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1 **N-acylation of L-amino acids in aqueous media: evaluation of the catalytic**  
2 **performances of *Streptomyces ambofaciens* aminoacylases**

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9

10 **Abstract:**

11 N-acylated amino acids are widely used as surfactants and/or actives in cosmetics and  
12 household formulations. Their industrial production is based on the use of the Schotten-  
13 Baumann chemical and unselective reaction. Faced to the growing demand for greener  
14 production processes, selective enzymatic synthesis in more environment-friendly conditions  
15 starts to be considered as a potential alternative. This study concerns the use of the  
16 aminoacylases from *Streptomyces ambofaciens* to selectively catalyse aminoacid acylation  
17 reaction by fatty acids in aqueous medium. The results demonstrated that, when using  
18 undecylenoic acid as acyl donor, these aminoacylases properly catalyse the acylation of 14 of  
19 the 20 proteogenic L-amino acids tested on their  $\alpha$  amino group with a great variability  
20 depending on the nature of the amino acid (polar or not, positively/negatively charged,  
21 aromatic or not...). More precisely, the following 9 amino acids were shown to be  
22 preferentially acylated by *S. ambofaciens* aminoacylases as follows: lysine > arginine >  
23 leucine > methionine > phenylalanine > valine > cysteine > isoleucine > threonine. Different  
24 fatty acids were used as acyl donors and, in most cases, the fatty acid length influenced the  
25 conversion yield. The kinetic study of  $\alpha$ -lauroy-lysine synthesis showed a positive influence  
26 of lysine concentration with  $V_{max}$  and  $K_m$  of 3.7 mM/h and 76 mM, respectively. Besides,  
27 the lauric acid had an inhibitory effect on the reaction with  $K_i$  of 70 mM. The addition of  
28 cobalt to the reaction medium led to a more than six-fold increase of the reaction rate. These  
29 results, achieved with the aminoacylases from *S. ambofaciens* represent an improved enzyme-  
30 based N-acylated amino acids production in order to provide an alternative way to the  
31 Schotten-Baumann chemical reaction currently used in the industry.

32

33

## 34 **1. Introduction:**

35 Amino acid surfactants (AAS) are one of the most important class of natural surface-active  
36 molecules [1]. They come from the condensation of an amino acid as acyl acceptor, and one  
37 or more non polar molecules such as long chain fatty acids as acyl donors to constitute  
38 amphiphilic molecules with high surface activity. This characteristic, in addition to their eco-  
39 compatibility, makes them molecules of choice for food, pharmaceutic and cosmetic  
40 industries [2]. Given the structural diversity of the 20 natural amino acids and the great  
41 variety of fatty acids that can be involved in acylation reaction, a wild range of surfactants  
42 with diverse properties and activities can be found. Indeed, depending on the amino acid  
43 radical and the acylated group (O-acylation or N-acylation), AAS can be classified on the  
44 basis of head group type with different commercial applications. For instance, cationic AAS  
45 like cocoyl arginine ethyl ester were described as disinfectants, antimicrobial agents and  
46 antistatic agents. Anionic AAS like N-cocoyl-L-glutamate and potassium N-cocoyl glycinate  
47 are used in cosmetics for several applications and non-ionic AAS for their good detergency  
48 and foaming properties (phenylalanine and leucine derivatives). Zwitterionic or amphoteric  
49 AAS like  $\epsilon$ -lauroyl-lysine, are widely used in creams and hair conditioners because of their  
50 low solubility in aqueous media [3,4]. The N-acylation position in the case of lysine as an  
51 acyl acceptor plays an important role in the property and activity of the synthesized AAS.  
52 Pérez et al. (2009) studied the influence of the N-acylated group of the lysine methyl and  
53 ethyl-ester on the antimicrobial activity [5]. They demonstrated that the N-acylation on  $\epsilon$ -  
54 position decreased considerably the antimicrobial activity as compared to what was observed  
55 when acylation occurred in  $\alpha$ -position. This was due to the important difference in pKa  
56 between amino groups. These pKa are of 8.8 and 6.2 for the amino group in position  $\epsilon$  and  $\alpha$ ,  
57 respectively. They also reported that the hemolytic activity of N- $\alpha$ -acyl lysine based  
58 surfactant was lower than the N $\epsilon$ -acyl lysine one, but both had much lower activity as

59 compared to the classical cationic quaternary ammonium surfactants like  
60 alkyltrimethylammonium salts [5,6]. These results showed the practical interest of N- $\alpha$ -  
61 acylation of amino acids and consequently, of a regioselective acylation when using an amino  
62 acid such as lysine, with two different amino groups.

63 The most common way to produce those AAS is *via* the Schotten-Baumann chemical reaction  
64 where the amino acid reacts with a fatty acid chloride under highly alkaline condition by  
65 using NaOH or Na<sub>2</sub>CO<sub>3</sub> in TFA/water or by using solvents like trimethylamine or N-  
66 methylmorpholine. In addition, the N-acylation of amino acids exhibiting functional groups at  
67 their side chain requires a preliminary step of protection in order to avoid side reactions [7].  
68 Even if this chemical way presents high synthesis yields, the production at industrial scale  
69 leads to large volumes of effluents requiring a very expensive reprocessing step. Nowadays,  
70 more and more industries try to develop sustainable technologies by switching from waste  
71 remediation and pollution control by end-of-pipe solution to waste prevention, reducing the  
72 environmental issues and the reprocessing cost [8]. One of the most interesting way to meet  
73 these aims is the use of biologically mediated reactions, more generally called bioconversions.  
74 Enzymes are catalysts of choice for such reactions to avoid the formation of by-products and  
75 salts. They can make the catalysis in mild conditions (pH and temperature), are relatively  
76 substrate specific and often show chemo-, regio- and even enantioselectivity. To date, while  
77 many articles and books have been dealing with O-acylation (esterification or  
78 transesterification), mainly using lipases, only few studies have been focused on the N-  
79 acylation reaction. Few authors have evaluated the performances of enzymes for the acylation  
80 of amino acids using even free fatty acids or triglycerides like soybean and palm oil [9–15].  
81 Lipases, especially *Candida antarctica* lipase B (CALB), *Rhizomucor miehei* lipase, and  
82 proteases are the most used biocatalysts. They are hydrolases that can perform synthesis  
83 reaction in non-aqueous media such as organic solvents or in solvent free condition in order to

84 shift the thermodynamic equilibrium towards the synthesis. However, amino acids are poorly  
85 soluble in organic solvents [14,16,17]. Attempting to solve this problem, several authors used  
86 amino acid derivatives using Cbz (carboxybenzyle) or tert-butyl ester in order to improve  
87 their solubility and to protect the carboxylic part, allowing an increase of the conversion rate  
88 [17–19]. But this method needs an additional chemical step of deprotection to give the final  
89 product [19]. Other authors used ionic liquids in order to improve the solubility of the acyl  
90 acceptor. Husson et al., 2009 were able to improve the reaction performance of CALB for the  
91 synthesis of N- $\epsilon$ -oleyl lysyl-serine by using 1-butyl-3-methylimidazolium  
92 hexafluorophosphate ([Bmim]<sup>+</sup> [PF6]<sup>-</sup>) instead of 2-methyl-2-butanol as reaction medium  
93 (initial rate was found to increase from 1 to 32 mM/h) [20]. However, the large scale use of  
94 neoteric media such as ionic liquids tends to be limited by several key drawbacks: their high  
95 viscosity, the complexity of their production but also their price as well as a lack of  
96 information on their toxicity [21]. As a catalytic alternative to lipases in anhydrous or almost  
97 anhydrous solvents, the use of aminoacylases obviously presents one key advantage: they can  
98 perform N-acylation in water while being present in a wide variety of biological matrices  
99 [22,23]. Only few studies investigated N-acylation of amino acids in aqueous media. Acylases  
100 produced by microorganisms were among the most used enzymes at industrial scale for the  
101 production of biomolecules. Microbial enzymes performing the amino acids amidation in  
102 aqueous or organic-aqueous medium were described by Koreishi et al, [10,11,24,25]. Four  
103 acylases from *Streptomyces mobaraensis* were identified and three of them were characterized  
104 for the N-amino acid acylation reaction. The first one is a monomeric aminoacylase metallo-  
105 enzyme of 100 kDa. This enzyme was able to catalyse the N-acylation of almost all the amino  
106 acids with much less specificity for glycine, cysteine, aspartic acid and histidine and no  
107 reaction with tyrosine and proline using 10 mM of ferulic acid as acyl donor in 70% glycerol  
108 reaction media. The production of N- $\alpha$ -feruloyl-L-lysine was improved by using 100 mM tris

109 HCl buffer (pH 7.5) without glycerol at optimum conditions [24]. The second enzyme was the  
110  $\epsilon$ -Lysine acylase (Sm-ELA) which was identified as a monomeric metallo-enzyme of around  
111 60 kDa. This enzyme showed a regioselectivity towards the N-acylation of the lysine side  
112 chain ( $\epsilon$ -position). It efficiently catalysed the N- $\epsilon$ -acylation of lysine with fatty and aromatic  
113 acyl groups in aqueous buffer [9,10]. The third acylase (Sm-PVA) was a dimeric enzyme of  
114 61 and 19 kDa identified as belonging to the Penicillin V acylase family. This latter was able  
115 to catalyse the N-acylation reaction of several amino acids with lauric acid showing a better  
116 activity with lysine in  $\epsilon$ -position, arginine and cysteine [11,26]. The last acylase (Sm-AA)  
117 was a monomeric protein of 55 kDa belonging to the peptidase M20 family. This enzyme  
118 showed efficient hydrolytic activity towards amino acids acylated with short or long chain  
119 fatty acids [25]. Recently, Dettori et al. (2017) described the ability of a crude extract from *S.*  
120 *ambofaciens* ATCC23877 to perform the N-acylation of lysine and peptides with oleic acid  
121 on their  $\alpha$  position unlike the CALB. By blast analysis, and using the genes encoding Sm-AA,  
122 Sm-ELA and Sm-PVA from *S. mobaraensis* as baits, 3 genes encoding putative acylases and  
123 sharing 86, 80 and 69% identity, respectively, were identified in the genome of *S.*  
124 *ambofaciens*. Another gene, presenting 55% identity with the sequence of Sm-AA was also  
125 identified. By gene deletions, and just keeping Sam-AA or Sam-ELA genes, it was shown  
126 that Sam-ELA enzyme showed hydrolytic specificity for N- $\epsilon$ -acetyl-L-lysine and that Sam-  
127 AA enzyme had specificity towards N- $\alpha$ -acetyl-L-lysine, while the crude extract was  
128 unspecific. By generating a mutant without Sam-ELA and Sam-AA genes, it was also  
129 demonstrated that 85% of the N-acylation ability from the crude extract was due to Sam AA  
130 and Sam-ELA [12]. The activity of these acylases from *S. ambofaciens* ATCC23877 was also  
131 evaluated for the synthesis of lauroyl-lysine after enzyme immobilisation [27]. However, to  
132 date, no data has been reported regarding the substrate specificity (acyl donor and acyl  
133 acceptor) of the acylases from *S. ambofaciens* ATCC23877.

134 The aim of this study was therefore to determine the substrate specificity of the aminoacylases  
135 from *S. ambofaciens* for the acylation reaction of the twenty proteinogenic amino acids with  
136 middle and long chain fatty acids. The influence of the operational conditions on reaction  
137 performances was also investigated using lauroyl lysine synthesis as a reference reaction.

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

### 139 **2.1. Aminoacylases of *S. ambofaciens* production**

140 In order to have enough *S. ambofaciens* ATCC23877 spores before starting the culture steps,  
141 a sporulation step was performed. The strains spores stored as suspensions at -80°C were  
142 streaked on Soy Flour Mannitol agar (SFM) filled. This later contain 20 g/L Mannitol, 20 g/L  
143 precooked Soy flour (Celnat) and 20 g/L Agar. After minimum 7 days of culture at 28°C, the  
144 spores of *S. ambofaciens* were collected by adding 2 mL of 20% (v/v) glycerol and by  
145 scratching the mycelium with a sterile handle before freezing the spore suspension at -20°C.  
146 In the same way the spores of *S. ambofaciens*  
147  $\Delta$ SAM23877\_1485::scar $\Delta$ SAM23877\_0977::scar strain was prepared. This strain was  
148 obtained by deletion of  $\Delta$ SAM23877\_1485 and  $\Delta$ SAM23877\_0977 genes coding for Sam-  
149 ELA and Sam-PVA respectively as described in [12]. The culture was done in 3 steps:  
150 germination, preculture and the culture. The first step was performed in the ICS medium  
151 (Insoluble complex medium). This medium contained sucrose 15 g/L, corn steep 5 g/L  
152 sterilized beforehand, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 10 g/L, KH<sub>2</sub>PO<sub>4</sub> 1 g/L, NaCl 3 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g/L  
153 and CaCO<sub>3</sub> 0.2 g/L. The medium was then sterilized at 121°C for 20 min. In a 250 mL baffled  
154 Erlenmeyer flask, 40 mL of this medium was inoculated with 100  $\mu$ L of spore suspension.  
155 After the germination, 20 mL of ICS medium were transferred in 200 mL of pre-culture  
156 medium contained in 2 L Erlenmeyer flask for the pre-culture step. The preculture medium  
157 composition was: Glucose 10 g/L, white dextrin 10 g/L, yeast extract 5 g/L, casein

158 hydrolysate 5 g/L, and CaCO<sub>2</sub> 1 g/L. The pH of the preculture medium was set at 6.5 before  
159 sterilization at 121°C for 20 min. The two first steps were performed for 48 h at 28°C and 250  
160 rpm orbital agitation using an INFORS AG AK 82 agitator. 30 ml of the preculture were then  
161 added in 950 ml of culture medium in 5 L Erlenmeyer flask for incubation. The culture was  
162 maintained for 7 days at 28°C and 200 rpm orbital agitation using an INFORS HT Multitron  
163 agitator. The composition of the culture medium was meat extract 40 g/L, soluble starch 40  
164 g/L, casein hydrolysate 20 g/L, MgSO<sub>4</sub> 20 g/L, and K<sub>2</sub>HPO<sub>4</sub> 2 g/L. The pH of the culture  
165 medium was set to 7 before sterilisation at 121°C for 20 min. All the components of the  
166 culture media were purchased from Sigma-Aldrich, except sucrose and yeast extract, from  
167 Fluka, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (99%), NaCl, CaCO<sub>3</sub> and acetonitrile, from Carlo Erba. At the end of the  
168 main culture, the bacterial cells were disrupted by first mixing the whole suspension for  
169 around 5 min minimum with an ULTRA-TURRAX T25 (Janke & Kunkel IKA Labortechnik)  
170 followed by 2 passages in a cell disrupter (Constant Cell disruption system, Cell-D) at 2  
171 kBars. The cellular debris were removed by centrifugation at 8600×g at 4°C for 20 min.  
172 Thereafter, the proteins including acylases were precipitated from the cell free extract by  
173 adding ammonium sulfate to the supernatant to 60% of saturation and agitation was  
174 maintained for 1 h at room temperature. The precipitate was recovered by centrifugation at  
175 8600×g at 4°C for 20 min and was dissolved in 25 mM Tris-HCl buffer, NaCl 50mM, at pH  
176 8. The solution was then diafiltrated and concentrated using a Cogent µScale tangential flow  
177 filtration system equipped with a 10kDa ultrafiltration membrane (Merck Millipore). Five dia-  
178 volumes were necessary for removing the salt. Salt removal was checked by following the  
179 conductivity evolution during the filtrations steps. The apparent specific activity of the *S.*  
180 *ambofaciens* crude extract was evaluated using lauroyl-lysine synthesis reaction. For this  
181 reaction lauric acid and L-lysine were used as substrates at equimolar concentration (0.1 M) in  
182 25 mM Tris-HCl buffer, NaCl 50mM, at pH 8, 45°C and at 500 rpm.



## 183        **2.2. Enzyme assays**

184        Substrate specificity of the acylases from *S. ambofaciens* toward acyl acceptor was evaluated  
185        by performing the condensation reaction of the 20 L- amino acids as acyl acceptors with 10-  
186        undecenoic acid as acyl donor. Reactions with taurine, 6-aminohexanoic acid, 6-amino-1-  
187        hexanol, ethanolamine, hydroxylamine, 4-amino-benzoic acid and carnosine as acyl acceptor  
188        and lauric acid as acyl donor were also performed. All acyl donors were from Sigma Aldrich  
189        except 4-amino-benzoic acid, which was from Merck.

190        Substrate specificity of the acylases from *S. ambofaciens* towards acyl donor was performed  
191        using lysine, arginine, methionine and phenylalanine with octanoic, 10-undecenoic, lauric and  
192        oleic acid as acyl donors. The influence of the acyl donor structure on the regioselectivity was  
193        evaluated using lysine as acyl acceptor and acetic, butyric, isobutyric, valeric, octanoic,  
194        phenylpropionic, benzoic and azelaic acid as acyl donors. All acyl donors were from sigma  
195        Aldrich except butyric acid and azelaic acid which were from Rectapur and Fluka Chemika,  
196        respectively. Substrate specificity evaluation was conducted in test tubes using 100 mM of  
197        each substrate in 2 mL of 25 mM Tris-HCl, 50 mM NaCl buffer, at 45°C, pH 8 and 250 rpm  
198        magnetic agitation. All reaction media were sonicated using an ultrasonic processor in order  
199        to improve reaction medium homogeneity.

200        The influence of substrate concentration was studied using lauroyl-lysine synthesis reaction.  
201        Concentrations from 25 to 250 mM of lysine or from 10 to 200 mM of lauric acid were used,  
202        the other substrate being fixed to 100 mM. The inhibition by the product of the reaction was  
203        investigated by adding different concentrations of a chemically produced  $\alpha$ -lauroyl-lysine  
204        (from GeneCust, France, with 97.21% purity). The reaction was conducted in 1 mL reaction  
205        media containing 100 mM of both substrates.

206 The effect of metal ions was investigated using CoCl<sub>2</sub>, ZnSO<sub>4</sub> (from Sigma Aldrich) and  
207 CuCl<sub>2</sub> (from Fluka Chemika). The reactions were performed in 3 mL of 25 mM Tris-HCl, 50  
208 mM NaCl buffer, at 45°C, pH 8 and 500 rpm magnetic agitation.

209 In all cases, the reaction is started after one night agitation, in order to check if the reaction  
210 occurs without enzyme, by adding 1 g/L of the produced enzyme mixture from *S.*  
211 *ambofaciens*. At appropriate times, 50 or 100 μL aliquots of the reaction mixture were  
212 withdrawn and diluted with 80% methanol for HPLC and LC-MS-MS analyses. For the  
213 regioselectivity evaluation, a TLC analysis was also performed. Finally, all reactions were  
214 stopped by freezing the mixture at -18°C. Each reaction was performed at least twice.

## 215 **2.3. Analytical methods**

### 216 **2.3.1. Qualitative analyses by TLC**

217 Qualitative analyses of the reaction media by TLC were performed on Kieselgel G 60 Plates  
218 20 cm × 20 cm (Merck, Darmstadt, Germany). The mobile phase was: butanol (60%)/acetic  
219 acid (20%)/water (20%) that permitted a slight but visible separation between the α and the ε  
220 monoacylated products. Knowing that lysine contains two amino groups allowed the detection  
221 by spraying a commercial solution of ninhydrin reagent (Sigma-Aldrich).

### 222 **2.3.2. Quantitative analyses by HPLC**

223 The quantification of reaction conversion yield was monitored using HPLC (LC 10 AD-VP,  
224 Shimadzu, France) equipped with a UV detector at 214 nm followed by a light-scattering low  
225 temperature evaporative detector (Shimadzu, France). Two columns were used: the first one  
226 was a C18 amide 125 x 2.1 mm (Altima®, Altech, France) maintained at 25°C. The mobile  
227 phase (0.2 mL/min flow rate) consisted of solvent A: methanol/water/TFA (60/40/0.1 v/v/v)  
228 and solvent B: methanol/TFA (100/0.07). A linear elution gradient was applied to reach 95%  
229 methanol/TFA after 6 min. This methanol concentration was maintained for 13 min and then

230 decreased in 1 min to reach the initial methanol/water ratio until the end of the run (35 min).  
 231 However, the use of this column did not allow the separation of the product and the fatty acids  
 232 when phenylalanine, leucine and isoleucine were used as acyl acceptors. In that cases a  
 233 second column, Force Biphenyl 3  $\mu\text{m}$  column 150 x 2.1 mm (Restek, USA), maintained at  
 234 35°C, was used. The same mobile phase composition and gradient as before were used.  
 235 Calibrations were performed using standard curve fatty acids and purified product in the case  
 236 of  $\alpha$ -lauroyl-lysine. The substrate conversion yield was determined using the following  
 237 equation: conversion yield (%) =  $(1 - ([\text{fatty acid}]_{\text{mol/L}} \text{ consumed} / [\text{fatty acid}]_{\text{mol/L}} \text{ initial}) \times$   
 238 100. Initial reaction rates were calculated using a third order polynomial model applied to the  
 239 derivate of the first experimental data points exhibited in the figures. The maximum velocity  
 240 ( $V_{\text{max}}$ ) and the Michaelis-Menten constant ( $K_m$ ) for each substrate were determined using a  
 241 standard Michaelis-Menten model based on the equation of the graphic representing  $\frac{1}{V} =$   
 242  $f\left(\frac{1}{S}\right) = \frac{1}{S} * \frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}}$  where V is the initial rate and S is the substrate concentration. In the  
 243 case of enzymatic substrate inhibition, inhibition constants ( $K_i$ ) and maximum velocity ( $V_{\text{max}}$ )  
 244 can be evaluated using  $V = \frac{V_{\text{max}} \times S}{K_m + S + \frac{S^2}{K_i}}$  and by neglecting Michaelis-Menten constant ( $K_m$  is  
 245 very low as compared to S).  $V_{\text{max}}$  and  $K_i$  were then determined according to the equation of  
 246 the graphic representing  $\frac{1}{V} = f(S) = \frac{S}{K_i * V_{\text{max}}} + \frac{1}{V_{\text{max}}}$ .

### 247 **2.3.3. Mass spectrometry analysis**

248 Qualitative and semi quantitative analysis of the different acyl-amino acids were carried out  
 249 on an HPLC–MS-MS system (ThermoFisher Scientific, San Jose, CA, USA) consisting in a  
 250 binary pump connected to a photodiode array detector (PDA) and a Linear Trap Quadrupole  
 251 mass spectrometer (LTQ). The system was equipped with an atmospheric pressure ionization  
 252 interface operating in positive electrospray mode (ESI+). Chromatographic separation was

253 performed by injecting ten microliters of the prepared samples on a C18 Altima column  
254 (Grace/Alltech, Darmstadt, Germany, 150 mm × 2.1 mm, 5 μm porosity) equipped with a C18  
255 pre-column (Grace/Alltech Darmstadt, Germany, 7.5 mm × 2.1 mm, 5 μm porosity) at 25°C.  
256 The flow rate was set at 200 μL.min<sup>-1</sup> and mobile phases consisted of solvent A:  
257 methanol/water/TFA (80:20:0.1, v/v/v) when lauric or oleic acid was used and  
258 methanol/water (50:50, v/v) when shorter fatty acid was used and solvent B methanol/TFA  
259 (100:0.1, v/v). Acylated products were eluted using a linear gradient from 0 to 100% of B for  
260 5 min and then an isocratic step at 100% of B for 10 min. Allure Biphenyl column 3 μm  
261 column 150-2.1 mm (Restek, USA), maintained at 35°C, was used to perform the separation  
262 of the product and the fatty acid when using phenylalanine, leucine and isoleucine following  
263 the same gradient as with the C18 column. Mass spectrometric conditions were as follows:  
264 spray voltage was set at +4.5kV; source gases were set for sheath gas, auxiliary gas and sweep  
265 gas at 30, 10 and 10, respectively (in arbitrary units min<sup>-1</sup>); capillary temperature was set at  
266 250°C; capillary voltage was set at 48V; tube lens, split lens and front lens voltages were set  
267 at 120 V, -34 V and -4.25 V, respectively. Ion optics parameters were optimized in ESI+  
268 mode by automatic tuning using a standard solution of oleylysine-serine at 0.1 g L<sup>-1</sup> infused in  
269 mobile phase (A/B: 50/50) at a flow rate of 5 μL.min<sup>-1</sup>. Full scan MS spectra were performed  
270 from 100 to 1000 m/z and additional MS<sup>2</sup> scans were realized to get structural information  
271 based on daughter ions elucidation. Raw data were processed using Xcalibur software  
272 program (version 2.1, <http://www.thermoscientific.com>). MS<sup>2</sup> fragmentation data were  
273 interpreted in the way to evaluate chemoselectivity and regioselectivity of the aminoacylases  
274 from *S. ambofaciens*.

## 275 **3. Results and Discussion**

### 276 **3.1. Enzyme production**

277 All the proteins used in this study were obtained from the same seven days culture of *S.*  
278 *ambofaciens* ATCC23877 after two days of germination and pre-culture steps. After this  
279 incubation time, the cells were completely disrupted and the proteins were precipitated using  
280 ammonium sulphate. The proteins were then re-suspended and ultrafiltration using a 10 kDa  
281 membrane was performed. The filtration step allowed the elimination of almost all  
282 ammonium sulphate (decrease of the conductivity from 30 to 6.5 mS / m) and 135 mL of  
283 crude extract containing  $21 \pm 0.7$  g/L protein were recovered from the 5 L culture medium.  
284 The apparent specific activity of the extracted proteins was evaluated using lauroyl-lysine  
285 synthesis reaction. Dettori et al. (2018) used this reaction for the evaluation of the activity of  
286 immobilized acylases from *S. ambofaciens* on mesoporous material [27]. However, in this  
287 study equimolar substrate concentrations of 0.1 M were used (the influence of the  
288 concentration of each substrate will be treated later). The specific activity of the acylases from  
289 *S. ambofaciens* was  $33.4 \pm 5.0$   $\mu$ M of  $\alpha$ -lauroyl-lysine / min / mg of protein. The influence of  
290 the pH when performing this reaction was firstly investigated in order to set a fixed pH range  
291 of the reaction media for the further reactions (data not shown). The results showed that the  
292 enzyme was much less active at pH lower than 6.5 and higher than 8.5 with highest activity  
293 around pH 8. So all the further reactions were conducted at pH 8.0.

## 294 **3.2. Substrate specificity**

### 295 **3.2.1. Acyl acceptor specificity**

296 As described in a previous study [12], acylases from *S. ambofaciens* were able to perform the  
297 N-acylation of lysine and peptides with oleic acid on the amine function located on  $\alpha$ -  
298 position. The specificity towards other amino acids have never been reported. 10-undecenoic  
299 acid was used as acyl donor in this study. It is a mono-unsaturated fatty acid having  
300 antifungal, antiviral and insect-repelling activity which makes it useful to treat superficial  
301 infection [28,29]. It was also described as having neuroprotective and radical-scavenging

302 activities [30]. The enzymatic acylation of amino acids with this fatty acid has never been  
303 reported before. All condensation reactions were carried out with 1 g/L of acylases mixture at  
304  $45^{\circ}\text{C} \pm 3^{\circ}\text{C}$  during 3 days. As shown in fig. 1, the aminoacylases from *S. ambofaciens*  
305 allowed the acylation of amino acids with conversion yields from 5 to 25%, except for  
306 tyrosine, tryptophane, histidine, proline, glutamic and aspartic acids for which the quantity  
307 produced was below the detection limit. The results obtained with tyrosine and tryptophane  
308 can be attributed to their very low solubility at pH 8 which is the optimal pH of these  
309 enzymes. In the case of non-polar aliphatic amino acids and also in the case of polar and non-  
310 charged amino acids with hydroxyl group, a relation between the side chain length and the  
311 conversion percentage was observed: the longer the chain length the higher the conversion  
312 yield. The best results were obtained with lysine, arginine, leucine and methionine with  $24 \pm$   
313  $1.5$ ,  $22 \pm 1.2$ ,  $23 \pm 0.14$  and  $20 \pm 0.27\%$  conversion yields, respectively, after 3 days.  
314 According to these results, the specificity of the aminoacylases from *S. ambofaciens* was very  
315 different from that described by Koreishi et al. for the aminoacylase (of 100 kDa molecular  
316 weight) and the penicillin V acylase (Sm-PVA) from *S. mobarensis* [11,24]. Indeed, these  
317 authors used ferulic acid (10 mM) as acyl-donor for the evaluation of the 100 kDa  
318 aminoacylase of *S. mobarensis* specificity towards amino acids in tris-HCl buffer pH 7.2,  
319 containing 70% glycerol, for 6 days, at  $37^{\circ}\text{C}$ . The best conversion yield was around 12% with  
320 phenylalanine and between 0 and 8% with the other amino acids. They were able to improve  
321 the conversion rate of lysine by using only tris HCl buffer (pH 7.5) [24]. The evaluation of  
322 Sm-PVA specificity was done using lauric acid as acyl-donor (7.5 mM) in tris-HCl buffer  
323 containing 78% glycerol, for 2 days, at  $37^{\circ}\text{C}$ . The best results were observed with lysine that  
324 was acylated on its  $\epsilon$  amino group, arginine and cysteine with 44, 32 and 32% conversion  
325 yields, respectively. 0 to 10% conversion yields were obtained with the other amino acids  
326 tested [11]. Recently, Takakura et al. (2019) identified a new aminoacylase from

327 *Burkholderia sp.* specific for the acylation of amino acids with lauric acid. The evaluation of  
328 the amino acids specificity was performed after 60 h at 25°C in 100 mM sodium borate buffer,  
329 pH 9.0, containing 100 mM sodium laurate and 200 mM amino acid. The results showed that  
330 the enzyme was able to catalyse the acylation of most of the amino acids, especially when  
331 arginine, phenylalanine, lysine or valine (89, 51, 28 and 23 % yield respectively) were used.  
332 Like with the aminoacylases from *S. ambofaciens* no product was observed when using  
333 aspartic and glutamic acid as substrates [31].

334 Fig.1:

335 The acylation reaction media were analysed by LC-MS-MS after 3 days of incubation in order  
336 to check the presence of the product and to evaluate the chemo and the regioselectivity of the  
337 aminoacylases from *S. ambofaciens*. The acylation of amino acids by 10-undecenoic acid led  
338 to the formation of mono-acylated products in almost all cases except with glutamic acid and  
339 proline where no product was detected. Probably, N-undecenoyl-proline was not synthesized  
340 because of the cyclic structure of the proline with no primary amino function available. The  
341 fragmentation of the parent ion  $[M + H]^+$  of each product generated daughter ions  
342 corresponding to  $[M - COOH + H]^+$  or  $[M - COOH - H_2O + H]^+$  or  $[M - COOH - 2 H_2O +$   
343  $H]^+$  which confirmed that the acylation took place on the amino group of the amino acids. In  
344 the case of some of the amino acids carrying a reactive side chain, daughter ions that came  
345 from the radical part of the acyl-acceptor were generated. Indeed, the fragmentation profiles  
346 of acylated serine and threonine, which present a potentially reactive hydroxyl group on their  
347 radical part, were very similar. The fragmentation of the parent ion generated daughter ions  
348 coming from the carboxyl group and hydroxyl group with no fragment coming from the  
349 amine group (Fig. 2). These fragments showed that the acylation reaction occurred only on  
350 amino acid amino groups. Cysteine also generated a daughter ion that corresponded to  $[M -$   
351  $CH_2 - SH]^+$  coming from the  $-SH$  group, thus confirming the acylation of the amino acid on

352 its amino group (Fig. 2). However, the LC-MS-MS analysis showed the presence of a mass  
353 (241 m/z) corresponding to the cystine that came from the condensation of two cysteine  
354 molecules with disulphide bond bridge formation which has a very low solubility in the buffer  
355 [32]. In terms of regioselectivity, the results of the acylation of lysine and arginine showed  
356 that the acylation occurred on the  $\alpha$ -position. Indeed, the acylation of lysine with fatty acids  
357 has already been described in the literature. Whatever the fatty acid involved, two distinct  
358 fragmentation profiles were observed for the  $\alpha$  and the  $\epsilon$ -acyl-lysine [12,33]. The authors  
359 showed that for  $\epsilon$  acylated lysine only one major daughter ion corresponding to  $[M - H_2O -$   
360  $COOH + H]^+$  was found, whereas two major daughter ions corresponding to  $[M - 2 H_2O +$   
361  $H]^+$  and  $[M - H_2O - COOH + H]^+$  were obtained for the  $\alpha$ -acylated lysine. In the present  
362 study, the fragmentation of the parent ion of undecenoyl-lysine (m/z = 313) produced two  
363 major daughter ions corresponding to  $[M - 2 H_2O + H]^+$  and  $[M - H_2O - COOH + H]^+$ ,  
364 showing that acylation took place on the  $\alpha$ -position. The fragmentation of the acylated  
365 arginine generated two major daughter ion corresponding to  $[M - NH_2 - C - NH_2 - NH]$  and  
366  $[M - NH_2 - C - NH_2 - NH - H_2O]$  which indicates also an acylation on the  $\alpha$ -position. This  
367 result was in perfect accordance with a previous study using oleic acid acid as acyl donor.  
368 Indeed, Dettori et al. (2017) used lysine and peptides containing lysine as acyl acceptors to  
369 show the ability of the aminoacylases from *S. ambofaciens* to perform the N-acylation  
370 reaction. They demonstrated that the extracted enzymes catalyse the acylation of the  
371 substrates, in alpha position without any acylation on the side chain despite the presence of  
372 Sam-ELA which is specific for the acylation of lysine epsilon amino group [9,12]. This result  
373 was related to the presence of the aminoacylase Sam-AA which is responsible of more than  
374 85% of the  $\alpha$ -aminoacylase activity of the extracted enzymes [34].

375 In order to extend the comprehension of the selectivity of the aminoacylases from *S.*  
376 *ambofaciens*, other molecules like taurine, hydroxylamine, 6-aminohexanoic acid, 4-amino-



377 benzoic acid, ethanolamine and the dipeptide carnosine were used as acyl-acceptor but no  
378 acylation reaction was observed (Tab.1). However, when 6-amino-1-hexanol was used, a  
379 small quantity of the product was detected by LC-MS-MS. This result suggested the negative  
380 influence of the carboxyl group at the end of the 6-aminohexanoic acid, which is replaced by  
381 a hydroxyl group on the 6-amino-1-hexanol. This may explain the lack of reaction when using  
382 the negatively charged amino acids (aspartic and glutamic acids). In order to understand the  
383 exact biological mechanism implicated, the structure of the aminoacylase has to be elucidated  
384 and a molecular modelling study has to be performed.

385 Reactions using glycine ethyl-ester, methyl-ester and tert-butyl-ester as acyl-acceptor were  
386 also performed but no conversion was observed. These results showed that the aminoacylases  
387 from *S. ambofaciens* have a completely different acyl-acceptor selectivity as compared to a  
388 lipase such as CALB. Indeed, several author described the ability of CALB to perform the  
389 acylation of molecules such as carnosine and 6-amino-1-hexanol in organic solvent and ionic  
390 liquid [35–37]. They also compared the acylation of amino acids and amino acid derivatives  
391 like glycine and glycine tert-butyl-ester, showing an improvement of the conversion rate due  
392 to their better solubility in organic solvent. Goujard et al. (2004) evaluated the performances  
393 of CALB for the N-acylation reaction of the glycine with arachidonic acid in acetonitrile.  
394 They demonstrated that this enzyme was not efficient for the acylation of the glycine.  
395 However, the use of an activated amino acid containing tert butyl group on its carboxylic part  
396 led to a significant increase in the conversion yield [19]. Those results are completely  
397 different from the ones obtained in the present study where an activated amino acid or fatty  
398 acid doesn't allow any improvement of the activity.

399 In order to check the enantio-selectivity of the enzyme, reactions using D-phenylalanine and  
400 L-phenylalanine were compared. No acylation of the D-phenylalanine was observed  
401 suggesting that aminoacylases from *S. ambofaciens* are very specific to the natural L-amino

402 acids. Koreishi et al. (2005) also showed that the 100 kDa aminoacylase from *S. mobaraensis*  
403 wasn't able to catalyse the hydrolysis of the N-acetyl-D-methionine while exhibiting a very  
404 high hydrolysis activity of the L-enantiomer, showing the same enantioselectivity as the one  
405 of the aminoacylases from *S. ambofaciens* [24].

406 Fig.2

407 Tab.1

### 408 **3.1.1. Acyl donor specificity**

409 The evaluation of the specificity towards fatty acids was first performed by using lysine,  
410 arginine, methionine, leucine and phenylalanine as acyl-acceptors as these substrates  
411 presented high reactivity with different radical groups. Four different fatty acids were used in  
412 equimolar conditions (0.1 M of each substrate). The synthesis of N-acyl-amino acids with  
413 various fatty acids is reported in fig.3.A. The aminoacylases from *S. ambofaciens* showed  
414 different activities towards the fatty acids, depending on the amino acid used. The conversion  
415 yields obtained for N-acyl-L-phenylalanine derivatives were similar whatever the fatty acid  
416 used, with values from 10 to 15%, after 24 h. Likewise, N-acyl-L-methionine products were  
417 obtained with similar conversion yields, however a slight decrease of the conversion yield  
418 was noted when using oleic acid as acyl donor. The results obtained with arginine, lysine and  
419 leucine showed an increase of the conversion yields when increasing the fatty acid chain  
420 length from C8 to C12, and then, a decrease when oleic acid was used. This result is  
421 consistent with the work of Wada et al. (2002) who studied the substrate specificity of the  
422 acylase I from pig kidney towards different fatty acids, using arginine as acyl-acceptor. They  
423 showed nearly the same profile as the one observed with the aminoacylases from *S.*  
424 *ambofaciens* with an increase of the conversion yield from C10 to C12 fatty acid, then a  
425 significant decrease with C18 [38]. The final samples were analysed by LC-MS-MS in order

426 to evaluate the influence of the chain length on the regioselectivity of the aminoacylases from  
427 *S. ambofaciens*. The MS<sup>2</sup> spectrum after fragmentation of the parent ion of the product of  
428 each N-acyl-amino acid did not change by changing the fatty acid confirming that the  
429 ionisation of the product occurred on its amino acid part. This result confirmed that in all  
430 cases the acylation of the amino acids on the amine function was preserved. However, the  
431 structure of the lysine is particular because of the presence of two reactive amino groups. The  
432 products resulting from lysine acylation with the different fatty acids always corresponded to  
433 N- $\alpha$ -acyl-lysine derivatives except for octanoic acid that led to the synthesis of both  $\alpha$  and  $\epsilon$ -  
434 acyl-lysine. As described in Fig.4, two products corresponding to the same molar mass were  
435 found with 1.5 min retention time difference. Those products presented different MS<sup>2</sup>  
436 fragmentation profiles where the highest peak corresponded to the  $\alpha$ -acylated lysine, the same  
437 as the one described previously, and the second one, with  $[M - H_2O - COOH + H]^+$  as major  
438 daughter ion, corresponded to the  $\epsilon$ -acylated lysine. The presence of  $\epsilon$ -octanoyl-lysine can be  
439 explained by the presence of Sam-ELA in the crude extract of *S. ambofaciens* which is similar  
440 to Sm-ELA from *S. mobarensis* described by Koreishi et al. with 80% sequence identity [10].  
441 Sm-ELA was able to catalyse the acylation of lysine on its  $\epsilon$ -amino group with 58.7%  
442 conversion yield by using 10 mM octanoic acid, in 100 mM Tris-HCl, pH 7.0 buffer, at 45°C.  
443 However, Sm-ELA also led to 100% conversion by using lauric acid in the same conditions,  
444 which would not be the case with the corresponding enzyme from *S. ambofaciens* which  
445 seemed not able to catalyse the acylation of  $\epsilon$ -amino group (fig. 1). A better acylation of the  
446 lysine  $\epsilon$ -position was observed with lauric acid using Sm-PVA compared with the lysine  $\alpha$ -  
447 position. No acylation occurred with octanoic acid [11]. The arginine presents also two free  
448 amine groups. But the guanidine side chain of this amino acid remains protonated under  
449 operational conditions (pH 8) preventing its acylation [6].

450 In order to check if there is a structural relation between the regio-selectivity and the fatty  
451 acid chain length, different short chain fatty acids (acetic acid, butyric acid and valeric acid)  
452 were used with lysine as acyl-acceptor (Tab.2). The regioselectivity was evaluated by  
453 combining TLC and LC-MS-MS analyses (data not shown). In all reactions performed with  
454 fatty acid chains shorter than eight carbons, both  $\alpha$  and  $\epsilon$  mono-acylated products were found.  
455 However, there was a relation between the chain length and the regioselectivity since short  
456 carbon chains seemed to favour acylation on the  $\epsilon$ -position. Also, regioselectivity towards  
457 amino group in  $\alpha$ -position was higher with butyric acid as compared to isobutyric acid. This  
458 kind of relation has never been reported before. With molecules containing cyclic group such  
459 as benzoic acid, 4 amino benzoic and homovanillic acid, no product was detected by LC-MS-  
460 MS analysis, except for the phenylpropionic acid. This result can be explained by the short  
461 distance between the carboxylic group and the cyclic group which may lead to a steric  
462 hindrance. For the phenylpropionic acid, the LC-MS-MS analysis showed the presence of  
463 both mono-acylated products thus confirming the previous results. Even if this fatty acid  
464 contains 9 carbon atoms, the presence of the phenyl group made it similar to short length fatty  
465 acids in terms of regioselectivity. Reaction with azelaic acid (C9), which is a dicarboxylic  
466 acid widely used in cosmetics, was conducted in the same conditions. The results of the LC-  
467 MS-MS analysis showed the presence of a monoacylated product corresponding to the pseudo  
468 mass 317 m/z and a diacylated product corresponding to the pseudo mass 487 m/z. The MS<sup>2</sup>  
469 fragmentation profiles of the monoacylated product, suggested that acylation occurred on the  
470  $\alpha$ -amino group of the lysine. Another mass corresponding to 444 m/z formed by the  
471 condensation of two lysine units with both carboxylic parts of the azelaic acid was also found.  
472 This product represents a bolaamphiphile amino acid surfactant which belongs to a product  
473 family widely used in household products and cosmetics [3]. These results were confirmed by  
474 TLC-MS analysis which showed the three products described before. These results are of

475 clear interest for further studies aiming to produce such products knowing that the enzymatic  
476 synthesis of bolaform surfactants using acylases in aqueous media has never been reported  
477 until now. Indeed, several authors used enzymes, such as lipases, in the synthesis of bolaform  
478 surfactants [39]. However, a chemical step have to be performed to recover the  
479 bolaamphiphilic surfactant. Valivety et al. (1998) performed the reaction of esterification of  
480 N-protected amino acids for the synthesis of bolaform surfactant using lipases. However, in  
481 order to recover the final product a chemical step had to be performed in order to remove the  
482 protection from the amino acids [39]. Consequently, it is the first time that the one-step  
483 enzymatic condensation on both carboxylic groups of a diacid is described.

484 In order to confirm the contribution of Sam-ELA to the acylation of the lysine with octanoic  
485 acid on its side chain, the gene sequence that express the production of Sam-ELA and Sam-  
486 PVA in *S. ambofaciens* was deleted. This allowed the expression of the sequence  
487 corresponding to Sam-AA, which was described as being of major importance in the N- $\alpha$ -  
488 acylation activity [34]. The transformed strain was cultivated and the enzyme was extract  
489 following the same steps as with the wild strain of *S. ambofaciens*. As shown in the fig.3 B,  
490 the presence of only Sam-AA allowed the N-acylation of the lysine on its  $\alpha$ -position with all  
491 fatty acid used even with octanoic acid with nearly similar conversion rate. Knowing that  
492 Sam-PVA is similar to Sm-PVA described by Koreishi et al., 2006 as being specific to the  
493 acylation of amino acids with lauric acid only, this result confirms that the  $\epsilon$ -acylation of  
494 lysine with octanoic acid observed with the aminoacylases from the non-transformed *S.*  
495 *ambofaciens* was due to the presence of Sam-ELA [11].

496 Fig.3:

497 Tab.2:

498 Fig.4:

### 499 3.2. Influence of substrate concentration on the synthesis of N- $\alpha$ -lauroyl-L-lysine

500 Lauroyl-lysine is one of the most used acylated derivative of amino-acids among healthcare  
501 products due to its interesting surface active property and biological activity. Several studies  
502 reported the acylation of lysine on its  $\epsilon$ -position by using lipases or acylases but very few  
503 enzymes were described as being able to catalyse the acylation of lysine on its  $\alpha$ -position  
504 [12,24]. In order to evaluate the influence of each substrate on the reaction rate, the  
505 concentration of one substrate was set to 100 mM while different concentrations of the  
506 substrate under study were used (from 25 to 250 mM for lysine and from 10 to 200 mM for  
507 lauric acid). The increase of lysine concentration from 25 to 150 mM led to an increase of the  
508 initial rate of the reaction. The curves of initial rates followed standard Michaelis-Menten  
509 model allowing the determination of  $K_m$  and  $V_{max}$  for the amino acid. According to the  
510 equation of Lineweaver and Burk, a  $V_{max}$  and  $K_s$  of around 3.1 mM / h and 69 mM were  
511 found, respectively (Tab.3). However, at concentrations higher than 175 mM, a decay in the  
512 initial rate was observed. This decay might be due to an inhibition by the amino acid.

513 The effect of the lauric acid concentration was conducted by using 100 mM lysine and  
514 concentrations from 0 to 200 mM of lauric acid. Between 10 and 50 mM lauric acid, a low  
515 increase in the conversion was observed but a decrease was observed at concentrations higher  
516 than 75 mM. This decrease can be attributed to mass transfer limitation. Wada et al., 2002  
517 also attributed the decrease of the activity by increasing lauric acid concentration when using  
518 acylase I from Pig Kidney in buffer/glycerol mixture, to the fatty acid saturation which is  
519 reached at around 10 mM [38]The second reason could be the inactivation or the inhibition of  
520 the enzyme by the reaction product [10,15]. In this case,  $V_{max}$  and  $K_i$  of around 4.4 mM h<sup>-1</sup>  
521 and 85 mM, respectively, were found (Tab.3). A  $K_m$  of around 2 mM was also determined.

522 Tab.3:

### 3.3. Influence of metal ions on the reaction rate

As described by Dettori et al., [12] the genes encoding the aminoacylases from *S. ambofaciens* present close similarities to the ones encoding acylases from *S. mobarensis*, which were described by Koreishi et al. [9,11,25]. Two of those identified acylases (Sm-AA and Sm-ELA) were described as belonging to a metallo-dependant family which can be influenced by the addition of metal ions. In order to evaluate the effect of metal ions on the activity of acylases from *S. ambofaciens*,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$ , described as the most influencing ones, were added in the reaction medium [10,11,24,25]. As shown in Fig.5A, for the same concentration of the three metal ions (0.1 mM), the addition of  $\text{Co}^{2+}$  ion led to a three time increase of the initial rate while  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  ions only led to a low increase of the initial rate. Very low conversion yield was observed when EDTA (metal scavenger) was used, showing that the observed activity originates from a metallo-enzyme. Indeed, even if the initial rate in the presence of EDTA was similar to the one without, a decrease of 75% of the conversion rate was observed. This result can be explained by a decay of the enzymatic stability when a chelating agent is used. The same result was reported by different authors with others metallo-enzymes, where a partial decrease of the activity was registered when using EDTA [10,24,31]. However, the conversion rate at thermodynamic equilibrium decreased to 11 and 17% using  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  ions, respectively at 0.1 mM, while it remained stable with  $\text{Co}^{2+}$ . The influence of  $\text{Co}^{2+}$  concentration was therefore investigated (Fig.5B). The increase of the Cobalt concentration up to 0.4 mM allowed an important increase (about 6 times) of the initial rate of the reaction (Tab.4). The thermodynamic equilibrium in those conditions was reached after only 4 h reaction time instead of around 72 h without  $\text{CoCl}_2$ . The LC-MS-MS analysis did not show any modification in the regioselectivity of the enzyme. Koreishi et al. 2005 also studied the influence of metal ions on the hydrolytic activity of *S. mobarensis* aminoacylaseS. Their results were similar to the ones of the present study with an

548 increase of around 3 times of the activity by adding 0.1 mM CoCl<sub>2</sub>. However, they observed a  
549 decrease of around 80% of the activity by adding Cu<sup>2+</sup> ion, which was not the case of the  
550 acylases from *S. ambofaciens* [24].

551 Fig.5:

552 Tab.4:

## 553 **Conclusion**

554 The performances of the aminoacylases from *S. ambofaciens* in terms of substrate specificity  
555 and regioselectivity were evaluated. This work showed for the first time a relationship  
556 between the side chain length of the amino acids, especially with non-polar aliphatic amino  
557 acids, and the conversion rate (from 6% for glycine to 23% for leucine). Furthermore, the side  
558 chain nature of the amino acid was shown to play an important role. Best results were  
559 obtained with positively charged amino acids (lysine and arginine) while no reaction was  
560 obtained with the negatively charged ones (aspartic and glutamic acids). The results  
561 concerning the acyl donor specificity showed that the regioselectivity was affected by the  
562 fatty acid chain length which was attributed to the presence of Sam-ELA in the crude extract  
563 of *S. ambofaciens*. Indeed, this enzyme is able to perform the acylation of the lysine on its  
564 side chain and appears to be also specific for short chain fatty acid. Moreover, for the first  
565 time, the possible synthesis of bolaamphiphile amino acid surfactant using azelaic acid and  
566 lysine was shown. The study concerning the synthesis of lauroyl lysine in various operational  
567 conditions showed an inhibition of the enzymes by the product of the reaction. In order to  
568 overcome this issue, the future development of processes using continuous flow reactors such  
569 as packed bed reactors with immobilized aminocylases from *S. ambofaciens* appears as a  
570 promising way. The results obtained in the presence of metal ions showed that it was possible  
571 to significantly increase the reaction by adding CoCl<sub>2</sub> that allowed to reach the



572 thermodynamic equilibrium in few hours. Further works are in progress to intensify the  
573 process increasing the conversion yield in order to develop a green process for the production  
574 of eco-friendly lipoaminoacids as bio-based surfactant using *S. ambofaciens* aminoacylases in  
575 aqueous media.

576

577

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## **Figures:**

Fig.1 Conversion yields for the synthesis reaction using various amino acids (100 mM) and 10-undecenoic acid (100 mM). Reaction conditions: 3 days, at 45°C, 250 rpm agitation, in 25 mM Tris-HCl buffer, NaCl 50mM, pH 8, using 1 g/L enzyme

Fig.2 MS<sup>2</sup> spectra after fragmentation of the parent ion of (A) N-10-undecenoyl-threonine (B) N-10-undecenoyl-cysteine synthesized using aminoacylases from *S. ambofaciens*

Fig.3 Conversion yields using various amino acids (100 mM) and fatty acids (100 mM). A. acylation of arginine, methionine, leucine and phenylalanine using the aminoacylases from *S. ambofaciens*, B. Acylation of lysine using the aminoacylases from *S. ambofaciens* and Sam-AA from transformed *S. ambofaciens*. Reaction conditions: 1 day, at 45°C, 250 rpm agitation, in 25 mM Tris-HCl buffer, NaCl 50mM pH 8, using 1 g/L enzyme

Fig.4 Mass analysis of octanoyl-lysine using aminoacylases from *S. ambofaciens* (A) single ion chromatogram of the acylated product [M+H]<sup>+</sup> parent ion (m/z = 273); (B) MS<sup>2</sup> spectrum after fragmentation of the parent ion at 5.79 min retention time; (C) MS<sup>2</sup> spectrum after fragmentation of the parent ion at 7.36 min retention time.

Fig.5: Influence of metal ions on the initial rate of the  $\alpha$ -lauroyl-lysine (C12K) synthesis reaction (0.1 M of lauric acid and lysine) using *S. ambofaciens* acylases in tris HCl 25mM NaCl 50 mM buffer pH 8 at 45°C, (A) Produced concentration of C12K during the time according to the metal ion used and the concentration of COCl<sub>2</sub> used (B)

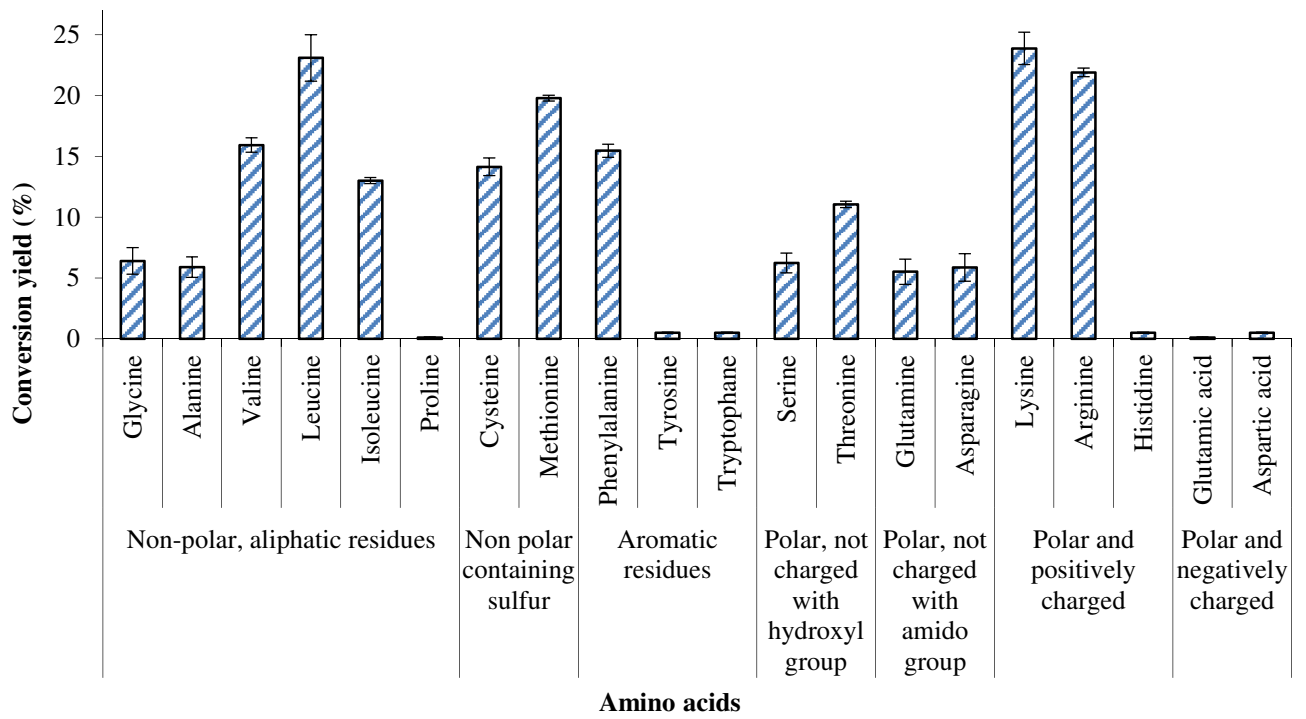
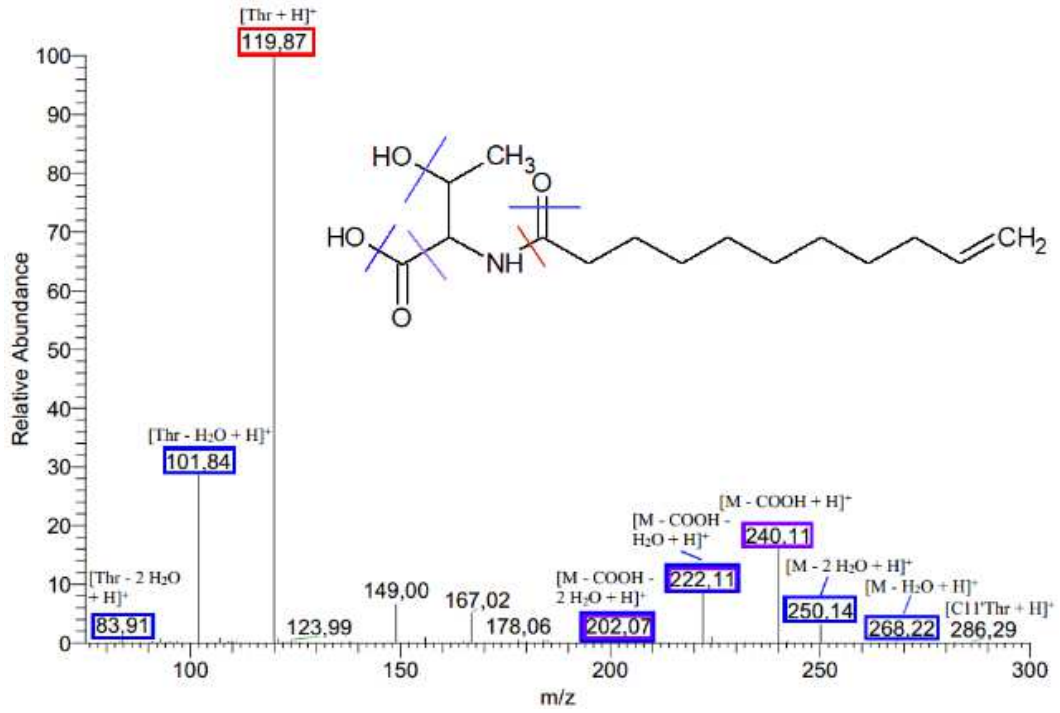


Fig.1

A



B

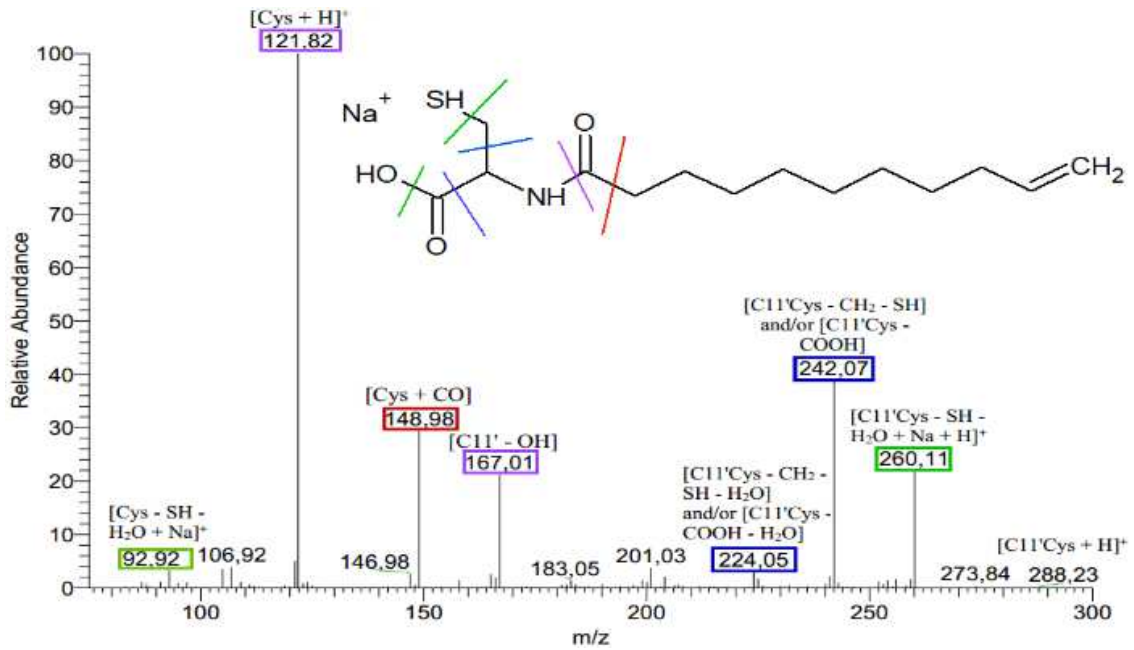


Fig.2

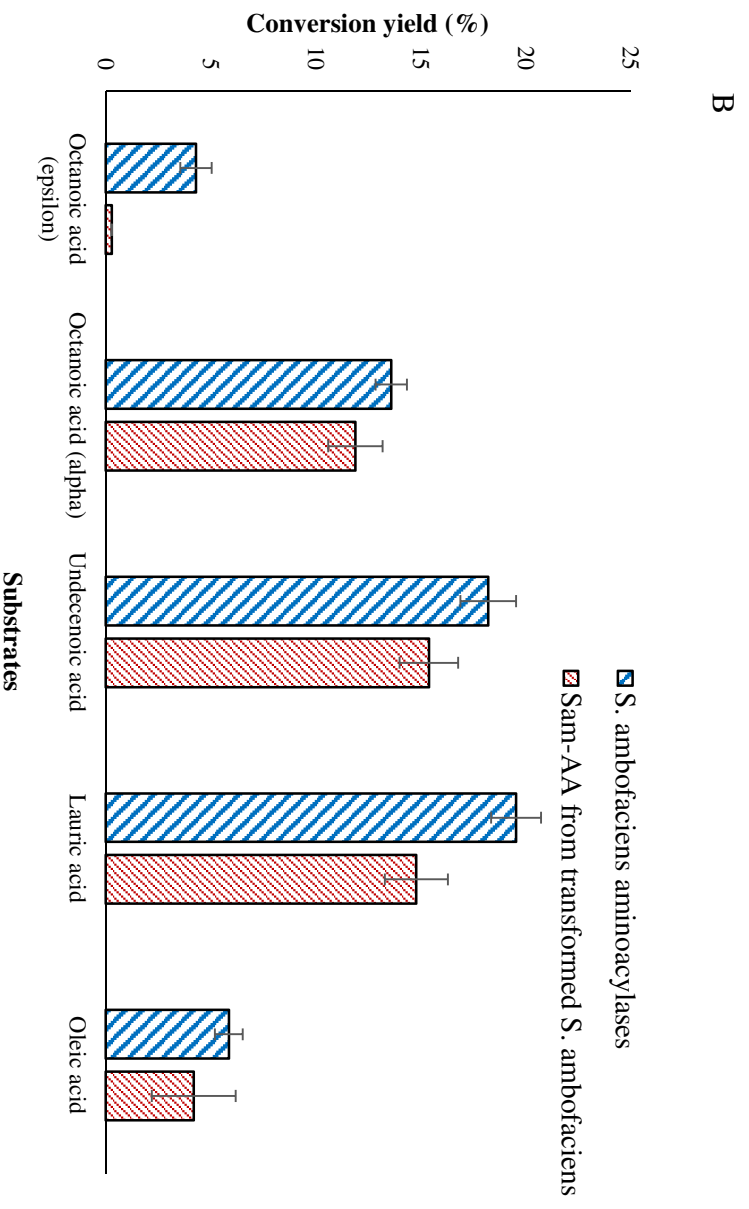
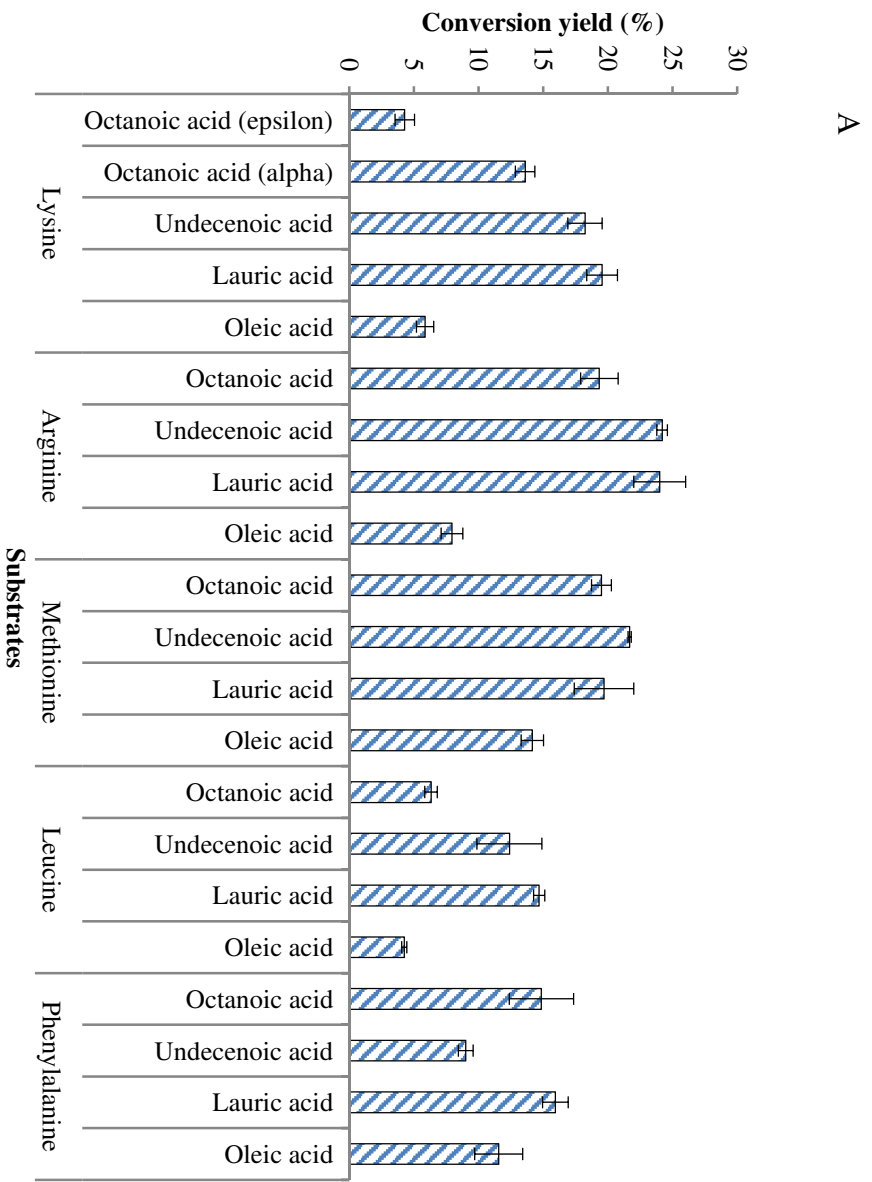
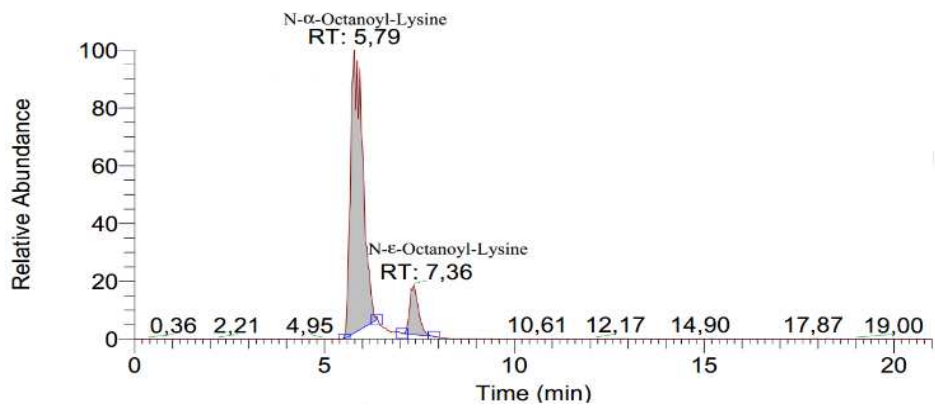
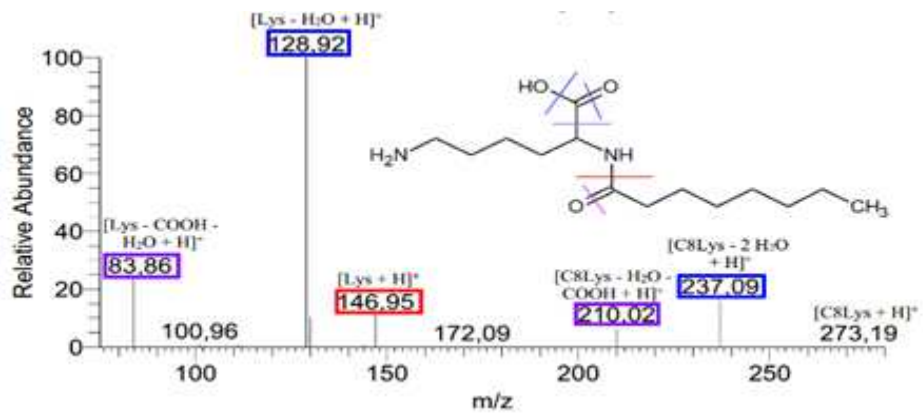


Fig. 3

A



B



C

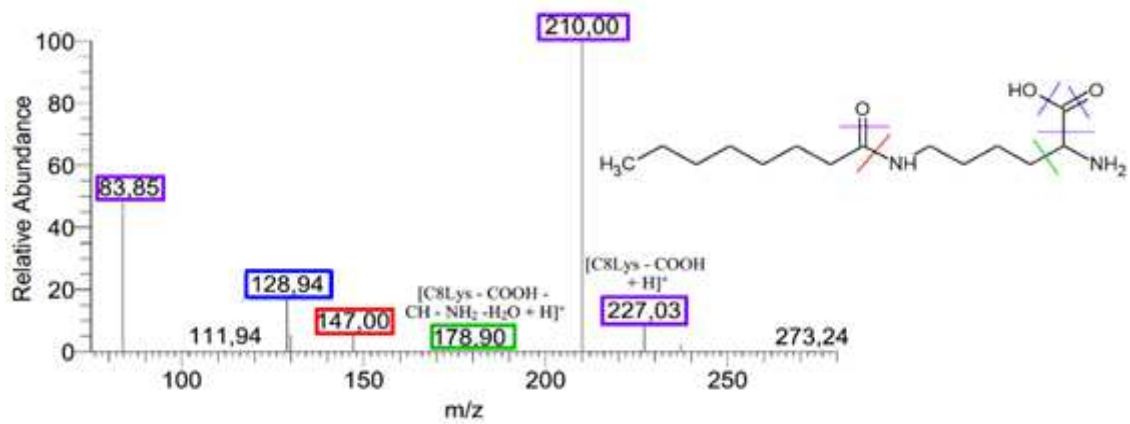
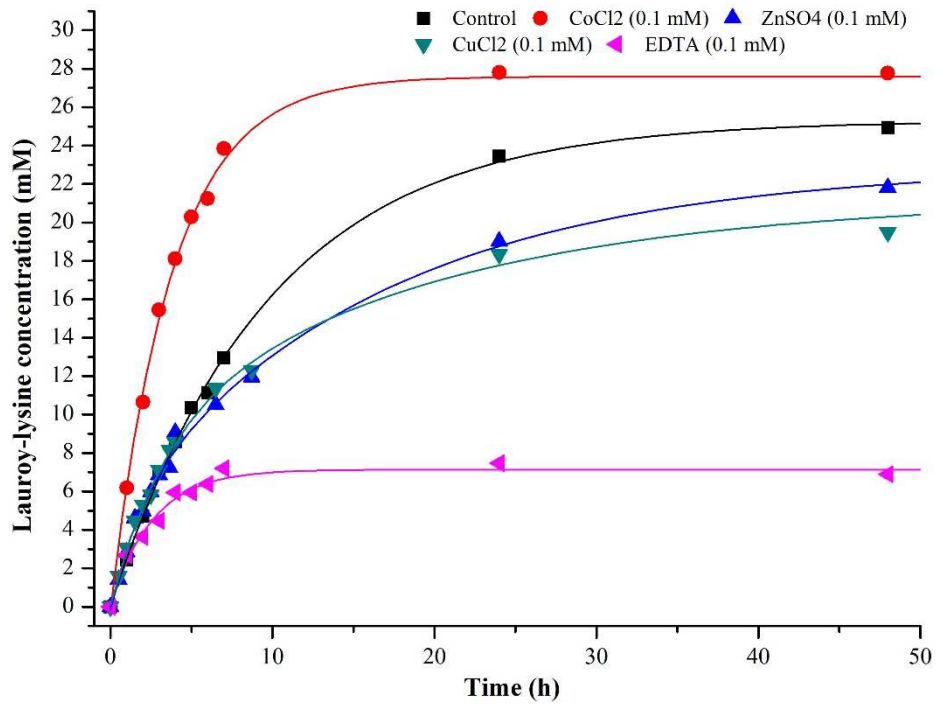


Fig.4



A



B

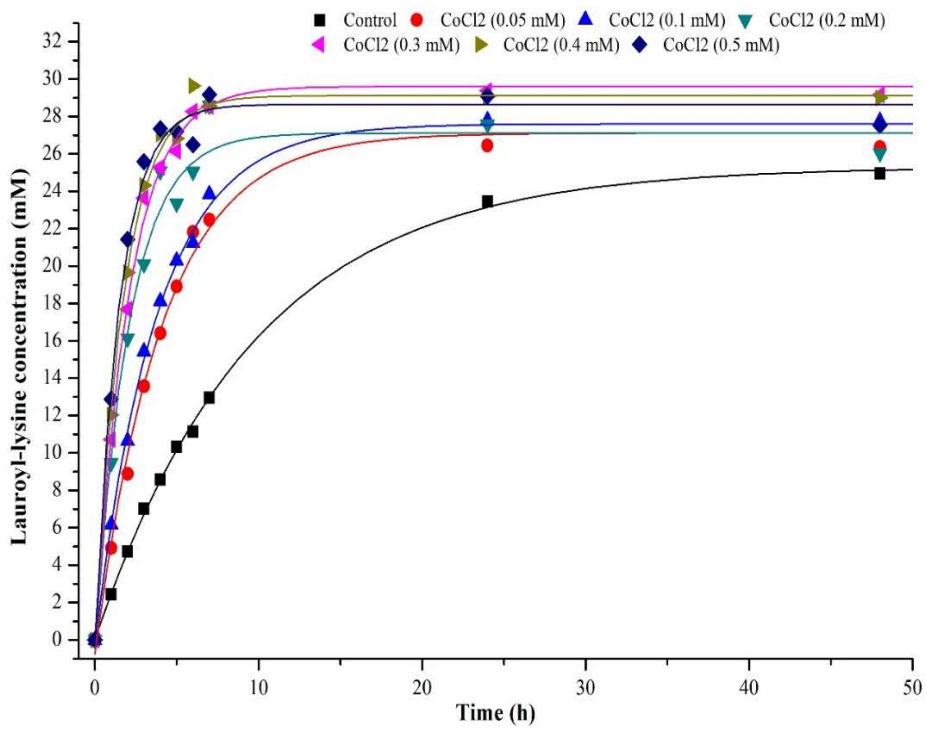
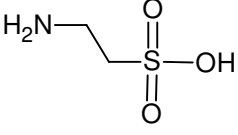
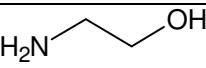
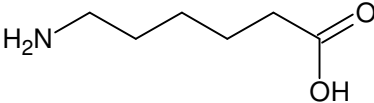
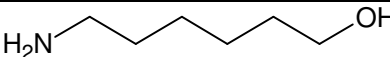
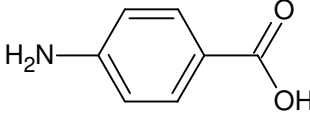
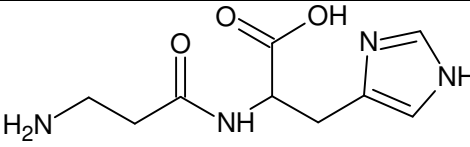
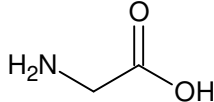
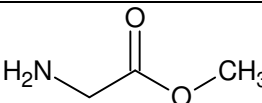
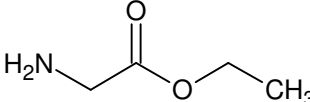
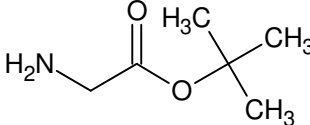


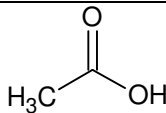
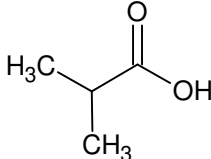
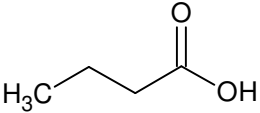
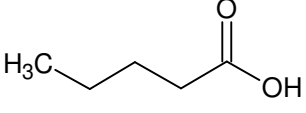
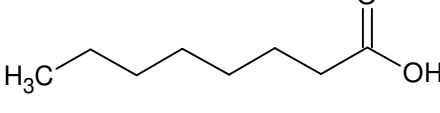
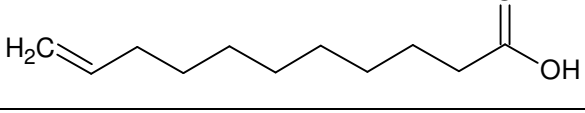
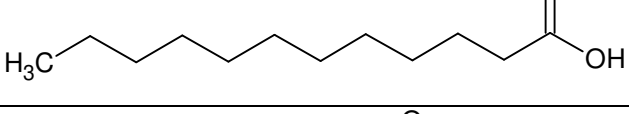
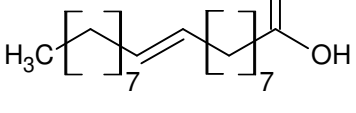
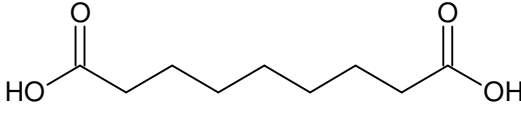
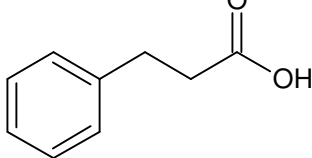
Fig.5

**Table :**

Tab.1 structure of the acyl receivers used

Fatty acid	Molecular structure	Reaction
Taurine		-
Hydroxylamine	$\text{H}_2\text{N}-\text{OH}$	-
Ethanolamine		-
6-aminohexanoic acid		-
6-amino-1-hexanol		+
4-amino-benzoic acid		-
Carnosine (N-β alanyl, L-histidin)		-
Glycine		+
Glycine methyl-ester		-
Glycine ethyl-ester		-
Glycine tert-butyl-ester		-

Tab.2 structure of the fatty acids used

Fatty acid	Molecular structure	Acylation position	
		Alpha	Epsilon
Acetic acid		+	+
Isobutyric acid		+	+
Butyric acid		+	+
Valeric acid		+	+
Octanoic acid		+	+
10-undecenoic acid		+	-
Lauric acid		+	-
Oleic acid		+	-
Azelaic acid <sup>a</sup>		+	-
Phenylpropionic acid		+	+

a: acylation on both carboxylic group

Tab.3: Kinetic parameters of the substrates concentrations for  $\alpha$ -lauroyl-lysine synthesis

	Lysine	Lauric acid
Vmax (mM/h)	$3.1 \pm 0.2$	$4.4 \pm 0.2$
Km (mM)	$69 \pm 7$	$2.1 \pm 0.3$
Ki (mM)	-	$85.5 \pm 7.8$

Tab.4 Initial rate evolution depending on the condition.

Metal ion	No metal ions	EDTA	CuCl <sub>2</sub>	ZnSO <sub>4</sub>	CoCl <sub>2</sub>	CoCl <sub>2</sub>				
Concentration (mM)		0.1	0.1	0.1	0.1	0.05	0.2	0.3	0.4	0.5
Initial rate (mM / h)	2.6 ± 0.4	2.4 ± 0.2	2.9 ± 0.4	3.2 ± 0.0	6.3 ± 0.6	4.6 ± 0.1	12.1 ± 0.2	14.2 ± 0.0	14.6 ± 1.8	15.8 ± 2.0

# Graphical abstract

