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Improved enzymatic galactose oleate ester synthesis in ionic liquids

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ABSTRACT

A fast and improved lipase-catalyzed synthesis of galactose oleate ester was performed in 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) ionic liquid with the addition of dimethylsulfoxide (DMSO) as a solubilizing agent and co-solvent; and Lipozyme RM IM (lipase from *Rhizomucor miehei* immobilized on macroporous anion exchange resin) as the biocatalyst. Different reaction parameters (type of solvent, type of enzyme, amount of enzyme, reaction time, temperature, stirring rate and substrate molar ratio) were studied. A high conversion (87%) was obtained after only 2 h at optimal synthesis conditions (1:20 DMSO:[Bmim][BF_4] ratio with 2% (w/w) Lipozyme RM IM, temperature 60 °C, stirring rate of 300 rpm and a molar ratio of galactose to oleic acid of 1:3).

1. Introduction

The application of carbohydrate fatty acid esters or carbohydrate esters in biotechnological industries as emulsifiers and surfactants for detergents, food, pharmaceuticals and cosmetics has been on the increase recently due to their specific properties. These esters are non-toxic, non-irritant, non-ionic, tasteless and odorless surfactants besides being synthesized from cheap and renewable materials. Harmless and biodegradable, carbohydrate esters have potential as antibacterial, insecticidal and antitumoral agents [1–3]. Besides chemical synthesis, an enzymatic synthesis has been chosen as a significant route to synthesize carbohydrate esters. Chemical synthesis produces a variety of carbohydrate esters and leads to low selectivity. Furthermore, chemical synthesis requires high temperatures and produces colored products with base catalysts. In this case, enzymatic synthesis offers more advantages over conventional chemical synthesis to produce carbohydrate esters. The possibility of substrate or product denaturation can be reduced or eliminated since enzymatic synthesis works well under mild conditions. Furthermore, monoester production can be increased due to enzyme's high regioselectivity [4,5].

Lipases extracted from various sources (e.g. yeast, molds or bacteria) demonstrate specific positional characteristics during esterification or trans-esterification reactions. Lipase-catalyzed esterifications have been largely reported to catalyze a wide range of regioselective and stereoselective transformations in organic solvents as reaction media [6,7]. Organic solvents (e.g. *tert*-butanol, acetone and acetonitrile) are often used since enzymes remain active in these solvents. However, the usage of organic solvents in sugar ester synthesis has some limitations due to low solubility of sugar in these solvents.

To overcome the solubility problem of sugar in organic solvents, organic salts or ionic liquids (ILs) are specifically used as substituting solvents. ILs are non-volatile, non-inflammable and thermally stable. They possess special characteristics in that their properties can be tuned depending on the composition of cations and anions. Commercial anhydrous ILs with tetrafluoroborate ([BF_4]), hexafluorophosphate ([PF_6]), bis(trifluoromethylsulfonyl)imide ([NTf_2]) and trifluoromethanesulfonate ([TfO]) anions have been recently used in carbohydrate ester synthesis as reaction media [8–10]. Enzyme stability and selectivity have been found to be high in anion based ILs. However, carbohydrate solubility remains a major problem in ILs [11–13]. The addition of co-solvents may improve the solubility of carbohydrates in ILs.

Galactose is an essential monosaccharide. It is a basic substrate for the biosynthesis of macromolecules in the body. Oleic acid is a monounsaturated long chains fatty acid and is widely used as a substitute for saturated fatty acids to reduce potential dietary problems. It also exhibits antioxidant properties by preventing

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lipoprotein oxidative modification that leads to atherosclerosis [14–16].

In this work, galactose oleate ester was specifically synthesized since there were only a few reports on the synthesis of sugar ester from galactose and oleic acid, especially in ILs as reaction media. Some researchers reported up to 60% conversion using galactose and divinyladipate as substrates with DMF as reaction medium with reaction time of 7 days at 35 °C [17]. In addition, long chain sugar esters are widely applied as surfactants and only monoester and diester are relevant for cosmetic applications [18]. An ester of galactose and oleic acid, i.e. galactose oleate is expected to exhibit positive characteristics from both substrates for use in the food industry and others.

The aim of this study is to synthesize sugar fatty acid esters by lipase-catalyzed esterification. Screening tests of several parameters were conducted by varying one parameter at a time. The reaction conditions of sugar fatty acid ester synthesis were optimized. Parameters for screening were solvent type, enzyme source, enzyme amount, reaction time, temperature, rotational speed and sugar to fatty acid molar ratio.

2. Materials and methods

2.1. Materials

Commercial ionic liquids, 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF_4]), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]), 1-butyl-3-methylimidazolium trifluoromethylsulfonylimide ([Bmim][NTf_2]), 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([Bmim][TfO]), solvents and chemicals (acetone, acetonitrile, hexane, methanol, molybdatophosphoric acid and tetrahydrofuran) were purchased from Merck, Germany. Commercial lipases, Novozym 435 (immobilized lipase B from *Candida antarctica*), Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*) and Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosus*) were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Free lipase from *Candida rugosa* and D-galactose were from Sigma–Aldrich, St. Louis, USA. 2-Methyl-2-butanol was from Fischer Scientific, chloroform was from J.T. Baker and oleic acid was from TCI Tokyo Kasei. Free lipase from *Geobacillus zalihae* strain T1 was obtained from UPM, Selangor, Malaysia. All chemicals were commercially available and of analytical grade unless otherwise specified.

2.2. Lipase catalyzed esterification

D-Galactose (0.05 mmol) was fully dissolved in 50 μL DMSO at 60 °C for 30 min. Oleic acid (0.1 mmol) and 1 mL of ionic liquid (IL) were added and stirred until a homogenous solution was obtained. Galactose and oleic acid were specifically chosen as substrates in this study since they are cheap, renewable and abundantly found in plant and animal products. Lipozyme RM IM (2%, w/w) was added into the mixture and the reaction was conducted in an Eppendorf Thermomixer at a fixed rotational speed (rpm), temperature (°C) and time (h). At the end of the reaction, 20 μL was withdrawn to measure the concentration of unreacted oleic acid.

2.3. Analysis and characterizations

Conversion of fatty acid was measured with a High Performance Liquid Chromatography (HPLC) system (Agilent 1200 Series) equipped with an Evaporative Light Scattering Detector (ELSD) and an Eclipse XDB-C18, 5 μm column (150 mm × 4.6 mm). 20 μL samples from the reaction mixture were withdrawn at initial and final reaction times, diluted with a 40 μL mixture of acetonitrile/methanol (1:1) and were centrifugated. The clear supernatant was filtered and injected into a HPLC. The mobile phase was 80:20 of acetonitrile/water (v/v) with a flow rate of 0.5 mL/min. Percentage of conversion was calculated from the remaining free fatty acids by comparing the sample's peak area with the standard curve of oleic acid (R_t 14–15 min). All experiments were run at least in duplicate and the percentage of error was less than 5%. The retention time for galactose oleate ester was 7.2 min.

The scanning electron microscopy of Lipozyme RM IM enzyme was performed by using JEOL JSM-6400 SEM (with Energy Dispersive X-Ray (EDX) (JEOL, Tokyo, Japan). A small amount of enzyme was placed on the SEM stub with carbon tape attached on stub's top surface. The sample was then coated with a platinum layer for better contrast.

3. Results and discussion

3.1. Effect of solvents

A suitable solvent is required to dissolve or suspend the starting material and to limit undesired side-reactions. Fig. 1(a) shows preliminary solvent screening. In organic solvents such as acetone, acetonitrile and 2-methyl-2-butanol, the maximum conversion was 40%. This could be due to the low solubility of carbohydrate and enzyme inactivation in polar organic solvents. Common imidazolium-based ionic liquids (ILs) with different polarities were screened to replace organic solvents as reaction media and worse results than organic solvents were obtained with conversion up to 25% (Fig. 1(a)). The lower conversion in ILs was also caused by the low solubility of galactose in ILs as most of the galactose remains undissolved. It was thought that a better conversion would be obtained if the carbohydrate was fully soluble in the reaction media and addition of a co-solvent may help to improve solubility and conversion [19]. As a result, DMSO, a very polar solvent that can completely solubilize galactose was introduced as a co-solvent. Other polar solvent (DMF and pyridine) were also investigated as a potential co-solvent but galactose was not fully soluble in either DMF or pyridine and these solvents were not investigated further. The best ratio of DMSO to IL was found to be 1:20 (v/v) since higher amount gave worse conversion.[1] This was not surprising since DMSO was known to deactivate enzymes [20]. However, when lower amount of DMSO was used, galactose was not fully solubilized and the enzymatic reaction was not investigated. Galactose was fully solubilized in DMSO before the beginning of the reaction. Even at 1:20 ratio, the reaction time has to be kept short (2 h) as signs of enzyme deactivation appeared as the reaction progressed. Noticeably, even at 2 h, the conversion was quite high (77%) since such high conversion was reported to be achieved only after 50 h for reaction performed in supersaturated sugar solution [21].

With addition of DMSO at a 1:20 ratio (DMSO:IL), the best result was obtained when the reaction was done in ([Bmim][BF_4]), Fig. 1(b). In terms of activity and stability, enzymes may prefer hydrophobic ILs ([Bmim][PF_6] and [Bmim][NTf_2]) compared to hydrophilic ILs ([Bmim][BF_4] and [Bmim][TfO]). Indeed, hydrophobic medium was able to protect essential water layer around protein molecules which increase stability of enzyme towards denaturing environment and decrease direct protein–ion interactions [22]. Protein conformation will also be kept active since hydrophobic ILs build ionic matrix to preserve enzyme molecules in tolerable microenvironments [23]. However, our result showed

[1] Conversion at different ratio of DMSO:IL ([Bmim][BF_4]) was investigated (1:20, 1:10, 1:5, 1:1). Conversion decreased as ratio of DMSO increased. The ratio of 1:20 gave the best result (77%).

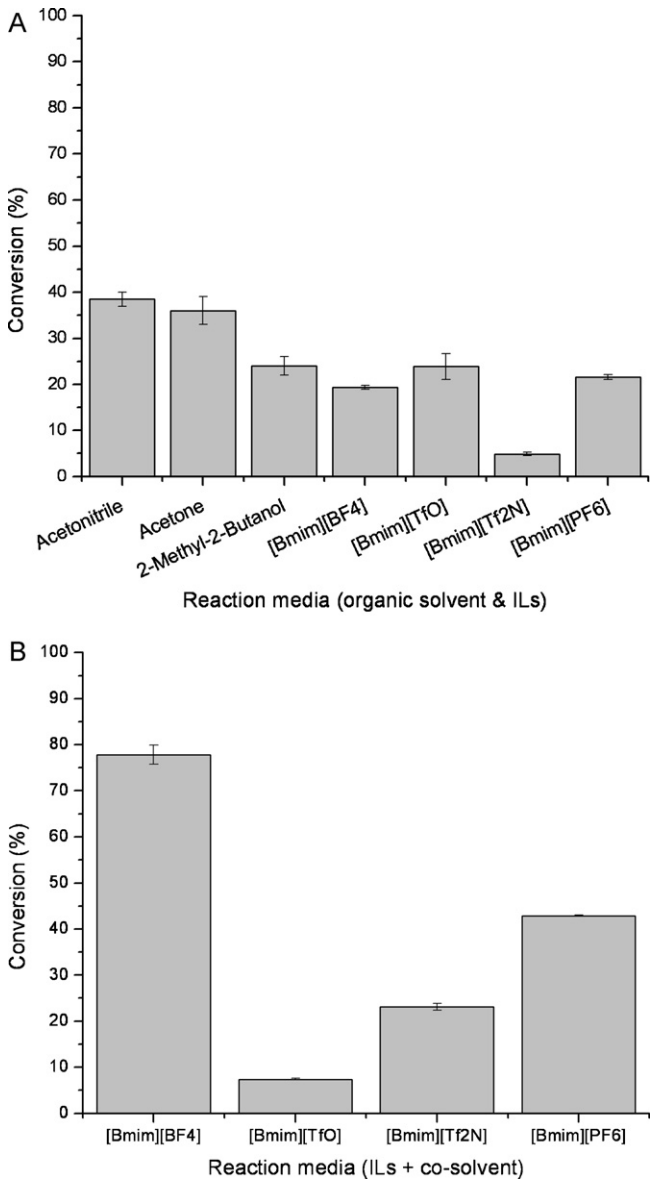

Fig. 1. (A) Effect of reaction media on synthesis of galactose oleate ester. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), at 60 °C. Conversions were compared once the steady state was reached after 24 h (organic solvents) and 2 h (ILs). (B) Effect of ILs as reaction media on synthesis of galactose oleate ester with DMSO as co-solvent. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) at 60 °C for 2 h.

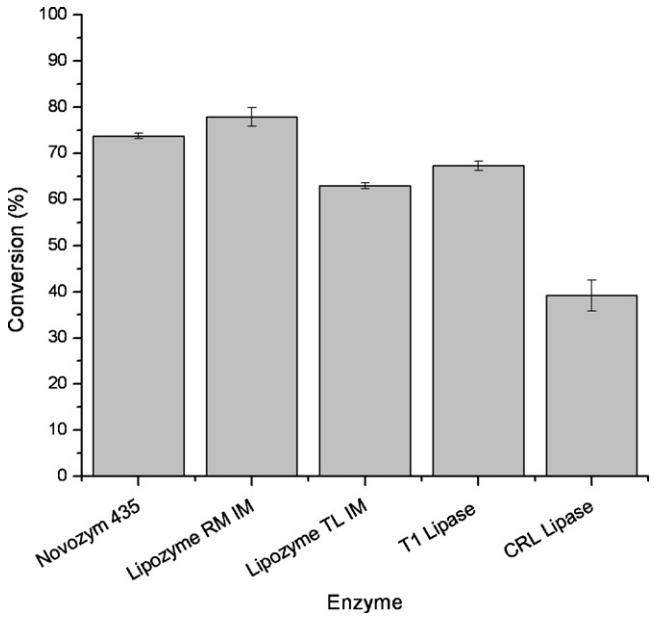

Fig. 2. Effect of different enzymes on synthesis of galactose oleate ester. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), enzyme (2%, w/w), DMSO:ILs (1:20) at 60 °C for 2 h.

that a hydrophilic IL, 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) was the best reaction media with conversion of 77% with the addition of DMSO as the co-solvent (Fig. 1(b)).

In our work, the high conversion observed in hydrophilic ILs could be due to better solubility of galactose. Hydrophobic ILs were found to be not suitable since conversions in [Bmim][PF_6] and [Bmim][NTf_2] were less than 50% even after the addition of a co-solvent. Hydrophobic ILs showed low conversion since hydrophilic substrate solubility is still a major problem in them, even though after addition of co-solvents [24]. A two-phase system was observed with the addition of hydrophobic ILs in the mixture composed of DMSO and galactose. Since DMSO was immiscible with hydrophobic ILs, this could be the reason of the low ester conversion. Ganske and Bornscheuer [13] reported that esterifications conducted in ILs and *t*-butanol mixtures showed conversions up to 60%; however, two phases were observed in the reaction media and the enzyme positioned at the interface without proper mixing. Moreover, only Novozym 435 showed good conversion compared to other lipases (Lipozyme RM IM (8%) and Lipozyme TL IM (33%)) which was in contrast with our results where a cheaper enzyme (Lipozyme RM IM) showed higher conversion than Novozym 435 (see Section 3.2). The use of two different ILs mixtures is actively reported in literature where high conversions up to 80% were obtained [11,12,19]. The supersaturated sugar solution method has also been used to solve solubility problem [21]. However, this method is time consuming, as we found out. Addition of DMSO as co-solvent was more effective since sugar was completely dissolved after short time of stirring.

3.2. Selection of enzyme

Lipases have different specificities towards carbohydrates. Therefore, four different commercial lipases and one new lipase were screened to find the most suitable biocatalyst to synthesize galactose oleate. Three different commercial immobilized and two free lipases were tested, and Lipozyme RM IM showed to be the best enzyme to catalyze the reaction with maximum conversion of 77% (Fig. 2). Novozym 435, which is usually the most effective biocatalyst in sugar ester synthesis, produced slightly lower results (73%). Since Lipozyme RM IM is cheaper than Novozym 435, it would be advantageous in future large-scale studies and applications in industry to use the former. Low conversions were obtained with free lipases (lipases from *G. zalihae* strain T1 [25] and *C. rugosa*) when compared to immobilized lipase due to reaction media used. Since hydrophilic medium was used, free enzymes were more exposed to environment and direct interaction leads to quick denaturation by water stripping [22]. In addition, interruption of intermolecular hydrogen bonds by strongly coordinating ILs anion and larger surface area of immobilized enzyme exposes enzyme molecules to a more homogenous substrate concentrations than free enzyme [26,27]. Activity of immobilized enzyme would be retained since immobilization boost enzyme's stability due to conformational rigidity and reduce exposure towards reaction media [28]. Moreover, catalytic integrity of enzyme will be maintained thanks to immobilization [22].

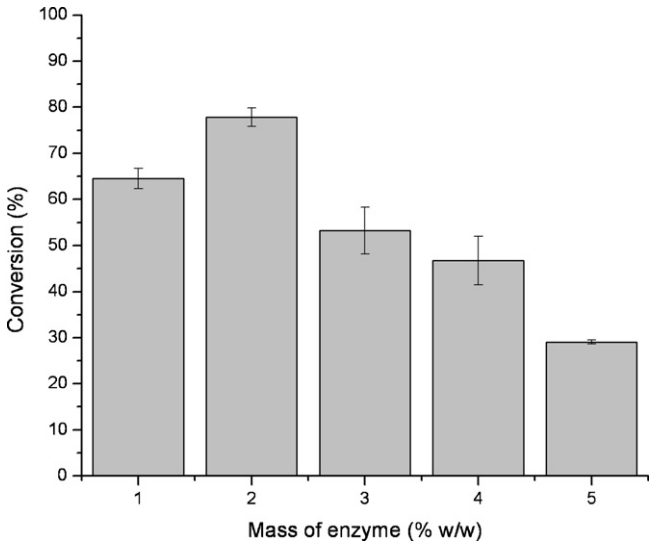

Fig. 3. Effect of enzyme amount on synthesis of galactose oleate ester with Lipozyme RM IM as biocatalyst. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), DMSO:ILs (1:20) at 60 °C for 2 h.

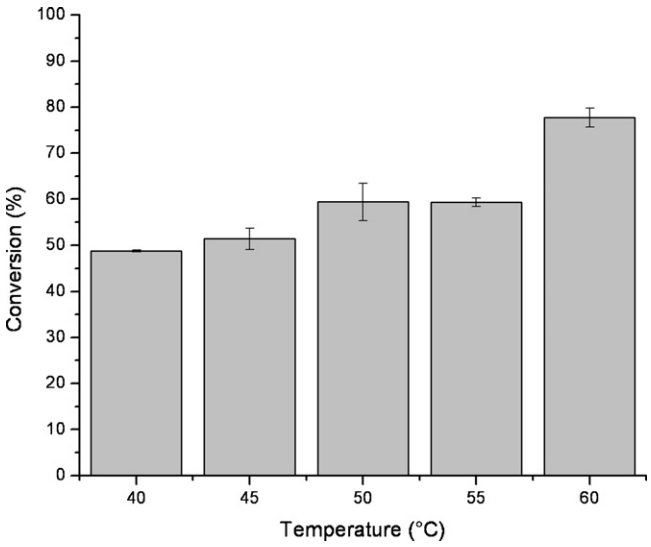

Fig. 5. Effect of reaction temperature on synthesis of galactose oleate ester. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) for 2 h.

3.3. Effect of amount of enzyme loaded

A minimal amount of enzyme was loaded into the reaction due to the high cost of commercial immobilized enzyme. The enzyme amount varied and conversions were compared once the steady state was reached after 2 h of reaction time. Our results (Fig. 3) indicate that conversion initially increased as amount of enzyme (w/w) was needed to reach the highest conversion of 77%. Indeed, above 2%, the conversion decreases with the increase of enzyme quantity what may be due to mass transfer limitation since the reaction mixture became more viscous. Moreover, above 2%, the conversion decreases with the increase of enzyme quantity may be due to the substrate limitation [29].

3.4. Effect of reaction time

The time course of galactose oleate ester formation is shown in Fig. 4. Usually, using organic solvents, the reaction profiles were studied for 72 h. However, in our work, the reaction kinetic was followed only for the first 4 h since maximum conversion was achieved more rapidly. This suggests that the equilibrium was reached in 120 min with the highest yield at a conversion of 77%. After 120 min, no significant increase in conversion was observed. Based on our results the reaction time to achieve high conversion can be considered as short since sugar ester synthesis usually requires a long period of incubation to achieve maximum yield [30]. The short reaction time was promoted by complete solubility of sugar since high initial concentration of galactose in the media leads to faster reaction. It was advantageous since enzyme denaturation caused by the addition of DMSO was limited. No enzyme activity was observed in pure DMSO after a long period of incubation.

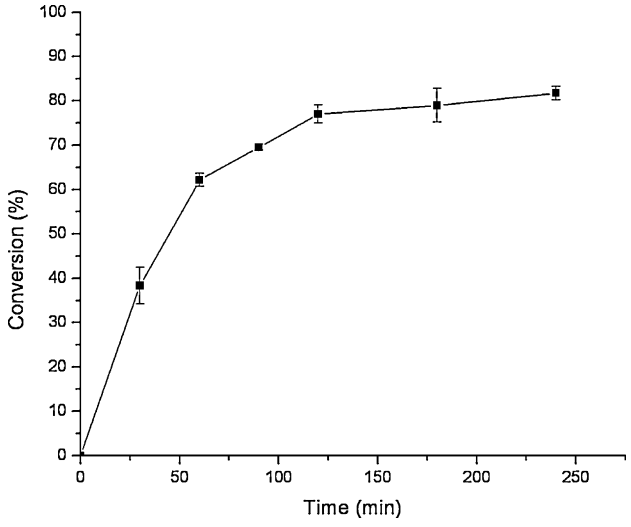

Fig. 4. Kinetic of the synthesis of galactose oleate ester. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) at 60 °C.

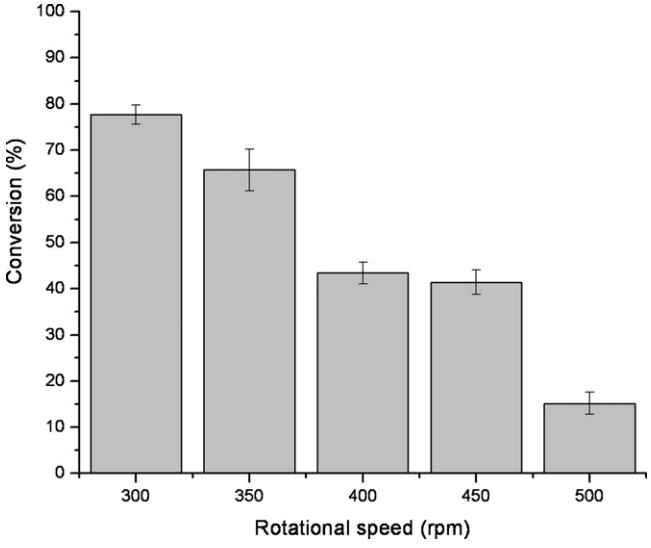

Fig. 6. Effect of stirring rate on synthesis of galactose oleate ester. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) at 60 °C for 2 h.

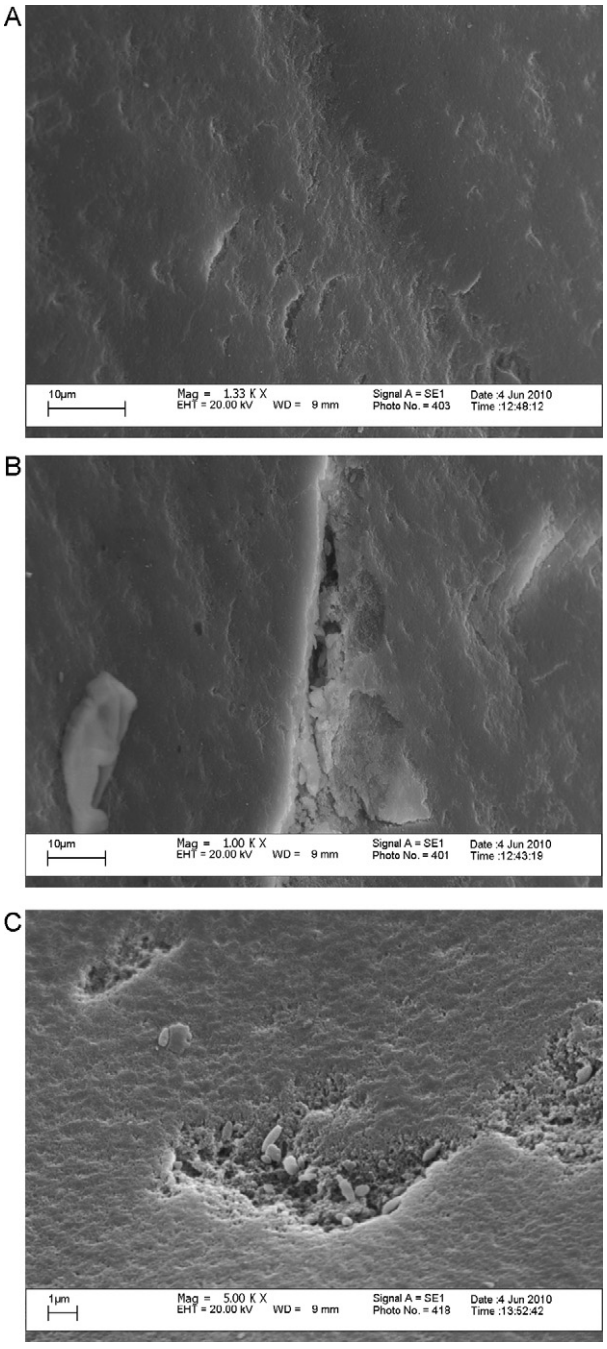

Fig. 7. SEM images of (A) Fresh Lipozyme RM IM; (B) Lipozyme RM IM after 700 rpm; (C) Lipozyme RM IM after 900 rpm. Reaction conditions: galactose (0.05 mmol), oleic acid (0.1 mmol), Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) at 60 °C for 2 h.

3.5. Effect of reaction temperature

Optimal reaction temperature is required because temperature exerts a great influence on both substrate solubility and rate of esterification. Fig. 5 shows the effect of reaction temperature on galactose oleate synthesis where conversion increases gradually with the temperature up to a maximum conversion of 77% at 60 °C. The temperature increase will improve the enzyme activity, the substrate solubility, reduce viscosity, thus promoting higher conversion [31]. In addition, reaction rates will increased with temperature due to a higher number of collision between enzyme and substrates molecules. However, it was reported that further increase in temperature, above 60 °C, led to thermal deactivation of the enzyme [1,21].

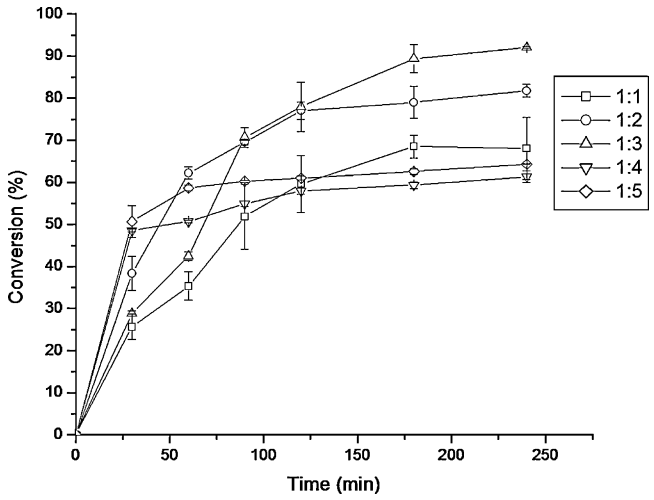

Fig. 8. Effect of galactose/fatty acid ratio on the synthesis of galactose oleate ester. Reaction conditions: Lipozyme RM IM (2%, w/w), DMSO:ILs (1:20) at 60 °C, galactose (0.05 mmol) and oleic acid (0.05 mmol (□); 0.1 mmol (○); 0.15 mmol (△); 0.2 mmol (▽); 0.25 mmol (◊)).

3.6. Effect of stirring rate

Stirring is an important parameter to increase the substrate mass transfer to the enzyme active site and to facilitate the product release from the enzyme. Reactions were conducted at stirring rates from 300 to 900 rpm. Results depicted on Fig. 6 indicate that a low stirring rate of 300 rpm was the optimum with maximum conversion of 77%. Lower conversions at higher stirring rates (up to 900 rpm) could be due to the damages observed on the surface of enzyme's support as suggested by our SEM experiments (Fig. 7). Consequently, free enzyme may have been released and denatured rapidly due to the hydrophilic environment [22]. Furthermore, a high stirring rate is not suitable and is undesirable in large-scale study.

3.7. Effect of substrate molar ratio

Increasing the substrate availability to the enzyme catalytic site can improve lipase-catalyzed reaction thus leading to higher conversions. Since enzyme-catalyzed reactions are reversible, one substrate is often used in considerable excess to displace the equilibrium towards the synthesis. In this study, the galactose:oleic acid molar ratio varied from 1:1 to 1:5 and reaction kinetics are depicted on Fig. 8. Conversion increased with the molar ratio from oleic 1:1 to 1:3. The highest conversion (87%) was reached for the molar ratio 1:3. Increasing fatty acid concentration may facilitate further acylation that leads to higher conversions [7]. When the molar ratio further increased up to 1:5, the conversion dropped. This may be due to the higher amounts of oleic acid introduced which limit mass transfer. Indeed, it was observed that the reaction media became more viscous at high amounts of oleic acid [22]. Limitation of galactose concentration in the reaction also contributes to low conversion as concentration of oleic acid increased [32]. Furthermore, remaining and excess oleic acid may cause difficulties for the product separation.

4. Conclusions

Sugar solubility is the major problem in sugar ester synthesis. This problem can be solved by using DMSO as the sugar solubilizing

agent. Only a very small amount of DMSO (1:20 (v/v) DMSO:IL) was required to reach a high conversion and limit enzyme deactivation. Moreover, high conversion (87%) was achieved in less than 2 h, to compare to conventional method that usually needs several hours or days. DMSO is a great solubilizing agent as galactose was completely dissolved after stirring for 10 min at 60 °C. Parameters affecting the sugar ester synthesis (reaction media, type of biocatalyst, amount of biocatalyst, reaction time, temperature, stirring rate and substrate molar ratio) were studied to achieve maximum conversion rate at the optimum conditions. [Bmim][BF_4] was the most suitable reaction media in combination with DMSO as co-solvent for lipase-catalyzed esterification. The highest conversion for the synthesis of galactose oleate ester (87%) was achieved under these conditions: 2% (w/w of substrates) of Lipozyme RM IM, reaction carried out at 60 °C for 2 h at a stirring rate of 300 rpm and with a 1:3 galactose:fatty acid molar ratio.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.12.004.

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