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Microemulsion-Based Organogels as an Efficient Support for Lipase-Catalyzed Reactions under Continuous-Flow Conditions

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ABSTRACT: In this work, microemulsion-based organogels (MBGs) containing lipase are reported as an efficient catalytic system for monoacylglyceride (MAG) synthesis. More specifically, *Candida antarctica* lipase B (CaLB) was immobilized on an MBG matrix (MBG_{CaLB}) formed with (hydroxypropyl)methyl cellulose as a gelling agent, and this catalyst revealed high conversion yields (expressed in grams of ester per hour per gram of enzyme) under both batch and continuous-flow conditions. Under flow conditions, 1:1 stearic acid/solketal (100 mM in *n*-heptane) could be converted to protected MAG (>99%) in a residence time of 11 min at 45 °C. A recycle study showed that the MBG_{CaLB} system can be recycled 15 times without activity loss, a number 2 times higher than under batch conditions catalyzed by immobilized lipase, in agreement with green chemistry protocols.

1. INTRODUCTION

Reverse micelles, or water-in-oil (w/o) microemulsions, have already been reported as an effective medium for enzyme immobilization,[1,2] as they have been successfully used in several cases to improve reaction yields using lower amounts of biocatalyst and to enhance enzymatic activity compared with conventional systems.[3−6] Reverse micelles are usually defined as spherical aqueous nanodroplets capped with a monolayer of surfactant and/or cosurfactant molecules and isotropically distributed within an oil.[7]

These structures form a thermodynamically stable and optically transparent liquid medium with large interfacial area that provides an aqueous domain where hydrophilic enzymes can be hosted, an interface where the active site of enzymes can be anchored, and a nonpolar organic phase where the hydrophobic substrates or products may be dissolved.[8,9] However, the main drawback of these systems is the difficulty in isolating the products due to the presence of surfactants that hinder phase separation.

Nevertheless, some microemulsions can be transformed to gels by adding a gelling agent, usually a biopolymer such as gelatin, agar, or a cellulose derivative,[10] to form so-called microemulsion-based organogels (MBGs). MBGs are rigid and stable in various nonpolar or relatively polar organic solvents and therefore can be used for several biotransformations in organic media, such as hydrolysis, esterification, and other syntheses.[11,12] The gel matrix formed by the gelling agent, such as a cellulose derivative, fully retains the surfactant, water, and enzyme components and can be handled as an immobilized biocatalyst that facilitates the diffusion of nonpolar substrates and products.[13] The network of the gel is considered to contain a bicontinuous phase that may coexist with conventional w/o microemulsion droplets containing the encapsulated enzyme.[10]

For industrial purposes, continuous-flow systems are preferred to batch reactors because they provide greater process control, higher productivity, and improvement of quality/purity and yield.[14,15] Several types of reactor can be used in continuous operation, among which packed-bed reactors (PBRs) are the most popular because of their high efficiency, low cost, and ease of construction, operation, and maintenance.[16,17]

In our continuous work on the development of more efficient biocatalytic systems,[3] here we report our results on the use of MBGs containing lipase under batch and continuous-flow conditions applied to the synthesis of various protected monoacylglycerol esters.

2. MATERIALS AND METHODS

2.1. Materials. Lipase B from *Candida antarctica* (CaLB), sodium bis(2-ethylhexyl) sulfosuccinate (AOT), and (*R*,*S*)-1,2-*O*-isopropylidene glycerol (solketal) were supplied by Fluka (Basel, Switzerland). CaLB had a specific activity of 9.2 units/mg of protein (1 unit corresponds to the amount of enzyme that liberates 1 μmol of butyric acid/min at pH 8.0 and 40 °C using tributyrin as the substrate). (Hydroxypropyl)methyl cellulose (HPMC) (viscosity 2600−5600 cP, 2% in H_2O, 20 °C) and stearic acid (≥99%) were purchased from Sigma (Germany). All other materials were at least reagent-grade. Millipore Milli-Q water was used for the preparation of gels and buffer solution.

2.2. Preparation of AOT/Isooctane Microemulsions and HPMC MBGs. For the preparation of microemulsions, the

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appropriate amount of CaLB was dissolved in 0.2 M Tris/HCl buffer (pH 7.5) to a final concentration of 30 mg/mL, and 970 μL of a 0.2 M AOT/isooctane solution and 30 μL of enzyme solution were added in 2 mL vials. The system was carefully homogenized until a clear solution was formed. The water content expressed in terms of the water to surfactant molar ratio (w_o) was 8. The MBGs were prepared by introducing the appropriate amount of AOT microemulsion containing the lipase to a second solution of HPMC in water.[18] In a typical experiment, 2 mL of AOT microemulsion containing 1.2 mg of lipase was gelled with 2.0 g of HPMC and 4.0 mL of water at room temperature. The esterification reactions took place under batch and continuous-flow conditions.

2.2.1. Batch Reactions. For either batch or flow experiments, stock solutions of the appropriate fatty acid source (stearic acid, fatty acid residue, or fatty sewer) with solketal (100 mM) in the appropriate solvent (heptane, isooctane, or toluene) were prepared. A 10 mL aliquot of each solution was added to a 20 mL glass flask, followed by the addition of 7 g of MBG (containing 1.2 mg of lipase). The system was incubated at 40−60 °C without stirring. Conversions were analyzed as described in section 2.3.

2.2.2. Continuous-Flow Reactions. The reaction mixture (fatty acid and solketal in the appropriate solvent) was initially stirred for 5 min at the appropriate reaction temperature. Esterification reactions were performed in a PBR (Asia Flow Reactor) equipped with an Omnifit column (cross-sectional area of 1.7671 cm^2 and height of 5 cm = reaction volume of 8.83 mL) containing 7 g of the MBG_{CaLB} (1.2 mg of lipase). Reaction parameters (temperatures between 40 and 60 °C; flow rates of 0.1−3.0 mL/min) were selected on the flow reactor controller. Initially, only pure solvents were pumped through, until the instrument was equilibrated at the desired reaction parameters and stable processing was ensured, after which the reaction mixture was pumped through the system.

2.3. Conversion Analysis. Process conversions were measured by determining the residual fatty acid by applying a modified Lowry and Tinsley method[19] and confirmed by GC analysis. More specifically, 0.3 mL of reaction medium was placed in vials with 1 mL of reaction solvent and 0.3 mL of a 5% solution of copper acetate/pyridine (pH 6−6.2). The mixture was vigorously stirred for 30 s. The supernatant was measured spectrophotometrically at 715 nm. Each reaction was measured in triplicate, and conversions were calculated by using calibration curves of the acid used.

For gas chromatography (GC), an HP Innowax column (30 m × 0.25 mm i.d. × 0.32 μm film thickness) mounted on a Hewlett-Packard (HP) model GC-6890C gas chromatograph was used. The injector and detector temperatures were 270 °C, and the oven temperature was constant at 120 °C for 1 min, then increased by 15 °C/min to 225 °C, and further increased by 5 °C/min to 260 °C, where it was held constant for 2 min.

3. RESULTS AND DISCUSSION

3.1. Batch Reactions. According to our previous work,[3] where AOT microemulsions containing CaLB [40 °C, 1:1 stearic acid/solketal (100 mM)] were used, maximum conversion yields were found after only 30 min of reaction (80% conversion of protected monoacylglycerol). We used these conditions in a first attempt to evaluate the performance of microemulsion-based organogels containing CaLB (MBG_{CaLB}), and the conversions were analyzed for up to 120 min of reaction (Figure 1). The results presented in Figure 1

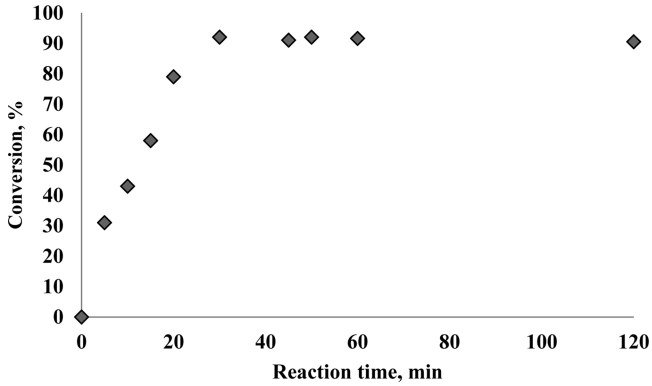

Figure 1. Reaction profile for the esterification reaction between solketal and stearic acid catalyzed by the MBG_{CaLB} system. Conditions: 1:1 stearic acid/solketal (100 mM), 40 °C, no stirring.

show that the immobilized biocatalyst presented excellent yields of the desired product after only 30 min of reaction, where the conversion reached a plateau. It is also important to note that the final conversion yield after 30 min was 90%. This yield is higher than the one achieved under the same reaction conditions by the related batch reaction in AOT microemulsion without the gelling agent.[3]

Water-in-oil microemulsions present some restrictions regarding the organic solvents used as far as enhanced solubility of reaction substrates is concerned. On the contrary, MBGs operate as heterogeneous catalysts making it possible to apply an adequate external solvent carrying the substrates for the desired transformation. In order to evaluate the influence of nonpolar solvents on the reaction mediated by MBG_{CaLB}, we performed the same reaction presented in Figure 1 using different solvents, and the results are shown in Figure 2.

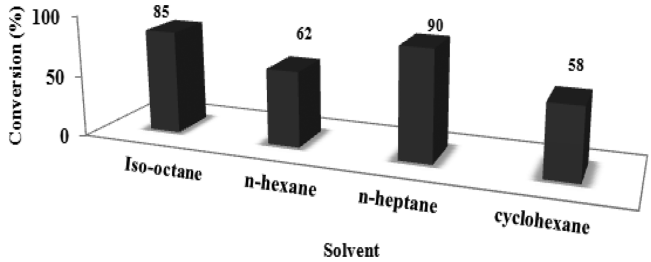

Figure 2. Evaluation of solvent effect on the reaction catalyzed by MBG_{CaLB}. Conditions: 1:1 stearic acid/solketal (100 mM), 40 °C, no stirring.

As observed in Figure 2, higher conversions were found for reactions performed in *n*-heptane. The same result was found for commercial immobilized enzymes, as described in our previous publications.[20−23] It is obvious that these results can be related to the solvents' log *P* values and their respective viscosities, which reflect their lipophilicity and can influence the enzyme stability and the contact surface between the enzyme and the substrate. Initial rate values were also calculated for the different solvents used, and the values are shown in Table 1.

The effect of the amount of enzyme loaded in the MBG_{CaLB} biocatalyst on the reaction rate was also a subject of analysis. As shown in Figure 3, the initial rate of the reaction increased as the amount of lipase in the biocatalyst was increased. The linearity observed under the studied conditions is consistent with a kinetically controlled enzymatic reaction, maintaining

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Table 1. Initial rates of the esterification reaction of solketal and stearic acid catalyzed by MBG_{CaLB} in different solvents[a]

solvent	initial rate ($mM \cdot min^{-1}$)
isooctane	20.2
n-heptane	27.3
n-hexane	12.4
cyclohexane	8.4

[a]Reaction conditions: 1:1 stearic acid/solketal (100 mM), 40 °C, no stirring.

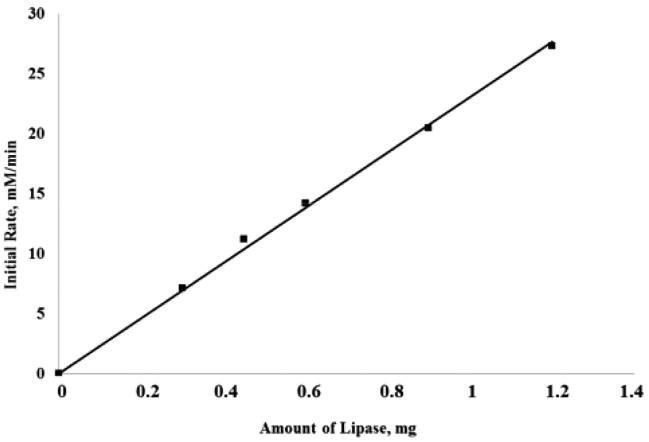

Figure 3. Effect of different concentrations of CaLB, calculated in terms of milligrams of commercial free lipase in the immobilized biocatalyst (MBG_{CaLB}), on the rate of the esterification of solketal (100 mM) with stearic acid (100 mM) in *n*-heptane. Conditions: 40 °C, no stirring.

the characteristics found for free microemulsion systems.[24] The linearity observed here has been reported before for *Rhizomucor miehei* lipase immobilized in similar AOT–HPMC MBGs for the catalysis of propyl laurate synthesis.[18]

It is important to emphasize here that both the amount of enzyme applied in this system and the reaction time required to reach equilibrium were much lower than for the reaction performed with the corresponding commercial immobilized lipase, Novozyme 435 (30 mg pt/g of support).[25] This fact can be more obvious in terms of productivity. Novozyme 435 showed a production of 6.5 (g of ester)·h^{-1}·(g of enzyme)$^{-1}$, while the MBG_{CaLB} system gave a value of 101.2 (g of ester)·h^{-1}·(g of enzyme)$^{-1}$, confirming the improvement in lipase performance.

3.2. Continuous-Flow Reactions. *3.2.1. Reaction of Solketal with Stearic Acid.* Aiming to further improve the productivity of the MBG_{CaLB} system, we also studied the use of MBG_{CaLB} under a continuous-flow regime (Scheme 1). Flow rates between 0.05 and 1.0 mL/min were tested at different temperatures in a flow reactor filled with MBG_{CaLB} using *n*-heptane as the reaction solvent containing stearic acid and solketal (1:1/100 mM), and the results are presented in Table 2. As can be seen, temperature plays a very important role in

Scheme 1. Monoacylglycerol synthesis under continuous-flow conditions with stearic acid and solketal (1:1/100 mM) in *n*-heptane and 1.2 mg of lipase

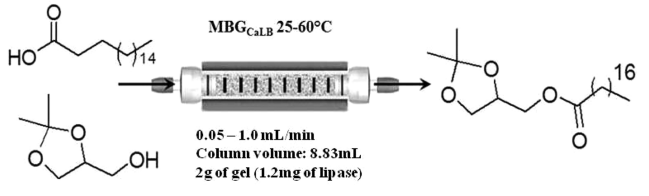

Table 2. Effect of residence time (t) on the conversion yields of the esterification reaction between solketal and stearic acid (1:1/100 mM) catalyzed by MBG_{CaLB} under continuous-flow conditions

	conversion (%)			
T (°C)	t = 5.5 min	11 min	55 min	110 min
25	8	12	21	25
35	38	42	56	55
40	59	58	81	86
45	69	93	>99	77
50	50	54	66	64

the esterification reaction catalyzed by MBG_{CaLB} under continuous-flow conditions. Conversions of 93 and >99% were found at residence times of 11 and 55 min, respectively, at 45 °C. This result is impressive since in only 11 min a productivity of 334 g·h^{-1}·(g of enzyme)$^{-1}$ was revealed, a much higher value (3.3-fold increase) than that found for the same reaction under batch conditions. At higher temperatures, a decrease in conversion yield was observed, as well as greater turbidity of the samples.

HPMC MBGs are stable up to 65 °C, and at that temperature the catalytic ability of the enzyme is well-preserved. However, at temperatures higher than 50 °C a decrease in the reaction rate can be observed.[26] This could be attributed to structural changes of the gel matrix caused by the temperature increase that may induce leakage of protein into the reaction medium.[26,27] Allied to the thermal effect, high flow rates can accelerate this process, resulting in leaching of the enzyme into the system.

In order to further improve the productivity generated by the reaction system as described in Table 2, increasing substrate concentrations were tested in *n*-heptane while maintaining the molar ratio of 1:1 (Table 3). The reaction system showed high

Table 3. Effect of increasing substrate concentrations in the monostearin synthesis catalyzed by the MBG_{CaLB} system under continuous-flow conditions at 0.5 mL/min (11 min residence time) and 45 °C

substrate concentration (mM)	conversion (%)	productivity g·h^{-1}·(g of enzyme)$^{-1}$
100	92	241
150	91	361
300	91	723
500	91	1205
750	54	225

conversions for increasing substrate concentrations up to 500 mM. For substrate concentrations of 500 mM, the final production was 1205 g·h^{-1}·(g of enzyme)$^{-1}$, a value that was the highest for the production of MAG. However, the recyclability of this system was limited to five cycles (data not shown), while for lower substrate concentrations (150 mM) the recyclability can reach 15 cycles, as shown in Figure 4.

According to Figure 4, the MBG_{CaLB} system could be reused for approximately 15 recycles without significant loss of enzyme

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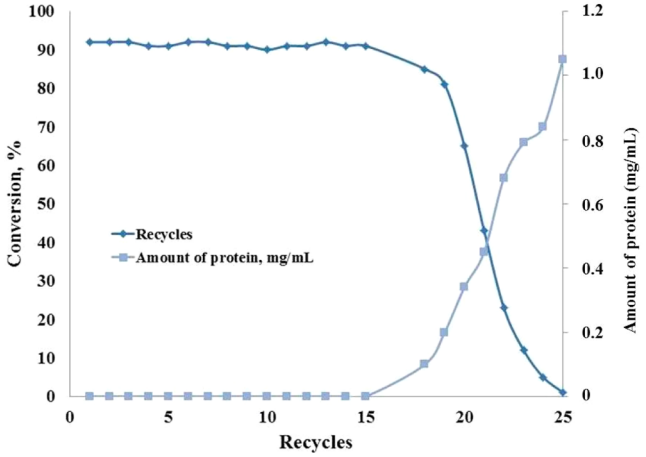

Figure 4. Recycling of the MBG_{CaLB} system under continuous-flow conditions.

performance. Upon further use of the catalyst (16th to 25th cycle), a decrease of the conversion yield was noticed as a result of the large amount of protein in the collected samples, as measured by the Bradford method.[28] The ability to reuse the catalyst 15 times is an important advantage of the MBG_{CaLB} system applied under continuous-flow conditions.

Taking into account the fact that the productivity is an important parameter, we compared the values obtained in this study for batch and continuous-flow reactions with the ones obtained using other commercial immobilized enzymes. The results (summarized in Figure 5) show that MBG_{CaLB} can be much more efficient than the most traditional immobilized enzymes used in the literature for esterification reactions under batch conditions. Under continuous-flow conditions, the results obtained for Novozyme 435 and MBG_{CaLB} are similar. Nevertheless, MBG_{CaLB} shows many advantages over commercial immobilized enzymes, such as the versatility of the amount of enzyme loaded on the biocatalyst, biocompatibility of the support, and reusability, among others.

3.2.2. Reactions with Other Fatty Acid Sources. The optimum conditions as determined by the study presented here for the reaction of stearic acid and solketal (45 °C, flow rate of 0.1 mL/min) were also applied for the esterification reactions of palm waste and a sample of fatty sewer as alternative fatty acid sources. The results are summarized in Table 4. As can be observed, high conversion yields could be obtained with palm waste as a fatty acid source, up to a concentration of 300 mM. The behavior of palm waste is not similar to our previous results, mainly because of the complexity of this residue, which contains several impurities that could decrease enzyme performance. The developed system seemed to be more affected by the fatty sewer sample since only at a concentration of 100 mM were good productivities achieved. Although the chemical compositions of the two acid sources are similar, the fatty sewer, being a raw material, probably presents some impurities that affect enzyme performance.

Table 4. Conversion yields (%) for the esterification reactions of solketal and two different acid waste sources catalyzed by MBG_{CaLB} under continuous-flow conditions with 1:1 acid and solketal in *n*-heptane and 1.2 mg of CaLB

	fatty acid source	
substrate concentration (mM)	palm waste	fatty sewer
100	83	80
150	83	75
300	80	63
500	51	45
750	37	29

4. CONCLUSION

In the work presented here, we have shown that CaLB lipase immobilized in an MBG matrix can efficiently be used to catalyze the production of protected MAG, since high productivities were obtained compared with commercial immobilized lipases. Under continuous-flow conditions, all of the reagents could be converted to protected MAG in a

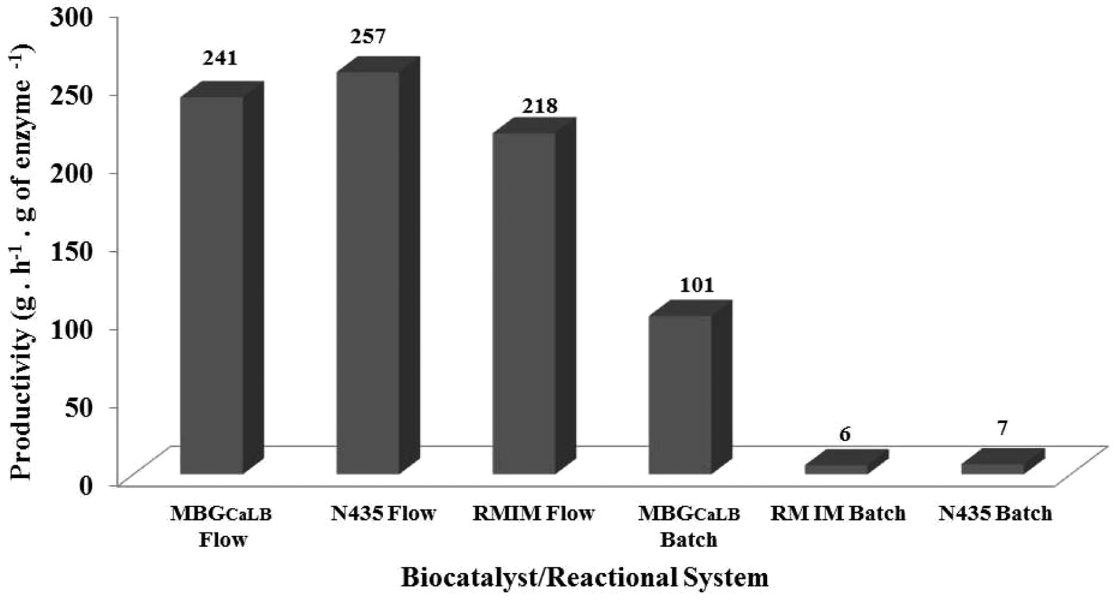

Figure 5. Relation between productivity and catalysis system for the esterification of stearic acid and solketal at 100 mM in *n*-heptane.

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residence time of 11 min at 45 °C, giving conversion yields of up to 99%. The study of the reuse of the system showed that it can be recycled 15 times without activity loss, a number 2 times higher than that for immobilized lipase under batch conditions.[a]

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Notes

The authors declare no competing financial interest.

ADDITIONAL NOTE

[a]Although there is a relatively small difference between the structures of the applied organic solvents, these differences impart intense chemical properties, such as viscosity and lipophilicity. More viscous solvents tend to reduce the surface contact between the biocatalyst and the substrate, resulting in lower conversions. However, the enzyme affinity for the solvent is also important. The lipophilicity is essential for the lipase interface formation to expose its active site and perform catalysis. In the case of microemulsions and reverse micelles, the enzyme is entrapped in an aqueous microdomain, with the active site facing the external surface. These nanostructures are sustained by the surfactant, in this case AOT. Nonpolar solvents require much larger amounts of AOT, which can be detrimental to the enzyme and at the same time generate a more viscous microemulsion. Although each reaction system (either batch or continuous flow) presents inherent advantages, each system in continuous flow showed a greater process control in this reaction and therefore was compared with the previous work done in batch mode.

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