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Highly efficient solvent-free synthesis of 1,3-diacylglycerols by lipase immobilised on nano-sized magnetite particles

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ABSTRACT

Recently, 1,3-DAGs (1,3-diacylglycerols) have attracted considerable attention as healthy components of food, oil and pharmaceutical intermediates. Generally, 1,3-DAG is prepared by lipase-mediated catalysis in a solvent free system. However, the system's high reaction temperature (required to reach the reactants' melting point), high substrate concentration and high viscosity severely reduce the lipase's activity, selectivity and recycling efficiency. In this report, *Mj*L (*Mucor javanicus* lipase) was found to have the best performance in the solvent-free synthesis of 1,3-DAGs of several common commercial lipases. By covalent binding to amino-group-activated NSM (nano-sized magnetite) particles and cross-linking to form an enzyme aggregate coat, *Mj*L's specific activity increased 10-fold, and was able to be reused for 10 cycles with 90% residual activity at 55 °C. 1,3-DAGs of lauric, myristic, palmitic, stearic, oleic and linoleic acid were prepared using the resulting immobilised enzyme, all with yields greater than 90%, and the reaction time was also greatly reduced.

1. Introduction

1, 3-DAGs (1,3-diacylglycerols) are known to be minor component of edible fats (D'Alonzo, Kozarek & Wade, 1982; Fureby, Tian, Adlercreutz, & Mattiasson, 1997). Recent studies have claimed that consumption of rich 1,3-DAG oil has beneficial effects on suppressing the accumulation of body fat and preventing increased body weight (Lo, Tan, Long, Yusoff, & Lai, 2008; Meng, Zou, Shi, Duan, & Mao, 2004; Morita & Soni, 2009; Reyes et al., 2008; Yanai et al., 2008). Additionally, 1,3-DAGs have attracted growing attention as intermediates for the synthesis of various compounds with pharmaceutical applications. When esterified with 1,3-DAG at the 2-position hydroxyl group, drugs, such as Niflumic Acid (a channel blocker), are often better absorbed and have fewer side effects than in the original form (Berger & Schneider, 1993; Mantelli, Speiser, & Hauser, 1985).

Due to their minor content in their natural form, the synthesis of 1,3-DAGs becomes important. 1,3-DAGs can be prepared both chemically and enzymatically through glycerolysis, partial alcoholysis and esterification. The enzyme mediated processes, usually carried out with lipase as a catalyst, have been found to be superior to conventional chemical methods due to their mild reaction conditions, higher yield and procedural convenience. Nevertheless, glycerolysis and partial alcoholysis tend to produce more MAG (monoacylglycerol) and 1,2-DAG than 1,3-DAG (Fureby et al., 1997; Garcia, Yang, & Parkin, 1996; Kristensen, Xu, & Mu, 2005a; Kristensen, Xu, & Mu, 2005b). Therefore, the ideal procedure to obtain a high yield of 1,3-DAG is through the esterification of glycerol and FFA (free fatty acid).

Unfortunately, glycerol is poorly soluble in most of the common hydrophobic organic solvents that lipase favours, such as *n*-hexane and toluene. Moreover, in solvent systems, side reactions such as acyl migration may occur and result in a reduction of the 1,3-DAG purity. It has been reported that in *t*-butanol, both glycerol and oleic acid are soluble, and their esterification catalysed by Novozym 435 (immobilised Candida antarctica lipase B produced by Novozymes A/S) achieved a reasonable yield of 1,3-DAG (Duan, Du, & Liu, 2010), but *t*-butanol could not dissolve most other FFAs, especially saturated long-chain ones. Worse still, most common commercial lipases other than Novozym 435 have notably low catalytic activity in *t*-butanol.

From the above discussion, the ideal method for 1,3-DAG preparation is clearly the solvent-free esterification of glycerol and FFAs. However, a solvent-free glycerol/FFA system has high substrate concentration and high viscosity, and requires a relatively high reaction temperature (to reach the FFAs' melting point to keep the reaction liquid), which severely impact the lipase's catalytic activity, selectivity and recycling efficiency. Therefore, until now, to the best of our knowledge, related studies have only solved

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problems, such as operation conditions or reaction equilibrium (Berger, Laumen, & Schneider, 1992; Rosu, Yasui, Iwasaki, & Yamane, 1999; Watanabe et al., 2003), whilst research on the key problem, the improvement of the heat resistance, activity and operational stability in the system of solvent-free glycerol/FFA esterification of the biocatalyst, has never been reported. In this work, common commercially available lipases were screened, and *Mj*L (*Mucor javanicus* lipase) was selected because it performed better. To enhance its performance further, *Mj*L was covalently bound to surface activated NSM (nano-sized magnetite) particles and later cross-linked to form a CLEA (cross-linked enzyme aggregate) coating structure. The resulting nano-sized magnetite immobilised lipase was used as catalyst for preparing 1,3-DAGs of lauric, myristic, palmitic, stearic, oleic and linoleic acid ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$), all with good yield (>90%) with shortened reaction time (from 24 h to 8 h or less). The immobilised *Mj*L was able to be reused for 10 cycles at 55 °C with only a 10% loss of activity. Thus, a highly efficient, widely usable and low-cost system for the synthesis of 1,3-DAG has been established.

2. Materials and methods

2.1. Materials

GA (glutaraldehyde, 25% water solution), APTES ((3-aminopropyl)triethoxysilane), glycerol, FFAs (free fatty acids: lauric, myristic, palmitic, stearic, oleic and linoleic acid, all purity >98%) and Candida rugosa lipase (aka CRL Type VII) were purchased from Sigma-Aldrich Co.; lipases from *Aspergillus niger*, *Pseudomonas fluorescens*, *M. javanicus*, *Rhizopus niveus*, *Burkholderia cepacia* (aka Lipase A, AK, M, N and PS, respectively) were purchased from Amano Enzyme Inc.; native and immobilised lipase from *Rhizomucor miehei* (aka Lipozyme RM and Lipozyme RM IM), together with immobilised Candida antarctica lipase B (Novozym 435) were purchased from Novozymes A/S. All other reagents referred in this article were of analytical grade.

2.2. Preparation of surface modified NSM

Fe_3O_4 particles with a diameter of 10–20 nm were prepared by coprecipitation (Molday, 1984), as shown in Fig. 1a-1, the NSM was modified with APTES according to Ma's work (Ma et al., 2003). Afterwards, as shown in Fig. 1a-2, 1 g of the APTES modified NSM, 2 mL of GA (25% aqueous solution) and 16 ml of PBS (phosphate buffer solution, 25 mmol L^{-1}, pH = 7) were mixed and stirred at 200 rpm and 25 °C for 2 h. The resulting solid was magnetically separated by a strong magnet (N52 Nd-Fe-B magnet, 60 × 60 × 40 mm, Yantai Metal Material Company, Shanghai, China), washed several times with PBS, and vacuum frozen for 12 h. Finally, a dry powder of surface activated NSM was obtained.

2.3. Lipase immobilisation

As shown in Fig. 1a-3, 1 g of activated NSM as prepared above and 1000 mg of *Mj*L crude powder (containing about 320 mg protein) was resolved in PBS (4 °C, 20 mL). The mixture was stirred at 200 rpm at 4 °C for 12 h, at which point 0–2.4 mL of GA (25% weight/weight) was added. This mixture was stirred for another 12 h to prepare CLEA as shown in Fig. 1b. Finally the insoluble solid was magnetically separated from the mixture, washed several times with PBS (pH = 7) to remove the unbound protein, and freeze-dried for 12 h. The resulting immobilised lipase was kept at 4 °C before use. The amount of lipase protein bound onto the carrier was calculated by a mass balance. The amount of lipase protein in the supernatant was determined by the Bradford method (Bradford, 1976) using BSA (bovine serum albumin) as a standard.

To investigate influence of pH's on immobilisation, PBS solutions at pH 5, 6, 7, 8 and 9 were used. To investigate protein concentration's influence, a series of *Mj*L solution were used, whose concentration ranged between 0 and 16 (mg protein)mL^{-1} were used.

2.4. Esterification of the fatty acids

Esterification of free fatty acid and glycerol was used to prepare 1,3-DAG. The general equation of the reaction is as follows:

$$\begin{aligned} &RCOOH + CH_2OHCHOHCH_2OH \\ &\rightarrow RCOOCH_2CHOHCH_2OCOR(1,3-DAG) \\ &\quad + RCOOCH_2CHOCORCH_2OH(1,2-DAG) + H_2O \end{aligned} \quad (1)$$

where R – represents the carbon chain of fatty acids. The detail reaction condition was: free fatty acid (100 mmol, approximately 20.0, 22.8, 25.6, 28.4, 28.2 and 28.0 g for lauric, myristic, palmitic, stearic, oleic and linoleic acids, respectively), glycerol (50 mmol, approximately 4.60 g), *Mj*L (in native or immobilised form, 1 g), and molecular sieves (4 Å, 10 g, for water removal) were adequately mixed in a 50 mL conical flask at atmospheric pressure, and reacted at 55 °C for lauric, oleic and linoleic acid, and 60, 65 and 70 °C for myristic, palmitic and stearic acid (to reach the three fatty acids' melting point), respectively, stirring at 200 rpm for 6–12 h. At 0.5 h of the reaction (during this time, conversion rate of FFA was beneath 5%), 50 μL of the reactant was sampled to determine the activity and selectivity.

2.5. Assay of lipase's activity and selectivity

Esterification of glycerol and oleic acid (that is the case when R- = $CH_3(CH_2)_7CH = CH(CH2)_6CH_2$– in Eq. (1) was used as a model reaction to assay lipase activity and selectivity. Activity of esterification was defined as the initial consumption rate of oleic acid (μmol) per minute (when conversion was under 5%), that is, 1 U_E = 1 (μmol oleic acid) min^{-1}. The specific activity of esterification was based on the total protein content, that is, (specific activity) = (activity of esterification)/(total protein in the lipase powder or loaded on the immobilisation carrier). Activity recovery = (total esterification activity of the immobilised lipase)/(total esterification activity of the lipase powder used for immobilisation)×100%. The protein content of the lipase solution was determined according to the Bradford method (Bradford, 1976), in which BSA was used as protein standard. The amount of bound protein of immobilised enzyme was indirectly determined by comparing the difference between the total amount of protein used and the amount of protein in the washing solution.

In analogy to the *ee* value used for the description of enantiomeric excess, a similar value for the regioisomeric excess was defined: *re* = [(1,3-DAG)%−(1,2-DAG)%]/[(1,3-DAG)% + (1,2-DAG)%]. The larger the *re* value is, the more 1,3-selective the lipase is. FFA and glycerides concentrations were determined by GC. The sample was analysed using a HP 1890 gas chromatograph (Hewlett-Packard Co. CA, US) equipped with a FID detector and a DB-17ht capillary column (30 m × 0.25 mm, Agilent Technologies Inc., CA, US). Hydrogen was used as a carrier gas. Both the injector and detector temperatures were 350 °C. The temperature program was as follows: initial temperature of 80 °C, then heating to 340 °C at 10 °C min^{-1}. The final temperature was 340 °C and was held for 15 min.

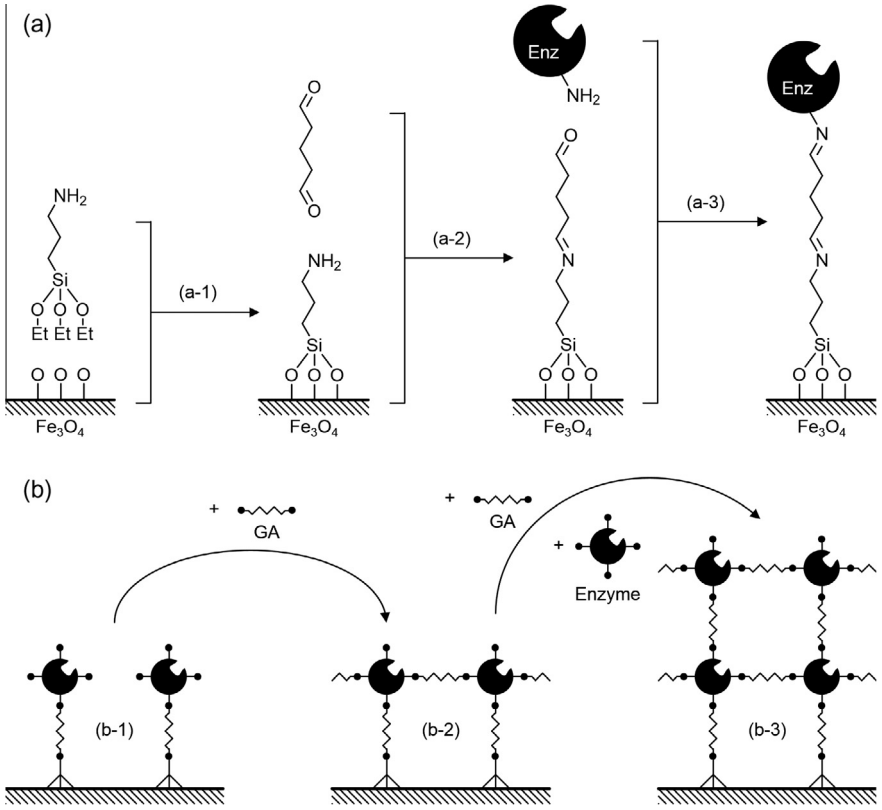

Fig. 1. Process of immobilisation. (a) Activation of NSM and binding the lipase onto the carrier. (a-1) The nano-sized Fe_3O_4 particle was modified by APTES; (a-2) GA was attached to the particle via the APTES branch; (a-3) lipase was immobilised onto the activated particle by covalent bonding of its surface amino group and the carrier's aldehyde group. (b) Cross linking and enzyme aggregate coating. (b-1) surface bonding; (b-2) cross-linking; (b-3) enzyme aggregate coating. In this figure, a circle with a notch denotes an enzyme molecule, and the tiny dots with sticks around the molecule denotes amino groups.

2.6. Assay of the lipase operational stability

The esterification activity of the lipase at 55 °C was determined in batched reactions, with the operation parameters stated in Section 2.4. After every cycle had finished, the old reactant and molecular sieves were removed (for native enzyme, the reaction mixture was centrifuged at 12,000 rpm for 10 min; for the immobilised enzyme, a strong magnet was applied for 10 min to completely precipitate the magnetic immobilised lipases), and fresh ones were added for the next batch.

2.7. Assay of lipase thermal stability

One gram of native or immobilised *Mj*L powder was dispersed in 20 g oleic acid by stirring at 200 rpm at a 55, 65 and 75 °C for 12 h, afterwards, the solid was recycled by centrifugation or magnetic separation. The activity of the heat-treated enzyme was then determined according to the method described in Section 2.5.

3. Results and discussion

3.1. Screening of commercial lipases

Esterification activity and regioisomer excess of common commercial lipases in native or immobilised form were investigated for use in 1,3-DAG solvent-free synthesis. As shown in Table 1, two immobilised lipases (Lipozyme RM IM and Novozym 435) had the highest activity. Amongst the native enzymes, *M. javanicus* lipase (*Mj*L) had the best activity and not very low selectivity (*re* = 75%). The lipases with good *re* values (90% or more) had very low activity. This was because the optimal temperature for most lipases is 37 °C, whilst their enzymatic activity was assayed at 55 °C. This temperature was too high for these lipases and inactivated them. Moreover, in the solvent-free system, the substrate concentration was very high, resulting in substrate inhibition. Therefore, *Mj*L was selected as the catalyst for 1,3-DAG preparation. It should be noted that although the immobilised lipases had good activity and at least acceptable selectivity, in the high-viscosity system containing strong hydrophilic substrate, glycerol, the lipase protein would desorbed from their carrier, causing the problem of mass loss during reuse. Worse still, similar to the native lipases, these immobilised lipases can be recycled from the reactant only by high-speed centrifugation (12,000 rpm as stated in Section 2.6), which is not a convenient way for separation. Besides, these commercial immobilised lipases lacked potential of further

Table 1
Lipase screening.

Enzyme	Source	Activity of esterification	*re*
		U_E (g solid)$^{-1}$	(%)
CRL Type VII	*Candida rugosa*	2.7	67
Lipase A	*Aspergillus niger*	6.4	95
Lipase AK	*Pseudomonas fluorescens*	13.0	95
Lipase M	*Mucor javanicus*	44.0	75
Lipase N	*Rhizopus niveus*	2.4	95
Lipase PS	*Burkholderia cepacia*	29.5	75
Lipozyme RM	*Rhizomucor miehei*	14.6	95
Lipozyme RM IM	*Rhizomucor miehei* (immobilised)	61.2	95
Novozym 435	*Candia antarctica* (fraction B, immobilised)	92.4	75

enhancement via special immobilisation techniques such as cross-linked enzyme aggregate and employing nano-sized magnetite carrier.

3.2. Immobilisation of MjL

pH strongly affected the results of assays of enzyme immobilisation based on covalent binding. Native *Mj*L powder was dissolved in PBS (25 mmol L^{-1}) at different pHs and mixed with the surface-activated NSM for 12 h. After that process, the resulting powder was separated magnetically and washed with PBS (25 mmol L^{-1}, pH = 7.0) because neutral pH was reported to be the optimum pH for *Mj*L (Ishihara, Okuyama, Ikezawa, & Tejima, 1975). As in Fig. 2a, the protein binding rate at acidic or alkaline pH was greater than that at neutral pH, as an aldehyde group of the carrier and amino group of the protein tended to react in the presence of acid or base. However, the specific activity at those pH values were the lowest, potentially because many *Mj*L molecules were deactivated during the 12-hour-long immobilisation process at far-from-optimum pHs.

A series of different concentrations of *Mj*L were used for immobilisation. The results are depicted as an adsorption isothermal curve (Fig. 2b). From the figure, it can be observed that as the protein concentration reached approximately 16 mg mL^{-1}, covalent

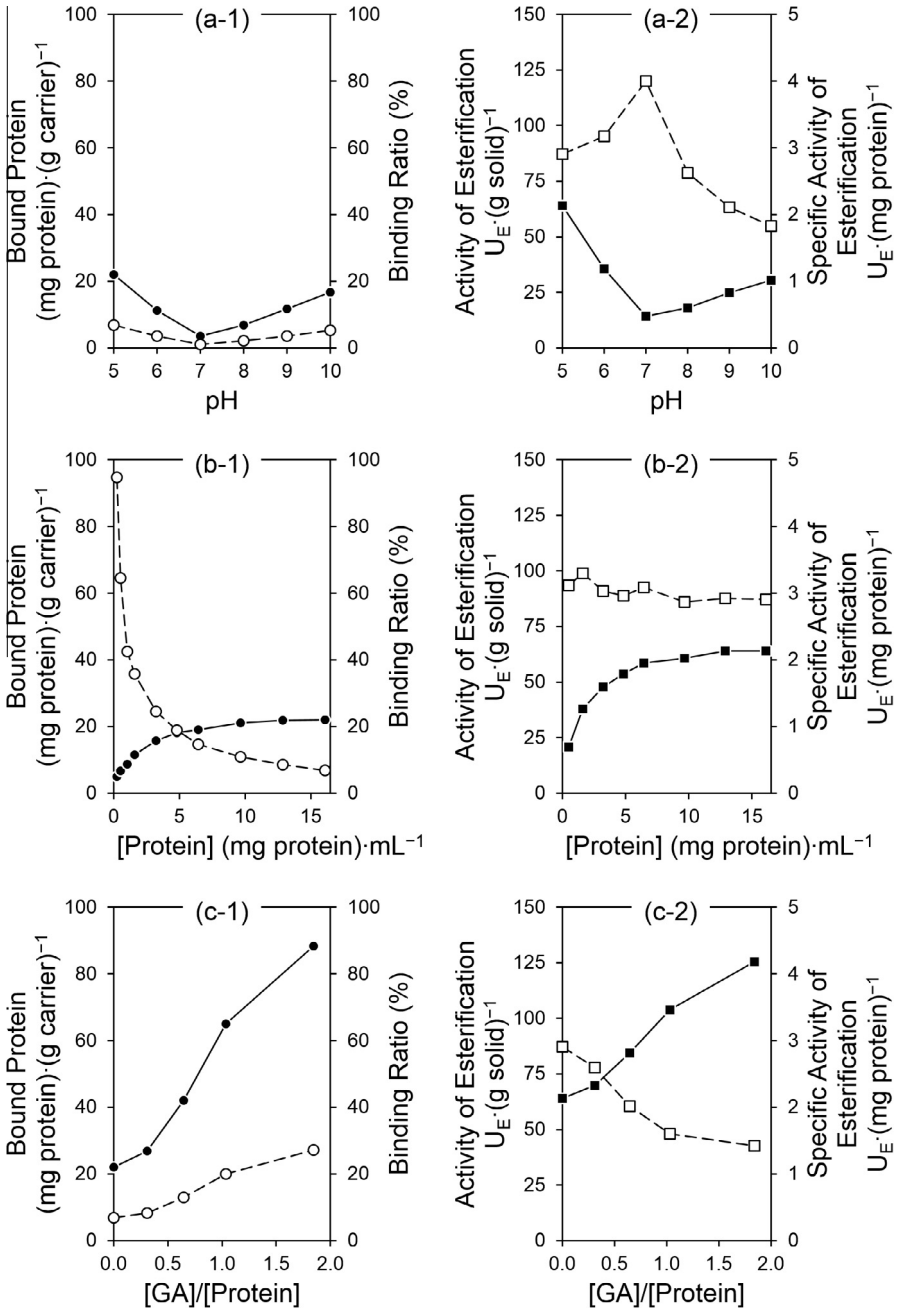

Fig. 2. Investigation of the immobilisation conditions. (a) pH influence. (b) Binding equilibrium curve of immobilisation. In this figure, the title of the horizontal axis, [Protein], stands for the total protein concentration of the lipase used for immobilisation. (c) Effect of GA cross-linking: In this figure, the horizontal axis represents the ratio of GA concentration vs. *Mj*L protein concentration; both concentrations are in mg mL^{-1}. Meaning of the symbols: —●— bound protein, - -○- -adsorption rate, —■— activity, - -□- - specific activity.

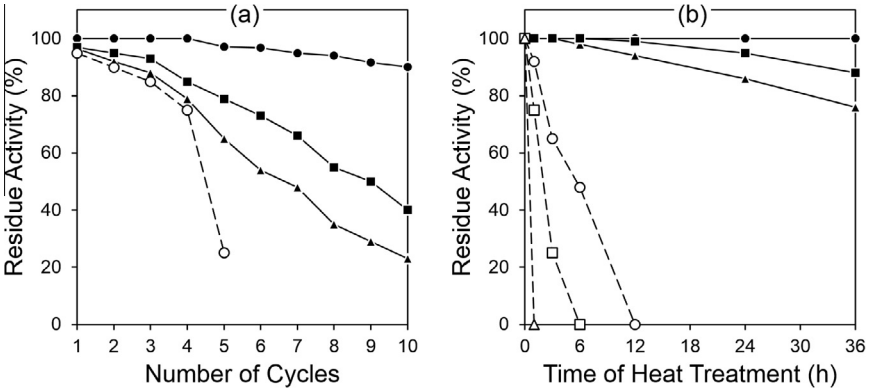

Fig. 3. Stability of the lipase. (a) Operational stability (at 55 °C) of the immobilised *Mj*L and other commercial lipases: - -○- - native *Mj*L (Lipase M), —●— immobilised *Mj*L, —■— immobilised CaL-B (Novozym 435), —▲— immobilised Rml (Lipozyme RM IM). (b) Thermal stability of *Mj*L in immobilised or native form: —●— Immobilised *Mj*L (55 °C), —■— immobilised *Mj*L (65 °C), —▲— immobilised *Mj*L (75 °C), - -○- - native *Mj*L (55 °C), - -□- - native *Mj*L (65 °C), - -△- - native *Mj*L (75 °C).

binding was saturated. It should be noted that in Fig. 3b, as bound protein increased, the specific activity did not decrease accordingly, indicating that the immobilisation method employed was able to avoid mass transfer limitations, which is one of the great advantages of nano-sized particles.

As Fig. 1b indicates, enzyme cross-linking and the aggregate coating method was proposed to attach more molecules and aggregates in the solution onto 'seed' enzyme molecules that had already been covalently attached onto the support. In our study, to further improve the activity and stability of the immobilised *Mj*L, we attempted to prepare the cross-linked enzyme-aggregate derivative and investigated the characteristics of the immobilised preparation. The experimental results (Fig. 2c) confirmed that as the amount of cross-linker (glutaraldehyde) increased, the amount of bound protein, together with esterification activity of the immobilised *Mj*L, increased also.

Through immobilisation onto nano-sized magnetite particle in optimum conditions (*Mj*L (22 (mg protein) mL^{-1}), carrier (1 g mL^{-1}), stirring 200 rpm for 12 h at 4 °C in PBS (25 mmol L^{-1}, pH = 5), 12 h cross-linking with GA (40 mg mL^{-1}) at 4 °C and 200 rpm stirring), the *Mj*L specific activity of esterification increased 10-fold (from 0.133 to 1.42 U_E (mg protein)$^{-1}$), and *re* increased from 75% to 90%. The activity recovery (esterification of oleic acid) of the immobilisation was 285%.

3.3. Stability of the immobilised MjL

As shown in Fig. 3a, the immobilised *Mj*L retained 90% residual activity after 10 reaction cycles, whilst native *Mj*L (Amano Lipase M) retained only 25% for 5 batches. The critical improvement of the stability of the lipase might be the result of the following: cross-linked enzyme molecules were tightly bound to each other; covalent binding minimised the amount of enzyme released from the carrier; and, the higher recycling efficiency of the magnetic separation reduced the mass loss of the catalyst.

The esterification activity of the native and immobilised *Mj*L after several hours of heat treatment at 55, 65 and 75 °C in oleic acid were determined (Fig. 3b). At 55 °C, the immobilised *Mj*L was able to keep 100% activity for 36 h compared with the full deactivation of native *Mj*L in less than 12 h. As the temperature rose to 65 and 75 °C, even the immobilised *Mj*L began to lose activity. Regardless, the thermal stability of the lipase was critically enhanced, enhancing the potential for DAG synthesis at high temperatures.

3.4. Preparation of 1,3-DAGs using immobilised MjL

Using the immobilised *Mj*L as the catalyst, 1,3-DAGs of lauric, myristic, palmitic, stearic, oleic and linoleic acid were synthesised.

Table 2
Preparation of 1,3-DAGs catalysed by the immobilised *Mj*L.

Free fatty acids		Reaction temperature	Reaction time	Conversion[a]	*re*	Glyceride composition[b]		
						TAG	DAG	MAG
		(°C)	(h)	(%)	(%)	(%mol)	(%mol)	(%mol)
By native MjL[c]								
Lauric acid	($C_{12:0}$)	55	24	92	73	0.0	88.7	11.3
Oleic acid	($C_{18:1}$)	55	24	90	75	0.0	86.4	13.6
Linoleic acid	($C_{18:2}$)	55	24	90	75	0.0	88.0	12.0
By Immobilised MjL								
Lauric acid	($C_{12:0}$)	55	6	97	90	0.0	99.0	1.0
Myristic acid	($C_{14:0}$)	60	9	96	90	0.0	98.9	1.1
Palmitic acid	($C_{16:0}$)	65	12	94	91	0.0	98.0	2.0
Stearic acid	($C_{18:0}$)	70	12	92	91	0.0	95.0	5.0
Oleic acid	($C_{18:1}$)	55	12	90	92	0.0	93.3	6.7
Linoleic acid	($C_{18:2}$)	55	12	91	92	0.0	94.4	5.6

[a] Conversion was calculated according consumption of fatty acid in the reactant.
[b] TAG = triacylglycerol, DAG = diacylglycerol, MAG = monoacylglycerol. The ratio of tri-, di-, and mono-acylglycerols was calculated based on the molar percentage of total glyceride. No triacylglycerol was detected in the analysis of the glyceride composition of the esterification product. This finding could be observed because in the reactant, molar ratio of glycerol vs. FFA was set to 1:2, and TAG could not be generated in this condition.
[c] There was no data on the esterification of myristic, palmitic and stearic acid catalysed by native *Mj*L because the reaction temperature required by these FFAs was sufficiently high that the native lipase would be inactivated during the reaction.

As shown in Table 2, all products' FFA conversion, DAG percentage from total glyceride and re value were greater than 90%. In contrast, the *re* of 1,3-DAG prepared with native *Mj*L was only 75%. The reaction time was reduced from over 24 h to less than 12 h. These results indicated that the selectivity of the immobilised lipase was quite reasonable, and the very reaction conditions (the glycerol/FFA solvent-free system) were quite suitable for highly efficient 1,3-DAG synthesis.

4. Conclusions

In this work, common commercial lipases were screened, and *M. javanicus* lipase was found to have most acceptable performance. Surface-activated nano-sized magnetite particle was employed as carrier for the immobilisation of *Mj*L via covalent-bonding. Further treatment by glutaraldehyde was employed to form a cross-linked enzyme aggregate. The resulting nano-sized magnetite immobilised lipase exhibited critical improvement in esterification activity, regioselectivity and thermal/operational stability in a high-viscosity and high-temperature system for 1,3-DAG preparation. Good yields of 1,3-DAGs of lauric, myristic, palmitic, stearic, oleic and linoleic acid synthesised by the immobilised lipase were achieved with *re* > 90%, and the reaction time was significantly reduced. It can be concluded that using the immobilised *Mj*L developed in this work in the solvent-free esterification of glycerol and free fatty acid is an effective system for 1,3-DAG synthesis.

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