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## Enzymatic synthesis of N-10-undecenoyl-phenylalanine catalysed by aminoacylases from *Streptomyces ambofaciens*

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1     **Enzymatic synthesis of N-10-undecenoyl-phenylalanine catalysed by**  
2                     **aminoacylases from *Streptomyces ambofaciens***

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16

17                    **Short title:** “Enzymatic synthesis of N-10-undecenoyl-phenylalanine”

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21

## 22 **Abstract:**

23 Due to its physico-chemical and biological activities, N-10-undecenoyl-phenylalanine  
24 (C11'F) is one of the most interesting lipoaminoacids used in cosmetic and pharmaceutical  
25 industries. Its production is currently based on the Schotten-Baumann chemical reaction,  
26 which shows some environmental issues in terms of effluents. As a possible biocatalytic  
27 alternative, this study presents the evaluation of the reactional and process conditions  
28 allowing the production of C11'F using aminoacylases from *Streptomyces ambofaciens*  
29 culture. These aminoacylases showed the best activity at 45°C and pH between 7 and 8 with a  
30 moderate thermal stability. The influence of substrates concentrations on the kinetic  
31 parameters of C11'F synthesis showed a more important impact of the phenylalanine  
32 concentration as compared to the 10-undecenoic acid concentration. As a reactional product,  
33 C11'F appeared to have an inhibitory effect on the enzymatic N-acylation. Cobalt addition  
34 allowed an eleven-fold increase of the reaction rate. Batch reactors were used with free  
35 aminoacylases without impact on the final C11'F concentration. For the first time,  
36 enzymatically produced C11'F was finally purified at the gram scale as a 99% purity white  
37 powder. The evaluation of the biological activity on melanocytes cultures showed the  
38 presence of skin lightening activity similar to the one obtained with the chemically produced  
39 C11'F.

40

## 41 **1. Introduction:**

42 Amino acids surfactants (AAS) are eco-compatible surface-active agent with very interesting  
43 techno-functional properties and, for some of them, with attractive bioactivities. There are  
44 molecules of choice for food, pharmaceutical and cosmetic industries [1]. Non-ionic AAS,  
45 like phenylalanine and leucine derivatives, are very attractive AAS as ingredients at industrial

46 scale because of their good detergency and foaming properties [2,3]. In addition to these  
47 properties, phenylalanine-based surfactants were described as presenting cytotoxicity against  
48 several human cancer cells thus widening their application scope [4]. Bisset et al., 2009  
49 described the ability of phenylalanine-fatty acid derivatives like N-10-undecenoyl-L-  
50 phenylalanine to reduce melanin production in melanocytes. In their study, an emulsion  
51 prepared by the combination of 1 % N-10-undecenoyl-phenylalanine with 5 % niacinamide  
52 increased significantly the efficiency in reducing the appearance of facial hyperpigmentation  
53 *in vivo* [5]. However, such AAS are produced at industrial scale using the Schotten-Baumann  
54 chemical reaction where the amino acid reacts with a fatty acid chloride under highly alkaline  
55 conditions by using NaOH in water and sulfuric acid to stop the reaction [4]. In some cases,  
56 some organic solvents are used to facilitate the reaction. In this last case a high purity powder  
57 with only some fatty acid traces is obtained. Despite a high yield of synthesis, the  
58 corresponding down-stream processing of the reaction medium leads to large volumes of  
59 saline effluents requiring an expensive reprocessing step for the industrials [6]. Nowadays, the  
60 environmental issues caused by such process encouraged the authorities to promote the  
61 industrial development of sustainable technologies by switching from waste remediation and  
62 pollution control by end-of-pipe solution to waste prevention thus reducing the reprocessing  
63 cost [7]. Enzymes represent an alternative for such reactions to avoid by-products and salts  
64 generation. Enzymes were widely used for acylation reaction especially for O-acylation but  
65 much less for N-acylation of amino acids. The ability of some enzymes to perform the N-  
66 acylation of amino acids were studied by only few authors using free fatty acids [8–15].  
67 Lipases and proteases are hydrolases that can perform the acylation reaction in non-aqueous  
68 media like organic solvents or in solvent-free conditions in order to shift the thermodynamic  
69 equilibrium of the reaction. However, the low solubility of amino acids in organic solvents  
70 limits the mass transfer of the substrates to the active site, which decreases the performance of

71 the reaction [8,16,17]. In this context, the use of acylases like aminoacylases that are able to  
72 catalyse the N-acylation reaction in aqueous media with better solubility of the substrates,  
73 especially of the acyl acceptor, has been yet considered [18,19]. Several enzymes were  
74 evaluated for amidation reaction but much less for the N-acylation of amino acids. The most  
75 used enzymes performing this reaction were described by Koreishi et al. who identified four  
76 acylases from *Streptomycess mobaraensis* [10,11,20,21]. Three of them were aminoacylases  
77 characterized as monomeric metallo-enzymes with different regioselectivities. Indeed, the  
78 aminoacylase of 100 kDa and the second one of 55 kDa (called Sm-AA) had regioselectivity  
79 towards the alpha amino group of the amino acid while the third one of 60 kDa (called Sm-  
80 ELA) was specific for the lysine epsilon amino group [10,12,20,21]. The fourth acylase was a  
81 penicillin V acylase (Sm-PVA), which is a dimeric enzyme able to perform the N-acylation of  
82 several amino acids with lauric acid showing a better activity with arginine, cysteine and  
83 lysine with a preference for the amino group in  $\Sigma$  position [11,22]. In another study, the  
84 ability of a crude extract from *Streptomycess ambofaciens* ATCC23877 to perform the N-  
85 acylation of lysine and peptides containing lysine on their alpha position was described [13].  
86 By genome comparison, a very close sequence identity between the genes encoding Sm-AA,  
87 Sm-ELA and Sm-PVA from *S. mobaraensis* and the ones responsible for the acylation  
88 activity from *S. ambofaciens* was shown, with 86, 80 and 69 % sequence identity respectively.  
89 Another gene was also identified with 55% identity with Sam-AA (Sam-AA like) but this had  
90 a very low  $\alpha$ -acylation activity. In addition, the authors demonstrated that the enzyme similar  
91 to Sm-AA, named SamAA, was responsible of 85 % of the N- $\alpha$ -acetyl-L-lysine hydrolysis  
92 activity [13]. However, no information is available until now about the condition influencing  
93 the activity of the acylases from *S. ambofaciens* ATCC23877. In a recent study, Bourkaib et  
94 al. (2020) described the substrate specificity of the crude extract of *Streptomyces ambofaciens*  
95 was investigated toward the 20 natural proteogenic amino acids and different fatty acids for

96 the enzymatic synthesis of many AAS in water. In the same study authors also investigated  
97 the impact of some operational conditions on the production of alpha-lauroyl lysine in batch  
98 reactor [23]. They also compared the performance of the crude extract from the wild strain of  
99 *S. ambofaciens* and the one from a mutant strain which express only Sam-AA. The results  
100 showed that the substrates specificity was similar between both strains confirming the  
101 position that Sam-AA is responsible of the N- $\alpha$ -acylation activity of the crude extract from  
102 the wild strain *Streptomyces ambofaciens*.

103 The aim of the present work was to focus on the enzymatic synthesis of one AAS, the N-10-  
104 undecenoyl-phenylalanine, using aminoacylase from a crude extract from *Streptomyces*  
105 *ambofaciens* culture. As above mentioned, the N-10-undecenoyl-phenylalanine (C11'F) is an  
106 AAS of direct industrial interest, used in cosmetics and pharmaceuticals as a skin lightening  
107 agent. To date, no study was reported on the enzymatic synthesis of this AAS in water and  
108 using enzymes. In this study, the influence of the operational conditions on the synthesis  
109 reaction of C11'F was investigated. An up-scaling of production using different reactor  
110 configurations as well as a convenient way to purify C11'F as a white powder was also  
111 presented. The inhibition of melanin production of the purified product was evaluated using  
112 melanocyte cell line and compared to the activity of the chemically produced C11'F.

## 113 **2. Materials and methods:**

### 114 **2.1. Aminoacylases of *S. ambofaciens* production**

115 The production of aminoacylases from *S. ambofaciens* was performed as described in detail in  
116 [23]. Aminoacylases from *S. ambofaciens* were finally obtained as a protein crude extract  
117 whose specific activity was evaluated using a lauroyl-lysine synthesis reaction. For this  
118 reaction, lauric acid and L-lysine were used as substrates at equimolar concentration (100

119 mM) in 25 mM Tris-HCl buffer, NaCl 50mM, at pH 8 and 45°C at 500 rpm magnetic stirring  
120 (2 mL reaction volume) in a simple glass tube.

## 121 **2.2. Enzymatic N-acylation of L-Phenylalanine assays**

122 The evaluation of the operational conditions influencing the activity of the crude extract of  
123 aminoacylases were conducted in test tubes of 2 mL final reaction medium containing 100  
124 mM of each substrate (except for substrate concentration influence) and 1 g/L enzymes  
125 concentration (except for enzyme concentration influence) prepared in 25 mM Tris-HCl, 50  
126 mM NaCl buffer, at 45°C (except for temperature influence), pH 8 (except for pH influence)  
127 and 500 rpm magnetic agitation.

128 The influence of substrate concentration was investigated by using concentrations from 25  
129 to 200 mM of L-phenylalanine and from 25 to 200 mM of 10-undecenoic acid setting the  
130 other substrate's concentration at 100 mM. The influence of the pH of the reaction was  
131 studied in the pH range [6-9.5] using NaOH 7.5 M and HCl 1 M to control the pH.  
132 Temperature effect on the reaction was evaluated in two steps. The first one was the  
133 evaluation of the optimal temperature of the enzymes by performing the synthesis reaction in  
134 a temperature range of [30-55°C]. The second step was to evaluate the stability of the  
135 enzymes by performing the standard reaction after incubation at different temperature (37, 45  
136 and 55°C) and during different incubation time (16 and 25 h). The enzymes concentration  
137 influence was evaluated by using (0.25, 0.5, 1 and 2 g/L) protein concentration at the standard  
138 conditions. The inhibition by the product of the reaction was investigated by adding different  
139 concentrations of a chemically produced commercial N-10-undecenoyl-phenylalanine. The  
140 relative activity was then calculated using the initials rates of the reactions with ( $V_{i\ C11'F}$ ) and  
141 without ( $V_{i0}$ ) the addition of the product (*relative activity* (%) =  $\frac{V_{i\ C11'F}}{V_{i0}}$ ).

142 We investigated the effect of metal ions by using 0.1 mM of CuCl<sub>2</sub> (from Fluka Chemika),  
143 CoCl<sub>2</sub>, ZnSO<sub>4</sub> (from Sigma), and Ethylene-Diamine-Tetra-acetic Acid (EDTA) in the  
144 standard conditions. The influence of CoCl<sub>2</sub> concentration was evaluated by using CoCl<sub>2</sub>  
145 concentrations in the range [0-0.5 mM]. In all case, the reaction was started after one-night  
146 agitation, in order to check if there was a reaction without enzymes, by adding 1 g/L of the  
147 crude extract of aminocaylases from *S. ambofaciens*.

148 At appropriate times, 50 or 100 μL aliquot of the reaction mixture was withdrawn and  
149 diluted with 80% Methanol for HPLC-ELSD analysis. Finally, all reactions were stopped by  
150 freezing directly the mixture at -18°C. Each reaction run was performed at least twice.

### 151 **2.3. Reactor configuration**

152 Different batch reactor with different reaction medium volumes and agitation systems  
153 were also evaluated and compared to the classic batch reaction performed in a standard vessel  
154 filled with 2 mL of the reaction medium. The first one was a 100 mL glass spinner flask with  
155 double sidearms (Wheaton<sup>®</sup> 356875 Celstir<sup>®</sup>) used as reactor equipped with suspended  
156 magnetic agitation filled with 20 mL of reaction medium final volume. The second one was a  
157 reactor of 500 mL equipped with automatic pH, temperature and agitation monitoring  
158 (OptiMax, METTLER TOLEDO<sup>®</sup>). The agitation module consisted of a four blades propeller,  
159 upwards (M8) of 45 mm diameter and 45° tilt. The reactor was filled with 300 mL of final  
160 reaction medium. In both cases the reaction medium contained 100 mM of each substrate and  
161 1 g/L enzymes concentration in 25 mM Tris-HCl, 50 mM NaCl buffer, at 45°C, pH 8 and 500  
162 rpm agitation. At appropriate times, 100 μL aliquot of the reaction mixture was withdrawn  
163 and diluted with 80% Methanol for HPLC-ELSD analysis. Finally, all reactions were stopped  
164 by freezing directly the mixture at -18°C. Each reaction was performed at least twice. The  
165 product of the reaction performed in the OptiMax was purified following the procedure  
166 described below.



167        **2.4. Purification of the final product:**

168    In order to get, for the first time, a pure enzymatically produced N-10-undecenoyl-  
169    phenylalanine, a purification process was developed. The 300 mL final reaction medium  
170    containing 1.53 g of the produced C11'F, residual 10-undecenoic acid (4.64 g) and L-  
171    phenylalanine (4.16 g) and the enzymes was used for the development of the purification  
172    protocol. The first stage was an ultrafiltration with a 10 kDa membrane (Amicon ultra-15;  
173    Merck) made of regenerated cellulose in order to eliminate small molecules coming from the  
174    aminoacylases crude extract. Two diafiltration steps and a 10-fold concentration step of the  
175    initial mixture were performed by centrifugation during 30 min and 5000 rpm, using a fixed  
176    angle rotor (Centrifuge 5804 R, Eppendorf). The second stage of the protocol consisted in  
177    removing the proteins by precipitation with methanol. To do this the 30 mL concentrated  
178    solution was diluted 10 times with a 80%/20% methanol/water solution. After 3 hours  
179    magnetic agitation the precipitated proteins were removed by centrifugation (20 min at 8400  
180    rpm and 15°C). The methanol present in the supernatant part was evaporated using a rotating  
181    evaporator (Laborota 4002 control, Heidolph, Germany). The third stage consisted in  
182    removing residual impurities and salts by size exclusion chromatography (BioSep™ SEC-  
183    S2000 300 × 21.2 mm, 5 µm particle diameter, Phenomex, USA). The elution of the 5 mL  
184    injected volume was performed using an isocratic system (45% acetonitrile) with a 5 mL/min  
185    flow rate, during 37 min. The mixture C11'/C11'F was collected after a 23 min elution time.  
186    The recovered mixture was then concentrated by evaporation for the next stage of purification  
187    by liquid chromatographic method. Different high pressure reverse phase liquid  
188    chromatography methods were tested using a “classical” C18 column (Grace/Alltech,  
189    Darmstadt, Germany, 150 mm × 2.1 mm, 5 µm particle size) or less classical C30 phases (LC  
190    Acclaim™ C30, 250 mm × 2.1 mm, 5 µm particle size, ThermoFisher scientific, USA) and  
191    also a more specific phase containing phenyl functional groups on its surface (Force Biphenyl

192 5  $\mu\text{m}$ , 150  $\times$  2.1 mm for analytic column and Ultra Biphenyl 5  $\mu\text{m}$ , 250  $\times$  21.2 mm for  
193 preparative column, Restek, USA). However, the very close physicochemical properties of the  
194 product and the residual fatty acid, like hydrophobicity ( $\log P$  C11' = 3.99,  $\log P$  C11'F =  
195 5.05), did not allow their proper separation. Also, although the biphenyl analytical scale  
196 column showed its capability to separate the two molecules with about 2 min difference in  
197 retention times, the same phase used in a preparative scale column did not allow any  
198 separation anymore even after several different trials. Actually, normal phase chromatography  
199 using Kieselgel G 60 powder under atmospheric pressure appeared to be the most suitable  
200 method leading to the pure product. The mixture 50% cyclohexane/50% ethyl acetate was  
201 selected to elute the residual fatty acid, selectively. The product was then eluted by adding  
202 around 1% (v/v) acetic acid to the previous eluting system. The elimination of the residual  
203 fatty acid and the recovery of the product were followed using Kieselgel G 60 Plates and  
204 iodine chamber. The collected fractions containing the product were concentrated using a  
205 rotary evaporator and dissolved again in water for the lyophilisation step, leading to a white  
206 powder. The product purity was assessed by HPLC-ELSD, LC-MS and proton NMR  
207 analyses.

## 208 **2.5. Analytical methods**

### 209 **2.5.1. Quantitative analyses by HPLC**

210 We monitored the reaction conversion by using the same analytical method as the one  
211 previously described in [23], based on the use of an HPLC (LC 10 AD-VP, Shimadzu,  
212 France) equipped with a UV detector at 214 nm followed by a light-scattering low  
213 temperature evaporative detector (Shimadzu, France). Calibrations were performed using a  
214 chemically synthesized N-10-uncenoyl-phenylalanine named Sepiwhite<sup>TM</sup> MSH kindly  
215 provided by SEPPIC, France. The substrate conversion yield was determined using the  
216 following equation: conversion (%) =  $\left(\frac{[\text{C11}'\text{F}]_{\text{mol/L}} \text{ produced}}{[\text{C11}']_{\text{mol/L}} \text{ initial}}\right) \times 100$ . We

217 calculated the initial rates of the reaction by using a third order polynomial model applied to  
218 the derivate of the first experimental data points exhibited in the figures. The maximum  
219 velocity (Vmax) and the Michaelis-Menten constant (Km) for each substrate were determined  
220 using a standard Michaelis-Menten model based on the equation of the graphic representing  
221  $\frac{1}{V} = f\left(\frac{1}{S}\right) = \frac{1}{S} * \frac{Km}{Vmax} + \frac{1}{Vmax}$  where V is the initial rate and S is the substrate concentration.

### 222 **2.5.2. Mass spectrometry analysis**

223 We performed the qualitative and semi-quantitative analysis of N-10-undecenoyl-  
224 phenylalanine by using an HPLC-MS-MS system (ThermoFisher Scientific, San Jose, CA,  
225 USA) consisting in a binary pump connected to a photodiode array detector (PDA) and a  
226 Linear Trap Quadrupole mass spectrometer (LTQ). An atmospheric pressure ionization  
227 interface operating in positive electrospray mode (ESI+) was used. The chromatographic  
228 separation and mass spectrometric conditions that were used as described in details in [23].  
229 Raw data were processed using Xcalibur software program (version 2.1,  
230 <http://www.thermoscientific.com>).

### 231 **2.6. Biological activity evaluation**

232 The evaluation of the melanin production by melanocytes was performed using B16 murine  
233 melanoma cells (B16F1 cells) following the method described by Chung et al., 2019 with  
234 some adaptations [24]. B16F1 cells were maintained in a CO<sub>2</sub> incubator in Dulbecco's  
235 modified Eagle medium (DMEM), without phenol red, supplemented with 10% fetal bovine  
236 serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated in CO<sub>2</sub> for  
237 24 h after being seeded in 96-well plate (6000 cells/well). Then cells were treated with  
238 Sepiwhite<sup>TM</sup> MSH (SEPPIC) or enzymatic N-10-undecylenoyl-phenylalanine initially  
239 dissolved in ethanol or the culture medium. Two different concentrations of each was tested  
240 (10 and 50 µg/mL) and kojic acid at 50 µg/mL in the culture medium was used as a positive

241 reference. 200  $\mu$ L of the cell culture medium was transferred to a 96-well plate after 72h  
242 incubation. The absorbance of the samples and standard curve of synthetic melanin (0 to  
243 500  $\mu$ g/mL) was measured at 450 nm. The inhibition activity (%) was expressed as  
244  $\frac{\text{concentration of melanin with the tested molecule}}{\text{concentration of melanin without tested molecule}} \times 100$ . In order to evaluate the cells viability,  
245 the latters were isolated and lysed in PBS buffer prior concentration determination using a  
246 protein assay kit (BCA Protein Quantitation Kit). The cells viability was expressed as  
247  $\frac{\text{concentration of proteins with the tested molecule}}{\text{concentration of proteins without tested molecule}} \times 100$ . The measured melanin contents were  
248 then normalised by the protein amount and expressed as  $\mu$ g/mg of cell's proteins. The  
249 evaluation was done at least 5 times and the statistical analysis was done using Student test to  
250 compare the results with the control (XLSTAT software, Addinsoft Inc., Paris, France).

251

## 252 **Results and Discussion**

### 253 **3.1. Influence of substrate concentration on enzymatic activity**

254 In a recent study [23], the ability of the aminoacylases from *S. ambofaciens* to catalyse the  
255 N-acylation of the amino acids on alpha amino group position was demonstrated. 10-  
256 undecenoic acid is a mono-unsaturated fatty acid described as having antifungal, antiviral and  
257 insect-repelling activity which makes it useful to treat superficial infections [25,26]. It was  
258 also described as having neuroprotective and radical-scavenging activity [27]. The N-undecyl-  
259 10-enoyl-L-phenylalanine (C11'F) generated by the condensation of the phenylalanine with  
260 the 10-undecenoic acid was described as having very interesting properties for cosmetic  
261 applications. However, the enzymatic synthesis of C11'F has never been reported. In order to  
262 study the operational conditions influencing the activity of the aminoacylases from *S.*  
263 *ambofaciens* the synthesis of C11'F was used as reference reaction. In this study all  
264 experiments were carried out using the same crude extract from *S. ambofaciens* containing 21

265  $\pm 0.7$  g/L of proteins [28]. The apparent specific activity of the proteins crude extract was  
266 evaluated using lauroyl-lysine synthesis reaction performed with an equimolar concentration  
267 of the lauric acid and lysine (100 mM). The reaction was performed using 1 g/L of enzymatic  
268 crude extract. The apparent specific activity of the acylases from *S. ambofaciens* was  $33.4 \pm 5$   
269  $\mu\text{M}$  of  $\alpha$ -lauroyl-lysine / min / mg of proteins.

270 All N-acylation (amidation) reactions were carried out in 25 mM Tris-HCl buffer, NaCl 50  
271 mM pH 8, containing 1 g/L acylases at  $45^\circ\text{C} \pm 3^\circ\text{C}$ . In order to evaluate the substrate  
272 concentration influence on enzyme activity different reactions were performed. First of all,  
273 the best substrate concentration, at equimolar conditions, was evaluated for the further  
274 parameters evaluation by using different concentration (25 mM, 50 mM, 100 mM and 200  
275 mM). The concentration produced after 48 h (Fig.1) showed a maximum reaction by using  
276 100 mM of both substrates while very low activity was observed in the other conditions. The  
277 influence of the substrate concentration on the performance of the aminoacylases from *S.*  
278 *ambofaciens* was then studied by fixing the concentration of one of the substrates at 100 mM  
279 and by varying the concentration of the other substrate from 25 mM to 200 mM. The  
280 influence of the concentration in 10-undecenoic acid was very low compared to the influence  
281 of phenylalanine concentration (Fig.2). The initial rate of the reaction was increased by the  
282 increase of the concentration of the fatty acid from 25 mM to 125 mM followed by a  
283 stabilization of the reaction rate. The evolution of the initial rate followed standard Michaelis-  
284 Menten model allowing the determination of  $K_m$  and  $V_{max}$  for the undecenoic acid.

285 According to the equation of the graphic representing  $\frac{1}{v} = f\left(\frac{1}{S}\right)$   $V_{max}$  and  $K_m$  of around  $0.50$   
286  $\pm 0.05$  mM / h and 19.2 mM were found. Above a C11' concentration of 175 mM a slight  
287 decrease of the activity was observed which can be due to a substrate inhibition. The  
288 influence of the amino acid was more pronounced than the fatty acid where a continuous  
289 increase of the initial rate was observed. Indeed, according to the equation of the graphic

290 representing  $\frac{1}{v} = f\left(\frac{1}{S}\right)$   $V_{max}$  and  $K_m$  of around  $0.50 \pm 0.05$  mM / h and 42 mM were found.  
291 Koreishi et al., 2005 observed nearly the same relationship when performing the reaction of  
292 N $\alpha$ -Feruloyl-L-lysine synthesis using the aminoacylase from *Streptomyces mobaraensis*.  
293 They showed, like in this study, that the activity was almost unaffected by the concentration  
294 of the acyl donor. However, the effect of L-lysine concentration on the activity was positively  
295 much more important [20].

296 Fig.1:

297 Fig.2:

### 298 **3.2. Influence of the pH on aminoacylases activity**

299 The pH of the reaction medium is one of the most important operational parameters affecting  
300 the performance of a reaction by changing even the ionisation state of the substrates and  
301 enzymes and, subsequently, the enzymes activity. The results showed that the activity  
302 remained nearly stable at pH range from 6.5 to 9 while no reaction was obtained at pH 6 and  
303 very low activity was measured at pH 9.5 (Fig.3 A). However, by following and comparing  
304 the kinetic of the reaction at pH 8-9 (Fig.3 B), the enzymes seems to be much less stable at pH  
305 8.5 and 9 than at pH 8, which is very important in the perspective of an extrapolation. For this  
306 reason, the pH of all the reaction performed using the aminoacylases from *S. ambofaciens* at  
307 45°C were set at a range of 7-8. Several studies evaluated the influence of the pH on the  
308 enzymatic activity and showed nearly the same pH range for optimum activity, which is  
309 widely used for industrial application. Koreishi et al., showed that the optimum pH of the  
310 aminoacylase (SmAA) from *Streptomyces mobaraensis* was between 7 and 8 while the  
311 second aminoacylase of 100 kDa had an optimum pH of 6.5 [20,21]. Wada et al., showed that  
312 the acylase I from Pig Kidney had an optimal pH for the reaction of around 7 and 7.5 [29].

313 Fig.3:

### 314 **3.3. Influence of the temperature**

315 The influence of the temperature of the reaction on the enzymatic activity is shown in fig 4.A.  
316 the best activity was obtained at 45°C while very low activity was observed at 55°C. The loss  
317 of the activity at 55°C can be related to a low stability of the enzymes due to a fast protein  
318 denaturation. In order to evaluate the thermal stability of the enzymes, the aminoacylases  
319 were incubated at different temperature (37, 45 and 55°C) during 16 and 25 h before starting  
320 the reaction at 45°C. As shown in fig.4.B, the enzymes lost around 80 % of the activity after  
321 16 h incubation at 37°C and around 90 % after 25 h and the higher is the incubation  
322 temperature, the higher is the loss of activity. The results at 55°C confirmed that the low  
323 activity observed when performing the reaction at this temperature was due to protein  
324 denaturation. Similar results were obtained by Koreishi, who studied the stability of the  
325 aminoacylase (SmAA) from *Streptomyces mobaraensis* by performing the hydrolysis of N-  
326 acetyl-L-methionine at 37 °C in Tris-HCl buffer at pH 7.5. They also showed that the  
327 enzymes lost almost all the activity after only one-hour incubation at 55°C [21].

328 Fig.4:

### 329 **3.4. Influence of protein concentration**

330 Reactions using different protein concentration in 25 mM Tris-HCl buffer, NaCl 50 mM  
331 containing 100 mM of both substrates at 45°C, pH 8 and 450 rpm magnetic agitation were  
332 conducted. By following the kinetics of the production of C11'F at the different protein  
333 concentrations, the increase of the quantity produced after 3 days was proportional to the  
334 protein concentration when a concentration between 0.25 and 1 g/L was used. However, the  
335 increase of the produced concentration was much less important from 1 to 2 g/L while the  
336 initial rate was proportional to the quantity of protein used (Fig.5 A and B). In all cases, a  
337 decrease of the activity after a certain time was observed. This can be attributed to a relatively

338 low thermal stability of the enzymes or to an inhibition by the product of the reaction  
339 knowing that the C11'F is soluble in the operational condition. In order to face the problem of  
340 thermal denaturation and to increase the amount of C11'F produced, a reaction was performed  
341 using 1 g/L of enzymes with an additional 1 g/L introduced to the reaction medium after 24 h.  
342 The kinetic of the reaction was compared to the ones with 1 and 2 g/L (Fig.5 C). The addition  
343 of enzymes during the reaction caused an increase of the production to reach the same  
344 concentration as with 2 g/L protein concentration. This means that the protein denaturation is  
345 not the only factor controlling the reaction. The second reason could then be the enzymes  
346 inhibition by the C11'F itself or that the thermodynamic equilibrium of the reaction was  
347 reached. The product inhibition of the reaction was evaluated by performing the reaction in 25  
348 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates and [0-10 mM] of  
349 C11'F at 45°C, pH 8 and 450 rpm magnetic agitation. The results (Fig.5 D) showed a clear  
350 product inhibition where a progressive decay of the initial activity of the enzymes was  
351 observed by increasing the concentration of the product.

352

353 Fig.5:

### 354 **3.5. Influence of metal activator on the reaction**

355 As described by Dettori et al., 2018, the sequence coding the aminoacylase from *S.*  
356 *ambofaciens*, named SamAA, is very close to the one coding the aminoacylase from *S.*  
357 *mobaraensis* (Sm-AA) described by Koreishi et al. in 2009 with 86 % sequence identity. This  
358 enzyme was described as belonging to a metal-dependant family which can be influenced by  
359 the addition of metal ions playing a key role in the catalytic process. In order to evaluate the  
360 effect of metal ions on the acylases from *S. ambofaciens* activity,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  which  
361 were described as the most influencing ones, were tested [10,11,20,21]. Ethylene-diamine-  
362 tetra-acetic acid (EDTA) was used as chelating agent to check the metal-dependency of the



363 enzyme. As shown in Fig.6, for the same concentration of the three metal ions (0.1 mM),  
364 Cobalt ion led to a three time increase of the initial rate unlike  $Zn^{2+}$  and  $Cu^{2+}$  ions allowing  
365 only a slight increase. No reaction was registered when EDTA was added, which confirmed  
366 that the aminoacylases involved in the N-acylation are metalloenzymes. However, by  
367 comparing the reaction course, the thermodynamic equilibrium was reached with  $CoCl_2$  after  
368 48h while the thermodynamic equilibrium of the control reaction and the reaction with  $Zn^{2+}$   
369 as metal ion was not yet reached after 72 h. For the reaction with  $Cu^{2+}$  the thermodynamic  
370 equilibrium was also reached after 48 h but with around 25% less activity than with  $Co^{2+}$  as  
371 metal ion. The influence of Cobalt concentration was therefore also investigated. The increase  
372 of the Cobalt concentration allowed an important increase of the initial rate of the reaction to  
373 reach around eleven-fold increase of the initial rate by using 0.5 mM of  $CoCl_2$  (Tab.1). When  
374 concentration from 0.3 to 0.5 mM of  $CoCl_2$  was used, the thermodynamic equilibrium was  
375 reached after only 24 h reaction time but the final product concentration was affected. Indeed,  
376 the increase of the  $CoCl_2$  concentration led to an increase in the conversion rate until 0.3 mM  
377  $CoCl_2$  with more than 20 mM C11'F concentration. A 16 mM C11'F concentration was  
378 reached when using a 0.5 mM  $CoCl_2$  concentration. Koreishi et al. 2005 studied the influence  
379 of metal ions on the hydrolytic activity of *S. mobaraensis* aminoacylase. Their results were  
380 similar to the ones observed in this study where three-fold increase of the activity by adding  
381 0.1 mM  $CoCl_2$  and only a slight increase by adding  $Zn^{2+}$  ion was observed. However, they  
382 showed a decrease of 80 % of the activity by adding  $Cu^{2+}$  ion (Koreishi et al., 2005a), which  
383 was not the case with the acylases from *S. ambofaciens*.

384 Fig.6:

385 Tab.1:

386

387 **3.6. Influence of reactor configuration**

388 In a perspective of process intensification and to evaluate the best way to produce the  
389 C11'F at gram scale, different reactor configurations were evaluated. CoCl<sub>2</sub> was not added in  
390 this part in order to avoid residual Co<sup>2+</sup> in the final product. Intensified designed reactors were  
391 used by different authors for enzymatic intensification allowing an enhancement of the  
392 activity [30,31]. In this kind of reactors free or immobilized enzymes can be used. The  
393 immobilization of the acylases from *S. ambofaciens* was evaluated on several supports with  
394 different characteristics (hydrophilic, hydrophobic and surface modified supports) and using  
395 different immobilisation technics (adsorption, covalent and entrapment) however the  
396 aminoacylases lost almost all of their activity (results not shown).

397 Different batch reactor configurations with different volume and agitation module were  
398 evaluated in order to reach a gram scale of pure enzymatic C11'F. As shown in fig.7 A, there  
399 is no influence of the reactor configuration on the final product concentration when  
400 performing the reaction in the same conditions. Indeed, the same final concentration was  
401 obtained when passing from 3 mL batch reactor with magnetic stirrer to the 300 mL batch  
402 reactor (Optimax) equipped with Rushton mechanical stirrer. The reaction using Optimax  
403 reactor was carried out seven days allowing to reach around 1.58 g of produced C11'F with  
404 around 4.6 g residual fatty acid (Fig.7 B). The final reaction mixture was used for the  
405 purification procedure in order to obtain the pure product.

406 Fig.7:

### 407 **3.7. Purification of the reaction product**

408 As detailed in the Materials and Methods section, the C11'F was purified using filtration and  
409 chromatographic methods. The first step consisting in an ultrafiltration on a 10 kDa allowed  
410 the selective passage of the product of interest and the residual fatty acid (331 Da and 184 Da  
411 respectively) while eliminating few impurities. As shown in fig.8 A, the three first steps of

412 diafiltration allowed the elimination of 64 % of the fatty acid with only 15 % loss of the  
413 product from a sample of the initial reaction medium composed of 300 mg of C11' and 127  
414 mg of C11'F. However, when more diafiltration steps were performed a higher loss of the  
415 C11'F was observed (fig.8.A). This partial separation of the C11' and the C11'F can be due to  
416 the amphiphilic property of the acylated amino-acid which can interact with the proteins  
417 standing in the retentate forming a polarization layer, while the apolar characteristic and the  
418 high quantity of the residual C11' increased its elimination in the permeate. However, after  
419 the elimination of around 64 % of the fatty acid, the membrane pores became more accessible  
420 for the C11'F, leading to a loss of the product. A compromise that allowed the partial  
421 elimination of the residual C11' while limiting the loss of the C11'F was found by applying  
422 two diafiltration steps and a concentration step. This led to the recovery of a solution  
423 containing around 1.1 g of the product and equivalent quantity of the fatty acid in addition to  
424 the aminoacylases used. The proteins were eliminated by precipitation with methanol, and the  
425 product was separated from the residual fatty acid by size exclusion chromatography. After  
426 lyophilisation 0.8 g of a 99 % pure white powder of enzymatically produced N-10-  
427 undecenoyl-phenylalanine was obtained, as determined by mass spectroscopy and proton  
428 NMR analysis (Fig.8 B).

429 Fig.8:

### 430 **3.8. Evaluation of the biological activity of the purified product**

431 N-10-undecenoyl-phenylalanine was described in the literature as having a lightening effect  
432 on the skin. This activity is due to an inhibition of the production of melanin by melanocytes  
433 as antagonist to alpha-melanocyte stimulating hormone (MSH) receptor [5]. The enzymatic  
434 and the chemical N-10-undecenoyl-phenylalanine solutions were prepared in ethanol and  
435 culture medium at different concentrations (10 and 50 µg/mL) for the bioactivity evaluation.  
436 As represented in Tab.2, inhibitory activity at 50 µg/mL was higher with enzymatic C11'F

437 than with the chemical one when it was solubilized in ethanol, while it was less active when it  
438 was solubilized in the medium. This can be explained by the solubility of the enzymatic  
439 C11'F (data not shown), which was slightly lower than the chemical one in the medium. The  
440 lower solubility of the enzymatic product in the medium might be due to the presence of silica  
441 particles traces from the last chromatographic step or acetic acid used to recover the product  
442 from the column. The acetic acid can influence the ionization of the product resulting in a  
443 precipitation of a part of the product in unbuffered medium. By comparing cells viability in  
444 those conditions, the enzymatic product prepared in medium showed less toxicity than the  
445 chemical one unlike the enzymatic product prepared in ethanol, which showed higher toxicity  
446 as compared to the chemical one. Knowing that ethanol and the medium do not show any  
447 effect on the cell growth, this effect is related to the presence of the product. This can also be  
448 explained by the slight solubility difference of the enzymatic and chemical C11'F. In ethanol,  
449 enzymatic C11'F showed higher cell toxicity than the chemical one but much better  
450 lightening activity due to its good solubility. In the medium, the enzymatically produced  
451 C11'F showed lower cell toxicity than the chemical one, which might be related to the lower  
452 solubility reducing the accessibility of the product to the cells and to the MSH receptor  
453 resulting in less toxicity and activity. At 10 µg/mL, according to statistical analysis, non-  
454 significant difference was observed between enzymatic and chemical C11'F prepared in  
455 ethanol and culture medium for the depigmentation activity, due to low concentration.  
456 However, cell toxicity was slightly more pronounced with enzymatic C11'F for no explained  
457 reason. Further study is needed in order to explain the reason of the observed differences.

458 Tab.2:

## 459 **Conclusion**

460 The aim of this study was to evaluate the operational conditions influencing the performances  
461 of the aminoacylases from *S. ambofaciens* for the N-acylation of the L-phenylalanine with 10-

462 undecenoic acid as acyl donor. The results showed a positive effect of the phenylalanine  
463 concentration on the aminoacylases activity, unlike what was observed with the 10-  
464 undecenoic acid. Investigations on operational conditions showed a good N-acylation activity  
465 at 45°C, pH [7-8] but low temperature stability. The inhibitory effect of the product on the  
466 conversion rate was also demonstrated showing the interest of immobilization aiming to use  
467 these aminoacylases in continuous flow reactors. The influence of the metal ions was also  
468 studied confirming that the aminoacylases used are metallo-dependant and showing a very  
469 interesting acceleration of the reaction by adding CoCl<sub>2</sub>. Different reactor configurations were  
470 evaluated but the batch reactor was the most suitable for the synthesis of N-10-undecenoyl-  
471 phenylalanine without any effect of the agitation mode. A purification procedure was  
472 developed leading to 0.8 g of N-10-undecenoyl-phenylalanine with more than 99% purity,  
473 under the form of a white powder. The biological activity of the product was tested on  
474 melanocyte cell cultures and showed a clear inhibition of the melanin production. When  
475 significant, the difference in the lightening activity and/or the cell toxicity observed between  
476 the C11'F of chemical origin or that issued from the enzymatic process was likely to be due to  
477 different impurities remaining in the two products. These results represent a step forward  
478 towards the development of a green bioprocess using *S. ambofaciens* aminoacylases in  
479 aqueous media, which could constitute a promising alternative to existing chemical processes  
480 for the production and the purification of lipoaminoacids (i.e. AAS) as biosurfactants.

481

## 482 **CRedit author statement**

483 **Mohamed Chafik Bourkaib:** Conceptualization, Investigation, Writing-Original Draft

484 **Stephane Delaunay:** Conceptualization, Methodology **Xavier Framboisier:** Resources,

485 Investigation **Catherine Humeau:** Investigation, Methodology, Writing - Review & Editing.

486 **Jerôme Guilbot:** Funding Acquisition, Validation **Cecile Bize:** Ressources, Investigation,  
487 Formal analysis, Writing - Review & Editing **Estelle Illoud:** Project administration, Writing  
488 - Review & Editing **Isabelle Chevalot:** Project Administration, Supervision, Writing -  
489 Review & Editing, Validation **Yann Guiavarc’h:** Funding Acquisition, Project  
490 Administration, Supervision, Formal Analysis, Writing - Review & Editing, Validation.

491

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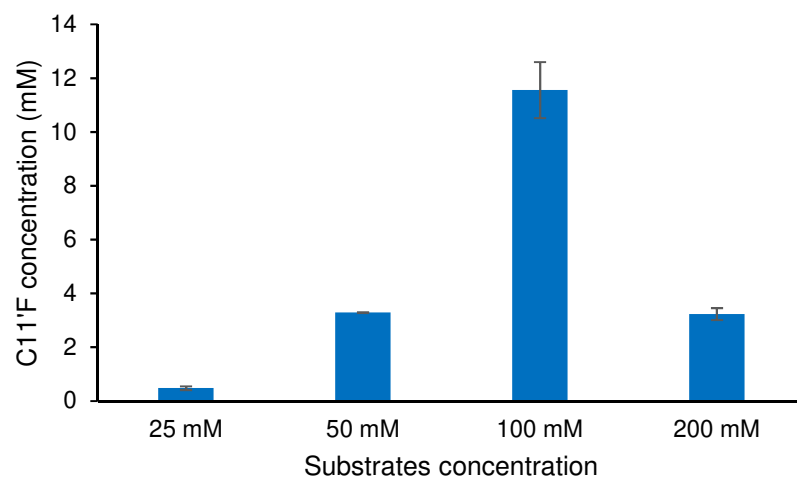
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### Figures



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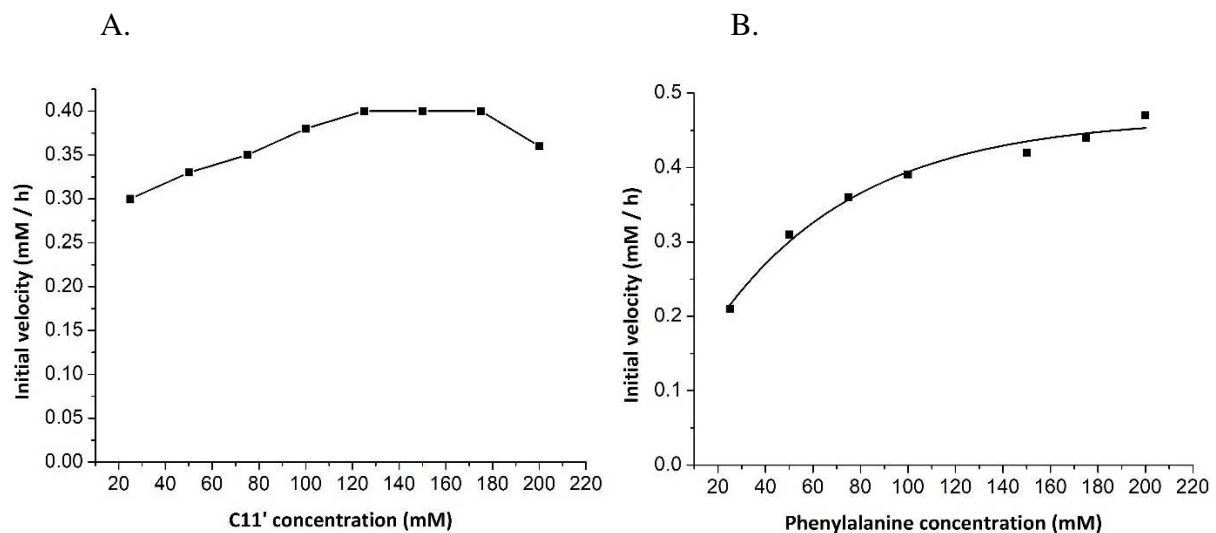
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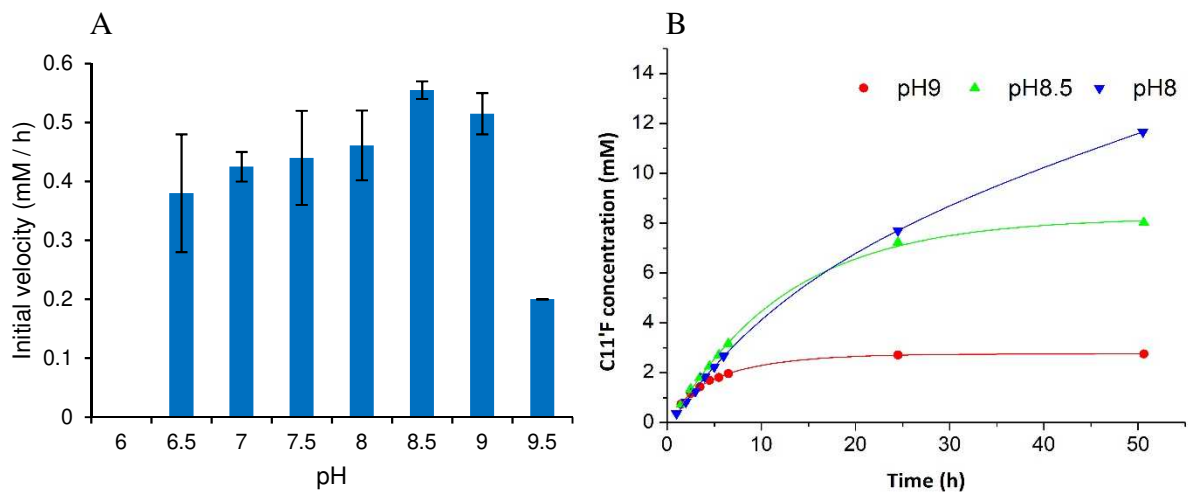
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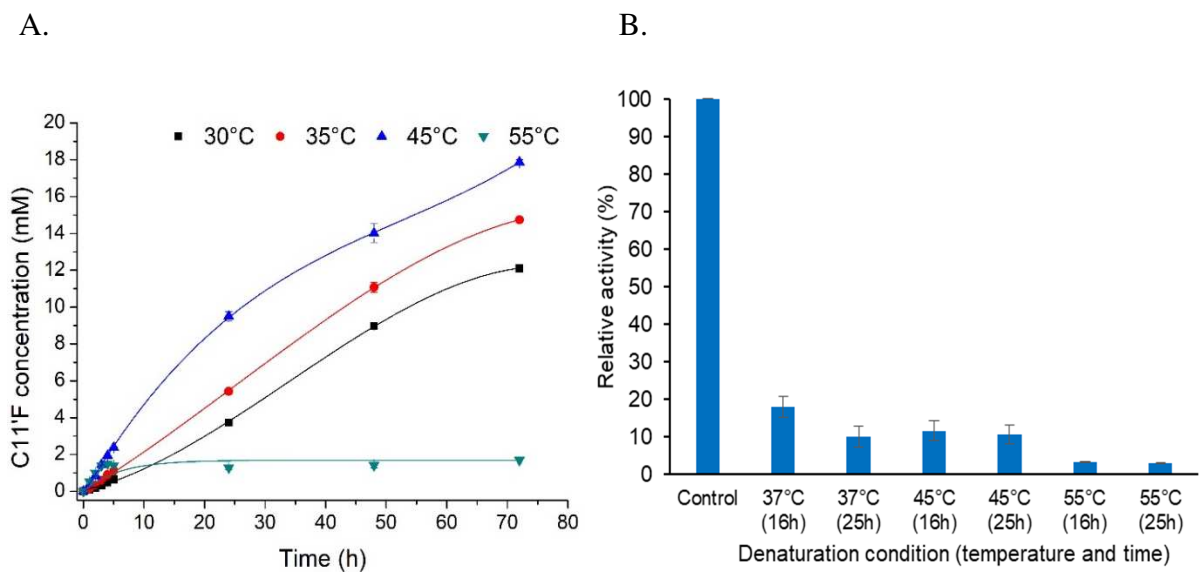
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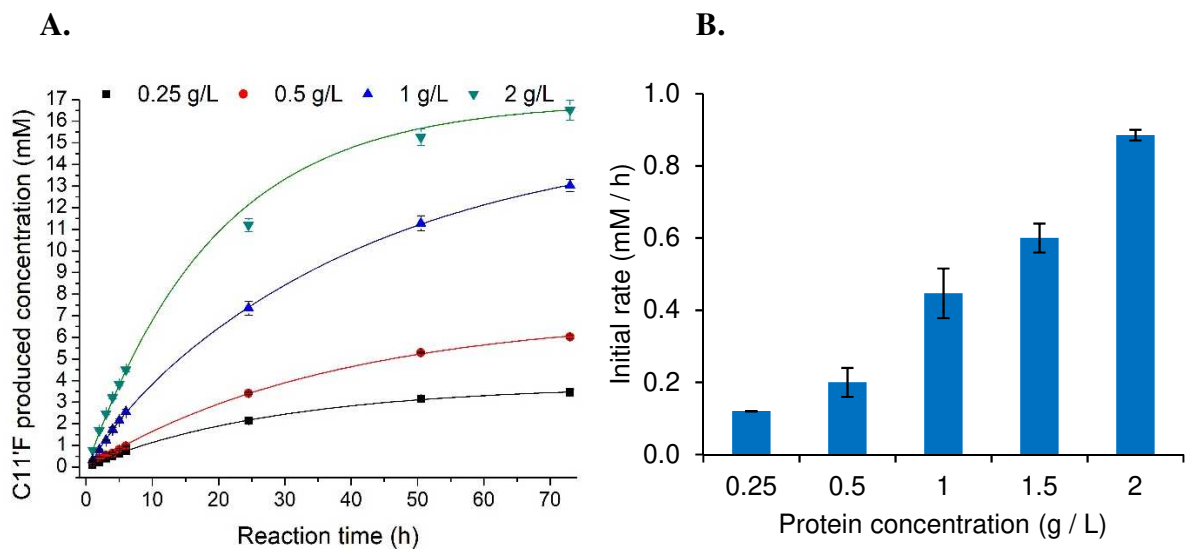
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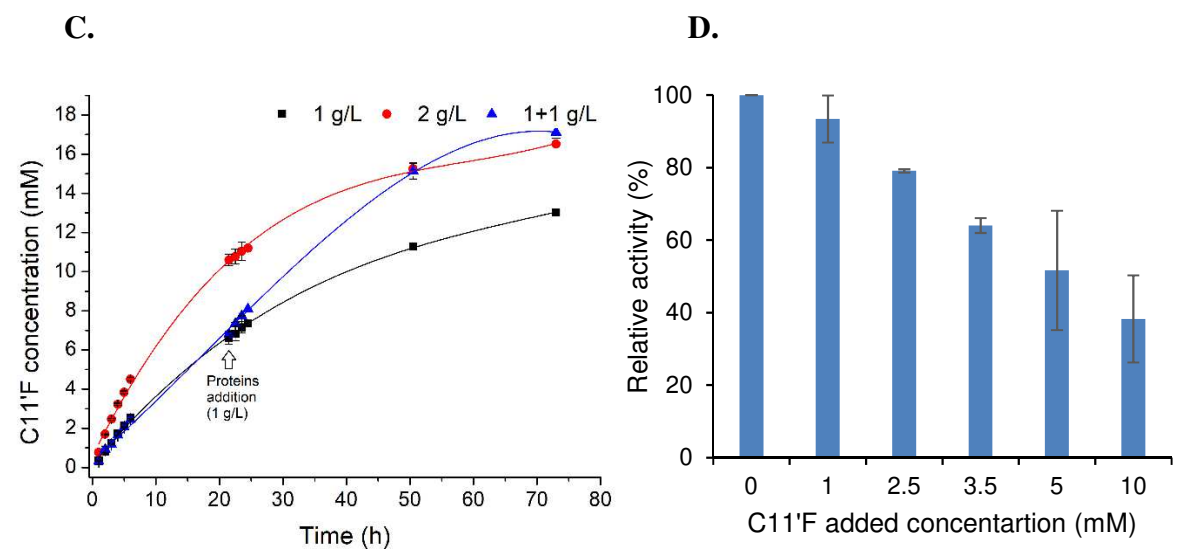
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696 Fig.5:

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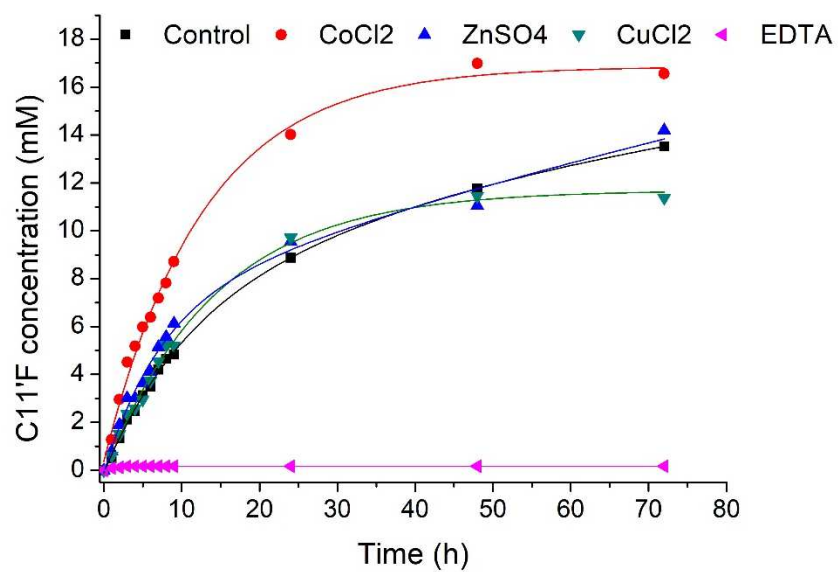
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706 Fig.6:

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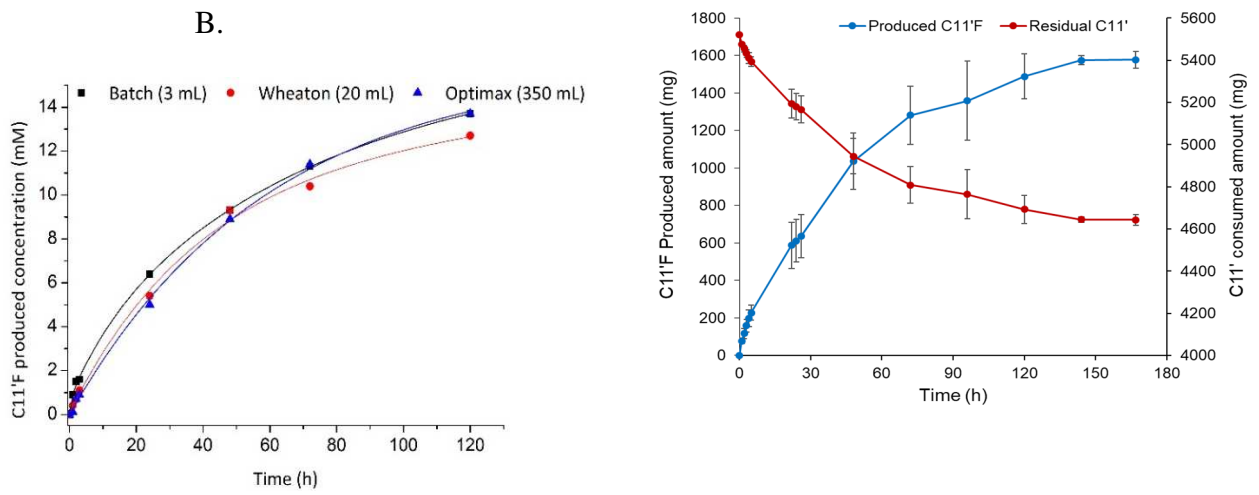
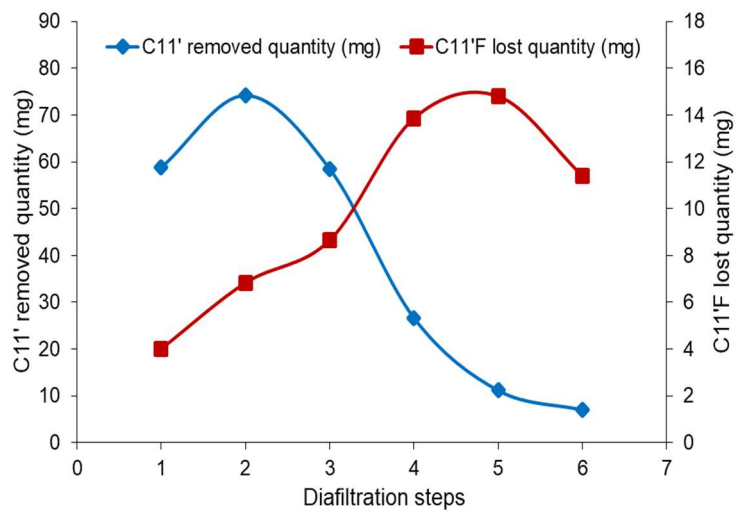
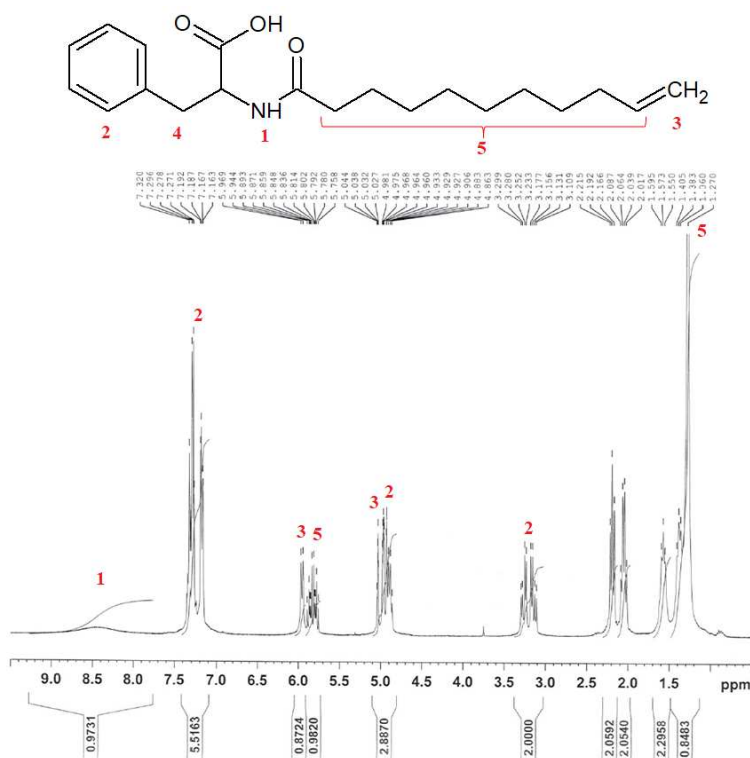


Fig.7:

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754 Fig.8:

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757 **Fig.1:** Influence of substrates concentration at equimolar condition on the synthesis of N-10-  
 758 undecenoyl-phenylalanine after 72 h in 25 mM Tris-HCl buffer, NaCl 50mM containing  
 759 1 g/L acylases at 45°C, pH 8 and 450 rpm magnetic agitation (n=2 or 3).

760 **Fig.2:** Influence of substrates concentration on the initial rate for the synthesis of N-10-  
 761 undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50mM containing 1 g/L acylases  
 762 at 45°C, pH 8 and 450 rpm magnetic agitation, A. Effect of 10-undecenoic acid concentration  
 763 on the initial rate of N-10-undecenoyl-phenylalanine synthesis reaction. A reaction mixture  
 764 containing 0.025-0.2 M of 10-undecenoic acid and 100 mM L-phenylalanine. B. Effect of L-  
 765 phenylalanine concentration on the initial rate of N-10-undecenoyl-phenylalanine synthesis

766 reaction. A reaction mixture containing 100 mM 10-undecenoic acid and 0.025–0.2 M of L-  
767 phenylalanine (n=2).

768 **Fig.3:** Influence of the pH on the enzymatic activity for the synthesis of N-10-undecenoyl-  
769 phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both  
770 substrates, 1 g/L acylases at 45°C, pH [6 - 9.5] and 450 rpm magnetic agitation, A. effect of  
771 the pH on the initial rate. B. Kinetic evolution of the synthesized N-10-undecenoyl-  
772 phenylalaninesynthesis reaction at pH 8, 8.5 and 9 (n=2).

773 **Fig.4:** Effect of the temperature on the enzymatic activity A. Influence of the reaction  
774 temperature on the enzymatic activity for the synthesis of N-10-undecenoyl-phenylalanine in  
775 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases  
776 at T°C [30-55°C], pH 8 and 500 rpm magnetic agitation B. Thermal stability of the enzymes  
777 after 16 and 25 h incubation time at 37, 45 and 55°C compared to fresh enzymes reaction  
778 conducted in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1  
779 g/L acylases at 45°C, pH 8 and 500 rpm magnetic agitation (n=2).

780 **Fig.5:** Influence of the protein concentration and the product concentration on the enzymatic  
781 activity for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl  
782 50 mM containing 100 mM of both substrates, 1 g/L acylases (except for the protein  
783 concentration influence) at 45°C, pH 8 and 450 rpm magnetic agitation, A. C11'F production  
784 kinetic at different enzymes concentration [0.25-2 g/L] B. Initial rate of the production at  
785 different protein concentration C. C11'F production kinetic with enzymes addition during the  
786 reaction compared to the reaction performed using 1 and 2 g/L initial enzymes concentration  
787 D. Influence of the C11'F added concentration on the initial rate of the reaction (n=2 for Fig.  
788 5A, 5B, 5C, 5D).

789 **Fig.6:** influence of different metal ions at 0.1 mM concentration on the enzymatic activity for  
790 the synthesis of N-10-undecenoyl-phenylalaninein 25 mM Tris-HCl buffer, NaCl 50 mM  
791 containing 100 mM of both substrates, 1 g/L acylases at 45°C, pH 8 and 500 rpm magnetic  
792 agitation.

793 **Fig.7:** scaling-up of the C11'F synthesis reaction in 25 mM Tris-HCl buffer, NaCl 50 mM  
794 containing 100 mM of both substrates, 1 g/L acylases at 45°C, pH 8 and 500 rpm magnetic  
795 agitation A. influence of batch reactor configuration B. kinetic of the C11'F production and  
796 the C11' consumption for the reaction performed in OptiMax reactor (n=2).

797 **Fig.8:** Details on C11'F purification process A. quantity of fatty acid and product removed at  
798 different diafiltration steps using a 10 kDa cut-off membrane and an initial solution of 25 mL  
799 of the final reaction mixture (300 mg of C11' and 127 mg of C11'F) B. proton NMR of the  
800 final purified product.

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#### Tables:

805 **Tab.1** effect of metal ion and the concentration of CoCl<sub>2</sub> on the initial rate of the reaction  
806 synthesis of N-10-undecenoyl-phenylalaninein and the C11'F produced concentration after  
807 48h in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L  
808 acylases at 45°C, pH 8 and 500 rpm magnetic agitation.

Metal ion	No metal ions	ZnSO <sub>4</sub>	CuCl <sub>2</sub>	CoCl <sub>2</sub>	CoCl <sub>2</sub>
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<b>Concentration</b> (mM)	0.1	0.1	0.1	0.05	0.2	0.3	0.4	0.5	
<b>Initial rate</b> (mM / h)	0.4 ± 0.08	0.73 ± 0.17	0.61 ± 0.05	<b>1.01 ± 0.26</b>	0.69 ± 0.18	1.64 ± 0.1	2.48 ± 0.7	3.22 ± 0.6	<b>4.34 ± 0.4</b>
<b>C11'F</b> <b>concentration</b> (mM)	11.8 ± 1.5	12.1 ± 3	11.5 ± 2.2	<b>17 ± 1.2</b>	15.7 ± 1.5	17.7 ± 2.5	<b>22 ± 0.35</b>	20 ± 2.9	17.2 ± 2.7

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**Tab.2** melanin production inhibition and cells viability evaluation.

Dilution solution	Concentration (µg/mL)	Depigmentation* (%)		p-value T test	Cells viability** (%)		p-value T test
		Chemical	Enzymatic		Chemical	Enzymatic	
		C11'F	C11'F		C11'F	C11'F	
Ethanol	50	9	<b>26</b>	0.0002 (***)	<b>84</b>	75	0.0001 (***)
	10	6	9	0.2101 (NS)	<b>98</b>	95	0.0100 (*)
Culture	50	<b>67</b>	48	0.0463 (*)	76	<b>82</b>	0.0018 (**)

<b>medium</b>	10	17	13	0.2514 (NS)	<b>100</b>	97	0.0205 (*)
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826 LS: limit of significance;  $0.05 < p < 0.1$ ;  $*0.01 < p < 0.05$ ;  $**0.001 < p < 0.01$  and  $***p < 0.001$ , n = 6.

827 \*: 97% inhibition of melanin production by melanocytes using Kojic acid as positive control

828 without any cell toxicity

829 \*\*: 100% cell viability correspond to the result with the control culture of melanocyte (without

830 any added molecules)

## 5\*13 cm Graphical abstract

