

**Kinetic study of 2-butanol O-acylation and  
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1 **Kinetic study of 2-butanol O-acylation and *sec*-butylamine N-acylation catalyzed by**  
2 ***Candida antarctica* lipase B.**

3

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12

13 **Abstract**

14 The aim of this work was to study the differential behavior shown by *Candida antarctica*  
15 lipase B during the O-acylation and N-acylation of monofunctional alcohols and  
16 monofunctional amines. To achieve this, 2-butanol and *sec*-butylamine were used as model  
17 molecules. Yields, kinetics and enantioselectivity were studied for both reactions. Although a  
18 steady-state ordered ternary complex bi-bi mechanism was obtained for the O-acylation of 2-  
19 butanol, a ping-pong bi-bi mechanism was obtained for the N-acylation in case of low *sec*-  
20 butylamine concentrations. The values of apparent kinetics parameters were calculated: the  
21 enantiomeric ratios (E) were evaluated and confirmed the preference of *Candida antarctica*  
22 lipase B for the (R)-enantiomer, which was consistent with the literature. The  
23 enantioselectivity was calculated for the alcohol ( $E \approx 3.17$ ) and for the amine ( $E \approx 1.34$ ).  
24 Concerning the O-acylation, the yields were found to be very similar for both enantiomers R  
25 and S. However, both initial rates and yields of the (R)-enantiomer N-acylation were higher  
26 than those of the (S)-enantiomer. In the last part of our study, the chemoselectivity of *Candida*  
27 *antarctica* lipase B was evaluated, showing that *Candida antarctica* lipase B was a  
28 chemoselective enzyme that preferentially catalyzed the O-acylation to the detriment of the  
29 N-acylation ( $C \approx 92$ , for the selective acylation of (R)-enantiomers). These results provide  
30 new insights for the synthesis of products issued from the selective acylation of  
31 multifunctional substrates such as amino-alcohols.

32

33 **Keywords:** O/N-acylation; *Candida antarctica* lipase B; Kinetic mechanism;  
34 Chemoselectivity; Enantioselectivity.

35

## 36 **1. Introduction**

37 Many molecules such as amino acid esters [1], glucamides [2] or ceramides [3] are derived  
38 from the selective acylation of multifunctional compounds like amino-alcohols. The method  
39 used for the chemical synthesis of these molecules is well established but is confronted to  
40 several limitations. It requires fastidious steps of hydroxyl group protection and deprotection  
41 for the control of chemoselectivity and stereoselectivity. The high temperatures required also  
42 preclude the use of fragile molecules and may cause coloration of final products. In addition,  
43 the coproduction of salts, and the use of toxic solvents (dimethylformamide, methanol, ...)  
44 that must be eliminated at the end of the reaction increase the cost of the processes. For these  
45 reasons, an interesting alternative is the use of biocatalysts which offers a clean way to  
46 perform chemical processes, under mild reaction conditions and with a high degree of  
47 selectivity.

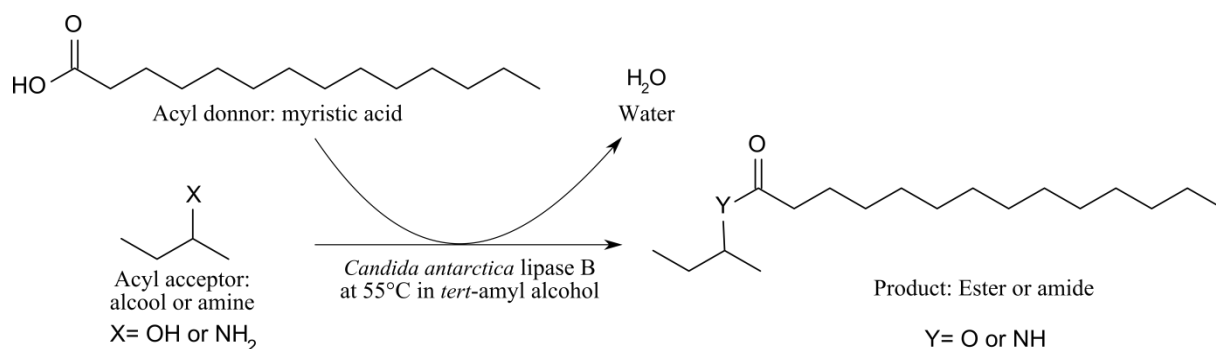
48 Enzymes have been used mostly for aqueous phase reactions. However, non-aqueous  
49 enzymology has potential applications in industry. The use of immobilized enzymes, in  
50 particular lipases, in organic media rather than aqueous media has several advantages such as  
51 the shift in thermodynamic equilibrium in favor of the synthesis over the hydrolysis reaction,  
52 the increased solubility of non-polar substrates, the elimination of side reactions, the ease of  
53 enzyme and product recovery and the increased enzyme thermostability.

54 Lipases can be used to catalyze a wide range of valuable synthesis reactions among which the  
55 acylation of primary alcohols and amines. Many models concerning the lipase-catalyzed  
56 acylation of primary alcohols in organic solvents or solvent-free systems have already been  
57 characterized and shown to kinetically proceed via a ping-pong bi-bi mechanism or  
58 sometimes an ordered bi-bi mechanism [4-6]. In some cases, substrate inhibition was  
59 observed. For instance, an ordered bi-bi mechanism with inhibition by both substrates was  
60 used to model the esterification of cetyl alcohol with oleic acid [7] and a ping-pong bi-bi

61 mechanism implying a competitive inhibition by substrates was described for the  
62 transesterification of isoamyl alcohol with ethyl acetate conducted in *n*-hexane as a solvent  
63 [8]. A ping-pong bi-bi mechanism with inhibition by the amine was also reported for the N-  
64 acylation of ammonia with oleic acid [9]. On the other hand, Arcos *et al.* did not identify any  
65 inhibition step when they proposed a ping-pong bi-bi mechanism to describe the lipase-  
66 catalyzed esterification of glucose with fatty acids [10].

67 Among lipases used in synthesis, *Candida antarctica* lipase B is well known for its ability to  
68 convert alcohols and amines into esters and amides [2,11]. Both alcohols and amines are  
69 nucleophiles that can play the role of acyl acceptor. However there is some difference  
70 between those two chemical groups that affects the behavior of *C. antarctica* lipase B toward  
71 O-acylation and N-acylation. For example, amines are more nucleophilic than alcohols, have  
72 a larger steric hindrance that may interfere with their positioning in the active site, have  
73 ability to realize more hydrogen bonds etc... Thus, understanding the kinetic mechanism and  
74 selectivity of the O-acylation or N-acylation of monofunctional alcohols or amines catalyzed  
75 by *C. antarctica* lipase B is necessary before extending such enzymatic processes to  
76 multifunctional molecules.

77 In this context, the behavior of *C. antarctica* lipase B toward the acylation of monofunctional  
78 amines and alcohols was studied in this work (Scheme 1). The kinetic mechanism and the  
79 enantioselectivity of the reaction were established for both substrates. Finally, the  
80 chemoselectivity of *C. antarctica* lipase B toward the O-acylation and N-acylation was  
81 evaluated.



82  
83 **Scheme 1.** Acylation of monofunctional alcohols and amines catalyzed by *C. antarctica*  
84 lipase B in *tert*-amyl alcohol.

## 85 2. Material and methods

### 86 2.1. Enzyme and chemicals

87 Novozym<sup>®</sup> 435 (immobilized *Candida antarctica* lipase B), was kindly provided by  
88 Novozymes A/S, Bagsvaerd, Denmark. R and S pure enantiomers (99%) of 2-butanol and  
89 *sec*-butylamine, as well as *tert*-amyl alcohol were purchased from Sigma-Aldrich (St Louis,  
90 USA) while myristic acid and acetic acid were from Fluka (St Quentin-Fallavier,  
91 Switzerland). All chemicals were dried over molecular sieves. Pure water was obtained via a  
92 Milli-Q system (Millipore, France). Acetonitrile was purchased from Carlo ERBA (Val-de-  
93 Reuil, France).

### 94 2.2. Enzymatic reactions

95 Initial rate measurements were performed at 55°C in *tert*-amyl alcohol according to a  
96 previously established procedure [12]. 2 ml of the reaction mixtures containing various  
97 amounts of substrates (25-350 mM) were incubated for 10 minute prior to addition of 10 g.l<sup>-1</sup>  
98 of *C. antarctica* lipase B for the acylation of 2-butanol with myristic acid and 50 g.l<sup>-1</sup> of *C.*  
99 *antarctica* lipase B for the acylation of *sec*-butylamine with myristic acid. 100 µl samples  
100 were taken at intervals and centrifuged at 14000 rpm. The supernatant was analyzed by LC-  
101 MS. The equilibrium synthesis yields were determined after a 48h reaction on the basis of the

102 limited starting substrate consumption. The equilibrium constants were then calculated  
103 according to equation 1.

$$104 \quad K_{\text{eq}} = (a_w \times a_p) / (a_{\text{myristic acid}} \times a_B) \quad (1)$$

105 where  $a_w$  and  $a_{\text{myristic acid}}$  are the thermodynamic activities of water and myristic acid,  
106 respectively.  $a_p$  and  $a_B$  are the thermodynamic activities of 1-methylpropyl myristate ester or  
107 myristic acid methylpropylamide, and of 2-butanol or *sec*-butylamine, respectively. The  
108 thermodynamic activity values used were estimated using the contribution method of  
109 predicting activity coefficients, UNIFAC (universal functional activity coefficient) [13].

110 The initial velocities were calculated from the linear relationship of the total concentration of  
111 products against reaction time (0-2 h for the 2-butanol esterification and 0-3 h for the *sec*-  
112 butylamine amidification).

### 113 2.3. Data analysis

#### 114 2.3.1 Determination of kinetic mechanisms

115 All initial rates data were fitted to all the kinetic models provided by the Enzyme Kinetics  
116 Module of Sigma Plot, "Enzyme Kinetics 2004 1.3" (Systat Software Inc., San Jose, USA),  
117 using nonlinear least-squares regression analysis.

118 Initial rate data corresponding to 2-butanol O-acylation were best fitted to the equation  
119 describing a steady-state ordered ternary complex bi-bi mechanism [14] (Eq. (2)).

$$120 \quad v = V_{\text{max}}[A][B] / (K_{\text{ia}}K_{\text{mB}} + K_{\text{mB}}[A] + K_{\text{mA}}[B] + [A][B]) \quad (2)$$

121 Initial rate data corresponding to *sec*-butylamine N-acylation for low concentrations of amine  
122 (<100 mM) and myristic acid (<175 mM) were best fitted to the equation describing a steady-  
123 state ping-pong bi-bi mechanism [14] (Eq. (3)).

$$124 \quad v = V_{\text{max}}[A][B] / (K_{\text{mB}}[A] + K_{\text{mA}}[B] + [A][B]) \quad (3)$$

125 A represents myristic acid as B represents 2-butanol in equation 2 and *sec*-butylamine in  
126 equation 3.  $K_{mA}$  and  $K_{mB}$  are the Michaelis constants for A and B, respectively.  $V_{max}$  is the  
127 maximum rate.  $K_{ia}$  is defined as the dissociation constant of A from the binary complex E.A.  
128 To allow visualization of fitting quality, the experimental data points are presented in plots  
129 along with theoretical lines fitted by “Enzyme Kinetics 2004 1.3” software. Moreover, linear  
130 regressions of these theoretical lines were used to calculate the apparent kinetics parameters  
131  $V_{max}^{app}$  and  $K_{mB}^{app}$ .

### 132 2.3.2 Evaluation of the enantioselectivity and of the chemoselectivity

133 The enantioselectivity can be described by the enantiomeric ratio (E). If the initial rates of the  
134 individual enantiomers acylations are measured separately at a certain substrate concentration,  
135 the ratio of initial rates depends on the substrate concentration and serves merely as a  
136 qualitative measure of enantioselectivity (Eq. (4)). If concentrations well below the  $K_m$  values  
137 are used (pseudo first-order kinetics) or if  $K_m^R = K_m^S$ , the ratio of initial rates will equal E and  
138 the catalytic efficiency ratio [15]. In this study, E-values were always calculated by using the  
139 catalytic efficiency ratio.

$$140 E = v_i^R/v_i^S = (V_{max}^R/(K_m^R + C_{S0})) / (V_{max}^S/(K_m^S + C_{S0})) \quad (4)$$

141 The chemoselectivity of *C. antarctica* lipase B was studied by comparing (R)-2-butanol O-  
142 acylation and (R)-*sec*-butylamine N-acylation, and then evaluated via the apparent catalytic  
143 efficiency ratio (Eq. (5)) [16].

$$144 C = (V_{max}^{app} \text{ O-acylation} / K_m^{app} \text{ O-acylation}) / (V_{max}^{app} \text{ N-acylation} / K_m^{app} \text{ N-acylation}) \quad (5)$$

### 145 2.4. HPLC analysis

146 Structural and quantitative analysis of reaction products were conducted using a LC/MS-ES  
147 system from Agilent (1100 LC/MSD Trap mass spectrometer VL) with a C18 Uptisphere 300



148 A OD column (250×4 mm, 5 μm; Interchim) for the analysis of esters and a C18 Prontosil  
149 120-5-C18-AQ column (250×4 mm, 5 μm; Bischoff Chromatography) for the analysis of  
150 amides. Both columns were eluted with acetonitrile/water/acetic acid (90:10:0.1, v/v/v) at  
151 room temperature and at a flow rate of 1 ml.min<sup>-1</sup>. Products were detected and quantified by  
152 differential refractometry using HP Chemstation software off-line for the processing.

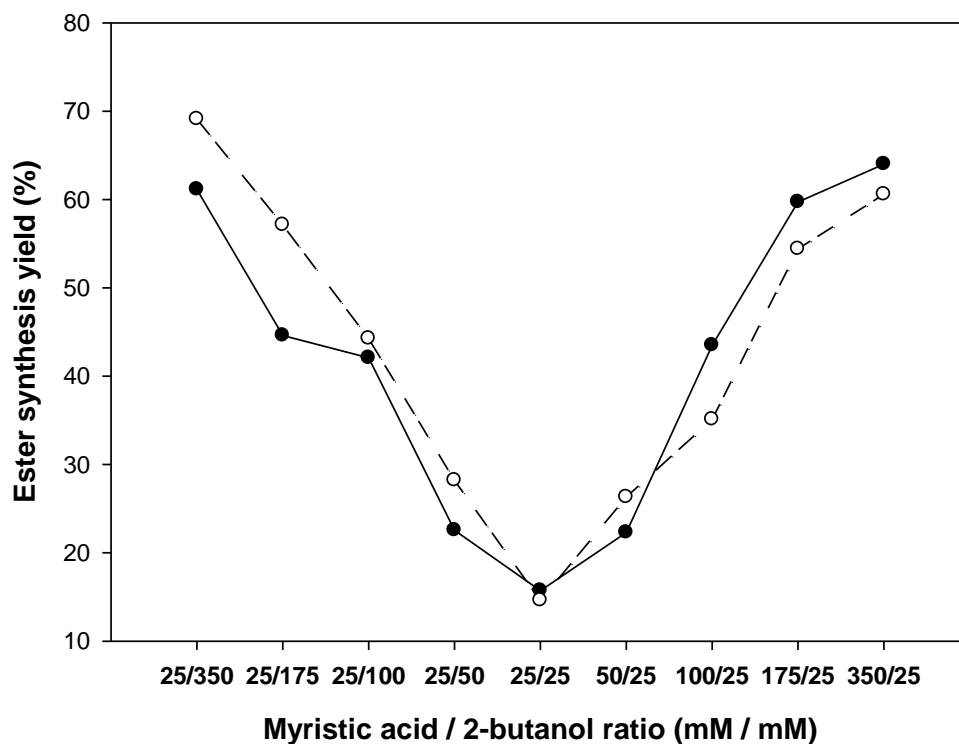
### 153 3. Results and discussion

#### 154 3.1. Kinetics and enantioselectivity of 2-butanol O-acylation.

155 The synthesis of 1-methylpropyl myristate ester catalyzed by *C. antarctica* lipase B from 2-  
156 butanol with myristic acid as an acyl donor was chosen as the model reaction for the O-  
157 acylation study. Pure (R)- and (S)-enantiomers of 2-butanol were used in separate  
158 experiments to investigate the effect of the concentration of both substrates on the initial rate  
159 values and to elucidate the kinetic mechanism of the reaction. From the HPLC analysis, the  
160 decrease of myristic acid concentration was always seen to be concomitant with the synthesis  
161 of 1-methylpropyl myristate ester which was identified by mass spectroscopy. In absence of  
162 enzyme, no product was detected within 2 days.

163 Both substrate concentrations were varied from 25 mM to 350 mM and Figure 1 shows the  
164 effect of the substrate molar ratio on the equilibrium ester synthesis yield based on the  
165 limiting initial substrate concentration, after 48 hours of (R)- or (S)-2-butanol acylation. We  
166 observed that the yield increased sharply when one substrate was used in excess, starting from  
167 about 15% ester synthesis under stoichiometric conditions to reach more than 60% ester  
168 synthesis under the conditions with 350 mM of myristic acid or 2-butanol. Under the best  
169 conditions used, corresponding to 350 mM of 2-butanol and 25 mM of myristic acid, up to  
170 69% conversion (17.2 mM) was obtained with the (S)-conformation, giving an equilibrium

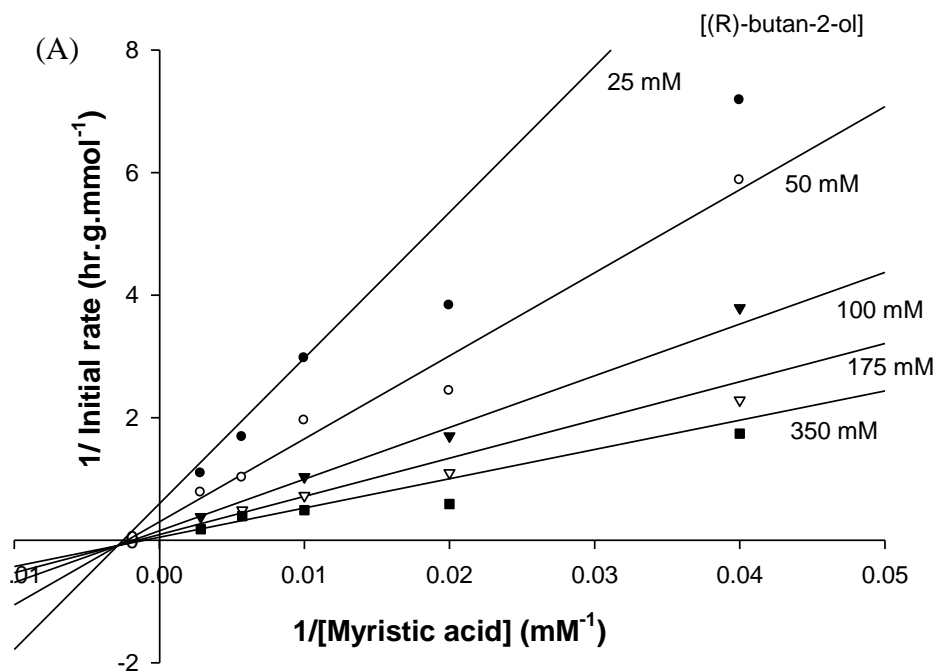
171 constant  $K_{eq}$  of 1.87, which favors synthesis. Moreover, no significant difference was  
172 observed between the (R)- and (S)-ester synthesis yields whatever the substrate molar ratio.



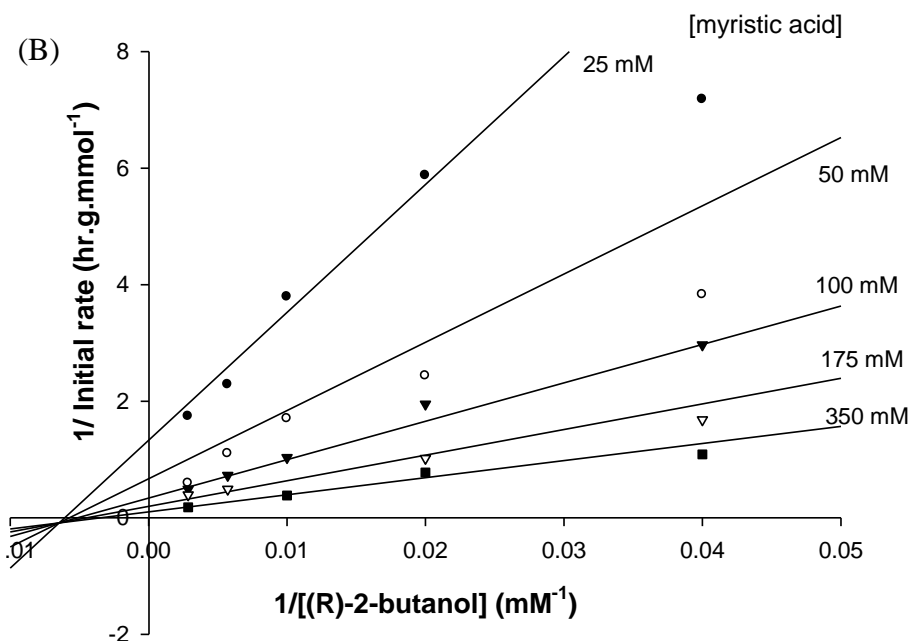
173  
174 **Fig. 1.** Effect of the myristic acid/2-butanol molar ratio on the equilibrium ester synthesis  
175 yield. Reactions were carried out in *tert*-amyl alcohol using (R)-2-butanol (●) or (S)-2-butanol  
176 (○) for 48 hours at 55°C with 10 g.l<sup>-1</sup> of *Candida antarctica* lipase B.

177  
178 Systematic analysis of the rates, which were dependent on the substrate concentration,  
179 revealed two Lineweaver-Burk double reciprocal plots, depending on whether myristic acid or  
180 (R)-2-butanol was parametric. The results are shown on Figure 2.

181



182



183 **Fig. 2.** Reciprocal initial rates versus reciprocal substrate concentrations. The synthesis was  
 184 carried out at  $55^{\circ}\text{C}$  using  $10\text{ g}\cdot\text{l}^{-1}$  of *Candida antarctica* lipase B. (A) The concentration of  
 185 (R)-2-butanol was fixed at 25 mM (●), 50 mM (○), 100 mM (▼), 175 mM (▽) and 350 mM  
 186 (■). (B) The concentration of myristic acid was fixed at 25 mM (●), 50 mM (○), 100 mM  
 187 (▼), 175 mM (▽) and 350 mM (■). The data represent the averages of triplicate runs whose  
 188 standard deviations were always lower than 15%.

189 The corresponding families of the double reciprocal plots were linear and gave intersecting  
190 patterns at the left of the y-axis (Figure 2A and Figure 2B), characterizing either a steady-state  
191 ordered ternary complex bi-bi mechanism or a Theorell-Chance mechanism. The probability  
192 that a Theorell-Chance system exists is very low and this mechanism was therefore excluded  
193 [14]. We did not observe any type of inhibition with the tested substrate concentrations. The  
194 initial rates data obtained with the (S)-enantiomer were also seen to fit with this kinetic model  
195 (data not shown).

196 In the literature, most of the models concerning the lipase-catalyzed acylation of primary  
197 alcohols are based on a ping-pong bi-bi mechanism [4-5]. This model was notably used to  
198 describe O-acylation reactions catalyzed by *C. antarctica* lipase B in organic solvents [8], in  
199 supercritical media [17] and in solid-gaz reactors [18]. However, the ordered bi-bi mechanism  
200 was reported in few studies dealing with transesterification reactions catalyzed by *C.*  
201 *antarctica* lipase B and carried out in solvent-free systems [6,19]. In fact, the catalytic  
202 mechanism of lipases is believed to be analogous to that proposed for serine proteases,  
203 involving the known active site triad of residues serine, histidine and aspartic acid. It has been  
204 suggested that a Michaelis-Menten complex formed between the acyl donor and the enzyme  
205 proceeds to give the first tetrahedral intermediate. This then collapses to give an acyl-enzyme  
206 intermediate and release the first product. Then the second substrate binds to the active site,  
207 allowing the formation of the second tetrahedral intermediate. Finally, the latter breaks down  
208 to give the ester product [20]. This corresponds to a ping-pong bi bi mechanism but in our  
209 situation, the first product is water, which is a small molecule that may already be present in  
210 the catalytic site and cannot be a cause of steric hindrance. So the second substrate can access  
211 to the active site before the release of the first product, leading to an ordered bi-bi mechanism.

212 The apparent kinetic parameters  $V_{\max}^{app}$  and  $K_{mB}^{app}$  were determined (Table 1).

213 **Table 1.** Apparent kinetic parameters of acylations using 175 mM myristic acid as acyl donor  
 214 and 25 – 350 mM substrate B as acyl acceptor in *tert*-amyl alcohol.

Reaction	Substrate B	$K_{mB}^{app}$ (mM)	$V_{max}^{app}$ (mmol.h <sup>-1</sup> .g <sup>-1</sup> )	Apparent Catalytic Efficiency ( $V_{max}^{app} / K_{mB}^{app}$ ) (l.h <sup>-1</sup> .g <sup>-1</sup> )
1	(R)-2-butanol	<b>150</b>	<b>4.62</b>	<b>0.031</b>
2	(S)-2-butanol	<b>207</b>	<b>2.5</b>	<b>0.012</b>
3	(R)- <i>sec</i> - butylamine	<b>619</b>	<b>0,21</b>	<b>3,36 x 10<sup>-4</sup></b>
4	(S)- <i>sec</i> - butylamine	<b>120</b>	<b>0,03</b>	<b>2.5 x 10<sup>-4</sup></b>

215

216

217 These results provide informations about the enantioselectivity of *C. antarctica* lipase B  
 218 toward the O-acylation. First, the apparent maximum rate ( $V_{max}^{app}$ ) of the ester production  
 219 starting from the (R)-conformation (4.62 mmol.h<sup>-1</sup>.g<sup>-1</sup>) was about 2-fold higher than the  
 220 apparent maximum rate obtained starting from the (S)-conformation (2.5 mmol.h<sup>-1</sup>.g<sup>-1</sup>).  
 221 Furthermore, the apparent Michaelis constant ( $K_{mB}^{app}$ ) of the (R)-enantiomer (150 mM) was  
 222 slightly lower than the  $K_{mB}^{app}$  of the (S)-enantiomer (207 mM), which indicated an affinity of  
 223 the lipase slightly favoring the (R)-enantiomer. Logically, the resulting catalytic efficiency for  
 224 the (R)-enantiomer acylation (0.031 l.h<sup>-1</sup>.g<sup>-1</sup>) was higher than the catalytic efficiency for the  
 225 (S)-enantiomer acylation (0.012 l.h<sup>-1</sup>.g<sup>-1</sup>). Moreover, an E-value of about 3.17 (Eq. (4)) was  
 226 obtained when using 175 mM of myristic acid, which signified that *C. antarctica* lipase B had  
 227 a preferential enantioselectivity toward the (R)-enantiomer O-acylation.

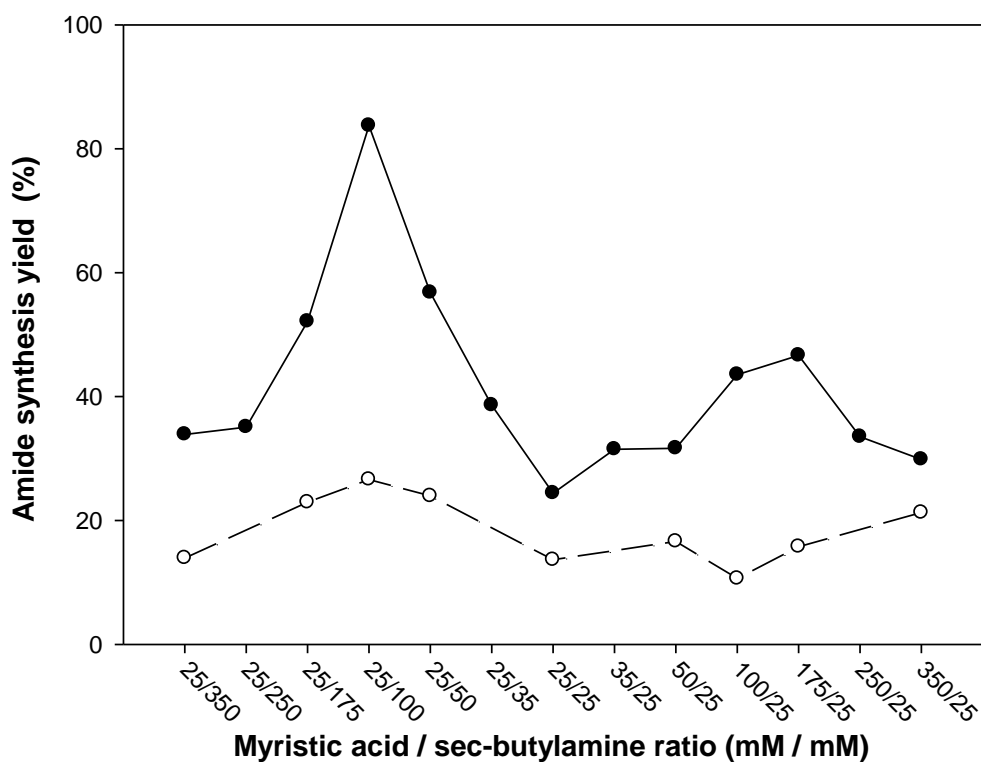
228 This result is consistent with several works, which have shown that *C. antarctica* lipase B  
 229 preferentially catalyzes the (R)-enantiomer acylation [21]. Some molecular modeling data

230 have revealed that this enantioselectivity may be related to the enzyme conformation which  
231 would allow the (R)-enantiomer to easier access the active site [22].

### 232 3.2. Kinetics and enantioselectivity of *sec*-butylamine *N*-acylation

233 The synthesis of myristic acid methylpropylamide catalyzed by *C. antarctica* lipase B from  
234 *sec*-butylamine with myristic acid as an acyl donor was chosen as the model reaction for the  
235 *N*-acylation study. Pure (R)- and (S)-enantiomers of *sec*-butylamine were used in separate  
236 experiments to investigate the effect of both substrate concentrations on the initial rate and the  
237 synthesis yield. From the HPLC analyses, the decrease in myristic acid concentration was  
238 always seen to be concomitant with the synthesis of myristic acid methylpropylamide, which  
239 was identified by mass spectroscopy. In absence of enzyme, no product was detected within 2  
240 days.

241 Figure 3 shows the effect of the myristic acid/*sec*-butylamine molar ratio on the equilibrium  
242 amide synthesis yield based on the limiting initial substrate concentration. Under the best  
243 conditions used (100 mM of (R)-*sec*-butylamine and 25 mM of myristic acid), 84% of amide  
244 synthesis (21 mM) was reached for the (R)-enantiomer acylation, giving an equilibrium  
245 constant  $K_{eq}$  of 14.37, strongly favoring synthesis, while the amide synthesis yield never  
246 exceeded 25% (6.2 mM) for the (S)-enantiomer acylation, whatever the myristic acid/*sec*-  
247 butylamine molar ratio.



248

249 **Fig 3.** Effect of the myristic acid/*sec*-butylamine molar ratio on the equilibrium amide  
 250 synthesis yield. Reactions were carried out in *tert*-amyl alcohol using (R)-*sec*-butylamine (●)  
 251 or (S)-*sec*-butylamine (○) for 48 hours at 55°C with 50 g.l<sup>-1</sup> of *Candida antarctica* lipase B.

252

253 Furthermore, the (R)-amide synthesis yields were higher than the (S)-amide synthesis yields  
 254 under all conditions tested. We also observed that the increase in one substrate concentration,  
 255 from 25 to 100 mM of *sec*-butylamine and from 25 to 175 mM of myristic acid, allowed to  
 256 enhance the amide synthesis yield. However, the use of a large excess of one substrate toward  
 257 the other, corresponding to concentrations higher than 100 mM of *sec*-butylamine or 175 mM  
 258 of myristic acid, caused the decrease in the amide synthesis yield. This was most likely due to  
 259 an inhibitor effect similar to an excess substrate inhibition that was not observed for the O-  
 260 acylation of 2-butanol (see section 3.1; Figure 1), probably due to an interaction between *sec*-  
 261 butylamine and myristic acid. Indeed, the presence of an amino substrate and a fatty acid in an  
 262 organic solvent generally leads to the formation of an ion-pair complex between both

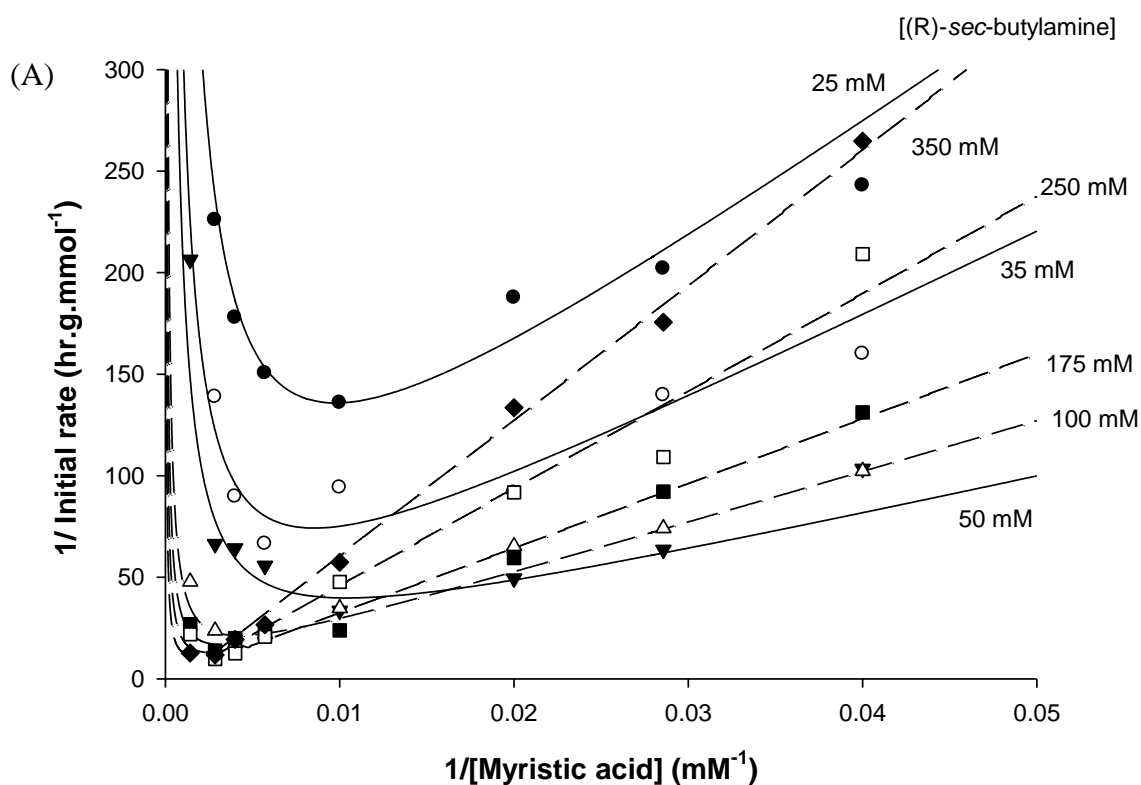
263 substrates, depending on the acido-basic conditions of the medium [12,19,23]. This salt  
264 complex can lead to the unreactivity of the ion forms of both substrates ( $\text{NH}_3^+$  amine form  
265 and  $\text{COO}^-$  fatty acid form) and therefore to the overestimation of the reactive substrate  
266 concentrations that are really available for the enzyme in the reaction medium. This ion-pair  
267 complex was already described by Maugard *et al* [12] as a limiting factor of the lipase-  
268 catalyzed acylation under conditions where it was less soluble than free substrates.

269 Using kinetic experiments, we then sought to identify the kinetic mechanism of the synthesis  
270 of myristic acid methylpropylamide from *sec*-butylamine and myristic acid catalyzed by *C.*  
271 *antarctica* lipase B. Systematic analysis of the rates, which were dependent on the substrate  
272 concentration, revealed two Lineweaver-Burk double reciprocal plots, depending on whether  
273 myristic acid or (R)-*sec*-butylamine was parametric (Figure 4). The corresponding families of  
274 the double reciprocal plots showed a set of solid parallel lines for low concentrations of  
275 substrates (inferior or equal to 100 mM of myristic acid or 50 mM of (R)-*sec*-butylamine)  
276 characterizing a steady-state ping-pong bi-bi mechanism. The initial rates data obtained with  
277 the (S)-enantiomer were also seen to fit with this kinetic model (data not shown).

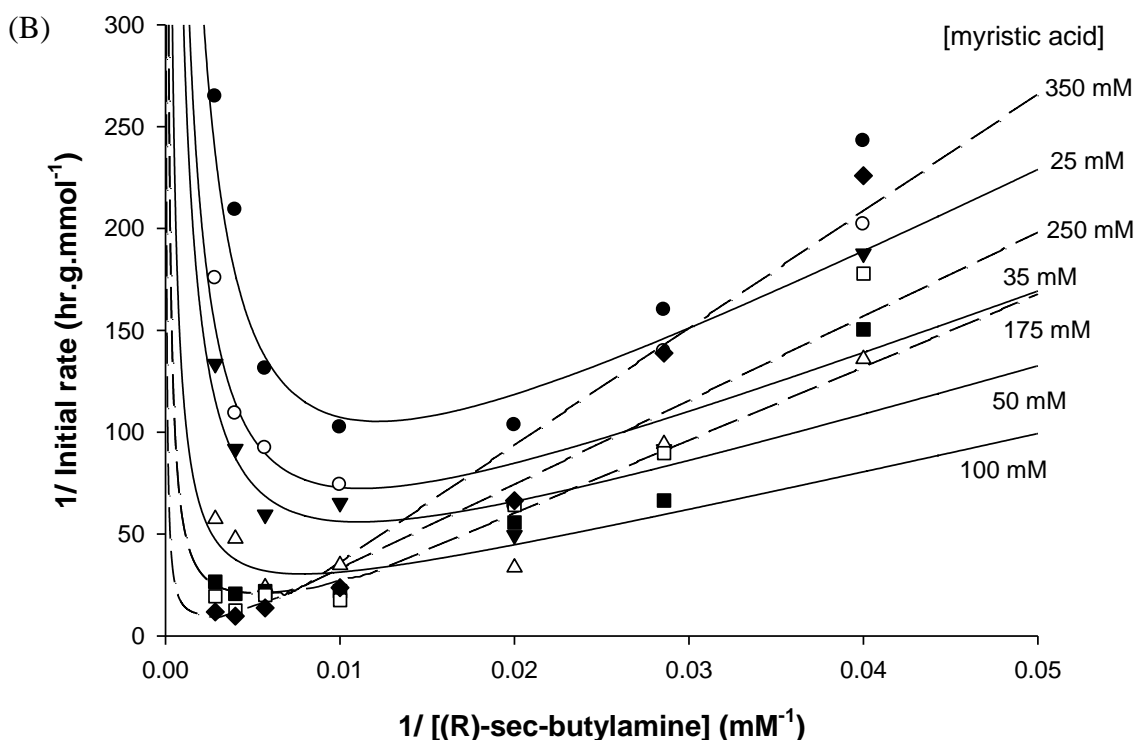
278 Moreover, for high concentrations of substrates (superior to 100 mM of myristic acid or (R)-  
279 *sec*-butylamine), a decrease in initial rates was observed: this was characterized on both  
280 lineweaver-Burk plots by an increase in  $1/\text{initial rate}$ -values as well as a loss of parallelism of  
281 the corresponding plots, which showed a set of dashed lines tending to intersect near the y-  
282 axis. Two hypotheses can be proposed to explain this phenomenon. First, it may be due to a  
283 steady-state ping-pong bi-bi mechanism implying a double substrate competitive inhibition,  
284 which would be enforced by the fact that the corresponding experimental data were best fitted  
285 with this model. Nevertheless, this hypothesis should probably be ruled out given that no  
286 inhibition by myristic acid was observed for 2-butanol O-acylation conducted under very  
287 similar conditions. The second and most likely hypothesis would be the previously proposed



288 formation of an ion-pair complex between (R)-*sec*-butylamine and myristic acid that would  
289 lead to the decrease in the substrates availability in the enzyme environment. As the formation  
290 of a complex salt cannot occur between myristic acid and 2-butanol, due to the impossibility  
291 for 2-butanol to form its unprotonated anionic form under the experimental conditions used,  
292 this phenomenon was not observed for 2-butanol O-acylation.



293



294

295 **Fig. 4.** Reciprocal initial rates versus reciprocal substrate concentrations. The synthesis was  
 296 carried out at 55°C using 50 g.L<sup>-1</sup> of *Candida antarctica* lipase B. (A) Myristic acid  
 297 concentration was varied from 25 to 350 mM and (R)-*sec*-butylamine concentration was fixed  
 298 at 25 mM (●), 35 mM (○), 50 mM (▼), 100 mM (△), 175 mM (■), 250 mM (□) and 350 mM  
 299 (◆). (B) (R)-*sec*-butylamine concentration was varied from 25 to 350 mM and myristic acid  
 300 concentration was fixed at 25 mM (●), 35 mM (○), 50 mM (▼), 100 mM (△), 175 mM (■),  
 301 250 mM (□) and 350 mM (◆). The data represent the averages of triplicate runs whose  
 302 standard deviations were always lower than 15%.

303

304 The apparent kinetic parameters  $V_{\max}^{app}$  and  $K_{mB}^{app}$  were determined (Table 1). These results  
 305 provide informations about the enantioselectivity of *C. antarctica* lipase B toward the N-  
 306 acylation. First,  $V_{\max}^{app}$  of the amide production starting from the (R)-conformation (0.21  
 307 mmol.h<sup>-1</sup>.g<sup>-1</sup>) was about 7-fold higher than the apparent maximum rate obtained starting from  
 308 the S conformation (0.03 mmol.h<sup>-1</sup>.g<sup>-1</sup>). On the other hand, the  $K_{mB}^{app}$  of the (R)-enantiomer  
 309 (619 mM) was about 5-fold higher than the  $K_{mB}^{app}$  of the (S)-enantiomer (120 mM), which  
 310 indicated an affinity of the lipase significantly favoring the (S)-enantiomer. Nevertheless, the  
 311 resulting catalytic efficiency for the (R)-enantiomer acylation ( $3.36 \times 10^{-4} \text{ l.h}^{-1}.\text{g}^{-1}$ ) was higher

312 than the catalytic efficiency for the (S)-enantiomer acylation ( $2.5 \times 10^{-4} \text{ l.h}^{-1}.\text{g}^{-1}$ ), which  
313 signified that *C. antarctica* lipase B had a preferential enantioselectivity toward (R)-  
314 enantiomer N-acylation, mostly due to a better catalysis rate toward the (R)-enantiomer. This  
315 was confirmed by calculating an E-value of 1.34 (Eq. (4)) when using 175 mM of myristic  
316 acid.

### 317 3.3. Chemoselectivity of *C. antarctica* lipase B

318 The kinetic data of 2-butanol O-acylation and *sec*-butylamine N-acylation showed in the first  
319 and second parts of our study that *C. antarctica* lipase B favored the O-acylation. The  $V_{\text{max}}^{\text{app}}$  of  
320 (R)-ester synthesis ( $4.62 \text{ mmol.h}^{-1}.\text{g}^{-1}$ ) was thus about 22-fold higher than the  $V_{\text{max}}^{\text{app}}$  of (R)-  
321 amide synthesis ( $0.21 \text{ mmol.h}^{-1}.\text{g}^{-1}$ ). On the other hand, the  $K_{\text{mB}}^{\text{app}}$  of (R)-*sec*-butylamine (619  
322 mM) was 4-fold higher than the  $K_{\text{mB}}^{\text{app}}$  of (R)-2-butanol (150 mM). This pointed out a better  
323 affinity of *C. antarctica* lipase B toward 2-butanol (Table 1, reaction 1). To more precisely  
324 evaluate the chemoselectivity of *C. antarctica* lipase B, (R)-2-butanol O-acylation and (R)-  
325 *sec*-butylamine N-acylation were conducted separately using 175 mM of myristic acid. The  
326 chemoselectivity ratio (C) was then calculated (Eq. (5)), giving a C-value close to 92. This  
327 value clearly confirmed that *C. antarctica* lipase B was chemoselective for the O-acylation  
328 under our synthesis conditions. In vivo, lipases are acyl-hydrolases that catalyse the  
329 hydrolysis of esters [24,25]. It seems thus coherent for *C. antarctica* lipase B to be  
330 chemoselective for the O-acylation of (R)-2-butanol rather than for the N-acylation of (R)-  
331 *sec*-butylamine. In addition, some studies dealing with the acylation of long difunctional  
332 compounds have shown similar results. In particular, Husson *et al.* reported a high  
333 chemoselectivity in rates exhibited by *C. antarctica* lipase B for the O-acylation of 6-amino-  
334 1-hexanol conducted in *tert*-amyl alcohol starting from oleic acid as an acyl donor [19].  
335 Furthermore, apart from an intrinsic chemoselectivity naturally due to the design and the in

336 vivo function of *C. antarctica* lipase B, the previously discussed possibility of a negative  
337 effect induced by the formation of an ion-pair complex between (R)-*sec*-butylamine and  
338 myristic acid may also explain the low chemoselectivity observed toward N-acylation in  
339 comparison with O-acylation.

#### 340 **4. Conclusion**

341 The kinetic behavior of *C. antarctica* lipase B toward 2-butanol O-acylation and *sec*-  
342 butylamine N-acylation starting from myristic acid as an acyl donor was investigated. The  
343 equilibrium yields and initial rates of the reactions were measured in order to identify the  
344 kinetic mechanisms and parameters which allowed us to understand the origin of the lipase  
345 selectivity. Under the best O-acylation conditions used, a similar synthesis yield (close to  
346 65%; 16 mM) was observed after 48 h for the conversion of (R)- or (S)-2-butanol into 1-  
347 methylpropyl myristate ester. On the other hand, optimal N-acylation conditions resulted in a  
348 significant preference for the conversion of (R)-*sec*-butylamine into myristic acid  
349 methylpropylamide, which reached about 84% (21 mM) when excess (R)-*sec*-butylamine was  
350 used, whereas only 25% of (S)-*sec*-butylamine was converted under optimal conditions.  
351 These results are of interest as they highlight the potential use of *C. antarctica* lipase B for the  
352 biotechnological selective acylation of amino-alcohols with high yields, which could be have  
353 applications in many areas, such as pharmaceuticals and cosmetics (the synthesis of ceramides  
354 [26] as potential anti-viral or anti-tumor drugs [27,28] or anti-oxidant stabilizers [29]), or for  
355 the environment, food, and agricultural industries (the synthesis of glucamide- or aminoacid-  
356 based surfactants [2,30]).

357 Regarding the kinetic studies of the reactions, the experimental data corresponding to the N-  
358 acylation of (R)-*sec*-butylamine and (S)-*sec*-butylamine were fitted with a steady-state ping-  
359 pong bi-bi mechanism for lower substrate concentrations. Interestingly, the kinetic data  
360 corresponding to the O-acylation of (R)-2-butanol and (S)-2-butanol were found to fit with a

361 steady-state ordered ternary complex bi-bi mechanism model. Concerning the  
362 enantioselectivity studies, *C. antarctica* lipase B was found to preferentially catalyze (R)-2-  
363 butanol O-acylation in terms of both catalytic activity and affinity: the calculated E-value of  
364 3.17 confirmed its preferential enantioselectivity for (R)-enantiomer O-acylation. *sec*-  
365 butylamine N-acylation also showed a preferential enantioselectivity of *C. antarctica* lipase B  
366 for the (R)-enantiomer, giving an E-value of 1.34. Nevertheless, (R)-*sec*-butylamine N-  
367 acylation was shown to occur with higher initial rates and yields than those of (S)-*sec*-  
368 butylamine N-acylation whereas the affinity of *C. antarctica* lipase B was lower for (R)-*sec*-  
369 butylamine than for (S)-*sec*-butylamine. The preferential enantioselectivity of *C. antarctica*  
370 lipase B for (R)-*sec*-butylamine N-acylation seemed in fact to find its origin mostly in the  
371 difference of catalysis rate and thus of catalytic activity rather than in the difference of affinity  
372 toward the (R)- and (S)-enantiomers. Finally, it was confirmed that  
373 *C. antarctica* lipase B is a chemoselective enzyme [2], exhibiting a preference for O-acylation  
374 rather than for N-acylation [19].

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