Lipase-catalyzed Solvent-free Synthesis of Polyglycerol 10 (PG-10) Esters

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Polyglycerol fatty acid esters, which have been widely used as emulsifiers in food, medicine and cosmetics industries, were the subject of solvent-free enzymatic synthesis in this study. There have been previous reports on enzymatic synthesis of various polyglycerol esters; however, this investigation extends the synthesis to PG-10 esters. The effects of substrate molar ratio, addition of emulsifiers to enhance mixing, and addition of molecular sieves or N_2 flushing for water removal, was investigated. The solvent-free synthesis using N_2 flushing leads to complete conversion of fatty acid, yielding a completely acid free product. The synthesis is validated for polyglycerol laurate and polyglycerol caprylate, both useful products in the cosmetic industry.

Keywords:
lipase, polyglycerol, PG-10, esters, solvent-free synthesis

Introduction

Partial esters of polyols, such as glycerol and polyglycerols (PG), are non-ionic surfactants used as emulsifiers in the food, detergents, and cosmetics industries. Especially polyglycerol esters (PGE) are becoming prominent in new products for surfactants, lubricants, cosmetics, food additives, etc. Polyglycerol esters also have excellent antifogging properties due to their controlled migration rate in the polymer, optimum surface-active properties, high thermal stability, and their compatibility with various polymers and additives. These properties extend their industrial application to food packaging and agriculture (antifogging agents in green houses).[1] Polyglycerol and its derivatives provide an alternative to the use of ethoxylated polyethylene glycol products that are widely used in home and personal care. In addition to delivering a wide range of properties, PG-10 esters also score better than ethoxylated compounds on the renewability of raw materials and biodegradability aspects. The market development towards products with improved biodegradability is not driven only by consumer wishes, but also by regulatory requirements.[2]

The commercial synthesis of the fatty acid esters of polyols is carried out mainly by direct esterification of the polyol, usually catalyzed by a homogeneous acid, such as sulfuric- and sulfonic acids. Alternatively, transesterification of triglycerides and polyols, catalyzed by alkaline hydroxides like NaOH, KOH, $Ca(OH)_2$, or the sodium salts of lower aliphatic alcohols, like methanol, is another lesser used approach. This technology possesses severe drawbacks, related to the generation of large amounts of by-products, high energy demand, and heterogeneity of the ester mixtures obtained.[3]

Availability of enzymes with high stability and industrial relevance has driven the growth of biocatalysis in the last decades. This development is strongly enabled due to the advances in molecular biology methods that has helped biocatalysis in becoming a potential alternative to chemical synthesis. The benefits of enzymes are increasingly being appreciated by different production sectors, e.g., pharmaceutical and fine-chemical industry, along with the more traditional sectors of food and detergents. Particularly, in the cosmetics industry, the use of biocatalysts leads to natural and green labelling, giving a distinct marketing advantage to the enzymatically synthesized products.[4] Novozym® 435 is a CALB lipase immobilized on an hydrophobic carrier (acrylic resin). CALB is a lipase originating from *Candida antarctica* exhibiting a very high degree of substrate specificity, with respect to both regio- and enantioselectivity. Exhibiting a broad temperature range, this enzyme also works very well in anhydrous conditions, thus showing immense potential for synthesis reactions. This lipase is highly robust and displays activity under a wide variety of conditions establishing its enormous importance for both hydrolysis and synthesis reactions. Therefore, its usage for (trans)esterification reaction is extensively reported.[5]

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Quite a number of studies have investigated the solvent free synthesis of polyglycerol polyricinoleate (PGPR), which is used as an emulsifier in the food industry, especially in chocolate coatings and chocolate bars. The procedure consists of two steps; firstly, the polymerization of ricinoleic acid to produce polyricinoleic acid followed by esterification of polyricinoleic acid with polyglycerol to produce PGPR. The step is mainly catalyzed by an immobilized lipase *of Candida rugosa*. For the second step, lipases from various microbial sources have been immobilized and their performance is compared among each other. Furthermore, a single-step process has also been investigated using Novozym® 435 for both steps, which considerably shortens the reaction time. The focus of these studies was to develop an optimized process with a short reaction time required for complete substrate conversion. Moreover, for final product, the emphasis was on producing PGPR compliant with the specifications set by the European Community and the recommendations of the European Food Emulsifiers Manufacturers Association.[6–10] Apart from PGPR, polyglyceryl laurate is also a well-known non-ionic surfactant widely used in cosmetics such as cleansing oil, shampoo, moisturizing lotion, and essence. There are two reported studies focusing on the synthesis of polyglycerol-3 laurate in solvent-free system using Novozym® 435.[11,12] Furthermore, transesterification of diglycerol, pentaglycerol and octaglycerol with oleic acid methyl esters has also been reported.[13]

Compared to PG-3 laurate synthesis in previous studies, the synthesis of polyglycerol-10 laurate and polyglycerol-10 caprylate using Novozym® 435 is investigated in this study. Particular emphasis is on comparison of different reaction conditions and reaction set-ups in order to investigate the feasibility of solvent-free synthesis of highly viscous compound.

Materials and methods

Chemicals

Polyglycerol-10 (PG-10) was kindly provided by Spiganord, Italy. The composition was as follows: hexaglycerol and higher > 40 %, tetraglycerol and higher > 50 %, diglycerol ≤ 10 %, glycerol ≤ 1 %. Technical grade lauric acid and caprylic acid were kindly provided by Oleon. PG-10 laurate (Mitsubishi Chemical Corporation) was kindly received from a cosmetics company. Novozym® 435 (*Candida antarctica* lipase immobilized on acrylic resin) with activity 10000 PLU g^{-1} and non-immobilized Lipozyme® CALB *Candida antarctica B* (5000 LU g^{-1}) was obtained from Novozymes A/S (Bagsvaerd, Denmark). Molecular Sieve UOP Type 3 Å was purchased from Merck. Other chemicals, namely, diethyl ether, 2-propanol, potassium hydroxide, phenolphthalein, and hydrochloric acid were of analytical grade and purchased from Merck.

Solvent-free polyglycerol laurate synthesis

Synthesis using molecular sieves for water removal

The equimass mixture of PG-10 and lauric acid (20 g each component) was mixed and incubated overnight at 65 °C in a 250-mL baffled flask at 200 rpm in the shaking incubator Innova 42. The reaction was started by the addition of 5 wt% (w/w lauric acid) Novozym® 435 at 65 °C. Molecular sieves (with 19.5 wt% water adsorption capacity) were added in 50 % extra dosage to ensure complete water removal. Addition of the product PG-10 laurate was also investigated in order to enhance the miscibility between the hydrophobic acid and hydrophilic PG-10. In selected tests, as indicated in results, the reaction mixture containing both substrates was premixed using ultraturex, and 3 g extra PG-10 and 1.2 g extra molecular sieves were also added.

Synthesis using N_2 flushing for water removal

First set of reactions using N_2 were carried out in a Schott Duran bottle with a cap. Equimass mixture of PG-10 and lauric acid (20 g each) were added to the bottle with 5 wt% of immobilized or non-immobilized lipase at 65 °C. N_2 was provided via a tube entering the bottle through the cap, which also had an outlet for removal of gas. N_2 entering the bottle in this manner provided limited mixing to the viscous reaction mixture, therefore a magnetic stirrer was added to stir the polyglycerol settled at the bottom of the flask (Fig. 1a). Second set of reactions was conducted in a 35-cm glass column with internal diameter of 20 mm. N_2 was bubbled through the sieve at the bottom of the column. This provided mixing to the reaction system, which consisted of PG-10, lauric acid and enzyme, and facilitated water removal (Fig. 1b).

Determination of acid value

Samples were withdrawn after specific time intervals and analyzed for acid value (AV) according to DIN 53402. The samples were dissolved in diethyl ether and 2-propanol (1:1 ratio), and titrated with 0.1 M ethanolic potassium hydroxide solution using phenolphthalein as an indicator.

The general formula for the AV is

$$\mathrm{AV}(\mathrm{mg_{KOH}\ g^{-1}}) = \frac{V \cdot C \cdot M_{\mathrm{KOH}}}{m} \qquad (1)$$

where V is the volume of potassium hydroxide solution (mL), C is the concentration of potassium hydroxide solution (mol L^{-1}), M_{KOH} is the molecular mass of potassium hydroxide (g mol^{-1}), m is the mass of sample (g).

Based upon acid value, the acid conversion was calculated as follows:

$$\text{Acid conversion}(\%) = \frac{AV_{initial} - AV}{AV_{initial}} \cdot 100 \quad (2)$$

Determination of saponification value

For the determination of the saponification number, 25 mL of 0.5 N alcoholic potassium hydroxide was mixed with 2 g of the sample. This mixture was refluxed for 1 h and then titrated with 0.5 N hydrochloric acid using phenolphthalein as an indicator.

$$SV = \frac{(V_{blank} - V_{sample}) \cdot N \cdot M_{KOH}}{m} \quad (3)$$

where V_{blank} and V_{sample} are the volumes of HCl required for titration (mL), N is the normality of alcoholic KOH, M_{KOH} is the molecular mass of potassium hydroxide (g mol^{-1}), m is the mass of sample (g).

The ester value was calculated by subtracting "acid value" from "saponification value". The ester value was then used to calculate the number of moles of KOH required to saponify the product and thus the moles of lauric acid attached to each mole of PG-10.

Determination of water content

The water content of samples was measured with Karl Fisher Titrino (720 KFS, Metrohm). A mixture of methanol and 1-propanol (1:1) was used as solvent and samples were titrated HYDRANAL™ – Composite 5.

Results and discussion

Synthesis using molecular sieves for water removal

Effect of substrate weight ratios and additives

The solvent-free synthesis of PG-10 laurate at two PG-10 and lauric acid (LA) weight ratio is shown in Fig. 2a. On increasing the weight ratio of PG-10 compared to LA, there was a decrease in the rate of the reaction, but the final fatty acid conversion was similar. This difference might be attributed to the poor miscibility of PG-10 with LA. Furthermore, as the reaction proceeded, the formation of PG-10 laurate product assisted the miscibility and similar final LA conversions (> 90 %) were achieved in both cases. In order to bridge the miscibility gap between the substrates, PG-10 laurate (final product) was added. The use of PG-10 laurate for better mixing of PG-10 into the apolar lauric acid phase, helped to increase the rate of conversion of lauric acid; however, the final lauric acid conversion did not improve. As presented in Fig. 2b, using 1:1 weight ratio and various weight % of PG-10 laurate as an additive, initially enhanced the LA conversion rate compared to solvent-free synthesis. The rate also increased with increasing PG-10 addition percentage. However, the final LA conversion remained in the range of 85 to 90 %. This corresponds to the acid values from 13 to 19 mg_{KOH} g^{-1}, thus limiting the direct applicability of these products in cosmetics applications. It is also to be noted that, although PG-10 laurate was added to facilitate the initial mixing of the substrates, its addition also shifted the equilibrium to the substrate side of the reaction, and thus no further improvement in final lauric acid conversion was exhibited.

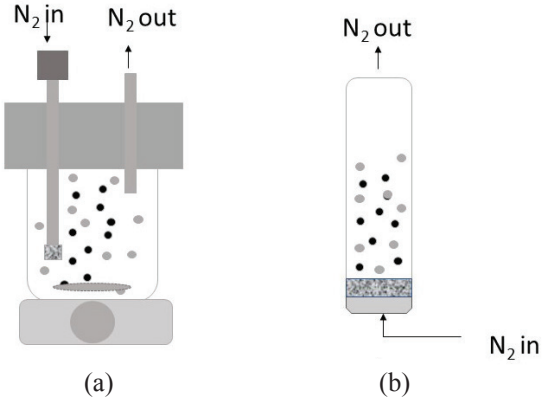

Fig. 1 – *Reaction using N_2 flushing for water removal (a): set-up consisting of Schott Duran bottle and stirrer, (b): set-up consisting of glass column with N_2 flushing from the bottom*

Effect of premixing and sequential addition of substrate and molecular sieves

To enhance the effectiveness of solvent-free reaction, the reaction mixture was premixed with ultraturex. Moreover, to decrease the acid values, extra PG-10 and molecular sieves were added in the course of reaction to investigate the limiting factor for incomplete conversion of lauric acid. Premixing helped in increasing the reaction profile, where after 100 h, > 90 % LA conversion was achieved for all conditions (Fig. 3a). In conditions 2 and 3, where both PG-10 and molecular sieves were added at 122 h, and condition 4, where only extra molecular

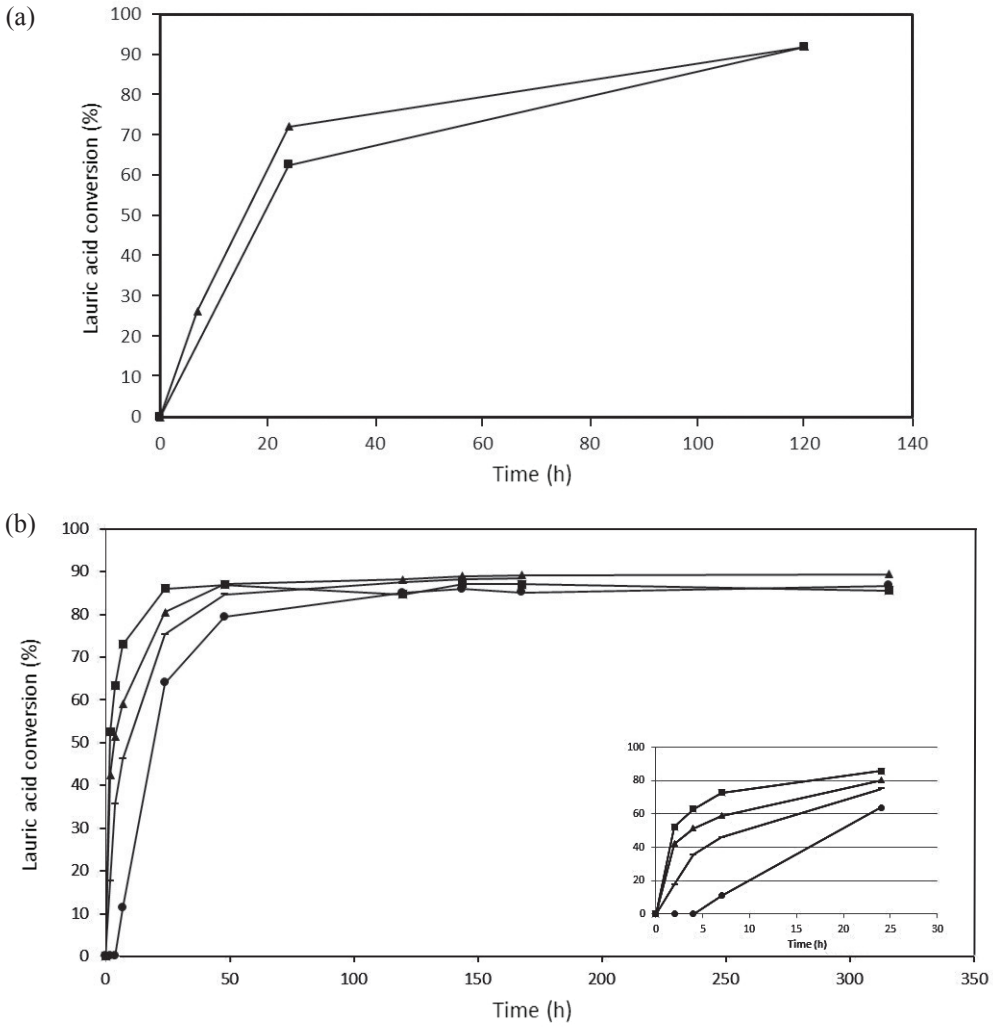

Fig. 2 – *(a) Conversion of lauric acid at 1:1 (▲) and 2:1 PG-10 and LA ratio (■) (b) conversion of lauric acid at 1:1 PG-10 and LA ratio (●) and addition of 5 (-), 10 (▲) and 25 (■) wt% PG-10 laurate. LA = lauric acid; PG = polyglycerol.*

sieves were added after 172 h, the final acid values were lower compared to the condition 1, where no extra additions were done. Moreover, the similar final acid values of conditions 2, 3, and 4 revealed that water removal is indeed the limiting factor for complete LA conversion. Although the ultraturex mixing and extra molecular sieves helped to obtain better conversions, it was evident from Fig. 3b that molecular sieves were not a very effective water removal tool in this viscous reaction system. To maintain the conformation of the catalytic site of enzymes, water is indeed essential; however, the amount of water necessary for enzyme activity, especially in the case of lipase, is very small. On the other hand, in esterification reaction, water is generated as a co-product, which affects the equilibrium conversion of the reactions; therefore, as the water content increased, lower equilibrium conversions were achieved.[6]

Synthesis with N_2 flushing

Continuously sparging nitrogen through the reaction medium is an effective way of water removal in organic phase enzymatic reactions. Additionally, this method can provide required mixing. On a large scale, N_2 can be recovered and reused after stripping off its water content. Therefore, N_2 sparging was provided as a method of water removal and mixing in a Schott Duran bottle. However, the viscosity of the reaction mixture (PG-10 and LA) and even higher viscosity of the product, which could be >20-fold in solvent-free environment, did not allow efficient mixing, therefore a magnetic stirrer was added.[11] This set-up resulted in 100 % fatty acid conversion, and at a much faster rate compared to molecular sieves (Fig. 4), but also caused destruction of the Novozym® 435 enzyme carrier. Previously, the mechanical disintegration of Novo-

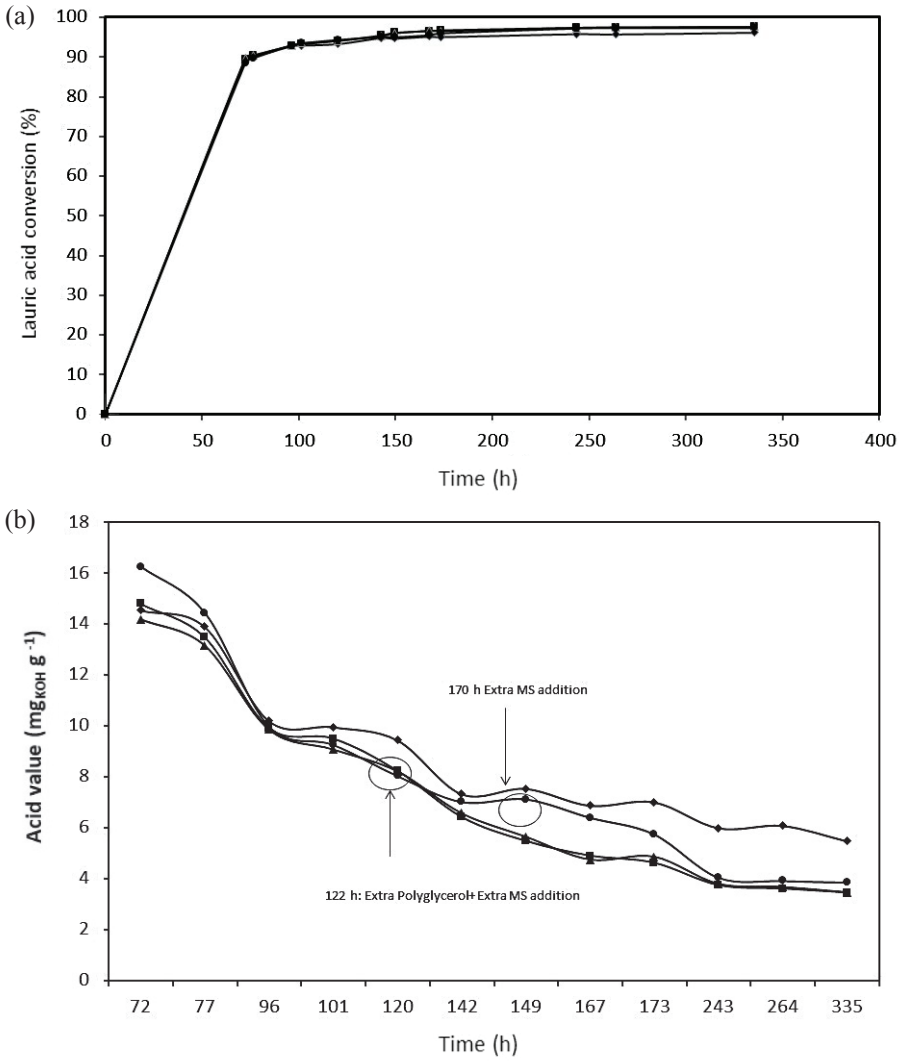

Fig. 3 – *(a) Conversion of lauric acid and at 1:1 PG-10 and LA weight ratio (♦) In condition 2 and 3, extra molecular sieves (MS) (▲) and PG-10 (■) were added at 122 h. In condition 4, only extra MS (●) was added at 170 h. (b) Represents the acid values in these 4 conditions.*

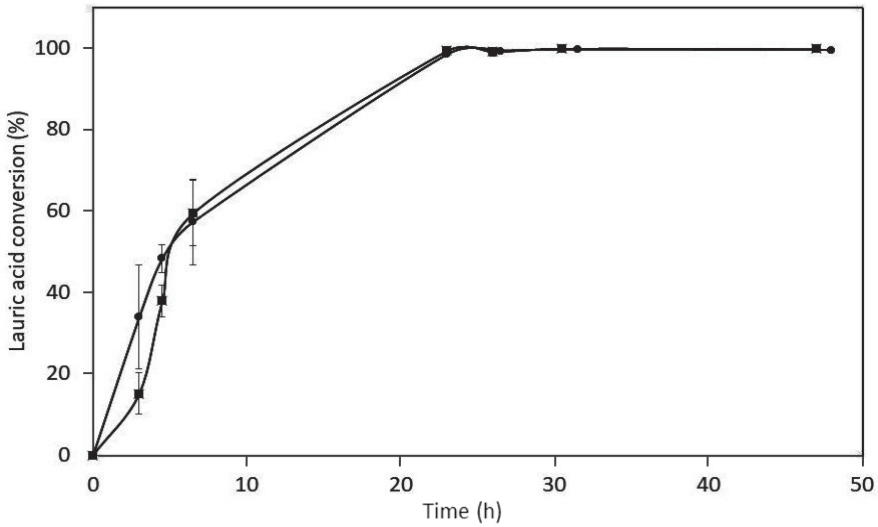

Fig. 4 – *Conversion of lauric acid 1:1 PG-10 and LA weight ratio using N_2 flushing for water removal in Schott Duran bottle using free (●) and immobilized (■) enzyme*

zym® 435 carrier has also been observed.[11] The use of free enzyme in order to avoid the destruction of enzyme support, also resulted in similar conversion profile (Fig. 4). Although the use of free enzyme was effective, its removal from the reaction mixture proved more difficult. On filtering the product over a 30 kDa membrane, there was still enzyme activity present in the permeate and it was not possible to filter the viscous product over a lower molecular weight cut-off membrane. Approximately 0.54 % residual water was found in both conditions of the free and immobilized enzyme at the end of the reaction.

To provide better mixing without destroying the enzyme carrier, the Schott Duran bottle was replaced with cylindrical glass reactor. The cylindrical reactors wherein gas is sparged, via a distributor, in the form of bubbles, into liquid or liquid solid suspension are also known as bubble column reactors. These reactors are commonly employed in the chemical, petrochemical, biochemical, and metallurgical industries. Excellent heat- and mass-transfer characteristics, lack of moving parts, and thus reduced wear and tear, higher durability of catalyst, ease of operation, compactness, and low operating and maintenance costs are some of the advantages of using such reactors.[14] The use of bubble column reactors has also been reported for solvent-free enzymatic polyglycerol-3laurate and oligoglycerol linoleate synthesis[11,15] and the present study extends the usage to polygylercol-10 laurate and polyglycerol-10 caprylate.

As evident from Fig. 5a, for 1:1 ratio, between 30 and 60 h were required to observe complete LA conversion. However, on increasing the ratio of PG-10 and LA, complete conversion was observed in about 20 h. This reaction time can still be shortened considerably by using higher reaction temperature to decrease viscosity and increase mass transfer. However, enzyme deactivation at such temperatures can occur rapidly. As reported by Wan *et al.*, at 3 wt% enzyme with an oligoglycerol/linoleic acid molar ratio of 1.5:1, the acid conversion was 95.5 % at 3 h operating at 90 °C.[15] Lipase catalyzed enzymatic production of polyglycerol-3 laurate has been reported in a bubble column reactor, where the reaction could be conducted at 75 °C (compared to > 90 °C in a stirred tank reactor), thus increasing the half-life of the enzyme by 2 times.[11] Apart from temperature, very high mixing is another solution to address the viscosity issue, but this also leads to destruction of enzyme by mechanical stress. Use of dissolved CO_2 to reduce viscosity is another method which can lead to the increase in reaction rate by a factor of 4, as demonstrated in a study by Brummund *et al.*[12] A 60 % LA conversion after 70 h at a low enzyme loading of 0.25 wt% and 60 °C at 1:1 PG-3 and LA ratio was reported.

In our study, 2:1 PG-10 and LA ratio exhibited best performance among the tested conditions. This could be attributed to the fact that, on increasing the ratio to 3:1, the highly viscous PG-10 delayed the reaction further. Wang *et al.* observed 1.5:1 oligoglycerol/linoleic acid as the optimum ratio, because increasing the oligoglycerol resulted in more viscous environment and less conversion, while increasing the acid (higher acid compared to alcohol) resulted in poor enzymatic performance due to the acidification of the micro-aqueous interface of the enzyme, which would inhibit the enzyme activity.[15,16] As evident from the results, the reactions with N_2 flushing led to faster acid conversion compared to shake flasks. The bubbling of N_2 ensures a convective flow field and creates an effective interaction in the multiple-phase reaction system to provide a high mass-transfer, and thus higher efficiency.[15] Moreover, this set-up also proved effective in water removal; compared to zeolites, the water concentration was below 0.5 wt% in all tests.

In addition to lauric acid, cylindrical column experiments also showed feasibility of producing PG-10 caprylate (Fig. 5b) under the same reaction conditions. For both tested ratios of PG-10 and caprylic acid, complete CA conversion was observed after 22 h with faster conversion observed for higher PG-10 and CA ratio. These results are quite similar to LA esterification. Polyglyceryl caprylate, an ester of polyglycerol with C8 fatty acid, reduces body odour by a unique activity-on-demand principle based on the lipase activity of skin microorganisms. In addition to its emulsifying properties, polyglyceryl caprylate offers a base level of inhibitory activity on microorganisms. Increased perspiration is associated with increased lipase activity on the skin, which releases caprylic acid from the polyglycerol ester by hydrolysis. Caprylic acid, in turn, is a stronger inhibitor of microbial growth, and thereby interferes with the formation of body odour when needed. This unique activity-on-demand principle in combination with excellent skin compatibility and eco-profile is another example of the flexibility of polyglycerol chemistry for modern cosmetics ingredients.[2]

The emulsifying properties of the esters depend upon their hydrophilic-lipophilic balance (HLB), the higher the HLB value, the greater the hydrophilicity of the surfactant. The combination of the number and type of hydrophobic “tail” and the free hydroxyl groups present in the polar “head” of the ester result in varying HLB values. Therefore, it is possible to select specific surfactants for targeted applications as a function of their HLB values. For practical purposes, and due to the strong hydropho-

bic character of the fatty acyl group, esters containing an OH/fatty chain ratio equal or higher than two are, in general, best preferred as emulsifiers. For a given fatty acid and esterification degree, the hydrophilic character increases with the molecular weight of the polyglycerol (higher hydroxyl/fatty acid ratio).[3,15] In this investigation, the degree of esterification was indirectly estimated using the saponification value, and it ranged from 3.7 to 2.8 on increasing the polyglycerol/LA ratio. Similar trends were observed for polyglycerol caprylate (Fig. 6). It is indeed obvious that decreasing the acid component would result in lower degree of esterification. Even though the aim here was to form polyglycerol-10 laurate monoesters, the resulting products were tri- and tetraesters. For lower polyglycerols, monoesters are preferred, whereas diesters and eventually even triesters of polyglycerols of high molecular weight could still have good emulsifying properties for certain applications. However, if the substrate polyglycerol is a mixture of cyclic and linear molecules, then it is more appropriate to reduce the carbon chain length of lipid substrate in order to produce an emulsifier with more hydrophilic properties.[13]

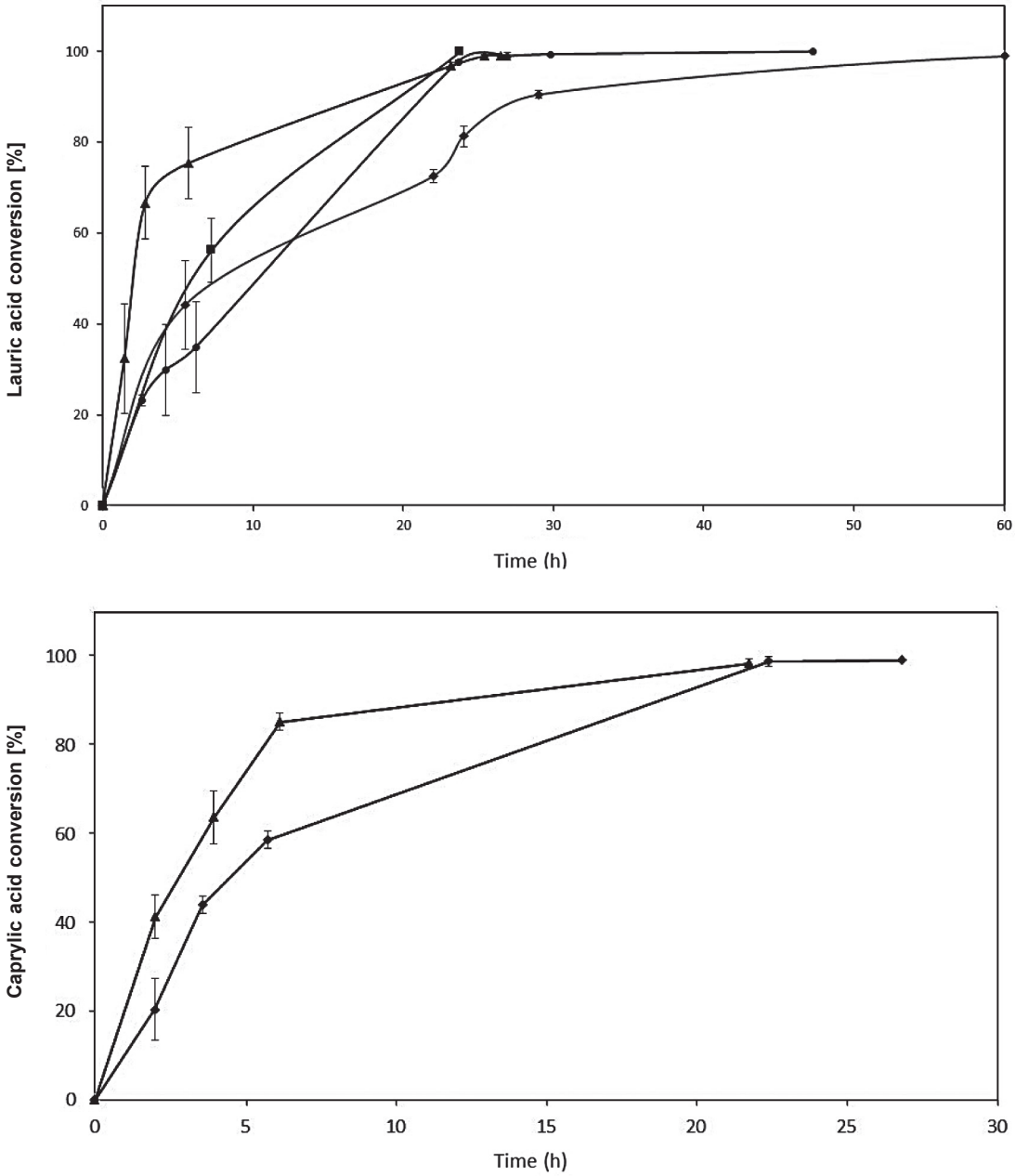

Fig. 5 – *(a) Conversion of lauric acid at 1:1 (♦), 1.5:1 (●), 2:1 (▲) and 3:1 (■) at various PG-10 and LA weight ratio (b) conversion of caprylic acid at 1:1 (♦), and 2:1 (▲) PG-10 and CA weight ratio using N_2 flushing for water removal in cylindrical glass column*

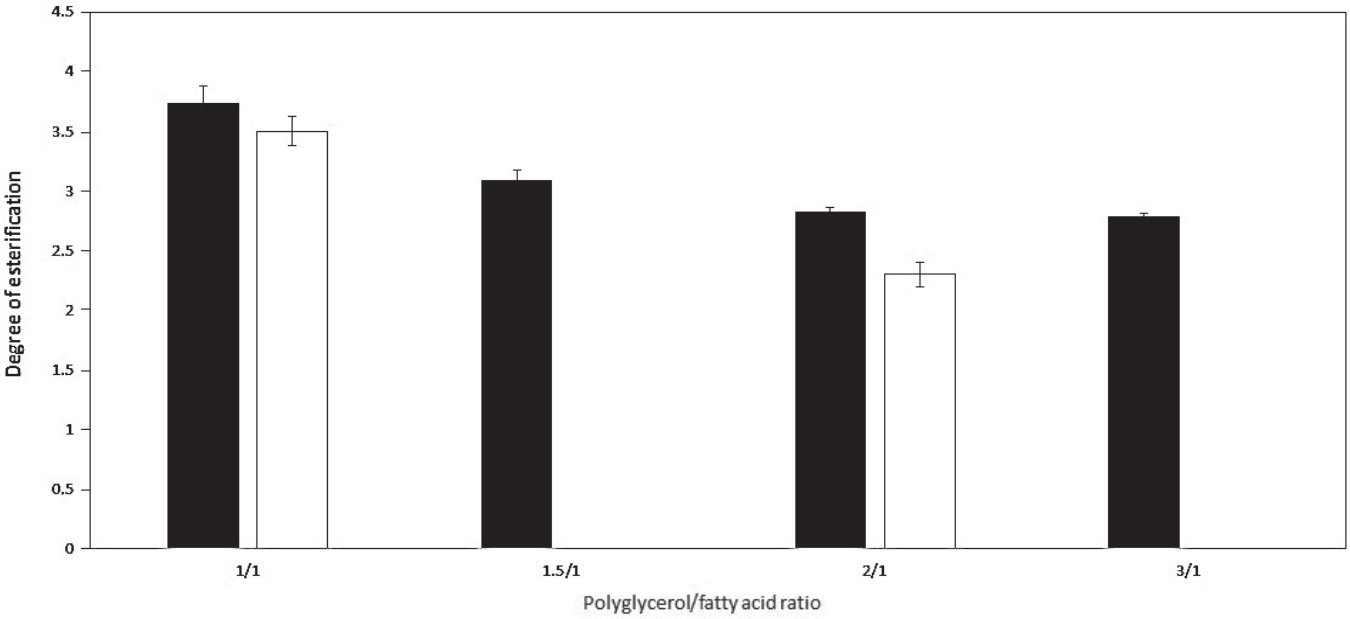

Fig. 6 – *Degree of esterification of each PG-10 molecule calculated by measuring saponification and acid value. Black bars indicate PG-10 laurate and white bars indicate PG-10 caprylate.*

Conclusions

The solvent-free enzymatic synthesis of PG-10 laurate and PG-10 caprylate was investigated. Removal of water generated during esterification and sufficient mixing between the hydrophobic fatty acid and hydrophilic PG-10 are two main factors to achieve complete fatty acid conversion. This was successfully demonstrated under mild reaction conditions in a cylindrical column reactor with nitrogen flushing at 65 °C and 5 wt% enzyme loading. Despite being highly viscous substrates and products, complete fatty acid conversions were observed. Polyglycerol esters are widely used in the food and cosmetics industries as emulsifiers, dispersants, thickeners, solubilizers, spreading agents, or emollients. The emulsifying properties of the PG esters depend basically upon the length of the polyglycerol chain, the degree of esterification, and the molecular weight of the fatty acid. Due to the highly complex composition of PG-10 products, further investigation into the separation and purification of accurate esterification products is needed.

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