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# Lipase catalyzed solvent free synthesis of monoacylglycerols in various reaction systems and coupling reaction with pervaporation for *in situ* water removal

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ABSTRACT

This work reports a practical comparison of simple reaction systems for enzymatic production of monoacylglycerols (MAG) from lauric- and caprylic acid. Two approaches namely shake flasks and packed bed reactor (from 60 g to 3 kg scale) were compared as well as water removal strategies such as adsorption on molecular sieves, $N_2$ stripping and hydrophilic pervaporation, were studied. In all the reactor systems high fatty acid conversion (>90%) was achieved in solvent free conditions. The product profile obtained showed 35 wt% - 50 wt % MAG in various systems and was related to final fatty acid conversion as well as reaction conditions and set-ups. The selectivity of products in the tested systems, ranged from 55 to 70 wt% mono-, 30–35 wt% di- and 1–10 wt% triacylglycerols. Packed bed reactor enabling enzyme reuse, was coupled with pervaporation, demonstrating practical applicability of zeolite based membranes for *in situ* water removal.

## 1. Introduction

The robustness, high selectivity and wide substrate range of lipases make them highly employable biocatalysts in fine chemical-, pharmaceutical- and agrochemical industry. Initially thought to be utilized in aqueous medium, today lipase catalyzed synthesis are conducted in some non-conventional media such as organic solvents, ionic salts and -liquids, supercritical fluids, and solvent-free systems. Specifically, solvent-free systems come with the advantages of lower costs, higher substrate concentration, and greater volumetric productivity [1]. One of the important applications of lipases is the synthesis of monoglycerides or monoacylglycerols (MAGs). MAGs are non-ionic surfactants consisting of a hydrophilic head (glycerol) and a hydrophobic tail (fatty acid), displaying excellent emulsifying properties, especially for combining oil and water phase [2]. Monoacylglycerol compounds account for approximately 75% of the world's annual emulsifier production and have a wide application in our day to day life such as food-grade additives, drug carriers, modifiers and synthetic intermediates [3]. For example, MAGs, such as monooleates, are suitable as emulsifying components in aqueous fiber finishes, as lubricant components, as fine mechanical oils, as water displacing oils, and in grinding and polishing pastes [4]. In addition, many kinds of mono-unsaturated and poly-unsaturated MAG have positive effects on human health such as preventing prostatic hyperplasia or cardiovascular disorders [3]. Moreover, some MAGs such as monolaurin, monomyristin, monocaprin, monoolein, and monolinolein are reported to be displaying antimicrobial activities. The antibacterial activity of MAGs is determined by the chemical structure and hydrophilic and lipophilic properties that are expected to interact with cell walls of both Gram-positive and Gram-negative bacteria [2].

With the wide range of applications, the demand for these esters increases each day as new applications are found. MAGs are commercially produced by glycerolysis of triacylglycerols (TAG) at high temperatures (220 °C-250 °C) using chemical catalysts, such as sodium-, potassium- or calcium hydroxide to accelerate the process. The drawbacks of this process are the lower MAG yield, the formation of undesirable by-products, the energy consumption caused by high reaction temperatures and the high capital investment. In addition, due to low MAG yield, an energy-intensive molecular distillation is always needed to purify these preliminary products, in order to obtain high-quality product with at least 90% MAG. But this additional separation process severely increases the overall production cost. Thus, there is an urgent requirement to improve TAG glycerolysis reactions to enhance MAG productivity [3]. During the past decade, extensive research has been

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conducted in the area of enzymatic synthesis of MAGs aiming at higher yields and mild reaction conditions, no side products, better quality of desired products and lower energy consumption [4].

Enzymatic synthesis of MAGs can be conducted by ethanolysis or glycerolysis of triglycerides/oils, direct esterification of fatty acid with glycerol, and transesterification of fatty acid methyl ester with glycerol. Various commercial or non-commercial free and immobilized lipases *e.g. Candida antarctica, Rhizomucor miehei, Burkholderia cepacia, Penicillium camembertii*, etc. have been employed to produce MAG in organic solvent medium [5-8], in solvent free systems [9-11], or using microemulsion as reaction media [12]. Applying green chemistry and clean technology systems in industry is the demand of today. Solvent free biocatalysis and pervaporation are green and clean technologies from which the industrial landscape can benefit. Application of pervaporation as green technology has matured a lot since past 20 years drawing the attention of academics, manufacturers as well as industries for its application [13]. Today, the implementation of PV at the industrial scale has been principally focused on the dehydration of organics, however water removal from chemical reactions using PV has also been carried out reporting successful outcomes. The advances on developments of PV combined with chemical and bio-chemical reactions have been comprehensively reviewed recently [14]. Reactions performed in solvent-free systems exhibit various advantages, such as higher volumetric productivities and easy product separation from the remaining substrates. Moreover, the elimination of solvent brings economic and environmental benefits [15].

In the present contribution, the results of solvent free MAG synthesis are reported for lauric- and caprylic acid. Different reaction systems, namely, batch reactors and packed bed reactors are employed at gram scale and 3 kg scale. Since water removal is highly important for shifting the equilibrium in esterification, various water removal approaches *viz.* molecular sieves adsorption, $N_2$ flushing and pervaporation, have been used. Moreover, to the best of our knowledge, this is the first study reporting the solvent free synthesis of monolaurin in packed bed reactor coupled with pervaporation for water removal.

## 2. Material and methods

### 2.1. Materials

Caprylic acid (C8, CA), Lauric acid (C12, LA) and glycerol used in the reactions as well as monolaurin and monocaprylin used as additives, were kindly provided by Oleon. Mono-, di-and triacyl glycerol of lauric - and caprylic acid used as analytical standard for HPLC were purchased from Merck. Immobilized lipase B from *Candida antarctica* (Lipozyme® 435, food-grade version of Novozym® 435, immobilized on macroporous acrylic resin, *personal communication with Novozymes*) and lipase from *Rhizomucor miehei* Novozym 40,086 (immobilized on macroporous anionic resin) were kind gifts from Novozymes. These enzymes are mentioned as CALB lipase and RM lipase, respectively, from here onwards. Molecular sieves 3 Å (particle diameter 1.6–2.5 mm) were purchased from VWR. Mitsui ceramic membrane zeolite type T (length 200 mm nominal area 56.5 $cm^2$) was purchased from DeltaMem AG.

### 2.2. Reaction in batch reactors with orbital shaking at ~60 g and ~2.5 kg scale

The solvent free substrate mixtures were incubated in 200 mL baffled Erlenmeyer flasks with approximately 60 g reaction mixture consisting of fatty acid and glycerol (0.13 mol each). The tests were conducted in a shaker incubator at 50 °C or 65 °C at 200 rpm. Enzyme loading of 0.75 wt% (of fatty acid), 1:1 molar ratio of glycerol and fatty acid and an excess of molecular sieves (43 wt% of the reaction mixture) were added to start the reaction. Samples were withdrawn at regular intervals and tests were stopped after 24 h. The reactions at kilogram scale (2.5 kg) were conducted in 5 L Scott Duran bottle equipped with a cap fitted for nitrogen flushing for water removal. The tests were conducted in a shaker incubator at 65 °C at 200 rpm. For lauric acid and glycerol, 9 mol of each substrate was used (final reaction weight 2.6 kg). Similarly, for caprylic acid and glycerol, 12 mol of each component was used with final reaction weight of 2.7 kg.

### 2.3. Reaction in packed bed reactor at 100 g and 3 kg scale

The packed bed reactor (PBR) experiments for 100 g scale synthesis were carried out in an Omnifit® borosilicate glass column (13 cm in length × 1 cm diameter) containing 0.5 g CALB lipase (0.75 wt% of used LA). It was connected to an in-house constructed stainless steel column (11 cm length × 2 cm diameter) containing 30 g of molecular sieves (activated overnight at 150 °C), to adsorb the produced water. A previously mixed 100 g substrate mixture (in a Schott Duran bottle) was continuously introduced into the reactor in downwards direction at a flow rate of 0.07 mL/min and then through the molecular sieves column, which was operated from the bottom to the top. The residence time in the packed bed for one pass was 10 min. Using a Watson Marlow 205S peristaltic pump, the feed was recirculated into the Schott Duran bottle until the end of the test period .

The 3 kg scale PBR consisted of an in-house made stainless steel column (15 cm in length × 3.4 cm diameter) connected to a 5 L stirred reaction vessel coupled to a pervaporation membrane (2 membrane units in series were used) (Fig. 1). The reaction was started by recirculating the reaction mixture over the enzyme column (containing 40 g CALB lipase) with a flow rate of 0.3 L/h. The residence time in the packed bed for one pass was 10 min. Flow rate through the membrane was 126 L/h (104 kg/h) and a vacuum of 20 mbar was applied. This system was evaluated only for monolaurin synthesis

### 2.4. Analytical Methods

Conversions of fatty acids were determined by measuring acid value. Samples were withdrawn after specific time intervals and analyzed for acid value (AV) according to DIN 53,402. 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 Acid Value (AV) is
* $AV\left(mg\frac{KOH}{g}\right) = \frac{VX\ C\ X\ m_{KOH}}{m}$
* where V is the volume of potassium hydroxide solution (mL)
* C is the concentration of potassium hydroxide solution (mol/L),
* $m_{KOH}$ is the molecular mass of potassium hydroxide (g/mol),
* m is the mass of sample (g).

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

$$Acid\ conversion\ (\%) = \frac{AV_{initial} - AV}{AV_{initial}}\ X\ 100$$

Mono-, di- and tri- acylated glycerols (MAG, DAG and TAG, respectively) were analysed by HPLC with ELSD detector and on an Alltima HP C18 HL 5 µm, 250 mm × 4.6 mm column (Alltech Grace). The eluents used were: (1) acetonitrile/methanol (90/10), (2) isopropanol. Glycerol was analysed by HPLC with RID detector and on a Monochrom 5 Diol 8 µm, 250 mm × 4.6 mm column. The eluents used were $dH_2O$/acetonitrile (5/95). The results are presented in terms of weight percent of all components in final reaction mixture and the selectivity of the products (MAG, DAG and TAG). The selectivity is calculated as follows:

$$Selectivity\ (\%) = \frac{\%MAG\ (mole)}{\%MAG\ (mole) + \%DAG(mole) + \%TAG(mole)}$$

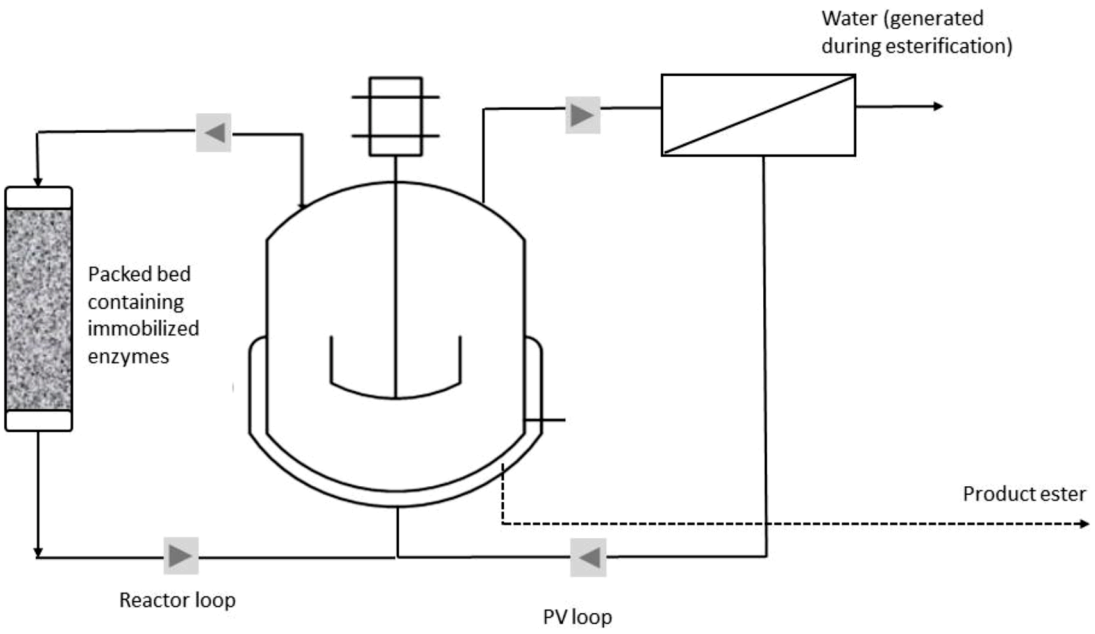



**Fig. 1.** Coupled reaction and pervaporation set-up for esterification and on-line water removal. The reaction vessel with a stirrer was connected to a packed bed reactor containing immobilized lipases (CALB), on the left and two zeolite membranes (Mitsui ceramic membrane zeolite type T: length 200 mm, nominal area 56.5 $cm^2$) was used in series for pervaporation, on the right. Whole set-up was maintained at 65 °C.

Determination of water (wt%) in the reaction was conducted using Karl-Fischer volumetric titrator (720 KFS Titrino, Metrohm Ltd, Herisau, Switzerland). A mixture of methanol/1-propanol (1:1 v/v) was used as solvent and Hydranal®-Composite 5 (Fluka 34,805) was used as the titrant.

## 3. Results and discussion

### *3.1. Solvent free synthesis of monolaurin and monocaprylin in batch reactors with orbital shaking at ~60 g and ~2.5 kg scale*

For monolaurin synthesis, the reaction at ~60 g scale showed 93% and 90% lauric acid (LA) conversion after 24 h at 65 °C and 50 °C, respectively for CALB lipase (Fig. 2a). Compared to 50 °C, higher reaction rate was observed at 65 °C but final conversions were similar for both temperatures . When employing RM lipase, high LA conversions (> 90%) are observed at 50 °C, but at 65 °C only a maximum of 70% LA conversion is observed. This could be attributed to the fact that the specified temperature optimum for CALB lipase is 30 °C- 60 °C, whereas for RM lipase, it is 30 °C - 50 °C, although it is very common for both immobilized CALB and RM lipases to be used at higher temperatures (>60 °C). Using CALB lipase for monocaprylin synthesis showed similar trends with final caprylic acid (CA) conversion of >90% after 24 h. However, for RM lipase, no conversion was observed (Fig. 2b). Besides conversion, the final composition in case of monolaurin synthesis, shows the monoacylglycerol (MAG) weight between 34% - 44 wt% with highest value obtained for CALB lipase at 65 °C, diacylglycerol (DAG) ranging from 28 to 34 wt% and < 10 wt% triacylglycerol (TAG) (Fig. 3a). For monocaprylin synthesis, the weight (%) of MAG is slightly higher (50 wt%) at 50 °C (CALB lipase) (Fig. 3b). Compared to weight, the selectivity (%) (calculated as explained in Section 2.4) of the lipases towards the MAG stayed highest in the order of 63–70% for MAG, 25–30% for DAG and less than 5% for TAG for both lauric- and caprylic acid (Fig. 4a and 4b). Using a Lipase from *Chromobacterium viscosum,* the ester molar fractions at equilibrium were found to be dependent on the fatty chain length in the absence of a solvent [16]. With C8, the monoester was the highest mole fraction, while for C18, the diester mole fraction was found to be the highest one. In the presence of a solvent added in sufficiently high concentrations, the ester mole fractions become independent of the chain length of the fatty acids. In the current study, although slightly high MAG (wt%) is observed with CA at 50 °C compared to LA, the selectivity (%) for MAG was 69% (CA) as compared to 65% (LA) employing CALB lipase. This could be due to the fact that the difference in the chain length of C8 and C12 is less compared to C8 and C18, therefore the values do not differ significantly, as confirmed in the following tests at ~2.5 kg scale.

In solvent free reactions, temperature may lower the viscosity thus affecting the mass transfer, enzyme activity, and product profile. For CALB lipase (Lipozyme®435), 50 °C was reported as the optimal temperature [17] and for RM lipase and best performance was observed between 54 and 55 °C for monolaurin synthesis [18]. However, in the present study, 65 °C was chosen for further investigation due to faster reaction rates, maximum fatty acid conversion and similar MAG profiles (at 50 °C and 65 °C) with CALB.

Glycerol and fatty acids have different polarities, therefore the homogenization between the reactants in a solvent free system is very limited. Addition of minor quantities of product acting as emulsifier, at the beginning of the reaction, would aid the mixing of the substrates. Addition of 5 wt% monolaurin or monocaprylin, however resulted in a very similar fatty acid conversion profile therefore depicting no significant impact of the added MAGs (Fig. 5a and 5b). The composition of products in terms of weight and selectivity was also in the same order as in the conditions where no MAG was added (data not shown). The information on the use of additives in enzymatic esterification reactions is quite limited. Addition of surfactants to homogenize oils with water in the enzymatic hydrolysis of oils has been described in a few studies [19, 20]. Recently, it was reported that addition of the product increased the fatty acid conversion rate (83% in 30 min with product addition as compared to 77% without addition) for monolaurin synthesis [21]. During the synthesis of Polyglycerol-10 laurate from lauric acid and polyglycerol in a solvent free medium, addition of 5 wt%, 10 wt% and 25 wt% PG-10 laurate product assisted in elevating the initial reaction rate but did not improve the final conversion. This system might have benefitted from viscosity decrease in the initial phase of the reaction but the presence of product shifted the equilibrium to the substrate side of the reaction, and thus no further improvement in final lauric acid

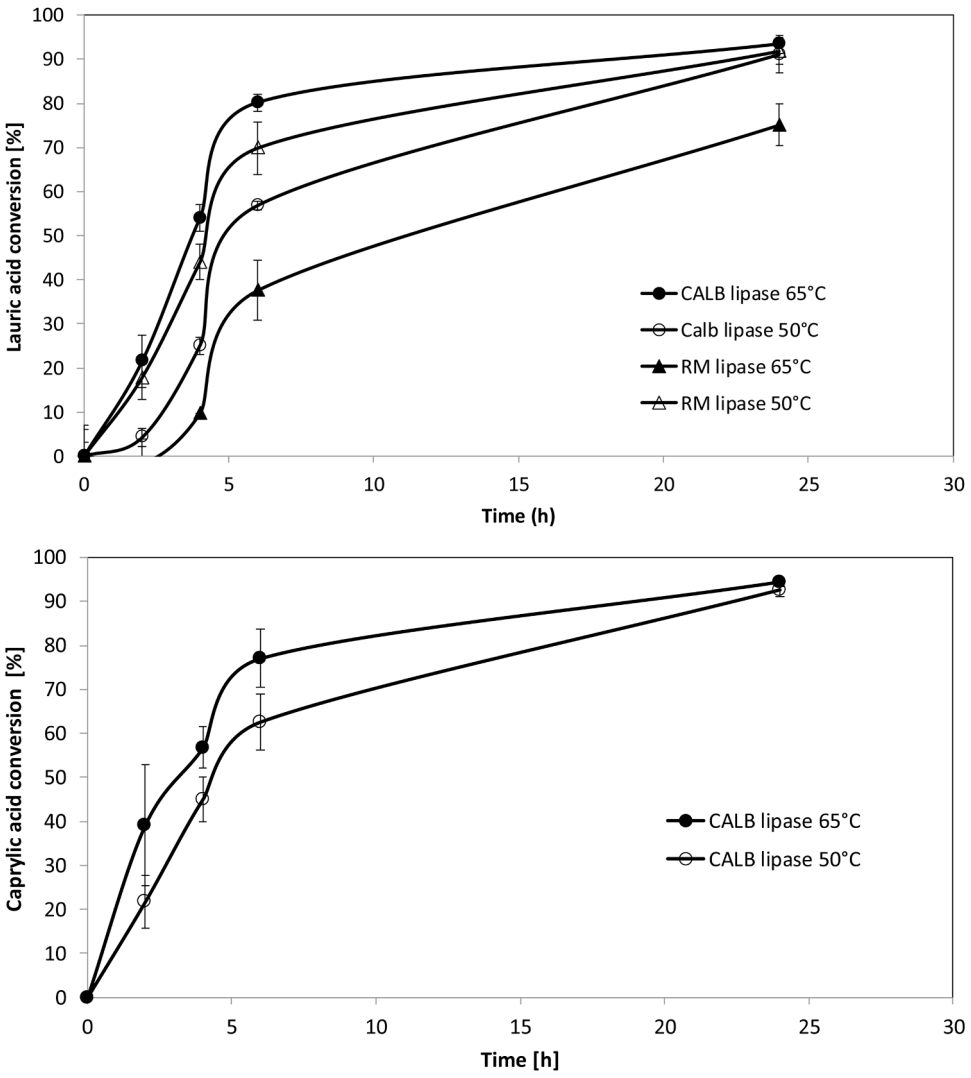



**Fig. 2.** (a) Time course for lauric acid conversion: CALB lipase at 65 °C (filled circle) and at 50 °C (open circle); by RM lipase at 65 °C (Filled triangle) and at 50 °C (open triangle). (b) Time course for caprylic acid conversion: CALB lipase at 65 °C (filled circle) and at 50 °C (open circle).

conversion was exhibited [22]. Apart from the product, other surfactants can be added to the reaction but it is essential to make an informed choice, because apart from providing mixing, the additive should be inert and can be used in the final product. For example, the use of Tween and soy lecithin as additive during the glycerolysis of fish oil with immobilized CALB (Novozym®435), resulted in the enzymatic modification of the additives. Such incidences can complicate the downstream processing and final recovery of the desirable products [23]. Demonstrating a positive influence on the phase behavior of the reaction mixture, Baum et al., reported, a 9% selectivity increase in synthesis towards MAG with the addition of 5 wt% of the amphoteric surfactant cocamidopropyl betaine (CAPB) compared to a solvent free reaction. Being an approved additive in cosmetic products, CAPB does not need to be removed from the reaction [24].

Upscaling the reaction to ~2.5 kg with nitrogen flushing, the reaction proceeded faster. As shown in Fig. 6a, >98% fatty acid (LA and CA) conversions are observed after 23 h. The product consisted of > 50 wt% MAG for LA and > 45 wt% for CA, with values of 30 wt% for DAG and 10 wt% for TAG (Fig. 6b). Observing higher MAG (wt%), for LA as compared to CA in upscaled reaction also confirms the fact that weight (%) MAG production was not related to fatty acid chain length, at least for the C8 and C12 acids. It is reported that various lipases display varying degrees of selectivity towards the substrates with which they interact and the stearic hindrance (branching, unsaturation and chain length) and electronic effects of the substrates are the two major factors that determine selectivity. However, it is difficult to generalize the effect of chain length on esterification as this depends on individual lipase preparation and its specificity [9]. In the current study, no particular difference between the C8 and C12 was observed for CALB lipase, presumably due to the fact that both C8 and C12 are still in the region of scissile binding site of CALB lipase. Comparing the two reaction systems ~60 g scale (water removal with molecular sieves adsorption) and ~2.5 kg scale (water removal by $N_2$ flushing), MAG selectivity for both LA and CA was lower in the later system (Fig. 6c). This could be attributed to two reasons: (i) higher fatty acid conversion at kg scale with $N_2$ flushing, because as the quantity of residual fatty acid decreases, each type of acyl glycerol increases [25], thus decreasing the selectivity particularly for MAG, (ii) better mixing and water removal in kg scale system. Compared to molecular sieves for water adsorption, nitrogen flushing is a more effective method and especially with low volatile compounds such as LA, CA and glycerol, this method presents better applicability. In addition, air and/or $N_2$ flushing yields relatively high convective mass transfer rates for water with a minimal influence on enzyme activity and stability [26].

Monoacylglycerol synthesis using lipases has been reported in both solvent free systems as well as in the presence of organic solvents such as

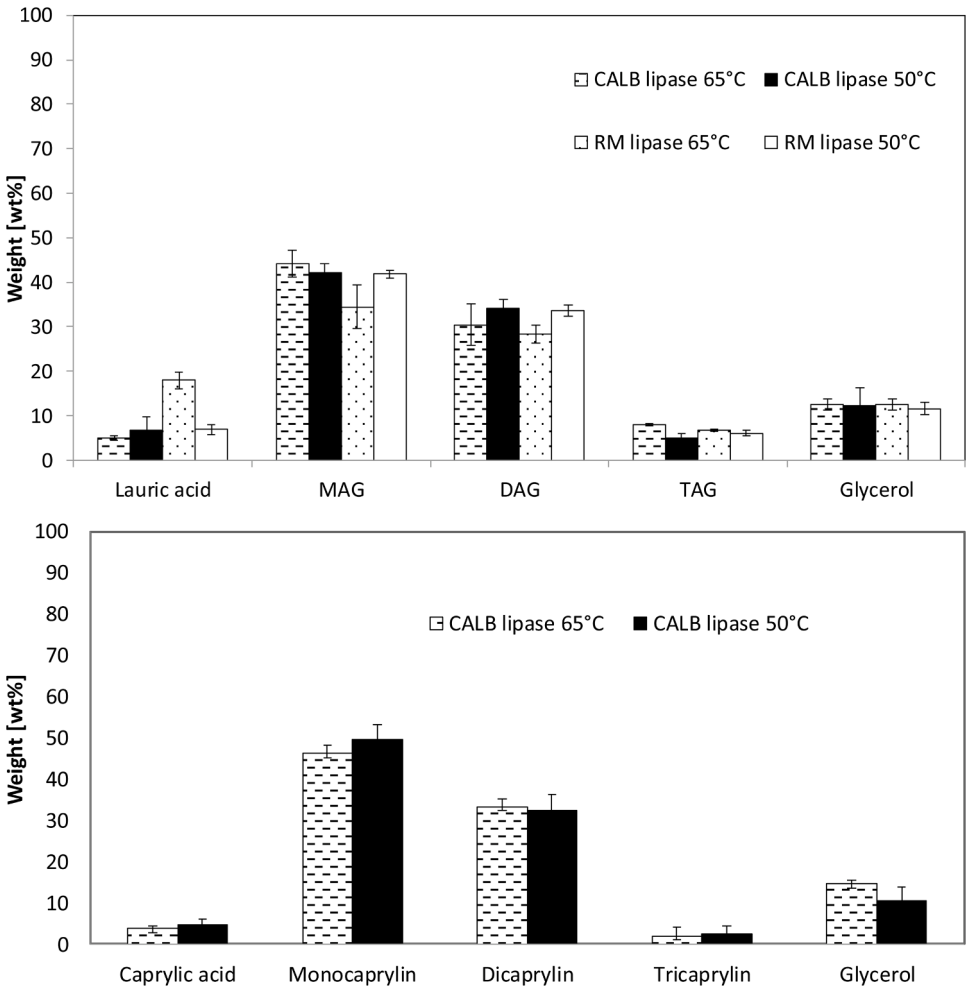



**Fig. 3.** (a) Weight (wt%) of reaction components after 24 h for monolaurin synthesis: CALB lipase at 65 °C (dashed bars) and at 50 °C (filled bars); RM lipase at 65 °C (dotted bars) and at 50 °C (empty bars). (b) Weight (wt%) of reaction components after 24 h for monocaprylin synthesis: CALB lipase at 65 °C (dashed bars) and at 50 °C (filled bars).

acetone, n-hexane/tert-butanol, hexadecane etc. [7,27,28]. The aim of adding solvent is to provide better mixing between hydrophilic glycerol and hydrophobic fatty acids and stimulate the production of MAG. These studies reported 66 to 81 wt% MAG with taking into account the residual fatty acid but the glycerol and additional solvent are not included in the calculation. Also for solvent free synthesis, the residual glycerol is most of the time not accounted for weight percent in the final converted reaction mixture. Therefore, comparison of precise values with those reported in literature has to be done with utmost care, because apart from differences in reaction systems, the way of calculating and reporting the data is different. For monolaurin synthesis, *Rhizomucor miehei* is reported to exhibit superior performance as compared to that of *Candida antarctica B*. This observation is supported by the fact that CALB has high activity for short and medium chain length fatty acids and decreasing activity for long-chain fatty acids. *Rhizomucor miehei* lipase on the contrary, has relatively low activity for short chain fatty acids and vice-versa. The scissile fatty acid binding site of CALB is relatively short (C13) and has a small hydrophobic area located at the wall of the binding funnel. In RM lipase it is located in a long (C18), well-defined hydrophobic crevice [15,29]. The use of immobilized lipases from *Rhizomucor miehei* resulted in 76% lauric acid conversion after 6 h with product profile consisting of 45 wt%, 27 wt% and 3 wt% MAG, DAG and TAG, with residual acid being 24% (without taking the residual glycerol into account). In principle, a higher conversion would have been possible on increasing the reaction time [18]. In the current study, it is indeed observed that > 90% lauric acid conversion can be achieved using longer reaction times (24 h) for both CALB and RM lipase (except with RM lipase at 65 °C) (Fig. 2a). However, for fair comparison, the enzyme loadings, which are eventually related to activity of a particular immobilized enzyme formulation, should be considered. Various studies employ enzyme loading ranging from 0.4 to 10 wt%. In the current study, we used 0.75 wt% (of the fatty acid) which is lower than 3 wt%, as reported by Pereira et al. [18]. The molar ratios of the substrates and especially higher glycerol to acid can increase the acid conversion without improving the selectivity towards MAG. Employing a glycerol to lauric acid ratio of 4:1, Mustafa et al., observed 93% LA conversion resulting in 50% MAG, 34.6% DAG and 8.4% TAG utilizing the 4 wt% immobilized *Rhizomucor miehei* lipase at 60 °C [21]. However, in longer term, excess glycerol inhibits the enzyme activity due to its polar nature as it can strip the water from the enzyme which eventually could lead to its inactivation. Moreover, higher molar fraction of acid compared to alcohol enhances the synthesis of di-and tri-laurin [18,30].

For monocaprylin synthesis in the current study, the non-reactivity of RM lipase could be due to the fact that RM lipase favour longer chain fatty acid. Moreover, the support material and immobilization method of the enzyme would have also an impact. For *Penicillium camembertii*, lipase immobilized on epoxy $SiO_2$-PVA in solvent-free media, high selectivity for MAG was observed for C14 and C16, whereas the fatty acid conversion was highest for C12. As stated before, this could also be attributed to final equilibrium since lower conversion eventually would lead to higher MAG selectivity [9]. In the current solvent free synthesis study, with a C8 and C12, the molar fractions of three

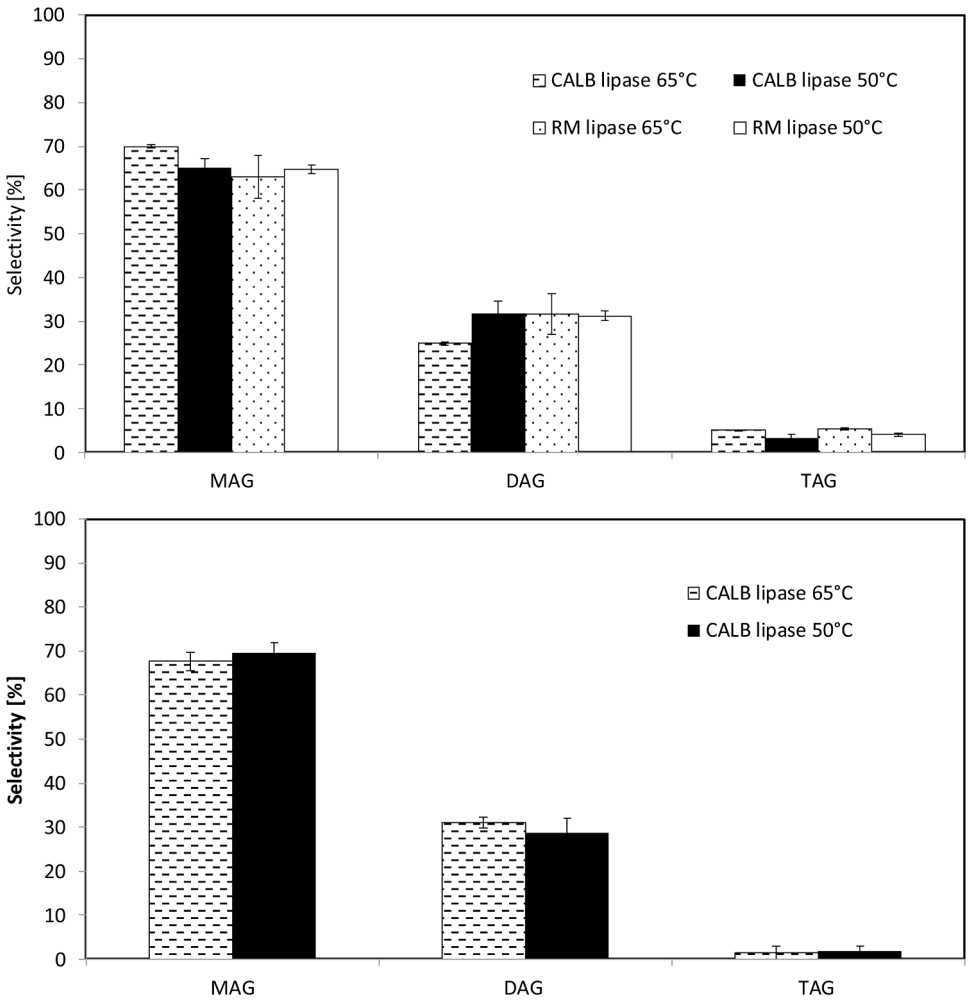



**Fig. 4.** (a) Selectivity (%) of reaction components after 24 h for monolaurin synthesis: CALB lipase at 65 °C (dashed bars) and at 50 °C (filled bars); RM lipase at 65 °C (dotted bars) and at 50 °C (empty bars). (b) Selectivity (%) of reaction components after 24 h for monocaprylin synthesis: CALB lipase at 65 °C (dashed bars) and at 50 °C (filled bars).

components namely MAG, DAG and TAG are similar using CALB for C8.

Compared to C12, C8 is relatively less studied for solvent free esterification of monocaprylin. There are however many studies that have used immobilized lipase to incorporate caprylic acid into different sources of oils in solvent or solvent-free media [31-33]. In a recent study, Guebara et al. reported 7%, 68% and 13% MAG, DAG and TAG, respectively at 2:1 CA and glycerol ratio at 50 °C by using *Rhizomucor miehei* lipase. Moreover, by increasing the CA/glycerol ratio to 2.58/1 and decreasing the temperature to 36 °C, more DAG could be synthesized thus indicating that the esterification reaction can be directed to obtain desired caprylin by adjusting reaction conditions. In this case, the tendency to produce dicaprylin was attributed to lipase sn-1,3 specificity [25]. In yet another study, employing RM lipase immobilized on three mesoporous materials, silica, alumina and titania, highest degree of caprylic acid esterification using a mixture of glycerol and water, was observed with hydrophobized silica because the water activity is lower at the solid surface of this carrier [34].

The WHO directive for the use of additive E471 (mono- and diglycerides of fatty acids) as biosurfactants requires a minimum content of 30% w/w MAG and at least 70% w/w of MAG and DAG, and below 10% w/w of TAG [35]. However, in some food emulsifier applications ≥ 80–90% MAG content is required because the final MAG concentrations would impact the texture or mouthfeel of the processed food product [7]. Considering this, the values obtained in the current study (Fig. 6b) for the esters of both LA and CA meet the requirements for additive E471 but downstream purification steps will be needed for their use in some food emulsifiers.

### 3.2. *Solvent free enzymatic synthesis of monolaurin and monocaprylin in packed bed reactor (PBR at 100 g scale)*

To make the use of immobilized enzymes economical, their recyclability is highly important. Mechanical stability of the immobilization support decreases in batch reactors equipped with stirrers, thus hampering their reusability. The disintegration and attrition of immobilization support leads to the release of the enzymes and consequent loss of its activity. Therefore, working in continuous flow, can protect the immobilized enzymes from mechanical stress. In most cases, packed bed reactors are the choice for biocatalyzed continuous flow processes. This involves the presence of the immobilized enzyme in a packed column where the reaction media is pumped throughout the column under a specific flow rate which will determine the residence time (reaction time) according to the column volume [36]. Therefore, in the next step, the reactions were studied in packed bed column initially at 100 g scale.

As shown in Fig. 7a, after 74 h, >80% LA conversion was observed, and it reached the equilibrium as the values stayed the same after 99 h. Therefore, at 99 h, the molecular sieves in the stainless steel column were replaced with 31 g of fresh molecular sieves (dried at 150 °C overnight), and the first sample after replacement was collected at 119 h. The conversion stayed in the same order until 124 h with a further

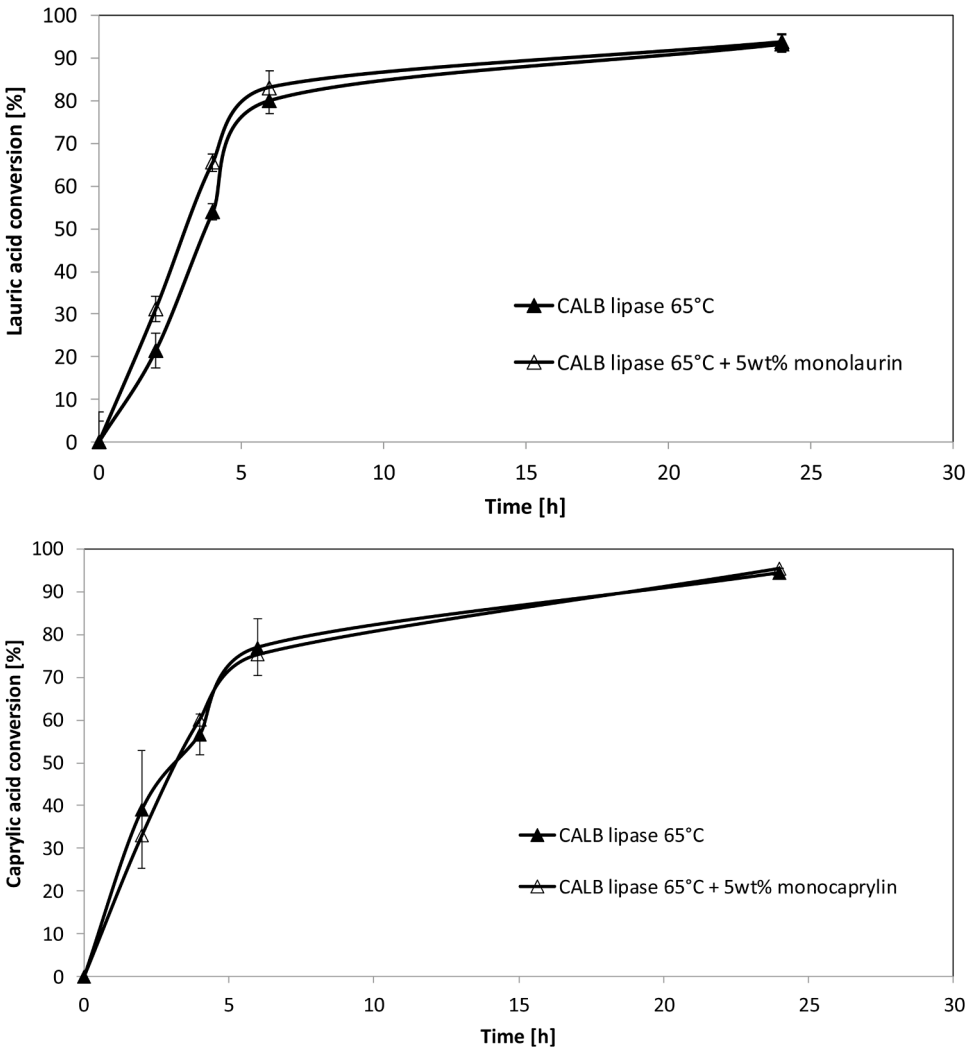



**Fig. 5.** (a) Time course for lauric acid conversion by CALB lipase at 65 °C: no additives (filled triangle) and with 5 wt% added monolaurin (open triangle). (b) Time course for caprylic acid conversion by CALB lipase at 65 °C: no additives (filled triangle) and with 5 wt% added monocaprylin (open triangle).

increase from 139 h onwards, with final conversion reaching >99% at 260 h. The water weight% (wt%) measured after 24 h was 1.2 wt%, which declined to 1 wt% and stayed stable. This trend implies that the water that was being produced during the reaction was also getting adsorbed by the molecular sieves. In trend with the LA conversion, the water percentage also stabilized around 99 h. When the molecular sieves were replaced, continuous decline in water (wt%) in the reaction mixture after 139 h was noticed which finally reached the value of 0.05 wt%. The MAG (wt%) at about 100 h (when LA conversion is 84%), was around 37 wt% with 28 wt% DAG and < 10 wt% TAG (Fig. 7b). In comparison to the batch reactors (~60 g) with orbital shaking (Section 3.1), the selectivity of MAG is lower in 100 g scale packed bed reactors and at the same time DAG and TAG display higher selectivity in PBR (Fig. 7c). This could be attributed to the fact that longer reaction time might lead to production of higher DAG and TAG since MAG can also act as substrate for further esterification to produce the latter two compounds. The results obtained for caprylic acid also exhibit similar trends. At equilibrium, CA conversion reached 75% after 48 h. After the replacement of molecular sieves, after a slight dip in conversion, an increasing trend was observed, and a conversion of >98% CA was observed after 192 h. The water (wt%) in monocaprylin reaction increased after 28 h (2.5 wt% water) as compared to monolaurin production (Fig. 8a). This is related to initial water present in the substrates, since the used C8 contained 0.85 wt% water and C12 had a lower amount of water (0.01 wt%). Moreover, to achieve the same reaction volume, in solvent free conditions and equimolar alcohol/acid ratio, more glycerol (40 wt%) is added to C8 reaction (because of its smaller molecular weight) as compared to C12 reaction where 30 wt% glycerol was added (initial water present in glycerol was 0.35 wt%). Similar observations as in C12 were made for final product profile in case of C8, implying that longer reaction times led to an increase in wt% of DAG and TAG (Fig. 8b and 8c).

Use of lipase in a packed bed reactor for solvent free esterification for the synthesis of monolaurin and monocaprylin has not been reported in the literature to the best of our knowledge. However, transesterification of methyl laurate and glycerol in a binary solvent system (*tert*-butanol/isopropanol 20:80, w/w) has been studied [17]. Employing a 1:6 substrate molar ratio (methyl laurate to glycerol), 80% methyl laurate conversion with 85 wt% MAG and 0.7 wt% DAG was reported. During 18 days of testing period, Lipozyme® 435 was found to be stable. MAG synthesis has also been studied by glycerolysis of palm olein in PBR in solvent medium (acetone/isooctane). Employing palm olein to glycerol molar ratio of 1:12 and immobilized lipase *Pseudomonas* sp., 70% MAG yield after 24 h of operation time was observed [37]. In another study, for monoolein synthesis in PBR, *Penicillium camembertii* (Lipase G) immobilized on polyvinyl alcohol hybrid composite ($SiO_2$-PVA) was employed. Using a oleic acid and glycerol molar ratio of 1:8 in a solvent free system, the continuous esterification reaction was carried out in

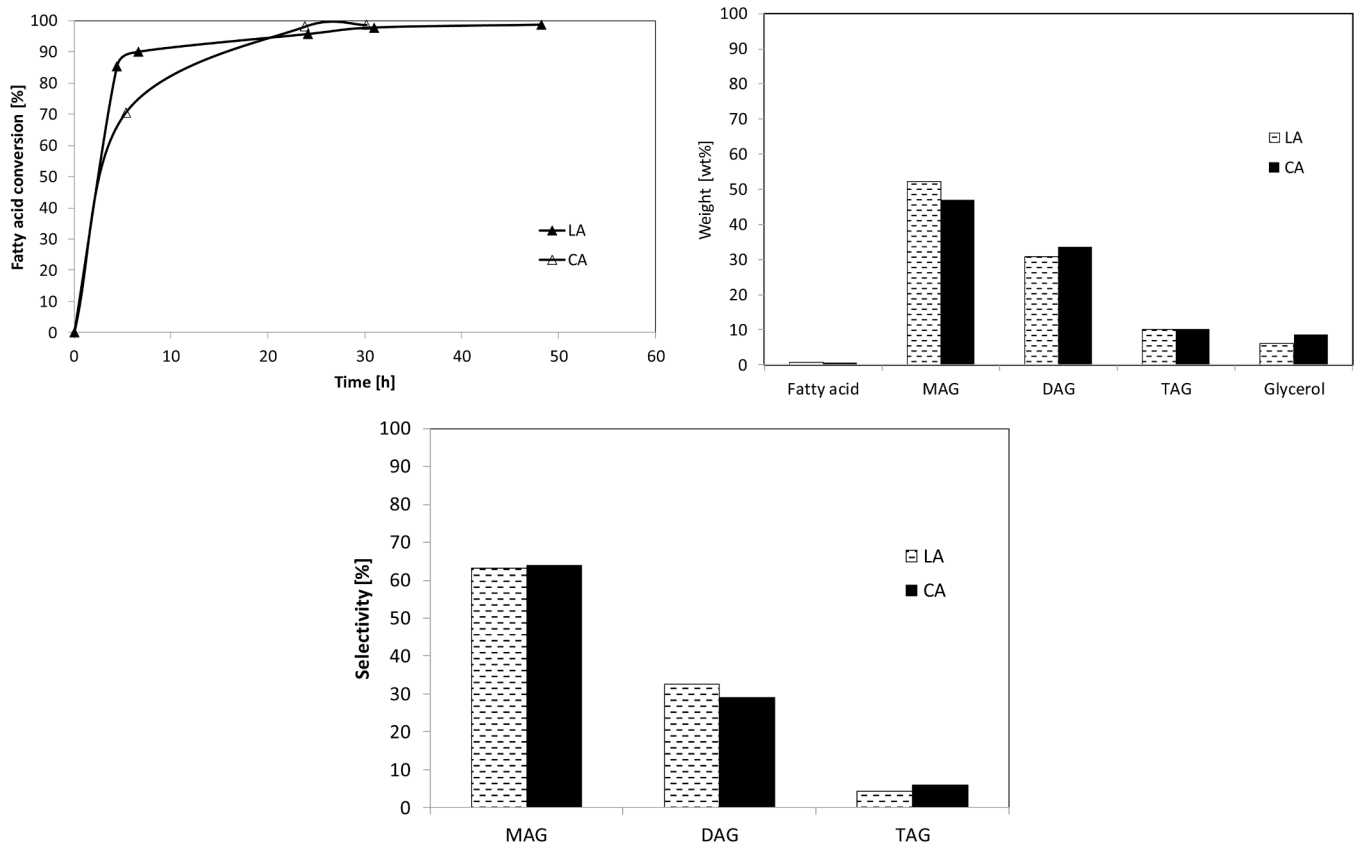


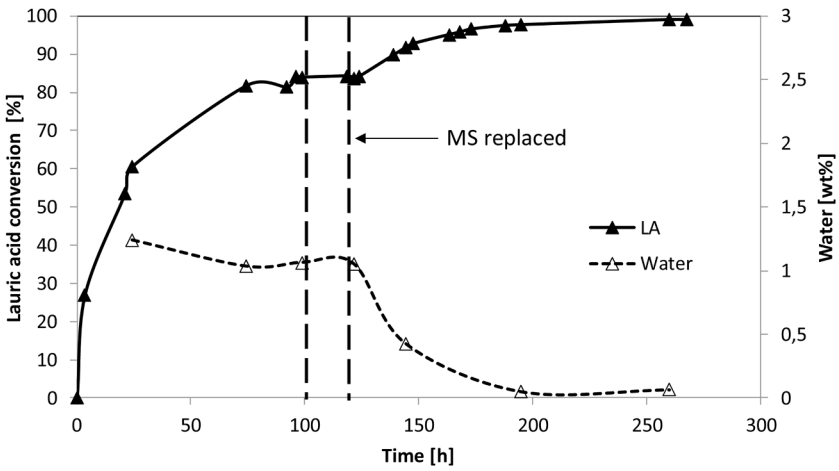

**Fig. 6.** (a) Time course for fatty acid conversion by CALB lipase at 65 °C with $N_2$ flushing at 2.5 kg scale: lauric acid (filled triangle) and caprylic acid (open triangle). (b) Weight (wt%) of reaction components after MAG synthesis by CALB lipase at 65 °C with $N_2$ flushing at 2.5 kg scale: lauric acid (dashed bars) and caprylic acid (filled bars). (c) Selectivity (%) of reaction components after MAG synthesis by CALB lipase at 65 °C with $N_2$ flushing at 2.5 kg scale: lauric acid (dashed bars) and caprylic acid (filled bars).

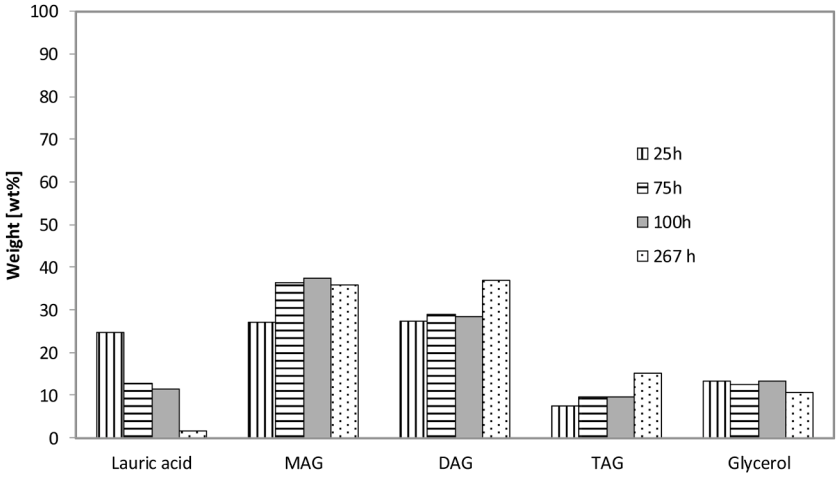
three columns in series with a molecular sieve column for water extraction. The steady state was reached in 72 h, and the MAG concentration remained at an average 35% up to 288 h operation, with a slight decrease after this time. During the steady state, DAG and free fatty acid concentrations were in the order of 15% and 53%, respectively. Employing the molecular sieve column enhanced the system by maintaining the water at the desirable levels during the course of the reaction [4]. Apart from MAG, the nutritional benefits of DAG have attracted much attention. A pilot scale (10 kg) production of oleic- and palmitic acid DAG using immobilized RML in a PBR was reported in 2007 [38]. The reaction at 66 °C using fatty acid/glycerol molar ratio of 2.14, yielded 48 wt% DAG and 14 wt% TAG. The enzyme did not show any significant activity losses or changes in fatty acid selectivity on DAG synthesis during the 10 pilot productions but displayed higher selectivity towards the production of oleic acid-enriched DAG. The same approach has been used for the synthesis of DAG-enriched stearic acid showing similar results even on the pilot scale [39].

### *3.3. Solvent free enzymatic synthesis of monolaurin in packed bed reactor coupled with pervaporation (PV-PBR at 3 kg scale) for in situ water removal*

The role of water is very important and crucial in lipase catalyzed solvent free synthesis. Water is absolutely necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes. On the other hand, in esterification, water content affects the equilibrium conversion thus by removing the produced water from the reaction medium it is possible to achieve higher productivity, higher equilibrium conversion, as well as higher reaction rates [40,1]. On a laboratory scale, use of zeolites or molecular sieves is widely used for water removal as also in the current study. Although theoretically the molecular sieves could also be re-used in subsequent reactions, it is important to note that this method for water removal might not be the most practical at industrial scale, since it brings more solids into the reaction, makes downstream processing more difficult and requires that the sieves are regenerated at high temperatures [41]. Therefore, other water removal methods such as pervaporation would be more appropriate. Pervaporation is the selective transport of liquid through a homogeneous nonporous membrane with simultaneous evaporation of permeates. It can be coupled with a reactor unit and enable selective removal of water. The main advantages of pervaporation are lower energy consumption in comparison to distillation by 75%, and 50% lower investment and operating costs. Another industrial advantage is an easy scale-up of the process and continuous operation [42,43].

The reaction in packed bed reactor and coupled pervaporation (PV-PBR) operated with 3 kg reaction mixture, showed steady increase in LA conversion till 67% after 25 h and this also corroborates with built up of water in the reactor in initial hours before the PV was started (at 19 h). The LA conversion increased steadily, till 95% after 256 h and the final water reached a lowest value of 0.8 wt% (Fig. 9a). The final product at 256 h consisted of 35 wt%, 37 wt%, 13 wt% of MAG, DAG and TAG, respectively. The residual fatty acid and glycerol in the final reaction mixture were 4 wt% and 11 wt%, respectively (Fig. 9b). As evident from Fig. 7b and 9b, the final components (wt%) is similar in both PBR (100 g scale with water removal by molecular sieves) and PV-PBR (coupled pervaporation), with similar profile for DAG and TAG. However, the increase in MAG (wt%) is slower in PV-PBR compared to PBR. On the other hand, DAG (wt%) for PBR stayed at 28 wt% till 100 h with only increase at a later stage to 36%. In PV-PBR, DAG started with 45 wt%



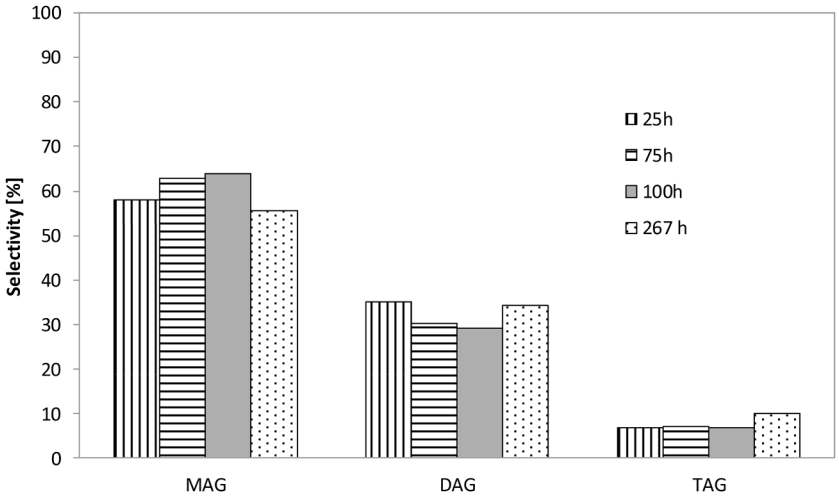
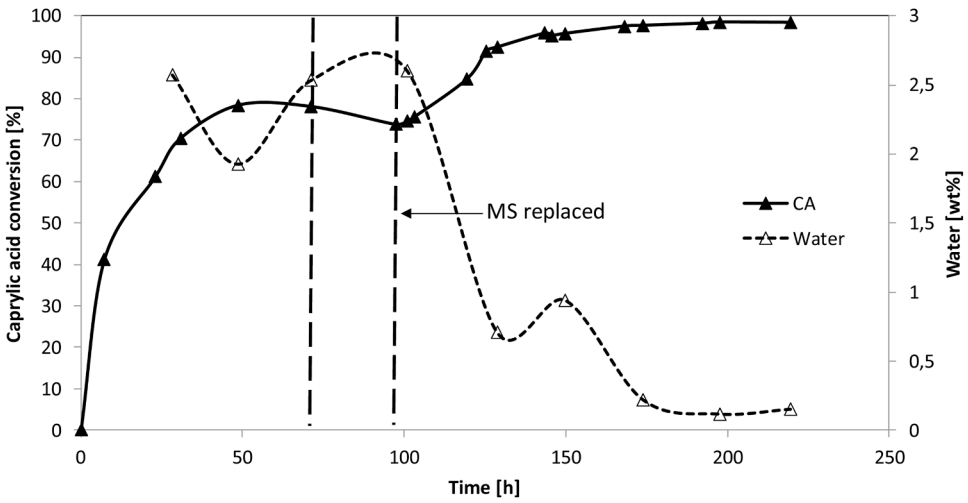
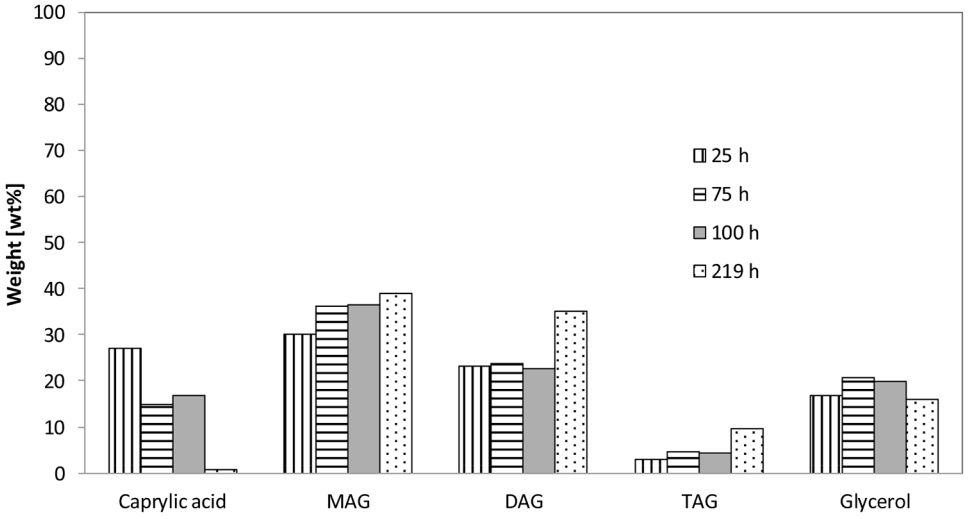




**Fig. 7.** (a) Time course for lauric acid conversion by CALB lipase at 65 °C in 100 g scale packed bed reactor: lauric acid conversion (filled triangle), water (wt%) (open triangle). MS: molecular sieves. (b) Weight (wt%) of reaction components for monolaurin synthesis by CALB lipase at 65 °C in 100 g scale packed bed reactor: after 25 h (vertical bars), 75 h (horizontal bars), 100 h (filled bars) and 267 h (dotted bars). (c) Selectivity (%) of reaction components for monolaurin synthesis by CALB lipase at 65 °C in 100 g scale packed bed reactor: after 25 h (vertical bars), 75 h (horizontal bars), 100 h (filled bars) and 267 h (dotted bars).

and gradually declined to 37 wt%. The major difference between PBR and PV-PBR is the time and method of water removal. PBR system was coupled to molecular sieve column from the beginning and a maximum of 1.24 wt% water was reached, whereas in PV-PBR the maximum water measured was 5 wt% at 15 h (PV started at 19 h). Systems with better water removal result in higher MAG production and therefore, PV-PBR showed higher DAG initially when water (wt%) was highest [4]. It has been reported that PV should not be started immediately, because an intensive water removal can overcome water creation thus eventually deactivating or inhibiting the enzyme [44]. However, when water is formed as a co-product in the same molar proportion as the main product, there is presence of at least two distinct liquid phases (organic and aqueous) and the distribution of water between the enzymatic solid and solution depends very much on the nature of both substrate and support material. Therefore, presence of water has a profound effect on the lipase performance, either directly, by affecting the hydration of the enzyme, or indirectly, by changing the nature of the reaction media and/or enzyme support materials [15]. As the water was eventually removed after starting the pervaporation, the weight of MAG also increased in PV-PBR. As evident from Fig. 7c and 9c, final MAG, DAG and TAG selectivity are similar for PBR and PV-PBR.

Comparing the two upscaled reactions, PV-PBR and the Scott Duran



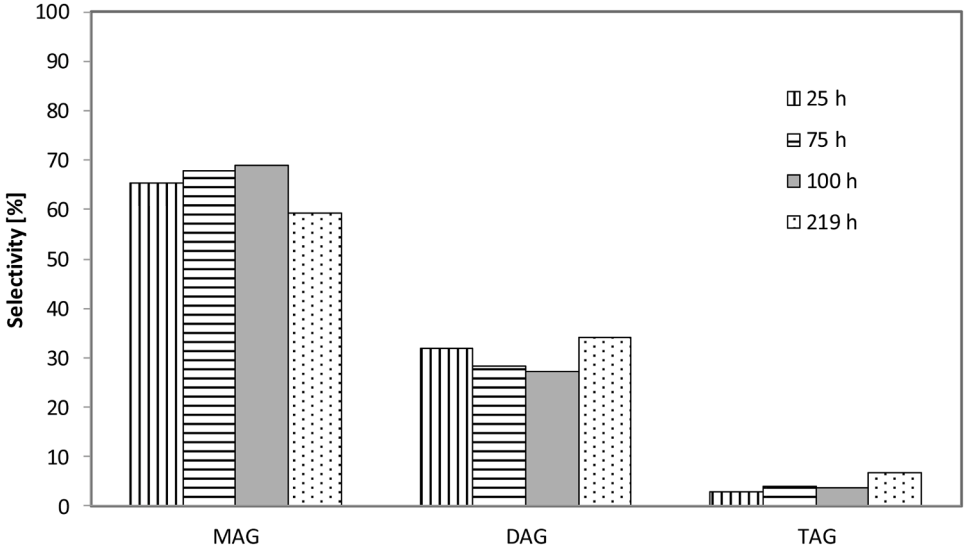




**Fig. 8.** (a) Time course for caprylic acid conversion by CALB lipase at 65 °C in 100 g scale packed bed reactor: caprylic acid conversion (filled triangle), water (wt%) (open triangle). MS: molecular sieves. (b) Weight (wt%) of reaction components for monocaprylin synthesis by CALB lipase at 65 °C in 100 g scale packed bed reactor: after 25 h (vertical bars), 75 h (horizontal bars), 100 h (filled bars) and 219 h (dotted bars). (c) Selectivity (%) of reaction components for monocaprylin synthesis by CALB lipase at 65 °C in 100 g scale packed bed reactor: after 25 h (vertical bars), 75 h (horizontal bars), 100 h (filled bars) and 219 h (dotted bars).

bottles with nitrogen flushing, clearly the latter is more efficient in terms of conversion and product profile having faster conversion and higher MAG selectivity. In such viscous systems, $N_2$ flushing might be a better option, however, immobilized enzyme would need filtration after the reaction in order to allow recycling. In this study, the aim was to achieve the lowest possible acid value (residual free fatty acid), therefore the (PV)-PBR reaction was operated on recirculation mode until maximum fatty acid conversion was obtained. Thus, the LA conversion rate in PBR and PV-PBR can be improved by adding additional substrates to keep the reaction rates sufficiently high.

In general, PV is especially attractive in a temperature-sensitive process because it can be operated at moderate temperatures.

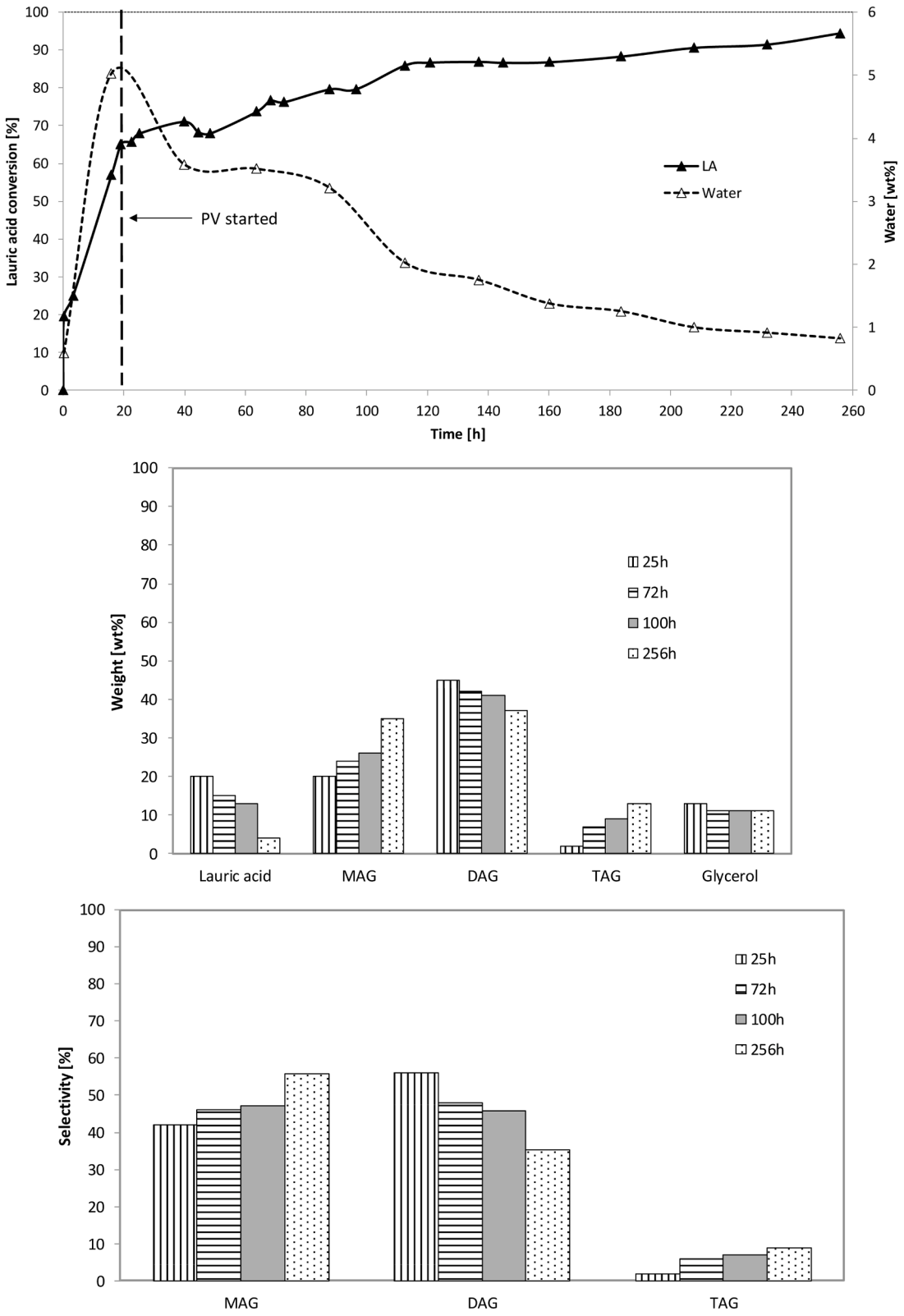



**Fig. 9.** (a) Time course for lauric acid conversion by CALB lipase at 65 °C in 3 kg scale packed bed reactor coupled with pervaporation: lauric acid conversion (filled triangle), water (wt%) (open triangle): Pervaporation started at 19 h. (b). Weight (wt%) of reaction components for monolaurin synthesis by CALB lipase at 65° in 3 kg scale packed bed reactor coupled with pervaporation: after 25 h (vertical bars), 72 h (horizontal bars), 100 h (filled bars), 256 h (dotted bars). (c) Selectivity (%) of reaction components for monolaurin synthesis by CALB lipase at 65° in 3 kg scale packed bed reactor coupled with pervaporation: after 25 h (vertical bars), 72 h (horizontal bars), 100 h (filled bars), 256 h (dotted bars).

Pervaporation has been conducted in lipase catalyzed esterification in solvent based/ionic media [45,46]. In solvent free lipase catalyzed system, sequential pervaporation as well as integrated pervaporation have been reported [47-49]. A system demonstrating lauryl stearate synthesis in cyclohexane medium where the *in situ* water removal improved the stearic acid conversion by 40% was reported by Zhang et al. [50]. This comprised of a novel "sandwich-like" catalytically active membrane consisting of a top layer immobilized with *Candida rugosa* lipase that was sealed in the middle of the membrane reactor and a vacuum (4~5 kPa) was applied at the permeate side.

Like any other membrane process, the separation and selectivity of the membrane during pervaporation is also highly important. The membrane should be able to withstand reaction conditions for a longer time. For an in-house developed two layered membrane, a decline in membrane selectivity was observed on increasing the reaction temperature from 30 °C to 50 °C during ethyl acetate synthesis. The membrane, consisting of a top layer of immobilized lipase (*Rhizomucor miehei*) and a dense bottom layer of sodium alginate (acting as a PV membrane), showed a decline in performance due to an increase in the chain mobility and free spacing of the polymer material, thus resulting in the transfer of large molecules such as esters and alcohols together with water and reducing membrane selectivity. Since alginate is a hydrophilic polymer, the presence of water caused swelling through the intramolecular spaces of the polymeric membrane [51]. In this respect, zeolite membranes are well suited for separating liquid-phase mixtures by pervaporation. Zeolite membranes have uniform, molecular-sized pores, and they separate molecules based on differences in the molecules' adsorption and diffusion properties. Since there are a large number of zeolite membranes that can be prepared, it is essential to test the performance and long term stability. NaA zeolites are the benchmark inorganic membrane material for PV dehydration applications [52] but they lack good performance and stability in acidic environment [53]. However, over the last decade, membranes made with several "newer" materials have become commercially available, including chabazite, T-type zeolites, and hybrid silica. The lower aluminum content is believed to make T-type zeolites less hydrophilic and more acid-stable than Linde Type A zeolites [54]. Furthermore, placing the membrane outside the reaction mixture has another advantage over its long term stability. In a chemically catalyzed esterification reaction between acetic acid and ethanol, a zeolite T membrane was immersed inside the reactor. After having a long term contact with acid, the separation factor declined, and could be restored by immersing the membrane in alkali solution [55]. The current study reports the results of *in situ* water removal by pervaporation integrated with monolaurin synthesis in a PBR for the first time, to the best of our knowledge.

## 4. Conclusions

Lipases offer a wide range of possibilities for synthesis of food and feed components such as monoglycerides (or monoacylglycerols). The enzymatic monoglyceride synthesis, which takes place between 60 °C and 80 °C, has been considered as an alternative to the chemical process for the production of MAG in the past decades. However, despite the number of studies and availability of immobilized commercial lipase preparations, their application for commercial scale synthesis are very limited. Therefore, it is essentially required to develop and demonstrate processes, even with established enzymes such as lipases, in order to broaden their industrial applicability. These examples would help to enable green chemistry on industrial scale thus having high societal and scientific impact. Moreover, the benefits of membrane process such as pervaporation in binary mixture separation are well established due to its high selectivity, efficiency and low-energy requirements [56]. Yet, practical examples are needed to enforce its implementation in a biocatalytic synthesis reaction. Currently pervaporation membranes made up of polymers, inorganics or mixed matrixes are commercially available providing a broad choice to industry in order to find a suitable membrane for their application. Some of the commercially available membrane have exhibited long-term operation (over 400 h) in dehydration of methanol, ethanol and isopropanol (at 120 °C) demonstrating high stability. Also mixed matrix membranes consisting of graphene oxide incorporated into a cross-linked poly (vinyl alcohol) (PVA) matrix showed 75% enhancement of the original permeation rates of pristine cross-linked PVA membranes [56]. In addition, the design aspect is also well covered with the availability of flat sheet, tubular modules and hollow fiber membranes. In fact, due to its minimal fouling and large effective surface area, hollow fiber configuration is recognized as the most advantageous geometry in comparison with flat sheet or tubular modules [57].

In the current study, it was demonstrated that MAG production from lauric acid and caprylic acid with glycerol can be done successfully using Lipozyme® 435 in solvent free conditions. Various reactor types and water removal methods showed a broad range of choices for process implementation. The absence of any volatile components in the reaction mixture, provide an opportunity for bubble column or a reactor with $N_2$ flushing while PV-PBR concept is particularly interesting from enzyme reuse point of view. In order to improve the reaction time in a PV-PBR, it would be interesting to evaluate the process in a feed and bleed system so that the fatty acid conversion rates can be maintained sufficiently high. Of course, in such a system, residual free fatty acid would be higher and demand elaborate downstream processing (DSP). Therefore, evaluation of this trade-off between better biocatalyst yield (by recycling the lipases) and DSP for residual acid removal can be made by conducting more tests and a techno-economic analysis.

## Authors' contributions

Yamini Satyawali contributed in project conceptualization, funding acquisition, planning of experimental work, data processing and interpretation of results. She is also the lead author of this manuscript. Lieve Cauwenberghs performed the experimental work and prepared samples for analytics. Miranda Maesen optimized the analytical methods and analyzed the samples. Winnie Dejonghe contributed in project conceptualization, funding acquisition, planning of experimental work, and interpretation of results. She also contributed with her inputs in the final draft of manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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