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Very Important Paper

Creating an Efficient Methanol-Stable Biocatalyst by Protein and Immobilization Engineering Steps towards Efficient Biosynthesis of Biodiesel

Shalev Gihaz,[a] Diána Weiser,[b, c, d] Adi Dror,[a] Péter Sátorhelyi,[c] Moran Jerabek-Willemsen,[e] László Poppe,*[b, d, f] and Ayelet Fishman*[a]

Two ternary sol–gel matrices, an octyltriethoxysilane-based aliphatic matrix and a phenyltriethoxysilane (PTEOS)-based aromatic matrix, were used to immobilize a methanol-stable variant of lipase from *Geobacillus stearothermophilus* T6 for the synthesis of biodiesel from waste oil. Superior thermal stability of the mutant versus the wildtype in methanol was confirmed by intrinsic protein fluorescence measurements. The influence of skim milk and soluble *E. coli* lysate proteins as bulking and stabilizing agents in conjunction with sol–gel entrapment were investigated. *E. coli* lysate proteins were better stabilizing agents of the purified lipase mutant than skim milk, as evidenced by reverse engineering of the aromatic-based system. This was also shown for commercial *Candida antarctica* lipase B (CaLB) and *Thermomyces lanuginosus* lipase (TLL). Uniform, dense, and nonaggregated particles imaged by scanning electron microscopy and a small particle size of 13 μm pertaining to the system comprising PTEOS and *E. coli* lysate proteins correlated well with high esterification activity. Combining protein and immobilization engineering resulted in a durable biocatalyst with efficient recycling ability and high biodiesel conversion rates.

Introduction

Expanding urbanization, a rise in living standards, and a growth in the world-wide population have led to a constantly increasing demand for energy sources. Simultaneously, concerns about the hazardous environmental effects of petroleum-based fuels have become more significant, and this has influenced the political, social, and environmental sectors.[1] Biodiesel offers a promising solution for the energy crisis, as it is a sustainable and renewable alternative to fossil fuels and can be obtained from a wide range of feedstocks. Also called fatty acid alkyl esters, biodiesel is the product of the transesterification of triglycerides from renewable sources such as plant oils (edible and nonedible) or animal fats and alcohol.[1d,2]

The most commonly used alcohol in the transesterification of triglycerides for biodiesel production is methanol owing to its low price, reactivity, and availability.[3] In this case, the fatty acid alkyl esters resulting from the reaction comprise a methyl group and are called fatty acid methyl esters (FAMEs).[1a] FAME production can be achieved chemically by using acid or base catalysts, which require higher temperatures and elevated alcohol-to-oil molar ratios. In addition, downstream processing of biodiesel from chemical synthesis is costly and challenging with respect to issues such as glycerol recovery, water and salt removal, and saponification of free fatty acids. Consequently, the chemical production of biodiesel is considered less environmentally friendly and high-energy demanding relative to biobased routes.[1c,2]

A "greener" method for biodiesel production is enzymatic transesterification. It involves the use of lipases under mild reaction conditions and low energy consumption by exploiting a variety of oil feedstocks.[4] Despite the benefits of high yields and product purity, the enzymatic synthesis of FAMEs is limited by the constrained stability of lipases in short-chain alcohols.[4,5] The main reason for this limited stability has been shown to be the stripping of structural water molecules by the

[a] S. Gihaz, Dr. A. Dror, Prof. Dr. A. Fishman
Department of Biotechnology and Food Engineering
Technion-Israel Institute of Technology
Haifa, 3200003 (Israel)
E-mail: afishman@tx.technion.ac.il

[b] Dr. D. Weiser, Prof. Dr. L. Poppe
Department of Organic Chemistry and Technology
Budapest University of Technology and Economics
Műegyetem rkp. 3
H-1111 Budapest (Hungary)
E-mail: poppe@mail.bme.hu

[c] Dr. D. Weiser, Dr. P. Sátorhelyi
Fermentia Microbiological Ltd.
Berlini út 47-49
Budapest, H-1045 (Hungary)

[d] Dr. D. Weiser, Prof. Dr. L. Poppe
SynBiocat Ltd
Lövőház u 19/1
H-1023 Budapest (Hungary)

[e] Dr. M. Jerabek-Willemsen
NanoTemper Technologies GmbH
Flößergasse 4
81369 München (Germany)

[f] Prof. Dr. L. Poppe
Biocatalysis and Biotransformation Research Group
Babes-Bolyai University of Cluj-Napoca
Arany János str. 11
RO-400028 Cluj-Napoca (Romania)

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alcohols, which thereby significantly alters the native conformation and conformational flexibility.[4] Attempts to handle the shortcoming of biocatalytic biodiesel synthesis have led to the development of several approaches for obtaining methanol-stable enzymes. Isolation of thermophilic enzymes and design by protein engineering are some of the common solutions to enhance the stability of lipases against the denaturing effects of short-chain alcohols.[5a,c,6] Recently, a lipase from the thermophilic bacteria *Geobacillus stearothermophilus* T6 (LipT6$_{WT}$) was mutated by using different protein-engineering approaches, and this resulted in several methanol-stable variants.[7] The triple mutant H86Y/A269T/R374W (LipT6$_{M}$) exhibited a half-life value of 324 min upon exposure to 70% methanol, which reflected a stability that was 87-fold enhanced relative to that of the wildtype. Its crystal structure revealed the formation of a branched network of hydrogen bonds on the enzyme's surface, which enhanced its resistance to the alcohol–water displacement phenomenon.[7b] Owing to its excellent performance in the transesterification of waste chicken oil relative to other soluble commercial lipases, this variant was chosen for further stabilization by immobilization in an attempt to create a potential industrially relevant biocatalyst.

Enzyme immobilization is one of the key approaches to integrate a biocatalyst into a large-scale economical process. The main immobilization techniques are covalent binding to a carrier, carrierless methods of cross-linking leading to enzyme aggregates, and entrapment (encapsulation in a matrix).[8] Entrapment-based methods have proven to be an easy and efficient way to obtain immobilized enzymes with enhanced thermal and solvent tolerance.[9] Relative to chemical catalysts, immobilized enzymes are high priced, mostly because purification of the enzyme represents a significant cost contribution. Recent work highlighted the importance of one-step purification and immobilization by using the crude cell extract for an entrapment-based immobilization process as an economical way to overcome this drawback.[8a,b,10]

One of the most well-studied entrapment methods is sol–gel immobilization, which involves the formation of a silica matrix with the enzyme present during polymerization. Some of the advantages of sol–gel-immobilized enzymes are enhanced thermostability, mechanical resistance, improved solvent tolerance, and high stability in storage.[11] Nevertheless, mass-transfer limitations of substrate influx and product efflux can become a challenging disadvantage while entrapping enzymes within an inert matrix.[8d,h] The sol–gel mechanism is based on hydrolysis (acid or base catalyst) of tetraalkoxysilane precursors [$Si(OR)_4$], which is followed by polycondensation to form a dense silica gel polymer network. The entire process is performed in the presence of the encapsulated enzyme. If the tetraalkoxysilane [$Si(OR)_4$] is applied as part of a blend with more hydrophobic silanes [$R'Si(OR)_3$ or $R'R''Si(OR)_2$] bearing various substituents, the hydrophobic nature of the matrix can be adjusted, and beneficial effects on lipase activity are observed.[12] Among the main interactions between the enzyme and the silica network are hydrogen bonding and ionic and steric (van der Waals) interactions; the latter can be attributed to the alkyl side chains or the aromatic rings found in several organosilane precursors [$R'Si(OR)_3$].[13] In addition, the incorporation of small molecules as additives and/or of natural polymers (e.g., saccharides, proteins, etc.) as bulking agents can assist in modulating the inner sol–gel environment.[11a,13a,14] Although proteins (such as bovine serum albumin or gelatin) have been evaluated as additives during sol–gel entrapment of hydrolases, no significant improvement in the enzyme activity by proteins have so far been reported.

A study on the open conformation of lipase from *Geobacillus thermocatenulatus* (PDB code: 2W22) having 95% sequence identity to the lipase from *Geobacillus stearothermophilus* T6 (PDB code: 4X6U) demonstrated that interfacial activation played an important role in the action of these bacterial thermoalkalophilic lipases.[15] Therefore, providing conditions that stabilize the catalytically active conformation with sufficient conformational flexibility for the entrapped enzyme molecules to accommodate the substrate and release the product is a crucial issue.

Entrapment within a suitable polymeric matrix is a generally applicable and robust enzyme immobilization method. However, many of the traditionally obtained polymeric and sol–gel matrices provide an environment that is too tight for the enzyme molecules, which thereby reduces the catalytic activity of the immobilized biocatalyst. In the case of immobilized enzymes, biocatalysis is performed in the heterogeneous phases; thus, partitioning and diffusion barriers are among the key factors governing the efficiency of the process. In this respect, surface properties, permeability, and diffusion path length of the enzyme carrier or entrapment matrix are critical points.[8,16]

Entrapping lipases within sol–gel matrices for the purpose of biodiesel production has been reported previously owing to the thermal and mechanical qualities of this immobilization method along with its simplicity and relativity low cost. Microbial lipases such as *Pseudomonas cepacia* and *Thermomyces lanuginosus* were immobilized in sol–gel matrices in a set of studies focusing on the design of organosilane side chains, the structure of the formed particles, the use of different feedstocks, and recyclability.[17]

Obtaining a methanol-stable lipase through protein engineering is only the first step in creating a commercially feasible biocatalyst. In the present study, our goal was to convert the methanol-stable lipase variant LipT6$_{M}$ into a durable, reusable, and economical catalyst for biodiesel synthesis by immobilization engineering of the sol–gel entrapment process. To that end, two ternary sol–gel matrices, an octyltriethoxysilane (OTEOS)-based aliphatic matrix and a phenyltriethoxysilane (PTEOS)-based aromatic matrix,[12a,b] were evaluated. These precursors were selected on the basis of a recent study on the entrapment of microbial lipases in electrospun polyvinyl alcohol matrices, which demonstrated that OTEOS and PTEOS could contribute to stabilization of the catalytically active forms of lipases during entrapment in a polymeric matrix.[18] In addition to the selection of two beneficial sol–gel precursors, the influence of skim milk (SM) and soluble *E. coli* lysate proteins as bulking and stabilizing agents in conjunction with sol–gel entrapment were investigated for the first time. Activity tests, microscopy imaging, particle-size analysis, and recycling tests

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were used to find the most promising immobilized lipase system. Thermal stability of the variant in methanol was studied by using intrinsic tryptophane/tyrosine fluorescence. Introducing this combined approach of protein and immobilization engineering enabled us to improve the biodiesel production capabilities of the soluble enzyme.

Results and Discussion

Thermostability analysis of lipase T6

Two of the most desired features required from industrial lipases are thermostability and organic-solvent tolerance. Obtaining such stable enzymes is possible by isolation from thermophilic organisms or through protein engineering.[19,20] A combination of these strategies was used by Dror et al. to generate a thermostable and methanol-stable variant of lipase T6, H86Y/A269T/R374W, termed LipT6$_M$.[7] To further characterize the mutant, the melting temperature (T_m) was measured with a NanoDSF device (differential scanning fluorimetry) by monitoring the shift in the intrinsic tryptophan fluorescence at the emission wavelengths of 330 and 350 nm. Pure lipase solutions (with and without methanol) were loaded into capillary glass tubes and a temperature gradient was introduced. The T_m thermograms (Figure 1) demonstrated the thermal superiority of LipT6$_M$ relative to that of LipT6$_{WT}$ in buffer and in methanol solutions. A gradual decrease in T_m for both enzymes was observed upon increasing the methanol concentration; nevertheless, LipT6$_M$ maintained higher stability during these stressful conditions.

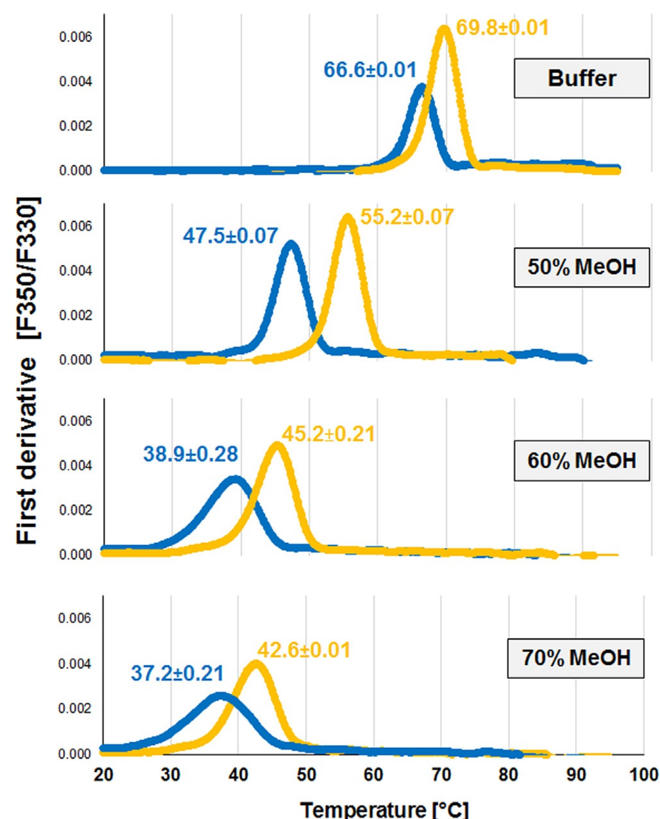

Figure 1. Melting temperature determination of LipT6$_{WT}$ (blue) and LipT6$_M$ (orange) in buffer and various methanol solutions. The melting scans show the first derivative of the fluorescence ratio (350 nm/330 nm). The numbers indicate the exact unfolding temperature T_m [°C].

Whereas most work dealing with thermostability focus on heat flow analysis methods such as differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), herein, for the first time, we measured the thermostability of LipT6 directly in organic solvents by using intrinsic fluorescence emission under a temperature gradient.[19b,21] In our previous study in which we used DSC, the T_m values of LipT6$_{WT}$ and LipT6$_M$ in buffer were 66.8 and 70.6 °C, respectively, which are identical to the ones obtained by NanoDSF.[7b] This tool is straightforward and highly correlative to traditional techniques, without the need for calibrating protein concentrations and measuring a reference cell in parallel. Nevertheless, it is noted that DSC is a direct measure of thermal transitions of a polymer. LipT6$_M$ was designed and screened for methanol stability and was found to be more stable owing to surface substitutions that formed a more branched hydration shell covering the enzyme's surface.[7b] Hydrolysis and transesterification assays previously showed that LipT6$_M$ was more active in 70% methanol, and the T_m measurement directly confirmed the enhanced thermal stability of the triple mutant. Consequently, all subsequent immobilization experiments were performed with recombinant LipT6$_M$, expressed in *E. coli* BL-21 cells. The systematic immobilization workflow is presented in Figure S1 in the Supporting Information.

Immobilization of LipT6$_M$_CE and LipT6$_M$ _HT by entrapment in sol–gel matrices

All of the soluble enzyme fractions and their mixtures with protein-containing additives as bulking agents utilized in this study are presented in Figure 2 for assessment of their protein content. For comparison of the effects of protein-containing additives on sol–gel immobilization of purified LipT6$_M$ (lane 3 in Figure 2), entrapment of two commercial lipases, the lipase from *Thermomyces lanuginosus* (TLL, lane 7 in Figure 2) and lipase B from *Candida antarctica* (CaLB, lane 9 in Figure 2), were also investigated.

The general mechanism of immobilization of LipT6$_M$ by entrapment in sol–gel matrices in the presence of protein-containing additives as bulking agents is presented in Figure 3.

Immobilization of proteins by the sol–gel process was first introduced in 1984 by Venton et al. by using a blend of tetraethoxysilane (TEOS) and a chemically modified organosilane [RSi(OEt)$_3$, R = 3-aminopropyl].[22] Throughout the last decades, this method has attracted much attention for entrapping enzymes as biocatalysts for organic chemistry processes.[23] The immobilization of lipases in a purely TEOS-based sol–gel matrix was found to be limited owing to the highly polar environment within the particle network. The hydrophilic surrounding decreased the activity of the entrapped lipase, which requires hydrophobic activation on an interface for its lid opening and substrate accessibility. Lipophilic substitutions on the central

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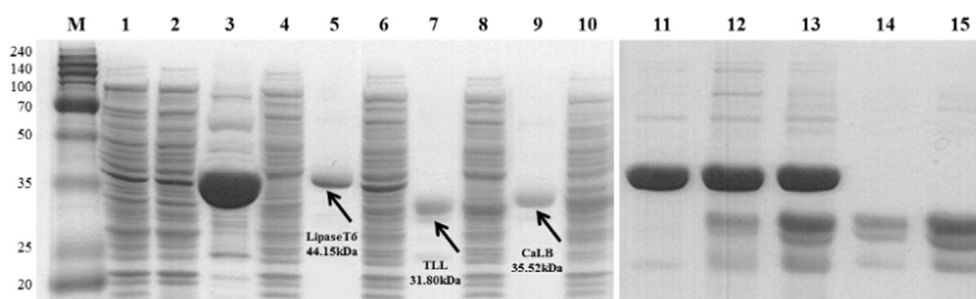

Figure 2. SDS-PAGE visualization of different immobilized fractions. The triple mutant was used throughout these experiments. M) Protein marker in kDa, 1) LipT6$_M$_CE cell extract, 2) LipT6$_M$_HT cell extract after heat treatment, 3) LipT6$_M$ purified lipase (5 mg mL^{-1}), 4) lysate—cell lysate without lipase, 5) LipT6$_M$_3% soluble purified lipase T6$_M$ at 3% (w/w) in buffer, 6) LipT6$_M$ + lysate—recombined mixture of lysate and 3% lipase T6$_M$, 7) TLL_3% soluble TLL at 3% concentration in buffer, 8) TLL + lysate—recombined mixture of lysate and 3% lipase TLL, 9) CaLB_3% soluble lipase CaLB at 3% concentration in buffer, 10) CaL-B + lysate—recombined mixture of lysate and 3% CaLB, 11) LipT6$_M$ (2 mg mL^{-1}), 12) LipT6$_M$ + SM-15 (1:15 lipase/skim milk *w/w*), 13) LipT6$_M$ + SM-30, 14) SM-15 control comprising SM suspended in buffer without enzyme, and 15) SM-30 control comprising SM suspended in buffer without enzyme. Protein samples (45 µg) were loaded into 15% acrylamide gel and stained with Coomassie blue.

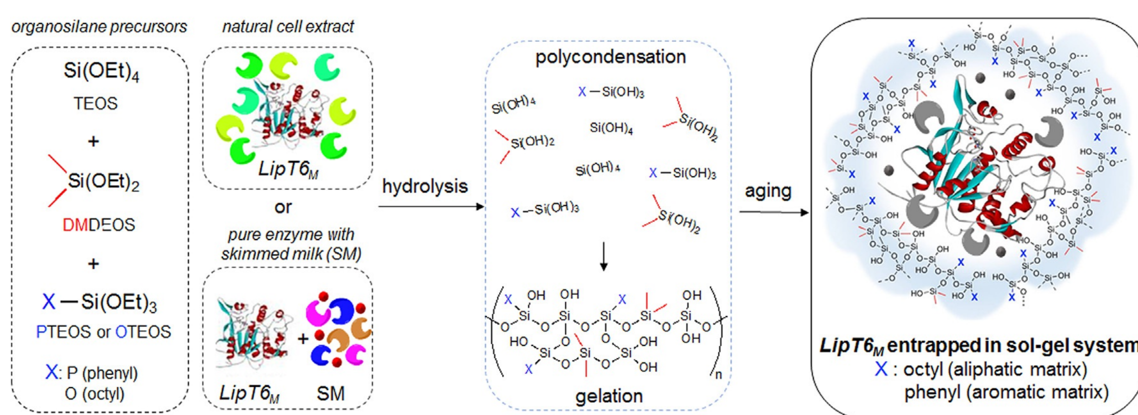

Figure 3. Schematic mechanism of immobilization of lipase T6$_M$ entrapped in sol–gel system in the presence of a protein containing additives as bulking agents. X substituents of the silica atom defines the type of substituted triethoxysilane (PTEOS: X = phenyl, OTEOS: X = octyl). Shapes in pale green and light green symbolize endogenous proteins of the *E. coli* host in cell extract. Shapes in orange, blue, and magenta represent milk proteins, and small beads in red symbolize small molecules such as lipids and sugars in the skimmed milk additive.

silica atom transformed the tetraalkoxysilanes into more hydrophobic monomers; this resulted in the formation of an appropriate environment for lipase activity and thus enhanced stability and activity.[11,13a,24]

In this study, entrapment of LipT6$_M$ was performed in two different ternary sol–gel matrices involving PTEOS (leading to an "aromatic" matrix) and OTEOS (leading to an "aliphatic" matrix) as the monosubstituted organosilane precursors, along with TEOS and dimethyldiethoxysilane (DMDEOS) as additional organosilane precursors of the ternary blends (Figure 3).[12a,b] Initially, the fraction comprising the crude cell extract was produced (LipT6$_M$_CE), quantified for protein content and hydrolytic activity (*para*-nitrophenyl laurate; *p*NPL hydrolysis), and immobilized by sol–gel entrapment. The same cell extract was treated in parallel at 50 °C for 15 min to remove the host proteins, as described previously.[7] The formed LipT6$_M$_HT fraction was immobilized in the same matrices. The esterification activity[25] of the dry immobilized biocatalysts was tested by using butyl laurate synthesis (Table 1).

The immobilized forms of LipT6$_M$_CE in either the aromatic or aliphatic matrix enabled higher esterification activity in hexane relative to that observed for any of the immobilized forms of the LipT6$_M$_HT fraction, despite the higher specific activity of the soluble form of the heat-treated enzyme system. This may be explained by the protective nature of one or more endogenous proteins of the *E. coli* host cells—present in

Table 1. Immobilization of LipT6$_M$_CE and LipT6$_M$_HT fractions in two sol–gel matrices.[a]

Soluble form fraction	protein conc.[b] [mg mL^{-1}]	lipase specific activity[c] [U_{NP} mg^{-1}]	Immobilized form sol–gel matrix	esterification activity[d] [U_{BL} g^{-1}]
LipT6$_M$_CE	30 ± 1.3	200 ± 10	aromatic	696 ± 176
			aliphatic	505 ± 86
LipT6$_M$_HT	25 ± 1.2	250 ± 17	aromatic	99 ± 25
			aliphatic	232 ± 35

[a] All data represent an average of four replicates. [b] Determined by Bradford protein assay. [c] Determined by *p*NPL hydrolysis assay. [d] Determined by butyl laurate esterification assay in hexane.

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the crude cell extract but lost in the heat treatment process—during the entrapment in the sol–gel matrices. LipT6$_M$_CE entrapped in the aromatic matrix showed higher enzymatic activity than the aliphatic alternative. Control experiments by entrapment of only the endogenous proteins of the host without enzyme (the *E. coli* host with an empty pET9a plasmid) showed no activity by the formed sol–gel particles.

Effect of aromatic versus aliphatic matrix on lipase activity

Immobilizing a crude enzyme preparation, whether as a cell extract or a heat-treated extract (Figure 2, lanes 1 and 2, respectively), is advantageous in terms of scale-up production and cost reduction, as expensive enzyme purification steps are avoided.[26] Thus, a systematic evaluation was performed in this study (Figure S1) to assess the potential of each fraction obtained during purification of LipT6$_M$ in becoming a promising biocatalyst immobilized in a ternary sol–gel matrix. It was found that immobilizing the LipT6$_M$_CE fraction, containing the enzyme within the full repertoire of endogenous soluble proteins, sugars, and nucleic acids of the *E. coli* host, was the most active system in combination with the aromatic matrix (Table 1). Immobilized heat-treated cell lysate (LipT6$_M$_HT, with higher specific activity in the soluble form) was generally much less active than the LipT6$_M$_CE fraction entrapped in any of the sol–gel matrices. However, the LipT6$_M$_HT fraction entrapped in the aliphatic matrix was more than twofold more active than the form entrapped in the aromatic polymeric network in the esterification assay. In such complex mixtures of proteins and other molecules surrounding the lipase during the entrapment, it is difficult to identify the exact interactions between the enzyme and the sol–gel network branches. Nonetheless, it is known that in the sol–gel polymerization process, enzyme molecules serve as templates for matrix formation.[11a,13a] Theoretically, the lipase can be trapped in this kind of immobilization system within two protective layers: one, an endogenous *E. coli* proteins assembly; two, the sol–gel inorganic matrix. It is most likely that host proteins stabilize the lipase during the stressful sol–gel condensation process by maintaining its hydration layer of surface-structured water molecules and serving as protectants without catalytic contribution. This hypothesis was described beforehand showing that bacterial protein mixtures could serve as a protective agent during the immobilization, drying, and lyophilization processes.[21a,c,27] Furthermore, during the esterification reaction, the lipase is masked from denaturing agents but is still flexible enough to perform maximal catalysis.

Although several researchers have reported on the positive correlation between the ratio of alkyl monomers in the matrix and the lipolytic activity, here we revealed an improvement in catalytic performance in the less preferable known network comprising aromatic side chains.[8d,11b,27c] This increase in activity in the aromatic environment relative to that in the aliphatic counterpart may be possibly explained by interaction of the lysate proteins with the matrix surface (statistically, representing most of the protein–network interactions inside the sol–gel particles). Bacterial cytoplasm proteins are mostly globular and have a hydrophobic core and a polar surface in aqueous solution. In the work reported by Purcar et al., OTEOS-modified silica films showed more hydrophobic properties than PTEOS-modified surfaces.[28] Subsequently, it can be interpreted that the microenvironment within the sol–gel aliphatic matrix is superior to that in the aromatic matrix in terms of lipophilic nature. Therefore, we suggest better conformation of the host proteins assembly on the particles' interphase in the aromatic surroundings, which is less favorable in the more lipophilic oil-like surface created in the aliphatic matrix. A similar link between aromatic silanes and improved lipase activity was shown previously by Zarkula et al. on lipase AK from *Pseudomonas fluorescens*. Their results presented an increase in acetylation activity of secondary alcohols with lipase AK in a PTEOS/TEOS sol–gel matrix.[29] Specific surface area determination by N_2 adsorption was not determined for the matrices, because quite recently it was demonstrated that values measured by this method did not correlate with biocatalyst activity. N_2 adsorption could provide the surface available for small and relatively nonpolar N_2 molecules but not for larger molecules with higher polarity. On the basis of scanning electron microscopy (SEM) and atomic force microscopy (AFM) data, however, the activity could be associated with the average length of the nanochannels, which was much shorter for a microstructured material than for a non-microstructured matrix.[29,30]

The opposite trend for the immobilized heat-treated cell lysate (LipT6$_M$_HT)—the activity of the LipT6$_M$_HT form entrapped in the aliphatic matrix including OTEOS was higher than that of the form entrapped in the aromatic matrix including PTEOS—could be rationalized on the basis of a recent study on the immobilization of CaLB in various ternary sol–gel systems that indicated that the bioimprinting effect of the partially hydrolyzed forms of OTEOS was stronger than that of partially hydrolyzed PTEOS during entrapment of CaLB in sol–gel systems.[31] If the beneficial effects of the endogenous proteins of the *E. coli* host were destroyed in the LipT6$_M$_HT form by heat treatment, then the stronger bioimprinting effect of the OTEOS precursor and the higher hydrophobicity of the aliphatic matrix could render the aliphatic matrix more effective.

Immobilization of pure LipT6$_M$ in a sol–gel matrix in the presence of skim milk

Skim milk (SM) is a common low-fat powder containing the major proteins comprising typical cow milk.[32] In the powder used in this research, three major proteins were present (Figure 2, lanes 4 and 5) that were the three subgroups of casein (α_1, α_2, and β).[33] SM is frequently used as a protective agent during dehydration of cell suspensions and enzyme solutions aimed for long-term storage by maintaining the hydrogen-bonding network on the enzyme surface.[21a,c,27b,d,34] Thus, a second approach was to test the effect of this inexpensive protein mixture as a bulking and stabilizing agent during the immobilization of pure LipT6$_M$ in the aromatic and aliphatic sol–gel matrices (Figure 4).[33,35] Purified LipT6$_M$ was mixed with SM powder in different weight ratios (w/w) with the compositions presented in Figure 2.

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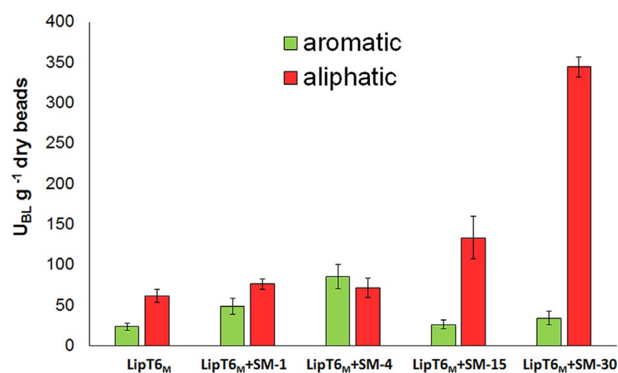

Figure 4. Esterification activity of sol–gel immobilized LipT6$_M$ in two matrices with different weight ratios of SM as a bulking agent. The reaction was performed with *n*-butanol and lauric acid as substrates in *n*-hexane at 45 °C (detected by GC–MS). The reactions were started by adding the dry sol–gel biocatalyst to the reaction mixture, and samples were collected every 15 min.

According to the results presented in Figure 4, the addition of SM in low ratios (1:1 and 1:4) did not have a major effect on lipase esterification activity in either of the sol–gel matrices relative to that observed for the pure lipase without additives (LipT6$_M$). The highest improvement in activity by the aromatic matrix was 3.6-fold upon adding a 4-fold amount of SM. In contrast, lipase immobilized within the aliphatic matrix in the presence of high ratios of the SM powder (1:15 and 1:30) turned out to be extremely active. A 5.6-fold improvement was obtained by adding a lipase-to-SM ratio of 1:30, which is a ratio similar to that of the original cell extract produced in this study (3 % w/w lipase, which is a 1:33 ratio of lipase/host proteins). Furthermore, control experiments confirmed that the SM powder or sol–gel matrix by itself possessed no hydrolytic or esterification activity.

The hydrophobic nature of lipases and their enhanced solubility in the presence of lipophilic agents (such as detergents, etc.) is well documented.[26,36] In parallel, the surface hydrophobicity of milk proteins was also studied and correlated to their emulsification abilities.[37] It was reported that milk caseins have high emulsification abilities and that their adsorption performance increases as the surface hydrophobicity rises.[38] It could be suggested that the nature of the aliphatic matrix, which is more hydrophobic than the aromatic synonym, had a beneficial effect on interaction of the milk proteins with the matrix.[11] Furthermore, the positive effect of sugar additives—also present in SM—during immobilization of bacterial β-galactosidase was also recently reported.[39]

In addition to their hydrophilic protective effect, the milk proteins and sugars can serve as templates for the pores of the particles and improve substrate transport to the trapped enzyme.[11a,13a] This structural role supports the significant morphological differences between LipT6$_M$ and LipT6$_M$ + SM-15 particles in the SEM analysis (Figure S2) and particle-size measurements following ultrasonication (Figure 5 and Table 2).[40]

The morphology of the particles containing purified lipase was different from that of the particles containing the cell extract (LipT6$_M$_CE and LipT6$_M$_HT) in terms of particle size, aggregation, and overall size distribution (Figure S2, Figure 5, and

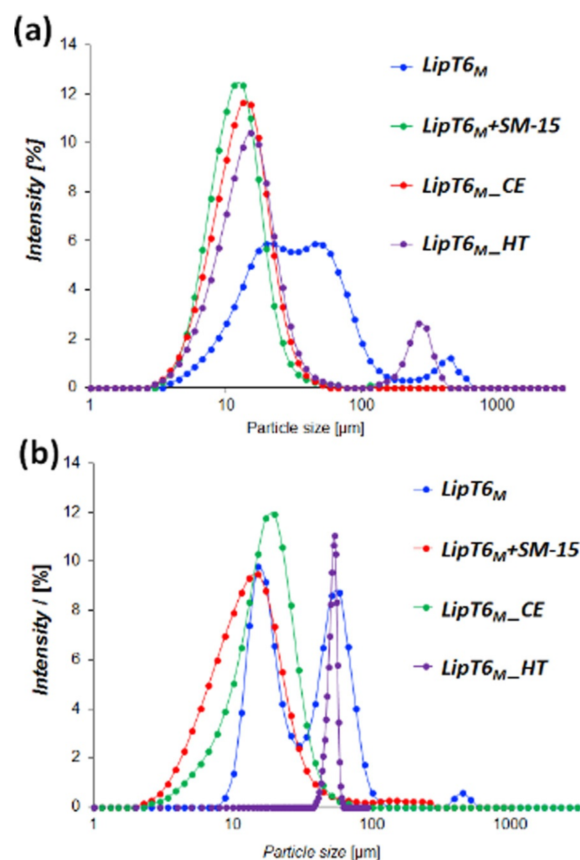

Figure 5. Particle-size distribution of various LipT6$_M$ biocatalysts entrapped a) in aliphatic (TEOS/DMDEOS/OTEOS) or b) in aromatic (TEOS/DMDEOS/PTEOS) sol–gel matrices after sonication for 600 s in methanol. The results are based on Mie scattering theory.

Table 2. Particle-size distribution and esterification activity of various LipT6$_M$ biocatalysts entrapped in aliphatic (TEOS/DMDEOS/OTEOS) and aromatic (TEOS/DMDEOS/PTEOS) sol–gel matrices.

Fraction	Sol–gel matrix	Mean particle size[a] [µm]	Esterification activity[b] [U_{BL} g^{-1}]
LipT6$_M$	aromatic	53	61
	aliphatic	37	23
LipT6$_M$ + *SM-15*	aromatic	12	133
	aliphatic	17	26
LipT6$_M$_CE	aromatic	13	696
	aliphatic	16	505
LipT6$_M$_HT	aromatic	39	99
	aliphatic	13	232

[a] Determined by Mie scattering after sonication for 600 s in methanol. [b] Determined by butyl laurate esterification assay in hexane.

Table 2). The pure immobilized lipase (LipT6$_M$) contained relatively low amounts of protein, which resulted in a higher particle size and lower esterification activity (Figure 5 a and Table 2), and the frequent presence of aggregates covered with a thin sol–gel layer, which remained relatively stable even upon ultrasonication (Figure 5 b). The relatively high diameter and aggregation effect of LipT6$_M$ can directly influence the substrate's diffusion towards the encapsulated biocatalyst; this explains its

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decreased apparent activity, which partially results from the increased path length of the microchannels.[8d,13a]

It is assumed that the shift in the superiority of the additive-enriched immobilized lipase T6$_M$ from the aromatic matrix (cell lysate) to the aliphatic matrix (in the pure form) was triggered by the nature of the supplemented proteins. The *E. coli* endogenous polar cytosolic proteins showed better activity in protecting LipT6$_M$ in the aromatic matrix, whereas the milk proteins had a major effect on lipolytic activity within the aliphatic matrix. The fact that supplementation of LipT6$_M$ with 1:30 milk proteins (LipT6$_M$+SM-30) did not result in an activity value similar to that of LipT6$_M$_CE, both having the same lipase/additive ratio, emphasizes the importance of the interaction of the additives with the inorganic network over their mere total added ratio. Notably, immobilizing the pure lipase in the presence of 1:4 SM (LipT6$_M$+SM-4) resulted in microstructural properties similar to those presented in the pure lipase without SM (LipT6$_M$), which hinted to a critical concentration of milk proteins that affects the morphology of the particles. A larger particle size was also observed in immobilized LipT6$_M$_HT (Figure S2 d, Figure 5 b, and Table 2), which contains a lower concentration of endogenous *E. coli* proteins.

Immobilization of high protein concentrations (within both matrices) promoted the creation of uniform, dense, relatively small, and nonaggregated particles with good diffusional accessibility according to SEM (Figure S2) and particle-size analyses (Figure 5).[13a] The smaller particle sizes of the sol–gel preparations with higher protein contents correlated well with their higher esterification activity (Table 2). Similar results were described recently by Paul et al., who emphasized the substrate–active site flux dependence on the morphology of the particles.[30]

All in all, the highest esterification activity obtained thus far was attributed to LipT6$_M$_CE immobilized in the aromatic matrix (696 $U_{BL}\,g^{-1}$). Therefore, the aromatic matrix was used in subsequent experiments, such as recycling efficiency and FAME production from different feedstocks. The superior influence of endogenous host proteins found in the cell extract versus SM proteins was next studied with other lipases by using the aromatic matrix.

Effect of endogenous host proteins on immobilization of lipases by entrapment in sol–gel matrices

Seeing the improvement in lipase activity following the addition of SM, it was our objective to evaluate, for the first time, the influence of endogenous proteins of the *E. coli* cell lysate (lysate). The study on the beneficial effect of bacterial cell lysate on the lipase activity of the entrapped LipT6$_M$ was extended to the investigation of two other common commercially available lipases (i.e., TLL and CaLB). This was achieved by recombining cell lysate (comprising extract from *E. coli* cells harboring a plasmid pET9a with no insert) with pure lipase solutions LipT6$_M$, TLL, and CaLB (lanes 6, 8, and 10 in Figure 2, respectively). The lysate enrichment with soluble lipase was performed at the same ratio as that of the original expression system of lipase T6$_M$ used in this research (3% w/w, based on the average ratio between the specific activity of LipT6$_M$_CE and LipT6$_M$). All mixtures (lanes 6, 8, and 10 in Figure 2) were immobilized in the aromatic matrix, and control systems contained buffer only instead of the *E. coli* lysate. In parallel, lysate (without lipase expression: lane 4 in Figure 2) was immobilized to ensure the absence of side reactions from *E. coli* native enzymes. In this reverse engineering approach, the recombined mixtures essentially mimicked the LipT6$_M$_CE system (as if the lipases were expressed in the same ratio as LipT6$_M$ by using the same expression system). Relative to that shown by the control group (3% lipase in buffer, immobilized in the aromatic matrix), all immobilized recombined mixtures showed better activity (Figure 6). The esterification activities of CaLB+lysate,

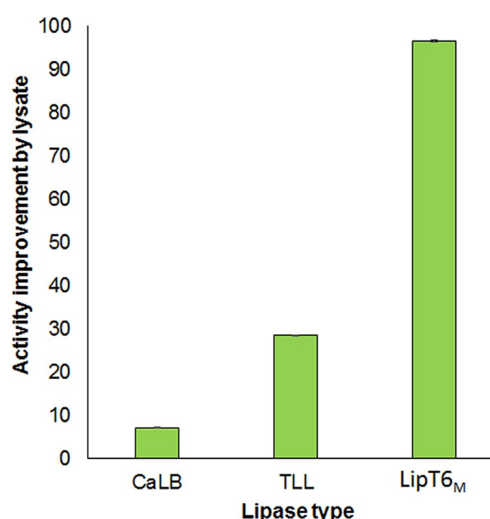

Figure 6. Relative enhancement effect of *E. coli* lysate on different lipases immobilized by entrapment in the aromatic sol–gel matrix. The values were calculated by dividing the activity value ($U_{BL}\,g^{-1}$) of immobilized lipase+lysate by the activity value of immobilized lipase without lysate (buffer only, 3% w/w enzyme). Results are mean ± standard deviation (SD) ($n=3$); SDs were lower than 0.3.

TLL+lysate, and LipT6$_M$+lysate, immobilized into the aromatic matrix, were improved by factors of 7, 28, and 96 relative to the activities of their synonym control groups (buffer only), respectively. Although the cell lysate had a positive effect on all three lipases, significant differences in the positive effect of the cytosolic bacterial protein mixture between the commercial lipases and our "in-house"-purified LipT6$_M$ were observed. The fact that different lipases showed significant improvements in the esterification activity highlights the protective and stabilizing effect of the cell lysate macromolecules on biocatalyst performance. The degree of improvement was enzyme dependent, and the most enhanced catalyst was LipT6$_M$+lysate. The relatively low increase in activity of the recombined mixtures of commercial lipases can be attributed partially to the unknown additives present in these CaLB and TLL preparations to stabilize the soluble form for long-term storage.[27b–d,41] Although the same amount of lipase is present in the mixtures, as evidenced by sodium dodecyl sulfate polyacrylamide gel ectrophoresis (SDS-PAGE) analysis, their activities in *n*-hexane

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along with their interactions with the sol–gel components and protective endogenous proteins are apparently different.

Recycling and biodiesel production by immobilized LipT6$_M$_CE

The ability to use the biocatalyst for several successive reaction cycles is an important feature in industrial applications, in general, and for large-scale biodiesel production, in particular. Sol–gel immobilization is considered an inexpensive, easy, and straightforward approach, and it is scalable for mass production of encapsulating enzymes.[12a,b,13a,27c] The use of the crude cell extract containing the lipase without the need for costly affinity purifications can reduce financial costs and avoid complicated downstream processing.[26,36a] Our most active system comprising LipT6$_M$_CE immobilized in the aromatic matrix was investigated for its recycling ability to explore its potential as an economic and stable biocatalyst for biodiesel production. It was previously shown that sol–gel networks have very low enzyme-leaching features and that the active protein within the matrix is maintained in a stable conformation.[11] Consecutive 2 h long cycles of lauric acid esterification at 45 °C were performed, as shown in Figure 7. Full conversion was obtained in the first 16 cycles, after which a gradual decline in activity became evident. The total mass loss (immobilized particles) in four independent recycling experiments was less than 4% (w/w), which indicated that the loss in activity had a major contribution to the decrease in conversion.

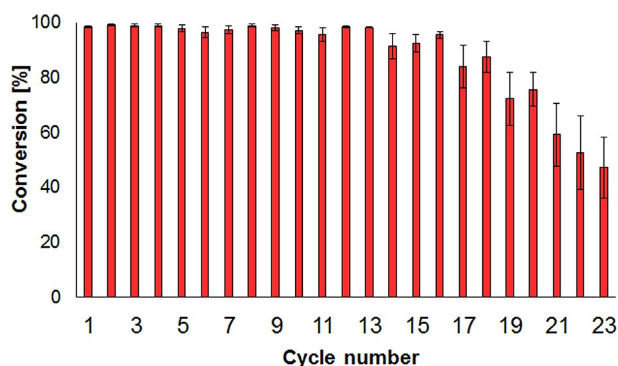

Figure 7. Recycling of sol–gel-immobilized LipT6$_M$_CE for lauric acid esterification. The 2 h reactions were performed in hexane at 45 °C by using *n*-butanol and lauric acid as substrates. Results are mean ± SD ($n=4$). Total mass loss of sol–gel particles was less than 4% w/w.

In total, the esterification reaction was performed over 23 consecutive cycles (2 h per each cycle), which resulted in 40.6 mmol butyl laurate (10.4 g) as the overall amount of product by 200 mg of immobilized crude LipT6$_M$ in 46 h (Figure 7). The stability of the immobilized lipase was attributed to the modified sol–gel environment, which entrapped the protein mixture under suitable conditions and enabled a significant improvement in catalysis in organic solvents.[11,12] The results presented herein are superior to those obtained by using recent lipase immobilization procedures involving amino-functionalized silica particles and resin adsorption.[42] Both of these works, involving the use of immobilized lipases from *Burkholderia cepacia* and *Rhizopus oryzae*, respectively, showed a decrease in activity after 6–10 cycles of reuse in biodiesel production.

Finally, enzymatic transesterification of two different oil feedstocks was studied with LipT6$_M$_CE immobilized within the aromatic and aliphatic matrices in a solvent-free system. A methanol/oil molar ratio of 4.5:1 was used on the basis of industrial requirements.[7b] The FAME formation profiles are presented in Figure 8. The highest conversion (80%) after 24 h was achieved

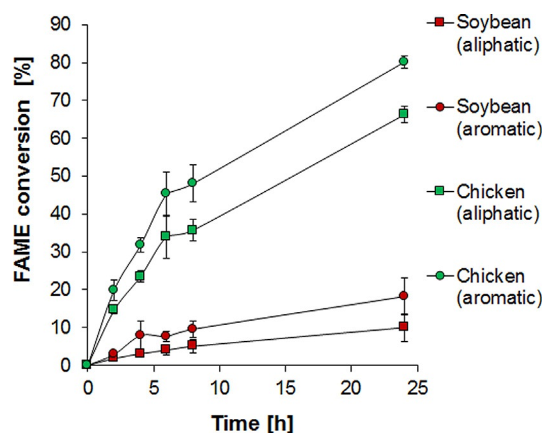

Figure 8. FAME biosynthesis from soybean and waste chicken oils by using immobilized LipT6$_M$_CE in aliphatic and aromatic sol–gel matrices. Reaction conditions: oil (2 g), water (5%), methanol/oil molar ratio of 4.5:1, and dry immobilized lipase (50 mg, 2.5% based on the oil weight), 1350 rpm, 45 °C. The results represent experiments performed in triplicate.

with LipT6$_M$_CE immobilized in the aromatic matrix by using waste chicken oil as the feedstock. LipT6$_M$_CE entrapped in the aliphatic matrix resulted in somewhat lower conversion (67%) after 24 h. With soybean oil as a substrate, a much lower conversion was obtained with LipT6$_M$_CE entrapped in the aromatic matrix, but the conversion in the aromatic matrix (18%) was higher than that in the aliphatic matrix (10%). Initial rates of FAME formation (first 6 h) from waste chicken oil were approximately 150 and 144 mg oil converted per hour for the aromatic and aliphatic particles, respectively. Subsequently, after 8 h of reaction the estimated rates decreased to 38 and 34 mg oil converted per hour for the aromatic and aliphatic particles, respectively.

As expected, a positive correlation between the esterification activity values and the formation of biodiesel was well observed. The better activity of LipT6$_M$_CE immobilized within the aromatic matrix could be rationalized by the balanced hydrophobicity of this matrix, which resulted in less severe partitioning effects. This balanced hydrophobicity enabled diffusion of the lipophilic substrate and product as well as sufficient mass transfer of more hydrophilic methanol and glycerol, which was clearly more hindered in the more hydrophobic aliphatic matrix. As mentioned earlier, the relatively high activity of LipT6$_M$ in its host lysate environment was due to the stabi-

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lizing surrounding of a network enriched with aromatic side chains and a conserved hydration layer.

The improved activity on waste chicken oil could most likely be attributed to the chemical (substrate variety distribution) and physical properties (water content, viscosity, etc.) of this oil, as previously reported.[7] This feedstock dependence of the biodiesel synthesis potential was previously described,[3,43] and whereas less research information is available on enzymatic transesterification of waste chicken oil, soybean oil and other edible vegetable oils have been largely utilized.[44]

The fact that the catalyst/substrate ratio was low (50 mg per 2 g, which is 2.5 % w/w), relative to that used in other work involving the use of 10 or 30 %, reinforces the economic potential of this sol–gel biocatalyst to produce biodiesel.[17b–d] The transesterification profile was similar to that obtained in the work of Hsu et al., who reported a higher activity rate in the first 6 h in the ethanolysis of grease with a sol–gel immobilized lipase.[17d] The decrease in the rate of the progress of transesterification on waste chicken oil can be attributed to the long exposure to methanol and lipase inactivation, as discussed in the work of Philkana et al.[17a] Another explanation such as particle aggregation causing diffusion limitations and glycerol inhibition can also contribute to the loss in activity in the advanced stages of the methanolysis reaction.[17c] Finally, by immobilizing LipT6$_M$ in this ternary sol–gel matrix, we succeeded in improving our previous results with soluble lipase while reducing purification costs and efforts.[7b]

Conclusions

The immobilization of the lipase T6 triple mutant in a sol–gel matrix with phenyl side chains was found to be highly beneficial in terms of recyclability and productivity. The highest activity was obtained by entrapment of the crude *E. coli* cell lysate containing the expressed enzyme, and therefore, costly purification efforts and downstream processing were avoided. This first work on the encapsulation of a methanol-stable engineered lipase demonstrates the additive contribution of protein engineering along with immobilization engineering towards a desired reaction and operating conditions. Evaluating two sol–gel matrices having different hydrophobic natures revealed diverse silica network preferences of the same enzyme in its pure form relative to its crude extract state. The presence of protective agents such as skim milk powder and *E. coli* soluble proteins was found to promote the esterification activity significantly, albeit to different extents depending on the properties of the sol–gel matrix.

Experimental Section

Details of the experimental and analytical methods are found in the Supporting Information.

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