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Influence of the water content in the synthesis of fructose laurate by immobilized lipase B of *Candida antarctica* (Novozym 435)

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Abstract

New synthesis conditions for a direct esterification of fructose by lauric acid with Novozym 435 as a catalyst are reported in this paper. With these new conditions, a conversion rate of 72 % was achieved, which is almost the same value as in the case of transesterification where no water as by-product is produced (74.6 %). The conversion rate of these reactions can be modeled as a function of the initial water content by a three-parameter power law for the esterification and by an exponential law for the transesterification. The influence of the water content on the mono over di-ester ratio will also be discussed to a lesser extent.

Keywords **:** esterification, lipase, surfactant, water content, modeling, Novozym 435, lipase B of *Candida antarctica*

1. Introduction

Fatty acid sugar esters are non-ionic, biodegradable, non toxic, non skin irritant, odourless and tasteless surfactants which allows their use in the food or the cosmetic industries [1-3]. In recent years, lipase-catalyzed synthesis of sugar esters has emerged as an interesting alternative to the chemical synthesis because of milder conditions using less toxic solvents [4-8]. The initial water content is important in the enzymatic reaction in several ways. If the water content is too high, hydrolysis of ester occurs instead of the synthesis. Several authors have demonstrated that a lack of water in the reaction mixture exhibits all biochemical reactions [9]. In 1993, Akoh [10] publishes results of oleic acid incorporation during the transesterification of methyl glucoside by methyl oleate in benzene/pyridine catalyzed by *Candida* sp. lipase (SP382) when water was added. More recently, the same information was published by Chamouleau *et al.* during the esterification of fructose by palmitic acid in 2-methyl-2-butanol catalyzed by Novozym 435, the incorporation of the water in the reaction phase was achieved by using saturated salt solutions atmospheres [6]. On the other hand in 2007, Yoo controled the water content by addition of a certain quantity of molecular sieves during the esterification of xylitol by oleic acid [11].

The main objective of this paper was to extent the works of Akoh and Chamouleau by investigating the influence of the water content for the lauric acid fructose ester synthesis in new conditions, in particular the influence on the conversion rate of the lauric acid and on the mono over di-esters ratio.

On the other hand, the behavior of the surfactant depends on the length of the fatty chain: a small chain (below C12) gives a hydrophilic surfactant while long chain surfactants (above C12) are lipophilics. Lauric acid fructose ester surfactants have been chosen because of the intermediate hydrocarbon chain length of lauric acid. Moreover, they exhibit a biological activity such as an inhibitory effect on the growth of *Streptococcus mutans* [3].

2. Materials and Methods

2.1. Enzymatic esterification with lipase Novozym 435

A mixture of fructose (0.5044 g, 2.8 mmol) and lauric acid (0.5609 g, 2.8 mmol) in 20 ml of 2-methyl-2-butanol was heated at 60 °C for 1 hour. The lipase (100 mg) was then added in 30 min either on its own or with 2 g of molecular sieves (4 Å as extruders 1,6 mm technical) (previously activated at 350 °C) or water (50, 100, 150 or 200 μl). The reaction mixture was incubated in an shaking water bath at 60 °C for 48 h at 100 rpm in an Erlenmeyer flask sealed with a screw cap. The mixture was then filtered and 40 ml of water was added to the filtrate. The aqueous phase was extracted with methylene chloride (2 x 10 ml), and the organic phase was removed under reduced pressure.

Purification was achieved by liquid chromatography using silicagel G60 (30 g) preconditioned in 70 ml of chloroform, eluted by 130 ml chloroform, 120 ml chloroform/methanol (90/10, v/v) and 120 ml chloroform/methanol/water (64/10/1, v/v/v).

All reactions were carried out in duplicate. A blank was carried out in the same conditions without enzyme.

2.2. Analysis

The determinations of the average and the error (using the Student-Fisher calculation at 95 % confidence level) were performed by the Statistic on One Variable Module Version 1.05 [12] which is a part of Gabriel Data Analysis software [13]. The data analysis of points were performed by the Statistic on Two Variables Module 1.31 [14] which is a part of the same software.

2.2.1. Water content

The water content of reaction media was determined by titration of about 1 g of this media using a Mettler Toledo DL31 Karl Fisher titrator [15].

2.2.2. Determination of conversion rate

10 mL absolute ethanol and 2 drops of phenolphatlein were added to 1 ml of a sample reaction as a quick method to determine the conversion rate. The free lauric acid was then titrated by KOH 0.02 M. All samples were titrated in duplicate.

2.2.3. Determination of monoester-diester ratio

HPLC analyses were performed using Agilent Technologies 1200 series system equiped with an ELSD detector (Alltech 330) (temperature: 84.5 °C, gas flow rate: 2.22 slpm). An Agilent Zorbax SB-C18 (150 x 4.6 mm, 3.5 µm) was used for analysis. HPLC gradient program (flow rate 0.5 ml min^{-1}): at 0 min 45/55 (% v acetonitrile/% v water), at 15 min 100/0, at 25 min 100/0, at 30 min 45/55, then holding for 10 min.

3. Results and Discussion

The reaction was carried out in different hydration conditions. First, without molecular sieves the reaction mixture was naturally enriched with water during the reaction. Secondly, with addition of different quantities of water and finaly with molecular sieves, the released water was trapped during the esterification reaction. All results were compared to the results of Akoh and Chamouleau. All experiments were carried out during 48 h because, it was previously observed that the conversion rate increased only 2 % from 48 h to 72 h.

Without molecular sieves the water content increased from 0.150 % to 0.207 % in 48 h and the conversion rate was only 21 % (Table 1) which is higher than the conversion rate obtained by Chamouleau, which was only 10.6 %. Akoh had a conversion rate of about 75 %, but it was a transesterification where no water was produced as by-product.

Table 1 : Conversion rate and diester over monoester ratio as a function of the water content

	With molecular sieves	Without molecular sieves	With water added			
			50 μl	100 μl	150 μl	200 μl
Water content (%)	0.14±0.01[*] 0.02±0.01[†]	0.150±0.005[*] 0.207±0.006[†]	0.27±0.02[*] 0.424±0.009[†]	0.434±0.006[*] 0.608±0.004[†]	0.65±0.04[*] 0.80±0.02[†]	0.87±0.02[*] 0.96±0.02[†]
Conversion rate (%)[‡]	72±6	21±3	10.1±0.8	7.2±0.8	5.3±0.7	4.5±0.9
Diester/ monoester (%/%)[¶]	30.2/69.8	2.6/97.4	1.4/98.6	nd/-	nd/-	nd/-

[*] Initial water content determined by Karl Fisher Titration. The data are the average of two different reactions and six data

[†] Water content after 48h determined by Karl Fisher Titration. The data are the average of two different reactions and six data

[‡] Based on the lauric acid conversion after 48 h. The data are the average of two different reactions and four data

[¶] Determined by HPLC - Two repetitions - See Figure 2. (nd: not detectable)

In the second set of experiments 50, 100, 150 and 200 μl of water were added to the initial mixture wich correspond to an initial water content of 0.27, 0.434, 0.65 and 0.87 % respectively. During 48 h the water content increased about 0.14% in average (see Table 1). The conversion rate of lauric acid decreased with water content as expected (Table 1) from 21 % without molecular sieves to 4.5 % with 200 μl water added (Figure 1).

This fact was also observed by Chamouleau for the synthesis of fructose palmitate [6] and by Yoo for xylitol oleate [11]. In the case of the work presented here, the equation of the conversion as a function of the initial water content follows a Herschel-Bulkley three-parameter power law equation:

$\text{Conversion}(\%) = 3{,}68625 + 0{,}80315 \text{ x } (\text{InitialWaterContent})^{-1{,}61746}$; $r = -0{,}9992$ (Figure 1) and can be used for predicting the conversion rate at 48 h.

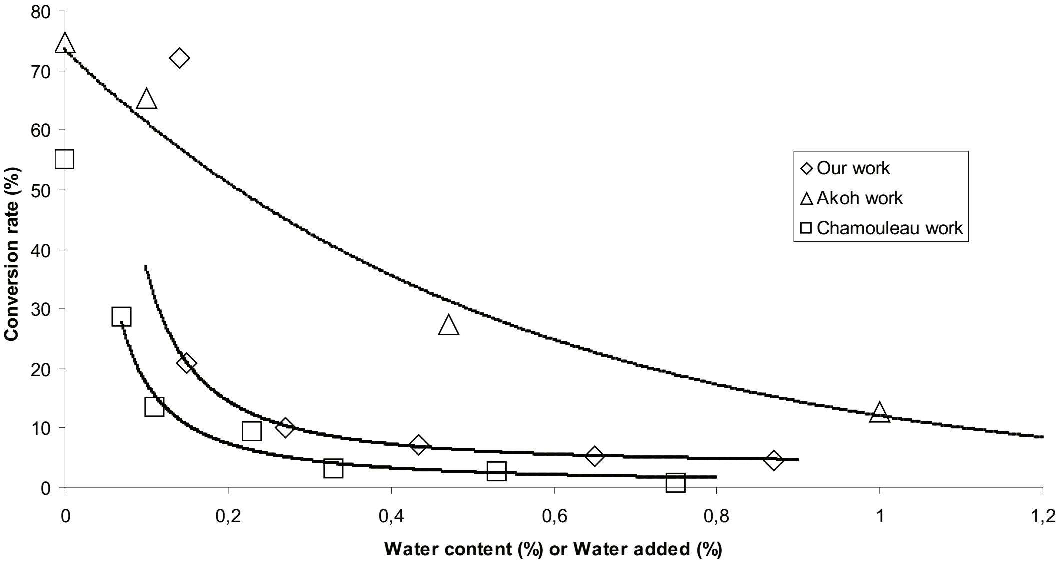

Fig. 1 : Conversion rate as a function of the initial water content (Chamouleau and our works) or water added (Akoh work)

It seems important to model also the results of Chamouleau and Akoh. As Chamouleau's reaction was also a esterification, the equation of the conversion as a function of the initial water content can be described also by a three-parameter power law equation which is Conversion(%) = 0,48347 + 0,82710 x $(\text{InitialWaterContent})^{-1,31602}$; r = -0,9844 (Figure 1). As Akoh's reaction was a transesterification, the best model was obtained with an exponential law Conversion(%) = 73.48444 x $0.16330^{\text{InitialWaterContent}}$; r = -0.99326 (Figure 1). When comparing the coefficients of the different three-parameter power laws, it can be concluded that the first coefficient is related to the experimental conditions if no water is present and the third (the exponent) the "efficiency" of the water (for decreasing the conversion rate). In this case the first coefficient indicates that the conditions presented here are better than those of Chamouleau's work, but the third term clearly shows that the decrease of the conversion rate is stronger in the new conditions than in Chamouleau's. This is due to the fact that the water was directly introduced into the medium whereas Chamouleau diffused the water using saturated salt solutions which is milder.

The last experiment corresponds to the use of molecular sieves in the reaction medium. As the produced water was trapped, the conversion rate increased to 72 % (Table 1) which is close to the best results of the transesterification of Akoh (74.6 % with no water added) [10] but much higher than that of Chamouleau which was only 55.1 % in presence of molecular sieves [6].

Futhermore, the quantitative HPLC study shows that the products formed are different (Figure 2). The synthesis of diester decreases from 30.7 % to 1.4 % when the initial water content increased from 0.14 % (molecular sieves) to 0.27 % (50 µl) respectively. A similar result in acetone with oleic acid has been found by Arcos *et al.* [16]. According to Chamouleau the enzymes loss of selectivity is due to the reduction of the hydration layer of proteins ; the lack of water close to the enzyme increases the hydrophobicity of the environment and therefore induces a decrease of fructose solubility. Under these conditions, the monoester becomes a better substrate than fructose [6]. Chamouleau only reports the kinetic for two points, with and without molecular sieves, as a complete study of several water additions (see Table 1 and Figure 2) is reported here.

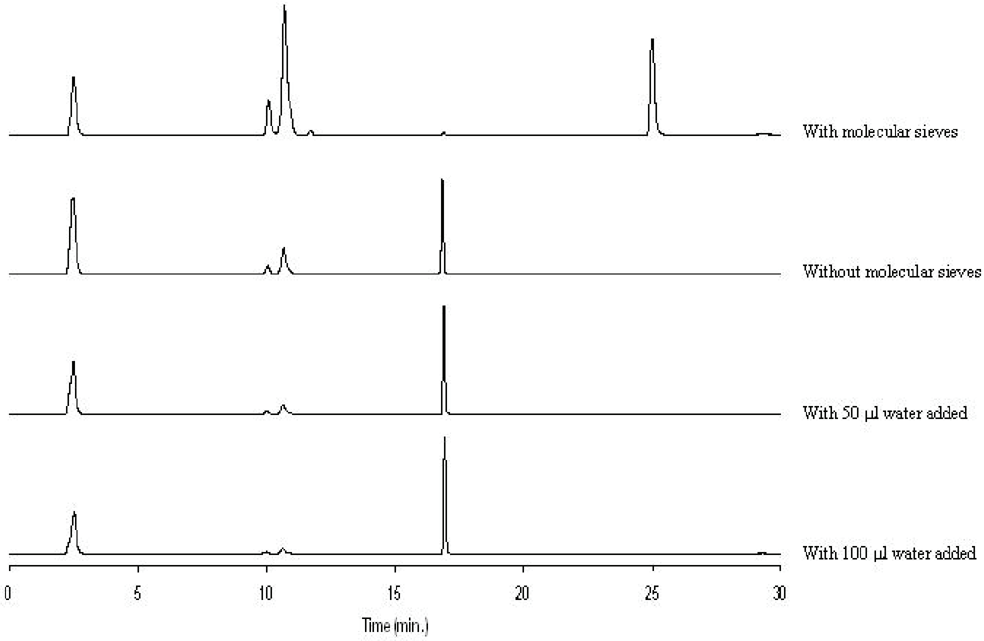

Fig. 2 : HPLC Chromatogram of 4 selected reactions mixture after 48 h. Conditions: see Materials and methods. The retention times are as follow: 2.4 mn fructose; 10.1 and 10.8 mn fructose monoesters; 16.8 mn lauric acid ; 24.5 mn fructose polyesters.

It is not possible to conclude if the phenomenom of decrease of the polyesters synthesis is the same when more than 50 µl of water are added because, in term in kinetics, the synthesis speed of diester is the square of the synthesis speed of monoester and as the yield decreases with addition of water, the HPLC detection of polyester was not assured (Figure 2).

Under, the same conditions, whatever the initial water concentration, the lipase of *Candida antarctica* only synthesized monoester glucose instead of monoester fructose (data not shown).

4. Conclusion

With the new synthesis conditions presented here using hermetic conditions, a best conversion rate was obtained for a direct esterification of fructose by lauric acid with Novozym 435 as a catalyst. Almost the same value as in case of transesterification, where no water as by-product was produced, was achieved (72% vs 74.6%). The conversion rate of this reaction can be modeled as a function of the initial water content by the following three-parameter power law $\text{Conversion}(\%) = 3{,}68625 + 0{,}80315 \text{ x } (\text{InitialWaterContent})^{-1{,}61746}$. Futhermore, it seems that the addition of water decreases the synthesis of polyesters.

5. Acknowledgements

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