ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DISSECTING THE EFFECT OF VISCOSITY OF DEEP EUTECTIC SOLVENTS ON THE STRUCTURE AND DYNAMICS OF THERMOSTABLE LIPASES

ZEYNEP KAVALCI

M.Sc. THESIS

DEPARTMENT OF BIOSTATISTICS AND BIOINFORMATICS

SUPERVISOR
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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

06.07.2023

Zeynep Kavalcı

PREFACE AND ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest appreciation to my advisor, Prof. Emel Timuçin for showing her constant support, sharing her broad knowledge, boosting unlimited motivation, and patience throughout my master's program. I was able to contribute to my work by considering her advice. Her expertise and encouragement helped me to complete this research, write this thesis and helped me progress step by step to become a scientist.

I am also grateful to Prof. Osman Uğur Sezerman for his kind support and immerse knowledge. Being able to take courses from him was amazing and very valuable. I would like to thank Dr. Aişe Ünlü for sharing her knowledge about wet lab with me and for her kindness and sincerity. I would also like to thank Assoc. Prof. Perinur Bozaykut Eker and Asst. Prof. Serkan Belkaya for showing kindness and being in my thesis jury. Thanks should also go to Dr. Mohamed Shehata. Thanks to his endless support and encouraging and motivating speeches, I was able to start this thesis and finish it well. I would like to extend my sincere thanks to you for helping me when I'm stuck and for always supporting me.

A special thanks to my friends Lara Naserikhojasteh and İlayda Amanoğlu. This would not be possible without your sweet prep talks and support. With our study dates, us staying up late, our tears and joy, we have come to this day. You were my consistent source of energy on this journey. I would like to show my appreciation to my dear friends Şimal Kayıkcı, Nisa Baltacı and Yağmur Poyraz for being close by when I needed some strong support. I would not have made it thus far in life without your motivation and assistance.

My words cannot express how much I am grateful to my parents, you have always served as my source of motivation and given me all I needed to achieve my goals. Thank you for all that you have done for me. I feel so grateful and blessed to have such supporters like you in my life.

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LIST OF ABBREVATIONS AND SYMBOLS

Å	Angstrom
API	Active Pharmaceutical Ingredient
Asp	Aspartic Acid
atm	Atmosphere
BTL2	*Bacillus thermocatenulatus* lipase 2
Ca^{+2}	Calcium Ion
CHARMM	Chemistry at Harvard Molecular Mechanics
ChCl	Choline Chloride
Cl^{-}	Chloride Ion
DCCM	Dynamic Cross Correlation Map
DES	Deep Eutectic Solvent
fs	Femtosecond
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
His	Histidine
K	Kelvin
MD	Molecular Dynamics
Na^{+}	Sodium Ion
NAMD	Nanoscale Molecular Dynamics
nm	Nanometer
NPT	Isothermal-Isobaric Ensemble
ns	Nanosecond
NVT	Canonical Ensemble
PDB	Protein Data Bank
RDF	Radial Distribution Function
R_G	Radius of Gyration
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SASA	Solvent Accessible Surface Area
Ser	Serine

TAG	Triacylglycerol
VMD	Visual Molecular Dynamics
Zn^{+2}	Zinc Ion

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ÖZET

Derin Ötektik Çözücülerin Viskozitesinin Termostabil Lipazların Yapısı ve Dinamiği Üzerindeki Etkisinin İncelenmesi

Lipaz, lipitlerin karboksilik ester bağlarının hidrolizini katalize eder. Bu enzim, katalitik karmaşıklığını gösteren, yalnızca tek bir aktif bölge ile çok çeşitli reaksiyonları katalize edebilir. Derin ötektik çözücü (DES), organik çözücülerin aksine toksik değildir, uçucu değildir, yanıcı değildir, ucuzdur, biyolojik olarak parçalanabilir ve hazırlanması kolaydır. Lipaz katalizli reaksiyonlar için etkili bir şekilde kullanılmıştır. Farmasötik, biyoyakıt ve zirai kimyasalların üretimi lipaz uygulamalarının örnekleridir. DES, bir hidrojen bağı alıcısı ve bir hidrojen bağı donörü karıştırılarak hazırlanabilir. Bir hidrojen bağı ağı oluştururlar ve bununla sıvı olarak kalır. DES, bir enzimatik reaksiyonda çözücü, substrat ve hem çözücü hem de substrat olarak kullanılabileceği için çok önemlidir. DES'ler çok viskozdur ve enzimlerin çalışması için esnekliğe ihtiyacı vardır. Bu çalışmada, aynı konsantrasyon oranlarına sahip iki tip DES (gliselin ve etalin) karşılaştırılmıştır. Bununla birlikte, aynı konsantrasyondaki farklı DES türlerinin lipazın stabilitesini nasıl etkileyeceği ve solvent yapısının nasıl değişeceği hesaplamalı yöntemlerle kontrol edildi. Sistemler hazırlandı, açık lipaz %100 gliserol, gliselin 2:1 ve 6:1, etalin 2:1 ve 6:1 içinde çözüldü. Ethalin bazlı DES, solvent viskozitesi ile lipaz hareketliliği arasında negatif bir korelasyon gösterirken gliselin bazlı DES, pozitif bir korelasyon gösterdi. Termoalkalofilik lipazlar, DES bazlı endüstriyel reaksiyonlarda umut vaat etmektedir. Bu tezin bulguları, çözücünün bileşiminin yanı sıra fizikokimyasal özellikleri uyarlayarak DES'lerde optimal lipaz stabilitesine ilişkin yeni bilgiler sağladı.

Anahtar Sözcükler: Termoalkalofilik Lipazlar, Derin Ötektik Solventler, Moleküler Dinamik Simülasyonlar, Viskozite, Termostabilite

ABSTRACT

Dissecting the Effect of Viscosity of Deep Eutectic Solvents on the Structure and Dynamics of Thermostable Lipases

Lipase catalyzes the hydrolysis of carboxylic ester bonds of lipids. This enzyme can catalyze a wide range of reactions with only a single active site, indicating its catalytic complexity. Deep eutectic solvent (DES) is non-toxic, non-volatile, non-flammable, inexpensive, biodegradable, and easy to prepare, unlike organic solvents. It has been effectively used for lipase-catalyzed reactions. Production of pharmaceuticals, biofuels and agrochemicals are examples of lipase applications. DES can be prepared by mixing a hydrogen bond acceptor and a hydrogen bond donor. They form a hydrogen bond network and with this, it stays as liquid. DES is very important as it can be used as a solvent, substrate and both solvent and substrate in an enzymatic reaction. DESs are very viscous, and enzymes need flexibility to work. In this study, two types of DES (glyceline and ethaline) are compared while having the same concentration ratios. With this, how different types of DES at the same concentration would affect the stability of lipase and how the solvent structure would change were checked using computational methods. The systems were prepared, open lipase was dissolved in glycerol 100%, glyceline 2:1 and 6:1, ethaline 2:1 and 6:1. Ethaline-based DES showed a negative correlation while glyceline-based DES showed a positive correlation between solvent viscosity and lipase mobility. Thermoalkalophilic lipases show promise in DES-based industrial reactions. Findings of this thesis provided novel insights into the optimal lipase stability in DESs by tailoring physiochemical properties as well as composition of the solvent.

Keywords: Thermoalkalophilic Lipases, Deep Eutectic Solvents, Molecular Dynamic Simulations, Viscosity, Thermostability

1 INTRODUCTION AND AIM

1.1 Lipases

Alongside plants and microorganisms ranging from yeast, bacteria to fungi, lipases can be found in all animal species (1). They are also known as triacylglycerol acyl hydrolases and are a member of serine hydrolase subgroup. As an enzyme that catalyzes the breakdown of the carboxylic ester bonds in lipids, which is refers to hydrolysis, lipases are classified as hydrolase enzymes. Given that it has just one active site yet can catalyze numerous processes, this enzyme is catalytically promiscuous (2). These qualities greatly increase its potential as biocatalysts. Christiaan Eijkmann made the initial discovery of lipases in the early 1900s. For the purpose of trying to break down lipids, he noticed that various bacteria could generate and secrete lipases into extracellular environments (3). After analyzing each lipase structure that has been identified to date, it can be said that all lipases have similar structural characteristics. A central beta sheet and connecting helices form the structure of the common alpha/beta hydrolase fold that is shared by all lipases (Figure 1) (4,5).

The lid domain in the catalytic cleft of *Bacillus thermocatenulatus* lipase plays a critical role in regulating the enzyme's activity and substrate specificity (6). The lid domain is a flexible loop or segment of the enzyme that covers the active site and acts as a "lid" to control access to the catalytic pocket. In the closed conformation, the lid domain shields the active site, limiting substrate accessibility and reducing enzymatic activity (7). However, upon interaction with specific substrates or ligands, the lid domain undergoes conformational changes, transitioning to an open state, which exposes the active site, allowing substrates to bind and initiate catalysis (6,8). This dynamic feature of the lid domain is essential for accommodating substrates of varying sizes and shapes and adapting to different reaction conditions. The regulatory role of the lid domain in BTL2 lipase ensures precise control over enzyme function, contributing to its versatility and efficiency in catalyzing a wide range of reactions, including hydrolysis and esterification.

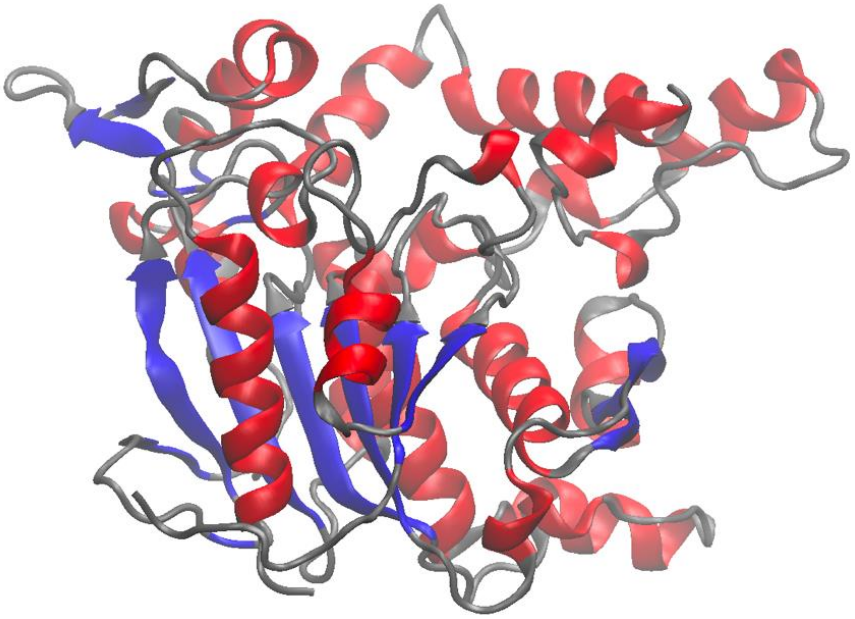

Figure 1 BTL2 open structure lipase (PDB ID: 2W22, 2.2 Å, R-factor = 0.182), represented in NewCartoon. Alpha helices are shown in red, beta sheets are blue and loops are gray.

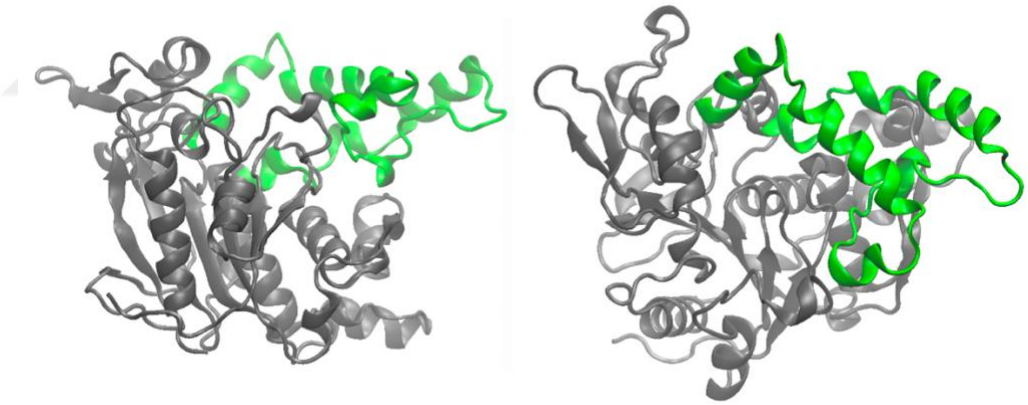

Figure 2 Two different views of the BTL2 lipase (2W22) structure. The lid domain is shown in green. The left panel shows the top side of catalytic cleft, the right panel shows the top view of the catalytic cleft.

1.2 Lipase Reactions

Lipids are composed of ester bonds, and ester bonds in lipids can be hydrolyzed (breakdown) or formed (synthesis) by lipases (9). Triglycerides, phospholipids, esters, and sterol esters are just a few of the many lipid substrates with which lipases can react

with (2). Depending on the enzyme source and nature, lipases have varying substrate specificities.

The breakdown and synthesis of lipids (Figure 3) are greatly influenced by lipase processes, which are critical for lipid metabolism, digestion, and different industrial applications (10). Despite having only a small amount of sequence homology, they share an alpha/beta hydrolase fold in the three-dimensional form (11). The variation among lipases is primarily caused by additional structural extensions with low homology that exist outside of this highly conserved fold of its central nucleus. The active region, consisting of residues in the oxyanion hole and a Ser-His-Asp catalytic triad, is most conserved (12). The catalytic triad of *Bacillus thermocatenulatus* lipase, an enzyme belonging to the family of serine hydrolases, plays a pivotal role in its enzymatic activity and substrate specificity. Lipase has a three-residue catalytic structure (a catalytic triad) in its structure, and this structure is of great importance (Figure 5). This catalytic triad consists of a serine, a histidine, and an aspartate or glutamate that functions as a nucleophile and is acidic (13). Serine 114 serves as the nucleophile, initiating the catalytic reaction by attacking the ester bond of the substrate, leading to hydrolysis. Asp318 acts as a general base, abstracting a proton from the hydroxyl group of Ser114, thereby facilitating the nucleophilic attack on the substrate. His359, in turn, acts as an acid-base catalyst, stabilizing the charge on the transition state during the reaction (6). The cooperative action of these three amino acids within the catalytic triad orchestrates the enzymatic activity of *Bacillus thermocatenulatus* lipase, allowing it to efficiently hydrolyze a wide range of substrates (6,12).

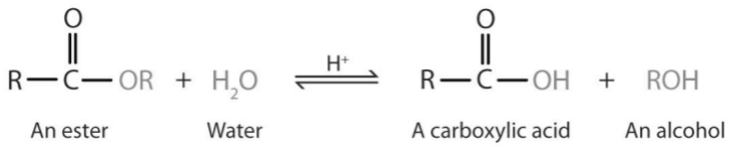

Figure 3 Hydrolysis and esterification reactions that are catalyzed by lipase.

1.2.1 Hydrolysis

Lipases catalyze hydrolysis reactions. In the hydrolysis reaction, the cleavage of ester bonds in lipids occurs with the addition of water molecules (14). The lipase

enzyme acts as a catalyst in this reaction and facilitates the breaking of the ester bond in the lipid molecule. As a result of this break, glycerol and free fatty acids are released (15). Hydrolysis processes are especially important during the digestive stages, as lipases are crucial to dissolving dietary lipids into components that can be absorbed (16).

1.2.2 Esterification and transesterification

The formation of ester bonds between a fatty acid and an alcohol molecule occurs through esterification and transesterification reactions, and lipase catalyzes these reactions. These processes, which are the reverse of hydrolysis, require getting rid of water molecules (17). In these processes, the lipase enzyme is responsible for condensing a fatty acid with an alcohol and forming an ester bond as a result. Making biodiesel, flavor esters, cosmetic esters, and other ester-based compounds are just a few examples of the many industrial operations that use these reactions (18,19).

1.2.3 Triacylglycerol

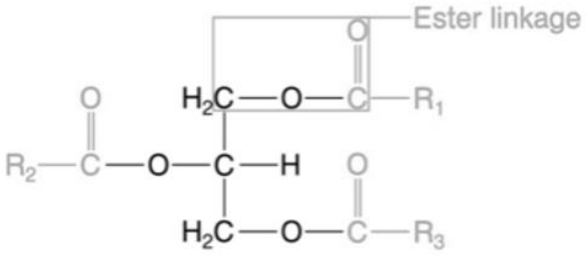

Figure 4 Structure of triacylglycerol (TAG).

Triacylglycerol (Figure 4), or TAG for short, is also known as triglycerides. It is also the most common type of lipid found in nature. As a highly important form of energy storage, it functions in many organisms, including humans (10). Triacylglycerol molecules consist of three fatty acid chains esterified into a glycerol molecule. Different results are possible depending on the chemical properties of the reactants and the presence of water in the environment, but triacylglycerols are their natural substrates (12). Understanding the structure and function of triacylglycerol is

of great importance, and by understanding it, lipid metabolism, energy balance, and the role of fats in the diet can be grasped. Triacylglycerols provide a concentrated form of energy and have a variety of physiological functions in the body (10).

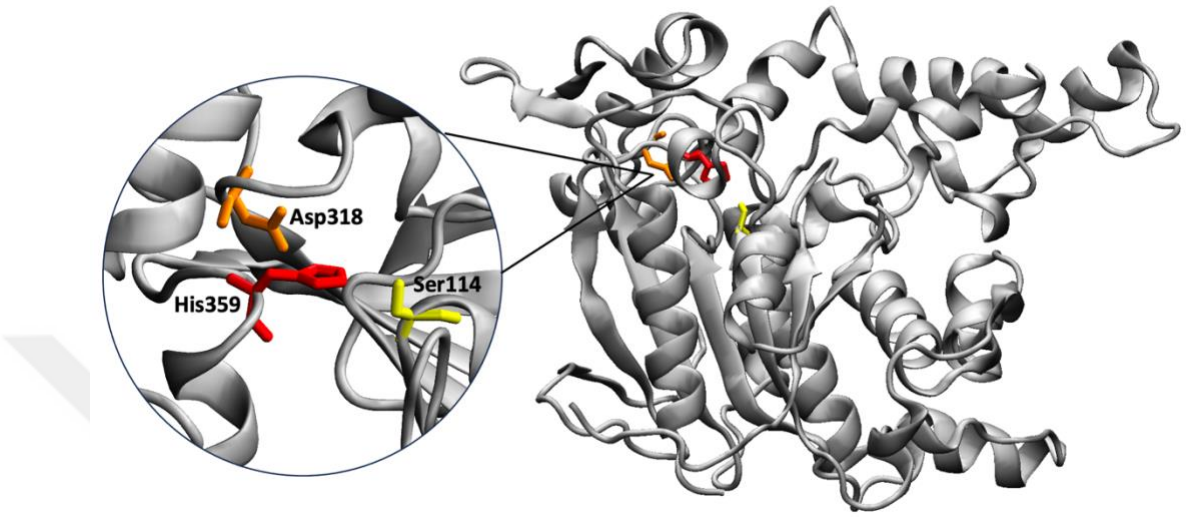

Figure 5 The catalytic triad, which is located in the active site of BTL2 lipase (gray). The residues Ser114 (yellow), Asp318 (orange), and His359 (red) are shown in licorice.

1.3 Thermoalkalophilic Lipases

Lipases are enzymes that catalyze the breakdown of fats and oils into glycerol and fatty acids. Thermoalkalophilic lipases are a special class of lipase enzymes that function best in environments with high temperatures and alkaline pH levels (7,20). Being able to have an optimal activity in high temperatures shows their thermophilic property, while having optimal activity in alkaline pH represents their alkalophilic feature. In reverse processes, lipases can also catalyze the formation of ester bonds. These enzymes can function and remain stable at above-normal temperatures, usually between 50 and 80 degrees Celsius and additionally, they typically work most effectively at alkaline pH levels between 8 and 10 (3,21). High temperature and alkaline environments are ideal for industrial applications of these enzymes. They are also renowned for maintaining exceptional stability in harsh conditions. They can survive at extreme pH levels, high temperatures, in and in the presence of organic

solvents, detergents and other denaturing agents, which denaturing is likely to happen for any other protein (21,22). This stability makes them valuable in various industrial processes. In addition to all of this, they have been extensively studied and modified through protein engineering techniques to enhance their stability, activity, and specificity (18). Mutagenesis, site-directed mutagenesis, and directed evolution approaches have been employed to create lipases with improved characteristics for specific industrial applications (23,24).

In the fields of biotechnology and industrial applications, there is a lot of interest in the thermoalkalophilic lipase of *Bacillus thermocatenulatus* called BTL2. This lipase, which comes from the bacteria *Bacillus thermocatenulatus*, has distinct qualities that make it different from other lipases. It is a thermoalkalophilic lipase and these lipases are extremely adaptable and useful in hostile environments because of their stability and activity throughout a broad range of temperatures and pH levels (9). This enzyme is ideally suited for a variety of industrial operations, including the creation of biodiesel, the formulation of detergents, and the processing of food, due to its capacity to function efficiently at high temperatures and an alkaline pH (3,22). Researchers continue to explore and optimize the properties of this lipase to unlock its full potential for industrial and biotechnological applications, aiming to harness its remarkable stability and catalytic efficiency for diverse practical uses (9).

1.4 Lipase In Industry

In order to enhance lipases' properties and broaden their industrial usage, a lot of research has been performed to gain insight into how their structure and function interact, (1). Due to their outstanding catalytic capabilities and affinity for lipid substrates, lipases are widely used in a variety of industries (18). These enzymes can overcome the challenges of sustainable and efficient processes, providing a range of benefits over traditional chemical approaches. The goal of ongoing studies and enzyme engineering is to improve lipase function and expand its industrial applications where a primary concern is product biocompatibility (25).

Examples of lipase applications include the production of medicines, biofuel, and agrochemicals (26). In order to obtain a renewable substitute for conventional diesel fuel, lipase enzyme can be utilized. Triglycerides found in vegetable or animal oils are converted by lipases to fatty acid methyl esters (biodiesel) and glycerol, in a procedure called transesterification. Lipases offer high catalytic activity and selectivity, enabling the efficient and sustainable production of biodiesel. Lipases are also essential ingredients in detergent compositions, particularly those used in laundry (27). They help remove greasy and oily stains from fabrics by hydrolyzing triglycerides into glycerol and fatty acids. Lipases have the ability to effectively remove oily stains, and by removing them, they help to clean the surface on which detergents act more effectively (28). These enzymes are also capable of being used to catalyze selective esterification, transesterification or dissolution processes in the manufacture of pharmaceutical intermediates and active pharmaceutical ingredients (APIs) (29).

The diverse applications of lipases in these industries demonstrate their versatility and interest as biocatalysts in a variety of situations. Lipases provide significant benefits over older chemical approaches, making operations more efficient and sustainable (30).

1.5 Green Solvents

Green solvents are also known as environmentally friendly solvents or sustainable solvents. It is a type of solvent that is arranged to work with minimum negative impact when it comes to both the environment and human health (31). These solvents have almost negligible toxicity, which is why they are preferred to other dangerous and volatile solvents that have a very bad effect on ecosystem and human health (32). They are generally non-carcinogenic, non-mutagenic or non-toxic to aquatic organisms, and because green solvents have low vapor pressure, they evaporate into the atmosphere more slowly (33). These features increase worker safety, reduce emissions, and reduce the risk of air pollution. Green solvents can naturally decompose into harmless compounds as they are biodegradable. The biodegradability of these solvents guarantees that they do not remain in the environment, reducing their effect on the

ecosystems. They can be produced from renewable resources such as agricultural waste and biomass (34).

Other than green solvents, organic solvents are effectively used in the synthesis of industrially useful compounds due to its lipase catalysis benefits in organic solvents (2,9). The benefits of organic solvents as a reaction medium for lipase-catalyzed processes cannot be ignored, but there are many disadvantages that limit their industrial usefulness and complicate their use. Organic solvents unfortunately are not environmentally friendly, as they can be flammable, poisonous, non-biodegradable and volatile, on the contrary, they can be quite dangerous due to these properties (35). In addition, they produce environmentally harmful chemical waste that cannot be ignored. Taking all this into account, the cost of lipase reactions increases due to the laborious and expensive nature of organic solvents (34,35).

The use of renewable energy sources lessens the dependence on fossil fuels and helps support sustainable resource use. Green solvents are generally designed to operate at lower temperatures and pressures, so they consume less energy during production (36). Reducing energy demand helps ensure overall ecological sustainability. Green solvents are becoming more common in a variety of industries, including pharmaceuticals, paints and coatings, cleaning products, agriculture, and others (37). Green solvent creation and application remain important areas of research and innovation for solving the sustainability problems of the chemical industry (9).

1.6 DESs

Deep eutectic solvents (DESs), a class of ionic liquids, are widely used in biocatalysis procedures (32). They are cheaper and more environmentally friendly substitutes for organic solvents. Deep eutectic solvents are a different class of solvents created by the precise coupling of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) (9). HBDs can be quaternary ammonium salts, amines, alcohols or organic acids, while metal salts, halides or other chemicals can be used for HBAs (2). In Figure 6, two different types of DES are shown. DESs can be used as solvents or

reagents in a variety of industrial applications and are a major focus of interest in sustainable solvent systems and green chemistry (38).

Following the development of DESs in 2001, they were mostly used in electrophoretic deposition (39,40). When the two components, HBD and HBA, are combined in precise proportions, they produce a eutectic mixture with a lower melting point than the individual components (39,41). Since it has this bizarre characteristic, DES can remain in a liquid state at relatively low temperatures. Given the occurrence of a thorough network of durable intermolecular hydrogen bonds among its components, DESs have the ability to remain liquid even after cooling the reaction to ambient temperatures (42).

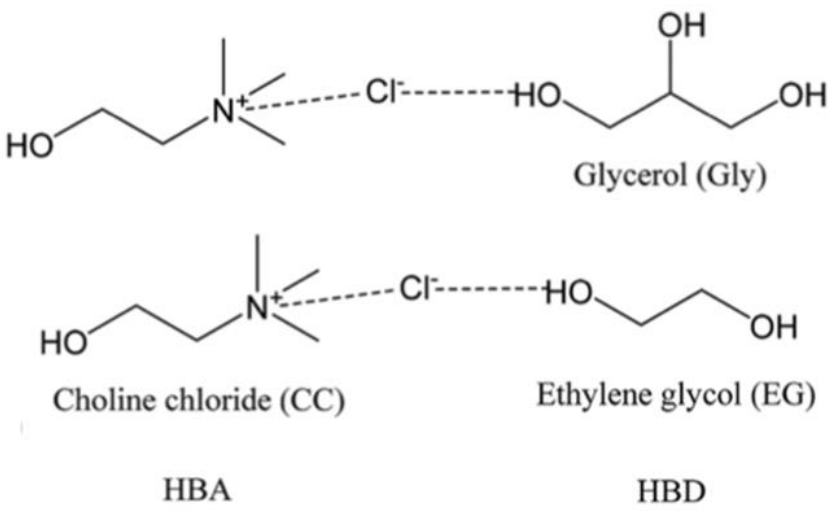

Figure 6 Two different types of DES, glyceline (glycerol-based DES) and ethaline (ethylene glycol-based DES), are shown as 2D structures. Choline chloride is the hydrogen bond acceptor (HBA), glycerol and ethylene glycol are the hydrogen bond donor (HBD).

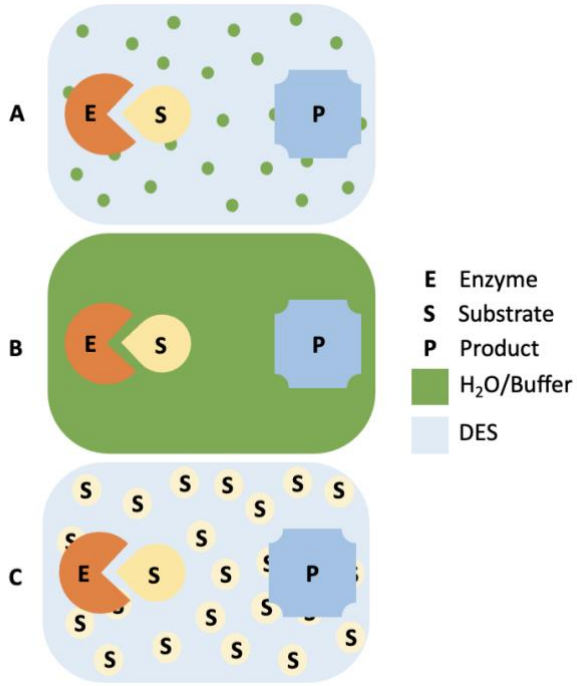

Figure 7 DES application types for biotransformation processes. (A) DES as co-solvent. (B) DES as reaction medium. (C) DES as 2-in-1 reaction medium.

Conflicting with standard solvents, DES is usually made from naturally occurring and biodegradable components, causing them to exist as environmentally beneficial substitutes (43). DESs are easy to produce, cheap, non-volatile, non-flammable, non-toxic, and biodegradable (44). These solvents have gained a lot of attention in recent years due to their unusual properties and possible uses in various industries. DES can be created in different kinds of ways, accomplishing in having the senses of adjustability and versatility. DES aspects have the ability to be adjusted to suit specific uses through selecting numerous combinations of HBD and HBA and varying their ratio (45). However, its most widely recognized disadvantage is its high viscosity (46,47). Deep eutectic solvents remain a case of current research, with a continued examination of their characteristics, uses, and potential benefits over conventional solvents. Many industries seeking more environmentally friendly and sustainable options find them attractive because of their distinctiveness and different possible applications for biotransformation (Figure 7) (38).

1.7 Aim

Understanding how lipases behave in DES and how the physicochemical properties of the DES solvents affect the lipase's stability and activity is crucial to the efforts of successful industrial applications of lipases. It is the aim of this study to meet the need to examine how *Bacillus thermocatenulatus* (BTL2) lipase stability and activity respond to different formulations of DES at different concentration levels which result in different viscosity values.

2 BACKGROUND

2.1 Lipase Reactions In DESs In Industry

The tunable properties of DES provide the potential to generate a wide range of DES with varying effects on a biocatalytic reaction system. The decision on the combination of HBAs and HBDs has a significant impact on the entire procedure (32).

Decreased enzyme stability and restricted reusability are few of the issues that conventional organic solvents that are employed in this process face. DES can improve the enzyme activity, increase the enzyme reusability and reaction rate by solubilizing and stabilizing lipases with its unique features (47). Green and sustainable chemistry practises can be promoted with DES and combination of lipases (2). DES is known for its biodegradable solvent grade and environmentally friendly properties (38). Hence, DES is important for decreasing the impact on environmental hazards caused by chemical processes. Efficient biocatalytic transformations are impacted by lipase solvation in DES resulting in employment of enzymatic processes instead of traditional chemical catalysts. Synthesis of fine chemicals and pharmaceutical intermediates, greatly depend on the lipase family for the most cases. Increased catalytic activity, improved stability and selectivity are few of the benefits of application of DES (2,9). In addition, racemic mixtures resolving, efficient and selective synthesis of chiral compounds and performing regio- and enantioselective transformations are enabled by lipase solvation in DES (8). With reasons listed above, utilization of lipase solvation in DES contributes to industrial applications by turning lipases into more selective, stable, active and reusable proteins. Also, properties that are special to DES assure more sustainable and environmentally friendly processes.

Solving lipase in deep eutectic solvents (DES) is used in many industrial applications and industries, and this use offers the industry many benefits and opportunities (38). The lipase enzyme catalyzes the esterification and transesterification processes, as well as a reaction that takes place in industry, such as the hydrolysis of lipids (48). This enzyme showed improved performance and stability

when dissolved and used in DES. For example, lipase-catalyzed transesterification is a frequently used technique for biodiesel production.

2.2 Previous Reports On Lipases And DESs

As the production of organic solvents became more and more expensive and the need for organic solvents in the industry increased, researchers sought solvents that were similar to organic solvents, less costly, green, easily and quickly produced to meet this need (49). The first application of DES was performed by Abbot et al. in 2003 (39).

Often investigated in an organic medium, lipases are popular choices for enzymatic reactions carried out in DESs. The lipase-catalyzed biotransformation reaction in DESs was first tested by Kazlauskas et al. in 2008 (48). They investigated the potential of certain lipases in the transesterification of ethyl valerate with butanol in various types of DES in this research. In conclusion, the outcome demonstrated that enzymes are mainly active in deep eutectic mixtures. Regardless of having a higher viscosity, mixtures of eutectic glycerol with choline chloride (also known as glyceline) were as effective as toluene in transesterification of ethyl valerate with 2-butanol. Subsequently, Zhao et al. validated this keenness to DES usage as an alternative solvent structure to organic solvents for biocatalysis reactions where the enzyme is lipase (43,50,51). Their study pointed out the worthiness of future researches of DES as a green solvent for enzymatic transesterification and thus biodiesel production (43). In this study, it was observed that the researchers evaluated the effect of the molar ratio of salt to glycerol on the activity and selectivity of lipase. As a result, it was found that reducing the glycerol molecule ratio in DES content caused a decrease in the activity values of lipase (43). As another example of DES researches, in 2012, Durand and his colleagues worked on the capability of DESs as a solvent environment for biocatalysis reactions using lipase as the enzyme (52). As a result of their studies, it was observed that the characteristics of the nucleophilic substrate as well as the components that make up the DES structure, namely ammonium salts and hydrogen bond donor) affect the reactivity performance in DES (43).

3 MATERIALS AND METHODS

3.1 Structure Preparation And System Generation

Crystal structure with clear and active conformational characteristics was obtained from thermoalkalophilic lipases (PDB ID: 2W22, 2.2 Å, R-factor = 0.182). The water and ligands in the obtained crystal structure were cleaned, but Zn^{2+} and Ca^{2+} ions were left without any restrains. It was then protonated via VMD to match the physiological pH of 7.0. As shown in Figure 8, the lipase crystal structure was placed in the center and solvated in cubic simulation boxes with 60 Å in all directions (x, y, z) created using PACKMOL (53).

Four lipase systems that were solved in two different DESs with different ChCl (choline chloride) molar ratios that resulted in different viscosities. In addition, we have generated another system which consists of only glycerol and lipase and this system will be used as a control. A total of 5 different systems were obtained, whose solvents were prepared in different concentrations with lipase in the center: 100% glycerol solvent, 2:1 glyceline-based DES solvent, 6:1 glyceline-based DES solvent, 2:1 ethaline- based DES solvent and 6:1 ethaline-based DES solvent. Since the ability to obtain eutectic nanostructures of glyceline and ethaline has an important place in this study, the distance between the components of both DES types was determined as 3.0 Å. This distance is also important to prevent any aggregation in the solvent structure and is determined to allow translational and rotational movements during balancing. Afterwards, the systems were neutralized at pH 7.0 using Na^+ and Cl^- counterions. Detailed information about the systems created is given in Table 1.

Table 1 Details of lipase solvated in DES.

Number of Residues (Number of Atoms)						
System	**Protein**	**ChCl**	**Glycerol**	**Ethylene Glycol**	**Ca^{+2}**	**Zn^{+2}**
Glycerol 100%	388 (5986)	0 (0)	8000 (112000)	0 (0)	1 (1)	1 (1)
Glyceline 2:1	388 (5986)	3000 (66000)	6000 (84000)	0 (0)	1 (1)	1 (1)
Glyceline 6:1	388 (5986)	1250 (27500)	7500 (105000)	0 (0)	1 (1)	1 (1)
Ethaline 2:1	388 (5986)	3000 (66000)	0 (0)	6000 (60000)	1 (1)	1 (1)
Ethaline 6:1	388 (5986)	1250 (27500)	0 (0)	7500 (75000)	1 (1)	1 (1)

3.2 Molecular Dynamics Simulations

Molecular dynamics (MD) simulations of a thermostable lipase (BTL2) in two different DES compositions formed by the choline/chloride (ChCl) as the hydrogen bond acceptor and either glycerol or ethylene glycol as the hydrogen bond donor were implemented. Energy minimization of 5 different systems with the open conformation of lipase was performed with the default minimizer in NAMD (54). This minimizer uses conjugate gradient and line search algorithm in principle, also the system was equilibrated with NVT (Canonical Ensemble) and then NPT (Isothermal-Isobaric Ensemble) ensembles. The minimization of the systems was determined to be 40000 steps, followed by an equilibration (NVT) of 0.25 ns (nanosecond). In this process, the atoms in the lipase structure were fixed. Then, the minimization and equilibration steps were repeated without fixing the lipase atoms. After all these steps, NPT was applied to the systems with a Langevin temperature of 298K and a Langevin pressure of 1 atm for 10 ns. The reason behind carrying out the NPT was to ensure the stability of the RMSD pattern of the backbone of all systems. CHARMM36 was used when applying force fields on each and every system (55). Timestep is set to 2 fs (femtosecond) and

periodic boundary conditions are applied in all dimensions. Production simulations were run using NAMD 2.14 and run for 300 ns at two different temperatures for each system, 298K and 373K.

3.3 Trajectory Analysis

The trajectories were analyzed to assess how the DES composition and alternating molar ratios affect the lipase structures. The residues of thermostable lipase structure (BTL2) were analyzed with the Root-Mean-Square-Fluctuation (RMSF) numerical measurement to investigate how the under different viscosity conditions are affected. Radius of Gyration (R_G) values was examined to observe changing center of mass of the protein structure for 300 ns. Solvent-Accessible Surface Area (SASA) values were calculated for the surface area of the thermostable lipase structure. Pairwise RMSD analysis was performed to compare all the frames in our structures. Plugins in VMD and related Tcl scripts were used for RMSF, R_g, SASA and RDF (Radial Distribution Function) analyzes (56). The MDAnalysis package was used for Pairwise RMSD (2D-Root Mean Square Deviation) analysis (57). PCA (Principal Component Analysis) and DCCM (Dynamic Cross Correlation Map) were done using an R package, Bio3D (58). Raw and normalized hydrogen bond count are calculated for protein-solvent and solvent-solvent. Counts of solvent molecules interacting with protein and active site were calculated and afterwards, the number of unique DES molecule number interacting with protein and active site were estimated.

4 RESULTS

4.1 Open Lipase Structures In DESs

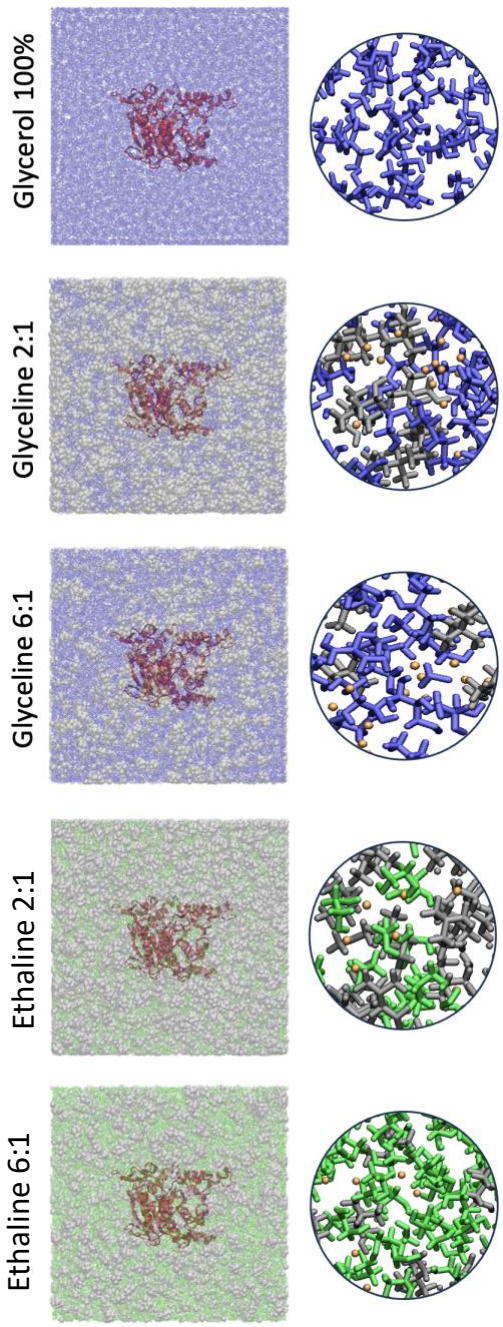

Figure 8 Snapshots of the MD systems are shown after PACKMOL, before MD simulations. The circle illustrations focus on the close-up representation of the solvent structure. Blue molecules are glycerol, green ones are ethylene glycol, gray molecules are choline, and the orange dots represent chloride ions.

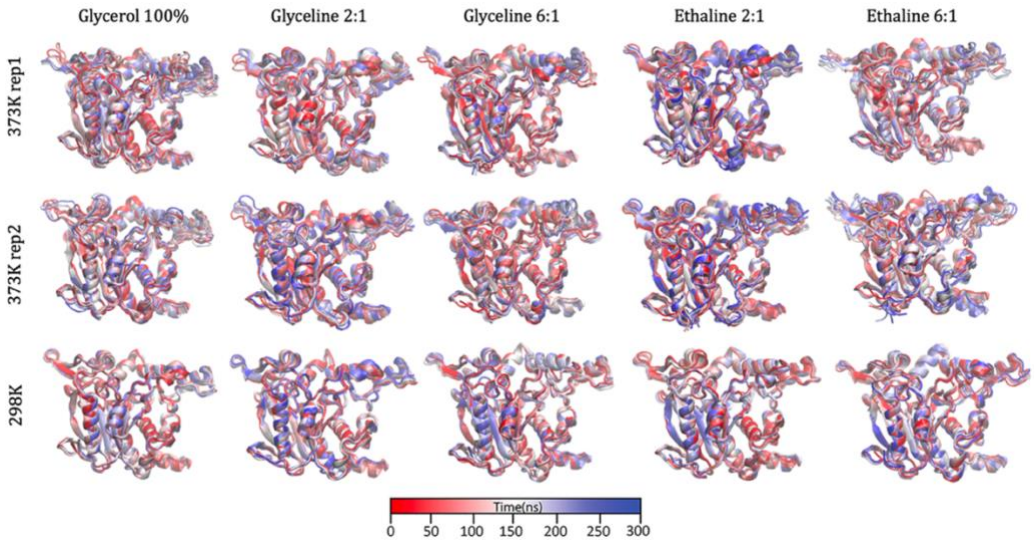

Figure 9 Reduced trajectory of the production simulations of BTL2 lipase (PDB ID: 2W22) performed at 298K and 373K (for 373K there are 2 replicated systems, rep1 and rep2).

To comprehend how the open structure of BTL2 lipase (PDB ID: 2W22) is affected, a total of 15 MD simulations were run for 300 ns. 10 of them were performed at 373K as 2 replicas, rep1 and rep2, and 5 of them were performed at 298K. In Figure 9, the reduced trajectories of the MD simulations are shown. Red color indicates start of the simulation and blue color indicates the end. Simulations were lasted for 300 ns. By looking at these, the movements of lipase can be observed. The lid domain of the lipase structure mainly has more movement in the higher temperature, 373K, compared to 298K.

4.2 Backbone Mobility

Looking at the pairwise RMSD results (Figure 10), it is seen that there is the most movement in the ethaline 6:1 system. Mobility is also observed in the glycerol 100% system and the glyceline 6:1 system. If the backbone mobility of the systems is compared in terms of temperature, the movement in the systems operated at 373K is more than the movements at 298K, respectively. Backbone mobility has progressed stably in other systems. Replicated simulations (rep1 and rep2) for the 373K were run to ensure consistency at the 373K and demonstrated the stability of the results.

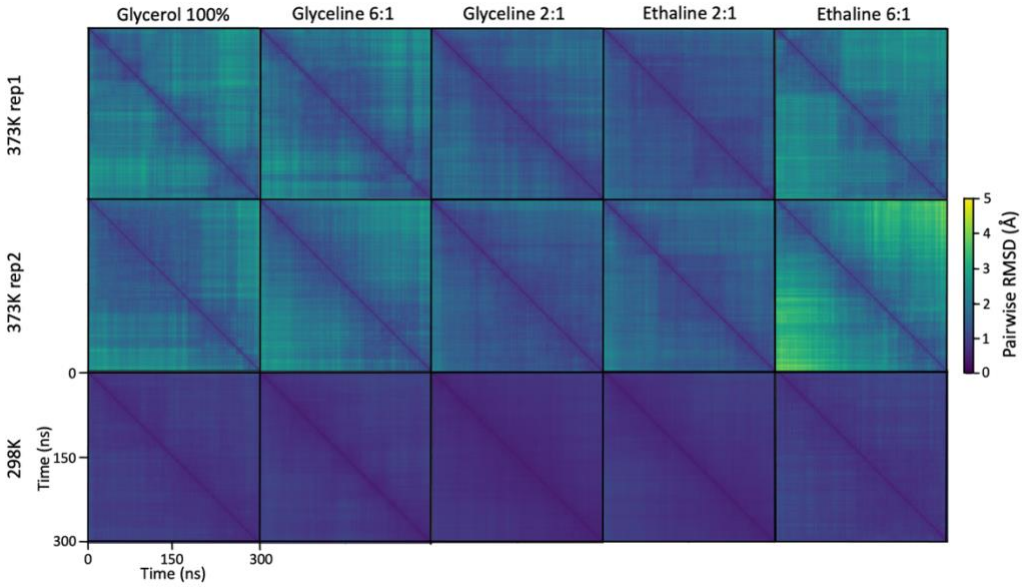

Figure 10 Pairwise RMSD graphs of the systems at two different temperatures.

The fluctuations of the backbone C-alpha atoms were examined in order to gauge how flexible the lipase backbone is in response to the change in solvent structure. Higher fluctuations are naturally expected at high temperature. When the RMSF graphs are examined (Figure 11), it is understood that the temperature with the highest fluctuation is 373K and there is activity in the lid domain. Overall, looking at the 298K values from both pairwise RMSD and RMSF graphs, the mobility is significantly low (<2Å) compared to the 373K. The mobility in the glyceline systems and the ethaline 6:1 system is greater than in other systems, which supports pairwise RMSD results.

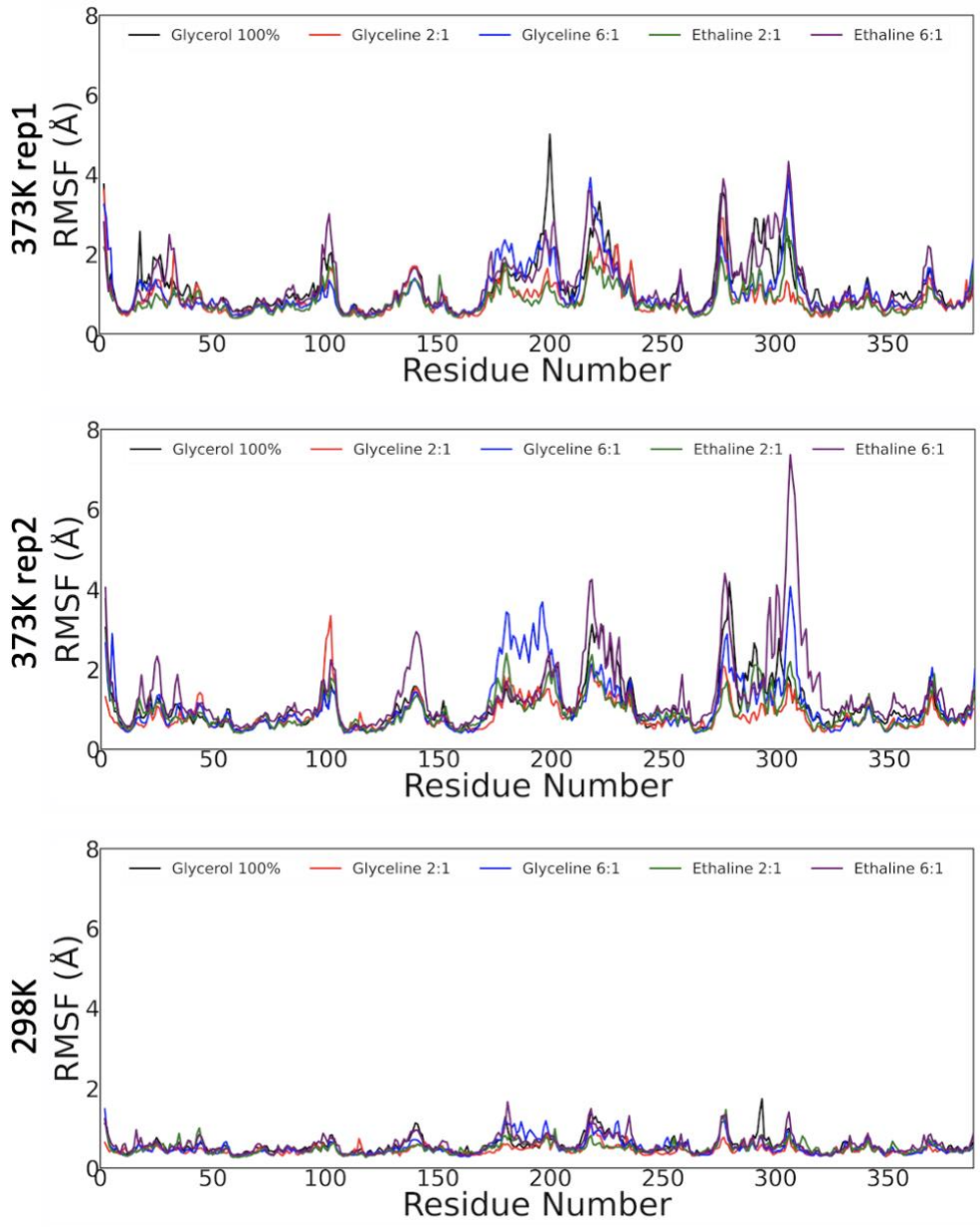

Figure 11 RMSF graphs of the systems at two different temperatures, 373K and 298K.

4.3 Shape Analysis

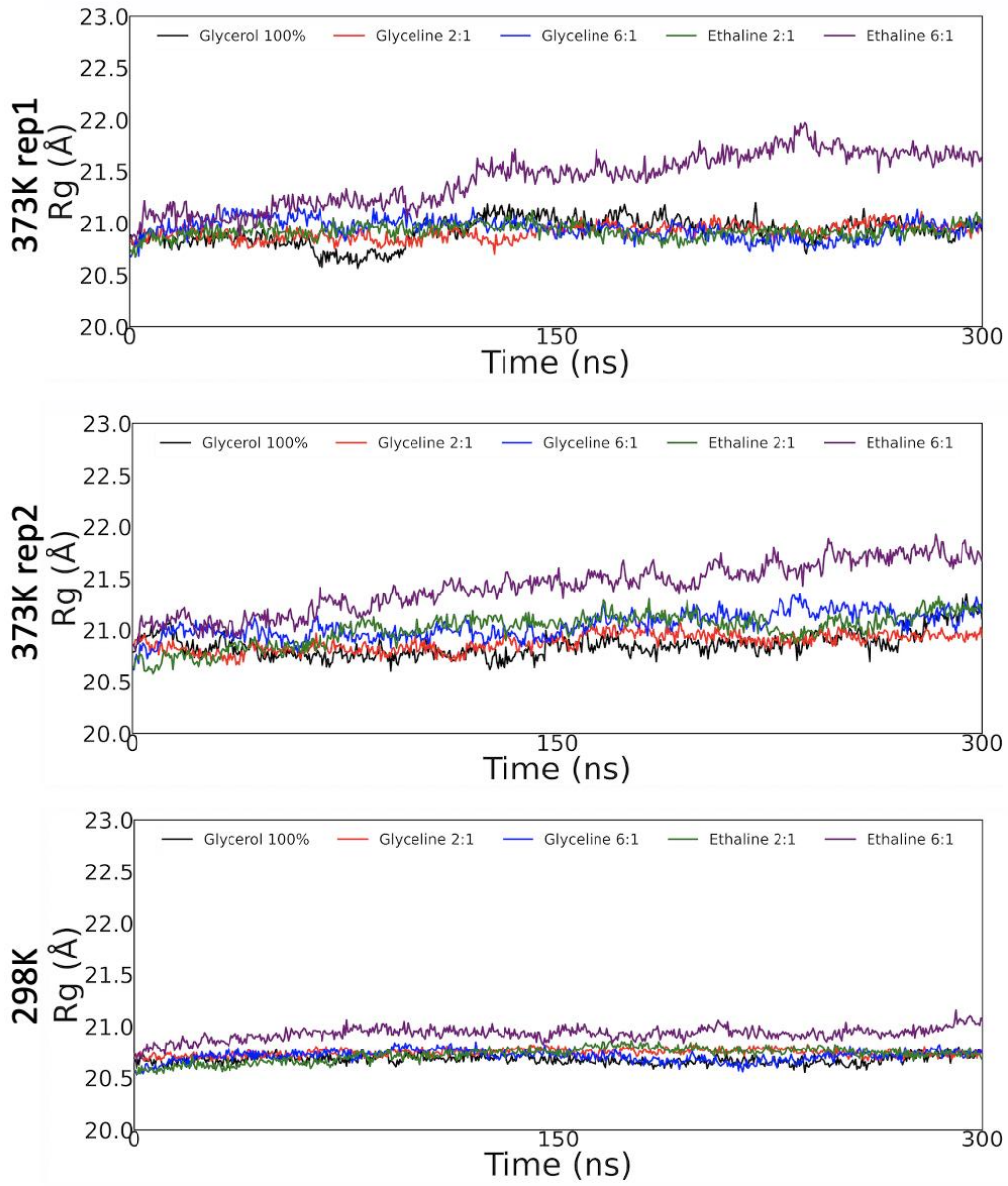

Figure 12 Rg of the systems at two different temperatures, 373K and 298K.

Radius of gyration (Rg) is evaluated for inspecting the lipase compactness in different solvents (Figure 12). In both temperatures, ethaline 6:1 has higher values, however in 373K, it has increased Rg profile. Still, lipase remained its packed conformation in spite of the high temperature.

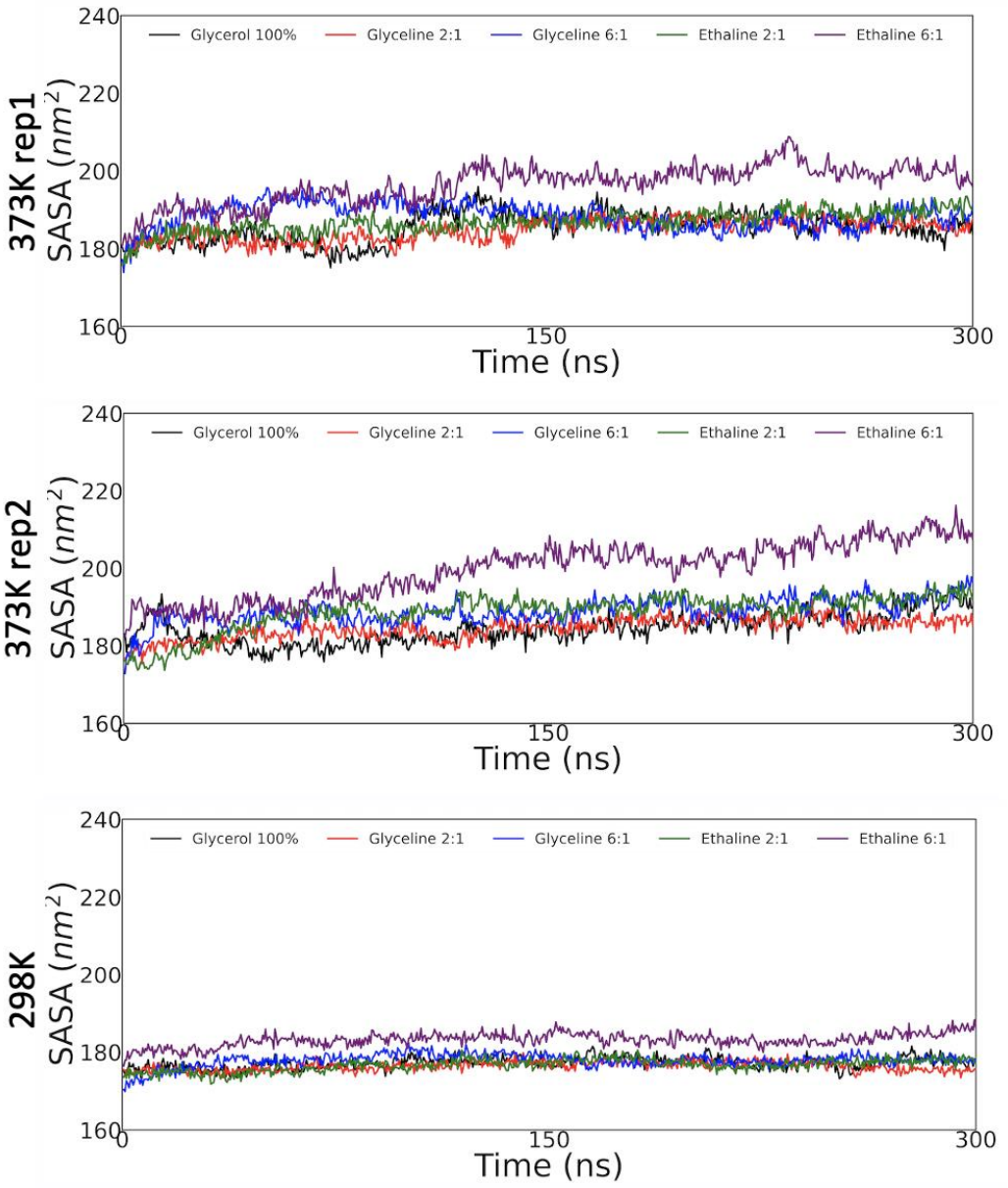

Figure 13 SASA graphs of the systems at two different temperatures, 373K and 298K.

By calculating solvent accessible surface area (SASA), the response of the lipase structure to different solvent constructions is measured (Figure 13). At low temperature, the lipase structure has slightly less SASA in all solvents than at high temperature. Again, ethaline 6:1 system has a higher value, as it has in Rg.

4.4 Center Of Mass Radial Distribution Function

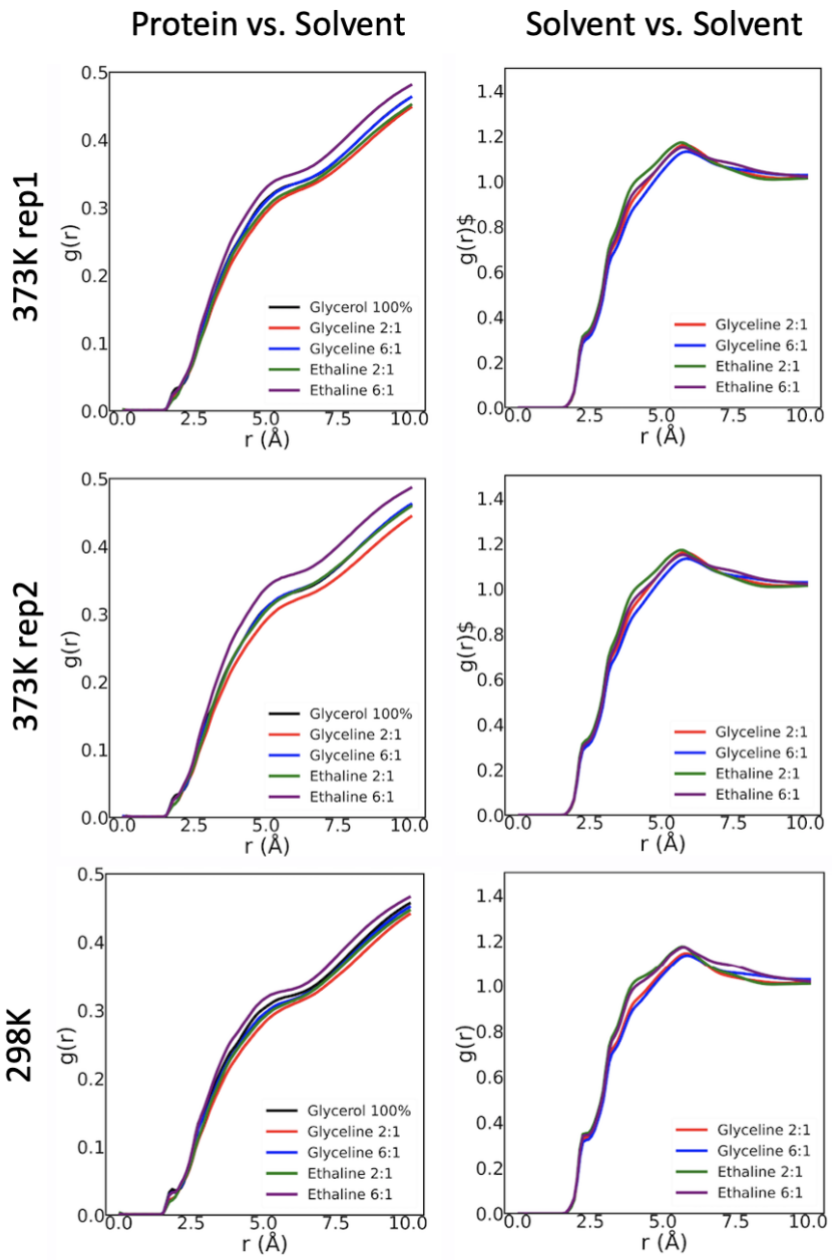

Figure 14 COM-COM RDF graphs of the systems at two different temperatures, 373K and 298K.

COM-COM RDF (Center of Mass Radial Distribution Function) analysis can be interpreted to calculate how many molecules there are when you integrate the area underneath. The distribution of solvent around lipase was investigated by computing the center of mass radial distribution functions (COM-RDF) to investigate how DES

composition and temperature impact the interactions between lipase and solvent. In the protein-solvent plot, the value approaches 1 as the simulation time progresses, which is to be expected, which means that the solvent molecules are surrounding the protein.

The highest g(r) value belongs to ethaline 6:1 system, regardless of the temperature. Ethaline 6:1 spends more time around protein, more likely to be there. The lowest is glyceline 2:1, spends less time around protein. The solvent nanostructure could be more intact in ethaline 2:1 and glyceline 2:1, so they don't spend a lot of time surrounding protein, when looking at around 2.5Å of the protein. Because there is a small peak at 2.5Å, it can be observed that the molecules are gathered there.

Furthermore, the following peaks were greater in size in ethaline 6:1, glycerol 100%, and glyceline 6:1 than in ethaline 2:1 and glyceline 2:1. In the case of ethaline 6:1, glycerol 100%, and glyceline 6:1, these data imply that the solvent molecules diffuse into the lipase core. The solvent structure does not change much in solvent-solvent graph, at least in terms of glyceline and ethaline.

4.5 Essential Dynamics Analysis

Principal Component Analysis (PCA) was performed on all trajectories to calculate the essential dynamics of lipase in different solvents (Figure 15). PC1 and PC2 explains an average of 40% of the lipase motion variation. PCA of ethaline 6:1 spanned the largest conformational space in 373 K rep2. Notably, glyceline 6:1 and glycerol 100% come second in terms of conformational space span.

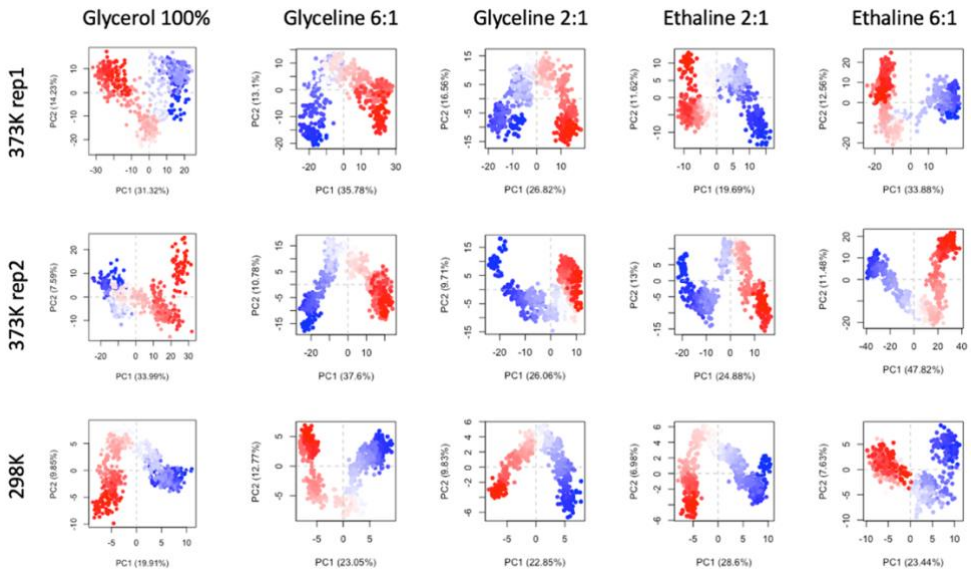

Figure 15 Principal component analysis (PCA) of lipase in different solvents.

4.6 Correlation Of Lipase Motions In Different Solvents

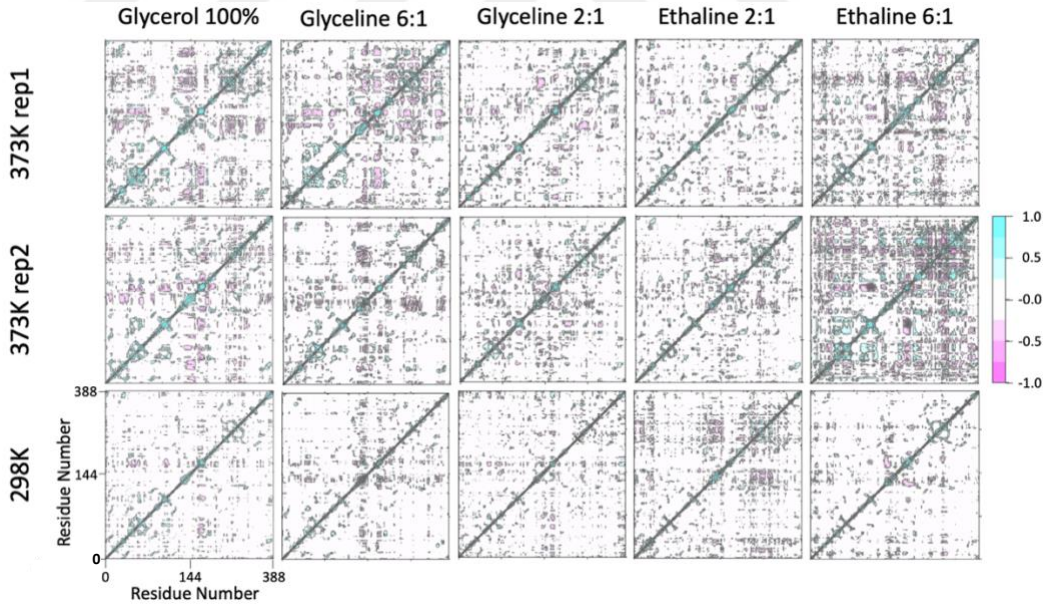

Figure 16 DCCMs of lipase structure in different solvents.

DCCMs are created to analyze the correlation of lipase movements in different organic solvents (Figure 16). A lot of movement is observed in ethaline 6:1 and glyceline 6:1 at high temperature. No movement is observed at low temperature as much as at high temperature. These results are parallel with pairwise RMSD.

4.7 Analysis Of Intermolecular Interactions

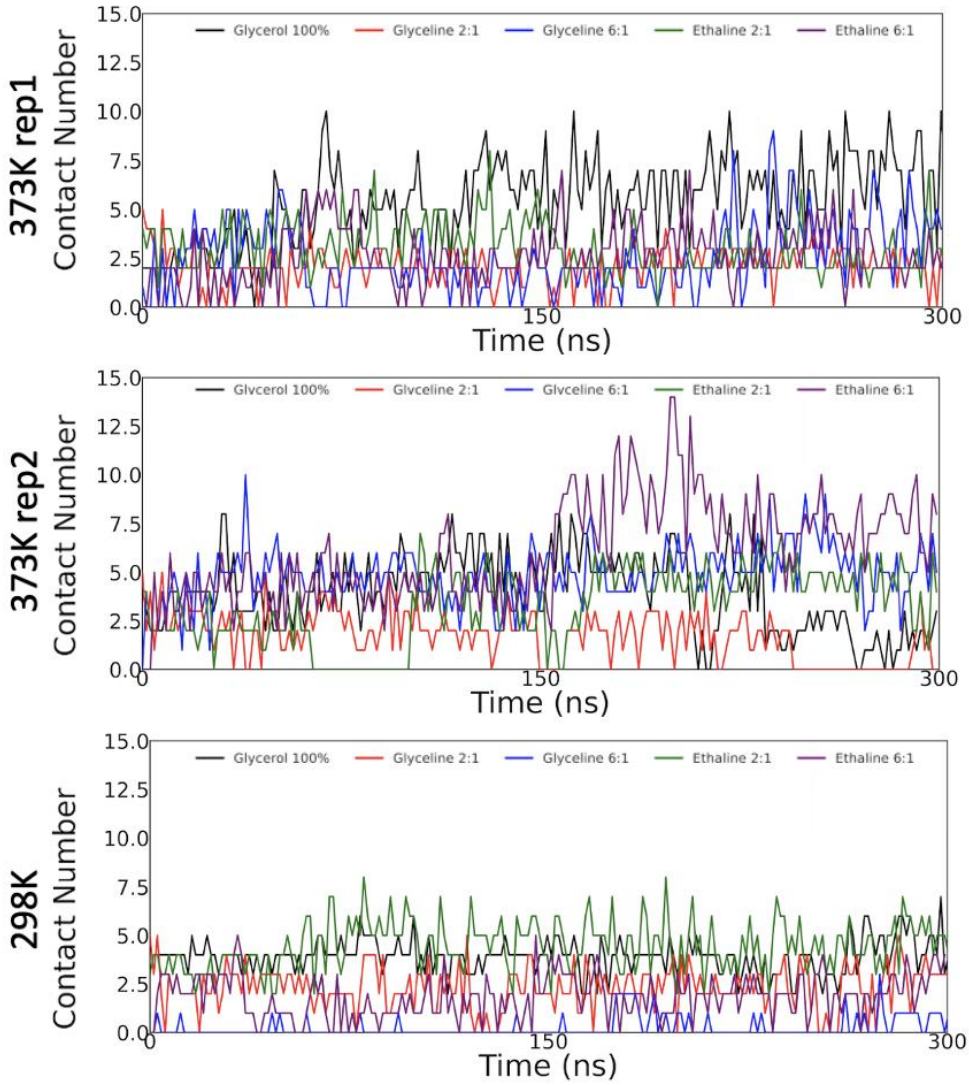

Figure 17 Contact number between solvent molecules and BTL2 lipase active site.

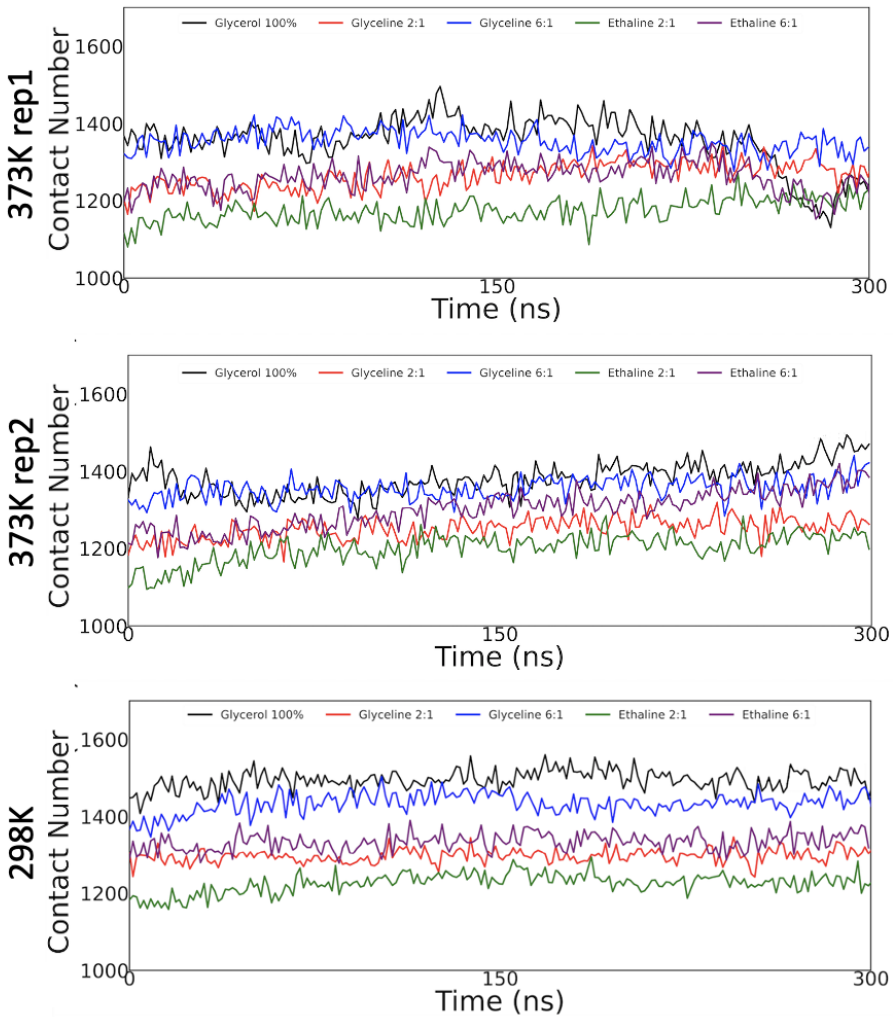

Figure 18 Contact number between solvent molecules and BTL2 lipase.

The number of solvent atoms interacting and contacting with the active site are shown in Figure 17. The interaction rate got higher when the temperature increased. In both replicas of 373K, glycerol 100% and ethaline 6:1 count is higher than other systems. At 298K, conversely, ethaline 6:1 is lower and ethaline 2:1 became the highest of all. Looking at the active site values from the contact number graphs, ethaline 2:1 at 298K is a bit intrusive. In 373K rep2, ethaline 6:1 appears to be the most interacting here, and it's the system that makes the most contact. 373 rep1 has high glycerol 100% values, but ethaline 6:1 is also high. Other systems avoided being on the active site. In Figure 18, solvent count interacting and contacting with protein is shown. At both temperatures, glycerol 100%, glyceline 6:1 and ethaline 6:1 have higher count.

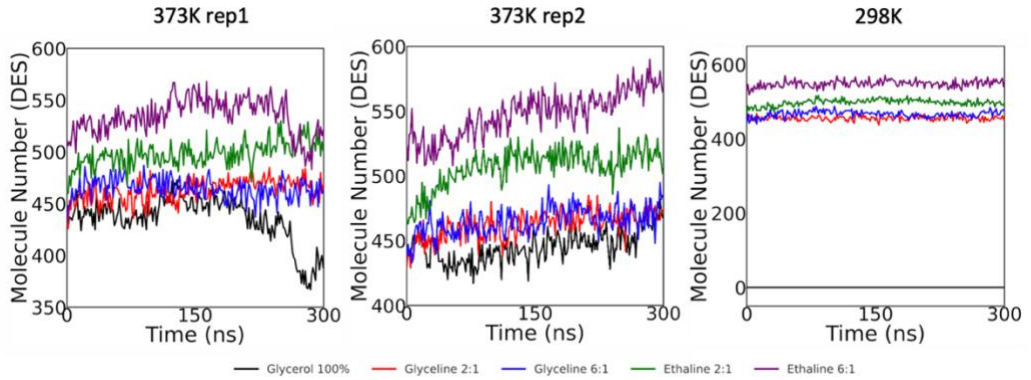

Figure 19 Number of solvent molecules in close contact with protein.

To further inspect the diffusion and distribution of solvent molecules to the lipase core, the number of solvent molecules in the immediate vicinity of the protein was calculated. In Figure 19, the values of the ethaline 6:1 and glycerol 100% systems decreased towards the end of the simulation. In the remaining systems, there is an overall increase, but in any case, the ethaline 6:1 system has the highest value.

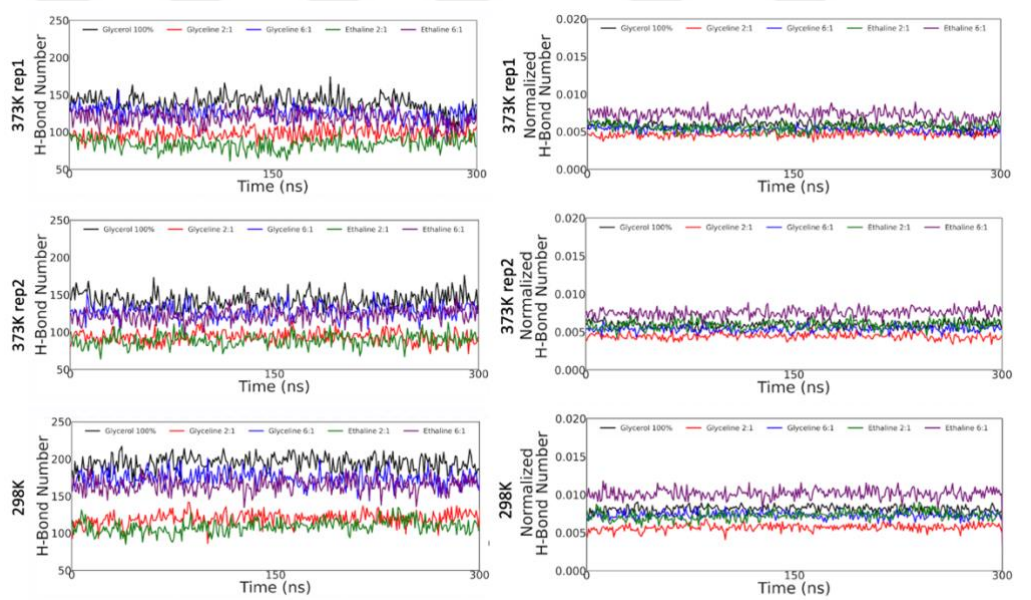

Figure 20 Raw and normalized hydrogen bond numbers of protein-solvent, in two different temperatures.

When the protein-solvent hydrogen bond number graphs are examined (Figure 20), ethaline 6:1 is higher than the others in the normalized values graph. The solvent

structure has a stronger structure in terms of hydrogen bonding, but it also establishes a stronger protein interaction than other systems. When raw count is normalized, the systems that have the worst interaction with protein and remain inert are ethaline 2:1 and glyceline 2:1 systems. In other words, systems with the lowest viscosity in glycerol-containing systems and the highest viscosity in ethaline systems remain inert.

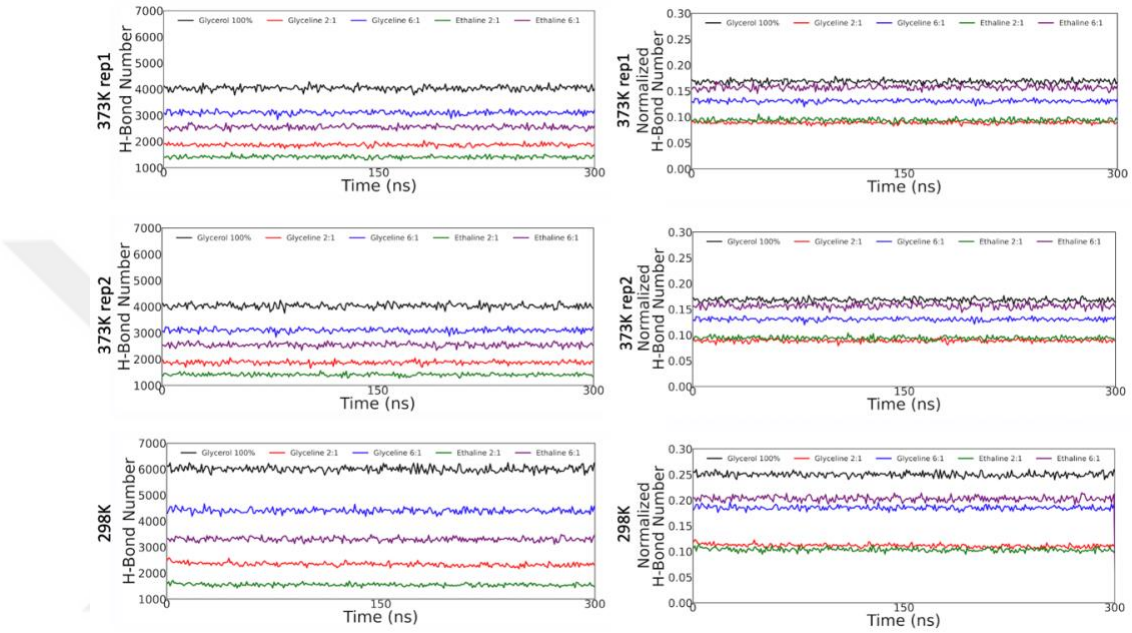

Figure 21 Raw and normalized hydrogen bond numbers of solvent-solvent, in two different temperatures.

Table 2 Hydrogen bond capacity of each component of DES.

Molecule	Hydrogen Bond Capacity
Glycerol	3
Ethylene Glycol	2
Choline Chloride	1

Looking at the normalized results from the hydrogen bond plots that show solvent-solvent results (Figure 21), it is seen that glycerol 100% and ethaline 6:1 equate to each other. The dividing number when normalizing in glyceline is higher than in other systems. The reason for this is that, as can be seen in Table 2 and Table 3, the number

of glycerol molecules and the hydrogen bonding capacity of glycerol are equal to 3 and are higher than ethylene glycol and ChCl. In both the raw count and the normalized count, glycerol 100% can establish more hydrogen bonding networks, both because of the excess number of molecules and because the hydrogen bond capacity per molecule is higher than other types of molecules. While normalizing, the number that the raw count is divided into is higher than in other systems, and as a result, although its value in raw count is the highest, it is lower in the normalized graph compared to the other systems. It is possible to have both HBD and HBA in real time with the capacity of 3 hydrogen bonds in the glycerol molecule. For example, you can establish 2 bonds as HBD and the remaining 1 bond as HBA or vice versa. The same is true for the ethylene glycol molecule, as can be seen from Table 2, this molecule also has the capacity to form 2 hydrogen bonds. The solvent-solvent interaction in the glycerol 100% system is quite strong.

Table 3 Total hydrogen bond capacity of each system.

System	Total Hydrogen Bond Capacity
Glycerol 100%	24000
Glyceline 2:1	21000
Glyceline 6:1	23750
Ethaline 2:1	15000
Ethaline 6:1	16250

5 DISCUSSION

5.1. Lipase in DESs

The thermoalkalophilic lipase of *Bacillus thermocatenulatus*, known as BTL2, has received significant attention in biotechnology and industrial applications. Lipases play a crucial role in a variety of industries due to their exceptional catalytic abilities and affinity for lipid substrates (43). The use of deep eutectic solvents (DES) opens up possibilities to produce a wide variety of DESs with varying effects on biocatalytic reaction systems. DES takes advantage of its unique properties, facilitating the dissolution and stabilization of lipases, leading to enhanced enzyme activity, enhanced enzyme reusability and accelerated reaction rates in the process (47).

Considering recent reports demonstrating DES applications on BTL2 lipase, the importance of investigating the effect of DES type and viscosity on lipase stability is studied in this thesis in order to improve lipase implementations in these promising solvents. MD simulations were run to investigate the effect of water content in DESs on the stability and dynamics of thermoalkalophilic lipases.

5.2 Viscosity of DES

A liquid's viscosity influences its ability to flow and its intermolecular frictional resistance. The viscosity of a fluid is how much resistance it must overcome to maintain its flow rate (59). It is concerned with the internal structure and molecular interactions of the liquid. Due to the molecular friction, the fluid flow difficulty shows high viscosity (60). Pa.s (Pascal seconds) or kg/(m.s) (kilograms per meter per second) and cP (centipoise) or mPa.s (pascals per million) can be used. Experimental viscosity value was searched for the glyceline 6:1 system but could not be found, so it was placed between glycerol 100% and glyceline 2:1 in the viscosity order based on the molar ratio.

Table 4 Viscosity of the DES systems in 298K and 373K.

System	Temperature	Viscosity	Temperature	Viscosity	References
Glycerol 100%	298K	~3400 mPa.s	373K	~100 mPa.s	(49)
Glyceline 6:1					
Glyceline 2:1		~800 mPa.s		~100 mPa.s	(49)
Ethaline 2:1		~80 mPa.s		~15 mPa.s	(60)
Ethaline 6:1		~50 mPa.s		~6 mPa.s	(60)

5.2.1 Viscosity and backbone stability of lipase

By looking at pairwise RMSD results (Figure 7) and viscosity values based on previous studies (Table 2), it can be observed that when the concentration of glycerol in glyceline increases, the viscosity also increases. However, it the quite the opposite for ethaline. As the concentration of ethylene glycol increases, the viscosity decreases. The viscosity order of the systems that are used in this thesis can be done like this: Glycerol 100% > Glyceline 6:1 > Glyceline 2:1 > Ethaline 2:1 > Ethaline 6:1

Backbone stability is explained in Pairwise RMSD (Figure 10). The movement in ethaline 6:1 is greater than 3 angstroms, 3 angstroms is not a huge movement, but the mobility in ethaline 6:1 exceeds 3 angstroms and that's not the case in any other system. After checking the pairwise RMSD (Figure 10) and RMSF (Figure 11) graphs, the following statement can be made. For systems consisting of ethylene glycol, the viscosity decreases as the concentration increases. When the temperature increases with it, the backbone stability and stiffness increase, thus decreasing the mobility. For systems containing glycerol, the viscosity increases as the concentration increases. With an increase in temperature, backbone stability and stiffness decrease, thus increasing mobility. Interestingly, these findings result in a behavioral difference

between systems with glycerol and with ethylene glycol in terms of viscosity. They have contrasting solvent structure.

5.2.2 Viscosity and hydrogen bond number

When the raw count graph is examined in terms of viscosity for systems containing glycerol, it can be concluded that the pattern of viscosity ranking from high to low can be seen here as well. In the glycerol molecule, there is a hydrogen bond network depending on the viscosity, as the viscosity increases, the probability of hydrogen bond formation increases. For ethaline systems, it can be commented that the low-viscosity ethaline 6:1 system made more hydrogen bonds than the high-viscosity ethaline 2:1 system. Therefore, it is concluded that the ethaline 6:1 system, which has the lowest viscosity among the systems in raw count, has a strong solvent structure and establishes a hydrogen bond network as much as the glycerol 100% system, which has the highest viscosity. The presence of a hydrogen-bond network within deep eutectic solvents (DESs) results in a reduction of the chemical potential of their components. Consequently, this characteristic renders DESs highly suitable as solvents for a significantly broader spectrum of reactions compared to what would be anticipated based solely on the individual components.

6 CONCLUSION

This thesis computationally analyzed a thermostable active lipase in different DESs with varying viscosities. It is clear from these simulations that ethylene glycol can also interact with the protein and does not disrupt its solvent structure at the time. E6:1 also makes a strong solvent bond; it can also interact with protein. Its viscosity is also the lowest of all. Glycerol, on the other hand, can form hydrogen bonds, but does not interact much with protein compared to ethaline 6:1. But if they are compared among themselves, glycerol 100 and glyceline 6:1, that is, at the concentrations where glycerol is the most, can go and interact with the protein the most. The more glycerol, the more it interacts with protein. Ethaline not only establishes a stronger solvent structure, but also has the lowest viscosity 6:1, establishes a strong hydrogen bond network within itself, and also has the strongest interaction with protein. It shows that ethylene glycol is more active with protein than glycerol, it can be said to be a more suitable DES component for lipase. Ethaline and glyceline-based DESs interact with the lipase without any significant distraction of their solvent structures. Ethaline-based DES showed a negative correlation while glyceline-based DES showed a positive correlation between solvent viscosity and lipase mobility. Thermoalkalophilic lipases show promise in DES-based industrial reactions. The findings of this thesis provided novel insights into the optimal lipase stability in DESs by tailoring physiochemical properties as well as the composition of the solvent.

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