Efficient Lipase-Catalyzed Production of Tailor-Made Emulsifiers Using Solvent Engineering Coupled to Extractive Processing

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ABSTRACT: Within the framework of enzymatic biosurfactant synthesis, the development of reaction processes leading to a selective and efficient production is of crucial interest. In this work, we proposed an enzymatic method for producing 1(3)-monooleoyl-*rac*-glycerol using a commercial immobilized lipase, the Lipozyme®. To avoid the blockage of the enzyme by the presence of insoluble glycerol, the latter was adsorbed onto dried silica gel. The selective monoester production (83%) was obtained using 2-methyl-2-butanol amended *n*-hexane (90:10, vol/vol) as reaction medium. The observed decrease in the substrate conversion, in a medium containing 2-methyl-2-butanol, was countered by coupling to the reaction an inline selective recovery method for the produced monoester by adsorption onto a silica gel column located at the outflow of the reaction vessel. This process leads to an efficient monooleoyl glycerol production with a high oleic acid conversion (71%) and to the recovery of a 100% pure monooleoyl glycerol.
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KEY WORDS: Adsorbed glycerol, emulsifier, esterification, extraction, *n*-hexane, 2-methyl-2-butanol, monooleoyl glycerol, silica gel adsorption, surfactant, thermodynamic equilibrium.

Fatty acid esters of polyols are of increasing economic interest in many industries involving a wide field of applications. For example, fatty acid esters of sugars show surface-active properties and very good emulsifying, stabilizing, or conditioning effects (1–5). In the food industry, propylene glycol monoesters of eicosapentaenoic and docosahexaenoic acids are potentially health-beneficial emulsifiers, reducing both thrombotic tendency and hypertriglyceridemia (2). In the pharmaceutical industry, some fatty acid esters of polyols are used as drug carriers. Alkyl polyglycosides are a new generation of surfactants in the detergent industry (3). Finally, monoesters of polyols are reported as being an important group of emulsifying agents used in the food and pharmaceutical industries (4,5).

Common industrial practice for producing these kinds of compounds involves esterification of polyols with fatty acids in the presence of a chemical catalyst. These chemical methods, which generally need drastic reaction conditions, result in a complex mixture of products. The use of enzyme as catalyst seems to be a good alternative for overcoming this problem. Indeed, enzyme-catalyzed esterification in nonconventional media under mild conditions allows esters with specific properties to be produced, taking advantage of the specificity of the biocatalyst.

While the highest enzyme esterification activity and good stability are generally observed in hydrophobic solvents like hexane, hydrophilic polyols are immiscible in this kind of reaction medium, resulting in very low esterification rates. In order to solve this solubilization problem, several solutions have been proposed. For example, in order to perform enzymatic esterification, the use of hexane-soluble 1,2-*O*-isopropylidene-*rac*-glycerol has been suggested (6,7). Moreover, several authors have proposed the solubilization of polyols in hydrophobic solvents through the formation of a complex with acylboronic acid, especially phenyl boronic acid (8–10). Finally, Berger *et al.* (11) reported a method for the immobilization of polar substrates onto a solid hydrophilic support, such as silica gel, for the synthesis of various mono- and diesters of diols. In a previous report, an explanation of the silica gel role was proposed based on the study of oleic acid esterification using silica gel adsorbed glycerol as substrate (12).

In order to improve the selectivity of the enzymatic esterification of polyols, different methods have been used such as using different lipases or different organic solvents or varying the ratio of the reactants. In this way, Berger *et al.* (13) selectively produced monoglyceride using a high ratio of glycerol to fatty acid. Kwon *et al.* (14) have demonstrated that lipase from *Mucor miehei* shows a specificity for the production of dipalmitoyl glycerol, whereas *Rhizopus delemar* lipase mainly produces monopalmitoyl glycerol.

The solvent engineering to produce tailor-made emulsifiers appears to be interesting (15). For example, Janssen *et al.* (16) have studied the influence of the nature of the organic solvent on product compositions during esterification of decanoic acid with various polyols in biphasic media, and have observed that the use of tertiary alcohols with log $P < 2$ favors the synthesis of monoesters. Liu and Shaw (2) reported the

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enzymatic esterification of fatty acids with propylene glycol and showed that the use of *n*-hexane supplemented with 2-methyl-2-butanol increases the specific production of monoesters. Finally, Ducret *et al.* (1), who worked on the esterification between oleic acid and sorbitol in a solvent-free system, demonstrated that the addition of 2-methyl-2-butanol causes an increase in the monoester production from 26 to 70%. However, the oleic acid conversion fell drastically from 95 to 32.5%.

In the present work, we propose a reaction system combining the use of some of the processes described above to selectively and efficiently produce monooleoyl glycerol in a monophasic medium 2-methyl-2-butanol amended *n*-hexane using a commercial immobilized lipase from *M. miehei,* the Lipozyme®. The choice of a monophasic system is based on the fact that a solvent-free medium does not allow selectivity (1) and that a monophasic system is more easily conducted in a continuous way. In order to avoid the blockage of the biocatalyst by the glycerol, a phenomenon described by Castillo *et al.* (8), this glycerol was adsorbed onto silica gel. The effect of the silica gel hydration state was also studied. The influence of the use of *n*-hexane supplemented with a tertiary alcohol, 2-methyl-2-butanol, on the reaction selectivity was tested. Finally, in order to overcome the low conversion observed with this process, an integrated downstream processing was used to recover selectively the product of interest. In others words, a method coupling a selective reaction with a selective fractionation was proposed for synthesizing preferentially monoglycerides at a high conversion.

MATERIALS AND METHODS

Materials. Immobilized lipase from *M. miehei*, Lipozyme® IM-46, was kindly offered by Novo Nordisk Industry A/S (Bagsvaard, Denmark). Glycerol, oleic acid (*cis*-9-octadecanoic acid), 1(3)-monooleoyl-*rac*-glycerol, 1,3-dioleoyl glycerol, 1,2-dioleoyl glycerol, trioleoyl glycerol, and silica gel (mesh 70–230) were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest available purity. All high-purity solvents used were purchased from PROLABO (France).

Methods. Substrates were adsorbed as described by Berger *et al.* (11) and Castillo *et al.* (8): 1 g of glycerol and 1 g of silica gel were carefully mixed until a homogeneous powder was obtained. In typical reactions, 0.8 g of silica gel-adsorbed glycerol and 200 mg of Lipozyme® were mixed in an assay tube containing a 30 mM solution of oleic acid in *n*-hexane (10 mL). The reaction mixture was shaken at 40°C, and several samples of the reaction (50 µL) were withdrawn and analyzed by high-performance liquid chromatography (HPLC). For the reactions in *n*-hexane implemented with 2-methyl-2-butanol 90/10, the same procedure was followed.

To study the effect of the water content on the conversion rate, the silica gel was dried in an oven at 90˚C. Water was removed from 2-methyl-2-butanol by 3Å molecular sieve (Merck, Darmstadt, Germany).

HPLC analysis. The *n*-hexane was extensively evaporated from the reaction samples in a thermostated bath at 90°C and resuspended in an equal volume of HPLC solvent. Product analyses were performed using an integrated system HP 1050 series II (Hewlett-Packard, Palo Alto, CA) with a C_{18} reverse-phase analytical column Spherisorb ODS-2 (5µ, 250 × 4.6 mm) thermostated at 45°C. The product compositions (mono-, di-, and triglycerides) were quantified at a wavelength of 210 nm with a detector UV/VIS 1050 series II. The eluent was a mixture of acetone/acetonitrile (50:50, vol/vol) mixture, isocratically run. Elution was carried out as previously described by Ergan and André (17), with a flow rate gradient of 0.5 mL/min for 7 min, then increased in 3 min to 3 mL/min and held there for 6 min before decreasing to 0.5 mL/min in 4 min. Consumption quantification of oleic acid was performed using the same HP 1050 series II (Hewlett-Packard), the same C_{18} column and an HP 1047A refractive index detector (Hewlett-Packard). Elution was carried out at 50°C with methanol/acetic acid (99.7:0.3, vol/vol) and a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Water content effect of the silica gel adsorbed glycerol on the conversion rate of the enzymatic esterification of oleic acid in n-*hexane*. Biphasic systems (water/organic solvent) solve the difficulty of realizing a reaction between a polar substrate and a hydrophobic one (16). Here, we have chosen the monophasic process, but in *n*-hexane the nonsolubility of glycerol leads to its adsorption onto the immobilized catalyst and consequently to a very low activity. As shown in our previous report (12), the lipase-catalyzed reaction of silica gel adsorbed-glycerol with fatty acids is possible in *n*-hexane. Silica gel plays the role of a "reservoir" for glycerol. In this work, we have focused our attention on the influence of the silica gel adsorbed-glycerol water content on the oleic acid conversion. Indeed, it is well known that the thermodynamic water activity of the reaction medium is one of the determinant factors for enhancing the position of the synthesis thermodynamic equilibrium. Indeed, the thermodynamic equilibrium of a general esterification synthesis

$$S1 + S2 \Leftrightarrow P + W \qquad [1]$$

is defined by:

$$K = (a_p \bullet a_w)/(a_{S1} \bullet a_{S2}) \qquad [2]$$

where a_p, a_w, a_{S1}, and a_{S2} are the thermodynamic activities of the product, the water, and the two substrates, respectively.

Since K is a constant, decreasing the thermodynamic water activity causes an increase in the product activity and consequently in the final product concentration.

The time curves of oleic acid conversion are presented in Figure 1. Here, it is observed that using dried silica gel adsorbed-glycerol (3.7% of water, w/w), a higher conversion at

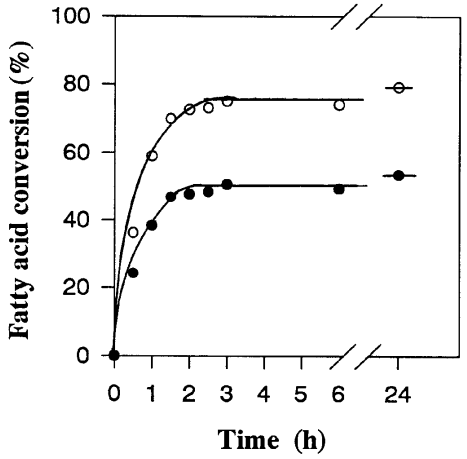

FIG. 1. Time course of oleic acid esterification with glycerol adsorbed on undried silica gel (●) and dried silica gel (○), in 10 mL of *n*-hexane, using an immobilized lipase (200 mg).

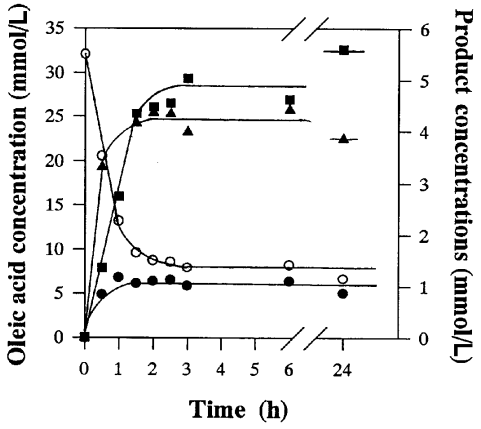

FIG. 2. Time course of triolein, diolein, and monoolein synthesis from oleic acid (30 mM) and adsorbed dry silica gel glycerol (0.8 g) in 10 mL of *n*-hexane, using 200 mg of immobilized Lipozyme. (○), Oleic acid; (●), monoolein; (▲), diolein; (■), triolein.

the thermodynamic equilibrium was obtained (79%), whereas a conversion of only 53% was reached with nondried silica gel adsorbed-glycerol (6.3% of water, w/w). Moreover, it was observed that dried silica gel adsorbed-glycerol gave the higher initial rate.

The product composition obtained in *n*-hexane with dried silica gel was analyzed and is presented in Figure 2. At the thermodynamic equilibrium, the mono-, di-, and trioleoyl glycerol represented, respectively, 8, 38, and 54% of the total product.

While the conversion rate of oleic acid is high in *n*-hexane, this reaction is not selective, and the reaction medium at the end of the reaction is composed of a mixture of three products, with the monooleoyl glycerol as a minor product. This being undoubtedly the most interesting product, we focused our attention on its selective production.

Oleic acid esterification with silica gel adsorbed-glycerol in a mixture of solvents. Figure 3 shows the product composition of the esterification of silica gel adsorbed-glycerol with oleic acid when the reaction is carried out in *n*-hexane supplemented with 2-methyl-2-butanol (90:10, vol/vol). The polarity of the reaction medium seems to have a drastic influence on the nature of the product composition. Indeed, at the thermodynamic equilibrium, the mono-, di-, and trioleoyl glycerol represented 83, 17, and 0%, respectively, of the total of the products. From a principally triglyceride production in *n*-hexane, the reaction was shifted to a principally monoglyceride production in *n*-hexane supplemented with 2-methyl-2-butanol.

We propose here to explain this result in terms of thermodynamic effect. Indeed, the synthesis of trioleoyl glycerol is a succession of three equilibrated reactions:

$$\text{glycerol + oleic acid} \Leftrightarrow \text{monooleoyl glycerol + water} \quad [3]$$

$$\text{monooleoyl glycerol+ oleic acid} \Leftrightarrow \text{dioleoyl glycerol + water} \quad [4]$$

$$\text{dioleoyl glycerol + oleic acid} \Leftrightarrow \text{trioleoyl glycerol + water} \quad [5]$$

The three respective equilibrium constants are:

$$K_1 = (a_{mono} \cdot a_w)/(a_{acid} \cdot a_{gly});$$
$$K_2 = (a_{di} \cdot a_w)/(a_{mono} \cdot a_{acid}); \quad [6]$$
$$K_3 = (a_{tri} \cdot a_w)/(a_{di} \cdot a_{acid})$$

where a_w, a_{acid}, a_{mono}, a_{di}, a_{tri} are the respective thermodynamic activities of water, oleic acid, monooleoyl glycerol, dioleoyl glycerol, and trioleoyl glycerol in the reaction medium.

It could be assumed that the solubilities of dioleoyl glycerol and particularly of trioleoyl glycerol are lower in the mixture of 2-methyl-2-butanol amended *n*-hexane (90:10,

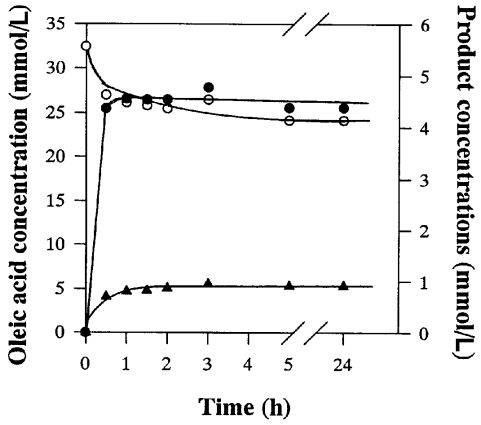

FIG. 3. Time course of the lipase-catalyzed esterification of oleic acid (30 mM) with glycerol adsorbed on dried silica gel (0.8 g) in 10 mL of a mixture of solvents (*n*-hexane/2-methyl-2-butanol 90:10, vol/vol). (○), Oleic acid; (●), monoolein; (▲), diolein.

vol/vol) than in pure *n*-hexane. The consequence is that in the solvent mixture, the thermodynamic activities of dioleoyl glycerol and of trioleoyl glycerol, a_{di} and $a_{tri,}$ are higher than in *n*-hexane, for the same concentrations. In other words, the activity coefficient (ratio of the thermodynamic activity to the concentration) of these two products is higher in 2-methyl-2-butanol amended *n*-hexane than in pure *n*-hexane. In consequence, reactions 2 and 3 could be thermodynamically disadvantaged by an increase in the solvent polarity. Moreover, one could assume that an increase in the solvent polarity leads also to a decrease in the water activity which is favorable to the three reactions. In fact, it would be supposed that the respective changes in solute activity coefficients would modify the selectivity of the reaction.

The monoglyceride production was favored in *n*-hexane supplemented with 2-methyl-2-butanol, but on the other hand, the oleic acid conversion rate in this solvent mixture (30%) is lower than in pure *n*-hexane (79%) (Fig. 4). It could be assumed that monooleoyl glycerol, being more soluble in *n*-hexane supplemented with 2-methyl-2-butanol than in pure *n*-hexane, leads to a better final monooleoyl glycerol concentration in the mixture of solvents. This is experimentally verified: the final monooleoyl glycerol concentration is five times higher in the mixture of solvents than in *n*-hexane. However, in 2-methyl 2-butanol amended *n*-hexane, unfavorable conditions for carrying out reactions 2 and 3 lead to a lower oleic acid conversion, reaction 1 being the only oleic acid consumer. Janssen *et al.* (16) did not observe this fact during esterification of decanoic acid with glycerol in various biphasic (aqueous/organic) solvent systems. This was, on the contrary, observed by Ducret *et al.* (1) and Valivety *et al.* (18). Calculations using methods such as UNIFAC to estimate solute activity coefficients would allow the evolution of the thermodynamic equilibrium position to be predicted. In this way, it is necessary to take into account the partition of water and of glycerol between solid phases and the solvent.

Enhancement of the oleic conversion rate by the selective recovery of monooleoyl glycerol. In order to improve the fatty acid conversion, a method usually proposed is water removal with a reduced pressure process (1). Water activity is maintained at a low value, and consequently the substrate conversion rate is high. Based on the same principle, we tested another way, in our opinion more attractive, which consists in maintaining as low as possible the thermodynamic activity of the second product, the monoester. We assessed the possibility of selectively extracting monooleoyl glycerol from the reaction medium by adsorbing it onto silica gel bed. This extractive system also allows a nonnegligible amount of the water produced to be eliminated. Consequently, integrated downstream processing can reduce production costs.

We carried out the reaction in a volume of 50 mL, containing 30 mM of oleic acid, 4 g of silica gel adsorbed-glycerol, prepared as previously described, and 1 g of Lipozyme®. When the thermodynamic equilibrium was reached at 37%, a value similar to the one observed previously, the medium was continuously pumped at 1 mL/min through a column packed with 4 g of dried silica gel and recycled into the reaction vessel. At the outflow of the separation column, it was checked that the totality of the monooleoyl glycerol was adsorbed onto the silica gel whereas the totality of the oleic acid and of the dioleoyl glycerol was recycled into the reaction vessel.

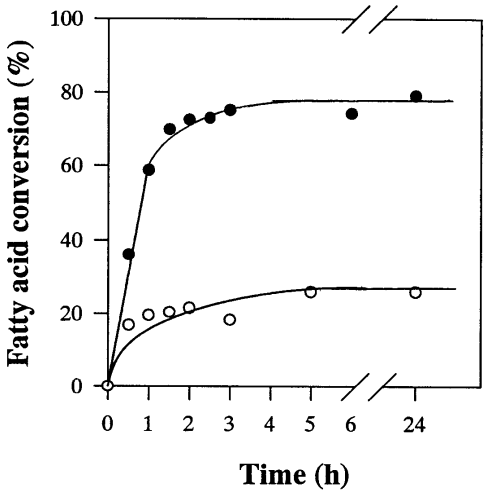

FIG. 4. Effect of the polarity of the solvent on the equilibrium of the reaction of esterification of oleic acid (30 mM) with adsorbed dried silica gel glycerol (0.8 g) catalyzed by immobilized Lipozyme (200 mg). (●), *n*-hexane; (○), *n*-hexane/2-methyl-2-butanol (90:10, vol/vol).

In this way one can observe a displacement of the thermodynamic equilibrium toward synthesis of monooleoyl glycerol from 37–72% in 2 h (Fig. 5). This process, coupling a selective reaction to a selective fractionation, allows monooleoyl glycerol to be produced and to be recovered selectively.

This work contributes to the development of an efficient process to produce enzymatically emulsifiers of monoglyceride type. Production of biosurfactants consists in making contact between a polar substrate, e.g., glycerol, and a hydrophobic substrate, oleic acid. This was realized by dissolving oleic acid in an apolar solvent, *n*-hexane, and by adsorbing glycerol onto silica gel. This process allows the enzyme blockage by glycerol adsorption to be avoided. With this system, providing that dried silica gel is used, a high oleic acid conversion (79%) is obtained, but no product selectivity is achieved and monooleoylglycerol represents only 8% of the product mixture.

On the other hand, *n*-hexane supplemented with 2-methyl-2-butanol (90:10, vol/vol) favors the production of principally monooleoyl glycerol (83%). A thermodynamically based explanation of this phenomenon is proposed. The solvent polarity increase would modify the thermodynamic activity coefficients, leading to unfavorable thermodynamic conditions for

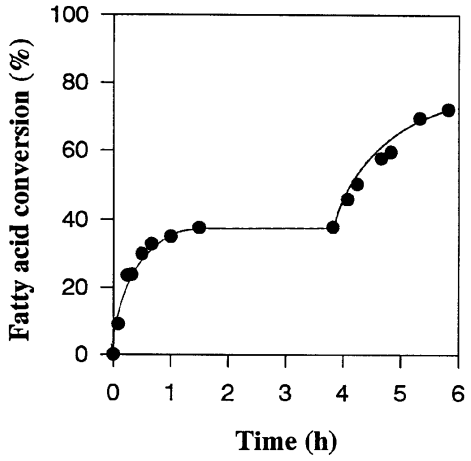

FIG. 5. Time course of the conversion yield of oleic acid on esterification reaction with adsorbed glycerol. Displacement of the thermodynamic equilibrium position toward synthesis of monooleoyl glycerol by on-line recovery of products.

di- and triglycerides formations. In order to prove this hypothesis, we are, at the moment, working on the prediction of the thermodynamic equilibrium, using UNIFAC to estimate the value of the activity coefficient of the solutes in different media.

To overcome the observed decrease in the oleic acid conversion, a process coupling reaction and monoglyceride recovery onto a silica gel column was tested. Residual oleic acid and the minor product, dioleyl glycerol (17%), are recycled into the reaction vessel. This process leads to an efficient monooleoyl glycerol production with a high oleic acid conversion (71%) and a high mono ester purity (83%). The efficient postreactional separation allows the recovery of 100% pure monooleoyl glycerol, this being the only one adsorbed on the column.

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