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Microwave Assisted Enzymatic Synthesis of Fatty Acid Sugar Ester in Ionic Liquid-*tert*-Butanol Biphasic Solvent System

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Microwave enhanced enzymatic synthesis of fatty acid sugar ester (FASE), glucose oleate in biphasic solvent system comprising of an ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate, [BMIM][BF_4] and *tert*-butanol were studied comparative to other conventional heating's. The effect of biphasic solvents in synthesizing glucose oleate and optimization of reaction parameters, in terms of temperature, time and lipase were screened in microwave reactor relative to water bath shaker and hot plate stirrer. In microwave, the enzyme screening showed Novozym 435 had the optimal activity at 60 ºC and time 0.5 h and maximum conversion of oleic acid to glucose oleate was 90 %. Microwave proved to be an efficient heating system for glucose oleate synthesis at much reduced time than conventional heatings.

Key Words: Fatty acid sugar ester, Enzymatic synthesis, Microwave, Ionic liquid, Biphasic solvent system.

INTRODUCTION

Fatty acid sugar ester are non-ionic surfactants which are generally used in food, detergent, cosmetic and pharmaceutical industries due to their excellent emulsifying, stabilizing, hygroscopic and detergency effects[1,2]. Most recently, enzymatic synthesis of fatty acid sugar ester have received many interests because of its high-regioselectivity and mild reaction conditions which minimize by-products formation relative to the conventional chemical processes[3-5]. Lipases are the most remarkable catalyst for acylations of the hydroxyl groups in monosaccharides and have been well established in biotransformation process[6,7].

Nevertheless, lipase-catalyzed synthesis had been successfully applied in esterification of fatty acid sugar ester; the solvent media used are normally flammable and toxic to environment. As an alternative, ionic liquids (ILs) have emerged as a promising neoteric solvent for enzyme stabilization and a desirable substituent for organic solvents. Previous studies showed enzyme stability and substrate solubility are influenced by the properties of ionic liquids like [BMIM][BF_4] and others[8,9]. Surprisingly, in the recent investigation of Novozym 435 catalyzed transesterification of glucose in pure [BMIM][BF_4] was found inactive[10]. Thus, a small amount of *tert*-butanol was added to create a biphasic solvent system and to facilitate the enzyme activity in sugar ester production.

However, the developed two phase system was hampered by the time consuming conventional method which only gave 60 % of product conversion[10]. Current studies aim on performing organic synthesis like fatty acid sugar ester in microwave reactor because of its advantages in terms of efficient heating, cleaner productions and most essentially, higher yield production in shorter reaction time[11,12]. For that reason, in this research the study was mainly focused on developing a technique of synthesizing the lipase-catalyzed esterification of glucose oleate using the microwave. To our best of knowledge, the application of microwave irradiation on enzymatic esterification with biphasic system has not been reported yet.

EXPERIMENTAL

A commercially known biocatalyst, Novozym 435 an immobilized *Candida antarctica* lipase B on a macroporous acrylic resin was bought from Novozymes (Denmark). The specific activity for Novozym 435 is 10,000 PLU/g catalysts. A free lipase from *Candida rugosa* (CRL), D-(+)-glucose, molecular sieves (3 Å) and [BMIM][BF_4] were purchased from Sigma Aldrich (USA). Tetrahydrofuran (THF) and *tert*-butanol

were obtained from Fisher Scientific (UK). Oleic acid was bought from TCI (Japan).

General procedure for the synthesis of glucose oleate in biphasic solvent system was adapted from the previous literature[10].

Microwave heating synthesis: Typically, 0.50 mmol of D-(+)-glucose and 0.25 mmol of oleic acid were placed in microwave sample vial. Followed by, 5 % (w/v) of enzyme, 10 % (w/v) of molecular sieves (3 Å, 4-8 mesh) as a water adsorbent, 200.0 µL of *tert*-butanol, 300.0 µL of [BMIM][BF_4] and a magnetic micro stir bar (10 mm × 3 mm) were added to the same vial. The reaction mixture was irradiated with microwave (CEM Corporation) with power of 290 W and stirred at a medium speed rate. Controls (without enzyme and ionic liquids) were prepared too. The reaction was performed in the sealed vial which then compressed on top with the microwave unit. All experiments were tested in triplicate.

At the end of the reaction, the enzyme and molecular sieves were removed to quench the esterification process. The product in *tert*-butanol was extracted out with 15 mL of THF from the ionic liquids, [BMIM][BF_4] phase. The higher solubility of sugar in *tert*-butanol made the major part of the product being formed in the *tert*-butanol (top layer) then [BMIM][BF_4] (bottom layer). A two mL of the combined extracts was separated and titrated against 0.1 M of sodium hydroxide (aq) in the presence of two drops of phenolphthalein. The oleic acid conversion to glucose oleate was determined as the colour of phenolphthalein changes from colourless to pink.

The amount of oleic acid remaining in sample was identified relative to the control based on the conversion equation reported in the present literature[3]. The glucose oleate in the combined extract was recovered with removal of the excess THF solvent by rotary evaporation. Lastly, the product (white solid/viscous transparent liquid) was collected then characterized qualitatively by thin layer chromatography (TLC) and Universal Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (FT-IR) (Perkin-Elmer, 100 series) analysis.

Conventional heating: Meanwhile, for the conventional method, the ester was synthesized in water bath shaker (Hotech Instruments Corporation) and shaken with the speed of 200 rpm. The similar procedures as described in microwave heating had been repeated for this study.

Product detection: TLC analysis was performed on TLC plastic sheet silica gel $60F_{254}$ (Merck, Germany) and developed in the solvent system containing chloroform (70): methanol (20): acetic acid (8): water (2) (v/v/v/v). The plate was than treated with a cerium reagent (25.0 g of molybdato phosphoric acid, 1.0 g of cerium(IV) sulphate, 80 mL of concentrated sulphuric acid and 1000 mL of distilled water) and visualize by heating[10]. Further determination of glucose oleate formation was carried out using FT-IR analysis. The samples in this analysis were subjected to a diamond pressing method.

RESULTS AND DISCUSSION

The formation of glucose oleate was confirmed with TLC by observing the product (blue spot) comparative to the substrates (black spot). Further characterization of product by FT-IR analysis showed all the relevant peaks for functional groups of glucose oleate were noticeable in the spectrum. The FT-IR results are presented in the Table-1. The presence of C-O bond was confirmed by the sharp peaks at frequency absorptions between 1300 and 1000 cm^{-1}. The existence of carbonyl group C=O (ester) was proved by the presence of sharp peak between 1735 and 1750 cm^{-1}. The significant absence of broad hydroxyl functional group peak between 3200 and 3650 cm^{-1} in the spectrum has proven that the esterification reaction occurred between the D-(+)-glucose and oleic acid. The FT-IR spectrum of D-(+)-glucose, oleic acid and glucose oleate are shown in Figs. 1-3, respectively.

TABLE-1
FT-IR ANALYSIS

Functional group	Frequency absorption (cm^{-1})
C-O stretching	1284, 1168, 1052
C=O	1730
Aliphatic C-H stretching	2954
CH_2 bending	1450
C=C stretching	1680-1700

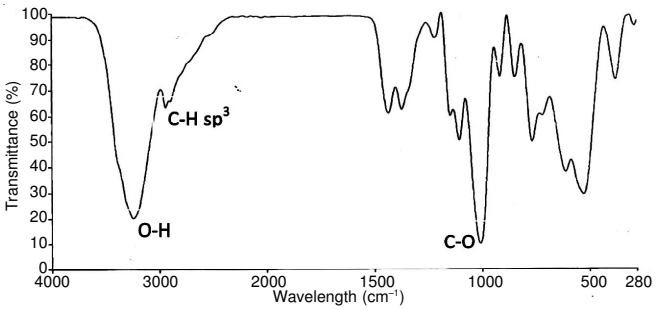

Fig. 1. FT-IR spectrum of D-(+)-glucose

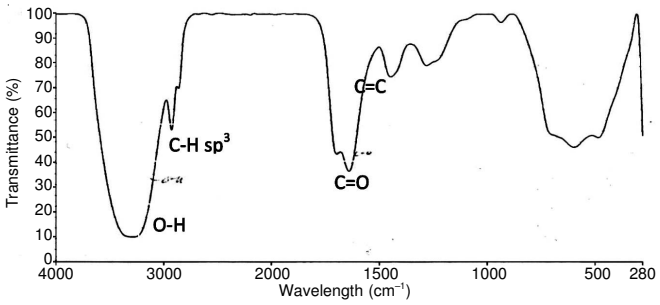

Fig. 2. FT-IR spectrum of oleic acid

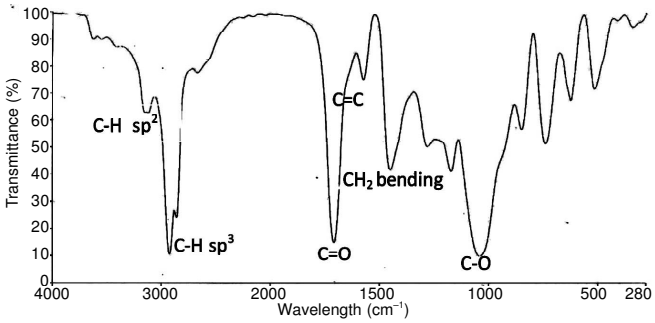

Fig. 3. FT-IR spectrum of glucose oleate

Influence of solvent system in lipase-catalyzed synthesis of glucose oleate: Initially, glucose oleate was synthesized in

a single phase containing [BMIM][BF_4]. There is no conversion found for this reaction media either in water bath shaker (Table-2, entry 14) or microwave irradiation (Table-3, entry 12). The solubility of D-(+)-glucose in [BMIM][BF_4] was very low. Meanwhile, synthesis of glucose oleate in pure *tert*-butanol shows the conversions were moderate in both water bath shaker (Table-2, entry 15) and microwave (Table-3, entry 11). Apparently, in the existence of *tert*-butanol, the D-(+)-glucose was seemed to be partially dissolved and the conversion was slightly improved. This outcome has been justified by In *et al.*, who underlined that high solubility of the sugar in the reaction media is needed for a high yield of fatty acid sugar ester[13].

TABLE-2
SYNTHESIS OF GLUCOSE OLEATE USING WATER BATH SHAKER (200 rpm) IN A (2:1) RATIO OF D-(+)-GLUCOSE AND OLEIC ACID[a]

Entry	Enzyme	Time (h)	Temperature (ºC)	Conversion (%)
1	CRL	24	40	41.80
2	CRL	48	40	43.84
3	CRL	72	40	45.13
4	CRL	24	60	43.99
5	CRL	48	60	49.27
6	CRL	72	60	52.47
7	Novozym 435	24	40	53.38
8	Novozym 435	48	40	54.85
9	Novozym 435	72	40	56.27
10	Novozym 435	24	60	60.70
11	Novozym 435	48	60	62.96
12	Novozym 435	72	60	69.80
13[b]	Novozym 435	24	60	71.00
14[c]	Novozym 435	24	60	n.d.[d]
15[e]	Novozym 435	24	60	24.37

[a]Reactions took place in biphasic solvent system. [b]Reaction took place in the hot plate with vigorous stirring. [c]Single phase containing [BMIM][BF_4]. [d]n.d. = not detected. [e]Single phase containing *tert*-butanol.

TABLE-3
SYNTHESIS OF GLUCOSE OLEATE USING MICROWAVE REACTOR IN A (2:1) RATIO OF D-(+)-GLUCOSE AND OLEIC ACID[a]

Entry	Enzyme	Time (min)	Temperature (ºC)	Conversion (%)
1	CRL	15	60	22.80
2	CRL	30	60	8.70
3	CRL	15	40	63.30
4	CRL	30	40	48.50
5[b]	CRL	15	60	12.18
6[b]	CRL	30	60	3.17
7	Novozym 435	15	60	65.10
8	Novozym 435	30	60	90.45
9	Novozym 435	15	40	48.20
10	Novozym 435	30	40	72.00
11[b]	Novozym 435	15	60	34.09
12[c]	Novozym 435	30	60	n.d.[d]

[a]Reactions took place in biphasic solvent system. [b]Single phase containing tert-butanol. [c]Single phase containing [BMIM][BF_4]. [d]n.d. = not detected.

However, the conversion of oleic acid to glucose oleate in single phase system (*tert*-butanol) was still lower compared to biphasic solvent system which contained both *tert*-butanol and [BMIM][BF_4]. The biphasic system has proven as an efficient reaction media in both classical heating (Table-2, entry 1-13) and microwave mode (Table-3, entry 1-4, entry 7-10). These conditions explained, lipases have a better stability or activity in the organic solvent with the presence of ionic liquids, [BMIM][BF_4] as an additive. The applications of ionic liquids in biocatalysis may provide more effective environment in terms of ionic liquids monarchy coated enzymes or substrate anchoring phenomenon[14]. The effect of reaction media on synthesis of glucose oleate is shown in Fig. 4.

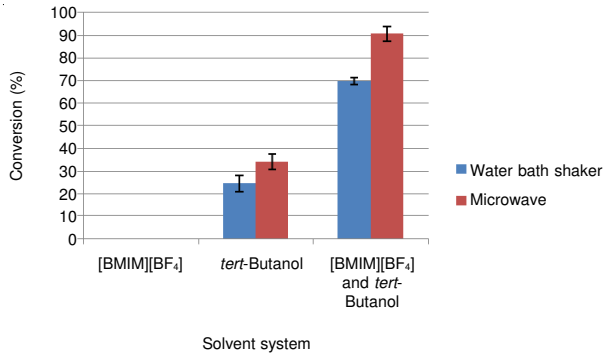

Fig. 4. Evaluation of esterification reaction in three different solvent media. The reaction mixtures comprised of D-(+)-glucose and oleic acid (2:1) and catalyzed by Novozym 435 at temperature; 60 ºC. The reactions were carried out in water bath shaker and microwave out at time; 24 h and 0.5 h, respectively

Stability of lipase under microwave irradiation: After 72 h of reaction time, synthesis glucose oleate in water bath shaker (200 rpm) was only 69.8 % (Table-2, entry 12). Meanwhile, in microwave, a maximum conversion of 90 % was achieved within 0.5 h when using Novozym 435 as the biocatalyst (Table-3, entry 8). It was thought that a low speed in water bath shaker might have caused low conversion of product. In view of this velocity, a reaction catalyzed by Novozym 435 was repeated with more vigorous stirring using a magnetic micro stir bar on a hot plate stirrer (CD-162, Stuart). Despite that, only 71 % conversion of product was obtained in 24 h (Table-1, entry 13). Though, dynamic stirring has been proven to give better conversion, yet the reaction time, 24 h is still not comparative with microwave reaction times, 15 and 30 min. Synthesis of glucose oleate with three different reactors are shown in Fig. 5.

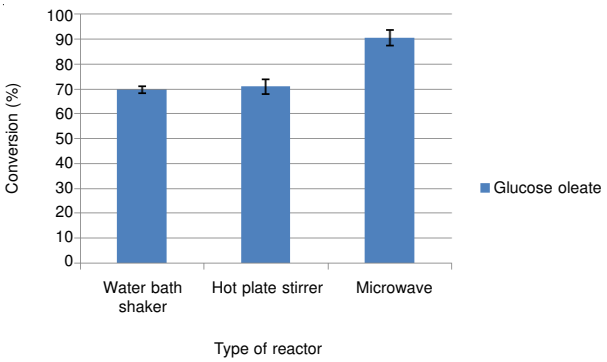

Fig. 5. Comparison of glucose oleate synthesis with three different reactors (water bath shaker, hot plate stirrer, microwave) at temperature; 60 ºC and time; 72, 24 and 0.5 h, respectively. The reactions were catalyzed by Novozym 435 and carried out with D-(+)-glucose and oleic acid (2:1) in biphasis system of [BMIM][BF_4] and *tert*-butanol

Effect of temperature: In microwave at 60 ºC, the CRL catalyzed esterification reaction was significantly lower than at 40 ºC (Table-3, entry 1-4). Conversely, at 60 ºC in water bath shaker, CRL exhibited the best catalytic activity for glucose oleate synthesis (Table-2, entry 4-6). The deactivation of CRL at higher temperature probably caused by a temperature fluctuation trend happened in the microwave irradiated reaction. For instance, when a reaction was subjected to 40 ºC heating in the microwave caused the temperature fluctuated to 60 ºC and above. While for reaction carried out at 60 ºC, the temperature has raised to 90 ºC. However, after a couple of seconds, the temperature started to drop as the power detection in microwave has commenced to reduce the heating temperature back to 60 or 40 ºC.

The incident above can be further explained by a superheating phenomenon that experienced by the polar solvents in the microwave[15]. Previous study reported, *tert*-butanol and [BMIM][BF_4] have moderately high dielectric constant of 12.47[16] and 11.7[17], respectively. In addition, they have low heat capacities in the range of 1-2[18]. These distinctive combinations have made them absorbed microwaves easily and being heated up rapidly above their boiling points[19].

The power operation was another important factor which influenced the temperature fluctuation in microwave. In this experiment, the esterification reaction was controlled at 290 W. Normally, high power usage caused instant temperature raised in microwave. This condition agreed with a literature attributed by Saxena *et al.*[20], where high power employed in microwave caused higher reaction rates to be exhibit in a very short time. The statement here is able to explain the incident that observed in this study whereby the significant temperature fluctuation in microwave is due to the very high power (290 W) usage as this would cause the high dielectric constant solvents like [BMIM][BF_4] and *tert*-butanol to absorb the microwave energy intensively and made the heat transferring between the polar substrates too rapid until the temperature in the microwave is hard to be control.

Therefore, too high power set-up will initiates the polar solvents to be heated very fast, as the environment in microwave gets hot immediately. Thus, reaching high temperature around 90 ºC within 0.5 min. Generally, high dielectric constant solvent needs only a small power to reach the desire temperature[12]. These circumstances has rationalized the synthesis of glucose oleate at 60 ºC with the power control 290 W in microwave is not suitable for biocatalyst like CRL. Free lipase like CRL easily denatured when the temperature fluctuated beyond 60 ºC where the peptide bonds and amino acid side chains became reactive and lead to undesirable reactions[21].

Nevertheless, the problem has no strong impact on immobilized lipase, Novozym 435 showed higher conversion at 40 ºC and even better at 60 ºC. Unlike, CRL has lower optimum reaction temperature of 40 ºC. These conditions illustrated that the immobilization of lipase on the acrylic resin could be attributed to higher thermo-resistance property[22] that made the Novozym 435 to have better tendency to withstand the extreme temperatures fluctuations[23] in microwave.

Effect of reaction time in microwave: There are two different reaction times being studied in microwave which were 15 and 30 min (Table-3, entry 1-4). The optimized time for CRL in microwave is 15 min. It seems that, CRL was inefficient in catalyzing the acylation reaction at 0.5 h. Most likely by the time of 0.5 h the CRL has denatured due to the drastic temperature fluctuation in the microwave. Otherwise, it was perhaps the CRL hydrolyzed the esters back into D-(+)-glucose and oleic acid[24]. For Novozym 435, the reaction time 15 min was insufficient to catalyze the esterification reaction to the completion. The immobilized lipase worked better at longer time (Table 2, entry 8) and 0.5 h was found to be the optimal reaction time for it. (Figs. 6 and 7) showed the respective graphs of esterification catalyzed by CRL and Novozym 435.

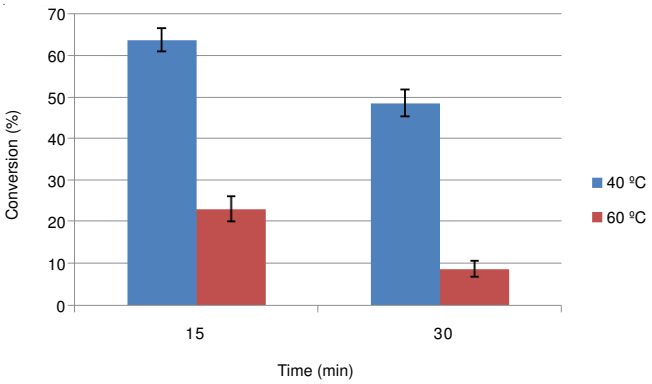

Fig. 6. Effect of reaction time and temperature on stability of lipase, CRL in microwave. The reaction were performed with D-(+)-glucose and oleic acid (2:1) in biphasis system containing [BMIM][BF_4] and *tert*-butanol at different reaction parameters

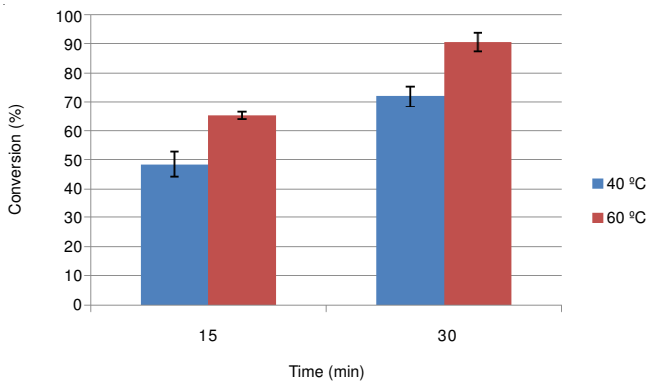

Fig. 7. Effect of reaction time and temperature on stability of Novozym 435 in microwave. The reactions were performed with D-(+)-glucose and oleic acid (2:1) in biphasis system containing [BMIM][BF_4] and *tert*-butanol at different reaction parameters

Synergism of microwave and biphasic solvent system on stability of Novozym 435: There was an advantage of choosing [BMIM][BF_4] and *tert*-butanol as the components of two phase system in the microwave reactor. In this research, the [BMIM][BF_4] and *tert*-butanol acted as a hydrophobic solvents which does not absorb the essential hydrophilic water layer from the enzyme vicinity. In conjunction to this phenomenon, an interesting behaviour has been reported by Hua *et al.*[19] in microwave enhanced synthesis. The Novozym 435 in microwave was activated by a superheating layer of water molecules that surrounded the enzyme particles. Such layers have a high dielectric constant that can be formed when the hydrophobic solvents presence as reaction media. Hence,

it's not surprising that in microwave, Novozym 435 most likely facilitated a much better conversion of glucose oleate than CRL.

Conclusion

The study shows clearly that microwave heating can be successfully applied to improve reaction conversion rate at a much lower reaction time. Novozym 435, the immobilized enzyme has better stability hence it gave a better conversion than CRL under the microwave irradiation conditions (60 ºC and 0.5 h). Biphasic solvent system of *tert*-butanol and [BMIM][BF_4] gave a better performance than *tert*-butanol or [BMIM][BF_4] alone. Microwave assisted enzymatic synthesis of fatty acid sugar ester in two phase systems is only a preliminary study. More microwave-based synthesis like esterification of other renewable monosaccharides and effect of ionic liquid hydrophobicity in microwave are currently under progress.

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