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Physicochemical Characterization of Lactose-Based Ester as Potential Pharmaceutical Biosurfactant

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Abstract

Enzymatic processes offer an alternative for the synthesis of biosurfactants through the employment of biocatalysts, which allow for a mild reaction condition and high selectivity. Fatty acid sugar esters, a group of biosurfactants, are produced by the esterification of sugars with fatty acids. They are odorless, non-toxic and non-irritant to the skin, making them suitable not only as emulsifiers for foods, but also in pharmaceuticals and cosmetics. Moreover, due to their high biodegradability and varied range of hydrophilic-lipophilic balance (HLB) values, the study and production of fatty acid sugar esters (FASEs) have attracted keen attention from many researchers. A biochemical approach has been implemented through the use of lipase immobilized on an inexpensive carrier of mica clay as biocatalyst (NER-CRL). The synthesis of FASEs or sugar-based biosurfactants was optimized via various reaction parameters before conducting product characterization and validation. In this study, an optimized esterification condition was employed for the synthesis of FASEs, specifically lactose ester. The synthesized lactose monocaprate with molecular formula $C_{22}H_{40}O_{12}$ was physicochemically identified to examine their efficacy for industrial application. Interestingly, it was found that the new synthesized lactose caprate (biosurfactant) derived from green enzymatic esterification of lactose and capric acid had calculated HLB value of 14.88, which is suitable for the preparation of oil-in-water (O/W) emulsions. Furthermore, this non-ionic biosurfactant (yellowish) was found to behave like a water-soluble surfactant and an O/W emulsifier which potentially used for food, pharmaceuticals and detergent industries.

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Introduction

The development of green surfactants based on natural renewable resources is a concept that is gaining recognition in pharmaceutical and cosmetics [1]. Instead of their application in industrial detergents, the oil-in-water surfactants also have been used as emulsifiers in food products and in the manufacture of medications for parenteral administration [2]. Amongst the surfactants, fatty acid sugar esters (FASEs) have attracted attention of biotechnological researchers due to many advantages such as availability in a wide range of hydrophilic-lipophilic balance (HLB) values, biodegradability, non-toxic, tasteless and non-irritancy to the skin and eyes [3]. FASEs that are synthesized from fatty acids and carbohydrates have broad applications in the food and pharmaceutical industries [4]. Various sugar esters are commercially available and are used in variety of applications in the food, pharmaceutical and personal care industries generally functioning as non-ionic emulsifiers. With regards to the sugar, lactose is a by-product in cheese production and is an inexpensive carbohydrate source [5]. The US produces 40 million pounds of lactose, a by-product per year. Thus, there is a need to find more value-added uses for lactose. Lactose esters can be synthesized by either chemical or enzymatic means. The chemical method leads to formation of unspecific products while the enzymatic means are better for synthesizing sugar monoesters. Due to poor selectivity of chemical syntheses, the enzymatic regioselectivity acylation of sugar offers an alternative to synthesize FASEs enzymatically, which allow a mild reaction condition and more selective towards substrates. Recently, our previous research proved that the lipase catalysed regioselective esterification of lactose sugar has shown a better alternative to chemical synthesis as it requires lower reaction temperatures and high operational enzyme stability comparatively, thereby producing higher yield of sugar ester [6]. However, the lactose esters produced under optimum enzymatic reaction condition and their physicochemical properties have not been reported before, therefore this work will be novel research leading to potential of new pharmaceutical ingredients. Furthermore, the use of low cost immobilized lipases that we prepared for this work had possessed efficient biocatalytic performance and more economically for the production of lactose ester over using high cost of commercial immobilized enzymes [7]. Thus, this work was aimed to synthesize FASEs of lactose monocaprate enzymatically under optimized reaction condition, followed by characterization of physicochemical properties of lactose monocaprate ester product as potential biosurfactant.

An immobilized lipase (NER-CRL) previously prepared was used in this experiment. Lactose monohydrate, capric acid, Tween 80, refractometer, and solvents used were of analytical grade (AR) and purchased from Sigma-Aldrich.

Under optimized esterification condition as previously reported 6,7, enzymatic esterification system consisted of lactose and capric acid (molar ratio of 2:1), molecular sieve and selected immobilized lipase, NER-CRL (containing 2.14 mg protein) in acetone (10.0 mL). The reaction mixture was incubated at 55°C for 48h, with continuous

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shaking at 250 rpm in a horizontal water-bath shaker. The reaction was terminated by the addition of 5.0 mL ethanol:acetone (1:1, v/v) mixture and the biocatalyst was filtered out. Five replicates of experiment were done and their reaction products were pooled prior to rotary evaporation. The amount of the lactose monoester synthesized was determined via HPLC.

The solvent was removed by rotary evaporation. The semi-solid product was then dissolved in a solution of methanol:acetonitrile (70:30, v/v) before being identified using TLC, GC-MS and UHPLC. Samples of lactose esters were separated on a thin layer of silica gel plate (TLC) with chloroform (100% vol) as an optimized developing solvent system. The plates were treated with cerium reagent and visualized under UV. The product was further analyzed qualitatively using UHPLC (Universiti Sains Islam Malaysia). The HPLC system (Agilent 1290 Infinity UHPLC) consisted of automatic injector, a 1200 Series ELSD detector and a software (Version Chemstation Revision B.0402) for control of the system and processing of signals. Aliquots of 1.0 µL of the reaction mixtures were injected into the column. For separation purpose, a RP-C18 column (particle size 5 µm), maintained at 40°C was used. The mobile phase was methanol: acetonitrile: water (50:35:15, v/v/v) with flow rate of 0.5 mL min-1. GC-MS analysis of product was performed on a Shimadzu (model GC-17 A., model MS QP5050A; Shimadzu, Tokyo, Japan) instrument using a nonpolar column (silica capillary column SGE BPXS, 30 m, 0.25 mm ID, 0.25 µm thickness). The carrier gas was helium at a flow rate of 2.9 mL/min with injection volume of 2.0 µL. The column temperature was programmed at 60°C. The analysis was conducted at Universiti Putra Malaysia.

Lactose caprate products were determined for their physicochemical properties such as hydrophilic-lipophilic balance (HLB) value, physical appearance, solubility, refractive index and irritancy test. HLB value was calculated from the partial weights of a hydrophilic group from the whole compound. The HLB number of a material was given by the following equation [8]:

$$\text{HLB value, } \text{HLB}_{\text{G}} = 20\left(\frac{M_H}{M}\right)$$

HLB number ranges from 1 to 20, corresponding to the most lipophilic and the most hydrophilic property. Other properties of the product were analyzed and compared with the commercially available surfactant product, namely Tween 80. Solubility of the product was determined in various solvents, while refractive index value was determined by refractometer at room temperature. The product was evaluated with the irritection test in order to predict their potential in causing dermal irritation. Standard volume dependent dose-response studies based on cosmetic protocol were performed with the Dermal Irritection Assay test method (Malaysian Palm Oil Board).

The selected immobilized lipase (NER-CRL) was employed as biocatalyst in esterification reaction. Under optimal reaction conditions, product of lactose caprate ester was successfully synthesized with 75 - 85% of ester conversion, at 48 h, temperature of 55°C, molar ratio of lactose to capric acid (2:1) in acetone. The development of products from the esterification reaction of lactose sugar and different fatty acids catalyzed by immobilized lipase were ascertained by TLC. Figure 1 illustrates the behavior of lactose esters on TLC. Five TLC plates (1, 2, 3, 4 and 5) representing the spots of the raw materials of lactose sugar (a) and fatty acids (b), while the spots of sugar esters (products) were marked as (c). The spots of lactose esters (c) seemed to appear at a bit lower level than fatty acids. In fact, the lactose esters were more polar than fatty acids (but less polar than pure lactose sugar) due to the dominant of multiple hydroxyl groups (OH) from lactose structure present in the esters. Each lactose ester had one principal spot, believed to be the monoester, which appeared close to the origin. Rf values of different sugar esters spots were; LC = 0.23, LD = 0.26, LL = 0.3, LM = 0.33, LP = 0.43, while the Rf values for different fatty acid spots were CA = 0.55, DA = 0.57, LA = 0.58, MA = 0.5, PA = 0.63. Depending on their chain length of esters, Rf values of different esters was ranged from 0.23 - 0.43.

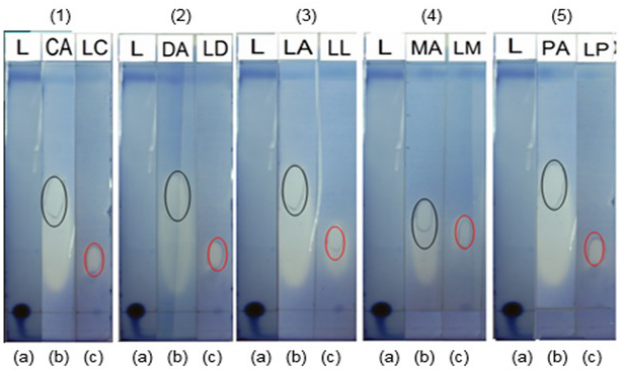

Figure: Thin layer chromatograms of lactose esters (lc: lactose caprylate; ld: lactose decanoate/caprate; ll: lactose laurate; lm: lactose myristate; lp: lactose palmitate) catalyzed by ner-crl. (l: lactose; ca: caprylic acid; da: decanoic acid/ capric acid; la: lauric acid; ma: myristic acid; pa: palmitic acid).

In addition, the substrates and ester product were also characterized by ultra high-performance liquid chromatography (UHPLC). The peaks at the retention time of 0.45-0.54, 0.96 and 4.26 minutes (Figure 2) correspond to lactose sugar (1), lactose monocaprate (2), and capric acid (3), respectively. The analysis showed lactose monocaprate ester to be the main product which appeared at the retention time very close to that of the free sugar, while the two minor peaks (*) appeared might be unknown trace. It was shown that successful amount of sugar ester, namely lactose monocaprate (0.9 min) was produced, with very low amount in trace level of unreacted lactose and capric acid (4.2 min) were also observed. This result was believed due to the presence of immobilized enzyme as biocatalyst in the esterification reaction system which highly selective towards reacting with substrates to produce specific product. As shown, the reaction catalyzed by this immobilized lipase was very selective towards the formation of monoester, namely lactose caprate. Products of the reaction were ascertained using gas chromatography-mass spectrometry (GC-MS)(Figure 3). The corresponding ester compound of lactose monocaprate ($C_{22}H_{40}O_{12}$) can be recognized by mass spectra (a), in which the characteristic of molecular ion fragmentation at *m/z* 495 (M^{-1}) is present. Other bonds cleavage occurred through some pathways and gave further fragmentation ions at*m/z* 43, 73, 99, 171 and 325 (b).

Characterization of the sugar esters is as important as the synthesis reaction in order to examine their efficacy as surfactants in industrial application. The physicochemical properties of sugar esters as surfactants are of critical importance in determining their economic values. One of the most important characteristics of a surfactant which is frequently used for predicting surfactant behavior is the hydrophilic-lipophilic balance (HLB)

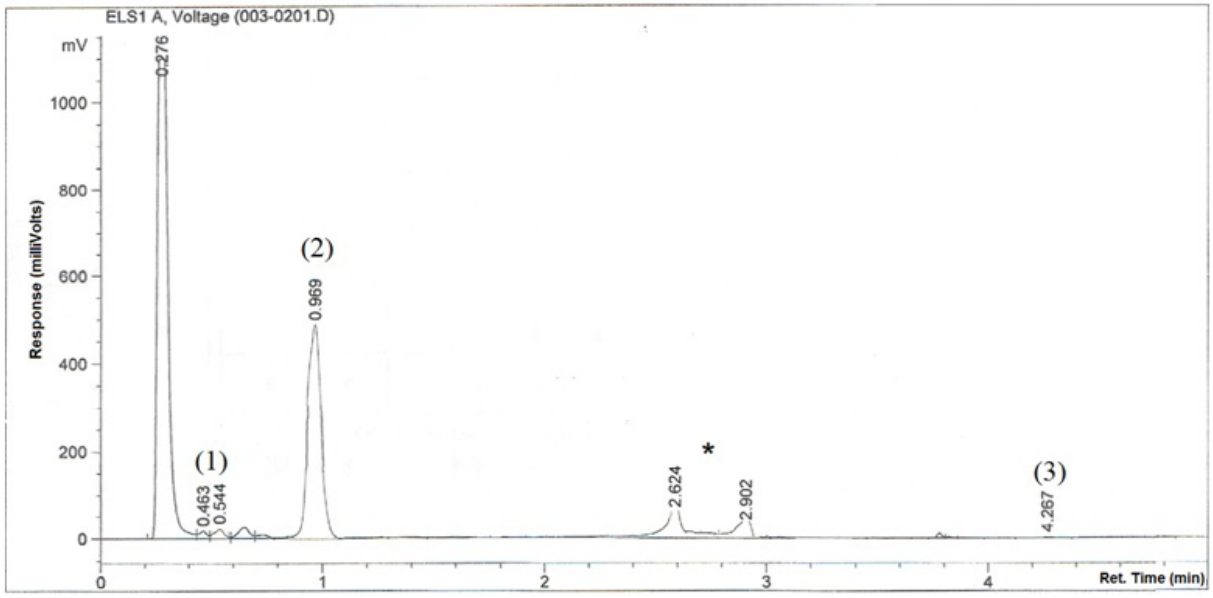

Figure 2: Uhplc chromatogram of lactose sugar (1), lactose monocaprate ester (2) and capric acid (3), respectively.

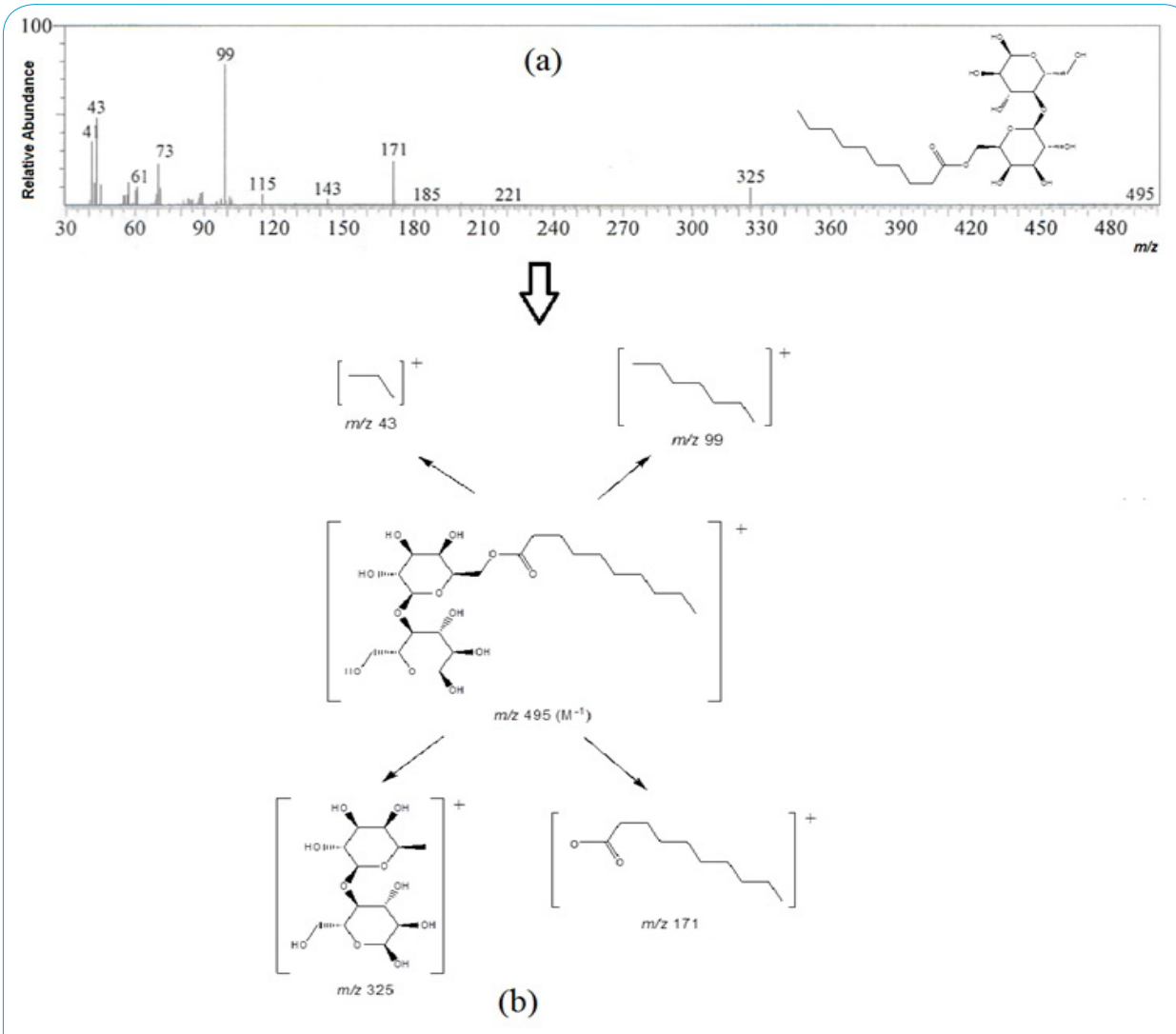

Figure 3: Mass spectra of lactose monocaprate ester (a) and its ion fragmentation scheme (b).

Characterization of the sugar esters is as important as the synthesis reaction in order to examine their efficacy as surfactants in industrial application. The physicochemical properties of sugar esters as surfactants are of critical importance in determining their economic values. One of the most important characteristics of a surfactant which is frequently used for predicting surfactant behavior is the hydrophilic-lipophilic balance (HLB) value. Furthermore, sugar esters of lactose monocaprate are relatively rare compound and research on their properties is limited, thus some physicochemical tests of lactose caprate esters were investigated. The properties of synthesized surfactant of lactose monocaprate ester are presented in Table 1. The corresponding physicochemical value of commercial surfactant is also included in the table for comparison purposes. The chemically prepared surfactant, sorbitan ester or commercially also known as Tween 80 is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid.

Properties	Sugar esters (Synthesized surfactant)	Tween 80 (Commercial surfactant)
Synonyms	Lactose monocaprate	Polyoxyethylene (20) sorbitan monooleate, Sorbitan ester
Molecular formula	$C_{22}H_{40}O_{12}$	$C_{64}H124O26$
HLB value [a]	14.88	15 ± 1.0
Physical state	Yellowish liquid	Oil-like liquid (viscous)
Solubilityin b : - water - methanol - ethanol - acetone - chloroform - isopropanol	Soluble Soluble Soluble Soluble Soluble Not analyzed	Soluble Soluble Soluble Not analyzed Not analyzed Soluble
Refractive index [b]	1.45	1.47
Odor	Fatty (slight)	Fatty (slight)
Irritancy test [c]	Only slightly irritant	Slightly irritant (in case of skin contact to more sensitive individuals)

Table 1: Physicochemical characteristics of synthesized sugar esters

[a]Hydrophile-lipophyle balance, as analyzed using the Griffin's method.
[b]The analyses were conducted at 25 °C
[c]The analyses were carried out at Efficacy Laboratory, Malaysian Palm Oil Board (MPOB), Advanced Oleochemical Technology Centre, Bandar Baru Bangi, Selangor.

Selections of surfactants are highly depending on their HLB requirement. The HLB system is particularly useful to identify surfactants for oil and water emulsification. Water-in-oil emulsions (w/o) require low HLB surfactants, while oil-in-water (o/w) emulsions often require higher HLB surfactants. Generally, formulators use surfactants for the functional properties they provide in products such as conditioning, wetting, foaming and providing detergency and emulsification. This study showed that the biosurfactant derived from lactose and capric acid had an HLB value of 14.88, which is very closed to the value of a commercial surfactant compound namely Tween 80. The hydrophilic moieties in the synthesized compound are multihydroxyl groups of disaccharide which consists of glucose and galactose saccharide units. Meanwhile, the lipophilic moieties in this compound are hydrocarbon chains of the fatty acids of C10. Mass of the whole surfactant molecule of lactose caprate is 496.25 g/mol. The higher HLB value of the lactose monocaprate ester has shown that the synthesized surfactant is more water soluble. Generally, surfactants with the low HLB values of 3-8 are better suited for the preparation of water-in-oil (w/o) emulsions, whereas the surfactants with the high HLB values of 9-18 are suitable for the preparation of oil-in-water emulsions (o/w) [10]. The system depends on the solubility of the surfactant related to the percentage by weight of the hydrophilic portions of the molecule, and is relatively independent of the nature of the fatty acid groups.

Color is an important indication of product composition, purity, and degree of deterioration. Hence, color measurement is used for quality monitoring, production control, and determination of final product conformance to predetermined color tolerance and of compliance with customer specifications [11]. Generally, the colors of esters are light yellow to brown color. This study had shown that the physical appearance of the lactose caprate is yellowish liquid, which is almost similar with the commercial surfactant (Tween 80) having an appearance of oil-like liquid but a liitle bit more viscous. The resultant lactose caprate ester synthesized by an enzymatic process had performed as a water soluble of hydrophilic surfactant, and has a potential to be upgraded for utilization as biodegradable surfactants.

On the other hand, refractive index is commonly used as a rapid method to monitor chemical reaction wherein the measurement is correlated to a chemical property. The measurements were carried out using refractometer on the basis of ratio of the speed of light in air to speed of light in the liquid. Refractive index value of lactose caprate esters (1.45) was observed to be almost similar as for Tween 80. According to Fereidoon [12], at constant temperature, the refractive index increased with increase in length of hydrocarbon chain as well as with the increase in the number of double bonds (unsaturation) in molecules. Conjugation and polymerization are reported as well to contribute in increase of refractive index in oil and fat [13]. Thus, Tween 80 was expected to exhibit higher refractive index compared to lactose caprate esters since the presence of polyethoxylated sorbitan compound together with the unsaturated oleic acid structure contributed to the longer total carbon chain length. Generally, chemically prepared surfactants have been known to give odorless or somehow, a slightly fatty odor. The enzymatically synthesized surfactants of sugar esters were found to have a slightly fatty odor as also shown by Tween 80.

Based on dermal irritection assay by means of cosmetic protocol (Table 1), the synthesized biosurfactant was found to possess only a slightly irritating (in minor case of skin contact to more sensitive individuals). Therefore, the overall results on the physicochemical studies reveal the suitability of the lactose monocaprate esters produced as potential biosurfactants in industrial applications specifically in detergency, pharmaceutical and food applications.

Conclusion

Sugar ester of lactose monocaprate, a biosurfactant was successfully synthesized via esterification of lactose sugar with capric acid under optimized reaction condition. In this work, immobilized lipase (NER-CRL) was used as biocatalyst for the enzymatic reaction. Both chemical and physicochemical properties of resultant lactose ester was identified and characterized by comparing with the commercial surfactant of Tween 80. The results have shown that synthesized lactose monocaprate ester had a hydrophilic-lipophilic balance (HLB) value of 14.88, thus performs a promising oil-in-water (O/W) emulsion to be used as potential biosurfactant in pharmaceutical application.

Competing Interests

The authors declare that they have no conflict of interest in this work.

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