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Lipase-catalyzed synthesis and characterization of novel lipidyl-cyclodextrins in solvent free medium

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ABSTRACT

The lipase-catalyzed amidation reaction between fatty acyl donors and mono-6-amino-permethylated β-cyclodextrin (MBCD-NH_2) was achieved with high yield in solvent free medium. The ability of *Mucor miehie* and *Candida cylindracea* lipases, free or immobilized, to catalyze amidation reaction was shown. Products were purified and characterized. To optimize the reaction, some reaction conditions were assessed like temperature, type of shaking and amount of enzyme, on a model amidation reaction between butyl caprylate and MBCD-NH_2. Among the biocatalysts tested, Lipozyme was found to be the most active, requiring low loading, being robust at 70 °C even under magnetic agitation.

1. Introduction

Targeted drug delivery is of particular importance for the treatment in many diseases. Therefore, one of the main challenges is the development of stable drug carriers enabling selective specific drug targeting. Cyclodextrins (CD) are recognized to have significant potential as drug carriers arising from inclusion complexes. The optimization of their pharmacological properties has led to the synthesis of numerous analogues. Amphiphilic derivatives were designed to improve the cell targeting of the drug-containing cyclodextrin cavities through their transportation in the organism, within self-assembling systems. Several amphiphilic cyclodextrins can self-assemble into water-soluble aggregates such as mono- or polydisperse micelles, or insert in lipid membranes and liposomes [1–4]. There are some examples of chemical synthesis of amphiphilic substituted cyclodextrins such as phospholipidyl cyclodextrins able to pass through the Blood–Brain Barrier without breaking its integrity [5] or such as cholesteryl cyclodextrins [6]. Among the different native cylodextrins, the β-cyclodextrins (β-CD) seem to be very popular due to their ability to host a large range of hydrophobic molecules in their size-selective toroidal cavity [7]. They are doughnut-shaped molecules composed of seven α-(1-4) linked D-glucopyranosides. The permethylated amphiphilic β-cyclodextrins (MBCD) were shown to self-organize in water with low Critical Micelle Concentration (CMC) to form fluctuating micellar fibers or micelles retaining inclusion capacity [5,8] and moreover are soluble in organic solvent.

To overcome the drawbacks encountered with the phospholipidyl-CDs synthesis, namely high cost, tedious synthesis and rather poor versatility of the chemical structures and stability, we turned to chemo enzymatic synthesis of lipidyl-cyclodextrins [9,10]. These lipidyl-cyclodextrins can be obtained through lipase-catalyzed reaction between mono-6-amino permethylated β-cyclodextrin (MBCD), ended by either an amine or an alcohol function and fatty acids, esters or vinyl esters. Indeed, lipases (EC 3.1.1.3) catalyze not only the hydrolysis but also the synthesis of amides or esters in non-aqueous media on a wide variety of substrates while maintaining their regio- and stereoselectivity under mild reaction conditions. Microbial lipases constitute the most important group of biocatalysts for biotechnological and organic chemistry applications [11–13]. Furthermore, lipases act on complex substrates such as milk fat [14]. Although lipases are extensively used to catalyze esterification or transesterification, the amidation catalyzed by lipase is less studied [15,16] and only few authors described cyclodextrins as substrates [17].

In order to optimize the reaction conditions, lipase-catalyzed amidation of MBCD-NH_2 and butyl caprylate in solvent free medium was chosen as a model reaction. Herein, the influence of several synthesis parameters such as temperature, biocatalyst loading, and agitation are presented using lipases from *Mucor*

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miehei (MML) and *Candida cylindracea* (CCL) either in free or immobilized form. The versatility of the lipase-catalyzed reaction has been assessed with three other acyl donors: ethyl caprylate, butyl caprate and vinyl laurate and the products were characterized.

2. Materials and methods

2.1. Enzymes and chemical materials

Native β-cyclodextrin (β-CD) was purchased from Wacker Chemicals (Germany). Immobilized enzymes were from Fluka Analytical (France): Lipozyme is the immobilized lipase from *Mucor miehei* on a macroporous ion-exchange resin (42 U g^{-1}), and the lipase from *C. cylindracea* immobilized in Sol-Gel-AK (15.1 U g^{-1}). Free forms of the lipase from *M. miehei* (MML, 4370 U mg^{-1}) and of *C. cylindracea* (CCL, 30,000 U g^{-1}) were purchased from Sigma and from Amano (AY30), respectively. Butyl caprylate (99% purity) was purchased from TCI Europe (Belgium). Other chemicals were purchased from Sigma, Acros (Belgium) and Fluka Analytical (France). All the solvents employed for the reactions were distilled once before use.

2.2. Preparation of mono-6-amino-deoxy-permethylated β-cyclodextrins (MBCD-NH_2)

The modified CD used as substrate in enzymatic reaction was the permethylated 6-amino-6-deoxy-β-cyclodextrin (MBCD-NH_2). It has been obtained from the native β-CD in four steps using usual method [18–22]. It was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 9:1 (v/v), R_f 0.50) with 30% yield.

2.3. General procedure for the enzymatic synthesis with butyl caprylate

Otherwise specified, MBCD-NH_2 (1.8–2.6 mg) was dissolved in butyl caprylate (1.5 ml) and vigorously mixed in a closed Eppendorf tube and placed in a heater mixer (Eppendorf Thermomixer) at 40 °C and 1000 rpm. The reaction was initiated by adding either 2 mg of the free form of MML or CCL, or 10 mg of the corresponding immobilized form. It was checked that no reaction took place in the absence of enzyme under the same reaction conditions. As none of the biocatalysts are soluble in the medium, reaction was stopped by centrifugation at 4000 rpm during 2 min or by filtration on 0.2 μm for immobilized lipases. All essays were carried out at least in duplicate. The progress of the reaction was analyzed by HPLC.

2.4. HPLC analysis

HPLC analysis was carried out with a LC Module 1 Waters instrument equipped with a Refractometer R410 Waters detector (internal temperature: 40 °C) and a GraceSmart RP18 column (250 mm × 4.6 mm, 5 μm, Grace), elution was performed at 1 mL/min (20 μL injected) using an isocratic eluent (acetonitrile/acetone/water 40:40:20). A calibration curve was made with the product of reaction, i.e. mono-6-caprylylamido mono-6-deoxy permethylated-β-cyclodextrine (caprylyl-MBCD), obtained after purification of the larger scale production from ethyl caprylate, retention time at 5.15 min, ($y = 2\,076\,132x + 1\,765\,407$ and $R^2 = 0.997$). Results shown correspond to the average between two HPLC analyses.

2.5. Larger scale with other acyl donors and purification

To isolate product of lipase-catalyzed amidation, the reaction was scaled up in Erlenmeyer shaking flask capped with 30 mL of acyl donor in an orbital shaker at 109 rpm. In this case, reactions were performed in 30 mL of acyl donors with 150 mg of MBCD-NH_2 and 100 mg of Lipozyme The purification was performed through a partial distillation on reduced pressure to remove fatty esters followed by a column chromatography on silica gel (CH_2Cl_2/CH_3OH 95:5 then 90:10) and an ion-exchange chromatography on Dowex 1 × 8 Cl^- eluted with water.

2.6. Structural identification of the reaction product

The structure was determined by ^1H and ^{13}C NMR (Bruker DRX 500 spectrometer) at 25 °C and mass spectrometry ESI-Q-Tof (Waters-Micromass spectrometer (Manchester, UK).

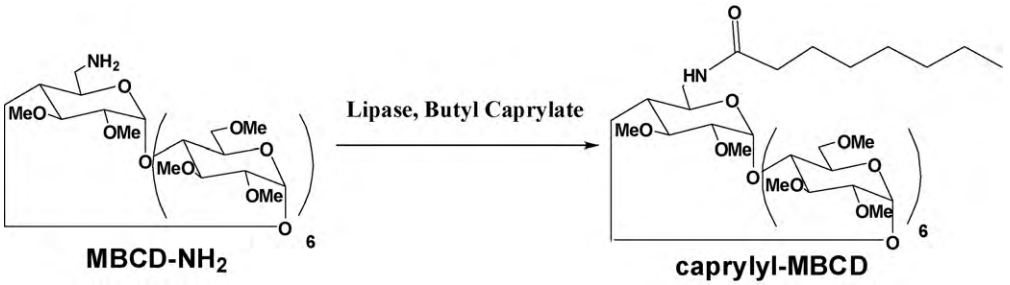
Caprylyl-MBCD: ^1H NMR (500 MHz, $CDCl_3$, TMS): $\delta = 0.9$ (t, 3H, $J = 7.3$ Hz, CH_{3aliph}), 1.2–1.4 (m, 8H, CH_{2aliph}), 1.6–1.7 (m, 2H, $CH_{2\beta}$), 2.1–2.3 (dt, 2H, $J = 2.8$ Hz, $J = 8.7$ Hz, $CH_{2\alpha}$), 3.1–4.0 (m, 42H; H_{2CD}, H_{3CD}, H_{4CD}, H_{5CD}, H_{6CD}), 3.4 (21H; $OCH_{3(2)}$), 3.5 (21H; $OCH_{3(3)}$), 3.7 (18H; $OCH_{3(6)}$), 5.1–5.2 (m, 7H, H_{1CD}), 6.0 (t, 1H, $J = 5.8$ Hz, NH_{CO}). ^{13}C NMR (125 MHz, $CDCl_3$, TMS): $\delta = 14.4$ (1C, CH_3), 22.9, 26.3, 29.4, 29.7, 32.1, 37.2 (6C, $CH_{2\alpha}$, $CH_{2\beta}$, CH_{2aliph}), 40.2 (1C, C_{6CD}), 59.1 ($OCH3_{(2)}$), 59.7 ($OCH3_{(3)}$), 59.8 ($OCH3_{(6)}$), 71.3–72.2 (6C, C_{6CD}), 82.2–82.5 (28C, C_{3CD}, C_{2CD}, C_{4CD}, C_{5CD}), 98.5–99.6 (7C, C_{1CD}); 173.5 (1C, *C*O). ESI-HRMS+ calculated for $C_{70}H_{125}NO_{35}Na$ $[M+Na]^+$ *m/z* 1562.7930, found 1562.7932.

Capryl-MBCD: ^1H NMR (500 MHz, $CDCl_3$, TMS): $\delta = 0.87$ (t, 3H, $J = 7.0$ Hz, CH_{3aliph}), 1.2–1.4 (m, 12H, CH_{2aliph}), 1.6–1.7 (m, 2H, $CH_{2\beta}$), 2.1–2.2 (dt, 2H, $J = 2.29$ Hz, $J = 7.78$ Hz, $CH_{2\alpha}$), 3.1–3.2 (m, 7H, H_{2CD}), 3.3–3.9 (m, 35H, H_{3CD}, H_{4CD}, H_{5CD}, H_{6CD}), 3.4 (s, 21H, $OCH_{3(2)}$), 3.5 (s, 21H, $OCH3_{(3)}$), 3.6 (s, 18H, $OCH_{3(6)}$), 5.1–5.2 (m, 7H, H_{1CD}), 6.0 (t, 1H, $J = 5.65$ Hz, NH_{CO}). ^{13}C NMR (125 MHz, $CDCl_3$, TMS): $\delta = 14.2$ (1C, CH_3), 22.8, 29.4, 29.5, 29.6, 29.8, 31.9 (6C, CH_2), 26.0 (1C, $CH_{2\beta}$), 37.0 (1C, $CH_{2\alpha}$), 39.9 (1C, C_{6CD}), 58.4 (7C, $OCH_{3(2)}$), 59.1 (7C, $OCH3_{(3)}$), 61.6 (6C, $OCH_{3(6)}$), 71.0 (7C, C_{5CD}), 71.7 (6C, C_{6CD}), 80.3–82.2 (21C, C_{3CD}, C_{2CD}, C_{4CD}), 99.0 (7C, C_{1CD}), 173.3 (1C, *C*O), ESI-HRMS+ calculated for $C_{72}H_{129}NO_{35}Na$ $[M+Na]^+$ *m/z* 1590.8243, found 1590.8234.

Lauryl-MBCD ^1H NMR (500 MHz, $CDCl_3$, TMS): $\delta = 0{,}87$ (t, 3H, $J = 6.90$ Hz, CH_{3aliph}); 1.25 (m, 16H, CH_{2aliph}), 1.61 (m, 2H, $CH_{2\beta}$), 2.13–2.19 (dt, 2H, $J = 3.05$ Hz, $J = 8.40$ Hz, $CH_{2\alpha}$), 3.11–3.24 (m, 7H, H_{2CD}), 3.38 (s, 21H, $OCH_{3(2)}$), 3.50 (s, 21H, $OCH_{3(3)}$), 3.63 (s, 18H, $OCH_{3(6)}$), 3.20–3.99 (m, 35H, H_{3CD}, H_{4CD}, H_{5CD}, H_{6CD}), 4.99–5.23 (m, 7H, H_{1CD}), 5.99 (t, 1H, $J = 5.65$ Hz, NH_{CO}). ^{13}C NMR (125 MHz, $CDCl_3$, TMS): $\delta = 15$ (1C, CH_3), 26.3 (1C, $CH_{2\beta}$), 23.1–32.3 (8C, CH_2), 37.3 (1C, $CH_{2\alpha}$), 40.3 (1C, C_{6CD}), 58.9 (7C, $OCH_{3(2)}$), 59.6 (7C, $OCH_{3(3)}$), 61.8 (6C, $OCH_{3(6)}$), 70.4 (7C, C_{5CD}), 71.9 (6C, C_{6CD}), 80.3–82.4 (21C, C_{2CD}, C_{3CD}, C_{4CD}), 99.2 (7C, C_{1CD}), 173.7 (1C, CO), ESI-HRMS+ calculated for $C_{74}H_{133}NO_{35}Na$ $[M+Na]^+$ *m/z* 1618.8556, found 1618.8545.

3. Results and discussion

In a previous work [9], we have reported that many lipases from different sources, fungi, microbial and animal, are able to catalyze in one step the amidation reaction between mono-substituted β-permethylated cyclodextrins such as MBCD-NH_2 and various acyl donors. Furthermore, as far as the acyl donors are liquids, the lipase-catalyzed reaction can be done without any solvent, by using an excess of acyl donors acting both as a substrate and a solvent for the CD. Based on this previous work, the screening led us to select two lipases widely used in the field of biotransformation: the lipase from *M. miehie* (MML) and the lipase from *C. cylindracea* (CCL) also known as *Candida rugosa*. Moreover these two lipases are commercially available under free and immobilized preparation. To optimize the reaction, some reaction conditions were assessed like temperature, type of shaking and amount of enzyme, on a model amidation reaction between butyl caprylate and MBCD-NH_2 (Scheme 1).



Scheme 1. Amidation reaction catalyzed by lipases between MBCD-NH_2 and butyl caprylate.

3.1. Comparison of the catalytic activities of *M. miehie* and *C. cylindracea* lipases

The time course of the reaction was followed for the lipase-catalyzed amidation between butyl caprylate and MBCD-NH_2 by MML, free or immobilized (Lipozyme) and by CCL free or immobilized (Fig. 1). For these first tests, the temperature was set at 40 °C since for many microbial lipases activity is maximal between 30 and 50 °C. Lipozyme seemed to be the most efficient. Indeed, the concentration of the final product, caprylyl-MBCD, reached a yield of about 70% at 100 h which was 1.5 times greater than the yield achieved with the free MML or the free or immobilized CCL. Moreover, there was no significant difference in the catalytic behaviour between the free and immobilized CCL. It can also be noticed that the initial rate was higher with Lipozyme than with the three other biocatalysts. However, reactions were not completed at this time whatever the enzyme used. This could be due to the low amount of biocatalyst, chosen arbitrarily, 2 mg for free form and 10 mg for the immobilized form or to some other parameters such as a temperature and agitation which can also greatly influence these reactions.

3.2. Effect of reaction temperature

It is well known that the increase of reaction temperature increases the activity; however care must be taken to the possible deactivation of enzyme. To this end, we tested different temperatures from 30 to 70° (Fig. 2). At 24 h, for the four enzymatic preparations, no optimal temperature reaction appeared since the amount of caprylyl-MBCD increases all over this range. There was no significant difference between the free form and immobilized form of CCL. The improvement of caprylyl-MBCD production was noteworthy for the MML and even more for the immobilized form, Lipozyme. The production increased almost linearly for the free MML between 30 and 70°. For Lipozyme, the amount of caprylyl-MBCD was first increased almost linearly between 30 and 50° and then more strongly between 50 and 70°. The concentration of the product was about 7-fold higher at 70 °C than at 30 °C.

We have then examined the progress of reaction at 70 °C (Fig. 3). The initial rate was higher with Lipozyme than with the three other biocatalysts. At 70 °C (Fig. 3) as at 40 °C (Fig. 1), there is no benefic effect of the immobilization of CCL. Conversely, a positive effect of the immobilization of MML was observed. While the results obtained with the free MML were barely higher than those of CCL, the reaction catalyzed by the Lipozyme seemed to be complete in 30 h, against more than 100 h at 40 °C.

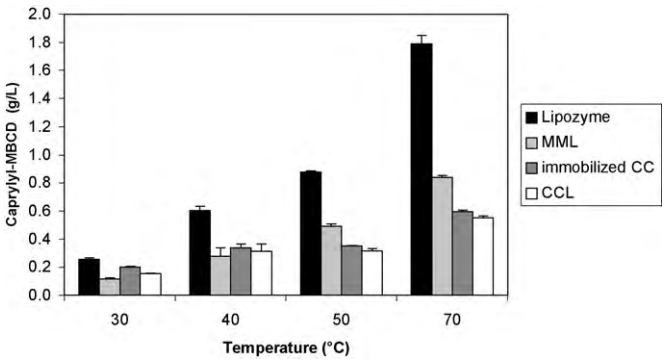


Fig. 2. Effect of reaction temperature at 24 h on lipase-catalyzed caprylyl-MBCD synthesis.

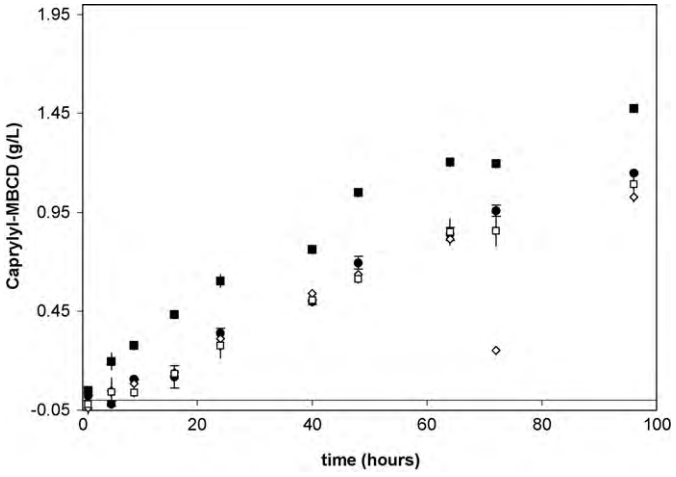


Fig. 1. Concentration of the caprylyl-MBCD as a function of time at 40 °C. The amidation reaction is catalyzed by (■) Lipozyme, (□) MML, (●) immobilized CCL, (◊) CCL.

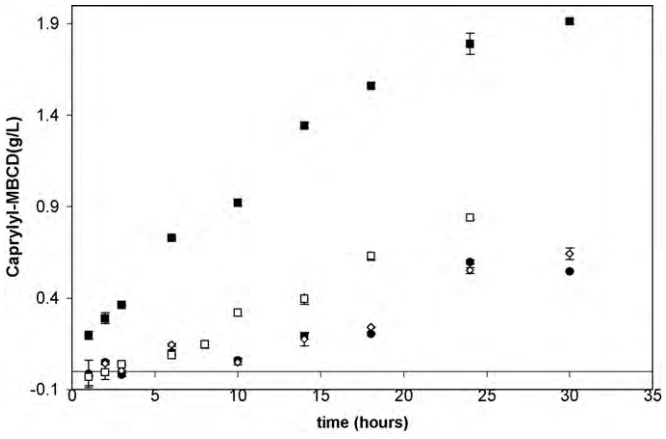


Fig. 3. Concentration of the caprylyl-MBCD as a function of time at 70 °C. The amidation reaction is catalyzed by (■) Lipozyme, (□) MML, (●) immobilized CCL, (◊) CCL.

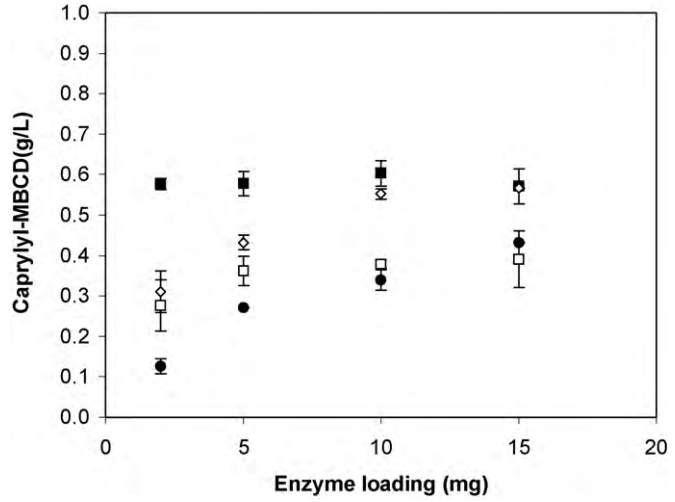


Fig. 4. Effect of the amount of lipase on the concentration of caprylyl-MBCD at 40 °C during 24 h: ■Lipozyme, □ MML, (●) immobilized CCL, (◊) CCL.

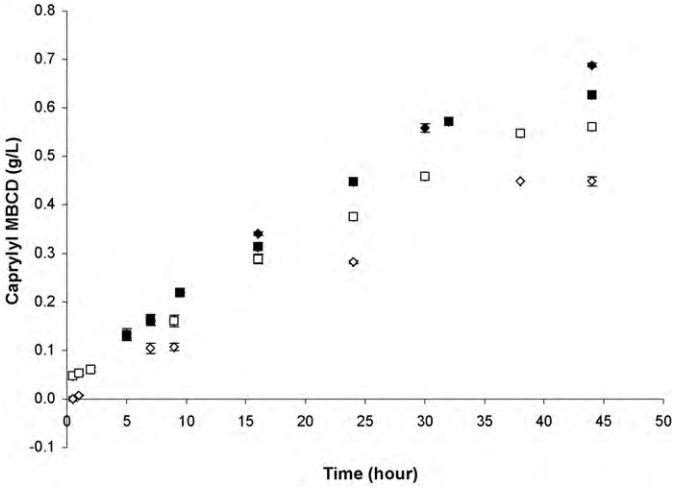


Fig. 5. Effect of the type of agitation on synthesis of caprylyl-MBCD at 40 °C with lipozyme (□) orbital shaking at 1000 rpm, (■) magnetic stirring and immobilized CCL: (◊) heater mixer, (♦) magnetic stirring.

3.3. Effect of the enzyme amount

To determine the optimum of enzyme to be used, the caprylyl-MBCD synthesis was performed with various amounts of enzyme loadings and the results obtained at 40 °C within 24 h were reported in Fig. 4. The efficiency of the catalyzed reaction increased with the increase in the amount of enzymatic catalyst from 2 to 10 mg for the free MML and CCL while 15 mg at least could be best with the immobilized CCL. However, in the case of Lipozyme 2 mg is enough to perform this reaction.

3.4. Effect of the type of agitation

A comparison has then been made between agitation in a heater mixer (Thermomixer), as used above and agitation in the presence of a magnetic stirring bar keeping the same volume of reaction. Due to the possible damage of free enzymes with this last kind of agitation, the experiments were performed only with the immobilized lipases (Fig. 5). Although there was no drastic effect of the type of agitation for the Lipozyme, best results were obtained in all cases with magnetic stirring. It can be pointed out that with this type of agitation, the activity of immobilized CCL ranged within the one of Lipozyme. The efficiency of the magnetic stirring for the immobilized CCL could certainly be explained by the fact that this preparation is in powder form while Lipozymes are macroscopic beads, less affected by the type of agitation.

3.5. Versatility of lipase-catalyzed lipophilization on MBCD-NH_2

From the optimization performed on butyl caprylate, we have selected the Lipozyme to catalyze the amidation on MBCD-NH_2 with other fatty acids in larger scale. Considering energy intake and enzyme reuse, reactions were done at 40 °C. Ethyl caprylate, butyl caprate and vinyl laurate were used as substrate in large excess. The solvent free medium allowed an easy purification of the product.

The yield (78%) obtained with butyl caprate as acyl donor is the best. The low yield obtained with the vinyl laurate (35%) can be explained by a competition with the formation of polyenyl derivatives of the MBCD [10]. The higher yield obtained with butyl caprate as compared to those with ethyl caprylate (60%) may be due to higher affinity of the lipase for longer acyl chains.

4. Conclusions

Lipophilization of a mono-amino-β-methylathed cyclodextrin was achieved through lipase-catalyzed process under mild reaction conditions. Solvent free media were used due to the solubilization of methylated cyclodextrins in acyl donors. The ability of *M. miehie* and *C. cylindracea* lipases, free or immobilized, to catalyze amidation reaction was shown with various acyl donors. Among the biocatalysts tested, Lipozyme was found to be the most active, requiring low loading, being robust at 70 °C even under magnetic agitation.

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