

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

Mohamed Chafik Bourkaib^a, Stephane Delaunay^a, Xavier Framboisier^a, Catherine Humeau^a, Jérôme Guilbot^b, Cecile Bize^b, Estelle Illous^b, Isabelle Chevalot^a, Yann Guiavarc'h^{a,*}

^a LRGP, UMR 7274 CNRS-Université de Lorraine, 2 avenue de la Forêt de Haye, TSA 40602, F-54518, VANDŒUVRE Cedex, France

^b R&D laboratory of Seppic, Air Liquide Healthcare Specialty Ingredients, 50 boulevard National - CS 90020, 92257, La Garenne Colombes Cedex, France

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 the reaction [8,16,17]. In this context, the use of acylases like aminoacylases that are able to catalyse the N-acylation reaction in aqueous media with better solubility of the substrates, especially of the acyl acceptor, has been yet considered [18,19]. Several enzymes were evaluated for

* Corresponding author.

E-mail address: yann.guiavarch@univ-lorraine.fr (Y. Guiavarc'h).

<https://doi.org/10.1016/j.procbio.2020.09.009>

Received 27 April 2020; Received in revised form 4 September 2020; Accepted 7 September 2020

Available online 10 September 2020

1359-5113/© 2020 Elsevier Ltd. All rights reserved.

amidation reaction but much less for the N-acylation of amino acids. The most used enzymes performing this reaction were described by Koreishi et al. who identified four acylases from *Streptomyces mobaraensis* [10, 11, 20, 21]. Three of them were aminoacylases characterized as monomeric metallo-enzymes with different regioselectivities. Indeed, the aminoacylase of 100 kDa and the second one of 55 kDa (called Sm-AA) had regioselectivity towards the alpha amino group of the amino acid while the third one of 60 kDa (called Sm-ELA) was specific for the lysine epsilon amino group [10, 12, 20, 21]. The fourth acylase was a penicillin V acylase (Sm-PVA), which is a dimeric enzyme able to perform the N-acylation of several amino acids with lauric acid showing a better activity with arginine, cysteine and lysine with a in ϵ position [11, 22]. In another study, the ability of a crude extract from *Streptomyces ambofaciens* ATCC23877 to perform the N-acylation of lysine and peptides containing lysine on their α position was described [13]. By genome comparison, a very close sequence identity between the genes encoding Sm-AA, Sm-ELA and Sm-PVA from *S. mobaraensis* and the ones responsible for the acylation activity from *S. ambofaciens* was shown, with 86, 80 and 69 % sequence identity respectively. Another gene was also identified with 55% identity with Sam-AA (Sam-AA like) but this had a very low α -acylation activity. In addition, the authors demonstrated that the enzyme similar to Sm-AA, named SamAA, was responsible of 85 % of the N- α -acetyl-L-lysine hydrolysis activity [13]. However, no information is available until now about the condition influencing the activity of the acylases from *S. ambofaciens* ATCC23877. In a recent study, Bourkaib et al. (2020) described the substrate specificity of the crude extract of *Streptomyces ambofaciens* was investigated toward the 20 natural proteogenic amino acids and different fatty acids for the enzymatic synthesis of many AAS in water. In the same study authors also investigated the impact of some operational conditions on the production of alpha-lauroyl lysine in batch reactor [23]. They also compared the performance of the crude extract from the wild strain of *S. ambofaciens* and the one from a mutant strain which express only Sam-AA. The results showed that the substrates specificity was similar between both strains confirming the position that Sam-AA is responsible of the N- α -acylation activity of the crude extract from the wild strain *Streptomyces ambofaciens*.

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

2. Materials and methods

2.1. Aminoacylases of *S. ambofaciens* production

The production of aminoacylases from *S. ambofaciens* was performed as described in detail in [23]. Aminoacylases from *S. ambofaciens* were finally obtained as a protein crude extract whose specific activity was evaluated using a lauroyl-lysine synthesis reaction. For this reaction, lauric acid and L-lysine were used as substrates at equimolar concentration (100 mM) in 25 mM Tris-HCl buffer, NaCl 50 mM, 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 ($V_{i\ C11'F}$) and without (V_{i0}) the addition of the product ($relative\ activity\ (\%) = \frac{V_{i\ C11'F}}{V_{i0}}$).

We investigated the effect of metal ions by using 0.1 mM of CuCl₂ (from Fluka Chemika), CoCl₂, ZnSO₄ (from Sigma), and Ethylene-Diamine-Tetra-acetic Acid (EDTA) in the standard conditions. The influence of CoCl₂ concentration was evaluated by using CoCl₂ 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 aminoacylases from *S. ambofaciens*.

At appropriate times, 50 or 100 μ L 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 μ L 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-phenylalanine, 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 (Bio-Sep™ SEC-S2000 300 × 21.2 mm, 5 µm particle diameter, Phenomex, 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 synthesized N-10-undecenoyl-phenylalanine named Sepiwhite™ MSH kindly provided by SEPPIC, France. The substrate conversion yield was determined using the following equation: conversion (%) = $\left(\frac{[C11'F]_{\text{mol/L}} \text{ produced}}{[C11']_{\text{mol/L}} \text{ initial}}\right) \times 100$. We calculated the

initial 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 (V_{max}) and the Michaelis-Menten constant (K_m) for each substrate were determined using a standard Michaelis-Menten model based on the equation of the graphic representing $\frac{1}{V} = f\left(\frac{1}{S}\right) = \frac{1}{S} * \frac{K_m}{V_{\text{max}}} + \frac{1}{V_{\text{max}}}$ 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 CO₂ 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 CO₂ 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-undecenoyl-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 72 h 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 $\frac{\text{concentration of melanin with the tested molecule}}{\text{concentration of melanin without tested molecule}} \times 100$. In order to evaluate the cells viability, the latter 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 $\frac{\text{concentration of proteins with the tested molecule}}{\text{concentration of proteins without tested molecule}} \times 100$. 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).

3. 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 μ M 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 $45^\circ\text{C} \pm 3^\circ\text{C}$. 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 K_m and V_{max} for the undecenoic acid. According to the equation of the graphic representing $\frac{1}{v} = f\left(\frac{1}{S}\right)$ V_{max} and K_m of around 0.50 ± 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 $\frac{1}{v} = f\left(\frac{1}{S}\right)$ V_{max} and K_m 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 N α -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].

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

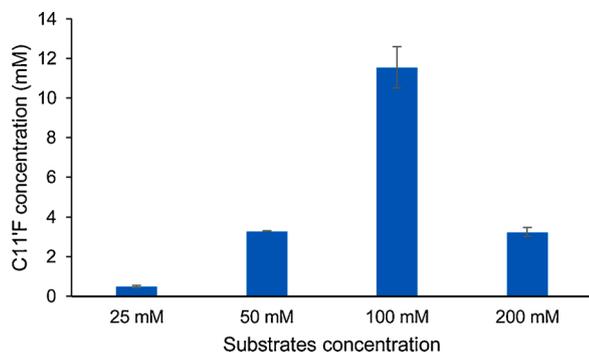


Fig. 1. Influence of substrates concentration at equimolar condition on the synthesis of N-10-undecenoyl-phenylalanine after 72 h in 25 mM Tris-HCl buffer, NaCl 50 mM containing 1 g/L acylases at 45°C , pH 8 and 450 rpm magnetic agitation ($n = 2$ or 3).

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].

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].

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

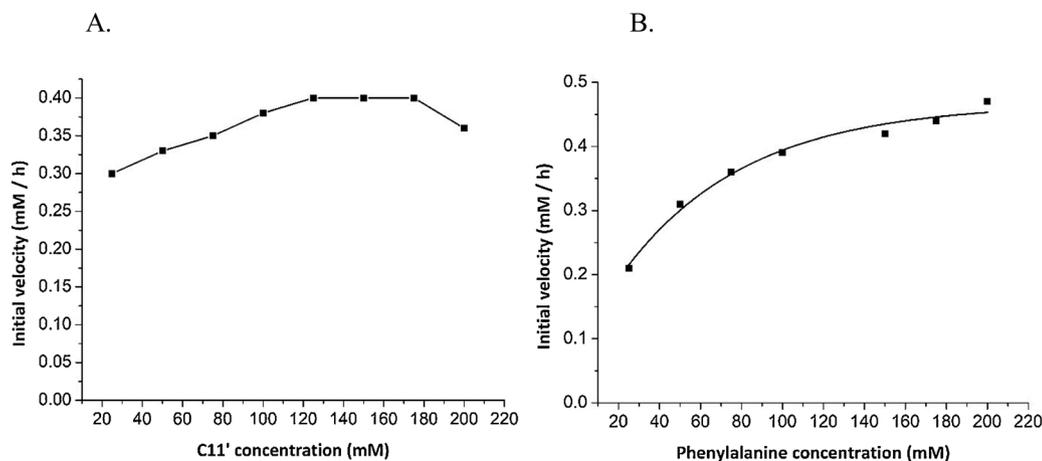


Fig. 2. Influence of substrates concentration on the initial rate for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 1 g/L acylases at 45 °C, pH 8 and 450 rpm magnetic agitation, A. Effect of 10-undecenoic acid concentration on the initial rate of N-10-undecenoyl-phenylalanine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

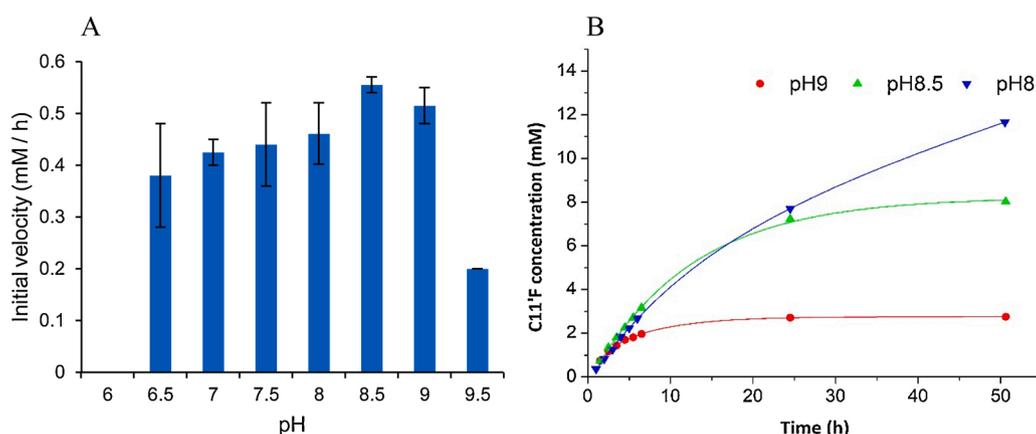


Fig. 3. Influence of the pH on the enzymatic activity for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases at 45 °C, pH [6 - 9.5] and 450 rpm magnetic agitation, A. effect of the pH on the initial rate. B. Kinetic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

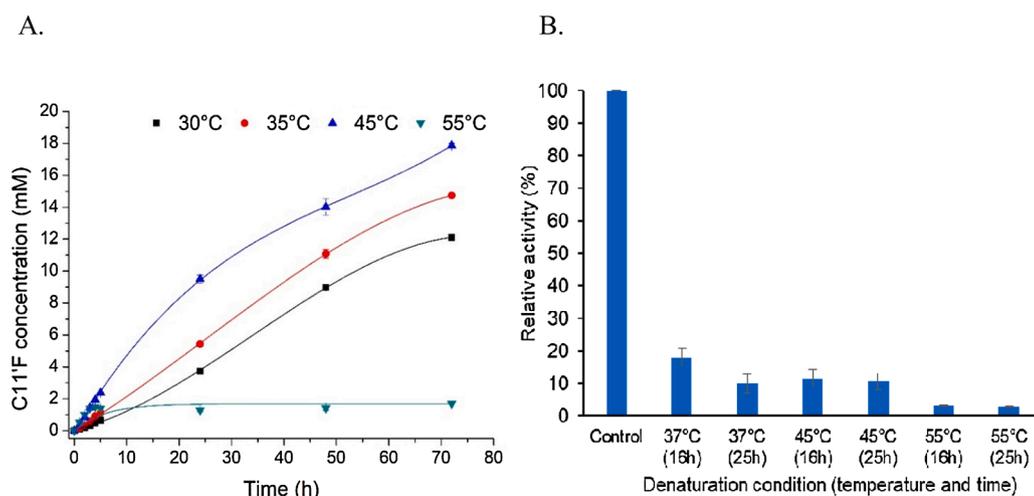


Fig. 4. Effect of the temperature on the enzymatic activity A. Influence of the reaction temperature on the enzymatic activity for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases at T °C [30–55 °C], pH 8 and 500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

enzymes was observed by increasing the concentration of the product.

3.5. Influence of metal activator on the reaction

As described by Dettori et al., 2018, the sequence coding the aminoacylase from *S. ambifaciens*, 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. ambifaciens* activity, Co^{2+} , Zn^{2+} and Cu^{2+} which were described

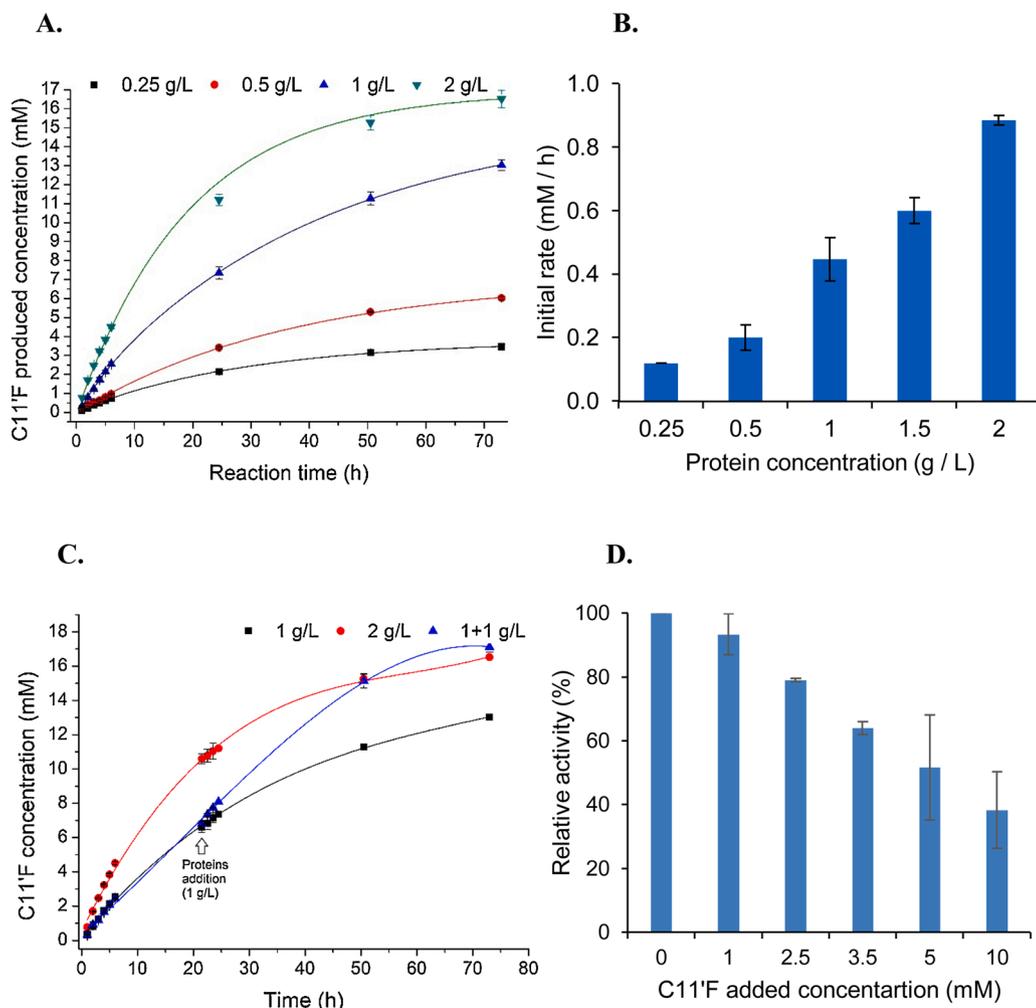


Fig. 5. Influence of the protein concentration and the product concentration on the enzymatic activity for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases (except for the protein concentration influence) at 45 °C,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

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 enzyme. As shown in Fig. 6, for the same concentration of the three metal ions (0.1 mM), Cobalt ion led to a three time increase of the initial rate unlike Zn²⁺ and Cu²⁺ ions allowing only a slight increase. No reaction was registered when EDTA was added, which confirmed that the aminoacylases involved in the N-acylation are metalloenzymes. However, by comparing the reaction

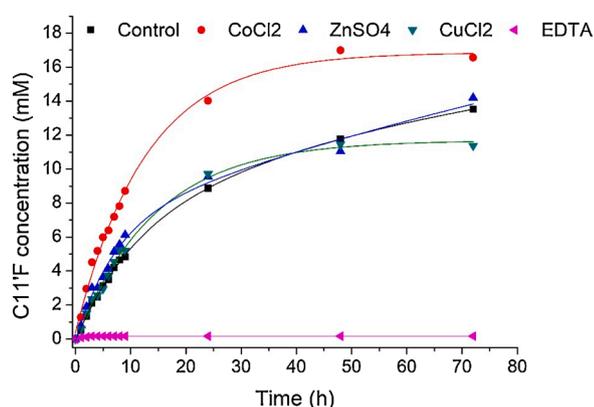


Fig. 6. influence of different metal ions at 0.1 mM concentration on the enzymatic activity for the synthesis of N-10-undecenoyl-phenylalanine in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases at 45 °C, pH 8 and 500 rpm magnetic agitation.

course, the thermodynamic equilibrium was reached with CoCl₂ after 48 h while the thermodynamic equilibrium of the control reaction and the reaction with Zn²⁺ as metal ion was not yet reached after 72 h. For the reaction with Cu²⁺ the thermodynamic equilibrium was also reached after 48 h but with around 25% less activity than with Co²⁺ as metal ion. The influence of Cobalt concentration was therefore also investigated. The increase of the Cobalt concentration allowed an important increase of the initial rate of the reaction to reach around eleven-fold increase of the initial rate by using 0.5 mM of CoCl₂ (Table 1). When concentration from 0.3 to 0.5 mM of CoCl₂ was used, the thermodynamic equilibrium was reached after only 24 h reaction time but the final product concentration was affected. Indeed, the increase of the CoCl₂ concentration led to an increase in the conversion rate until 0.3 mM CoCl₂ with more than 20 mM C11'F concentration. A 16 mM C11'F concentration was reached when using a 0.5 mM CoCl₂ concentration. Koreishi et al. 2005 studied the influence of metal ions on the hydrolytic activity of *S. mobaraensis* aminoacylase. Their results were similar to the ones observed in this study where three-fold increase of the activity by adding 0.1 mM CoCl₂ and only a slight increase by adding Zn²⁺ ion was observed. However, they showed a decrease of 80 % of the activity by adding Cu²⁺ ion (Koreishi et al., 2005a), which was not the case with the acylases from *S. ambofaciens*.

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

Table 1

effect of metal ion and the concentration of CoCl_2 on the initial rate of the reaction synthesis of N-10-undecenoyl-phenylalanine and the C11'F produced concentration after 48 h 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				
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

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. ambifaciens* 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.

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 diafiltration allowed the elimination of 64 % of the fatty acid with only 15 % loss of the product from a sample of the initial reaction medium composed of 300 mg of C11' and 127 mg of C11'F. However, when more diafiltration steps were performed a higher loss of the C11'F was observed (Fig. 8.A). This partial separation of the C11' and the C11'F can be due to the amphiphilic property of the acylated amino-acid which can interact with the proteins standing in the

retentate forming a polarization layer, while the apolar characteristic and the high quantity of the residual C11' increased its elimination in the permeate. However, after the elimination of around 64 % of the fatty acid, the membrane pores became more accessible for the C11'F, leading to a loss of the product. A compromise that allowed the partial elimination of the residual C11' while limiting the loss of the C11'F was found by applying two diafiltration steps and a concentration step. This led to the recovery of a solution containing around 1.1 g of the product and equivalent quantity of the fatty acid in addition to the aminoacylases used. The proteins were eliminated by precipitation with methanol, and the product was separated from the residual fatty acid by size exclusion chromatography. After lyophilisation 0.8 g of a 99 % pure white powder of enzymatically produced N-10-undecenoyl-phenylalanine was obtained, as determined by mass spectroscopy and proton NMR analysis (Fig. 8 B).

3.8. Evaluation of the biological activity of the purified product

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

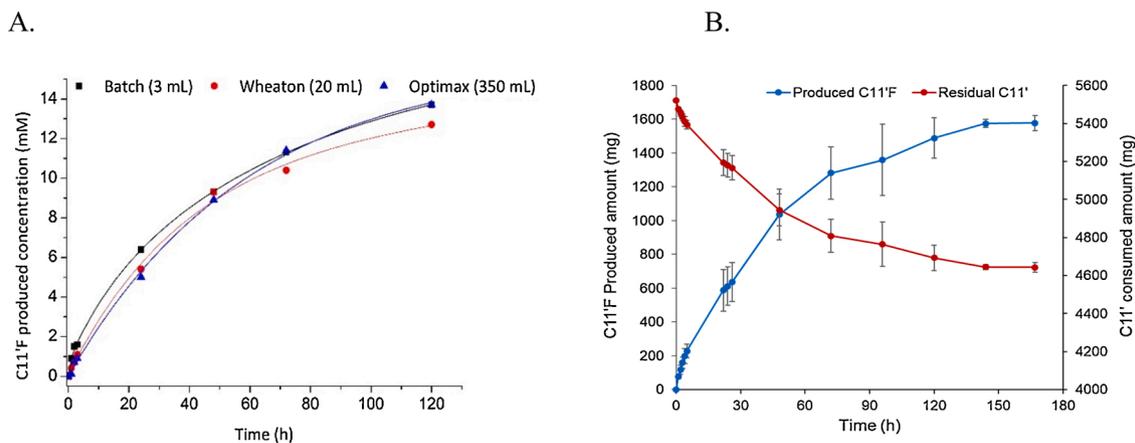


Fig. 7. scaling-up of the C11'F synthesis reaction in 25 mM Tris-HCl buffer, NaCl 50 mM containing 100 mM of both substrates, 1 g/L acylases at 45 °C, pH 8 and 500 rpm magnetic agitation A. influence of batch reactor configuration B. kinetic of the C11'F production and the C11' consumption for the reaction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

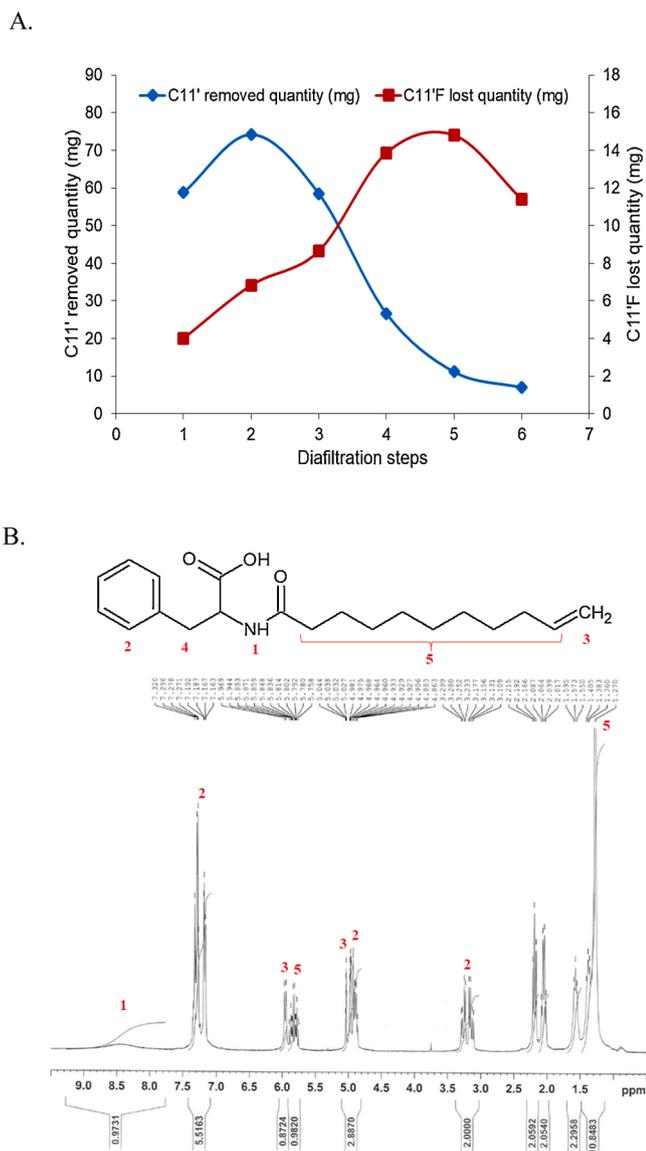


Fig. 8. Details on C11'F purification process A. quantity of fatty acid and product removed at different diafiltration steps using a 10 kDa cut-off membrane and an initial solution of 25 mL of the final reaction mixture (300 mg of C11' and 127 mg of C11'F) B. proton NMR of the final purified product.

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

Table 2
melanin production inhibition and cells viability evaluation.

Dilution solution	Concentration ($\mu\text{g/mL}$)	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 (*)
	50	67	48	0.0463 (*)	76	82	0.0018 (**)
Culture medium	50	17	13	0.2514 (NS)	100	97	0.0205 (*)

LS: limit of significance; $0.05 < p < 0.1$; $*0.01 < p < 0.05$; $**0.001 < p < 0.01$ and $***p < 0.001$, $n = 6$.

* 97% inhibition of melanin production by melanocytes using Kojic acid as positive control without any cell toxicity.

** 100% cell viability correspond to the result with the control culture of melanocyte (without any added molecules).

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 $10 \mu\text{g/mL}$, 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.

4. 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-undecenoic acid as acyl donor. The results showed a positive effect of the phenylalanine concentration on the aminoacylases activity, unlike what was observed with the 10-undecenoic acid. Investigations on operational conditions showed a good N-acylation activity at 45°C , pH [7–8] but low temperature stability. The inhibitory effect of the product on the conversion rate was also demonstrated showing the interest of immobilization aiming to use these aminoacylases in continuous flow reactors. The influence of the metal ions was also studied confirming that the aminoacylases used are metallo-dependant and showing a very interesting acceleration of the reaction by adding CoCl_2 . Different reactor configurations were evaluated but the batch reactor was the most suitable for the synthesis of N-10-undecenyl-phenylalanine without any effect of the agitation mode. A purification procedure was developed leading to 0.8 g of N-10-undecenyl-phenylalanine with more than 99% purity, under the form of a white powder. The biological activity of the product was tested on melanocyte cell cultures and showed a clear inhibition of the melanin production. When significant, the difference in the lightening activity and/or the cell toxicity observed between the C11'F of chemical origin or that issued from the enzymatic process was likely to be due to different impurities remaining in the two products. These results represent a step forward towards the development of a green bioprocess using *S. ambofaciens* aminoacylases in aqueous media, which could constitute a promising alternative to existing chemical processes for the production and the purification of lipoaminoacids (i.e. AAS) as biosurfactants.

CRediT authorship contribution statement

Mohamed Chafik Bourkaib: Conceptualization, Investigation, Writing - original draft. **Stephane Delaunay:** Conceptualization, Methodology. **Xavier Framboisier:** Resources, Investigation. **Catherine Humeau:** Investigation, Methodology, Writing - review & editing. **Jérôme Guilbot:** Funding acquisition, Validation. **Cecile Bize:** Ressources, Investigation, Formal analysis, Writing - review & editing. **Estelle Illous:** 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.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgement

This work was mainly supported by the French National Research Agency (ANR) through the “ISEAPIM3” project (ANR-15-CE07-0023-01), and partly by the “Impact biomolécules” project of the « Lorraine Université d'Excellence (LUE) » (PIA-ANR) program. We also thank Emilien GIROT from LRGP for giving us access to the Optimax reactor and for his valuable advices.

References

- [1] C. Rondel, I. Alric, Z. Mouloungui, J.-F. Blanco, F. Silvestre, Synthesis and Properties of Lipoamino Acid-Fatty Acid Mixtures: Influence of the Amphiphilic Structure, *J. Surfactants Deterg.* 12 (2009) 269–275, <https://doi.org/10.1007/s11743-009-1121-y>.
- [2] D.B. Tripathy, A. Mishra, J. Clark, T. Farmer, Synthesis, chemistry, physicochemical properties and industrial applications of amino acid surfactants: A review, *Comptes Rendus Chim.* 21 (2018) 112–130, <https://doi.org/10.1016/j.crci.2017.11.005>.
- [3] D. Yea, S. Lee, S. Jo, H. Yu, J. Lim, Preparation of Environmentally Friendly Amino Acid-Based Anionic Surfactants and Characterization of Their Interfacial Properties for Detergent Products Formulation, *J. Surfactants Deterg.* 21 (2018) 541–552, <https://doi.org/10.1002/jsde.12051>.
- [4] M. Sreenu, R.B.N. Prasad, P. Sujitha, C.G. Kumar, Synthesis and Surface-Active Properties of Sodium N-Acylphenylalanines and Their Cytotoxicity, *Ind. Eng. Chem. Res.* 54 (2015) 2090–2098, <https://doi.org/10.1021/ie503764v>.
- [5] D.L. Bissett, L.R. Robinson, P.S. Raleigh, K. Miyamoto, T. Hakozaiki, J. Li, G. R. Kelm, Reduction in the appearance of facial hyperpigmentation by topical N-undecyl-10-enoyl-L-phenylalanine and its combination with niacinamide, *J. Cosmet. Dermatol.* 8 (2009) 260–266, <https://doi.org/10.1111/j.1473-2165.2009.00470.x>.
- [6] M.C. Bourkaib, H. Randriamalala, L. Dettori, C. Humeau, S. Delaunay, I. Chevalot, Y. Guiavarc'h, Enzymatic synthesis of geranyl acetate in packed bed reactor in supercritical carbon dioxide under various pressure-temperature conditions and reactor configurations, *Process Biochem.* 71 (2018) 118–126, <https://doi.org/10.1016/j.procbio.2018.05.008>.
- [7] R.A. Sheldon, J.M. Woodley, Role of Biocatalysis in Sustainable Chemistry, *Chem. Rev.* 118 (2018) 801–838, <https://doi.org/10.1021/acs.chemrev.7b00203>.
- [8] D. Montet, F. Servat, M. Pina, J. Graille, P. Galzy, A. Arnaud, H. Ledon, L. Marcou, Enzymatic synthesis of N-ε-acyllysines, *J. Am. Oil Chem. Soc.* 67 (1990) 771–774, <https://doi.org/10.1007/BF02540489>.
- [9] E.L. Soo, A.B. Salleh, M. Basri, R.N. Zaliha Raja Abdul Rahman, K. Kamaruddin, Optimization of the enzyme-catalyzed synthesis of amino acid-based surfactants from palm oil fractions, *J. Biosci. Bioeng.* 95 (2003) 361–367, [https://doi.org/10.1016/S1389-1723\(03\)80068-3](https://doi.org/10.1016/S1389-1723(03)80068-3).
- [10] M. Koreishi, R. Kawasaki, H. Imanaka, K. Imamura, K. Nakanishi, A novel ε-lysine acylase from *Streptomyces mobaraensis* for synthesis of N-ε-acyl-L-lysines, *J. Am. Oil Chem. Soc.* 82 (2005) 631–637, <https://doi.org/10.1007/s11746-005-1121-2>.
- [11] M. Koreishi, D. Zhang, H. Imanaka, K. Imamura, S. Adachi, R. Matsuno, K. Nakanishi, A Novel Acylase from *Streptomyces mobaraensis* that Efficiently Catalyzes Hydrolysis/Synthesis of Capsaicins as Well as N-Acyl-L-amino Acids and N-Acyl-peptides, *J. Agric. Food Chem.* 54 (2006) 72–78, <https://doi.org/10.1021/jf052102k>.
- [12] M. Koreishi, R. Kawasaki, H. Imanaka, K. Imamura, Y. Takakura, K. Nakanishi, Efficient N-ε-lauroyl-L-lysine production by recombinant ε-lysine acylase from *Streptomyces mobaraensis*, *J. Biotechnol.* 141 (2009) 160–165, <https://doi.org/10.1016/j.jbiotec.2009.03.008>.
- [13] L. Dettori, F. Ferrari, X. Framboisier, C. Paris, Y. Guiavarc'h, L. Hôtel, A. Aymes, P. Leblond, C. Humeau, R. Kapel, I. Chevalot, B. Aigle, S. Delaunay, An aminoacylase activity from *Streptomyces ambofaciens* catalyzes the acylation of lysine on α-position and peptides on N-terminal position, *Eng. Life Sci.* 18 (2017) 589–599, <https://doi.org/10.1002/elsc.201700173>.
- [14] C. Bernal, F. Guzman, A. Illanes, L. Wilson, Selective and eco-friendly synthesis of lipoaminoacid-based surfactants for food, using immobilized lipase and protease biocatalysts, *Food Chem.* 239 (2018) 189–195, <https://doi.org/10.1016/j.foodchem.2017.06.105>.
- [15] Y. Takakura, Y. Asano, Purification, characterization, and gene cloning of a novel aminoacylase from *Burkholderia sp.* strain LP5_18B that efficiently catalyzes the synthesis of N-lauroyl-L-amino acids, *Biosci. Biotechnol. Biochem.* 83 (2019) 1964–1973, <https://doi.org/10.1080/09168451.2019.1630255>.
- [16] P. Villeneuve, Lipases in lipophilization reactions, *Biotechnol. Adv.* 25 (2007) 515–536, <https://doi.org/10.1016/j.biotechadv.2007.06.001>.
- [17] R. Valivety, P. Jauregi, I. Gill, E. Vulfson, Chemo-enzymatic synthesis of amino acid-based surfactants, *J. Am. Oil Chem. Soc.* 74 (1997) 879–886, <https://doi.org/10.1007/s11746-997-0232-8>.
- [18] A. Ferjancic-Biagini, T. Giardina, M. Reynier, A. Puigserver, Hog Kidney and Intestine Aminoacylase-Catalyzed Acylation of L-Methionine in Aqueous Media, *Biocatal. Biotransformation.* 15 (1997) 313–323, <https://doi.org/10.3109/10242429709003197>.
- [19] S.R. Morcelle, C.S. Liggieri, M.A. Bruno, N. Priolo, P. Clapés, Screening of plant peptidases for the synthesis of arginine-based surfactants, *J. Mol. Catal. B Enzym.* 57 (2009) 177–182, <https://doi.org/10.1016/j.molcatb.2008.08.013>.
- [20] M. Koreishi, F. Asayama, H. Imanaka, K. Imamura, M. Kadota, T. Tsuno, K. Nakanishi, Purification and Characterization of a Novel Aminoacylase from *Streptomyces mobaraensis*, *Biosci. Biotechnol. Biochem.* 69 (2005) 1914–1922, <https://doi.org/10.1271/bbb.69.1914>.
- [21] M. Koreishi, Y. Nakatani, M. Ooi, H. Imanaka, K. Imamura, K. Nakanishi, Purification, Characterization, Molecular Cloning, and Expression of a New Aminoacylase from *Streptomyces mobaraensis* That Can Hydrolyze N-(Middle/Long)-chain-fatty-acyl-L-amino Acids as Well as N-Short-chain-acyl-L-amino acids, *Biosci. Biotechnol. Biochem.* 73 (2009) 1940–1947, <https://doi.org/10.1271/bbb.90081>.
- [22] D. Zhang, M. Koreishi, H. Imanaka, K. Imamura, K. Nakanishi, Cloning and characterization of penicillin V acylase from *Streptomyces mobaraensis*, *J. Biotechnol.* 128 (2007) 788–800, <https://doi.org/10.1016/j.jbiotec.2006.12.017>.
- [23] M.C. Bourkaib, S. Delaunay, X. Framboisier, L. Hôtel, B. Aigle, C. Humeau, Y. Guiavarc'h, I. Chevalot, N-acylation of L-amino acids in aqueous media: Evaluation of the catalytic performances of *Streptomyces ambofaciens* aminoacylases, *Enzyme Microb. Technol.* 137 (2020) 109536, <https://doi.org/10.1016/j.enzmictec.2020.109536>.
- [24] S. Chung, G.J. Lim, J.Y. Lee, Quantitative analysis of melanin content in a three-dimensional melanoma cell culture, *Sci. Rep.* 9 (2019) 780, <https://doi.org/10.1038/s41598-018-37055-y>.
- [25] W.-L. Gao, N. Li, M.-H. Zong, Highly regioselective synthesis of undecylenic acid esters of purine nucleosides catalyzed by *Candida antarctica* lipase B, *Biotechnol. Lett.* 33 (2011) 2233, <https://doi.org/10.1007/s10529-011-0685-6>.
- [26] D. Shi, Y. Zhao, H. Yan, H. Fu, Y. Shen, G. Lu, H. Mei, Y. Qiu, D. Li, W. Liu, Antifungal effects of undecylenic acid on the biofilm formation of *Candida albicans*, *Int J. Clin. Pharmacol. Ther.* 54 (2016) 343–353, <https://doi.org/10.5414/CP202460>.
- [27] E. Lee, J.-E. Eom, H.-L. Kim, D.-H. Kang, K.-Y. Jun, D.S. Jung, Y. Kwon, Neuroprotective effect of undecylenic acid extracted from *Ricinus communis* L. through inhibition of μ-calpain, *Eur. J. Pharm. Sci.* 46 (2012) 17–25, <https://doi.org/10.1016/j.ejps.2012.01.015>.
- [28] J.M. Walker, The Bicinchoninic Acid (BCA) Assay for Protein Quantitation, in: J. M. Walker (Ed.), *Protein Protoc. Handb.*, Humana Press, Totowa, NJ, 2009, pp. 11–15, https://doi.org/10.1007/978-1-59745-198-7_3.
- [29] E. Wada, M. Handa, K. Imamura, T. Sakiyama, S. Adachi, R. Matsuno, K. Nakanishi, Enzymatic synthesis of N-acyl-L-amino acids in a glycerol-water system using acylase I from pig kidney, *J. Am. Oil Chem. Soc.* 79 (2002) 41–46, <https://doi.org/10.1007/s11746-002-0432-7>.
- [30] J.M. Bolivar, J. Wiesbauer, B. Nidetzky, Biotransformations in microstructured reactors: more than flowing with the stream? *Trends Biotechnol.* 29 (2011) 333–342, <https://doi.org/10.1016/j.tibtech.2011.03.005>.
- [31] M.P.C. Marques, P. Fernandes, Microfluidic Devices: Useful Tools for Bioprocess Intensification, *Molecules.* 16 (2011) 8368–8401, <https://doi.org/10.3390/molecules16108368>.