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The Effect of NaCl on the Stability of Ab3 Lipase from *Sphingobacterium* sp. that Co-Expressed with LEA K Using *In silico* Approach

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Abstract: This study explores how sodium chloride (NaCl) influences the binding affinity and stability of the Ab3 lipase-LEA K complex when interacting with olive oil, using molecular docking techniques. The docking simulations, conducted with Auto Dock Vina, reveal that the presence of NaCl significantly enhances the binding affinity of the enzyme-substrate complex, increasing it from-6.55 kcal/moL in the absence of salt to-7.81 kcal/moL with salt. This improvement in binding affinity is accompanied by the formation of additional hydrogen bonds and hydrophobic interactions, indicating that NaCl induces a favourable conformational change in the enzyme complex. Notably, NaCl facilitates interaction with His287, a key residue in the catalytic triad, suggesting improved accessibility and orientation of the active site, which is crucial for catalytic efficiency. These findings underscore the role of NaCl in stabilizing the enzyme complex and enhancing its structural integrity and activity, which has significant implications for industrial applications. Specifically, in industries where processes occur under high-salt conditions, such as food processing and biofuel production, the ability of NaCl to enhance lipase stability and performance could lead to more efficient and cost-effective operations, reducing the need for frequent enzyme replacement and improving overall process efficiency.

Keywords: Sodium Chloride, Ab3 Lipase-LEA K-Complex, Binding Affinity, Enzyme Stability, Catalytic Triad, Hydrogen Bonds, Hydrophobic Interactions, High-Salt Environments

Introduction

Lipases are vital enzymes with many biotechnological applications due to their ability to catalyze the hydrolysis of fats into glycerol and free fatty acids. They are widely used in food processing, pharmaceuticals, detergents and biofuels (Fatima *et al*., 2021). The efficiency of lipases, however, is greatly impacted by environmental conditions, with factors like temperature, pH and the presence of salts such as sodium chloride (NaCl) playing crucial roles in their activity and stability (Martin del Campo *et al*., 2023; Kiat, 2020).

The production of lipase by the bacterium *Sphingobacterium* sp. has demonstrated significant potential in industrial applications due to its robust enzyme activity. However, its stability in saline environments, which are common in many industrial processes, remains a major challenge. High salt concentrations often lead to enzyme denaturation or inactivation, limiting its practical utility (Ng *et al*., 2022).

To address this issue, coexpression with stress-responsive proteins such as Late Embryogenesis Abundant protein K (LEA K) has been proposed. LEA K is known for its ability to stabilize proteins and membranes under stressful conditions, including high salinity and desiccation. In this study, we employ an *In silico* approach to investigate how NaCl influences the stability of lipase from *Sphingobacterium* sp. when co-expressed with LEA K,

providing insights into strategies to enhance enzyme stability in saline environments.

One of the most significant groups of enzymes in biotechnology, lipases have numerous uses in a variety of sectors. They are employed in the food business to create flavours, in detergents to dissolve lipids and in the pharmaceutical sector to create chiral intermediates. Because lipases may operate in non-aqueous settings and catalyze a wide range of reactions, such as esterification and transesterification, which are essential for the generation of biodiesel, they are incredibly versatile (Sellami *et al*., 2022).

Although lipases are important for industry, their sensitivity to environmental conditions frequently makes them difficult to use. One common ingredient in many industrial processes, NaCl, can have a substantial impact on the stability and activity of lipases; high concentrations of NaCl can cause protein denaturation, loss of enzyme activity and decreased catalytic efficiency (Li *et al*., 2021; Sarmiento *et al*., 2015). Therefore, knowing how salt affects lipase stability is essential for enhancing its industrial applicability.

Gram-negative *Sphingobacterium* sp. has garnered interest due to its capacity to generate lipases with distinct characteristics. These lipases are useful for a variety of industrial processes because they show notable activity in a broad range of circumstances. *Sphingobacterium* sp. lipases, like those of other microbial lipases, can be inactivated by high salt concentrations (Sarmiento *et al*., 2015). Therefore, it is imperative to do research into improving these enzymes' stability in such environments.

A class of proteins known as Late Embryogenesis Abundant (LEA) proteins are abundantly expressed in plants during the later stages of seed development and are linked to resistance to desiccation. Under stressful situations like high salt levels, dehydration and high temperatures, it has been demonstrated that LEA proteins, in particular LEA K, preserve other proteins and cellular structures (Ling *et al*., 2016; Pedrosa *et al*., 2015). They are good options for enhancing the stability of enzymes like lipases in harsh environmental circumstances because of their capacity to stabilize proteins (Pedrosa *et al*., 2015).

Molecular simulations and protein modelling are two examples of *In silico* techniques that have proven to be useful in the investigation of the stability of enzymes under diverse circumstances. These methods enable the investigation of interactions between proteins and environmental elements, including NaCl, as well as the prediction of structural alterations and the identification of crucial residues involved in stability (Wang *et al*., 2007). This study's application of *In silico* methods offers an economical and successful way to investigate the possible advantages of LEA K coexpression on lipase stability in saline settings (Biswas *et al*., 2022). According to Ng *et al*. (2022), the activity of lipase from *Sphingobacterium* sp. co-expressed with LEA K increased significantly, from 70.3-91.5 U/mg. Building on this, the current study utilizes *In silico* methods to investigate the effects of NaCl on the stability of lipase from *Sphingobacterium* sp. when co-expressed with LEA K. The objective is to enhance the stability of lipases for various biotechnological applications by understanding the molecular interactions and structural changes induced by NaCl.

Materials

The following materials and software tools were utilized in this study to conduct protein-ligand docking and analyze the interactions between olive oil (represented by oleic acid) and the Ab3 lipase-LEA K peptide complex.

Software and computational tools: Auto dock vina v1.2.5 (Trott and Olson, 2010) was used for molecular docking simulations. Ligand structure refinement and molecular optimization were performed using Avogadro (Hanwell *et al*., 2012). Molecular visualization and interaction analysis were carried out using PyMOL v3.0 (DeLano, 2002) and BIOVIA Discovery Studio 2024 (Biovia, 2024).

Ligand preparation: Triolein (PubChem CID: 445639) was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and refined using Avogadro for optimal geometry and energy minimization before docking simulations.

Protein preparation: The Ab3 lipase-LEA K peptide complex was retrieved from the Protein Data Bank (PDB) (https://www.rcsb.org/) and prepared for docking. The protein structure was converted into PDBQT format using auto dock tools for compatibility with auto dock vina.

Molecular docking parameters: Docking simulations were performed using auto dock vina v1.2.5 with a grid box dimension of 30×40×35 Å. The grid box center was set at x = 5.1420, y = 0.0952, z = -8.5396. A total of 1,000 independent docking runs were conducted, and all other docking parameters were set to default.

Methods

Using auto dock vina version 1.2.5 (Trott and Olson, 2010), protein-ligand docking was used to examine the binding affinity of olive oil to the Ab3 lipase-LEA K peptide complex both with and without sodium chlorite (salt). Oleic acid, the predominant fatty acid in olive oil, was chosen as a representative model compound for this study due to its abundance and significant role in olive oil's chemical profile. The SMILES string for triolein was obtained from PubChem (CID: 445639) and refined using Avogadro software. This simplification enabled the effective simulation of interactions between the Ab3 lipase-LEA K complex and olive oil, focusing on oleic acid's most relevant molecular contributions while maintaining computational feasibility and biological relevance (Hanwell *et al*., 2012). To prepare

docking, the PDB files for the protein complex and ligands were transformed and saved as PDBQT files. The Ab3 lipase-LEA K peptide complex and ligands were docked molecularly inside a grid box of 30×40×35 Å. The box's centre along x, y and z was found at 5.1420, 0.0952 and 8.5396, respectively. Every docking simulation had a thousand runs, and the default values were applied to all other docking parameters. With the lowest binding free energies, the best-docked conformations were chosen, and PyMOL version 3.0 (DeLano, 2002) and BIOVIA discovery studio version 2024 (Maharani *et al.*, 2024) were used to display their interactions.

Results

Protein-Ligand Docking with and without Salt

Molecular docking was performed to determine the binding affinity of olive oil to the Ab3 lipase-LEA K complex with and without salt. Based on the findings, the binding affinity of olive oil with Ab3 lipase-LEA K complex increased from 6.6-7.8 kcal/moL in the presence of salt Table (1). The ligand showed no interaction with any amino acids that constitute the catalytic triad of the Ab3 lipase (Ser163, Asp257 and His287) in the absence of salt. As shown in Fig. (1a), the Ab3 lipase-LEA K complex interacted with the olive oil by forming one hydrogen bond between Gly91 and the substrate. Ten hydrophobic interactions were observed in the lipase-olive oil interface, and the interacting residues included Tyr17, Ile104, Tyr95, Phe103, Phe291 and Phe292. On the other hand, increased hydrogen bonds and hydrophobic interactions were observed in the presence of salt. As shown in Fig. (1b), the Ab3 lipase-LEA K complex interacted with olive oil by forming two hydrogen bonds with Gly91 and His287, by which the latter constitutes the catalytic triad of Ab3 lipase. Twelve hydrophobic interactions were observed in the lipase-olive oil interface, and the interacting residues included Tyr17, Ile104, Tyr95, Ile20, Tyr22, Met211, Ala288, Phe291 and Phe292.

Significant variations in binding affinity, bond types and interaction lengths are found when the Ab3 lipase-LEA K complex and olive oil are subjected to a binding interactions study, both in the presence and absence of NaCl. These findings are compiled in Table (1). When salt was present, the Ab3 lipase-LEA K complex's binding affinity with olive oil significantly improved. The binding affinity was measured at -6.55 kcal/moL in the absence of salt, which suggests a moderately strong interaction. NaCl, on the other hand, raised the binding affinity to -7.81 kcal/moL, indicating that the presence of salt strengthens and stabilizes the contact between the olive oil and the enzyme complex.

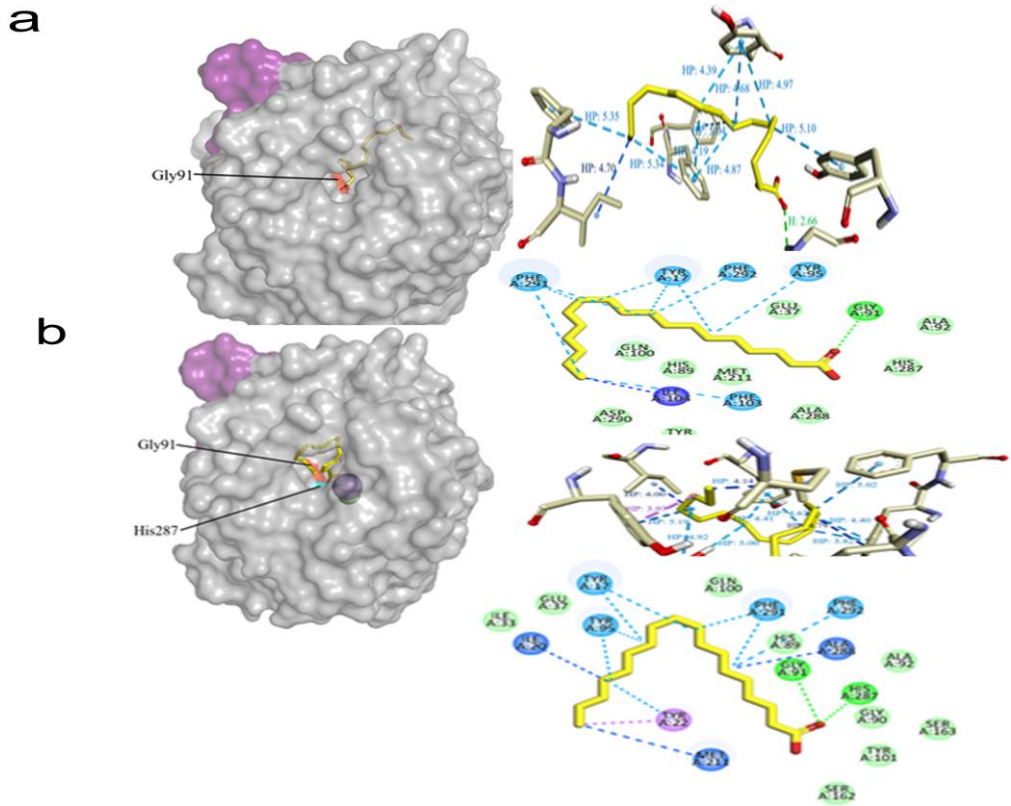

Fig. 1: Molecular surface views of the Ab3 lipase-LEA K complex with olive oil (a) without salt (b) with salt. (Left panel) (The surface of Ab3 lipase is shown in grey, LEA K peptide is shown in purple, and olive oil is shown in yellow stick forms). 3D and 2D interaction with hydrogen bonds and other non-bonded interactions. (Right panel) (H: Hydrogen Bond; HP: Hydrophobic interaction)

Table 1: Molecular docking interactions between olive oil and Ab3 lipase-LEA K peptide complex with and without salt

Protein	Ligand	Binding affinity (kcal/moL)	Bond types	From	To	Distance (Å)
Ab3 lipase-LEA K complex (without salt)	Olive oil	-6.55	Hydrogen bond	Gly91	Olive oil	2.66
			Alkyl hydrophobic	Olive oil	Ile104	4.70
			Pi-Alkyl hydrophobic	Tyr17	Olive oil	4.39
			Pi-Alkyl hydrophobic	Tyr17	Olive oil	4.68
			Pi-Alkyl hydrophobic	Tyr17	Olive oil	4.97
			Pi-Alkyl hydrophobic	Tyr95	Olive oil	5.10
			Pi-Alkyl hydrophobic	Phe103	Olive oil	5.35
			Pi-Alkyl hydrophobic	Phe291	Olive oil	5.34
			Pi-Alkyl hydrophobic	Phe291	Olive oil	4.19
			Pi-Alkyl hydrophobic	Phe291	Olive oil	4.87
			Pi-Alkyl hydrophobic	Phe292	Olive oil	5.04
Ab3 lipase-LEA K complex (with salt)	Olive oil	-7.81	Hydrogen bond	GLY91	Olive oil	1.99
			Hydrogen bond	His287	Olive oil	2.81
			Pi-Sigma hydrophobic	Olive oil	Tyr22	3.93
			Alkyl hydrophobic	Olive oil	Met211	4.14
			Alkyl hydrophobic	Olive oil	Ile20	4.00
			Alkyl hydrophobic	Ala288	Olive oil	4.79
			Pi-alkyl hydrophobic	Tyr17	Olive oil	4.41
			Pi-alkyl hydrophobic	Tyr17	Olive oil	4.62
			Pi-alkyl hydrophobic	Tyr22	Olive oil	5.19
			Pi-alkyl hydrophobic	Tyr95	Olive oil	4.92
			Pi-alkyl hydrophobic	Tyr95	Olive oil	5.00
			Pi-alkyl hydrophobic	Phe291	Olive oil	3.82
			Pi-alkyl hydrophobic	Phe291	Olive oil	4.40
			Pi-alkyl hydrophobic	Phe292	Olive oil	5.02

Bond Types and Interactions

Without Salt

Gly91 of the lipase and olive oil were found to have a single hydrogen bond with a bond distance of 2.66 Å. The precise binding of the enzyme to the substrate depends on this interaction. Pi-Alkyl hydrophobic interactions with residues Tyr17, Tyr95, Phe103, Phe291 and Phe292 were noted. These interactions took place at distances between 4.19 and 5.35 Å, with Phe291 and olive oil having the closest Pi-Alkyl contact (4.19 Å). The existence of these hydrophobic contacts highlights the significance of non-polar residues in maintaining the lipase-substrate complex.

With Salt

In our previous study, we demonstrated that the presence of Na^+ ions enhanced the specific activity of Ab3 lipase by 30%, achieving 91.5±0.1 U/μg (Ng *et al*., 2022). These findings highlight the critical role of Na^+ ions as cofactors in enzymatic activity. Two hydrogen bonds were found when NaCl was present. The first was compared to the no-salt condition and involved a much shorter binding distance of 1.99 Å between Gly91 and olive oil. With a bond distance of 2.81 Å, His287 was involved in the sec hydrogen bond. In the presence of salt, the bond lengths are shorter, indicating stronger and more stable connections.

Tyr22 and olive oil exhibited a Pi-sigma hydrophobic contact at 3.93 Å upon the addition of salt, which was not seen in the no-salt condition. Tyr17, Tyr22, Tyr95, Phe291 and Phe292 were among the residues involved in the Pi-Alkyl and Alkyl hydrophobic interactions. The closest contact was found between Phe291 and olive oil, with bond lengths ranging from 3.82-5.19 Å. A more compact and stable binding arrangement is indicated by the occurrence of new contacts and closer bond lengths in the salt state.

Discussion

The study's findings show that sodium chloride (NaCl) has a significant effect on the binding interactions between olive oil and the Ab3 lipase-LEA K complex. According to molecular docking research, NaCl dramatically alters the kinetics of the interaction between the enzyme complex and its substrate and increases the binding affinity.

Interaction with the Catalytic Triad

The ligand did not bind with the catalytic triad residues (Ser163, Asp257 and His287) of the Ab3 lipase in the absence of NaCl, indicating that the active site may not be accessible or in the best place in a non-saline environment. Because the enzyme may be less successful in binding and processing the substrate in these

circumstances, there is a chance that the catalytic efficiency will be decreased (Patil *et al*., 2010). However, in the presence of NaCl, the ligand and His287- a crucial residue in the catalytic process- formed a hydrogen bond. The interaction suggests that NaCl causes an alteration in the enzyme's structure, hence improving the accessibility and orientation of the active site for catalysis. Since it aids in stabilizing the transition state throughout the catalytic process, the creation of a hydrogen bond with a catalytic residue is a reliable sign of increased enzyme activity and stability (Vagenende *et al*., 2009; Wolkers *et al*., 2001).

Hydrogen Bonding and Hydrophobic Interactions

The Ab3 lipase-LEA K complex and olive oil formed two hydrogen bonds with salt in the presence of NaCl, up from one hydrogen bond without salt. This rise implies that NaCl improves the strength and specificity of the enzyme and substrate interaction. The extra hydrogen bond probably contributes to the higher binding affinity seen in the presence of salt. Hydrogen bonds are essential for preserving the structural stability of protein-ligand complexes (Petukh *et al*., 2015).

In addition, the study found that in the presence of NaCl, the number of hydrophobic contacts increased from ten in the absence of salt to twelve. Because hydrophobic interactions help keep water molecules out of the protein-ligand interface, they are crucial for the stability of proteins, particularly in aquatic settings. The increase in these connections when NaCl is present suggests that salt improves the binding interface's overall structural integrity in addition to stabilizing the enzyme complex (Kumar and Nussinov, 2002). The presence of extra residues in these hydrophobic contacts in saline circumstances, such as Tyr22, Met211 and Ile20, implies that NaCl facilitates a more broad and stable binding network, which adds to the overall increase in binding affinity and stability of the enzyme (Bansal *et al*., 2021; Satti *et al*., 2019).

Impact of NaCl on Binding Affinity

Oleic acid was used as a model compound to represent olive oil in computational modelling due to its dominance in olive oil composition (55-83%) and its relevance to enzyme interactions. This simplification aligns with standard practices in molecular docking studies and facilitated the analysis of NaCl's stabilizing effects on the Ab3 lipase-LEA K complex while also emphasizing oleic acid's role in enhancing binding affinity and stability. The binding affinity between olive oil and the Ab3 lipase-LEA K complex increased significantly in the presence of NaCl, with the binding energy improving from -6.55-7.81 kcal/moL. This enhancement underscores the beneficial effect of NaCl on the enzyme-substrate interaction. The observed improvement raises the possibility that NaCl plays a critical role in stabilizing the enzyme structure, resulting in stronger and more advantageous binding interactions (Satti *et al*., 2019). This stabilization mechanism is particularly relevant for enzymes like lipases, which are frequently subjected to fluctuating environmental conditions during industrial processes. For instance, NaCl may shield unfavourable electrostatic interactions, promote hydration shell stability or induce conformational rigidity, thus enhancing the enzyme's functional integrity. Such stabilization is essential for ensuring the robustness and activity of lipases in high-salt environments, such as those encountered in biofuel production or food processing (Bansal *et al*., 2021). The change in binding energy, which represents a significant difference in interaction strength, highlights the potential of NaCl to optimize enzymatic performance. Given the exponential relationship between binding energy and binding affinity, this improvement could substantially increase enzymatic efficiency under salt-stressed conditions (Satti *et al*., 2019). Furthermore, NaCl's effect may offer economic and operational advantages in industrial settings. Compared to other stabilizing agents, NaCl is inexpensive, readily available and easy to integrate into existing workflows. This positions NaCl as a valuable additive for enhancing the stability and activity of enzymes, particularly in processes where high salt concentrations are unavoidable. These findings align with previous studies demonstrating the role of salts in enzyme stabilization (Vagenende *et al*., 2009; Wolkers *et al*., 2001). Additionally, the broader applicability of NaCl to stabilize lipases in diverse industrial conditions could reduce enzyme degradation, improve reaction efficiency and lower operational costs (Satti *et al*., 2019). This makes NaCl a promising candidate for enhancing enzymatic processes in high-salt environments.

Role of Hydrogen Bonds and Hydrophobic Interactions

Shorter bond lengths imply a tighter and more stable binding conformation. The study also shows a considerable increase in the quantity and strength of hydrogen bonds in the presence of salt. The stability and specificity of enzyme-substrate interactions are known to depend on hydrogen bonds, and the observed increase in binding affinity is probably due to the amplification of these bonds in the presence of NaCl (Chen *et al*., 2022; Patil *et al*., 2010).

Furthermore, Tyr22 and olive oil have a Pi-Sigma contact, which is one of the novel hydrophobic interactions that NaCl encourages to occur. Protein structural stability depends on hydrophobic interactions, particularly in situations like high salinity where water activity is decreased (Jaeger *et al*., 1999; Patil *et al*., 2010). The synthesis of these extra hydrophobic bonds in the presence of salt implies that NaCl not only maintains the stability of already-existing contacts but also promotes the development of new ones that improve the overall

stability of the complex between the enzyme and the ligand. The concept that NaCl improves the structural stability of the complex is further supported by the observation that bond lengths are shorter and new contacts are introduced under saline circumstances, indicating a more compact and advantageous binding confirmation (Mi *et al*., 2021).

Implications for Industrial Applications

The industrial use of lipases is significantly affected by these discoveries, especially in high-salt conditions. Lipases co-expressed with LEA K may be especially well-suited for applications in saline settings, such as enzymatic fat degradation in food processing or the manufacture of biodiesel from salt-tolerant algae, based on the increased stability and binding affinity seen in the presence of NaCl (Jia *et al*., 2020; Wu *et al*., 2014). The capacity to forecast and improve the stability of enzymes by adding NaCl or other stabilizing agents may enable the creation of more resilient enzyme formulations that can function in challenging environments and for extended periods. This would increase process efficiency and lower costs related to enzyme replacement and downtime (Jia *et al*., 2020).

Our findings suggest that NaCl, at appropriate concentrations, interacts positively with the lipase-LEA K complex, enhancing its structural stability and binding affinity. This stabilizing effect is particularly evident when the lipase is co-expressed with LEA K, a stress-responsive protein that protects enzymes under harsh conditions. While high concentrations of NaCl can typically destabilize enzymes, the presence of LEA K mitigates this effect, allowing the enzyme to maintain its functionality in saline environments. This highlights the critical role of LEA K in enabling lipase activity under conditions that would otherwise inhibit it, offering potential solutions for industrial applications requiring enzyme stability in high-salinity conditions.

Conclusion

This study provides compelling evidence that sodium chloride (NaCl) significantly influences the stability and binding affinity of the Ab3 lipase-LEA K complex with olive oil. Molecular docking simulations demonstrated that NaCl enhances the binding affinity by promoting the formation of additional molecular interactions. Specifically, NaCl facilitates the establishment of stronger hydrogen bonds and hydrophobic interactions, which are critical for maintaining the structural integrity and functionality of the enzyme complex. These interactions stabilize the lipase-LEA K complex, counteracting the destabilizing effects typically associated with saline environments.

The findings underscore the dual role of NaCl: While high salt concentrations generally disrupt enzyme activity, the presence of NaCl in the context of the LEA K-stabilized complex enhances the enzyme's performance. By enabling the lipase to remain stable and active in high-salinity conditions, NaCl plays a pivotal role in extending the applicability of Ab3 lipase for industrial processes that operate under such challenging conditions. This study provides mechanistic insights into how NaCl interacts with the lipase-LEA K complex and highlights the potential of leveraging NaCl and coexpression strategies to engineer enzymes with enhanced salt tolerance. These insights pave the way for designing more robust biocatalysts suitable for industrial applications where high salinity is a common stressor.

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Author's Contributions

Ibrahim Muhammad and Ng Siau Ning: Conceptualized and designed the research idea and analyzed data. Conducted the experiments, wrote the main manuscript, reviewed and edited.

Rashidah Abdul Rahim: Conceptualized and designed the research idea and analyzed data. Supervision, resources, reviewing and editing.

Shinya Ikeno, **Nurul Izza Ismail and Hasni Arsad:** Contributed LEA peptide, the analytical tools and analyzed data.

Ethics

This article is original content that hasn't been published before. The corresponding author (Rashidah Abdul Rahim) attests that all authors have reviewed and approved the study. This article does not contain any studies with human participants or animals performed by any of the authors. The research complies with ethical guidelines and standards.

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