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LIPASE-CATALYSED SYNTHESIS OF BIOSURFACTANTS BY TRANSACYLATION OF N-METHYL-GLUCAMINE AND FATTY-ACID METHYL ESTERS.

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Abstract : The enzymatic synthesis of a range of amide surfactants by transesterification reactions between various amines and fatty-acid methyl esters (Diester®) a low-cost natural and renewable raw material is described. The transacylation reaction catalysed by a commercial lipase from *Candida antarctica* (Novozym®) at 90°C, yielded 100 % conversion of fatty-acid methyl esters with 80 % amide synthesis in less than 20 hours. Yield and chemoselectivity of the reaction are under the control of the fatty-acid methyl ester / amine molar ratio.

INTRODUCTION

N-acyl-N-alkyl-glycamines are biosurfactants that contain amide bonds and constitute a new class of amphiphilic molecules, greatly appreciated by surfactant manufactures because the amide bond is chemically and physically very stable in alkaline media. They can be obtained mainly using inexpensive, optically pure, environmentally friendly and renewable resources, such as fatty esters and sugar derivatives[1].

The chemical synthesis of these molecules is not very specific, and requires hydroxyl group protection / deprotection steps. The high temperature of the chemical reaction also prevents the use of fragile molecules and may cause coloration of final products. It is often necessary at the end of the reaction to remove the salts coproduced with the amide (as in the case of the Schotten-Baumann reaction), in order to preserve good detergency of the product.

In this context, amide surfactant synthesis following an enzymatic approach presents several advantages. There are no by-products and no need for protection / deprotection of the reagent because enzymes are regio and stereoselective. Enzymatic synthesis of amide bonds may be carried out by reverse hydrolysis or by transacylation in organic media, using either proteases or lipases as catalysts. However, proteases are not efficient catalysts for such reactions, because they are specific for certain amino acids and more sensitive to organic solvents[2].

In contrast, lipases were more efficient than proteases, because they hardly hydrolyse the synthesised amide bond[3]. Lipases have been used for the synthesis of chiral amides[4], peptides[5], fatty amides[6], N-acyl-amino acids[7] and for aminopropanol acylation[8]. However, the corresponding yields are too low for

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development at the industrial scale. In fact for biocatalysis to be competitive, the surfactants must be produced at low cost, preferably from cheap renewable materials and at high yields.

The present study describes a process of enzymatic synthesis of glycamides by transacylation reaction, catalysed by immobilized lipase from *Candida antarctica* (Novozym®) in organic media. Low-cost substrates were chosen to guarantee low-cost final products. The effect of the ratio amine/fatty-acid methyl esters on selectivity and amide yield was determined. Extension to the synthesis of other amides was investigated to propose other routes for non-food valorization of plant oils.

RESULTS AND DISCUSSION

Synthesis of glucamide surfactants by fatty-acid methyl ester transacylation.

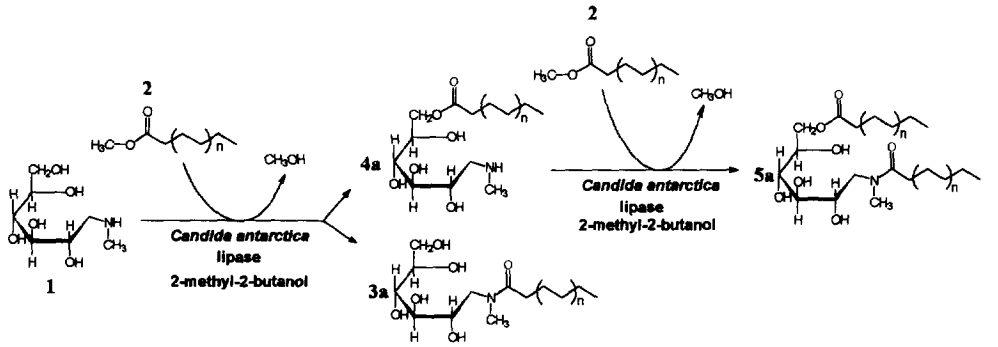
The synthesis of glucamide by transacylation using substrates from natural sources was tested. N-methyl-glucamine **1** was chosen because it is easily obtained by reductive amination of glucose in the presence of methylamine. Fatty-acid methyl ester **2** (Diester® from Sidobre Sinnova), is produced by Colza oil methanolysis. It is essentially composed of 59.3 % oleic acid methyl ester (C18:1), 22 % linoleic acid methyl ester (C18:2), 11 % linolenic acid methyl ester (C18:3), 5.2 % palmitic acid methyl ester (C16), 1.9 % gadolic acid methyl ester (C20:1), 0.5 % erucic acid methyl ester (C22:1) and 0.1 % myristic acid methyl ester (C14). The two substrates of the reaction (N-methyl-glucamine **1** and fatty-acid methyl ester **2**) are molecules of different polarities and solubilities. Fatty-acid methyl ester **2**, is soluble in hydrophobic solvents, while N-methyl-glucamine is poorly soluble in such solvents. 2-methyl-2-butanol (polar protic solvent) was selected for glucamide synthesis because this alcohol partially dissolves N-methyl-glucamine, it is a non-toxic solvent and is not a substrate for lipase. *Candida antarctica* lipase (Novozym®) was chosen as catalyst because this immobilized enzyme is commercially available, stable in 2-methyl-2-butanol and can be easily recovered[8].

The reaction was run at 90°C, under 500 mBar. To shift the reaction equilibrium toward synthesis, the coproduced methanol was rapidly removed by distillation. The decrease of fatty-acid methyl ester **2** concentration was seen to be concomitant with the synthesis of products (Figure 1). During the first hours, amide (**3**) and ester (**4**) were synthesised. Then, the esters **4** produced during the first hours of the reaction, completely disappeared at the end of the reaction. At the same time, new products were synthesised with 10 % yield, and were identified as amide-esters (**5**), probably formed from the esters **4** (Scheme 1). After 10 hours of reaction, 100 % of the fatty-acid methyl esters were completely transformed and amide yield reached 80 %.

In order to improve the selectivity and the amide yield, reactions were run using various fatty-acid methyl ester / N-methyl-glucamine molar ratios as shown in Table 1. We can observe that the yield of compound **5**, can be reduced by using higher amine concentrations in the reaction mixture. But in these

conditions, the excess amine will increase the cost of the surfactants produced and may introduce problems of purification of the mixture.

Consequently, optimum conditions for selective amide production correspond to reaction at 90°C under atmospheric pressure, using a Fatty-acid methyl ester / amine ratio of **1**. In these conditions a mixture of surfactants was obtained which contained 129 g/l of amides **3** (80 % w/w), 25 g/l of amide-esters **5** (15 % w/w) and 7 g/l of N-methyl-glucamine **1** (5 % w/w) (Figure 2). In these proportions, for industrial preparations, the separation of the different compounds is not necessary and the mixture can be used directly in cosmetic formulations.



Scheme 1

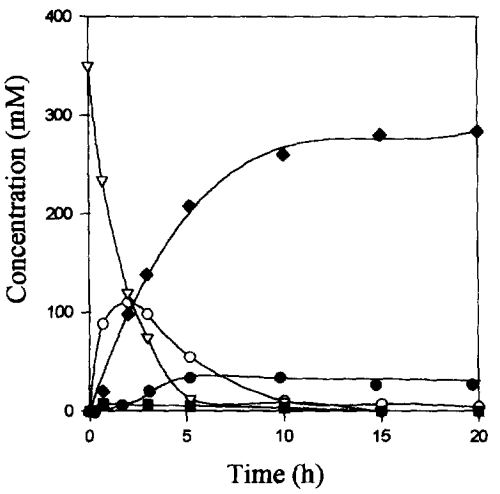


Figure 1. Condensation of N-methyl-glucamine (350 mM) with Fatty-acid methyl esters (350 mM). The reaction was run in 2-methyl-2-butanol at 90°C under 500 mBar with 10 g/l of Novozym®. ◆ amide, O ester, ● amide-ester, ■ acid, ∇ Fatty-acid methyl esters.

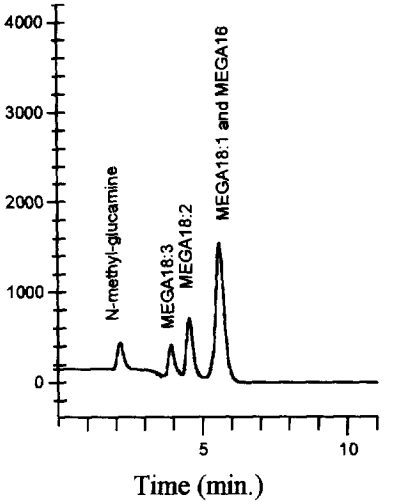


Figure 2. HPLC Analysis of the products of N-methyl-glucamine acylation by fatty-acid methyl esters. N-methyl-glucamine was eluted at 2 minutes, followed by acyl-N-methyl-glucamides (MEGA at 4, 4.5 and 5.5 minutes). The amide-esters are not shown. Column C18 (250 x 4 mm), methanol/water/TFA (90/10/0.3, v/v/v), 1 ml/min., 40°C. The products were detected using a differential refractometer.

Table 1. Chemoselective acylation of N-methyl-glucamine *versus* fatty-acid methyl ester / N-methyl-glucamine molar ratio.

Fatty-acid methyl esters / N-methyl-glucamine (mM/mM)	Amide-ester **5a** (%)	Ester **4a** (%)	Amide **3a** (%)	Total amine conversion (%)
350/175	30	20	50	100
350/350	10	0	80	90
350/700	4	0	96	50

(Reactions were carried out in 2-methyl-2-butanol at 90°C under 500 mBar with 10 g/l of Novozym®)

In conclusion, an efficient method has been developed for the lipase-driven synthesis of stable amide-bond surfactants such as N-methyl-glucamides from fatty-acid methyl esters (Diester®) and N-methyl-glucamine. Various other amines were tested (N-methyl-glucamine, N-methyl-galactamine, N-octyl-glucamine). In all cases, the reactions yielded more than 80 % amide. The efficiency of the synthesis with fatty-acid methyl esters (Diester®) allows to further industrial development to be envisaged on a large scale and at low cost and the valorization of excess Colza oil production.

EXPERIMENTAL PROCEDURES

Biological and Chemical material

Novozym® SP 435 (lipase from *Candida antarctica* immobilised on an acrylic resin), was a gift from Novo Industries (Denmark). The solvents, all pure, were from Fluka. N-methyl-glucamine (Sigma Chemical Co.), N-methyl-galactamine (Aldrich) and N-octyl-glucamine (Aldrich) were more than 99 % pure. Fatty-acid methyl esters (Diester®), was from Sidobre Sinova.

General procedure for the enzymatic reaction

Amine, Fatty-acid methyl esters and Novozym® were mixed in 2-methyl-2-butanol. The reactions were carried out in 25 ml flasks mechanically stirred on a rotary evaporator (Büchi) which was used as a reactor under reduced pressure and at controlled temperature. In standard conditions, 3.5 mmoles of Fatty-acid methyl esters were reacted with 3.5 mmoles of N-methyl-glucamine and 100 mg of Novozym® in 10 ml of 2-methyl-2-butanol. The reaction was run at 90°C under 500 mBar for 20 hours.

HPLC Analysis

Analyses were performed with an HPLC system from Hewlett Packard (processor, pump, UV detector and injector model 1050, differential refractometer (RI) model 1047A), equipped with an Ultrasep C18 (250 x 4 mm, 6 µ) reverse-phase column from ICS, France. 25 µl of the proper dilution of the reaction mixture were injected. A mixture of methanol/water/TFA, 90/10/0.3 (v/v/v) was used as eluent at 40°C with a flow rate of 1 ml/min. Products were detected using a differential refractometer.

Purification of reaction products

At the end of the reaction, the biocatalyst was removed by filtration and the solvent evaporated under reduced pressure. The remaining oil was separated into amides, monoesters of N-alkyl-glycamine and amide-esters of N-alkyl-glycamine, by chromatography using a silica gel (60 H, Merck) column (30 cm x 20 mm). The concentrated oil sample was diluted in a minimum volume of chloroform/methanol (9/1, v/v) and was deposited at the top of the column previously equilibrated with chloroform/methanol (9/1, v/v). The column was eluted with chloroform/methanol mixtures from 9/1 to 7/3 (v/v). All the fractions obtained were analysed by HPLC before structural analysis.

Structural analysis of major compounds

Carbon 13 Nuclear Magnetic Resonance (^{13}C NMR) spectra were recorded using an AC 250 MHz spectrometer from Brüker, with an internal reference of tetramethylsilane. Infra-Red (IR) spectra were recorded using a Perkin Elmer IRFT 1760-x spectrometer for KBr pellets. Mass spectra were obtained by chemical ionisation (DCI/NH_3), using a NERMAG R10-10 spectrometer.

1-deoxy-1-[methyl(1-oxo-9-octadecenyl)amino]-D-glucitol, (oleoyl-N-methyl-glucamide)(3a):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-N) = 1620 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.7 (CH_2OH), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{49}NO_6$: C, 65.42 ; H, 10.66. Found : C, 65.53 ; H, 10.74. Mass (DCI/NH_3) : 461 (M + H^+), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-methylamino-6-(1-oxo-9-octadecenyl)-D-glucitol, (6-O-oleoyl-N-methyl-glucamine)(4a):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and ν (CO-O) = 1735 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 178 (CO-O), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 65.7 (CH_2O), 49(CH_2NH), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{49}NO_6$: C, 65.42 ; H, 10.66. Found : C, 65.45 ; H, 10.84. Mass (DCI/NH_3) : 461 (M + H^+), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[methyl(1-oxo-9-octadecenyl)amino]-6-(1-oxo-9-octadecenyl)-D-glucitol, (N-O-dioleoyl-N-methyl-glucamide)(5a):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} , ν (CO-O) = 1730 cm^{-1} and ν (CO-N) = 1620 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 176 (CO-O), 130-129.7 (4 CH=CH), 22-36 (28 CH_2), 14 (2 CH_3), 70-73 (4 CH-OH), 65.7 (CH_2O), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{43}H_{81}NO_7$: C, 71.25 ; H, 11.18. Found : C, 71.33 ; H, 11.05. Mass (DCI/NH_3) : 725 (M + H^+), 460 (M - 265 + H^+), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[methyl(1-oxo-9-octadecenyl)amino]-D-galactitol, (oleoyl-N-methyl-galactamide)(3b):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and (CO-N) = 1620 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.7 (CH_2OH), 51(CH_2N), 37.5 (CH_3N). Anal. Calcd for $C_{25}H_{49}NO_6$: C, 65.42 ; H, 10.66. Found : C, 65.49 ; H, 10.55. Mass (DCI/NH_3) : 461 ($M + H^+$), 196 (CH_2OH-$(CHOH)_4$-CH_2-NH-CH_3+ H^+).

1-deoxy-1-[octyl(1-oxo-9-octadecenyl)amino]-D-glucitol, (oleoy-N-octyl-glucamide)(3c):

IR : ν (OH) = 3500 cm^{-1}, ν (CH) = 2800-2900 cm^{-1} and (CO-N) = 1620 cm^{-1}.

^{13}C NMR/$CDCl_3$ (δ in ppm) : 175 (CO-N), 130 (2 CH=CH), 22.7-35.8 (14 CH_2), 14 (CH_3), 70-73 (4 CH-OH), 63.749.7 (CH_2N), 50 (C_7H_{15}-$\underline{CH_2}$-N), 25.2-34.15 (6 CH_2), 14 (CH_3). Anal. Calcd for $C_{32}H_{63}NO_6$: C, 68.81 ; H, 11.29. Found : C, 68.63 ; H, 11.10. Mass (DCI/NH_3) : 559 ($M + H^+$), 294 (CH_2OH-$(CHOH)_4$-CH_2-NH-C_8H_{17} + H^+).

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