The control of Novozym® 435 chemoselectivity and specificity by the solvents in acylation reactions of amino-alcohols

Florian Le Joubioux, Nicolas Bridiau, Yesmine Ben Henda, Oussama Achour, Marianne Graber, Thierry Maugard

The control of Novozym® 435 chemoselectivity and specificity by the solvents in acylation reactions of amino-alcohols

Florian Le Joubioux, Nicolas Bridiau, Yesmine Ben Henda, Oussama Achour, Marianne Graber, Thierry Maugard*

UMR 7266 CNRS-ULR, LIENSs, Equipe Approches Moléculaires, Environnement-Santé, Université de La Rochelle, Avenue Michel Crépeau, 17042 La Rochelle, France.

Author for correspondence (Fax: +33 546458277; E-mail: tmaugard@univ-lr.fr)

Abstract

The current work describes the differential behavior of Novozym® 435 (immobilized *Candida antarctica* lipase B) in *O*-acylation and *N*-acylation catalysis of bifunctional amino-alcohols acyl acceptors. We perfomed acylation experiments on three amino-alcohols (alaninol, 4-amino-1-pentanol and 6-amino-1-hexanol) using myristic acid as an acyl donor. Two organic solvents (*tert*-amyl alcohol and *n*-hexane) and one ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate: Bmim [PF_6]) were used to determine the effect of the solvent. The influence of the amino-alcohol carbon chain length between the alcohol and amino groups on chemoselectivity C ($k_{cat,\ app\ O\text{-acylation}}/k_{cat,\ app\ N\text{-acylation}}$) was highlighted. *N*-acylation is improved using alaninol, a short chain amino-alcohol (no mono-*O*-acylation in *tert*-amyl alcohol and C = 0.12 in *n*-hexane) whereas *O*-acylation is improved using 4-amino-1-pentanol and 6-amino-1-hexanol which are amino-alcohols with longer chain (C = 10.5 in *tert*-amyl alcohol and C = 539 in *n*-hexane). On the other hand, the production of the acylated amino-alcohols after 96 hours of reaction was shown to be strongly affected by the solvent nature and the amino-alcohol structure: starting from alaninol as an acyl acceptor, the yield of amide synthesis reaches up to 98% in *tert*-amyl alcohol using 0.7 equivalents of myristic acid while the yield of amido-ester synthesis reaches up to 88% in Bmim [PF_6] using 1.75 equivalents of myristic acid.

Keywords: *N*-acylation; *O*-acylation; Novozym® 435; Organic solvent; Ionic liquid; Chemoselectivity

1. Introduction

Acylated amino-alcohols such as ceramides, glucamides and amino-acid derivatives have found important applications, such as in pharmaceutical and cosmetic industries, as potential anti-viral or anti-tumor drugs [1-3], anti-oxidant stabilizers [4] and as active ingredients in hair and skin care products [5]. In addition, these compounds can also be used as surfactants in environmental, food and agricultural industries [6-8].

Many processes of chemical acylation of amino-alcohols have been developed so far but these methods have faced several limitations. One of the most serious restrictions during chemical processes is the necessity for fastidious steps of alcohol or amino group protection and deprotection, which are essential to control the chemoselectivity and stereoselectivity of acylation reactions [9-10].

Biocatalysis is considered as an interesting alternative for the preparation of synthetic compounds: it offers a clean way to perform chemical processes under mild reaction conditions, with a high degree of selectivity [11-12]. Lipases (E.C. 3.1.1.3) in particular provide several advantages when used in anhydrous organic media [13-15]. These include shifting of the thermodynamic equilibrium in favour of synthesis over hydrolysis reaction, increasing the solubility of non-polar substrates, eliminating side reactions, making enzyme recovery easier and increasing enzyme thermostability [16-18]. Lipases are excellent biocatalysts in *O*-acylation, transesterification and *N*-acylation reactions in the synthesis of various acylated derivates used in pharmaceutical, cosmetic and food industries [19-22]. They have, therefore, been widely studied over the past two decades and a large amount of their key properties in biocatalysis have been highlighted [15, 23-25]. Recently, a new proton shuttle reaction mechanism was proposed to explain chemoselectivity for lipase-catalyzed *N*-acylation of amino-alcohols [26]. However, it is still necessary to optimize the output of

lipase-catalyzed reactions and to understand this type of selectivity in different reaction conditions.

The properties of lipases (activity, chemoselectivity, regioselectivity and stereoselectivity) can be modulated by many parameters such as genetic or chemical modification, enzyme immobilization [15, 27-29]. Solvent nature also particularly influences lipase-catalyzed acylation reactions. Recent studies have shown that the solvent affects the activity and selectivity of enzymes in a complex way, involving many interactions between the solvent and both the substrate and the enzyme [30-35]. Furthermore, the use of lipases in organic solvents makes many synthetic reactions possible that do not occur in the natural media of these enzymes. However, these solvents can display certain disadvantages, such as volatility and/or toxicity towards the environment [36], particularly when they are used on a large scale. An alternative to these organic solvents is the use of ionic liquids. Ionic liquids have recently emerged to replace organic solvents in biocatalytic transformations, especially in the case of polar substrates like amino-alcohols that are difficult to dissolve in organic solvents [37]. Moreover, they show unique properties, including no vapor pressure and capacity to be recycled and to prevent the thermal deactivation of enzymes [30, 38]. These properties can be useful in lipase-catalyzed biotransformation. In many cases, these media have been shown to improve the efficiency of lipase-catalyzed acylation reactions [31, 39–41] and used to perform both ester and amide synthesis [38, 42-44].

Despite the attractive properties of organic solvents and ionic liquids, few studies have been devoted to the lipase-catalyzed acylation of bifunctional substrates, exhibiting both amino and alcohol groups, such as ethanolamine, diethanolamine, 2-amino-1-butanol, 6-amino-1-hexanol, serine and other amino-alcohols with variable carbon chain lengths [45-50]. In such reactions, the lipase catalyzes *O*-acylation or *N*-acylation with a chemoselectivity that is largely dependent on the amino-alcohol structure [26].

Among lipases used in organic synthesis, *Candida antarctica* lipase B is well known for its ability to convert alcohols and amines into esters and amides in various solvents [8, 51] and seems to be the ideal enzyme for the acylation of compounds such as amino-alcohols. Moreover, commercially available immobilized *Candida antarctica* lipase B, such as Novozym® 435, shows many advantages such as long-term stability at moderately high temperatures and a tolerance for polar and non-polar solvents [52]. In the present work, we investigated two organic solvents and one ionic liquid as reaction solvents in the acylation of three amino-alcohols, catalyzed by Novozym® 435, with myristic acid **1** as an acyl donor. In order to determine the optimum conditions for selective *N*-acylation and *O*-acylation of amino-alcohols and to maximize both the yield and the selectivity, the effects of amino-alcohol structure and solvent nature were compared.

2. Materials and methods

2.1. Materials

Novozym® 435 (*Candida antarctica* lipase B immobilized on acrylic resin), was kindly provided by Novozymes A/S, Bagsvaerd, Denmark. **(±)**-alaninol **2** (98%) and 6-amino-1-hexanol **6** (≥97%), as well as *tert*-amyl alcohol (99%) and 1-butyl-3-methylimidazolium hexafluorophosphate (Bmim [PF_6]) (≥97%) were purchased from Sigma-Aldrich (St Louis, USA) while **(±)**-4-amino-1-pentanol **4** was from Santa Cruz Biotechnology (USA). Myristic acid **1** and acetic acid were from Fluka (St Quentin-Fallavier, Switzerland). All chemicals were dried over molecular sieves. Pure water was obtained via a Milli-Q system (Millipore, France). Acetonitrile, methanol, *n*-hexane, chloroform and 1-butanol were purchased from Carlo ERBA (Val-de-Reuil, France).

2.2. Enzymatic acylation procedure

105 *2.2.1. General procedure for lipase catalysed amino-alcohol acylation*

106 In all cases, reactions were carried out at 55°C in screw-caped tubes. 2 ml reaction mixtures
107 containing various amounts of amino-alcohol (25-350 mM) and 175 mM of myristic acid **1** as
108 an acyl donor were incubated for 10 minutes prior to addition of 5 g l^{-1} of Novozym® 435.
109 Reactions were conducted for 96 hours. Initial rate measurements were also performed
110 according to a previously established procedure [53]. The initial rates were calculated from
111 the linear relationship of the total concentration of products against reaction time (0-1 hour in
112 *n*-hexane and 0-2 hours in *tert*-amyl alcohol or Bmim [PF_6]).

113 *2.2.2. Procedure for amino-alcohol acylation in tert-amyl alcohol*

114 When using *tert*-amyl alcohol as a reaction solvent, 100 µl samples were taken at intervals
115 and centrifuged at 18,000 g for a minute. The supernatants were then analyzed by LC-MS,
116 leading to the determination and quantification of remaining substrates and synthesized
117 products.

118 *2.2.3. Procedure for amino-alcohol acylation in n-hexane*

119 When using *n*-hexane as a reaction solvent, various samples containing the same
120 concentration of reactants and enzyme were prepared and incubated under the same
121 conditions. Reactions were then conducted in parallel and withdrawn at different times to
122 determine the reaction progress. 6 ml of a methanol/chloroform (50/50, v/v) mixture were
123 then added in each sample and the reaction medium was homogenized. 500 µl samples were
124 taken and centrifuged at 18,000 g for a minute. The supernatants were then analyzed by LC-
125 MS, leading to the determination and quantification of remaining substrates and synthesized
126 products.

2.2.4. Procedure for amino-alcohol acylation in 1-butyl-3-methylimidazolium hexafluorophosphate

When using 1-butyl-3-methylimidazolium hexafluorophosphate (Bmim [PF_6]) as a reaction solvent, various samples containing the same concentration of reactants and enzyme were prepared and incubated under the same conditions. Reactions were then conducted in parallel and withdrawn at different times to determine the reaction progress. 6 ml of 1-butanol, a solvent that is immiscible with Bmim [PF_6], were added in order to extract substrates and products from Bmim [PF_6]. 500 µl samples were taken from 1-butanol extracts and centrifuged at 18,000 g for a minute. The supernatants were then analyzed by LC-MS, leading to the determination and quantification of remaining substrates and synthesized products.

The partition coefficient between Bmim [PF_6] and 1-butanol was determined for all compounds (myristic acid **1** and acylated products) using the following procedure: a solution of Bmim [PF_6] with a known concentration of each compound was prepared and then extracted in 1-butanol. The partition coefficient was calculated as the ratio of the final quantity determined by HPLC to the known initial quantity. All samples were performed in duplicate and the averages of duplicate partition coefficients were mentioned in Table 1. Finally, it was used to correct the concentration values of all compounds in 1-butanol extracts.

Table 1

Partition coefficient of myristic acid **1** and acylated products in a Bmim [PF_6]/1-butanol biphasic system.

Compound	Partition coefficient[a]
Myristic acid **1**	0.84
N-myristyl 2-amino-1-propanol **3a**	0.84
O-myristyl 2-amino-1-propanol **3b**	0.88
O,N-dimyristyl 2-amino-1-propanol **3c**	0.99
N-myristyl 4-amino-1-pentanol **5a**	0.82
O-myristyl 4-amino-1-pentanol **5b**	0.90
O,N-dimyristyl 4-amino-1-pentanol **5c**	0.99

N-myristyl aminohexanol **7a**	0.83
O-myristyl aminohexanol **7b**	0.87
O,N-dimyristyl aminohexanol **7c**	0.99

[a] The partition coefficient was calculated as the ratio of the final concentration determined by HPLC to the known initial concentration.

2.3. Analytical methods

2.3.1. HPLC-MS analysis

Structural and quantitative analysis of reaction products were conducted using a LC/MS-ES (liquid chromatography–mass spectrometry) system from Agilent (1100 LC/MSD Trap mass spectrometer VL) with a C18 Prontosil 120-5-C18-AQ reversed-phase column (250×4 mm, 5 μm; Bischoff Chromatography, Germany). The elution of reaction samples was carried out at room temperature and at a flow rate of 1 ml min^{-1}, using a mobile phase consisting in a mixture of two solvents: acetonitrile/water/acetic acid (77/23/0.1, v/v/v) (A) and methanol/acetic acid (100/0.1, v/v) (B). The following variations of the mobile phase were used during the time interval of the analysis: 100% solvent A from 0 to 20 minutes; a linear gradient reaching 0% solvent A and 100% solvent B from 20 to 23 minutes; 100% solvent B from 23 to 80 minutes; a linear gradient reaching back 100% solvent A and 0% solvent B from 80 to 82 minutes; 100% solvent A from 82 minutes to the end of the run at 90 minutes. Products were detected and quantified by differential refractometry and UV detection at 210 nm. An external calibration was performed with pure myristic acid. Then, calibrations for individual acylation products were obtained after mass balance in reaction conditions enabling to obtain only these products with myristic acid as an acyl donor. Low-resolution mass spectral analyses were obtained by electrospray in the positive detection mode. Nitrogen was used as the drying gas at 15 l min^{-1} and 350 °C at a nebulizer pressure of 4 bars. The scan range was 50–1000 m/z using five averages and 13,000 m/z per second resolution. The capillary voltage was 4000 V. Processing was done offline using HP Chemstation software.

172

173 *2.3.2. Acylated-amino-alcohols purification and analysis for identification*

174 In order to perform the purification and characterization of acylated products, 20 ml reaction
175 mixtures containing 50 mM of the amino-alcohol and 175 mM of myristic acid **1** in *tert*-amyl
176 alcohol were incubated for 24 hours in presence of 15 g l^{-1} of Novozym® 435. Purified
177 products were then characterized by ^{1}H NMR (nuclear magnetic resonance spectroscopy) and
178 IR (infrared spectroscopy) after purification via preparative HPLC using a ProntoPrep C18
179 reversed-phase column (250×20 mm, 10 µm; Bischoff Chromatography, Germany) eluted via
180 the mobile phase given in section 2.3.1, at room temperature and at a flow rate of 5 ml min^{-1}.
181 ^{1}H NMR were recorded on a JEOL-JNM LA400 spectrometer (400 MHz), with
182 tetramethylsilane as an internal reference. Samples were studied as solutions in $CDCl_3$.
183 Infrared (IR) spectra were recorded from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using a
184 100 ATR spectrometer (Perkin-Elmer, United States).

185 ***N*-myristyl 2-amino-1-propanol 3a**: m/Z (LR-ESI^+) $C_{17}H_{36}NO_2$ (M + H^+), found: 286.4,
186 calculated for: 286.48. IR ν_{max} (cm^{-1}): 3100-3500 (O-H, alcohol and N-H, amide), 2800-3000
187 (CH of myristyl chain), 1638 (C=O, amide), 1543 (N-H, amide). ^{1}H NMR (400 MHz, $CDCl_3$,
188 δ ppm): δ 0.88 (t, 3H, *J*= 6.06Hz, -CH_2-C<u>H_3</u>), 1.17 (d, 3H, *J*= 6.06Hz, –CH-C<u>H_3</u>), 1.25 (m,
189 20H, -C<u>H_2</u>- of myristyl chain), 1.63 (m, 2H, -C<u>H_2</u>-CH_2-CO-NH- of myristyl chain), 2.19 (t,
190 2H, *J*= 6.06Hz, -CH_2-C<u>H_2</u>-CO-NH- of myristyl chain), 3.04 (s, 1H, -O<u>H</u>), 3.27 (dd, 1H, *J*=
191 5Hz, *J*= 10Hz, –CH-C<u>H_2</u>-OH), 3.46 (dd, 1H, *J*= 3.7Hz, *J*= 11Hz, –CH-C<u>H_2</u>-OH), 4.07 (m,
192 1H, -C<u>H</u>-), 5.7 (s, 1H, -N<u>H</u>-).

193 ***O,N*-dimyristyl 2-amino-1-propanol 3c**: m/Z (LR-ESI^+) $C_{31}H_{62}NO_3Na$ (M + Na^+), found:
194 518.6, calculated for: 518.85. IR ν_{max} (cm^{-1}): 3301 (N-H, amide), 2800-3000 (CH of myristyl
195 chain), 1737 (C=O, ester), 1643 (C=O, amide), 1542 (N-H, amide). ^{1}H NMR (400 MHz,

$CDCl_3$, δ ppm): δ 0.88 (t, 6H, *J*= 7.6Hz, 2x -CH_2-C$\underline{H}_3$), 1.16 (d, 3H, *J*= 7.6Hz, –CH-C$\underline{H}_3$), 1.25 (m, 40H, -C$\underline{H}_2$- of myristyl chain), 1.6 (m, 4H, 2x -C$\underline{H}_2$-CH_2-CO- of myristyl chain), 2.14 (t, 2H, *J*= 7.2Hz, -CH_2-C$\underline{H}_2$-CO-O- of myristyl chain), 2.32 (t, 2H, *J*= 7.2Hz, -CH_2-C$\underline{H}_2$-CO-NH- of myristyl chain), 4 (dd, 1H, *J*= 4.4Hz, *J*= 10.7Hz, –CH-C$\underline{H}_2$-O-), 4.13 (dd, 1H, *J*= 4.9Hz, *J*= 10Hz, –CH-C$\underline{H}_2$-O-), 4.29 (m, 1H, -C$\underline{H}$-), 5.54 (d, 1H, *J*= 7.3Hz, C-N$\underline{H}$-CH_2-).

***N*-myristyl 4-amino-1-pentanol 5a:** m/Z (LR-ESI^+) $C_{19}H_{40}NO_2$ ($M + H^+$), found: 314.2 calculated for: 314.53. IR ν_{max} (cm^{-1}): 3200-3500 (O-H, alcohol and N-H, amide), 2800-3000 (CH of myristyl chain), 1639 (C=O, amide), 1545 (N-H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, *J*= 6.58Hz, -CH_2-C$\underline{H}_3$), 1.14 (d, 3H, *J*= 6.23Hz, –CH-C$\underline{H}_3$), 1.25 (m, 20H, -C$\underline{H}_2$- of myristyl chain), 1.53 (m, 4H, –CH-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-OH), 1.63 (m, 2H, -C$\underline{H}_2$-CH_2-CO-NH- of myristyl chain), 2.14 (t, 2H, *J*= 7.27Hz, -CH_2-C$\underline{H}_2$-CO-NH- of myristyl chain), 2.94 (s, 1H, -O$\underline{H}$), 3.67 (m, 2H, –CH_2-C$\underline{H}_2$-OH), 4.06 (m, 1H, -C$\underline{H}$-), 5.28 (s, 1H, -N$\underline{H}$-).

***O*-myristyl 4-amino-1-pentanol 5b:** m/Z (LR-ESI^+) $C_{19}H_{40}NO_2$ ($M + H^+$), found: 314.2 calculated for: 314.53. IR ν_{max} (cm^{-1}): 3291 (N-H, amine), 2800-3000 (CH of myristyl chain), 1736 (C=O, ester), 1557 (N-H, amine). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, *J*= 6.99Hz, -CH_2-C$\underline{H}_3$), 1.14 (d, 3H, *J*= 8Hz, –CH-C$\underline{H}_3$), 1.25 (m, 20H, -C$\underline{H}_2$- of myristyl chain), 1.52 (m, 4H, –CH-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-O-), 1.62 (m, 2H, -C$\underline{H}_2$-CH_2-CO-O- of myristyl chain), 2.22 (t, 1H, *J*= 7.16Hz, -CH_2-C$\underline{H}_2$-CO-O- of myristyl chain), 2.29 (t, 1H, *J*= 7.5Hz, -CH_2-C$\underline{H}_2$-CO-O- of myristyl chain), 3.43 (m, 1H, –CH_2-C$\underline{H}_2$-O-), 3.69 (m, 1H, –CH_2-C$\underline{H}_2$-O-), 4.1 (m, 1H, -C$\underline{H}$-).

***O,N*-dimyristyl 4-amino-1-pentanol 5c:** m/Z (LR-ESI^+) $C_{33}H_{66}NO_3Na$ ($M + Na^+$), found: 546.2 calculated for: 546.9. IR ν_{max} (cm^{-1}): 3304 (N-H, amide), 2800-3000 (CH of myristyl

chain), 1732 (C=O, ester), 1640 (C=O, amide), 1546 (N-H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, *J*= 7.43Hz, 2x -CH_2-C$\underline{H}_3$), 1.14 (d, 3H, *J*= 6.83Hz, –CH-C$\underline{H}_3$), 1.25 (m, 40H, -C$\underline{H}_2$- of myristyl chain), 1.53 (m, 4H, –CH-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-O-C), 1.6 (m, 4H, 2x -C$\underline{H}_2$-CH_2-CO- of myristyl chain), 2.14 (t, 4H, *J*= 7.08Hz, -CH_2-C$\underline{H}_2$-CO- of myristyl chain), 3.68 (m, 2H, –CH_2-C$\underline{H}_2$-O-C), 4.07 (m, 1H, -C$\underline{H}$-), 5.27 (d, 1H, *J*= 6.86Hz, -N$\underline{H}$-).

***N*-myristyl aminohexanol 7a:** m/Z (LR-ESI$^+$) $C_{20}H_{42}NO_2$ (M + H^+), found: 329.5 calculated for: 328.56. IR ν_{max} (cm^{-1}): 3385 (O-H, alcohol), 3314 (N-H, amide), 2800-3000 (CH of myristyl chain), 1634 (C=O, amide), 1534 (N-H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, *J*= 7.5Hz, -CH_2-C$\underline{H}_3$), 1.25 (m, 20H, -C$\underline{H}_2$- of myristyl chain), 1.51 (m, 2H, -C$\underline{H}_2$-CH_2-CO-O- of myristyl chain), 1.59 (m, 4H, –CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-OH), 2.26 (t, 2H, *J*= 7.65Hz, -CH_2-C$\underline{H}_2$-CO-OH of myristyl chain), 2.72 (s, 1H, -O$\underline{H}$), 3.25 (t, 2H, *J*= 7.07Hz, –CH_2-C$\underline{H}_2$-OH), 3.63 (t, 2H, *J*= 7.29Hz, –CH_2-C$\underline{H}_2$-NH-CO-CH_2), 5.41 (s, 1H, -N$\underline{H}$-).

***O*-myristyl aminohexanol 7b:** m/Z (LR-ESI$^+$) $C_{20}H_{42}NO_2$ (M + H^+), found: 329.5 calculated for: 328.56. IR ν_{max} (cm^{-1}): 3400 (N-H, amine), 2800-3000 (CH of myristyl chain), 1736 (C=O, ester), 1544 (N-H, amine). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, *J*= 7.28Hz, -CH_2-C$\underline{H}_3$), 1.25 (m, 20H, -C$\underline{H}_2$- of myristyl chain), 1.55 (m, 2H, -C$\underline{H}_2$-CH_2-CO-O- of myristyl chain), 1.62 (m, 4H, –CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-NH_2), 2.28 (t, 2H, *J*= 7.65Hz, -CH_2-C$\underline{H}_2$-CO-O- of myristyl chain), 2.81 (s, 2H, -N$\underline{H}_2$), 3.64 (t, 2H, *J*= 6.47Hz, –CH_2-C$\underline{H}_2$-NH_2), 4.04 (t, 2H, *J*= 6.47Hz, –CH_2-C$\underline{H}_2$-O-CO-CH_2).

***O,N*-dimyristyl aminohexanol 7c:** m/Z (LR-ESI$^+$) $C_{34}H_{67}NO_3Na$ (M + Na^+), found: 560.7, calculated for: 560.93. IR ν_{max} (cm^{-1}): 3298 (N-H, amide), 2800-3000 (CH of myristyl chain), 1726 (C=O, ester), 1635 (C=O, amide), 1547 (N-H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, *J*= 6.48Hz, 2x -CH_2-C$\underline{H}_3$), 1.25 (m, 40H, -C$\underline{H}_2$- of myristyl chain), 1.5 (m, 4H, -C$\underline{H}_2$-CH_2-CO- of myristyl chain), 1.6 (m, 4H, –CH_2-C$\underline{H}_2$-C$\underline{H}_2$-CH_2-O-C), 2.15 (t,

2H, *J*= 7.8Hz, -CH_2-C$\underline{H_2}$-CO-NH-), 2.29 (t, 2H, *J*= 7.8Hz, -CH_2-C$\underline{H_2}$-CO-NH-), 3.24 (q, 2H, *J*= 6.5Hz, –CH_2-C$\underline{H_2}$-NH-), 4.06 (t, 2H, *J*= 5.9Hz, –CH_2-C$\underline{H_2}$-O-CO-CH_2), 5.4 (s, 1H, -N$\underline{H}$-).

2.3.3. Analysis of the ionization state of substrates in tert-amyl alcohol

The ionization state of myristic acid **1** in *tert*-amyl alcohol was investigated using infrared spectroscopy analysis. Infrared (IR) spectra of samples containing 175 mM of myristic acid **1** and from 0 to 250 mM of alaninol **2** were recorded from 1500 to 1800 cm^{-1} with a resolution of 4 cm^{-1} using a 100 ATR spectrometer (Perkin-Elmer, United States). Before the interpretation of data, a treatment (base line correction, smoothing and normalization min–max) was applied to spectra.

2.4. Titration of Novozym® 435 active sites

In order to determine the amount of immobilized *Candida antarctica* lipase B (Novozym® 435) active sites, a suicide inhibitor (4-methylumbelliferyl hexylphosphonate) was used according to the method developed by Fujiia *et al.* [54-55]. This inhibitor was added to 10 mg of immobilized lipase (immobilized on beads of acrylic support), to a final concentration of 50 μM. In this sample, acetonitrile was added to final volume of 1 mL. 100 μL sample solution was adding to 900 μL of buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0), then fluorescence intensity was analyzed using a luminescence spectrometer (Luminescence Spectrometer Model LS-50B, PerkinElmer, MA, USA). The excitation wavelength was λ=360 nm and the emission wavelength was λ=445 nm. The active site amount was establish from the linear relationship between fluorescence intensity and the concentration of the leaving group 4-methylumbelliferone. Finally, the relationship between fluorescence intensity and the amount of beads containing immobilized lipase was linear. The resulting active *Candida antarctica* lipase B load on beads was found to be 4.7% (%weight/weight) which equals 1.4 μmol of lipase active site per gram of beads.

2.5. Determination of the kinetic parameters

The kinetic parameters from amino-alcohol acylation were determined using Lineweaver-Burk reciprocal plots of initial rates versus amino-alcohol concentrations. The apparent maximum rates ($V_{max,app}$) of myristic acid conversion, *O*-acylation and *N*-acylation were obtained by using y-intercept which correspond to the $1/V_{max,app}$ value. The apparent catalytic constants ($k_{cat,app}$) were then calculated as the ratio of the $V_{max,app}$ to the total amount of Novozym® 435 active sites in the reaction medium, which was previously determined by titration (1.4 μmol of lipase active sites per gram of Novozym® 435: see section 2.4). In parallel, the apparent Michaelis constants ($K_{m,app}$) were obtained by using x-intercept which correspond to the $-1/K_{m,app}$ value.

2.6. Evaluation of the chemoselectivity

The chemoselectivity of Novozym® 435 during the acylation of amino-alcohols was studied by comparing the alcohol group *O*-acylation and the amino group *N*-acylation, and then calculated via the apparent catalytic efficiency ratio (Eq. (1)) [56], which was transformed into an apparent catalytic constant ratio (Eq. (2)) owing to the fact that there is a unique $K_{m,app}$ for each bifunctional amino-alcohol.

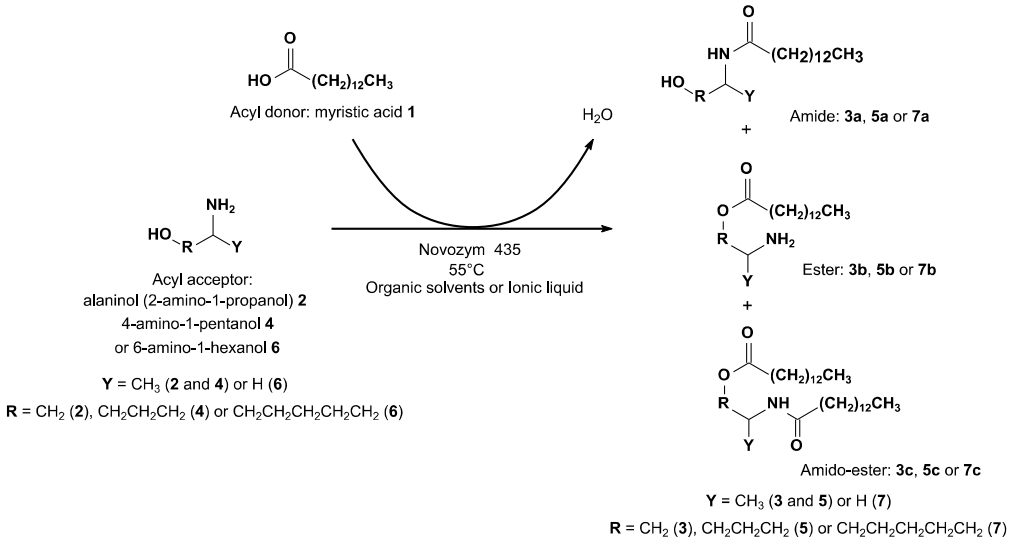
$$C = (k_{cat,\ app\ O\text{-acylation}} / K_{m,app}) / (k_{cat,\ app\ N\text{-acylation}} / K_{m,app}) \quad (1)$$

$$C = k_{cat,\ app\ O\text{-acylation}} / k_{cat,\ app\ N\text{-acylation}} \quad (2)$$

3. Results and discussion

3.1. Effect of the solvent on the apparent kinetic parameters and the chemoselectivity of Novozym® 435 in the acylation of amino-alcohols

Two organic solvents (*tert*-amyl alcohol and *n*-hexane) and one ionic liquid (1-Butyl-3-methylimidazolium hexafluorophosphate; Bmim [PF_6]) were used for the selective acylation of alaninol (**2**), 4-amino-1-pentanol (**4**) and 6-amino-1-hexanol (**6**) using myristic acid **1** as an acyl donor (Scheme 1). Bmim [PF_6] was chosen as a model ionic liquid on the basis of several earlier studies which revealed the efficiency of Bmim [PF_6] for *O*-acylation, transesterification and *N*-acylation reactions catalyzed by *Candida antarctica* lipase B [57, 58]. The two organic solvents were chosen from previous works found in the literature, which have demonstrated the efficacy of these solvents during the acylation of amino-polyol substrates catalyzed by lipases [8, 53, 59].



Scheme 1. Acylation of three amino-alcohols by Novozym® 435 (immobilized *Candida antarctica* lipase B).

All experiments were performed in media containing various amounts of the amino-alcohol (25-350 mM) as an acyl acceptor and 175 mM of myristic acid **1** as an acyl donor. In the absence of enzyme, no product was detected within 2 days. LC-MS analysis demonstrated that the decrease in myristic acid **1** concentration is always concomitant with the synthesis of acylated products. The purification and the structural elucidation by MS, IR and NMR analyses identified the structure of the acylated products.

The kinetic parameters for the acylation of amino-alcohols **2**, **4** and **6** were determined using Lineweaver-Burk reciprocal plots.

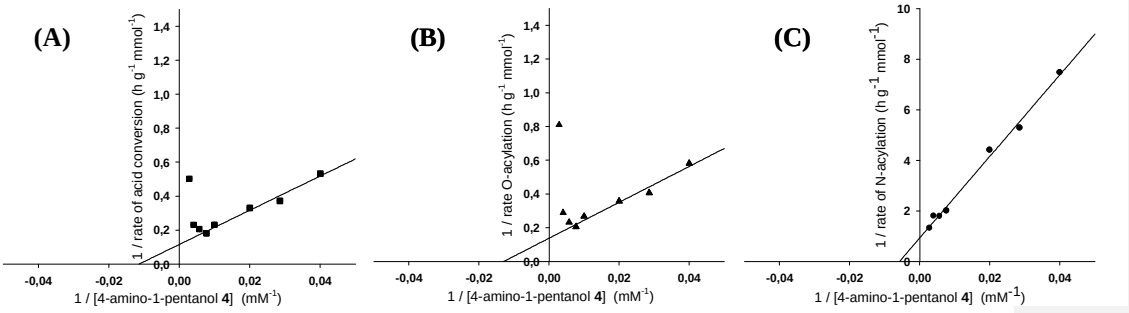


Fig. 1. Reciprocal initial rates of myristic acid conversion (**A**), 4-amino-1-pentanol *O*-acylation (**B**) and 4-amino-1-pentanol *N*-acylation (**C**) versus reciprocal 4-amino-1-pentanol concentrations. Reactions were carried out at 55°C using a fixed concentration of myristic acid (175 mM) and 5 g l^{-1} of Novozym® 435 in 2 ml of *tert*-amyl alcohol. The data represent the averages of triplicate runs whose standard deviations were always lower than 15%.

As shown on Fig. 1, which describes the acylation of 4-amino-1-pentanol **4** in *tert*-amyl alcohol, a decrease in the initial rates of myristic acid **1** conversion, *O*-acylation and *N*-acylation, occurred when amino-alcohol was in excess (≥ 175 mM). This phenomenon was

also noticed on the acylation kinetic profile of alaninol **2** and 6-amino-1-hexanol **6**, in both organic solvents and ionic liquid and could be attributed to an inhibitor effect similar to excess substrate inhibition. However, it was not observed in a previous work in which we performed the *O*-acylation of 2-butanol starting from myristic acid as an acyl donor under similar experimental conditions [60]. Thus, the decrease in initial rates is most likely due to an interaction between myristic acid **1** and the amino group of the amino-alcohol. Indeed, the presence of an amino substrate and a fatty acid substrate in an organic solvent generally leads to the formation of an ion-pair complex between both substrates, depending on the acido-basic conditions of the medium [46, 53, 59]. This salt complex makes the ion forms of both substrates non reactive (NH_3^+ amine form and COO^- fatty acid form) and therefore leads to the overestimation of the substrate concentrations that are actually available to the enzyme in the reaction medium. Maugard et al. [53] previously described this ion-pair complex as a limiting factor in lipase-catalyzed acylation, under conditions where it is less soluble than free substrates. To verify this hypothesis, the composition of the medium, in particular the carbonyl species was analyzed by IR spectroscopy at the start of the reaction using various concentrations of amino-alcohol **2** in *tert*-amyl alcohol as a reaction solvent. When myristic acid **1** alone was totally solubilized, only one carbonyl band was observed at 1710 cm^{-1} (Table 2, entry 1), corresponding to the acid form. When the concentration of amino-alcohol **2** was increased in the media, a decrease in the area of the carbonyl acid band (1710 cm^{-1}) was concomitant with an increase in the area of the carboxylate band (1562 cm^{-1}) (Table 2, entries 2 to 5). This demonstrates the formation of an ion-pair complex between substrates **1** and **2**.

Table 2

Quantification of IR spectrum bands of mixtures containing 175 mM of myristic acid **1** and various concentrations of alaninol **2** in *tert*-amyl alcohol.

Entry	[Alaninol 2]	IR band

	(mM)	1710 cm^{-1} (-COOH) (Area in %)	1562 cm^{-1} ($-COO^-$) (Area in %)
1	0	100	0
2	50	64	36
3	100	26	74
4	175	7	93
5	250	5	95

Due to the inhibitor effect of the ion-pair complex at high substrate concentrations, the apparent kinetic parameters ($k_{cat,app}$ and $K_{m,app}$) were obtained from Lineweaver-Burk reciprocal plots corresponding to amino-alcohols concentrations lower than 175 mM. Indeed, no decrease in initial rates and de facto no inhibition due to the ion-pair complex formation was observed for these amino-alcohol concentrations. Thus, for amino-alcohol **4** acylation in *tert*-amyl alcohol (Fig. 1), the $k_{cat,app}$ values were 86 min^{-1} for *O*-acylation (Fig. 1B) and 13 min^{-1} for *N*-acylation (Fig. 1C) and 99 min^{-1} for myristic acid **1** conversion (Fig. 1A) and the $K_{m,app}$ value of amino-alcohol **4** acylation was 75 mM (Fig. 1A). The apparent kinetic parameters, the chemoselectivity ratio (C), as well as the log P values of amino-alcohols, are summarized in Table 3. The log P value is defined as the logarithm of the partition coefficient of a given compound in a standard octanol/water biphasic system [61]. This parameter characterizes the hydrophobicity of a compound: the higher the log P value, the more hydrophobic the compound [62, 63].

Table 3

Apparent kinetic parameters and chemoselectivity ratios of the Novozym® 435 catalyzed acylation of amino-alcohols **2**, **4** and **6** carried out at 55°C in *n*-hexane, *tert*-amyl alcohol or Bmim [PF_6] as a reaction solvent, using 175 mM of myristic acid **1** as an acyl donor.

Entry	Solvent	Amino-alcohol	$k_{cat,app}$ of myristic acid 1 conversion (min^{-1})	$k_{cat,app}$ of *O*-acylation (min^{-1})	$k_{cat,app}$ of *N*-acylation (min^{-1})	$K_{m,app}$ (mM)	C [c]

1	*n*-hexane		357	14	114	18.7	0.12
2	*tert*-amyl alcohol	**(±)-**alaninol **2** (log P -0.96)	58	nd[a]	51	182	<0.001
3	Bmim [PF_6]		33	2	27	69.7	0.07
4	*n*-hexane		241	231	0[b]	35.3	>1000
5	*tert*-amyl alcohol	**(±)-**4-amino-1-pentanol **4** (log P -0.43)	99	86	13	75	6.61
6	Bmim [PF_6]		38	27	0[b]	72.2	>1000
7	*n*-hexane		551	539	1	53	539
8	*tert*-amyl alcohol	6-amino-1-hexanol **6** (log P -0.01)	136	126	12	63	10,5
9	Bmim [PF_6]		44	41	0[b]	45	>1000

[a] *Not detected.*
[b] *The acylated product was detected at a concentration too low to be quantified.*
[c] *The chemoselectivity ratio calculated via the apparent catalytic constant ratio (Eq. (2)).*

Table 3 provides a number of key information. In order to facilitate the comparison of the parameters described in this table, the effect of solvent nature on the $K_{m,app}$, $k_{cat,app}$ and chemoselectivity is discussed separately.

- *Effect on the $K_{m,app}$*

The $K_{m,app}$ of amino-alcohol **2** in *tert*-amyl alcohol (182 mM) was 2- and 2.5-fold higher than the $K_{m,app}$ of long chain amino-alcohols **4** (75 mM) and **6** (63 mM), respectively (Table 3, entries 2, 5 and 8). This indicates a lower affinity of Novozym® 435 for short chain amino-alcohol **2**. The same phenomenon is observed using Bmim [PF_6] (Table 3, entries 3, 6 and 9). In contrast, the $K_{m,app}$ of short chain amino-alcohol **2** in *n*-hexane (18.7 mM) was 1.5- and 2-fold lower than the $K_{m,app}$ of long chain amino-alcohols **4** (35.3 mM) and **6** (53 mM), respectively (Table 3, entries 1, 4 and 7), which indicates a switch in the affinity of Novozym® 435 in favour of the short chain amino-alcohol **2**.

To date, a clear consensus has not yet emerged on the parameters to quantitatively describe solvents and their influence on enzymatic reactions. However, it is well known that solvent

hydrophobicity is a key factor, whose influence can be evaluated using the log P value [62, 64-65]. The solvent effect can be analysed using the probable relationship between enzyme activity and substrate solvation. In a hydrophilic solvent such as *tert*-amyl alcohol (log P 0.89) or Bmim [PF_6] (log P -2.36), the solvation of the highly hydrophilic amino-alcohol **2** (log P – 0.96) is favoured to the detriment of its availability for the enzyme. Thus, the affinity of the lipase for amino-alcohol **2** is lower than its affinity for other amino-alcohols in hydrophilic solvents, resulting in a higher $K_{m,app}$ value (Table 3). In contrast, in a hydrophobic solvent such as *n*-hexane (log P 3.90), the solvation of an amino-alcohol is favoured when its hydrophilicity decreases, the solvation of the more hydrophilic amino-alcohol **2** is therefore not favoured and this benefits its availability for the enzyme. Thus, the affinity of the lipase for amino-alcohol **2** is higher than its affinity for other amino-alcohols in hydrophobic solvents, resulting in a lower $K_{m,app}$ value (Table 3). In addition, Novozym® 435 consisting in *Candida antarctica* lipase B immobilized on a positively charged acrylic polar resin, solvation of the biocatalyst would not be favoured in a hydrophobic solvent: as a concequence, this promote biocatalyst availability for polar substrates such as amino-alcohols in *n*-hexane, resulting in a lower $K_{m,app}$ value. These results are in accordance with several studies that have demonstrated that the use of various supports (polar or apolar) allows to modulate activity and selectivity of a particular immobilized lipase used in various solvents [17].

- *Effect on the $k_{cat,app}$ of myristic acid conversion*

In this work, the $k_{cat,app}$ of myristic acid conversion is a representative parameter of the biocatalyst activity for the amino-alcohol acylation. Indeed, the $k_{cat,app}$ of myristic acid conversion corresponds to the sum of $k_{cat,app}$ of mono-*N*-acylation, mono-*O*-acylation and amido-ester synthesis.

n-Hexane was the most effective reaction solvent, in terms of $k_{cat,app}$ of myristic acid **1** conversion, to enhance the activity of Novozym® 435, regardless of the three amino-alcohols

411 tested. Catalytic activity was lowest using Bmim [PF_6]. The $k_{cat,app}$ of myristic acid **1**
412 conversion from the acylation of amino-alcohol **2** in *n*-hexane (357 min^{-1}) was 6- and 11-fold
413 higher than the $k_{cat,app}$ of myristic acid **1** conversion in *tert*-amyl alcohol (58 min^{-1}) and in
414 Bmim [PF_6] (33 min^{-1}), respectively (Table 3, entries 1 to 3). These results are not surprising
415 given that *tert*-amyl alcohol and Bmim [PF_6] are the most polar of the three solvents used.
416 Indeed, the polarity of solvents is well known to dramatically influence the catalytic activity
417 of enzymes [64-69]. Extremely or intermediary hydrophilic polar solvents, such as *tert*-amyl
418 alcohol, interact with the absolute amount of water, indispensable in the acquisition and the
419 maintenance of enzyme conformation. These solvents strip off water molecules from the
420 enzyme, resulting in either a change in the conformation and flexibility of the enzyme or a
421 negative effect on the transition state stability and, thus, on catalytic activity. Apart from their
422 potential interactions with water molecules, hydrophilic polar solvents also interact with the
423 secondary structure of the enzyme via multiple hydrogen bonds and via other strong
424 interactions. This can lead to an alteration in the protein conformation and thus a negative
425 effect on catalytic activity.

426 However, the low $k_{cat,app}$ of myristic acid **1** conversion obtained in Bmim [PF_6] (Table 3,
427 entries 3, 6 and 9) can also be explained by limiting factors such as diffusion and mass-
428 transfer limitations associated with the high viscosity of this ionic liquid [70, 71]. Moreover,
429 anions such as [PF_6] can strongly interact with the positively charged sites in the *Candida*
430 *antarctica* lipase B structure, potentially causing conformational changes [72]. In addition, it
431 must be taken into consideration that ionic liquids form ion-pairs that can interact with both
432 the carboxyl group of fatty acid and amino groups of amino-alcohols. On a one hand, these
433 interactions could modulate the ionization state of the substrates and the enzyme and thus
434 affect catalytic activity. On the other hand, they may stabilize substrate ionic form and
435 promote as a result the formation of an ion-pair complex between both substrates. This would

cause higher limitation of substrate availability in Bmim [PF_6] than in *n*-hexane and *tert*-amyl alcohol.

- *Effect on the chemoselectivity*

The chemoselectivity of the Novozym® 435-catalyzed acylation of the three amino-alcohols was evaluated using the chemoselectivity ratio (C) (Eq. (2)). The acylation of amino-alcohol **2** in *n*-hexane and Bmim [PF_6] gave similar C values, close to 0.1 (Table 3, entries 1 and 3), as no ester **3b** was detected in *tert*-amyl alcohol (Table 3, entry 2). In contrast, C values of 539 and 10.5 were obtained for the acylation of amino-alcohol **6** in *n*-hexane and *tert*-amyl alcohol, respectively (Table 3, entries 7 and 8), when only trace amounts of amide **7a** were detected in Bmim [PF_6] (Table 3, entry 9) (the experimental detection limit by LC-MS analysis was 2 μM). Similarly, C value of 6.61 was observed for the acylation of amino-alcohol **4** in *tert*-amyl alcohol (Table 3, entry 5) while amide **5a** was detected as trace amounts not only in Bmim [PF6] but also in *n*-hexane (Table 3, entries 4 and 6). According to these data, Novozym® 435 shows higher selectivity towards *N*-acylation of amino-alcohol **2**, which has two carbons between the alcohol and amino groups. Moreover, we can also conclude that Novozym® 435 is more chemoselective for *O*-acylation of substrates **4** and **6**, which are long chain amino-alcohols exhibiting four and six carbons between their alcohol and amino groups, respectively.

In addition, the fact that the higher $k_{cat,\ app}$ of *O*-acylation was obtained with amino-alcohol **6** (Table 3, entries 7 to 9) in comparison with amino-alcohol **4** (Table 3, entries 4 to 6) can be explained not only by the increase in the carbon chain length but also by the fact that amino-alcohol **6** is a primary alcohol while amino-alcohol **4** is a secondary one. Indeed, it has been widely demonstrated in the litterature that lipase-catalyzed acylation of primary alcohols generaly occurs with a higher catalytic activity than the acylation of secondary alcohols [73-75].

3.2. Effect of the solvent and of the amino-alcohol concentration on the production of acylated amino-alcohols catalyzed by Novozym® 435

- *Time-course of alaninol acylation in tert-amyl alcohol, n-hexane and Bmim [PF6]*

Prior to study the Novozym® 435-catalyzed acylation of amino-alcohols **2**, **4** and **6** in terms of production at a fixed time, the reaction was carried out in *n*-hexane, *tert*-amyl-alcohol and Bmim [PF_6], using 35 mM amino-alcohol and 175 mM myristic acid **1**, and monitored over time.

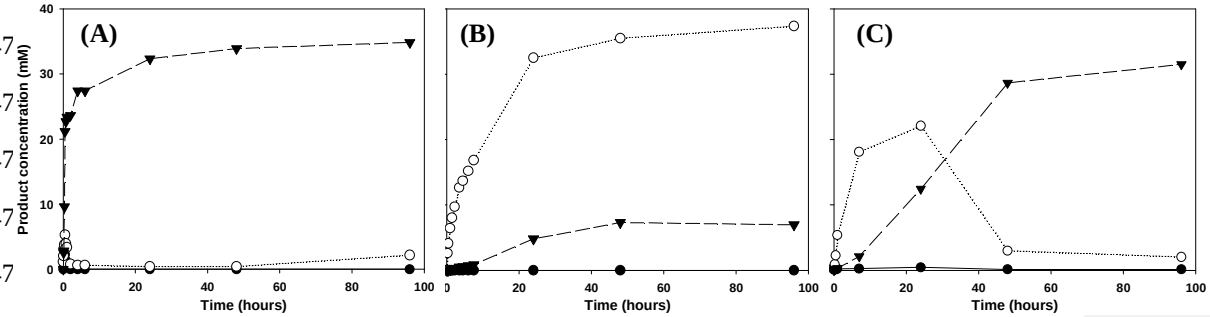


Fig. 2. Enzymatic acylation of alaninol **2** (35 mM) by myristic acid **1** (175 mM) in *n*-hexane (A), *tert*-amyl alcohol (B) and Bmim [PF_6] (C): ester (●), amide (○) and amido-ester (▼). Reactions were carried out at 55°C using 5 g l^{-1} of Novozym® 435.

Fig. 2 shows the time-course of alaninol **2** acylation. During this reaction conducted in Bmim [PF_6] (Fig. 2C), ester **3a** was detected only as trace amounts. On the other hand, amide **3a** was the main product synthezised in *tert*-amyl alcohol at a stationary state reached after 96 hours with a yield close to 100% (based on starting alaninol **2**) (Fig. 2B), whereas it was predominant only over the first 30 minutes and 24 hours of reactions effected in *n*-hexane and Bmim [PF_6], respectively (Fig. 2 A and C). After these times, amide **3a** was then consumed to the benefit of amido-ester **3c** synthesis, which became the main product after 96 hours with

yields close to 99% and 90% in *n*-hexane and Bmim [PF_6], respectively (based on starting alaninol **2**). Interestingly, this stationary phase was also reached after 96 hours for reactions involving amino-alcohols **4** and **6**, whatever the solvent (data not shown). On the other hand, the time-course of the increase in amido-ester concentration was clearly concomitant with the decrease in amide concentration.

These results suggest that the synthesis of the amido-ester proceeds in two steps which might follow two hypothetic sequences:

- (1) either the *N*-acylation of alaninol **2** occurs first and is then followed by the *O*-acylation of the amide synthesized in the first step;
- (2) or the *O*-acylation of alaninol **2** occurs first and is then followed by the *N*-acylation of the ester synthesized in the first step. This second hypothesis would, however, necessarily imply a reason to make the ester undetectable all along the reaction time-course. Two possibilities would explain this: either the rate of ester *N*-acylation is at least as fast as the rate of alaninol **2** *O*-acylation, or the ester is transacylated into the amide via a kinetically controlled process involving the formation of the acyl enzyme from the ester that would be released by the attack of the amine, as reported by Volker Kasche works to describe the mechanism of amidase–catalyzed amine acylation using esters as acyl donors [76].

Moreover, we focused in a previous work on the kinetic analysis of Novozym® 435-catalyzed acylation of a mono-alcohol and a mono-amine structurally related to alaninol **2** carried out in *tert*-amyl alcohol under similar experimental conditions. We demonstrated then that (R)-2-butanol *O*-acylation rate was 22-fold faster than (R)-*sec*-butylamine *N*-acylation rate [60]. On this basis, we would expect the ester intermediate to accumulate in the reaction medium

510 before being converted into the amido-ester, which is, however, not the case: this ester
511 intermediate is detected at best as trace amounts whatever the reaction time or the solvent
512 tested. In addition, we have also shown that the *N*-acylation rates obtained for the enzymatic
513 acylation of bifunctionnal compounds such as 1-methoxy-2-propylamine and 1,2-
514 diaminopropane, catalyzed by Novozym® 435 in *tert*-amyl alcohol at 55°C, were 8- and 38-
515 fold higher, respectively, than the *N*-acylation rate of the related mono-amine (*sec*-
516 butylamine) [77]. These bifunctionnal compounds are structurally related to alaninol **2** but
517 exhibit no alcohol group in their structure and thus cannot possibly be *O*-acylated. For this
518 reason, we formulated the following postulate for amino-alcohol acylation: the presence of an
519 alcohol group in β-position of the amino group of the acyl acceptor would result in the
520 enhancement in the rate of *N*-acylation. This may be due to the formation of an intramolecular
521 interaction between the amino group and the alcohol group located in β-position, which is
522 strengthened by the fact that this interaction could not occur for amino-alcohols **4** and **6**,
523 considering the longer distance between both functional groups, giving as a result low rates of
524 *N*-acylation. In order to confirm and develop this new postulate, molecular modelling studies
525 were conducted to precisely understand the Novozym® 435-catalyzed *N*-acylation mechanism
526 using a methoxy-amine, a di-amine, a short chain amino-alcohol or a long chain amino-
527 alcohol as an acyl acceptor. These studies recently led to propose a new proton shuttle
528 reaction mechanism to explain chemoselectivity for Novozym® 435-catalyzed *N*-acylation of
529 alaninol **2** and 4-amino-1-pentanol **4** [26]. As a consequence, all these results seem to rule out
530 the hypothetic sequence 2 and suggest that the amide is a better substrate than the amino-
531 alcohol for enzymatic *O*-acylation, especially when using *n*-hexane: the highest amido-ester
532 **3c** synthesis rate of 7 $mmol.h^{-1}.g^{-1}$ was obtained in this solvent.

533 Finally, to identify optimal, highly selective conditions required to produce monoacylated and
534 diacylated compounds, the influences of the amino-alcohol concentration and substrate molar

ratio **r** ([myristic acid **1**]/[amino-alcohol]) were investigated. All experiments were performed using the same conditions as in section 3.1. Figs. 3, 4 and 5 show the product distribution in *n*-hexane, *tert*-amyl alcohol and Bmim [PF_6] solvents, respectively, after 96 hours of Novozym® 435-catalyzed acylation of amino-alcohols **2**, **4** and **6**. This reaction time was chosen to reach a stationary phase for the production of acylated amino-alcohols under all experimental conditions such as previously described.

- *Production of acylated amino-alcohols in n-hexane*

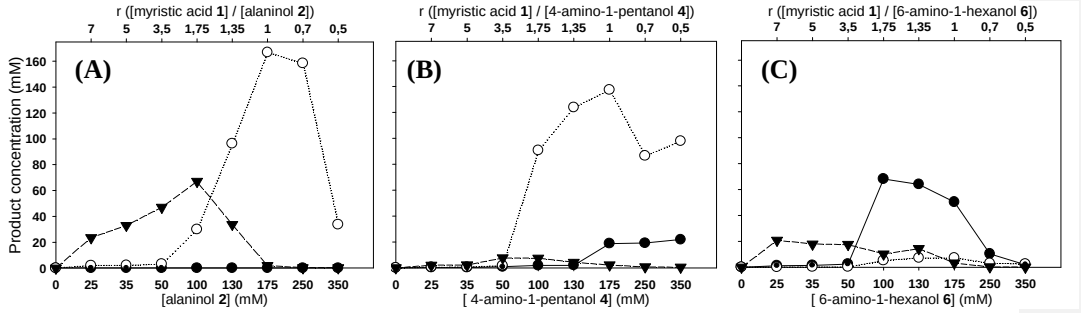


Fig. 3. Effect of amino-alcohol concentration and substrate molar ratio **r** on the enzymatic production of acylated amino-alcohols in *n*-hexane, at 96 hours: ester (●), amide (○) and amido-ester (▼). Reactions were carried out at 55°C using amino-alcohols **2** (A), **4** (B) or **6** (C), with a fixed concentration of myristic acid **1** (175 mM) and 5 g l^{-1} of Novozym® 435.

The data in Fig. 3 demonstrate that the use of *n*-hexane gives two major products, depending on the **r**-value. At initial amino-alcohol concentrations inferior or equal to 100 mM (**r** ≥ 1.75), the selective production of amido-esters **3c**, **5c** or **7c** is improved, up to 49 mM for amido-ester **3c** production (56% yield based on starting myristic acid **1**). Amino-alcohol concentrations superior to 100 mM (**r** < 1.75) enhances the production of monoacylated compounds. The synthesis of amides from amino-alcohols **2** and **4** reached 166 mM for amide **3a** and 138 mM for amide **5a** (95% and 79% yields based on starting myristic acid **1**) under

556 stoichiometric conditions (Fig. 3A and 3B). Moreover, ester **3b** was not detected and ester **5b**
557 was negligible. Hence, stoichiometric conditions are the optimal conditions for acylation of
558 amino-alcohols **2** and **4**, and favour both the synthesis yield and the chemoselectivity of amide
559 production. On the other hand, the production of ester **7b** from the longest amino-alcohol **6** (6
560 carbons) (Fig. 3C) was improved and reached up to 68 mM (39% yield based on starting
561 myristic acid **1**) using 100 mM starting amino-alcohol **6**. Again, this change in the
562 chemoselectivity of Novozym® 435 at 96 hours, which is shown to be dependent on the
563 carbon chain length between the alcohol and amino groups of the amino-alcohol, was in
564 accordance with its chemoselectivity under initial velocity conditions, previously described in
565 section 3.1. An interesting result of this study, which should be highlighted, is the decrease in
566 the global conversion yield observed when a large excess (350 mM) of amino-alcohol (**r** <
567 0.7) is used. This is true for all amino-alcohols tested. The decrease could be explained by the
568 formation of a non-reactive ion-pair complex between myristic acid **1** and the amino-alcohol
569 as also described in section 3.1. Other studies have already demonstrated that the use of non-
570 polar solvents such as *n*-hexane promotes and stabilizes the formation of an ion-pair complex
571 because polar amino-alcohols are slightly solubilized while ion-pair complex forms of amino-
572 alcohols are highly solubilized in these solvents [46]. This is due to the carboxylic acid
573 function playing the role of phase transfer agent for the amino-alcohol, which improves its
574 solubility. The formation of this complex would indeed drastically affect the availability of
575 both substrates for the enzyme, especially in excess of the amino-alcohol.

576 • *Production of acylated amino-alcohols in tert-amyl alcohol*
577

578

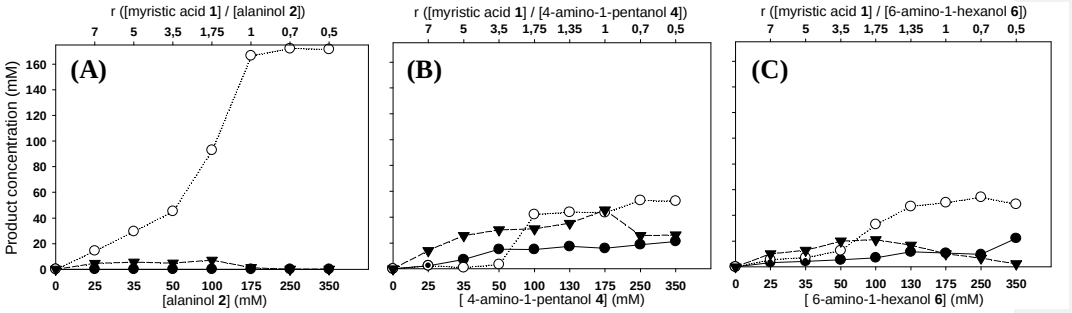


Fig. 4. Effect of amino-alcohol concentration and substrate molar ratio **r** on the enzymatic production of acylated amino-alcohols in *tert*-amyl alcohol, at 96 hours: ester (●), amide (○) and amido-ester (▼). Reactions were carried out at 55°C using amino-alcohols **2** (A), **4** (B) or **6** (C), with a fixed concentration of myristic acid **1** (175 mM) and 5 g l^{-1} of Novozym® 435.

Similar experiments were performed using *tert*-amyl alcohol as a polar protic solvent (Fig. 4). The acylation of amino-alcohol **2** in *tert*-amyl alcohol (Fig. 4A) leads predominantly to the production of amide **3a** and was enhanced by an increase in amino-alcohol **2** concentration, reaching a maximum value of 172 mM (98% yield based on starting myristic acid **1**) for initial amino-alcohol **2** concentrations superior or equal to 250 mM. However, ester **3b** was not detected and the production of amido-ester **3c** was negligible. Acylation of amino-alcohols **4** and **6** (Fig. 4B and 4C) studies were also performed: esters **5b** and **7b** were detected and quantified although their production remained low. On the other hand, the production of amido-esters **5c** and **7c** were predominant and reached maxima of 30 mM and 20 mM (34% and 23% yields based on starting myristic acid **1**), respectively, for concentrations in long chain amino-alcohol lower than 50 mM (**r** > 3.5). The production of amides **5a** and **7a** was improved for concentrations higher than 50 mM (**r** < 3.5). Under the best conditions used (250 mM starting amino-alcohol **4** or **6**), production close to 53 mM of amides **5a** or **7a** (30% yield based on starting myristic acid **1**) was reached.

On the other hand, in contrast to the results obtained in *n*-hexane, no decrease of global yield was observed in *tert*-amyl alcohol when a large excess (350 mM) of amino-alcohol (r < 0.7) was used. This could be explained by the likely complete solubilization of substrates in this solvent which would make the ion-pair complex formation less favoured and less stable than in *n*-hexane. Both substrates would be therefore more available for the enzymatic reaction than in *n*-hexane.

In summary, amide production at 96 hours is improved in *tert*-amyl alcohol for all amino-alcohols tested, although acylation catalyzed by Novozym® 435 of amino-alcohols exhibits higher $k_{cat,app}$ of *O*-acylation than $k_{cat,app}$ of *N*-acylation as described in section 3.1. These results suggest that Novozym® 435 favours the synthesis of the amide as a thermodynamic product whereas the ester, which is preferentially synthesized under initial velocity conditions, can be considered as a kinetic product.

- *Production of acylated amino-alcohols in Bmim [PF_6]*

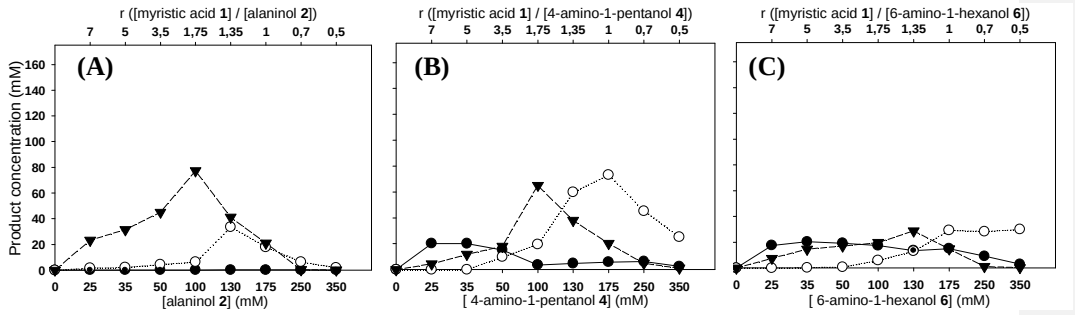


Fig. 5. Effect of amino-alcohol concentration and substrate molar ratio **r** on the enzymatic production of acylated amino-alcohols in Bmim [PF_6], at 96 hours: ester (●), amide (○) and amido-ester (▼). Reactions were carried out at 55°C using amino-alcohols **2** (A), **4** (B) or **6** (C), with a fixed concentration of myristic acid **1** (175 mM) and 5 g l^{-1} of Novozym® 435.

Finally, Bmim [PF_6] was tested as a solvent (Fig. 5). The use of 100 mM amino-alcohol (low excess of myristic acid **1**; **r** = 1.75) in Bmim [PF_6] gave the highest amido-ester production of all our experiments, reaching 77, 65 and 20 mM of amido-esters **3c**, **5c** and **7c** (88%, 74% and 23% yields based on starting myristic acid **1**), respectively. However, when amino-alcohol concentration was higher than 130 mM (**r** < 1.35) a sharp decrease in amido-ester production was observed. In contrast, the production of esters **5b** and **7b** (Fig. 5B and 5C) never exceeded 20 mM (11% yield based on starting myristic acid **1**) and the production of amides was enhanced when amino-alcohol concentration increased from 100 to 175 mM (1.75 > **r** > 1), reaching up to 74 mM of amide **5a** (42% yield based on starting myristic acid **1**) under stoichiometric conditions.

4. Conclusion

In this work, we firstly evaluated the effect of reaction solvent on the activity and chemoselectivity of Novozym® 435 (immobilized *Candida antarctica* lipase B) for the acylation of three amino-alcohols (alaninol **2**, 4-amino-1-pentanol **4** and 6-amino-1-hexanol **6**) using myristic acid **1** as an acyl donor. Our results suggest that the nature of the solvent affects both the availability of substrates and the catalytic activity of the enzyme. In contrast, we also found that the chemoselectivity of Novozym® 435 under initial velocity conditions is mainly affected by amino-alcohol structure rather than by the reaction solvent. In particular, we have shown that the $k_{cat,app}$ of *N*-acylation is improved when using the short chain amino-alcohol alaninol **2** whereas the $k_{cat,app}$ of *O*-acylation is improved when using the longer chain amino-alcohols 4-amino-1-pentanol **4** and 6-amino-1-hexanol **6**. This demonstrates the strong influence of substrate structure on the chemoselectivity of Novozym® 435, and provides new insights into the selective synthesis of amides or esters produced from the acylation of bifunctional substrates.

644 Furthermore, we also investigated the impact of amino-alcohol structure, solvent nature and
645 substrate molar ratio on enzymatic production obtained at a stationary state after 96 hours of
646 reaction. From a general point of view, our work clearly demonstrates clearly that the
647 production of acylated amino-alcohols catalyzed by Novozym® 435 after 96 hours is
648 markedly influenced by three main parameters:

649 • Reaction solvent: *tert*-amyl alcohol improves the production of amides **3a**, **5a** and **7a**
650 while Bmim [PF_6] favours the formation of amido-esters **3a** and **5a**.
651 • [myristic acid **1**]/[amino-alcohol] molar ratio (**r**): under stoichiometric conditions and
652 in excess of the amino-alcohol (**r** ≤ 1) in *tert*-amyl alcohol and *n*-hexane, the
653 production of monoacylated amino-alcohols is improved in most cases. On the other
654 hand, a large excess of myristic acid **1** (**r** > 1) enhances amido-ester production in *n*-
655 hexane and Bmim [PF_6].
656 • Amino-alcohol structure: chemoselective production of monoacylated amino-alcohols
657 is markedly affected by the amino-alcohol structure. Using *n*-hexane and in excess of
658 amino-alcohol, amide production from alaninol **2** and ester production from the longer
659 chain amino-alcohol 6-amino-1-hexanol **6** were favoured.

660 Together with our previously published work [60, 77], these data give a clearer understanding
661 of the parameters affecting the Novozym® 435-catalyzed acylation of amino-alcohols, which
662 is important in order to maximize both the yields and the chemoselectivity of these reactions.
663 In addition, *n*-hexane and *tert*-amyl alcohol used as solvents in this work can potentially be
664 replaced by other organic solvents with similar properties but considered as more
665 environmentally friendly, such as for example: cyclopentane and *tert*-butanol.

666 As prospect, it is also conceivable to test the influence of other parameters to modulate lipase
667 properties, for example the use of various supports to immobilize *Candida antarctica* lipase

668 B. Indeed, the use of various immobilization protocols to produce a biocatalyst starting from a
669 particular lipase has been shown to significantly affecting its catalytic activity and selectivity,
670 depending on the solvent used [28-29]. On the other hand, it would also be interesting to test
671 other lipases as biocatalysts, such as for instance *Rhizomucor miehei* lipase which has already
672 been used to acylate amino-alcohol substrates [5].

673

Acknowledgments

This study was supported by the Centre National de la Recherche Scientifique and the French ANR (National Research Agency) through the EXPENANTIO project (CP2P program: Chimie et Procédés pour le Développement Durable).

679 **References**

680 [1] M. Bakke, M. Takizawa, T. Sugai, H. Ohta, P. Herold, J. Org. Chem. 63 (1998) 6929-
681 6938.

682 [2] H. Garg, N. Francella, K.A. Tony, L.A. Augustine, J.J. Jr Barchi, J. Fantini, A. Puri,
683 D.R. Mootoo, R. Blumenthal, Antiviral. Res. 80 (2008) 54-61.

684 [3] M. Fillet, M. Bentires-Alj, V. Deregowski, R. Greimers, J. Gielen, J. Piette, V. Bours,
685 M.P. Merville, Biochem. Pharmacol. 65 (2003) 1633-1642.

686 [4] J.F. Molina, Household and personal care today 2 (2008) 12-15.

687 [5] L. Lassalle, F. Yvergnaux, FR Patent 2,855,823 (2004).

688 [6] E.L. Soo, A.B. Salleh, M. Basri, R.N.Z.R.A. Rahman, K. Kamaruddin, J. Biosci.
689 Bioeng. 95 (2003) 361-367.

690 [7] A. Singh, J.D. Van Hamme, O.P. Ward, Biotech. Adv. 25 (2007) 99-121.

691 [8] T. Maugard, M. Remaud-Simeon, P. Petre, P. Monsan, Tetrahedron 53 (1997) 5185-
692 5194.

693 [9] K. Fukunaga, M. Yoshida, F. Nakajima, R. Uematsu, M. Hara, S. Inoue, H. Kondo, S.
694 I. Nishimura, Bioorg. Med. Chem. Lett. 13 (2003) 813-815.

695 [10] J. Yin, H. Liu, C. Pidgeon, Bioorg. Med. Chem. Lett. 8 (1998) 179-182.

696 [11] J. Tao, J. H. Xu, Curr. Opin. Chem. Biol. 13 (2009) 43-50.

697 [12] B.M. Nestl, B.A. Nebel, B. Hauer, Curr. Opin. Chem. Biol. 15 (2011) 187-193.

698 [13] F. Björkling, S.E. Godtfredsen, O. Kirk, Trends Biotechnol. 9 (1991) 360-363.

699 [14] K.E. Jaeger, S. Ransac, B. Dijkstra, C. Colson, M. Van Heuvel, O. Misset, Federation
700 of European Microbiological Societies Microbiology Reviews 15 (1994) 29-63.

701 [15] D. Sharma, B. Sharma, A.K. Shukla, Biotechnol. 10 (2011) 23-40.

702 [16] H. Ogino, H. Ishikawa, J. Biosci. Bioeng. 91 (2001) 109-116.

703 [17] E.P. Hudson, R.K. Eppler, D.S. Clark, Curr. Opin. Biotechnol. 16 (2005) 637-643.

704 [18] N. Doukyu, H. Ogino, Biochem. Eng. J. 48 (2010) 270-282.

705 [19] E.M. Anderson, K.M. Larsson, O. Kirk, Biocatal. Biotransform. 16 (1998) 181-204.

706 [20] R. Sharma, Y. Chisti, U. Chand Banerjee, Biotechnol. Adv. 19 (2001) 627-662.

707 [21] R.N. Patel, J. Liposome Res. 11 (2001) 355-393.

708 [22] R. Aravindan, P. Anbumathi, T. Viruthagiri, Indian Journal of Biotechnology 6 (2007)
709 141-158.

710 [23] L. Alberghina, M. Lotti, Chem. Phys. Lipids 93 (1998) 1-216.

711 [24] T. Ema, Curr. Org. Chem. 8 (2004) 1009-1025.

712 [25] L. Chebil, C. Humeau, A. Falcimaigne, J-M. Engasser, M. Ghoul, Process Biochem.
713 41 (2006) 2237-2251.

714 [26] P.O. Syren, F. Le Joubioux, Y. Ben Henda, T. Maugard, K. Hult, M. Graber,
715 ChemCatChem 5 (2013), doi: 10.1002/cctc.201200751.

716 [27] A.M. Gumel, M.S.M. Annuar, T. Heidelberg, Y. Chisti, Process Biochem. 46 (2011)
717 2079-2090.

718 [28] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente,
719 Enzyme and Microbial Technol. 40 (2007) 1451-1463.

720 [29] O. Barbosa, C. Ortiz, R. Torres, R. Fernandez-Lafuente, J. Mol. Catal. B: Enzym. 71
721 (2011), 124-132.

722 [30] F. Van Rantwijk, R.M. Lau, R.A. Sheldon, Trends Biotechnol. 21 (2003) 131–138.

723 [31] P. Lozano, T. De Diego, D. Carrie, M. Vaultier, J.L. Iborra, 23 (2001) 1529–1533.

724 [32] B. Fu, P.T. Vasudevan, Energ. Fuel. 24 (2010) 4646-4651.

725 [33] M. Graber, V. Leonard, Z. Marton, C. Cusatis, S. Lamare, J. Mol. Catal. B: Enzym.
726 52-53 (2008) 121-127.

727 [34] V. Leonard, S. Lamare, M.D. Legoy, M. Graber, J. Mol. Catal. B: Enzym. 32 (2004)
728 53-59.

729 [35] Z.Q. Duan, W. Du, D.H. Liu, Bioresource Technol. 102 (2011) 11048-11050.

730 [36] R. Irimescu, K. Kato, J. Mol. Catal. B: Enzym. 30 (2004) 189–94.

731 [37] R. Madeira Lau, F. Van Rantwijk, K.R. Seddon, R.A. Sheldon, Org. Lett. 2 (2000)
732 4189–4191.

733 [38] F.J. Hernández-Fernández, A.P. De Los Ríos, L.J. Lozano-Blanco, C. Godínez, J.
734 Chem. Technol. Biot. 85 (2010) 1423-1435.

735 [39] P. Lozano, T De Diego, D. Carrie, M. Vaultier, J.L. Iborra, J. Mol. Catal. B: Enzym.
736 21 (2003) 9–13.

737 [40] R.A. Sheldon, R. Madeira Lau, M.J. Sorgedrager, F. Van Rantwijk, K.R. Seddon,
738 Green Chem. 4 (2002) 147–151.

739 [41] C. Roosen, P. Müller, L. Greiner, L. Appl. Microbiol. Biotechnol. 81 (2008) 607-614.

740 [42] V. Gotor-Fernandez, V. Gotor, Curr. Org. Chem. 10 (2006) 1125–1143.

741 [43] N. Jain, A. Kumar, S. Chauhan, S.M.S. Chauhan, Tetrahedron 61 (2005) 1015–1060.

742 [44] C. Pilissão, P. De O. Carvalho, G. Da, M. Nascimento, J. Brazil. Chem. Soc. 21 (2010)
743 973-977.

744 [45] M. Fernandez-Perez, C. Otero, Enzyme Microb. Technol. 33(2003) 650–660.

745 [46] M. Fernandez-Perez, C. Otero, Enzyme Microb. Technol. 28 (2001) 527–536.

746 [47] V. Gotor, R. Brieva, F. Rebolledo, J. Chem. Soc., Chem. Commun. (1988) 957-958.

747 [48] T. Furutani, M. Furui, H. Ooshima, J. Kato, Enzyme Microb. Technol. 19 (1996) 578–
748 584.

749 [49] L.T. Kanerva, E. Vanttinen, T.T. Huuhtanen, M. Dahlqvist, Acta chem. Scand. 46
750 (1992) 1101–1105.

751 [50] O. Torre, V. Gotor-Fernandez, V. Gotor, Tetrahedron Asymmetr. 17 (2006) 860–866.

752 [51] S. Tawaki, A.M. Klibanov, Biocatal. Biotransform. 8 (1993) 3-19.

753 [52] J. A. Laszloa, M. Jacksona, R. M. Blancob, J. Mol. Catal. B: Enzym. 69 (2011) 60–65.

754 [53] T. Maugard, M. Remaud-Simeon, D. Petre, P. Monsan, Tetrahedron 53 (1997) 7587-
755 7594.

756 [54] R. Fujiia, Y. Utsunomiyaa, J. Hiratake, A. Sogabeb, K. Sakataa, BBA-Mol. Cell. Biol.
757 L. 1631 (2003) 197–205.

758 [55] J. A. Laszloa, M. Jacksona, R. M. Blancob. J. Mol. Catal. B-Enzym. 69 (2011) 60–65.

759 [56] A. Fersht, Enzyme Structure and Mechanism, second ed., W. H. Freeman and
760 Company, New York, (1985) 98-120.

761 [57] S.J. Nara, J.R. Harjani, M.M. Salunkhe, Tetrahedron Lett. 43 (2002) 2979.

762 [58] K.P. Dhake, Z.S. Qureshi, R.S. Singhal, B.M. Bhanage, Tetrahedron Letters 50 (2009)
763 2811–2814.

764 [59] E. Husson, C. Humeau, F. Blanchard, X. Framboisier, I. Marc, I. Chevalot, J. Mol.
765 Catal. B: Enzym. 55 (2008) 110-117.

766 [60] F. Le Joubioux, O. Achour, N. Bridiau, M. Graber, T. Maugard, J. Mol. Catal. B:
767 Enzym. 70 (2011) 108-113.

768 [61] G. Carrea, G. Ottolina, S. Riva, Trends Biotechnol. 13 (1995) 63-70.

769 [62] S. Hazarika, P. Goswami, N.N. Dutta, A.K. Hazarika, Chem. Eng. J. 85 (2002) 61-68.

770 [63] W.S. He, J.J. Li, X.X. Pan, Y. Zhou, C.S. Jia, X.M. Zhang,Bioresource Technol.
771 (2012) 114: 1-5.

772 [64] C.C. Akoh, L.N. Yee, J. Mol. Catal. B: Enzym. 4 (1998) 149-153.

773 [65] G. De Crescenzo, A. Ducret, M. Trani, R. Lortie, J. Mol. Catal. B: Enzym. 9 (2000)
774 49-56.

775 [66] A. Zaks, A.M. Klibanov, J. Biol. Chem. (1988) 263: 3194.

776 [67] S. Hari Krishna, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001)
777 193-201.

778 [68] P.V. Iyer, L. Ananthanarayan, Process Biochem. 43 (2008) 1019-1032.

779 [69] M. Liaquat, J. Mol. Catal. B: Enzym. 68 (2011) 59-65.

780 [70] P. Hara, U. Hanefeld, L.T. Kanerva, Green Chem. 11 (2009) 250–256.

781 [71] Z. Yang, W. Pan, Enzyme Microb. Technol. 37 (2005) 19–28.

782 [72] J.L. Kaar, A.M. Jesionowski, J.A. Berberich, R. Moulton, A.J. Russell, J. Am. Chem.
783 Soc. 125 (2003) 4125-4131.

784 [73] G. Ljunger, P. Adlercreutz, B. Mattiasson, Biotechnol. Lett. 16 (1994) 1167-1172.

785 [74] P. Ciuffreda, L. Alessandrini, G. Terraneo, E. Santaniello, Tetrahedron Asymmetr.
786 14 (2003) 3197-3201.

787 [75] G.D. Yadav, P.S. Lathi, Enzyme Microb. Tech. 38 (2006) 814-820.

788 [76] V. Kasche, Enzyme Microb. Technol. 8 (1986), 4-16.

Mis en forme : Français (France)

789 [77] F. Le Joubioux, Y. Ben Henda, N. Bridiau, O. Achour, M. Graber, T. Maugard, J.
790 Mol. Catal. B: Enzym. 85-86 (2013) 193-199.

791