Enzymatic Performance of *Aspergillus oryzae* α-Amylase in the Presence of Organic Solvents: Activity, Stability, and Bioinformatic Studies

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ABSTRACT: Enzymatic reactions can be modulated by the incorporation of organic solvents, leading to alterations in enzyme stability, activity, and reaction rates. These solvents create a favorable microenvironment that enables hydrophobic reactions, facilities enzyme-substrate complex formation, and reduces undesirable water-dependent side reactions. However, it is crucial to understand the impact of organic solvents on enzymatic activity, as they can also induce enzyme inactivation. In this study, the enzymatic performance of *Aspergillus oryzae* α-amylase (Taka-amylase) in various organic solvents both experimentally and computationally was investigated. The results demonstrated that ethanol and ether sustain Taka-amylase activity up to 20% to 25% of the organic solvents, with ether providing twice the stability of ethanol. Molecular dynamics simulations further revealed that Taka-amylase has a more stable structure in ether and ethanol relative to other organic solvents. In addition, the analysis showed that the loop located near the active site in the AB-domain is a vulnerable site for enzyme destabilization when exposed to organic solvents. The ability of Taka-amylase to preserve the secondary loop structure in ether and ethanol contributed to the enzyme's activity. In addition, the solvent accessibility surface area of Taka-amylase is distributed throughout all enzyme structures, thereby contributing to the instability of Taka-amylase in the presence of most organic solvents.

KEYWORDS: Amylase, organic solvents, enzymatic performance, enzyme stability, in silico

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Introduction

Most enzymes are used in industrial applications as biocatalysts in various industrial sectors, including[1] energy, food production, or fine chemicals.[1,2] Nowadays, bioinformatics has made significant efforts toward enhancing the performance, robustness, and substrate specificity of enzymes, as well as developing innovative catalytic activities.[3] Due to the capability of enzymes to reduce processing time, low-energy input, effective cost, and non-toxic characteristics, the use of enzymes in the industry is progressively elevated, eg, the protease for peptides synthesis, invertase for oligosaccharides production, or lipase for synthesizing fat (lipid, triglyceride) into free fatty acids (FFA) and monoglycerides.[4-6] Enzymes exhibit remarkable stability in an aqueous environment with minimal stabilization energy because of the almost complete enthalpy-entropy compensation.[7] Furthermore, several methods have been developed to improve thermostability and catalytic activity, such as covalent attachment, adsorption, encapsulation, and cross-linking techniques. Adsorption involves the physical adsorption of the enzyme onto the support material, whereas encapsulation includes trapping the enzyme within a polymeric matrix or microcapsule.[8] Cross-linking involves the formation of covalent bonds between the enzyme and the support material, as well as covalently attaching the enzyme to its substrate.[9,10]

The use of an organic solvent in an industry is applied as non-aqueous solvents for biocatalysts—eg, isopropyl ether as water-immiscible solvents and 2-propanol as water-miscible solvents.[11] Moreover, organic solvents significantly improve reaction stability compared with free-organic solvent reactions.[12] The stability of the enzymes as biocatalysts in organic solvent makes their applications proper in enzymatic reactions. Most of the reactions in the industry are performed in organic solvents because the easy solubility of non-polar compounds determined the biocatalysts' pace.[13]

Enzymatic activity in the presence of organic solvents indicates that water molecules are deflected from the enzyme's surface and replaced by co-solvent molecules. H-bonds between protein and organic solvent increase, whereas H-bonds between enzyme and water decrease. Polar amino acid side chains become rigid and interact with amino acids inside the protein structure. Non-polar amino acid side chains become more flexible, increasing hydrogen bonds inside enzyme structure compared with enzyme structure in water media by reorientating

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polar amino acid side chains from the surface to the core. Water in water-miscible solvents like organic solvents could equilibrate for some of the shortcomings in water properties. The mixture can speed up reaction rates, provide an environment for reaction in which hydrophobic reaction is involved in the formation of enzyme and substrate, and overcome unwanted water-dependent side reactions.[14] Organic solvents affect biocatalyst conformational stabilities, including interactions with the hydration layer essential for catalysis and protein structure changed by direct contact either hydrophobic or H-bonding with protein solvation sites.[15] The performance of biocatalytic reactions using organic solvents ethylene glycol, methanol, and acetone has a beneficial effect in the catalysis of haloalkane dehalogenase.[16]

Enzymes that are active in organic solvents catalyzed some beneficial reactions. Factors for maintaining enzyme activity in non-aqueous media have been considered, such as increasing the enzyme by pH and accomplishing while the enzyme is dissolved in the buffer before dehydration before its suspension in the organic solvent.[13] A study on enzyme activity in organic solvents has been carried out. The results show that the lipase enzyme activity is more stable in the organic solvent hexane, acetone, benzene, and toluene.[5,17] Medium engineering of biocatalysts shows a modification of reaction products, increased stability, or variations in enzymatic mechanisms, eg, in lipase, haloalkane dehalogenase, and α-amylase from *Thermotoga maritima*.[18,19] The α-amylase (1,4-α-D-glucan glucanohydrolase) belongs to endoamylases which catalyze the cleavage of 1,4-α-glycosidic bonds of starch.[20] It needs a specific amount of calcium ion in its activity.[21] The enzyme is involved in converting starch to α-limit dextrins, oligosaccharides, maltose, and glucose together with other amylolytic enzymes.[22] The α-amylases, which act at a random location along the starch chain and act anywhere on the substrate,[23] are considered the leading enzyme and are employed in starch processing industries to hydrolysis polysaccharides. This enzyme produced from fungal and bacterial sources has dominated industrial applications.[24] α-Amylase constitutes one of the most significant enzymes in industrial applications like textile, food, detergent, and bioclinical industries.[25] In biotechnology, α-amylase expands in many fields such as clinical, medicinal, and analytical chemistry.[20] The high stability of α-amylase is essential because it is demanded in its use in industry. Organic solvent enhanced the hydrolytic and alcoholic activity of the lipase enzymes at low concentrations, even when both activities drastically dropped when the organic solvent concentration was increased.[19]

The study on *Aspergillus niger* α-amylase (Taka-amylase) demonstrated that the stabilizing effect of organic solvents increased proportionally with their concentration, and the mechanism of stabilization was influenced by the interaction between the solvent and the enzyme's active site. In addition, the introduction of organic solvents to aqueous enzyme solutions resulted in the strengthening of hydrophobic interactions among non-polar amino acid residues, leading to a higher resistance to unfolding and thermal denaturation. As a result, the enzyme displayed improved stability in diverse conditions.[26]

This study aims to evaluate the performance of α-amylase activity and stability in the presence of organic solvents in vitro and in silico. For in vitro assay, various organic solvents and concentrations are treated with Taka-amylase. The activity and stability were evaluated by the Fuwa method, whereas the behavior of this enzyme in various organic solvents was studied through molecular dynamics (MD) simulation. The results of this study are expected to be an essential asset to provide the potential for organic solvents that involve this enzyme.

Materials and Methods

Materials

Aspergillus oryzae α-amylase was used as a source of enzyme and commercially purchased. All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) and Merck (Darmstadt, Germany) in pro-analysis grade, except if specifically mentioned.

Activity

According to Fuwa,[27] the reaction contained 100 µL enzyme 1 mg/mL in organic solvent (methanol, acetone, ether, ethanol, and butanol) and soluble starch 100 µL 0.2% and was incubated for 10 minutes at 50°C. The reaction was halted by adding 100 µL of 1 M HCl. Next, 100 µL of an iodine solution was added, followed by 1.6 mL of water. The absorbance of the starch-iodine complex formed was measured at 600 nm. One unit of amylase activity is defined as a 10% decrease in the color of the starch-iodine complex after 10 min of incubation at 50°C.

Stability assay

A mixture of 100 µL enzyme 1 mg/mL in various concentrations of organic solvents was incubated for a different time interval (0 to 40 min) at 50°C. Subsequently, 100 µL of 0.2% soluble starch solution was added. The reaction was reincubated at 50°C in 10 min and halted by adding 100 µL of 1 M HCl. 100 µL of an iodine solution was then added, followed by 1.6 mL of water, and the absorbance of the starch-iodine complex formed was measured at 600 nm.[28]

System preparation of molecular dynamics simulation

The 3-dimensional (3D) structure of α-amylase with PDB code 7TAA was cleaned and removed from other organic molecules through the ambpdb program from the AMBER package.[29] Hydrogen atoms were added to the structure, and the residue side chain protonation state was carried out using the

Table 1. Comparison of α-amylase activity in the presence of organic solvents.

CONCENTRATION (%)	ORGANIC SOLVENT				
	METHANOL	ETHANOL	BUTANOL	ACETONE	ETHER
0	100%	100%	100%	100%	100%
15	n.d	15.20 ± 0.13%	n.d	n.d	14.88 ± 0.22%
20	n.d	29.25 ± 0.01%	n.d	n.d	20 ± 0.19%
25	n.d	18.48 ± 1.48%	n.d	n.d	n.d
30	n.d	n.d	n.d	n.d	n.d

Abbreviation: n.d., no activity detected.

100% activity of α-amylase was 500 U.mg^{-1}.

H++ program (http://biophysics.cs.vt.edu/), resulting in a topology file and coordinates which were converted back into a PDB structure on ambpdb. The prepared structure was then put into a box of water and a 20% solvent box containing ethanol, methanol, butanol, ether, and acetone using the Packmol software.[30] Solvent volume was determined from the gap of total box volume with a dimension of 14 Å minus the protein volume. The number of solvent particles is calculated based on the solute's partial specific volume and the solvent's concentration. The topology, coordinates, and PDB files were created on the Amber program using the ff14SB force field.

Molecular dynamics simulation

Molecular dynamics simulations were performed on Ubuntu 20.04.2 LTS with Intel R Xeon Gold b230@2.10 GHz × 40 and GPU Nvidia RTX 3090. The obtained topology and coordinate files were prepared for minimization using constrained minimization. Each hydrogen atom and the molecule were given a constraint of 10 kcal and then released slowly. Each system underwent a 10 000 steps minimization, which consisted of 500 steps of steepest descents and 9500 steps of conjugate gradients each. Next, the system was slowly heated to a temperature of 323 K. The equilibration protocol was the Langevin protocol with NVT ensemble (fixed volume and temperature) with 50.000 steps and 2 fs time step (dt) with restraint 5 kcal and followed by the relaxation process under NPT protocol (fixed pressure and temperature). The NPT method is employed to obtain system equilibrium in MD simulation, which was then followed by the production stage. The simulation duration was 100ns and runs continuously using several script files. On completion, all trajectory files were merged using ccptraj in AmberTools18, allowing for a comprehensive analysis of the system's dynamics and properties. The analysis includes the computation of Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), Principal Component Analysis (PCA), Solvent Accessible Surface Area (SASA), Radius of Gyration, and secondary structure analysis.

Results

In vitro analysis

To determine enzymatic activity in the organic solvent, the highest activity of α-amylase in various dilution times was assessed. The enzyme that showed the highest activity in various dilution times was 500 U/mg. To identify the required amount of organic solvent for α-amylase activity, methanol, acetone, ether, ethanol, and butanol were used in different percentages (15%, 20%, 25%, and 30%) with 0.2% soluble starch as a substrate.

Table 1 illustrates the α-amylase activity levels in different organic solvents. Ethanol and ether exhibited significant α-amylase activity, whereas methanol, acetone, and butanol showed no detectable activity. The enzyme's activity in the absence of organic solvent was considered 100% activity. The enzyme noticeably maintained its activity at 20% of ethanol and ether. However, the presence of 25% of ethanol resulted in a decrease in α-amylase activity. In the presence of 20% solvent concentrations or higher, conformational changes occurred in the enzyme, disrupting its native non-covalent interaction. This phenomenon can be attributed to the propensity of hydrophilic organic solvents, which tend to remove water from the enzyme surface as the temperature increases.[12]

The effect of various organic solvents on the stability of amylase was studied and showed the half-life in 10 minutes (Figure 1). To determine enzymatic stability, ether and ethanol were selected based on their demonstrated enzymatic activity in comparison to other organic solvents that has been carried out. Both ether and ethanol exhibited enzymatic activity. Hence, enzyme stability reactions were performed with an organic solvent with significant α-amylase activity, ethanol, and ether. The finding revealed that α-amylase displayed higher stability in the presence of ether compared with ethanol. Interestingly, the enzyme's stability in the presence of ether was similar to that of the untreated α-amylase.

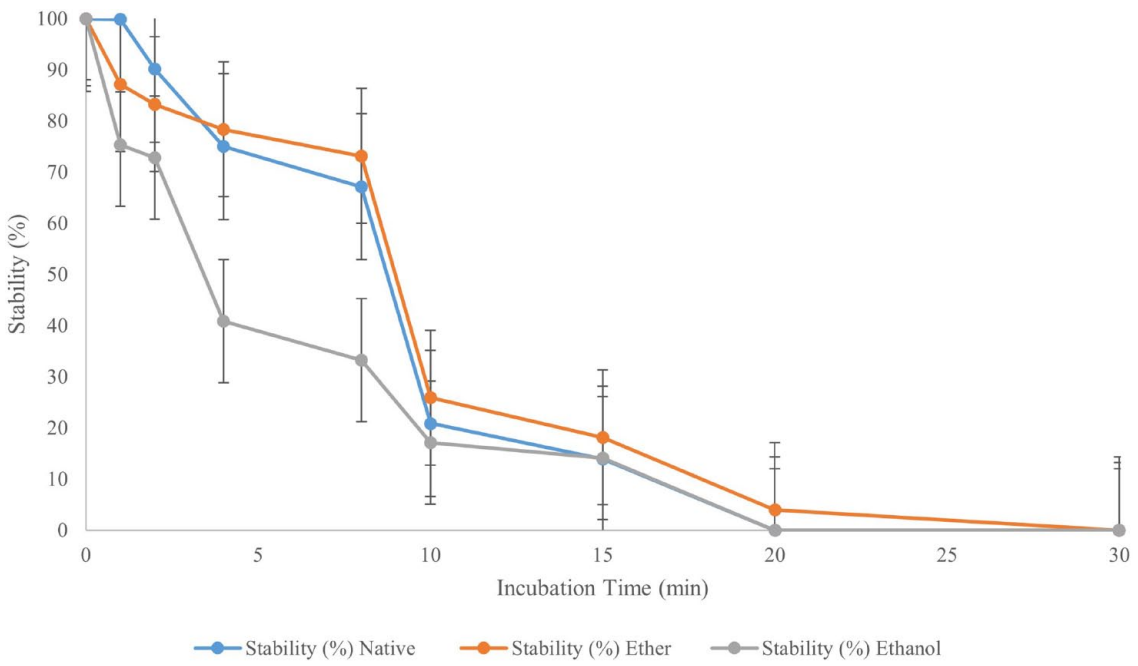

Figure 1. Stability of α-amylase in the presence of different organic solvents.

Molecular dynamics analysis

Molecular dynamics simulations were performed using 6 different solvent systems: acetone, butanol, ethanol, ether, methanol, and water. The RMSD value for all systems showed a value less than 3 Å. The enzyme in water system exhibited the highest stability, with an RMSD range of 1 to 1.5 Å, followed by ethanol (Figure 2). This finding was in agreement with the hydrolysis reaction occurring in water, establishing water as the positive control in this simulation.[31] Ethanol also demonstrated stable RMSD throughout the simulation, starting from the initial stages. Interestingly, ether also maintained a stable RMSD from 40 ns until the end of the simulation, although a high deviation occurred at the beginning of the simulation. It was indicated that the enzyme was not equilibrated well, but it was able to adapt and preserve its structure. In contrast, butanol, methanol, and acetone did not reach new stable conditions, as evidenced by fluctuating RMSD values. The RMSF revealed 2 distinct high peaks in the residue numbers 20 to 40 and 150 to 170 (Figure 3). These regions are located in the loop of the A/B domain, near the active site. These loops play a crucial role in substrate binding during hydrolysis processes.[32,33]

Among the solvents studied, butanol exhibited the highest fluctuation in loops 20 to 40, followed by acetone. Conversely, ether showed greater fluctuation in loops 150 to 170, followed by methanol. The high fluctuation indicated that the loop was very flexible. Interestingly, the visual inspection of the structural trajectories found that ether could maintain its secondary structure, particularly in the loop 150 to 170 compared with butanol, methanol, and acetone. However, in the last frame of MD simulation, butanol could not maintain the helix structure due to the loss of hydrogen bond (Figure 4). Besides, the radius of gyration, SASA analysis, NAMD energy, and hydrogen bond analysis throughout 100 ns MD showed vary in trend. Nevertheless, in RMSD trend value, water is the most stable during the simulation, followed by ethanol which converges to a value of 1.5 Å during the simulation time. Although ether initially increases in the first 40 ns of the simulation, it ultimately stabilizes at 2 Å until the simulation ends (Supplemental Figure S1). The distance and PCA analysis also showed that ethanol, ether, and water have good stability (Supplemental Figures S2 and S3). The Dictionary of Secondary Structure of Protein (DSSP) of amylase in all 6 different solvent systems is shown in Supplemental Figure S4.

Discussion

Enzyme stability generally exhibits a greater enhancement in organic solvents compared with water-based solvents.[17] The stability of an enzyme is evaluated by determining its half-life, which is derived from the decline in enzymatic activity. Enzyme stability in this study measures the remaining activity after incubation at 50°C for various periods.[22] Several assays and evaluations can be used to determine the activity profile of an enzyme. In this study, we used spectrophotometric assay which was evaluated by the Fuwa method and the structural instability evaluation was carried out using MD simulation. It can provide valuable insight into the structural integrity and conformational stability which can impact its activity. In some conditions, enzymes are also thermostable in organic solvents due to their conformational rigidity in a dehydrated state and the absence of prevalent covalent reactions responsible for the irreversible thermal inactivation of enzymes in aqueous solutions.[33] Studies about thermostability in organic solvents suggested that water is

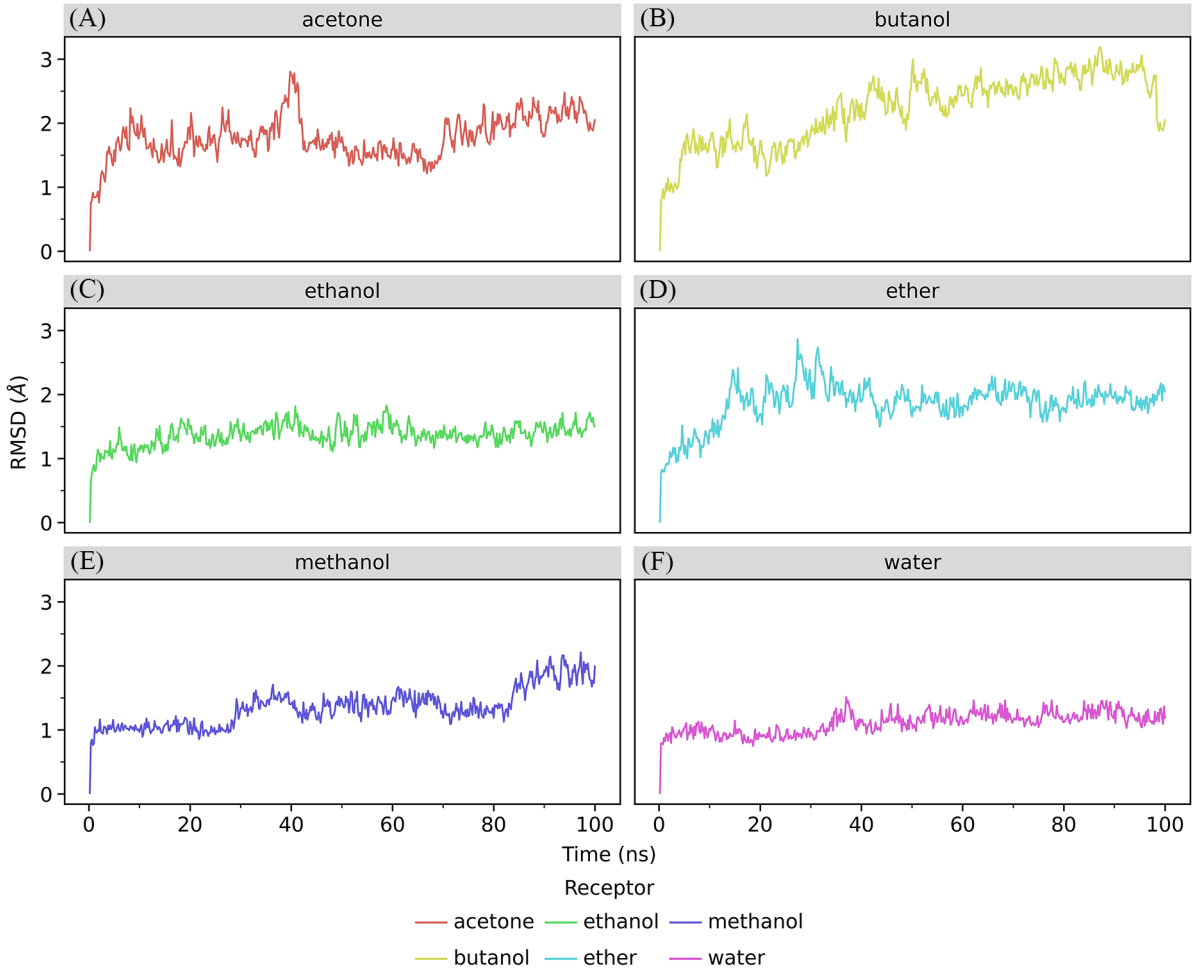

Figure 2. RMSD profile of all systems. Aceton (A), butanol (B), ethanol (C), ether (D), methanol (E), and water (F).

involved in all processes causing irreversible thermal inactivation of enzymes. Thermostability in cytochrome oxidase, ATPase,[34] terpene cyclase,[35] chymotrypsin, and lipase[36] show higher thermostability in organic solvents than in water. Recombinant thermostable T1 lipase from *Geobacillus zalihae* wild-type showed rising activity by 25% (v/v) in ethanol, *n*-heptane, *n*-hexane, acetonitrile, and methanol as well as its mutant. The stability of mutant lipase increased by 25% (v/v) in dimethyl sulfoxide (DMSO), *n*-heptane, and *n*-hexane.[37]

In this study, α-amylase showed inactivation in most organic solvents tested. Shafiei and coworkers also reported that butanol decreased the activity of α-amylase from *Nesterenkonia* sp.[38] Structural changes in the domain nearby active site A/B were revealed to be the causal effect of inactivation. However, in the presence of ether or ethanol, α-amylase was able to maintain its activity. The in silico study indicated that the distance of these water, ethanol, and ether systems have high density and are also tight. The PCA analysis also revealed that the 3 systems have a close distribution. The radius of gyration and SASA analysis showed the same pattern with RMSD. However, the energy calculated by NAMD, which consists of electrostatic and van der Waals interaction, showed that methanol and ethanol have the highest energy, and the hydrogen bond remained relatively constant throughout the simulation (Supplementary Material). Hence, the most rational reason for the activity and stability of this enzyme was the ability to maintain the secondary loop structure and the overall distribution deviation. This finding aligns with the enzyme's activity and stability assay in ether and ethanol.

Among organic solvents tested, Taka-amylase activity can be detected in the presence of ethanol and ether. In silico analysis revealed that the loop nearby the active site located in AB-domain is a vulnerable site of enzyme destabilization against organic solvents. The α/β class protein enolase and lipase superfamily also showed that the loop contributed as modular components of variability. Loops are usually located at the entry of the active site and play a major role in substrate selectivity and recognition. It also can facilitate the binding of the substrate into their binding site.[39] In Taka-amylase, the most rational reason for the activity and stability of the enzyme was the ability to maintain the secondary loop structure and the overall distribution deviation.

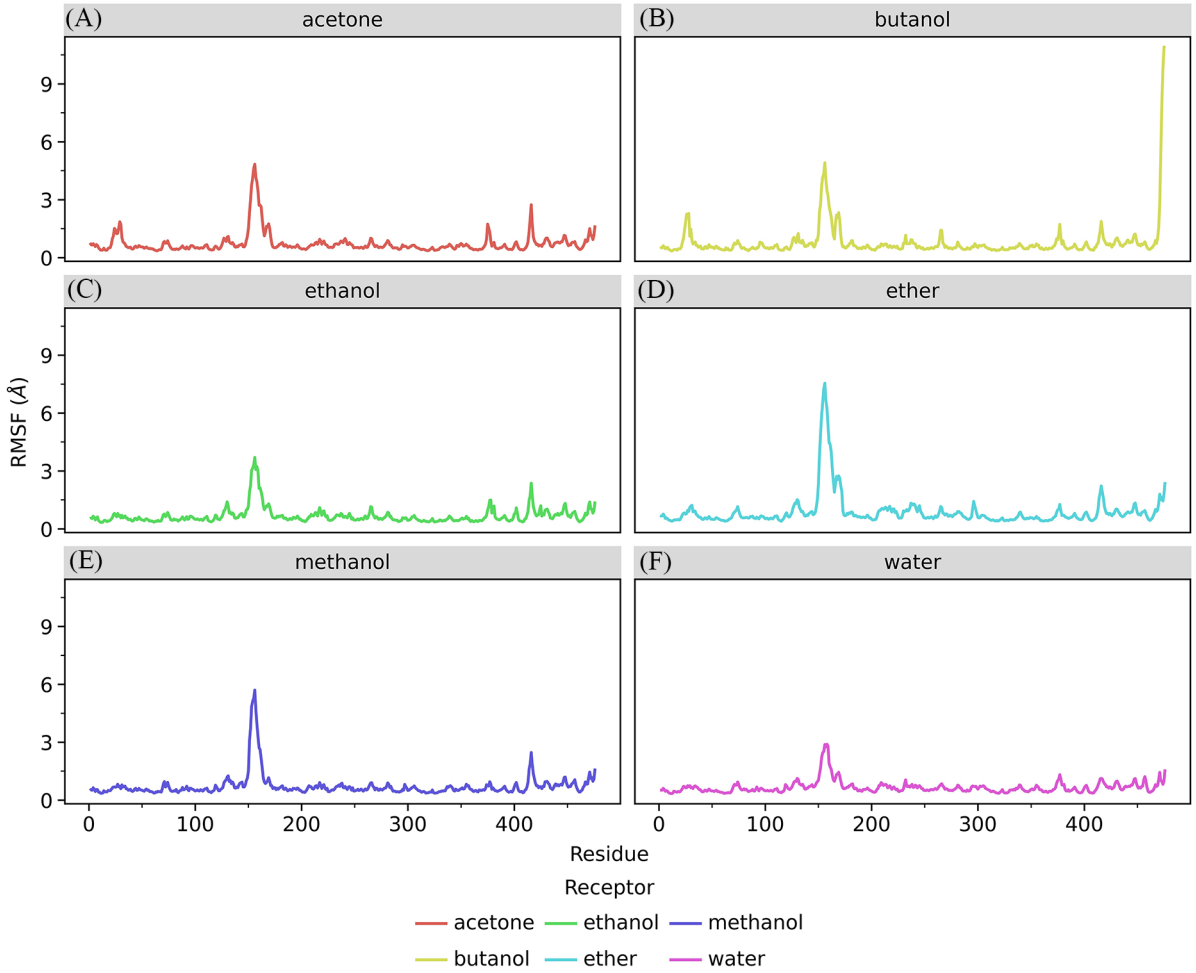

Figure 3. RMSF profile of all systems. Aceton (A), butanol (B), ethanol (C), ether (D), methanol (E), and water (F).

Conclusions

The use of enzymes is essential in the industry and some special characteristics are needed to increase the activity, especially in industry. Organic solvents are commonly used in the industry because the biocatalysts' speed can be determined by their solubility. In this study, various organic solvents are evaluated for Taka-amylase activity and stability, followed by their structural stability through MD simulation. It was shown that the activity increased in ethanol and ether, and the thermal stability analysis revealed that ether can provide twice more than others. Molecular dynamics simulations showed that this phenomenon occurs because Taka-amylase can maintain the secondary structure of the loop near the active site and the deviation of the structure remains stable throughout the simulation. These findings could provide valuable insight into the structure of Taka-amylase, offering potential avenues for enzyme modification and enhancing its application in biotechnology within the industrial sector.

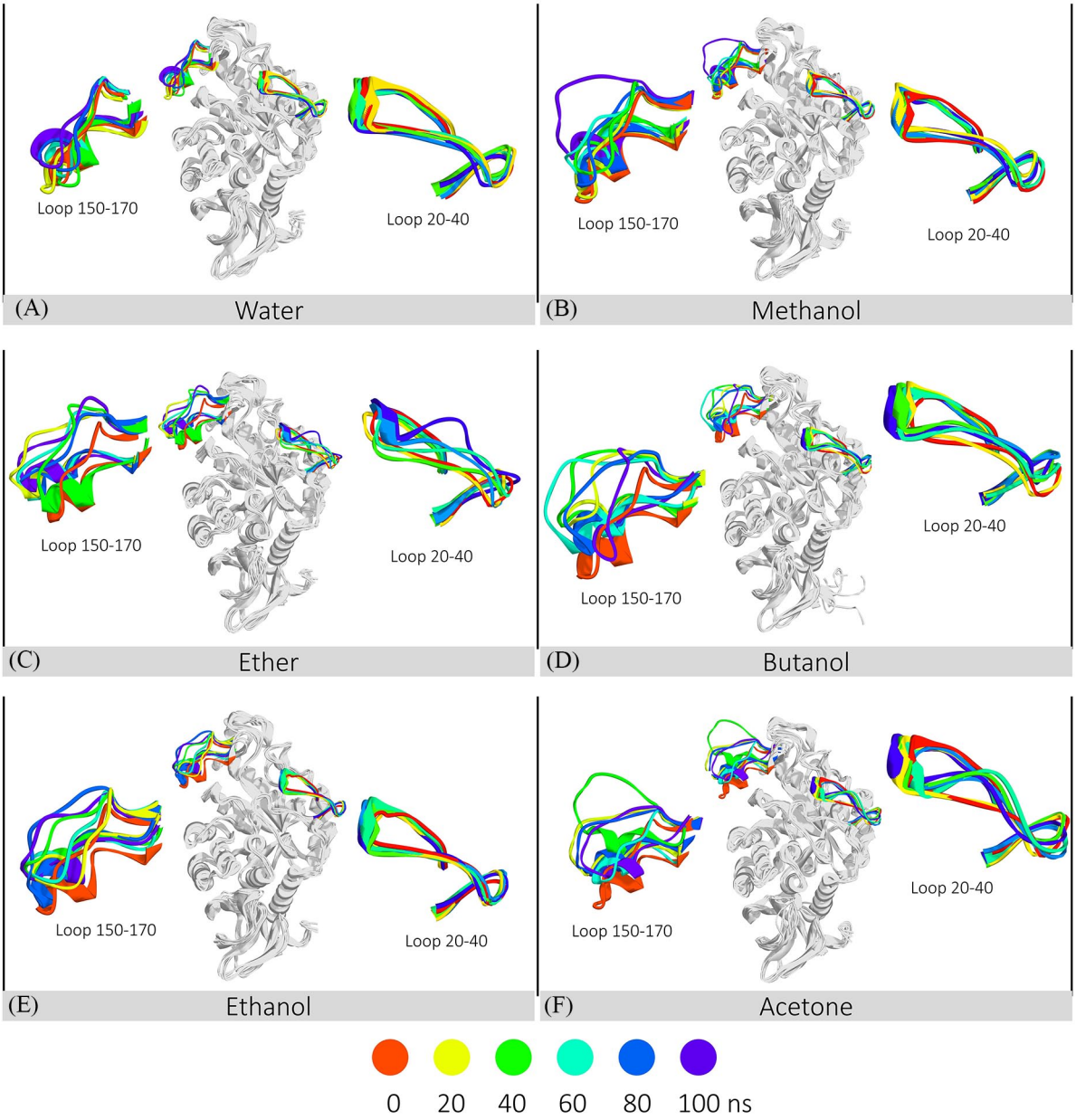

Figure 4. Time evolution snapshot every 20 ns highlighted in loops 20 to 40 and loops 150 to 170. Water (A), methanol (B), ether (C), butanol (D), ethanol (E), and acetone (F).

Author Contributions

Conceptualization and supervision were conducted by KH, MY, and TS; methodology was developed by KH, UB, ZSM, MA, and MTN; the original draft was written by UB, INM, ZSM, MTN, and MA; KH, MY, UB, and TS were involved in reviewing and editing the manuscript; funding was acquired by TS. All authors have reviewed and approved the final version of the manuscript.

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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