ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 21 (2003) 167–174

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Wax esters synthesis from heavy fraction of sheep milk fat and cetyl alcohol by immobilised lipases

Andrea Salis, Vincenzo Solinas, Maura Monduzzi*

Dipartimento di Scienze Chimiche, Università di Cagliari, Cittadella Monserrato, S.S. 554 Bivio Sestu, Monserrato, 09042 Cagliari, Italy

Received 18 March 2002; accepted 19 July 2002

Abstract

Wax esters were obtained from lipase-catalysed alcoholysis of triglycerides with cetyl alcohol, using *n*-hexane as solvent. The heavy triglyceride fraction (HTF), obtained by fractionation of sheep milk fat, was used as raw material. In the natural fat mixture GC analysis showed that palmitic, myristic, stearic and oleic acids are the most abundant fatty acids which are useful to produce wax esters. Reactions were tested for different amounts of Lipozyme RMIM catalyst, and the optimum concentration of 10 mg catalyst/ml solution has been determined. The formation of the four main products, i.e. cetyl myristate, cetyl palmitate, cetyl oleate and cetyl stearate, was determined by HPLC/ELSD quantitative analysis. The optimum water activity in the reaction medium $a_w = 0.35$ in the case of Lipozyme RMIM, and $a_w = 0.53$ for Novozym 435 was found. Lipozyme RMIM (immobilised *sn*-1,3-specific lipase from *Rhizomucor miehei*) was more active than Novozym 435 (immobilised nonspecific lipase-B from *Candida antarctica*) towards wax esters production. The acyl migration of 2-monoglycerides was suggested as a crucial step to explain the higher yields produced by the 1,3-specific lipase.

Keywords: Wax ester; Alcoholysis; Lipase; Sheep milk fat

1. Introduction

In the last decade the growing demand for 'natural' food and in general for environmentally friendly processes has widened the use of enzymes as biocatalysts, particularly in the modification of natural lipids [1].

Lipase-catalysed processes have attracted attention because of the mild reaction conditions under which they occur. The specificity and selectivity of these biocatalysts are other crucial factors. Through lipase-catalysed reactions, many interesting lipid based molecules and mixtures have been produced, but only a few have led to commercial products. In principle, because of the selectivity of many lipases, it is possible to obtain products which are difficult to make by more conventional chemical reactions. In fact, in most cases, a deeper understanding of the reaction mechanisms and of the optimum reaction conditions (temperature, pH, molar ratio of reactants, etc) is needed to develop and improve the catalyst performance and applications. In addition, the cost of using enzymes implies that only low-volume, high-value products can be produced.

Among transesterification reactions, alcoholysis of triglycerides is simple and rather important to the fat and oil industries. Moreover, the starting material is cheap. Alcoholysis involves the exchange of an acid moiety between an ester molecule and

* Corresponding author. Tel.: +39-70-675-4385; fax: +39-70-675-4388.
E-mail address: monduzzi@unica.it (M. Monduzzi).

1381-1177/02/$ – see front matter
PII: S1381-1177(02)00124-8

an alcohol. Schuch and Mukherjee [2,3] found that lipase-catalysed alcoholysis of triacylglycerols with long-chain alcohols yielding wax esters is by far the fastest of the transesterification reactions.

Waxes are esters of long-chain fatty acids and long-chain alcohols; natural waxes originate from animals and vegetables. They can be constituted by saturated and unsaturated wax esters. The former can come from beeswax, whereas, traditional raw materials for unsaturated wax esters are sperm whale and jojoba oil. Commercial waxes have a wide range of applications as lubricants, polishes, plasticizers, coating materials in the medical and food industries and as raw materials in cosmetic and other chemical industries.

Different authors have reported synthesis of wax esters from different raw materials [4–10], among which also milk fat has been recently tested [11]. High yields of wax esters have been obtained by the use of lipases from *Rhizopus miehei* or *Candida antarctica* in solvent-free systems at 60 °C [12].

The present work focuses on sheep milk fat, and aims to ascertain the feasibility and flexibility of wax ester production in very mild reaction conditions, and using the two commercial immobilised lipases Lipozyme RMIM and Novozym 435, in the presence of *n*-hexane as solvent. Among the organic solvents, *n*-hexane can be easily removed because of its low boiling point.

Sheep milk is more concentrated in fats, proteins and sugars than cow milk. Actually, it is used only for cheese production, but it may represent an important renewable source of raw materials for food and nonfood applications. In particular, it contains about 8% of fats, twice than that of cow milk, so that there is great interest in its utilisation for new purposes. In this work the heavy triglyceride fraction (HTF) of the sheep milk fat, obtained through a separation based on crystallisation at 5 °C, was used.

Commercial lipase preparations are nowadays widely employed in many investigations. They are convenient, readily available, and allow work to be verified and extended by other researchers. In addition, the use of these preparations simplifies calculations of process economics for potential scale-up developments. Moreover, the presence of nonproteinaceous material which commercial lipases contain (often more than 80 wt.%) has been shown to play a synergic role in the enzymatic reaction while the specific enzyme activity is not affected [13].

Here, the influence of enzyme concentration and water activity on the wax ester production from cetyl alcohol (hexadecanol, a typical fatty alcohol present in natural waxes) and the HTF mixture has been investigated for the immobilised *sn*-1,3-specific lipase from *Rhizomucor miehei* and for the immobilised nonspecific lipase-B from *C. antarctica* to ascertain the optimum reaction conditions, and also to deepen on possibly different reaction mechanisms for the two lipases. HPLC/ELSD quantitative analyses of cetyl alcohol conversion and wax esters yield have been used.

2. Experimental

2.1. Chemicals

Cetyl alcohol, stearyl alcohol, tripalmitin, tristearin and fatty acid cetyl ester standards were from Sigma (St. Louis, MO). HPLC-grade dichloromethane, acetonitrile were from Merck (Darmstadt, Germany). Immobilised lipases Novozym 435 from *C. antarctica* and Lipozyme RMIM from *R. miehei* are kind gifts from Novo Nordisk—Bioindustriale s.r.l. (Milano, Italy).

2.2. Fractionation of sheep milk fat

The sheep milk fat was separated in fractions differing in the melting point. This procedure is necessary to decrease the amount of short-chain fatty acids, which are not suitable to wax esters production. Fats of sheep milk cream have been dissolved in acetone at 40 °C. At 20 °C proteins and phospholipids precipitate and the resulting clear solution contains mainly triglycerides (TGs). Different fat fractions can be obtained by crystallisation at different temperatures; the heavy triglyceride fraction (HTF) has been obtained by crystallisation at 5 °C.

2.3. Adjustment of initial a_w of enzymes and substrate solutions

Immobilised enzymes and substrate solutions were equilibrated over a saturated salt solution in a closed bottle for 3–4 days at 25 °C, to obtain a defined initial

a_w. Saturated $MgCl_2$, $Mg(NO_3)_2$ and NaCl (a_w = 0.33, 0.53, 0.75, respectively) salt solutions were used. Water content, at the different water activities, was determined by Karl Fischer titration on a 684 KF Coulometer from Metrohm (Herisau, Switzerland).

2.4. Wax esters production

Because natural waxes are constituted by long-chain fatty acid, product formation was determined for the most abundant long fatty acids (chain length ≥ 14C atoms) in the HTF mixture, namely palmitic acid (PA), myristic acid (MA), stearic acid (SA) and oleic acid (OA), as shown in Table 1. The alcoholysis reactions were carried out with an alcohol/TG molar ratio 3/1 (stoichiometric conditions) and at a temperature of 35 °C. An amount of 40.9 mg of HTF (average molecular weight = 706.1 g/mol) were dissolved in 5 ml of hexane, pre-equilibrated at the different water activities, and then mixed with 42.2 mg of cetyl alcohol. The reaction mixtures were introduced in a closed vial of 10 ml, equilibrated at 35 °C before the addition of the enzyme preparation, and then mixed through a magnetic stirrer. Aliquots of 20 ml were withdrawn and diluted to the final volume of 200 μl by adding 20 μl of internal standard (stearyl alcohol in hexane) and 160 μl of *n*-hexane for HPLC analysis. A total of 50 mg of enzyme preparation was generally used except for the study of the optimum enzyme concentration where 10, 20, 30, 50, 70 and 90 mg were used.

Table 1
Fatty acid composition of the heavy triglyceride fraction (HTF) compared to original sheep milk fat (SMF) and cow milk fat (CMF)

Atoms of C:no of insaturations	CMF (%mol/mol)[a]	SMF (%mol/mol)	HTF (%mol/mol)
C2:0	0.1	–	–
C4:0	2.3	10.5	5.8
C6:0	2.8	9.3	5.5
C8:0	2.0	6.7	4.5
C9:0	0.4	1.1	–
C10:0	4.2	12.7	11.4
C11:0	0.5	0.4	–
C12:0	4.4	5.7	6.4
C14:0	12.2	10.4	13.9
C15:0	2.3	1.4	1.4
C16:0	30.6	18.0	28.3
C16:1	1.3	0.5	–
C17:0	2.3	1.8	0.7
C18:0	10.1	5.2	11.2
C18:1	21.0	13.7	10.1
C18:2	2.8	1.6	0.8
C18:3	0.6	1.0	0.0
MA + PA + OA + SA	73.9	47.3	63.5

[a] Data obtained from Poisson et al. [11].

2.5. Derivatisation of HTF in fatty acid methyl esters

About 0.2 g of fat was dissolved in a HCl 2% solution in methanol. The resulting mixture was heated in water bath for 40 min then extracted by 1 ml of hexane. The top phase containing the fatty acid methyl esters was analysed by GC technique.

2.6. GC analysis

GC analyses were performed with a HP 5890 gas chromatograph. The instrument was equipped with a split–splitless injector, a TCD and was fitted with a 60 m × 0.2 mm i.d., coated with a 0.2 μm film of Supelcowax. Carrier gas was helium, flow rate was 2.6 ml/min and head pressure was 60.2 psi. Samples were injected with a syringe at an oven temperature of 40 °C. After an isothermal step of 3 min, the GC oven was heated at the rate of 10 °C/min up to 220 °C and left at this temperature for 30 min.

2.7. HPLC analysis

HPLC analysis was performed using a Lichrospher 100 RP-8 end capped, 5 μm, column (Merck, Darmstadt, Germany) and monitored by an evaporative light scattering detector Sedex 75 (Sedere, France). Analysis were carried out at 35 °C, at constant flow of 2.0 ml/min with the following solvent gradient. After 5 min of running pure CH_3CN, a linear gradient to 20% CH_2Cl_2 was achieved over 10 min. This mixture was run for 5 min when the gradient to pure CH_3CN was achieved over 4 min. This was run for one additional minute. A good resolution was obtained for cetyl alcohol, stearyl alcohol (the internal standard) and the investigated wax esters. Retention times of 4.0 min for cetyl alcohol (CAL), 4.5 min for stearyl alcohol (SAL), 10.9 min for cetyl myristate (CM), 12.7 min for cetyl palmitate (CP), 13.5 min for cetyl oleate (CO), and 14.6 min for cetyl stearate (CS) were measured. Short-chain esters were not assigned.

2.8. Conversion and yield calculations

The cetyl alcohol conversion and the wax esters' yields were determined according to calibration curves obtained with internal standard method. The following relationships were used to calculate the CAL conversion (Eq. (1)), and the wax esters yield (Eq. (2)):

$$\text{conv}_{(\text{CAL})} = \frac{\text{mol}_{(\text{CAL})_0} - \text{mol}_{(\text{CAL})_t}}{\text{mol}_{(\text{CAL})_0}} \times 100 \quad (1)$$

$$\text{yield}_{\text{WE}} = \frac{\text{mol}_{(\text{CM})} + \text{mol}_{(\text{CP})} + \text{mol}_{(\text{CO})} + \text{mol}_{(\text{CS})}}{\text{mol}_{(\text{MA})_0} + \text{mol}_{(\text{PA})_0} + \text{mol}_{(\text{OA})_0} + \text{mol}_{(\text{SA})_0}} \times 100 \quad (2)$$

where $\text{mol}_{(\text{CAL})_t}$ and $\text{mol}_{(\text{CAL})_0}$ are the amount of CAL at the time 't' and at the initial time ($t = 0$), $\text{mol}_{(\text{FA})_0}$ is the initial amount (moles) of each fatty acid as obtained from its wt.% in the HTF mixture on the basis of an average molecular weight of 706.1 of the TGs. All experiments (calibrations, water activity determinations, wax ester productions, and instrumental analysis) were carried out at least in triplicate. Errors never larger than 5% were determined.

The response of the evaporative light scattering detector is not linear with the amount of sample, but it is exponential or sometimes sigmoidal. Detailed calibration curves for the reactants and the most important products were determined to estimate the error of the measurements.

3. Results and discussion

3.1. Composition of HTF

As already stated in the introduction, sheep milk is an important raw material because of its composition. In the second and in the third column of Table 1, the fatty acid composition of cow milk fat (CMF) compared to sheep milk fat (SMF) is reported, as determined by GC analysis of fatty acid methyl esters. The content in long fatty acid, the only important for wax ester production, is too low for SMF, so that its fractionation in heavy and a light fraction is needed. From the fractionation process, described in the previous section, a fraction named as HTF is obtained. Its composition is reported in the fourth column of Table 1. The HTF fraction is richer in the heaviest and saturated fatty acids than sheep milk fat, namely myristic acid (MA, C14:0), palmitic acid (PA, C16:0), stearic acid (SA, C18:0) and oleic acid (OA, C18:1); they represent 63% (mol) of the fatty acid content of HTF. From the heavy fraction composition an average molecular weight of 706.1 g/mol was calculated.

Fractionation process may be improved to obtain higher contents of long-chain fatty acids, but this method does not allow the complete removal of the short-chain acids which occur in the TGs. Only a quantitative hydrolysis of TGs, followed by a separation of long-chain from short-chain acids can produce fractions of fatty acids having the desired chain length. A similar process, although interesting, is actually beyond the aim of this work.

3.2. Effect of enzyme concentration

The yield of CP, obtained from the reaction of cetyl alcohol (CAL) with the tripalmitin (TP) standard, in the presence of the 1,3-specific lipase, according to:

$$3\text{CAL} + \text{TP} \rightleftarrows 3\text{CP}$$

was used to ascertain the optimum enzyme concentration. Reactions were performed at 35 °C and with $a_w = 0.53$. Fig. 1 shows activity of Lipozyme RMIM (i.e. μmol of product formed/min) versus enzyme concentration. From 2 to 10 mg/ml, there is a significant increment of enzyme activity. Further increase of enzyme concentration is shown to produce a minor increment of enzyme activity. Therefore, a lipase concentration of 10 mg/ml, which seems to be the best compromise between the highest enzyme activities and the lowest consumption of precious catalyst, was chosen to investigate the activity of both enzymes in the wax ester production from the HTF mixture.

3.3. Screening of the enzymes

The alcoholysis reactions for the HTF mixture were carried out as described in the previous section, at 35 °C and with $a_w = 0.53$. Fig. 2 monitors the total wax ester production with time for both enzymes. Reactions were carried out in batch conditions for 720 min, but equilibrium conditions were obtained

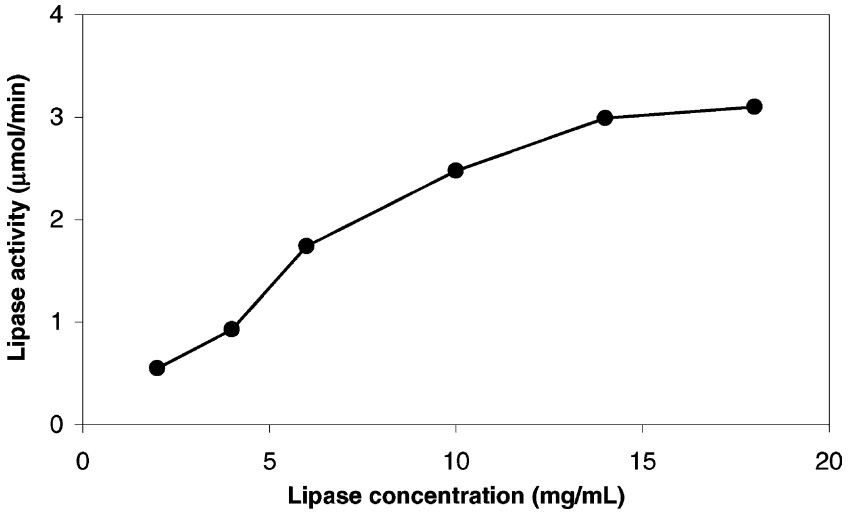


Fig. 1. Lipozyme RMIM activity (μmol of product/min) as a function of the amount of enzyme used.

already after 300 min. The HPLC analysis of the drawings at various reaction times allowed to evaluate the amounts of CAL conversion and the CM, CP, CS and CO wax ester formation. The yields for these wax esters, obtained after 300 min of reaction time, from the alcoholysis reaction of the HTF mixture in the presence of Lipozyme RMIM, immobilised *sn*-1,3-specific lipase from *R. miehei*, and of Novozym 435, immobilised nonspecific lipase-B from *C. antarctica*, are reported in Table 2. The trends shown in Fig. 2, as well as the data reported in Table 2, indicate significant differences in the activity of the two catalysts.

The comparison of our results with those obtained for cow milk fat and oleyl alcohol in solvent-free systems at 60 °C [11] gives evidence of a significant increase of the rates of the transesterification reactions in the presence of *n*-hexane at 35 °C, and particularly for the Lypozyme RMIM catalyst. Moreover, the trend

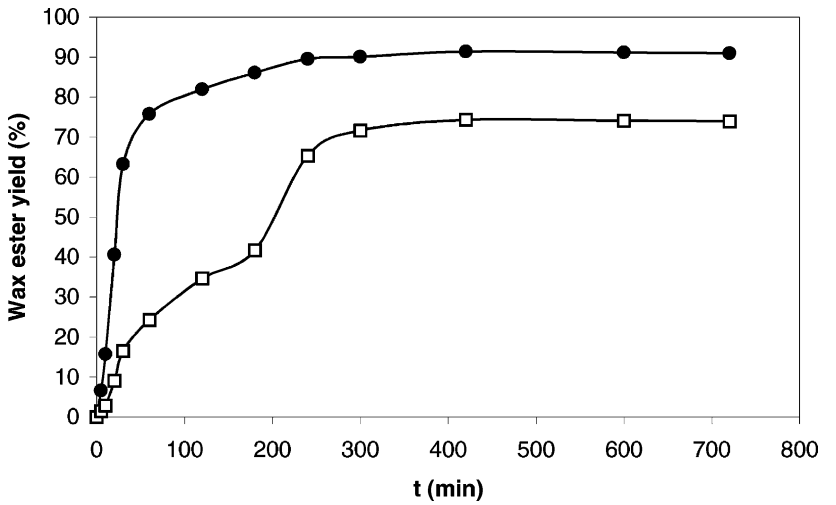


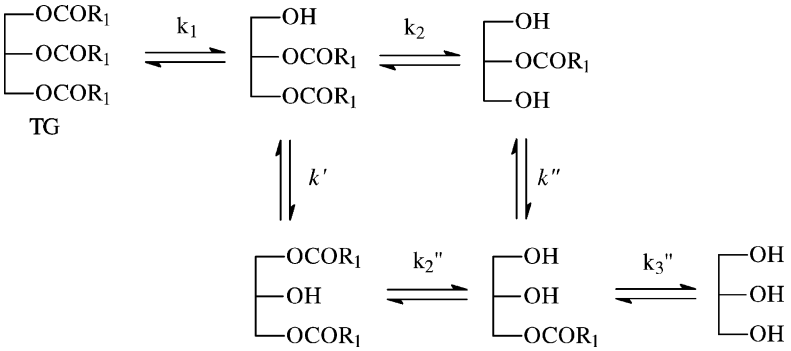
Fig. 2. Progress of wax ester synthesis from HTF and cetyl alcohol, at 35 °C and $a_w = 0.53$, for the two lipases tested: (●) Lipozyme RMIM; (□) Novozym 435.

Table 2
Wax ester yields after 300 min of alcoholysis between HTF and cetyl alcohol, catalysed by the two lipase preparations tested, at 35 °C and $a_w = 0.53$

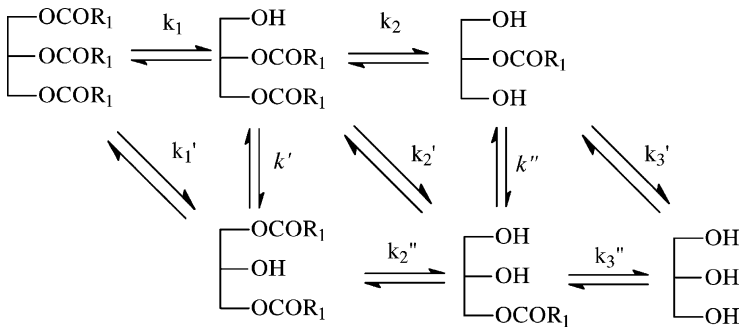
	CM (mol%)	CP (mol%)	CS (mol%)	CO (mol%)
Lipozyme RMIM	20.6	41.9	16.6	15.1
Novozym 435	15.7	31.9	12.6	11.5

shown by the two catalysts is rather different and in apparent contrast with the results reported in literature. Indeed, Steinke et al. [8] for the transesterification of crambe oil or camelina oil with oleyl alcohol, at 60 °C and in solvent-free systems, obtained higher conversion with Novozym 435 than Lipozyme RMIM. Evidently, the presence of the organic solvent, plays a crucial role. The solvent favours the reaction by lowering the viscosity of the reaction medium, which, in turn, determines more effective interactions between catalyst and reagents.

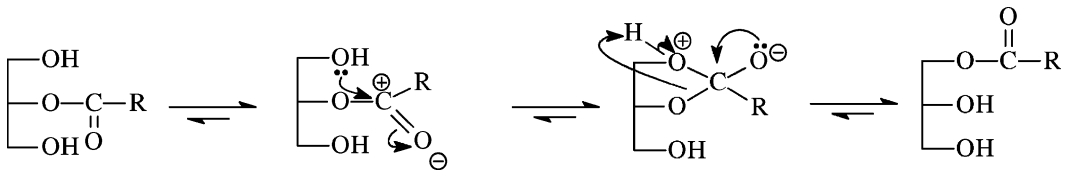
Turning the attention to our reactions, and particularly to the wax ester yield shown in Fig. 2, the higher enzymatic activity of the 1,3-specific lipase with respect to the nonspecific one must be related not only to effective catalyst-reagents interactions favoured by the solvent, but also to possible different reaction mechanisms, which, again, are solvent dependent. Let us consider the catalysed alcoholysis processes shown in Scheme 1 for the 1,3-specific lipase and in Scheme 2 for the nonspecific lipase. TGs react with alcohol to produce diglycerides (DG), monoglycerides (MG) and free glycerol in three different steps, and also 3 mol of wax esters. 1,2-DGs and 2-MGs, which form initially, are likely to undergo, because of thermodynamic reasons [14,15], acyl migration processes to produce 1,3-DGs and 1-MGs according to Scheme 3. To explain our and Steinke results [8], it can be suggested that, in solvent-free media, k' and k'' (acyl migration processes) are the lowest rate constants of the whole catalytic process. In other words, acyl migration is the



Scheme 1. Enzymatic action of Lipozyme RMIM (product and inverse kinetic constants are not reported to simplify the scheme).



Scheme 2. Enzymatic action of Novozym 435 (product and inverse kinetic constants are not reported to simplify the scheme).



Scheme 3. Acyl migration process.

rate determining step. Indeed, acyl migration is expected to be a slow process in the absence of a solvent, as demonstrated in the case of monoolein in the absence and in the presence of 5 wt.% of water [16], and a fast process in the presence of a solvent as an aliphatic hydrocarbon [17]. Thus, the nonspecific Novozym 435 is not significantly influenced by the absence of solvent, it acts indifferently in all the position of glycerol backbone. On the contrary, 1,3-specific Lipozyme RMIM is unable towards position β in 1,2-DGs and 2-MGs, thus, it must wait acyl migration in the α position before catalysing the alcoholysis. In our case, the presence of *n*-hexane should increase the acyl migration rate constants significantly, so that acyl migration is not the rate determining step. The different rates of the processes depend on the different equilibria that occur among the enzymes, the reagents and the various intermediate products. However, it cannot be excluded that the higher temperatures used in the solvent-free systems may modify the activity of the two enzymes differently, for instance through different diffusion rates towards specific catalytic sites.

This conclusion is further supported by the results of Millqvist et al. [17] on the study of acyl migration phenomenon of monoolein. The attempts made to rationalise the effects in terms of different solvent polarity parameters, such as dipole moment, dielectricity constant, etc. gave no satisfactory correlations. However, mono- and di-glycerides, dissolved in aliphatic hydrocarbons, like *n*-hexane, were found to undergo extensive acyl migration toward 1- and 1,3-isomers [15].

3.4. Cetyl alcohol conversion at different water activities

Alcoholysis reactions do not involve water in the reaction, but nevertheless, water control is important for understanding what happens in the reaction medium. Indeed, even if the reaction medium is an organic solvent, water is important to retain enzymes in the active conformation. In addition, the amount of water influences acyl migration, that it has been shown to be very important in our experiments.

The transesterification reaction between CAL and the HTF mixture was performed at three different values of water activity, namely a_w = 0.33, 0.53, and

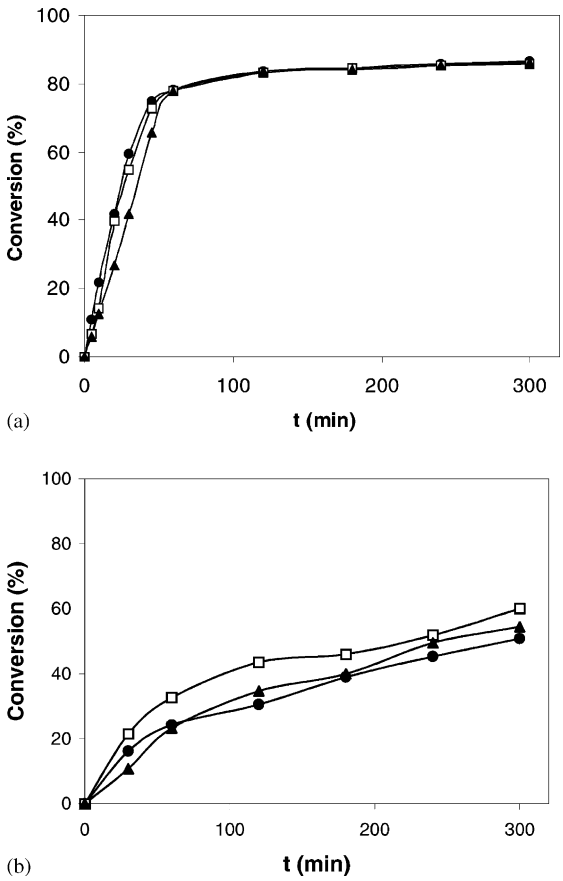


Fig. 3. Cetyl alcohol conversion at different water activities. (●) a_w = 0.33; (□) a_w = 0.53; (▲) a_w = 0.75. (a) Lipozyme RMIM; (b) Novozym 435.

0.75. The results are shown in Fig. 3a and b where the CAL conversion is reported for the two lipases, Lipozyme RMIM, Novozym 435, respectively.

In the case of the 1,3-specific lipase, significant differences of CAL conversion are observed only during the initial stage (less than 60 min), where the conversion trend is inversely dependent on a_w. For longer reaction times, the conversion curves coincide. This result is in agreement with Millqvist et al. [17]. This author found that in some organic solvents (*n*-hexane included), the acyl migration rate decreased with increasing water activity. A possible justification of this fact might be the formation of hydrogen bonds between water molecules and the hydroxyl groups on the glycerol backbone, with the effect of decreasing acyl migration rate through a stabilisation of the 2-MG isomer.

In the case of nonspecific Novozym 435, acyl migration is not important for its activity, thus, the trend shown in Fig. 3 brings about the conclusion that there is an optimum of water activity for this lipase, that is $a_w = 0.53$.

4. Conclusions

In this work, the formation of wax esters was obtained in very mild experimental conditions by the use of two different commercial immobilised lipases in *n*-hexane. High-values of conversion towards wax ester production were observed. In addition by-products formed at a very negligible extent. Remarkably, the use of the 1,3-specific Lipozyme RMIM allowed a wax ester yield 20 mol% higher than that obtained by the nonspecific lipase. A solvent dependent, fast acyl migration process is suggested as a possible explanation of the observed trend.

Acknowledgements

MURST (Italy), Project 8, Law 488/2000, and Consorzio Sistemi Grande Interfase (CSGI-Firenze) are acknowledged for support.

References

[1] P. Adlercreutz, Biotechnol. Genet. Eng. Rev. 12 (1994) 231.
[2] R. Schuch, K.D. Mukherjee, J. Agric. Food. Chem. 35 (1987) 1005.
[3] R. Schuch, K.D. Mukherjee, Naturforsch 42 (1987) 1285.
[4] J.P. Chen, J.B. Wang, H.S. Liu, Biotechnol. Lett. 17 (1995) 1177.
[5] M.L. Hallberg, D. Wang, M. Härröd, J. Am. Oil Chem. Soc. 76 (1999) 183.
[6] K.D. Mukherjee, I. Kiewitt, J. Agric. Food Chem. 346 (1988) 1333.
[7] B.K. De, D.K. Bhattacharyya, C. Bandhu, J. Am. Oil Chem. Soc. 76 (1999) 451.
[8] G. Steinke, R. Kirchhoff, K.D. Mukherjee, J. Am. Oil Chem. Soc. 77 (2000) 361.
[9] G. Steinke, S. Scönwiese, K.D. Mukherjee, J. Am. Oil Chem. Soc. 77 (2000) 367.
[10] M. Trani, F. Ergan, G. Andrè, J. Am. Oil Chem. Soc. 68 (1991) 20.
[11] L. Poisson, S. Jan, J.C. Vuillemard, C. Sarazin, J.P. Sèguin, J.N. Barbotin, F. Ergan, J. Am. Oil Chem. Soc. 76 (1999) 1017.
[12] N. Goma-Doncescu, M.D. Legoy, J. Am. Oil Chem. Soc. 74 (1997) 1137.
[13] M.A. Bjurlin, S. Bloomer, M.J. Haas, J. Am. Oil Chem. Soc. 78 (2001) 153.
[14] B. Serdarevich, J. Am. Oil Chem. Soc. 44 (1967) 381.
[15] B.J. Sjursnes, T. Anthonsen, Biocatalysis 9 (1994) 285.
[16] S. Murgia, F. Caboi, M. Monduzzi, H. Ljusberg-Wahren, T. Nylander, Progr. Colloid Polym. Sci. 120 (2002) 41.
[17] A. Millqvist Fureby, C. Virto, P. Adlercreutz, B. Mattiasson, Biocatal. Biotransform. 14 (1996) 89.