This is the peer reviewed version of the following article Chemistry Select.2018. 3(2), 748-752, which has been published in final form at https://doi.org/10.1002/slct.201702332. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

One-Step Solvent-Free Production of a Spermaceti Analogue Using Commercial Immobilized Lipases

Dr. Mar Serrano-Arnaldos, Prof. Josefa Bastida, Dr. Fuensanta Máximo(*), Dr. Salvadora Ortega-Requena and Dr. Claudia Montiel.

Department of Chemical Engineering. University of Murcia. 30071 Murcia. Spain.

(*) Corresponding author.

Tel: +34 868 88 7367. Fax: +34 868 88 4148. e-mail: fmaximo@um.es.

Abstract: The enzymatic synthesis of fine chemicals is a promising approach to overcome the major problems of the traditional chemical routes and implement eco-friendly and low energy consuming industrial processes. This work introduces for the first time the one-step production of an analogue of the sperm whale's spermaceti in a vacuum, solvent-free system. With the aim of easing the industrial feasibility of the process, two commercial biocatalysts with appropriate technical properties were tested. Results pointed out that under optimal conditions, both commercial immobilized derivatives (Lipozyme® RM IM and CalB immo Plus) gave excellent results as they can be reused up to 15 batches without a noticeable loss of their activity, obtaining conversions of ~98% in two hours. This green and sustainable process might be economically competitive and leads to a product that meets the all specifications required for its cosmetic use and that can be labeled as "natural", increasing its added value.

Introduction

In the last decades, new green processes based on the use of biocatalysts have been developed; being one of the most studied the enzymatic synthesis of esters by means of lipases (triacylglycerol ester hydrolase E.C.3.1.1.3). Lipases are hydrolytic enzymes that are able to catalyze esterification, transesterification and alcoholysis reactions in anhydrous media. Due to their wide range of application, during the last years several studies on the biocatalytic synthesis of wax esters from fatty acids and long-chained alcohols have been published. These reactions should be performed with a very low presence of water in reaction media, in order to push the chemical equilibrium towards the synthesis. In this way, organic solvents,[1–3] ionic liquids[4,5] and supercritical fluids[6–8] have been employed, but either for the unnecessary severe operation conditions or for the risk of finding traces of the reaction media on the product, these methods are unsuitable for obtaining cosmetic or pharmaceutical compounds. Therefore, the enzymatic synthesis of these products can be faced with great success by operating under vacuum using a solvent-free system, as it requires mild reaction conditions and purification steps are considerably reduced.[9,10]

The spermaceti is a solid wax rich in high-molecular-weight esters, extracted from the cranial cavity of the sperm whale *Physeter macrocephalus* L. (*P. catodon* L.). Several authors have tackled its study and characterization, being Wellendorf[11] the one who established its centesimal composition: 15% cetyl laurate, 37% cetyl myristate, 38% cetyl palmitate and 8% cetyl stearate, even if slight differences in this proportion can be found depending on the age and gender of the sperm whale.[12] The spermaceti is a crystalline bright white solid at room temperature and its physical properties are a consequence of its high content in long-chained esters, with a minimum percentage of triglycerides.[11–13] It has been used for years in the manufacture of candles and lubricants, and also in some pharmaceutical formulations, mainly ointments. Actually, it is chiefly employed in cosmetic industry as a moisturizer and as a carrier agent for some fragrances and active ingredients due to its similarity with the natural oils produced by human skin.

Since the 1980s, the sperm whale is a protected species and its hunting has been banned, so new industrial processes had to be developed in order to find a synthetic pathway for obtaining this ester mixture. The most common method is based on the reaction between the carboxylic acid and the alcohol at high temperature (120-160 ºC) using an acid catalyst such as H_2SO_4, HCl, etc., but currently the trend is replace them with heterogeneous catalysts of various nature.[14–16] These methods present many disadvantages: handling with very corrosive and hazardous chemical compounds, high energy consumption and the deterioration of the synthesized product.[17] Additionally, the extreme reaction conditions favor the formation of numerous by-products that might have a negative effect on the process economy, as this entails the need of complicated separation and purification processes to recover the ester of interest. Consequently, some researchers have tried to overcome these issues with promising results by carrying out the synthesis of the main components of spermaceti separately by using microwave irradiation and an acid catalyst of activated Indian bentonite, but this process required long reaction times, non-stoichiometric substrates and harsh reaction conditions under the presence of organic solvents to achieve maximum yields.[18] On the other hand, previous work has demonstrated the feasibility of the synthesis of each one of these cetyl esters under atmospheric pressure using the well-known Novozym® 435, with good outcomes and avoiding the complicated task of formulating an appropriate and stable biocatalyst for this synthetic purpose.[19] Nevertheless, there are other commercial alternatives, developed by Novozymes and other manufacturers, which can be evaluated in order to implement the enzymatic process at industrial scale.

Thus, this work compares the synthesis of a mixture of cetyl esters similar to natural spermaceti by using two commercially available lipase-based catalysts: Lipozyme® RM IM and CalB immo Plus. The differences between these enzymatic preparations lie not only in the lipase immobilized, the technique of immobilization and the carrier used, but also in their prices, being CalB immo Plus 2.5-3 times more expensive than Lipozyme® RM IM. According to the authors previous experience, the process has been performed under vacuum conditions, as pushing the reaction equilibrium towards the ester synthesis by eliminating the water present in reaction media significantly increases the final conversion.[10,19–22]

Results and Discussion

Influence of the biocatalyst concentration

The optimization of the biocatalyst concentration was performed for each ester of the spermaceti separately, and reagents were added in equimolecular ratio. The amounts of immobilized derivative tested were comprised between 0.125 and 1 g, which represents a concentration of 0.625%-5% (w/w) referred to the substrates. All reactions were conducted at 70 ºC, the lowest operation temperature in common for all the substrates, as the highest melting point is ~69 ºC for the stearic acid.[23]

Table 1 gathers the conversion values after 60 min in the synthesis of cetyl laurate (CL), cetyl myristate (CM), cetyl palmitate (CP) and cetyl stearate (CS). From a general overview of these data, it is obvious that an increase of the concentration of biocatalyst clearly improves reaction rate until the 1 g, when the reaction media has become saturated of catalytic species. Nevertheless, this effect on the kinetics of the process is not clearly proportional to the amount of enzyme present in the reaction medium. The conversion increases between 1.3 and 1.5 times when the catalyst amount is doubled from 0.125 g to 0.250 g, however this increase is only 1-2% when it goes from 0.5 g to 1 g (Table 1).

Table 1. Conversion values after 60 min of reaction during the synthesis of the spermaceti's esters separately (70 ºC, 25 cm^3/min N_2, 350 rpm, 20 g of substrates, vacuum).

		Amount of biocatalyst (g)			
Ester	**Biocatalyst**	**0.125**	**0.25**	**0.5**	**1**
CL	*Lipozyme® RM IM*	67.00 ± 0.41	88.03± 0.71	96.59 ± 1.34	98.16 ± 0.68
	CalB immo Plus	68.38 ± 0.75	92.44 ± 2.21	97.30 ± 1.37	98.70 ± 0.66
CM	*Lipozyme RM® IM*	65.95 ± 1.23	90.98 ± 1.17	96.62 ± 0.53	97.30± 0.08
	CalB immo Plus	71.19 ± 1.38	90.84 ± 0.57	97.75 ± 1.29	98.68 ± 0.42
CP	*Lipozyme® RM IM*	61.81 ± 1.59	91.00 ± 1.30	96.71 ± 0.06	97.18 ± 0.87
	CalB immo Plus	68.90 ± 0.05	89.29 ± 0.51	97.49 ± 1.26	98.34 ± 0.78
CS	*Lipozyme® RM IM*	60.56 ± 1.99	87.60 ± 0.43	95.25 ± 1.63	95.78 ± 0.69
	CalB immo Plus	65.20 ± 2.43	87.99 ± 1.32	95.28 ± 0.60	97.66 ± 0.84

Furthermore, during the study of the substrate specificity of different lipases in the hydrolysis of various esters with substitutions on the alcohol or the acid part of the substrates, Naik et al.[24] found out that, even if Rml and CalB were in general more active on the alcohol-substituted substrates than on acid-substituted ones, CalB showed good activity on the hydrolysis of most of the large acid-branched molecules. This study showed also that Rml, for its part, achieved good yields in the hydrolysis of the ester with the largest linear acid part. This evidence would also support experimentally observed results during the cetyl esters synthesis, as Lipozyme® RM IM exhibited a high activity despite of the non-hydrophobic nature of the resin Duolite ES 562.

In order to study the influence of the chain length of the substrates during the synthesis, reaction rates were calculated for a fixed amount of both immobilized derivatives, and results are showed in Table 2. As a general trend, reaction rate is slower as the carbon chain length increases. This effect is observed for both immobilized derivatives being 21.58 and 19.97 % for Lipozyme® RM IM and CalB immo Plus respectively. It is only noticeable for the highest quantities of biocatalyst assayed, as the rate determining step might be the mass transfer and diffusion of substrates and products, while for the lowest amounts tested the limiting step may be related to the kinetics of the process.

Table 2. Reaction rate calculated at 15 min of operation time, for the higher amount of lipase assayed (1 g of biocatalyst, 70 ºC, 25 cm^3/min N_2, 350 rpm, 20 g of substrates, vacuum).

	Reaction rate (Δ(AV)/min)	
	Lipozyme® RM IM	*CalB immo Plus*
CL	7.60 ± 0.38	7.71 ± 0.04
CM	6.58 ± 0.21	7.20 ± 0.42
CP	6.31 ± 0.06	6.31 ± 0.27
CS	5.96 ± 0.67	6.18 ± 0.35

It is important to highlight that the final conversion after 360 min achieved by both biocatalyst was higher than 98%, with AVs ≤3 mg KOH/g. So, taking all this considerations into account, the optimum amount of biocatalyst selected for CalB and Rml biocatalysts was 0.5 g (a concentration of 2.5% (w/w) referred to substrates).

Influence of the temperature

Best reaction temperature was determined by performing assays at 60, 70 and 80 ºC with the optimum concentration of biocatalyst selected: 0.5 g. These experiments were carried out using as substrates the mixture of four fatty acids and cetyl alcohol. As it can be observed in Figure 1, there is not apparent denaturation of the enzymes for the range of temperature studied, and the reaction time required to reach the final conversion is slightly shorter as the temperature increases for CalB and Rml biocatalysts. This positive effect might be explained by two main reasons: on one hand, the enhancement of the reaction rate experienced by all kind of chemical reactions when process' temperature increases; on the other hand, the reduction of the viscosity of the reaction media with temperature eases mass transfer.[25] Consequently, 70 ºC was selected as the optimum temperature in all cases, as a higher temperature significantly increases energy consumption and a longer conditioning time is required when operating at 60 ºC. Under the best operation conditions, CalB immo Plus only required 2 hours to reach a conversion of ~98%, while the conversion obtained

after a reaction time of 6 hours, when the equilibrium of the reaction was achieved, was of ~98.5%. For Lipozyme® RM IM the final conversion was of 98.1%.

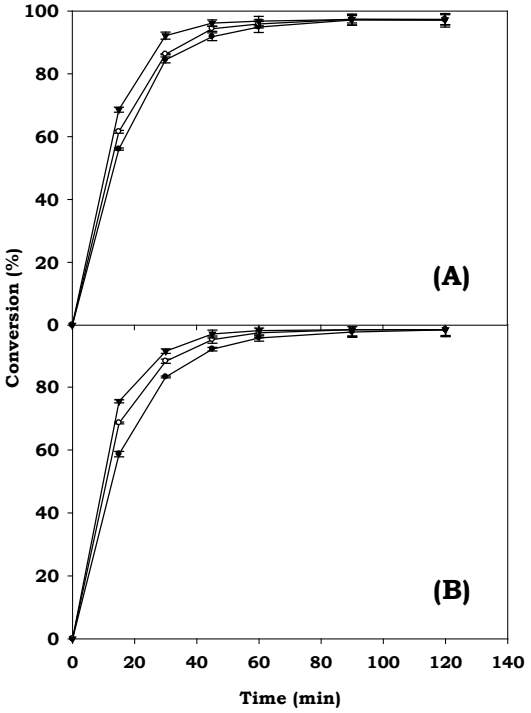


Figure 1. Influence of the reaction temperature in the synthesis of spermaceti for (A) Lipozyme® RM IM (B) CalB immo Plus (0.5 g of enzyme, 25 cm^3/min N_2, 350 rpm, 20 g of substrates, vacuum, 60 ºC (●); 70 ºC (○); 80 ºC (▼)).

Reuse of the biocatalysts

Stable and active biocatalysts which can be recovered and reused are crucial to make economically competitive an enzymatic process for industrial applications, as enzymes usually are the major responsible of the process cost.[26] On that purpose, both commercial derivatives were tried in 15 consecutive batches under the optimum conditions previously determined. All the assays were carried out only for 1 hour, as short reaction times makes easier to appreciate variations on the conversion achieved, and hence, on the immobilized derivative activity, and using as substrates the mixture of four fatty acids and cetyl alcohol. Table 3 shows the conversion obtained after 15 reuses compared with the one reached in the first use. These results prove the excellent operational stability of all the lipase preparations under the experimental conditions.

As it has been described in the Experimental section, once finished the reaction time set, the product was removed from the reaction vessel, and the biocatalyst was rinsed with acetone, filtered and air dried, but this post-treatment of the biocatalyst is only interesting from a scientific point of view in order to determine the weight of immobilized derivative recovered and to not influence the initial AV of the next reaction mixture. Thus, the rinsing and drying steps can be avoided during the industrial production of these esters as long as the synthesized product does not change between batches, reducing costs, processing time and possible loses of biocatalyst. During these assays, no damage of the biocatalyst due to the mechanical stirring was observed, as it is corroborated by the amount of biocatalyst recovered after the 15 uses (Table 3), as the process of recovering and rinsing is the main responsible for the variations of weight measured. These results predict a good long-term reusability of these commercial biocatalysts for the synthesis of spermaceti at industrial scale.

Table 3. Conversion and amount of biocatalyst in the first use and recovered after 15 batches (0.5 g biocatalyst, 70 ºC, 25 cm^3/min N_2, 350 rpm, 20 g of substrates, vacuum).

	Conversion (%)		Amount of biocatalyst (g)	
Biocatalyst	**First use**	**15 uses**	**First use**	**15 uses**
Lipozyme® RM IM	96.12 ± 1.64	95.71 ± 1.27	0.5002 ± 2.10^{-4}	0.5084 ± 8.10^{-3}
CalB immo Plus	96.32 ± 0.93	95.39 ± 1.38	0.5003 ± 1.10^{-4}	0.4077 ± 15.10^{-3}

Characteristics of the enzymatic spermaceti

Considering the fact that natural spermaceti composition may present slight differences depending on the sample and that specific legal standards for the use of spermaceti in cosmetic industry are not established, data sheets for the main producers of this compound are nowadays a good reference to set the minimum requirements for its use in cosmetic formulations. Table 4 compares the physicochemical characterization of the in-lab spermaceti with the specifications of two manufacturers of this cetyl esters mixture through classic chemical synthesis, putting into evidence that the enzymatic product accomplishes the standards of the commercialized spermaceti. HPLC analysis confirms the absence of unreacted or side products (Table 5), avoiding the need of undergoing a host of reprocessing steps to meet the demand of an odor and colorless product.

In addition, the cosmetic compounds obtained via enzymatic processes have the distinctive feature of being catalogued as "natural" according guidelines approved by the Council of Europe expert committee on cosmetics,[27] which makes the biotechnological product more attractive to a market more and more concerned with the personal and the environmental care.

Table 4. Physicochemical characterization of the enzymatic spermaceti.

	Commercial product 1[(a)]	**Commercial product 2[(b)]**	**Enzymatic product**
Color	white	white	white
Odor	n.d.	bland	bland
Acid Value (mg KOH/g)	≤1.5	≤5	1.7- 2.2
Saponification value (mg KOH/g)	105-120	109-120	108-125
Hydroxyl value (mg KOH/g)	n.d.	n.d.	1-12
Iodine value (g I_2/100 g)	≤1	≤1	0.2
Water content (ppm)	n.d.	n.d.	3.6

(a) Imperial-Oel-Import, (b) Jeen International Corporation.

Table 5. HPLC analysis of the enzymatic spermaceti.

Ester	CL	CM	CP	CS
Retention time (min)	5.469	7.224	9.639	12.778
Composition of the sample (%)	18.8	39.4	34.8	7.0
Composition of the natural spermaceti (%)	15.3	37.8	38.8	8.2

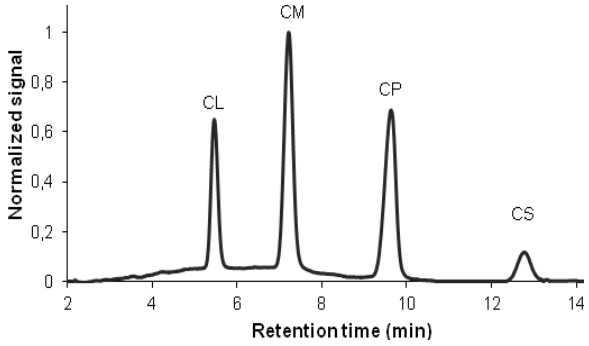


Figure 2. Chromatogram of biotechnological spermaceti.

Conclusions

This work demonstrates the feasibility of obtaining a mixture of cetyl esters similar to natural spermaceti by using a one-step solvent-free enzymatic process. Both commercial immobilized lipases tested, Lipozyme® RM IM and CalB immo Plus, did not exhibited significant differences in their activity and gave the best results at 70 ºC in a concentration of 2.5% (w/w), as final conversion was almost achieved within 2 hours. The high conversion yields in a short reaction time, the stability and reusability of the biocatalysts, the mild operation conditions and the fact that is unnecessary the reprocessing of the ultra-pure product are promising factors to turn economically profitable the process towards the classic chemical route. This method is also environmental friendly, as the sperm whales are not involved in the production process, the reaction media did not contained any organic solvent and little waste is generated. In addition, the labeling "natural" of the enzymatic spermaceti makes the biotechnological product more attractive to a market more and more concerned with the personal and the environmental care.

Supporting Information Summary

Supporting information file includes the Experimental Section and the associated references.

Acknowledgements

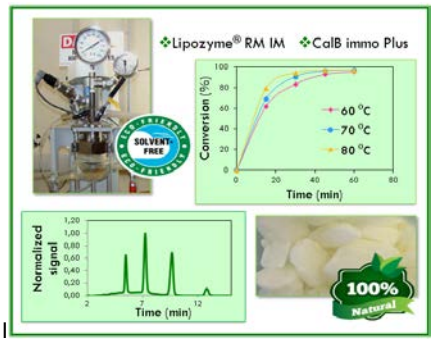
This work has been funded with support from the Spanish MICINN (CTQ2015-66723-R) and the European Commission (FEDER/ERDF). M. Serrano-Arnaldos had a pre-doctoral scholarship (FPI) from MINECO. We wish to acknowledge D. Ramiro Martínez Gutiérrez (Novozymes Spain S.A.) who kindly supplied the biocatalysts.

Keywords: *Candida antarctica* • enzymatic synthesis • *Rhizomucor miehei* • solvent-free • spermaceti.

References

[1] R. Awang, M. Basri, S. Ahmad, A.B. Salleh, *Biotechnol. Lett.* **2004**, *26,* 11–14.
[2] H.C. Chen, C.-H. Kuo, H.H. Chen, Y.-C. Liu, C.J. Shieh, *J. Am. Oil Chem. Soc*. **2011**, *88,* 1917–1923.
[3] C.-H. Kuo, H.-H. Chen, J.-H. Chen, Y.-C. Liu, C.-J. Shieh, *Int. J. Mol. Sci.* **2012,** *13*, 11694–11704.
[4] T. De Diego, P. Lozano, M.A. Abad, K. Steffensky, M. Vaultier, J.L. Iborra, *J. Biotechnol.* **2009**, *140*, 234–241.
[5] T. Itoh, *J. Synth. Org. Chem. Jpn.* **2009**, *67*, 143–155.
[6] Ž. Knez, C.G. Laudani, M. Habulin, E. Reverchon, *Biotechnol. Bioeng.* **2007**, *97*, 1366–1375.
[7] C.-H. Kuo, H.-Y. Ju, S.-W. Chu, J.-H. Chen, C.-M.J. Chang, Y.-C. Liu, C.-J. Shieh, *J. Am. Oil Chem. Soc.* **2012**, *89*, 103–110.
[8] M. Zarevucka, Z. Wimmer, *Int. J. Mol. Sci.* **2008**, *9*, 2447–2473.
[9] G. Hills, *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 601–607.
[10] M.C. Montiel, M. Serrano, M.F. Máximo, M. Gómez, S. Ortega-Requena, J. Bastida, *Catal. Today* **2015**, *255*, 49–53.
[11] M. Wellendorf, *Nature* **1963**, *198*, 1086–1087.
[12] D.W. Rice in *Encyclopedia of Marine Mammals, Second Edition*, (Eds.: W.F. Perrin, B. Würsig, J.G.M. Thewissen), Academic Press, London, **2009,** pp. 1098–1099.
[13] T. Horiguchi, Y. Takase, Y. Arai, H. Ageta, *Nat. Med*. **1999**, *53*, 105–108.
[14] H. Firouzabadi, N. Iranpoor, S. Farahi, *J. Mol. Catal. -Chem*. **2008**, *289*, 61–68.
[15] K. Mantri, K. Komura, Y. Sugi, *Synth.-Stuttg*. **2005**, 1939–1944.
[16] K. Mantri, K. Komura, Y. Sugi, *Green Chem.* **2005**, *7*, 677–682.
[17] L. Deng, X. Wang, K. Nie, F. Wang, J. Liu, P. Wang, T. Tan, *Chin. J. Chem. Eng.* **2011**, *19*, 978–982.
[18] B. Vijayakumar, G. Nagendrappa, B.S.J. Prakash, *Indian J. Chem. Technol.* **2009**, *16*, 377–381.
[19] M. Serrano-Arnaldos, M.F. Maximo-Martin, M.C. Montiel-Morte, S. Ortega-Requena, E. Gomez-Gomez, J. Bastida-Rodriguez, *Bioprocess Biosyst. Eng*. **2016**, *39*, 641–649.
[20] A. Bodalo, J. Bastida, M.F. Maximo, M.C. Montiel, M.D. Murcia, S. Ortega, *Biochem. Eng. J.* **2009**, *44*, 214–219.
[21] J.L. Gomez, J. Bastida, M.F. Maximo, M.C. Montiel, M.D. Murcia, S. Ortega, *Biochem. Eng. J.* **2011**, *54*, 111–116.
[22] S. Ortega-Requena, A. Bódalo-Santoyo, J. Bastida-Rodríguez, M.F. Máximo-Martín, M.C. Montiel-Morte, M. Gómez-Gómez, *Biochem. Eng. J.* **2014**, *84*, 91–97.
[23] The PubChem Project. https://pubchem.ncbi.nlm.nih.gov/ (accessed 24.04.17).
[24] S. Naik, A. Basu, R. Saikia, B. Madan, P. Paul, R. Chaterjee, J. Brask, A. Svendsen, *J. Mol. Catal. B-Enzym.* **2010**, *65*, 18–23.
[25] F. Hasan, A.A. Shah, A. Hameed, *Enzyme Microb. Technol.* **2006**, *39,* 235–251.
[26] P. Tufvesson, U. Tornvall, J. Carvalho, A.J. Karlsson, R. Hatti-Kaul, *J. Mol. Catal. B-Enzym.* **2011**, *68,* 200–205.
[27] J. Newerli-Guz, *Zesz. Nauk.* **2012**, *74,* 36–42.

Entry for the Table of Contents



This work introduces for the first time the one-step production of an analogue of the sperm whale's spermaceti, using an eco-friendly and low energy consuming process. This green and sustainable process might be economically competitive and leads to a product that meets the all specifications required for its cosmetic use and that can be labeled as "natural", increasing its added value.

WILEY-VCH

Chemistry SELECT

Supporting Information

© Copyright Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2018

One-Step Solvent-Free Production of a Spermaceti Analogue Using Commercial Immobilized Lipases

Mar Serrano-Arnaldos, Josefa Bastida, Fuensanta Máximo,* Salvadora Ortega-Requena, and Claudia Montiel

Supporting Information

Chemicals

Lipozyme® RM IM was kindly donated by Novozymes Spain S.A. Lipozyme® RM IM is a *Rhizomucor miehei* (Rml) biocatalyst prepared by immobilizing on the weak anionic exchange resin Duolite ES 562 (phenol-formaldehyde copolymers), with a declared activity of 275 IUN/g (interesterification units). CalB immo Plus was purchased from c-LEcta GmbH (Leipzig, Germany) and Purolite Corporation (Pennsylvania, USA). It is prepared by adsorption of CalB on the DVB/methacrylate Purolite's carrier ECR1030M (9550 ± 500 PLU/g). Lauric acid (99 %) was acquired from Acros Organics (Geel, Belgium). Myristic acid (98 %), palmitic acid (98 %), stearic acid (95 %) and cetyl alcohol were all from Sigma-Aldrich (Missouri, USA). All other chemicals were analytical reagent grade.

Synthesis under vacuum

All reactions were carried out using a Parr 5101 series low-pressure reactor (Parr Instrument Company, Illinois, USA). The reaction vessel is round-bottomed, glass jacketed and has a total volume of 100 mL. An appropriate mixing was achieved through a four-bladed impeller set at 350 rpm, as literature suggests that using speeds within this range in viscous media is a good compromise between avoiding the mechanical destruction of the carrier and reducing mass transfer limitations.[1–4] All reactions were carried out in a solvent-free medium containing 20 g of substrates (see Table 1) with a continuous dry N_2 input 25 cm^3/s at 213 mbar. The reaction course was followed by determining the acid value (AV) of samples of ~0.5 mL extracted during the synthesis.

Table 1.- Amounts of fatty acids and cetyl alcohol added to de reactor.

	Susbstrate				
	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Cetyl alcohol
Product					
CL	9.05 g 0.045 mol	-	-	-	10.95 g 0.045 mol
CM	-	9.70 g 0.042 mol	-	-	10.30 g 0.042 mol
CP	-	-	10.28 g 0.040 mol	-	9.72 g 0.040 mol
CS	-	-	-	10.80 g 0.038	9.20 g 0.038 mol
Spermaceti	1.51 g 0.0075 mol	3.73 g 0.0163 mol	3.84 g 0.0150 mol	0.81 g 0.0028 mol	10.11 g 0.042 mol

The optimization experiments were performed within 6 hours, while the reuse of the biocatalyst was studied by recycling the immobilized derivative several times after one hour of use. For that purpose, the bulk of the product was removed and the biocatalysts were recovered by rinsing two or three times with 10 mL of acetone, filtering and air drying before using them in another batch, with new fresh reagents.

Acid value determination

The acid value (AV) is defined as the amount of KOH (in milligrams) necessary to neutralize the free carboxyl groups present in 1 g of sample.[5]

Conversion can be defined based in AV measurements as:

$$\text{Conversion } (\%) = \frac{\text{AV}_0 - \text{AV}_i}{\text{AV}_0} \text{ x } 100$$

Where AV_0 and AV_i are the acid values at the beginning of the reaction and at a given time during it, respectively.

Results are the mean of two data and they are graphically represented with their standard deviation.

Saponification value measurements

The saponification value represents the milligrams of potassium hydroxide needed to saponify all the free and esterified fatty acids in 1 g of sample.[6]

Hydroxyl value measurements

The hydroxyl value is defined as the milligrams of potassium hydroxide used to neutralize the acetic acid remaining when the hydroxyl groups contained in 1 g of sample are acetylated.[7]

Iodine value measurements

The iodine value is used to determine the grade of unsaturation of a substance, and it corresponds to the grams of iodine consumed per 100 g of sample.[8]

Water content determination

The amount of water contained in 1 g of sample was determined by a coulometer model 899 equipped with an 860 KF Thermoprep oven, both from Metrohom (Herisau, Switzerland).

High performance liquid chromatography (HPLC)

HPLC analysis was carried out using a modular system from Waters (Massachusetts, USA), with a 717PLUS automatic injector and a 600 E quaternary-gradient pump. The system was equipped with a 2996 photodiode array detector and an XBridge C18 column (4.6 mm × 150 mm), all from Waters. The analysis was performed in an isocratic elution, at 35 ºC using a mobile phase of acetonitrile/acetone (75/25 v/v) at a flow rate of 2 mL/min.

References

[1] Q. Lei, S. Ba, H. Zhang, Y. Wei, J.Y. Lee, T. Li, *Food Chem.* **2016,** *199,* 364–371.
[2] C. Otero, P. Márquez, M. Criado, E. Hernández-Martín, *J. Am. Oil Chem. Soc.* **2012**, *9,* 81–90.
[3] L.F. Peng, X.B. Xu, H.L. Mu, C.E. Hoy, J. Adler-Nissen, *Enzyme Microb. Technol.* **2002**, *31,* 523–532.
[4] G. Trubiano, D. Borio, A. Errazu, *Enzyme Microb. Technol.* **2007**, *40,* 716–722.
[5] ASTM D974–02e1, Standard test method for acid and base number by color-indicator titration, ASTM International, West Conshohocken, PA, 2002. doi:10.1520/D0974-02E01.
[6] ASTM D5558-95(2011), Standard Test Method for Determination of the Saponification Value of Fats and Oils., ASTM International, West Conshohocken, PA, 2011. doi:10.1520/D5558-95R11.
[7] ASTM D1957-86(2001), Standard Test Method for Hydroxyl Value of Fatty Oils and Acids (Withdrawn 2007)., ASTM International, West Conshohocken, PA, 1986. doi:10.1520/D1957-86R01.
[8] ASTM D5554-95, Standard Test Method for Determination of the Iodine Value of Fats and Oils., ASTM International, Conshohocken, PA, 1995. doi:10.1520/D5554-95.