

Lipase Catalyzed Synthesis of Aromatic Esters of Sugar Alcohols

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Abstract—Commercially available lipases (*Candida antarctica* lipase B, Novozyme 435, *Thermomyces lanuginosus* lipase, and Lipozyme TL IM), as well as sol-gel immobilized lipases, have been screened for their ability to acylate regioselectively xylitol, sorbitol, and mannitol with a phenolic ester in a binary mixture of *t*-butanol and dimethylsulfoxide. HPLC and MALDI-TOF MS analysis revealed the exclusive formation of monoesters for all studied sugar alcohols. The lipases immobilized by the sol-gel entrapment method proved to be efficient catalysts, leading to high conversions (up to 60%) in the investigated acylation reactions. From a sequence of silane precursors with different nonhydrolyzable groups in their structure, the presence of octyl and *i*-butyl group was most beneficial for the catalytic activity of sol-gel entrapped lipases in the studied process.

Keywords—Lipase, phenolic ester, specificity, sugar alcohol, transesterification.

I. INTRODUCTION

IT is well recognized that phenolic acids or phenolic acid esters exhibit antioxidative activity, peroxy radical scavenging activity, and they are inhibitors of carcinogenesis. Moreover, sugar esters of various phenolic acids, isolated from plants or synthesized as phenolic alkylglycoside esters, are pharmacologically active compounds with demonstrated antimicrobial, antiviral, anti-inflammatory, and antitumor action [1,2]. In addition, sugar esters are non-ionic and biodegradable surfactants that have excellent emulsifying, stabilizing, or conditioning properties. They are widely used in food, cosmetic, pharmaceutical, and detergent industries [3]. The solubility of natural antioxidants, such as ascorbic acid or phenolic acids, in water is usually high. The hydrophilic character of these antioxidants reduces their effectiveness in stabilizing fats and oils, and it has been reported as a serious disadvantage if an aqueous phase is also present. Therefore, the modification of these compounds *via* esterification with aliphatic molecules (fatty acids or alcohols) can be used as a tool to adjust solubility in oil-based formula and emulsions. Esterification by a fatty alcohol with the carboxylic function results in the formation of more lipophilic constituents. However, application of the monoacyl sugar alcohols to the preparation of oil-in-water (O/W) emulsions has scarcely been investigated [4]-[6]. The traditional chemical synthesis of sugar or polyol esters is based on a high temperature and high-pressure esterification reaction between sugars and fatty acids with acidic and metal catalysts, leading to complex and expensive reaction setup, large amount of raw materials and complex mixtures of products [7].

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In the recent years, application of enzymes has emerged as an interesting alternative, since enzymatic synthesis has several advantages over chemical synthesis, such as high regio- and stereoselectivity, mild reaction conditions, avoiding the use of toxic catalyst, and low energy consumption [8]. Aliphatic mono- and diesters of arabitol, ribitol, xylitol, and sorbitol with lauric acid were synthesized using immobilized *Candida antarctica* lipase in acetone [9], while synthesis of sorbitol monoesters with aromatic acids were reported only by chemical catalysis [10].

In the present work, our aim was to investigate various commercial lipases and sol-gel immobilized lipases in transesterification reactions of sugar alcohols holding five or six hydroxy groups of different sterical orientation (such as xylitol, sorbitol, and mannitol), with a phenolic ester as acyl donor, in organic reaction medium.

II. EXPERIMENTAL PART

A. Materials

Vinyl laurate and dimethyl sulfoxide (DMSO, 99.5%) were from Fluka. Sodium fluoride, 2-propanol, *n*-hexane, *n*-hexadecane (> 98%), and acetonitrile (99.5%) were obtained from Merck. Native lipase (Lipozyme from *Candida antarctica* B, and Lipolase from *Thermomyces lanuginosus*), as well as immobilized (Novozyme 435 from *C. antarctica* and Lipozyme TL IM from *T. lanuginosus*) lipases were gifts from Novozymes (Denmark). Xylitol, 1-octanol ($\geq 98\%$), 3-(4-hydroxyphenyl)-propionic acid methyl ester (HPPME, 97%), tertiary-butanol (*t*-BuOH, $\geq 99\%$), molecular sieves (4Å, 4-8 mesh), polyethyleneglycol (PEG), and 2,5-dihydroxybenzoic acid ($\geq 98\%$) were from Sigma Aldrich. Mannitol, sorbitol, *n*-decosane were obtained from Acros Organics. Silane precursors tetramethoxysilane (TMOS, 98%), methyl- (MeTMOS, 98%), propyl- (PrTMOS, 98%), vinyl- (VTMOS, 98%), octyl- (OcTMOS, 97%), 3-aminopropyl- (3-NH₂PrTMOS, 97%), and phenyl-trimethoxysilane (PhTMOS, 97%) were purchased from Brunschwig Chemie (The Netherlands). Dimethyl-(DMeDMOS, 96%) was from Fluka, methyl-phenyl-dimethoxysilane (MePhDMOS, 95%) from Aldrich and *i*-butyl-trimethoxysilane (*i*-BuTMOS, 97%) was from Brunschwig Chemie.

B. General Procedure for Immobilization by Sol-Gel Entrapment

The immobilization procedure was the same as previously described [11]. In a 4 mL glass vial, lipase solution (780 μ L), 4% w/v PEG (200 μ L), 1M NaF (100 μ L), and isopropyl alcohol (200 μ L) were mixed, and a mixture of silane precursors of different molar ratio (total 6 mmol) were added under continuous stirring. The stirring was maintained until

gelation was observed. The resulting gel was kept at room temperature for 24 hours to complete polymerization. The bulk gel was washed with isopropyl alcohol (5 mL), distilled water (5 mL), again isopropyl alcohol (5 mL) and n-hexane (5 mL), filtered, and subsequently dried room temperature (48 h).

The Bradford method [12] was used to measure the protein concentration of solutions with dissolved protein and bovine serum albumin (BSA) was used as the standard. The amount of bound protein onto silica supports was determined indirectly, as the difference between the initial total protein subjected to immobilization and the amount of protein recovered in the washing solutions after immobilization. The immobilization yields were calculated as the ratio of the amount of bound protein to the amount of protein subjected to immobilization.

C. Acylation of 1-Octanol by Vinyl Laurate

Acylation have been made in 2 mL glass vials, charged with a mixture of 1-octanol (0.5 mmol), vinyl laurate (1.5 mmol), as internal standard *n*-hexadecane (30 μ L), free or sol-gel immobilized lipase from *T. lanuginosus* (1 mg) and *n*-hexane (1 mL). Reactions were performed at 40°C in a Memmert Modell 500 incubator with controlled temperature, using a rotator (Stuart SB2) for parallel reactions. The enzyme was removed by centrifugation (10 min, 20,000 x g). Samples taken at different time intervals were analyzed by gas-chromatography. The conversions were calculated using a calibration curve of 1-octanol, realized by the internal standard method. Specific activities were calculated as the average amount of formed ester (in μ mol) in one minute interval by 1 mg of protein. The reaction without enzyme did not give any product using the same conditions. The protein content of the native lipases was determined by the Bradford method [12].

D. Acylation of 1-Octanol by HPPME

Free, commercially immobilized (10 mg) or sol-gel immobilized lipase from *C. antarctica* or *T. lanuginosus* (20 mg) was added to a mixture of 1-octanol (0.05 mmol), HPPME (0.15 mmol), *n*-decosane (1 mg), *t*-BuOH (1 mL). Reactions were carried out and monitored as described in 2.3. The reaction without enzyme did not give any product using the same conditions.

E. Acylation of Sugar Alcohols by HPPME

The reaction mixture consisted of 0.05 mmol sugar alcohol (xylitol, mannitol, sorbitol), 0.15 mmol HPPME, free, commercially immobilized, or sol-gel immobilized lipase from *C. antarctica* and *T. lanuginosus* (the enzyme amount was equivalent to 1 total activity unit, calculated from the reaction of 1-octanol with HPPME), molecular sieves (20 mg, 4Å, 4-8 mesh), and a mixture of 10% DMSO/*t*-BuOH (1 mL). First, the sugar alcohol has been solubilized in DMSO and the HPPME dissolved in *t*-BuOH and were added at 50°C.

Reactions were performed in 2 mL glass vials at 50°C for 72 h in a Memmert Modell 500 incubator with controlled

temperature, using a rotator (Stuart SB2) for parallel reactions. Samples were taken at different times, the solid enzyme was removed by centrifugation (10 minutes, 20,000 x g). Two mL DMSO was added subsequently to the samples and stirred at 50°C for 1 h. A control reaction was performed under the same conditions without enzyme. All the experiments were carried out in duplicate. The progress of the transesterification was monitored by High Pressure Liquid Chromatography (HPLC), and Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was employed for the identification of products.

F. Gas Chromatography

A Focus GC (Interscience) gas chromatograph was used, equipped with a flame ionization detector and a Restek, 30m x 0,25 mm x 0,25 μ m column.

The analysis conditions were set as follows: oven temperature: 100°C (2 min) to 300°C (3 min) with 10°C/min heating rate, injector temperature 275°C, detector temperature 300°C, carrier gas (helium) flow 1.5 mL/min.

G. High Pressure Liquid Chromatography

The reaction mixture was analyzed by reverse phase (RP) HPLC on an Atlantis T3 3 μ m, 2.1 mm x 100 mm column (Waters) thermostated at 30°C, using a HPLC system (Waters) equipped with UV dual wavelength detector and autosampler. The eluting components were detected at 220 nm. The compounds were eluted using an acetonitrile gradient from 2% to 98%, containing 0.1% trifluoroacetic acid, at a flow rate of 0.5 mL/min.

H. Matrix-Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry

For the MALDI-TOF MS analysis, 1 μ L of the reaction mixture was mixed with 9 μ L of matrix solution (10 mg 2,5-dihydroxy benzoic acid in 1 mL acetonitrile: water, 1:1). Two μ L of this mixture was subsequently transferred to a target plate and dried under a stream of dry air. Measurements were performed on an Ultraflex Workstation (Bruker Daltonics, Germany), running in the positive mode and equipped with a 337 nm laser. Ions were accelerated with a 25 kV voltage after a delayed extraction time of 200 ns. Detection was performed in the reflector mode. The lowest laser intensity needed to obtain a good quality spectrum was applied. The machine was calibrated using a mixture of maltodextrins (Avebe, The Netherlands) with known molecular masses. FlexControl and FlexAnalysis software packages (Bruker Daltonics, Germany) were used for acquisition and processing of the data.

III. RESULTS AND DISCUSSION

Compared to the well-studied aliphatic esters, the synthesis of aromatic esters is more difficult to be accomplished by lipase-catalyzed reactions. More than being non-natural substrates for lipase, the acyl donor compounds containing an aromatic moiety fit poorer in the active site of lipases, resulting in lower conversions [18].

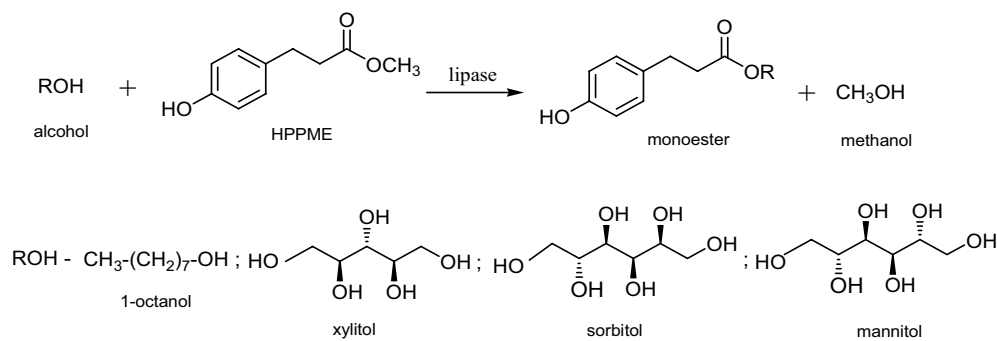


Fig. 1 Reaction scheme of lipase catalyzed acylation of the primary alcohol and sugar alcohols by HPPME

However, selection of the most appropriate lipase and optimization of the reaction parameters should allow better performances. In our study, sugar alcohols and 1-octanol were acylated by the phenolic ester HPPME (Fig. 1). The selection of the reaction medium is always difficult for highly polar compounds, like sugars or sugar derivatives. *t*-BuOH has often been chosen due to the need for innocuous solvents in the food industry and good solvation capability for sugar esters. This solvent was also selected for our study. It was able to solubilize the phenolic methyl ester and sugar alcohol esters, but this was not the case for the sugar alcohol, needing a more hydrophilic co-solvent (DMSO) to achieve a reasonable solubility [13].

A. Catalytic efficiency of native and immobilized lipases in acylation reactions with HPPME

Acylation of 1-octanol by the phenolic ester HPPME, in *t*-BuOH as reaction medium has been investigated with commercially available native and immobilized lipases from *T. lanuginosus* and *C. antarctica*. The scope was to evaluate the specificity of different lipases for the phenolic ester, using 1-octanol, well known for its reliability in lipase-catalyzed biotransformations, as the alcohol substrate. Vinyl laurate was selected as reference acylation agent, since vinyl esters are largely used in lipase-catalyzed acylations. The conversions and activities calculated from gas chromatographic analysis data, after 1 hour reaction time (Table I), show that all tested enzymes catalyzed the formation of the octyl ester of 3-(4-hydroxyphenyl)-propionic acid. However, all tested biocatalysts showed much lower activity in the reaction with HPPME, attesting that aromatic acids are poorer substrates than aliphatic acids for the active site of lipases. The important difference concerning specificity is demonstrated by the specific activities of the investigated lipases, which have been

found at least 5,000 times higher in the acylation reaction with vinyl laurate. The same microbial lipases have been subjected to immobilization by the sol-gel entrapment method, using binary or tertiary precursor mixtures of tetramethoxysilane and one or two trimethoxysilanes with the general formula $\text{R-Si}(\text{OCH}_3)_3$, R being an alkyl or aryl nonhydrolyzable group. Admitting that the effect of silane precursor nature and molar ratio should be the same for both lipases, the study of this influence was carried out using the native lipase that showed higher activity against the HPPME substrate. The catalytic efficiency of sol-gel immobilized *T. lanuginosus* lipase preparations, obtained with different silane precursors, was investigated in the acylation reaction of 1-octanol, using as acyl donor either the phenolic ester (HPPME), which is not a natural substrate for the lipase, and a reactive aliphatic fatty acid ester (vinyl laurate) as test reaction. The same reactions have been carried out also with native *T. lanuginosus* lipase, to allow the calculation of immobilization efficiency, expressed by the relative specific activity values of the immobilized enzyme. As shown in Table II, all preparations obtained by the sol-gel entrapment technique were active in transesterification reactions involving vinyl laurate as acyl donor, but most of them did not show any activity in reactions with HPPME. Even for the immobilized lipases active against both substrates, the specific activity values were much higher in the reaction with the aliphatic fatty acid ester. The probable reason for lower specificity against the phenolic ester substrate could be steric hindrance and thereby, limiting the access to the enzyme active site. It might originate from unfavorable resonance effect and steric hindrance of the aromatic, bulky, and rigid phenyl ring. The immobilized biocatalysts obtained with different silane precursors demonstrated a considerable

TABLE I

RESULTS IN THE ACYLATION OF 1-OCTANOL BY VINYL LAURATE IN N-HEXANE (40°C) AT 1 MINUTE, AND BY HPPME IN T-BUOH (40°C) AFTER 1 H REACTION TIME, CATALYZED BY NATIVE AND IMMOBILIZED COMMERCIAL LIPASES FROM *THERMOMYCES LANUGINOSUS* AND *CANDIDA ANTARCTICA*

Biocatalyst	Substrate	Conversion (%)	Activity ^a (U/mg)	Specific activity ^a (U/mg protein)	Relative specificity ^b
<i>Candida antarctica B</i>	Vinyl laurate	1	36	356	19,620
	HPPME	2	0.10	1.09	
Novozyme 435	Vinyl laurate	2	11	n.d.	-
	HPPME	1	0.03	n.d.	
<i>Thermomyces lanuginosus</i>	Vinyl laurate	1	13	133	5,040
	HPPME	5	0.15	1.59	
Lipozyme TL IM	Vinyl laurate	1	5	n.d.	-
	HPPME	3	0.07	n.d.	

^aActivity unit (U) expressed as micromole product/min for the reaction with vinyl laurate, and micromole product/h for the reaction with HPPME.

^bCalculated as the ratio of specific activity in the acylation with vinyl laurate, related to the specific activity in the acylation with HPPME.

n.d. not determined for the immobilized commercial enzymes.

influence of the nonhydrolyzable group structure on the catalytic efficiency. In the test reaction of 1-octanol acylation by vinyl laurate, some sol-gel immobilized preparations showed higher specific activities compared to the native enzyme, while others were less active. It is not surprising to have a partial inactivation of the enzyme during the immobilization process, as the immobilization conditions can generate modifications of the active site conformation, or unfolding of the enzyme. In the specific case of sol-gel entrapment employing methoxysilanes as precursors, inhibition can be a consequence of methanol resulted from the polycondensation reaction [14]. The presence of octyl nonhydrolyzable group in the sol-gel

matrix, which induces increased hydrophobicity of the matrix, resulted in the highest activity values. The best efficiency was shown by the biocatalyst obtained with OctMOS:TMOS at a 1:1 molar ratio, without any immobilization additive. In addition the preparations containing octyl groups were the only biocatalysts showing catalytic activity in reactions with HPPME as acyl donor. Lipases are well known as enzymes being activated in hydrophobic environments, and the hydrophobic groups belonging to the immobilization matrix could act in this way [15]. A possible explanation of the absence of activity for most of the immobilized preparations could be the more significant restriction of the bulky phenyl ring access into the sol-gel matrix, and the impossibility to reach the active center.

TABLE II

INFLUENCE OF THE SILANE PRECURSOR NATURE ON THE IMMOBILIZATION YIELD AND SPECIFIC ACTIVITY OF NATIVE AND IMMOBILIZED *THERMOMYCES LANUGINOSUS* LIPASE, IN THE ACYLATION REACTION OF 1-OCTANOL USING VINYL LAURATE AND HPPME AS ACYL DONORS. THE REACTIONS WERE PERFORMED AT 40°C, IN N-HEXANE (ACYLATION BY VINYL LAURATE), AND T-BUOH (ACYLATION BY HPPME)

Silane precursor (molar ratio)	Immobilization yield (%)	Substrate	Specific activity ^a (U/mg protein)	Relative activity ^b
Native enzyme	-	Vinyl laurate	133	1.00
		HPPME	1.59	1.00
PhTMOS:MeTMOS:TMOS (1,6:0,4:1)	67	Vinyl laurate	245	1.84
		HPPME	n.d.	-
OcTMOS:TMOS (1:1)	91	Vinyl laurate	298	2.24
		HPPME	0.18	0.11
OcTMOS:TMOS (2:1)	87	Vinyl laurate	326	2.45
		HPPME	0.17	0.10
OcTMOS:TMOS (1:1) additive free	93	Vinyl laurate	609	4.58
		HPPME	0.53	0.33
MeTMOS:TMOS (1:1)	98	Vinyl laurate	8	0.06
		HPPME	n.d.	-
PrTMOS:TMOS (1:1)	89	Vinyl laurate	162	1.22
		HPPME	n.d.	-
PhTMOS:TMOS (1:1)	87	Vinyl laurate	198	1.49
		HPPME	n.d.	-
VTMOS:TMOS (1:1)	91	Vinyl laurate	207	1.55
		HPPME	n.d.	-
DMeDMOS:TMOS (1:1)	81	Vinyl laurate	259	1.95
		HPPME	n.d.	-
PhMeDMOS:TMOS (1:1)	91	Vinyl laurate	74	0.55
		HPPME	n.d.	-
(3-NH ₂ Pr)TMOS:TMOS (1:1)	96	Vinyl laurate	10	0.07
		HPPME	n.d.	-
iBuTMOS:TMOS (1:1)	96	Vinyl laurate	112	0.84
		HPPME	n.d.	-

^aActivity unit (U) expressed as μmol product/min for the reaction with vinyl laurate, and μmol product/h for the reaction with HPPME.

^bCalculated as the ratio of specific activity of immobilized enzyme, related to the specific activity of the native enzyme.

n.d. is not detectable.

B. Regioselective Synthesis of Aromatic Monoesters of Sugar Alcohols

The wide substrate specificity of lipases is generally recognized, as well as their higher specificity for alcohol-branched, than for acid-branched substrates [16]. The specificity of the native, commercially immobilized, and sol-gel immobilized lipases from *T. lanuginosus* and *C. antarctica* towards polyhydroxylic substrates has been investigated in transesterification reactions of three sugar alcohols (xylitol, mannitol, and sorbitol) with HPPME in 10% DMSO/ *t*-BuOH. The chosen solvent mixture represented a compromise to ensure reasonable enzyme activity and increased sugar alcohol solubility, as well. The obtained results were evaluated in comparison to those of 1-octanol acylation by the same aromatic ester, considering the primary alcohol as the ideal substrate for lipase. The enzymatic reaction was monitored at regular time intervals by HPLC. An example for the acylation of xylitol by *C. antarctica* after 72 h is shown in Fig. 2.

The excellent regioselectivity of all investigated lipases was demonstrated by MALDI-TOF MS analysis, which showed the exclusive formation of monoesters in the acylation reaction of sugar alcohols by HPPME (Table III). In mass spectra the *m/z* values at 323.3 and 353.3 correspond to the molecular weights of sodium adducts of HPPME monoesters of xylitol, mannitol, and sorbitol, respectively (the last two compounds having the same molecular mass), while the *m/z* values of the appropriate diesters at 471 and 501 were not detected. Synthesis of aromatic esters of 1-octanol and sugar alcohols was catalyzed by native and immobilized *T. lanuginosus* lipase, although the yields remained relatively low after 72 h reaction time (Fig. 3). Using the same conditions, the yields were much higher for the monohydroxylic alcohol than for polyols, as expected.

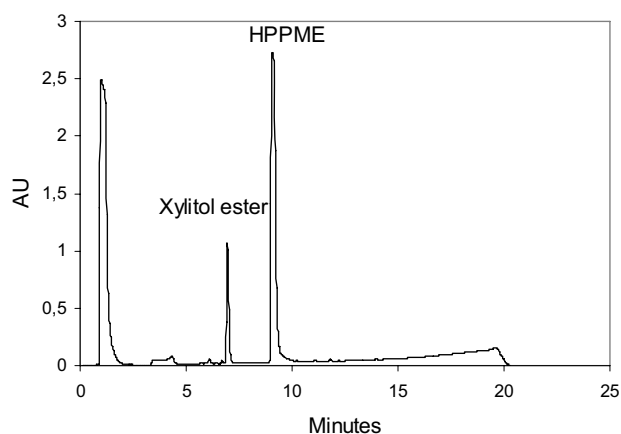


Fig. 2 HPLC chromatogram of the xylitol acylation product by HPPME, catalyzed by *Candida antarctica* B lipase, in 10% DMSO/*t*-butanol, at 50°C and 72 h reaction time. Chromatographic analysis conditions were set as described in Experimental part

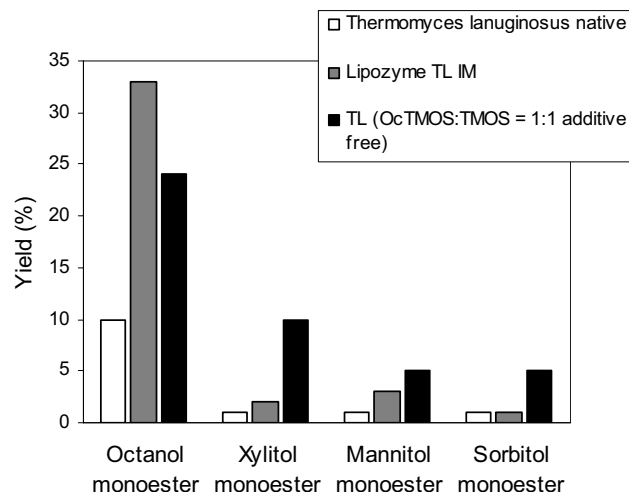


Fig. 3 Monoester yields after 72 h reaction time, of the acylation of 1-octanol (in *t*-BuOH at 40°C), xylitol, mannitol, and sorbitol (in 10% DMSO/*t*-butanol at 50°C), by HPPME, catalyzed by native, commercially immobilized, and sol-gel immobilized lipases from *Thermomyces lanuginosus*

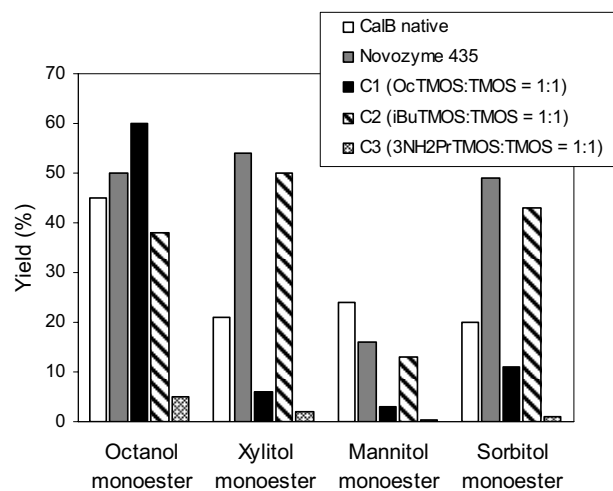


Fig. 4 Monoester yields after 72 h reaction time, of the acylation of 1-octanol (in *t*-BuOH at 40°C), xylitol, mannitol, and sorbitol (in 10% DMSO/*t*-butanol at 50°C), by HPPME, catalyzed by native, commercially immobilized, and sol-gel immobilized lipases from *Candida antarctica*

It is important to notice that, in the acylation reaction of sugar alcohols, the best performing (as resulted from Table II) sol-gel entrapped lipase, containing a hydrophobic (octyl)-nonhydrolyzable group in the silica matrix, showed higher catalytic efficiency than the same native and commercially immobilized enzyme. Lipases have a hydrophobic lid that obstruct the transport of the substrate to the active site, which can be favorably displaced during or following immobilization [17].

As results from Fig. 4, the lipase from *C. antarctica* was a much better catalyst for synthesis of phenolic esters from sugar

alcohols than *T. lanuginosus* lipase. Particularly Novozyme 435 demonstrated excellent catalytic performances, displaying the highest yields after 72 h reaction time. Considering the sol-gel immobilized *C. antarctica* B lipase preparations, the presence of *i*-butyl nonhydrolyzable group was the most valuable for the catalytic activity, compared to other silane precursors and showed comparable activity as the commercial immobilized *C. antarctica* lipase B (Novozyme 435).

IV. CONCLUSIONS

The investigated lipases proved to be efficient not only for acylation of a primary alcohol by an aromatic ester, but they also accepted sugar alcohols as substrates, achieving high conversions. Monoacyl sugar alcohol esters with a phenolic acid residue regiospecifically bound to one of the primary hydroxyl groups of the sugar alcohol have been synthesized. Octyl 3-(4-hydroxyphenyl)-propionate was also synthesized through lipase-catalyzed acylation of the appropriate primary alcohol by the phenolic ester. Lipase immobilization by sol-gel entrapment method and selection of the suitable silane precursors resulted in entrapped lipase composites with higher specific activity than the native enzyme in the case of *T. lanuginosus*, and similar to the best commercially available immobilized lipase in the case of *C. antarctica*. It can be noticed that the two lipase types showed different affinity towards the nonhydrolyzable group of the silane precursor.

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TABLE III
IDENTIFICATION OF MONOESTERS AND/OR DIESTERS BY MALDI-TOF MASS SPECTROMETRY AFTER INCUBATION OF MANNITOL, SORBITOL, AND XYLITOL WITH HPPME, CATALYZED BY *CANDIDA ANTARCTICA* LIPASE B IN 10% DMSO/T-BUOH AT 50°C, AFTER 72 HOURS REACTION TIME

Acceptor	m/z			
	[monoester + Na] ⁺	[monoester + K] ⁺	[diester + Na] ⁺	[diester + K] ⁺
Mannitol	353.3	369.3	n.d.	n.d.
Sorbitol	353.3	369.2	n.d.	n.d.
Xylitol	323.3	339.2	n.d.	n.d.