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

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Short title: "Enzymatic synthesis of N-10-undecenoyl-phenylalanine"

Abstract:

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

1. Introduction:

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

scale because of their good detergency and foaming properties [2,3]. In addition to these properties, phenylalanine-based surfactants were described as presenting cytotoxicity against several human cancer cells thus widening their application scope [4]. Bisset et al., 2009 described the ability of phenylalanine-fatty acid derivatives like N-10-undecenoyl-L-phenylalanine to reduce melanin production in melanocytes. In their study, an emulsion prepared by the combination of 1 % N-10-undecenoyl-phenylalanine with 5 % niacinamide increased significantly the efficiency in reducing the appearance of facial hyperpigmentation *in vivo* [5]. However, such AAS are produced at industrial scale using the Schotten-Baumann chemical reaction where the amino acid reacts with a fatty acid chloride under highly alkaline conditions by using NaOH in water and sulfuric acid to stop the reaction [4]. In some cases, some organic solvents are used to facilitate the reaction. In this last case a high purity powder with only some fatty acid traces is obtained. Despite a high yield of synthesis, the corresponding down-stream processing of the reaction medium leads to large volumes of saline effluents requiring an expensive reprocessing step for the industrials [6]. Nowadays, the environmental issues caused by such process encouraged the authorities to promote the industrial development of sustainable technologies by switching from waste remediation and pollution control by end-of-pipe solution to waste prevention thus reducing the reprocessing cost [7]. Enzymes represent an alternative for such reactions to avoid by-products and salts generation. Enzymes were widely used for acylation reaction especially for O-acylation but much less for N-acylation of amino acids. The ability of some enzymes to perform the N-acylation of amino acids were studied by only few authors using free fatty acids [8–15]. Lipases and proteases are hydrolases that can perform the acylation reaction in non-aqueous media like organic solvents or in solvent-free conditions in order to shift the thermodynamic equilibrium of the reaction. However, the low solubility of amino acids in organic solvents 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 ∑ 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 α-acylation activity. In addition, the authors demonstrated that the enzyme similar
91 to Sm-AA, named SamAA, was responsible of 85 % of the N-α-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-α-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 pharmaceutics 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

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

2.2. Enzymatic N-acylation of L-Phenylalanine assays

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

The influence of substrate concentration was investigated by using concentrations from 25 to 200 mM of L-phenylalanine and from 25 to 200 mM of 10-undecenoic acid setting the other substrate's concentration at 100 mM. The influence of the pH of the reaction was studied in the pH range [6-9.5] using NaOH 7.5 M and HCl 1 M to control the pH. Temperature effect on the reaction was evaluated in two steps. The first one was the evaluation of the optimal temperature of the enzymes by performing the synthesis reaction in a temperature range of [30-55°C]. The second step was to evaluate the stability of the enzymes by performing the standard reaction after incubation at different temperature (37, 45 and 55°C) and during different incubation time (16 and 25 h). The enzymes concentration influence was evaluated by using (0.25, 0.5, 1 and 2 g/L) protein concentration at the standard conditions. The inhibition by the product of the reaction was investigated by adding different concentrations of a chemically produced commercial N-10-undecenoyl-phenylalanine. The relative activity was then calculated using the initials rates of the reactions with ($Vi_{C11'F}$) and without (Vi_0) the addition of the product ($relative\ activity\ (\%) = \frac{Vi\ C11'F}{Vi\ 0}$).

We investigated the effect of metal ions by using 0.1 mM of $CuCl_2$ (from Fluka Chemika), $CoCl_2$, $ZnSO_4$ (from Sigma), and Ethylene-Diamine-Tetra-acetic Acid (EDTA) in the standard conditions. The influence of $CoCl_2$ concentration was evaluated by using $CoCl_2$ concentrations in the range [0-0.5 mM]. In all case, the reaction was started after one-night agitation, in order to check if there was a reaction without enzymes, by adding 1 g/L of the crude extract of aminocaylases from *S. ambofaciens*.

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

2.3. Reactor configuration

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

2.4. Purification of the final product:

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

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

2.5. Analytical methods

2.5.1. Quantitative analyses by HPLC

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

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

2.5.2. Mass spectrometry analysis

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

2.6. Biological activity evaluation

The evaluation of the melanin production by melanocytes was performed using B16 murine melanoma cells (B16F1 cells) following the method described by Chung et al., 2019 with some adaptations [24]. B16F1 cells were maintained in a CO_2 incubator in Dulbecco's modified Eagle medium (DMEM), without phenol red, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated in CO_2 for 24 h after being seeded in 96-well plate (6000 cells/well). Then cells were treated with Sepiwhite™ MSH (SEPPIC) or enzymatic N-10-undecylenoyl-phenylalanine initially dissolved in ethanol or the culture medium. Two different concentrations of each was tested (10 and 50 µg/mL) and kojic acid at 50 µg/mL in the culture medium was used as a positive

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

Results and Discussion

3.1. Influence of substrate concentration on enzymatic activity

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

± 0.7 g/L of proteins [28]. The apparent specific activity of the proteins crude extract was evaluated using lauroyl-lysine synthesis reaction performed with an equimolar concentration of the lauric acid and lysine (100 mM). The reaction was performed using 1 g/L of enzymatic crude extract. The apparent specific activity of the acylases from *S. ambofaciens* was 33.4 ± 5 µM of α-lauroyl-lysine / min / mg of proteins.

All N-acylation (amidation) reactions were carried out in 25 mM Tris-HCl buffer, NaCl 50 mM pH 8, containing 1 g/L acylases at 45°C ± 3°C. In order to evaluate the substrate concentration influence on enzyme activity different reactions were performed. First of all, the best substrate concentration, at equimolar conditions, was evaluated for the further parameters evaluation by using different concentration (25 mM, 50 mM, 100 mM and 200 mM). The concentration produced after 48 h (Fig.1) showed a maximum reaction by using 100 mM of both substrates while very low activity was observed in the other conditions. The influence of the substrate concentration on the performance of the aminoacylases from *S. ambofaciens* was then studied by fixing the concentration of one of the substrates at 100 mM and by varying the concentration of the other substrate from 25 mM to 200 mM. The influence of the concentration in 10-undecenoic acid was very low compared to the influence of phenylalanine concentration (Fig.2). The initial rate of the reaction was increased by the increase of the concentration of the fatty acid from 25 mM to 125 mM followed by a stabilization of the reaction rate. The evolution of the initial rate followed standard Michaelis-Menten model allowing the determination of Km and Vmax for the undecenoic acid. According to the equation of the graphic representing $\frac{1}{V} = f\left(\frac{1}{S}\right)$ Vmax and Km of around 0.50 ± 0.05 mM / h and 19.2 mM were found. Above a C11' concentration of 175 mM a slight decrease of the activity was observed which can be due to a substrate inhibition. The influence of the amino acid was more pronounced than the fatty acid where a continuous increase of the initial rate was observed. Indeed, according to the equation of the graphic

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

Fig.1:

Fig.2:

3.2. Influence of the pH on aminoacylases activity

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

Fig.3:

3.3. Influence of the temperature

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

Fig.4:

3.4. Influence of protein concentration

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

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

Fig.5:

3.5. Influence of metal activator on the reaction

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

In a perspective of process intensification and to evaluate the best way to produce the C11'F at gram scale, different reactor configurations were evaluated. $CoCl_2$ was not added in this part in order to avoid residual Co^{2+} in the final product. Intensified designed reactors were used by different authors for enzymatic intensification allowing an enhancement of the activity [30,31]. In this kind of reactors free or immobilized enzymes can be used. The immobilization of the acylases from *S. ambofaciens* was evaluated on several supports with different characteristics (hydrophilic, hydrophobic and surface modified supports) and using different immobilisation technics (adsorption, covalent and entrapment) however the aminoacylases lost almost all of their activity (results not shown).

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

Fig.7:

3.7. Purification of the reaction product

As detailed in the Materials and Methods section, the C11'F was purified using filtration and chromatographic methods. The first step consisting in an ultrafiltration on a 10 kDa allowed the selective passage of the product of interest and the residual fatty acid (331 Da and 184 Da 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

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

Tab.2:

Conclusion

The aim of this study was to evaluate the operational conditions influencing the performances 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 $CoCl_2$. 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.

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

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Figures

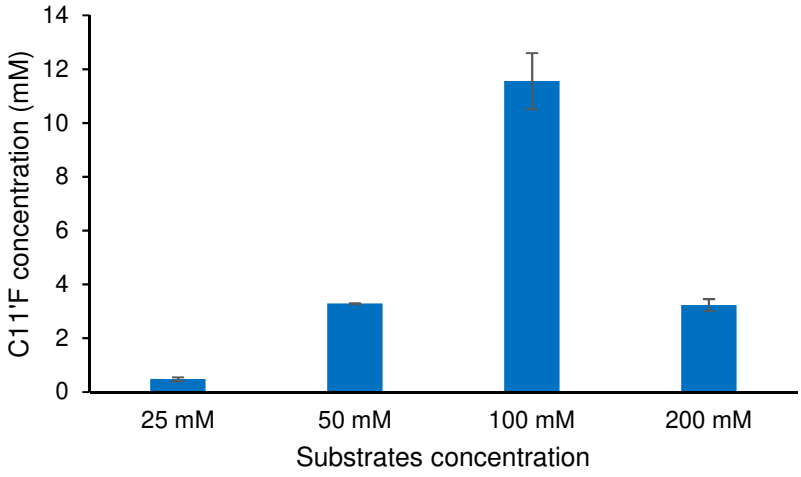


Fig.1 :

A. B.

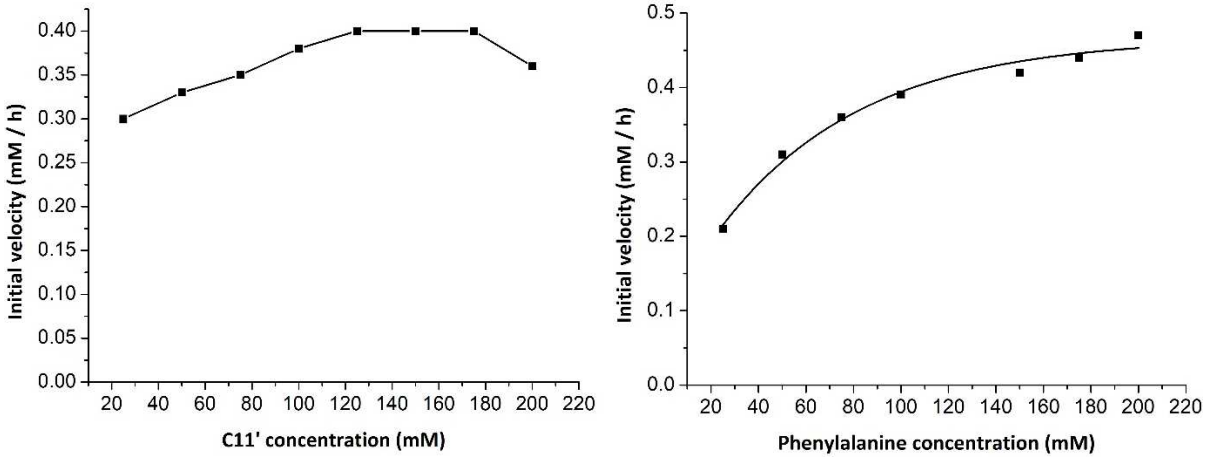

Initial velocity (mM / h)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
20 40 60 80 100 120 140 160 180 200 220
C11' concentration (mM)
Initial velocity (mM / h)
0.0
0.1
0.2
0.3
0.4
0.5
20 40 60 80 100 120 140 160 180 200 220
Phenylalanine concentration (mM)

Fig.2:

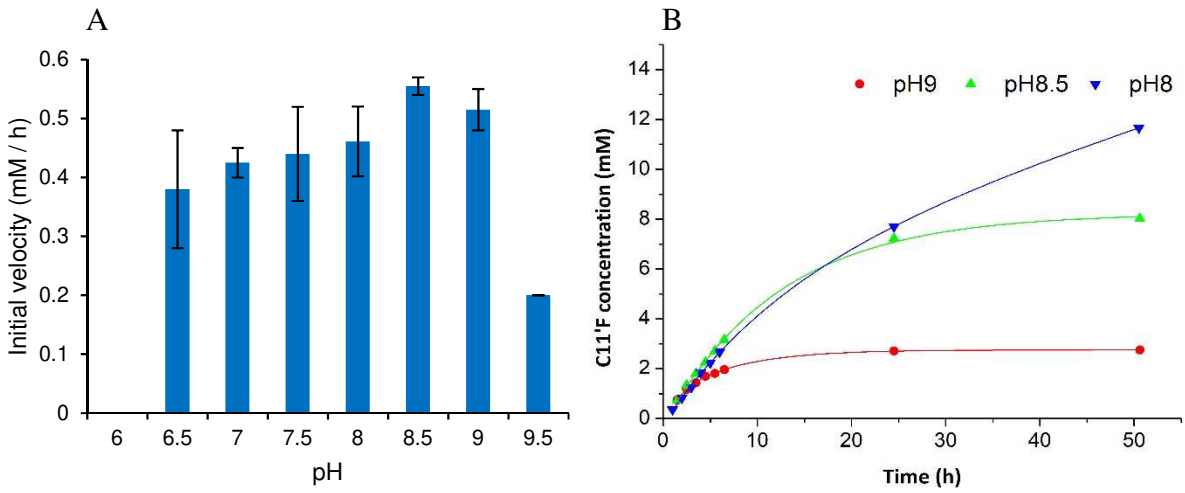

A
Initial velocity (mM / h)
0.6
0.5
0.4
0.3
0.2
0.1
0
6
6.5
7
7.5
8
8.5
9
9.5
pH
B
C11'F concentration (mM)
14
12
10
8
6
4
2
0
pH9
pH8.5
pH8
0
10
20
30
40
50
Time (h)

Fig.3:

A. B.

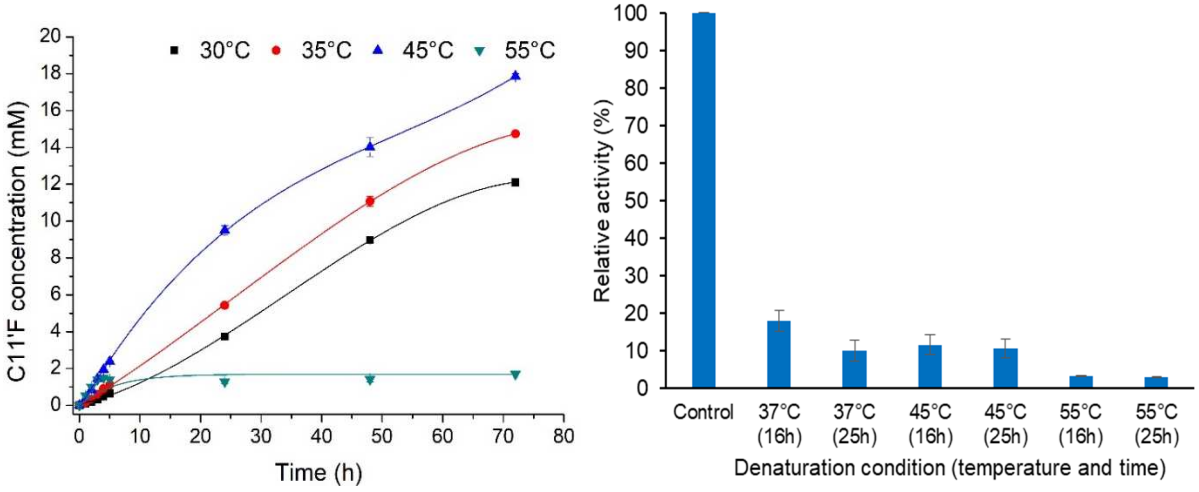

30°C
35°C
45°C
55°C
C11F concentration (mM)
Time (h)
Relative activity (%)
Control
37°C (16h)
37°C (25h)
45°C (16h)
45°C (25h)
55°C (16h)
55°C (25h)
Denaturation condition (temperature and time)

Fig.4:

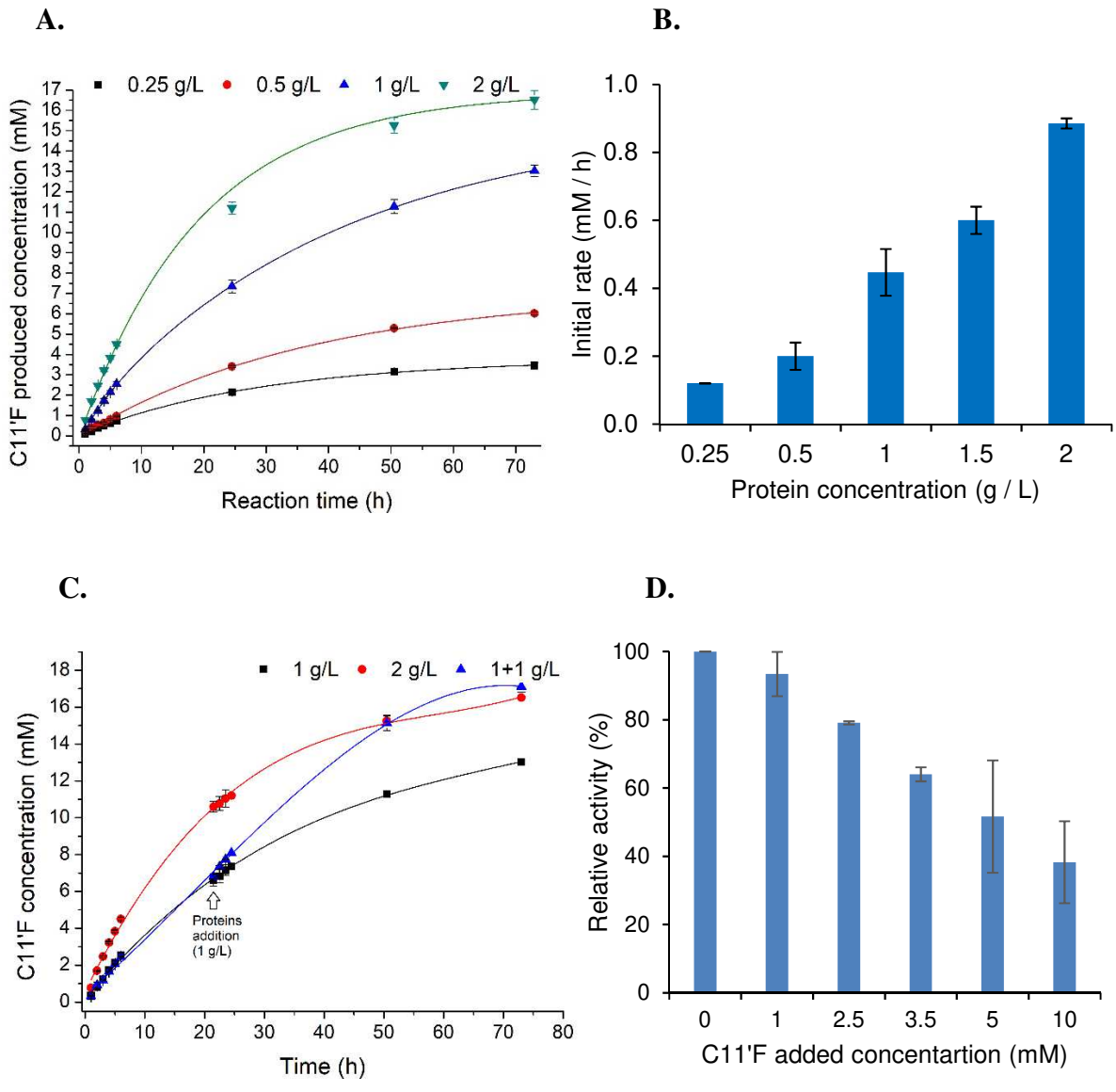

A.
B.
0.25 g/L
0.5 g/L
1 g/L
2 g/L
C11'F produced concentration (mM)
Reaction time (h)
Initial rate (mM / h)
Protein concentration (g / L)
C.
D.
1 g/L
2 g/L
1+1 g/L
C11'F concentration (mM)
Proteins addition (1 g/L)
Time (h)
Relative activity (%)
C11'F added concentartion (mM)

Fig.5:

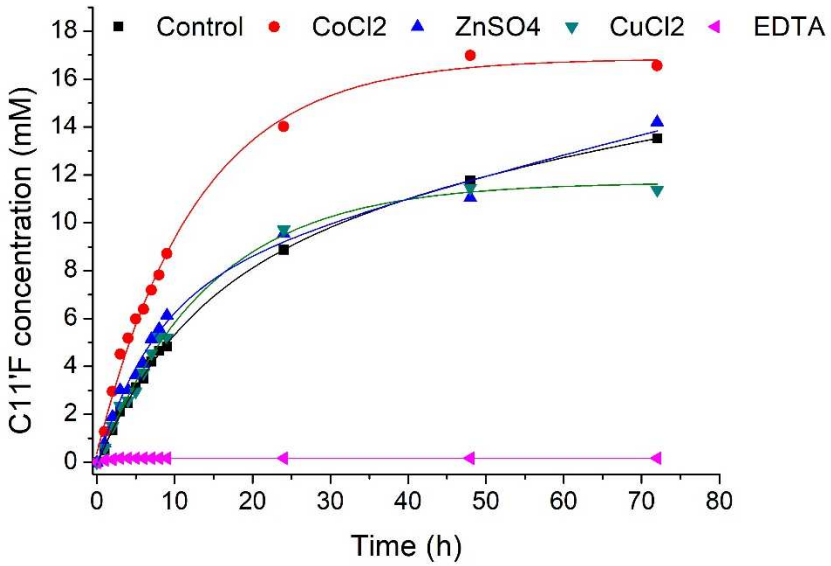
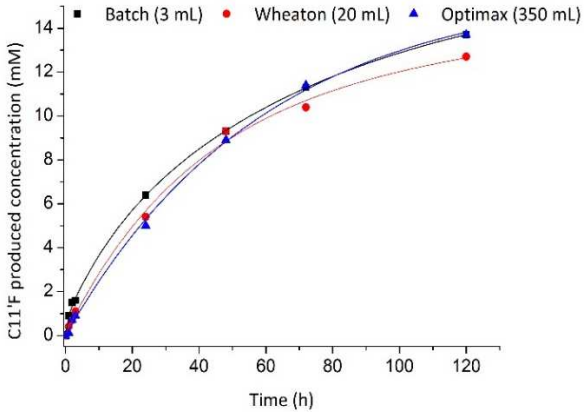

Control
CoCl2
ZnSO4
CuCl2
EDTA
C11'F concentration (mM)
Time (h)
0
2
4
6
8
10
12
14
16
18
0
10
20
30
40
50
60
70
80

Fig.6:

A.

B.


Batch (3 mL)
Wheaton (20 mL)
Optimax (350 mL)
C11'F produced concentration (mM)
Time (h)

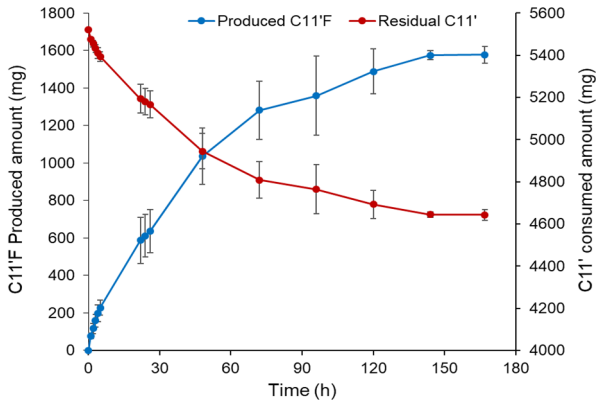

Produced C11'F
Residual C11'
C11'F Produced amount (mg)
C11' consumed amount (mg)
Time (h)

Fig.7:

740

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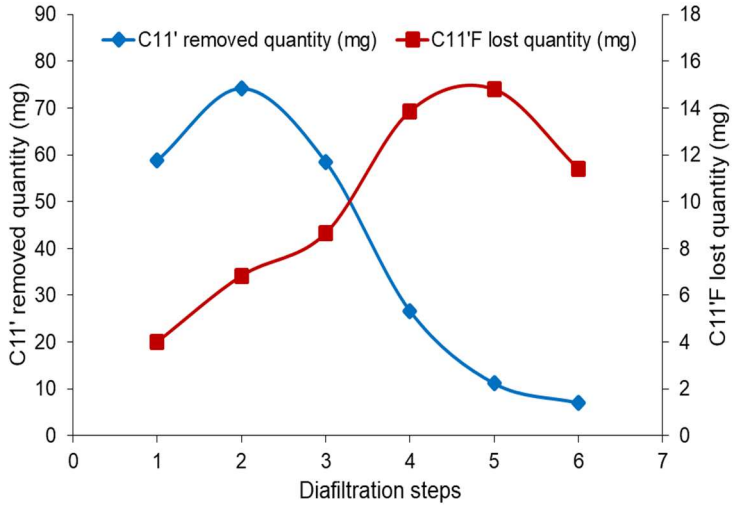
749

750 A.



751

752 B.

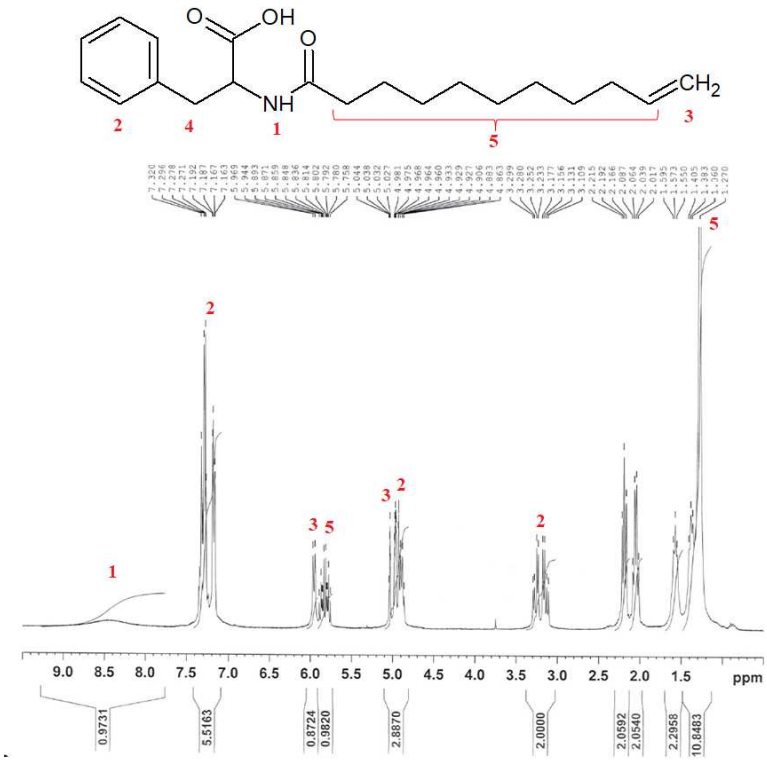


Fig.8:

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

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

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

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

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

Tables:

Tab.1 effect of metal ion and the concentration of $CoCl_2$ on the initial rate of the reaction synthesis of N-10-undecenoyl-phenylalaninein and the C11'F produced concentration after 48h in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases at 45°C, pH 8 and 500 rpm magnetic agitation.

Metal ion	No metal ions	$ZnSO_4$	$CuCl_2$	$CoCl_2$	$CoCl_2$

Concentration (mM)		0.1	0.1	0.1	0.05	0.2	0.3	0.4	0.5
Initial rate (mM / h)	0.4 ± 0.08	0.73 ± 0.17	0.61 ± 0.05	**1.01 ± 0.26**	0.69 ± 0.18	1.64 ± 0.1	2.48 ± 0.7	3.22 ± 0.6	**4.34 ± 0.4**
C11'F concentration (mM)	11.8 ± 1.5	12.1 ± 3	11.5 ± 2.2	**17 ± 1.2**	15.7 ± 1.5	17.7 ± 2.5	**22 ± 0.35**	20 ± 2.9	17.2 ± 2.7

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 C11'F	Enzymatic C11'F		Chemical C11'F	Enzymatic C11'F	
Ethanol	50	9	**26**	0.0002 (***)	**84**	75	0.0001 (***)
	10	6	9	0.2101 (***NS***)	**98**	95	0.0100 (*)
Culture	50	**67**	48	0.0463 (*)	76	**82**	0.0018 (**)

medium	10	17	13	0.2514 (***NS***)	**100**	97	0.0205 (*)

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