

STUDYING THE DYNAMICS OF KERATINASES IN IONIC LIQUIDS FOR SUSTAINABLE HUMAN HAIR KERATIN EXTRACTION

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Submitted by:

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To the

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Shahbad Daultpur, Main Bawana Road, Delhi-110042

November, 2025

*Dedicated to My Parents,
Husband and Beloved Son,
Reyansh*



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CANDIDATE'S DECLARATION

I, Priya Rai, 2K19/PHDBT/02, hereby certify that the work which is being presented in the thesis entitled **"Studying the dynamics of keratinases in ionic liquids for sustainable human hair keratin extraction"** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from August, 2019 to November, 2025 under the supervision of Prof. Yasha Hasija.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the student has incorporated all the corrections suggested by the examiners in the thesis and statement made by the candidate is correct to the best of our knowledge.

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Signature of Supervisor

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Signature of External Examiners



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CERTIFICATE BY THE SUPERVISOR(s)

Certified that **Priya Rai (2K19/PHDBT/02)** has carried out their search work presented in this thesis entitled “**Studying the dynamics of keratinases in ionic liquids for sustainable human hair keratin extraction**” for the award of **Doctor of Philosophy** from Department of Biotechnology, Delhi Technological University, Delhi, under my supervision. The thesis embodies results of original work, and studies are carried out by the student herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution.

A handwritten signature in blue ink, which appears to read "Yasha Hasija".

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Date: 28 NOVEMBER, 2025

ABSTRACT

The increasing concern regarding degradation of keratinaceous wastes, specifically human hair, because of its exceptional durability, has currently made it an emerging environmental problem. Keratin is a fibrous structural protein found abundantly in hair, nails, feathers, and other animal tissues; it owes its recalcitrant nature to tightly packed polypeptide chains stabilized by strong disulfide linkages. This physicochemical stability causes keratin-rich wastes to persist in the environment, contributing to their accumulation in landfills and drainage systems, hence causing problems in sanitation, blocking urban drainage, and ecological imbalance. Decomposition of human hair waste, which is mainly generated from barber shops and salons, is very slow under natural conditions and may release fine particulates that pose respiratory health risks upon inhalation. Other significant sources of keratin-rich wastes come from slaughterhouses, poultry farms, and wool processing units, improper waste disposal from which may facilitate the proliferation of pathogens, thereby jeopardizing environmental and public health.

Amongst all, biological approaches to keratinous waste management have come out to be of great significance due to their eco-friendly potential compared to conventional chemical and physical treatments. Certain microorganisms have developed special enzymatic mechanisms for the degradation of keratin. These organisms secrete a class of serine- or metalloproteases, keratinases (EC 3.4.21/24/99), which are able to hydrolyze the resistant structure of keratin into simpler peptides and amino acids. Unlike regular proteases, which cannot degrade keratin, the activity of keratinases involves disruption of disulfide bonds, thereby allowing bioconversion of keratinous materials. Besides playing a significant role in the natural recycling of keratin, keratinases provide potential for various industrial applications such as organic fertilizer, animal feed additives, cosmetics ingredients, and biomedical materials. Current research interest is generally focused on studies of their catalytic mechanism, screening of novel keratinases with high activity, and the application of protein engineering to modify their functionality and stability for industrial applications.

Human hair, containing about 65–95% keratin depending on geography and individual genetics, has recently become a promising biomaterial. The excellent biocompatibility, low immunogenicity, and spontaneous self-assembly driven by its high cysteine content make human hair keratin an attractive biomaterial for regenerative medicine, drug delivery, and tissue engineering applications. In this context, various functional materials, such as hydrogels, scaffolds, and films, have been successfully prepared with human hair keratin, illustrating the great value of human hair keratin as a renewable biopolymer. The extraction of keratin remains difficult due to the characteristics of insolubility and strong

structural organization, so environmentally benign methods are under exploration to assist the recovery of keratin.

Ionic Liquids (ILs), sometimes referred to as "green" or "designer solvents," are usually defined as salts that are liquid below 100 °C and are composed of a wide range of combinations of organic cations and inorganic or organic anions. Tunable ILs allow millions of possible variants, each with a specific solvating capability. They are characterized by non-volatility, non-flammability, low vapor pressure, and excellent thermal stability, presenting themselves as potentially viable alternatives to conventional organic solvents. Their multifunctional interactions, such as electrostatic, hydrogen bonding, van der Waals, and π - π interactions, allow the disruption of covalent and non-covalent bonds present in complex biomolecules, therefore allowing the dissolution of recalcitrant materials like keratin. Various ILs have been used for the dissolution of human hair, including tetramethylammonium hydroxide (TMAOH), tetrabutylammonium hydroxide (TBAH), choline hydroxide (Ch-OH), and several imidazolium-based ILs, such as [BMIM]Cl, [BMIM]Br, and [AMIM]Cl. Due to their higher extraction efficiency, reusability, and mild operating conditions when compared to water and other organic solvents, this class of solvent now represents a sustainable tool in biopolymer recovery.

Given that both keratinases and ILs degrade human hair, an integrated approach using enzymatic hydrolysis together with IL-mediated dissolution might offer an eco-friendly route for extracting keratin. The present study investigates the stability and possible compatibility of keratinases in a range of ILs to find the best match for combined use. Computational and experimental investigations so far have indicated that ILs can both stabilize and destabilize protein conformation depending on their ionic structure and thus have to be analyzed systematically for enzyme performance.

In this context, our study focuses on three bacterial keratinases-ker6 from *Bacillus cereus*, kerC from *Bacillus subtilis*, and kerF from *Stenotrophomonas maltophilia*-to investigate their conformational stability in IL systems previously reported to dissolve hair. Protein sequences were retrieved from the UniProt database. Since no experimentally determined structures existed in the Protein Data Bank for these enzymes, high-confidence tertiary structure predictions were generated using AlphaFold2, a state-of-the-art artificial intelligence system capable of resolving protein structures from amino acid sequences with near-atomic accuracy. Subsequent stereochemical validation was performed using the PDBSum server to verify structural integrity and active site analysis via PyMOL. Six ionic liquids-Tetrabutylammonium hydroxide, Tetramethylammonium hydroxide, Choline hydroxide, 1-butyl-3-methylimidazolium bromide, 1-butyl-3-methylimidazolium chloride, and 1-allyl-3-methylimidazolium chloride-were shortlisted based on their documented ability to disrupt the keratin matrix. Their three-dimensional structures were collected from the PubChem database, and system preparation was carried out using the CHARMM-GUI web interface, ensuring correct atom typing and force field

compatibility for all components. Molecular dynamics simulations were then performed to analyze the stability of these keratinases in water and in six selected human hair-degrading ILs. The simulation parameters, including root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and intra-protein hydrogen bonding, were evaluated to assess conformational stability and solvent interactions.

The results showed that molecular stability and residue-level flexibility of keratinases were strongly dependent on the solvent used. Results from molecular dynamics simulations of three keratinases-ker6, kerC, and kerF-in water and different ionic liquids have shown that this solvent dependency affects enzyme structural stability that is important for sustainable extraction of keratin. ker6 exhibited the highest stability in tetramethylammonium hydroxide (TMAOH), characterized by reduced backbone fluctuations, increased intra-protein hydrogen bonding, and compact structure as reflected by the low radius of gyration and solvent-accessible surface area. In contrast, kerC showed optimal stability in 1-allyl-3-methylimidazolium chloride (AMIMCl), maintaining low structural deviations and compactness, while kerF remained stable in both AMIMCl and 1-butyl-3-methylimidazolium bromide (BMIMBr), showing uniform maintenance of structural integrity. Other tested ionic liquids resulted in increased fluctuations and destabilization of these keratinases, underlining the specificity in enzyme-solvent interactions.

Understanding how keratinases behave in various ILs will help to shed light on the feasibility of combining these biocatalysts with IL extraction systems for sustainable and efficient recovery of human hair keratin. The outcome of such integrated biocatalytic and green solvent systems may pioneer new methodologies for waste valorization, reduce environmental contamination, and facilitate circular bioeconomy strategies by converting underutilized keratinaceous wastes into high-value biomaterials.

Collectively, this thesis provides an advanced understanding of microbial keratinase behavior in designer solvents regarding biowaste valorization and offers a scientific basis for sustainable, scalable keratin extraction. The molecular insights that have been gained are promising directions for the rational design of enzyme-IL combinations, hence potentiating the upcycling of human hair into next-generation biomaterials for applications such as wound healing, drug delivery, tissue engineering, and environmental remediation.

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LIST OF ABBREVIATIONS

HHK	HUMAN HAIR KERATIN
HHW	HUMAN HAIR WASTE
ILs	IONIC LIQUIDS
TBAH	TETRABUTYL AMMONIUM HYDROXIDE
TMAOH	TETRAMETHYL AMMONIUM HYDROXIDE
CHOH	CHOLINE HYDOXIDE
AMIMCL	1-ALLYL-3-METHYLIMIDAZOLIUM CHLORIDE
BMIMCL	1-BUTYL-3-METHYLIMIDAZOLIUM CHLORIDE
BMIMBR	1-BUTYL-3-METHYLIMIDAZOLIUM BROMIDE
PNS	PERIPHERAL NERVOUS SYSTEM
ECM	EXTRACELLULAR MATRIX
KAPNPs	KERATIN ASSOCIATED NANOPARTICLES
RMSD	ROOT MEAN SQUARE DEVIATION
RMSF	ROOT MEAN SQUARE FLUCTUATION
SASA	SOLVENT ACCESSIBLE SURFACE AREA
R _g	RADIUS OF GYRATION
FESEM	FIELD EMISSION SCANNING ELECTRON MICROSCOPY
XRD	X-RAY DIFFRACTION
FTIR	FOURIER TRANSFORM INFRARED SPECTROSCOPY
KOH	POTASSIUM HYDROXIDE
NaOH	SODIUM HYDROXIDE
Ca(OH) ₂	CALCIUM HYDROXIDE
SDS	SODIUM DODECYL SULPHATE
SFE	STEAM FLASH EXPLOSION
pLDDT	PREDICTED LOCAL DISTANCE DIFFERENCE TEST
MCS	MAXIMUM COMMON SUBSTRUCTURE
LINCS	LINEAR CONSTRAINT SOLVER
PLIP	PROTEIN-LIGAND INTERACTION PROFILER

CHAPTER 1

INTRODUCTION

1.1. Keratinaceous wastes

The solid organic byproducts produced from various animal related industries and human activities are generally referred to as keratinaceous wastes. It is mainly composed of resilient fibrous proteins present in wool, horns, nails, hooves, hairs and beaks. The major producers of keratinaceous wastes are slaughterhouses, fur industries, wool industries, tanneries, and barber shops. Because of the high amount of crosslinking present in these wastes owing to large amount of disulfide and hydrogen bonds, they are environmentally persistent and are resilient to the various common chemical, physical and biological treatment methods of degradation. Because of their environmental persistence they accumulate in our nature and leads to disposal problems resulting in pollution of water, air and soil [1].

The growing demands of agriculture, food, and textile industries across the globe have drastically increased the production of keratinaceous byproducts such as poultry feathers, animal hair, wool, horns, hooves, and human hair. Major waste-generating sectors include poultry farms, which alone contribute millions of tons of feathers annually, while slaughterhouses, leather processing plants, barber shops, and wool industries produce massive amounts of hair, nails, and hides. For example, every year more than 20,000 tons of feathers are disposed by poultry farming, and globally, about 24 billion chickens are processed, resulting in several billion pounds of feather waste. Such waste, if disposed of improperly, results in soil, air, and water pollution, encourages the proliferation of microbes that cause diseases, and is a health risk to people in nearby communities. Besides, human-derived keratinous wastes, such as hair from barber shops and nails, present urban waste management challenges and may provoke respiratory problems linked to the slow decomposition and dispersion of hair dust. Although there are conventional methods for their disposal, including incineration, landfilling, and composting, they often result in greenhouse gas emission, inefficient nutrient recovery, and persistent environmental hazards. Thus, sustainable strategies for keratinaceous waste management are important to reduce its ecological and public health impacts, while offering opportunities to valorize these wastes into bioproducts with value via microbial or enzymatic biotechnology [2] [3].

1.1.1. Major sources of keratinaceous wastes

1.1.1.1. Poultry industry: Feathers and by-products

Poultry is the most significant source of keratinous waste worldwide. Huge quantities of feathers, beaks, and claws are produced as wastes during poultry reprocessing. More than 20,000 tons of chicken feathers are estimated to be dumped every year through poultry farms alone, with the total amount exceeding 8.5 billion tons annually worldwide. Among these, feathers are the most abundant and highly recalcitrant to degradation, as they contain as much as 90% β -keratin. Due to a lack of proper disposal methods, these wastes cause landfill problems, as well as air and water pollution, serving as ideal niches for the growth of pathogenic microorganisms. Large amounts of keratin-rich solid residues are also generated by poultry slaughterhouses and meat-processing plants, which demand special treatment to dispose of safely [4] [5] [6].

1.1.1.2. Leather and tannery industries: Hair, horns and hooves

Tanneries or leather processing units generate huge amounts of keratinous waste in the form of animal hair, horns, and hoof clippings that are removed prior to and during the cleaning and processing of hides. Both solid wastes like hair, horns, and hoofs and liquid wastes generated from leather industries contain keratin and contribute to pollution if not disposed of properly. Traditional methods of open dumping or burning usually result in acute air, soil, and water pollution, besides the potential occupational hazard to workers in these industries [7] .

1.1.1.3. Wool, fur and textile industries

Shearing and cleaning of sheep wool, goat fur, and other animal fibers generate a very significant amount of keratin-based waste. These activities produce wool dust, rejected clippings, scouring wastes, and off-cuts that are regularly discarded or incinerated. Key contributors involve sheep, goats, cattle, and other livestock; wool contains more than 95% protein, the majority of it in the form of α -keratin. Such wastes are often considered to be low-value byproducts but can still provide significant logistical and environmental management issues as a result of mass accumulation [8].

1.1.1.4. Slaughterhouses and meat processing: Mixed keratin waste

Slaughterhouses generate various types of keratinous wastes apart from feathers and hair, such as hooves, horns, bristles, and animal nails. Huge amounts of solid residues rich in keratin are released by the processing of livestock, poultry, and other animals, which remain recalcitrant in landfills and pose the potential for pollution through the leaching of ammonia and other nutrients [8].

1.1.1.5. Barber shops, salons, and human-related activities such as hair and nails

Barber shops, salons, and household grooming activities in both urban and rural areas produce tonnes of human hair and nail clippings. The small quantities produced by

individuals add up to immense municipal human hair wastes, especially in densely populated regions. Its inappropriate disposal causes blockages in the drainage system of municipalities, soil pollution, and allergenic dust in open dumps [9].

1.1.1.6. Other animal wastes: beaks, scales and stratum corneum

Other keratin wastes include bird beaks, reptilian and fish scales, and the stratum corneum (outermost skin layers) from animal husbandry. These more minor sources are still of environmental relevance because of the highly cross-linked keratin structures, which further complicate their degradation in natural environments [3].

Comprehensive studies indicate that poultry feathers account for about 40% of total keratinous biomass, human hair for 25%, and animal wool for another 15%. The leading generators are concentrated in countries with intensive poultry and livestock processing sectors, including the United States, China, India, and Brazil. In addition to industrial sources, municipal solid waste streams are increasingly recognized as contributors to the keratin waste burden.

While keratinaceous wastes arise from a diverse range of animal-based sources—including poultry feathers, wool and fur from textile industries, slaughterhouse byproducts, and solid residues from the leather sector—one particularly significant yet understudied fraction is human hair waste. Worldwide, hair salons, barber shops, and households collectively generate vast quantities of human hair as a municipal solid waste stream. It is estimated that approximately 300,000 tons of human hair are discarded annually, accumulating rapidly in urban and rural environments alike. Owing to the densely packed, highly cross-linked structure of keratin proteins, waste human hair resists natural biodegradation, persisting in landfills, clogging drainage systems, and contributing to dust-borne allergens and other urban sanitation issues.

Despite being biodegradable in principle, human hair's slow disintegration rate and high protein content make its disposal an environmental challenge, but also present unique opportunities for resource recovery. Human hair is distinguished by its high nitrogen and sulfur content, mechanical robustness, and biocompatibility, all of which have fueled growing research interest in its valorization for applications in biomaterials, fertilizers, water purification, and composite manufacturing. Notably, India alone is a major producer and exporter of human hair, with substantial commercial and scientific efforts directed at its recycling and conversion into value-added products [9].

1.2. Scale of Human Hair Waste Generation

Human hair waste (HHW) is increasing with the growth in population and globalization, further supported by the boom in the beauty industry. The global generation of human hair wastes is estimated to be in millions of tons annually, due to continuous hair

shedding among people and the similarly rapid generation of cut hair coming from salons and barber shops. Recent estimates show that worldwide, about 2.2 billion kg of hair wastes are generated every year from haircutting salons alone, most of which end up in landfills or being incinerated; incineration releases methane and toxic gases into the atmosphere.

For instance, Europe generates an estimated 72 million kilograms of hair waste annually, most of which are never recycled or put to other uses. According to various studies, the UK alone sends about 6.7 million kilograms of human hair to landfills annually, which is enough to fill dozens of football stadiums. In India, rural and urban barbers alike generate high volumes; Kerala's more than 36,000 barber shops and beauty parlors discard nearly 827 tons of hair every year, with its disposal method varying from landfilling and burning to open dumping, depending on local regulations and infrastructure. Growing urbanization and changes in lifestyles have only accelerated this pace, as the frequency of haircuts increases and the scale of beauty services expands [9] [10].

Scientific estimates put global hair production at around 6.9×10^5 tons (~690,000 tons) per year from natural hair growth, haircuts, and salon waste, assuming an average production of about 80-100g of hair per person annually. This figure accounts for only direct human cuttings and does not include those shed naturally or through routine grooming activities. The total value of traded human hair, primarily used in wigs, extensions, and commercial products, has also soared to reach USD 149.4 million in 2020, adding demand and driving the generation of international waste flows in their wake [11] [12].

Given that most countries lack effective collection and recycling systems, hair waste has become an integral part of municipal solid waste streams. Disposal methods vary around the world, but most of the developing world burns hair along with other household refuse or sends it into sewers, clogging pipes, increasing nitrogen levels in wastewater, and contributing generally to ecological deterioration. Incineration results in toxic gas emissions of sulfur dioxide, ammonia, and phenolic compounds; accumulation in landfills emanates greenhouse gases and leads to dust hazards [13] .

Although it is environmentally persistent, hair waste is increasingly viewed as an important keratinous resource, arising from its renewability, abundance, and potential for upcycling into biotechnological products such as fertilizers, biomaterials, or pollution control products through value addition. Present sustainability efforts have targeted landfill diversion of hair waste into recycling streams or value addition, with the dual purpose of reducing ecological impact while capturing unique hair chemical properties for a wide range of applications [14].

In short, human hair has turned into a significant worldwide solid waste problem owing to enormous annual production, together with the inefficiency of the disposal systems and constant environmental threats. Extensive data from various regions of Europe, India, and the UK reveal the magnitude and growing importance of finding more viable management and valorization of human hair wastes.

1.3. Environmental and health implications of improper disposal

The improper disposal of human hair waste, whether open dumping, landfill burial, or incineration, poses a severe threat to the environment and public health. Human hair can resist decay for many years, and in landfills, it decays very slowly, emitting chemical byproducts such as ammonia and sulfur dioxide, which are major contributors to air pollution and foul odors. It can leach out microplastics, heavy metals, and nitrogen into the land and water adjacent to the landfills, causing secondary pollution. This causes ecological stress and algal blooms that could potentially affect aquatic life [9].

Burning hair is one of the most common disposal practices in many parts of the world and is frequently done along with household solid waste. In addition, incineration generates highly toxic emissions, such as sulfur dioxide, ammonia, phenols, and volatile organic compounds, which, beyond deteriorating air quality, increase respiratory irritation, allergic reactions, and chronic pulmonary conditions among exposed populations. Case-control studies on cough, breathlessness, and obstructive lung disease revealed significantly higher rates among hairdressers and employees in hair-reprocessing industries, reflecting the occupational health burden from chronic exposure to these air pollutants .

Unmanaged hair waste in urban areas fills municipal drainage systems, causing blockages and subsequent flooding of urban areas. Due to its high nitrogen content, it spikes nitrate levels in sewage, promoting water eutrophication and aquifer contamination in cases where runoff enters the groundwater supplies. Many studies among workers in these salons and barbershops indicate that most of these establishments do not have efficient means of disposing of hair, which is why it is usually mixed with municipal wastes and goes into unscientific disposal. Open dumping not only leads to unsightly, persistent waste but also produces airborne dust. Hair dust is capable of acting as an allogenic particulate, exacerbating respiratory allergies and urban air pollution, especially in highly populated neighborhoods.

Consequently, long-term ecological degradation has been further highlighted in rural India around hair dumps and reprocessing sites by changes in the elemental profile of soil and water, toxic metal accumulation, and a decline in the diversity of aquatic microorganisms, which in turn threatens food safety and community health. Epidemiological analyses confirm elevated levels of heavy metals in the hair and serum

of landfill workers due to bioaccumulation from chronic environmental exposure, with implications for systematic health risks among vulnerable populations [15] [16].

Improperly disposed human hair waste gives rise to the linked health and environmental hazards of air and water pollution, toxic emissions, nitrogen loading, respiratory disease, and ecosystem degradation. Such risks require structured waste collection, safe recycling methods, and greater awareness of hair persistence in the environment.

1.4. Overview of Keratin as a structural protein

Keratin is a tough, fibrous structural protein that is abundant in epithelia of vertebrates, including hair, nails, wool, feathers, hooves, horns, and the outermost layers of the skin. It plays a central role in building biological barriers and appendages and in maintaining mechanical stability, resilience, and resistance to environmental insults. The keratins belong to the superfamily of intermediate filaments, which represent an essential part of the cytoskeleton and underlie tissue structural integrity [17].

Structurally, all keratins are characterized by their high content of cysteine, glycine, and serine amino acids, providing ample opportunity for disulfide bonding along with hydrogen bonding. This cross-linking underlies the insolubility and chemical inertness of keratin, as well as its tensile strength and elasticity. There are two main keratin types based on secondary structure: α -keratins are found predominantly in mammals, forming hair, wool, nails, and the stratum corneum; β -keratins are found predominantly in avian and reptilian tissues, including feathers, beaks, and scales. The α -keratin subunits associate to form right-handed coiled-coil structures, which further assemble into 7–10 nm diameter intermediate filaments, whereas β -keratin structures appear as pleated sheets that build up into 3–4 nm filaments [18].

Keratin filaments exhibit remarkable diversity in their hierarchical complexity: the epithelial cells of mammals, for example, use both acidic (Type I) and basic (Type II) keratin chains to generate heterodimeric coiled-coil motifs that assemble into higher-order filament networks. Molecular-level studies have defined the axial orientation and length of the coiled-coil rod domains, established key crosslinking interactions such as lysine–lysine and cysteine–cysteine bonds, and provided an understanding of filament–matrix network and mesh structure that is important for tissue function. There has also been a strong emphasis on elucidating keratin's toughness towards mechanical, thermal, and chemical insults, and its slow biodegradation—a set of properties that render keratin a model structural biopolymer [19].

Apart from the biological architecture, there are evolutionary and functional purposes for keratins in animal adaptation, wound healing, and cellular proliferation. The insolubility in both water and organic solvents ensures anatomical protection that endures. This, together with its ability as a protein to resist enzymatic attack and the

stabilization of tissues under stress, points to the special place occupied by keratin as a structural protein in biology and its great potential in biomaterials science, tissue engineering, and biotechnological applications [20] [21].

1.5. Resistance to degradation: crosslinking and insolubility

Most of the resistance of keratin to chemical, enzymatic, and physical degradation is a consequence of its highly crosslinked and insoluble molecular structure. The abundance of covalent disulfide bonds, S-S, between cysteine residues is the major structural feature of keratin that provides such strength. These disulfide bridges form complex cross-links within and between the protein chains, establishing a tight and stable network that denies access by proteolytic enzymes to the peptide backbone [22].

In addition to the disulfide bonds, keratin is extensively stabilized by hydrogen bonding and hydrophobic interactions along its α -helical or β -sheet conformations. These noncovalent interactions enhance the packing density and mechanical strength of the protein fibers considerably, contributing to the impressive water and organic solvent insolubility of keratin. Due to such a structure, keratin resists the action of most common proteases like trypsin and pepsin and hence can hardly be degraded by regular enzymatic processes [23].

Generally, degradation of keratin needs a two-step mechanism: a sulfitolysis process wherein the disulfide bonds are reductively cleaved, loosening up the rigid protein conformation, followed by the proteolytic cleavage of the now-exposed peptide chains by specialized keratinases or proteases. This explains the synergy wherein the keratinase-producing microorganisms usually secrete disulfide reductases or depend on exogenous reducing agents for effective keratin breakdown, underlining the challenge that exists in efficient keratin hydrolysis [24] [25].

Insolubility and recalcitrance are among the major hindrances of recycling and bioconversion of keratin due to the presence of a cross-linked matrix. Advanced pretreatment methods, involving chemical reduction or oxidation or use of ionic liquids, are hence required prior to enzymatic attack. Its dense cross-linking is also responsible for the thermal and chemical stability of keratin, making this protein suitable in harsh environmental conditions for carrying out protective biological functions but a challenge in waste management and valorization.

Recent advances in biomaterials have taken advantage of the cross-linking density of keratins in engineering mechanically robust hydrogels and biocomposites with tunable degradation rates. For instance, hydrogels based on human hair keratin cross-linked through thiol-ene chemistry showed slow enzymatic degradation kinetics, consistent with their degree of crosslink density. Balancing crosslinking and proteolytic access remains

important for the development of sustainable waste treatments and biomedical uses of keratin [26].

In summary, resistance to degradation in keratin becomes a direct result of extensive disulfide crosslinking with hydrogen bonding and hydrophobic interactions, conferring on it chemical inertness and insolubility. Such a molecular characteristic requires specific biochemical or chemical methods for its effective breakdown and utilization.

1.6. Amino acid Composition and biological value

Keratin is one of the major structural proteins within vertebrates, possessing a typical amino acid profile that imparts its mechanical strength, resilience, and functional specificity across tissues such as hair, nails, feathers, and wool. It is characteristically rich in sulfur-containing amino acids, especially cysteine, with an approximate amount of 10-14% of total residues in hard keratins such as hair and nails.

Extensive disulfide bonds are established through cysteine's thiol groups, forming covalent cross-links that underlie the rigidity, chemical stability, and resistance of keratin to enzymatic degradation. In addition to cysteine, keratin contains large amounts of hydrophobic amino acids glycine, alanine, valine, and leucine, which favor close packing and fiber formation due to hydrophobic interactions. Polar amino acids, such as serine, threonine, and glutamic acid, participate in hydrogen bonding networks that stabilize characteristic secondary structures, like α -helical and β -sheet, of keratin filaments.

The overall amino acid content differs among keratin types and tissue sources. For instance, cysteine and proline are more abundant in α -keratins of hair and wool than in softer skin keratins, reflecting a greater mechanical need. Human scalp hair keratin uniquely contains a complex mixture of the amino acids lysine, arginine, histidine, and tyrosine, which affect properties like hydrophilicity, UV protection, and trace metal interactions. Analysis of human hair keratin also reveals the presence of all 21 amino acids in varying amounts, which therefore affects hair's tensile strength and other biological functions [27] [28].

Biologically, the amino acid composition of keratin underpins the tough, fibrous structures that provide physical protection, barrier function against pathogens, and cellular integrity in epithelial tissues. The sulfur content also influences nutritional and recycling value of keratin wastes, with cysteine and other amino acids being of value for further bioconversion uses. Such biochemical properties make keratin not only important to the biology of organisms but also an attractive focus for sustainable biomaterial uses and strategies for the reuse of proteins.

In conclusion, the amino acid composition that dominantly contains cysteine, glycine, alanine, and serine defines the mechanical robustness, resistance against degradation, and biological function of keratin across tissues. The richness in these amino acids therefore underpins both the structural functions and the opportunities for the biotechnological exploitation of sources containing keratin [29].

1.7. Amino Acid composition of Human Scalp hair.

Here is a summary and tabular representation of the amino acid content of human scalp hair keratin, as reported by Rashaid et al. (2015), based on GC/MS analysis of 64 subjects as shown in Table 1.1 [30]. The values are given as mean concentration in mg amino acid per mg hair for the 14 most abundant and acid-resistant amino acids.

Table 1.1 Amino acid content of human scalp hair keratin, based on GC/MS analysis of 64 subjects, with 33 male and 31 females.

Amino Acid	Mean $\pm$ 95% CI (Male, n=33)	Mean $\pm$ 95% CI (Female, n=31)
Alanine (Ala)	12.5 $\pm$ 2.0	12.4 $\pm$ 2.9
Glycine (Gly)	3.6 $\pm$ 0.6	4.1 $\pm$ 1.0
Valine (Val)	6.8 $\pm$ 0.8	9.8 $\pm$ 2.1
Leucine (Leu)	15.2 $\pm$ 1.6	15.8 $\pm$ 3.2
Isoleucine (Ile)	4.4 $\pm$ 0.6	4.8 $\pm$ 0.9
Proline (Pro)	9.8 $\pm$ 1.0	12.7 $\pm$ 2.5
Serine (Ser)	1.8 $\pm$ 0.7	1.8 $\pm$ 0.6
Threonine (Thr)	2.8 $\pm$ 0.7	2.3 $\pm$ 0.5
Aspartic Acid	9.0 $\pm$ 1.4	10.3 $\pm$ 2.2

(Asp)		
Cysteine (Cys)	3.1 ± 1.0	2.8 ± 1.0
Glutamic Acid (Glu)	8.5 ± 1.6	9.3 ± 1.6
Phenylalanine (Phe)	2.3 ± 0.2	3.4 ± 0.6
Lysine (Lys)	7.7 ± 1.1	7.8 ± 1.3
Tyrosine (Tyr)	6.0 ± 0.6	5.7 ± 0.7

The table above summarizes the amino acid abundance for both male and female subjects, demonstrating slight variation between sexes, with phenylalanine, valine, and proline showing the most significant differences.

The cysteine content, important for disulfide cross-linking and mechanical integrity, averages about 3 mg per mg hair for both sexes in this study, reinforcing the high sulfur profile previously reported for keratin.

These results set a modern benchmark for the biological value and molecular diversity of hair keratin.

1.8. Current management and utilization strategies

1.8.1. Conventional disposal methods and issues

1.8.1.1. Landfilling

Incineration of keratin waste, which includes hair and feathers, is widely used for volume reduction and the control of pathogens. High temperatures over 850°C transform keratinous material into ash, carbon dioxide, and water while eradicating most infectious agents. Although incineration reduces bulk wastes, the process generates a number of toxic gases, including ammonia, hydrogen sulfide, sulfur dioxide, phenols, and dioxins, which further contribute to air pollution and respiratory problems among the local population. This is particularly problematic in areas with poor filtration and emission controls [31].

1.8.1.2. Incineration

Incineration of keratin waste, like hair and feathers, is a common method of volume reduction and pathogen control in several areas. At high temperatures of over 850°C, the keratinous material is reduced to ash, carbon dioxide, and water, with the elimination of most infectious agents. While it reduces the bulk of waste, incineration generates noxious fumes of ammonia, hydrogen sulfide, sulfur dioxide, phenols, and dioxins, adding to air pollution and respiratory problems among the residents of that area. The emissions are more problematic in areas where proper filtration and controls on emissions are lacking, thus raising environmental and public health concerns [32].

1.8.1.3. Composting

Composting of keratin-rich residues, especially when it comes to animal hair and feathers, can be considered an organic approach to managing such wastes. Although composting can indeed provide nutrient-rich fertilizers, the inherent recalcitrance of keratin slows down decomposition, requiring pretreatment such as mechanical grinding or chemical pretreatment to improve breakdown. Even under these conditions, poor or incomplete composting will lead to odors, proliferation of pathogens, and incomplete conversion of keratin into valuable compost. Pre-treatment with chemicals is effective but may interfere with the composting process and produce toxic byproducts [33].

1.8.1.4. Mechanical Grinding

Keratinous wastes such as feathers, hair, and hooves are mechanically ground into smaller sizes to enhance either post-processing treatment or disposal. This process physically breaks the recalcitrant keratin matrix into smaller pieces, thereby increasing the surface area available for microbial attack or further chemical treatments. The ground keratin waste is usually used as a protein source in animal feed or for soil amendment. Nevertheless, some heat generated during the grinding process can degrade thermolabile amino acids and decrease the nutritional quality of the whole processed material. Additionally, there is still incomplete biochemical resistance of keratin after its mechanical processing; hence, supplementary treatment is needed for full valorization [34].

1.8.1.5. Other methods and their challenges

Other methods of disposal involve open dumping, burning, and chemical hydrolysis. Open dumping and crude burning of hair and feathers in rural or remote regions contribute to local air and soil pollution; this may facilitate the spread of airborne pathogens or allergenic particles. Chemical hydrolysis with strong acids or alkalis is effective in degrading keratin but has significant drawbacks in the context of energy consumption, environmental toxicity, and loss of valuable amino acids. This limits its

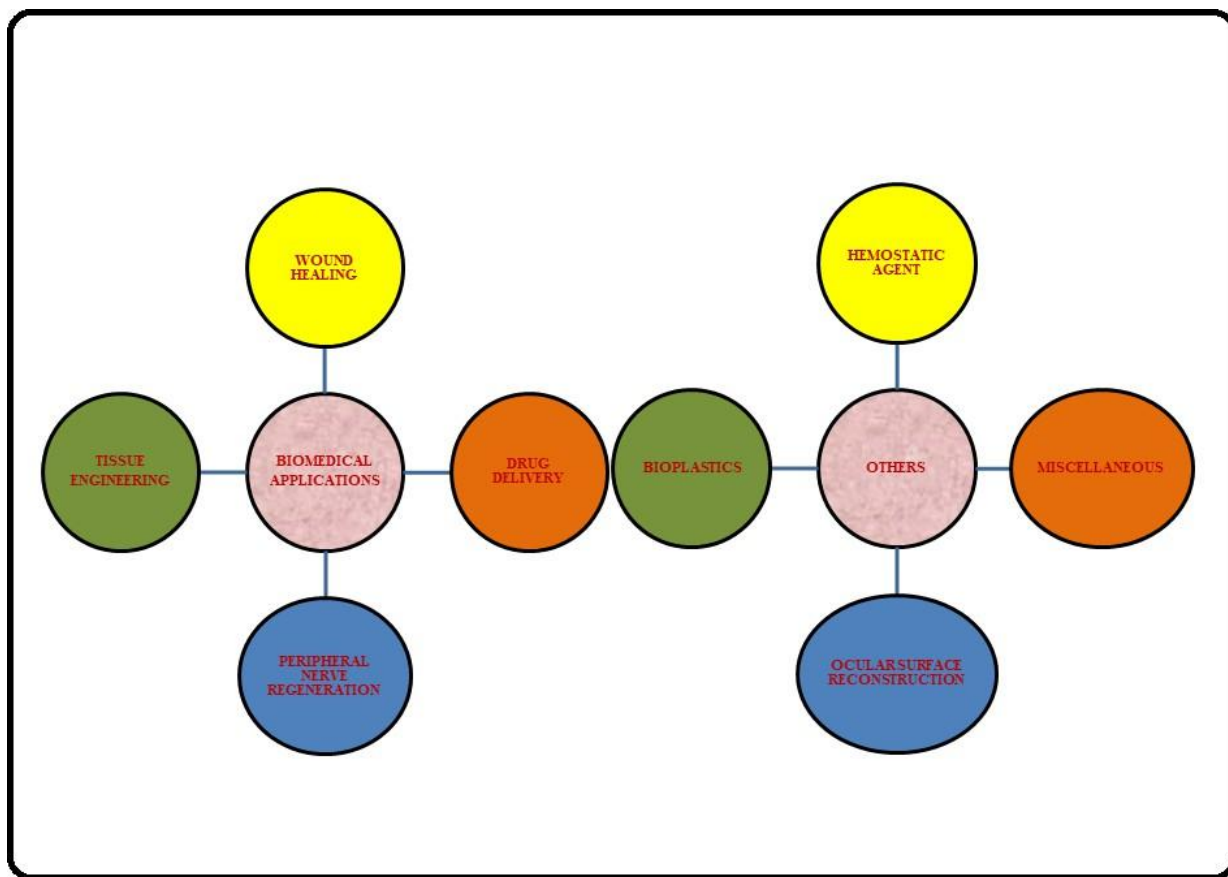
scaling up or sustainability. In general, each of these methods has problems with environmental safety, efficiency, cost, and secondary pollution [35].

The shortcomings of the existing methods reveal critical challenges associated with keratin waste: persistence, pollution, and incomplete degradability. These issues have motivated research toward alternative solutions like enzymatic hydrolysis, microbial valorization, and green solvent-based extraction methods for better sustainability and value recovery.

1.9. Applications of Human hair keratin

A schematics representation of the different applications of human hair keratin (HHK) is shown in figure 2.1.

Figure 2.1 Different applications of human hair keratin



1.9.1. Wound healing

Poor wound healing has been a major medical concern and has spurred significant research efforts in tissue engineering for the development of an effective skin substitute for acute and chronic wounds. A major area of pursuit in regenerative medicine has been

to design scaffold systems that are similar in structure and function to natural skin tissue. Human hair keratin biomaterials have been the focus of significant study in the last decade as candidates for a host of regenerative applications. These biomaterials are uniquely positioned through their combination of excellent biocompatibility, biodegradability, and solid mechanical strength. Besides, keratin sourced from human hair is a sustainable biomaterial, making it an environmentally friendly choice. The biocompatibility of keratin favors cell attachment and growth, while the biodegradability allows its gradual replacement at the wound site without any adverse long-term effect. Their robust mechanical properties ensure that scaffolds prepared from keratin will provide adequate structural support throughout the healing process. These features make biomaterials from human hair keratin quite effective in advanced wound care, addressing both acute and chronic open wounds. They provide physiological support to facilitate tissue regeneration and accelerate the healing process by promoting cell proliferation, migration, and tissue remodeling. For all these reasons, such keratin-based biomaterials are being increasingly regarded as superior alternatives to conventional dressings, promising better healing with fewer complications and improved patient experience. Their sustainability adds further to their value as a component in future innovations in wound management, with increasing calls for biomedical materials that respect the environment [36] [37].

Several works have been dedicated to the investigation of keratin biomaterials, which were derived from human hair for wound-healing applications and which proved to be effective in tissue repair. As an example, Park et al. demonstrated that human hair keratin dramatically enhances near-complete regeneration of the epithelial layer, enhancing the overall efficiency of wound healing. In another work, Sadeghi et al. fabricated a hydrogel made of clindamycin, CMC, and keratin; by increasing the content of keratin, several functional properties of the hydrogel were improved. In particular, the higher the level of keratin, the better the water vapor transmission rate to optimize moisture balance required for wound healing, the better the mechanical stability of the hydrogel, and the longer the delivery of the antibiotic drug. Moreover, they detected that increasing keratin concentration positively affects fibroblast behavior, including better attachment, proliferation, and spreading compared to pure CMC hydrogels. These findings have emphasized the important role of keratin in enhancing the biological and structural properties of wound dressings supportive of accelerated healing while maintaining stable and protective conditions around the wound. Such keratin-based materials offer, therefore, very promising multifunctional platforms for advanced wound management by combining antimicrobial action, biocompatibility, and enhanced cell interactions required for effective tissue regeneration [38].

Lin et al. emphasized that human hair keratin biomaterials provide biodegradability, biocompatibility, and an optimal pore structure, which support hASC adhesion,

proliferation, and differentiation both in cell culture and animal models. In the in vivo experiments using ICR mice, the treatment of wounds with keratin scaffolds in combination with hASCs resulted in a significant enhancement of wound healing with improved epithelialization compared to scaffolds only, stem cells only, or untreated controls. The enhanced tissue remodeling further supports that the scaffold architecture and cell compatibility are crucial for the regenerative outcome. Synergistic effects of keratin biomaterials with hASCs thus provide evidence that these composites offer superior support to the skin tissue regeneration and remodeling process. Human hair keratin scaffolds engineered with stem cells, therefore, have emerged as the most promising approach for the next-generation wound care and regenerative medicine applications having the capability to provide fast closure of wounds and superior quality of the tissue with respect to conventional therapies [39].

Kim et al. showed that the keratin hydrogel derived from human hair significantly accelerated the complete skin repair process on full-thickness skin wounds of mice in 21 days, without scabbing or contraction, while untreated controls evidenced residual scarring. Histological examinations confirmed that mice treated with the keratin hydrogel evidenced double re-epithelization rates, rates of tissue remodeling, and overall tissue repair compared to a control group. Continuing with the above-described work, Li et al. prepared insulin-conjugated keratin hydrogel (Ins-K) and demonstrated its application in rat wound models. The Ins-K hydrogel offers enhanced hemostatic capability and faster wound closure, especially within two weeks of healing, compared to both keratin-only and insulin-only treatments. Histopathological analysis in the Ins-K group exhibited organized tissue structures and a significant reduction of TGF- β levels. TGF- β is known to mediate the control of fibroblast proliferation and prevention of excessive scarring. These results together demonstrated that the integration of insulin into keratin hydrogel enhanced not only the speed of wound healing and repair but also reduced scar formation, showing that the natural biological properties of keratin could be optimized by drug integration for further advances in wound care applications [40].

Poranki et al. studied the human hair-derived keratin hydrogels for their therapeutic potential, mainly gamma-keratose, in treating thermal and chemical burns using both mouse and swine models. Their in vitro studies revealed that gamma-keratose hydrogel significantly supported fibroblast viability by 125% 24 hours after thermal injury, while alpha-keratose and crude keratose could only maintain 46% and 65%, respectively. In their in vivo studies, gamma-keratose hydrogel-treated burns achieved complete closure within 18 days, which is 6 days faster than burns treated with either chitosan or saline controls. The improved healing was due to the composition of gamma-keratose-degraded alpha-keratin proteins-found to enhance cell survival, promote re-epithelialization, and protect viable tissue within burn wounds. The promising results hence indicate that gamma-keratose hydrogels offer improved cell rescue and tissue regeneration capabilities

and position them as potent interventions for managing severe burn injuries by accelerating closure and supporting improved cellular recovery and skin remodeling [41].

Gao et al. investigated the regenerative capabilities of recombinant human hair keratin proteins, including RKNP37 and RKNP81, and keratin nanoparticles for the healing of full-thickness wounds in rats. Their in vitro study demonstrated that both the RKNP proteins stimulated HaCaT keratinocyte proliferation in a dose-dependent manner, but RKNP81 exhibited a remarkable cell growth of 310% at 1.000 mg/mL compared to 250% for RKNP37. Follow-up in vivo studies then revealed that the RKNP81-treated wounds closed faster than either RKNP37 or KNP, as evidenced by improved epithelialization and collagen matrix organization within two weeks, along with neovascularization. Importantly, all keratin biomaterials proved biocompatible, displaying no signs of inflammatory response or systemic toxicity. The study ends with the conclusion that especially RKNP81 presents higher regenerative potential in wound repair, combining effective biodegradability and low immunogenicity with strong support for tissue remodeling [42].

Chen et al. investigated the efficacy of human hair keratin hydrogels for the treatment of impaired wounds caused by radiation, with particular relevance for oncology patients treated by radiotherapy and surgery. The in-vitro irradiation of HaCaT keratinocyte cells with 5 Gy of gamma rays showed that the keratin hydrogel treatment, after 48 hours, promoted cell viability to approximately 120% as opposed to around 60% in non-treated and irradiated cells; these differences were found to be statistically significant ($p < 0.01$). In vivo studies in irradiated rat wound models showed progressive wound closure in keratin hydrogel-treated groups, with the wounds consistently reducing their size every week up to complete healing, whereas untreated irradiated wounds demonstrated very little repair. These results support the potential of keratin hydrogels to neutralize or at least counteract the noxious effects of radiation on cell viability and tissue regeneration and should therefore be considered a promising treatment modality for the management of complex wounds in patients exposed to radiotherapy. According to this study, keratin is able to restore cell vitality and induce orderly, successful healing of lesions in challenging clinical situations [43].

Veerasubramanian et al. developed a multifunctional hydrogel composed of konjac glucomannan (KGM), human hair keratin (KER), and Avena sativa extract (OAT) for enhanced diabetic wound healing in rats. In vitro studies confirmed that this composite hydrogel is nontoxic to fibroblast cells and possesses antimicrobial properties against common wound pathogens such as *Staphylococcus aureus* and *Escherichia coli*. Wounds treated with the KGM+KER+OAT hydrogel demonstrated greatly accelerated healing marked by faster re-epithelialization, a thicker epidermis layer, and mature collagen deposition by day 12. Histological analyses showed enhanced collagen synthesis within the wound bed, consistent with complete wound closure by day 16. Impressively,

KGM+KER+OAT accelerated healing by four days compared with KGM+KER and by eight days compared with untreated controls. This demonstrates that the incorporation of oat extract significantly enhances the hydrogel's biological activity and makes KGM+KER+OAT a better combination formulation for diabetic wound dressing than simpler formulations [44].

Ponrasu et al. explored hydrogel scaffolds comprising morin, psyllium, and human hair keratin (PSH + KER + MOR) as a novel biomaterial that assists in the enhancement of skin wound healing in diabetic Wistar rats. Cell viability in vitro remained consistent at approximately 82% for all groups, including PSH + KER, PSH + KER + 0.5% MOR, and PSH + KER + 1% MOR, at the end of 48 hours, which demonstrated the biocompatibility of the hydrogels. The antimicrobial test revealed that the addition of morin renders the hydrogel bacteriostatic to *Staphylococcus aureus*; however, PSH + KER scaffolds were not as effective against gram-negative bacteria such as *Escherichia coli*. In vivo studies of wound healing, conducted every four days, showed that PSH + KER + MOR hydrogel accelerated wound contraction and re-epithelialization time in diabetic rats by enhancing collagen synthesis and deposition, which is very important in the process of tissue remodeling. The morin-loaded scaffolds also significantly outperformed simpler PSH + KER scaffolds and controls, supporting faster and more efficient healing in diabetic wound models [45]. The different applications of human hair keratin for wound healing is listed in Table 1.2.

Table 1.2 Applications of human hair keratin based biomaterials in wound healing

Type of Keratin	Wound Dressing	Reference
Keratin scaffold seeded with hASCs	Keratin scaffold seeded with hASCs	
Keratin-based hydrogel	Keratin-based hydrogels (100 μ L)	
Keratin hydrogel conjugated insulin	Keratin, insulin, and the Ins-K hydrogels	
Keratin-hydrogel	Keratin-hydrogel	
Recombinant human hair keratin proteins (RKNP37 and RKNP81) and keratin	RKNP37, RKNP81, or KNPs and fixed with Tegaderm film	

nanoparticles (KNP)		
Keratin hydrogel	Keratin hydrogel	
Konjac glucomannan-keratin hydrogel scaffold loaded with Avena sativa extracts	KGM + KER scaffolds, KGM + KER + OAT scaffolds	
Keratin hydrogel (KER) supplemented with Psyllium seed husk (PSH) or Morin (MOR)	PSH + KER scaffolds, PSH + KER + 0.5% MOR scaffolds, PSH + KER + 1% MOR scaffolds	

1.9.2. Drug delivery

Human hair keratin has been recognized as a very versatile biomaterial for tunable drug delivery systems in regenerative medicine due to its inherent biocompatibility, cysteine-rich structure, and capability to form chemically modified hydrogels with precisely controlled therapeutic release profiles. Upon extraction in its reduced form as kerateine, keratin forms disulfide-crosslinked hydrogels whose crosslinking density can be finely tuned by chemical alkylation of thiol groups using reagents such as iodoacetamide. This modulation enables the precise control over hydrogel mechanical properties, degradation rates, and erosion kinetics. Such degradable hydrogels thus provide a customizable release platform for various therapeutics, from small-molecule antibiotics such as ciprofloxacin (~300 Da) to mid-sized growth factors like recombinant human insulin-like growth factor-1 (rhIGF-1, ~7 kDa) and large osteoinductive proteins such as recombinant human bone morphogenetic protein-2 (rhBMP-2, ~26 kDa). Notably, these modifications maintain natural cellular compatibility and non-toxicity of keratin, as demonstrated by robust adhesion and proliferation comparable between MC3T3-E1 pre-osteoblasts on keratin hydrogels and fibronectin-coated controls. By synchronizing degradation-mediated drug release with specific timelines of tissue repair, keratin-based hydrogels marry biological advantages inherent to natural polymers with synthetic-like control over material performance, offering a flexible personalized therapeutic platform for applications in wound healing and tissue engineering [46].

To date, human hair keratin has proved to be one of the most versatile biomaterials for advanced drug delivery systems, mainly because of its responsiveness to tumor microenvironment conditions, such as pH changes, levels of glutathione, and enzymatic activity. Hao et al. (2019) demonstrated the wound healing efficacy of recombinant

keratin nanoparticles, RKNP81, through the promotion of angiogenesis and the formation of vascular networks, thus attaining 56% wound closure in vivo within 7 days. For treating cancer, Bhattacharjee et al. (2020) developed dual-drug loaded keratin nanoparticles of approximately 50 nm in size, co-encapsulating rutin and quercetin. These nanoparticles were able to kill 92% of HeLa cancer cells in 24 hours synergistically by targeting the oncogene H-Ras P21. Moreover, these nanoparticles showed sustained drugs release-88% of the drugs released in 30 hours-and a very high encapsulation efficiency of 91% for rutin and 86.5% for quercetin. These keratin-based nanoparticles therefore combine biocompatibility with targeted therapeutic delivery and properties of controlled release, making them promising candidates for applications in wound healing and cancer nanomedicine [42].

In the field of diabetes management, keratin nanoparticles loaded with indoloquinoline derivatives were formulated by Theivendren et al. (2020), showing high encapsulation efficiency of 89%. Such nanoparticles exerted better efficacy compared with conventional metformin treatment through the activation of AMP-activated protein kinase and suppression of protein tyrosine phosphatase 1B in adipocytes, both being highly important regulators of glucose metabolism [47]. Separately, Wang et al. (2020) prepared mucoadhesive gastric-retentive nanoparticles by blending keratose (KOS) and keratine (KTN). These nanoparticles achieved tunable release profiles for amoxicillin, where KTN-rich nanoparticles possessed a drug loading capacity of 15.7%. Moreover, pH-responsive changes in the zeta potential of these nanoparticles enable the regulation of interactions with the gastric mucosa, enhancing retention time. These keratin-based nanoparticulate systems together illustrate a strategy toward better treatment of diabetes due to targeted drug delivery and enhanced bioavailability.

Yuan et al. (2020) developed mesoporous silica nanoparticles coated with a keratin-poly-dopamine (PDA) layer that enabled controlled release of 71.3% of the encapsulated anticancer drug doxorubicin (DOX) under conditions of high glutathione (GSH) and acidic pH, typical of tumor microenvironments. This design successfully reduced toxicity toward normal cells while enhancing drug release in tumor tissues. Similarly, Shen et al. (2020) engineered charge-reversal DOX-loaded nanoparticles exhibiting a shift in zeta potential from -9.5 mV to +5.1 mV under reductive environments, promoting enhanced uptake by tumor cells. In another approach, Li et al. (2018) reported keratin-catechin nanoparticles approximately 67 nm in size for intraperitoneal DOX delivery, which achieved a significant tumor growth inhibition of 63.3%, nearly 3.4 times higher than free catechins. These keratin-based nanoparticles demonstrate smart stimuli-responsive drug release and improved therapeutic efficacy, making them promising candidates for targeted cancer therapy [48].

The technical advances in keratin-based nanomedicine are highlighted by Gnanamani et al. (2020), who fabricated gold nanoparticles functionalized with keratin and albumin. This decreased hemolysis rates to just 2%, as compared to 8% in the case of non-coated gold nanoparticles, and developed stability for up to six months. The work revealed the effectiveness of keratin in improving drug-loading capacity up to 89%, achieving microenvironment-sensitive drug release, and reducing systemic toxicity. Examples of such inventions make keratin a very promising component for treatments both in oncology and in regenerative medicine [49].

Recent advances in nanomedicine highlight keratin's enormous utility in cancer therapeutics using precisely engineered nanoparticle systems. One of the leading works demonstrated the formulation of rutin-quercetin nanoparticles (Ru-Qr NPs) with human hair keratin to enhance chemotherapeutic targeting and efficacy. These Ru-Qr NPs were very stable, as evidenced by spectroscopic and particle sizing analyses, and exhibited regular morphologies in the range of 67–89 nm. Molecular docking analysis indicated strong potential interaction, which showed a binding energy of -8.2 kcal/mol at the active site of the H-Ras P21 proto-oncogene, suggesting a possible inhibitory effect on cancer signaling. In vitro biodistribution profiles further established a preferential accumulation of NPs in kidneys, liver, and spleen within 72 h, hence supporting controlled and organ-targeted drug exposure. More interestingly, these NPs did not reach the brain, hence minimizing the central nervous system toxicity. The cytotoxicity test demonstrated that Ru-Qr NPs induced 95.6% death in HeLa cell cultures at 50 μ g/mL while still sustaining high viability (78%) in normal liver cells, thus confirming their tumor-selective action. This is supported by an inherent characteristic of keratin, where its biocompatibility and stimuli responsiveness against tumor microenvironment signals, such as pH and GSH, facilitate targeted and controlled drug release, reducing adverse side effects. In summary, keratin-based Ru-Qr NPs establish a robust foundation in the design of environment-friendly, tumor-responsive nanomedicine platforms with superior therapeutic precision [50] [51] [52].

Human hair keratin has been harnessed as a sustainable and cytocompatible resource for the synthesis of rod-shaped nanoparticles for targeted drug delivery, exemplified by advances in recent years using acidic hydrolysis methods. These KNP were characterized as amorphous by FTIR and XRD and exhibit a smaller size and higher aspect ratio versus their alkaline-prepared counterparts, maximizing surface area and drug interaction potential. Their strong negative zeta potential of -33.8 mV at pH 8.0 enabled effective electrostatic adsorption of the cationic anticancer agent berberine, achieving a high loading capacity of 168.05 mg/g while maintaining $> 90\%$ viability in stem and VERO cell lines. Upon berberine adsorption, the surface charge decreases to -10.14 mV, preparing the system for photothermal activation. By leveraging the photothermal responsiveness of keratin under an 808 nm laser, a sustained drug release

was achieved at 82% over a period of 24 hours; this, in turn, resulted in selective tumor cell cytotoxicity through combined chemotherapy and thermal effects. The KNPs/BERB system presents dose-dependent toxicity toward HT-29 cells in vitro, offering a dual-modality oncology platform. This strategy, apart from overcoming the limitation of natural solubility of berberine, capitalizes on the biodegradability of keratin and valorization of human hair waste, hence promoting an eco-friendly approach toward precision cancer therapy with minimized off-target side effects [53] [54].

1.9.3. Tissue engineering

Tissue engineering is an interdisciplinary field combining cell biology, materials science, reactor engineering, and medicine to repair damaged tissues and organs when traditional treatments are inadequate. Central to this approach is a porous three-dimensional scaffold that supports cell proliferation, migration, and differentiation while providing structural support for the new tissue. Keratin, obtained from hair and wool, is an important biomaterial used for the construction of scaffolds. It is biocompatible, biodegradable, possesses intrinsic biological activity, and has cell-binding sequences such as EDS and LDV, which interact with integrins, making self-assembly into complex 3D networks quite feasible; these are crucial for the mimicry of native extracellular matrices. Covalent disulfide bonds give mechanical strength to keratin. Non-immunogenicity and a high resistance to degradation-in the absence of native keratinase enzymes-impart durability to the scaffolds in vivo. Hydrogels of keratin, normally prepared by using cross-linking agents such as transglutaminase or glutaraldehyde, are hydrophilic, which favored fluid absorption and retention, hence mimicking several properties of the ECM. Biocompatibility, along with its strong mechanical properties and effective ECM mimicry, guarantees the success of biomaterials in tissue regeneration [55] [56] [57].

Human hair keratin has become a unique biomaterial in tissue engineering due to its natural biocompatibility, biodegradability, and special cell-adhesive motifs such as LDV, EDS, and RGD, which help overcome a broad range of regenerative challenges. In diabetic wound care, hydrogels combining keratin with konjac glucomannan and *Avena sativa* extract have shown a remarkable increase in dry weight up to 7.5-fold when soaked in phosphate-buffered saline, hence promoting faster reepithelization by means of immunomodulatory and antioxidant effects while managing exudate formation in wounds with high efficiency. Injectable keratin hydrogels loaded with deferoxamine reduce the healing time from 14 to 10 days in tail amputation and liver injury models through enhancement of clot formation and vascular growth. In periodontal therapy, antibiotic-eluting keratin films demonstrate rapid water absorption within 4 h and maintain their swollen state for more than 100 h, allowing localized drug delivery. In urinary tract reconstruction, oxygen-generating scaffolds prepared from keratin, gelatin, silk fibroin, and calcium peroxide can maintain O₂ release for more than two weeks, and

this is useful in the regeneration of muscle and epithelial tissue. Bone tissue engineering employs freeze-dried scaffolds made from keratin, jellyfish collagen, and hydroxyapatite to facilitate stem cell osteogenic differentiation without the use of external inducers. Electrospun mats prepared from blends of polycaprolactone, keratin, and calcium carbonate have significantly enhanced the proliferation of osteoblasts. Bilayer gelatin methacrylate-keratin/chitosan scaffolds for skin repair enhance tensile property and improve keratinocyte differentiation, further augmented with bacterial cellulose/keratin composites that enhance the adhesion of fibroblasts. In nerve regeneration applications, keratin sponges stimulate axon growth and Schwann cell adhesion, while injectable keratin hydrogels support neural stem cell differentiation, hence facilitating spinal cord repair. Skeletal muscle regeneration utilizes collagen/keratin hydrogels to induce new muscle fiber formation within 15 days, while keratin/gelatin scaffolds stimulate myogenin mRNA expression, hence accelerating muscle development. In vascular tissue engineering, phosphobetainized keratin/polycaprolactone nanofibrous mats are utilized in catalyzing nitric oxide production, encouraging endothelial cell adhesion, and inhibiting excessive smooth muscle proliferation [58] [59] [60]. In table 1.3 the different applications of human hair keratin in tissue engineering is enumerated.

Table 1.3 Applications of human hair keratin based biomaterials in tissue engineering.

Composition	Biomaterial Type	Properties and Function	Applications
Keratin and fibrinogen	Hydrogels	Suitable for controlled protein delivery; supports cell proliferation and migration.	Skin tissue regeneration
Phosphobetainized keratin (PK) and poly(ϵ -caprolactone) (PCL)	Nanofibrous mats	Catalyzes nitric oxide (NO) generation; enhances endothelial cell adhesion/migration; suppresses smooth muscle cell proliferation; anticoagulant.	Vascular tissue engineering
Keratin and chitosan	Hydrogels	>80% cell viability; negligible cytotoxicity against L929 fibroblasts; tunable mechanical properties.	General tissue engineering

Collagen and keratin	Hydrogel	Promotes myogenesis; generates de novo muscle fibers in mice within 15 days.	Skeletal muscle regeneration
Keratin and gelatin	Scaffolds	Accelerates myogenesis; enhances myogenin mRNA expression and myotube development.	Skeletal muscle regeneration
γ -PGA and keratin	Electrospun nanofibrous scaffolds	Supports mouse fibroblast proliferation and adhesion in vitro.	General tissue engineering
Fibroin, keratin, and vanillin	Spongy scaffolds	Vanillin enables dose-dependent antibacterial activity against E. coli and S. aureus; promotes cell infiltration.	Infected tissue engineering
Keratin (alone)	Sponges	Promotes axon extension and Schwann cell adhesion; supports peripheral nerve regeneration.	Nerve regeneration
Keratin (modified)	Injectable hydrogels	Enables minimally invasive delivery; adapts to complex tissue defects; supports neural stem cell differentiation.	Neural tissue engineering
Keratin, konjac glucomannan, Avena sativa	Hydrogel bandage	7.5-fold dry weight increase in PBS; accelerates re-epithelialization; anti-inflammatory/antioxidant properties.	Diabetic wound healing
Keratin with deferoxamine (DFO)	Injectable hydrogel	Reduces wound healing time from 14→10 days; decreases wound persistence to 11%.	Hemostasis and wound healing
Keratin with	Films	Rapid water absorption (4 hrs); sustained swelling (>100 hrs);	Periodontal

glycerol/antibiotics		local antibiotic elution.	regeneration
Keratin, gelatin, silk fibroin, calcium peroxide	Oxygen-generating scaffold	Sustained O ₂ release (>2 weeks); promotes muscle bundle/epithelial regeneration in dog urethral defects.	Urinary tract reconstruction
Keratin, jellyfish collagen, hydroxyapatite	Freeze-dried scaffold	Enables osteogenic differentiation of stem cells without induction agents.	Bone regeneration
PCL, keratin, CaCO ₃	Electrospun scaffold	Enhances Saos-2 osteosarcoma cell proliferation; superior to pure PCL.	Bone engineering
GelMA + keratin/chitosan nanofibers	Bilayer scaffold	Increases tensile strength; promotes HaCaT proliferation/keratinocyte differentiation; mimics dermal-epidermal structure.	Skin tissue engineering
Bacterial cellulose (BC)/keratin	Nanocomposite	Enhances cutaneous fibroblast adhesion.	Functional skin construction

1.9.4. Peripheral nerve regeneration

The PNS is responsible for transmitting motor and sensory signals between the CNS and the tissues/ organs of the body. Peripheral nerve injury due to trauma, compression, and other insults may range from neuropraxia, a block of axoplasmic flow with normal axon morphology, to axonotmesis and neurotmesis, where there is disruption of either the axon or both the nerve and nerve sheath, respectively. While some inherent regenerative capacity exists within the PNS, this is often insufficient in the most severe injuries, delayed treatments, and/or when nerve gaps exceed 3 cm. The slow rate of nerve regeneration (averaging only 1-3 mm per day) often results in the degeneration of distal target organs, muscle atrophy, fibrosis, and possible lifelong disability coupled with

decreased quality of life. Gold-standard treatments, including nerve anastomosis and autologous nerve grafting, present several challenges that include donor site morbidity, limited graft availability, immune rejection, diameter mismatches, and surgical complications. As a result, alternative strategies have been the focus of research; these approaches include the use of artificial nerve conduits and biomaterials capable of bridging nerve gaps and promoting axonal regeneration. Hydrogels and membranes fabricated from natural and synthetic polymers represent promising biomaterial candidates due to their biocompatibility and ability to mimic the native ECM. However, key issues relating to immune responses, unpredictable degradation rates, toxicity concerns, limited raw materials, and high costs limit their wide clinical translation. These challenges necessitate further innovation in biomaterial design for successful peripheral nerve repair [61] [59].

In the realm of peripheral nerve regeneration, human hair keratin has attracted extensive attention as a biomaterial due to its rare amalgamation of desirable attributes. Keratin is an abundant, naturally derived polymer that not only demonstrates outstanding biocompatibility and non-immunogenicity, but also features intrinsic cell-adhesive motifs, such as LDV and RGD, commonly found in extracellular matrix proteins; these are pivotal for promoting cell adhesion and supporting the growth, proliferation, and migration of reparative cells like Schwann cells and neurons. Research has demonstrated that keratin-based scaffolds—membranes, sponges, and hydrogels—support the advancement of neural and bone marrow stem cells and are structurally stable even at heightened temperatures, offering considerable advantages over more delicate biomaterials. However, pure keratin's mechanical fragility has prompted the creation of composite scaffolds, notably with chitosan, a polysaccharide renowned for its strength and inherent regenerative effects. These keratin-chitosan composites amalgamate keratin's biological activity with chitosan's mechanical resilience, creating scaffolds better able to bridge nerve gaps and withstand physiological stress. Advances have further amplified keratin's regenerative effects by incorporating neuroactive agents such as curcumin into keratin/chitosan hydrogels. Curcumin is recognized for enhancing Schwann cell proliferation and migration, safeguarding dorsal root ganglia, and imparting robust anti-inflammatory and antioxidant effects. When embedded within keratin-based hydrogels, curcumin is released gradually at the injury site, maximizing local therapeutic efficacy while minimizing systemic exposure. Collectively, these strategies position human hair keratin composites as highly promising solutions in the challenging landscape of peripheral nerve repair, offering biocompatibility, tunable mechanics, and sustained therapeutic delivery in one adaptable platform [62].

Human hair keratin has been validated for peripheral nerve regeneration through various innovative approaches and animal studies. Keratin has been shown to induce axonal outgrowth in dorsal root ganglion neurons *in vivo* and enhance Schwann cell adhesion,

proliferation, and migration, and the secretion of neurotrophic factors in vitro. In addition, keratin biomaterials have an immunomodulatory function, which elicits M2 (anti-inflammatory) macrophage polarization, a very important process in tissue repair and functional recovery after spinal cord injury. Recombinant keratin variants, 33A and 35, especially keratin 33A, showed strong anti-inflammatory effects and supported M2 polarization to facilitate nerve repair in rat models. Nerve grafts developed with the combination of activated Schwann cells and human hair keratin have shown significantly improved nerve conduction and motor function after sciatic nerve injury in rats, presumably due to the increased expression of growth factors. Recently, keratin/chitosan hydrogels have also been developed as platforms for localized and sustained curcumin delivery, maintaining its release for up to 10 days and leading to improved nerve regeneration in animal experiments. Such hydrogels exhibited excellent cytocompatibility, histocompatibility, and biodegradability. Studies have confirmed that keratin sponge dressings and hydrogels alleviate motor deficits and contribute to nerve recovery without any induction of major inflammation or organ toxicity. All these studies together suggest human hair keratin-based biomaterials and their composites as an exciting, multifunctional platform for peripheral nerve repair, incorporating biological activity, immunomodulation, and therapeutics to improve regeneration and function [63].

1.9.5. Hemostatic agent

Hemostatic agents have an important role in the arrest of bleeding by facilitating blood clot formation at injury sites. Hemostasis is a complex biological process that involves the activation of platelets and key proteins, including fibrinogen and thrombin, which converge to form a stable clot. While the innate hemostatic responses of the body are often sufficient in minor injuries, severe trauma and major surgical procedures can overwhelm these mechanisms, creating life-threatening risks that need the application of special hemostatic agents. Over the last decades, several new agents, adhesives, and sealants have been developed for uncontrolled bleeding in surgical, trauma, and battlefield applications. An ideal hemostatic solution is yet to be found since it should rapidly arrest hemorrhage and combine strong mechanical support, superior adhesion, ease of use independent of wet or dynamic environments, and finally be biodegradable, biocompatible, and antibacterial. The most important areas of research are into biomaterials that combine the compatibility of natural materials with the tunable features of synthetic compounds. Many studies point to biomaterials based on human hair keratin as one of the most promising hemostatic platforms for both trauma and surgical management due to their unique chemical and biological properties [60].

Keratin derived from human hair has become a highly versatile and biologically compatible hemostatic agent for bleeding control. Functionally, keratin can directly activate platelets and coagulation proteins, increasing platelet adhesion and aggregation,

which is crucial for rapidly forming blood clots at injury sites. Its versatility allows it to be processed into sponges, powders, films, and composite scaffolds, all of which physically entrap blood cells and foster robust clot development. Experimental evidence shows that keratin-based sponges can absorb more than 90% water and maintain cell viability, whereas keratin/alginate formulations absorb upwards of 1600% of their weight in liquid and markedly shorten clotting times. Crosslinked keratin scaffolds with alginate or chitosan further enhance liquid absorption while maintaining excellent biocompatibility. Importantly, keratin-associated protein nanoparticles (KAPNPs) fabricated from human hair have a spherical shape and exhibit marked biocompatibility with cells and rapid clotting. Recent animal studies show that human hair-derived keratin hydrogels are comparable, and in some aspects superior, to leading commercial hemostats, such as QuickClot and HemCon, as reflected by higher survival rates and favorable tissue healing. A unique advantage in the case of keratin is its natural abundance and rapid extraction from waste, conferring sustainability benefits. Collectively, these studies confirm the two major promises of keratin as an advanced and tissue-friendly hemostatic agent [64] [65] [66].

1.9.6. Ocular surface reconstruction

Ocular surface reconstruction aims at the repair and regeneration of the cornea, conjunctiva, and limbus to restore vision and alleviate pain subsequent to trauma, infections, chemical injury, surgical complications, or autoimmune diseases. Key goals include the restoration of a clear vision, minimization of pain, and the prevention of further ocular damage, particularly in cases when the surface has been highly compromised. Traditionally, human amniotic membrane has represented the biological gold standard in such reconstructions, often in company with anti-inflammatory drugs such as dexamethasone for greater efficacy in healing and minimizing inflammation. However, despite wide usage, the amniotic membrane presents a number of disadvantages: variable biomechanical strength, suboptimal transparency, inconsistency between donors, and a possibility of infection. These disadvantages, along with the postoperative management issues, have made researchers and clinicians pursue other biomaterials that better mimic the native ocular structure and regenerate it. Human hair-derived keratin films represent one of the most promising substitutes due to their biocompatibility, transparency, and standardized quality. Animal model studies show that keratin films support rapid epithelial healing, maintain corneal transparency, and minimize inflammatory responses, making them strong candidates for next-generation treatments of corneal and conjunctival regeneration [67] [68] [69].

Human hair keratin has gained increasing attention for its use as an advanced biomaterial in ocular surface reconstruction due to its excellent biocompatibility, biodegradability, and potential to support cell proliferation, adhesion, and migration. The mechanical properties of keratin-based scaffolds, membranes, and fibrous mats

closely mimic those of the native ocular surface and have provided a stable scaffold for epithelial regeneration and seamless tissue integration. Recent studies using keratin films from human hair, especially combined with anti-inflammatory medication such as dexamethasone, have shown strong therapeutic benefits. These films maintain essential transparency and optimal biomechanical properties, while their capacity for controlled drug release minimizes the need for frequent administration, thereby improving patient compliance and comfort. In the *in vivo* rabbit model, keratin films allow complete ocular surface healing, preventing abnormal vascularization and performing at least as well as, if not better than, conventional amniotic membranes. An inherent anti-inflammatory effect of the keratin biomaterials provides a favorable healing milieu, promoting epithelial cell growth without significant immune reactions. With properties that meet key requirements for transparency, mechanical strength, cell compatibility, and the control of inflammation, such keratin-based biomaterials represent a very attractive alternative for ocular surface repair. Ongoing research is under way to further develop these keratin-based platforms for optimal clinical use and improved patient outcomes [70] [71] .

1.9.7. Bioplastics

Human hair-derived keratin is a raw material that has gained increasing attention for the production of bioplastic films, serving as an environmental alternative toward conventional petroleum-based plastics. Extraction of keratin from human hair waste and its transformation into films, usually called "kertics," initiates the process. Fabrication by solution casting of the keratin protein dissolved in specific solvents into silicone molds is required. To date, plasticizers such as glycerol, ethanediol, diethylene glycol, and triethylene glycol have been added during processing to adjust physical and mechanical properties for specific applications. Addition of these plasticizers effectively enhances flexibility and reduces brittleness; hence, these plasticizers play a relevant role in the adjustment and control of the morphology of the bioplastic films. The keratin bioplastics prepared so far have been stable and biodegradable, in line with the concerns related to the pollution of the environment by synthetic plastics. Besides its natural abundance from human hair wastes, keratin should be a sustainable and economic feedstock for the production of bioplastics to be applied in packaging and biomedical devices, among other uses. Green processing with renewable proteins yields flexible, biodegradable films that can be used to help solve the problem of plastic waste and to contribute to circular economy principles [72] [73].

The keratin bioplastic films produced from human hair exhibit several notable characteristics. Analytical techniques, including Field Emission Scanning Electron Microscopy (FESEM), X-Ray Diffraction (XRD), and Fourier Transform Infrared Spectroscopy (FTIR), confirm their uniform surface morphology, structural integrity, and preservation of keratin's native chemical structure. These films are biodegradable,

as demonstrated by their ability to be broken down by keratinophilic fungi, underscoring their environmental friendliness. The versatility of human hair keratin bioplastics makes them attractive for multiple applications, notably in packaging and disposable products. Their inherent biocompatibility also opens avenues for biomedical uses such as wound dressings and tissue scaffolds. Furthermore, the properties of these “kertics” can be finely tailored by varying the type and concentration of plasticizers like glycerol, ethanediol, and diethylene glycol, allowing customization for specific needs. This tunability enhances their potential as sustainable, customizable materials that address the urgent demand for alternatives to petroleum-based plastics. Their biodegradability and sustainable sourcing from waste human hair position these films as a next-generation solution in efforts toward reducing plastic pollution and advancing green materials science [74] [75] [76].

1.9.8. Miscellaneous

Human hair keratin films, which are structurally analogous to the human nail plate, have been developed as innovative biological models for the study of onychomycosis and evaluation of drug penetration and permeation. These films offer a practical alternative to human nails, which are often difficult to obtain in sufficient size for laboratory testing [77] [78] [79] [80].

Beyond biomedical applications, keratin from human hair also finds application in the construction industry for the preparation of plasters for wall and other building structures. This addition may enhance the setting behavior of construction materials and their mechanical properties. In addition, biodegradable keratin-based materials have been used to fabricate several parts in electrical devices such as resistors, capacitors, and inductors, indicating that keratin can be applied in the development of sustainable electronics. Keratin biomaterials also serve as active biosorbents in environmental remediation through heavy metal ion removal from contaminated water. This normally occurs through active polar sites on keratin surfaces, which attract and bind charged metal ions, hence making keratin a useful resource in pollution control. Altogether, these listed applications demonstrate the versatility and sustainability of human hair keratin, making it a very useful and innovative material in biomedical, construction, electronic, and environmental areas [81].

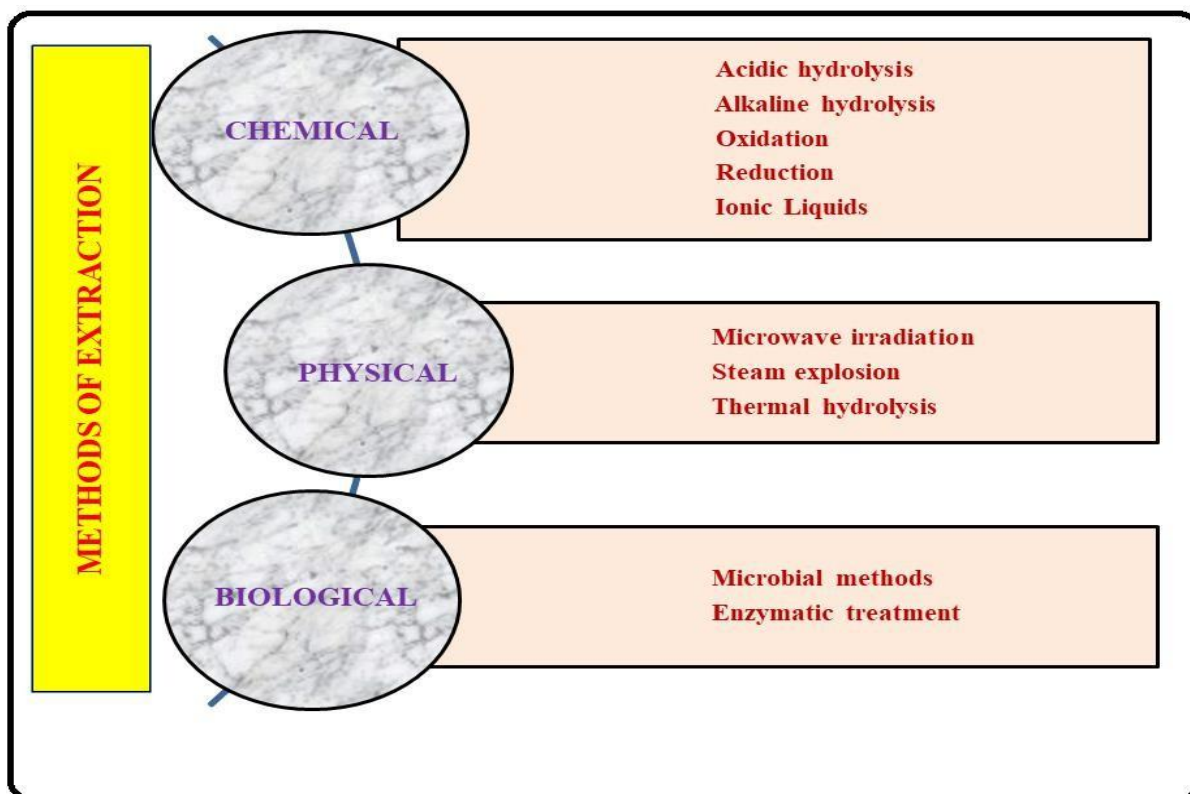
1.10. Extraction of keratin: challenges and solutions.

1.10.1. Traditional chemical and physical extraction methods

Keratin is a robust and fibrous structural protein, which widely occurs in several keratin-rich wastes, including human hair, wool, feathers, horns, and hooves [82]. Among these keratinous wastes, human hair waste is considered one of the most abundant but not yet well valorized wastes generated globally from salons, barber shops, and households. The

inappropriate disposal of this keratinous biomass is not only causing environmental pollution but also concomitantly squandering the keratin biomaterial value. Keratin from hair was demonstrated to have excellent physicochemical properties, including mechanical strength, biocompatibility, and biodegradability, which makes it suitable for applications ranging from biomedicine to agriculture, cosmetics, and materials science [83]. However, this tight structure of hair keratin is stabilized by numerous disulfide and hydrogen bonds, which restricts the efficiency of keratin extraction [84] [85]. In recent years, chemical, biological, and physical extraction methods have been proposed and developed for the recovery of high-value keratin from human hair wastes. Generally, acid or alkali hydrolysis is employed in chemical extraction methods; biological extraction employs keratinolytic enzymes from microorganisms, whereas physical treatment may involve thermal hydrolysis or ultrasonic-assisted methods. The type of extraction method generally influences the yield, molecular weight, and functional properties of keratin [86]. Valorization of human hair wastes via optimized keratin extraction has great environmental and economic advantages, catalyzing sustainable material innovation and reduction, with the increased demand for ecofriendly and renewable biomaterials [87] [88] [89]. This section provides a critical review of the various methods for the extraction of keratin from keratin-rich wastes, focusing on human hair as shown in Figure 1.2.

Figure 1.2 Different methods of extraction of keratin



1.10.1.1. Chemical Methods

Most chemical methods for keratin extraction generally involve the disruption of strong intermolecular bonds within keratin fibers using chemical agents such as acids, alkalis, and oxidizing or reducing chemicals. These treatments effectively break disulfide and hydrogen bonds, allowing keratin to be solubilized from sources like human hair and wool for further use. General methods can include direct alkaline hydrolysis using sodium hydroxide, or acidic treatments-both methods cleave cystine disulfide bridges and degrade the fiber structure. Alternatively, oxidative and reductive methods may employ reagents such as peracetic acid, thioglycolic acid, or 2-mercaptoethanol, which selectively reduce or oxidize the crosslinks in keratin. Chemical extraction methods are widely employed owing to the high efficiency and relatively high yields of keratin. However, these treatments have been reported to alter the native molecular structure of keratin and change some functional properties such as molecular weight and bioactivity. Optimizing conditions such as reagent concentration, temperature, and duration is crucial to balance extraction efficiency and preservation of keratin functionality for downstream applications in biomedicine and materials science [2].

1.10.1.1.1. Acidic hydrolysis

The usual way to obtain keratin from any keratin-rich tissue is through the application of acidic hydrolysis. In this process, hair or wool is subjected to strong acidic solutions like hydrochloric acid or sulfuric acid; the protein then undergoes hydrolytic cleavage. This cleavage breaks down the complex protein structure of keratin and permits its extraction. After hydrolysis, the acidic solution must be neutralized, and the resulting keratin product is generally dried for stabilization. Hydrolysis time is a critical consideration, as longer hydrolysis times increase keratin yields but simultaneously promote fragmentation into much lower molecular weight peptides. This might not be desirable in various value-added applications. Disadvantages of this method include the damage to labile amino acids such as tryptophan, reducing the nutritional value of the peptides obtained. The process also produces significant amounts of acidic waste, causing corrosiveness, equipment degradation, and challenges with environmentally safe disposal. Therefore, great care in optimizing acid concentration and hydrolysis time will be required in order to balance extraction efficiency, preserve keratin quality, and address environmental concerns [90].

1.10.1.1.2. Alkaline hydrolysis

Alkaline hydrolysis is a chemical process commonly used for extracting keratin from keratin-rich tissues by treating them with hydroxide-based compounds such as calcium hydroxide ($\text{Ca}(\text{OH})_2$), potassium hydroxide (KOH), or sodium hydroxide (NaOH). This method generally preserves the amino acid profile better than acidic hydrolysis, as there

is minimal amino acid loss. However, alkaline treatment can trigger side reactions including decarboxylation, desulfuration, and deamination, which may degrade certain components such as cystine residues, impacting the keratin's mechanical properties. The extracted keratin often exhibits a brown color and lower molecular weight, which limits its ability to form films effectively. Efficiency depends on several parameters including the type of alkali—lithium hydroxide (LiOH) tends to be more effective than KOH, which is superior to NaOH—the pH levels, temperature, reaction duration, and alkali concentration. Moderate heating enhances the extraction rate, but excessive heat can damage sensitive amino acids. Acid and alkali combined treatments sometimes offer better yields and functionality than either method alone. Despite its extraction efficiency, the resulting weakening in mechanical strength restricts alkali-extracted keratin's applicability in areas requiring robust film-forming capabilities. Alkaline hydrolysis extracts keratin from keratinous tissues by treating them with hydroxide compounds like calcium hydroxide, potassium hydroxide, or sodium hydroxide. This method better preserves amino acids compared to acidic hydrolysis, with less amino acid loss. However, side reactions such as decarboxylation, desulfuration, and deamination can occur, leading to degradation of cystine residues and reduced mechanical strength. The extracted keratin is often brownish and less capable of forming films, especially with lower molecular weight fractions. Extraction efficiency depends on factors including the alkali type, pH, temperature, reaction time, and concentration. Lithium hydroxide is more effective than potassium or sodium hydroxide for hydrolysis. Heating improves extraction but excessive temperatures can damage amino acids. Combining acid and alkali treatments can yield better extraction results. Despite its efficient extraction, the weakened mechanical properties limit the use of alkali-extracted keratin in applications like film formation.

1.10.1.1.3. Oxidation

Oxidative extraction of keratin involves the use of strong oxidizing agents such as performic acid, peracetic acid, hydrogen peroxide, peroxyacetic acid, and peroxyformic acid to cleave the disulfide bonds present within keratin fibers. These disulfide covalent bonds are converted into cysteic acid residues, leading to the formation of soluble keratin fractions known as keratoses. These keratoses, stabilized by non-covalent interactions, exhibit hygroscopic and water-soluble properties. Keratoses are categorized based on their solubility in ammonia and tissue origins: α -keratoses from the cortex, soluble in ammonia and precipitating at acidic pH; β -keratoses from the cuticle, insoluble in ammonia; and γ -keratoses, which are ammonia-soluble but do not precipitate at acidic pH. Tissue types differ in their keratose composition; for instance, wool contains around 60% α -, 10% β -, and 30% γ -keratoses. The oxidation process converts disulfide bonds into various oxidized groups, including sulfonates and cysteine oxides. However, a major disadvantage is the degradation of sensitive amino acids like tryptophan, methionine,

cysteine, and others due to harsh oxidation conditions. Moreover, achieving higher keratin yields, often exceeding 50%, requires prolonged exposure to high concentrations of oxidants, sometimes up to three days, which can adversely affect the quality and characteristics of the extracted keratin. Therefore, optimizing oxidation conditions is essential to balance yield, functionality, and preservation of keratin integrity.

1.10.1.1.4. Reduction

The reduction method is generally accepted as the most prevalent chemical method of keratin extraction from wool, feathers, hair, horns, and hooves. This process utilizes aqueous solutions containing reducing agents such as 2-mercaptoethanol and other thiols—including dithiothreitol, sodium thioglycolate, and thioglycolic acid—to cleave the disulfide bonds inherent in the structure of keratin. These reducing agents are very often used with denaturing compounds such as urea (sometimes to concentrations of up to 8 M) or thiourea, as well as surfactants such as sodium dodecyl sulfate (SDS) and sodium hydrogen sulfite, which enhance the solubility and stability of keratin in solution. The presence of denaturants disrupts hydrogen bonding and the protein framework, thereby increasing the effectiveness of the reducing agents, while surfactants prevent keratin aggregation. Although the commonly utilized thiols, such as 2-mercaptoethanol, are highly effective, the attendant toxicity and cost have given rise to more frequent use of substitutes such as sodium disulfite, which, though less toxic and less expensive, often yields lower efficiencies. For example, a solution containing 0.125 M sodium metabisulfite, 0.05 M SDS, and 2 M urea effectively cleaved disulfide linkages in wool by yielding approximately 49% dissolution with resulting keratin hydrolysates that could self-assemble. However, for keratin sources with lower cysteine content, such as feathers and hooves, the extraction yields usually reported are well below those observed for wool because of their often unique biochemical composition.

1.10.1.1.5. Ionic Liquids

Ionic liquids are a class of molten salts composed of large organic cations with an organic or inorganic anion counterpart that remain liquid at temperatures below 100 °C and usually at ambient temperature [91] [92] [93] [94]. These solvents show high polarity, excellent solvation capability, and remarkable thermal and chemical stability, which makes them particularly suitable for the dissolution of natural polymers like keratin. In contrast to classic acidic or basic extraction agents, ILs offer a greener and safer alternative, having negligible vapor pressure, being recyclable, and showing low flammability [95]. The strong polar nature of these agents promotes the disruption of the intermolecular hydrogen bonds of keratin fibers and, consequently, increases its solubilization efficiency [96] [97] [98] [99][100]. However, effective dissolution also demands the cleavage of disulfide bonds within the protein molecule in cysteine residues; therefore, compounds like sodium sulfite (Na_2SO_3), sodium bisulfite (NaHSO_3), SDS,

and urea are often added to the IL systems with the aim of accelerating such a reduction process and improving the extraction rate [101] [102] [103] [104].

According to recent reports, chloride-based ionic liquids, such as 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) and 1-allyl-3-methylimidazolium chloride ([Amim]Cl), are highly effective at dissolving keratin because of their strong nucleophilic nature and high chloride ion content, which serve to effectively break the hydrogen bonding within the protein network [105]. Xie et al. showed that [Bmim]Cl was capable of solubilizing wool keratin in the temperature range between 100 and 130 °C after 10–24 hours with a yield of up to 11%. Although the yield was relatively low, the extracted keratin showed better thermal stability than native wool fibers [106] [107]. This original work was followed by several other studies where imidazolium-based ILs were combined with sodium sulfite (Na₂SO₃) and resulted in feather keratin extraction yields close to 75% at 90 °C in 60 minutes. Ji et al. then further showed that ILs with chloride anions performed better than those containing BF₄⁻, Br⁻, or PF₆⁻ anions; for example, [Bmim]Cl produced a yield of 57% at 120 °C (1:6 w/w ratio) and up to 78.5% when the IL-to-feather ratio was increased to 1:10. The corresponding yield for 1-ethyl-3-methylimidazolium diethyl phosphate ([Emim]DEP) under similar conditions was 70.2%. Temperature played an important role in extraction efficiency; for instance, Ghosh et al. achieved optimal yields (57%) at 120 °C in [Bmim]Cl, whereas higher temperature (150–180 °C) resulted in reduced recovery, presumably due to some of the amino acids remaining dissolved in the IL phase. Similarly, extraction studies on human hair confirmed the solubilization of keratin using [Amim]Cl with yields of around 19%. The structure of the regenerated keratin indicated a transition from α -helical to β -sheet structures, reflecting the interruption of the native conformation upon dissolution. Overall, these studies have proven that ionic liquids, more precisely chloride-based ionic liquids combined with appropriate reducing co-solvents, are controllable, green, and efficient media for keratin extraction, where IL composition, processing temperature, reaction [108] [109] [110] [111] [104] .

1.10.1.2. Biological Methods

Biological methods for extracting keratin have some advantages compared to traditional chemical ones. Most of the processes take place under mild reaction conditions, requiring lower energy input while causing less destruction to essential amino acids; hence, the functional properties of the protein are maintained. In addition, most are considered to be more environmentally friendly since fewer harsh chemicals are used, and less toxic by-product formation takes place. These processes utilize either microorganisms with keratinolytic activity or specific enzymes known as keratinases to break down the strong keratin structure. It relies on the reduction of disulfide bonds besides the hydrolysis of peptide bonds, allowing for effective solubilization and recovery. Keratin fractions recovered using these processes usually possess a lower molecular weight and often

provide comparable or higher recovery levels than chemical processes. It is relatively costly to produce the enzyme or grow microorganisms compared to inexpensive chemical reagents.

1.10.1.2.1. Microbial methods

Microbial keratin extraction is based predominantly on bacteria and fungi that synthesize keratinolytic enzymes capable of degrading the highly recalcitrant keratin structure. Among these, bacteria from the genus *Bacillus*, particularly *Bacillus licheniformis*, are often reported to be highly efficient degraders, with frequent isolation from habitats with high amounts of keratin, including soil, poultry litter, and feather waste. Potent keratinolytic capabilities have been realized with other bacterial species, including *Amycolatopsis*, *Chryseobacterium*, *Streptomyces*, and *Staphylococcus*. Fungal species also contribute significantly to this category, especially thermotolerant fungi like *Chrysosporium tropicum* and *Microsporum gypseum*, which have shown remarkable potential for keratin degradation, although most fungi capable of keratinolysis are mesophilic in nature. In practical extraction protocols, combinations of bacteria and fungi are sometimes employed, and denaturing additives like urea may be incorporated to improve the keratin yield and to regulate the molecular weight distribution. Functionally, microorganisms can be grouped by their keratinolytic activity into those that display strong proteolysis that directly hydrolyzes keratin fibers and others which act through the indirect degradation of the associated non-keratin protein matrix, thereby liberating keratin effectively [112].

1.10.1.2.2. Enzymatic treatment

The most efficient biological strategies for keratin extraction involve enzymatic hydrolysis, which employs specialized enzymes called keratinases that can degrade the unique, tightly packed structure of keratin. Unlike more conventionally used proteases such as pepsin and trypsin, keratinases display superior catalytic activity toward insoluble keratin substrates, making them essential for bioconversion processes. These enzymes are produced by a wide range of microbial groups, including bacteria, actinomycetes, and keratinophilic fungi, and may be intracellular or secreted into the extracellular environment-the latter often favoring recovery and large-scale application. Microorganisms from genera *Bacillus*, *Streptomyces*, *Aspergillus*, and *Lysobacter* are among the well-recognized high producers of keratinase. The effectiveness of enzymatic hydrolysis may be further improved by addition of denaturing agents or surfactants, which facilitate the destruction of the compact keratin matrix of feathers, wool, and hair. Keratinases exhibit remarkable stability over a wide pH range and also over the temperature range, enabling their application under conditions in which many enzymes lose their activity. They degrade keratin to form low-molecular-weight peptides and amino acids under severe reaction conditions. These may then be used for fertilizer,

animal feed, and soil conditioner formulations. In contrast, operation under controlled conditions can result in the production of higher molecular weight fractions suitable for biomedical and biomaterial applications, such as hydrogel scaffolds in wound healing, regenerative medicine, and targeted drug delivery. Indeed, at an industrial level, several keratinase-based preparations, including Savinase, have been successfully utilized for the eco-friendly processing of keratin waste in the textile, detergent, and leather industries.

1.10.1.3. Physical Methods

Physical methods of extraction employ mechanical or thermal energy that disrupts the dense keratin matrix from sources such as human hair, wool, or feathers. Methods such as microwave irradiation, steam explosion, and thermal hydrolysis involve the use of increased temperature or sudden changes in pressure that weaken intermolecular bonds, promoting solubilization and release of keratin proteins from the fibrous material [34].

1.10.1.3.1. Microwave irradiation

Microwave irradiation has emerged as an innovative physical method for extraction of keratin, significantly improving the efficiency of the process and energy consumption. It influences the kinetic process of protein dissolution by providing fast and uniform heating directly to keratinous substrates, such as wool, feathers, or hair, thereby reducing the activation energy for the process and allowing homogeneous energy absorption at the molecular level. As a result, it leads to faster degradation of keratin and its extraction, which usually takes place in seconds or minutes [113]. Experimental investigations have revealed that microwave treatment at temperatures between 150–180 °C can provide up to 60% of keratin from wool; feathers subjected to 160–200 °C for 20 minutes resulted in yields of 71.83%. The key advantages of microwave-assisted extraction are speed and equal heat distribution, which is quite impossible in the case of conventional thermal treatment. At the same time, the serious disadvantage of this approach is a significant loss of essential amino acids, including cysteine, whose concentration may decrease from 9.4 mol% to about 0.5 mol% after prolonged action. Despite these drawbacks, microwave irradiation appears as one of the promising energy-saving technologies; however, further research is required to explain the detailed action of electromagnetic fields on the complex structure of keratin [114] [115] .

1.10.1.3.2. Steam explosion

The steam explosion process is a hydrothermal method that consists of high-pressure saturated steam treatment of keratin-rich biomass such as wool or feathers, for short times typically ranging from 1 to 10 min at high temperatures in the 180–230 °C range. After this steam treatment, the system rapidly depressurizes and the explosive decompression physically ruptures the dense keratin fibers and increases access to the

protein for extraction. Initially developed for wool, the technique has subsequently been extended to other keratinous materials. For example, treatment of wool with saturated steam at 164.2 °C has been shown to render up to 80% of this biomass digestible by pepsin. Although steam explosion is effective in breaking disulfide bridges and reducing the crystallinity of wool, it often fails to break all disulfide and hydrogen bonds completely, which limits the effectiveness of the extraction. The intensive conditions during the treatment also strongly reduce the cysteine content of the keratin recovered and may affect its quality as well. Improved variants such as steam flash explosion (SFE) have been described that allow processing to take place in an even shorter time with improved digestibility and higher solubilization at lower temperatures but which still may result in reduced cystine content and partial degradation of the keratin's chemical structure.

1.10.1.3.3. Thermal hydrolysis

It is a physical method of keratin extraction by treatment of keratinous biomass, such as feathers, wool, or hair, with water under rigorously controlled temperature and pressure in a sealed pressure vessel. Generally, it consists of two steps: first, denaturation of the keratin protein matrix, and second, cleavage of the disulfide bonds holding the fibrillar structure together. The temperature and duration of treatment are the critical factors defining the rate and extent of dissolution of keratin. A yield of about 70% of keratin recovery can be realized with this process. Hydrolysis degradation reduces the protein into oligopeptides, and hence, it is very effective for the production of lower-molecular-weight keratin fractions. Because of its high efficiency and scalability, this is an attractive industrial-scale process for the extraction of keratin protein from protein-rich wastes.

1.11. Scope of the study

Keratin derived from human hair is an abundant, renewable, and valuable biomaterial with immense potential for biomedical applications such as wound healing, tissue engineering, drug delivery, and cosmetic formulations. Keratin-based materials exhibit excellent biocompatibility, biodegradability, mechanical durability, and bioactivity, making them well-suited for developing advanced biomaterials like hydrogels, scaffolds, and films that can promote skin regeneration, nerve repair, and controlled drug release. Despite these advantages, efficient and sustainable extraction of keratin from human hair remains a significant challenge due to the recalcitrant nature of keratinous waste.

Traditionally, chemical and enzymatic methods have been used for keratin extraction, but they often involve harsh conditions or are limited by enzyme stability and efficiency. Ionic liquids (ILs), as eco-friendly solvents with unique physicochemical properties, have shown promise in dissolving keratin and enhancing enzymatic hydrolysis. Meanwhile, keratinases—specialized proteolytic enzymes—can catalyze keratin degradation but their activity and stability in non-

conventional solvents like ILs are not well understood, hindering the development of optimized extraction protocol.

This work is motivated by the need to bridge the gap in understanding the molecular interactions and stability of keratinases in ILs, which could facilitate the design of environmentally sustainable and efficient processes for human hair keratin extraction. Identifying ILs that maintain or enhance keratinase stability can enable synergistic use of ILs and enzymes, reducing reliance on harsh chemicals and improving process sustainability. Ultimately, this study supports advancing green biotechnology approaches that valorize human hair waste into high-value keratin biomaterials for biomedical and industrial applications.

1.12. Objectives of the study

1. Identification of promising keratinases for human hair keratin degradation & structure prediction using advanced computational tools.
2. To select ionic liquids with reported efficiency in human hair degradation & prepare computational simulation systems incorporating these.
3. Investigating the stability, flexibility, dynamic behavior of keratinases in different ionic liquid environments for identifying appropriate enzyme-solvent systems.

1.13. Literature Review

This section enlists the various work done by researcher in the field of hair hydrolysis:

Rodrigues et al., 2008 have used tetramethylammonium hydroxide for the dissolution of human hair in their work entitled, “A fast method for the determination of 16 elements in hair samples by inductively coupled plasma mass spectrometry (ICP-MS) with tetramethylammonium hydroxide solubilization at room temperature”, developed a rapid procedure for the solubilization of human hair samples using TMAOH and determined the concentration of 16 trace elements by ICP-MS. Their method was able to efficiently dissolve hair at room temperature, enhancing sample preparation for subsequent analysis. The results emphasized that the choice of solvent was critical for the efficient hydrolysis of human hair [116].

Chen et al., 2014; Wang et al., 2014 have monitored the dissolution of human hair in various ionic liquids in their research work, “What happens during natural protein fibre dissolution in ionic liquids,” and “Blend films of human hair and cellulose prepared from an ionic liquid,”. Chen et al., investigated systematically the dissolution of natural protein fibers, such as human hair, wool, and silk, in different ionic liquids (ILs), carefully monitoring the dissolution mechanism and efficiency. The study evidences that ILs efficiently disrupt the robust structure of keratin in human hair and underlines the tunable properties of ILs for the selective solubilization and processing of protein-based biomaterials. Their findings thus point out the IL potential for green and sustainable

recovery and modification of keratin from human hair. Wang et al., prepared blend films of human hair keratin and cellulose dissolved in imidazolium-based ionic liquids to result in homogeneous and processable biomaterial composites. In this work, efficient extraction of keratin from hair using ionic liquids was realized, and their capability for breaking strong intermolecular bonds in protein fibers was demonstrated while preserving the wanted material properties. The technique has not only optimized valorization of human hair waste but also illustrated the design of advanced biomaterials based on keratin-cellulose with promising biomedical applications [117].

Singh and Prasad, 2019 in their work, “Multi-tasking hydrated ionic liquids as sustainable media for the processing of waste human hair: A biorefinery approach,” investigated the solubility of waste human hair in various hydrated ionic liquids. They explored the use of hydrated ionic liquids, namely tetra-n-butylammonium hydroxide (TBAH), as environmentally friendly solvents for the processing of waste human hair in an integrated biorefinery system. Their work showed effective dissolution and fractionation of hair for the simultaneous extraction of value-added biopolymers such as keratin and melanin. The work epitomizes the flexibility and efficiency of hydrated ILs in degrading resistant hair matrices under mild conditions, with prime emphasis on environmental benefits and recyclability, which constitute key parameters in green extraction strategies for keratinase research [118].

From these research works, six ionic liquids were identified with proven efficacy for hydrolysis of human hair.

Laba et al., 2014 in their research work, “Biodegradation of hard keratins by two *Bacillus* strains,” mentioned *Bacillus cereus* to be a producer of keratinases for degradation of hard keratins [119].

Kang et. al., in their work entitled, “Metagenomic analysis of a keratin-degrading bacterial consortium provides insight into the keratinolytic mechanisms” identified kerF from *Stenotrophomonas maltophilia* and kerC from *Bacillus subtilis* to be promising keratinases for hydrolysis of hard hair keratins [120].

Research gap: Previous research has not explored both ionic liquids and keratinases in the same study for the hydrolysis of human hair. While keratinases and ionic liquids separately have proven efficient for the hydrolysis of human hair, their combination has not been considered to date to see if ionic liquids enhance the activity of keratinases. This work seeks to fill a gap that still exists in the literature regarding the molecular interactions and stability of keratinases in ionic liquid environments, crucial for the development of green and efficient human hair keratin extraction processes. The identification of ionic liquids that stabilize or enhance the function of keratinases may

allow their synergistic use and further minimize the use of harsh chemicals, ensuring greater sustainability of the eco-friendly process.

CHAPTER 2

METHODOLOGY

2.1 Identification of keratinases and sequence retrieval from UniProt

A comprehensive literature review was conducted to identify microbial keratinases with potential efficacy for degradation of human hair keratin. The search focused on peer-reviewed articles, and reviews to ensure the selection of enzymes with established keratinolytic activity. Based on this analysis, three candidate keratinases—ker6, kerC, and kerF—were selected for further study. These enzymes are produced by *Bacillus cereus*, *Bacillus subtilis*, and *Stenotrophomonas maltophilia*, respectively, and have been reported in previous studies to exhibit significant activity against recalcitrant keratin substrates.

To facilitate structural and functional analysis, the amino acid sequences of these keratinases were retrieved from the UniProt database [121] [122]. UniProt (Universal Protein Resource) is a globally recognized, comprehensive database that provides curated protein sequence and functional information for millions of proteins from diverse organisms. It integrates data from several sources, including Swiss-Prot, TrEMBL, and PIR, and offers detailed annotations such as protein function, domain structure, post-translational modifications, and links to other biological databases [123] [124] [125]. UniProt is widely used in bioinformatics and molecular biology research due to its reliability, regular updates, and extensive cross-referencing, making it an essential resource for accurate protein sequence retrieval and downstream computational analyses [126] [127]. The UniProt accession numbers for ker6, kerC, and kerF are F8SVT0, E21QH8, and A0A1L4AJA4, respectively.

As no experimentally determined three-dimensional structures for these enzymes were available in the Protein Data Bank (PDB)—the primary global repository for macromolecular structures determined by experimental techniques such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and cryo-electron microscopy (cryo-EM)—computational modeling was required [128] [129] [130]. The PDB serves as a critical resource for the scientific community, providing open access to detailed atomic coordinates, structural annotations, and related metadata for proteins, nucleic acids, and complex assemblies. Each entry in the PDB is thoroughly curated and includes information on experimental conditions, resolution, and validation metrics, ensuring the reliability and reproducibility of structural data. The database is widely used for structural biology, drug discovery, molecular modeling, and bioinformatics research, enabling scientists to analyze protein folding, function, and interactions at the molecular level [131] [132] [133] [134]. The absence of keratinase structures in the PDB

necessitated the use of advanced computational tools to generate accurate three-dimensional models for further structural and functional studies.

2.2 Modeling of keratinases using AlphaFold2

To address the absence of experimentally determined three-dimensional structures for the selected keratinases in the Protein Data Bank (PDB), their tertiary structures were predicted using AlphaFold2, a state-of-the-art deep learning-based tool that has revolutionized the field of protein structure prediction. AlphaFold2, developed by DeepMind, utilizes advanced neural network architectures and attention mechanisms to predict protein folding with unprecedented accuracy, often achieving results comparable to those obtained through experimental techniques such as X-ray crystallography or cryo-electron microscopy [135] [136] [137]. The system was trained on vast datasets of known protein structures and sequences, enabling it to learn complex spatial relationships and evolutionary patterns that govern protein folding [138] [139] [140].

For this study, the modeling was performed using the AlphaFold2 notebook available through [ColabFold](https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) at colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb.

ColabFold provides a user-friendly and computationally efficient interface for running AlphaFold2 predictions on Google Colaboratory, making high-quality structure prediction accessible without the need for specialized hardware. AlphaFold2 generates not only the predicted three-dimensional coordinates of protein atoms but also confidence metrics such as the predicted Local Distance Difference Test (pLDDT) score, which helps assess the reliability of different regions within the model. The pLDDT score is a widely used confidence metric generated by AlphaFold to evaluate the reliability of predicted protein structures on a per-residue basis [141] [142] [143]. This score is expressed on a scale of 0 to 100, where higher values correspond to greater confidence in the accuracy of the local atomic positions. A pLDDT value above 80 is generally interpreted as indicating that the residue is modeled with high structural accuracy and is likely to be reliable for downstream structural or functional analyses. Intermediate scores between 50 and 70 typically reflect moderate confidence in the modeled residue positions, suggesting these regions may be flexible or less well-defined. In contrast, residues with scores below 50 are generally regarded as uncertain, often corresponding to intrinsically disordered regions or parts of the protein lacking a stable structure under physiological conditions [144] [145] [146]. Thus, the pLDDT score not only guides interpretation of AlphaFold2 predictions but also provides valuable clues about potential disorder, flexibility, or structural ambiguity along the protein chain. During the modeling the well-established alpha helical and beta sheet parts were considered leaving out some of the less ordered loop regions as revealed by the pLDDT plot of the modeled structures [147].

The remarkable accuracy and speed of AlphaFold2 have made it a transformative tool in computational biology, enabling researchers to model proteins for which no experimental structures exist, facilitate functional annotation, guide mutagenesis studies, and support drug discovery efforts. In this work, the high-confidence models generated by AlphaFold2 served as the foundation for subsequent structural analyses and molecular dynamics simulations, ensuring robust and reliable insights into the properties of the selected keratinases.

The modeled keratinase structures were further analyzed using PDBsum, a well-established online tool that offers comprehensive graphical and textual summaries of protein structures [148] [149]. PDBsum provides detailed structural information including schematic diagrams of secondary structure elements like alpha helices and beta sheets, domain organization, and functional motifs or active sites. It also generates topology diagrams, ligand-binding site maps, and interaction plots, all critical for understanding the key structural features and potential functional regions of the protein. By leveraging PDBsum, the quality and structural characteristics of the predicted keratinase models could be systematically assessed [150]. A noteworthy feature of PDBsum is its generation of Ramachandran plots, which provide essential stereochemical evaluation of the protein model [151] [152] [153]. The Ramachandran plot graphically displays the phi (ϕ) and psi (ψ) backbone dihedral angles of each amino acid residue, showing the conformational space occupied by the protein's backbone. This plot classifies residues into favored, allowed, and disallowed regions based on empirical data from high-quality protein structures. Residues clustered in favored regions indicate correct stereochemistry, while those in disallowed zones may signify unusual backbone conformations or potential modeling errors [154] [155] [156]. By analyzing the Ramachandran plot, one can gauge the overall stereochemical quality of the keratinase models, confirming that most residues adopt energetically favorable conformations that support structural integrity and biological function [157] [158] [159]. This plot thus complements other PDBsum analyses, enhancing the confidence in the predicted keratinase tertiary structures for further biochemical and molecular studies [160] [161] [162] [163].

For visualization and in-depth inspection, the predicted tertiary structures were explored using PyMOL 2.5, a widely used molecular graphics system renowned for its versatility and high-quality rendering capabilities. PyMOL allows for interactive manipulation of protein models, enabling users to rotate, zoom, and highlight specific regions or residues of interest. It supports a variety of visualization styles, such as cartoon, surface, and stick representations, which facilitate the examination of structural details. PyMOL also offers tools for measuring distances, angles, and generating publication-quality images, making it an essential platform for both structural analysis and scientific communication. The combined use of PDBsum and PyMOL 2.5 ensured a comprehensive and reliable

structural characterization of the keratinases, providing a robust foundation for subsequent molecular dynamics simulations and functional studies [164] [165].

2.3 Shortlisting of the ionic liquids.

Six ionic liquids with documented efficiencies in human hair degradation were identified through an extensive review of the scientific literature. The selected ionic liquids included Tetrabutylammonium hydroxide (TBAH), 1-allyl-3-methylimidazolium chloride [AMIM]Cl, 1-butyl-3-methylimidazolium bromide [BMIM]Br, 1-butyl-3-methylimidazolium chloride [BMIM]Cl, Tetramethylammonium hydroxide (TMAOH), and Choline hydroxide (Ch-OH). These compounds were chosen based on their reported ability to disrupt the robust keratin structure of human hair, making them suitable candidates for further investigation in keratin extraction protocols.

To obtain accurate chemical structures and related information for these ionic liquids, the PubChem database was utilized [166] [167] [168]. PubChem is a freely accessible, comprehensive chemical information resource maintained by the National Center for Biotechnology Information (NCBI). It provides detailed data on millions of chemical compounds, including their molecular structures, physicochemical properties, biological activities, safety profiles, and links to scientific literature [169] [170] [171]. PubChem is widely recognized for its reliability, regular updates, and integration with other major scientific databases, making it an essential tool for researchers in chemistry, biochemistry, and related fields. The database assigns a unique Compound Identifier (CID) to each chemical, facilitating precise retrieval and cross-referencing [172] [173] [174] [175].

For this study, the PubChem CIDs for the selected ionic liquids were as follows: Tetrabutylammonium hydroxide (TBAH) – 2723671, Tetramethylammonium hydroxide (TMAOH) – 60966, Choline hydroxide (Ch-OH) – 31255, 1-butyl-3-methylimidazolium bromide ([BMIM]Br) – 2734236, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) – 2734161, and 1-allyl-3-methylimidazolium chloride ([AMIM]Cl) – 11321106. The chemical structures and relevant data retrieved from PubChem were subsequently used for molecular dynamics simulation studies involving these ionic liquids.

2.4 Preparation of the simulation files using CHARMM-GUI

The chemical structures of the selected ionic liquids, initially obtained from the PubChem database, were processed using the PDB Reader & Manipulator tool, a critical step for preparing the molecules for molecular dynamics simulations within the CHARMM framework. The PDB Reader & Manipulator is an essential module within CHARMM-GUI designed to read, verify, and modify biomolecular structures and ligands for compatibility with various simulation engines including CHARMM,

GROMACS, and NAMD. This tool converts molecular files into simulation-ready formats by ensuring that all atom types, charges, connectivity, and residue names conform accurately to the CHARMM force field requirements [176] [177].

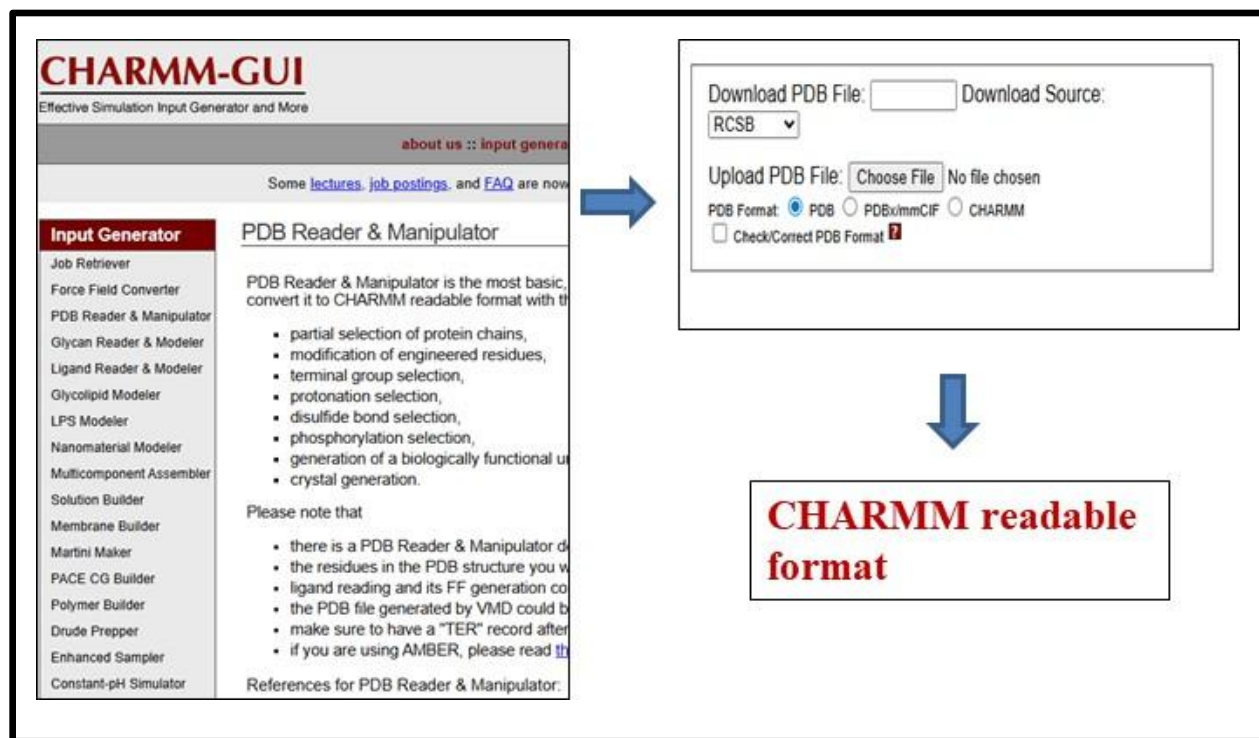


Figure 2.1 The PDB Reader & manipulator tool of CHARMM-GUI

Beyond simple file conversion, the PDB Reader & Manipulator provides advanced features such as automatic correction and verification of the PDB format, generation of missing atomic coordinates based on internal coordinate data, and adjustment of chain identifiers and connectivity information. It seamlessly identifies and substitutes nonstandard residues or ligands with corresponding CHARMM force field parameters when available. Additionally, it allows user-driven chemical modifications, including changes to protonation states or bond orders, facilitated through an interactive 2D sketchpad editor that helps verify and optimize ligand structures before 3D coordinate generation.

For the ionic liquids, this step ensured correct atom typing, charge assignment, and bonded connectivity, which are crucial for the accurate representation of these compounds in subsequent simulations. The tool also integrates molecular structure verification using maximum common substructure (MCS) searches paired with distance geometry approaches to preserve accurate 3D molecular conformations while optimizing geometry. By processing the ionic liquids through PDB Reader & Manipulator, the molecular files became fully compatible input for CHARMM-based molecular dynamics

simulations, enabling precise and reliable computational modeling of the keratin extraction system involving these specialized solvent molecules. This preparation step is fundamental to ensuring the chemical fidelity and simulation accuracy required for high-quality molecular dynamics studies [177] [178] [179].

To prepare the input files and configure the simulation systems, the CHARMM-GUI web-based graphical platform was employed. CHARMM-GUI, (accessible at <http://www.charmm-gui.org>) is a widely used, user-friendly interface designed to streamline the setup of molecular dynamics simulations for a variety of software packages, including CHARMM, NAMD, GROMACS, and AMBER [180] [181] [182]. Since its launch in 2006, CHARMM-GUI has become an indispensable resource for the molecular modeling community, offering a suite of specialized modules that facilitate the construction of both all-atom and coarse-grained models. The platform supports the assembly of complex biomolecular systems, such as proteins, nucleic acids, membranes, and solvent environments, under a range of physiological and experimental conditions [183] [184] [185].

CHARMM-GUI's intuitive workflow guides users through each stage of system preparation, from uploading molecular structures and defining simulation parameters to generating topology and coordinate files required for production runs. Its robust automation and error-checking features minimize manual intervention and reduce the likelihood of setup errors, making it accessible to both novice and experienced researchers. By leveraging CHARMM-GUI, the process of building, solvating, and parameterizing the ionic liquid systems was greatly simplified, ensuring reproducibility and consistency throughout the molecular dynamics simulations [179] [186] [187] .

For the setup of molecular dynamics simulation environments involving ker6, kerC, and kerF, a systematic workflow within CHARMM-GUI was followed to ensure accurate and reproducible system construction. To model the aqueous simulation settings, the Solution Builder module of CHARMM-GUI was utilized. This specialized tool allows for the straightforward preparation of solvated protein systems, enabling precise definition of water molecules, ionic strength, and box geometry [188]. In figure 2.2 a step wise chart displaying the snapshots of different steps involved in the preparation of the aqueous solution simulation system of keratinase is shown.

CHARMM-GUI

Effective Simulation Input Generator and More

STEP 1

about us :: input

Some [lectures](#), [job postings](#), and [FAQ](#)

Input Generator

- Job Retriever
- Force Field Converter
- PDB Reader & Manipulator
- Glycan Reader & Modeler
- Ligand Reader & Modeler
- Glycolipid Modeler
- LPS Modeler
- Nanomaterial Modeler
- Multicomponent Assembler
- Solution Builder
- Membrane Builder
- Martini Maker
- PACE CG Builder
- Polymer Builder
- Drude Prepper

Solution Builder

Solution Builder helps you to generate

1. solvate your molecule or
2. generate a water box only for other

Solution Builder also guides you to determine the total charge of the system. Ions can be added in the simulation box to neutralize the total system charge, i.e., the initial configuration of ions is then determined by interactions. By simply changing a variety of parameters, please note that

If you are not familiar with the force field, please refer to the [CHARMM-GUI](#) [FAQ](#) for more information.

STEP 2

Download PDB File:

Download Source: RCSB

Upload PDB File:

ker6_144b6...model_3.pdb

PDB Format: ☒ PDB ☐ PDBx/mmCIF ☐ CHARMM
☐ Check/Correct PDB Format

STEP 4

PDB Info
CHARMM PDB
Solvator
PBC Setup
Input Generator

Title:

PDB ID:

Type:

Experimental Method:

STEP 5

PDB Info
CHARMM PDB
Solvator
PBC Setup
Input Generator

Original PDB File: [ker6_144b6_relaxed_rank_1_model_3.pdb \(view structure\)](#)

Individual Chains: [ker6_144b6_relaxed_rank_1_model_3_proa.pdb](#)

CHARMM Input: [step1_pdbreader.inp](#)

CHARMM Output: [step1_pdbreader.out](#)

CHARMM PDB: [step1_pdbreader.pdb \(view structure\)](#)

CHARMM CRD: [step1_pdbreader.crd](#)

CHARMM PSF: [step1_pdbreader.psf](#)

STEP 6

PDB Manipulation Options:

☐ Terminal group patching: First Last

PROA: ☐ Cyclic peptide?

☐ Preserve hydrogen coordinates:

☐ Mutation:

☐ Protonation state:

☐ Disulfide bonds:

☐ Phosphorylation:

STEP 7

System Size:

Box Type:

Crystal Type:

System Size:

Crystal Angle:

STEP 8

Force Field Options:

☒ CHARMM36m

☐ WYF parameter for cation-pi interactions

☐ Hydrogen mass repartitioning

☐ Multi-site Ca²⁺

Input Generation Options:

The input generation scheme has been changed recently. Please

☐ NAMD

☐ GROMACS

☐ AMBER

☐ OpenMM

☐ CHARMM/OpenMM

☐ GENESIS

☐ Desmond

☐ LAMMPS

☐ Tinker

Equilibration Input Generation Options:

☒ NVT Ensemble

Dynamics Input Generation Options:

☒ NPT Ensemble

☐ NVT Ensemble

Temperature: K

STEP 9

Figure 2.2 Steps involved in the preparation of keratinase aqueous simulation using Solution Builder Tool.

For simulations containing ionic liquids, the Multicomponent Assembler module within CHARMM-GUI was employed [189] [190]. This tool supports the creation of complex systems with multiple components, facilitating the incorporation of desired concentrations and ratio of ionic liquids alongside the protein of interest. All ionic liquid-containing systems were assembled to maintain a final concentration of 0.5 M, ensuring consistent comparative conditions. A step wise chart displaying the snapshots of different steps involved in the preparation of the ionic liquid simulation system of keratinase is shown in figure 2.3.

CHARMM-GUI
Effective Simulation Input Generator and More

STEP 1

Input Generator

- Job Retriever
- Force Field Converter
- PDB Reader & Manipulator
- Glycan Reader & Modeler
- Ligand Reader & Modeler
- Glycolipid Modeler
- LPS Modeler
- Nanomaterial Modeler
- Multicomponent Assembler
- Solution Builder
- Membrane Builder
- Martini Maker
- PACE CG Builder
- Polymer Builder
- Drude Prepper
- Enhanced Sampler
- Constant-pH Simulator
- Free Energy Calculator
- LBS Finder & Refiner

Multicomponent Assembler

Multicomponent Assembler allows users to create proteins in an aqueous solution can be built easily. In most cases, the required files for your component are provided.

[Expand Help](#) [Collapse Help](#)

[How to use this module](#)

[Supported Component Types](#)

[File Naming Rules](#)

Recent Updates

- Preprint citation updated to full publication.
- Please see the [Update Log](#) for more information.

Future Development

- Auto-generate missing parameters with CGO.
- More periodicity options.

References for Multicomponent Assembler:

STEP 2

Multicomponent Assembler

STEP 1 STEP 2 STEP 3 STEP 4 STEP 5

Structures Read: 2
Read PSF/CRD Input: [step1_1_waterbox.inp](#)
Read PSF/CRD Output: [step1_1_waterbox.out](#)
Radius Info: [step1_1_waterbox.inp](#)

System Size Determination Options:

Calculate system size using:
☒ Box XYZ length
☐ Volume fraction

Give components using:
☒ Number of components
☐ Ratio of components

Box XYZ length (Å): 50

☐ Generate a membrane for this system

[Calculate System Size](#)

Component ID	PSF Name	Molecular Volume	# Components	Component Type	Initial Position
1	brntrpochrom	206.79 Å ³		Solvated Component	Set Position
2	keratin	30047.80 Å ³		Solvated Component	Set Position

STEP 3

Solvation Options:

☒ Include Water
☒ Include Ions

Ion Placing Method: [Distance](#)

☒ Basic Ion Types
 NaCl [Add Simple Ion Type](#)

☐ More Ion Types

Formula: Cation: Anion: Concentration: Neutralizing:
 KCl: K⁺: Cl⁻: 0.15: *

Calculate Solvent Composition Using:
☒ Volume Ratio + Pre-Mixing Density
☐ Post-Mixing Concentration

[Calculate Solvent Composition](#)

Component ID	Name	Molecular Volume	Molecular Mass	Volume Ratio	Pre-Mixing Density (g/L)	Count
1	K ⁺					81
2	Cl ⁻					305
3	water	23.34 Å ³	18.02 g/mol	1	993.824	28202

STEP 4

Multicomponent Assembler

STEP 1 STEP 2 STEP 3 STEP 4 STEP 5

Waterbox Generation Input: [step3_1_waterbox.inp](#)
 Waterbox Generation Output: [step3_1_waterbox.out](#)
 Waterbox Generation: [step3_1_waterbox.out](#) ([view structure](#))
 Ion Generation Input: [step3_4_ions.inp](#)
 Ion Generation Output: [step3_4_ions.out](#)
 Ion Generation PDB: [step3_4_ions.pdb](#) ([view structure](#))
 Assembly Input: [step3_assembly.inp](#)
 Assembly Output: [step3_assembly.out](#)
 Assembly PSF: [step3_assembly.psf](#)
 Assembly CRD: [step3_assembly.crd](#)
 Assembly PDB: [step3_assembly.pdb](#) ([view structure](#))

System Size:

Box Type: Rectangle
 Crystal Type: CUBIC
 System Size: A: 100, B: 100, C: 100
 Crystal Angle: Alpha: 90.0, Beta: 90.0, Gamma: 90.0

Periodic Boundary Condition Options:

☒ Generate grid information for PME FFT automatically
☐ Explicit grid information for PME FFT

STEP 5

Force Field Options:

CHARMM36m

☐ WYF parameter for cation-π interactions
☐ Hydrogen mass repartitioning
☐ Multi-site Ca²⁺

Input Generation Options:

The input generation scheme has been changed recently. Please check your system, input, and options.

☐ More CHARMM minimization during input generation

☐ NAMD
☐ GROMACS
☐ AMBER
☐ OpenMM
☐ CHARMM/OpenMM
☐ GENESIS
☐ Desmond
☐ LAMMPS
☐ Tinker

Equilibration Input Generation Options:

☒ NVT Ensemble

Dynamic Input Generation Options:

☒ NPT Ensemble
☐ NVT Ensemble
 Temperature: 303.15 K

STEP 6

3D visualization of the simulation box showing the protein (keratinase) and the surrounding ionic liquid and water molecules.

Figure 2.3 Steps involved in the preparation of keratinase simulations in ionic liquids using Multicomponent Assembler Tool.

Each simulation was confined within a cubic boundary box, the dimensions of which were selected based on the size and solvation requirements of each keratinase. Specifically, ker6 was placed in a box measuring $105 \times 105 \times 105 \text{ Å}^3$, kerC in a $100 \times 100 \times 100 \text{ Å}^3$ box, and kerF within a $115 \times 115 \times 115 \text{ Å}^3$ box. The solvation process employed the

TIP3P water model, a widely accepted and empirically validated model for simulating liquid water in biomolecular systems.

A buffer distance, or "center-edge distance," of 10 Å was maintained between the protein and the box boundary to minimize artifacts from periodic boundaries and to allow adequate space for solvent and ion distribution. To ensure charge neutrality within all simulation systems, sodium and chloride counter-ions were added at appropriate quantities, determined by the overall charge of the protein and additional solutes present.

After system assembly and parameterization, molecular dynamics simulations were carried out using GROMACS software, specifically the 2020.3 release [191] [192] [193] [194]. The CHARMM36 force field was selected for these simulations due to its well-established accuracy in representing protein and ionic liquid interactions. All system setups were thoroughly validated for steric clashes and correct atom assignment prior to simulation runs, ensuring robust and meaningful results for subsequent structural and functional analyses [195] [196] [197] [198].

Interatomic interactions were computed using distinct approaches for short-range and long-range effects. Van der Waals forces were represented by the Lennard-Jones potential, incorporating a force-switching function applied across the 1.0–1.2 nm range. For electrostatic calculations beyond this distance, the Particle Mesh Ewald (PME) algorithm was implemented with a direct-space cutoff of 1.2 nm. The energy minimization procedure employed the steepest descent algorithm, configured with a convergence criterion of 1000 kJ/mol/nm and an upper limit of 5000 iterations. The neighbor search list underwent refreshment every 10 computation cycles. Bond constraints involving hydrogen atoms were enforced using the Linear Constraint Solver (LINCS) protocol throughout this phase.

The equilibration phase employed a leap-frog integration scheme with a 1-femtosecond timestep, spanning 125 picoseconds total duration. Neighbor list updates occurred every 20 integration steps. Temperature regulation was achieved through a Nosé-Hoover thermostat ($\tau = 1.0$ ps) applied uniformly to solute and solvent components, maintaining the system at 303.15 K. Pressure control utilized the Parrinello-Rahman barostat set to 1.0 bar with a 5.0 ps time constant and 4.5×10^{-5} bar⁻¹ compressibility. Hydrogen-involved bonds remained constrained via the LINCS algorithm. Full periodic boundary conditions in all dimensions ensured spatial consistency throughout the subsequent 200-nanosecond production simulation.

2.5 Analysis of the Molecular Dynamics Trajectories.

To assess the structural changes of ker6 induced by different solvent environments, including pure water and various ionic liquids, the molecular dynamics simulation

trajectories were thoroughly analyzed [199]. This assessment leveraged a suite of key geometrical and dynamic parameters that collectively provide a comprehensive view of the protein's structural stability and conformational dynamics throughout the simulation. These parameters included the root mean squared deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), indicating the compactness of the protein structure; solvent-accessible surface area (SASA), and hydrogen bond analysis.

A central metric in this analysis is the root mean squared deviation (RMSD), which quantifies the average positional deviation of protein atoms compared to a reference configuration, typically the initial or equilibrated structure [200] [201] [202]. Calculated from the atomic coordinates, RMSD expresses how far the simulated structure departs from its starting state as a function of time [203] [204]. Lower RMSD values imply minimal fluctuation, indicating that the protein maintains a stable conformation and has likely reached equilibration. By contrast, higher RMSD values are indicative of larger structural shifts, which can signal regions of instability or biologically significant conformational changes—making RMSD a fundamental indicator for probing protein stability during MD simulations [205] [206] [207]. Consistently low RMSD values throughout the trajectory suggest the structural robustness and dynamic equilibrium of ker6 under specific solvent conditions, whereas marked increases may point to unfolding events or conformational transitions [208].

To assess the structural dynamics and local fluctuations of ker6 during molecular dynamics simulations, RMSF (Root Mean Square Fluctuation) is an essential parameter that complements RMSD analysis by providing residue-specific insights. RMSF measures how much each residue deviates, on average, from its reference position—usually the mean atom coordinate—over the course of the simulation [209] [210] [211].

Specifically, RMSF quantifies the average fluctuation of atomic positions relative to their mean during the simulation, offering a detailed view of the flexibility of individual amino acids. It is calculated using the sum of squared deviations of each residue's atomic coordinates, typically focusing on the alpha carbon (Ca) atoms for simplicity and relevance to backbone conformational changes, divided by the total number of time points, and then taking the square root of this mean [212] [213] [214].

Mathematically, RMSF involves averaging the positional deviations of each residue's atoms from their mean positions over all sampled time frames of the simulation. Peaks in a RMSF plot indicate residues with high mobility, often corresponding to flexible loops or solvent-exposed regions, while low RMSF values suggest residues in more rigid, structurally constrained regions. This analysis allows researchers to identify key dynamic sites that may influence protein function or stability under different solvent environments [207] [215].

By integrating RMSF results with RMSD data, a comprehensive understanding of structural stability and local flexibility emerges. RMSD reflects the overall structural deviations, indicating whether the protein is equilibrated or undergoing global conformational changes, while RMSF pinpoints specific residues or regions responsible for localized motions or stability, crucial for correlating molecular behavior with biological activity [216].

Solvent Accessible Surface Area (SASA) is a crucial parameter frequently used in protein dynamics studies to evaluate protein folding and structural stability. SASA represents the total surface area of a protein that is accessible to solvent molecules, effectively depicting how much of the protein's surface can interact with the surrounding solvent. It is defined by constructing a hypothetical spherical probe, typically approximating a solvent molecule like water, which "rolls" over the van der Waals surface of the protein atoms, generating a map of accessible surface area [217][218] [219].

Based on SASA values, amino acid residues are classified as either buried (low SASA) or exposed to the solvent (high SASA). The burial of hydrophobic residues within the protein core is a key driving force for proper folding and maintaining stable protein conformations, while residues with higher SASA values tend to be more solvent-exposed and flexible. The degree of exposure of residues correlates directly with their interactions with both the protein core and surrounding solvent, influencing the protein's folding pathway and conformational behavior [220] [221] [222].

Monitoring changes in SASA during molecular simulations helps researchers understand structural rearrangements such as unfolding/refolding events, surface exposure variations of active or binding sites, and dynamic shifts in hydrophobic residue burial—all of which are integral to the protein's function and stability. By analyzing SASA profiles, it becomes possible to identify regions of the protein that undergo significant conformational changes and assess their impact on solvent interactions and protein behavior within different environments [223] [224] [225].

Overall, SASA serves as a geometric and biophysical indicator that provides valuable insight into protein folding, stability, and interactions, playing an essential role in interpreting molecular dynamics simulations and guiding experimental and computational investigations of protein structure-function relationships.

The radius of gyration (R_g) serves as a measure of how compact a protein's structure is by describing the spatial distribution of its atoms around the center of mass [226] [227] [228]. It is computed as the root mean square distance of all atoms from this central point. Smaller R_g values correspond to tightly folded, compact protein conformations, whereas larger values suggest the protein adopts a more extended or unfolded shape

[229] [230] [231]. In molecular dynamics simulations, tracking changes in R_g over time helps evaluate protein stability and conformational shifts in varied environments. Additionally, different categories of proteins tend to have characteristic R_g values that reflect their typical degree of compactness and structural organization [232] [233].

Intra-protein hydrogen bonds play a crucial role in maintaining protein structure, guiding the folding process, and ensuring functional integrity [234] [235] [236]. Molecular dynamics (MD) simulations provide a powerful tool to analyze these hydrogen bonding networks, both within a protein (intramolecular) and between proteins or other molecules (intermolecular). These simulations reveal how specific residues participate in stabilizing the overall 3D conformation and how hydrogen bonds fluctuate and evolve over time at an atomic scale [237] [238] [239] [240]. Accurate analysis of hydrogen bonds in protein complexes, often performed with software such as GROMACS, requires careful definition of index groups to distinguish between intra- and inter-protein hydrogen bonding interactions. This precision allows researchers to better understand the dynamic contributions of hydrogen bonds to protein stability and interactions throughout the simulation [241] [242] [243].

All these metrics were computed using specific GROMACS command-line tools: 'gmx rmsd' for RMSD calculations, 'gmx rmsf' for residue-level fluctuations, 'gmx gyrate' for radius of gyration, 'gmx sasa' for solvent-accessible surface area, and 'gmx hbond' for hydrogen bond analysis [194] [244] [245] [246] [247]. This comprehensive analysis enabled a detailed understanding of how different solvents influence the structural integrity and dynamics of ker6 during the simulations.

2.6 Interaction analysis

We performed a detailed interaction analysis using the Protein-Ligand Interaction Profiler (PLIP) from the MD simulation of last 100 ns to investigate the interactions between ker6 and ionic liquid molecules used as solvent in each simulation system. It is a novel web service for fully automated detection of relevant non covalent interactions in 3D structures, freely available at projects.biotec.tu-dresden.de/plip-web [248] [249]. It returns a list of detected interactions, covering seven interaction types (hydrogen bonds, hydrophobic contacts, pi-stacking, pi-cation interactions, salt bridges, water bridges and halogen bonds). This analysis reveals how the nature and extent of protein-solvent interactions influence the stability of the protein in each ionic liquid simulation system [250] [251] [252].

CHAPTER 3

RESULTS

This section depicts the main findings of the study, following the methodology outlined earlier. It comprises the structural modeling and validation of selected keratinases, followed by detailed analysis of their dynamics and stability under different solvent environments using molecular dynamics simulations. The data are systematically reported for each enzyme and solvent combination by quantitative metrics such as RMSD, RMSF, radius of gyration, solvent-accessible surface area, and hydrogen bonding analysis. The interaction analysis of different ionic liquids with keratinases is also described. These results collectively provide insights into the relative behavior and stability of keratinases in both aqueous and ionic liquid media, thereby establishing the ground for further interpretation in the final conclusion section.

3.1. Keratinases identification and sequence retrieval

Previous research identified three keratinases—ker6 from *Bacillus cereus*, kerC from *Bacillus subtilis*, and kerF from *Stenotrophomonas maltophilia*—as promising candidates for degrading human hair keratin. Since their structural data were not available in the Protein Data Bank (PDB), computational modeling was undertaken using AlphaFold2. The protein sequences used for modeling were obtained from the UniProt database with accession numbers F8SVT0, E21QH8, and A0A1L4AJA4, as shown in table 3.1 corresponding to ker6, kerC, and kerF, respectively.

Table 3.1 Keratinases with their corresponding UniProt ID

UniProt ID	Keratinase name
F8SVT0	ker6
E21QH8	kerC
A0A1L4AJA4	kerF

The amino acid sequences of the three selected keratinases, as retrieved from the UniProt database, are presented here for reference in Table 3.2. These sequences, corresponding to UniProt accession numbers F8SVT0 (ker6), E21QH8 (kerC), and A0A1L4AJA4 (kerF), form the basis for the structural modeling and subsequent analyses described in this study. The sequences are shown in single-letter amino acid code, highlighting the primary structure of each enzyme that underpins their functional properties.

Table 3.2 Single letter amino acid code of the three keratinases; ker6, kerC, and kerF.

Keratinase	Amino acid sequence
ker6	SSTDYVPNQLIVKFKQNASLSNVQSFHKSVGANVLSKDDKLGF EVLVQFSKGTVEKINSYKNNPDVEYAEPNYYVHAFWTPNDPYF KNQYGLQKIAPQAWDSQRSDPGVKVAIIDTGVQGSHPDLASK VIYGHDIYDNDNTSDDGNGHGHGTHCAGITGALTNNSIGIAGVAP QTSIYAVRVLDNQSGTLDVAQAQGIREAADSGAKVISLSLGAPN GGTALQQAVQYAWNKGSVIVAAAGNAGNTKANYPAYYSEVI AVASTDQSDKKSSFSTYGSWVDVAAPGSNIYSTYKGSTYQSLS GTSMATPHVAGVAALLANQGYSENTQIRQIIESTTDKISGTGTYW KNGRVNAYKAVQYAKQLQENKAS
kerC	MRGKKVWISLLFALALIFTMAFGSTSPAQAAGKSNGEKKYIVG FKQTMSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKA VKELKKDPSVAYVEEDHVAQAYAQSVPYGVVSQIKAPALHSQG FTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNS HGTHVAGTVAALNNSVGVVLGVAPSASLYAVKVLGADGSGQYS WIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVV VVAAGNEGTSNGSSTVGYPGKYPSVIAVGAVNSSNQRAFSS VGSELDVMAPGVSIQSTLPGNKYGA YNGTSMASPHVAGAAALI LSKHPNWTNTQVRSSLENTTTKLGDIFYGKGLINVQAAAQ
kerF	MRGKKVWISLLFALALIFTMAFGSTSPAQAAGKSNGEKKYIVG FKQTMSTMSAAKKKDVISEKGGKVQKQFKYVDAASATLNEKA VKELKKDPSVAYVEEDHVAQAYAQSVPYGVVSQIKAPALHSQG FTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNS HGTHVAGTVAALNNSVGVVLGVAPSASLYAVKVLGADGSGQYS WIINGIEWAIANNMDVINMSLGGPSGSAALKAAVDKAVASGVV VVAAGNEGTSNGSSTVGYPGKYPSVIAVGAVNSSNQRAFSS VGSELDVMAPGVSIQSTLPGNKYGA YNGTSMASPHVAGAAALI LSKHPNWTNTQVRSSLENTTTKLGDIFYGKGLINVQAAAQ

3.2. Three-Dimensional Protein Structure Modeling Using AlphaFold2

3.2.1. ker6

In figure 3.1 the plot illustrates the pLDDT for the keratinase model, ker6, generated using AlphaFold2, which is a key metric for estimating residue-level confidence in the predicted tertiary structure. The plot shows pLDDT scores ranging from 0 to 100 across all sequence positions, with five ranked model predictions overlaid for comparison. Most regions exhibit consistently high pLDDT scores above 90, indicating strong model

confidence and suggesting well-defined structural regions throughout the majority of the protein. However, specific positions display sharp drops in pLDDT, corresponding to areas of lower confidence—these typically represent flexible loops, termini, or regions lacking homologous templates in structure databases. The agreement across all five ranked models further supports the robustness of the prediction. Such results collectively demonstrate that the AlphaFold2 model for ker6 is highly reliable in most regions, providing a sound basis for downstream structural and functional analysis in subsequent simulations.

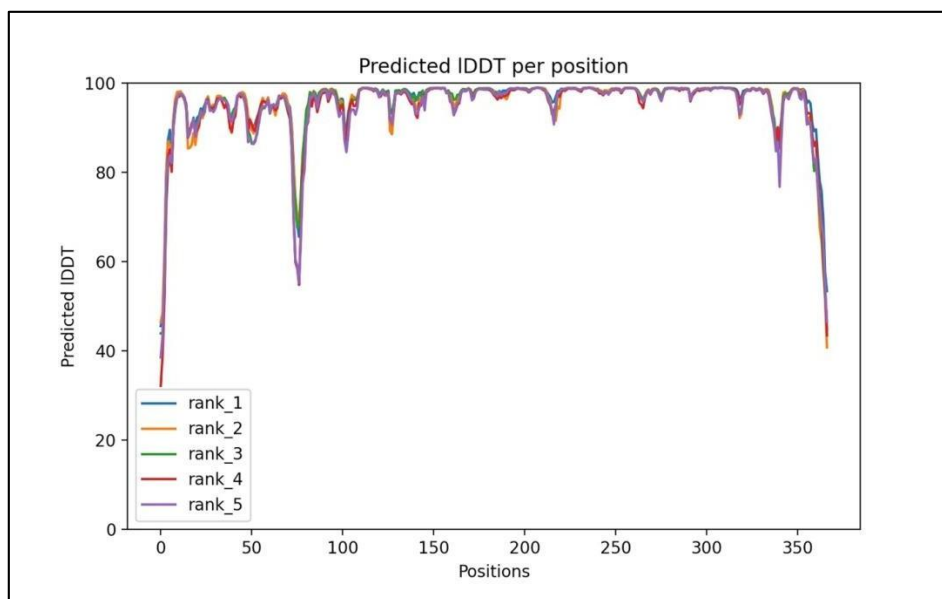


Figure 3.1 Predicted IDDT scores for five ranked AlphaFold2 generated models of ker6. The x-axis denotes the individual amino acid positions of ker6 from N-terminal to C-terminal, and the y-axis denotes the per-residue predicted scores of the amino acids in a range from 0-100.

3.2.2. kerC

The pLDDT plot shown in figure 3.2 represents the predicted Local Distance Difference Test scores for each residue position in the keratinase structure, kerC, modeled using AlphaFold2, where the y-axis quantifies per-residue model confidence (0–100) and the x-axis represents the amino acid sequence positions. This analysis includes five ranked AlphaFold2 models (rank_1 to rank_5), which display nearly identical profiles, highlighting the reproducibility and robustness of the structural prediction. The majority of the sequence positions exhibit pLDDT scores above 90, signifying high confidence in the model for those regions and suggesting they are well-ordered structurally. However, select positions—including notable drops around residues 60 and 220—display substantially lower pLDDT values near 40–50, which typically correspond to flexible loops, terminal ends, or potentially disordered regions with limited structural template

support. Taken together, this confidence profile demonstrates that the AlphaFold2 generated keratinase model, kerC, is highly reliable in most core regions, providing a solid foundation for molecular dynamics simulations, while pinpointing less certain and potentially flexible segments that may warrant targeted investigation or future protein engineering.

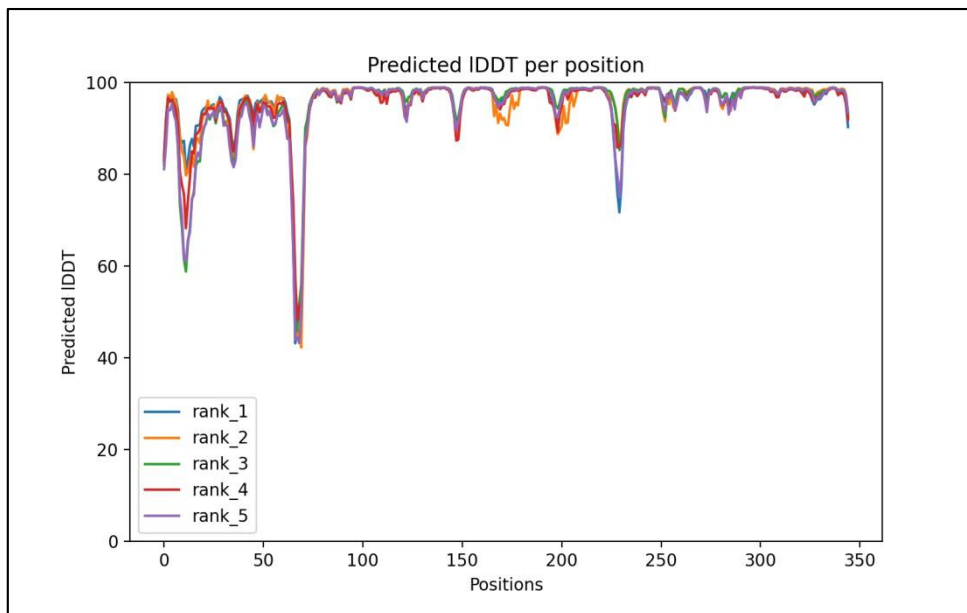


Figure 3.2 Predicted IDDT scores for five ranked AlphaFold2 generated models of kerC. The x-axis denotes the individual amino acid positions of kerC from N-terminal to C-terminal, and the y-axis denotes the per-residue predicted scores of the amino acids in a range from 0-100.

3.2.3. kerF

The plot shown in figure 3.3 depicts the pLDDT scores for each residue position in a keratinase model, kerF, generated by AlphaFold2. The y-axis represents the pLDDT scores, ranging from 0 to 100, serving as a quantitative measure of confidence in the predicted 3D coordinates for each amino acid. Across the protein sequence, the majority of positions have high pLDDT scores, often exceeding 90, which reflects a robust and confident structural prediction in these regions. Noticeable drops in pLDDT scores are observed at certain positions, particularly at the N- and C-termini, as well as in several internal sequence segments—these dips likely correspond to flexible loops or disordered regions, or to regions lacking homologous templates. In this analysis, the five ranked AlphaFold2 models (rank_1 to rank_5) show overall consistent trends, with only minor variations at specific locations, confirming the stability and reliability of the predicted structure. Together, these data indicate that the AlphaFold2 predicted kerF model

provides a highly credible representation of keratinase structure suitable for subsequent computational analyses.

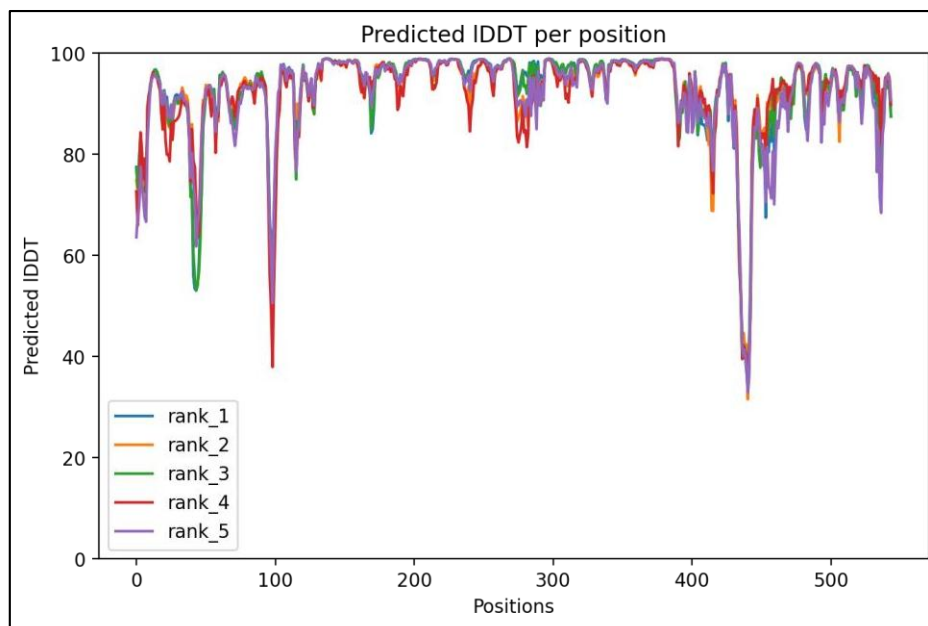


Figure 3.3 Predicted IDDT scores for five ranked AlphaFold2 generated models of kerF. The x-axis denotes the individual amino acid positions of kerF from N-terminal to C-terminal, and the y-axis denotes the per-residue predicted scores of the amino acids in a range from 0-100.

3.3. PyMOL generated image

The AlphaFold2 generated models of the three keratinases were visualized with the help of PyMOL 2.5. In the subsequent images of the different keratinases as shown in figure 3.4 the helix, sheets and loops are represented by red, yellow and green colors respectively.



Figure 3.4 Color Code for Keratinases

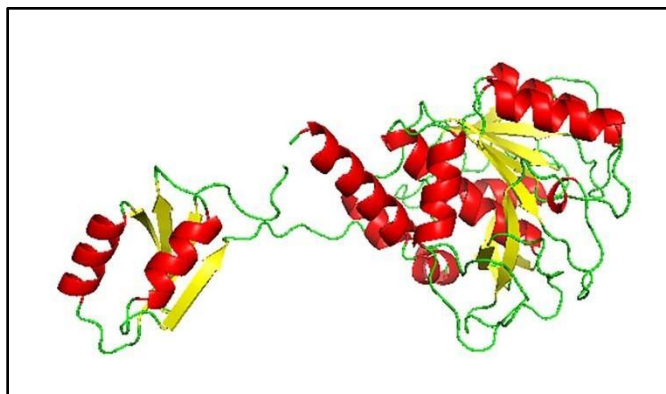


Figure 3.5 PyMOL generated image of modeled structure of ker6

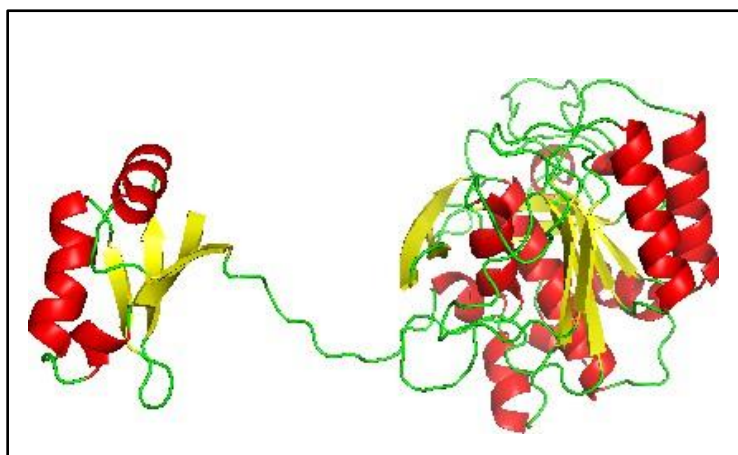


Figure 3.6 PyMOL generated image of modeled structure of kerC

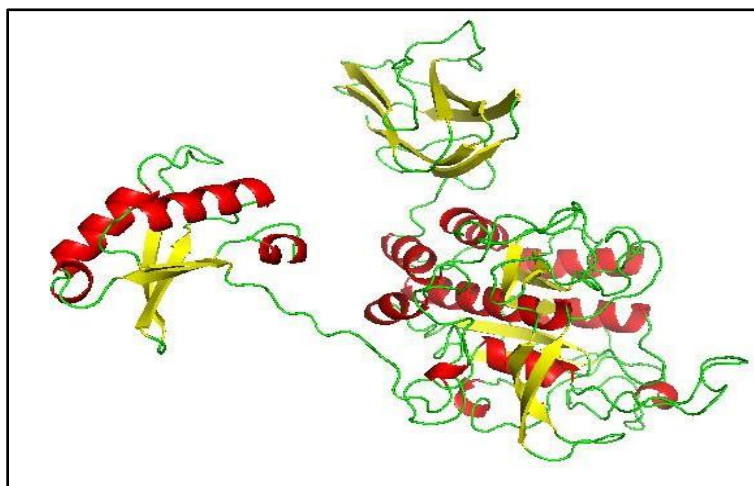


Figure 3.7 PyMOL generated image of modeled structure of kerF

In the figures 3.5, 3.6, and 3.7, the PyMOL visualized images of the AlphaFold2 generated models of ker6, kerC and kerF are shown respectively. The analyzed secondary

structural content in ker6 comes out to be helix (32.97%), loop (47.14%), and sheet (19.89%) as calculated with the help of PyMOL. Similarly for kerC, the analyzed secondary structural content are helix(33.04%), sheets(20.57%), and loop(46.37%) and for kerF they are (22.79%), sheets(23.16 %), and loop(54.04%).

3.4. Structure verification of modeled keratinases.

Modeled structures of keratinases generated using AlphaFold2 were verified with the help of PDBsum.

3.4.1. ker6

The Ramachandran plot of the AlphaFold2-modeled ker6 keratinase in figure 3.8, as generated by PDBSum, provides a comprehensive assessment of the stereochemical quality of the predicted protein structure. In this plot, each residue is mapped according to its backbone dihedral angles (phi and psi), allowing visual identification of conformational preferences and possible steric clashes. The analysis revealed that 90.3% of the residues (287 out of 318 non-glycine, non-proline residues) are located within the most favoured regions (A, B, L), reflecting excellent stereochemical quality and strongly supporting the accuracy of the structural model. An additional 7.9% of residues reside within the additionally allowed regions, and only 1.6% in generously allowed regions, with a single residue (0.3%) falling within the disallowed regions—well within the acceptable threshold for a high-quality model. Notably, none of the glycine or proline residues, which have unique conformational properties, fall in energetically unfavorable regions. The overall distribution closely matches the expected standards for reliable protein models, with the vast majority of residues clustered in energetically preferred zones. According to common validation criteria, an ideal model is expected to have more than 90% of residues in the most favoured regions; the current ker6 keratinase model meets this criterion, underscoring its suitability for further computational analyses and simulations.

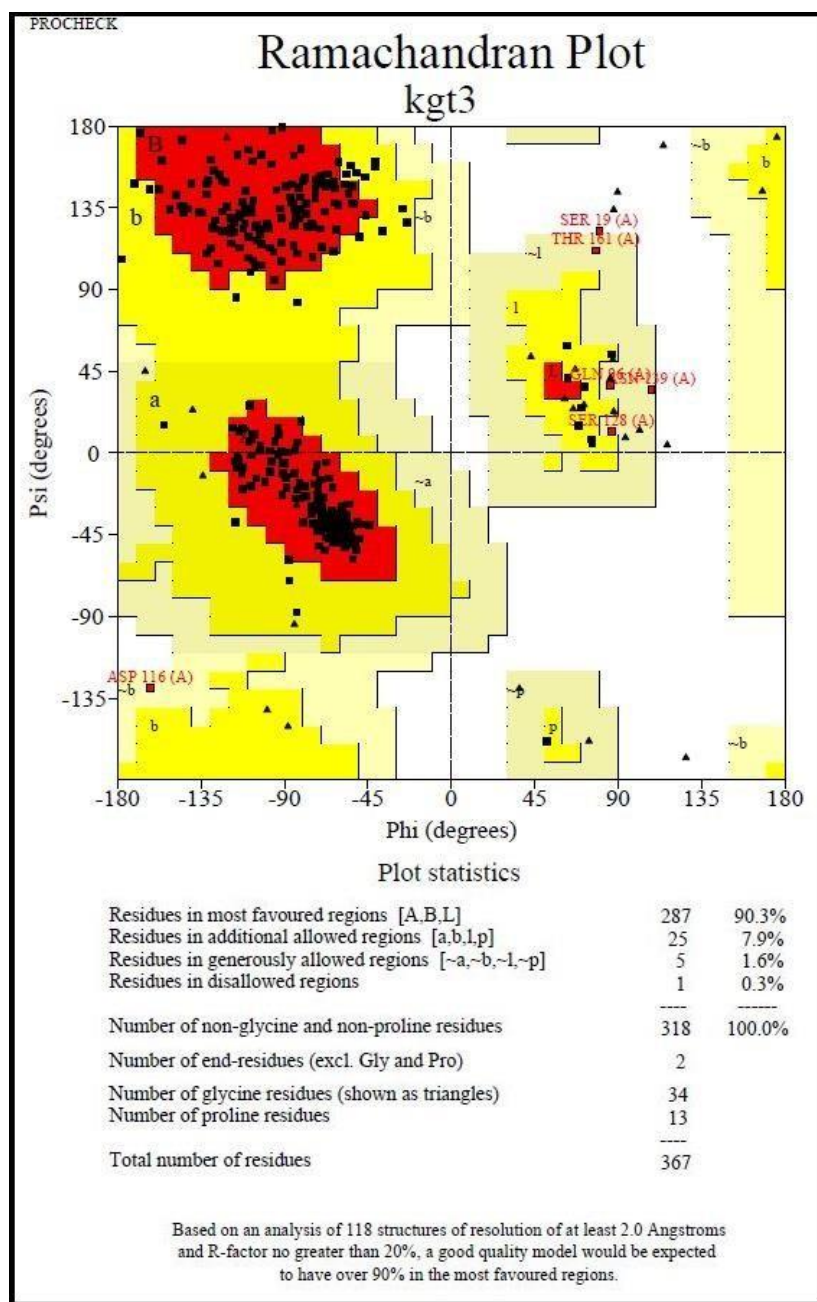


Figure 3.8 Ramachandran plot statistics generated by PDBsum for modeled structure of ker6.

3.4.2. kerC

The Ramachandran plot statistics for the AlphaFold2-predicted kerC keratinase structure shown in figure 3.9, as analyzed by PDBSum, further confirm the reliability of the modeled backbone geometry. Out of 292 non-glycine, non-proline residues, 90.1% (263

residues) are situated within the most favoured regions (A, B, L), satisfying the quality benchmark for accurate protein structures. An additional 8.2% (24 residues) occupy the additionally allowed regions, while 1.7% (5 residues) are found in generously allowed zones; notably, there are no residues in disallowed regions, indicating the absence of serious stereochemical outliers. The plot also separates end-residues, glycine, and proline positions—amino acids with distinct backbone conformational constraints—from core statistics, ensuring a fair assessment. Taken together, these results reinforce the high stereochemical quality and structural integrity of the kerC keratinase model, making it suitable for subsequent computational and experimental studies, and supporting confident functional annotation based on its predicted conformation.

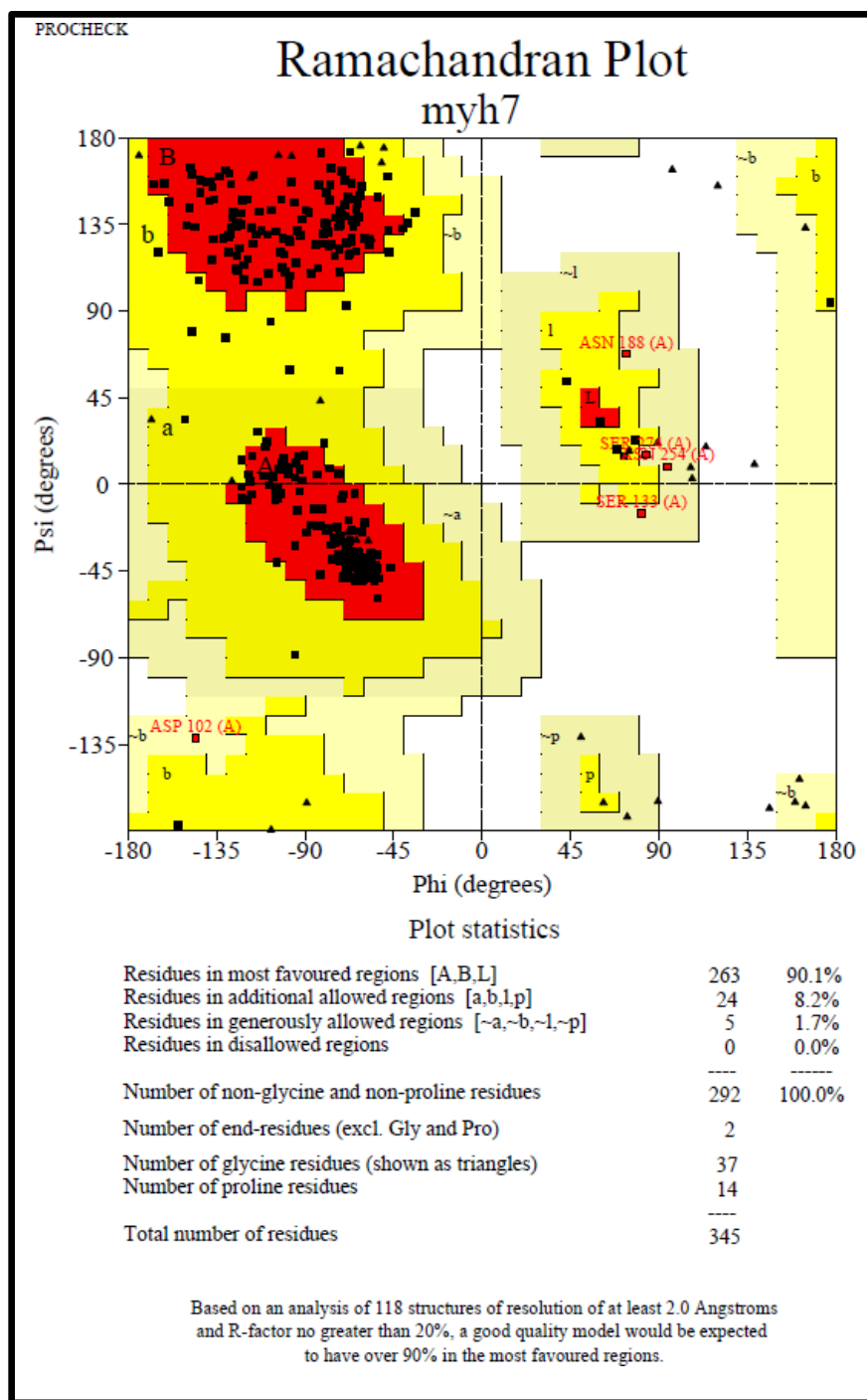


Figure 3.9 Ramachandran plot statistics generated by PDBsum for modeled structure of kerC.

3.4.3. kerF

The Ramachandran plot analysis for the AlphaFold2-modeled kerF keratinase structure, evaluated with PDBSum, demonstrates excellent backbone stereochemistry as presented in figure 3.10. Of the 446 non-glycine, non-proline residues analyzed, 90.4% (403 residues) are located in the most favoured regions (A, B, L), clearly satisfying accepted criteria for high-quality structural models. Additionally, 9.2% (41 residues) are found in the additional allowed regions and only 0.4% (2 residues) in generously allowed regions, while none fall in disallowed zones. The plot also identifies end residues, glycine, and proline, which are interpreted separately due to their distinctive backbone conformational properties. This distribution is consistent with robust modeling, reflecting minimal geometric outliers and strong structural integrity. The results for kerF, alongside other keratinases analyzed, reinforce the reliability of the AlphaFold2 predictions for downstream computational and functional studies.

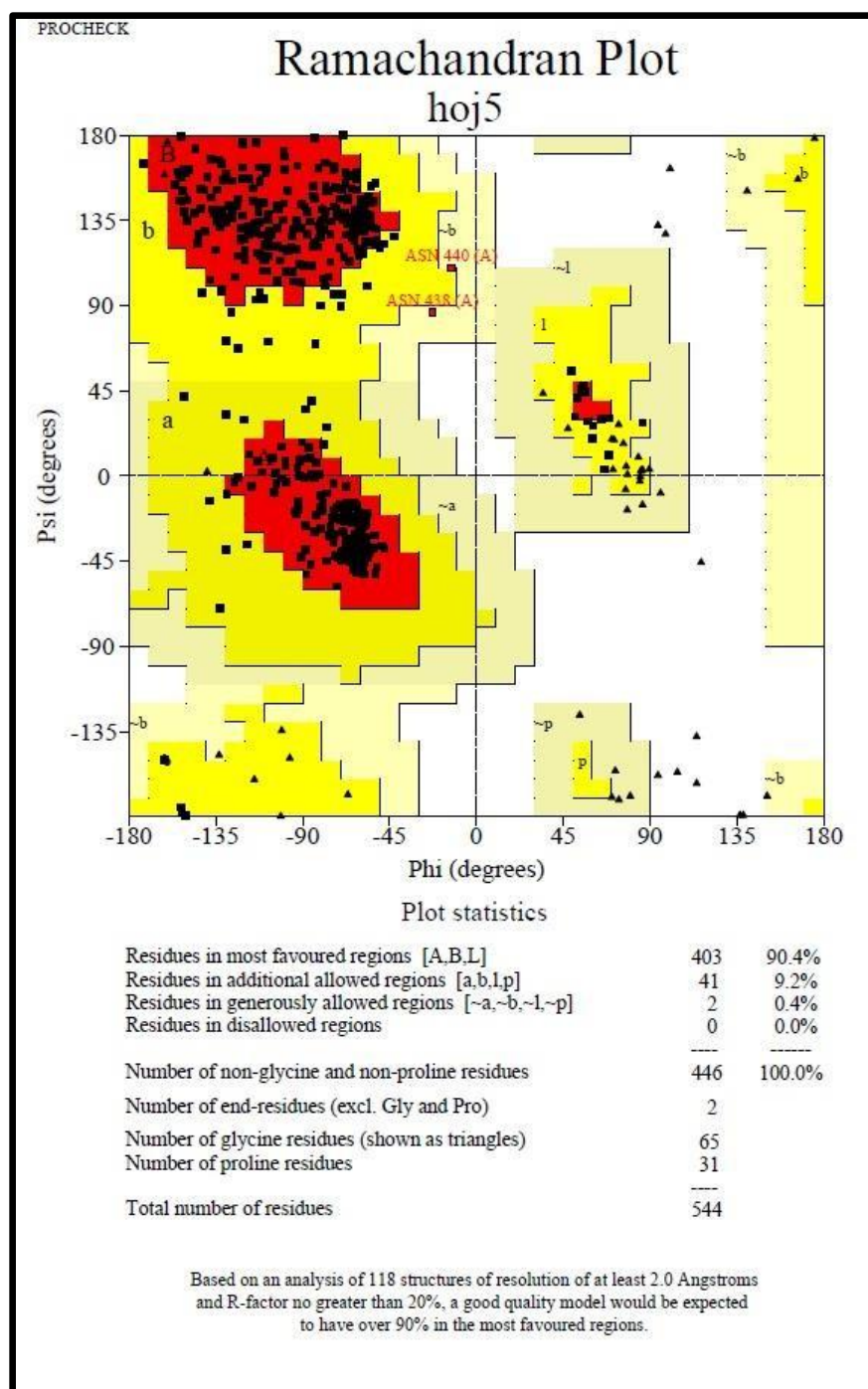


Figure 3.10 Ramachandran plot statistics generated by PDBsum for modeled structure of kerF.

3.5. Shortlisted ionic liquids

The chemical structures and details of the shortlisted ionic liquids downloaded from PubChem database are shown as follows:

3.5.1. Tetrabutylammonium hydroxide (TBAH)

Table 3.3 PubChem data for TBAH

PubChem CID	2723671
Molecular Formula	$C_{16}H_{36}N.HO$ / $C_{16}H_{37}NO$
Molecular weight	259.47 g/mol

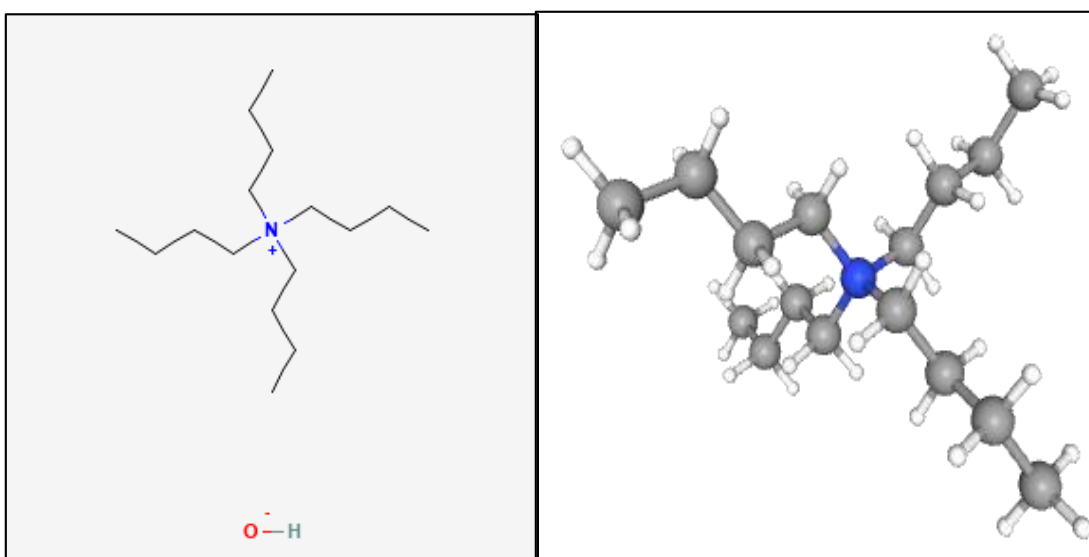


Figure 3.11 2D and 3D structural representations of TBAH downloaded from PubChem.

3.5.2. Tetramethylammonium hydroxide (TMAOH)

Table 3.4 PubChem data for TMAOH

PubChem CID	60966
Molecular Formula	$C_4H_{12}N.HO$ / $C_4H_{13}NO$
Molecular weight	91.15 g/mol

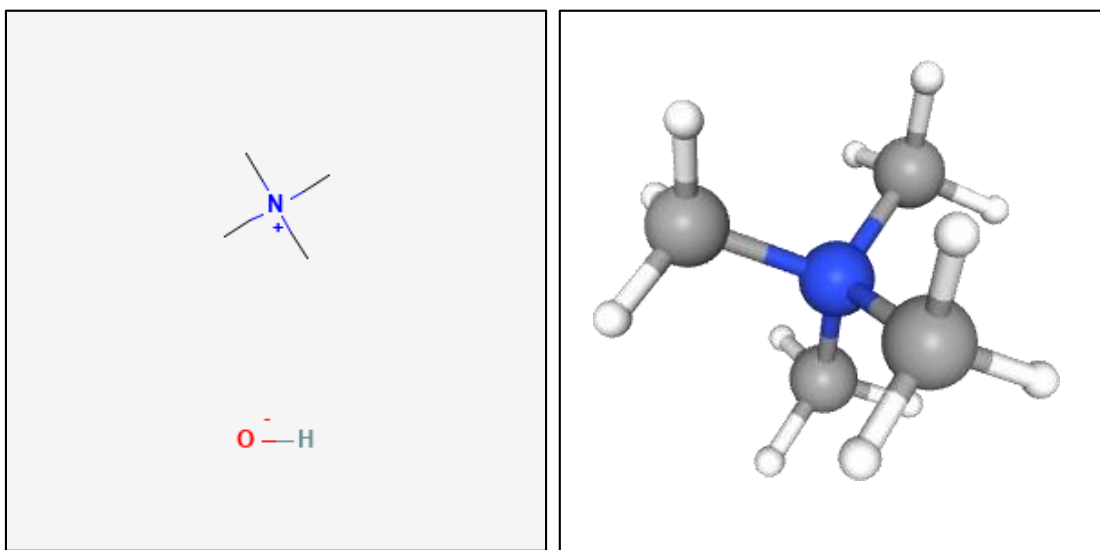


Figure 3.12 2D and 3D structural representations of TMAOH downloaded from PubChem.

3.5.3. Choline hydroxide (Ch-OH)

Table 3.5 PubChem data for Ch-OH

PubChem CID	31255
Molecular Formula	C ₅ H ₁₄ NO.HO / C ₅ H ₁₅ NO ₂
Molecular weight	121.18 g/mol

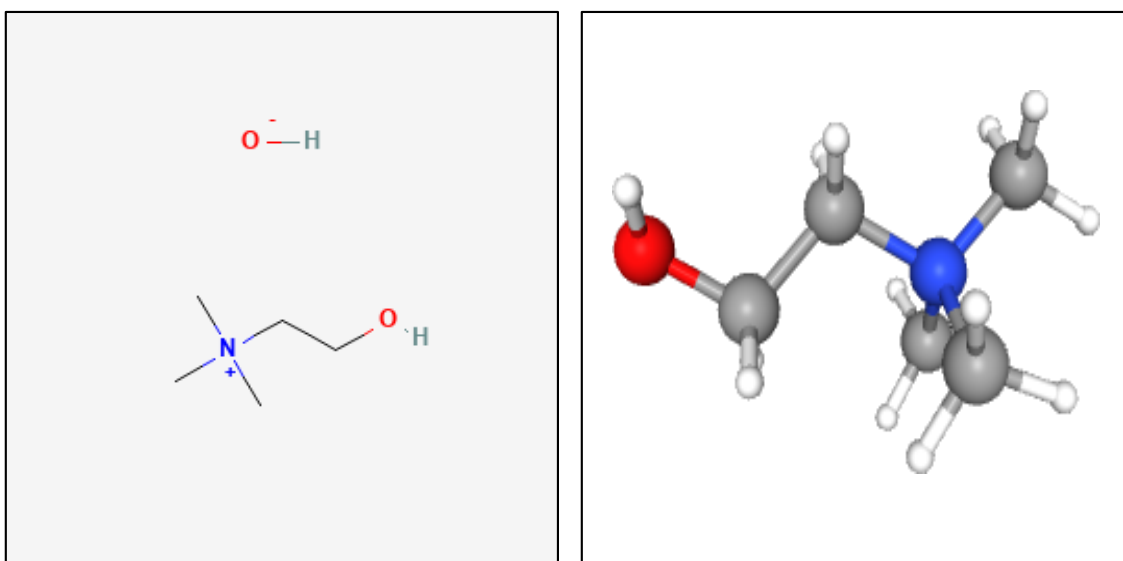


Figure 3.13 2D and 3D structural representations of Ch-OH downloaded from PubChem.

3.5.4. 1-butyl-3-methylimidazolium bromide ([BMIM]Br)

Table 3.6 PubChem data for [BMIM]Br

PubChem CID	2734236
Molecular Formula	C ₈ H ₁₅ BrN ₂
Molecular weight	219.12 g/mol

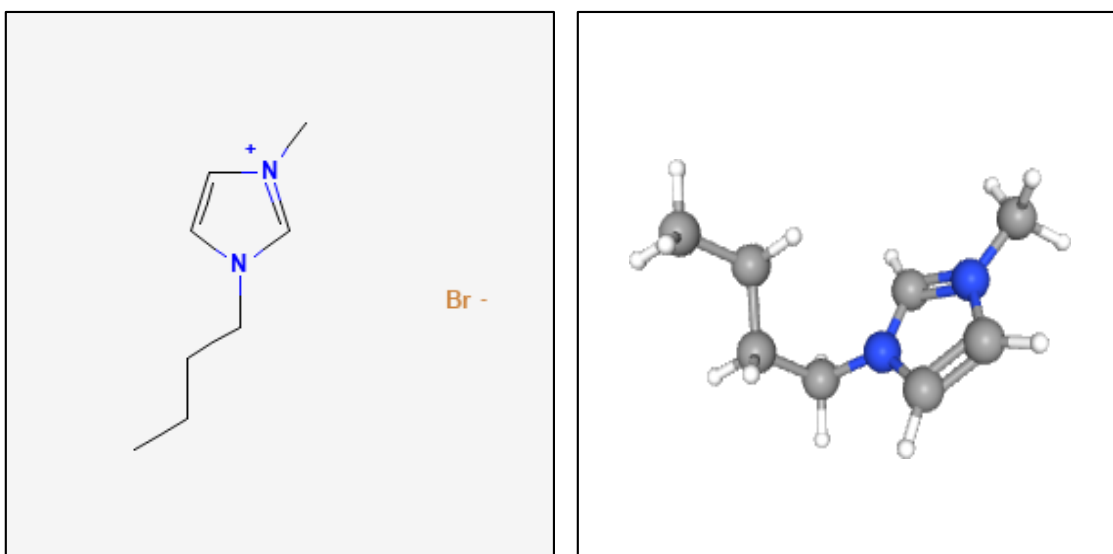


Figure 3.14 2D and 3D structural representations of [BMIM]Br downloaded from PubChem

3.5.5. 1-butyl-3-methylimidazolium chloride ([BMIM]Cl)

Table 3.7 PubChem data for [BMIM]Cl

PubChem CID	2734161
Molecular Formula	C ₈ H ₁₅ ClN ₂
Molecular weight	174.67 g/mol

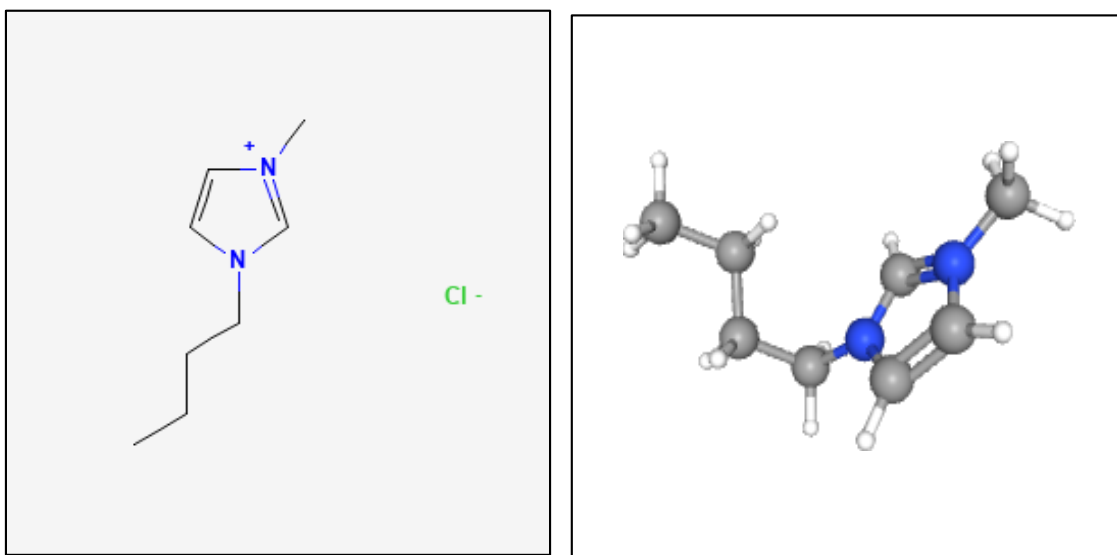


Figure 3.15 2D and 3D structural representations of [BMIM]Cl downloaded from PubChem

3.5.6. 1-allyl-3-methylimidazolium chloride ([AMIM]Cl)

Table 3.8 PubChem data for [AMIM]Cl

PubChem CID	11321106
Molecular Formula	C ₇ H ₁₁ ClN ₂
Molecular weight	158.63 g/mol

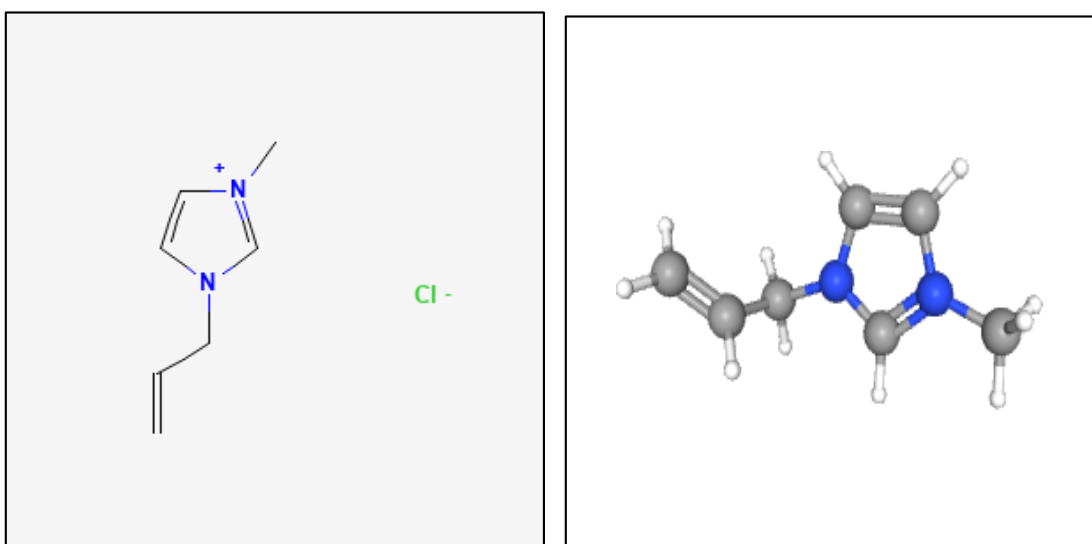


Figure 3.16 2D and 3D structural representations of [AMIM]Cl downloaded from PubChem

3.6. Analysis of the molecular dynamics simulations parameter

3.6.1. ker6

3.6.1.1. Root Mean Square Deviation (RMSD)

The root mean square deviation (RMSD) analysis of keratinase ker6 shown in figures 3.17 and 3.18 reveals substantial insights into its backbone stability across water and six ionic liquids during 200 ns molecular dynamics simulations, serving as a classical metric to quantify conformational changes relative to the initial model structure. The results demonstrate that ker6 in water exhibits marked instability throughout the simulation, with persistent RMSD fluctuations often surpassing 1 nm, indicating ongoing structural rearrangements and a lack of equilibrium. In contrast, ker6 in TMAOH undergoes initial RMSD fluctuations during the first 40 ns but stabilizes thereafter, maintaining RMSD close to or slightly above 1 nm for the remainder of the trajectory, thus suggesting that TMAOH facilitates a more stable protein environment after an adaptation period. TBAH provides a comparatively stabilizing influence, with ker6 RMSD fluctuating within a lower range (0.35–0.85 nm), while AMIMCL shows progressive increases in RMSD later in the simulation, pointing toward delayed instability. BMIMBR is associated with pronounced RMSD peaks throughout, frequently exceeding 1.2–1.75 nm, and signifies the least stable solvent environment for ker6 among those studied. In contrast, BMIMCL and CHOH induce relatively lower RMSD values throughout much of the simulation, indicating enhanced backbone stability compared to water and BMIMBR. Collectively, these analyses establish that TMAOH is a comparatively superior solvent for maintaining ker6 structure and stability, while water and other ionic liquid lead to continual conformational disruptions.

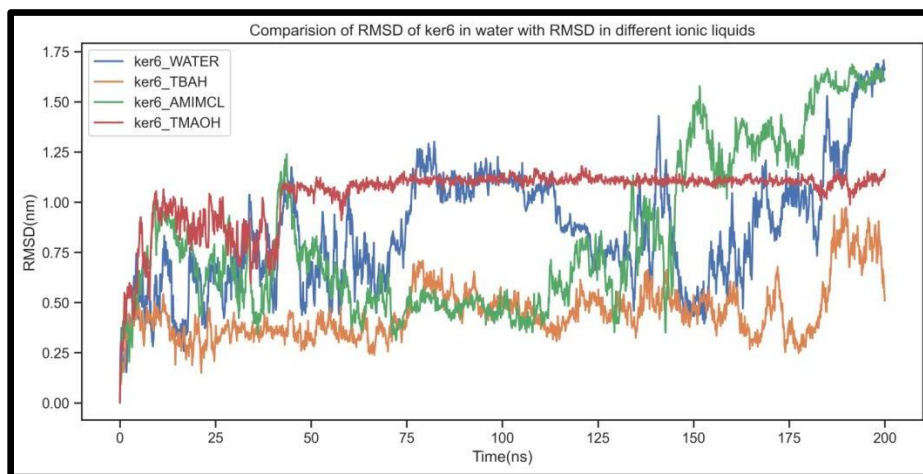


Figure 3.17 Root Mean Square Deviations (RMSD) of backbone of ker6 in water, TBAH, AMIMCL, and TMAOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

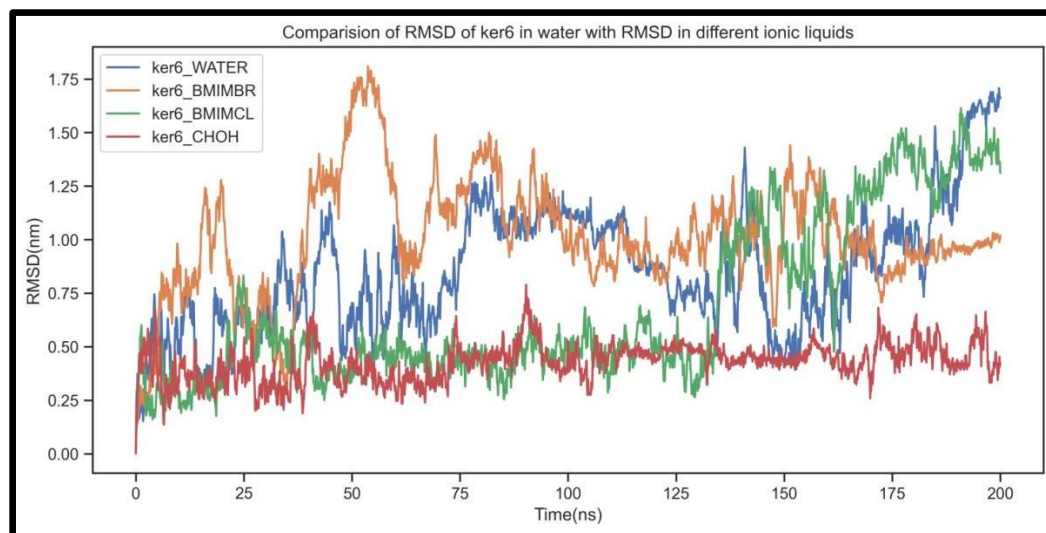


Figure 3.18 Root Mean Square Deviations (RMSD) of backbone of ker6 in water, BMIMBR, BMIMCL, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

3.6.1.2. Root Mean Square Fluctuation (RMSF)

The root mean square fluctuation (RMSF) analysis of keratinase ker6 was performed for the last 100 ns of molecular dynamics simulations across water and six ionic liquids to elucidate the residue-wise flexibility characteristics indicative of local protein stability. As visualized in figures 3.19 and 3.20, residues in the N-terminal region (1-70), comprising a mix of helices, loops, and sheets, displayed the most pronounced fluctuations in all solvent conditions, signifying their inherent conformational flexibility. Similarly, C-terminal residues (350-367), which are entirely helical, demonstrated notable RMSF peaks, reflecting dynamic structural behavior in these terminal regions. Across all systems studied, ker6 in water and TBAH exhibited the highest RMSF values throughout these flexible regions and, in general, over many other residues, revealing that both water and TBAH contribute to increased backbone mobility and reduced local stability in the enzyme structure. Simulations in BMIMBR, BMIMCL, CHOH, and AMIMCL also showed appreciable residue fluctuations, especially in the highly dynamic terminal segments and some internal loops, although the degree of movement was moderately diminished compared to water and TBAH, indicating intermediate stability. In contrast, the lowest overall RMSF values were consistently observed in TMAOH, both in the highly flexible terminal zones and throughout most of the ker6 sequence, indicating that TMAOH exerts the greatest stabilizing effect and minimizes local fluctuations more effectively than any other solvent tested. Taken together, these results confirm that the dynamic instability of ker6 is associated with water and TBAH,

while TMAOH stands out as an exceptionally stabilizing medium, making it particularly suitable for solvent environments aimed at preserving keratinase structural integrity over extended timescales.

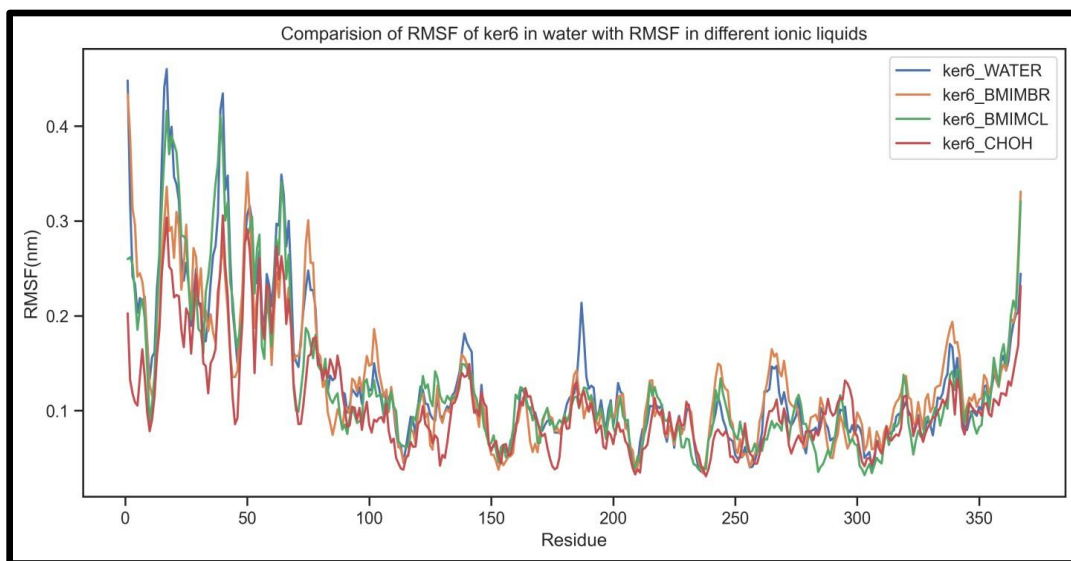


Figure 3.19 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of ker6 in water, BMIMBR, BMIMCL, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

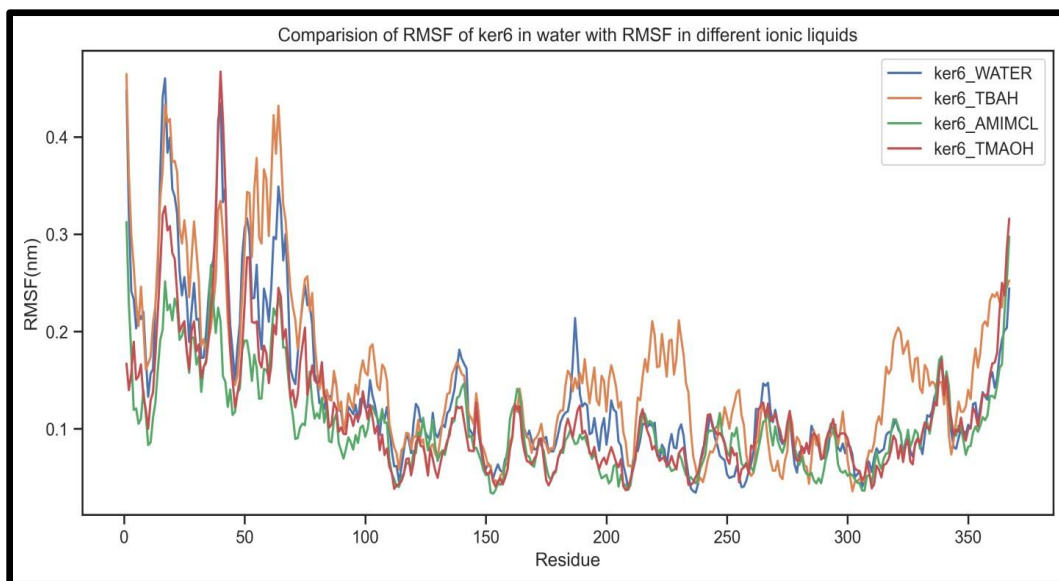


Figure 3.20 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of ker6 in water, TBAH, AMIMCL, and TMAOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

3.6.1.3. Hydrogen Bonds

The comprehensive analysis of intra-protein hydrogen bonds in keratinase ker6 presented in figures 3.21, 3.22 and 3.23 during the final 100 ns of molecular dynamics simulations in water and six different ionic liquids reveals critical insights into the relationship between hydrogen bonding patterns and protein stability. When comparing the total number of hydrogen bonds across these solvent systems, it is evident that ker6 forms remarkably fewer intra-protein hydrogen bonds in water and AMIMCL, which directly correlates with diminished structural stability and increased backbone flexibility as previously observed in RMSF and RMSD data. By contrast, ker6 maintains a consistently higher number of hydrogen bonds when simulated in TMAOH and CHOH, suggesting that these ionic liquids provide environments that reinforce the internal hydrogen bonding network, thereby stabilizing secondary structure elements and limiting conformational drift. Similarly, BMIMBR and BMIMCL support an intermediate level of hydrogen bonding, contributing to moderate stability relative to water, AMIMCL, TMAOH, and CHOH, but with more dynamic variation during the simulation. The underlying differences in hydrogen bond formation can be attributed both to the inherent hydrophobicity of each ionic liquid and to the nature and frequency of protein-solvent interactions unique to each system, wherein the presence of ionic liquid molecules may promote or disrupt intra-molecular interactions depending on their charge distribution, chemical structure, and affinity for the protein surface.

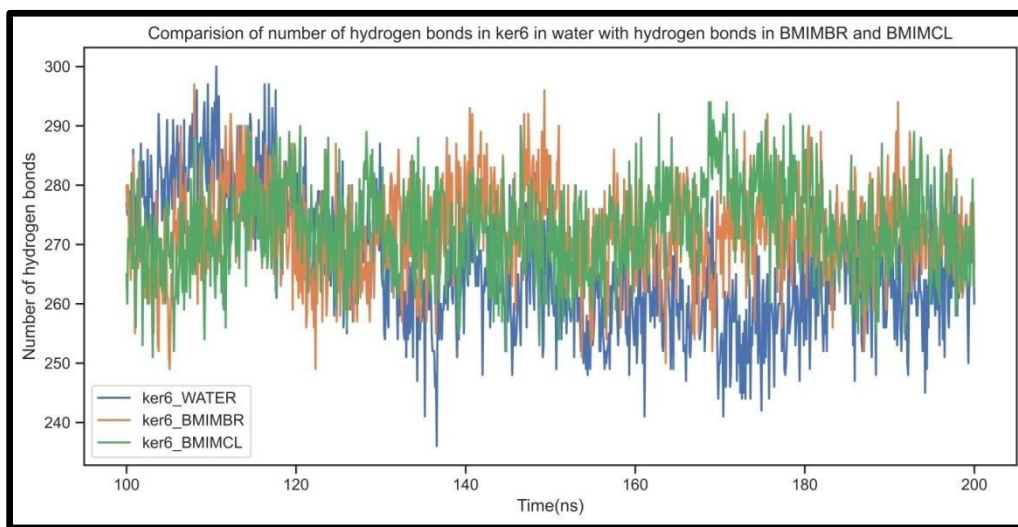


Figure 3.21 : Intra-protein hydrogen bonds in ker6 in water, BMIMBR, and BMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

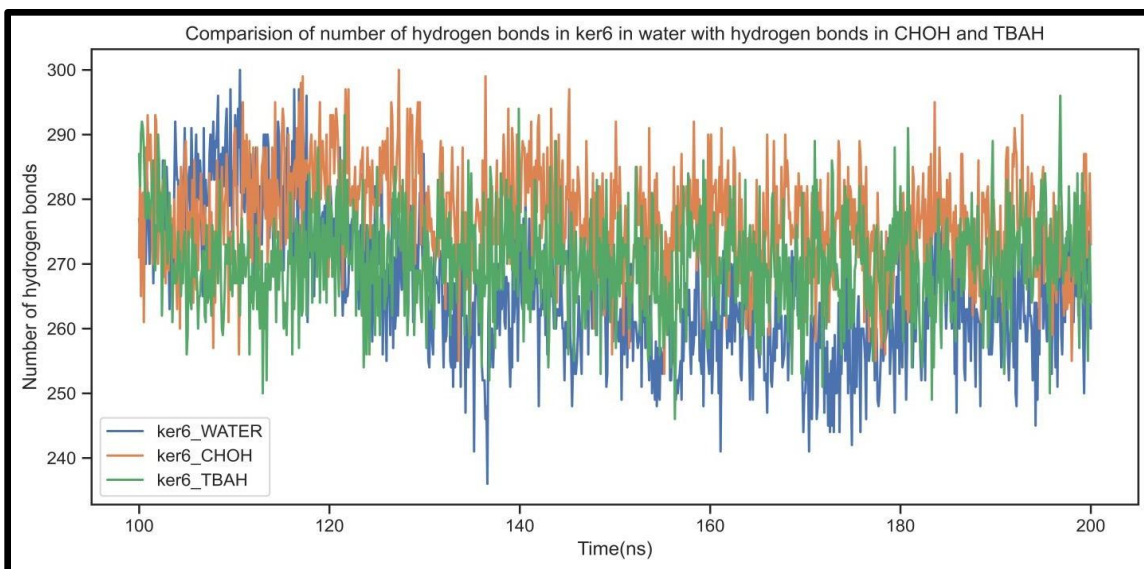


Figure 3.22 Intra-protein hydrogen bonds in ker6 in water, CHOH, and TBAH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

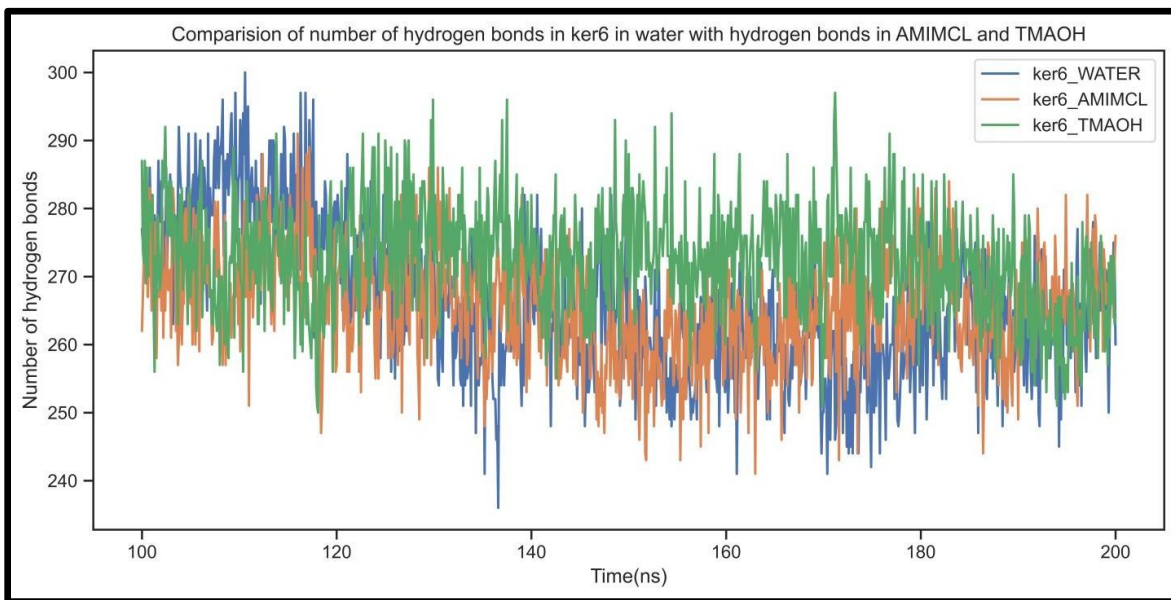


Figure 3.23 Intra-protein hydrogen bonds in ker6 in water, AMIMCL, and TMAOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

3.6.1.4. Solvent Accessible Surface Area (SASA)

The analysis of solvent-accessible surface area (SASA) for keratinase ker6 over the last 100 ns of molecular dynamics simulations in water and six different ionic liquids presented in figures 3.24, 3.25, and 3.26 uncovers important trends regarding protein-solvent interactions and conformational stability. Across all systems, TMAOH consistently exhibits the lowest SASA values, indicating a more compact conformation and reduced solvent exposure throughout the simulation window; this suggests heightened protein stability, as fewer solvent molecules can interact with ker6. CHOH and TBAH also promote conformational compactness, with SASA values moderately higher than TMAOH but lower than all other solvents, highlighting their favorable impact on keratinase stability. Among the remaining systems, AMIMCL presents intermediate SASA values while water shows even greater solvent exposure, signifying less structural protection and comparatively lower stability. In contrast, BMIMBR and BMIMCL display the highest SASA readings, implying that ker6 becomes most accessible to solvent molecules and, consequently, most prone to conformational fluctuations in these ionic liquids. The hierarchy that emerges from the comparison—least SASA in TMAOH, followed by CHOH, TBAH, AMIMCL, water, BMIMBR, and highest in BMIMCL—underscores the superior stabilizing influence of TMAOH and the destabilizing, solvent-exposing effect of BMIMBR and BMIMCL.

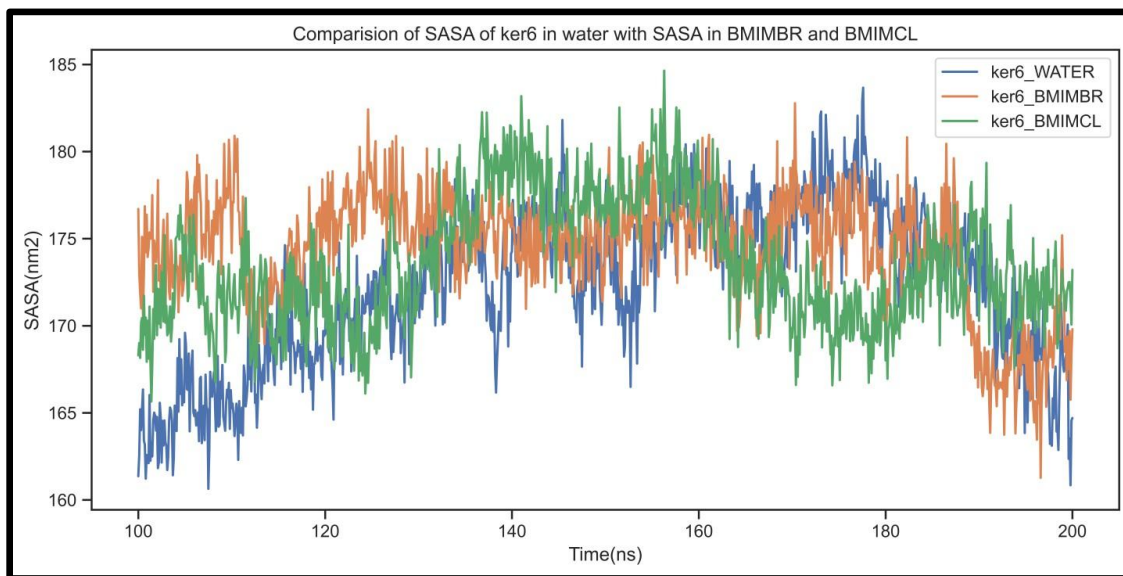


Figure 3.24 Solvent Accessible Surface Area (SASA) of ker6 in water, BMIMBR, and BMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

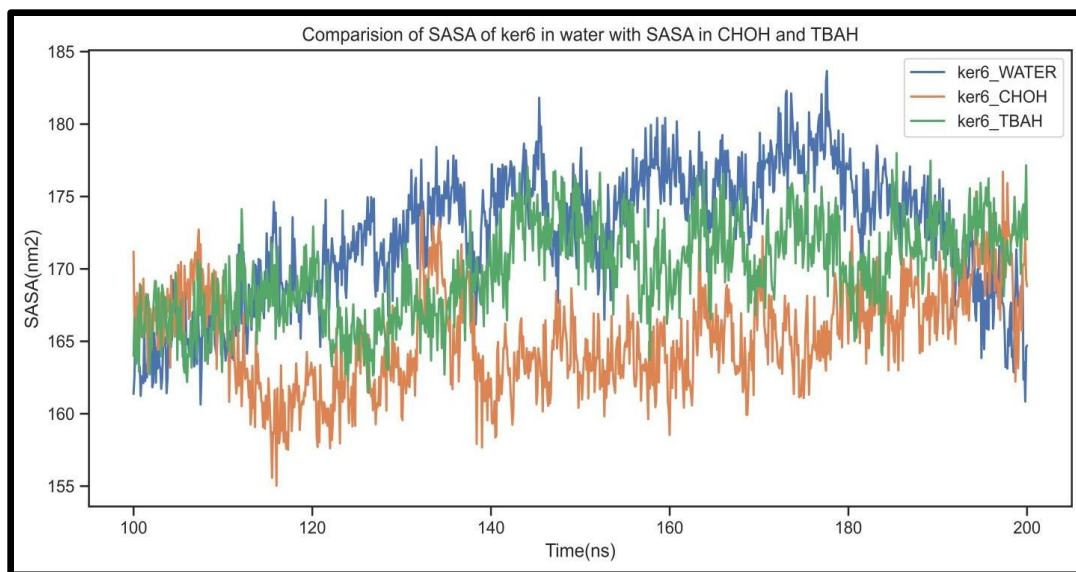


Figure 3.25 Solvent Accessible Surface Area (SASA) of ker6 in water, CHOH, and TBAH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

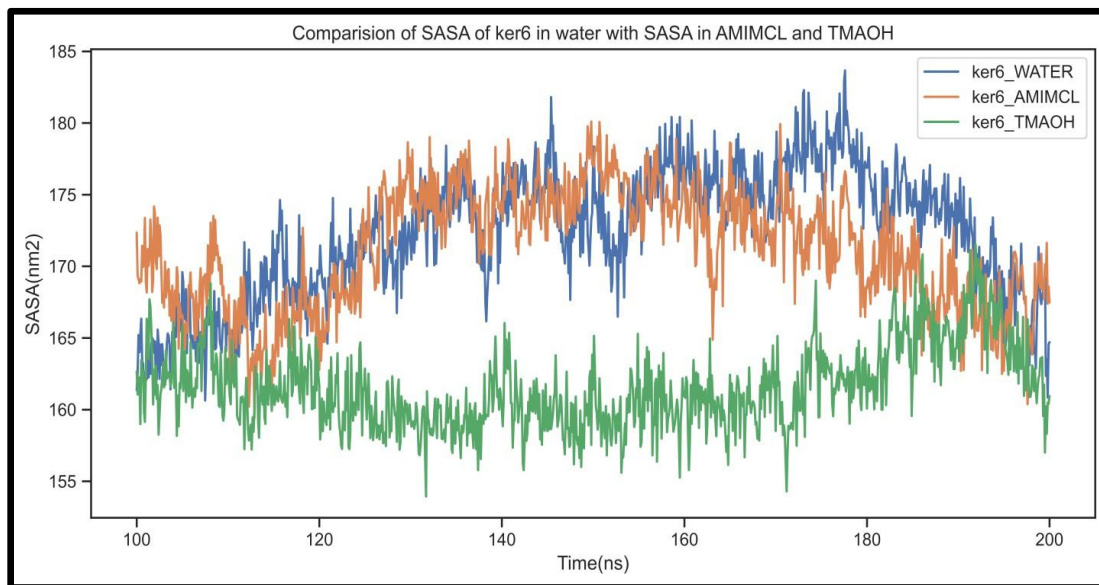


Figure 3.26 Solvent Accessible Surface Area (SASA) of ker6 in water, AMIMCL, and TMAOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

3.6.1.5. Radius of gyration (Rg)

The evaluation of the radius of gyration (Rg) of keratinase ker6 over the last 100 ns of molecular dynamics simulations in the presence of water and six different ionic liquids as visualized in figure 3.27 provides important insights into changes in global protein compactness and stability. Analysis shows that ker6 displays the highest Rg fluctuations when simulated in BMIMCL, followed by noticeable but slightly less extensive variations in BMIMBR, water, and TBAH, with all these environments associated with episodes of increased Rg values that point to transient expansions and decreased compactness of the enzyme's fold. Conversely, the ionic liquids CHOH, AMIMCL, and particularly TMAOH support a much more consistent and stable Rg profile over time, with TMAOH exhibiting the lowest and most uniform Rg values throughout, indicating that ker6 adopts its most compact and structurally resilient conformation in this solvent. The data thus emphasize that TMAOH and, to a lesser extent, CHOH and AMIMCL, effectively preserve the compactness of ker6 and minimize global unfolding, while water, BMIMBR, BMIMCL, and TBAH encourage conformational flexibility or expansion, which are less favorable for the maintenance of long-term protein stability in solution.

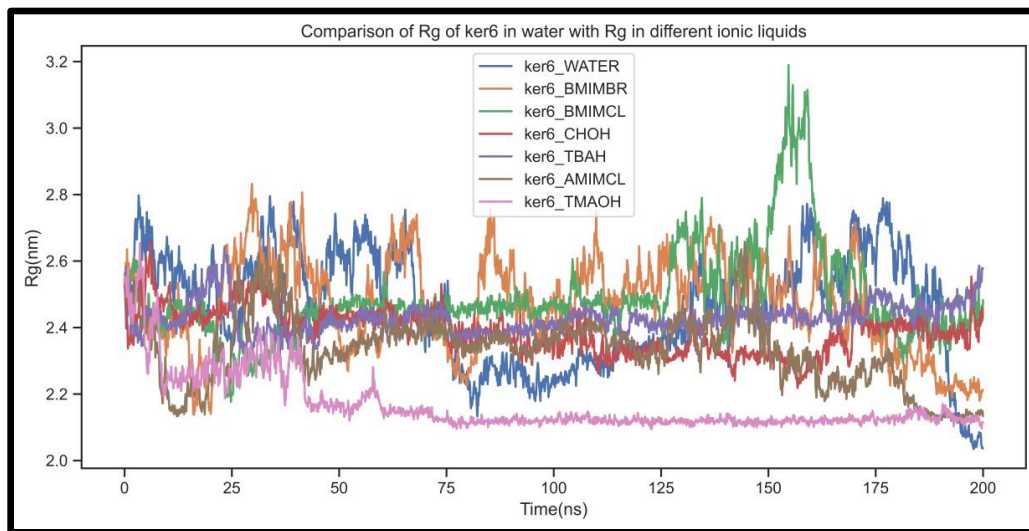


Figure 3.27 Radius of Gyration of ker6 in water and different ionic liquids. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the Rg in nm.

3.6.2. kerC

3.6.2.1. Root Mean Square Deviations (RMSD)

The RMSD analysis of kerC in water and a range of ionic liquids demonstrates pronounced differences in structural dynamics and stability depending on the solvent environment. As interpreted from the figures 3.28, 3.29, and 3.30, in water, kerC shows significant initial fluctuations, followed by stabilization for a substantial portion of the simulation between 1.0 and 1.2 nm, with occasional spikes suggesting transient conformational changes. Among the ionic liquids, AMIMCL stands out by conferring the highest structural stability; after an initial increase, the RMSD becomes relatively constant near 1.0–1.2 nm through the latter stages, indicating minimal deviation from native-like conformations. Conversely, TMAOH leads to the greatest instability, as evidenced by a sharp RMSD rise above 2 nm after 175 ns, pointing to considerable unfolding events late in the trajectory. TBAH is associated with persistent RMSD undulations, at times exceeding 1.4 nm, implicating ongoing disruptions to protein stability, while CHOH, despite marked early fluctuations, permits a steadier RMSD profile beyond 150 ns, suggesting delayed stabilization. In the BMIM-based solvents, both BMIMCL and BMIMBR are characterized by higher-amplitude fluctuations, with BMIMBR showing particularly prominent and frequent spikes in RMSD that can be as high as 1.7–1.9 nm, reflecting an especially dynamic and unstable protein environment. Quantitative evaluation over the final 20 ns reinforces these observations: AMIMCL exhibits the lowest mean RMSD (~0.95 nm), water and TBAH display moderate mean values (about 1.00 and 1.03 nm, respectively), while CHOH and especially TMAOH yield substantially elevated values (mean RMSD of 1.44 and 1.85 nm, with TMAOH reaching a maximum exceeding 2 nm). Collectively, these results underscore the remarkable stabilizing potency of AMIMCL for kerC, while highlighting the destabilizing effects of TMAOH and the persistent flexibility induced by BMIM-type ionic liquids.

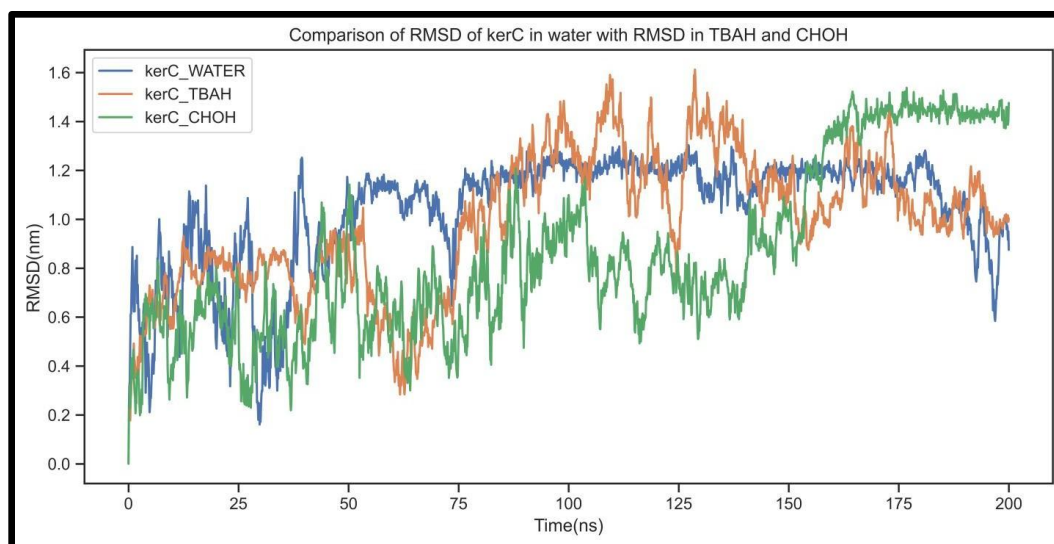


Figure 3.28 Root Mean Square Deviations (RMSD) of backbone of kerC in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

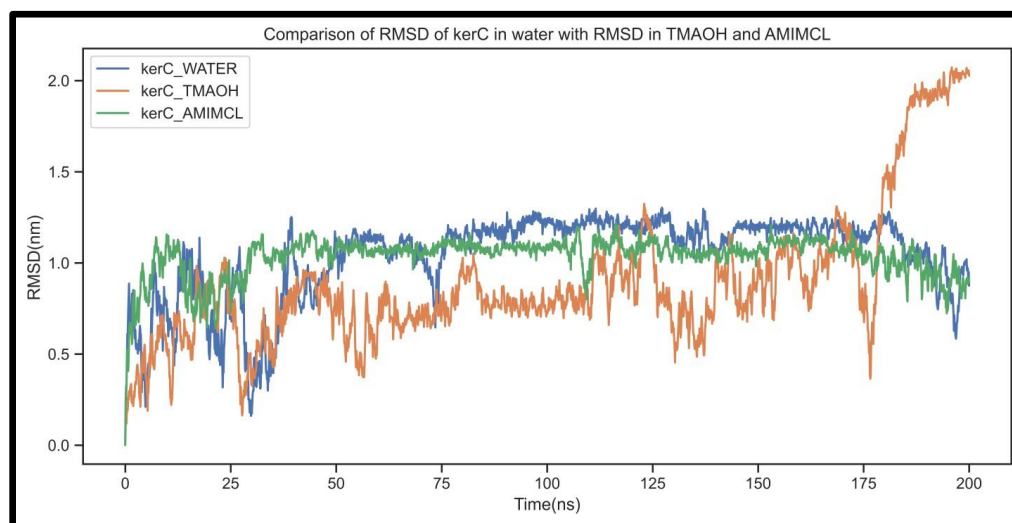


Figure 3.29 Root Mean Square Deviations (RMSD) of backbone of kerC in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

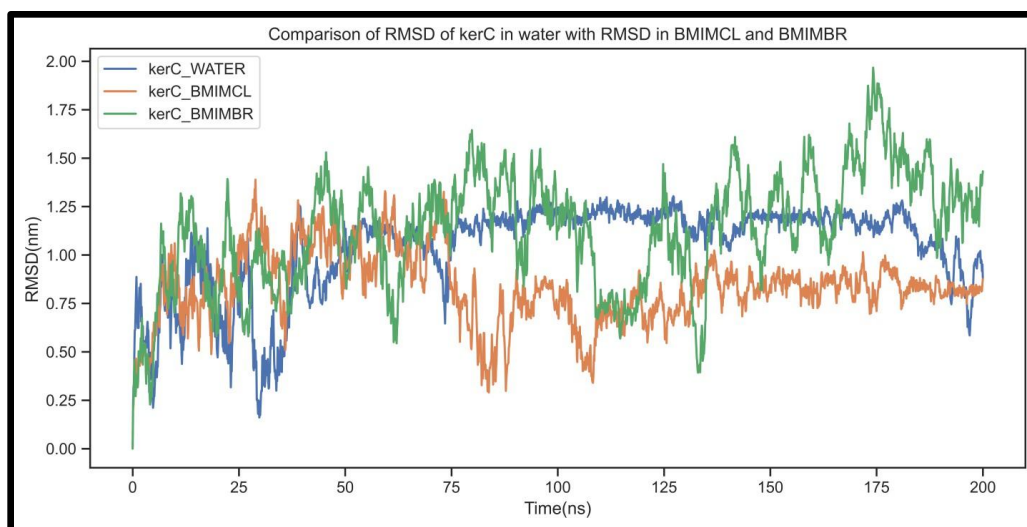


Figure 3.30 Root Mean Square Deviations (RMSD) of backbone of kerC in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

3.6.2.2. Root Mean Square Fluctuations (RMSF)

Analysis of the root mean square fluctuation (RMSF) data for kerC in water and ionic liquids as presented in figures 3.31, 3.32, and 3.33, reveals distinct patterns of residue-level flexibility that are highly dependent on the solvent. In water, kerC exhibits the greatest local fluctuations, particularly across the N-terminal residues, where RMSF regularly reaches or surpasses 0.4 nm, indicating elevated backbone mobility and local disorder in aqueous conditions. The introduction of ionic liquids such as TBAH, CHOH, TMAOH, BMIMCL, BMIMBR, and AMIMCL universally dampens these high-amplitude motions, with a clear reduction in RMSF throughout significant portions of the sequence, most notably within the first 70 residues and again at the C-terminus, supporting the broad stabilizing effect of these environments compared to water. TBAH is especially effective in lowering fluctuations, yielding the lowest RMSF values across nearly all residue positions and thus conferring the greatest rigidity to the backbone. CHOH and BMIMCL offer an intermediate level of stabilization, consistently showing diminished loop mobility relative to water but retaining slightly greater flexibility than TBAH. The effect of AMIMCL is non-uniform: while this solvent reduces flexibility at the N-terminus, several loop and exposed regions in the second half of the sequence exhibit elevated RMSF, at times matching or exceeding the flexibility observed in water. TMAOH and BMIMBR behave variably as well, with pockets of reduced as well as heightened mobility, the latter most apparent in the C-terminal residues. Across all environments, the lowest RMSF values are observed in regions corresponding to predicted secondary structural elements—suggesting that solvent type principally modulates the motion of flexible loops and disordered termini rather than rigid structural

cores. Taken together, these results demonstrate that here ionic liquids generally reduce the global and local fluctuations of kerC relative to water, but the magnitude and locations of these effects depend on both the specific solvent and the structural context within the protein, emphasizing the need for tailored solvent selection in the pursuit of stable yet catalytically competent keratinase formulations for biotechnological applications.

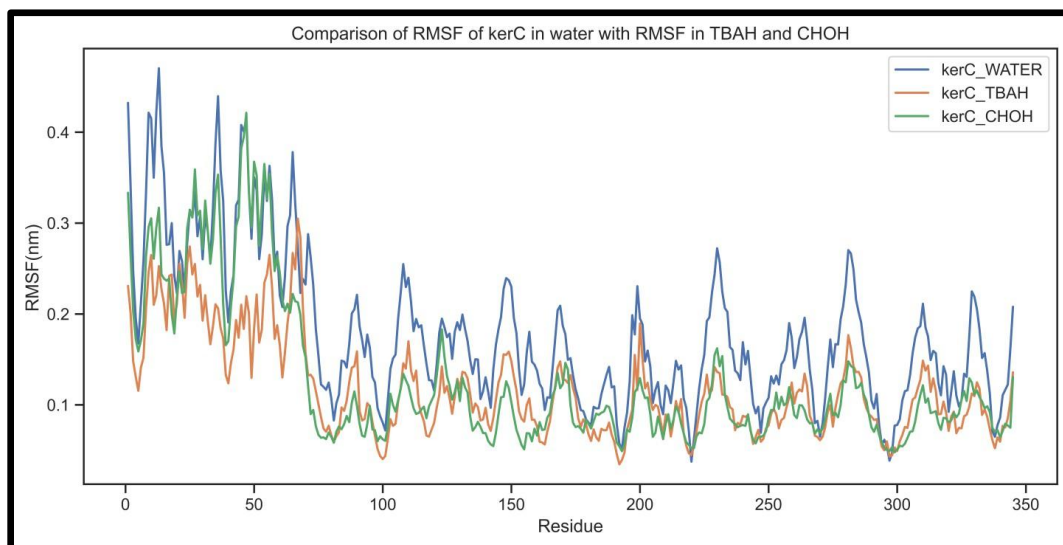


Figure 3.31 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of kerC in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

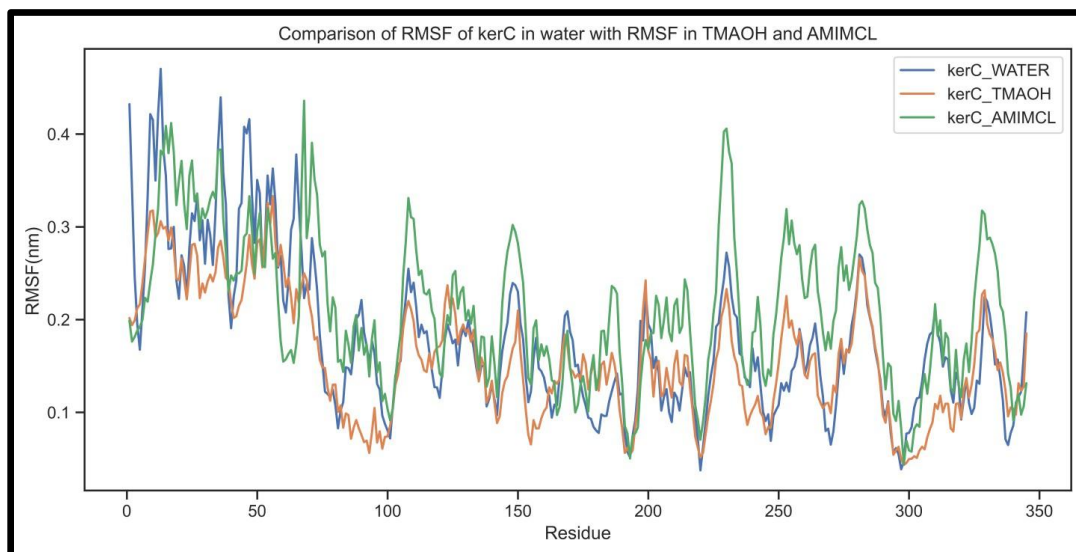


Figure 3.32 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of kerC in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

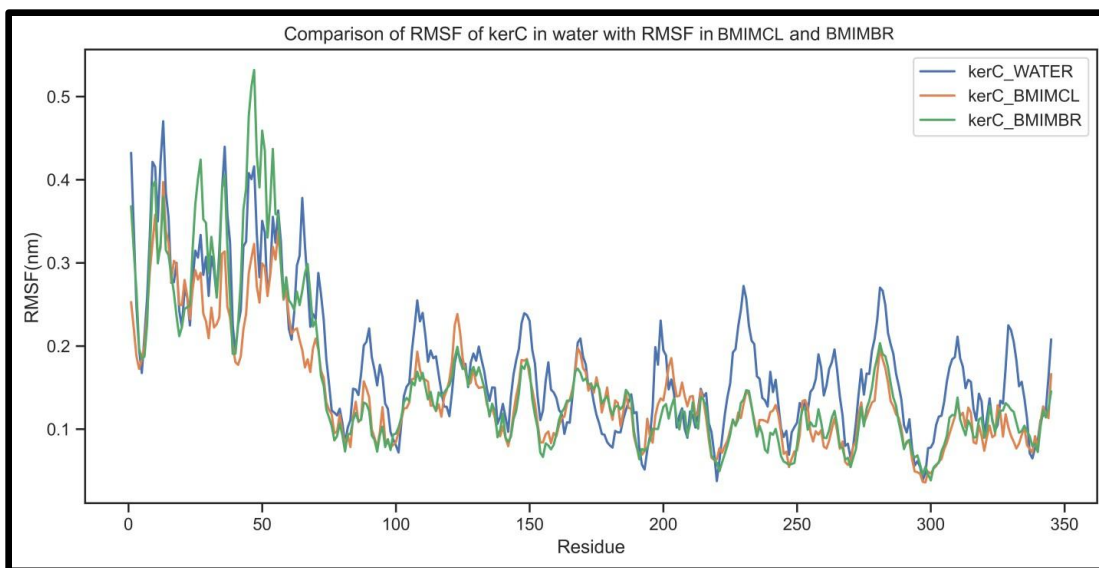


Figure 3.33 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of kerC in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

3.6.2.3. Hydrogen Bonds

The intramolecular hydrogen bonding pattern of kerC was analyzed over the 100–200 ns interval of the molecular dynamics simulations conducted in water and six different ionic liquids (TBAH, CHOH, TMAOH, AMIMCL, BMIMCL, and BMIMBR) as shown in figures 3.34, 3.35, and 3.36. The temporal profile of hydrogen bond numbers indicates that kerC sustains a consistently high and stable network of internal hydrogen bonds across all solvent systems, with minor oscillations corresponding to short-lived conformational rearrangements. Among the studied media, the TBAH and BMIMCL systems displayed marginally higher average hydrogen bond counts—around 246 and 243, respectively—relative to water, which averaged approximately 239. This slight increase suggests that these two ionic liquids enhance the internal hydrogen-bond stability of kerC, potentially through favorable polarity effects or specific molecular interactions. In contrast, CHOH and AMIMCL yielded the lowest mean values, both near 237, reflecting a modest reduction in internal stabilization. Despite these subtle differences, the overall fluctuation range (roughly 220–270 hydrogen bonds) remained narrow across all systems, signifying that the protein's secondary and tertiary architecture remained largely intact. These small solvent-dependent variations are consistent with trends observed in other structural metrics, indicating that while kerC's

hydrogen bonding responds to its ionic environment, its structural framework remains resilient even under less favorable solvent conditions.

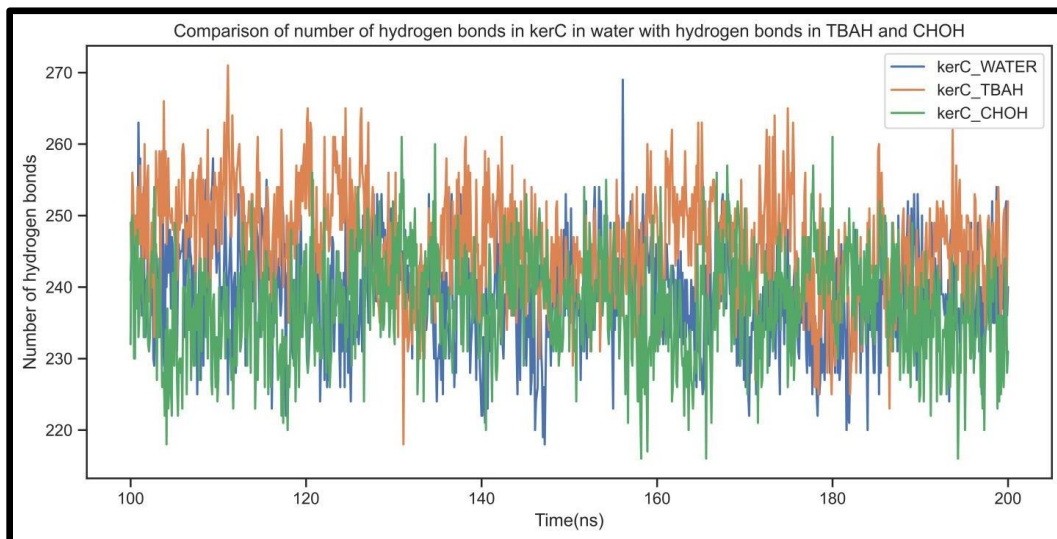


Figure 3.34 Intra-protein hydrogen bonds in kerC in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

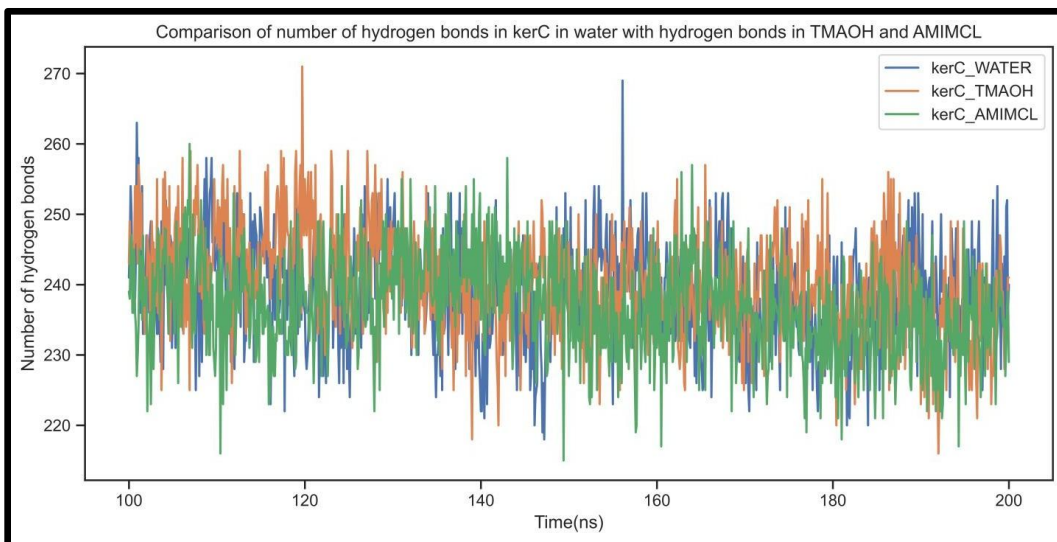


Figure 3.35 Intra-protein hydrogen bonds in kerC in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

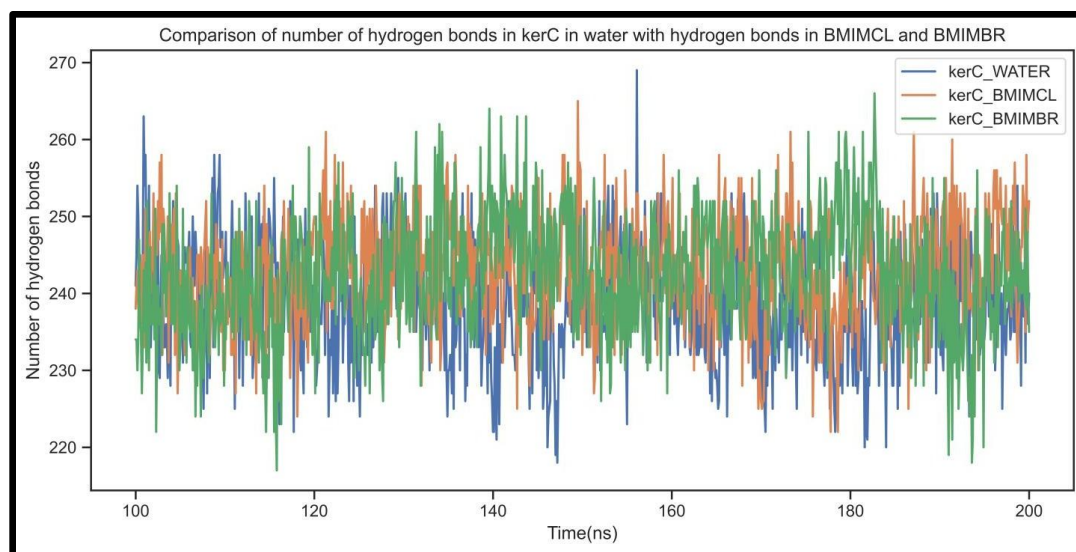


Figure 3.36 Intra-protein hydrogen bonds in kerC in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

3.6.2.4. Solvent Accessible Surface Area (SASA)

Figures 3.37, 3.38, and 3.39, presents the solvent accessible surface area (SASA) profiles of kerC simulated in water and six human hair-degrading ionic liquids. The time-dependent SASA plots reveal noticeable differences in protein–solvent interactions across the various environments. In water, kerC maintains an average SASA of approximately 154 nm². A pronounced increase is seen in TBAH and BMIMBR, with mean values near 158 nm² and 162 nm², respectively, indicating greater solvent exposure and potential surface relaxation, which may correspond to partial destabilization of the protein exterior. Conversely, CHOH and TMAOH exhibit moderate SASA values of about 156 nm², suggesting intermediate solvent interaction levels—higher than in water but less pronounced than in TBAH and BMIMBR. The lowest SASA is recorded for BMIMCL, averaging around 153 nm², implying additional surface compaction, while AMIMCL maintains a SASA of roughly 154 nm², consistent with a compact and stable structural state. These observations align well with RMSD trends, reinforcing that increased SASA generally reflects enhanced conformational flexibility, whereas reduced SASA denotes a more stable, tightly folded protein core.

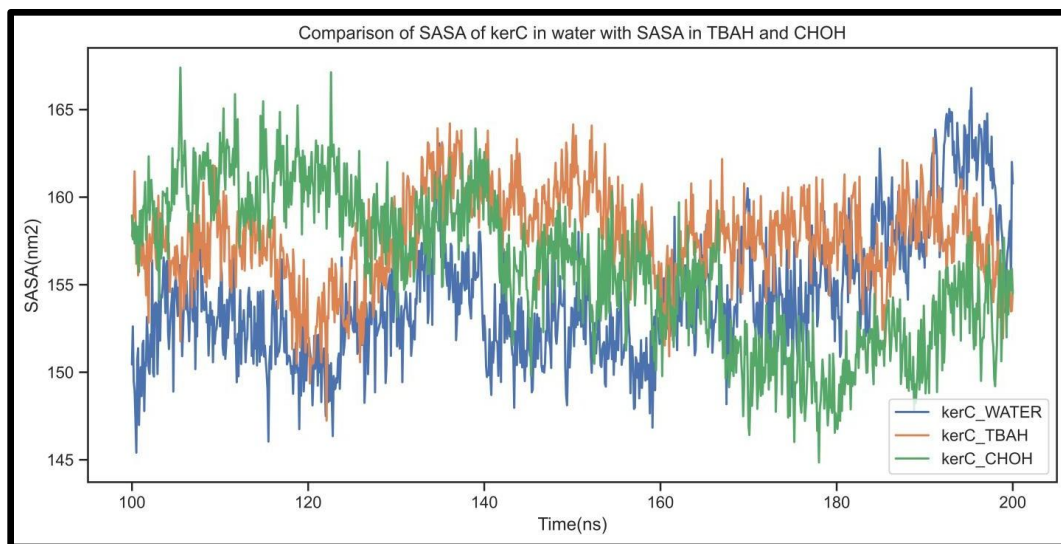


Figure 3.37 Solvent Accessible Surface Area (SASA) of kerC in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

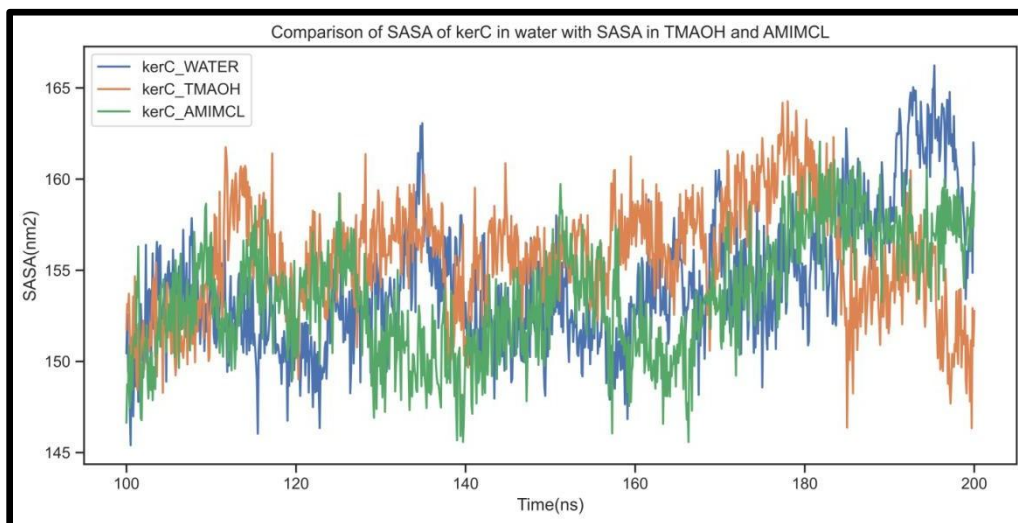


Figure 3.38 Solvent Accessible Surface Area (SASA) of kerC in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

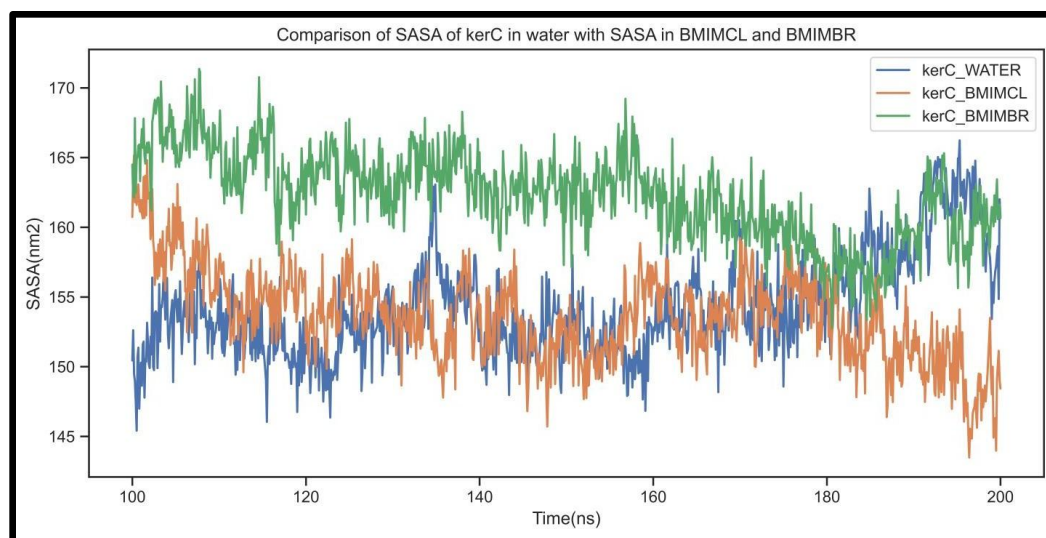


Figure 3.39 Solvent Accessible Surface Area (SASA) of kerC in water, BMIMCL and, BMIMBR . The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

3.6.2.5. Radius of gyration (Rg)

Figures 3.40, 3.41, and 3.42, illustrates the variation in the radius of gyration (Rg) for kerC across water and six ionic liquid systems, providing a comparative measure of the protein's compactness and stability during the 100–200 ns segment of the molecular dynamics simulations. The results reveal that kerC maintains a highly compact conformation in water, as evidenced by its low and stable average Rg of approximately 2.09 nm. In contrast, most ionic liquids lead to an evident expansion in the protein structure, indicating partial unfolding or reduced compactness. The most pronounced increases appear in BMIMBR and TBAH, with average Rg values near 2.49 nm and 2.42 nm, respectively, suggesting considerable destabilization under these solvent conditions. Notably, AMIMCL exhibits an Rg pattern closely comparable to that of water, with an average of about 2.06 nm, implying that this ionic liquid effectively preserves the structural integrity and compactness of kerC. Moderate structural expansion is observed in TMAOH, CHOH, BMIMCL, and BMIMBR, with mean Rg values around 2.21 nm, 2.27 nm, 2.21 nm, and 2.49 nm, respectively, pointing to increased structural flexibility. The relatively low Rg values in water and AMIMCL highlight their role in maintaining kerC's native folded state, while BMIMBR and TBAH present the least stabilizing environments, promoting considerable deviation from the protein's compact configuration.

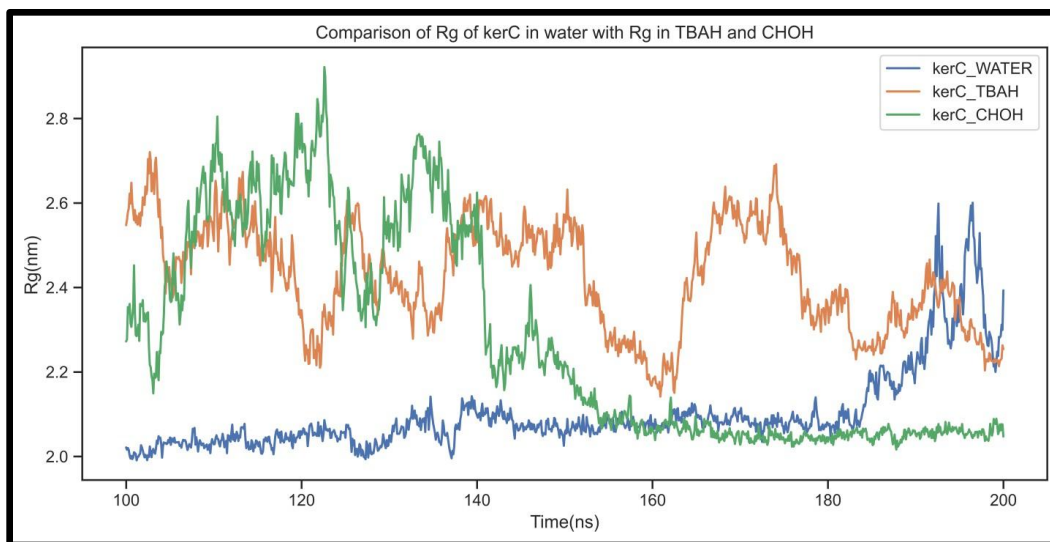


Figure 3.40 Radius of Gyration of kerC in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the Rg in nm.

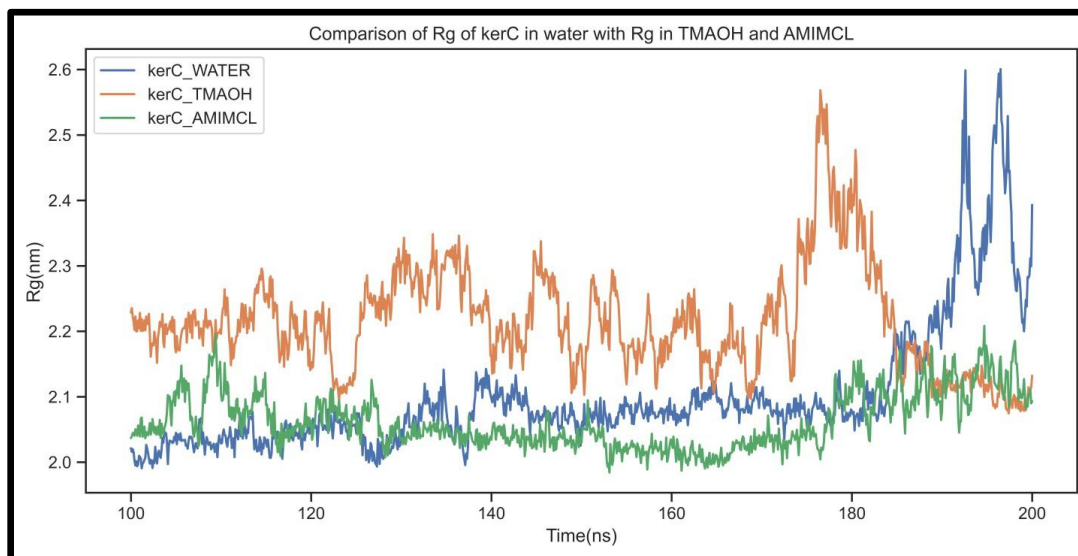


Figure 3.41 Radius of Gyration of kerC in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the Rg in nm.

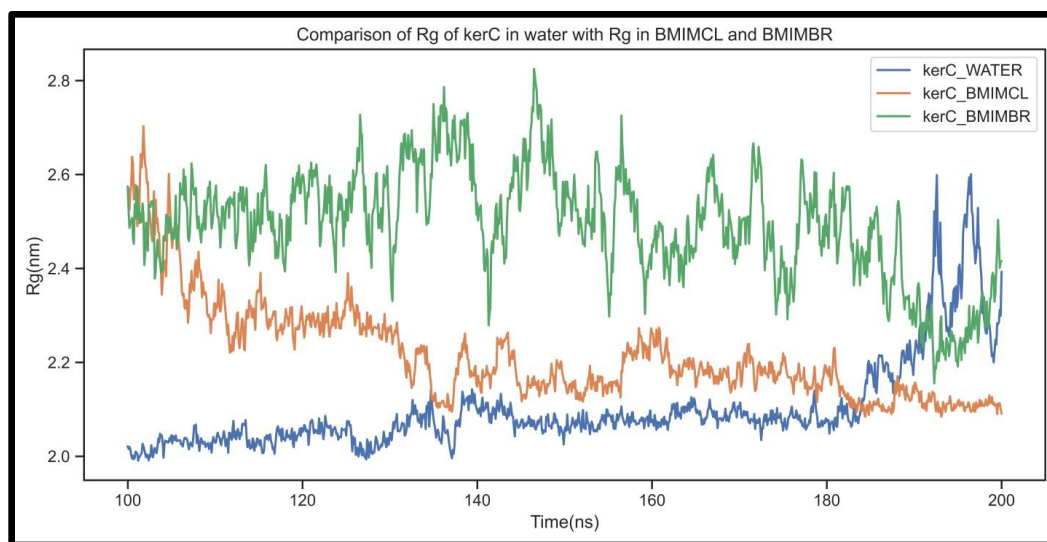


Figure 3.42 Radius of Gyration of kerC in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the Rg in nm.

3.6.3. kerF

3.6.3.1. RMSD

Figures 3.43, 3.44, and 3.45, presents the RMSD (root mean square deviation) trajectories of kerF over 200 ns molecular dynamics simulations performed in water and six ionic liquids: TBAH, TMAOH, CHOH, BMIMCL, BMIMBR, and AMIMCL. The data show that kerF achieves structural equilibration within the initial 50 ns across all solvent systems, although the extent and pattern of fluctuations vary depending on the environment. In water, the RMSD stabilizes rapidly and remains consistently low from 60 ns onward, with backbone deviations fluctuating between approximately 1.0 and 1.25 nm. This behavior signifies that kerF maintains strong structural stability and limited conformational variation in aqueous conditions. Among the ionic liquids, AMIMCL and BMIMBR exhibit RMSD trajectories most comparable to water, with both systems displaying steady profiles and final deviations indicating structural retention. BMIMBR, in particular, shows minimal variation in the 125–200 ns window, reflecting enhanced conformational stability, whereas AMIMCL demonstrates higher initial fluctuations that gradually diminish over time, pointing to progressive stabilization. Conversely, kerF experiences greater RMSD fluctuations and elevated deviation levels in BMIMCL, TBAH, TMAOH, and CHOH environments, suggestive of reduced stability and more extensive structural rearrangements.

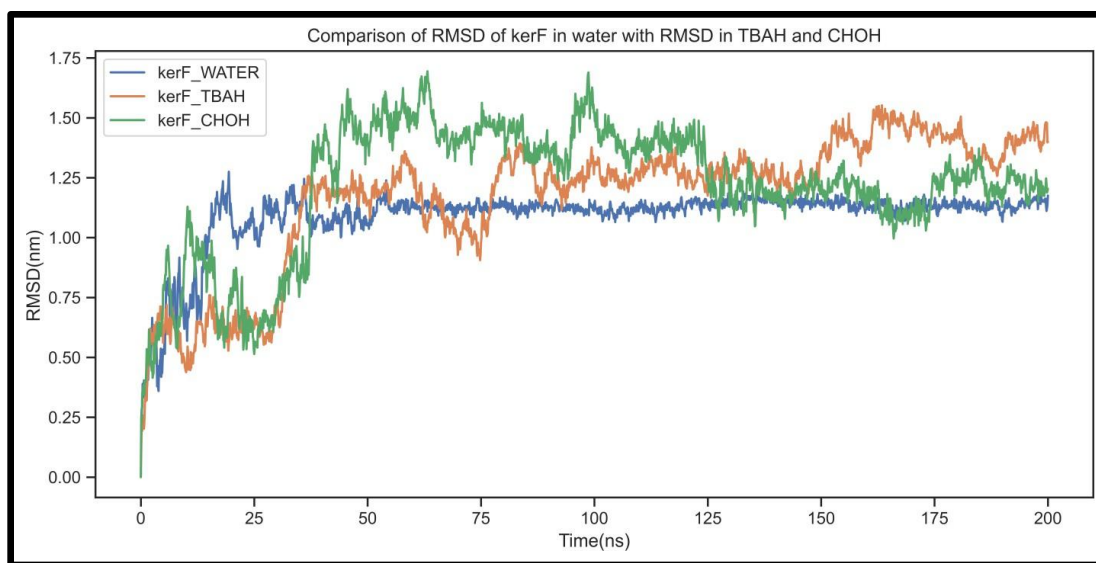


Figure 3.43 Root Mean Square Deviations (RMSD) of backbone of kerF in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

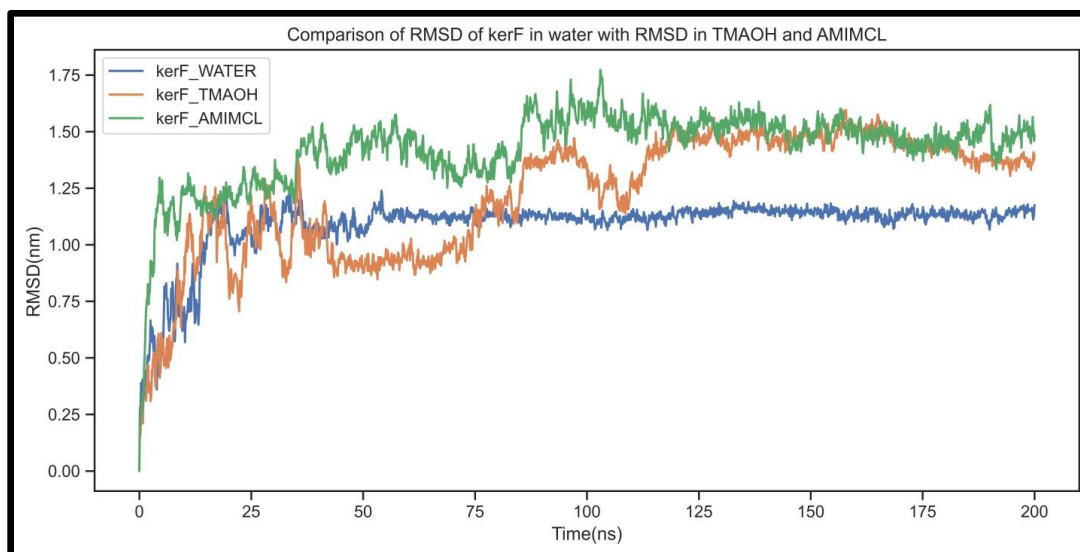


Figure 3.44 Root Mean Square Deviations (RMSD) of backbone of kerF in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

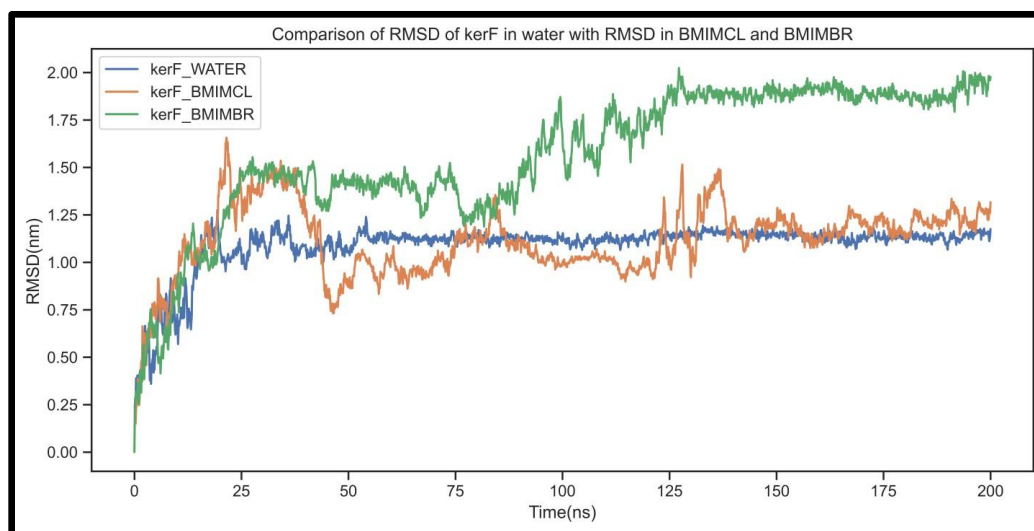


Figure 3.45 Root Mean Square Deviations (RMSD) of backbone of kerF in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSD in nm.

3.6.3.2. RMSF

Figures 3.46, 3.47, and 3.48, illustrates the root mean square fluctuation (RMSF) profiles of kerF, offering a detailed view of residue-specific flexibility across different solvent environments. This analysis provides key insights into how ionic liquids influence protein dynamics relevant to structural stability and functional behavior. The RMSF trends observed for kerF in water and the six ionic liquids reveal a highly environment-dependent modulation of residue motions. In aqueous conditions, distinct peaks appear in the N-terminal region (residues 1–100), where fluctuations often exceed

0.5 nm, indicating considerable mobility and structural disorder typical of looped or solvent-exposed segments. Among the ionic liquids, AMIMCL notably enhances flexibility in both the N-terminal and C-terminal regions, with RMSF maxima approaching 0.5 nm beyond residue 500, implying localized destabilization and increased solvent interaction. Conversely, TMAOH markedly suppresses mobility throughout most of the protein, yielding lower RMSF values across the structural core and secondary elements, though minor peaks persist in the initial residues, reflecting retained localized motion. Both BMIMCL and BMIMBR demonstrate considerable backbone stabilization relative to water, with BMIMBR showing the strongest suppression of fluctuations in terminal segments and an overall reduction in flexibility, suggesting a superior stabilizing role. TBAH and CHOH produce intermediate effects—TBAH notably decreases fluctuations in the first 100 residues by around 0.1–0.2 nm compared to water, while CHOH causes moderate dampening yet allows sporadic peaks within loop and terminal regions. Across all systems, residues forming α -helices and β -sheets exhibit low RMSF values (<0.2 nm), underscoring their intrinsic rigidity

regardless of solvent composition. In contrast, loop regions and flexible linkers, particularly near the termini, show solvent-sensitive behavior with their motions strongly modulated by the ionic environment. Overall, these results indicate that while kerF remains dynamically flexible in water, ionic liquids variably tune residue-level motions—either reinforcing structural rigidity or promoting local flexibility depending on their chemical characteristics.

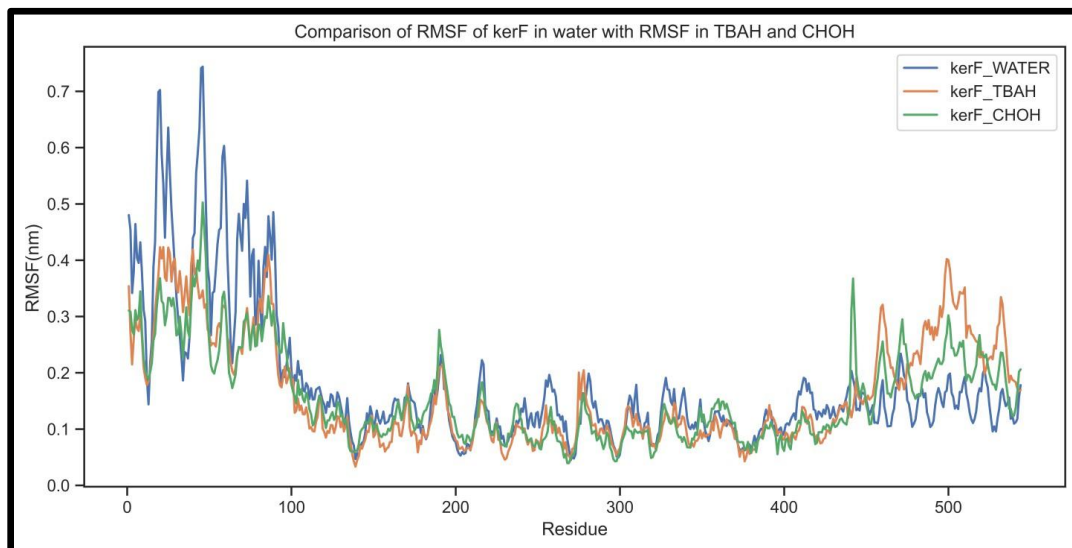


Figure 3.46 Root Mean Square Fluctuations (RMSF) of $C\alpha$ backbone atoms of kerF in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

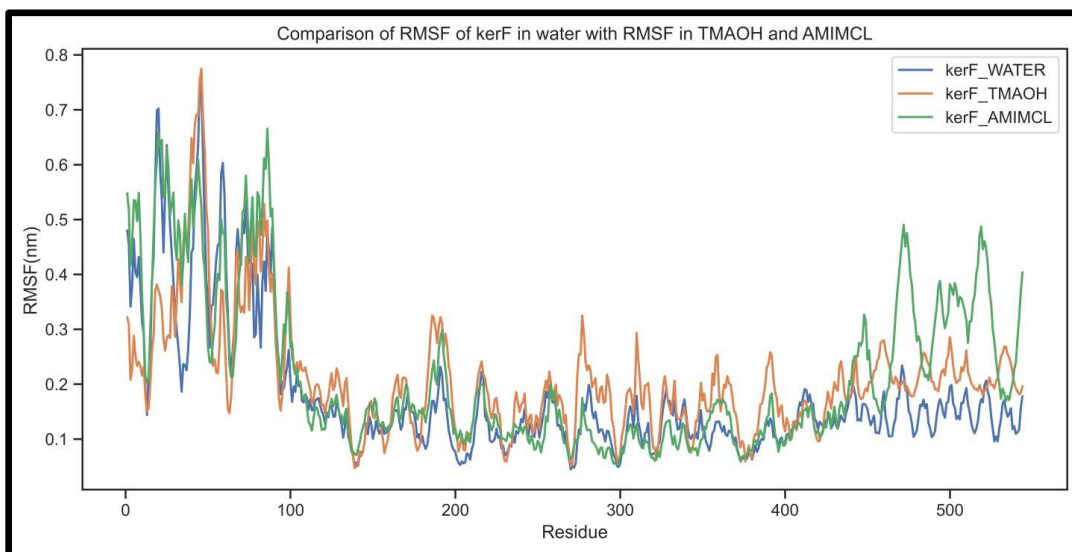


Figure 3.47 Root Mean Square Fluctuations (RMSF) of $C\alpha$ backbone atoms of kerF in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

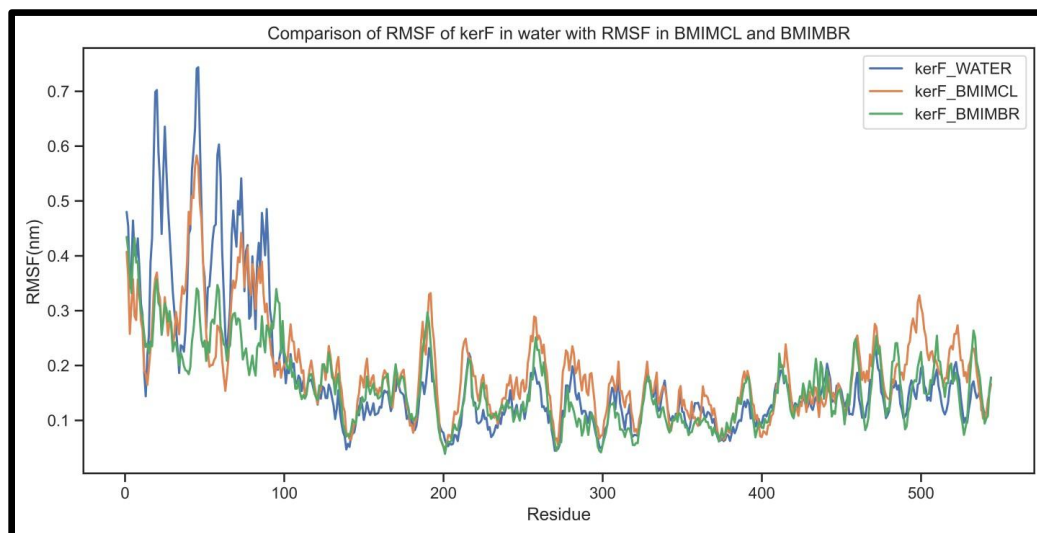


Figure 3.48 Root Mean Square Fluctuations (RMSF) of C α backbone atoms of kerF in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the RMSF in nm.

3.6.3.3. Hydrogen bonds

The intra-protein hydrogen bond analysis of kerF in water and six ionic liquid systems, shown in figures 3.49, 3.50, and 3.51 highlights the structural stabilization trends observed during the 100–200 ns segment of the molecular dynamics simulations. In aqueous conditions, kerF sustains a consistently high number of internal hydrogen bonds, averaging approximately 372, which serves as a baseline for structural stability. A similar pattern is noted in AMIMCL, where the mean value (~375) closely mirrors that of water, supporting prior RMSD and RMSF observations that identify AMIMCL as a highly stabilizing environment for kerF. Slight increases in hydrogen bond numbers are observed in TBAH and TMAOH, averaging around 379 and 374, respectively, indicating modestly enhanced backbone stabilization. In contrast, CHOH, BMIMCL, and BMIMBR exhibit reduced mean hydrogen bond counts of about 366, 363, and 359, respectively, suggesting minor weakening of intramolecular interactions. Notably, BMIMBR shows greater fluctuations in hydrogen bond numbers during the initial and mid-simulation periods but displays a progressive rise toward the final phase, consistent with the RMSD data that point to improved stabilization at later stages. Overall, these comparative profiles confirm that AMIMCL and BMIMBR provide favorable environments for maintaining kerF's internal hydrogen bonding network, thereby contributing to its structural persistence and potential catalytic efficiency.

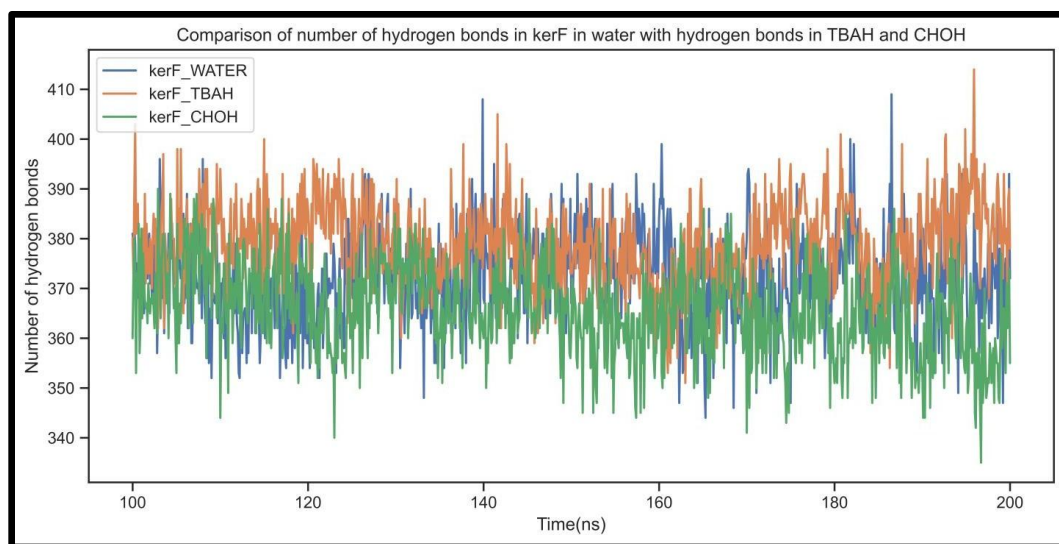


Figure 3.49 Intra-protein hydrogen bonds in kerF in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

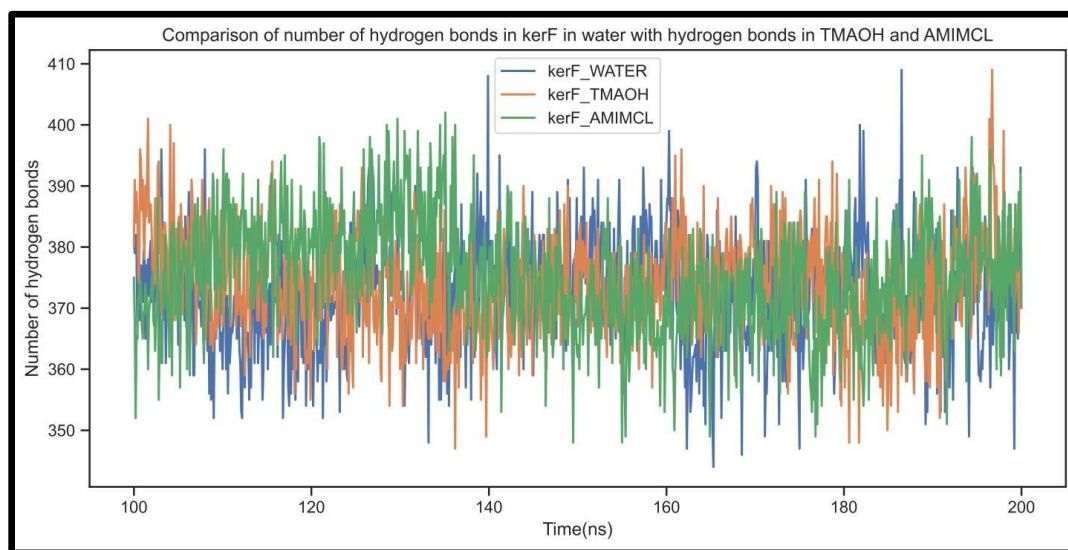


Figure 3.50 Intra-protein hydrogen bonds in kerF in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

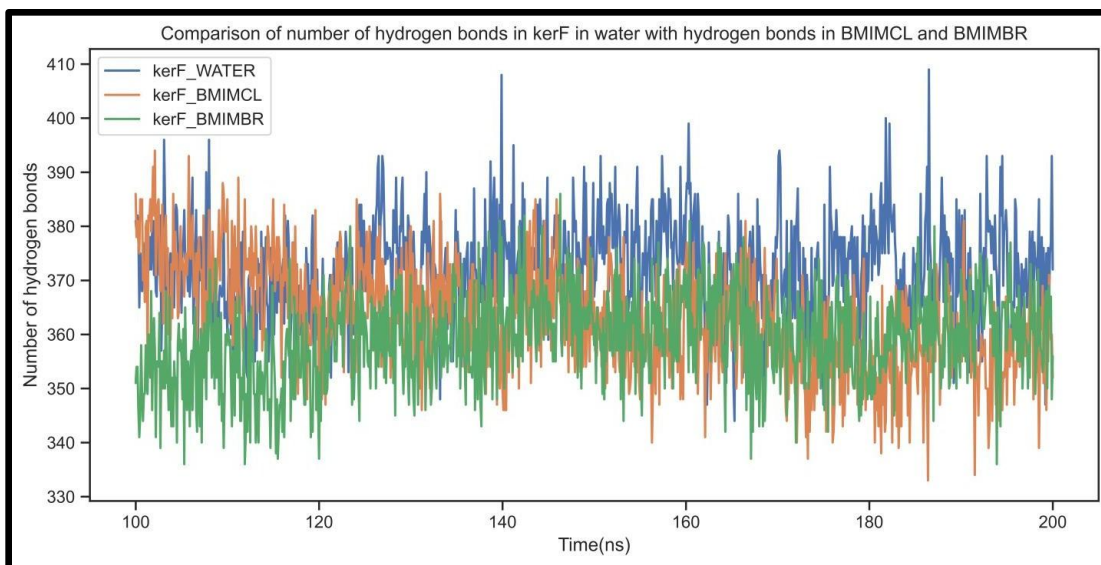


Figure 3.51 Intra-protein hydrogen bonds in kerF in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of intra-protein hydrogen bonds.

3.6.3.4. Solvent accessible surface area

The solvent accessible surface area (SASA) analysis of kerF in water and various ionic liquid environments, shown in figures 3.52, 3.53, and 3.54, illustrates the clear influence of solvent composition on the protein's conformational behavior. In water, kerF displays the lowest mean SASA value ($\sim 227 \text{ nm}^2$), reflecting a tightly packed and structurally stable conformation. By contrast, substantial increases are observed in TBAH ($\sim 236 \text{ nm}^2$) and BMIMBR ($\sim 246 \text{ nm}^2$), indicating enhanced solvent exposure and greater structural flexibility. Moderate elevations in SASA are also noted in AMIMCL ($\sim 234 \text{ nm}^2$) and BMIMCL ($\sim 240 \text{ nm}^2$), particularly during the later stages of the simulation, suggesting slight expansion and increased domain motion. Interestingly, TMAOH exhibits occasional SASA values lower than those in water, with an overall average of approximately 224 nm^2 , implying a more compact structural state. Collectively, these observations highlight that the solvent environment, particularly the type of ionic liquid, exerts a significant influence on the surface accessibility and conformational stability of kerF.

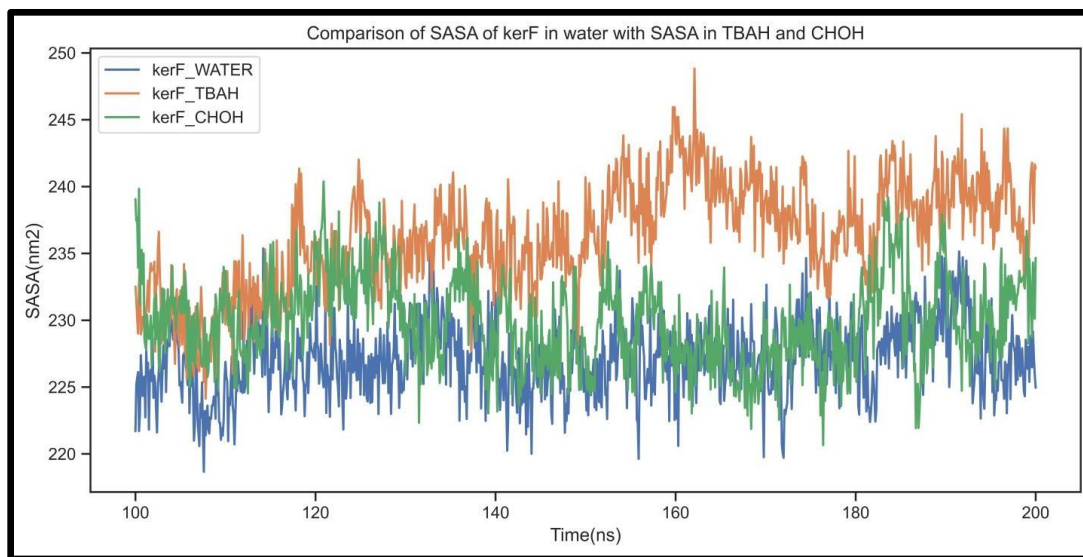


Figure 3.52 Solvent Accessible Surface Area (SASA) of kerF in water, TBAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

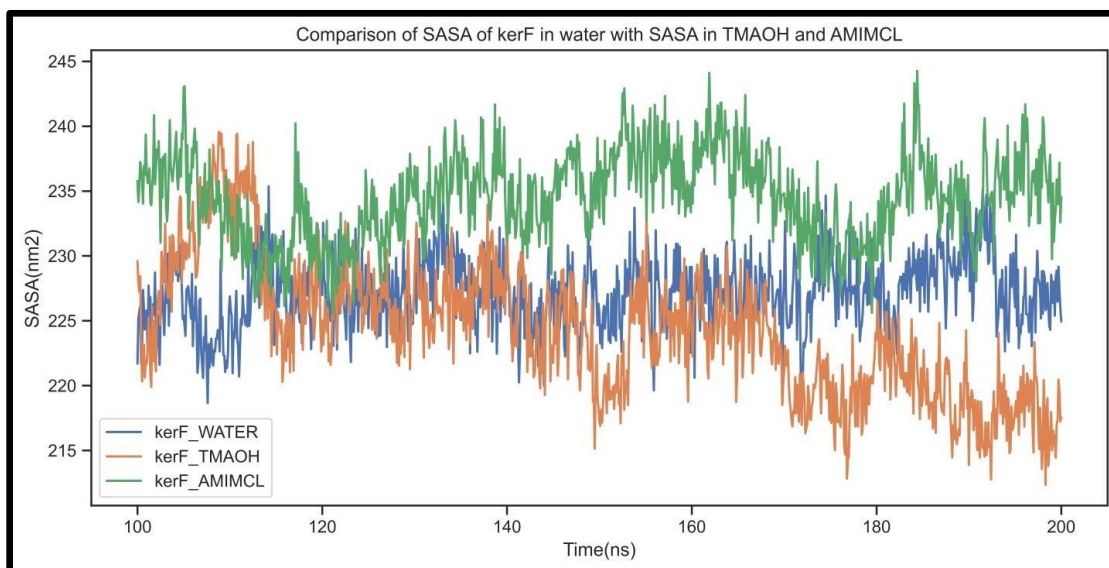


Figure 3.53 Solvent Accessible Surface Area (SASA) of kerF in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

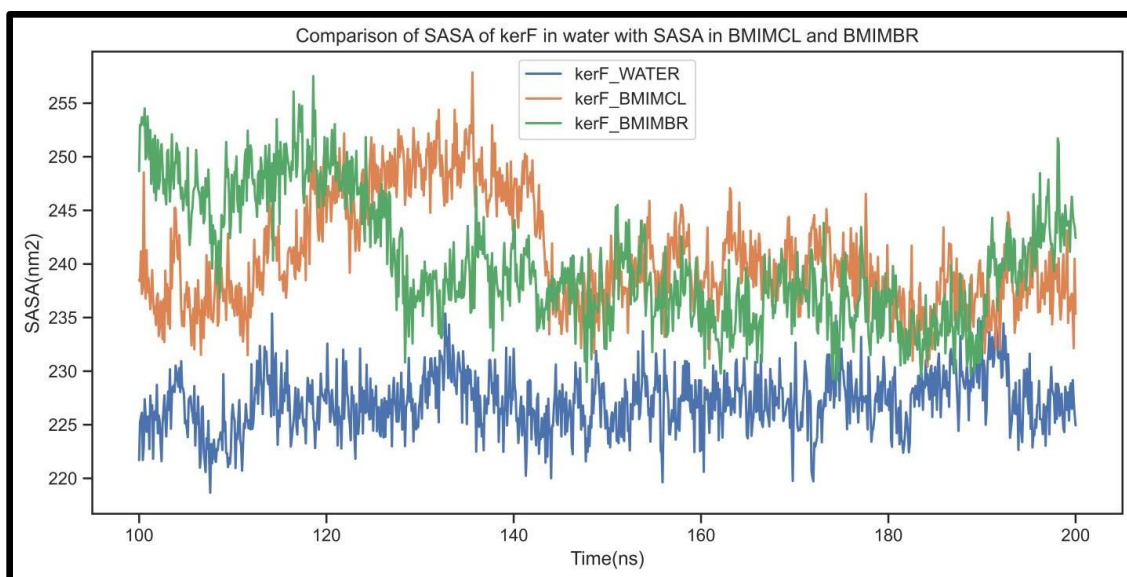


Figure 3.54 Solvent Accessible Surface Area (SASA) of kerF in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the number of SASA in nm².

3.6.3.5. Radius of gyration

Figures 3.55, 3.56, and 3.57, depicts the radius of gyration (Rg) analysis of kerF in water and six human hair-degrading ionic liquids, providing valuable information on the protein's compactness and structural stability during the 100–200 ns timeframe of molecular dynamics simulations. In water, kerF retains a compact and well-folded conformation, with Rg values remaining stable around 2.5 nm, indicative of structural robustness under aqueous conditions. In the presence of ionic liquids, however, notable alterations are observed in the Rg trajectory, reflecting the sensitivity of kerF's conformational dynamics to solvent effects. The mean Rg values for kerF in water, TBAH, CHOH, TMAOH, AMIMCL, BMIMCL, and BMIMBR are approximately 2.5, 2.6, 2.5, 2.5, 2.8, 2.6, and 2.6 nm, respectively. The slightly higher Rg recorded in AMIMCL suggests an adaptive structural expansion that may enhance functional flexibility within this environment. By contrast, lower Rg values across other systems signify tighter conformational packing, although their RMSD and RMSF patterns imply that such compactness may not correspond to improved stability or functional adaptability. Distinct behavior is observed in BMIMCL and BMIMBR: BMIMCL displays an early transient spike in Rg (up to ~3.01 nm), followed by gradual stabilization toward 2.6–2.7 nm in later stages, denoting an initial expansion phase succeeded by compaction. BMIMBR,

meanwhile, sustains moderately elevated R_g values (~ 2.7 nm) with less fluctuation, indicative of a more stable yet less compact conformational state.

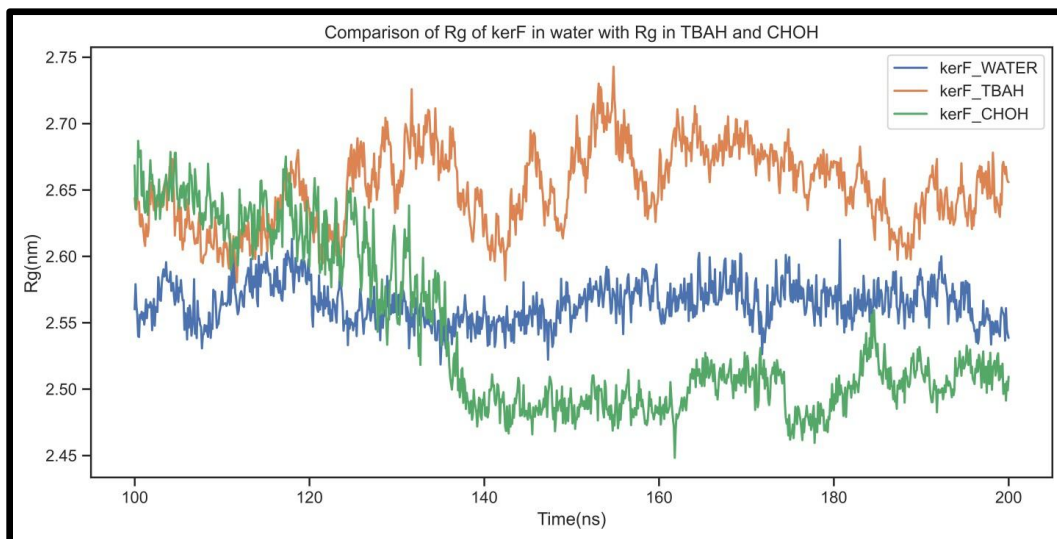


Figure 3.55 Radius of Gyration of kerF in water, THAH, and CHOH. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the R_g in nm.

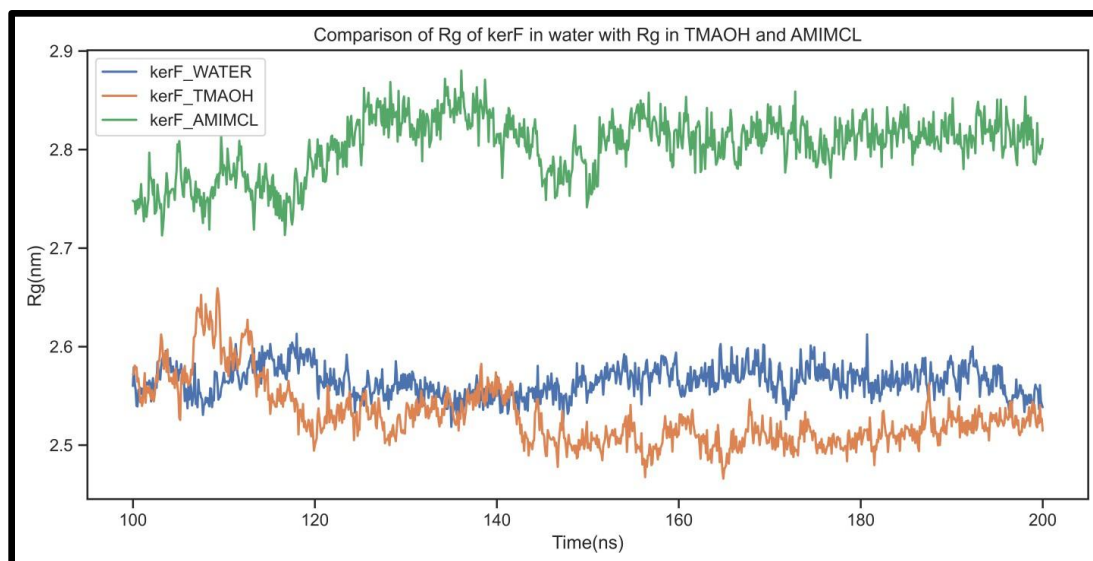


Figure 3.56 Radius of Gyration of kerF in water, TMAOH, and AMIMCL. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the R_g in nm.

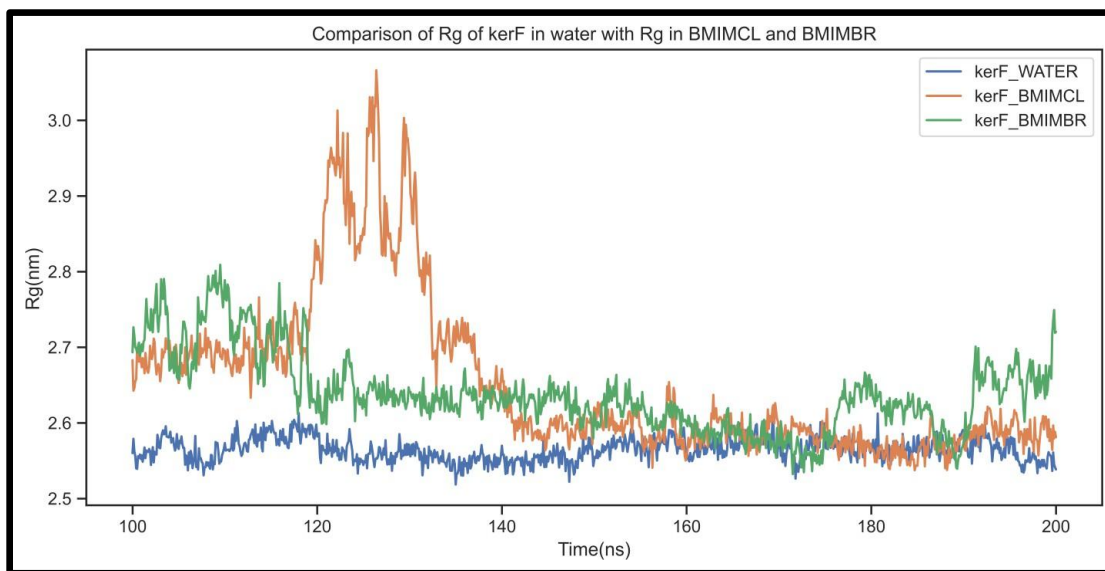


Figure 3.57 Radius of Gyration of kerF in water, BMIMCL, and BMIMBR. The x-axis denotes the duration of molecular dynamic simulations in nanoseconds and the y-axis presents the Rg in nm.

3.7. Interaction analysis

3.7.1. ker6

Table 3.9 Type and number of interactions observed between ker6 and different ionic liquid molecules using PLIP over the last 100 ns MD runs.

TYPES OF INTERACTIONS	IONIC LIQUIDS					
	BMIMBR	BMIMCL	CHOH	TBAH	AMIMCL	TMAOH
Hydrogen Bonds	0	0	6	0	0	0
Hydrophobic interactions	17	22	0	34	5	0
Salt bridges	0	0	0	0	0	0
π stacking	1	3	0	0	1	0

Table 3.9 illustrate the type and number of interactions observed between ker6 and the ionic liquid molecules used as solvent in each simulation system. TMAOH stands out with no detectable protein–solvent interactions. It appears to be the reason for ker6 to stay highly stable. Extensive hydrophobic interactions are observed for ker6 in TBAH

(34), BMIMCL (22), and BMIMBR (17), contributing to higher structural fluctuations and reduced stability.

3.7.2. kerC

Table 3.10 Type and number of interactions observed between kerC and different ionic liquid molecules using PLIP over the last 100 ns MD runs.

TYPES OF INTERACTIONS	IONIC LIQUIDS					
	BMIMBR	BMIMCL	CHOH	TBAH	AMIMCL	TMAOH
Hydrogen Bonds	21	20	22	23	20	19
Hydrophobic interactions	2	2	2	3	3	2
Salt bridges	0	0	0	0	0	0
π stacking	1	1	1	1	0	1

Table 3.10 shows that kerC forms numerous hydrogen bonds and several hydrophobic interactions in all ionic liquids, with occasional π stacking but no salt bridges. The interactions suggest robust interaction with TBAH showing the highest hydrogen bonding.

3.7.3. kerF

Table 3.11 Type and number of interactions observed between kerF and different ionic liquid molecules using PLIP over the last 100 ns MD runs.

TYPES OF INTERACTIONS	IONIC LIQUIDS					
	BMIMBR	BMIMCL	CHOH	TBAH	AMIMCL	TMAOH
Hydrogen Bonds	15	17	15	15	15	16
Hydrophobic interactions	1	2	2	0	2	3
Salt bridges	0	0	0	0	0	0
π stacking	0	0	0	0	0	0

Table 3.11 summarizes protein-ligand interactions detected by PLIP for kerF in various ionic liquids. Hydrogen bonds dominate across all ionic liquids, with minimal hydrophobic interactions and no salt bridges or π stacking observed. The results indicate that solvents are interacting with kerF, primarily via hydrogen bonding, regardless of the ionic liquid environment.

CHAPTER 4

CONCLUSION, FUTURE SCOPE AND SOCIAL IMPACT

4.1. Conclusion

This thesis presents an exhaustive study of the stability and potential of microbial keratinases in combination with ionic liquids (ILs) for the sustainable extraction of keratin from human hair waste. Using an integrated computational approach, three bacterial keratinases-ker6 (*Bacillus cereus*), kerC (*Bacillus subtilis*), and kerF (*Stenotrophomonas maltophilia*)-were structurally modeled using AlphaFold2 because of the lack of experimentally resolved structures. Subsequently, these enzyme models were validated and examined to detect their conformational integrity. Six different ionic liquids, all of which have previously been reported to dissolve human hair, have been selected to test their compatibility and stabilizing effect on the keratinases via molecular dynamics (MD) simulations under physiologically relevant conditions.

Detailed analysis of structural parameters such as RMSD, RMSF, Rg, SASA, and intraprotein hydrogen bonding showed that stability of keratinases is clearly dependent on the solvent system used. Of note, ker6 showed maximum stability in TMAOH, which was represented by minimal backbone fluctuation, higher hydrogen bonding, and structural compactness. kerC exhibited high stability in AMIMCl, while kerF maintained its structural integrity in both AMIMCl and BMIMBr. In contrast, most other ionic liquids promoted structural destabilization in these keratinases, indicating the importance of specific enzyme-solvent pairing.

The results highlight the potential of coupling green solvents with enzyme biocatalysis as a means to valorize recalcitrant keratinous wastes, particularly human hair, whose resistance to degradation and poor disposal habit presents a growing environmental concern. This approach not only ensures the efficient and selective recovery of high-value keratin biopolymers with functional and biomedical applications but also furthers the cause of circular bioeconomy principles. More importantly, the work introduces comparative approaches and underlines the importance of systematic enzyme-solvent matching in the context of enhancing protein stability, efficiency, and subsequent keratin extraction. The molecular understanding developed here represents a scientific basis on which future rational design of sustainable keratin extraction systems can be attempted.

This work cumulatively advances the understanding of protein dynamics in designer solvents, establishes a framework for integrated biocatalytic-IL extraction processes, and offers actionable perspectives for the sustainable management of human hair waste at both laboratory and industrial scales.

4.2. Future Scope

The future scope of this research on microbial keratinases and ionic liquids for sustainable human hair keratin extraction is broad and promising, with several key directions:

- **Enhanced Enzyme Discovery and Engineering:** Continued research is needed to discover and characterize new keratinases with improved activity, stability, and substrate specificity. Protein engineering and directed evolution methods can be applied to optimize keratinases for specific ionic liquid environments and industrial conditions, increasing their efficiency and robustness for keratin degradation.
- **Integrated Bioprocess Development:** The combined use of keratinases and ionic liquids represents a novel eco-friendly approach for keratinous waste valorization. Future work can focus on scaling up these integrated processes, optimizing reaction conditions, solvent recycling, and enzyme reuse. This will enable the development of sustainable industrial bioprocesses for high-yield keratin extraction and valuable by-product generation.
- **Exploration of Diverse Ionic Liquids and Solvents:** Expanding the range of tested ionic liquids and other green solvents will help identify the best solvent-enzyme pairs. Tailoring solvent properties to maximize enzyme stability while enhancing keratin dissolution can lead to designer solvent systems customized for different keratinases and feedstocks.
- **Broader Applications of Extracted Keratin:** The valorized keratin obtained through enzymatic-ionic liquid extraction can be further developed into biomaterials for biomedical applications such as wound healing, drug delivery, and tissue engineering, as well as biodegradable composites, fertilizers, and feed additives. Research can investigate functionalization and material processing of keratin for diverse uses.
- **Environmental and Economic Impact Studies:** Life cycle assessments and techno-economic analyses are needed to evaluate the environmental sustainability and economic viability of enzyme-ionic liquid extraction technology compared to conventional methods. This will support industrial adoption by demonstrating benefits in waste reduction, pollution mitigation, and circular bioeconomy integration.
- **Molecular Mechanism Insights and Simulation Advances:** Enhanced molecular dynamics simulations and experimental studies can elucidate keratinase-ionic liquid interactions at atomic detail. Understanding enzyme conformational dynamics in green solvents will aid rational design of more stable enzyme variants and improved solvent formulations for keratin extraction.
- **In conclusion,** this pioneering work establishes a scientific basis and opens multiple avenues for advancing microbial keratinase research, green solvent technology, and sustainable keratinous waste bioconversion, thereby contributing to innovative environmental biotechnology solutions and circular resource management.

4.3 Social Impact

The work presented in this thesis addresses an increasingly visible but often neglected solid-waste problem, which is related to the accumulation of keratinaceous residues, especially human hair, in municipal and industrial streams. By focusing on human hair waste as a valuable feedstock rather than as an intractable pollutant, the study helps in alleviating the problems of blocked drainage systems, unsanitary waste dumps, unpleasant odour, and the increasing amount of hair dust that aggravates respiratory conditions in densely populated urban and peri-urban areas. In this regard, the research helps support cleaner cities, improved public hygiene, and lessened health risks for both workers in grooming-related occupations and communities living near the informal disposal sites.

At the environmental level, the development of enzyme–ionic liquid–based strategies for human hair keratin extraction offers an alternative to conventional waste management routes such as landfilling or open burning that have a pronounced impact on greenhouse gas emissions, toxic fumes, and long-term soil and water contamination. Replacing aggressive chemical extraction processes with greener combinations of microbial keratinases and carefully selected ionic liquids reduces the use of aggressive reagents and minimizes secondary effluents while envisaging better resource recovery from a renewable protein-rich waste stream in full agreement with the dictates of green chemistry and circular bioeconomy, where persistent biowastes are upcycled into high-value products and not regarded as liabilities.

Socioeconomically, the concept of valorizing human hair waste into functional keratin-based biomaterials opens avenues for new local enterprises and value chains, particularly in countries like India, where hair collection and trade are already significant. Environmentally benign keratin extraction pipelines, informed by the stability data generated for ker6, kerC, and kerF in different ionic liquids, can underpin small- to medium-scale units that convert barbershop and salon hair into keratin for applications in wound dressings, scaffolds, cosmetic formulations, and agriculture. Such endeavors could provide gainful employment to waste collectors, processors, and small industries while concurrently relieving the burden on municipal waste management infrastructures.

Keratin derived from human hair is particularly attractive in the biomedical and personal-care sectors due to its origin from humans, biocompatibility, and low immunogenicity. Advancing toward conditions that are greener and safer in extraction enhances social acceptability for keratin-based products applied in wound healing, tissue engineering, drug delivery, and skin or hair-care formulations. This will translate to cheaper, locally sourced biomaterials supporting public health, especially in low- and middle-income settings where importation of biopolymer-based products might be unaffordable. The

thesis thus contributes foundational knowledge enabling ethically and environmentally responsible production of keratin-derived materials with direct societal benefits.

The work also has implications for sustainable agriculture and environmental remediation, since keratin hydrolysates and keratin-based formulations could be developed as slow-release nitrogen sources, soil conditioners, or components of biofertilizers. By enabling controlled, eco-friendly extraction of keratin, this work supports pathways through which human hair waste is diverted from landfills to applications that restore soil health, reduce reliance on synthetic agrochemicals, and improve nutrient cycling. This strengthens linkages between urban waste streams and rural productivity, contributing to the broader goals of food security and environmental stewardship. At the policy and awareness level, the thesis underscores that advanced computational and molecular methods, such as AlphaFold2-based modeling and molecular dynamics simulations, are not ivory tower exercises but can underpin concrete, socially relevant interventions in waste management and green technology design. By showing how protein stability data in ionic liquids guide practical solvent–enzyme choices for keratin extraction, the work will help bridge the gap between theoretically oriented research and implementable solutions. In this way, this work should be able to stimulate stakeholders at municipal authorities, regulators, and industry to support research-driven sustainable technologies for managing keratin-rich wastes and contribute to a better-informed, science-supported discourse on environmental protection and resource recovery.

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LIST OF PUBLICATIONS

- P. Rai and Y. Hasija, “Ionic liquids for stability of ker6, a potential keratinase for human hair keratin extraction,” *J. Mol. Liq.*, vol. 415, p. 126365, Dec. 2024, <https://doi.org/10.1016/j.molliq.2024.126365>.
- Rai, P., & Hasija, Y. (2025). Structural stability of two promising keratinases in human hair degrading ionic liquids: paving the way for more efficient and sustainable keratin extraction. *Journal of Biomolecular Structure and Dynamics*, 1–18. <https://doi.org/10.1080/07391102.2025.2586043>

CONFERENCE PRESENTATIONS

- Priya Rai and Yasha Hasiha, “ Computational analysis of degradation of keratin derived from keratinaceous wastes” presented at “International conference on Recent Advanves in Biotechnology, (icRAB-2022)”.
- Priya Rai and Yasha Hasija, “Exploring keratinases for efficient keratin recovery” presented at “International Conference on Multidisciplinary Innovation in Academic Research, (MIAR-2025)”.
- Priya Rai and Yasha Hasija, “ Molecular dynamics simulations based stability Assessment of keratinases in ionic liquids for sustainable keratin extraction” presented at “Indian conference on Bioinformatics”.



Ionic liquids for stability of ker6, a potential keratinase for human hair keratin extraction

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ABSTRACT

In this study, the stability of ker6, a keratinase produced by *Bacillus cereus*, was investigated in water and six distinct ionic liquids through molecular dynamic simulations. The analysis of ker6 stability involved occupations of root mean square deviation (RMSD), radius of gyration (Rg), solvent accessible surface area (SASA), root mean square fluctuation (RMSF), and intra-protein hydrogen bonds. Notably, the simulations revealed reduced fluctuations in RMSD and RMSF, an increased number of intra-protein hydrogen bonds and lower values of Rg and SASA in Tetramethylammonium hydroxide (TMAOH) simulation system all pointing towards the stability of ker6. Our computational analysis identified TMAOH as the most stable environment for ker6.

Ker6 is a keratinase recognized from literature as a potential candidate for the degradation of human hair keratin. We have modeled its structure using AlphaFold2. The six ionic liquids taken here for simulation are selected based on their documented efficiency in human hair degradation. This study aligns with the growing interest in identifying ionic liquids that enhance protein stability, particularly in the context of extracting valuable biomaterials like human hair keratin in an environmental sustainable way. The findings suggest that TMAOH could serve as a promising solvent for keratin extraction from human hair when utilizing ker6, thereby making the extraction process more environmentally friendly.

1. Introduction

Ionic liquids (ILs) are liquid salts having many fascinating properties that prompt numerous researchers to explore their utilization in various fields [1]. Melting points of ionic liquids are generally lower than 100 °C. They are often referred to as "solvents of the future" because of the diverse areas of applications, particularly as alternatives for conventional organic solvents in industrial processes [2]. According to estimates, there are over a million separate combinations of cations and anions, each of which results in a switch in the chemical and physical properties of the corresponding ILs, resulting in ionic liquids being called "designer solvents" [3]. Under normal conditions, they usually exhibit negligible vapour pressure, i.e., are non-volatile, and are generally non-flammable, leading them to be identified as "green solvents" [4,5]. Advantages such as the low vapour pressure at room temperature and superior thermal stability of ILs relative to traditional solvents make them nearly ideal solvents for extraction procedures [6]. Studies have shown the ability of ionic liquids to be superior to water and various other traditional solvents in stabilizing or destabilizing the

three-dimensional structure of DNA or proteins [7]. The combination of various interactions of ionic liquids, such as electrostatic, hydrogen bonding, van der Waals, and π -interactions, is favorable for the dissolution of highly recalcitrant materials because both covalent and non-covalent interactions in these materials are easily broken, with associated stabilization in solution form [8].

Keratinases (EC 3.4.21.24/30) are a category of hydrolytic enzymes capable of catalyzing keratin degradation [9,10]. They are usually serine- and metalloproteases. When compared to other biopolymers such as cellulose, collagen, and starch, the extraction of keratin is a very challenging process because of its complex structure, making it recalcitrant to general proteases. Notwithstanding some studies in the field of keratinase structural as well as biochemical characterization, and isolating keratinase-producing microorganisms, more research is required in this area to increase the application of keratinase in various fields. This can be done by deciphering their mechanism of action, pointing out and recognizing more keratinases, and applying protein engineering to increase the activity of the enzymes. Diverse physical and chemical strategies to treat keratinous wastes are present, but they are

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Structural stability of two promising keratinases in human hair degrading ionic liquids: paving the way for more efficient and sustainable keratin extraction

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Abstract

Keratin derived from human hair has garnered significant attention for its biomedical applications. While ionic liquids (ILs) and keratinases independently facilitate hair hydrolysis, their synergistic use remains largely unexplored. Here, we computationally investigate the possibility of combining the use of ionic liquids and keratinases. We present here the structure of kerF and kerC, two keratinases identified from literature as promising candidates for human hair degradation, predicted using AlphaFold2. Subsequently, we performed molecular dynamics simulations to investigate the stability of these keratinases in water and six ionic liquids, chosen based on their recorded human hair degradation efficiency. The stability analysis was conducted using key metrics such as root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (R_g), solvent accessible surface area (SASA), and number of intra-protein hydrogen bonds. Computational data indicate that kerF exhibits stability in both water and two ionic liquids: 1-Allyl-3-methylimidazolium chloride [AMIM]Cl, and 1-butyl-3-methylimidazolium bromide [BMIM]Br. kerC remains stable in [AMIM]Cl. The results suggest the potential utility of these two keratinases in environmentally friendly keratin extraction processes from human hair waste. This study aims to identify suitable keratinases and advances the understanding of their stability in non-conventional ionic liquid solvent systems, supporting the development of sustainable, eco-friendly strategies for the extraction of valuable human hair keratin.

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PROFILE

I am a passionate and results-driven computational biologist with a Ph.D. in Biotechnology (2019–2025). My research focuses on evaluating the stability of various keratinases in different ionic liquids for sustainable keratin recovery from human hair. I have a strong foundation in computational and molecular biology. I have published researches in high-impact, peer-reviewed journals. I bring extensive experience in bioinformatics.

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Publications

Category	Title	Journal/Publisher	Year
Research Articles	Ionic liquids for stability of ker6, a potential keratinase for human hair keratin extraction	Journal of Molecular Liquids	2024
	Structural stability of two promising keratinases in human hair degrading ionic liquids: paving the way for more efficient and sustainable keratin extraction	Journal of Biomolecular Structure and Dynamics	2025