Spectroscopic Kinetic Monitoring and Molecular Dynamics Simulations of Biocatalytic Ester Hydrolysis in Non-Aqueous Solvent

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Abstract

The use of enzymes as catalysts is becoming an increasingly important tool in chemical synthesis, given the mild conditions and high chemoselectivity that can be achieved through enzyme-catalyzed reactions. However, a major limitation in the use of enzymatic biocatalysis is the degradation of enzyme structure and activity in non-aqueous media. Lipase is an esterase found in the human liver that breaks ester bonds in lipids. In this study, we explore the effects of various concentrations of non-aqueous organic solvents on lipase activity and analyze the relationship between various properties of solvents and the kinetics of enzymatic hydrolysis through spectroscopic monitoring of a synthetic colorimetric substrate, 4-nitrophenyl acetate. The influence of non-aqueous solvent environments on protein stability is further explored with molecular dynamics simulations on a 1 nanosecond (ns) timescale.

Introduction

Lipases—a subclass of esterases—are enzymes produced primarily by the pancreas that catalyze the hydrolysis of ester bonds in lipids.[1] [Fig. 1A]

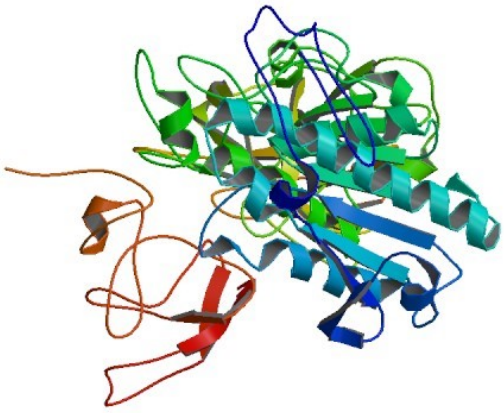

Figure 1A: Crystal Structure of lipase [PDB: 1N8S]

In addition, lipases have been previously reported to catalyze esterification, interesterification, and transesterification reactions in non-aqueous media.[2] For this reason, lipases are used as industrial biocatalysts in the food, detergent, and pharmaceutical sectors, and they also have potential applications in the leather, textile, cosmetic, and paper industries.

Enzymatic biocatalysis has attracted important applications in chemical synthesis due to greater levels of chemoselectivity, increased atom economy, and reduced production of hazardous by-products that are found in common reagents in synthesis. In particular, biocatalytic cascades—combinations of multiple enzymatic steps—shorten multi-step syntheses by eliminating intermediate steps and reduce hazardous waste. Engineered enzymatic biocatalysts have been recently reported in the chemical synthesis of islatravir.[3] *Candidaantarctica* lipase has been reported to catalyze alcoholysis, ammonolysis, and per hydrolysis reactions in organic media.[4] In addition, biocatalytic processes have been used for the synthesis of chiral intermediates for drugs

such as Formoterol and Omapatrilat.[5] Deacylations of phenolic esters can be conducted under acidic or basic conditions, but the utilization of enzymatic catalysis to allows the same reaction to be conducted under normal pH conditions. Understanding the effect of non-aqueous solvation on lipase activity is largely applicable in organic synthesis—where an enzyme is required for chemoselective reactions, and where ester-hydrolysis reactions are not necessarily amenable to being conducted in the enzyme's native buffered aqueous solution for solubility or reactivity reasons. Understanding the effect that different solvents have on the retention of catalytic activity in enzymes is critical in informing the choice of reaction conditions for conducting biocatalytic chemical reactions.

The active site of pre-gastric lipase consists of a serine nucleophile (Ser152), which is part of three amino acid residues that make up the catalytic triad [Fig. 1B], the other two being His263 and Asp176.[6] These residues form a charge-transfer relay network which serves to increase the nucleophilicity of Ser152. Macromolecular stability can be studied using molecular dynamics—a biophysics tool used to link protein structure and its dynamics—which allows us to determine the stability of lipase in non-aqueous solvents.[7,8]

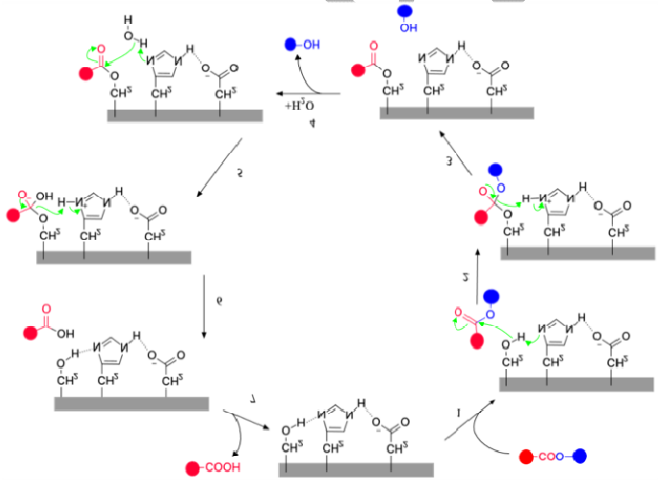

Figure 1B: Ser-His-Asp catalytic triad coordinate hydrolysis of an ester bond

The effects of both aqueous and non-aqueous solvents on enzyme activity are well-documented, and the impact of organic solvent on the catalytic activity of the enzyme can be attributed to changes in reactive intermediates in the active site or alterations in the noncovalent interactions that define an enzyme's secondary and tertiary structure. In aqueous solvents, water acts as a nucleophile to resolve the acyl-enzyme intermediate and reform the catalytic triad. Non-aqueous solvents change the physical properties of the solution, such as dielectric charge, polarity, and hydrophobicity, which would, in turn, affect acyl-enzyme stability and the rate of enzymatic hydrolysis.

Nitrophenyl esters are commonly used as colorimetric substrates to determine lipase activity.[9] Enzymatic cleavage of the nitrophenyl ester bond releases nitrophenol, which can be tracked spectroscopically via absorbance at a wavelength of 400 nanometers (nm) [Fig. 1C].

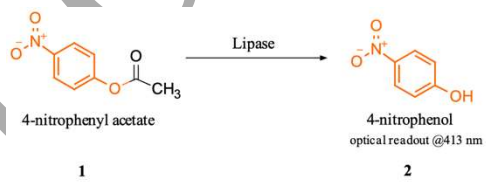

Figure 1C: Lipase cleaves the ester bond in 4-nitrophenyl acetate, releasing 4-nitrophenol, which can be tracked spectroscopically at 400 nm. This allows for the quantification of lipase activity.

Here, we synthesize 4-nitrophenyl acetate from the acylation of 4-nitrophenol, and utilize UV-visible (UV-vis) spectroscopy to screen and quantify lipase activity in seven organic solvents using 4-nitrophenyl acetate as a colorimetric probe. Enzymatic hydrolysis of the ester bond liberates 4-nitrophenol, which gives an optical readout that can be tracked on a spectrophotometer. We hypothesize that the greatest retention of enzymatic activity would be observed in solvent systems whose physical properties most closely mimic water, and that higher concentrations of non-aqueous solvent will decrease enzymatic activity. The library of common organic solvents screened was chosen based on their miscibility with water: methanol, ethanol, isopropanol, acetonitrile, acetone, N,N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). Lipase

activity was monitored at 5%, 10%, 20%, and 40% concentrations of each organic solvent.

Results

UV-vis data were analyzed to determine respective Beer's Law graphs of 4-nitrophenol at 1.00 nM, 10.0 nM, and 50.0 nM. Extinction coefficients were derived from Beer's Law graphs and used to calculate the concentration of nitrophenol as a function of absorbance at 413 nm. The rate of enzymatic hydrolysis in each solvent was obtained by determining the initial velocity of lipase, which was calculated using the slope of the line-of-best-fit of the concentration of 4-nitrophenol as a function of time [Fig. 2]. Consistent with our hypothesis, lipase activity generally decreases in higher concentrations of organic solvent. This loss of enzymatic activity is most apparent in methanol, where an increase in methanol concentration from 5% to 40% caused a decrease of enzymatic activity by 46%.

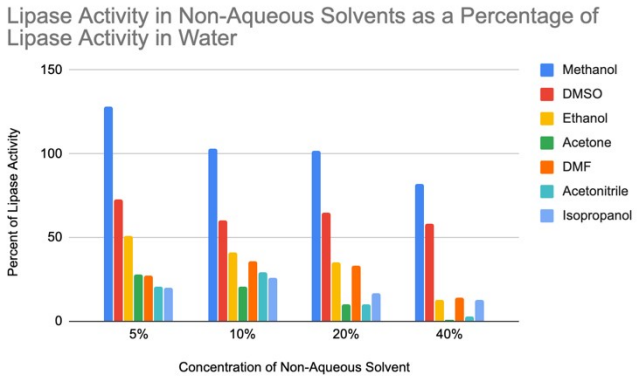

Figure 2: Lipase activity in non-aqueous solvents is graphed as a percentage of lipase activity in water.

Contrary to our initial expectations, lipase has higher levels of activity in lower concentrations of methanol than in water, with 128% lipase activity at 5% methanol, 103% lipase activity at 10 % methanol, and 102% enzyme activity at 20% methanol. Lipase activity for all other organic solvents was lower than that of water at all concentrations. A solvent system of 40% acetone results in the largest decrease in lipase activity, with only 1% of lipase activity compared to lipase in water. Average decrease was calculated by dividing the difference in lipase activity between 5% and 40% solvent by the number of changes in concentration (3). 5% methanol has the least detriment on lipase activity—increasing lipase activity by 28% compared to lipase in water. Methanol also results in the largest decrease in lipase activity in increasing concentrations with an average decrease of 15.33% and a net decrease in lipase activity by 46% between 5% and 40% methanol. On the other hand, isopropanol results in the smallest decrease in lipase activity in increasing concentrations with an average decrease of 2.33% and a net decrease in activity by 7% between 5% and 40% isopropanol.

It was also observed that alcohol solvents (methanol, ethanol, and isopropanol) tend to follow a trend in which a decrease in polarity corresponds to a decrease in lipase activity [Fig. 3]. Methanol, being the most polar of the solvents screened, displays the highest lipase activity, followed by ethanol and isopropanol (least polar). Methanol is the most polar of the three and has properties most similar to water, so its dipole interactions with lipase most closely mimic those of water. Other notable observations are the relatively unchanged rates of lipase activity despite increased concentrations of DMSO, in contrast with the drastic decreases in lipase activity after slight increases in the concentration of DMF and acetonitrile.

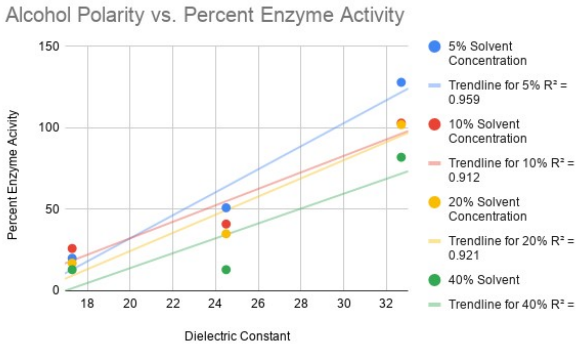

Figure 3: Percentage of lipase activity compared to water as a function of the alcohol's dielectric constant. Each line represents a different concentration of solvent (blue = 5%; red = 10%; yellow = 20%; green = 40%), and each alcohol is represented by its dielectric constant (ε 17.9 = isopropanol; ε 24.5 = ethanol; ε 32.7 = methanol).

Molecular dynamics simulations were also performed using GROningen MAchine for Chemical Simulations (GROMACS) to probe changes in

lipase stability in water, methanol, and acetonitrile [Fig. 4A, 4B, 4C].

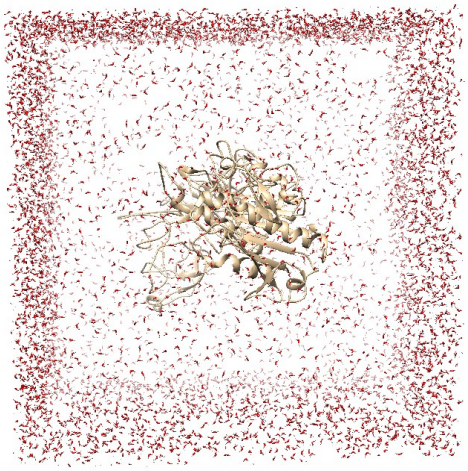

Figure 4A: Structure of lipase in water solvation shell.

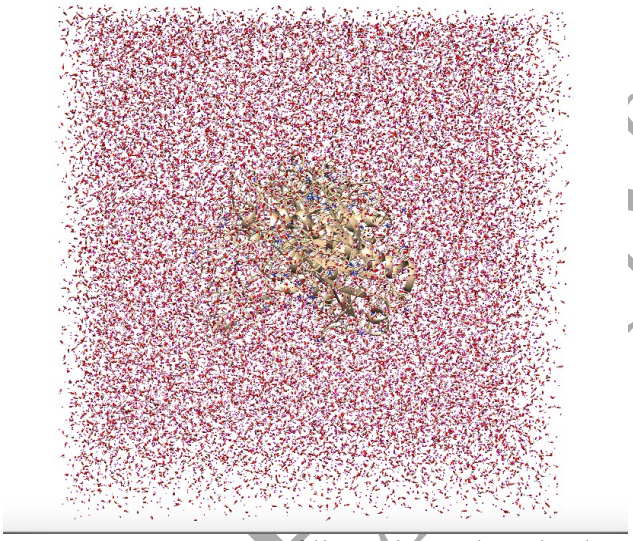

Figure 4B: Structure of lipase in methanol solvation shell.

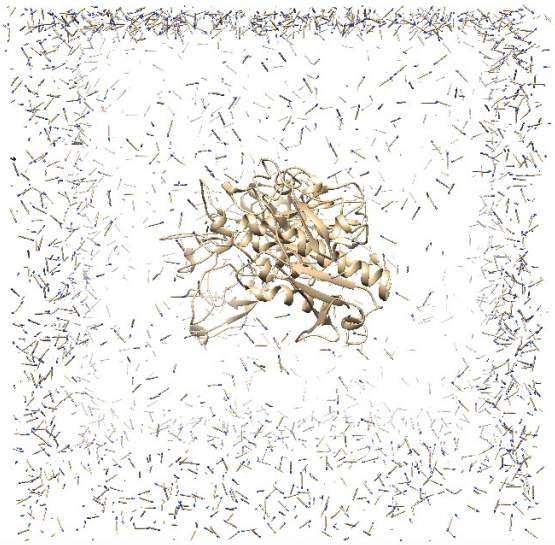

Figure 4C: Structure of lipase in acetonitrile solvation shell.

We were unable to perform these calculations for every solvent because we could not obtain all solvent topology files. Root mean square deviation (RMSD) values for lipase were derived from GROMACS and graphed as a function of time [Fig. 5A], as well as averages for each solvent [Fig. 5B]. Over time, the RMSD of lipase in acetonitrile increases, while the RMSD of water and methanol decreases over time. Lower RMSDs indicate less deviation of atomic positions, and further examination showed lipase in methanol to have the lowest average RMSD of 0.9365 Å, while lipase in water has the highest average RMSD of 1.61 Å.

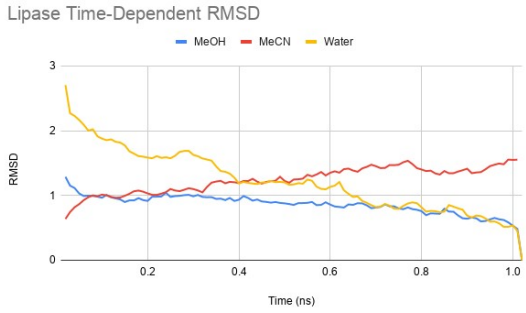

Figure 5A: RMSD values of lipase are graphed as a function of time in methanol, acetonitrile, and water. The RMSD of lipase in acetonitrile increases over time, while the RMSD of lipase in methanol and water decreases over time.

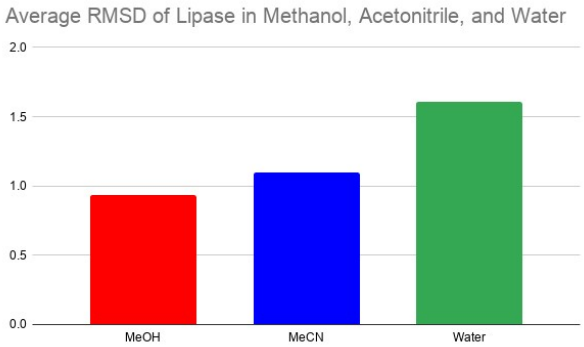

Figure 5B: Average RMSD values of lipase in methanol, acetonitrile, and water.

Discussion

Here, we screened the kinetics of catalytic deacylation of 4-nitrophenyl acetate by UV-vis spectroscopy in different organic solvents in efforts to determine the extent of retention of catalytic ability of lipase in non-aqueous solvent systems. A general decrease in lipase activity is observed with increasing concentrations of non-aqueous solvent, which is consistent with our hypothesis. It was determined that increasing methanol concentrations decrease lipase activity, but the significant increase of lipase activity in low concentrations of methanol might be attributed to nucleophilic competition of methanol for the acyl-enzyme intermediate.

Lower lipase activity in acetone, acetonitrile, and DMF can likely be attributed to solvent molecules disrupting crucial hydrogen bond interactions with lipase that may otherwise be present with an alcohol solvent. Similar to DMSO, these three solvents lack hydrogen-donor properties that both water and methanol possess, which likely affects the activity of lipase. Decreased lipase activity in acetone may also be explained by low polarity of acetone compared to water or methanol. Since high catalytic activity in DMSO compared to DMF and acetonitrile cannot be rationalized on the basis of polarity, it seems that loss of enzymatic activity in these solvents is most likely attributed to differences in the degree of disruption in the hydrogen bonding network that defines secondary and tertiary structure in the enzyme. Competition for amide hydrogen-bond networks have been reported with both DMSO and DMF in the literature.[10]

Molecular dynamics simulations suggest that lipase destabilizes in water, as evidenced by lipase's high average RMSD in water. In addition, the time-dependent RMSD behavior of lipase in methanol is similar to that of lipase in water, suggesting that the conformational state and stability of the enzyme in methanol is similar to that of the enzyme in water. This is consistent with our spectroscopic data since lipase displays higher enzymatic activity in low concentrations of methanol than in water. The RMSD of lipase in acetonitrile increases over time, which may be attributed to a net destabilization of key noncovalent interactions that define the secondary or tertiary structure of lipase. Additionally, lipase has the lowest average RMSD in methanol, which indicates that lipase experiences the least deviation in atomic positions in methanol compared to water and acetonitrile. While further mechanistic studies would be needed to identify whether this translates into greater catalytic activity, these initial MD simulations seem to indicate that the three-dimensional stability of the enzyme in methanol is more similar to its native aqueous environment, in comparison to a solvent such as acetonitrile, and this is consistent with the observation that lipase activity is highest in methanol compared to any other organic solvent.

It was observed that in 5% methanol, lipase activity was 28% higher compared to lipase activity in water. While low concentrations of methanol seem to accelerate the rate of enzymatic hydrolysis of the 4-nitrophenyl acetate ester bond, a trend of decreasing enzymatic activity with increasing concentrations of non-aqueous solvent was still observed. However, at all concentrations of organic solvent, enzymatic catalysis in methanol exhibited the greatest rate of deacylation of the substrate. Therefore, aqueous methanol solutions may be a desirable solvent system to use when conducting lipase-catalyzed deacylation reactions on organic compounds. The results from this research inform future efforts in involving lipase in the deacylation of other, similar substrates in a water-methanol co-solvent.

Materials and Methods

Chemical Synthesis

4-nitrophenyl acetate was synthesized via acetylation of 4-nitrophenol. 4-nitrophenol (1.00g, 1

eq., 7.2 mmol) was added to a round-bottom flask equipped with a Teflon stir bar, along with acetic anhydride (11g, 15 eq., 107.8 mmol), triethylamine (0.73g, 1 eq., 7.2 mmol), in ethyl acetate. The progress of the reaction was monitored to completion via thin-layer chromatography (TLC). Unreacted acetic anhydride was quenched with methanol, and the crude material was concentrated in a rotary evaporator. The crude product was extracted 3 times in ethyl acetate over saturated sodium bicarbonate to remove excess acetic acid. The combined organic layers were dried over anhydrous magnesium sulfate, concentrated *in vacuo* and purified on silica gel flash chromatography with a gradient of 0-25% ethyl acetate in hexanes to yield off-white crystals of 4-nitrophenyl acetate in 73% yield [Fig. 6A].

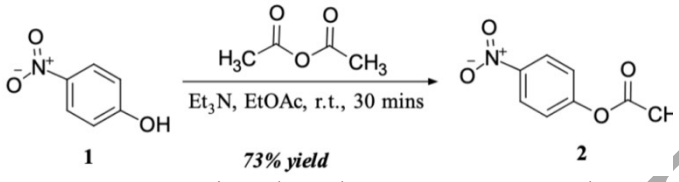

Figure 6A: 4-nitrophenyl acetate **2** was synthesized in 73% yield via acetylation of 4-nitrophenol **1**.

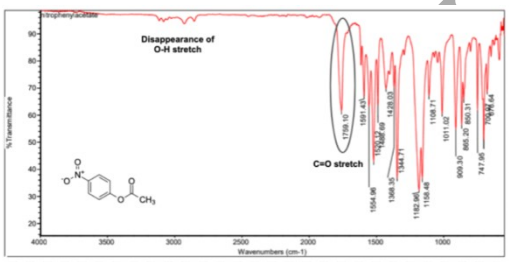

Figure 6B: FT-IR spectrum of 4-nitrophenyl acetate.

4-nitrophenyl acetate was characterized via ^{1}H NMR (Nanalysis NMReady 60 MHz ^{1}H NMR spectrometer in chloroform-D), Fourier-transform infrared (FT-IR) spectroscopy (Thermo Scientific iS5 Nicolet FT-IR spectrometer, iD5 ATR assembly) [Fig. 6B], and UV-vis spectroscopy (BioRad SmartSpec 3000 UV-vis spectrophotometer, quartz cuvette). 4-nitrophenol was characterized via FT-IR and UV-vis spectroscopy. ACS grade 4-nitrophenol was purchased from Reagent Inc. and used without further purification. ACS grade acetic anhydride was purchased from ChemSavers Inc. All solvents used were ACS grade.

4-nitrophenyl acetate: (^{1}H NMR, 60 MHz, $CDCl_3$): δ 7.21-8.34 (dd, 4H, J = 8.3Hz), 2.35 (s, 3H); FT-IR (ATR, cm^{-1}): 2900-3100 (Ar-H), 1759.10 (C=O), 1591.43, 1554.96, 1520.12, 1368.35.

4-nitrophenol: FT-IR (ATR, cm^{-1}): 3317.81 (O-H), 2900-3100 (Ar-H), 1612.64, 1586.58, 1486.66, 1319.95.

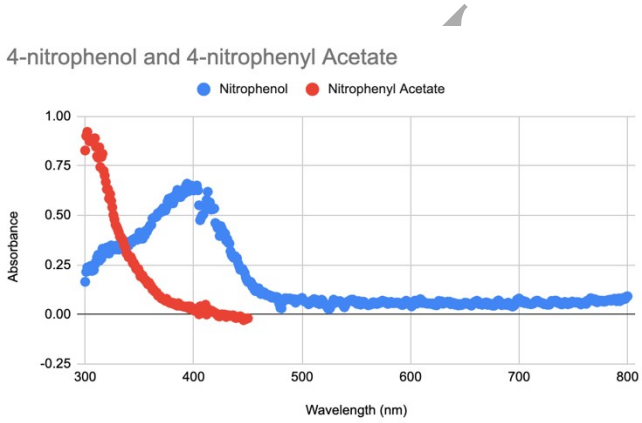

Figure 7: Overlaid UV-vis spectra of 4-nitrophenol (blue) and 4-nitrophenyl acetate (red).

Preparation of Enzyme Solutions

Enzyme buffers at pH 8 were prepared with 15 mM Tris base and 100 mM sodium chloride in deionized water. Then, solvent solutions of concentrations 5%, 10%, 20%, and 40% were prepared for each solvent (DMSO, Ethanol, Methanol, Isopropanol, Acetone, DMF, and Acetonitrile) in buffer. Pre-gastric lipase was then dissolved in each of the solutions to produce a (0.5 mg/mL) solution.

UV-Vis Spectrophotometry

A full UV-vis spectrum of 4-nitrophenol revealed two peaks: one around 413 nm and one at 310 nm. A UV-Vis spectrum of 4-nitrophenyl acetate revealed a peak at around 300 nm. We chose to track 4-nitrophenol at 413 nm because 4-nitrophenyl acetate does not absorb at that wavelength, which allowed for much simpler calculations when determining the hydrolysis kinetics of lipase [Fig. 7]. The extinction coefficients of 4-nitrophenol at 413 nm were determined via Beer's Law for each solvent system sampled.

A 1 mM solution of 4-nitrophenyl acetate in 5% DMSO (for the dissolution of nitrophenyl acetate) was prepared. 1 mL of substrate solution

and 1 mL of a buffered enzyme solution were mixed in a cuvette. To ensure that observed changes in absorbance during the experiments were a result of enzymatic hydrolysis, separate controls of substrate-only and enzyme-only solutions were prepared. The cuvettes were placed in a UV-vis spectrophotometer and tracked at 413 nm every 10 seconds for 3 minutes.

Molecular Dynamics

To quantify the stability of the enzyme in a non-aqueous environment, molecular dynamics simulations were performed to analyze lipase stability in non-aqueous solvent models. Molecular dynamics simulations of lipase (PDB: 1N8S) were conducted using GROMACS.[11,12] Solvent models for methanol and acetonitrile were used from the GROMACS molecule topologies website, and the water model was used directly from the software. The GROMOS96 43a2 forcefield was used, and the lipase was placed in a cubic solvent box at a distance of 1.0 nm from the solvent molecules. Energy minimization was then run to minimize the structure and clear clashes between the solvent atoms and lipase. Equilibration was run in two parts to reach the thermodynamic requirements of the structure. The final step was run in 50000 steps using Berendsen pressure coupling.

Molecular dynamics simulations were performed on a Dell Poweredge 710 server with a 24 core Intel Xeon X5660 processor @ 2.80GHz and 32GB RAM. Results were analyzed using Visual Molecular Dynamics and MDWeb Server.[13,14]

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Supplementary Information

SI-1 Molecular dynamics simulation of lipase (PDB: 1N8S) in water.

SI-2 Molecular dynamics simulation of lipase (PDB: 1N8S) in acetonitrile.

SI-3 Molecular dynamics simulation of lipase (PDB: 1N8S) in methanol.