incorporated into the 2-position of glycerol and calculated with the following equation:

$$\text{Acylmigration } \% = \frac{\frac{m_{d12}}{M_{d12}} + \frac{m_t}{M_t}}{\left(\frac{m_{CLA}}{M_{CLA}} + \frac{m_{m1}}{M_{m1}} + 2\cdot\frac{m_{d12}}{M_{d12}} + 2\cdot\frac{m_{d13}}{M_{d13}} + 3\cdot\frac{m_t}{M_t}\right)} \times 100 \quad (1)$$

In Eq. (1), m_{m1}, m_{d12}, m_{d13}, m_t and m_{CLA} denote the mass fractions of 1-mono- (1-MG), 1,2-di- (1,2-DG), 1,3-di- (1,3-DG), tri- conjugated linoleoyl glycerol (TG) and CLA in the reaction mixture, respectively, and M represents the molecular mass of corresponding species.

2.6. Reusability of Novozym 435 under the designed protocol

To test the thermostability of Novozym 435 in the vacuum-driven N_2 bubbling system and evaluate the commercial interest of this approach, a model reaction was performed with 5 mmol glycerol and 10 mmol CLA and 55.4 g l^{-1} Novozym 435. The reaction was allowed to progress at 45°C and 4 mbar for 3 h for each cycle. The enzyme remaining in the reactor after the removal of product by vacuum filtration was again employed for the next batch under otherwise identical conditions.

The resulting product was subjected to a preparative 20 × 20 cm TLC plate to collect 1,3-diglyceride of CLA (Cossignani, Damiani, Simonetti, & Mañes, 2004). The fatty acid composition of the product was thus determined by the GC analysis of its methylated product and compared with the composition of the starting fatty acid profiles.

3. Results and discussion

3.1. Strategy consideration and protocol design

The production of partial glycerides constitutes an important aspect of lipid processing. Enzymatic approaches of industrial interest are glycerolysis of triglycerides and direct esterification from fatty acid and glycerol (Bornscheuer, 1995; Watanabe et al., 2004). Glycerolysis of natural oils and fats, giving products with different fatty acid profiles, is a currently used method in the food industry. To prepare pure partial glycerides containing a single fatty acid profile, selective esterification of fatty acid with glycerol is a feasible alternative due to the natural unavailability of homogeneous triglycerides. Theoretically, a 1,3-diglyceride could be prepared by direct esterification of glycerol with fatty acids catalyzed by 1,3-specific lipase. In fact, to some extent the reaction and the composition of final product are governed by both reaction rate and reaction equilibrium. Therefore a judicious reaction design encompasses the essence of the art of processing.

Since the 1,3-diglyceride is a product midway between mono- and triglyceride, a high accumulation of 1,3-diglyceride should be based on the idea of a fast further esterification of 1-monoglyceride and a retarded formation of triglyceride. The former largely depends on a higher esterification-catalytic activity from the intrinsic nature of the enzyme, efficient interaction between substrates and enzyme, and a rapid dehydration leading to the shift of equilibrium, while the latter results from the acylmigration of 1,3-diglyceride from 1(3)- to 2-position of glycerol backbone and the further acylation of the resulting product. *C. antarctica* lipase has proved to be one of the proper candidates by our primary screening (Guo & Sun, 2004). With the same amount of enzyme loading, the incorporation rate of fatty acid for the CAL catalyzed reaction is about 3-fold of that for RML, and the maximum yield of 1,3-diglyceride for RML is only 64% instead of over 90% for CAL catalyzed reaction (data not shown in detail). Little reaction in TLL catalyzed esterification could be ascribed to the aggregation of enzyme granules, due to adhering to glycerol, which established a bigger obstacle for the transport of hydrophobic fatty acids through the glycerol layer surrounding the enzyme. Therefore Novozym 435 was used in following studies.

It is known that acylmigration is a thermodynamically controlled non-enzymatic reaction, so acylmigration could not be avoided but minimized. To create an efficient reaction system, we have designed a vacuum-driven

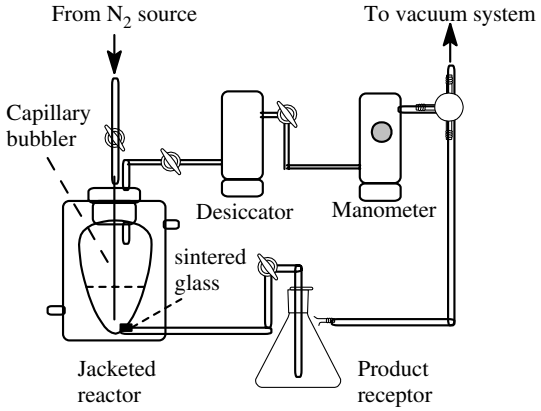

Fig. 1. Schematic diagram of vacuum-driven N_2 bubbling reactor.

N_2-bubbling protocol, as shown in Fig. 1, in which the core part is a pear-shaped flask equipped with N_2 introduction through a capillary by applying vacuum. This idea arises from the fact that vacuum distillation with a little introduction of air can elicit boiling and vigorous convection. On the other hand, with this design, water could be more efficiently removed by applied high vacuum and pressure of nitrogen.

The distinct advantages of the protocol include: the gravitational accumulation of glycerol and its adherence to enzyme are avoided due to the introduction of N_2 to the reactor bottom to assure a favourable dispersion of glycerol in the fatty acid phase, which has proven to be the main reason to retardant of the reaction (Watanabe et al., 2003; Castillo, Dossat, Alain, Condoret, & Combes, 1997). This constrained convection could also promote the effective interaction of hydrophilic glycerol, hydrophobic fatty acid and bead-bound lipase. Another virtue is that this design has the capacity to remove the generated water more efficiently than the vacuum without air introduction because of the pressure fraction, which makes the esterification work well at lower temperature. Because the introduction of N_2 is through a capillary, the nitrogen consumption is very little, with less cost increase, in view of industrial application. The introduction of nitrogen also provides a preventive inert environment for thermo- or oxygen-sensitive compounds and enzyme. Compared to other mixing modes, the mild stirring in this design will diminish the damage to enzyme resulting from the strong shear of mechanical stirring and help to prolong the half-life of enzyme, which also might help to reduce acylmigration (Bloomer, Adlercreutz, & Mattiasson, 1991).

The above claims are supported by from the results in Table 1. Compared to the other two protocols, incubation with molecular sieves gave only 80% conversion of CLA after 10 h and achieved a maximum yield of 1,3-diglyceride of 44% at 16 h. This result is identical to previous work (Arcos, Otero, & Hill, 1998), and is believed to result from mass transfer limitation and inefficiency of water removal due to the high viscosity of the reaction mixture. Obviously, this operation is of less industrial interests because of its slow reaction rate and the difficulty and labour of molecular sieve recovery and reuse. Both of the vacuum approaches achieved higher fatty acid incorporation rate in a short reaction time; however, the vacuum N_2-bubbling protocol gave the highest yield of 1,3-diglyceride (91.6%) and lowest acylmigration (0.81%) among the three protocols tested; the HPLC chromatogram of the glyceride composition of reaction mixture at 3 h is presented in Fig. 2. The slight retarding of the vacuum magnetic stirring to achieve the maximal yield of 1,3-diglyceride might result in mass transfer limitation, as discussed in the following section. We suggest that, for the vacuum magnetic stirring protocol, vigorous agitation might be the main reason leading to the acceleration of acylmigration, yielding more triglyceride. The difference of acylmigration and yield of 1,3-diglyceride between the two methods became more significant after

Table 1
Comparison of incorporation of CLA (Ip), acylmigration (Am) and yield of 1,3-DG (Yd) with different operation protocols[a]

Time (h)	Magnetic stirring under vacuum			Vacuum-driven N_2 bubbling			Incubation with molecular sieves		
	Cn	Am	Yd	Cn	Am	Yd	Cn	Am	Yd
1/3	0.00	0.00	0.00	0.51	0.00	0.56	1.07	0.00	0.00
2/3	1.24	0.92	0.95	0.53	0.00	0.58	3.28	0.00	0.24
1	47.3	1.31	30.0	10.1	0.00	7.21	2.54	0.00	1.12
2	75.3	1.11	74.8	98.5	0.00	76.1	25.3	0.74	1.21
3	97.7	1.91	86.0	***98.6***	***0.81***	***91.62***	39.6	1.84	3.52
4	***99.5***	***2.79***	***89.1***	98.6	1.86	87.6	49.8	1.56	3.79
6	100	4.77	75.4	100	3.25	80.5	67.4	3.18	12.6
8	100	6.46	68.5	100	4.24	79.9	74.2	2.62	23.3
12	100	8.54	64.9	100	5.43	75.6	80	4.47	36.6
16	100	10.6	57.5	100	5.97	68.6	***80***	***6.66***	***43.9***

[a] The esterification of 0.138 mol l^{-1} glycerol and 0.277 mol l^{-1} CLA at 50 °C catalyzed by 55.4 g l^{-1} Novozym 435 reaction was performed with three operational modes, respectively, that is, incubating with 221 g l^{-1} molecular sieves and shaking at 240 rpm, magnetic stirring at 400 rpm under vacuum (4 mbar) and vacuum-driven N_2 stirring (4 mbar). The data of each protocol to obtain its maximal yield of 1,3-diglyceride are marked in bold and italic. Abbreviations: Cn, conversion of CLA (mass%); Am, acylmigration (mol%) and Yd, yield of 1,3-diglyceride (mass%).

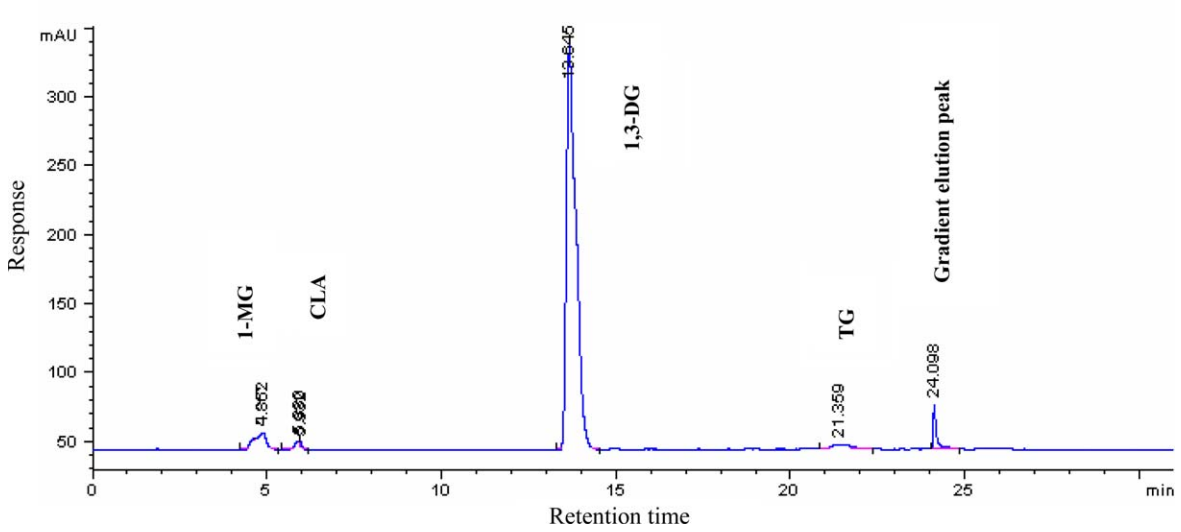

Fig. 2. HPLC chromatogram of the reaction mixture catalyzed by Novozym 435 in 3 h. Reaction conditions were the same as in Table 1.

3 h, i.e., the yield of 1,3-diglyceride for the vacuum magnetic stirring showed a faster decline (Table 1).

3.2. Effect of mass transfer

Enzymatic esterification from fatty acid and glycerol is a three-phase reaction consisting of hydrophobic and hydrophilic phase, as well as solid-bound enzyme. To better understand the reaction behaviour of this complicated system, the effect of mass transfer should be properly assessed. It could be speculated that enzymatic reaction should occur at the interface of oil phase and glycerol phase. Mass transfer between the two phases is a function of the interfacial area, depending on the mixing effect and the two phase volume ratio. In the case of the vacuum-driven N_2-bubbling protocol, mixing effect depends on vacuum pressure and introducing N_2. The contribution of a proper amount of N_2 to the increase of reaction rate has been observed in this work (data not shown), but is not easily quantified. Therefore, to operate the system in the absence of external mass resistance, the introduction of N_2 has been carefully adjusted and optimized to assure a good mixing effect in the further experiments of in this study. If no mass transfer limitation occurs, the dependency of reaction rate upon the enzyme concentration should be linear. This has been verified by conducting the reaction at different enzyme loadings (Fig. 3). Fig. 3 shows that, from 7 to 42 g l^{-1}, the increase of enzyme loading results in a linear increase of the conversion of CLA and yield of 1,3-diglyceride. The evident decline of the yield of 1,3-diglyceride at higher enzyme concentration might be primarily ascribed to substantial increase of acylmigration, as indicated in Fig. 3. The results suggest that the external transfer limitation could be eliminated by optimizing operation, and also indicate an optimized enzyme dosage range, of 40–70 g l^{-1}.

A linear relationship between the reaction rate and enzyme concentration is in consonance with a kinetically

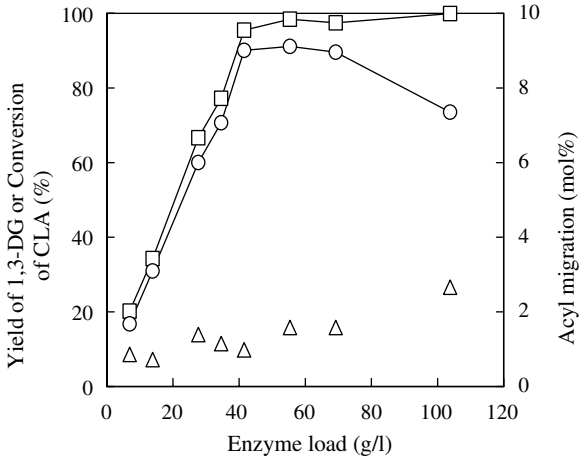

Fig. 3. Effect of enzyme loading on the conversion of CLA (□), yield of 1,3-DG (○), and acyl migration (△). The reaction was conducted at 50 °C and 10.7 mbar for 3 h with the concentration of CLA 0.277 mol l^{-1} and glycerol 0.138 mol l^{-1}.

controlled enzymatic reaction. However, this linearity alone does not rule out the possibility of internal mass transfer limitations on the rate, especially, in the current case, glycerol and fatty acid having poor compatibility, lipase residing inside the macroporous beads (Novozym 435) and glycerol having high viscosity. There exist two extreme issues for this system: one is that fatty acid first contacts the immobilized enzyme and fill the micropores of the carrier and glycerol diffuses in the fatty acid phase; the other is that the fatty acid follows after glycerol and diffuses through glycerol. In each case, to evaluate the effect of mass transfer we could compare its time constant for reaction (t_r) and diffusion (t_d) defined as follows (Yadav & Devi, 2002):

$$t_r = c_0/r_{c0} \quad (2)$$

$$t_d = D/(k_{SL})^2 \quad (3)$$

where c_0 is the substrate concentration in bulk organic phase (mol l^{-1}), r_{c0} the initial reaction rate, D the diffusivity of substrate in solvent and k_{SL} the solute–liquid mass transfer coefficient in solvent (cm s^{-1}). The mass transfer coefficient can be estimated by the Sherwood number: $k_{SL} = 2\ D/d$, where d is the diameter of the support particles (Perry & Green, 1984). Wilke-Chang correlation (Perry & Green, 1984) (Eq. (4)) (an empirical modification form of the Einstein–Stokes equation) was used to estimate the diffusion of glycerol in CLA or CLA in glycerol.

$$D_{AB} = 7.4 \times 10^{-8} \frac{(\phi M_B)^{1/2} T}{\eta_B V_A^{0.6}} \quad (4)$$

where D_{AB} is the diffusion coefficient of solute A in solvent B (cm^2/s), M_B molecular weight of solvent B, T temperature (K), η_B viscosity of solvent B, V_A the molar volume of solute A at its normal boiling temperature ($cm^3/(g\ mol)$) and ϕ association factor of solvent B, (dimensionless). Considering the high molecular association occurring in glycerol and the little association in CLA, as well as unavailability of their ϕ values, 2.60, (ϕ of water) and 1.0, (ϕ of hydrocarbons), are used in the calculation, respectively.

It was observed that, at 50 °C and the concentration of glycerol 0.138 and CLA 0.277 mol l^{-1}, the initial reaction rate was 3.79 mol l^{-1} s^{-1} (by conversion of CLA). So the reaction time should be 7.31×10^3 s according to Eq. (2). The diffusion coefficient of glycerol in CLA calculated by Eq. (4) is 2.34×10^{-6} cm^2 s^{-1}, the diffusion time could be accordingly estimated to be 3.5×10^2 s by Eq. (3). Obviously $t_r >> t_d$, indicating that the reaction is not influenced by mass transfer if Novozym 435 is first mixed with the fatty acid. For the diffusion of CLA in glycerol at 50 °C, the calculated value of t_d is 8.37×10^3 s, and at 25 °C it is 7.2×10^4 s. Comparable value of the reaction and diffusion time at 50 °C showed that the effect of diffusion should be seriously taken into account when Novozym 435 first contacts glycerol. A lower temperature (e.g., 25 °C), the reaction is principally dependent on the diffusion rate since the diffusion time is markedly longer than the reaction time.

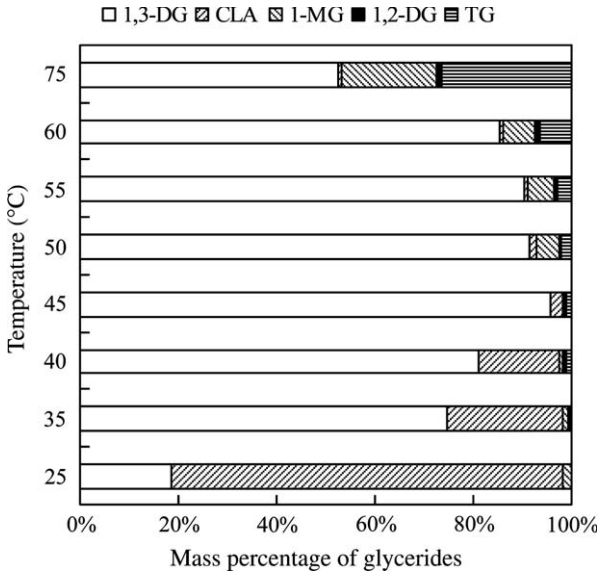

Fig. 4. Temperature dependency of the esterification of CLA with glycerol. The reaction was conducted at the concentration of CLA 0.277 mol l^{-1} and glycerol 0.138 mol l^{-1} for 3 h with 10.7 mbar vacuum and enzyme loading 55.36 g l^{-1}.

The differing effects of mass transfer in the two cases are actually related to the sample addition order and operation mode, as mentioned by Watanabe et al. (2003). Adopting magnetic stirring makes the immobilized lipase liable to aggregating with glycerol at the bottom of the reactor. To solve this problem, Watanabe et al. (2003) suggested that the immobilized enzyme should be first mixed with fatty acid, following the addition of glycerol with vigorous agitation. However, for the protocol designed in this work, aggregation of Novozym 435 can be essentially eliminated, because the introduction of N_2 to the bottom and its agitation make it impossible for the bulky accumulation of glycerol at the bottom of the reactor. In practice, the immobilized lipase might contact both fatty acid and glycerol and the esterification should be controlled diffusionally and kinetically. Therefore, the significant increase of reaction rate, indicated in Fig. 4, is suggested to result from the acceleration of diffusion and intrinsic enhancement from enzyme with the increasing temperature. On the other hand, the increasing solubility of glycerol in fatty acid is also believed to contribute to the increase of reaction rate (Watanabe et al., 2003). The negative effect of enhanced temperature is the substantial occurrence of acylmigration and the increase of triglyceride content (Fig. 4), which agrees with the report that temperature plays crucial role in producing acylmigration (Xu et al., 1998). Thus, according to the results in Fig. 4, the maximum yield of 1,3-DG could be expected to be achieved at a temperature between 45 and 55 °C.

3.3. Parameter optimization

Besides temperature and enzyme loading, other important factors to influence the conversion of CLA and yield of 1,3-DG are reaction time, substrate ratio and system vacuum (Fig. 5). Inspection of the profile change of glycerides reveals that the esterifying rate of fatty acid increases very rapidly to nearly total conversion in the first 3 h (Fig. 5A), and then the yield of desired product 1,3-DG steadily decline with the reaction control by dynamic equilibrium being changed to thermodynamic control, resulting in the increase of acylmigration and triglyceride. In agreement with previous reports (Rosu et al., 1999), the content of 1,2-DG remains low throughout the reaction and no 2-monoglyceride was detected. The main undesirable product is 1-MG. The results suggested that, to achieve a good yield of the product (an intermediate product of a multiple-step reaction), choice of a proper stop time is very important.

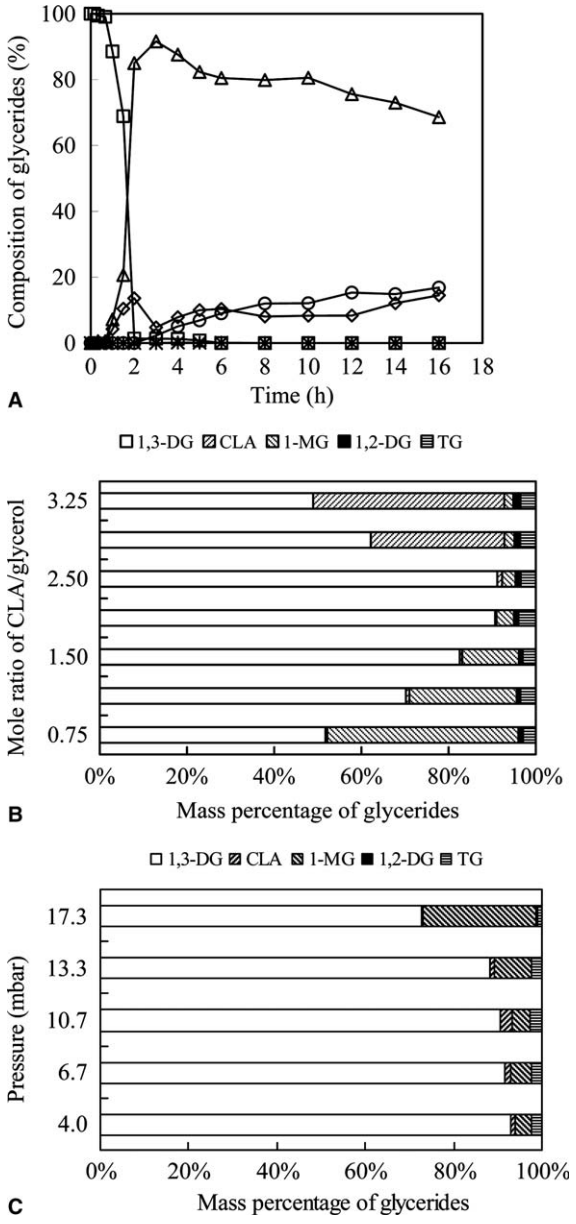

Fig. 5. Effects of reaction time (A), substrate ratio (B) and vacuum pressure (C). Reaction conditions: A, 50 °C, 10.7 mbar, 0.277 mol l^{-1} of CLA, 0.138 mol l^{-1} of glycerol and 55.36 g l^{-1} of Novozym 435, the relative content (%) of CLA (□), 1,3-DG (△), 1-MG (◇), TG (○) and 1,2-DG (∗); B, the reaction was carried out for 3 h with a fixed glycerol concentration of 0.138 mol l^{-1} and the other conditions were the same as in A; C, the reaction was conducted for 3 h with the other conditions identical to those in (A).

Fig. 5B shows that higher yield of 1,3-DG could be attained in the range of mole ratios from 2:1 to 2.5:1. At lower mole ratio of CLA/glycerol, a considerable percentage of 1-MG was not further esterified due to the deficiency of free CLA. However, unesterified fatty acid was present in the reaction mixture at the much higher mole ratio. In the range 2:1–2.5:1, the yield of 1,3-DG was more than 90%, and the contents of other impurities was all less than 4%, indicating that this range could be a recommended optimal condition.

Table 2
Reusability of Novozym 435 in repeated batch reaction with designed operational protocol

Recycle time	1	2	3	4	5	6	7	8	9	10
Conversion (%)	98.8	99.3	98.7	98	98.6	98	97.8	97.5	97.8	97.3
Yield of 1,3-DG (%)	95.8	96.1	95.5	94.2	95.0	94.4	94.2	93.3	93.2	92.8

In the tested range of system pressure, high conversions of CLA were obtained (Fig. 5C). However, the yield of 1,3-DG decreased with the system pressure employed, and the maximal yield 93.6% was achieved at 4 mbar (3 mmHg). Inefficient mixing of the reaction mixture at higher pressure (> 13.3 mbar) has been observed during operation, which is perhaps the main reason for the deceasing yield of 1,3-DG. Not surprisingly, no any activity drop of Novozym 435 was observed at lower water activity under higher vacuum, which is believed to be related to the unique structure of *C. antarctica* lipase B (Uppenberg, Hansen, Patkar, & Jones, 1994). The previous work has shown that this enzyme has a special structure enabling it to bind the essential water for catalysis and consequently can work well at low water activities or even in water deprived environment (Piyatheerawong, Iwasaki, Xu, & Yamane, 2004).

3.4. Reusability of enzyme and perspective for industrial application

Important for the application potential of the designed protocol on a commercial scale is, of course, the reusability of the employed biocatalyst. It is evident from Table 2 that the recovered immobilized lipase can be used at least 10 times without significant loss of activity. Even at the 10th consecutive batch reaction, the conversion of CLA and the yield of the desired product were still up to 97.3% and 92.8%, respectively. Excellent exhibition of Novozym 435, to catalyze the synthesis of 1,3-DG in repeated batch reactions, might benefit from less harm to the biocatalyst agitated by N_2 bubbling and the inert atmosphere provided by the designed protocol. The occurrence of glycerol and the absence of water-deprived media in the solvent-free system seem to help the enzyme to retain its activity. Not surprisingly, no obvious difference between the fatty acid composition of the starting fatty acid and the yielded product was observed, indicating a protective effect from the protocol. Therefore, according to the results in Table 2, it is estimated that at least 146 kg 1,3-DG products of 92–96% purity could be produced by this reaction design employing 1 kg Novozym 435 without any further purification. The above results also indicat the industrial potential of the operation protocol developed in this work.

4. Conclusion

The original motivation for this study is to establish a facile and efficient approach, with practical application

interests, through strategy evaluation and protocol design, and to achieve a better understanding of the behaviour of enzymatic multiple-phase reaction in a solvent-free system by mass transfer investigation. It turns out that mass transfer and reaction control are closely related to the operation modes adopted. The vacuum-driven N_2 bubbling protocol is able to create an effective interaction between the different phases of the enzymatic esterification of fatty acid and glycerol, and to eliminate the mass transfer resistance. A protective atmosphere created by N_2 introduction makes this method a universal protocol for all PUFA reaction systems. High purity of the desired product, bulky productivity in short time, less consumption of N_2 and excellent stability of enzyme give the protocol industrial application potential.

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