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Enzymatic Synthesis and Characterization of Sucrose Erucate

The enzyme catalyzed synthesis is a environmentally friendly route compared to traditional syntheses. The lipase-catalyzed synthesis of sucrose erucate was achieved in a solvent mixture of *t*-butanol and dimethyl sulfoxide (4 : 1) by esterification of sucrose with erucic acid using the immobilized *Thermomyces lanuginosus* lipase. Various process parameters like temperature, substrate molar ratio, solvent, time, and enzyme loading were studied. The optimal conditions for the esterification reaction obtained were 10 %(w/w) enzyme loading, a molar ratio sucrose/fatty acid 1 : 1, mild reaction conditions (50 °C and atmospheric pressure) and reaction time (40 h with 55.6 % conversion). The sugar esters were characterized for surfactant properties at different concentration. Sucrose erucate showed a surface tension of (32.73 ± 0.01) mN m^{-1}at a critical micellar concentration of 9.8×10^{-5} mol L^{-1} and good emulsification power.

Key words: Sucrose erucate, *Thermomyces lanuginosus* lipase, esterification, surfactant

Enzymatische Synthese und Charakterisierung von Saccharose-Erucat. Die enzymkatalysierte Synthese ist ein umweltfreundliches Verfahren im Vergleich zu herkömmlichen Synthesen. Die lipasekatalysierte Synthese von Saccharose-Erucat wurde in einem Lösungsmittelgemisch aus *t*-Butanol und Dimethylsulfoxid (4 : 1) durch Veresterung von Saccharose mit Erucasäure unter Verwendung der immobilisierten *Thermomyces lanuginosus*-Lipase erreicht. Es wurden verschiedene Prozessparameter wie Temperatur, Substrat-Molverhältnis, Lösungsmittel, Zeit und Enzymbeladung untersucht. Die optimalen Bedingungen für die erhaltene Veresterungsreaktion betrugen 10 % (Gew./Gew.) Enzymbeladung, ein Molverhältnis von Saccharose zu Fettsäure von 1 : 1, milde Reaktionsbedingungen (50 °C und atmosphärischer Druck) und eine Reaktionszeit von 40 Stunden für eine Umwandlung von 55,6 %. Die Zuckerester wurden hinsichtlich ihrer Tensideigenschaften bei unterschiedlicher Konzentration charakterisiert. Saccharose-Erucat zeigte eine Oberflächenspannung von (32,73 ± 0,01) mN m^{-1} bei einer kritischen Mizellenbildungskonzentration von 9.8×10^{-5} mol L^{-1} und ein gutes Emulgiervermögen.

Stichwörter: Saccharose erucate, *Thermomyces lanuginosus*, Veresterung, Tensid

1 Introduction

Surfactants are attaining enormous growth owing to the rising demand of end user applications such as cosmetics, pharmaceuticals, food and beverage, biotechnology, industrial cleaning, leather, textiles, paint coating and others [1–3]. Biosurfactants are produced by microbial fermentation or enzyme catalyzed reaction using different substrates like fatty acids, proteins, carbohydrates, glycolipids, lipopeptides, lipoproteins etc. With regard to their high levels of chemo-, regio- and stereoselectivity, cleanness and ease of administration the enzyme catalyzed reactions are an alternative to several reactions which are difficult to perform with the chemical catalysts. Enzymatic processes are considered as "green" because of the mild operating conditions and high selectivity without any byproduct [4]. In the context of green process the use of a renewable feedstock is also an important concern. This has led to the use of naturally obtained raw materials like carbohydrates from agricultural crops and fatty acids from vegetable oils as substrate.

Many sugars are obtained from agricultural resources. Among these sucrose is especially interesting because of its large production scale across the globe [3]. Sucrose is a naturally occurring compound which is mainly obtained from sugar-cane, sugar-beet and sugar-palm. It is a nonreducing saccharide containing D-glucose and D-fructose, glycosidically linked through their anomeric carbon atoms. The bulk of sucrose is used as a sweetener in food industry. Apart from human consumption a very low quantity of sucrose is directly used as an industrial raw material. Erucic acid a monounsaturated omega-9 fatty acid is obtained from rapeseed oil or mustard oil.

Sugar fatty acid esters are nonionic surfactants obtained by esterification or transesterification of sugar and fatty acid/fatty acid methyl ester. Esters are made from renewable natural resources and contain neither sulfates nor phosphates, and have a number of advantages over conventional surfactants like low toxicity, readily biodegradability etc. [5].

Currently, conventional chemical production of sucrose esters is carried out by the transesterification reaction between sucrose and methyl ester in the presence of an alkaline catalyst like potassium carbonate [6], alkali soap [7, 8], and anhydrous disodium hydrogen phosphate [9]. The process requires a high reaction temperature and a polar aprotic solvent like dimethylsulfoxide (DMSO), dimethylformamide (DMF). In chemical processing, it is difficult to achieve a regioselective acylation of sucrose due to the similar reactivity of the three primary and the eight secondary hydroxyl groups. In addition, there is the problem of intramolecular migration of the acyl group during processing. Intramolecular migration can be prevented by protection and deprotection but this complicates the synthesis. In the chemical processes colored side products are formed and hence numerous purification steps are required. The sugar fatty acid ester synthesized by a chemical process may not be employed in food application because of the usage of toxic organic solvents such as tetrahydrofuran or DMF during processing.

In the present work, the key parameters of sucrose fatty acid ester synthesis catalyzed by the lipase enzyme were investigated. The percentage of the investigated esterification was 60 % at the optimized conditions which are the temperature, the sugar-fatty acid ratio, the time and the enzyme loading. Additionally, this report encompasses different surfactant properties of sugar ester like reduction in surface tension, interfacial tension, foamability, wetting properties and emulsification.

2 Experimental Procedure

2.1 Materials

The immobilized *Thermomyces lanuginosus* lipase (Lipozyme TLIM) was obtained as a gift sample from M/s Brenntag Ingredients (India) Private Limited, Gurgaon, India, which was produced by submerged fermentation of a genetically modified *Thermomyces lanuginosus* microorganism and is immobilized in granulate form. Its activity was determined as 250 IUN g^{-1}. Erucic acid was obtained as a gift sample from M/s Godrej Industries Ltd., Mumbai, India. Sucrose, *t*-butanol and dimethyl sulfoxide were procured from M/s Himedia Ltd., Mumbai, India. All used chemicals were of analytical reagent grade.

2.2 Esterification reaction

The enzymatic synthesis of sucrose erucate was carried out in an organic solvent using Lipozyme TLIM. The synthesis experiments were conducted in a 250 ml conical flask by addition of 0.01 mol of sucrose and 0.01 mol of erucic acid. A mixture of approximately 25 ml *t*-butanol and DMSO (4 : 1) was used to dissolve the substrate. Activated molecular sieves (12 % w/w) were added to the reaction mixture to absorb the moisture. The reaction mixture was stirred for 30 min at 40 °C on orbital shaker. The substrate was charged after complete dispersion of enzyme (10 % w/w). The reaction mixture was stirred in orbital incubator shaker with 210 rpm for 48 h. The reaction temperature was maintained at 50 °C. The solvents were stored over molecular sieves (3 Å) for 24 h before use to remove moisture present in solvent. The reaction progress was checked by thin layer chromatography (TLC) and by checking the acid value of reaction mixture.

After completion, the reaction mixture was transferred to a beaker and mixed with 2 times its volume of the same solvent which was used in the reaction. The reaction mixture was kept for separation by gravity to remove the molecular sieves and the immobilized enzyme. The supernatant was collected and washed with hexane to remove the residual fatty acid. For a complete separation of fatty acid, the product was purified by column chromatography using silica gel (300 – 400 mesh). The elution phase was a mixture of chloroform and methanol (80 : 20 v/v), and the separation was monitored by thin-layer chromatography. The remaining solvent was evaporated under reduced pressure.

2.3 TLC analysis

The formation of sucrose erucate was confirmed by TLC, on silica gel (Make: Merk DC Kieselgel 60 F_{254}) as the stationary phase. Migration of different components took place from the solvent baseline towards the solvent front using a mobile phase which consists of chloroform, methanol, acetic acid and water in a ratio of 35 : 10 : 4 : 1 (v/v) [10]. About 5 µl samples were applied to baseline. The migrated sucrose esters were visualized by bial's reagent (orcinol/ferric chloride diluted with ethanol 4 volume) after spraying or dipping the TLC plate, and heating in hot air oven at 120 °C for 10 min [11].

2.4 Spectroscopic analysis using Fourier Transformed Infrared (FTIR)

The functional group analysis of the sucrose ester was carried out by using FTIR spectroscopy. The FTIR spectra were recorded on a Miracle 10 Shimadzu 8000 equipped with single reflection. Attenuated total reflection (ATR) accessory for liquid sample and diffuse reflectance spectroscopy (DRS) were used for powder samples. A regular scanning range of 4 000 – 400 cm^{-1} was used for 45 repeated scans. All the spectra were recorded with transmission mode.

2.5 Spectroscopic analysis using Mass Spectrometry

Mass spectra were acquired on the Thermo Finnigan LCQ Advantage mass spectrometer system. The scan range was adjusted to 500 – 1 000. A solution of sucrose erucate in methanol was infused into the electrospray ionization source. The molecular weight was determined by the intensity of the peak.

2.6 Surfactant Properties

The surface tension of surfactant solutions at an air-liquid interface and interfacial tension of liquid-liquid interface was determined by Krüss Tensiometer (Model: K100) using a standard Wilhelmy platinum plate (PL01) as the probe for measurement. The temperature was maintained at (25 ± 0.5) °C by a thermostatic bath during measurement. The accuracy of the measurements was controlled by the surface tension measurement of distilled water before each set of measurements. The interfacial tension was measured against *n*-heptane. The critical micelle concentration (CMC) was determined based on the variations in surface tension with respect to concentration as per the standard methods [12]. From the plot of the change in surface tension values against surfactant concentration, CMC was determined from the point of slope change. Wetting properties were measured by the canvas disk method [13]. All the measurements were performed in triplicate for accuracy. Emulsification power was evaluated according to Subrahmanyam and Achaya [14]. The separation of two phases was observed visually. Foamability of surfactant solution at different concentration was measured using a Ross-Miles apparatus [15].

3 Results and Discussion

3.1 Effect of solvent

The equilibrium constant of the esterification reaction depends on the concentrations of both reactants. To facilitate the conversion the reactants should be soluble in the reaction medium in which the enzyme is active and stable [16]. Sucrose is soluble in polar solvents like DMSO, DMF and hardly soluble in organic solvents, but the removal of these solvents is difficult due to their high boiling point, also enzymes were inactivated by polar solvents. Different solvent systems were screened for the esterification reaction. The use of a solvent mixture favored the conversion as shown by Ferrer [17]. The percentage of esterification was observed maximum (Fig. 1) in *t*-butanol and DMSO (4 : 1). With increasing the proportion of DMSO the conversion was decreased due to the altered structural stability in solvent [18]. Many authors reported the use of DMSO in combination with other solvents, as reaction media for enzymatic esterification [17, 19].

3.2 Effect of molar ratio

The reaction was performed at different molar ratios of sucrose and erucic acid. As shown in Fig. 2, the maximum

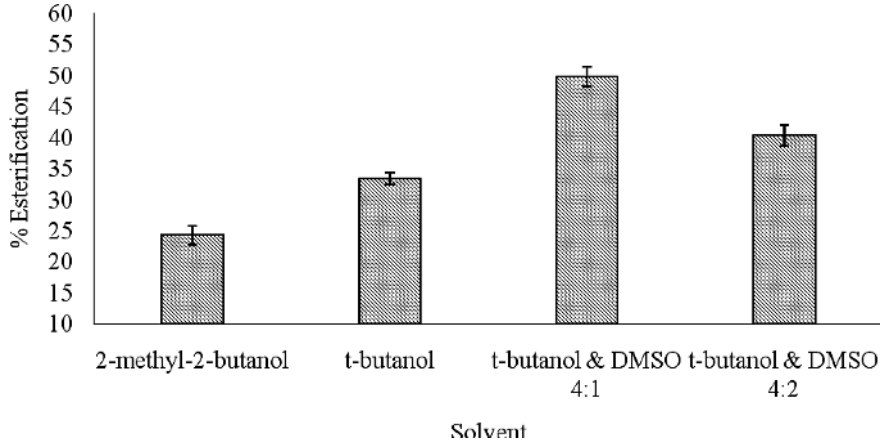

Figure 1 Effect of solvent on lipase-catalyzed esterification of sucrose with erucic acid; conditions described in the experimental section

conversion (52% w/w) was observed at a sucrose and erucic acid ratio of 1:1. The conversion was decreased after a further increase in molar ratio, it may be due to the effects of substrate inhibition on enzyme activity. The excess of fatty acid causes difficulty with product separation at the end of the process [20].

3.3 Effect of temperature

The effect of temperature on the esterification reaction is shown in Fig. 3. The reaction temperature may affect the solubility of the reactants and that of the product, the rate of reaction and the position of equilibrium. Under the optimized conditions, the conversion of sucrose erucate was increased with temperature. The highest yield (52.47%) was achieved at 50 °C. Similar results were also reported by Martins [21] for the production of butyl butyrate via TLIM-catalyzed reaction. It is reported that an increase in temperature may cause a loss of enzyme activity [22]. For temperatures higher than 50 °C the yield of sucrose erucate decreased as the activity of lipase decreased.

3.4 Effect of time

The time course profile of esterification is shown in Fig. 4. As expected, the conversion increased with time, reaching 50.82% at 35 h. After 35 h a plateau was observed, indicating that the conversion does not further increase.

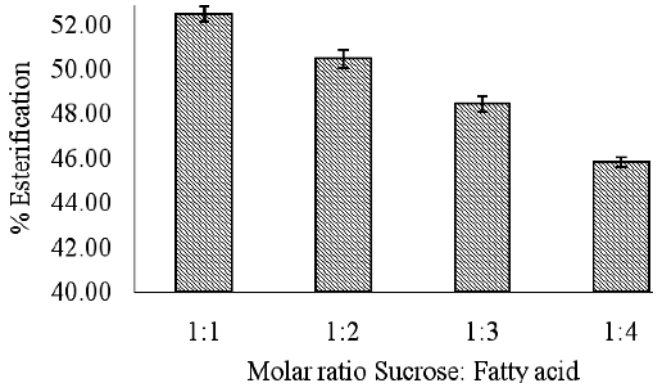

Figure 2 Effect of molar ratio on lipase-catalyzed esterification of sucrose with erucic acid; conditions described in the experimental section

3.5 Effect of enzyme concentration

The yield of sucrose erucate depended on the enzyme concentration. In order to find the effective enzyme concentration a number of experiments were performed in the enzyme concentration rage of 2 to 12%. When the concentration of enzyme is 10% the conversion reached 50.32%. A further increase of the enzyme concentration increased the conversion only marginally to 51.47%. At enzyme concentrations more than 10%, the active sites required for reaction became more than the substrate molecules, resulting

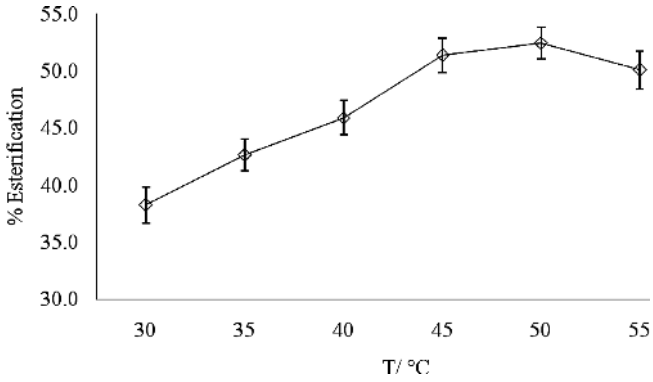

Figure 3 Effect of temperature on lipase-catalyzed esterification of sucrose with erucic acid; conditions described in the experimental section

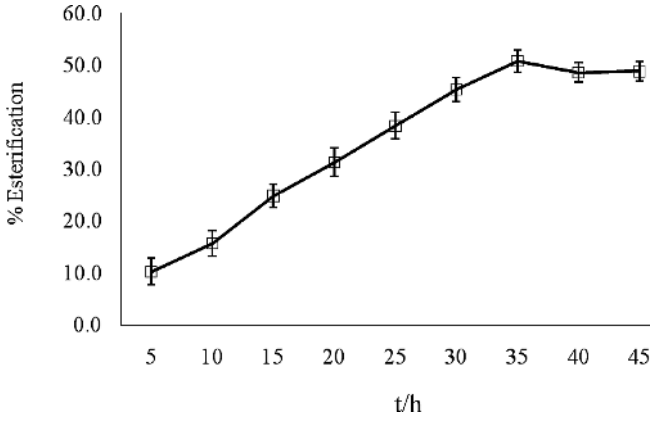

Figure 4 Effect of time on lipase-catalyzed esterification of sucrose with erucic acid; conditions described in the experimental section

in no further conversion. Thus, from the economic point of view, a lipase concentration of 10% would be an appropriate choice for the esterification reaction.

3.6 Characterization of sucrose erucate

3.6.1 TLC of sucrose erucate

The presence of the desired sugar ester was confirmed by TLC. The difference in retention factor (R_f) of the substrate and that of the product indicated the progress of reaction. An intense spot of sucrose erucatewas observed in R_f value 0.48. Similar R_f value of 0.5 for sugar ester was reported by Neta [23], hence it was possible to conclude the formation of sugar ester.

3.6.2 FTIR analysis of sucrose erucate

The formation of ester was confirmed by comparison FT-IR spectra of erucic acid and sucrose (substrate) and sucrose erucate (product). Compared to the spectra of the erucic acid, the major change was the presence of a peak at 1737.86 cm^{-1} corresponding to the carbonyl group of the ester formed after esterification of erucic acid. The intensity of the strong absorption band at 1710.86 cm^{-1} due to the stretching of the carbonyl of the acid group was decreased after esterification (Fig. 7). The OH of sucrose showed the broad absorption band in the range of 3250–3550 cm^{-1}. After esterification there was a marked reduction in the broadness of this trough and a narrow peak was seen at 3375.43 cm^{-1} which indicated the consumption of O–H group of sucrose.

3.6.3 Mass spectra of sucrose erucate

The mass spectrum of purified sucrose erucate is shown in Fig. 8. The molecular ion peak [M] at m/z of 661.19 in negative ionization mode represents the molecular mass of sucrose monoerucate.

3.6.4 Surfactant properties of sucrose erucate

Table 1 shows that the synthesized sucrose erucate considerably reduces the surface tension of water from (72.5 ± 0.003) mN m^{-1} to (32.73 ± 0.01) mN m^{-1}. The interfacial tension between aqueous solution and *n*-heptane is also significantly lowered by sucrose erucate. This indicates that the sucrose erucate emulsifies the two immiscible phases by lowering the surface tension. The wetting time is measured as the time that is required to sink the canvas disk in surfactant solution. The results show the reduction of the wetting time with the increase of the concentration of surfactant. In aqueous solution the surfactant molecules aggregate in micelles at a particular concentration, known as critical micelle concentration (CMC). The CMC value is practically important as

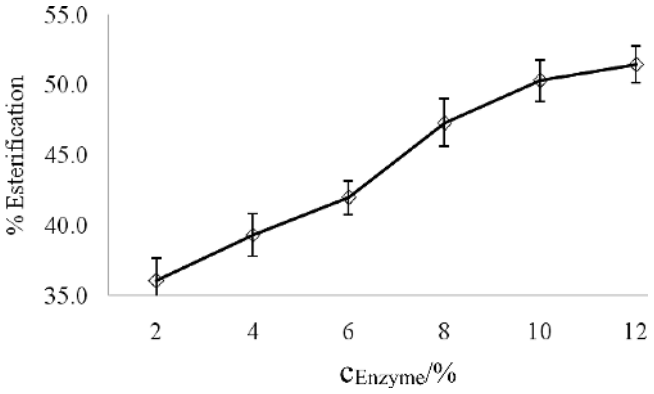

Figure 5 Effect of enzyme concentration on lipase-catalyzed esterification of sucrose with erucic acid; conditions described in the experimental section

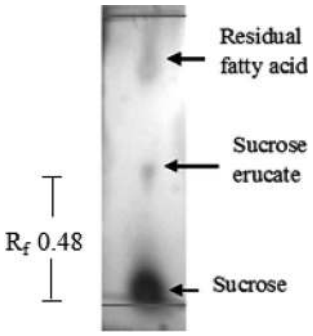

Figure 6 TLC of sucrose erucate

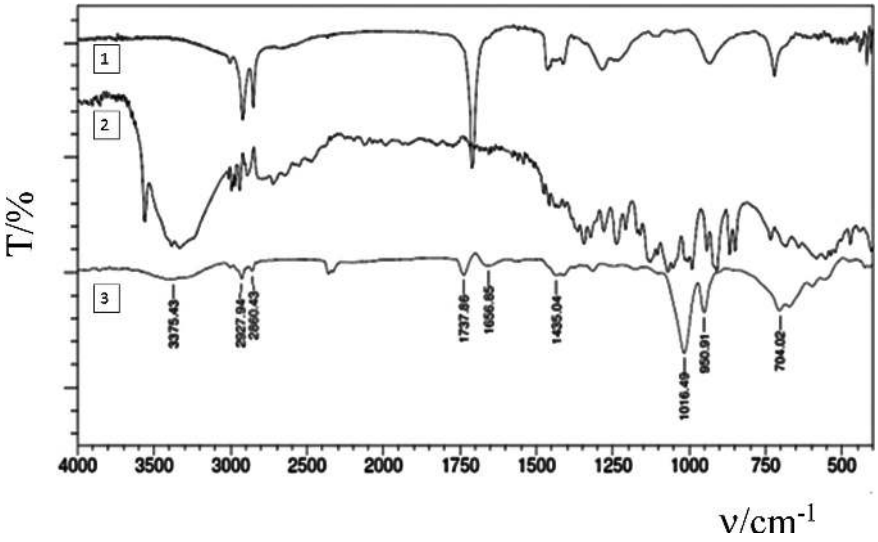

Figure 7 IR spectra of erucic acid (1), sucrose (2) and sucrose erucate (3)

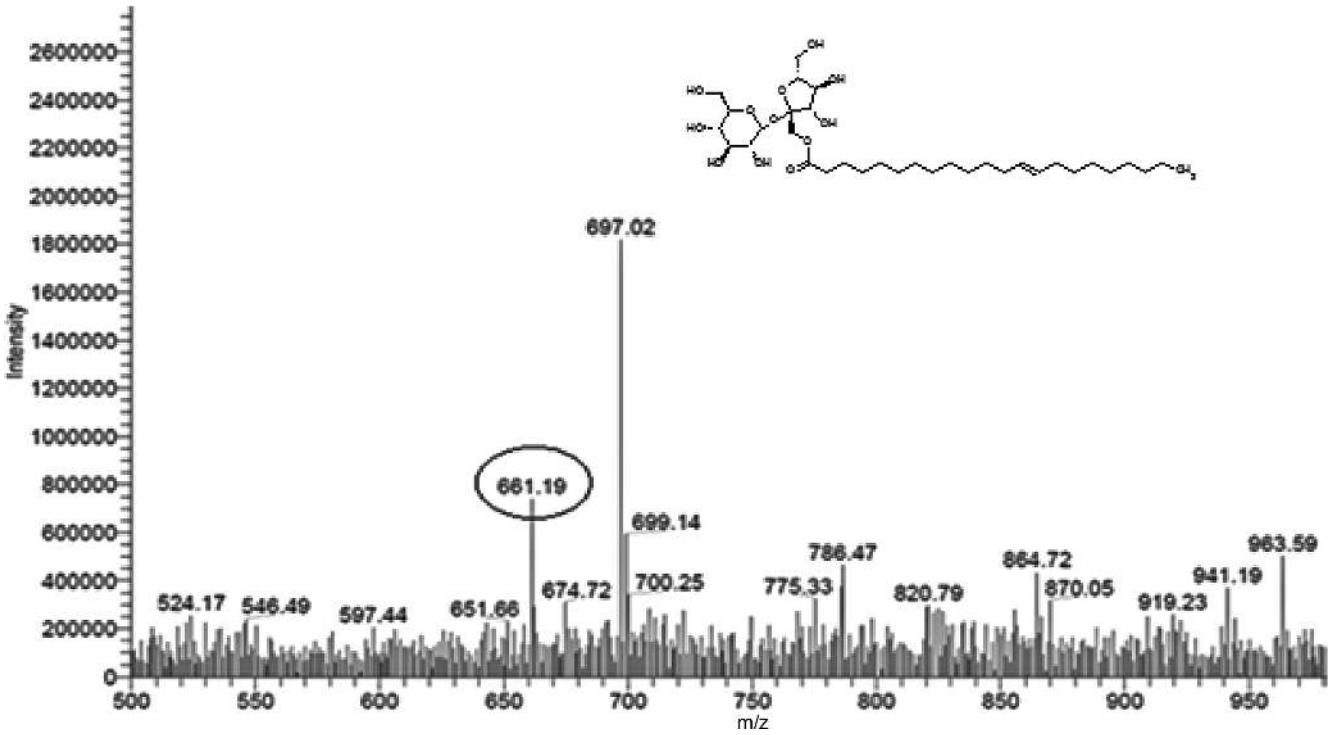

Figure 8 Mass spectrum of sucrose erucate

Concentration/wt.%	SFT/mNm^{-1}	IFT/mNm^{-1}	Wetting time/s
0.1	34.94 ± 0.007	2.37 ± 0.09	261
0.5	30.89 ± 0.04	1.82 ± 0.07	242
1	30.21 ± 0.01	1.97 ± 0.07	194

Table 1 Surface tension (SFT), interfacial tension (IFT) and wetting time of sucrose erucate at different concentrations

Surfactant	CMC/10^{-5} mol L^{-1}	γ_{cmc}/mN m^{-1}	Γ_{max}/10^{-6} mol m^{-2}	a_{min}/Å^2	ΔG/kJ mol^{-1}
Sucrose erucate	9.8	32.73	1.3744	120.82	33.63

γ_{cmc}: surface tension at CMC; a_{min}:surface area per molecule at interface; Γ_{max}: maximum surface excess concentration; ΔG: Gibbs free energy of adsorption

Table 2 Surfactant properties of sucrose erucate

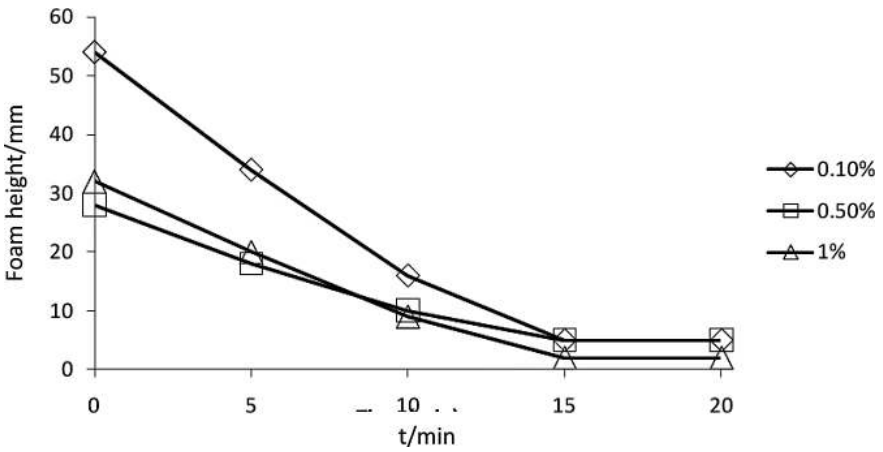

Figure 9 Foam stability of sucrose erucate at different concentration

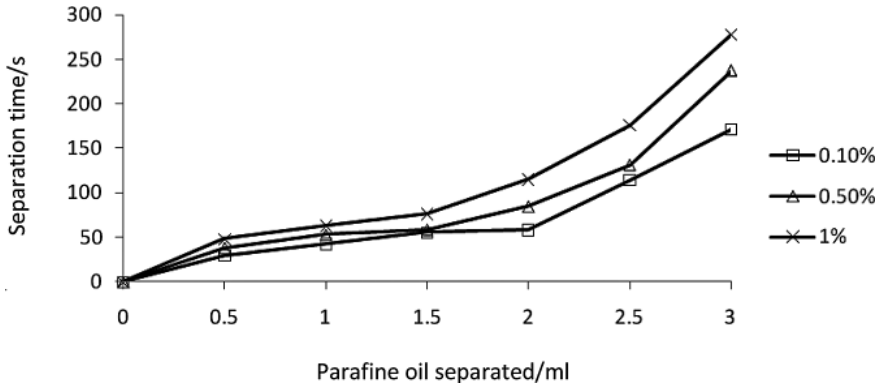

Figure 10 Emulsifying properties of sucrose erucate at different concentrations

it defines the minimum concentration of surfactant required to get desired surfactant properties. The CMC value of sucrose erucate is 9.8×10^{-5} mol L^{-1} and the surfactant properties are summarized in Table 2. The sugar fatty acid ester molecules contain a long chain of fatty acid and sugar molecules. So, these molecules can adsorb at interfaces and form micelles. A surfactant with a low CMC value shows better surfactant properties at low concentration.

The foamability and the oil-water emulsion stability of sucrose erucate were also studied at different concentrations. The foaming power of sucrose erucate was the best in the beginning of the experiment (approximately < 1 min), but over the time the foam height decreased indicating a stability loss of the foam.

The emulsification property of surfactant solution was measured at different concentrations. The aqueous solution of surfactant shows a better stability of emulsion at 1% surfactants concentration. The disaccharide fatty acid esters showed better emulsifying properties due to the presence of a long carbon chain [24, 25].

4 Conclusion

This study demonstrated a green route for the synthesis of sugar fatty acid esters using lipase as catalyst. The described process is efficient and environmentally compassionate as it uses renewable raw material for the synthesis of biosurfactants. The composition of solvent, reaction temperature, enzyme concentration and molar ratio of substrates were major factors affecting the conversion. Under the above mentioned optimized conditions 55.6% conversion was achieved. In addition, this investigated sucrose erucate synthesis at mild conditions is considered to be safe, environmentally friendly and of low cost. Sucrose erucate showed a better emulsion stability and lowers the SFT, hence it finds possible applications in pharmaceutical, food and cosmetic industry.

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