Immobilized Lipase-Catalysed Synthesis of Fructose Oleate Ester in Ionic Liquid [Bmim][TfO] and *tert*-Butanol Solvent Systems

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Fructose oleate is one of the most sought-after sugar esters, but its production is challenging due to the poor solubility of its substrates. However, 1-butyl-3-methylimidazolium (Bmim)-based ionic liquids can solubilize a wide range of substrates and prevent denaturation of enzymes as their physical and chemical properties can be easily tailored. In this work, the enzymatic synthesis of fructose esters using immobilized lipase was investigated using a series of Bmim-based ionic liquids and *tert*-butanol as solvents. The reaction conditions were optimized for different esterification parameters, such as fatty acid chain length, enzyme loading, reaction temperature and time. The immobilized lipase was found to be more stable in [Bmim][TfO] and had a high affinity towards the monounsaturated acyl donor, oleic acid. Optimum ester conversion (73%) was achieved under ideal reaction conditions comprising low enzyme loading (5% w/w) and a reaction temperature of 60 °C for 72 hours. The synthesis of fructose oleate in [Bmim][TfO] under these optimized conditions has great potential for use in many applications.

Keywords: Fructose oleate ester; surfactant; sugar ester; lipase; ionic liquids

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Sugar fatty acid esters (SFAEs) are an interesting class of surfactants produced via the esterification reaction between a sugar and a fatty acid. These non-ionic and odourless surfactants have been used in many industries, including food, cosmetics, detergents and pharmaceuticals, due to their excellent functionalities in detergency, stabilization and emulsification [1–3]. The Food and Drug Administration (FDA) previously approved sucrose fatty acid esters as safe for use in the food industry [4]. Interestingly, studies have found that fructose esters possess a higher interfacial tension value as well as better emulsion stability and initial foaming ability when compared to sucrose esters [3,5]. However, the production of fructose esters remains a challenging task. SFAEs are usually synthesized at high temperatures in the presence of mineral or heterogeneous solid acid catalysts. The process may produce undesirable sugar ester isomers due to the substitution of the alkyl group on unintended positions, and cause product discolouration after long reaction times [6,7]. Moreover, some of the by-products may be toxic and non-biodegradable.

Enzymatic synthesis of SFAEs catalyzed by free or immobilized lipase isolated from *Candida rugosa* [8,9], *Candida antarctica* [10–13], *Candida cylindracea* [14], *Bacillus subtilis* [15] or *Rhizomucor miehei* [16,17] is remarkably more selective. The enzymatic synthesis can be conducted under mild reaction conditions with less toxic or non-toxic solvents. However, poor substrate solubility and inactivation of enzymes are the major contributing factors that affect conversion efficiency. Several researchers have tried to solve the solubility issue and prevent deactivation of the enzymes while gaining a high conversion by rate using protecting group [16,18], mixed organic solvent [19–21] or solvent-free [17,22] strategies. However, these reaction systems had high volatility for the mixed organic solvent system, and high viscosity and low miscibility for the solvent-free system. Hence, the focus of the present study is on the enzymatic synthesis of SFAE using an alternative solvent system with ionic liquids to mitigate these drawbacks.

Bmim-based ionic liquids have been amply demonstrated to be a superior solvent for esterification reactions, producing higher ester conversions compared to organic solvents [23–25]. In our previous study, we described a fast and improved synthesis of galactose oleate ester in a Bmim-based ionic liquid under optimal synthesis conditions [26]. Nevertheless, there have been no reported studies on the synthesis of sugar esters from fructose and oleic acid, specifically in 1-butyl-3-methylimidazolium (Bmim)-based ionic liquids as a reaction media, to address the above issues. Enzyme-catalysed synthesis of fructose oleate has been commonly performed in organic reaction media and thus constrained by substrate solubility [27–29]. Therefore, Bmim-based ionic liquids were selected as

an alternative solvent due to their high solubility and thermal stability, low volatility and tuneable chemical composition by selecting different cations, anions, and substituents [30–32]. These properties may be ideal for the lipase-catalysed esterification of fructose oleate as some of the issues, including substrate solubility, enzyme selectivity or stability and conversion rate, may be resolved.

In this study, fructose oleate production via enzymatic synthesis using immobilized lipase, Novozym 435, was performed in three ionic liquids [Bmim][BF_4], [Bmim][TfO], [Bmim][PF_6] and tert-butanol, and the results were compared. The effects of several esterification parameters such as fatty acid chain length, enzyme loading, reaction temperature and time on the conversion of fructose oleate esters were also studied and further optimized to obtain the maximal product yield in a cost-effective manner.

MATERIALS AND METHODS

Materials

Commercial immobilized lipase, Novozyme 435 (*Candida antarctica* B, 10,000 propyl laurate units g^{-1}) was purchased from Novo Nordisk A/S (Copenhagen, Denmark). 1-butyl-3-methyl-imidazolium hexafluoro phosphate [Bmim][PF_6], 1-butyl-3-methyl-imidazolium tetrafluoroborate [Bmim][BF_4], 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][TfO], and tert-butanol were purchased from Merck (Darmstadt, Germany). Lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, and fructose were purchased from Sigma-Aldrich (USA). All chemicals and solvents used in this study were of analytical grade unless otherwise stated.

Fructose Solubility Study

0.9 g (0.5 mmol) fructose was weighed into four screw-cap glass vials. To each vial was added 1 mL of one of the four solvents, 1-butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF_4], 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6], 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][TfO] and tert-butanol. The vials were stirred at 200 rpm for 6 hours at room temperature (28 ± 1 °C). The resulting mixtures were classified and recorded based on their physical appearance, including whether they were soluble (clear liquid), slightly soluble (slightly milky liquid) or insoluble (milky or no change).

Enzymatic Synthesis of Fructose Ester

In general, the lipase-catalysed synthesis of fructose oleate was performed in a solid-phase system containing equimolar amounts (0.5 mmol) of fructose and fatty acid and 1 mL of solvent to stabilize the catalytic phase for the action of the lipase. Approximately 15 % (w/w) of a molecular sieve (3 Å) was added to remove the water formed during esterification. The direct esterification of fructose and fatty acid was started by adding 5 % (w/v) of immobilized lipase. The reaction mixture was incubated at the desired temperature and agitated at 200 rpm in a water bath for up to 72 hours. After the reaction was complete, tetrahydrofuran (THF) was added to the reaction mixture which was then filtered to recover the enzyme. The extraction filtrate containing the ionic liquid could be reused, while the THF was removed by vacuum evaporation. 1 mL of deionized water was then added to the system, and the suspension was filtered. The white semi-solid product was loaded onto a silica gel column and eluted with methanol/acetonitrile (75:25, v/v). The purified sample was freeze-dried and subjected to further analysis.

In this study, the effects of five independent variables on the esterification were studied by varying the type of the solvent ([Bmim][BF_4], [Bmim][PF_6], [Bmim][TfO] and *tert*-butanol), fatty acids chain length (C_{12}-C_{18}), amount of enzyme loading (5-25%), reaction temperature (25-60 °C) and reaction time (up to 72 hours). The percentage of fatty acid conversion during esterification was then quantified.

Quantification of Fatty Acid Conversion

Samples were taken from the reaction mixture at defined intervals, and the reaction was terminated by dilution with 5 mL of ethanol/acetone (50/50, v/v). The immobilized lipase was then removed by filtration. The remaining free fatty acid in the reaction mixture was quantified by titration with 0.1 M NaOH to an endpoint of pH 10 using an Autotitrator (808 Titrando System, Metrohm, Malaysia). The amount of acid reacted was calculated using Equation (1). The ester formed was expressed as equivalent to the conversion of the acid.

$$\text{Percentage of conversion (\%)} = \frac{V_{\text{control}} - V_{\text{sample}}}{V_{\text{control}}} \times 100\% \quad (1)$$

where $V_{control}$ is the volume of 0.1 M NaOH needed to titrate the control, and V_{sample} is the volume of 0.1 M NaOH needed to titrate the sample.

Identification of Fructose Ester

The product was analysed using Fourier-transform infrared (FTIR) spectroscopy to identify the fructose ester. A drop of the sample was placed between two NaCl plates to form a thin film. The plates were put into the sample holder and analysed directly using the spectrophotometer (Perkin-Elmer Model 1650, USA). The ester absorption bands were evaluated to determine the presence of fructose ester in the product. The product of the reaction synthesis was also analysed using a high-performance liquid chromatography (HPLC) system (Merck, Darmstadt, Germany) equipped with evaporative light-scattering detectors (PL-ELS 1000, Polymer Laboratories Darmstadt, Germany) and a Hypersil™ ODS C_{18} column. The analysis was performed using a filtered mobile phase of methanol:acetonitrile:water (75:20:5, v/v/v) at a flow rate of 0.25 ml/min. The column temperature was kept constant at 40 °C during analysis.

RESULTS AND DISCUSSION

Influence of Solvent on the Synthesis of Fructose Oleate

Bmim-based ionic liquids have become increasingly popular in replacing conventional organic solvents [26, 33, 34]. Aside from reducing the solubility issues usually encountered by conventional organic solvents, we previously found that the structural stability of enzymes was also increased in Bmim-based ionic liquids [35]. Only the enzyme's surface was affected due to the presence of the alkyl chain of the $[Bmim]^+$ cation that allowed preservation of the essential water layer around the enzyme. The inner part of the enzyme remained unaffected, thus reducing direct protein-ion interactions and preserving its catalytic function. These properties make Bmim-based ionic liquids ideal for enzymatic reactions of sugar fatty acid esters (SFAEs). However, there have been no detailed analyses of fructose esters in ionic liquids involving [Bmim][PF_6], [Bmim][BF_4] and [Bmim][TfO] as reaction media for enzymatic esterification. Therefore, prior to synthesis, the solubility of fructose in ionic liquids was investigated and compared with *tert*-butanol as shown in Figure 1.

Fructose was found to be more soluble in [Bmim][BF_4] and [Bmim][TfO] compared to [Bmim][PF_6] and *tert*-butanol. The solubility of fructose depends on the hydrophilicity of the solvent; [Bmim][BF_4] and [Bmim][TfO] are hydrophilic while [Bmim][PF_6] is a hydrophobic ionic liquid. The percentage conversion of fructose oleate as determined by the volumetric method was found to be correlated with the solubility of fructose in the solvent. Figure 2 illustrates that both hydrophilic ionic liquids produced higher product yields than their counterparts. The product yield was highest in [Bmim][TfO] (58%) followed by [Bmim][BF_4] (33%), *tert*-butanol (14%) and [Bmim][PF_6] (10%). Similar results were reported by Tukel et al. [36] as the percentage conversion of fructose stearate ester by Novozyme 435 decreased in the order of [Bmim][TfO]>[Bmim][BF_4]>tert-butanol>[Bmim][PF_6]. A previous study on the free-energy of the reaction also indicated that hydrophilic anions such as $[BF_4]^-$ and $[TfO]^-$ provided better solvation to polar substrates such as fructose, compared to hydrophobic anions such as $[PF_6]^-$ [37]. Also, the high viscosity of [Bmim][PF_6] may have limited the mass transfer of the substrate to the enzyme's active site, leading to a decrease in ester conversion. Hence, [Bmim][TfO] was the chosen solvent for the following experiments.

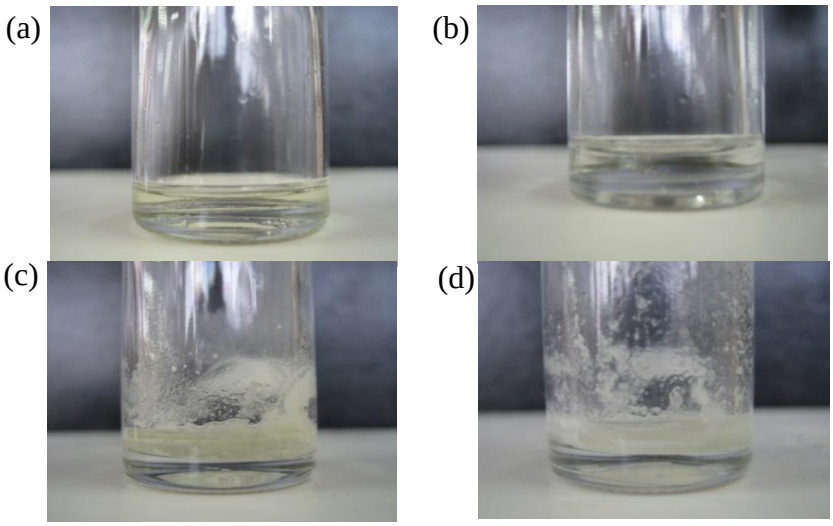

Figure 1. Solubility of fructose in different solvents: (a) [Bmim][BF_4], (b) [Bmim][TfO], (c) [Bmim][PF_6], and (d) tert-butanol.

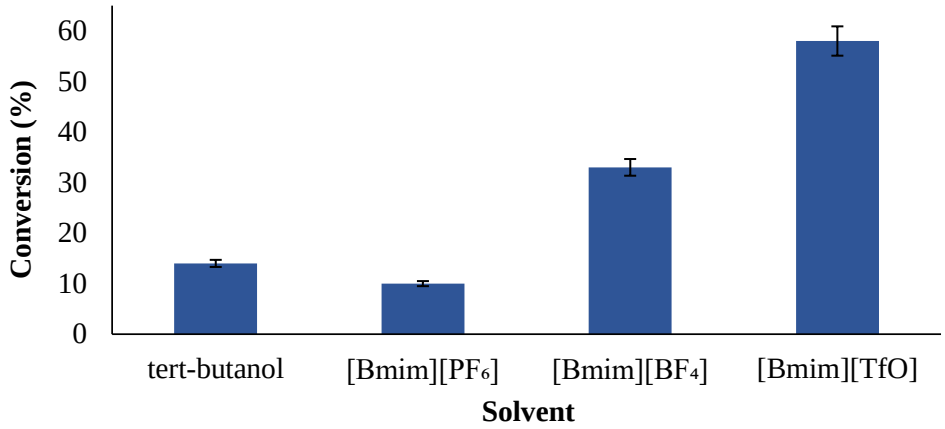

Figure 2. Effect of solvent on the synthesis of fructose oleate ester. Reaction conditions: 0.5 mmol fructose, 0.5 mmol oleic acid, 15% (w/v) molecular sieve 3 Å, 5% (w/v) Novozym 435, 50°C, 200 rpm, 24 hours.

Influence of the Fatty Acid Chain as Acyl Donor

The effect of fatty acid chain length (C_{12}-C_{18}) as an acyl donor in the synthesis of fructose esters is illustrated in Figure 3. Ester conversion was found to be inversely proportional to the saturated fatty acid chain length, with lauric acid, C_{12} producing the best yield (58%), followed by myristic acid, C_{14} (47%), palmitic acid, C_{16} (47%) and stearic acid, C_{18} (41%). This result is consistent with a recent study showing that the immobilized enzyme Novozym 435 exhibited substrate specificity for short-chain fatty acids [38]. Srilatha et al. also observed a similar result for the Nb_2O_5-catalyzed esterification of fatty acids with chain lengths of more than eight carbons [39]. Generally, it was found that the increased fatty acid chain length had a retarding effect on the conversion rate. This finding is probably due to steric effects and the polarity of the aliphatic system, which hindered the access of long-chain saturated fatty acids to the active sites [38, 40, 41]. In contrast, a short-chain saturated fatty acid has a smaller size and lower viscosity as the interaction between molecules are relatively smaller [42]. Therefore, it can easily access the enzyme's active site, leading to an increase in ester conversion.

It is interesting to note that oleic acid ($C_{18:1}$), did not follow this trend, as it gave the highest ester conversion of 61% at 24 hours reaction time. This monounsaturated fatty acid showed higher solubility and lower viscosity than long-chain saturated fatty acids in the reaction mixture containing [Bmim]TfO], which resulted in lower friction against the enzyme molecule and a higher rate of conversion. Oleic acid has also been shown to be more stable than other unsaturated fatty acids [43], which may broaden the range of potential applications of fructose oleate ester in many areas. Hence, oleic acid was selected as the acyl donor for the following experiments.

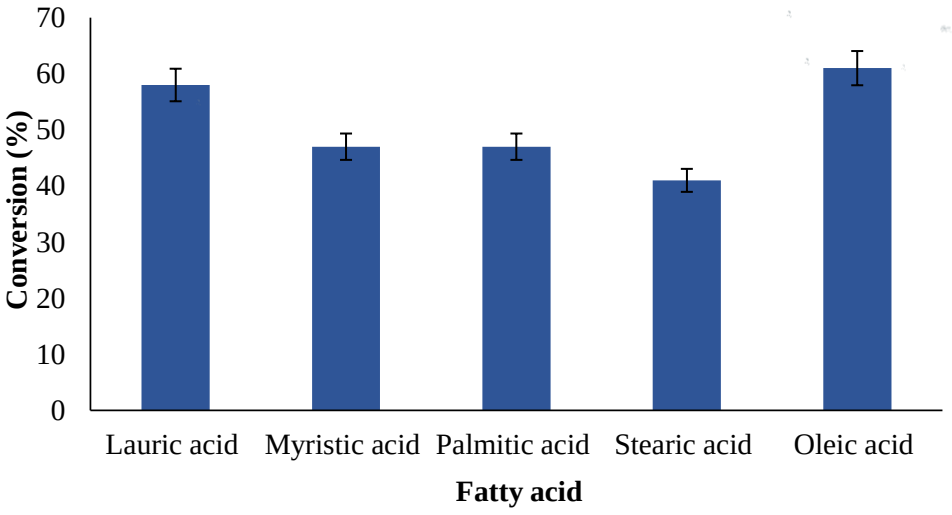

Figure 3. Effect of fatty acid chain length on the synthesis of fructose esters. Reaction conditions: 0.5 mmol fructose, 0.5 mmol fatty acid, 1 mL [Bmim][TfO] 15 % (w/v) molecular sieve 3 Å, 5 % (w/v) Novozym 435, 60 °C, 200 rpm at 24 hours.

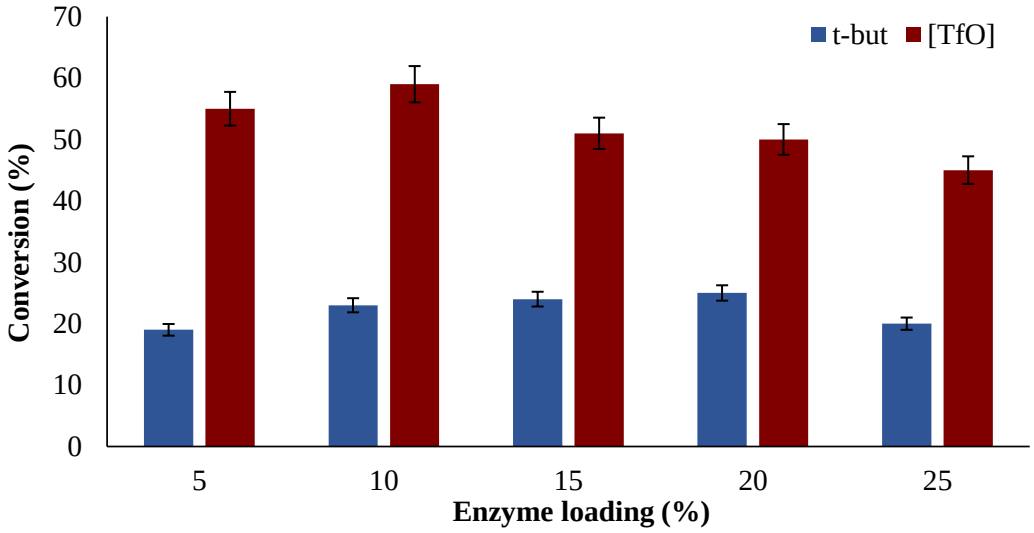

Figure 4. Effects of enzyme loading on the synthesis of fructose oleate ester in [Bmim][TfO] and *tert*-butanol solvent systems. Reaction conditions: 0.5 mmol fructose, 0.5 mmol fatty acid, 15 % (w/v) molecular sieve 3 Å, Novozym 435, 60 °C, 200 rpm at 24 hours.

Influence of Enzyme Loading on Esterification

Direct esterification can reduce the high production cost of SFAEs using conventional methods by eliminating protection and deprotection steps during the process. Determining the optimal enzyme loading in the reaction mixture is essential to optimize ester conversion. The results of the direct esterification of fructose and oleic acid in [Bmim][TfO] and *tert*-butanol with different amounts of immobilized lipase (5-25 % w/w), are shown in Figure 4. A lipase loading of 10% showed the highest ester conversion (59%) in [Bmim][TfO]. Conversely, only 25% ester conversion was achieved with 20% lipase in the *tert*-butanol solvent system after 24 hours of reaction time. Hence, a lower enzyme concentration was required to synthesis fructose oleate ester in [Bmim][TfO] compared to *tert*-butanol.

A comparable esterification trend was also reported for the synthesis of lipase-catalysed fatty acid esters in a mixture of methanol and 1-butanol [44]. The optimal yield was observed as the enzyme loading was increased to 20 %, due to the high availability of the active site to accommodate the substrate [45–47]. An increase in enzyme concentration above the optimal point resulted in decreased ester conversion. Under these conditions, the viscosity of the reaction medium will increase due to the aggregation of excess enzymes, which restricts the accessibility of the reactant to the active site [47]. Additionally, there was no significant difference in ester conversion at enzyme loading levels of 5 and 10 %. Therefore, 5 % (w/w) of immobilized lipase was chosen for further experiments.

Influence of Temperature on Esterification

Temperature also plays an essential role in the enzymatic synthesis of fructose ester due to its direct influence on product yield. The effect of reaction temperature (from 25 to 60 °C) on the immobilized lipase synthesis of fructose oleate ester is shown in Figure 5. The percentage of ester conversion was found to be directly proportional to the temperature. Initially, poor ester conversion was observed at low temperatures due to the high viscosity of the reaction medium which increased the mass transfer resistance and reduced enzyme activity. Then, the ester conversion rate exhibited a sharp increase at temperatures above 40 °C. The highest conversion was obtained at 60 °C, which produced a yield of approximately 49 % in the [Bmim][TfO] solvent system. This was attributable primarily to the higher reactant solubility as the temperature increased [17], leading to a large amount of reactant available for the ester conversion.

A previous study reported that the enzymatic esterification of methyl glucoside and palmitic acid using a similar immobilized lipase in a [Hmim] [TfO]/ 2M2B bisolvent system produced the highest yield at 45 °C [48]. The optimum production of fatty acid ethyl ester using the same lipase in a solvent-free system was achieved at 40 °C [49]. The immobilized lipase was found to be denatured when the reaction temperature exceeded 45 °C. Hence, increasing the temperature beyond the optimum point leads to decreased ester conversion due to thermal deactivation of the enzyme. These results suggest that the [Bmim] [TfO] ionic liquid used in this study increased the thermal stability of the immobilized lipase at higher temperatures of up to 60 °C. At these higher temperatures, more effective molecular collisions occur, thus increasing the esterification rate [50, 51].

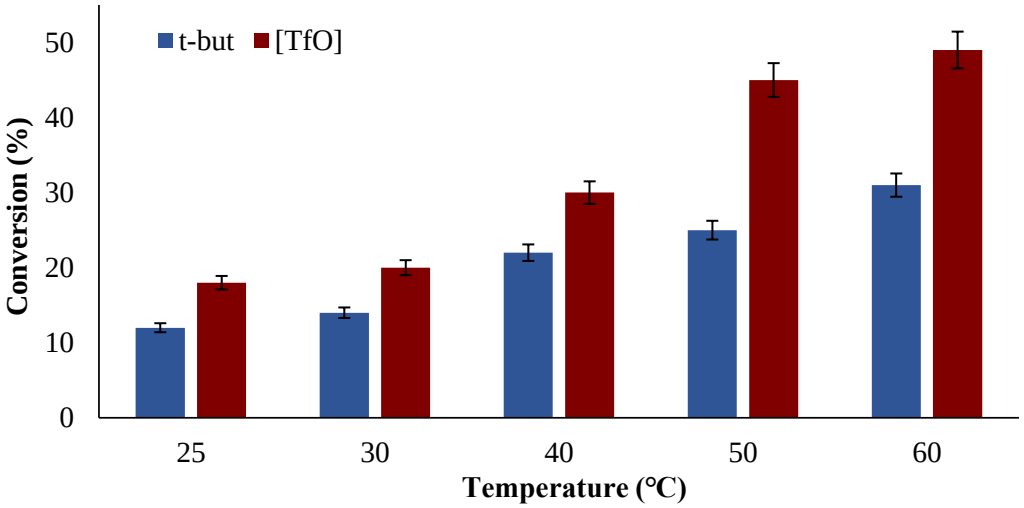

Figure 5. Effects of reaction temperature on the synthesis of fructose oleate ester in the [Bmim][TfO] and tert-butanol solvent systems. Reaction conditions: 0.5 mmol fructose, 0.5 mmol fatty acid, 15 % (w/v) molecular sieve 3 Å, 5 % (w/v) Novozym 435, 200 rpm, 24 hours.

Influence of Reaction Time on Esterification

The effect of reaction time on esterification was studied by varying the time from 6 to 72 hours. It was found that the ester conversion rate increased with reaction time (Figure 6). A gradual increase in esterification rate was observed in the *tert*-butanol solvent system, with the highest ester conversion (41 %) at 72 hours. In contrast, a significant increase in esterification rate was observed in the [Bmim][TfO] solvent system after 6 hours, and the maximum conversion (73 %) was achieved at 72 hours. These results indicate that the [Bmim][TfO] solvent system could provide a high esterification yield and conserve the catalytic activity of the immobilized lipase over a longer period.

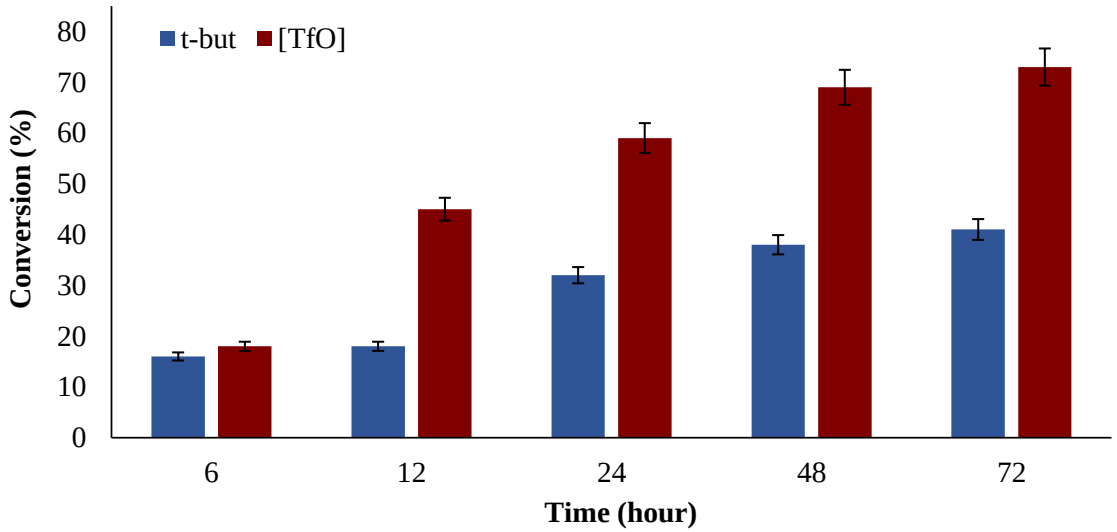

Figure 6. Effects of reaction time on the synthesis of fructose oleate ester in the [Bmim][TfO] and tert-butanol solvent systems. Reaction conditions: 0.5 mmol fructose, 0.5 mmol fatty acid, 15 % (w/v) molecular sieve 3 Å, 5% (w/v) Novozym 435, 60 °C, 200 rpm.

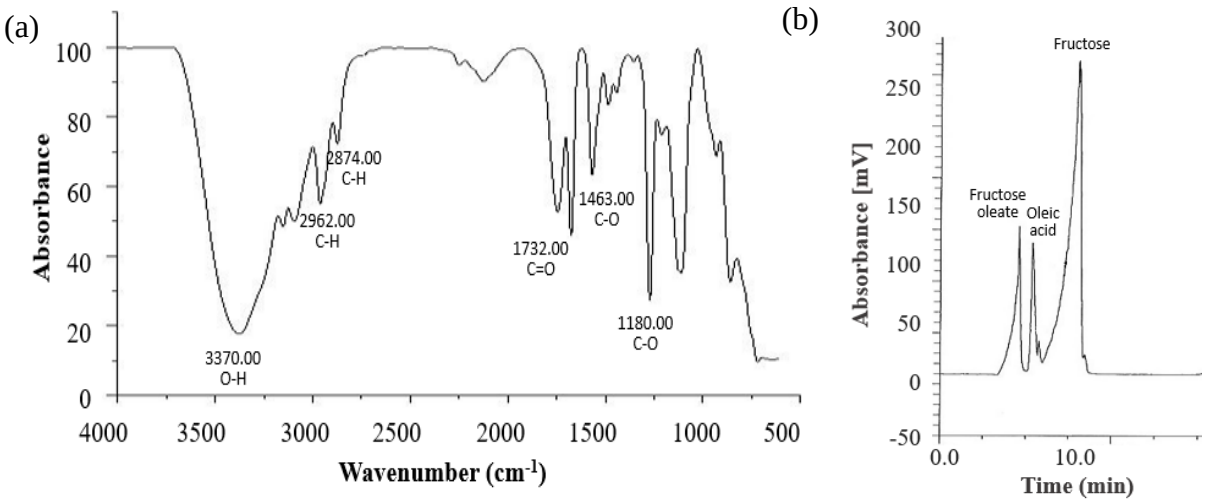

Figure 7. (a) FTIR spectrum of fructose oleate, and **(b)** HPLC chromatogram of the reaction mixture containing fructose oleate, oleic acid and fructose.

Product Identification

The presence of an ester was confirmed by Fourier Transform infrared spectroscopy (FTIR) analysis (Figure 7(a)). The FTIR spectrum of fructose oleate displayed absorption bands at 3363-3390 cm^{-1}, indicating the presence of OH bonds. The C-H stretching peak for a hydrocarbon chain was present at 2874-2962 cm^{-1} (C-H bond in $-CH_2$ or $-CH_3$). A significant peak at 1732 cm^{-1} was assigned to the C=O (ester) bond and another at 1463 cm^{-1} to $-CH_2$ and $-CH_3$ bonds. Meanwhile, the peak at 1180 cm^{-1} was due to C-O (ester) adsorption and another at 743 cm^{-1} to the CH_2 bond. The ester product was further analysed using high performance liquid chromatography (HPLC). Fructose oleate was eluted at a retention time of 4.61 min followed by oleic acid and fructose at 5.40 and 8.08 min, respectively (Figure 7(b)).

CONCLUSION

This study demonstrated the feasibility of the enzymatic synthesis of fructose oleate in Bmim-based ionic liquids. The immobilized lipase (Novozyme 435) exhibited higher stability in the [Bmim][TfO] solvent system and a higher affinity towards oleic acid as the acyl donor. The highest ester conversion (73%) was achieved under optimum reaction conditions with equimolar amounts of fructose and oleic acid (0.5 mmol) and 5 % (w/w) enzyme loading. [Bmim][TfO] was shown to improve the thermal stability of the immobilized lipase up to 60 °C over a long duration (72 hours), which broadens its range of potential applications.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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