# LIPASE-CATALYZED SYNTHESIS OF LONG-CHAIN FATTY ACID ESTERS IN A BENCH-SCALE PACKED BED BIOREACTOR

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## ABSTRACT

Long-chain fatty acid esters are useful functional molecules responding to the requirements of numerous fields of application in cosmetic, pharmaceutical and lubricant industry. In the present work, lipase-catalysed production of n-octyl oleate by esterification of oleic acid by 1-octanol in dense $CO_2$ reaction medium was performed in a bench-scale packed bed bioreactor, in order to obtain suitable reaction performance data for up scaling. Lipase from *Rhizomucor miehei* (Lipozyme RM IM) was used as the biocatalyst. The screening and optimisation of various process parameters were developed. The effect of these reaction variables was evaluated on the ester yield both independently and in combination. Operating at the optimum reaction conditions, higher ester yield compared with the one obtained at atmospheric pressure was detected. The enzyme activity was also studied. Indeed, carrying out the enzymatic reaction in the continuously operating bioreactor, a long-term enzyme lifetime was observed and a decrease of the Lipozyme lifetime was not registered over 1 month. This behaviour was explained because the negative effect of depressurisation/pressurisation cycles on biocatalyst activity was avoided. The ester content was quantified by calculating the residual fatty acid in the reaction mixture. The investigation on the catalytic behaviour of the immobilized enzyme showed a high synthetic activity and selectivity (>99.0 %) in supercritical $CO_2$. n-Octyl oleate was successfully synthesized in supercritical carbon dioxide by enzymatic reaction.

## INTRODUCTION

Although lipase-catalysed esterification has significant benefits, the industrial application of the technology has been slow. Many examples of biocatalytic synthesis have been documented, though few were optimised in bioreactor in spite of their importance for large-scale preparation [1-3]. For widespread industrial use to occur the process has to be technically and economically feasible. Packed-bed bioreactors have been extensively investigated for use in industrial scale applications. The ratio between substrate and enzyme is much lower in a packed bed-reactor than in conventional batch reactors, and it results in higher reaction performance. Moreover, the stirred-tank reactor might not be suitable for long-term and industrial-scale production because enzymes immobilized on resins cannot be loaded into the reactor at a high density and would be susceptible to breaking by the stirring shear force, while a conventional packed bed bioreactor is generally suitable for long-term continuous operations. Furthermore, the packed-bed reactor is one of the most commonly

employed for solid–fluid contacting in heterogeneous catalysis, because (i) it facilitates the contact and subsequent separation; (ii) it allows reuse of the enzyme without the need for a prior separation; (iii) it permits the use of a continuous mode of operation; and (iv) it is more cost effective than the batch operation [4-6]. In general, PBR resulted to have many advantages for large-scale process, such as obtaining a high yield with low cost, and ease of design, operation and maintenance [7].

In the present work, results obtained in a preliminary study of the feasibility of using a continuous flow packed-bed reactor containing immobilized lipase from *Rhizomucor miehei* to affect the n-octyl oleate synthesis by continuous esterification of long-chain fatty acid by 1-octanol accomplished in dense carbon dioxide have been presented.

Lipases are well suited for applications in $CO_2$ because their catalytic feature involves a lipid-water interface. This characteristic gives lipases an inherent affinity for hydrophobic media. It is well known that the catalytic efficiency of enzymes decreases as the hydrophilicity of the solvent increases [8]. The stability of the enzyme was investigated, being one of the critical factors for the successful operation of PBR. In non-aqueous media enzymology, the stability of enzyme was often affected by the solvent-induced inactivation. For lipase, such inactivation was usually effectively overcome by the use of appropriate immobilization technique [9]. There are several advantages, which make immobilized enzymes attractive for industrial applications of this reaction. For example, immobilized preparations usually exhibit higher operational stability, easier recycling for subsequent reuse and higher biocatalytic density, and offer economic benefits in terms of being able to produce a greater quantity of product per unit of enzyme consumed. In addition, immobilized enzyme is usually superior to crude enzyme for repeated usage of enzyme due to the easy recovery of the immobilized form [10]. Thus, by using the immobilized biocatalyst, it was intended to supply the system with enzyme with sufficient operational stability for continuous application. At present, the only disadvantage of the use of lipase in industrial applications is the cost of the enzyme. To overcome this problem, the lipase is employed in immobilized form because it would allow the re-utilization of the enzyme.

Furthermore, in this investigation, the performance of the packed-bed bioreactor was evaluated as a function of a few critical parameters, such as pressure and dilution ratio. The solvent concentration was varied first to determine if addition of solvent had an effect on n-octyl oleate formation, and secondly to obtain the minimum concentration of solvent needed for optimum incorporation. However, the easily tuneable solvating power of SC-$CO_2$ facilitates a relatively easy separation of reactants, products, and catalysts after reaction. The high volatility of $CO_2$ allows it to be completely and easily removed from the product, resulting in an overall solvent-free reaction, required for industrial manufacture, cosmetic and pharmaceutical applications, as n-octyl oleate uses.

## I - MATERIALS, METHODS and PROCEDURES

### Materials

Alcohols [1-Octanol (purity 98 %) and Ethanol (purity ≥99.9 %)] were supplied by Aldrich Chemical Co. (Milwaukee, WI). Oleic Acid (extra pure) and Sodium Hydroxide solution (0.1 N) were purchased from Merck (Darmstadt, Germany). Phenolphthalein was given from Kemika (Zagreb, Croatia). The enzyme [Lipozyme IM, lipase from *Rhizomucor miehei* immobilized on a macroporous anion exchange resin] was kindly donated from Novo Nordisk AS (Copenhagen, Denmark).

**Analytical methods**

The ester content was quantified by calculating the residual fatty acid amount in the reaction mixture. Using a volumetric method [11] a 0.1 g sample of the reaction mixture was diluted in 20 mL of 0.1 %wt phenolphthalein solution in absolute ethanol and tritaded with standardized sodium hydroxide solution of 0.1 M in water.

The amount of water included in the enzyme was measured, using a Mettler Toledo DL31 Karl Fisher coulometric titrator. It resulted that Lipozyme, commercially available from Novo Nordisk A/S, included approximately 3.8 %wt of water. The initial water content of the substrates was determined by colorimetric titration as well; 1-octanol and oleic acid initial water content were evaluated at 0.21 %wt and 0.17 %wt, respectively.

**Procedures**

A Packed-Bed Reactor (PBR) has been used to perform biosynthetic experiments in continuous mode. The high-pressure PBR, consisting of a stainless steel tube with internal volume of about 10 mL (75 cm in length and 4 mm inner diameter) was designed to operate up to 50 MPa and 473.15 K. The apparatus was equipped with a thermostated separator, whit internal volume of about 40 mL, which allowed collecting the final product samples. The column was packed with the biocatalyst and the upper and lower ends of the column were layered with cotton wool (1.0 and 2.0 cm in thickness, respectively). Two supply lines allowed to feed the liquid solution and $CO_2$, and one discharge line allowed to release the final reaction product. $CO_2$ from the supply tank was cooled and then pumped into the enzyme bed by a high-pressure pump (PM-101, NWA GmbH, Lorrach, Germany). The $CO_2$ output was measured with wet gas low-volume flow meter (Amco, Elster Handel GmbH, Mainz-Kastel, Germany). The substrates mixture before the injection was mixed by means of a sludge digester rotating mixing and heating system (Rotamix 550 MMH, Tehtnica, Železniki, Slovenia). A high-pressure pump (Solvent Delivery System CONSTA METRIC 3000, liquid flow up to 9.99 mL/min, LDC Analytical, Riviera Beach, FL, USA) was used for substrates feeding upwards the column at varying flow rates (0.05÷1.4 mL/min). The tubular reactor was connected to the rest of the apparatus through high-pressure valves on the $CO_2$ feed line (mod. 10VRMM-2812, Autoclave Engineers, Pennsylvania, USA) and on the substrates feed line and on the downstream line (mod. 10V-2081, Autoclave Engineers, Pennsylvania, USA), which was used to regulate the system operative pressure. A micro metering valve (mod. 10VRMM-2812, Autoclave Engineers, Pennsylvania, USA) on the discharge line provided to laminate the outgoing flow during release. The effluent from the reactor was pooled in the separator through a valve. Then, samples of the effluent stream were collected from the separator by means of high-pressure valve (mod. 10V-2081, Autoclave Engineers, Pennsylvania, USA) into a glass trap at periodic intervals and analysed. After start-up of the reactor, a space-time (for a liquid phase system, the reactor space-time is equal to the residence time of the fluid in the reactor) of 30÷60 min was allowed to elapse before effluent samples were taken for use in the analysis of the reaction kinetics. Thus, the experimental points correspond to quasi steady-state values of the effluent composition. The heating in all the apparatus was obtained using four electrical wires (mod. 020901, 020903, 020904, having respectively a length of 4 m, wrapped around on the reactor tube, 2 m, lodged on the inlet, 1 m, on the separator and on the outline, HORST GmbH, Lorsch, Germany). Temperature of the reactor and the separator was monitored by means of two PID controllers (mod. Grado 901, Hengstler, Aldingen, Germany), with an overall accuracy of the temperature measurement estimated within ±0.3 K. Digital thermometers (mod. GTH 1150, Greisinger Electronic GmbH, Regenstauf, Germany) with an overall accuracy within ±0.5 K

equipped with a K type thermocouple (WATLOW, Missouri, USA) controlled the inlet and outlet temperature. Pressure in the tubular reactor was measured by using a Digibar manometer (mod. PE 200, HBM GmbH, Darmstadt, Germany), with an accuracy within ±0.01 MPa. Two digital manometers (Transmitter for CPH 6200 0-400 bar WIKA GmbH, Klingenberg, Germany) controlled the pressure of the outlet and the separator, with an overall accuracy within ±0.05 MPa. All reaction experiments in PBR apparatus were performed at 323.15 K, using an equimolar substrates solution (63 mmol) and about 6 g of biocatalyst, varying pressure conditions and modulating the carbon dioxide/substrates molar ratio.

## II - RESULTS AND DISCUSSION

The intrinsic effect of pressure on enzymes carrying out biocatalysis in dense carbon dioxide is an important factor. Pressure is likely to affect the reaction performance by changing either the rate constant or the reactants solubility. The pressure effect was investigated in the range between 9 and 20 MPa. Data characterizing the dependence of the extent of esterification on the reactor space-time and pressure are shown in Fig. 1. This inspection reveals that increasing the pressure up to 10 MPa a maximum reaction yield was achieved, though at higher value of pressure the conversion of free fatty acid (FFA) into fatty acid ester (FAE) decreases. A straightforward explanation of this behaviour could be found on the basis that at higher pressures, a higher solvent concentration lessens the molar fraction of substrates. In the tested cases, the asymptotic limits correspond to conversion between 79÷93% of the free fatty acid originally fed to the reactor to its esterified form (see Fig. 1). As shown in Fig. 1, relatively short space times of greatest industrial interest, namely less than 0.5÷1 h, are sufficient to obtain an effluent in which the reaction solution contain significant extents of esterification. As the length of time the mixture of substrates is in contact with the immobilized enzyme increases, the extents of incorporation appear to approach asymptotic limits that are pressure-dependent. The differences in the asymptotes may reflect shifts in the positions for the several equilibria associated with the esterification reactions. From the results collected investigating the pressure effect, it seems reasonable that the pressure does not influence directly the Lipozyme RM IM function, i.e. decreasing the stability of the enzyme and hence decreasing catalytic activity. Change in pressure affects instead the density dependent physical properties, such as partition coefficient, dielectric constant and Hildebrandt solubility parameter, of dense $CO_2$, modulating indirectly enzyme activity, specificity and stability. Indeed, an increase in pressure leads to enhanced fluid density improving solvating power of the fluid. The solubility of dense $CO_2$ in the substrates liquid phase markedly increases up to 10 MPa. At pressures higher than 10 MPa, higher fluid density is counter-balanced by the substrates mole fraction decrease in the enzyme phase, wherein the catalysis takes place and the negative effect of substrate mole fraction decrease is strongly predominant on the high and positive substances solubility. Hence, two factors other than the density might contribute to the observed decreased yield with increased pressure above 10MPa: one factor is the decreased partition rate of substrates in the liquid-phase; the other factor is the increased reaction volume.

Flow rate of solvent, i.e. $CO_2$, is an important factor for a sufficient product concentration at the column reactor outlet, and the substrates liquid flow rate likewise. These two parameters are synergic; therefore, they were investigated simultaneously, in term of dilution ratio, i.e. $CO_2$ flow rate on the liquid substrates flow rate. As it is shown in Fig. 2 the bio-conversion rises up to a maximum as the dilution ratio is increased up to 1.2E-02. Over this value, the conversion level decreases in a linear fashion, (see right side of the graph in Fig. 2). Indeed,

increasing $CO_2$ flow rate over the liquid substrates flow rate, mass transfer enhances because of more rapid diffusion of solutes and higher number of local clustering of solutes and solvent. However, too high solvent concentration lessens the reaction performance due to decrease of substrates molar fraction at constant reactor volume. On the other hand, increasing the residence time by slowing down the substrates flow rate, the linear flow velocity passing through the packed bed becomes small and, as a result, the effect of mass transfer within the liquid film adjacent to the immobilized enzyme resin surface would affect the reaction rate. Furthermore, with an increase in residence time, namely, with decrease in circulation flow rate, the n-octyl oleate concentration is markedly decreased since the reverse reaction of hydrolysis is enhanced by the increase in the water content due to the insufficient water removal. Thus, faster substrates flow rate around a catalyst support at a high superficial velocity reduces the external diffusion resistance, although increasing the substrates flow rate, a price must be paid to achieve this high superficial velocity in terms of a larger pressure drop across the column.
Therefore, by modulating opportunely the $CO_2$/substrates flow ratio mass transfer the reaction performance can be easily and widely tuned.

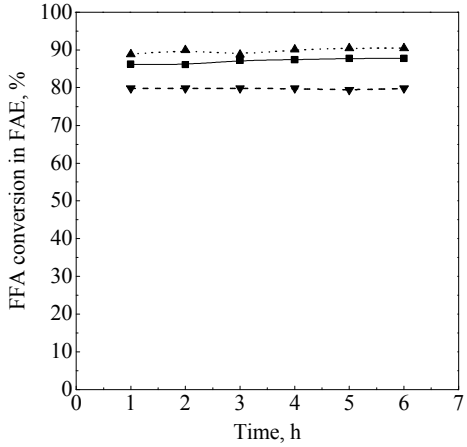



**Figure 1. Pressure effect on the total conversion of free fatty acid (FAA) in fatty acid ester (FAE). Pressure: ▲ 10 MPa; ■ 9 MPa ; ▼ 20 MPa.**

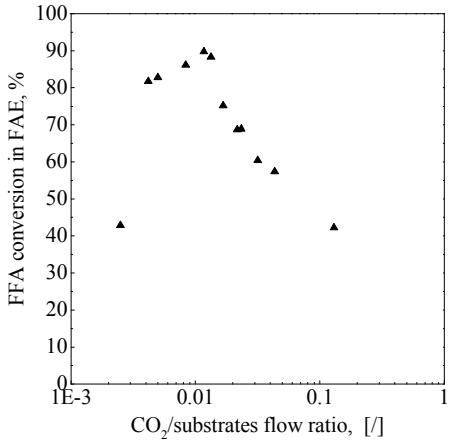
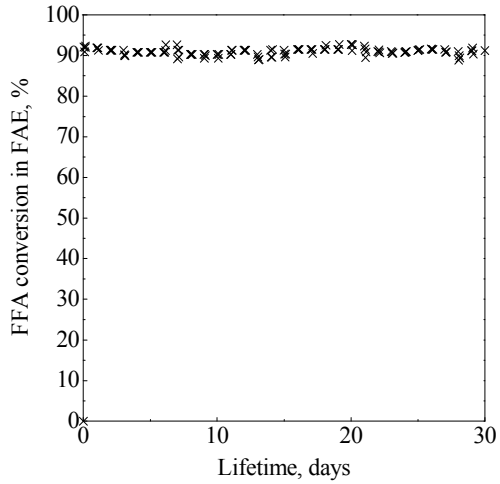



**Figure 2. Dilution ratio effect on the total conversion of free fatty acid (FAA) in fatty acid ester (FAE).**

The suitability of the designed tubular reactor was checked in relation to its behaviour over long time continuous operation. A semi-continuous mode [12, 13] was proposed, which consisted of cycles of esterification reaction and rinsing of the catalyst, enabling the maintenance of a high conversion value for long-term period. Indeed, by rinsing the enzymatic support, optimal hydration of the catalyst was maintained. The washing step has enabled all the water produced adsorbed onto the enzymatic support to be eliminated; optimal hydration of the catalyst has been conserved and initial conversion restored. Thus, the enzyme preparation was daily rinsed by $CO_2$ and conditioned overnight before any reaction run. The immobilised lipase was not replaced during the total 30-days operation. In this way, it was registered no loss in the Lipozyme RM IM activity after 30 days of bioconversion at high pressure, by using carbon dioxide as the both reaction medium and rinsing agent. Firstly, the esterification reaction was carried out for 7 hours; the conversion yield was about 93%. Next, the biocatalyst bed was rinsed with the $CO_2$ for 1 hour. The esterification reaction was then restored by pumping the substrates solution through the catalyst bed. The re-use performance with successive operations up to the 30 runs is shown in Fig. 3, wherein the conversion of 1-octanol-oleic acid ester in the effluent as a function of operation time is plotted.




As shown in Fig. 3, the conversion did not drop significantly after 30 days of reaction. The esterification reaction, carried out for one month in a succession of 7-h synthesis steps followed by 1 hour of washing steps at a flow rate of reaction performance, enables to minimize the loss in enzyme stability and retain the same reaction performance.

**Figure 3. Lipozyme RM IM lifetime.**

## CONCLUSIONS

The long-term stability of both operational system and biocatalyst confirmed the suitability and applicability of the proposed tubular packed-bed reactor for scale-up of the n-octyl oleate. The immobilised lipase from *Rhizomucor miehei* stably operated for at least 4 weeks by intermittent washings with $CO_2$ and the productivity of the desired fatty acid on the order of kg/L-reactor day was achieved.
Further engineering studies will be necessary to evaluate process economics, as well as the desirability of incorporating various purification steps to remove the free fatty acids from the products.

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