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1 **Continuous lipase-catalyzed production of pseudo-ceramides in a packed-**
2 **bed bioreactor**

3 **Florian Le Joubioux, Nicolas Bridiau*, Mehdi Sanekli, Marianne Graber, Thierry**
4 **Maugard**

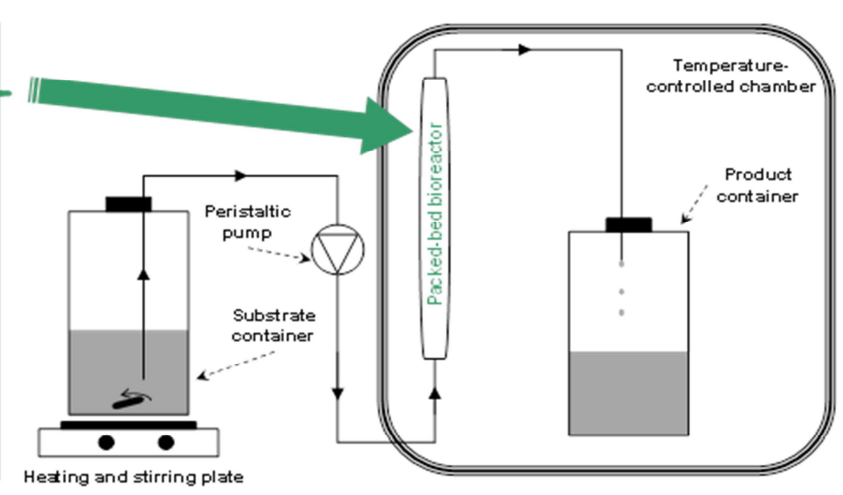
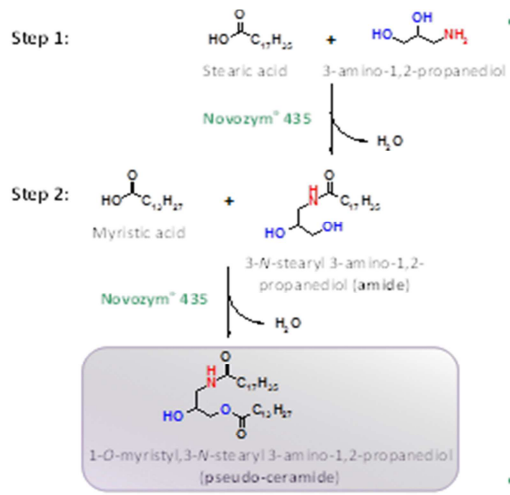
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11
12

13 Abstract

14 Ceramides are spingolipid compounds that are very attractive as active components in both
15 the pharmaceutical and the cosmetic industries. In this study, the synthesis of ceramide
16 analogs, the so-called pseudo-ceramides, was carried out using for the first time a two-step
17 continuous enzymatic process with immobilized *Candida antarctica* lipase B (Novozym[®]
18 435) in a packed-bed bioreactor. The first step involved the selective *N*-acylation of 3-amino-
19 1,2-propanediol using stearic acid as the first acyl donor (i). This was followed by the
20 selective *O*-acylation of the *N*-stearyl 3-amino-1,2-propanediol synthesized in the first step,
21 with myristic acid as the second acyl donor, to produce a *N,O*-diacyl 3-amino-1,2-
22 propanediol-type pseudo-ceramide, namely 1-*O*-myristyl,3-*N*-stearyl 3-amino-1,2-
23 propanediol (ii). The process was first optimized by evaluating the influences of three factors:
24 feed flow rate, quantity of biocatalyst and substrate concentration. Under optimal conditions
25 an amide synthesis yield of 92% and a satisfying production rate of almost 3.15 mmol h⁻¹
26 g_{biocatalyst}⁻¹ (1128 mg h⁻¹ g_{biocatalyst}⁻¹) were obtained. The second step, *N*-acyl 3-amino-1,2-
27 propanediol *O*-acylation, was similarly optimized and in addition the effect of the substrate
28 molar ratio was studied. Thus, an optimal pseudo-ceramide synthesis yield of 54% and a
29 production rate of 0.46 mmol h⁻¹ g_{biocatalyst}⁻¹ (261 mg h⁻¹ g_{biocatalyst}⁻¹) were reached at a 1:3 ratio
30 of amide to fatty acid. In addition, it was demonstrated that this two-step process has great
31 potential for the production of *N,O*-diacyl 3-amino-1,2-propanediol-type pseudo-ceramides on
32 an industrial scale. It was shown in particular that Novozym[®] 435 could be used for more than
33 3 weeks without a drop in the yield during the first step of 3-amino-1,2-propanediol *N*-
34 acylation, proving that this biocatalyst is very stable under these operational conditions. This
35 factor would greatly reduce the need for biocatalyst replacement and significantly lower the
36 associated cost.

37 **Keywords:** pseudo-ceramide, biocatalysis, lipase, continuous bioprocess, packed-bed
38 bioreactor

39 **1. Introduction**

40 Ceramides are natural compounds derived from the *N*-acylation of sphingosine and are key
41 intermediates in the biosynthesis of all complex sphingolipids. Like their synthetic analogs,
42 they have been widely used in the cosmetic and pharmaceutical industries. Indeed, due to
43 their major role in preserving the water-retaining properties of the epidermis [1], ceramides
44 have a wide range of commercial applications in the cosmetic industry as active ingredients
45 included in hair and skin care products. Moreover, ceramides can be used as active
46 components in dermatological therapy: they are effective in restoring the water content of dry
47 skin and in relieving atopic eczema [2]. In addition, it has been demonstrated that they have
48 commercial applications in the pharmaceutical industry as potential anti-viral or anti-tumor
49 drugs [3, 4] and anti-oxidant stabilizers [5].

50 As a result of these numerous commercial applications, there is a growing interest in the
51 development and optimization of new processes for ceramide synthesis. Ceramide synthesis is
52 usually performed by acylation of the amino group of a sphingosine, a sphinganine or their
53 derivatives [6–8]. However, due to the high cost of sphingoid bases, whose chemical
54 synthesis is complex, other approaches have been developed to synthesize ceramide analogs,
55 called pseudo-ceramides, by the selective acylation of multifunctional compounds like amino-
56 alcohols. All these compounds are presently synthesized by chemical procedures which
57 require fastidious steps of alcohol group protection and deprotection for the control of
58 chemoselectivity, regioselectivity and stereoselectivity [6–10]. Moreover, these procedures
59 often require high temperatures that may preclude the use of fragile molecules and may cause
60 coloration of the end products. In addition, the coproduction of salts and the use of toxic
61 solvents (dimethylformamide, methanol, etc.) that must be eliminated at the end of the
62 reaction tend to increase the cost of the processes.

63 In order to overcome these disadvantages, several studies focused on developing enzymatic
64 syntheses of pseudo-ceramides through immobilized lipase-catalyzed acylation or
65 transacylation reactions carried out in an organic solvent or in a solvent-free system [11–13].
66 Indeed, using lipases (E.C. 3.1.1.3) in the process can be both more effective, due to a higher
67 selectivity, and more eco-compatible, due to the limited number of steps required for the
68 synthesis [14–18]. Lipase-catalyzed acylation in organic media provides several advantages
69 such as shifting the thermodynamic equilibrium toward synthesis rather than hydrolysis,
70 increasing the solubility of non-polar substrates like fatty acids, eliminating side reactions,
71 making enzyme recovery easier and increasing enzyme thermostability [19].
72 Various studies have been devoted to the lipase-catalyzed acylation of multi-functional
73 molecules similar to the substrates used as precursors for the synthesis of pseudo-ceramides.
74 These molecules have both amino and alcohol groups, such as ethanolamine, diethanolamine,
75 2-amino-1-butanol, 6-amino-1-hexanol, serine and other amino-alcohols of variable carbon
76 chain length [20–28]. In such reactions, it has been shown that the lipases used can catalyze
77 the chemoselective acylation of these substrates in a highly efficient and chemoselective
78 manner. Some of these studies have already demonstrated the feasibility of selectively
79 synthesizing pseudo-ceramide-type compounds using heterogeneous solvent-free media in a
80 batch bioreactor, with productivity close to $15 \text{ g}_{\text{pseudo-ceramide}} \text{ g}_{\text{biocatalyst}}^{-1}$ [12, 26]. Based on
81 these studies, lipases seem to be the ideal biocatalysts for the synthesis of pseudo-ceramide
82 compounds.
83 On the other hand, despite the many synthetic processes that have already been developed,
84 also in batch reactors [6-13, 29], ceramides are still not easy to produce for industrial
85 applications. The price of the cheapest synthetic ceramide is close to 2000 €/kg, and
86 ceramides with a fatty acid composition similar to that found in the skin cost several hundred
87 thousand €/kg. So, it would be extremely beneficial to develop an alternative cost-efficient

88 method to produce this valuable product with a high yield and productivity. In recent years,
89 the use of continuous-flow technology has become an innovative, promising and attractive
90 alternative for the highly selective production of pure chemical compounds with a good level
91 of productivity. Packed-bed bioreactors are the most frequently used and the best continuous
92 production systems. They offer several advantages over a batch reactor: they are easy to use,
93 can be controlled and operated automatically, they reduce operating costs, provide a better
94 control of the operating conditions and products, leading to a significant enhancement in the
95 productivity of the biocatalyst and an improvement in quality (less secondary products) and
96 yield [30, 31]. Such systems have a low reactor volume due to the high enzyme/substrate ratio
97 maintained in the catalytic bed. In addition, the enzyme/substrate ratio is higher in packed-bed
98 bioreactors than in conventional batch bioreactors, thus shortening the reaction time and
99 potentially limiting side reactions, thereby improving selectivity.

100 Starting from this overview, the aim of our work was to develop for the first time a
101 continuous process for the efficient enzymatic production of 1-*O*,3-*N*-diacyl 3-amino-1,2-
102 propanediol-type pseudo-ceramides. These diacylated derivatives of 3-amino-1,2-propanediol
103 have been considered in various studies as pseudo-ceramides for two reasons: i) their structure
104 includes a polar head, two lipophilic carbon chains and an amide bond, and is thus very close
105 to natural ceramide structure; ii) they have been demonstrated to have restructuring effects
106 very similar to those of natural ceramides at the level of the uppermost skin layer, the so-
107 called *stratum corneum* [12, 26, 32].

108 The process developed in this work was performed using a packed-bed bioreactor containing
109 immobilized *Candida antarctica* lipase B (Novozym[®] 435). In order to control the
110 chemoselectivity of the reaction, the process was divided into two steps (scheme 1): *N*-stearyl
111 3-amino-1,2-propanediol **3a** (amide) was obtained in the first step from the *N*-acylation of 3-
112 amino-1,2-propanediol **1** using stearic acid **2a** as a first acyl donor. In the second step, 1-*O*-

113 myristyl,3-*N*-stearyl 3-amino-1,2-propanediols **4** (pseudo-ceramide) was produced from the
114 *O*-acylation of the *N*-stearyl 3-amino-1,2-propanediol **3a** (amide) produced in the first step
115 using myristic acid **2b** as a second acyl donor.

116 **Scheme 1.**

117 **2. Material and methods**

118 *2.1. Enzymes and chemicals*

119 Novozym[®] 435 (immobilized *Candida antarctica* lipase B) was kindly provided by
120 Novozymes A/S, Bagsvaerd, Denmark. (±)-3-amino-1,2-propanediol (97%), lauric acid
121 (≥99%), stearic acid (95%), linoleic acid (≥99%) and *tert*-amyl alcohol (99%) were purchased
122 from Sigma Aldrich (St Louis, USA) while myristic acid (≥98%) and oleic acid (97%) were
123 purchased from Fluka (St Quentin-Fallavier, Switzerland). All chemicals were dried over
124 molecular sieves. Pure water was obtained via a Milli-Q system (Millipore, France).
125 Acetonitrile, methanol, *n*-hexane and chloroform were purchased from Carlo ERBA (Val-de-
126 Reuil, France).

127 *2.2. Continuous process using a packed-bed bioreactor system for the Novozym[®]* 128 *435-catalyzed synthesis of 1-O,3-N-diacyl 3-amino-1,2-propanediol-type* 129 *pseudo-ceramides*

130 *2.2.1. Packed-bed bioreactor system*

131 Fig. 1 schematically shows the packed-bed bioreactor system used for the continuous two-step
132 enzymatic synthesis of 1-*O*,3-*N*-diacyl 3-amino-1,2-propanediol-type pseudo-ceramides
133 catalyzed by immobilized *Candida antarctica* lipase B (Novozym[®] 435) (scheme 1). For each
134 step, the reaction mixture (substrates and solvent) was first homogenized for 15 min at 55°C

135 while stirring at 250 rpm. The process was then started by percolating the reaction mixture
136 into a column packed with Novozym[®] 435 by means of a peristaltic pump (Minipuls
137 Evolution Peristaltic Pump from Gilson Inc., USA). Several stainless steel columns of
138 variable length and an inner diameter of 5 mm were used at the laboratory scale, while one
139 125 mm long column with a 10 mm inner diameter and a second that was 5 mm in length with
140 an inner diameter of 50 mm were used to scale-up the reactor design. Throughout the process,
141 the reaction medium leaving the bioreactor was continuously pooled into a product container
142 which, together with the column packed with Novozym[®] 435, was placed in a temperature-
143 controlled chamber at 55°C to promote the synthesis reaction and ensure the solubility of the
144 acylated products. Each step was carried out until the substrate container was empty,
145 indicating the end of the process. The concentration of the remaining substrates and acylated
146 products in the product container were then determined by LC/MS-ESI analysis.

147 **Fig. 1**

148 *2.2.2. First step: N-acylation of 3-amino-1,2-propanediol*

149 In the first step, the reaction mixture contained 3-amino-1,2-propanediol **1**, a fatty acid
150 (stearic acid **2a**, myristic acid **2b**, lauric acid **2c**, oleic acid **2d** or linoleic acid **2e**), which was
151 used as an acyl donor, and a *tert*-amyl alcohol/*n*-hexane (50:50 v/v) mixture used as the
152 reaction solvent.

153 *2.2.3. Second step: O-acylation of N-acyl 3-amino-1,2-propanediol*

154 In the second step, the reaction mixture contained the *N*-stearyl 3-amino-1,2-propanediol **3a**
155 produced during the first step, myristic acid **2b**, which was used as an acyl donor and a *tert*-
156 amyl alcohol/*n*-hexane (50:50 v/v) mixture used as the reaction solvent.

157 *2.3. HPLC/MS analysis*

158 To monitor the reaction, a 500 μl sample was taken from the product container when the
159 continuous process was complete, after 1 h of reaction. The study of the operational stability
160 of Novozym[®] 435 in the continuous packed-bed bioreactor was carried out in a slightly
161 different manner: 500 μl samples were taken from the packed-bed output at different times
162 over a 3-week period. In each case, 500 μl of a methanol/chloroform (50:50 v/v) mixture were
163 added to each sample in order to homogenize the reaction medium at room temperature.
164 Structural and quantitative analyses of the reaction products were then conducted on these
165 samples using a LC/MS-ES system from Agilent (1100 LC/MSD Trap mass spectrometer
166 VL) with a C18 Prontosil 120-5-C18-AQ reversed-phase column (250 \times 4 mm, 5 μm ; Bischoff
167 Chromatography, Germany). The elution of the reaction samples was carried out at room
168 temperature and at a flow rate of 1 ml min⁻¹ using a gradient that was derived from two eluent
169 mixtures (Table 1). The products were detected and quantified by differential refractometry
170 and UV detection at 210 nm. Quantification was performed against external calibration lines
171 prepared using the appropriate acylated products as standards. These standards were
172 synthesized using operating conditions in which only a specific standard could be formed
173 using a given acyl donor, then purified and structurally characterized. Low-resolution mass
174 spectral analyses were obtained by electrospray in the positive detection mode. Nitrogen was
175 used as the drying gas at 15 l min⁻¹, 350 °C and at a nebulizer pressure of 4 bars. The scan
176 range was 50–1000 m/z using five averages and 13,000 m/z per second resolution. The
177 capillary voltage was 4000 V. Processing was done offline using HP Chemstation software.

178 **Table 1**

179 *2.4. Purification and characterization of reaction products*

180 The reaction products were purified with a preparative HPLC system from Agilent (1200
181 LC/MSD) using a ProntoPrep C18 reversed-phase column (250 \times 20 mm, 10 μm ; Bischoff

182 Chromatography, Germany) eluted according to the gradient given in Table 1, at room
183 temperature and at a flow rate of 5 ml min⁻¹. The purified products were then characterized by
184 ¹H NMR and infrared (IR) spectroscopy. The ¹H NMR chemical shift values were recorded
185 on a JEOL-JNM LA400 spectrometer (400 MHz), with tetramethylsilane as an internal
186 reference. The samples were studied as solutions in CDCl₃. IR spectra were recorded from
187 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹ using a 100 ATR spectrometer (Perkin-Elmer,
188 United States).

189 2.4.1. *N*-stearyl 3-amino-1,2-propanediol **3a**

190 m/z (LR-ESI⁺) C₂₁H₄₄NO₃ (M + H⁺), found: 358.2, calculated for: 358.58. IR ν_{max} (cm⁻¹): 3312 (O-
191 H, alcohol and N-H, amide), 2800-3000 (CH of stearyl chain), 1633 (C=O, amide), 1544 (N-H,
192 amide). ¹H NMR (400 MHz, CDCl₃, δ ppm): δ 0.88 (t, 3H, *J*= 6.03Hz, -CH₂-CH₃), 1.25 (m, 28H, -
193 CH₂- of stearyl chain), 1.63 (m, 2H, -CH₂-CH₂-CO-NH- of stearyl chain), 2.21 (t, 2H, *J*= 7.57Hz, -
194 CH₂-CH₂-CO-NH- of stearyl chain), 3.42 (m, 2H, -CH-CH₂-OH), 3.54 (m, 2H, -CH-CH₂-NH-), 3.75
195 (m, 1H, -CH-), 5.84 (s, 1H, -NH-).

196 2.4.2. *N*-myristyl 3-amino-1,2-propanediol **3b**

197 m/z (LR-ESI⁺) C₁₇H₃₆NO₃ (M + H⁺), found: 302.1, calculated for: 302.47. IR ν_{max} (cm⁻¹): 3298 (O-
198 H, alcohol and N-H, amide), 2800-3000 (CH of myristyl chain), 1634 (C=O, amide), 1546 (N-H,
199 amide). ¹H NMR (400 MHz, CDCl₃, δ ppm): δ 0.88 (t, 3H, *J*= 6.55Hz, -CH₂-CH₃), 1.25 (m, 20H, -
200 CH₂- of myristyl chain), 1.63 (m, 2H, -CH₂-CH₂-CO-NH- of myristyl chain), 2.21 (t, 2H, *J*= 8Hz, -
201 CH₂-CH₂-CO-NH- of myristyl chain), 3.42 (m, 2H, -CH-CH₂-OH), 3.56 (m, 2H, -CH-CH₂-NH-), 3.76
202 (m, 1H, -CH-), 5.88 (s, 1H, -NH-).

203 2.4.3. *N*-lauryl 3-amino-1,2-propanediol **3c**

204 m/z (LR-ESI⁺) C₁₅H₃₂NO₃ (M + H⁺), found: 274.2, calculated for: 274.43. IR ν_{max} (cm⁻¹): 3307 (O-
205 H, alcohol and N-H, amide), 2800-3000 (CH of lauryl chain), 1631 (C=O, amide), 1545 (N-H, amide).
206 ¹H NMR (400 MHz, CDCl₃, δ ppm): δ 0.88 (t, 3H, *J*= 7Hz, -CH₂-CH₃), 1.26 (m, 16H, -CH₂- of lauryl

207 chain), 1.62 (m, 2H, $-\underline{\text{CH}}_2\text{-CH}_2\text{-CO-NH-}$ of lauryl chain), 2.23 (t, 2H, $J= 7.23\text{Hz}$, $-\text{CH}_2\text{-}\underline{\text{CH}}_2\text{-CO-NH-}$
208 of lauryl chain), 3.43 (m, 2H, $-\text{CH-}\underline{\text{CH}}_2\text{-OH}$), 3.56 (m, 2H, $-\text{CH-}\underline{\text{CH}}_2\text{-NH-}$), 3.76 (m, 1H, $-\text{CH-}$), 5.92
209 (s, 1H, $-\text{NH-}$).

210 2.4.4. *N-oleyl 3-amino-1,2-propanediol 3d*

211 m/z (LR-ESI⁺) $\text{C}_{21}\text{H}_{42}\text{NO}_3$ ($\text{M} + \text{H}^+$), found: 356.2, calculated for: 356.57. IR ν_{max} (cm^{-1}): 3342 (O-
212 H, alcohol and N-H, amide), 2800-3000 (CH of oleyl chain), 1632(C=O, amide), 1546 (N-H, amide).
213 ¹H NMR (400 MHz, CDCl_3 , δ ppm): δ 0.88 (t, 3H, $J= 6.55\text{Hz}$, $-\text{CH}_2\text{-}\underline{\text{CH}}_3$), 1.27 (m, 12H, $\text{CH-CH}_2\text{-}$
214 $\underline{\text{CH}}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_3$ of oleyl chain), 1.31 (m, 8H, $\text{CH-CH}_2\text{-}\underline{\text{CH}}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$
215 $\text{CH}_2\text{-CO-NH}$ of oleyl chain), 1.64 (m, 2H, $-\underline{\text{CH}}_2\text{-CH}_2\text{-CO-NH-}$ of oleyl chain), 2.01 (m, 4H, $-\underline{\text{CH}}_2\text{-}$
216 $\text{CH=CH-}\underline{\text{CH}}_2\text{-}$ of oleyl chain), 2.22 (t, 2H, $J= 7.24\text{Hz}$, $-\text{CH}_2\text{-}\underline{\text{CH}}_2\text{-CO-NH-}$ of oleyl chain), 3.41 (m,
217 2H, $-\text{CH-}\underline{\text{CH}}_2\text{-OH}$), 3.53 (m, 2H, $-\text{CH-}\underline{\text{CH}}_2\text{-NH-}$), 3.72 (m, 1H, $-\text{CH-}$), 5.34 (m, 2H, $-\text{CH}_2\text{-}\underline{\text{CH}}=\underline{\text{CH-}}$
218 $\text{CH}_2\text{-}$ of oleyl chain), 5.94 (s, 1H, $-\text{NH-}$).

219 2.4.5. *N-linoleyl 3-amino-1,2-propanediol 3e*

220 m/z (LR-ESI⁺) $\text{C}_{21}\text{H}_{40}\text{NO}_3$ ($\text{M} + \text{H}^+$), found: 354.1, calculated for: 354.56. IR ν_{max} (cm^{-1}): 3303 (O-
221 H, alcohol and N-H, amide), 2800-3000 (CH of linoleyl chain), 1634 (C=O, amide), 1548 (N-H,
222 amide).

223 2.4.6. *1-O-myristyl,3-N-stearyl 3-amino-1,2-propanediol 4*

224 m/z (LR-ESI⁺) $\text{C}_{35}\text{H}_{70}\text{NO}_4\text{Na}$ ($\text{M} + \text{Na}^+$), found: 590.2, calculated for: 590.94. IR ν_{max} (cm^{-1}): 3651
225 (O-H, alcohol), 3200-3400 (O-H, alcohol and N-H, amide), 2800-3000 (CH of stearyl and myristyl
226 chains), 1720 (C=O, ester), 1650 (C=O, amide), 1546 (N-H, amide). ¹H NMR (400 MHz, CDCl_3 , δ
227 ppm): δ 0.88 (t, 6H, $J= 6.3\text{Hz}$, 2x $-\text{CH}_2\text{-}\underline{\text{CH}}_3$), 1.25 (m, 48H, $-\text{CH}_2\text{-}$ of stearyl and myristyl chains),
228 1.62 (m, 4H, 2x $-\underline{\text{CH}}_2\text{-CH}_2\text{-CO-}$ of stearyl and myristyl chains), 2.21 (t, 2H, $J= 7.11\text{Hz}$, $-\text{CH}_2\text{-}\underline{\text{CH}}_2\text{-}$
229 CO-O- of myristyl chain), 2.34 (t, 2H, $J= 7.78\text{Hz}$, $-\text{CH}_2\text{-}\underline{\text{CH}}_2\text{-CO-NH-}$ of stearyl chain), 3.53 (dd, 1H,
230 $J= 4.88\text{Hz}$, $J= 14.15\text{Hz}$, $-\text{CH-}\underline{\text{CH}}_2\text{-NH-}$), 3.56 (dd, 1H, $J= 4.88\text{Hz}$, $J= 14.15\text{Hz}$, $-\text{CH-}\underline{\text{CH}}_2\text{-NH-}$), 3.94

231 (m, 1H, -CH-), 4.05 (dd, 1H, $J = 5.49\text{Hz}$, $J = 10.98\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{O}-$), 4.15 (dd, 1H, $J = 5.12\text{Hz}$, $J =$
232 11.46Hz , $-\text{CH}-\underline{\text{CH}}_2-\text{O}-$), 5.95 (t, 1H, $J = 5.2\text{Hz}$, -NH-).

233 3. Results and discussion

234 The continuous enzymatic synthesis of 1-*O*,3-*N*-diacyl 3-amino-1,2-propanediol-type pseudo-
235 ceramides catalyzed by immobilized *Candida antarctica* lipase B (Novozym[®] 435) was
236 conducted in a packed-bed bioreactor system (Scheme 1, Fig. 1) in two steps. *N*-acyl 3-amino-
237 1,2-propanediol (amide) was obtained from the *N*-acylation of 3-amino-1,2-propanediol **1** in
238 the first step (step 1). In the second step (step 2), 1-*O*,3-*N*-diacyl 3-amino-1,2-propanediol
239 (pseudo-ceramide) was then produced from the *O*-acylation of the *N*-acyl 3-amino-1,2-
240 propanediol (amide) synthesized in step 1. In order to promote both the synthesis and the
241 solubility of the products, all the reactions were carried out at 55°C.

242 A *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) was chosen as the reaction solvent on the
243 basis of previous work that demonstrated the capacity of these two solvents to promote the
244 selective Novozym[®] 435-catalyzed synthesis of amide and amido-ester products starting from
245 various amino-alcohols as substrates [33].

246 Regarding the choice of the appropriate acyl donors to use at each step of the process, we
247 decided first to base our selection on the structure of natural ceramides, which are mostly
248 composed of long-chain saturated fatty acids. C18:0 fatty acids are indeed one of the most
249 abundant fatty acids incorporated in the natural ceramides located in the outer layer of the
250 skin, namely the *stratum corneum* [34–36]. For this reason we chose stearic acid **2a** as the
251 first acyl donor for step 1 (*N*-acylation). Myristic acid **2b**, on the other hand, was chosen as
252 the second acyl donor for step 2 (*O*-acylation) to mimic the structure of the sphingoid bases
253 found in natural ceramides from human skin (18 carbons for the most common sphingoid
254 bases) [34–37]. To achieve this, the C14 carbon chain of myristic acid **2b** was conjugated to

255 the C3 carbon chain of 3-amino-1,2-propanediol **1** via an ester bond, giving a chain of 18
256 atoms with 17 carbons and 1 oxygen.

257 In a preliminary study, the two reactions were conducted under stoichiometric conditions
258 using a substrate concentration of 100 mM at a flow rate of 250 $\mu\text{l min}^{-1}$ for step 1, and a
259 substrate concentration of 50 mM at a flow rate of 125 $\mu\text{l min}^{-1}$ for step 2. Two stainless steel
260 columns, one 95 mm in length with an inner diameter of 5 mm, the other 145 mm in length
261 with an inner diameter of 5 mm, were packed with 430 and 875 mg of Novozym[®] 435 to
262 constitute the catalytic beds for steps 1 and 2, respectively. After production under these non-
263 optimized conditions and purification, the products of each step were analyzed by IR and
264 NMR spectroscopy. It was thus demonstrated that *N*-stearyl-3-amino-1,2-propanediol (amide
265 **3a**) was selectively produced at step 1 with a 76 % yield and a production rate of 2.65 mmol
266 $\text{h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ (948 $\text{mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$), while 1-*O*-myristyl,3-*N*-stearyl 3-amino-1,2-
267 propanediol (amido-ester **4**) was produced at step 2, also selectively, with a 24 % yield and a
268 production rate of 0.1 mmol $\text{h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ (58 $\text{mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$). Indeed, no secondary
269 product was detected for both steps. These results confirmed that step 1 is exclusively
270 chemoselective for the *N*-acylation of 3-amino-1,2-propanediol while step 2 is regioselective
271 for the *O*-acylation of the primary alcohol function in position 1. This corroborates the results
272 obtained in a preliminary study which demonstrated the same selectivity for the two steps of
273 the same process performed in a batch bioreactor (data not shown). Furthermore, these results
274 are also in agreement with data already published, regarding the Novozym[®] 435-catalyzed
275 acylation of substrates structurally related to 3-amino-1,2-propanediol, carried out in similar
276 organic solvents. These works were performed in a batch bioreactor using myristic acid as the
277 acyl donor. First, the acylation of alaninol (2-amino-1-propanol) demonstrated the
278 chemoselectivity for the *N*-acylation, with the production of 2-*N*-myristyl 2-amino-1-propanol
279 only [27, 28], which is similar to the results obtained at step 1 of the continuous process.

280 Secondly, the *O*-acylation of 1,2-propanediol was regioselective for the primary alcohol
281 function in position 1 [28].

282 All these preliminary results showed that the selectivity of both steps of the process does not
283 need to be controlled during its implementation. Nevertheless, despite being encouraging in
284 terms of yield and production rate, they were not satisfying enough to envisage scaling up the
285 process. Starting from this fact, we thus concentrated our efforts on optimizing both steps of
286 the process. For that purpose, the influences of feed flow rate, quantity of biocatalyst,
287 substrate concentration and substrate molar ratio were examined. These parameters are likely
288 to have a significant effect on the yield and productivity of a continuous enzymatic process.

289 *3.1. Optimization of the process*

290 *3.1.1. Effect of feed flow rate*

291 The feed flow rate plays an essential role in the continuous operation because it is related to
292 the residence time of the substrates and products in the column. In order to achieve a higher
293 synthesis yield for each step of the process, a sufficient residence time is needed to ensure that
294 the substrate is interacting with the enzyme's active site. We thus examined the effect of feed
295 flow rate on both synthesis yield and production rate (Fig. 2).

296 **Fig. 2**

297 During the first step, the flow rate was varied from 125 to 1000 $\mu\text{l min}^{-1}$ (Fig. 2A). The amide
298 **3a** yield was relatively constant and close to 80% from 125 to 500 $\mu\text{l min}^{-1}$. In parallel, the
299 amide **3a** production rate was shown to increase to a maximum value close to 6 mmol h^{-1}
300 $\text{g}_{\text{biocatalyst}}^{-1}$ (2145 $\text{mg h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$). On the other hand, the amide yield and production
301 decreased to 37% and 5.2 $\text{mmol h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$ at a flow rate of 1000 $\mu\text{l min}^{-1}$. These results
302 could be explained by the reduction in the substrate residence time within the packed-bed
303 bioreactor, which was very likely caused by the increase in flow rate. Thus, at 1000 $\mu\text{l min}^{-1}$,

304 the residence time was probably not sufficient for the *N*-acylation reaction to reach
305 thermodynamic equilibrium, which resulted in a lower yield. From these results, 500 $\mu\text{l min}^{-1}$
306 was considered as the optimum flow rate for step 1.

307 During the second step, the flow rate was varied from 125 to 500 $\mu\text{l min}^{-1}$ (Fig. 2B). Again, a
308 relatively constant yield of pseudo-ceramide **4** of roughly 25% was obtained using flow rates
309 within this range, with a maximum yield of 30% at a flow rate of 250 $\mu\text{l min}^{-1}$. The reduction
310 in the substrate residence time in the packed-bed bioreactor caused by the increase in the flow
311 rate thus had no effect on the yield, as was already observed for the first step. In contrast, the
312 production rate was shown to increase in conjunction with the faster flow rate, reaching a
313 maximum value of 0.38 $\text{mmol h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$ at a flow rate of 500 $\mu\text{l min}^{-1}$. However, this flow
314 rate gave the lowest yield (22%). For this reason 250 $\mu\text{l min}^{-1}$ was taken as a compromise
315 optimum flow rate value to achieve both the higher yield of 30% and a good production rate
316 of 0.26 $\text{mmol h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$ (148 $\text{mg h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$) in the second step.

317 3.1.2. Effect of the quantity of biocatalyst

318 The effect of the quantity of biocatalyst on both yield and production was investigated using
319 various quantities of Novozym[®] 435 packed into the packed-bed continuous reactor (Fig. 3).

320 **Fig. 3**

321 During the first step, the quantity of biocatalyst was varied from 215 to 1800 mg (Fig. 3A).
322 The lowest biocatalyst quantity of 215 mg resulted in the lowest amide **3a** yield obtained in
323 this study (17%). Starting from this value, the amide yield increased as a function of the
324 quantity of biocatalyst rising to 87% for 875 mg of Novozym[®] 435. Nevertheless, when the
325 quantity of biocatalyst was doubled (1800 mg), the amide **3a** yield did not exceed 85%. From
326 these results, we concluded that the thermodynamic equilibrium of the reaction was already
327 attained at 875 mg of biocatalyst. In parallel, amide **3a** production dramatically increased

328 within the range 215-430 mg, rising to $1.38 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ ($493 \text{ mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$),
329 whereas the yield did not exceed 79% and thermodynamic equilibrium was not reached. The
330 optimum quantity of biocatalyst for this step thus seems to be 875 mg because this represents
331 the best compromise between a high amide yield of 87% and the low cost of Novozym[®] 435,
332 despite the non-optimal production rate.

333 During the second step, the quantity of biocatalyst was varied from 430 to 2700 mg (Fig. 3B).
334 There was a degree of similarity in terms of the change in both the yield and the production
335 rate of pseudo-ceramide **4** and amide **3a**. The yield of pseudo-ceramide **4** increased to 24%
336 when the quantity of biocatalyst was increased from 430 mg to 875 mg but it did not exceed
337 25% when the quantity of Novozym[®] 435 was doubled (1800 mg). From these results we
338 concluded that the thermodynamic equilibrium of the reaction had already been reached at
339 875 mg of biocatalyst, as highlighted for the first step of *N*-acylation. In parallel, the
340 production rate of pseudo-ceramide **4** continuously decreased as the quantity of biocatalyst
341 was increased, falling from an initial rate of $0.18 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ ($102 \text{ mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$)
342 to barely $0.02 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ for a 16% yield with 2700 mg of Novozym[®] 435. This loss
343 of both yield and productivity may be explained by the fact that step 2 of pseudo-ceramide
344 synthesis consists in a reverse hydrolysis and is consequently accompanied by the production
345 of water molecules that gradually accumulate in the reaction medium. So, by increasing the
346 amount of biocatalyst, a greater quantity of synthesis product (pseudo-ceramide) and water
347 molecules is produced which are then in contact with the biocatalyst, resulting in competition
348 between the pseudo-ceramide hydrolysis reactions. For this reason the decrease in both the
349 yield and the production rate of pseudo-ceramide **4**, observed when using a large quantity of
350 immobilized lipase, may indicate that pseudo-ceramide hydrolysis is under thermodynamic
351 control while pseudo-ceramide synthesis is under kinetic control. An increase in the quantity

352 of biocatalyst would then promote the thermodynamic reaction, i.e. hydrolysis, to the
353 detriment of the synthesis.

354 To complete this part of the study, it is noteworthy that the optimum quantity of biocatalyst
355 for steps 1 and 2 was 875 mg, which represented the best compromise that comprised a high
356 synthesis yield (87% amide **3a** synthesis and 24% pseudo-ceramide **4** synthesis), an average
357 production rate and a lower cost of Novozym[®] 435.

358 3.1.3. Effect of substrate concentration

359 The effect of substrate concentration on both synthesis yield and production rate was
360 investigated using various concentrations of acyl acceptor and acyl donor under
361 stoichiometric conditions (Fig. 4). The results could not be interpreted when the substrate
362 concentration was higher than 100 mM due to the turbidity of the reaction mixture. This
363 resulted in a partial solubility of the amphiphilic amide **3a** produced in step 1, or used as a
364 substrate in step 2 in the *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) reaction solvent.
365 Indeed this partial substrate solubility caused plugging problems in the packed-bed bioreactor,
366 which precluded the development of a continuous process under these conditions.

367 **Fig. 4**

368 The use of substrate concentrations below 100 mM during the first step appeared to have very
369 little impact on the yield of amide **3a**, which had an average value of 82% ($\pm 5\%$). However,
370 the production rate of amide **3a** significantly and continuously increased in conjunction with
371 the increase in substrate concentration, reaching $0.75 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ (268 mg h^{-1}
372 $\text{g}_{\text{biocatalyst}}^{-1}$) at 100 mM of amino-diol **1** and fatty acid **2a**. Based on these results the amide
373 production rate seemed to depend directly on the substrate concentration, while the yield was
374 constant. Besides, 100 mM is without contest the optimum substrate concentration as it

375 corresponds to the highest concentration that could be used and didn't involve any problems
376 with partial substrate solubility.

377 During the second step, the yield of pseudo-ceramide **4** followed a bell-shaped curve,
378 reaching the best yield of 24% at 50 mM of substrate but decreasing to 12 and 17% for
379 substrate concentrations of 25 and 100 mM, respectively. The decrease in yield for the lowest
380 substrate concentrations can be explained by a dilution of the substrates in the reaction
381 medium. The decrease in yield for the highest substrate concentrations, however, is probably
382 due to the decrease in enzyme/substrate ratio occurring in the catalytic bed when the substrate
383 concentration is increased. Indeed, the thermodynamic equilibrium of the reaction may not be
384 reached if this ratio is too low, and this could lead to a decrease in yield. Furthermore, the
385 production rate of pseudo-ceramide **4** appeared to increase from 0.02 to 0.15 mmol h⁻¹
386 g_{biocatalyst}⁻¹ when the substrate concentration was increased from 25 to 75 mM. However, this
387 rate was not enhanced by further increasing substrate concentration to 100 mM i.e. the
388 increase in substrate concentration did not compensate for the low yield obtained at this
389 concentration. So, in contrast to what was previously described for amide **3a** synthesis at step
390 1, the production rate at step 2 seems to depend on both substrate concentration and synthesis
391 yield.

392 To conclude, 75 mM was the optimum substrate concentration at step 2 for the simple reason
393 that it provided the best compromise between a pseudo-ceramide yield close to the maximum
394 (23%) and an optimum production rate of 0.15 mmol h⁻¹ g_{biocatalyst}⁻¹ (85 mg h⁻¹ g_{biocatalyst}⁻¹).
395 Nevertheless, despite the high production rate obtained, these results were not satisfying
396 enough in terms of pseudo-ceramide yield and we consequently decided to optimize our
397 process by varying the substrate molar ratio in order to improve the yield in step 2.

398 *3.1.4. Effect of substrate molar ratio*

399 The effect of substrate molar ratio on both the synthesis yield and the production rate of
400 pseudo-ceramide **4** (step 2) was investigated using various myristic acid **2b** concentrations
401 and a fixed *N*-stearyl 3-amino-1,2-propanediol **3a** concentration of 50 mM. The effect of
402 increasing the amide **3a** concentration was not tested due to the low solubility of this
403 compound above 50 mM and at 55°C in the *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v)
404 reaction solvent. The substrate molar ratio of fatty acid **2b** to amide **3a** was varied within the
405 range 1-5 (Fig. 5).

406 **Fig. 5**

407 Starting from values of 24% and $0.1 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ at a molar ratio of 1, the synthesis
408 yield and production rate of pseudo-ceramide **4** were shown to increase concomitantly with
409 the molar ratio, reaching 53% and $0.22 \text{ mmol h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$ ($125 \text{ mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$),
410 respectively, at a molar ratio of 3. This was the optimum value since a further increase in
411 substrate molar ratio led to a fall in the values of these parameters to levels close to those
412 obtained at a substrate molar ratio of 1. These results are very similar to those described by
413 Xu et al. with lipase-catalyzed interesterification reactions between triglycerides of rapeseed
414 oil and capric acid, which demonstrated that the substrate molar ratio has a double function: a
415 higher concentration of the acyl acceptor will push the reaction equilibrium toward the
416 synthesis reaction and cause an increase in the theoretical maximum product yield, whereas a
417 higher free fatty acid content will increase the possibility of an inhibition effect and require a
418 longer reaction time to reach equilibrium [38]. Nevertheless, the results are interesting since
419 the pseudo-ceramide synthesis yield was enhanced by a factor of 2 compared to all the
420 previous results, and there was no decrease in the production rate.

421 Based on these encouraging results, we tested the best operational conditions identified so far:
422 the flow rate was (only) doubled to $250 \text{ } \mu\text{l min}^{-1}$, and we chose a substrate molar ratio of

423 myristic acid **2b** (150 mM) to *N*-stearyl 3-amino-1,2-propanediol **3a** (50 mM) of 3, a stainless
424 steel column 145 mm in length with a 5 mm inner diameter packed with 875 mg of
425 Novozym[®] 435 to constitute the catalytic bed. Under these optimized conditions, pseudo-
426 ceramide **4** was still produced with a yield of 54% but the production rate was doubled,
427 reaching 0.46 mmol h⁻¹ g_{biocatalyst}⁻¹ (261 mg h⁻¹ g_{biocatalyst}⁻¹).

428 To complete the study we wanted to scale-up our process. We thus decided to test various
429 acyl donors in the first stage to evaluate the possibility that our process could be used for the
430 synthesis of different pseudo-ceramides. The stability of Novozym[®] 435, which was an
431 essential condition prior to considering any further scale-up, was also investigated.

432 *3.2. Scale up of the process*

433 *3.2.1. Variation of the acyl donor nature*

434 In this part, the nature of the acyl donor was varied and evaluated at step 1 of the process. *N*-
435 acylation of 3-amino-1,2-propanediol was thus performed to compare five acyl donors, three
436 saturated fatty acids of various chain length (C12-C18) and two unsaturated C18 fatty acids.
437 The conditions previously optimized in terms of feed flow rate, substrate concentration,
438 quantity of biocatalyst and bioreactor design were used in the process. Fig. 6 shows the yields
439 of *N*-stearyl-, *N*-myristyl-, *N*-lauryl-, *N*-oleyl- and *N*-linoleyl-3-amino-1,2-propanediol
440 (amides **3a**, **3b**, **3c**, **3d** and **3e**, respectively) obtained after continuous Novozym[®]-435-
441 catalyzed *N*-acylation of 3-amino-1,2-propanediol **1** using stearic acid **2a**, myristic acid **2b**,
442 lauric acid **2c**, oleic acid **2d** and linoleic acid **2e** as acid donors, respectively.

443 **Fig. 6**

444 We observed that the yields obtained with saturated fatty acids **2a**, **2b** and **2c** ranged from
445 87% with lauric acid **2c** to 95% with myristic acid **2b**, which indicated that acyl chain length

446 had no significant effect on the amide yield. In addition, the use of unsaturated fatty acids **2d**
447 (C18:1) and **2e** (C18:2) gave yields of 85% and 80%, respectively. These results were barely
448 lower than the yield of 92% obtained using a saturated C18 fatty acid, stearic acid **2a**. Thus,
449 the presence of one or two unsaturations on the carbon chain of the acyl donor did not appear
450 to have a significant influence on the amide yield. To conclude this part of the study, an amide
451 yield superior or equal to 80% was obtained with every fatty acid used as an acyl donor at
452 step 1. Furthermore, this amide yield was shown to correspond to a mass production of amide
453 that was higher than $800 \text{ mg h}^{-1} \text{ g}^{-1}$. From these results, it would clearly be feasible to produce
454 a range of differently functionalized pseudo-ceramides with high yields starting from any of
455 the five fatty acids tested in order to obtain compounds with various properties and
456 applications.

457 3.2.2. Stability of Novozym[®] 435

458 The operational stability of immobilized *Candida antarctica* lipase B (Novozym[®] 435) in the
459 continuous packed-bed bioreactor was studied over a 3-week period, during which the
460 continuous *N*-acylation of 3-amino-1,2-propanediol **1** was carried out using lauric acid **2c** as
461 the acyl donor (Fig. 7).

462 **Fig. 7**

463 Novozym[®] 435 was found to be highly stable under these conditions since no decrease was
464 observed in *N*-lauryl 3-amino-1,2-propanediol **3c** yield after twenty-two days, with an average
465 yield of $91\% \pm 3\%$; the productivity was of the order of 113 g of amide per g of Novozym[®]
466 435. This high stability may be partly related to the reaction solvent used. Indeed, water is
467 produced during a reverse hydrolysis reaction so controlling water activity will consequently
468 be of great importance, especially in a continuous process. According to the literature, a polar
469 solvent such as *tert*-amyl alcohol can be used to control water activity in a continuous

470 acylation process [39, 40]. The *tert*-amyl alcohol polarity would thus enable the water
471 produced to be evacuated, resulting in a partial drying of the immobilized lipase. As a result,
472 optimal water activity would be maintained inside the reactor and optimum enzymatic activity
473 would remain stable for a long time.

474 The excellent stability of Novozym[®] 435 in the continuous packed-bed bioreactor allowed us
475 to envisage further large scale pseudo-ceramide production given that the cost of the
476 biocatalyst would not be a limiting factor.

477 3.2.3. Scale up of the bioreactor design

478 In order to perform a future scale-up of the packed-bed bioreactor to a pilot scale, the
479 influence of reactor design on the yield and production rate of pseudo-ceramide **4** (step 2) was
480 studied using two stainless steel columns of different geometries: column A was 125 mm in
481 length with a 10 mm inner diameter and column B was 5 mm in length with a 50 mm inner
482 diameter. Both columns were packed with 3300 mg of Novozym[®] 435 to constitute the
483 catalytic bed, which was roughly a four-fold scale up in terms of the optimized quantity of
484 875 mg of biocatalyst determined at the laboratory scale. In both cases, the flow rate was
485 varied from 100 to 1200 $\mu\text{l min}^{-1}$ to change the residence time of the substrates (Fig. 8).

486 **Fig. 8**

487 It is interesting to note that optimum pseudo-ceramide **4** yields of close to 30% were obtained
488 in both cases at different flow rates, depending on the type of column used. Thus, the optimal
489 yield was obtained for column A at a flow rate of 800 $\mu\text{l min}^{-1}$ (residence time of 12.5
490 minutes), which corresponded to the highest production of 0.23 $\text{mmol h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$ (131 mg
491 $\text{h}^{-1} \text{g}_{\text{biocatalyst}}^{-1}$), and the optimal yield was obtained for column B at a flow rate of 200 $\mu\text{l min}^{-1}$
492 (residence time of 50 minutes), which corresponded to a production rate of only 0.05 mmol h^{-1}

493 $^1 \text{g}_{\text{biocatalyst}}^{-1}$ ($28 \text{ mg h}^{-1} \text{ g}_{\text{biocatalyst}}^{-1}$). These results demonstrate that the use of a column with a
494 large diameter and a short length, such as column B, does not improve productivity.

495 In an enzymatic packed-bed bioreactor, two transport phenomena occur. The first involves the
496 transfer of the substrate from the bulk liquid phase to the surface of the immobilized
497 biocatalyst as a result of the formation of a fictitious laminar film. The second is the
498 simultaneous diffusion of the substrate and its reaction within the biocatalyst particles.
499 Internal diffusion limitations within porous carriers indicate that the slowest step is the
500 penetration of the substrate into the interior of the catalyst particle. On the other hand,
501 external mass transfer limitations occur if the rate of transport by diffusion through the
502 laminar film is rate limiting [41]. According to the literature, external mass transfer in packed-
503 bed reactors can be improved by decreasing linear velocity, which is generally enhanced by
504 decreasing the flow rate of the substrate or by changing the column reactor length-to-diameter
505 ratio (L/d) [42–45]. In this work, for a given flow rate of $800 \mu\text{l min}^{-1}$, linear velocity values
506 of 17 and 0.7 mm s^{-1} were obtained for columns A ($L/d = 12.5$) and B ($L/d = 0.1$),
507 respectively. Thus, the very low linear velocity obtained for column B under these conditions
508 increased the risk of external mass transfer limitation, which most likely explains the low
509 yield obtained for column B (17%) compared to column A (linear velocity 24 times higher
510 than column B). Moreover, as described above, when we used a 145 mm long column with a
511 5 mm inner diameter, the optimal yield was obtained at a flow rate of $250 \mu\text{l min}^{-1}$ (see
512 section 3.1.1), giving a linear velocity of 21 mm s^{-1} . Interestingly, this is of the same order as
513 the value obtained for column A (17 mm s^{-1}) and confirms that a high linear velocity is
514 needed to minimize external mass transfer limitation and favor synthesis.

515 These results show that it is essential to use a long column with a small diameter such as
516 column A (125 mm in length and 10 mm inner diameter) or the column used in other parts of
517 this work (145 mm in length and 5 mm inner diameter). These columns both have a L/d ratio

518 within the range 12.5-29, which for this reason could be taken as an optimum L/d reference
519 range to maintain an optimum yield and productivity in our continuous process. In addition, it
520 is also necessary to have an adequate flow rate that produces a sufficiently high linear velocity
521 (close to 20 mm s⁻¹) to facilitate external mass transfer.

522 3.2.4. Economic evaluation of the process

523 The final objective of this work was to perform an economic evaluation of our continuous
524 process under the optimal synthesis conditions for the two steps of the process. The economic
525 viability of an enzymatic synthesis process is determined by several key variables including
526 the manufacturing cost, the environmental cost, and the selling price and marketing cost for
527 the product. The term “manufacturing cost” is used to describe the total costs involved in the
528 manufacture of a synthetic product, which includes the cost of the biocatalyst, the chemicals,
529 the solvents, the equipment, the energy and other operational costs. In our case, we observed
530 the economic impact of three parameters which directly influence the manufacturing cost: the
531 cost of the biocatalyst, the substrates and the organic solvents (reaction solvents and solvents
532 used for the purification of the synthesis products).

533 In order to achieve a better assessment of the economic cost, we drew up a balance sheet of
534 the two steps of the process. Under our optimized experimental conditions used at a 4-fold
535 scale-up, an amide yield of 90% and a production rate of 1821 mg h⁻¹ were obtained at step 1
536 (*N*-acylation) using 3300 mg of biocatalyst packed into the bioreactor. Assuming a biocatalyst
537 lifespan of 3 weeks, a productivity of 918 g amide was obtained. Similarly, for step 2 (*O*-
538 acylation), a pseudo-ceramide yield of 30% and a production rate of 432 mg h⁻¹ were obtained
539 (see section 3.2.3), which corresponds to a productivity of 218 g of pseudo-ceramide. To
540 evaluate the cost effectiveness of the proposed process, the cost of pseudo-ceramide
541 production was calculated by considering the second step as the limiting step of the process in

542 terms of production and yield. So, given the price of the biocatalyst (Novozym[®] 435), the
543 substrates (3-amino-1,2-propanediol **1**, stearic acid **2a** and myristic acid **2b**) and the solvents
544 (*tert*-amyl alcohol, *n*-hexane and purification solvents), we calculated the cost of producing
545 one kg of pseudo-ceramide under our optimal conditions: 21 € of biocatalyst, 351 € of
546 substrates and 1,422 € of organic solvents. Suppliers quoted prices of about 2000 €/kg for the
547 cheapest synthetic ceramide compounds. In consequence, the cost of the biocatalyst,
548 substrates and organic solvents represent 1%, 18% and 71% of the product price, respectively.
549 The cost of the biocatalyst is usually one of the essential factors of the economic cost of an
550 enzymatic synthesis process due to the high price of biocatalysts (Novozym[®] 435: 1100 €/kg).
551 However, it is noteworthy that the pseudo-ceramide productivity of our continuous process in
552 packed-bed bioreactor ($69 \text{ g}_{\text{pseudo-ceramide}} \text{ g}_{\text{biocatalyst}}^{-1}$) was approximately 5-fold higher than the
553 results obtained in a process already developed for the synthesis of pseudo-ceramides in a
554 batch bioreactor ($15 \text{ g}_{\text{pseudo-ceramide}} \text{ g}_{\text{biocatalyst}}^{-1}$) [26], which shows that this method greatly
555 reduces the economic cost of the biocatalyst. These results are thus encouraging in terms of
556 the future development of this continuous process on a pilot scale but also demonstrate the
557 need to recover and reuse the organic solvents as this could potentially have a significant
558 impact on the cost effectiveness. Moreover, the production of pseudo-ceramides with a purity
559 close to 99%, like some commercial ceramides, would require the development of a
560 purification method applicable on a large scale, such as liquid extraction or low pressure
561 liquid chromatography.

562 **4. Conclusion**

563 In this work, we developed a new efficient continuous process for the selective Novozym[®]
564 435-catalyzed synthesis of pseudo-ceramides, conducted in a packed-bed bioreactor. To our
565 knowledge, only batch bioreactors had indeed been used so far to develop the lipase-catalyzed
566 synthesis of pseudo-ceramides or ceramides [11-13, 27]. Our process involved two steps for

567 the optimization of the selective diacylation of 3-amino-1,2-propanediol **1** conducted in a *tert*-
568 amyl alcohol/*n*-hexane mixture (50:50 v/v), starting from two fatty acids as acyl donors:
569 stearic acid **2a** (step 1) and myristic acid **2b** (step 2).

570 During the first step, the *N*-acylation of 3-amino-1,2-propanediol **1**, the operational conditions
571 of flow rate, quantity of biocatalyst and substrate concentration were optimized and an
572 excellent synthesis yield of 92%, associated with a very good production rate of 3.15 mmol h⁻¹
573 g_{biocatalyst}⁻¹ (1128 mg h⁻¹ g_{biocatalyst}⁻¹) were obtained. During the second step, which involved
574 the *O*-acylation of the *N*-stearyl 3-amino-1,2-propanediol **3a** produced in the first step, we
575 optimized the same operational conditions as in the first step together with the substrate molar
576 ratio. Under the best conditions identified, the desired pseudo-ceramide, i.e. 1-*O*-myristyl,3-
577 *N*-stearyl 3-amino-1,2-propanediol **4**, was produced at a satisfying yield of 54% and a
578 production rate of 0.46 mmol h⁻¹ g_{biocatalyst}⁻¹ (261 mg h⁻¹ g_{biocatalyst}⁻¹).

579 These results clearly demonstrate that this two-step process has great potential for the
580 industrial scale production of *N,O*-diacyl 3-amino-1,2-propanediol-type pseudo-ceramides,
581 and in particular the 1-*O*-myristyl,3-*N*-stearyl 3-amino-1,2-propanediol **4** synthesized in this
582 work. This assumption is first strengthened by the fact that the productivity of pseudo-
583 ceramide synthesis for this process was approximately improved by a factor 5, compared to
584 the results obtained in a process already developed in a batch bioreactor [26]. On the other
585 hand, we have shown that various fatty acids could be used as acyl donors in step 1 of our
586 process, so its use for the synthesis of different pseudo-ceramides can be seriously envisaged.

587 Finally, in order to better assess the economic cost of pseudo-ceramide production we drew
588 up a balance sheet of the two steps of the process at a 4-fold scale-up. So, given the suppliers'
589 quoted prices of about 2000 €/kg for the cheapest synthetic ceramide compounds, the cost of
590 the biocatalyst, substrates and organic solvents used for synthesis and purification represented
591 1%, 18% and 71% of the product price, respectively. These results are encouraging in terms

592 of the future development of this continuous process on a pilot scale, especially at the level of
593 the cost of the biocatalyst (Novozym[®] 435 can operate for more than 3 weeks without a drop
594 in yield during step 1). But they also demonstrate the need to recover and reuse the organic
595 solvents and to work on the development of the purification process as this could potentially
596 have a significant impact on the cost effectiveness.

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602 **References**

- 603 [1] L. Coderch, O. López, A. de la Maza, J.L. Parra, *Am. J. Clin. Dermatol.* 2 (2003) 107–
604 129.
- 605 [2] M. Kerscher, H.C. Korting, M. Schäfer-Korting, *Eur. J. Dermatol.* 1 (1991) 39–43.
- 606 [3] M. Fillet, M. Bentires-Alj, V. Deregowski, R. Greimers, J. Gielen, J. Piette, V. Bours, M.-
607 P. Merville, *Biochem. Pharmacol.* 10 (2003) 1633–1642.
- 608 [4] H. Garg, N. Francella, K.A. Tony, L.A. Augustine, J.J. Barchi Jr, J. Fantini, A. Puri, D.R.
609 Mootoo, R. Blumenthal, *Antiviral Res.* 1 (2008) 54–61.
- 610 [5] J.-F. Molina, *Househ. Pers. Care Today.* (2008) 12–15.
- 611 [6] S.H. Cho, L.J. Frew, P. Chandar, S.A. Madison, US Patent 5,476,671 A (1995).
- 612 [7] M. Philippe, D. Semeria, European Patent 884,305 A1 (1998).
- 613 [8] J.W.H. Smeets, P.G. Weber, US Patent 5,631,356 A (1997).
- 614 [9] H.-J. Ha, M.C. Hong, S.W. Ko, Y.W. Kim, W.K. Lee, J. Park, *Bioorg. Med. Chem. Lett.*
615 7 (2006) 1880–1883.
- 616 [10] E. Rochlin, US Patent CA2,373,286A1 (2000).
- 617 [11] M. Bakke, M. Takizawa, T. Sugai, H. Ohta, *J. Org. Chem.* 20 (1998) 6929–6938.
- 618 [12] L. Couturier, D. Taupin, F. Yvergnaux, *J. Mol. Catal. B Enzym.* 1 (2009) 29–33.
- 619 [13] R.M. De Pater, J.W.J. Lambers, J.W.H. Smeets, US Patent 561,004,0 A (1997).
- 620 [14] M.J. Haas, P.S. Fox, T.A. Foglia, *Eur. J. Lipid Sci. Technol.* 2 (2011) 168–179.
- 621 [15] M. Kapoor, M.N. Gupta, *Process Biochem.* 4 (2012) 555–569.
- 622 [16] B.M. Nestl, B.A. Nebel, B. Hauer, *Curr. Opin. Chem. Biol.* 2 (2011) 187–193.
- 623 [17] D. Sharma, B. Sharma, A.K. Shukla, *Biotechnology.* 1 (2011) 23–40.
- 624 [18] A. Tomin, G. Hornyánszky, K. Kupai, Z. Dorkó, L. Üрге, F. Darvas, L. Poppe,
625 *Process Biochem.* 6 (2010) 859–865.
- 626 [19] N. Doukyu, H. Ogino, *Biochem. Eng. J.* 3 (2010) 270–282.

- 627 [20] M. Fernández-Pérez, C. Otero, *Enzyme Microb. Technol.* 6 (2001) 527–536.
- 628 [21] M. Fernández-Pérez, C. Otero, *Enzyme Microb. Technol.* 5 (2003) 650–660.
- 629 [22] T. Furutani, M. Furui, H. Ooshima, J. Kato, *Enzyme Microb. Technol.* 8 (1996) 578–
630 584.
- 631 [23] V. Gotor, R. Brieva, F. Rebolledo, *J. Chem. Soc. Chem. Commun.* 14 (1988) 957–
632 958.
- 633 [24] L.T. Kanerva, M. Kosonen, E. Vääntinen, T.T. Huuhtanen, M. Dahlqvist, M.M. Kady,
634 S.B. Christensen, *Acta Chem. Scand.* 11 (1992) 1101–1105.
- 635 [25] O. Torre, V. Gotor-Fernández, V. Gotor, *Tetrahedron Asymmetry.* 5 (2006) 860–866.
- 636 [26] L. Lassalle, F. Yvergnaux, European Patent 20,040,767,243 (2008).
- 637 [27] F. Le Joubioux, Y. Ben Henda, N. Bridiau, O. Achour, M. Graber, T. Maugard, J.
638 *Mol. Catal. B Enzym.* 85-86 (2013) 193–199.
- 639 [28] P.O. Syren, F. Le Joubioux, Y. Ben Henda, T. Maugard, K. Hult, M. Graber, *Chem.*
640 *Cat. Chem.* 5 (2013) 1842–1853.
- 641 [29] T. Takanami, H. Tokoro, D. Kato, S. Nishiyama, T. Sugai, *Tetrahedron Lett.* 46
642 (2005) 3291-3295.
- 643 [30] S.-W. Chang, J.-F. Shaw, C.-K. Yang, C.-J. Shieh, *Process Biochem.* 9 (2007) 1362–
644 1366.
- 645 [31] A. H-Kittikun, W. Kaewthong, B. Cheirsilp, *Biochem. Eng. J.* 1 (2008) 116–120.
- 646 [32] L. Couturier, F. Yvergnaux, *Int. J. Cosmetic Sci.* 31 (2009) 209-224.
- 647 [33] F. Le Joubioux, N. Bridiau, Y. Ben Henda, O. Achour, M. Graber, T. Maugard, J.
648 *Mol. Catal. B Enzym.* (2013) 99–110.
- 649 [34] C. Bijani, PhD Thesis, 2010.
- 650 [35] R. Schnaar, A. Suzuki, P. Stanley, in: A. Varki, R.D. Cummings, J.D. Esko, H.H.
651 Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), *Essentials of*

652 Glycobiology, 2nd edition, Cold Spring Harbor Laboratory Press. New York, 2009, pp.
653 129–142.

654 [36] P.W. Wertz, M.C. Miethke, S.A. Long, J.S. Strauss, D.T. Downing, J. Invest.
655 Dermatol. 5 (1985) 410–412.

656 [37] W. Zheng, J. Kollmeyer, H. Symolon, A. Momin, E. Munter, E. Wang, S. Kelly, J.C.
657 Allegood, Y. Liu, Q. Peng, H. Ramaraju, M.C. Sullards, M. Cabot, A.H. Merrill Jr,
658 Biochim. Biophys. Acta. 12 (2006) 1864–1884.

659 [38] X. Xu, S. Balchen, C.-E. Høy, J. Adler-Nissen, J Am Oil Chem Soc. (1998) 1573–
660 1579.

661 [39] S. Colombié, R.J. Tweddell, J.S. Condoret, A. Marty, Biotechnol. Bioeng. 3 (1998)
662 362–368.

663 [40] W.F. Slotema, G. Sandoval, D. Guieysse, A.J.J. Straathof, A. Marty, Biotechnol.
664 Bioeng. 6 (2003) 664–669.

665 [41] Y.H. Chew, C.T. Lee, M.R. Sarmidi, R.A. Aziz, F. Razali, Food Bioprod. Process. 4
666 (2008) 276–282.

667 [42] A.P. Ison, A.R. Macrae, C.G. Smith, J. Bosley, Biotechnol. Bioeng. 2 (1994) 122–130.

668 [43] Y. Iwasaki, T. Yamane, in: H.W. Gardner and T.M. Kuo (Eds.), Lipid biotechnology,
669 Marcel Dekker, Inc. New York, 2002, pp. 421–422.

670 [44] M. Linde Damstrup, PhD Thesis, 2008.

671 [45] E. Szomanski, Proc. Third Australas. Conf. Hydraul. Fluid Mech. (1970) 117–123.

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675 **Scheme 1.** Two-step process for the selective enzymatic synthesis of 1-*O*,3-*N*-diacyl 3-amino-
676 1,2-propanediol-type pseudo-ceramides catalyzed by Novozym[®] 435 in a packed-bed
677 bioreactor.

678 **Fig. 1.** Experimental setup for the continuous Novozym[®] 435-catalyzed acylation reaction
679 conducted in a packed-bed bioreactor system.

680 **Fig. 2.** Effect of flow rate on the synthesis yield (Δ) and production rate (\bullet) of **amide 3a**
681 (**step 1, A**) and **pseudo-ceramide 4 (step 2, B)**. The reactions were carried out at 55°C in a
682 *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) using substrate concentrations of 100 (A:
683 amino-diol **1** and stearic acid **2a**) and 50 mM (B: amide **3a** and myristic acid **2b**) under
684 stoichiometric conditions. Stainless steel columns 95 mm in length with an inner diameter of
685 5 mm (A), and 145 mm in length with a 5 mm inner diameter (B), were packed with 430 (A)
686 and 875 mg (B) of Novozym[®] 435 to constitute the catalytic beds.

687 **Fig. 3.** Effect of the quantity of biocatalyst on the synthesis yield (Δ) and production rate (\bullet)
688 of **amide 3a (step 1, A)** and **pseudo-ceramide 4 (step 2, B)**. The reactions were carried out at
689 55°C in a *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v), at a flow rate of 125 $\mu\text{l min}^{-1}$ and
690 substrate concentrations of 100 (A: amino-diol **1** and stearic acid **2a**) and 50 mM (B: amide
691 **3a** and myristic acid **2b**) under stoichiometric conditions. Stainless steel columns with an
692 inner diameter of 5 mm and of variable length, in which various quantities of Novozym[®] 435
693 could be packed, were used as the catalytic beds.

694 **Fig. 4.** Effect of substrate concentration on the synthesis yield (Δ) and production rate (\bullet) of
695 **amide 3a (step 1, A)** and **pseudo-ceramide 4 (step 2, B)**. The reactions were carried out at
696 55°C in a *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) at a flow rate of 125 $\mu\text{l min}^{-1}$ and

697 various substrate concentrations, from 10 to 100 mM, under stoichiometric conditions (**A**:
698 amino-diol **1** and stearic acid **2a**; **B**: amide **3a** and myristic acid **2b**). A stainless steel column
699 145 mm in length with an inner diameter of 5 mm was packed with 875 mg of Novozym[®] 435
700 to constitute the catalytic bed.

701 **Fig. 5.** Effect of substrate molar ratio on the synthesis yield (Δ) and production rate (\bullet) of
702 **pseudo-ceramide 4 (step 2)**. The reactions were carried out at 55°C in a *tert*-amyl alcohol/*n*-
703 hexane mixture (50:50 v/v) at a flow rate of 125 $\mu\text{l min}^{-1}$, various substrate molar ratios from
704 1 to 5 and a fixed amide **3a** concentration of 50 mM. A stainless steel column 145 mm in
705 length with an inner diameter of 5 mm was packed with 875 mg of Novozym[®] 435 to
706 constitute the catalytic bed.

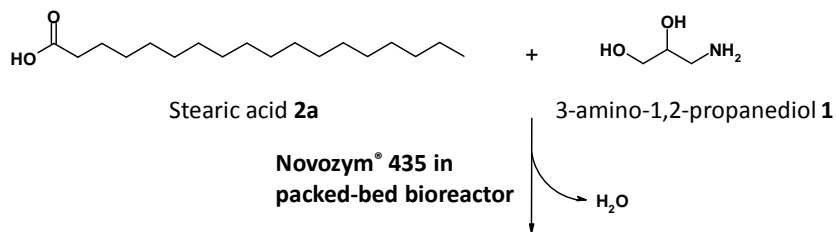
707 **Fig. 6.** Effect of the nature of the fatty acid used as an acyl donor on the synthesis yield
708 (histogram) and production rate (\bullet) of the **amide (step 1)**, using 3-amino-1,2-propanediol **1** as
709 the acyl acceptor and various fatty acids as acyl donors. The reactions were carried out at
710 55°C in a *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) at a flow rate of 500 $\mu\text{l min}^{-1}$ and a
711 substrate concentration of 100 mM, under stoichiometric conditions. A stainless steel column
712 145 mm in length with an inner diameter of 5 mm was packed with 875 mg of Novozym[®] 435
713 to constitute the catalytic bed.

714 **Fig. 7.** Continuous Novozym[®] 435-catalyzed synthesis of **amide 3c (step 1)** over a 3 week
715 period using 3-amino-1,2-propanediol **1** as the acyl acceptor and lauric acid **2c** as the acyl
716 donor. The reaction was carried out at 55°C in a *tert*-amyl alcohol/*n*-hexane mixture (50:50
717 v/v), at a flow rate of 250 $\mu\text{l min}^{-1}$ and a substrate concentration of 50 mM, under
718 stoichiometric conditions. A stainless steel column 145 mm in length with an inner diameter
719 of 5 mm was packed with 875 mg of Novozym[®] 435 to constitute the catalytic bed.

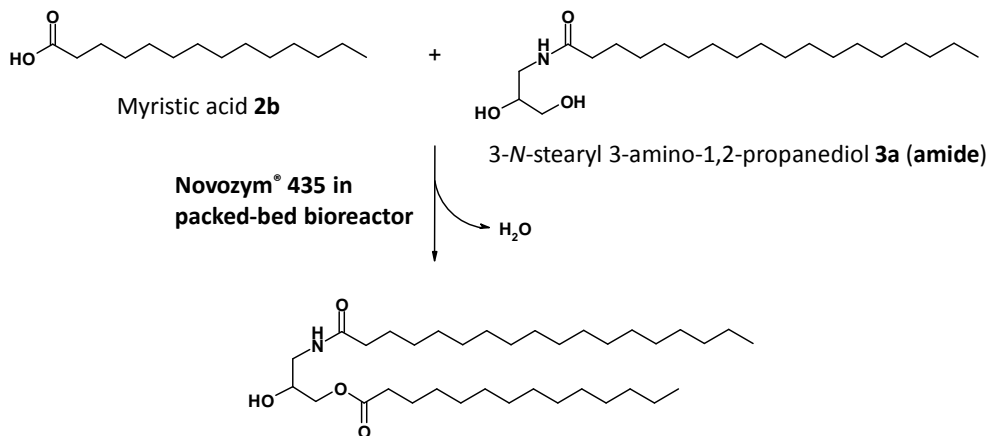
720 **Fig. 8.** Effect of reactor design on the synthesis yield (Δ) and production rate (\bullet) of **pseudo-**
721 **ceramide 4 (step 2)** using column A (125 mm in length and 10 mm inner diameter) or
722 column B (5 mm in length and 50 mm inner diameter). The reactions were carried out at 55°C
723 in a *tert*-amyl alcohol/*n*-hexane mixture (50:50 v/v) with 150 mM myristic acid **2b** and 50
724 mM amide **3a**. Stainless steel columns 125 mm in length with a 10 mm inner diameter (**A**)
725 and 5 mm in length with a 50 mm inner diameter (**B**) were packed with 3300 mg of
726 Novozym[®] 435 to constitute the catalytic beds.

727

Step 1:



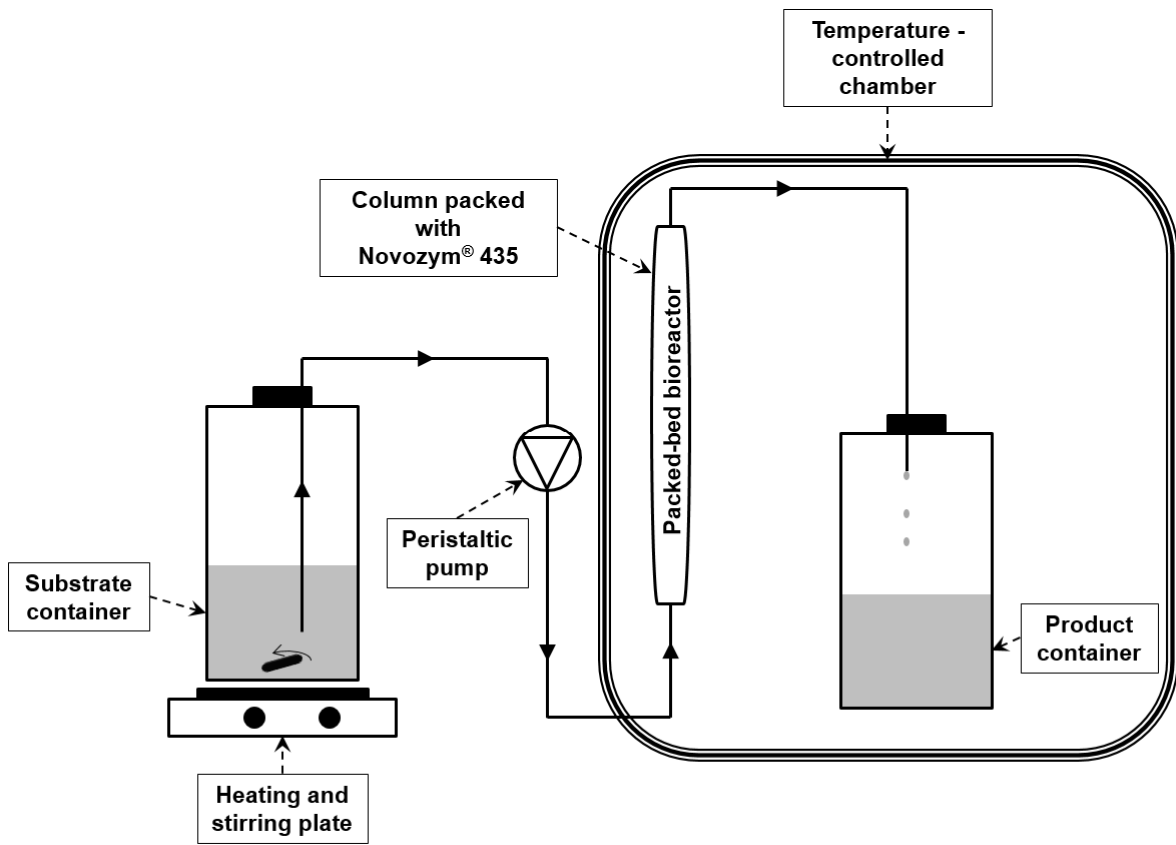
Step 2:



1-*O*-myristyl,3-*N*-stearyl 3-amino-1,2-propanediol **4** (pseudo-ceramide)

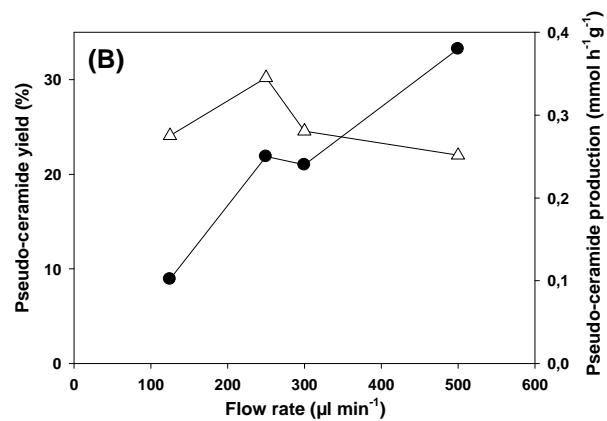
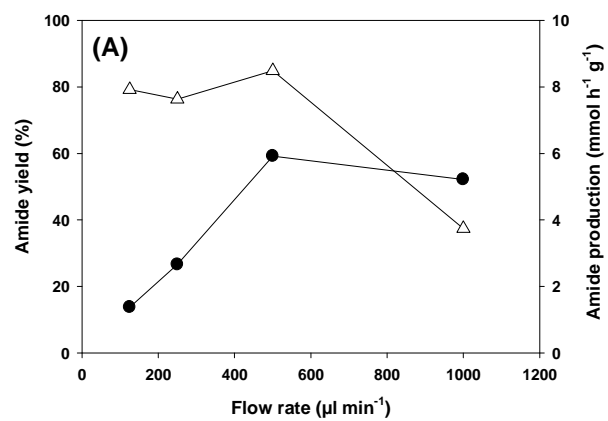
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729 **Scheme 1.**

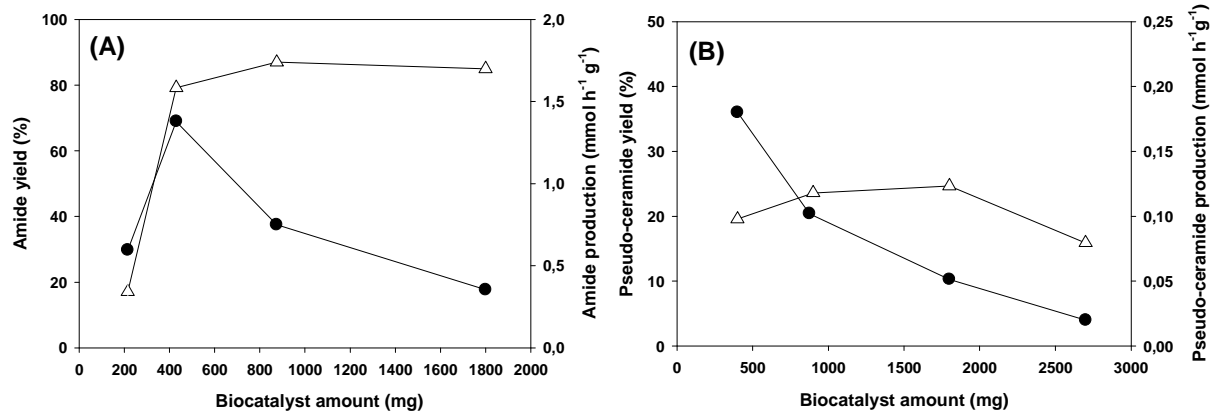


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731 **Fig. 1.**

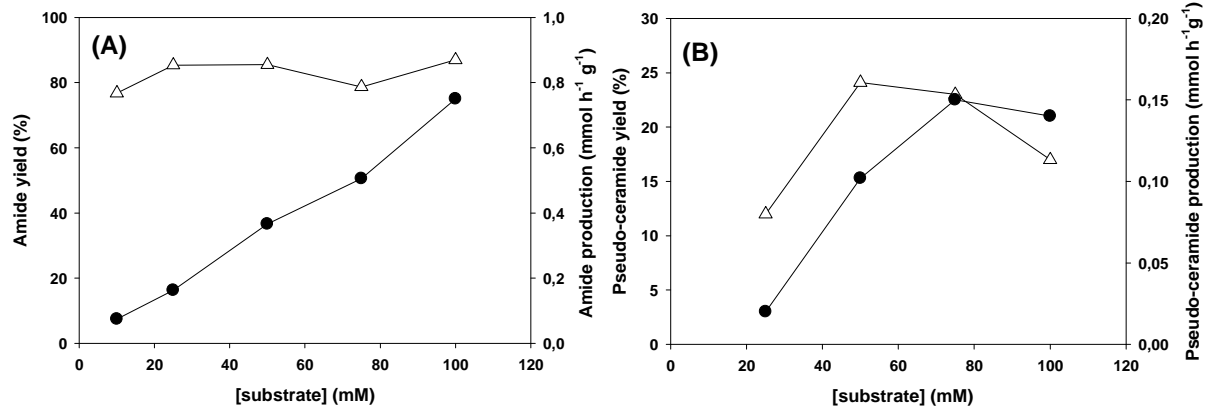


732
733 **Fig. 2.**



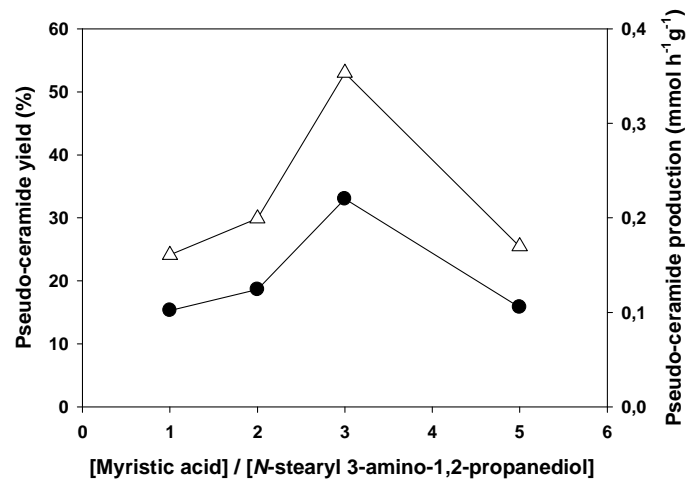
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735 **Fig. 3.**



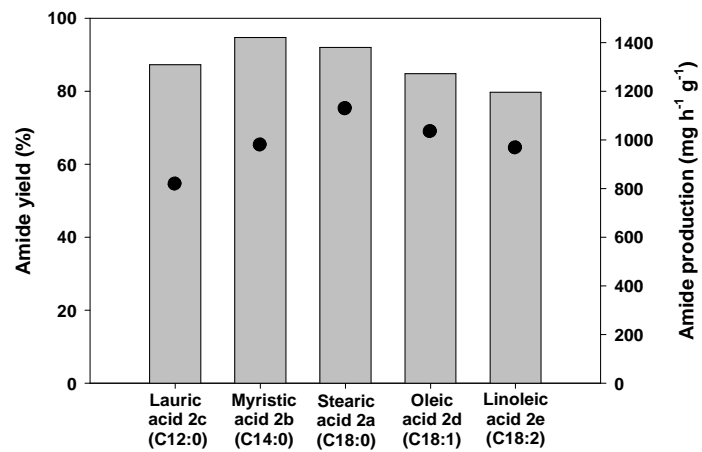
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737 **Fig. 4.**



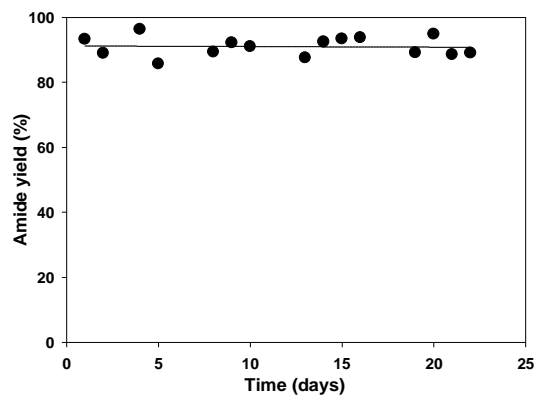
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739 **Fig. 5.**



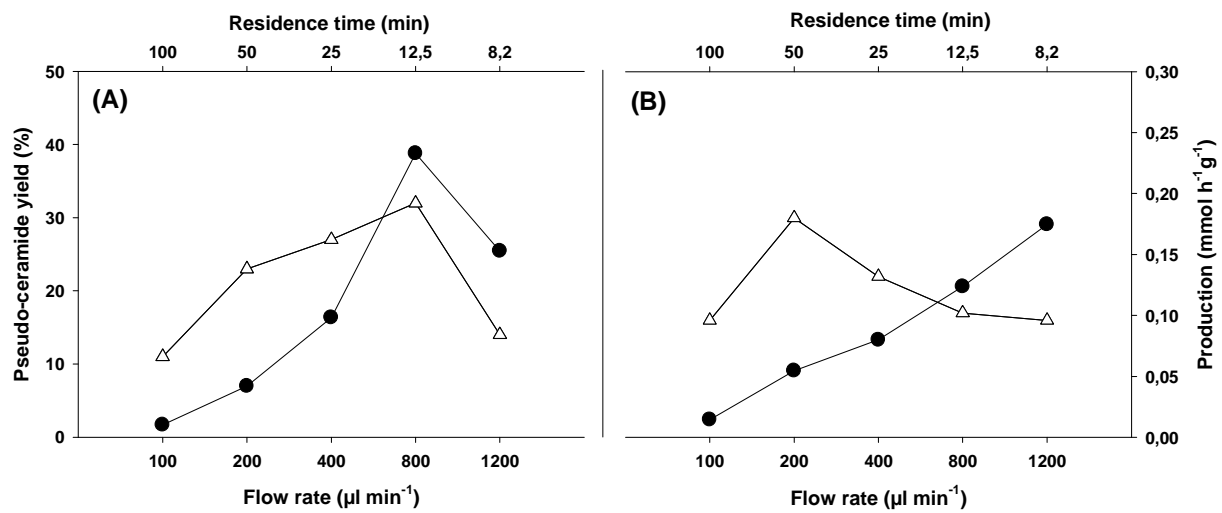
740

741 **Fig. 6.**



742

743 **Fig. 7.**



744
745 **Fig. 8.**

746

747

748 **Table 1**

749 Elution gradient for HPLC analysis

Time (min)	Solvent A: acetonitrile/water/acetic acid (77:23:0.1 v/v/v) (%)	Solvent B: methanol/acetic acid (100:0.1 v/v) (%)
0	100	0
90	100	0
93	0	100
143	0	100
145	100	0
153	100	0

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