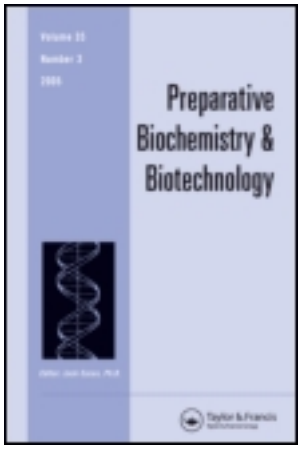

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(TRANS)ESTERIFICATION OF MANNOSE CATALYZED BY LIPASE B FROM *Candida antarctica* IN AN IMPROVED REACTION MEDIUM USING CO-SOLVENTS AND MOLECULAR SIEVE

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□ *Four co-solvents (dimethylformamide [DMF], formamide, dimethyl sulfoxide [DMSO], and pyridine) were tested with* tert*-butanol (tBut) to optimize the initial rate (v_0) and yield of mannosyl myristate synthesis by esterification catalyzed by immobilized lipase B from* Candida antarctica. *Ten percent by volume of DMSO resulted in the best improvement of v_0 and 48-hr yield (respectively 115% and 13% relative gain compared to pure tBut). Use of molecular sieve (5% w/v) enhances the 48-hr yield (55% in tBut/DMSO [9:1, v/v]). Transesterification in tBut/DMSO (9:1, v/v) with vinyl myristate leads to further improvement of v_0 and 48-hr yield: a relative gain of 85% and 65%, respectively, without sieve and 25% and 10%, respectively, with sieve, compared to esterification. No difference in v_0 and 48-hr yield is observed when transesterification is carried out with or without sieve.*

Keywords acylation, initial rate, myristic acid, novozym 435, sugar ester, vinyl ester

INTRODUCTION

Sugar esters are nonionic surfactants with multiple uses in the cosmetic, food, and pharmaceutical industries. Their enzymatic synthesis is generally preferred to chemical synthesis which is less selective toward the various

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hydroxyl groups and requires fastidious protection and deprotection steps. Due to the drastic conditions generally used (pH and high temperature), chemical synthesis often leads to generation of side products, rendering the recovery of the ester difficult. The range of substrates accessible to enzymatic synthesis is more restricted due to the selectivity of the enzymes. Numerous articles report the use of lipases to catalyze the synthesis of sugar esters.[1–3] Glucose is the main substrate exploited in these studies. Other sugars such as mannose (Man) have an interest, mainly in the medical field. Mannose-based vaccine or drug carrier can be interesting for increasing immunogenicity[4,5] and tumor targeting.[6]

For glucose-based esters synthesis, co-solvents and molecular sieve are frequently used to improve the product yield. In the case of mannose-based esters, pure water-miscible solvents such as acetonitrile, acetone, 2-methyl-2-propanol, and 2-methyl-2-butanol, without any co-solvent, are rather used.[7–9]

In the present study, the influence of the presence of various co-solvents and the effect of their proportion on the esterification of Man by myristic acid (C14Ac), catalyzed by the immobilized lipase B of *Candida antarctica,* is investigated. Dimethylformamide (DMF), formamide, dimethyl sulfoxide (DMSO), or pyridine is added to *tert*-butanol (tBut) and their effect on the initial rate (v_0) and yield of the reaction is examined. The effect of DMSO and pyridine on the sugar solubility and on the denaturation of the lipase is also studied to better understand the impact of these co-solvents on the reaction's v_0 and yield. The influence on these two parameters of the use of molecular sieve to remove water from the esterification medium is also investigated. The esterification is compared to transesterification of Man with vinyl myristate.

EXPERIMENTAL

Materials

Immobilized lipase B from *Candida antarctica* (Novozym 435) was kindly supplied by Novozymes (Denmark). Its activity measured by hydrolysis of *p*-nitrophenyl butyrate (*p*NPB test), is 69 U/g (1 unit corresponds to the amount of enzyme required to hydrolyze 1 μmol of pNPB/min). D-(+)-Mannose (>99%), myristic acid (>99%), pyridine (99%), formamide (99%), dimethylformamide (99.5%), dimethyl sulfoxide (99.5%), formic acid (FA, puriss p.a. for mass spectrometry), and molecular sieve 3 Å (8–12 mesh) were purchased from Sigma Aldrich (USA). Vinyl myristate (>99%, stabilized with MEHQ) was obtained from TCI Europe. Chloroform (stabilised with ~0.5% of ethanol), the HPLC-grade acetonitrile (ACN), and methanol (MeOH) were from Scharlau (Spain). Liquid

chromatography–mass spectroscopy (LC-MS) grade ACN and MeOH were from Biosolve (Netherlands).

Optimization of the Sugar and Fatty Acid Concentrations for the Enzymatic Esterification

The esterification reaction is shown in Figure 1a. tBut was added to Man (apparent concentrations from 0.05 to 0.60 *M*) and C14Ac (0.25 *M*) and the mixture was magnetically stirred and heated at 60°C in a water bath. After 30 min, the reaction was started by adding 20 mg of Novozym 435. The reactions were carried out for 48 hr. Aliquots were withdrawn over time, diluted in ACN/MeOH (1:1, v/v), centrifuged (5 min at 13,000 rpm), and analyzed by HPLC. In a second set of experiments, reactions were carried out with the optimal Man concentration found earlier (0.10 *M*) and C14Ac concentrations varying from 0.05 *M* to 1.20 *M*. The optimal fatty acid concentration was determined. These optimal reagent concentrations were established as the reference conditions for the esterification reaction (0.10 *M* Man and 0.60 *M* C14Ac).

Enzymatic Synthesis of Mannosyl Myristate by Transesterification

The procedure used was the same as described earlier for the reference esterification reaction but with vinyl myristate (0.60 *M*) used as acyl donor instead of C14Ac.

Influence of Co-Solvents on the Synthesis of Mannosyl Myristate by (Trans)Esterification

The co-solvent (formamide, DMF, DMSO, or pyridine) percentage in tBut varied from 0 (reference reaction) to 40% v/v. Reactions and analysis

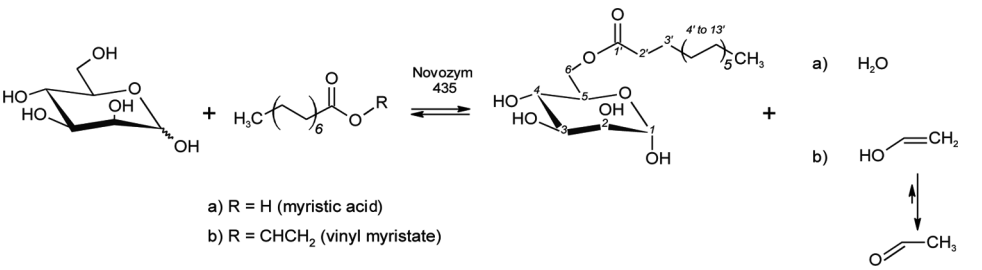

FIGURE 1 Synthesis of mannosyl myristate catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) by (a) esterification or (b) transesterification.

were carried out in the same manner as described for the optimization of the Man concentration.

Influence of the Co-Solvents on the Sugar Solubility and Enzyme Activity

The solubility of Man in various solvent mixtures (tBut with different percentages by volume of DMSO or pyridine) was determined using the following procedure: solutions of 0.10 *M* Man (apparent concentration) and 0.60 *M* C14Ac were magnetically stirred at 60°C for 30 min. After centrifugation, the supernatant was diluted five times with ACN/MeOH (1:1, v/v), analyzed by HPLC–evaporative light-scattering detector (ELSD), and quantified by external calibration.

To study the influence of the co-solvents on the enzyme activity, Novozym 435 was submitted to pure tBut (blank) or various co-solvents (DMSO or pyridine) percentages by volume in tBut for 5 hr. The enzyme was then recovered by filtration, rinsed with tBut, dried, and used for esterification under the reference conditions (in pure tBut). The v_0 and 48-hr yield obtained for Novozym 435 pretreated with the co-solvents were compared to those obtained with the blank.

Influence of Molecular Sieve on the Synthesis of Mannosyl Myristate by (Trans)Esterification

The (trans)esterification reactions were repeated twice under the reference conditions and in tBut with 10% v/v DMSO or 40% v/v pyridine with 5% w/v of molecular sieve 3 Å (preactivated by drying 4 hr at 250°C before use). The amount of sieve used was more than 50 times the amount necessary to absorb the water produced by the reaction if its yield was 100%.

High-Performance Liquid Chromatography (HPLC)

The HPLC analyses were performed on an Agilent Technologies 1200 series HPLC coupled to an evaporative light-scattering detector (ELSD). The column, a Zorbax 300 SB C18 (3.5 µm, 4.6 × 150 mm, Agilent), was thermostatted at 30°C. The flow rate was 0.8 mL/min and a linear gradient of milliQ water (0.1% FA) and ACN (0.1% FA) starting at 30% ACN and increasing to 100% ACN in 5 min was used. Then 100% ACN was maintained during 5 min. The ELSD parameters were 40°C and 3.5 bars N_2. Standards for external calibration consisted of commercial Man and purified mannosyl myristate (see later discussion). The HPLC-ELSD quantification allowed determination of the reactions 48-hr yield (%) and v_0

($mM_{ester}\ L^{-1}\ h^{-1}\ g_{Enz}^{-1}$). v_0 corresponds to the slope of the graph representing the ester concentration as a function of reaction time within the first 5 hr (or less if nonlinear). Esterification and transesterification reactions were duplicated and determined the standard deviations (SD) were always smaller than 10% and 3% for v_0 and the yields, respectively.

LC-MS (negative mode) analyses of the reaction medium were performed on an Agilent 1100 system coupled to a HCT mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The sample was diluted in MeOH with 0.1% FA in order to obtain a total concentration of 5 μg/mL. Analyses were performed on an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm; 3.5 μm). The elution (flow of 0.2 mL/min) was achieved with a linear gradient of ACN (0.1% FA) and milliQ water (0.1% FA) starting at 50% ACN and ending at 100% ACN in 20 min; 100% of ACN was maintained for 5 min. The ESI parameters were 40 psi (N_2), 9 L/min (N_2), and 365°C. The ionic trap was set to scan from $m/z = 160$ to $m/z = 1000$ with a target mass of 300 m/z. The trap was emptied after 200 ms or as soon as a total ionic current of 100 000 was attained. Results obtained (retention time [min], observed m/z, attribution): (1.7 min, 179 m/z, $[Man\text{-}H]^-$); (11.7 min, 389 m/z and 435 m/z, [mannosyl myristate–H]$^-$ and [mannosyl myristate + HCOOH–H]$^-$); (19.3 min, 227 m/z, $[C14Ac\text{-}H]^-$). The LC-MS analyses of the reference reaction medium after 48 hr showed that only Man monoester was produced; no peaks or ions were detected for higher esters such as di- or triesters.

Purification and Chemical Characterization of the Mannosyl Myristate

After synthesis under the reference conditions, the ester was purified by flash chromatography on a silica gel (60 Å/40–63 μm) column using a mixture of chloroform/MeOH/water (65:15:1.5, v/v/v). The purity of the collected fractions was checked by HPLC-ELSD.

MS and MS-MS spectra (negative mode) were acquired on the Bruker HCT mass spectrometer (see earlier description): ESI parameters (10 psi (N_2), 4 L/min (N_2), and 300°C), respectively. The scan range was adjusted to m/z 160–460 and the target mass was set to m/z 180, 228, and 390 for Man, C14Ac, and mannosyl myristate, respectively. Solutions of mannose, myristic acid, and mannosyl myristate at 5 μg/mL (diluted in water or MeOH with 0.1% formic acid [FA]) were infused into the electrospray ionization (ESI) source at a flow rate of 240 μL/min. For C14Ac, no FA was used. The fragmentation of mannosyl myristate was examined in the negative mode by recording of MS-MS experiments on ions at m/z 389 $[M\text{-}H]^-$ and m/z 435 $[M + HCOOH\text{-}H]^-$. The MS and MS-MS data (Table 1)

TABLE 1 ESI MS Results for C14Ac, Man, and Mannosyl Myristate and MS-MS Experiments for Mannosyl Myristate

MS data, Molecule	Detected ions (m/z)	Attribution
C14Ac	227	$[M\text{-}H]^-$
	455	$[2\,M\text{-}H]^-$
Man	179	$[M\text{-}H]^-$
	225	$[M + HCOOH\text{-}H]^-$
Mannosyl myristate	389	$[M\text{-}H]^-$
	435	$[M + HCOOH\text{-}H]^-$
	779	$[2\,M\text{-}H]$
Mannosyl myristate MS-MS data, parent ion (m/z)	**Fragment ions**	**Proposed fragmentation**
389 $[M\text{-}H]^-$	161	$[M\text{-}C_{14}H_{28}O_2\text{-}H]^-$
	227	$[C_{14}H_{27}O_2]^-$
	269	$[M\text{-}C_4H_8O_4\text{-}H]^-$
	371	$[M\text{-}H_2O\text{-}H]^-$
435 $[M+HCOOH\text{-}H]^-$	161	$[M\text{-}C_{14}H_{28}O_2\text{-}H]^-$
	227	$[C_{14}H_{27}O_2]^-$
	269	$[M\text{-}C_4H_8O_4\text{-}H]^-$
	329	$[M\text{-}C_2H_4O_2\text{-}H]^-$
	389	$[M\text{-}H]$

confirmed the molecular mass of the mannosyl myristate and also contributed to confirm its structure.

The infrared spectrum of the KBr pellet of the purified mannosyl myristate was recorded on a Bruker IFS 25 spectrometer (Karlsruhe, Germany) and revealed the expected characteristic bands (3200–3600 cm^{-1} (-OH), 2850–2920 cm^{-1} (-CH_3 and -CH_2), and 1735 cm^{-1} (ester)). The nuclear magnetic resonance (NMR) spectra (^1H, ^{13}C, $^1H/^1H$ COSY, HSQC, HMBC) were recorded in a 6:1 (v/v) mixture of DMSO-d_6/D_2O at 600 MHz (^1H) and 150 MHz (^{13}C) with a Varian instrument. Data are reported as follows: chemical shift in ppm [multiplicity (bs: broad singlet, dd: double doublet, t: triplet, m: multiplet), number of H, coupling constants in Hertz, attribution].

6-*O*-Tetradecanoyl-D-mannose: ^1H RMN (DMSO-d_6/D_2O) 4.81 (bs, 1H, H-1), 4.21 and 3.96 (dd, 2H, $J = 11.4$ Hz, $J = 6.6$ Hz, H-6), 3.67 (t, 1H, $J = 7.5$ Hz, H-6), 3.52 (bs, 1H, H-2), 3.48 (dd, 1H, $J = 9$ Hz, $J = 2.4$ Hz, H-3), 3.34 (t, 1H, $J = 9.6$ Hz, H-4), 2.25 (t, 2H, $J = 7.2$ Hz, H-2′), 1.48 (m, 2H, H-3′), 1.48 (m, 20H, H-4′ to H-13′), 0.82 (t, 3H, $J = 6.6$ Hz, H-14′) ; ^{13}C RMN (DMSO-d_6 / D_2O) 173.4 (C-1′), 94.5 (C-1), 71.9 (C-2), 70.8 (C-3), 70.8 (C-5), 67.6 (C-4), 64.6 (C-6), 33.8 (C-2′), 24.9 (C-3′), 22.5-33;9 (C-4′ to C-13′), 14.4 (C-14′).

The relative configurations at C-1 (α and β anomers) were established from their ^1H and ^{13}C chemical shifts and from the examination of their ^1H multiplicity. The anomeric ratio (α/β) was determined by ^1H-NMR,

which showed that the α anomer is the most important (90%) in the conditions of the analysis. The NMR analyses have demonstrated that only 6-*O*-tetradecanoyl-D-mannose was obtained. Two spin systems were revealed in the $^1H/^1H$ COSY spectrum: CH-1 to CH-6 and CH_2-2′ to CH_3-14′. The connectivity between those two spin systems was established by key HMBC correlation, the most noteworthy being between H_2-6 at δ_H 4.21 and 3.96 and C-1′(δ_C 173.4), and between H_2-2′ at δ_H 2.25 and C-6 (δ_C 64.6). Our result is in accordance with many other studies that have demonstrated the high regioselectivity of *Candida antarctica* lipase B for primary hydroxyl group of osidic molecules.[10]

RESULTS AND DISCUSSION

Optimization of the Sugar and Fatty Acid Concentrations for the Enzymatic Esterification

The solubility of Man in tBut containing 0.60 *M* C14Ac at 60°C determined by HPLC-ELSD is 0.060 *M* (SD = 10%, $n = 4$). As Man is consumed during the reaction, more may dissolve; the apparent Man concentration range tested is 0.05–0.60 M. The v_0 given in Figure 2, estimated thanks to the HPLC-ELSD peak area obtained for the ester, shows that v_0 is not significantly influenced by the Man concentration. The area of the ester observed after 48 hr is constant as of 0.10 *M* Man. That concentration was chosen for the further experiments.

Figure 3 indicates that for the concentration range of C14Ac (0.10–1.20 *M*) the maximum v_0 and ester concentration at 48 hr are attained at 0.60 *M,*

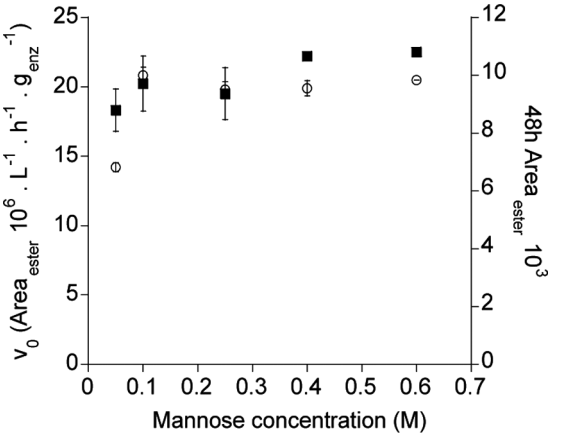

FIGURE 2 Optimization of the Man concentration for the esterification (0.25 *M* C14Ac, 0.2% w/v Novozym 435, 60°C, means of two independent experiments): (■) v_0 and (○) 48-hr ELSD $Area_{ester}$.

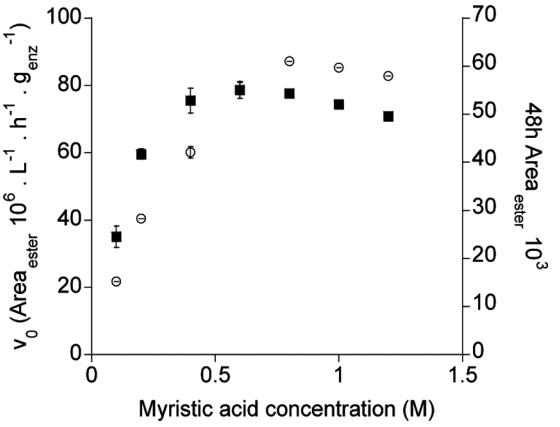

FIGURE 3 Optimization of the C14Ac concentration for the esterification (0.10 *M* Man, 0.2% w/v Novozym 435, 60°C, means of two independent experiments): (■) v_0 and (○) 48-hr ELSD Area$_{ester}$.

acid and this was chosen for the rest of the study. This corresponds to a molar excess of six of the acyl donor compared to the acceptor.

Influence of Co-Solvent Addition on the V_0 and Yield of the Enzymatic Esterification

The choice of solvent is critical for the enzymatic synthesis of sugar fatty esters. Indeed, the solvent must allow the lipase to maintain its catalytic activity and must be able to dissolve both sugar and lipid, two substrates differing highly in polarity. A majority of authors claim that the activity of the lipase is best correlated with the solvent's log P,[11–13] but some have found the best relation with its empirical Dimroth–Reichardt parameter (E_T),[14] others with the dielectric constant[7,8] or with the log $S_{w/o}$,[15] and some could not find any relation with any parameter. It thus seems that until now no universal parameter has been found that allows selection of the best solvent for a synthesis. Enzymatic catalyzed ester synthesis is often done in a pure solvent. In this work, we aimed at optimizing the v_0 and yield of the esterification of Man by C14Ac catalyzed by Novozym 435 by adding a co-solvent to tBut in which the lipase B from *Candida antarctica* shows good catalytic activity and tBut is also too sterically hindered to be used as a substrate by the enzyme. The four co-solvents (DMF, formamide, DMSO, and pyridine) were chosen as they allow good solubilization of sugars but generally cannot be used pure for enzymatic catalysis as they tend to inactivate the lipase.[16]

Man esterification without co-solvent (reference reaction) gave an 48-hr yield of 45% and a v_0 of 1.7 mM_{ester} L^{-1} h^{-1} g_{Enz}^{-1} (Figures 4a and 4b). A small addition (5% v/v) of any of the co-solvents, except pyridine, increased

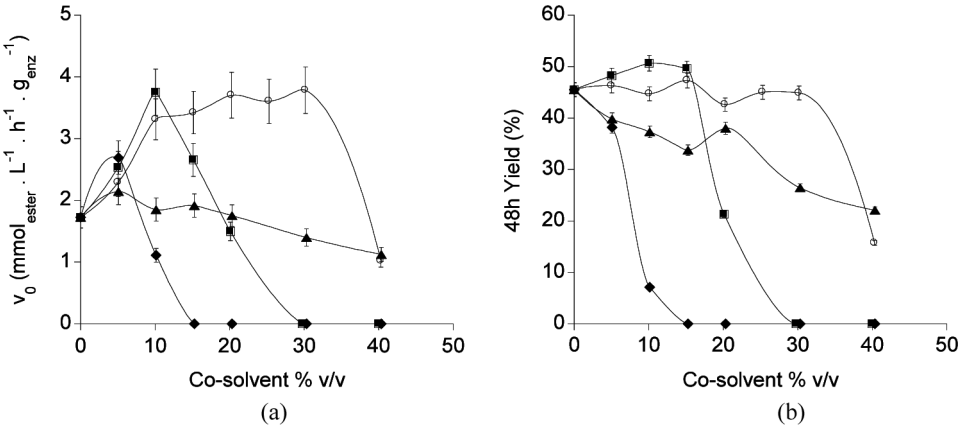

FIGURE 4 Influence of co-solvent percentage by volume of tBut on the v_0 (a) and 48-hr yield (b) of the esterification (0.10 *M* Man, 0.60 *M* myristic acid, 0.2% w/v Novozym 435, 60°C, means of two independent experiments): (○) DMF, (■) DMSO, (▲) pyridine, and (◆) formamide.

the v_0 (Figure 4a). The 48-hr yield (Figure 4b) was slightly improved only with 10% v/v DMSO (48-hr yield of 51%, relative gain of 13%). However, added at high proportions, all of the co-solvents have a negative impact on the v_0 and yield. The v_0 as a function of the % v/v of co-solvent added (Figure 4a) presents a maximum at 5% v/v formamide, 10% v/v DMSO, and between 10 and 30% v/v DMF. For these maxima, the relative v_0 gains are of 55% for formamide and 115% for DMSO and DMF, with DMSO being the most interesting as the optimum is reached at only 10% v/v. The v_0 and 48-hr yield diminish with percentages of DMSO higher than 10% v/v and reach zero at 30% v/v. At 40% v/v of DMF, the v_0 diminishes by 40% compared to the reference. These results are in accordance with the observations of Watanabe et al.,[8] who reported no synthesis of mannosyl laurate by Novozym 435-catalyzed esterification of Man in pure DMSO or DMF medium. Pyridine's relative gain is the lowest of all the solvents tested, while it was shown to be the most effective of the four for the Novozym 435-catalyzed synthesis of myristyl glucuronate from myristyl alcohol and glucuronic acid in tBut (unpublished results). The HPLC-ELSD chromatogram showed no formation of higher (di-, tri, etc.) esters, whichever the two-solvent mixture used.

Influence of the Co-Solvents on the Mannose Solubility and the Enzyme Denaturation

To better understand the effect of pyridine and DMSO on v_0 and yield, their impact on the solubility of Man and on the denaturation of Novozym 435 was studied. The dissolved Man concentration was measured for tBut

containing 0.60 *M* of C14Ac and various percentages (0 to 40% v/v) of DMSO or pyridine (Figure 5). The solubility increased with the percentage of co-solvent and reached a constant maximum value from 15% to 40% v/v of co-solvent corresponding to solubilization of approximately 85% and 75% of the Man introduced in the medium, respectively, for DMSO and pyridine. The v_0 of the mannosyl ester production was already optimal at 10% v/v of DMSO, slightly lower than the 15% v/v minimum DMSO percentage allowing the maximum solubilization of the sugar. Pyridine also improved the solubility of the Man in the medium, but, contrary to DMSO, it had no positive effect on the reaction's v_0. This confirms that the sugar solubility is not the only factor that explains the positive effect of the co-solvents.

The possible denaturing effect of the co-solvents on Novozym 435 was studied. For all the percentages by volume of pyridine, no significant effect of the preincubation of Novozym 435 was observed on the v_0 and the yield (results not shown). This indicates that pyridine does not denature the *Candida antarctica* lipase B. The results are in accordance with Degn and Zimmermann,[12] who report the use of Novozyme 435 in reaction medium consisting of tBut and pyridine in proportions 55:45 (v/v). On the contrary, the preincubation with tBut/DMSO (Figure 6) led to a decrease of both 48-hr yield and v_0. The higher the DMSO percentage is, the lower is the enzyme efficiency. At 30% v/v and above, the catalytic activity is completely lost (no ester synthesized after 48 hr). This is probably due to the

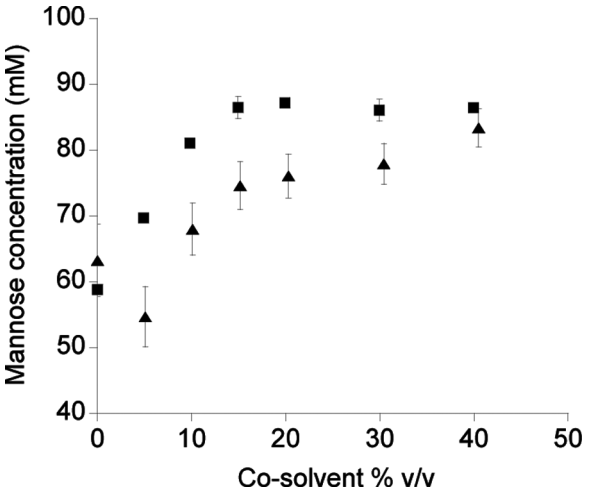

FIGURE 5 Influence of co-solvent percentage by volume of tBut on the Man solubility (0.10 *M* Man apparent concentration, 0.60 *M* C14Ac, 60°C, 30 min, means of two independent experiments): (■) DMSO and (▲) pyridine.

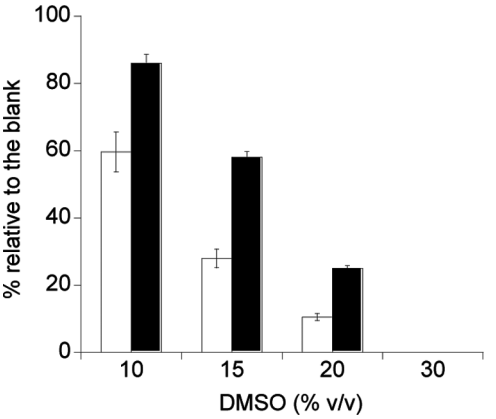

FIGURE 6 Influence on v_0 (black) and 48-hr yields (white) of the incubation of Novozym 435 in mixtures of tBut/DMSO before the esterification performed under the reference conditions (0.10 *M* Man, 0.60 *M* C14Ac, 0.2% w/v Novozym 435, 60°C, means of two independent experiments).

inactivation of the lipase by DMSO, which can cause unfolding of proteins and disrupt the hydration shell of the enzyme.[17]

Influence of Molecular Sieve on the Enzymatic Esterification

The reactants, the enzyme, and the solvents initially contain a small amount of water, which is also a by-product of the esterification. Water will affect the equilibrium position of the reaction and its elimination must favor synthesis rather than hydrolysis. Many methods have been employed to remove water, such as synthesizing under reduced pressure, under reflux, in membrane reactors, in solvents forming low-boiling-point azeotropes with water, and with use of desiccants. In this work, desiccants were chosen and a molecular sieve used, as it has already been studied by many researchers for lipase catalyzed synthesis,[8,12,18] and as Cauglia and Canepa[19] have shown, it gives better results than $CaSO_4$, $CaCl_2$, and $MgSO_4$ for Novozym 435-catalyzed esterification of glucose.

Blanks with molecular sieve but no lipase showed that the desiccant used was not able to catalyze the esterification of Man by C14AC in the three media tested (tBut, tBut/DMSO 9:1 v/v, tBut/pyridine 6:4 v/v), as no peak corresponding to the ester was detected by HPLC-ELSD (data not shown). Furthermore, the addition of the sieve to the enzyme did not lead to a qualitative change of the HPLC-ELSD chromatogram of the reaction media whatever the time of synthesis, and no higher esters were detected. Recently, some researchers have found entirely different results with the same enzyme whilst studying the transesterification with vinyl laurate of fructo-oligosaccharides.[20] They used a 4-Å molecular sieve, and

while they observed only monosubstitution in the absence of the sieve, they detected many multiple substituted products when using the desiccant alone. Furthermore, the product distribution depended on the percentage of DMSO in the tBut. They hypothesized that the catalytic activity was due to the strong acidic sites present on the zeolites constituting the sieve. Although the acidic sites catalytic activity is low, products can be obtained as high temperature and long reaction times were used.

Figure 7a shows that the v_0 of esterification is not influenced by the presence of molecular sieve except for the reaction medium containing 10% v/v of DMSO as co-solvent, for which a 50% v_0 increase is observed with the sieve. We can assume that at the beginning of the reaction, the amount of water produced (that would be trapped by the molecular sieve) is too small to have an influence on v_0. With DMSO, the increase is probably due to the faster reaction (producing more water) or to the higher initial content of free water. The 48-hr yield for the esterification (Figure 7b) is enhanced by the use of molecular sieve (∼55% in tBut/DMSO 9:1 v/v and in tBut/pyridine 6:4 v/v and ∼80% in pure tBut). It traps the water produced and displaces the equilibrium of the reaction toward synthesis rather than hydrolysis.

Comparison of Esterification and Transesterification of Mannose for the Mannosyl Myristate Synthesis

Lipase can catalyze ester synthesis by esterification or transesterification. The latter avoids the major drawback of esterification, namely, the

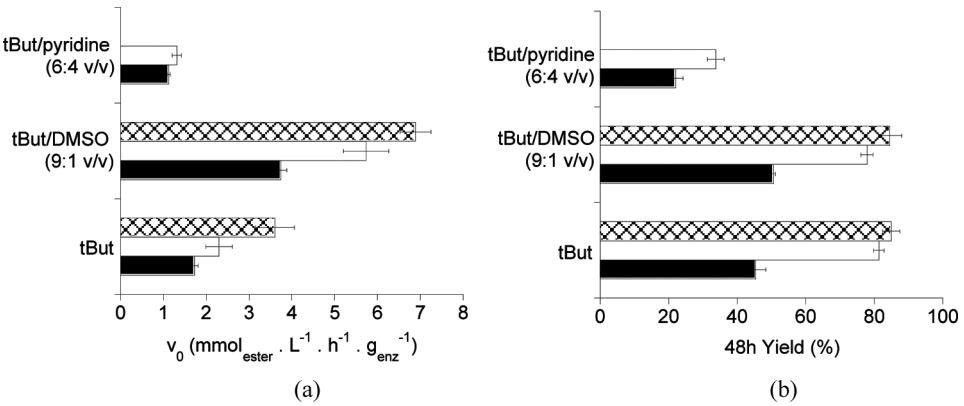

FIGURE 7 Influence of molecular sieve on the v_0 and 48-hr yield of the (trans)esterification (0.10 *M* Man, 0.60 *M* C14Ac or vinyl myristate, 0.2% w/v Novozym 435, 60°C, means of two independent experiments): esterification (black: without sieve; white: with 5% w/v molecular sieve 3 Å); transesterification (squared: without sieve).

production of water, which causes ester hydrolysis. In this study, it was decided to compare the results obtained by esterification with C14Ac to those obtained by transesterification with vinyl myristate. A vinyl ester was preferred to alkyl (methyl, ethyl) esters, as Ferrer et al.[21] demonstrated that the rate of transesterification with the former was about 20 to 100 times faster than with the alkyl esters. Vinyl esters release vinyl alcohol, which is nearly irreversibly converted by tautomerization into acetaldehyde (Figure 1b). Some lipases are inactivated by acetaldehyde, which plays the role of alkylating agent in Maillard type reactions leading to Schiff bases. *Candida antarctica* lipase B is remarkably stable to this product.[22]

In pure tBut without a molecular sieve, the transesterification leads to better results than the esterification with C14Ac, with the former leading to respectively 110% and 85% higher v_0 and 48-hr yield (Figures 7a and 7b). This can be due to the water produced during esterification and to the fact that the tautomerization of the vinyl alcohol to acetaldehyde favors the ester production for the transesterification reaction. Figure 8 shows that the influence of the DMSO percentage on the transesterification v_0 and 48-hr yield follows the same trend as for esterification, with the optimum also being at 10% of DMSO. At that percentage, the transesterification still gives better results than the esterification but to a slightly smaller extent than in pure tBut: the v_0 and 48-hr yield are respectively enhanced by 85% and 65%.

The transesterification was also carried out with 5% w/v of molecular sieve 3 Å in pure tBut or with 10% v/v of DMSO and contrary to the results for the esterification of Man with C14Ac, the desiccant has no significant

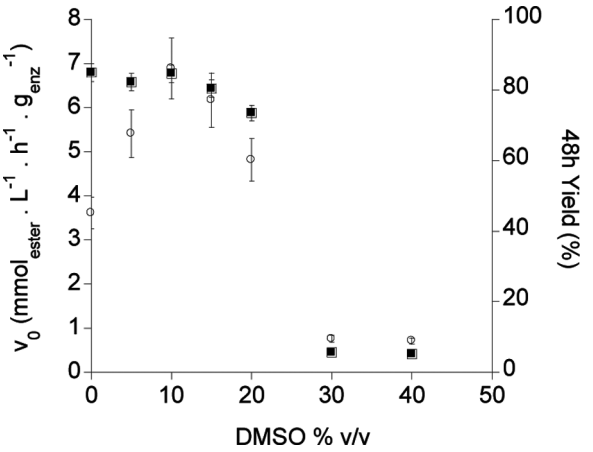

FIGURE 8 Influence of DMSO percentage by volume of tBut on the v_0 and 48-hr yield of the transesterification (0.10 *M* Man, 0.60 *M* vinyl myristate, 0.2% w/v Novozym 435, 60°C, means of two independent experiments): (○) v_0 and (■) 48-hr yield.

effect on v_0 and 48-hr yield (results not shown). This can be explained by the fact that no water is produced during the reaction and that the water initially present in the media does not cause any significant sugar ester hydrolysis.

With 5% w/v of molecular sieve in pure tBut, transesterification leads to respectively 80% higher v_0 and to the same 48-hr yield as those obtained by esterification. With 5% w/v of molecular sieve in tBut/DMSO (9:1, v/v), the transesterification leads to respectively 25% and 10% higher v_0 and 48-hr yield than those obtained by esterification. In both cases, the transesterification v_0 is greater than that of the esterification but to a lesser extent than without the sieve. The effect on the 48-hr yield is very small and is much less marked than in the absence of sieve.

CONCLUSIONS

Of the four co-solvents (DMF, formamide, DMSO, and pyridine) tested in combination with tBut for mannosyl myristate synthesis by esterification catalyzed by immobilized lipase B from *Candida antarctica*, DMSO allowed the best improvement of v_0 and 48-hr yield (respectively 115% and 13% relative gain compared to the reference), and pyridine never significantly improved the v_0 and even had a negative effect on 48-hr yield with percentages as low as 5% v/v. Solubility measurements demonstrated that both DMSO and pyridine help to solubilize Man. Experiments also showed that DMSO has a denaturing effect on Novozyme 435, and as a consequence, its percentage in the medium must be kept relatively low (<15% v/v). Contrary to DMSO, pyridine does not denature the enzyme, but it does not improve the reaction's v_0 and 48-hr yield. One hypothesis would be that pyridine could deprotonate the carboxylic function of myristic acid and thus reduce its availability. By trapping the water, use of molecular sieve in the esterification medium allows enhancement of the 48-hr yield (55% in tBut/DMSO [9:1, v/v]) by displacing the equilibrium of the reaction toward synthesis rather than hydrolysis. Mannosyl myristate was also obtained by transesterification with vinyl myristate. Without a molecular sieve, the transesterification leads to higher v_0 and yield than the esterification (no water production and tautomerization of the vinyl alcohol to acetaldehyde), but it requires an additional step for the vinyl ester production and increases the cost of the reagent compared to the fatty acid. This step can be avoided by using a molecular sieve and the solvent mixture (tBut/DMSO 9:1 v/v), as the esterification under these conditions gives v_0 and yield close to those of the transesterification. However, this option also presents disadvantages. For example, stirring of the medium becomes more difficult and the mass transfer can be limited. The use of the sieve can also

require bigger reactors, and an excess of sieve may remove the water in the enzyme vicinity essential for its activity.[23] Other authors have reported a change in the selectivity of the reaction,[20] and some have noted the possibility of adsorption and degradation of their ester (6-unsaturated acyl-L-ascorbates) by the sieve.[24] In the future, it will be interesting to optimize the concentration of the sieve and to study its effect on the mannosyl myristate (adsorption, degradation and ease of purification).

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