ISOLATION AND CHARACTERIZATION OF A NOVEL PROTEASE POSSESSING ANTI-DIARRHEAL PROPERTIES FROM *BACILLUS CLAUSII* STRAIN UBBC07

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY
in

Biochemistry

By

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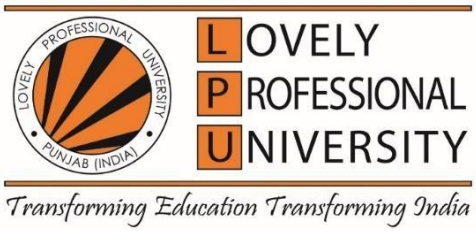
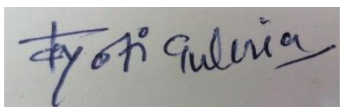


LOVELY PROFESSIONAL UNIVERSITY, PUNJAB
2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled "Isolation and characterization of a novel protease possessing anti-diarrheal properties from *Bacillus clausii* strain UBBC07" in fulfillment of my degree of **Doctor of Philosophy (Ph.D.)** is the outcome of research work carried out by me under the supervision **Dr. Minhaj Ahmad Khan**, working as **Professor,** in the **Department of Biochemistry/ School of Bioengineering and Biosciences,** of Lovely Professional University, Punjab, India. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made whenever the work described here has been based on the findings of other investigators. This work has not been submitted in part or in full to any other University or Institute for the award of any degree.



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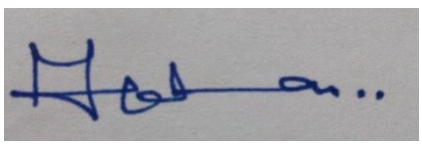
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Isolation and characterization of a novel protease possessing anti-diarrheal properties from *Bacillus clausii* strain UBBC07" submitted in fulfillment of the requirement for the reward of the degree of **Doctor of Philosophy (Ph.D.)** in the **Department of Biochemistry/ School of Bioengineering and Biosciences**, is a research work carried out by Jyoti Guleria, Registration No. 42100314, is a bonafide record of her original work carried out under my supervision and that no part of the thesis has been submitted for any other degree, diploma or equivalent course.



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ABSTRACT

Continuous exploitation of antibiotics has put pressure on the healthcare industry to develop new drugs with the narrow-inhibitory spectrum and selective toxicity to conquer present resistant pathogenic strains. Bacteria and some viruses cause diarrhea and are more common in children and infants in poor countries. Imprudent usage of medicines is associated with developing resistance, unwanted costs, and disease prevalence among the masses **(Bruzzese et al., 2018).** Researchers have reported bacteria, viruses, and some protozoa to be the causative agent of gastroenteritis, including *Clostridium difficile*, *Bacillus cereus*, enterotoxigenic *Escherichia coli* (ETEC), *Shigella, Salmonella,* norovirus, rotavirus, etc. Every year diarrhea is listed among the top diseases which accounts for the maximum fatalities all around the world. If not treated in time the loss of fluids during diarrhea can be fatal, especially among children below five **(Hartman et al., 2023)**. The disease isn't just the result of a pathogenic infection but different types of diarrhea can be induced, when some other disease is infecting the host (autoimmune, HIV, etc.). A major problem associated with diarrhea treatment is the diversity of pathogens which varies according to geographical regions. Physicians usually discourage patients from using antibiotics continuously as it may cause the diseases to become resistant to the therapy. Antimicrobial peptides can be a solution to conventional antibiotics because bacterial peptides are less likely to become resistant to pathogens due to their unique mode of action **(Solanki et al., 2021).** Strains of Genus *Bacillus* are categorized under probiotics and are used in the food and healthcare sectors. One of the important bacteria of the genus *Bacillus* is *Bacillus clausii* which is reported to produce antimicrobial peptides, extracellular proteins, and proteases whose application is reported to counteract diarrhea pathogens and their toxins **(Bueno et al., 2022)**. Proteases themselves have limitations including protease degradability, host toxicity, and loss of activity in harsh pH environments emphasizing a need to create a stable medicine out of the proteases. So far in-depth research gap in isolating unique proteases from *Bacillus clausii* provides a need to explore the species more for stable healthcare solutions. This requires extensive study on protease synthesis techniques, and further analysis of proteases' efficacy in eukaryotic/ animal models **(Lakshmi et**

al., 2017). Considering the present restraints in drug design for enteric infections/ diarrhea, the objectives of this study were designed.

As a result of systematic initial screening and literature analysis, commercially available Gram-positive *Bacillus clausii* strain UBBC07 was selected for protease isolation. The strain was optimized for the synthesis of extracellular antimicrobial compounds, and antimicrobial activity was tested against several diarrhea pathogens. The production of antimicrobial compounds started after 80 hours of incubation and reached its peak at 96 hours of incubation (12 mm zone of clearance), the activity started declining after 110 hours of incubation. The objectives were focused on the isolation and identification of newly isolated protease, physicochemical characterization, and MIC estimation using *Bacillus cereus* and *Salmonella enterica.*

The sequence of the newly isolated protease was identified by MALDI-TOFF mass spectroscopy. The molecular mass and the sequence were identified as a hydrolytic metalloprotease of the Dinb protein family. Physicochemical analysis at different pH showed the protein to be a neutral protease. The protease exhibited strong bactericidal activity against *Bacillus cereus* and *Salmonella enterica.*

In vivo studies also explored antimicrobial aspects of purified protease in *Drosophila melanogaster*. *In vivo* assay including the survival assays and enzymatic assays supported the *in vitro* analysis, confirming the bactericidal properties of protease.

Further, genetic engineering can help design a more stable molecule for drug formulations.

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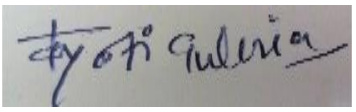

Jyoti Guleria

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LIST of ABBREVIATIONS

AMP	Antimicrobial Peptide
ACN	Acetonitrile
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
CLSI	Clinical and Laboratory Standards Institute
CADD	Computer-Aided Drug Design
CDNB	1-Chloro-2,4-Dinitrobenzene
CFU	Colony Forming Unit
DinB family	DNA damage-inducible protein
FESEM	Field Emission Scanning Electron Microscopy
GRAS	Generally Recognized as Safe
GSH	reduced glutathione
GST	Glutathione S-Transferase
GUI	Graphical User Interphase
LC/MS	Liquid Chromatography/ Mass Spectrometry
MALDI-TOFF	Matrix-Assisted Laser Desorption Ionization–Time ofFlight
MD	Molecular Dynamics
MDR	Multidrug Resistance
MH broth	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration

PBS	Phosphate Buffer Saline
PIPER	FFT-Based Docking with Pairwise Potentials
PyMOL	Python Molecular
QSAR	Quantitative Structure-Activity Relationship
RMSD	Root Mean Square Fluctuation
RMSD	Root-Mean-Square Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide GelElectrophoresis
SEM	Mean Standard Error
TcdB	*Clostridium difficile* toxin B
TFA	Trifluoroacetic Acid

Chapter 1

Introduction & Review of Literature

1.1 INTRODUCTION

1.1.1 Acute Gastroenteritis

Acute gastroenteritis (AGE), can be defined as the onset of diarrhea with vomiting, fever, and/or abdominal pain **(Bueno et al., 2022)**. Diarrhea can be cured with proper medication yet considered an important infection contributing to child mortality all around the world. According to the World Health Organization (WHO), up to 5 billion diarrhea cases are reported yearly **(Hartman et al., 2023)**, **(Motamedi et al., 2021)**. Diarrhea pathogens infect organisms by consuming unhygienic food and water, but this is one type of diarrhea associated with specific diarrhea pathogens. Gastroenteritis can be induced as a result of other disorders. Gut disorders can result due to different pathophysiological mechanisms, including gut epithelial abnormalities, inflammation, malabsorption and electrolyte imbalance, drug side effects, postoperative disorders, and gut-brain disorders. Diseases that affect the stomach are collectively called enteropathies **(Pecoraro et al., 2017).** Autoimmune diseases like Crohn's disease and celiac disease cause problems in the intestine. Hypertension drug like Olmesartan is reported to be associated with atrophic enteropathy, and nonsteroidal drugs (NSAID) cause ulcerous enteritis. Patients undergoing chemotherapy or being exposed to radiotherapy always develop intestinal disorders **(Gómez-Escudero et al., 2021).** Diseases like tuberculosis, human immunodeficiency virus, and *Helicobacter pyroli* infection also cause diarrhea. Food allergens, lymphangiectasis, malnutrition, and idiopathic sprue also contribute to the onset of diarrhea. Acute gastroenteritis (AGE), alters the normal microbiota composition of the gut, developing a state of dysbiosis. To restore the microbial balance of the gut, antibiotics are given **(Urdaci et al., 2018).** Continuous use of medication is not recommended by doctors, as most of the treatments for diarrhea are antibiotics-based medicines which increases the possibility of the creation of resistant species. Another important issue that helps the disease to persist is the cost of these treatments, which not all can afford.

One of the popular alternative medicines against diarrhea is the spore suspension of *Bacillus clausii* **(Lakshmi et al., 2017)**. *Bacillus clausii* strains in these suspensions may vary from country to country. It is sold as an over-the-counter

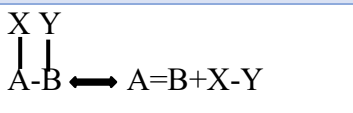
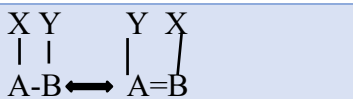
medicine. The *Bacillus clausii* is a Gram-positive, spore-forming, aerobic bacterium belonging to the genus *Bacillus*. Based on the outcomes from the use of *B. clausii* spore suspension has become one of the most promising bacteria in diarrhea control, which has been popularised in the past two decades. Acute gastroenteritis is known to alter the normal microflora of the gut, creating a state of gut dysbiosis, here the application of the bacterial spore suspension is considered to restore the microbial balance in the gut and counteract the pathogenic bacteria. The bacteria have been studied for their immunomodulatory properties and antibiotic resistance. Serine protease from *Bacillus clausii* has shown bacteriocin properties **(Ripert et al., 2016)**. With the advancement in pharmaceutical science, researchers are looking for new methods of drug delivery. Finding protease-based hydrolytic proteins is one of the novel areas of research in healthcare.

1.1.2 Enzymes

Enzymes are the bioactive molecules that work to catalyze the reactions in all living organisms. These complex protein molecules have been utilized commercially to attain the same benefits as they provide inside living bodies. The discovery of enzymes dates back to mid mid-nineteenth century when Louis Pasteur proposed the concept of fermentation happening in yeast due to the presence of some molecules **(Bordenave, 2003)**. Enzymes can be of proteinaceous or non-proteinaceous nature. Their main purpose of existence is to catalyze a reaction on the cellular level.

Today enzymes are majorly explored for their commercial use in various industries including, healthcare, the food industry, leather, detergent, textile, and agriculture **(Chapman et al., 2018)**. The science of studying enzymes for their usefulness is called enzyme technology. The field majorly deals with the isolation, purification, analysis, and characterization of enzymes that can have future commercial values. All enzymes are classified by the International Union of Biochemists (IUB) under six main categories, and this classification is given according to the reaction they catalyze **(Table 1)**.

Table 1. IUB categorization of enzymes according to their reaction-catalyzed

IUB Number	Enzyme Class	Type	Reaction Catalysed
1	Oxidoreductases	$A^- + B \longleftrightarrow A + B^-$	Oxidation- reduction
2	Transferases	$A\text{-}B + C \longleftrightarrow A + B\text{-}C$	Group transfer
3	Hydrolases	$A\text{-}B + H_2O \longleftrightarrow A\text{-}H + B\text{-}OH$	Hydrolysis
4	Lyases		Elimination of one group by the cleavage of a bond or the addition of a group to the double bond
5	Isomerases		Reaction involving isomerization
6	Synthases (Ligases)	$A\text{-}B + C \longleftrightarrow A + B\text{-}C$	Reaction joining together two molecules coupled with hydrolysis of high energy phosphate bonds

This chapter outlines the historical overview of the enzyme's origin, its general nature, classification, and applications. Particularly emphasis is placed on the classification of the bacterial protease. In addition, the production and purification of hydrolytic proteases and the determination of their antimicrobial activity are also illustrated in the following section. The utilization and usage of *Bacillus spp*. and its antimicrobial compounds in various fields are also described here. The chapter also addresses the use of probiotic *Bacillus sp*. and its safety issues. The objectives of the research are discussed at the end of this Chapter.

1.2 REVIEW OF LITERATURE

1.2.1 Drug Discovery in Diarrhea

Diarrhea is a serious public health problem. A range of pathogens from bacterial to viral origin are reportedly involved in the onset of the disease. Major causal pathogens include norovirus, astrovirus, rotavirus, *Shigella, Giardia, Enterococcus, Staphylococcus, Clostridium difficile,* etc. **(Motamedi et al., 2021)**. The mechanism of infection varies according to the causal organism. It can be classified as acute, chronic, inflammatory, and noninflammatory diarrhea. Acute and chronic diarrhea are classified according to the duration of infection, if diarrhea lasts for less than 14 days it is recognized as acute diarrhea, while infection persisting above 14 days is chronic diarrhea **(Gómez-Escudero et al., 2021).** Inflammatory diarrhea can be caused by the bacteria that release cytotoxins (*Escherichia coli, Clostridium difficile)* or by some glycoproteins released by the viruses. The attachment of toxins to the gut epithelial layer is the reason behind the inflammation. Contrary to this noninflammatory diarrhea is caused by the group of bacteria that release enterotoxin (*Vibrio cholerae etc.)* or by viruses. A common mechanism of infection in diarrhea involves the pathogen's attachment to the mucosal lining of the intestine, and evasion of host defense systems **(Khan et al., 2021)**. Regular use of antibiotics and antidiarrheal agents is not recommended as it may cause certain side effects. Similarly, the excess use of antibiotics in medicine, as an antimicrobial agent in the food industry, or as a growth promoter in animal husbandry has increased the overall burden of pathogen resistance in humans. The formation of multidrug-resistant organisms is the collateral damage of the scenario.

Protease-based drug formulation can be a new route in modern medicine. Proteases are a diverse class of enzymes that are involved in the catalysis of biological reactions by hydrolyzing the peptide bonds in proteins. Cleaved peptide fragments are active protein molecules that are themselves involved in enzymatic activities. Among all the prokaryotes and eukaryotic organisms, proteases function as proteins, biological messengers, or hormones **(Scarcella et al., 2022)**.

1.2.2 Proteases

Proteases are a class of enzymes that cleave peptide bonds in long protein molecules and release small peptide fragments. Proteases take part in metabolic and biological pathways like apoptosis, tissue differentiation, hormone release, and injury healing. They are an important part of the innate immune response by invading the infection molecules like tumor cells, bacterial toxins, etc. Proteases also activate many cell signaling pathways and are associated with the activation of hormones by catalyzing the proteins, where small peptides act like activators of intracellular pathways **(Solanki et al., 2021)**. These abilities make protease a good target for pharmaceutical research.

1.2.2.2 Classification Based on the Source of Extraction

1.2.2.2.1 Plant Proteases

Proteases are an important part of the plant life cycle, as they are always active during the life cycle of a plant to maintain all physiological functions. Hundreds of diverse proteases with commercial value have been reported from different plant sources. These include actinidin, bromelain, cardosins, caprifig coagulant, cyprosins, cucumisin, dubiumin, ficin, hieronymain, lettucine, onopordosin, oryzasin, papain, etc. **(Troncoso et al., 2022)**. Among these bromelains, ficin, keratinases, and papain, are a few of the well-characterized proteases.

A. Bromelain

The first isolation of bromelain has been reported from the pineapple plant. The enzyme is categorized under cysteine protease, with optimum activity at pH 5 to 9. Bromelain has a main application in the food industry in the preparation of fish concentrates and gelatin desserts. The enzyme is stable up to the temperature of 70 ºC.

B. Ficin

Ficin is a 26 kDa protease, extracted from the latex of fig plant. The enzyme has a major use in dissolving the scrap film to recover silver

C. Keratinases

Keratinases as the name suggests can break keratin molecules, which has a huge application in the textile industry in the digestion of wool. Also, this ability of keratinase is utilized in wastewater management to degrade hair and similar substances.

D. Papain

Papain is one of the oldest hydrolytic proteases reported, and it is present in the *Carica papaya* fruit. The enzyme has many isozymes that contribute to its diverse specificity for various substrates. The optimum pH of this enzyme lies between 5 and 9, and recordedtemperature stability is up to 80 ºC. In the food industry, papain has a role in meat andalcoholic beverage processing.

1.2.2.2.2 Animal Proteases

The isolation of animal protease involves the sacrifice of animals; hence such proteases are not considered for commercial use, due to the ethics involved. Yet, researchers have reported some proteolytic enzymes of animal descent, which are trypsin, chymotrypsin, pepsin, and rennins **(Gurumallesh et al., 2019).**

A. Trypsin

Trypsin is one of the most popular enzymes involved in the hydrolysis of proteins. Trypsin is a serine protease. One of the unique abilities of this protease is to inhibit the gut enzymes of hematophagous insects, which makes it a good biocontrol agent. Trypsin is used in bacterial media preparation and various medical aids.

B. Chymotrypsin

Chymotrypsin is a pancreatic hydrolase, that digests the protein sequences from the carboxyl side of aromatic amino acids. In the pancreas, the enzyme is stored as an inactive precursor form called chymotrypsinogen, activated by trypsin. Due to its unique protein-targeting abilities, the enzyme is used in sophisticated analytical and diagnostic methodologies.

C. Pepsin

Pepsin is a hydrolytic enzyme of the stomach, present in all vertebrates. The enzyme is also present as its precursor called pepsinogen, which is when required by the body gets activated by self-catalysis under the influence of stomach acid (HCl). Pepsin is an acidicprotease with an optimal pH of 1-2. The enzyme is explored for its properties in the food industry and medicine.

D. Rennin

Rennin is found in the stomach of nursing mammals, as an inactive precursor called prorenin. Autocatalysis of prorennin releases an active rennin, with the help of pepsin. The unique hydrolytic property of rennin involves the breakdown of κ- casein to generate para- κ- casein and C- terminal glycopeptide. Dairy industries use rennin to generate stable byproducts of dairy.

1.2.2.2.3 Microbial Proteases

The major protease producers among the microbes are fungi, bacteria, viruses, and actinomycetes.

A. Bacteria

Bacterial proteases are structurally and functionally more diverse than mammalian proteases **(Razzaq et al., 2019).** These proteases are classified according to their catalytic sites and their mode of function and the most diverse form of microbial proteases is found in *Bacillus*.

B. Fungi

Fungi also consist of a diverse range of proteolytic enzymes, which include acidic, neutral, and alkaline proteases **(Souza et al., 2015)**. Compared to bacterial proteases fungal proteases exhibit lower heat tolerance and low rate of reaction. These proteases are preferred over plant, animal, and microbial proteases in the food and dairy industry dueto their reduced bitterness.

C. Actinomycetes

Enzymes present inside the actinomycetes include amylase, lipase, cellulase, xylanase, asparaginase, etc. **(Katti et al., 2021, Devi et al., 2022).** This plethora of proteases is synthesized by the actinomycetes to fulfill their nutritional requirements **(Souza et al., 2015)**. Major species among actinomycetes that produce protease are *Streptomyces* and *Nocardia.* Especially marine actinomycetes have been explored more to isolate the protease of pharmaceutical importance **(Tuyen Do et al., 2021)**. Enzymes like amylase from actinomycetes are used in food, brewery, textile, and distillation industries.

D. Virus

Viral proteases have received more popularity in recent years due to their unique properties in viral-host interaction, disease onset in humans, their use in retroviral

therapies, and many more **(Zephyr et al., 2021)**. Viral proteases include serine, cysteine, and aspartic proteases. Research majorly involves studying the interaction of viral proteases with other protective molecules to design drugs to combat viral infections **(Ozen et al., 2019)**.

1.2.3 Metalloprotease of Genus *Bacillus*

Among the diverse bacterial diaspora species of *Bacillus* have played an important role in the rise of the enzyme industry, as they produce a diverse range of unique bacteria. Examples: amylase, arabinose, cellulase, chitinase, maltase, mannanase, xylanases, proteases, etc. **(Harwood et al., 2022).** The dominance of microbial enzymes at the industrial level comes with several advantages, like easier cultivation, no ethical regulation involved, easier genetic manipulation, and stability at a diverse range of temperatures and pH. Another unique property of *Bacillus* is the release of extracellular proteins which have huge therapeutic value **(Avila et al., 2019)**. *Bacillus* is reported to produce a variety of hydrolytic enzymes which include, a serine protease, metalloprotease, esterase, etc.

Different *Bacillus* species to produce metalloprotease are *B. clausii, B. halodurans, B. subtilis, B. megaterium, B. thermoproteolyticus, B. thuringiensis,* etc. The metalloproteases show the maximum activity between pH 3 to 8, as most of the proteases of this family belong to either acidic or neutral proteases **(Hasan et al., 2021)**. *Bacillus thermoproteolyticus* is one of the earlier bacteria from which extracellular metalloprotease named thermolysin-like metalloprotease was isolated. Thermolysin a 40 kDa protease, is associated with the pathogenesis of several diseases. chelators like EDTA can inhibit these proteases **(Lambré et al., 2024)**. *Bacillus anthracis* produces a lethal anthrax factor which is also a metalloprotease of 90 kDa molecular mass. The enzyme is associated with the cleavage of mitogen-activated protein kinase (MAPK) leading to disruption of signaling pathways in host cells. Neutral metalloprotease isolation has been reported from *Bacillus cereus,* which had an application in the textile industry **(He et al., 2021).** *Bacillus cereus* SV1 strain produced a calcium-dependent metalloprotease (35.5 kDa), which was degrading casein protein. The enzyme had an optimum pH of 8.0 and temperature stability at 55ºC **(Mahnashi et al., 2022)**.

Though the unique properties of metalloproteases of different *Bacillus* species have industrial significance, the potential for target functionality of *Bacillus clausii*

metalloprotease makes it a good target for the healthcare sector **(Ripert et al., 2016)**. A comparison of the proteolytic activity and substrate specificity of *Bacillus licheniformis* with the metalloprotease of *Bacillus clausii* has proved *B. clausii's* enzymes to be more effective.

1.2.4 Factors Affecting the Protease Production

Decades-long research has increased our understanding of the processes limiting the production of bacterial proteases. All physiological parameters like temperature, pH, nutrients, and the media contribute to the release and the commercial production of enzymes. A brief description of these contributing factors is as follows:

A. The Carbon Source

The synthesis of extracellular proteases requires an additional energy source **(Sharma et al., 2019)**. Usually, growth media is supplemented with carbon compounds, but additional use of sugars (glucose, sucrose) increases the protease production. The additional sugars can lead to catabolite repression in bacterial cells, where the expression of constitutive and inducible genes may change leading to changes in the production of extracellular enzymes as well. Carbon sources like inhibit protease production in many *Bacillus* species including, *B. licheniformis, B. subtilis,* and *B. amyloliquifaciens* **(Wei et al., 2021)**. Higher carbon source concentration in a liquid culture will produce more amino acids followed by a decrease in pH, which will not favor the growth of alkaline proteases, while the contrary will be useful for the synthesis of acidic proteases **(Kieliszek et al., 2021).**

B. Nitrogen Source

An important factor associated with the production of proteases is nitrogen source. Nitrogen is an important constituent of nucleic acids hence synthesis of various proteases will depend on its concentration **(Hernandez et al., 2020).** A low carbon-to-high nitrogen ratio is associated with the synthesis of serine and metalloproteases. Nitrogen sources like corn steep liquor, meat extract, peptone, protein hydrolysate, and yeast extract are reported to increase the proteolytic activities of proteases **(Kieliszek et al., 2021)**. The excess amounts of nitrogen source leads to the synthesis of amino acids which can ultimately cause a decrease in the protease synthesis. Different amino acids have different effects on protease synthesis **(Hou et al., 2017)**. For example, the abundance of threonine and isoleucine in *Bacillus megaterium* is reported to inhibit the

synthesis of neutral proteases.

C. Inorganic Salts

As cofactors are essential for the activity of proteases the presence of Ca^{2+}, Zn^{2+}, and Mn^{2+} can make a significant change in the synthesis of proteases.

D. pH and Temperature

Optimal pH is the basic requirement of protease synthesis **(Wei et al., 2021)**. Usually, bacterial growth pH is also the pH of protease synthesis but exceptions exist. For example, for the synthesis of acidic and alkaline proteases pH will vary compared to the initial pH of bacterial culture. Like pH, temperature may vary according to the protease requirements **(Worsztynowicz et al., 2020)**. Most of the protease grow at a constant temperature but some are synthesized under a constant change of temperature at regular time intervals.

E. Time of Incubation

The synthesis rate of protease is always directly proportional to the time of incubation. Bacillus is a sporulating bacteria, known to release extracellular compounds when grown under stress **(Ji et al., 2021)**. The initiation of stress conditions requires bacterial incubation for three to four days, producing proteases.

F. Choice of the Substrate for Proteolytic Activity Confirmation

The proteolytic activity estimation is performed during the optimization of protease synthesis **(Habicher, et al., 2019)**. This process utilizes natural and synthetic substrates, whose degradation predicts the presence of proteolytic substances **(Suberu et al., 2019).** Selection of substrates depends upon the need for protease which a researcher wants to isolate. Proteins like hemoglobin, casein, gelatin, peptone, peptides, etc. are some common protease substrates. More unconventional substrates may include bacteria, and their toxins when it comes to isolating a protease that could inhibit pathogenic bacteria. Processes like zymography, UV, and visible spectrophotometry are some methods of activity analysis.

1.2.5 Applications of Proteases in Biotechnology

Microbial proteases, especially metalloproteases contribute to half of the sales of the commercial enzyme industry **(Avila et al., 2019)**. Some examples include TLP-ste variant Boilysin (Groningen corporation, The Netherlands), Thermolysin (Merck), Neutrase (Novo Nordisk), and Thermoase PC10F. All these proteases are used extensively in food, leather, brewery, textile, and other industries **(**Harwood **et al.,**

2022).

A. Food Industry

Thermolysin-like protein-1 (TLP-1) has found its use in enzyme-based peptide synthesis. Researchers were studying the role of thermolysin, in synthesizing aspartame (artificial sweetener) precursor N-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester (Z-Asp-Phe-OMe), synthesis **(Alsoufi et al., 2019)**, **(Lambré et al., 2024)**. Enzyme neutrase was used in 1995 for the synthesis of compounds like Celite-545 and Polyamide-PA6. Celite 545 is a food-grade compound used in the filtration of other food materials **(Patel et al., 2019), (Oyewole et al., 2022).** For the synthesis of aspartame precursor Z-Asp-Phe-Ome, the food industry has explored many processes like a solid-to-solid synthesis of water-soluble organic solvent systems **(Kühn et al. 2002)**. Another metalloprotease named vimelysin isolated from *Vibrio sp.* T1800 showed a higher yield of aspartame at low temperatures **(Hasan, et al.2021).** *Pseudomonas aeruginosa* produces a protease named Pseudolysin which was also used in peptide synthesis **(Rival et al. 2000), (Capasso et al., 2024).** Many metalloproteases in combination with other proteases have been used as flavor enhancers in the food industry. The main enzyme used is Neutrase, which enhances the tenderisation of fermented sausages **(Hasan, et al.2021)**.Neutrase and thermolysin have also been reported to be used to produce novel peptides by the hydrolysis of proteins. The purpose of this methodology is to create bioactive peptides with increased antioxidant activity **(Shen et al. 2010), (Lunde et al., 2019)**.

B. Pharmaceutical Industry

Collagenase *Clostridium histolyticum* is a class of bacterial enzymes having application in medicine. Enzyme collagenase is used to treat abnormal tightening of tissues beneath the skin surface majorly in hands and palms (Dupuytren disease). The hydrolytic ability of collagenase relieves the pain by hydrolyzing the collagen of diseased areas **(Pasquale et al., 2019).** This bacterial enzyme class is used in the treatment of herniated discs and to isolate the pancreatic discs **(Frederick et al., 2020).** Collagenases isolated from *Vibrio sp.* are used in the treatment of ulcers, tissue healing, and burn treatment **(Barzkar et al., 2024).** Proteases of *Achromobacter lyticus* classified under the M23 family as beta-lytic metalloprotease are reported to have strong antimicrobial activity as their defense system **(Hioki et al., 2021), (Razew et al., 2022).** Lysostaphin isolated from *Staphylococcus simulans* is shown to treat endophthalmitis, an eye disorder

induced by methicillin-resistant *S. aureus* in a rat model **(Priya et al., 2019), (Moghri et al., 2021), (Jayakumar et al., 2021).** Aspartyl proteases associated with many viral infections like HIV are studied to target them to create antiretroviral therapies **(Majerová et al., 2021), (Kryštůfek et al., 2021).** The concept behind the utilization of viral proteases lies in the fact that these proteases are associated with the onset of infection in the host, and they are the ones targeting the humoral immune response. Hence studying their structure and mode of interaction provides us with the creation of inhibitors that would block these proteases when entered inside the host.

C. Other Applications

Metalloprotease is utilized in the baking industry to create dough of the desired consistency, which is used to generate a variety of bread (**Kermasha et al., 2021).** In the brewing industry, proteases are used to enhance the nitrogen content in the fermentation of beer. For silver recovery from X-ray films metalloproteases are utilized **(Chellappan et al., 2023).** Neutrase is insensitive to many plant protease inhibitors and, thus utilized in breweries. Metalloproteases (alkaline proteases) are used in the dehairing of animal skin and wool in the textile and leather industry **(Khambhaty et al., 2020), (Hasan et al., 2022).**

1.2.6 Proteases and Antimicrobial Resistance

Though antimicrobial proteases are a new concept in drug therapy, still the possibility of developing resistance among pathogenic strains exists. As antimicrobial proteases are a diverse group of biomolecules with unique structures and modes of action, they require huge modifications in the cell wall and membrane structures of target pathogens to escape the attack of such proteases **(Razzaq et al., 2019).** An exception to this concept, a few studies of pathogen resistance against antimicrobial protease have been reported. Proteases with lytic activities are reported from various microbes, but only a few have reached the approval of becoming a drug. Retroviral therapy is one of its kind supporting the use of proteases in creating novel drug treatments, but the problem lies in the treatment of bacterial infections **(Bond et al., 2020).** For almost a century antibiotics have been exclusively considered for bacterial infection treatment, which has already led the world towards the formation of superbugs and more deadly infection formations. The discovery of Penicillin by Alexander Fleming in 1928 is considered as rise of a golden age for antibiotics **(Kalyani et al., 2024).** However, its extensive use during World War II had simultaneously initiated the downfall of antibiotic

applications. Eventually, more antibiotic discovery and synthesis followed, but none of them stood well against resistance. This scenario resulted in the creation of multidrug-resistant (MDR) strains in various plant, and animal pathogens **(Gongora et al., 2020), (Sharma et al., 2021).** Diarrhea which is a communicable disease and spreads due to poor hygiene practices, also persists due to drug-resistant pathogens. Both developed and developing countries keep on facing diarrhea outbreaks due to resistant pathogens. As the causative agents of diarrhea are not limited to bacteria only, some classes of viruses have also been the causal pathogen of infection, it becomes very important to have treatments designed to escape resistance, as targeting such a broad range of pathogens is itself a difficult task. Presently, available treatment for diarrhea is expensive and limited majorly to antibiotics. The cost factor of the present treatment makes it difficult for patients from poor countries to afford it. Diarrhea outbreaks in nursing homes or particular geographical areas are reported each year. Infants and children are the main victims of the disease accounting for millions of deaths every year. Acute gastroenteritis is worsened in immunocompromised patients (HIV and AIDS) **(Basile et al., 2021)**. Human immunodeficiency virus itselfis reported to behave as an enteric pathogen and to release enterotoxins in the human gut **(Cohen et al., 2022).**

1.2.7 Antibiotic Resistance Origins and Mechanisms

1.2.7.1 Basic Biochemistry of Antibiotics

Communicable infections have been one of the major causes of death all around the world. Their continuous outbreaks are due to the formation of new resistant pathogens under the influence of antibiotics. There are approximately six classes and subclasses of antibiotics according to their mode of action **(Rourke et al., 2020)**. Several antibiotics don't fit the classification due to their unique modes of action. For years, researchers have reported resistant microbes concerning each class of antibiotic. A broad spectrum of antibiotics keeps failing in their job as most of them have similar enzymes, common modes to attack pathogens, or sometimes common structures. These similarities simply make the pathogens invincible. Beta-lactam antibiotics are the most commonly isolated or created class of antibiotics**.** Bacteria have evolved to change their porin proteins to reduce the affinity of the Beta-lactam ring to penicillin-binding proteins. Another class of antibiotics is macrolides. Macrolides work by targeting the

50S subunit of bacteria, causing the inhibition of protein synthesis in bacteria **(Patel et al., 2021).** Earlier they replaced methicillin which was used to eliminate Gram-positive bacterial infections eventually resistant strains against macrolides were discovered.

1.2.7.2 Transmission of Resistance

The most common survival technique used by bacteria is the formation of a resistant plasmid. Also, resistance is transferred to the next generation during cell division. With the advancement of technology, techniques like whole genome sequencing have identified the potential genes that are responsible for causing resistance in microorganisms. One common mechanism of causing antibiotic resistance against sulphonamide and trimethoprim **(Pavelquesi et al., 2021)** is gene amplification. The creation of complete or partial gene knockout libraries after saturation mutagenesis of bacterial genomes can help to predict mutant strains that show resistance in responses to antibiotics. The phenomenon of increasing the copy number of target genes can help to escape antibiotic targeting, as the increased copy number is proportional to reduced antibiotic concentration in the cell **(Nicoloff et al., 2019)**.

1.2.7.3 Genetic Origin of Antibiotic Resistance

The concept that genes and genetic factors are responsible for acquiring resistance in microorganisms has led researchers to infiltrate microbial genetics. Phenomenons like homologous gene transfer (HGT), heterologous expression, and DNA repair mechanisms have been extensively studied to inquire about microbial resistance **(Jian et al., 2021), (Lerminiaux et al., 2018).** Studies have shown that acquiring resistance affects energy uptake in microorganisms making multidrug-resistant microbes short-lived, showing unusual growth patterns. Regardless of all these theories, real-time events are always known to contradict laboratory results. Contributing to these events a short cell division time and rapid multiplication processes make the microbes invincible.

1.2.7.4 Conveyance of Resistance Genes

One of the most common ways of transmitting the resistance to the next generation in bacteria is through horizontal gene transfer (HGT) **(Nicoloff et al., 2019)**. Horizontal gene transfer (HGT) is reported as a continuous phenomenon of bacterial evolution. However, the frequency of gene transfer reported from past decades is very different

when compared to the gene transfer that happened for hundreds of years. Massive exploitation of antibiotics against diseases has created a selection pressure on microbes, compared to what was naturally happening for thousands of years. The frequency of gene transfer is seen to be significantly high in the natural environment when compared to laboratory conditions **(Lerminiaux et al., 2018)**. Normal microflora and the pathogens in the gut of humans and animals have been reported to inherit resistant genes and are continuously sharing them by gene transfer **(Jian et al., 2021)**. In the case of viruses, the phenomenon of drug resistance appears to be very intimidating. The genetic shift and drift phenomenon simply help viruses to acquire resistance against medical treatments. Viruses are reported to have evolved more frequently when compared to bacteria **(Zephyr et al., 2021)**. The structural changes in surface glycoproteins and receptors make it very difficult for one treatment to wipe away viral infections. Using antibiotics below prescribed treatment, discontinuous use of drugs is also known to provoke resistance in microbes.

1.2.7.5 Anthropogenic Activities in Causing Resistance

The selfish and irresponsible actions of mankind have led to the downfall of antibiotic therapy. Since the discovery of the first antibiotic in 1940, to manufacturing, synthesizing, and identifying new antibiotics, the whole genre of this therapy has been over-exploited. One of the main reasons behind the failure of antibiotic therapy was the beginning of World War II, where Penicillin was used beyond limits to treat wounded soldiers. Other than humans, antibiotics are used to treat animals, aquacultures, and the food industry to sustain longer shelf-life **(Kalyani et al., 2024)**. All this has led to the biomagnification of antibiotic-based compounds in ecosystems creating catastrophic situations. Waste handling techniques vary among developed and developing countries. Careless disposal of industrial waste, biocides, and other non-degradable compounds into the ecosystem has worsened the situation. A classic example of irresponsible disposal dates back to 2008 when a Hyderabad (India) based pharmaceutical company dumped ciprofloxacin (50 kg/day) into a nearby river **(Dixit et al., 2024)**. The genetic analysis of microbes from sewage treatmentplants reveals that they are the reservoirs of resistant microorganisms **(Sharma et al., 2022)**. This situation is not limited to humans and animals only. Plant systems have also become the victims of resistance. Excessive use of pesticides, insecticides, and weedicides has led to the creation of plant-resistant

pathogens which eventually might turn into a famine situation around the world. Restricted and responsible use of antibiotics has become an obligation of present times to mankind **(Uddin et al., 2021).**

1.2.6 Measures to Control Drug Resistance

History of mankind has reported multiple outbreaks of microbial infections. Epidemic and pandemic diseases simply create a downfall in a country's economy, leaving a long-lasting impact worldwide. An outbreak of food poisoning in Germany due to *Escherichia coli* resulted in the deaths of 50 people, leaving almost 5000 people affected **(Köckerling et al., 2017).** The outbreak did not involve any resistant strain, yet causing jeopardy to thousands. Over a million cases of multiple drug resistance were reported in Europe only, with 25000 reported deaths **(Mestrovic et al., 2022).** According to the Center for Disease Control (CDC) $55 billion annually on antimicrobial resistance (AMR), where healthcare costs up to $20 billion and the lost productivity devours $35 billion **(Dadgostar et al., 2019).** Worldwide it costs over 300 billion USD to treat resistant infections only **(Dadgostar et al., 2019)**. The condition is worsened in a developing country like India. Poverty, overpopulation, and poor hygiene conditions throughout the country (urban and rural areas) have created multidrug-resistant microbes has a surprising rate. It costs almost 6 billion dollars to the Indian economy to treat resistant organism infections **(Tamhankar et al., 2020).** Reports have shown increased mortality rates in Delhi-based hospitals due to the presence of a novel metallo β-lactamase-resistance gene (blaNDM-1) in resistant strains.

1.2.9 Responsibilities to Tackle Microbial Resistance

The development of antibiotic resistance is yet not a well-understood phenomenon, because laboratory results never seem to mimic the natural environment outcomes. The question that still exists is what are the stages or the environmental factors that are contributing to developing resistance? The microflora of humans, animals, and plants are acquiring resistance from inside the organism's body, but providing a natural environment to study the inside situation is not possible in many aspects. It is the responsibility of research to design modern diagnostic techniques and instruments to study MDR. Finding answers to the microbial resistance mechanism and authenticating these results is also the responsibility of research. Researchers need to be trained with modern diagnostic techniques, and technology transfer among countries to battle

multiple drug resistance a few steps to begin with (**Sharma et al., 2022**). Other than microbes understanding the genetic diversity of host organisms is also very important to develop new therapies.

1.2.9.1 Communal Awareness

Literacy rates of any country affect the rates of infections, their treatments, and the occurrence of drug-resistant pathogens. Half-knowledge is always dangerous. It becomes the duty of the government to educate people and spread awareness to the masses regarding bacterial infections and resistance (**Uddin et al., 2021**). Organizing awareness campaigns, educating students in schools, and patient counseling in clinics are the contributing factors in tackling drug resistance. As the current decade is a decade of technology, hence governments should launch various health apps to spread awareness about infectious diseases, summarizing highly resistant diseases and asking patients to submit the details of their infections online. One such online program was started in Europe called the e-Bug program to aware kids of the prospects and consequences of antibiotics.

1.2.9.2 Public Health and Hygiene

Many countries all around the world suffer from the lack of proper resources. When food is the only priority for such people health and hygiene come secondary to them. These people don't have access to clean water, houses, or sanitary conditions. Poor waste management, lack of sewage treatment plants, and zero knowledge about the consequences of excessive use of antibiotics worsen the situation here. Governments of these countries have a great responsibility on their shoulders to provide the people with basic amenities like food, education, and a healthcare system **(Köckerling et al., 2017)**. Awareness about the use of antibiotics, proper law enforcement, and providing a good budget for the healthcare system are some of the few important duties for the governments of these countries.

1.2.9.3 Progression of Antibiotic Drugs

For the past 50 years, researchers have been working to find new antibiotics, but mostly have ended up modifying the existing antibiotics. The absence of novelty has resulted in thecreation of resistant microbes **(Dadgostar et al., 2019**). As research is expensive, time-consuming, and unpredictable, it never appears a cup of tea for pharmaceutical companies, because these are profit-making organizations. Here comes the

responsibility of a country's government to establish institutes, hire professionals, and provide funds for the research **(Mestrovic et al., 2022).**

1.2.9.4 State of Old Antibiotics

Antibiotics that are considered obsolete by pharmaceutical companies should be reinvestigated. Governments should invite start-ups to have new ideas and methodologies that can be later implemented to study these antibiotics. One of the very famous examples of rejected antibiotics is daptomycin, which was aborted by a company, later when it was researched again it became an effective medicine against Gram-positive infections **(Karas et al., 2020).**

1.2.9.5 Authorizing Antimicrobial Drug Usage

One of the most common problems faced by almost all countries around the world is the illegal use of antibiotics for things that are not meant to use those drugs **(Nicoloff et al., 2019)**. Unethical use of antibiotics which are developed to treat humans, when used in animal husbandry or aquacultures simply creates biomagnification problems ultimatelyaffecting humans. Surveys have shown that 50% of manufactured antibiotics are used in unimproved activities like feeding animals to induce their growth hormones. The government should devise strict rules and restrictions on the distribution and use of antibiotics **(Jian et al., 2011)**.

1.2.9.6 Alternative Treatments to Antimicrobials

Designing novel drug therapies as an alternative to replace antibiotic function has become the need of the hour. Alternatives to antibiotics involve adjuvants, probiotics, vaccination, etc. **(Benevides et al., 2019)**. one of the modern therapies to replace antibiotics is peptide-based drug therapy. Peptides are small protein molecules which can be taken orally or intravenously. Studies have shown the absence of resistance development with protease-based drug therapies **(Ripert et al., 2016).** So far thousands of antimicrobial peptides have been isolated from sources like plants, animals, and microorganisms. The only drawback with these compounds is less success in clinical trials which is holding back the progress. Developing alternatives to antibiotics requires information about all kinds of beneficial, naturally occurring compounds that could be studied further. Nutraceuticals, prebiotics, and probiotics, genetically modified compounds can be seen as the most promising alternatives to design therapies **(Sachdeva et al., 2020)**.

One of the important steps that government research organizations can take is to set goals to develop a particular type of medicine in a certain timeline. Such ways can boost research. The most common approach for governments is to come together at international levels, as cooperation inresearch is the first step to success.

1.3 Gaps in the Current Research

Antimicrobial resistance is a global phenomenon, affecting millions of people worldwide. High levels of resistance can be seen in the case of communicable diseases and pathogens. Studies show a void in the development of new antibiotics in the past 30 years **(Figure 1), (Benevides et al., 2019)**. The problem related to drug resistance persists, making researchers search for novel drug therapies. One important duty that lies with researchis to provide patients an affordable, cost-effective medication with no side effects. Considering all these problems, thorough research has been performed to find the bestalternative to design targeted drug therapy. Researchers have demonstrated the safety profile and suitable efficacy of *B. clausii* against diarrhea but the problem that persists here is the consortium of all four strains of *Bacillus clausii* is required to make the drug effective. Hence research is required to identify a novel *Bacillus clausii* strain that will have a bactericidal impact on diarrhea pathogens **(Lakshmi et al., 2017)**, **(Bueno et al., 2022).** The spore suspension of *B. clausii* creates cost implications for developing countries making it inaccessible to many patients. Out of four strains of *Bacillus clausii,* only one (O/C)has been reported for its proteolytic activity **(Ripert et al., 2016)**. Research about the bacteriocin activity of proteases from *Bacillus clausii* has already been reported.Finding more novel proteases from *Bacillus clausii* can help to counteract antibiotic resistance and superbug-like microbe. Research is required to extract effective proteases that could target diarrhea pathogens and create stable drug formulations out of them. Literature related to *Bacillus clausii* has not reported much about novel protease isolation. Even the so-far reported protease has not been explored for its antidiarrheal potential, multidrug resistance, or efficacy of bactericidal proteases in eukaryotic models. Hence research is required to find new, unique proteases that couldcounteract the diarrhea disease and can have a future as a stable drug formulation.

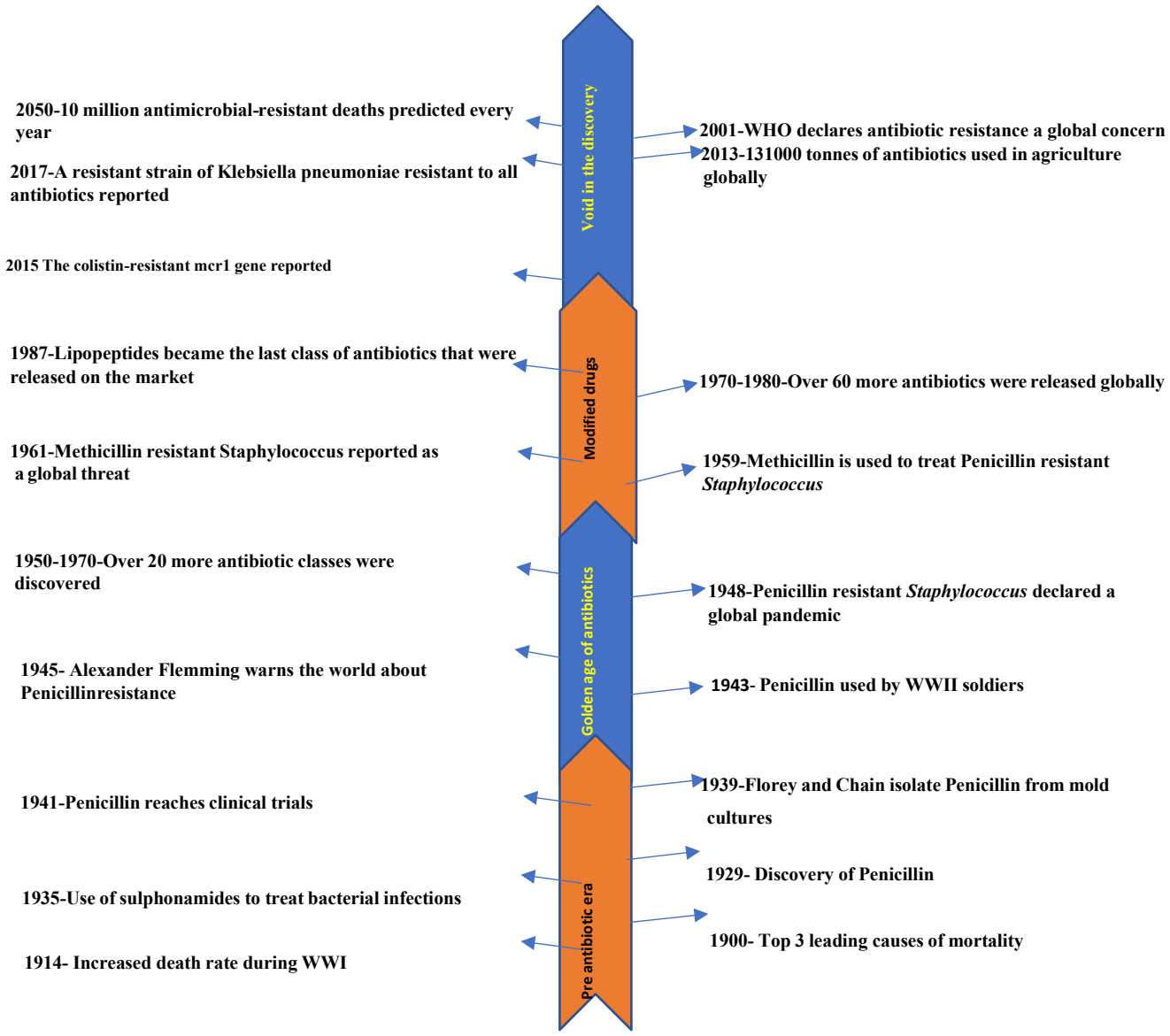


Figure 1: Timeline showing the antibiotic resistance.

Considering the above-discussed challenges, it was decided to procure an indigenous *Bacillus clausii* strain which is already reported to have antimicrobial properties, and which will be optimized to isolate novel protease with strong hydrolytic activity against a broad spectrum of diarrhea pathogens. The protease will be tested through *in vitro* and *in vivo* experiments to study its efficacy and activity against diarrhea pathogens. By keeping in mind all the research gaps following objectives were designed for the study:

1.4 Objectives of the Present Research Project

1. To isolate the novel protease from the *Bacillus clausii* exhibiting anti-diarrheal activity.
2. *In silico* analysis of functional peptides/ proteins of *Bacillus clausii* possessing anti-diarrheal properties.
3. To study the anti-diarrheal activity of newly isolated protease using *Drosophila*.

Chapter 2

Objective 1: To isolate the novel protease from the *Bacillus clausii* exhibiting anti-diarrheal activity

2.1 INTRODUCTION

Microbial proteases have been explored for their role in healthcare and pharmaceutical industries. Researchers have reported the influence of a nutritious diet in the release of gut proteases. Most of the gut microbiota utilize host nutrients and protein tosynthesize proteases. A balanced diet can be influenced by the consumption of probiotics which can balance the gut microbiota **(Yi et al., 2018).** Probiotic-based microorganisms such as *Bacillus* and *Bifidobacterium* are reported to release extracellular compounds like peptides (clausin, subtilisin, iturin), and proteins which are reported to have antimicrobial properties **(Taggar et al., 2021)**.

Our study aimed to explore the antimicrobial potential of the probiotic bacteria *Bacillus clausii* UBBC07, one of the most common, commercially available over-the-counter medicines, which is effectively used to treat diarrhea **(Dubey et al., 2021).** The strain was first time isolated and reported by Unique Biotech Limited, India in 2005. In our primary study, the antimicrobial/ bacteriostatic activity of *Bacillus clausii* UBBC07 was further explored from the sporulating culture. The bacteriostatic nature of extracellular secretions was confirmed by agar well diffusion assay against a set of diarrhea-causing Gram-positive and Gram-negative pathogens. Further proteolytic activity in partially purified protein precipitates was confirmed with gel overlay assay against a set of diarrhea-causing organisms. The ~23kDa protein band in tricine SDS-PAGE coinciding with the zone of clearance was considered the target protein which was further studied. MS/MS spectroscopy with MALDI-TOFF analysis confirmed the molecular weight and protein sequence.

2.2 MATERIALS AND METHODS

2.2.1 Reagent and Chemical: All the chemicals used in the study were purchased from Sigma-Aldrich.

A. Luria Bertani broth (gram/liter) (pH 7.0)

Tryptone	10
Yeast extract	5
NaCl	10
Distilled water	1litre

B. Luria Bertani media (gram/litre) (pH 7.0)

Tryptone	10
Yeast extract	5
NaCl	10
Agar	15
Distilled water	1litre

C. Muller Hinton broth (gram/liter) (pH 7.0)

Beef extract	300
Casein hydrolysate	17.5
Starch	1.5

All media were sterilized by autoclaving at 121°C for 15 minutes.

2.2.2 METHODOLOGY

2.2.2.1 Selection of Bacteria

The *Bacillus clausii* strain UBBC07 an over-the-counter medicine under the name **Bactolac** was procured from the LPU's Uni-hospital pharmacy in Jalandhar, India. The standard strains used in this study were, *Enterococcus faecalis* (MTCC 3159), *Staphylococcus aureus* (MTCC 1430), *Escherichia coli* (MTCC 293), *Pseudomonas putida* (MTCC 1194), *Enterobacter cloacae* (MTCC 7724), *Salmonella enterica* (MTCC 1164), *Bacillus cereus* (MTCC 6629). All test strains were obtained from the CSIR-Institute of Microbial Technology, Chandigarh.

2.2.2.2 Screening for Antimicrobial Compounds

The presence of antimicrobial compounds was examined as described by Maqbool et al., 2020 **(Aquaro et al., 2020)**. The standard strain of *Bacillus clausii* UBBC07 was grown in Muller Hinton broth at different temperatures. The test strains i.e. *Enterococcus faecalis* (MTCC 3159), *Staphylococcus aureus* (MTCC 1430), *Escherichia coli* (MTCC 293), *Pseudomonas putida* (MTCC 1194), *Enterobacter cloacae* (MTCC 7724), *Salmonella enterica* (MTCC 1164), *Bacillus cereus* (MTCC 6629) were grown in Luria Bertani broth and grown on Petri dish by spread plate method. All test strains were grown at 37°C. For optimization of *Bacillus clausii* UBBC07 a range of temperatures i.e. 25°C, 30°C, 37°C with continuous shaking at 180 rpm were tested for the presence of antimicrobial secretions. The cell culture was removed at fixed time intervals and centrifuged at 10,000 rpm for 20 minutes and cell-

free supernatant was tested for antimicrobial activity. 100 µl of centrifuged supernatant was added to the 6 mm deep well on overnight grown test strain plates. The presence of a clearance zone determined the effectiveness of the bacterial supernatant. The Clinical and Laboratory Standards Institute (CLSI) guidelines were followed for each experiment.

2.2.2.3 Antimicrobial Compound Synthesis

After optimizing the *Bacillus clausii* UBBC07 for antimicrobial compound synthesis, the primary culture of bacteria was grown from a single colony in MH broth and a secondary culture was initiated in 2 litre flasks. Inoculum was used in a 1:100 ratio (inoculum: broth). The culture was grown for 96 hours at 30°C/ 180 rpm, to achieve the maximum release of antimicrobial compounds.

2.2.2.4 Acid Precipitation

Protein was partially purified by acidic precipitation method **(Caminero et al., 2023).** All experiments related to target protein extraction and purification were performed at 4 ºC. The sporulated culture was then centrifuged at 10,000 rpm, for 20 minutes to separate the cells from the clear supernatant. The supernatant was treated with 1N HCl in a 1:100 (HCl: supernatant) ratio for 1 hour with constant stirring. The supernatant was again centrifuged at 10,000 rpm, for 20 minutes to separate the protein precipitates from the supernatant. Precipitated protein was washed with absolute ethanol (molecular grade; Merck), centrifuged at 10,000 rpm, for 10 minutes, and decanted the supernatant. The protein pellet was dried and stored at -80 °C till further use.

2.2.2.5 SDS-PAGE and Gel Overlay Assay

All the chemicals used in the SDS-PAGE were of molecular grade and purchased from Thermo Fisher Scientific.

A. Reagents for SDS-Polyacrylamide Gel

- 30% Acrylamide solution: 29% Acrylamide (W/V) + 1% Bis-acrylamide (W/V)
- 1.5 M Tris buffer (pH-8.8) for resolving gel
- 1 M Tris buffer (pH-6.8) for stacking gel
- Sodium dodecyl sulfate (SDS) 10% (W/V)
- Ammonium per sulfate: 10% (W/V)

B. SDS-PAGE Running Buffer (1X) 1 liter

- Glycine: 14 g
- Tris: 3.03 g
- SDS: 1 g (W/V)

C. SDS-PAGE Loading Dye 4X (10 ml)

- 1M Tris buffer (pH-6.8): 2 ml
- Glycerol: 2 ml
- SDS (20%): 4 ml
- Bromophenol Blue: 1 mg
- β-mercaptoethanol: 0.8 ml

2.2.2.6 SDS PAGE Sample Preparation and Gel Electrophoresis

Partially purified protein was mixed with 4X loading dye in a 1:1 ratio and kept at room temperature for two hours. Protein samples and markers were loaded on 15% SDS-PAGE gel in duplicate. A protein marker was used in the range of 250 kDa to 10 kDa. The gel was run at 4 ºC at a constant voltage of 50 V in stacking gel and at 90 V in resolving gel.

2.2.2.7 Gel Overlay Assay

After completing the process, the gel was divided into two parts, one part having protein sample only and another part having protein and protein marker. The gel with a protein marker in it was stained with a staining solution and another part of the gel was prepared for zymography. The second part of the gel was washed 3 times with 0.1M Tris buffer (pH 7.6) and 40% isopropanol. Each wash was done for 30 minutes in a rocker shaker at 4 ºC. These washing were performed to remove excess SDS from the gel, which might hinder the protein activity. Two more washes with 0.1M Tris buffer (pH 7.6) were done for 30 minutes each. The gel was gently picked from the container and placed on an overnight-grown target bacteria plate. The plates were incubated at 37 ºC for 24 hours and observed for the presence of a zone of clearance.

2.2.2.8 Protein Purification

All the chemicals used in the protein purification were of molecular grade and purchased from Thermo Fisher Scientific.

2.2.2.8.1 Reagent and Chemical

A) Protein Precipitation Solution

Acetonitrile 50%

Ammonium bicarbonate (20 mM) 1.58 g

Distilled water 1000 ml

B) Elution buffer 0.1 M (pH 7.0)

Tris 12.11 g

Distilled water 1000 ml

C) BSA Estimation Reagents

1) Bovine Serum Albumin

Dissolved 1.5 mg of BSA in 1 ml of phosphate buffer saline.

2) Reagent A

Solution A: Dissolve 20g Sodium carbonate in 0.1M Sodium hydroxide in 1000 ml of distilled water.

Solution B: Dissolve in 5 g of $CuSO_4 \cdot 5H_2O$ in 1000 ml of distilled water.

Solution C: 1% Sodium potassium tartrate

Reagent A: 48 ml of solution A + 1 ml of solution B + 1 ml of solution C

3) Reagent B

1 part Folin ciocalteu: 49 parts of distilled water

D) 0.1M Phosphate buffer saline 1X (pH 7.2)

NaCl 8g

KCl 0.2 g

Na_2HPO_4 1.44 g

KH_2PO_4 0.24 g

Distilled water 1000 ml

2.2.2.8.2 Protein Purification

The target protein band was extracted from SDS-PAGE as described by Sakuma et al., **(2021).** The excised gel pieces were treated with the destaining solution for two hours, with periodical vertexing. The protein sample was then subjected to centrifugation (4000 rpm/ 5 minutes) and removed the supernatant. The sample was mixed with 500 µl of 50:50 acetonitrile and 20 mM ammonium bicarbonate to submerge the gel pieces

thoroughly and incubate for 20 minutes. The sample solution was centrifuged and the supernatant was decanted. Elution buffer was added to the gel and incubated for 4 to 16 hours to elute out the protein.

To calculate the purified protein concentration standard Bovine Serum Albumin (BSA) curve was used. A standard solution of Bovine Serum Albumin (BSA) was prepared in phosphate buffer saline using 1.5 mg/ml of protein. From this stock, working stocks of 0 to 1500 µg/ ml were prepared. The reaction mixture was prepared by adding 40 µl of BSA working stock to 160 µl of water. To this 900 µl of reagent A was added and incubated for 10 minutes at room temperature. To the same solution, 100 µl of freshly prepared reagent B was added and incubated for thirty minutes at room temperature. Absorbance was taken using a spectrophotometer at 660 nm. Phosphate buffer saline was used as blank and the graph was plotted as absorbance against concentration.

2.2.2.9 Mass Spectrometry and Protein Identification

All the chemicals used in the protein purification were of molecular grade and purchased from Thermo Fisher Scientific.

2.2.2.9.1 Reagent and Chemical

A) Dithiothreitol (100 mM)

Dithiothreitol 150 g

Distilled water 1000 ml

B) 250 mM Iodoacetamide

Iodoacetamide 36.8 mg

Ammonium bicarbonate (100 mM) 1ml

C) Trypsin

D) Chymotrypsin

E) Glu-C

F) Trifluoroacetic acid 0.1%

G) Acetonitrile

2.2.2.9.2 Mass Spectrometry

The protein identification was performed by mass spectroscopy **(Noor et al., 2021).** 100 mM of dithiothreitol was added to the purified protein and the solution was incubated at 56 °C for an hour. After the first incubation was completed, 250 mM Iodoacetamide was added to the same solution, and the reaction mixture was kept at room temperature in the dark for 45 minutes. After reduction, trypsin (20 ul) was added

to the sample, and the mixture was incubated at 37 °C, overnight. Other enzymes used for protein digestion included chymotrypsin and Glu-C. Though no digested products were observed with Glu-C. Digested peptides were extracted using 100 ul of 0.1% Trifluoroacetic acid in water and 100% acetonitrile (1:1 ratio of 0.1% TFA in water and 100% ACN) and vacuum dried and dissolved in 5µl of Tris buffer. External calibration of the instrument was done with standard peptide (PEPMIX Mixture) supplied by Bruker, with masses ranging from 1046 to 3147 Da. Further analysis was done with FLEX ANALYSIS SOFTWARE (Version 3.3) in reflectron ion mode with an average of 500 laser shots at a mass detection range between 500 to 5000 m/z for obtaining the MS /MS. The masses obtained in the MS/MS were submitted for Mascot search in the "Red Algae" database to identify the protein.

2.2.2.10 Stability Analysis of Purified Protein

2.2.2.10.1 Reagents and Chemical

A) 0.1 M Sodium citrate buffer (pH 3) 1 litre

Sodium Citrate dihydrate 25.703 g

Citric Acid 2.421 g

Distilled water 1000 ml

Adjusted the pH with 1N HCl

B) Sodium citrate buffer (pH 5)

C) 0.1M Tris-Cl buffer (pH 7.2) 1 litre

Tris 12.1g

Distilled water 1000 ml

Adjusted the pH with 1N HCl

D) Tris-Cl buffer (pH 7.4)

E) Tris-Cl buffer (pH 9)

F) Tris-Cl buffer (pH 10)

G) Tris-Cl buffer (pH 11)

H)1% Sodium dodecyl sulphate

I) 1% Tween-20

J) 1% Tween-80

K) 1% Urea

L) 1% Triton X-100

2.2.2.10.2 Stability Analysis

Protein's sensitivity to various physicochemical factors like temperature, pH, reducing agents and surfactants were analyzed **(Taggar et al., 2021).**

A) Effect of Temperature

The purified protein was subjected to a range of temperatures, i.e. 40 °C, 50 °C, 60 °C, 70 °C, and 100 °C for 30 minutes and 121 °C for 20 minutes. Protein activity was tested using a well diffusion assay. Test bacteria used in the study were *Bacillus cereus* and *Salmonella enterica*. Partially purified protein without any temperature treatment was used as a control. 50 µl of protein samples were loaded into the wells on an LB agar plate and incubated at 37 °C for 24 hours to observe the zone of clearance.

B) Effect of pH

To study the pH sensitivity the purified protein sample was added to different buffers i.e., citrate phosphate buffer (pH 3 and 5), and Tris-HCl (pH 7.2, 7.4, 9, 10, and 11), followed by incubation at 37 °C for 2 hours. Treated and untreated control protein samples were added to *Bacillus cereus* and *Salmonella enterica* plates, using agar well diffusion assay, and observed for the zone of clearance.

C) Effect of Detergents

Surfactants and detergents used in the analysis included Sodium dodecyl sulfate, Tween-20, Tween-80, Urea, and Triton X-100. Each detergent was prepared as 1% and used in a 1:1 ratio with protein. The mixtures were incubated for 2 hours at 37 °C and tested for protein activity by agar well diffusion assay, followed by overnight incubation for up to 24 hours to observe and compare the zone of clearance against *Bacillus cereus* and *Salmonella enterica.* All physicochemical analysis assays were performed in triplicates with twoindependent sets of experiments to obtain conclusive results.

2.2.2.11 *In Vitro* Analysis of Antimicrobial Activity of Dinb Protein

2.2.2.11.1 Agar Well Diffusion Assay

To confirm the bactericidal activity of the purified protein, test strains were grown on LB media by spread plate method. 6 mm wide wells were made with the help sterile borer,and 50 µl of the purified protein was added to respective wells, and incubated at 37 ºC for 24 hours.

2.2.2.13 Determination of Minimum Inhibitory Concentration (MIC)

A) Inoculum Preparation

A single colony of *Bacillus cereus* and *Salmonella enterica* was inoculated in Luria Bertani broth and grown overnight. From the primary grown bacteria, a secondary culture was initiated. Cells were grown till optical density (600 nm) reached between 0.8 to 1. Cells were normalized to 10^5 CFU/ml and used further in the experiment.

B) Assay

Micro-dilution broth assay was performed using a 96-well microtiter plate **(Yi et al., 2018)**. 100 µl of sterile Luria Bertani broth was distributed in the first eight wells and 100 µl of the purified protein (1.8 mg/ ml) was added to the first well. The content of the first well was mixed well and 100 µl of this mixture was added to the second well, making it a two-fold dilution. The process was repeated till well 8. To each well 100 µl of test pathogen cells (10^5 CFU/ml) were distributed. The microtiter plate was incubated for 16 hours at 37 °C. 10 µl of reaction mixture from each well was spread on LB agar plates to observe the growth of bacteria. The lowest concentration which hampered the bacterial growth was concluded to be the minimum inhibitory concentration.

2.2.2.14 Time Kill Kinetics

The assay is an *in vitro* assay, used to measure the changes in cell growth and inhibition against a particular bacteriostatic or bactericidal agent within a given time interval.

A) Inoculum Preparation

Inoculum of both bacteria was prepared as described in 2.3.2.13

b) Inoculation and Determination of Microbial Population

The antibacterial properties of purified protein were assessed by counting the colony number of pathogenic bacteria, collected at different time intervals **(Jangra et al., 2019).** The 96-well microtiter plate was inoculated with 100 µl of 10^5 CFU/mL of *Bacillus cereus* and *Salmonella enterica* separately with double the MIC concentration of purified protein (2×9.78 µM) in LB broth. The sample fractions from 96 well plates were collected at different time intervals i.e. 0 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours. Collected sample fractions were plated on LB agar plates, using the spread plate method. Plates were incubated at 37 °C for up to 24 hours and bacterial colonies were calculated. All the experiments were performed in triplicates with two

independent sets of experiments to obtain conclusive results.

C) Microbial Log10 Reduction Calculation:

The primary bacterial population of both strains was in the control culture and was converted into a $\log_{10}$ population. Further, bacterial colonies that appeared on LB media plates were calculated and also converted to $\log_{10}$ values. All colony-forming units were calculated in duplicates, converted into $\log_{10}$, and then mean and variance were calculated.

The $\log_{10}$ reduction was calculated by the following formula:

$\log_{10}$ Reduction (LR) = Mean $\log_{10}$ (Initial microbial population) - Mean $\log_{10}$ (surviving microbial population)

The standard error of the mean was calculated by using the formula given below:

Standard Error = Square root of [(Variance of the $\log_{10}$ measured initial microbial population/ Number of replicates) + (Variance of the $\log_{10}$ surviving microbial population/Number of replicates)]

Percentage reduction in growth was calculated using the formula given below

Percent reduction (%) = 100 X (1 - 10LR)

2.2.2.15 Action Mechanism Study

An action mechanism study was performed as described by Wilson et al., **(2017).** Extracellular and intracellular changes in *Bacillus cereus* and *Salmonella enterica* were studied using a compound microscope. Inoculum was prepared using a single colony for individual bacteria and inoculating it in LB broth. The secondary culture was grown till $O.D._{600nm}$ reached between 0.8-1. Cells were normalized to 2×10^6 CFU/ml. Cell concentrations of 2×10^6 CFU/ml of both test pathogens were added to 2×MIC (9.78 μM) of purified protein, and incubated for 4 hours at 37 °C. Cells were thrice washed with 0.1 M of 1X phosphate buffer saline (pH 7.4) and stored in 1X PBS only. Gram-stained samples were observed under 40 X and 100 X magnification of a compound microscope. The test was performed in triplicates.

2.2.2.16 Scanning Electron Microscopy

Scanning electron microscopy was used to examine the protease-treated bacterial cells. For both test bacteria (*Bacillus cereus* and *Salmonella enterica*) 2×10^6 CFU/ml bacterial cells were mixed with 2×MIC (9.78 μM) of purified protease, incubated for

four hours, followed by treating the cells with 1X phosphate buffer saline.

Specimen Preparation for SEM

Treated and untreated control cells were washed with PBS three times followed by centrifugation at 3000 rpm for 5 minutes **(Nalezinková et al., 2024)**. Buffer was removed from the microcentrifuge tubes and the cells were incubated with 200 µl of 10 % formalin for 24 hours at 4 ºC. The cell samples were removed from 4 ºC and gently washed with 1X PBS three times. The cell pellets were dehydrated with the treatment of ethanol of variable concentrations i.e. 30 %, 50 %, 70 %, 90%, and 100 %. The ethanol was used from low to high concentration, followed by 10-minute incubation each time and 1-hour incubation with 100 % ethanol. Completely dehydrated samples were taken to the SEM facility for observation. The dehydrated samples were placed on a sample grid and gold coating was done to observe the external bacterial surface. The SEM JOEL JSM-7610F Plus model was used for analysis.

2.2.2.17 Statistical Analysis

The statistical analysis of data was done using Sigma Plot 12.0 and one-way analysis of variance (ANOVA). Values of probability i.e. $p > 0.05$ were considered non-significant while $p < 0.001$ was taken as significant.

2.3 RESULTS

2.3.1 Antimicrobial Compound Production Kinetics

The antimicrobial activity of *Bacillus clausii* UBBC07 was tested against *Salmonella enterica* (as the indicator strain). The synthesis of antimicrobial compounds started after 90 hours of incubation. It reached its maximum levels during 96 hours and gradually reduced after 120 hours. The antimicrobial compounds were secreted after cells entered the sporulation phase (during the stationary phase) **(Figure 2), (Figure 3).** The maximum activity of secreted compounds was calculated as the maximum zone of clearance.

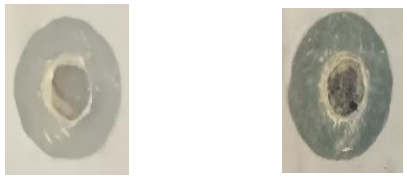


Figure 2a. ***Bacillus cereus*** **2b.** ***Salmonella enterica***

Figure 2. Agar well diffusion assay with 96 hours incubated supernatant in Muller Hinton broth, a zone of clearance appeared against both pathogens, *Bacillus cereus* and *Salmonella enterica.*

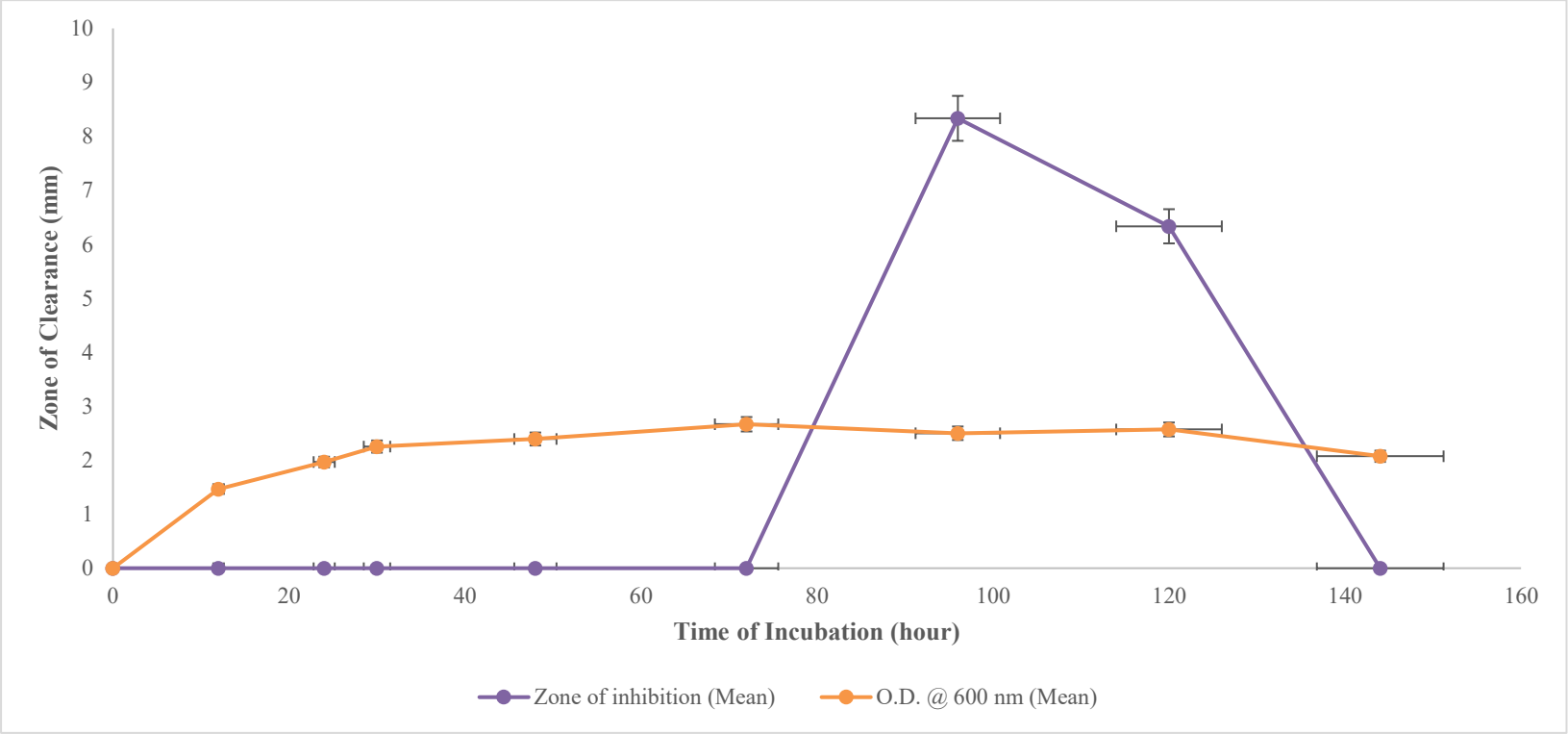


Figure 3. Bacterial growth and production kinetics of *Bacillus clausii* UBBC07; the antimicrobial compound production was expressed in millimeters. Data was presented as Mean ± SEM of two independent experiments with duplicates each time.

2.3.2 Identification, Purification, and Structural Characterization of Antimicrobial Compound

2.3.2.1 Gel Overlay Assay

Partially purified protein was run 15% Tricine SDS PAGE and one part of the gel was subjected to Coomassie blue staining and zymography was performed with another part of the gel. The test strains used were *Bacillus cereus* and *Salmonella enterica.* A zone of clearance appeared near 25 kDa when compared to the protein marker on Petri dishes **(Figure 4).**

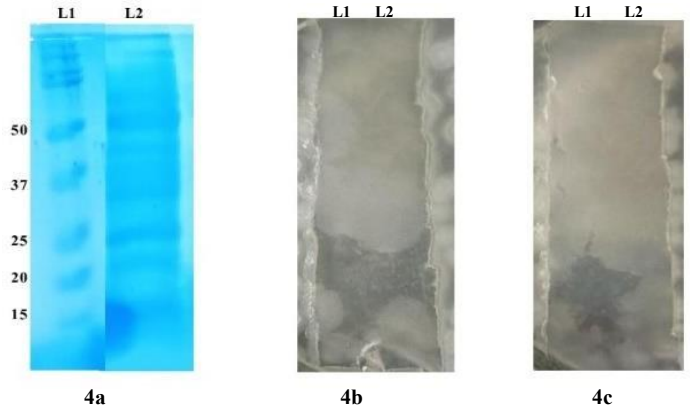


Figure 4 (a) Coomassie-stained gel of partially purified extracellular proteins (L2) along with protein marker (L1). (4b, 4c) Gel overlay assay on 1% agar showing clearance zone against both *Bacillus cereus* and *Salmonella enterica* respectively confirming the antimicrobial nature of the purified protein.

2.3.2.2 Protein Purification

After confirming the target protein band, it was excised, and treated with protein elution reagents, and the purified protein was collected **(Figure 5a).** The slope y = 0.0007x + 0.0981 of the standard curve was used to calculate the purified Dinb proteinconcentration **(Figure 5b).**

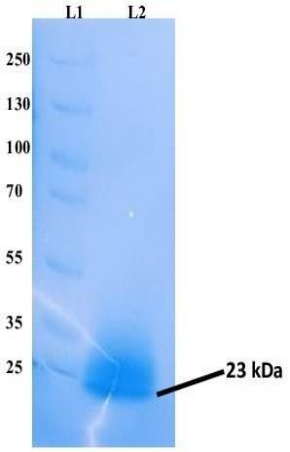


Figure 5a. Purified protein on 15% SDS PAGE.

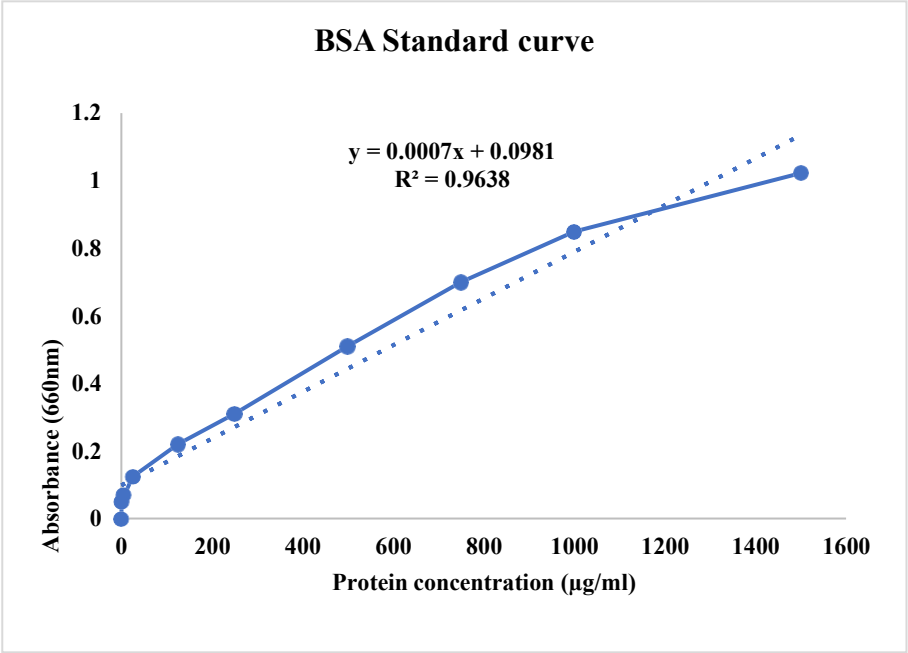
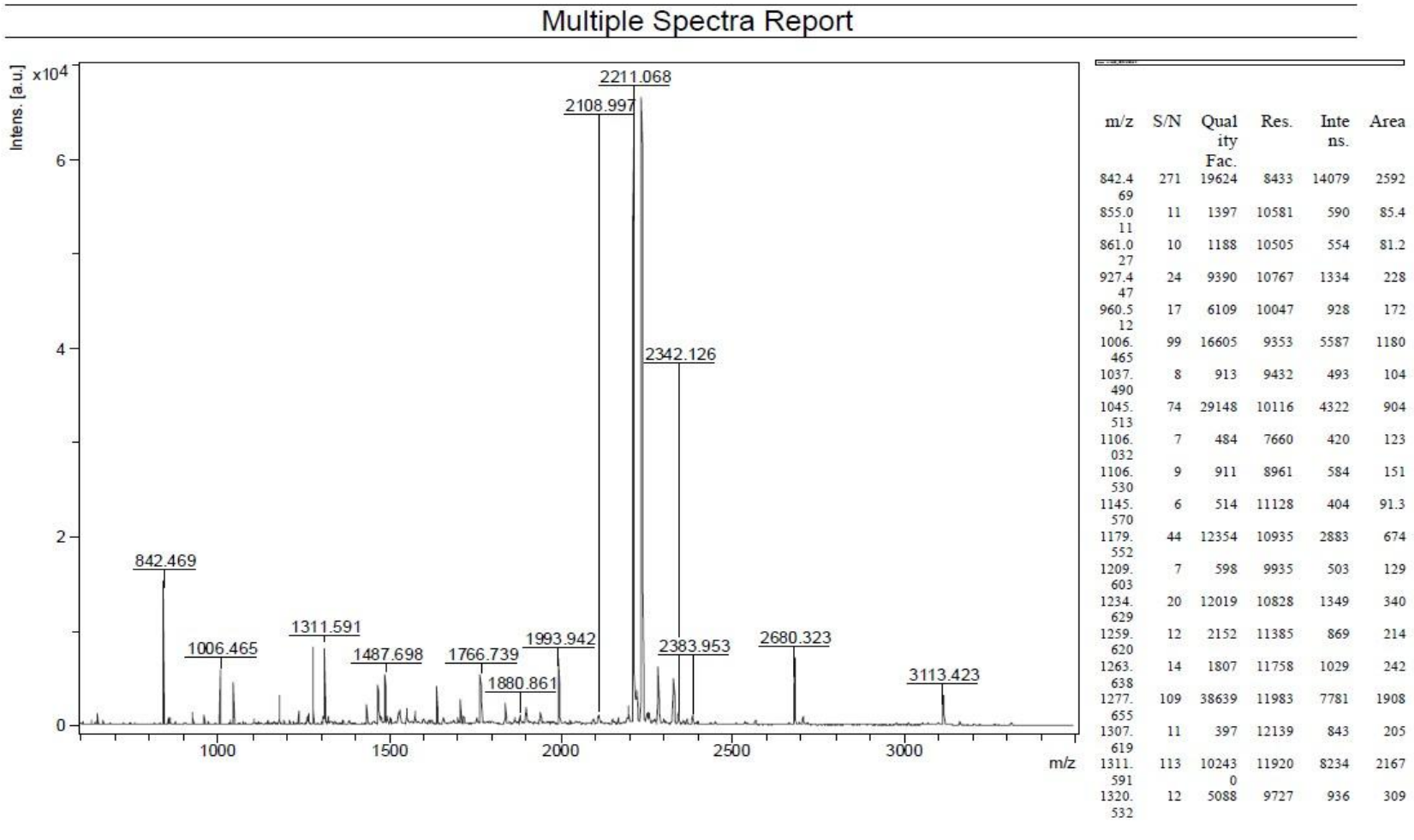


Figure 5b. Standard curve of Bovine serum albumin.

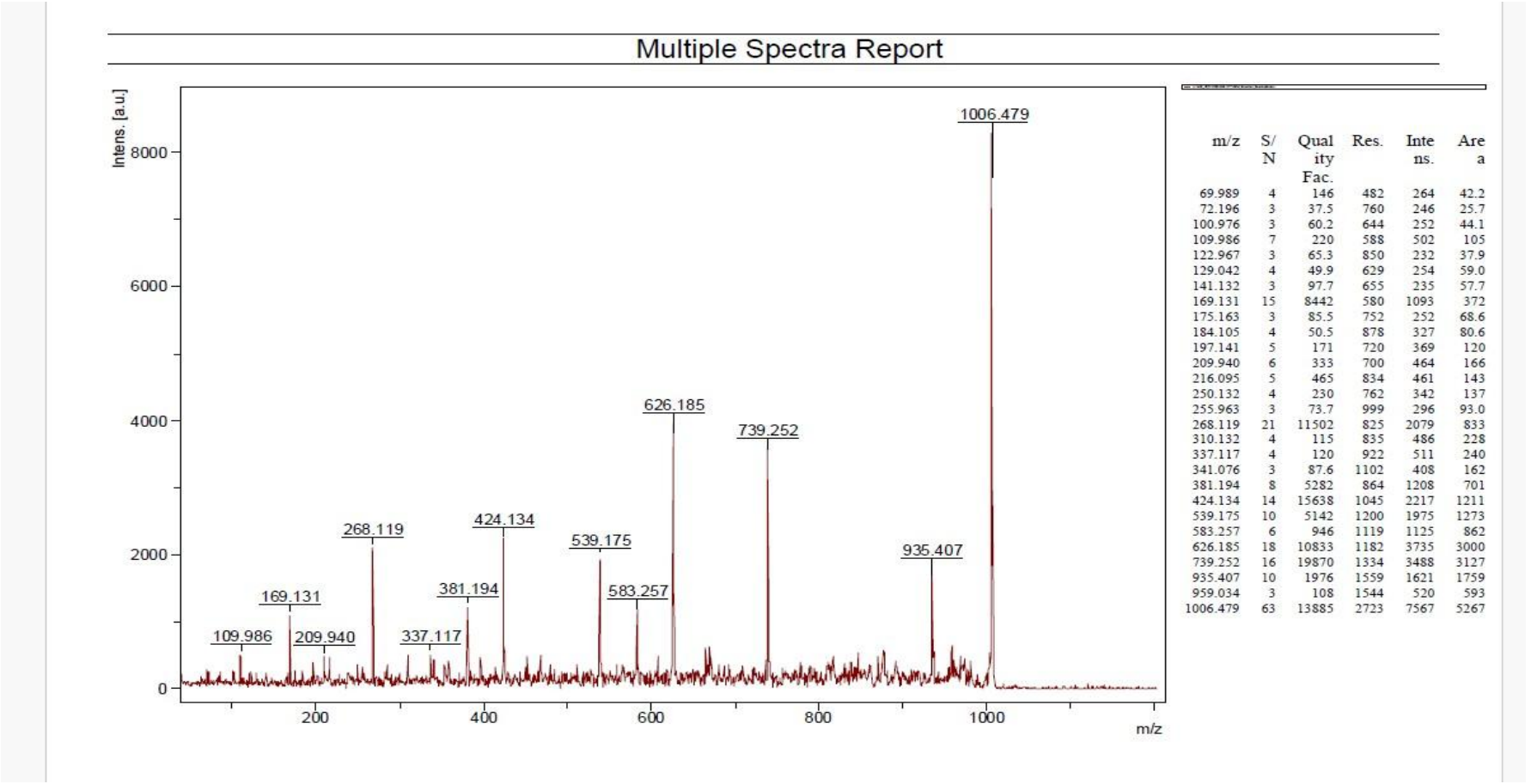
2.3.3 Protein Molecular Mass and Sequence Determination

Mass spectrometry analysis of the target protein **(Figure 6a, b, c, d, e, f, g)**, showed the molecular mass of the target protein to be 23460 Daltons. Peptide mass fingerprinting revealed the mass detection range between 500 to 5000 m/z. The obtained peptide sequences were analyzed using FLEX ANALYSIS SOFTWARE (Version 3.3) using Mascot search in the "*Bacillus*" database to identify the protein. The identified peptide fragment sequences of the target protein showed 36% sequence similarity to the DinB family protein (Accession number AQA268P3I3) of *Souchella clausii* (KSM-16) (**Figure 7**).



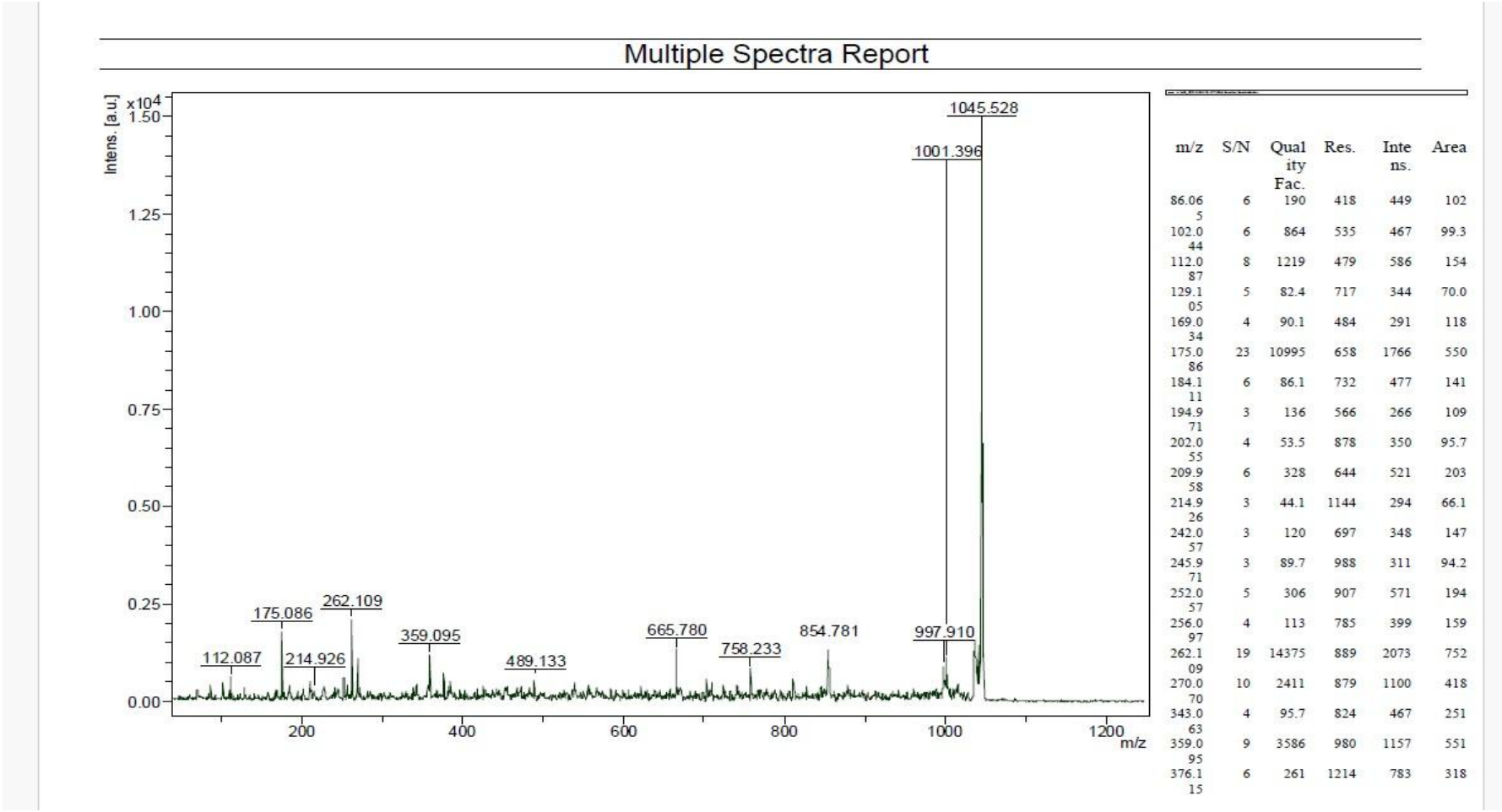
m/z	S/N	Quality Fac.	Res.	Intens.	Area
842.469	271	19624	8433	14079	2592
855.011	11	1397	10581	590	85.4
861.027	10	1188	10505	554	81.2
927.447	24	9390	10767	1334	228
960.512	17	6109	10047	928	172
1006.465	99	16605	9353	5587	1180
1037.490	8	913	9432	493	104
1045.513	74	29148	10116	4322	904
1106.032	7	484	7660	420	123
1106.530	9	911	8961	584	151
1145.570	6	514	11128	404	91.3
1179.552	44	12354	10935	2883	674
1209.603	7	598	9935	503	129
1234.629	20	12019	10828	1349	340
1259.620	12	2152	11385	869	214
1263.638	14	1807	11758	1029	242
1277.655	109	38639	11983	7781	1908
1307.619	11	397	12139	843	205
1311.591	113	102430	11920	8234	2167
1320.532	12	5088	9727	936	309

Figure 6a



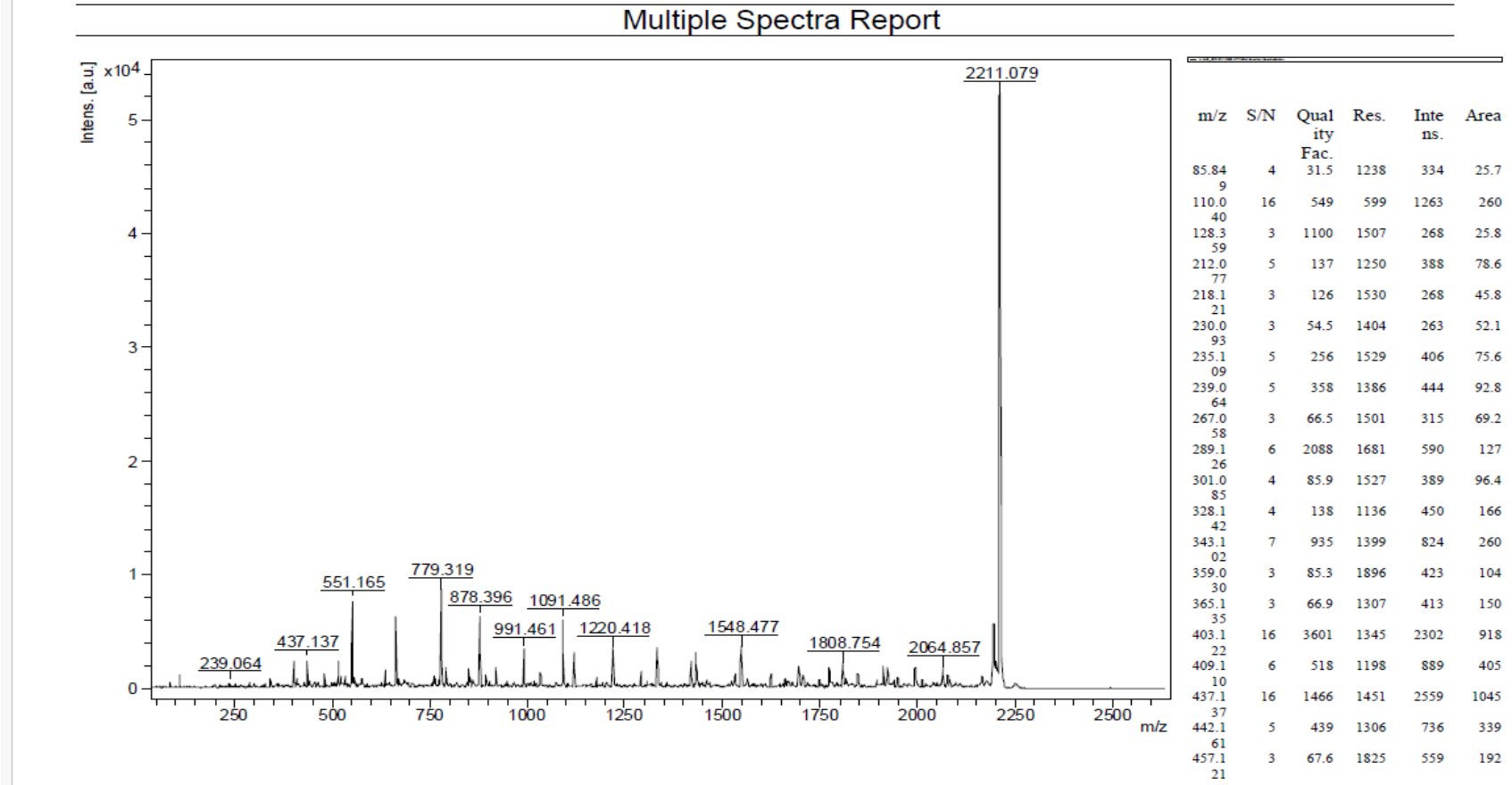
m/z	S/N	Quality Fac.	Res.	Intens.	Area
69.989	4	146	482	264	42.2
72.196	3	37.5	760	246	25.7
100.976	3	60.2	644	252	44.1
109.986	7	220	588	502	105
122.967	3	65.3	850	232	37.9
129.042	4	49.9	629	254	59.0
141.132	3	97.7	655	235	57.7
169.131	15	8442	580	1093	372
175.163	3	85.5	752	252	68.6
184.105	4	50.5	878	327	80.6
197.141	5	171	720	369	120
209.940	6	333	700	464	166
216.095	5	465	834	461	143
250.132	4	230	762	342	137
255.963	3	73.7	999	296	93.0
268.119	21	11502	825	2079	833
310.132	4	115	835	486	228
337.117	4	120	922	511	240
341.076	3	87.6	1102	408	162
381.194	8	5282	864	1208	701
424.134	14	15638	1045	2217	1211
539.175	10	5142	1200	1975	1273
583.257	6	946	1119	1125	862
626.185	18	10833	1182	3735	3000
739.252	16	19870	1334	3488	3127
935.407	10	1976	1559	1621	1759
959.034	3	108	1544	520	593
1006.479	63	13885	2723	7567	5267

Figure 6b



m/z	S/N	Quality Fac.	Res.	Intens.	Area
86.065	6	190	418	449	102
102.044	6	864	535	467	99.3
112.087	8	1219	479	586	154
129.105	5	82.4	717	344	70.0
169.034	4	90.1	484	291	118
175.086	23	10995	658	1766	550
184.111	6	86.1	732	477	141
194.971	3	136	566	266	109
202.055	4	53.5	878	350	95.7
209.958	6	328	644	521	203
214.926	3	44.1	1144	294	66.1
242.057	3	120	697	348	147
245.971	3	89.7	988	311	94.2
252.057	5	306	907	571	194
256.097	4	113	785	399	159
262.109	19	14375	889	2073	752
270.070	10	2411	879	1100	418
343.063	4	95.7	824	467	251
359.095	9	3586	980	1157	551
376.115	6	261	1214	783	318

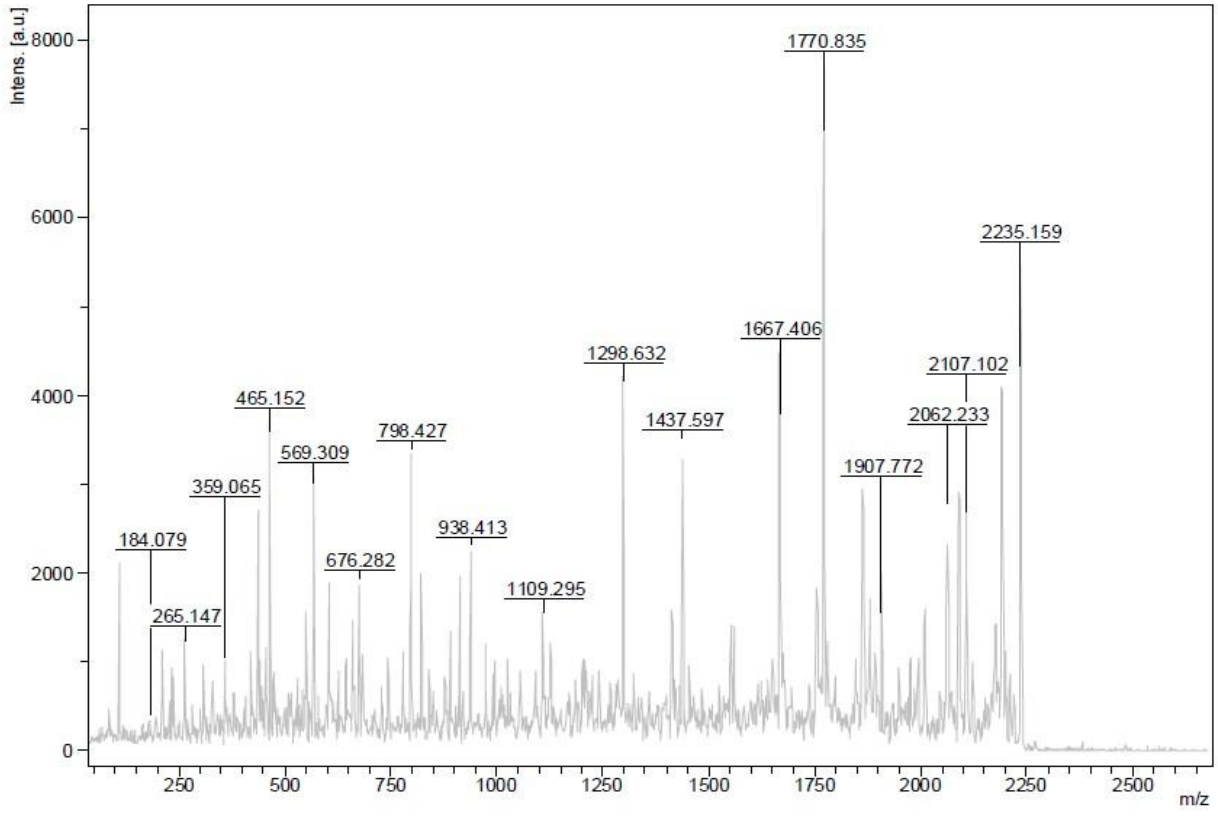
Figure 6c



m/z	S/N	Quality Fac.	Res.	Intens.	Area
85.849	4	31.5	1238	334	25.7
110.040	16	549	599	1263	260
128.359	3	1100	1507	268	25.8
212.077	5	137	1250	388	78.6
218.121	3	126	1530	268	45.8
230.093	3	54.5	1404	263	52.1
235.109	5	256	1529	406	75.6
239.064	5	358	1386	444	92.8
267.058	3	66.5	1501	315	69.2
289.126	6	2088	1681	590	127
301.085	4	85.9	1527	389	96.4
328.142	4	138	1136	450	166
343.102	7	935	1399	824	260
359.030	3	85.3	1896	423	104
365.135	3	66.9	1307	413	150
403.122	16	3601	1345	2302	918
409.110	6	518	1198	889	405
437.137	16	1466	1451	2559	1045
442.161	5	439	1306	736	339
457.121	3	67.6	1825	559	192

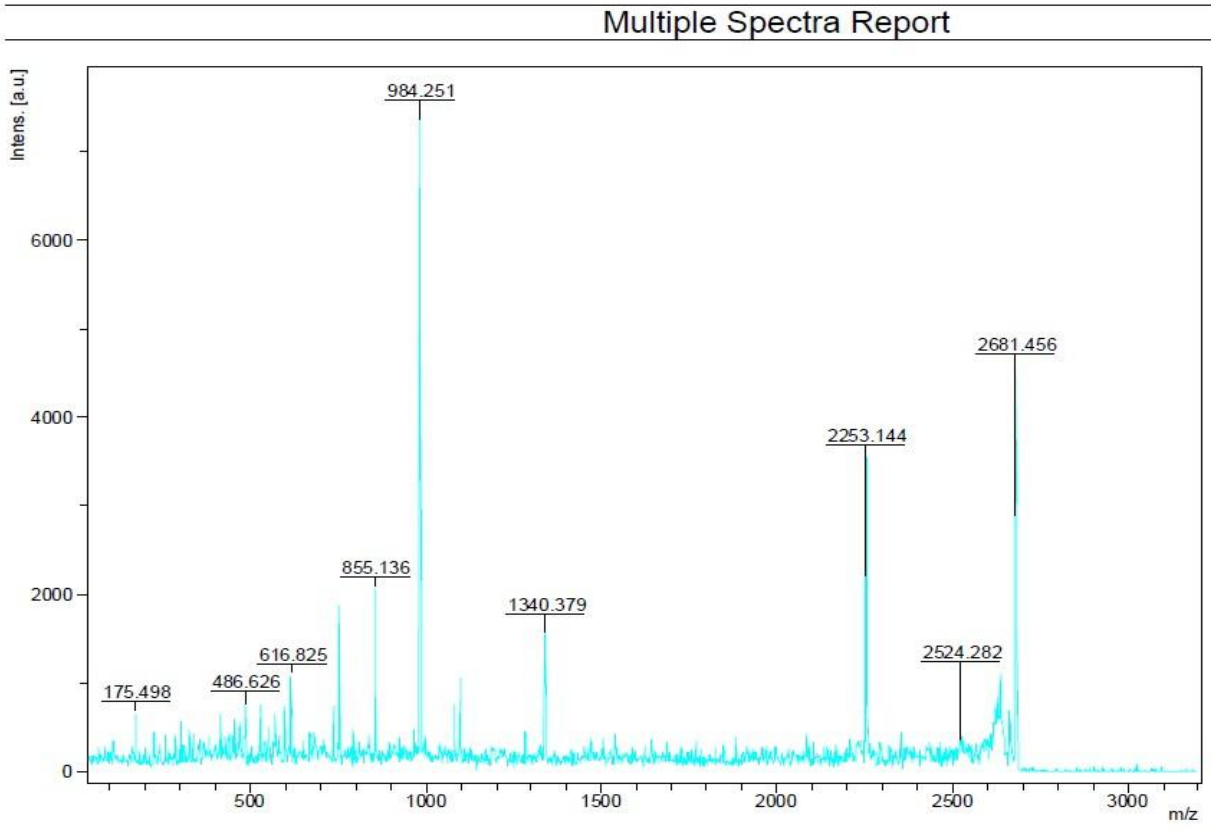
Figure 6d

Multiple Spectra Report



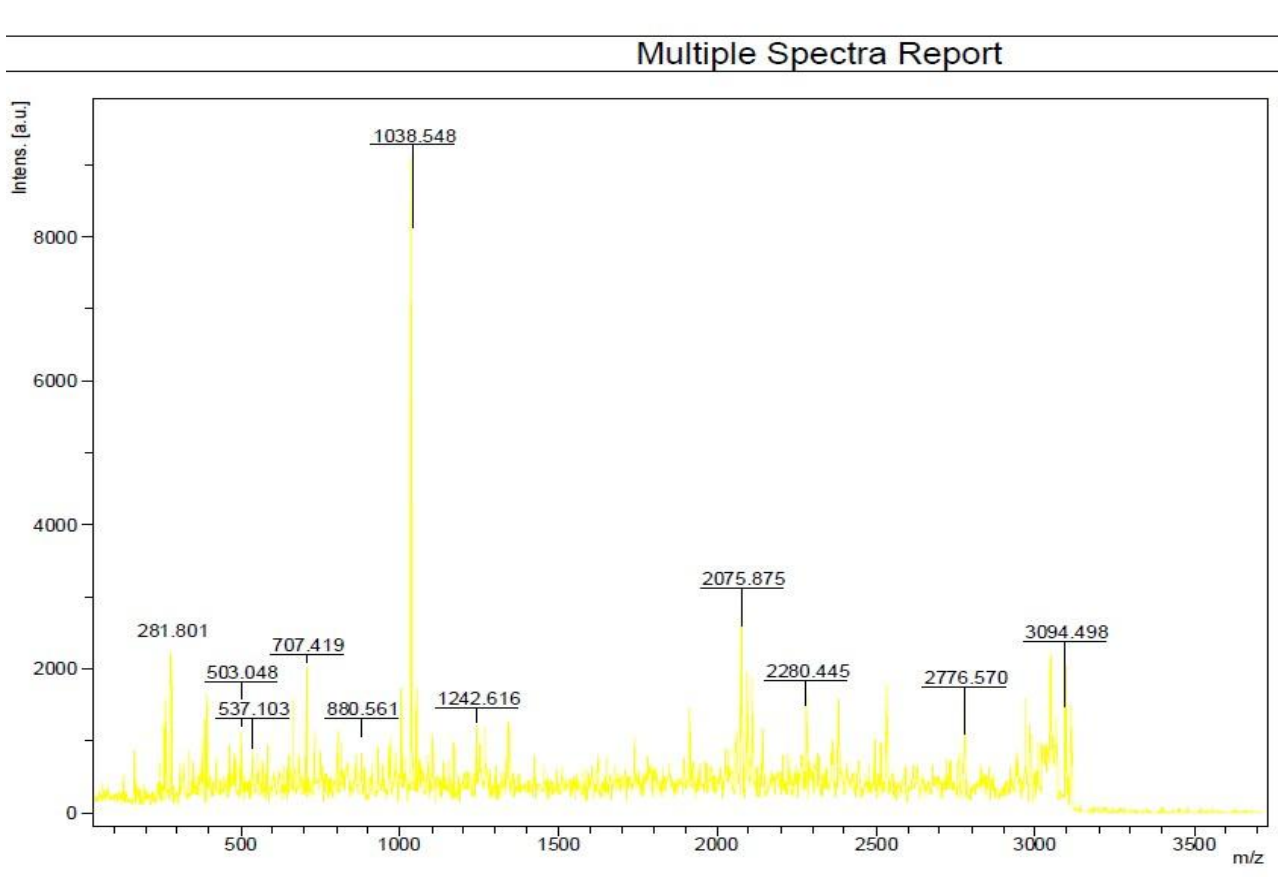
m/z	S/N	Quality Fac.	Res.	Intens.	Area
86.193	4	107	661	444	64.2
110.061	20	3095	463	2058	548
180.087	3	126	1302	334	54.1
184.079	3	203	818	339	89.8
197.181	4	114	1208	399	77.1
212.072	10	2142	1154	1110	244
230.085	5	1218	942	544	160
235.086	9	670	1623	1057	185
239.121	7	1904	861	812	274
252.094	3	84.7	1034	360	107
265.147	10	4316	1095	1161	346
283.113	4	99.2	1121	514	162
301.011	4	192	1918	466	91.9
310.115	7	517	1462	937	251
331.040	5	654	1185	750	268
343.091	3	269	1314	474	159
359.065	7	1140	1391	986	330
362.067	4	192	1720	631	173
365.113	3	40.9	2003	505	120
381.067	4	186	2108	638	152
409.161	3	142	1788	592	181
421.136	6	768	1966	1107	319
437.177	14	2836	1393	2641	1124
442.462	5	477	1280	992	466
447.172	4	297	1480	670	276
456.191	6	544	1107	1134	641
458.056	3	59.1	1177	646	345
465.152	18	11463	1309	3538	1731
473.137	5	103	1140	941	540
477.095	4	291	1899	769	268
516.190	3	64.1	1378	689	365
530.164	4	511	1707	798	354
551.176	7	1808	1830	1636	712
569.309	12	12018	1493	2946	1638
605.277	7	3453	1943	1771	826
627.149	3	213	1888	873	440
644.143	4	56.3	2113	1143	534
661.381	5	390	1515	1436	970

Figure 6e



m/z	S/N	Quality Fac.	Res.	Intens.	Area
67.775	3	710	999	279	20.7
86.233	3	48.0	778	278	34.1
110.269	4	47.6	599	358	73.9
164.096	3	143	1502	277	35.2
175.498	7	633	1166	627	110
225.502	5	93.6	1185	444	102
239.504	3	62.0	1217	300	71.5
258.540	5	257	1607	401	79.1
286.556	5	167	970	410	151
288.526	3	49.8	2005	276	49.5
303.561	6	1103	1638	543	127
308.478	3	66.3	1916	321	65.3
314.533	4	61.5	1583	341	85.8
326.494	5	154	1478	529	149
338.503	4	213	1290	400	135
357.541	4	42.5	1611	363	105
368.501	3	64.2	1443	358	119
382.561	4	52.9	1619	386	120
416.593	5	501	1748	610	195
430.514	3	73.3	1710	385	131
439.567	4	40.1	2203	462	125
455.549	5	153	1771	580	204
473.665	4	84.2	1377	527	251
486.626	6	250	1636	709	294
529.745	5	410	1461	720	373
552.639	4	42.4	1767	490	221
570.673	5	95.6	2055	664	269
599.766	5	325	1719	703	366
616.825	7	652	1553	1077	645
668.837	3	37.4	1759	509	301
737.030	5	146	1832	768	499
754.019	12	2925	1912	1982	1275
855.136	12	6343	1797	2031	1671
984.251	44	43442	1923	7300	6947
1082.264	4	99.7	2488	667	574
1099.209	6	520	2543	1036	893
1283.403	3	35.3	2557	468	521
1340.379	10	3281	1632	1504	2835

Figure 6f



m/z	S/N	Quality Fac.	Res.	Intens.	Area
166.851	5	887	915	824	175
247.524	4	121	1606	669	126
253.577	3	43.9	1834	512	86.5
259.636	7	2396	1567	1114	227
266.734	10	4191	1480	1514	336
281.801	14	3181	1331	2302	606
309.052	4	431	2025	677	130
319.069	3	243	1286	533	168
322.094	4	414	1698	724	175
337.160	5	1001	1709	807	204
352.343	4	251	1566	710	207
371.396	3	69.6	1739	610	171
380.471	6	1007	1747	1048	300
389.473	7	1580	2241	1321	303
395.525	9	1013	2202	1697	404
423.657	4	437	1734	775	255
467.805	5	150	2101	1004	308
478.893	4	141	2104	810	256
485.966	3	80.4	1812	760	284
503.048	5	1033	1776	1125	448
537.103	3	48.9	1610	823	393
554.103	3	112	2552	819	257
583.228	4	276	1712	888	445
651.394	3	167	1713	868	508
668.312	6	454	2238	1506	699
685.433	3	138	2123	848	430
707.419	7	2966	1773	2024	1283
733.461	4	198	2697	1211	531
806.520	3	271	2053	1071	708
819.534	3	95.2	2810	940	464
880.561	3	44.9	2388	988	639
972.611	3	97.6	3051	1052	619
1005.518	5	132	1989	1615	1537
1038.548	23	9014	2331	8032	6860
1054.590	5	565	1919	1751	1861
1105.449	3	41.9	2688	1160	956
1242.616	3	117	2778	1174	1139
2075.875	6	95.6	2575	2510	6422

Figure 6g

Figure 6. Mass spectra of peptide mass fingerprinting, showing the ionization peaks corresponding to the peptide sequences.

MASCOT Search Results

Protein View: A0A268P3I3

DinB family protein OS=Shouchella clausii OX=79880 GN=CHH72_06370 PE=4 SV=1

Database:	bacillus_clausii
Score:	39
Expect:	1,6
Monoisotopic mass (M_r):	23460
Calculated pI:	9,10

Sequence similarity is available as **an NCBI BLAST search of A0A268P3I3 against nr**.

Search parameters

MS data file:	peaklist.xml
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P,
Fixed modifications:	**Carbamidomethyl (C)**
Variable modifications:	**Oxidation (M)**
Mass values searched:	67
Mass values matched:	6

Protein sequence coverage: 36%

Matched peptides shown in ***bold red***.

```
  1 MPTGTASVYS MKGIMLATNS KLSHRPSASI ITPPFLLKIN AIFSCFRYTL
 51 TILPKGANTM TYAAFSYARM ANEQTLQTIP AEKHDIIPAG FKNSVHWNYG
101 HILVIADHVL GHAPTFEKTI PKEYYHPFAK GSSPLQWTDK VPSIETLQEA
151 AAKQQQAAQK LATETGGTEK TAPFTLRGQS FQTVDELLSI VAFHEGMHYR
201 TLLHYSNLFS N
```

Unformatted sequence string: **211 residues** (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
1 - 21	2220.2705	2219.2632	2219.0636	89.9	1	-.MPTGTASVYSMKGIMLATNSK.L + 2 Oxidation (M)
1 - 21	2235.8717	2234.8644	2235.0585	-86.8	1	-.MPTGTASVYSMKGIMLATNSK.L + 3 Oxidation (M)
119 - 130	1493.8307	1492.8234	1492.7714	34.8	1	K.TIPKEYYHPFAK.G
161 - 170	1006.5265	1005.5192	1005.4978	21.3	0	K.LATETGGTEK.T
178 - 200	2680.5178	2679.5105	2679.2751	87.8	0	R.GQSFQTVDELLSIVAFHEGMHYR.T + Oxidation (M)
201 - 211	1308.6275	1307.6202	1307.6510	-23.5	0	R.TLLHYSNLFSN.-

No match to: 842.6334, 855.1732, 861.1891, 927.6057, 960.6711, 1037.6553, 1045.6791, 1106.2053, 1106.7046, 1145.7502, 1179.7379, 1209.7943, 1234.8243, 1259.8198, 1263.8384, 1277.8584, 1311.8007, 1320.7433, 1365.8369, 1383.8754, 1434.9360, 1468.0229, 1469.9292, 1475.9663, 1487.9395, 1502.9617, 1526.9349, 1530.9449, 1550.9757, 1575.9911, 1637.0496, 1639.0767, 1658.0067, 1707.9943, 1717.0736, 1755.0978, 1767.0217, 1839.1638, 1881.1565, 1899.2480, 1941.2030, 1994.2494, 2109.3152, 2167.3323, 2193.3823, 2197.4338, 2211.3965, 2223.5334, 2249.3582, 2255.7336, 2258.4629, 2273.3896, 2283.4968, 2325.4766, 2328.4451, 2342.4683, 2367.5833, 2384.3008, 2705.5388, 3113.9568, 3161.9600

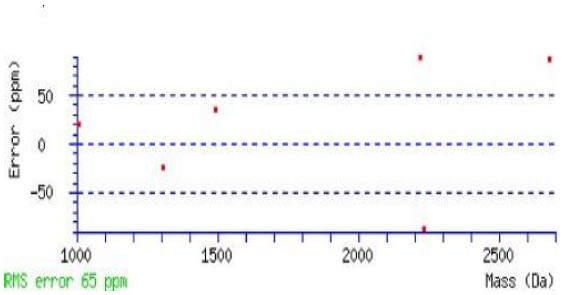


Figure 7. Protein analysis summary from MASCOT software.

2.3.4 Effects of Temperature, pH, and Detergents on Purified Protein Activity

Various physicochemical parameters like temperature, pH, and detergents were studiedon the efficacy of purified protein against pathogens *Bacillus cereus* and *Salmonella enterica.* Untreated purified protein was taken as a control for all experiments with 100 % stability. The average mean and standard deviation were used to describe proteinstability.

2.3.4.1 Effects of Temperature

Untreated purified protein was taken as a control for all experiments with 100 % stability. The average mean and standard deviation were used to describe protein stability. The protein remained stable at 37 ºC showing 100 % activity. At 40 ºC 96.8 % activity was retained against *Bacillus cereus.* At 50 and 60 ºC, 73 % of activity wasretained (**Figure 8**). At 70 ºC 50% activity, at 100 ºC 28 % activity was retained. Protein when subjected to autoclave at 121 ºC for 20 minutes completely lost its activity. Similar results were obtained for protein treatment when tested with *Salmonella enterica.* The protein retained 100 % activity from 37 ºC to 50 ºC. At 60 ºC, 80 % activity was retained, at 70 ºC and 100 ºC the activity was reduced to 65% and 37 % respectively. Also, protein treatment at 121 ºC for 20 minutes retained no activity. ANOVA analysis showed $p < 0.0007$ and $p < 0.0006$ for *Bacillus cereus* and *Salmonella enterica* respectively.

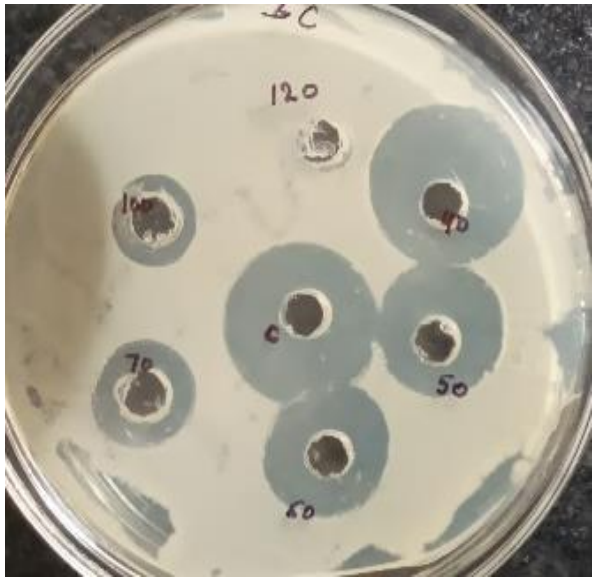


Figure 8a

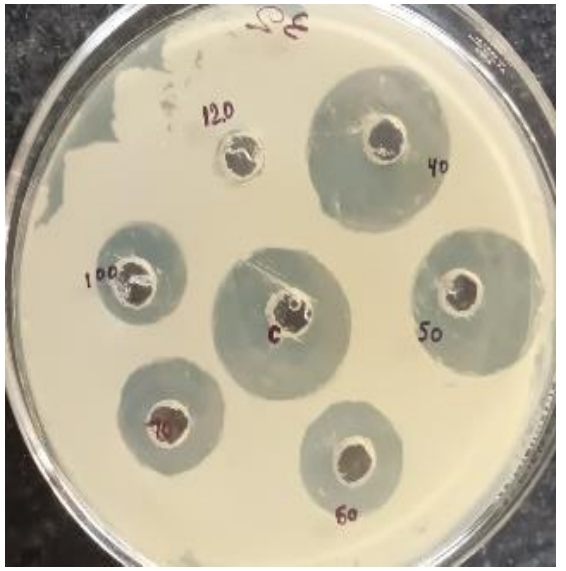


Figure 8b

Figure 8 (a, b). Zone of clearance on *Bacillus cereus* and *Salmonella enterica* plates, with test protein samples treated at different temperatures.

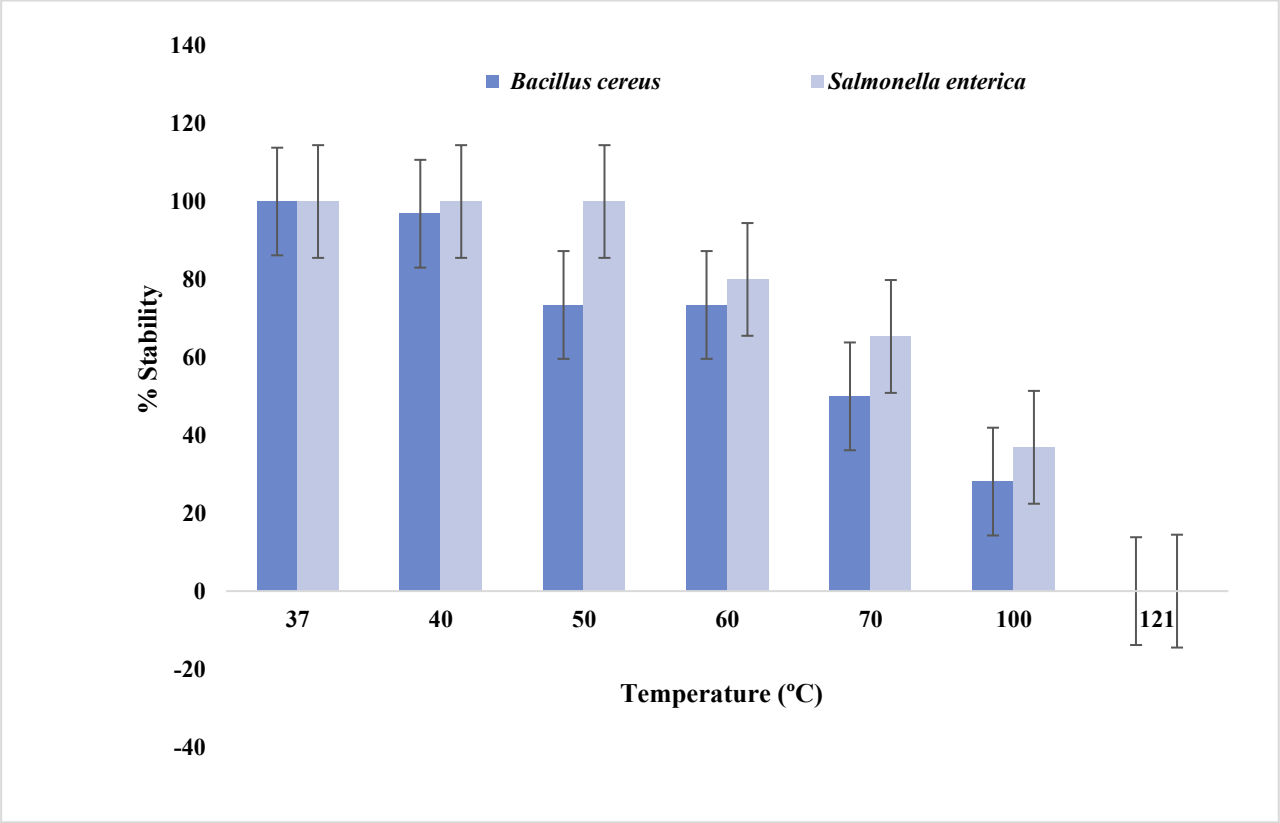


Figure 8c. Protein stability comparison after the treatment at a range of temperature for 30 minutes, and at 121 ºC for 20 minutes.

2.3.4.2 Effects of pH

Protein had 100 % stability at pH 7.2 and 7.4. For pH 3, 5, and 9, the protein decreased activity by 92%, 74.6%, and 85.7% against *Bacillus cereus* (**Figure 9**). Proteinretained 75% of its activity at pH 10, and 11. Similarly, *Salmonella enterica* protein showed 98.33% stability at pH 3 and 86.66 % at pH 5, and 9. A decrease to 78% and 73% for pH 10 and 11 respectively was observed. This concluded the protease to have an optimal activity between pH 7.2 to 7.4. The p values for *Bacillus cereus* and *Salmonella enterica* were $p < 0.001$ and $p < 0.004$.

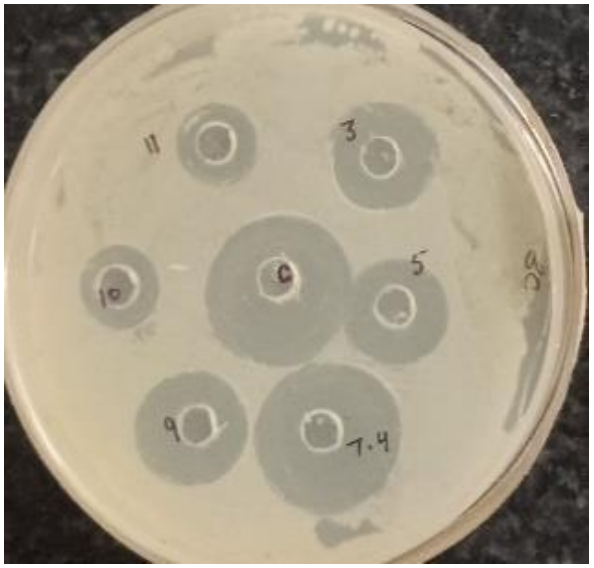


Figure 9a

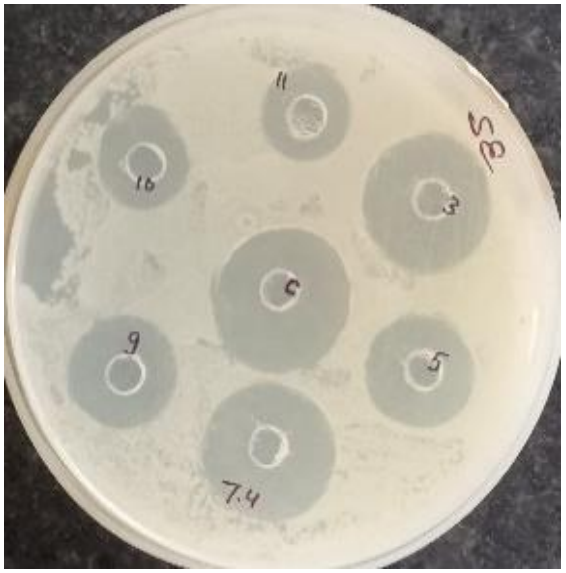


Figure 9b

Figure 9 (a, b). Zone of clearance on *Bacillus cereus* and *Salmonella enterica* plates, with test protein samples treated at different pH.

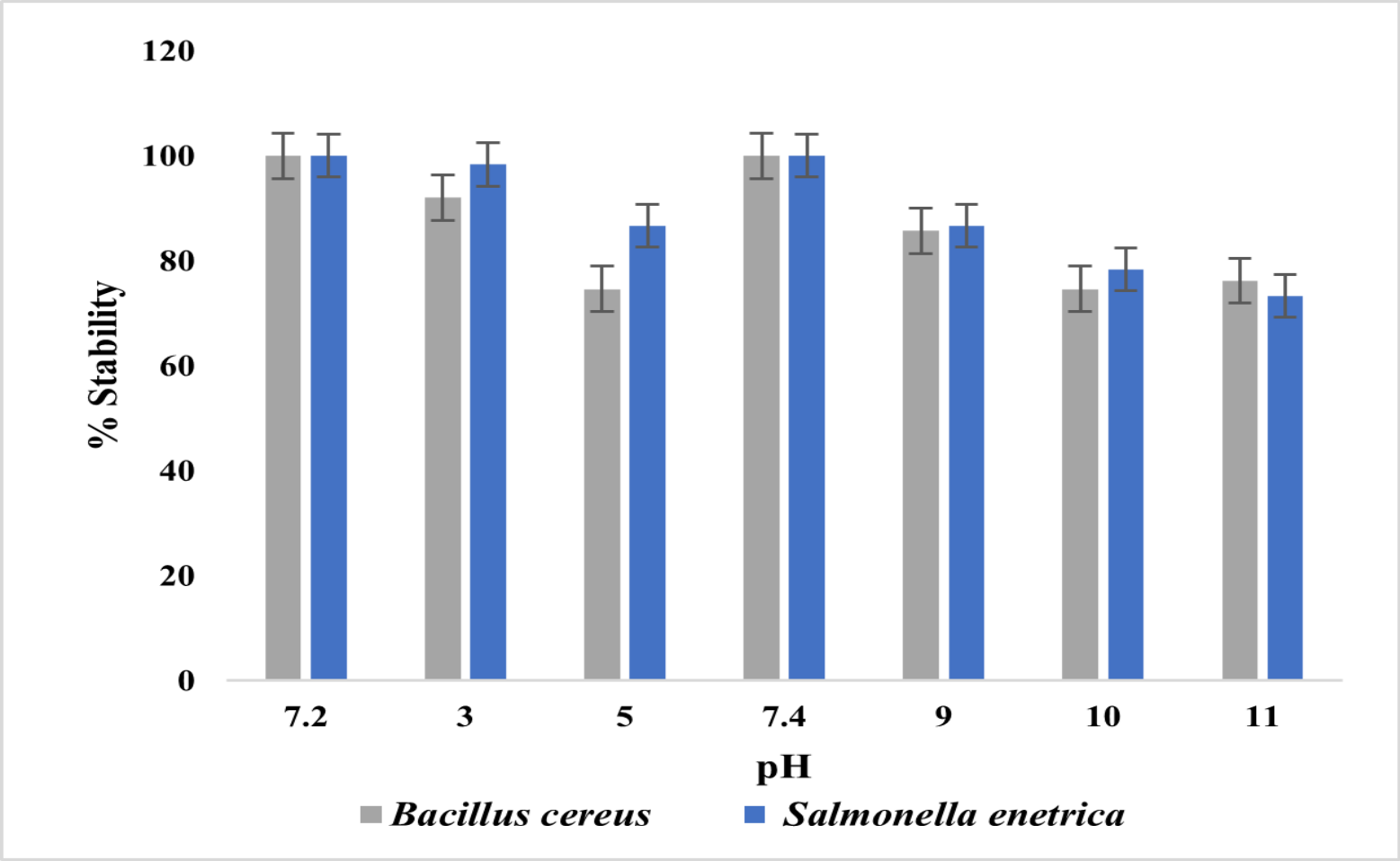


Figure 9c. Protein stability at different pH, the purified protein was treated with various buffers for 2 hrs at 37 ºC, and stability was observed as the zone of clearance against the pathogens.

2.3.4.3 Effects of Detergents

Protein treatment with Tween 20 and Tween 80, retained 98 % activity against *Bacillus cereus,* with urea 42 % activity retained. With Triton X-100 and sodium dodecyl sulfate, 93 % activity was recorded. Similarly for *Salmonella enterica* 98% activity wasretained with Tween 20 and Tween 80 **(Figure 10).** With urea, Triton X-100, and sodium dodecyl sulfate 49 %, 97 %, and 94.3 % of activities were retained. *p* values for *Bacillus cereus* and *Salmonella enterica* were < 0.00009 and < 0.00005.

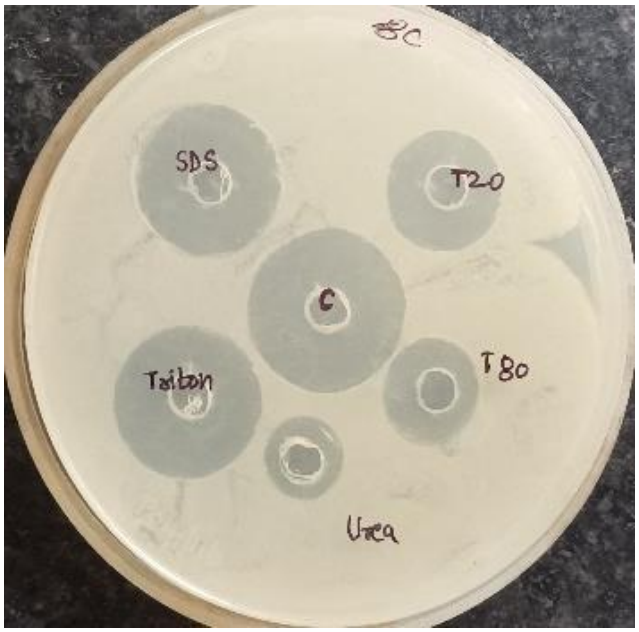


Figure 10a

Figure 10b

Figure 10 (a, b). Zone of clearance on *Bacillus cereus* and *Salmonella enterica* plates, with test protein samples treated with different detergents.

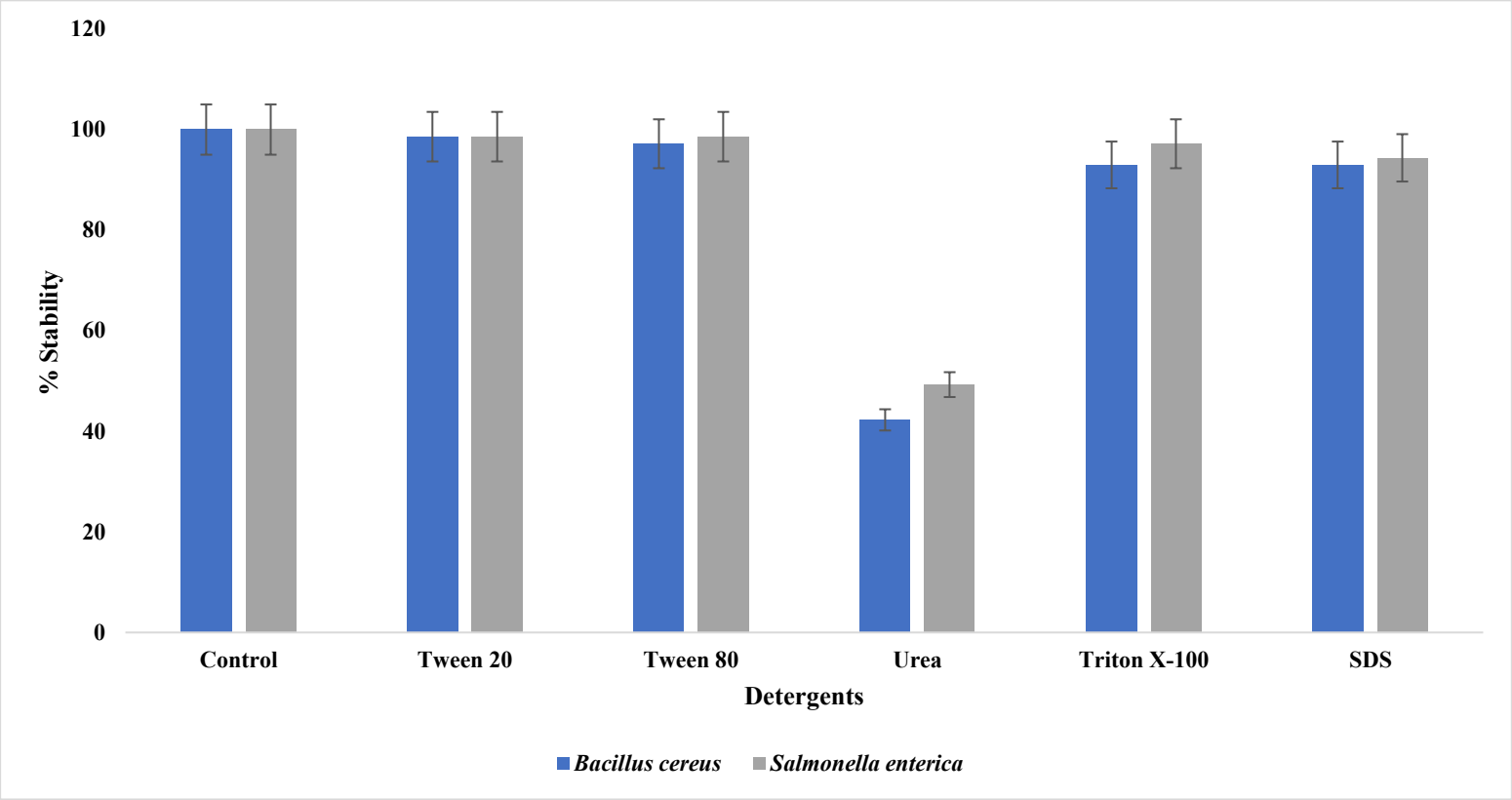


Figure 14 c. 100 µl of purified protein (22.4 µg) was treated with 1% detergents and reducing agents in 1:1 ratio for 2 hours at 37 ºC. An untreated pure protein sample was used as a control for all experiments.

2.3.5 *In Vitro* Analysis of Antimicrobial Activity

2.3.5.1 Agar Well Diffusion Assay

The Antimicrobial activity of purified protein Dinb protein was tested against various Gram-positive and Gram-negative diarrhea pathogens, including *Bacillus cereus, Salmonella enterica, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Pseudomonas putida,* and *Enterobacter cloacae.* The protein was active against Gram-positive and Gram-negative bacteria **(Table 2).**

Table 2. The bactericidal potential of purified protein against diarrhea pathogens, 50 µl of the purified protein was added to respective wells, and incubated at 37 ºC for 24 hours.

S. NO.	MTCC number of strains	Name of the test strain	Zone of clearance (mm)
1	MTCC 6629	*Bacillus cereus*	7
2	MTCC 1164	*Salmonella enterica*	6
3	MTCC 3159	*Enterococcus faecalis*	7
4	MTCC 1430	*Staphylococcus aureus*	8
5	MTCC 293	*Escherichia coli*	8
6	MTCC 1194	*Pseudomonas putida*	6
7	MTCC 7724	*Enterobacter cloacae*	5

2.3.5.2 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of purified protein against both target pathogens remained the same i.e. 9.78 µM (**Table 3**), **(Table 4).**

Table 3. The calculation of MIC values of purified protein (90 µg/ 100 µL) against *Bacillus cereus* and *Salmonella enterica.*

S. No.	1X Phosphate buffer (µl)	Protein (µl)	Bacterial culture (1×10^5 cfu/ml) (µl)	Dilution factor	Incubation for 16 hrs. and spread 10 µl on LB plates
Well 1	100	100	100	0	No growth
Well 2	100	100 µl of well 1	100	2^{-1}	No growth
Well 3	**100**	**100 µl of well 2**	**100**	**2^{-2}**	**No growth**
Well 4	100	100 µl of well 3	100	2^{-3}	Bacterial Growth
Well 5	100	100 µl of well 4	100	2^{-4}	Bacterial Growth
Well 6	100	100 µl of well 5	100	2^{-5}	Bacterial Growth
Well 7	100	100 µl of well 6	100	2^{-6}	Bacterial Growth
Well 8	100	100 µl of well 7	100	2^{-7}	Bacterial Growth

Table 4: MIC values of purified protein against *Bacillus cereus* and *Salmonella enterica* were obtained with micro-dilution broth assay. The lowest protein concentration (22.4 µg/ 100 µl) which hampered the bacterial growth was concluded to be the minimum inhibitory concentration.

S. No.	MTCC number of strains	Name of the test strain	MIC (µM) of protein
1	MTCC 6629	*Bacillus cereus*	9.78
2	MTCC 1164	*Salmonella enterica*	9.78

2.3.6 Time kill kinetics assay

Time kill kinetics assay was performed to analyze the killing of pathogenic strain cells (*Bacillus cereus* and *Salmonella enterica)* based on a variable time profile **(Table 5)**. A sharp decline in $\log^{10}$ (CFU/mL) from 20% to zero and 50% to zero for *Bacillus cereus* and *Salmonella enterica* respectively was obtained within four hours of treatment (**Figure 11**) confirming the antibacterial potential of purified protein.

Table 5. Time kill kinetics of *Bacillus cereus* and *Salmonella enterica*

S. No.	Time (in minutes)	CFU/ml for *Bacillus cereus* (Control)	CFU/ml for *Bacillus cereus* (Treatment)	CFU/ml for *Salmonella enterica* (Control)	CFU/ml (Treatment)
1	0	1×10^5	1×10^5	1×10^5	7.5×10^4
2	30	1×10^5	8.6×10^2	2×10^5	6×10^3
3	60	1×10^6	2.8×10^2	1×10^6	4×10^3
4	120	5×10^6	1×10^1	3.2×10^7	2.8×10^2
6	240	2×10^7	0	9×10^7	0
7	300	5×10^7	0	6×10^9	0

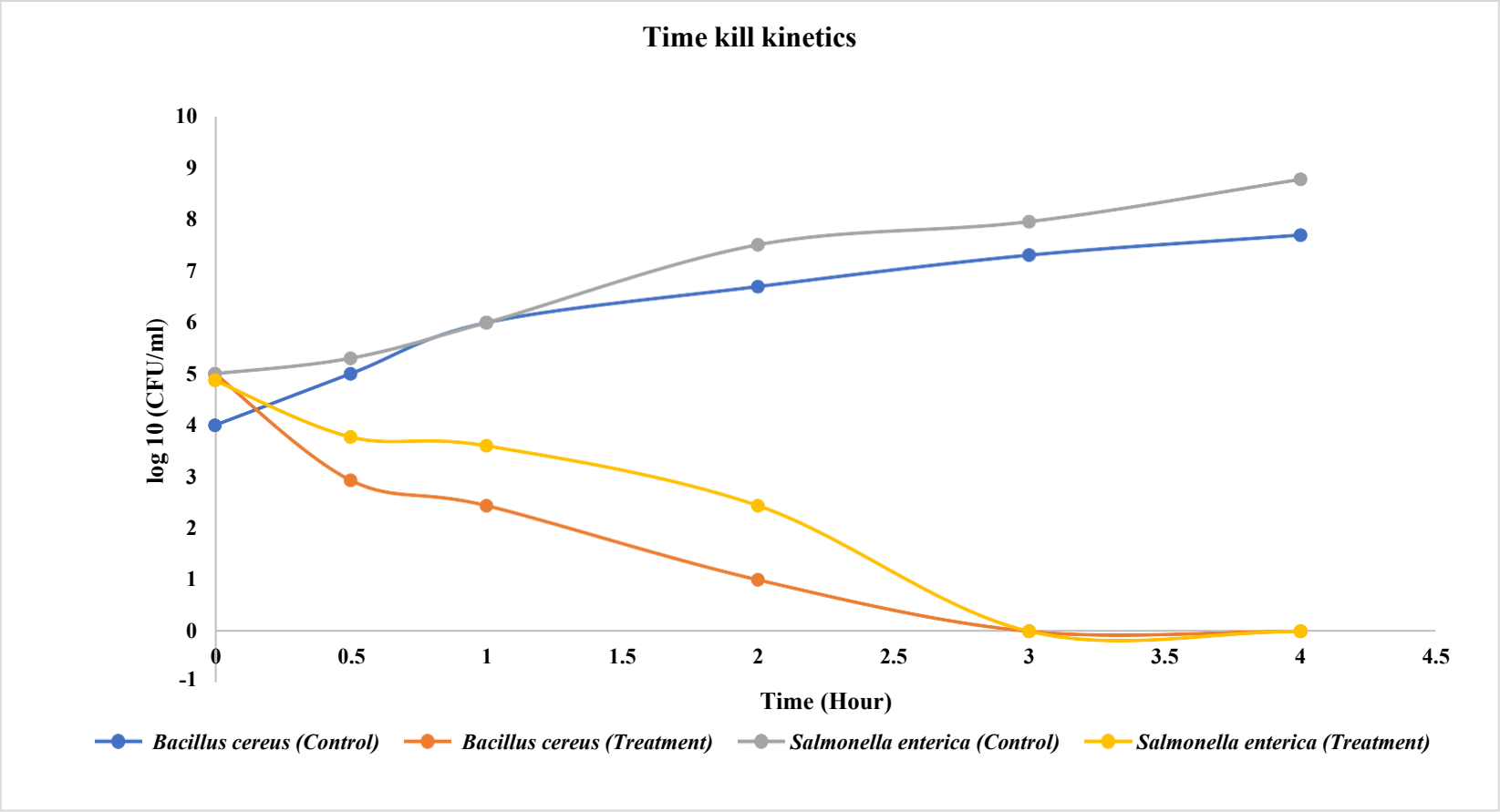


Figure 11. Time kill kinetics of purified protein against *Bacillus cereus* and *Salmonella enterica.* 2×MIC (9.78 µM) of protein was used against 2×10^6 CFU/ml of bacterial strains. Results were represented as a mean of two independent sets.

2.3.7 Action Mechanism Studies

Protein-treated bacterial cells were observed using compound microscopy. The cell membrane integrity was taken as a major of cell viability. Control and treatment cells were washed, Gram-stained, and observed for cell surface changes. The set of treatment cells from both bacteria appeared damaged and showed debris formation around healthy cells when compared to the control set. This confirmed the proteolytic property of purified protein (**Figure 12**).

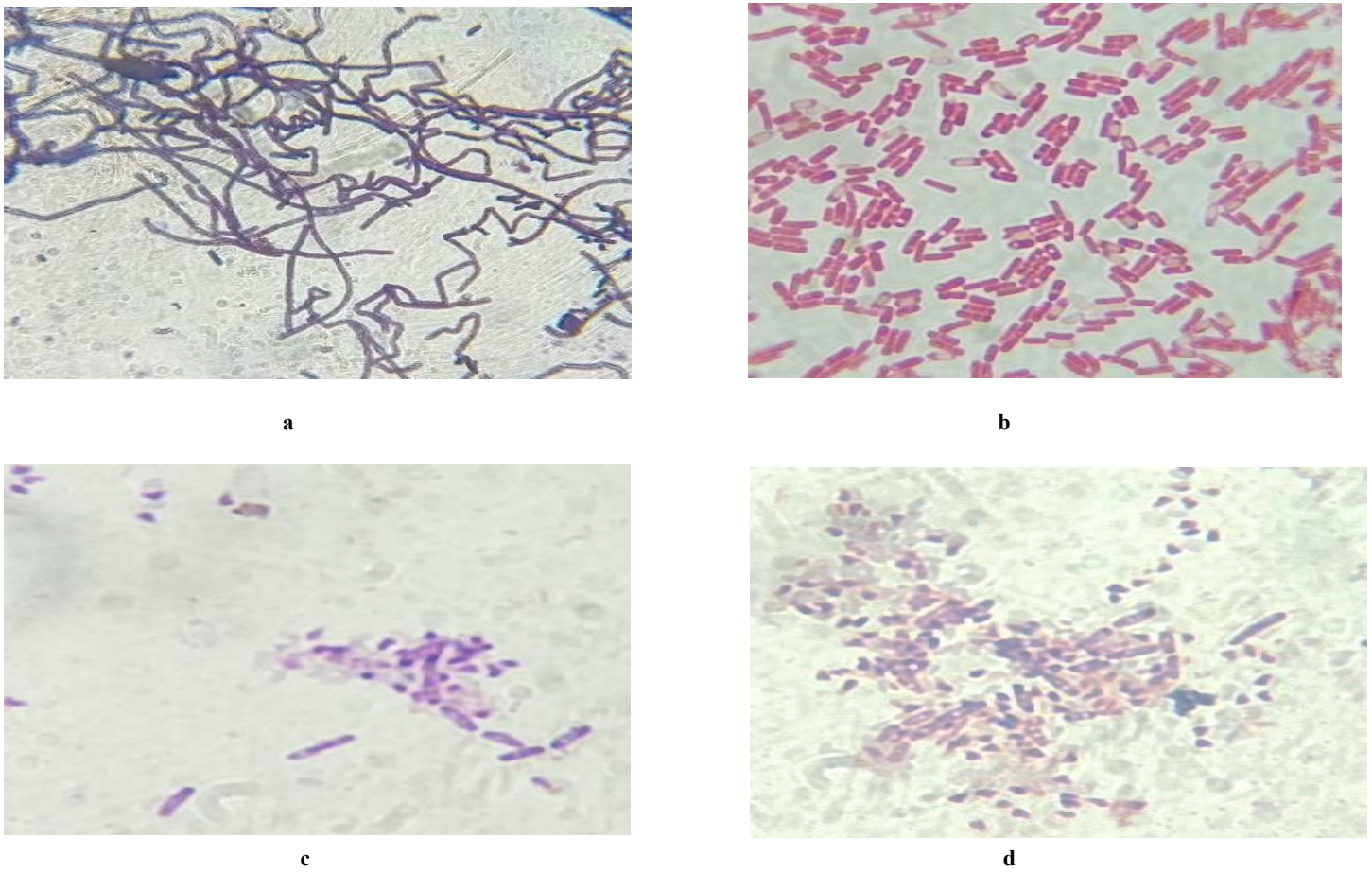


Figure 12. Gram-staining of *Bacillus cereus* and *Salmonella enterica.* (a, b) Untreated control group cells of *Bacillus cereus* and *Salmonella enterica* respectively. (c, d) 2×10^6 CFU/ml *Bacillus cereus* and *Salmonella enterica* cells treated with purified protein showed disrupted cells and debris.

2.3.8 Field Emission Scanning Electron Microscopy

Bacterial cell imaging **(Figure 13 a, b, c, d)** clearly showed a damaged surface in protease-treated cells when compared to control (healthy) cells. In both Gram-positive and Gram-negative bacteria protease appeared to cause degradation of bacterial cell surface.

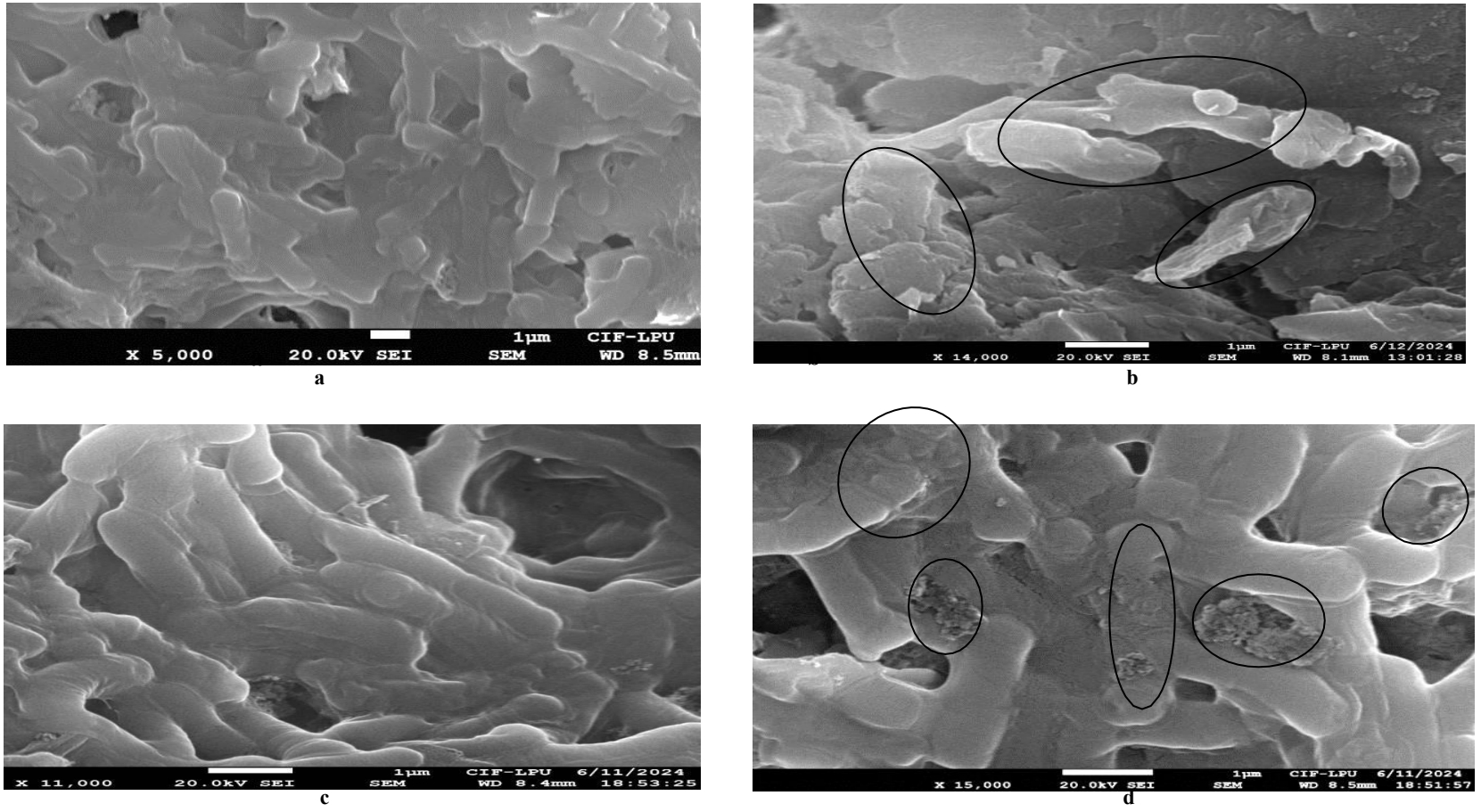


Figure 13. FESEM images of *Bacillus cereus* and *Salmonella enterica.* (a, b) Untreated control group cells of *Bacillus cereus* and *Salmonella enterica* respectively (c, d) 2×10^6 CFU/ml *Bacillus cereus* and *Salmonella enterica* cells treated with purified protein, showed cell degradation and debris formation.

2.5 Discussion

In our study, we identified and isolated a protein of the DinB protein family that showed inhibitory action against *Bacillus cereus* and *Salmonella enterica.* Cell-free supernatant from 96-hour incubation, as well as the purified protein, hindered the target *Bacillus cereus* and *Salmonella enterica.* The protein's molecular mass and sequence determination were done by MALDI-TOFF spectroscopy. Peptide mass fingerprinting confirmed the protein sequence similarity with 36% homology to the DinB protein family. The MS spectroscopy analysis confirmed the protein to be a metalloenzyme with strong protease activity, which was further supported by the *in vitro* experiments. Enzymatic degradation was not compatible with the remaining protein sequence, hence only partial protein sequence was determined. This outcome also concluded that the isolated target protein must have some unique amino acid sequence, which did not find any protein similarity in the *Bacillus* database using MASCOT software.

Gram-positive *Bacillus cereus* is a known cause of food poisoning and a causative agent of diarrhea **(Dubey et al., 2022)**. Likewise, all serotypes of *Salmonella enterica* cause diarrhea, which may appear fetal in many cases **(Zhang et al., 2023)**. In this study, both target pathogens' growth was constrained with a minimum inhibitory concentration of 9.78 μM of purified protein. The protein's ability to inhibit a broad range of bacteria and resistance to variable temperature, pH, and detergent treatment makes it suitable for drug design. The proteolytic action of protease was observed after time-kill kinetics against pathogenic bacteria. Under 100X magnification of a compound microscope treated cells showed debris formation, while the control bacterial cells remained intact. FESEM analysis of protease-treated bacterial cells confirmed the bacterial cell degradation. The images confirmed that the protease was disintegrating the bacterial cell membrane, ultimately degrading the whole cell **(Taggar et al., 2021)**.

Chapter 3

In silico analysis of functional peptides/ proteins of *Bacillus clausii* possessing anti-diarrheal properties

3.1 INTRODUCTION

Extracting peptide-based molecules of medicinal value is a very long, probability-based process. Peptide-based drug therapies are an excellent choice to conquer multidrug-resistance. But the problem lies in selecting the best molecule from a pool of proteins. Previously drug designing was based on the fact of "whatever works" rather than understanding the mode of action of drugs. For many years, antimicrobial agents of any kind were incorporated into medications since they were intended to target the disease as wholesome. But at present with the advancements in science and technology researchers have developed ways to explore the biosynthetic pathways of a drug molecule, its action on target organisms, and the aftermath of the therapies. Bioinformatic tools have increased our understanding of the biochemistry of molecules. Bioinformatic techniques are not only useful for discovering novel therapies but can also be used in studying their stability, mode of action, and overall impact. One of the rational studies is the use of a computational approach like computer-aided drug design (CADD) **(Figure 14), (Rajkishan et al., 2021)**. CADD is classified under two main divisions i.e. structure-based drug designing and ligand-based drug designing. The structure-based technique is used when all the information about receptor structure is available, while the latter is applicable in ligand-based drug designing. Drug designing combines computational drug designing with chemical biology to identify and improve drug molecules **(Bharatam et al., 2021)**. Chemical biology explores the biochemistry of drug molecules in biological systems. However, computer-aided drug design explores the target structures to identify the potential drug targets. The use of CADD has reduced the time and expense of finding a new drug target and revolutionized the pharmaceutical industry **(Camacho et al., 2018).** Our study employed structure-based drug designing, as the protein data bank had 3-dimensional structures of toxin proteins and peptides. Structure-based drug designing can be performed in two ways i.e. virtual screening and *de novo* analysis. Virtual screening utilizes the existing ligand molecule libraries to find suitable targets against target protein molecules, while *de novo* analysis uses the receptor molecules binding site to identify ligands that would interact with stability **(Maia et al., 2020), (Stokes et al., 2020)**. A complete process of virtual screening involves data collection from compound

databases, protein-protein docking, and selection of lead compounds. The central part of virtual screening is protein-protein docking. Docking can very accurately predict the symmetry of ligands with the target protein molecules. It follows the concept of lock and key principles. The docking software achieves this precision by using a scoring function (part of the software algorithm), which evaluates the binding energies henceforth the experimental assays of protein-protein complexes. The scoring function itself includes several energy functions which may vary from software to software **(Batool et al., 2019).** Further to understand the structure-function relationship of the protein-protein interactions, molecular dynamics (MD) simulation software is used. MD simulations have been applied successfully to study the pathogenesis of diseases, protein mutations, and virtual screening. The former study will take years to confirm the outcomes of *in vitro* and *in vivo* experiments, however *in silico* research related to disease pathogenesis will be completed in MD simulations within a few days thus saving a lot of time for *in vitro* experiments **(Liu et al., 2018)**. Researchers have reported the accuracy of MD simulations over conventional protein-protein docking in predicting the binding energy, and flexibility of receptor protein. The use of MM-PBSA or MM-GBSA calculations by MD simulations improves the accuracy of binding free energy evaluation between receptors and ligands **(Poli et al., 2019), (Tuccinardi et al., 2021)**. The primary aim of our study was to isolate a novel protein possessing anti-diarrheal properties from a natural source, but to make the selection process easier *in silico* drug designing was incorporated. This study aimed to collect peptides/ protein structures reported to have bactericidal effects against diarrhea pathogens, from the *Bacillus clausii* strain and then perform the virtual screening of bacterial toxins (receptors) with peptides (ligands) of *Bacillus clausii* species. The study aimed to provide insight into the amino acid composition and the mode of action, of the existing peptides so that the newly isolated protein/peptide structural similarity to the existing ones will help us to sort potential targets to inhibit diarrhea pathogens.

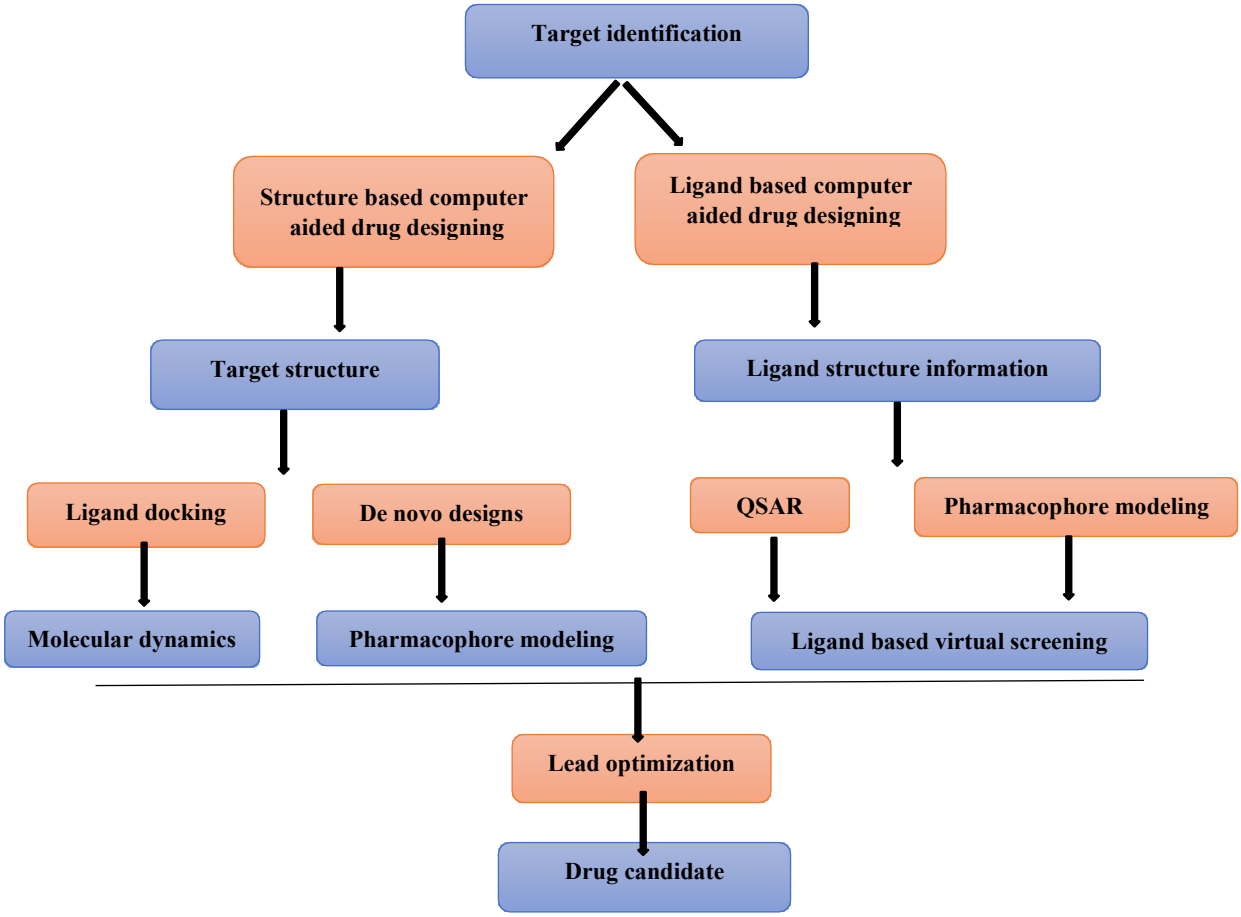


Figure 14: Schematic diagram showing different steps of computer-aided drug designing (CADD).

3.2 MATERIALS AND METHODS

3.2.1 Identification and Selection of Proteins

The target peptides and proteins were chosen after a vast literature search **(Table 6).** Originally only one peptide of the extracellular origin with complete sequence information i.e. clausin has been reported from *Bacillus clausii* so far **(Ahire et al., 2020).** The available peptide sequence was run in the homology search database, BLAST: Basic Local Alignment Search Tool from the official website; https://blast.ncbi.nlm.nih.gov/Blast.cgi, to identify the proteins with sequence similarity. pBLAST sorted the proteins according to their sequence homology to the clausin and calculated the E-value or statistical significance of the query with the database.

Table 6. List of selected peptides

S. No.	Peptide name	Sequence	Molecular weight (Dalton)	Source bacteria
1	Clausin	FTAVSFATPGAGETGAFNAFA	2034.21	*Bacillus clausii*
2	Gallidermin 1/ nisin	MEKAFDLDLEVVHTKAKDVQPDFTSVSFCTPGC GETGSFNSFCC	4792.37	*Alkalihalobacillus*
3	Gallidermin 2/ nisin	MEKAFDLDLEVVHTKAKDVEPDFTSVSFCTPGC GETGSFNSFCC	4792.37	*Bacillaceae*

3.2.2 3D Structures of Ligand Proteins from Phyre^2

After retrieving the protein sequences with E- value 0.001, a 3-dimensional structure ofall the target proteins was created in Phyre^2 (Protein Homology/ analogY Recognition Engine V2.0) (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) **(Tochukwu et al., 2019)**. Phyre^2 is a web-based collection of tools that predicts the 3-dimensional structure of proteins. The tool is a freely available online tool. The amino acid sequencewas copied on the homepage of Phyre^2. Phyre search was clicked. Results were downloaded to the computer.

3.2.3 Selection of Diarrhea Causing Toxin

The diarrhea-causing toxins were chosen based on the literature survey. Selection criteria included severity of infection, persistence, infected population count, andmorbidity **(Gómez-Escudero et al., 2021), (Cohen et al., 2022)**. Nine bacteria and viruses were chosen for the study including *Clostridium difficile, Enterobacter sp., Shigella,* rotavirus, and norovirus. Fifteen toxins of nine pathogens were selected for the study **(Table 7).** Sequences of all toxins were obtained from the Protein data bank (https://www.rcsb.org/) and 3-dimensional images were created with the help of Phyre^2 software.

Table 7. List of diarrhea pathogens

S.NO.	Diarrhea causing pathogenic bacteria	PDB ID	Molecular weight (kDa)
1	*Clostridium difficile* toxin A	4R04	209.64
2	*Clostridium difficile* toxin B	6OQ5	270.18
3	*E. coli* Heat stable toxin A	1EHS	1.19
4	*E. coli* Heat stable toxin B	1EHS	5.11
5	*E. coli* Heat labile toxin A	1LTA	21.62
6	*E. coli* Heat labile toxin B	1LTA	11.79
7	*Salmonella enterica*	7EE4	13.85
8	*Shigella dysenteriae* toxin A	1DM0	31.53
9	*Shigella dysenteriae* toxin B	1DM0	7.69
10	*Shiga*-like toxin *E. coli* subunit A	1R4P	33.2
11	*Shiga*-like toxin *E. coli* subunit B	1R4P	7.82
12	Norovirus GII.4 VP1	5IYN	33.72
13	Rotavirus NSP	3MIW	6.5
14	*Vibrio cholera* toxin A	1S5B	27.12
15	*Vibrio cholera* toxin B	1S5B	11.61

3.2.4 Protein-Protein Docking

Protein-protein docking is a part of structure-based drug designing, where a potential drug target is chosen from the existing or already reported molecules. Molecular docking is used to study protein-peptide complex interaction and to scale down the most stable conformation. To study protein-protein molecule interaction, ClusPro 2.0 software was used (https://cluspro.bu.edu/login.php) **(Lensink et al., 2017), (Alekseenko et al., 2020)**. The software predicted the interactions in the native state of both test proteins. Stability was calculated as a total of all four interactions i.e. Hydrophobic interaction, hydrogen bonding, Vander waal interactions, and electrostatic interactions. The docking server ClusPro 2.0 follows an algorithm of three major steps, the first step was sampling billions of structural conformations using rigid body docking; the second step was to calculate the root-mean-square deviation (RMSD) which was performed to generate 1000 lowest-energy structures, the method was called clustering; the third step was to develop a refined structure based on lowest calculated energy **(Figure 15).**

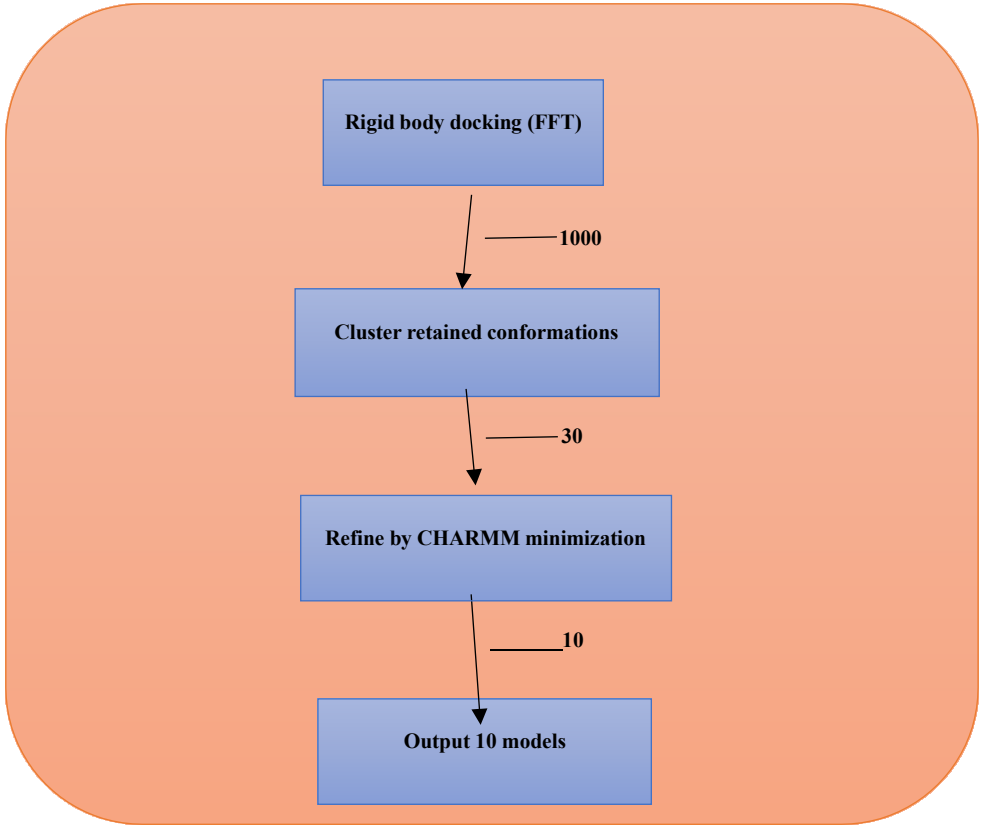


Figure 15. Flowchart of ClusPro algorithm.

ClusPro uses the PIPER docking algorithm. It is the PIPER algorithm that considers covalent and non-covalent energies to determine the most stable complex formation. The formula used to calculate the energy between protein and ligand is:

$$\mathbf{(E = w1_{Erep} + w2_{Eattr} + w3_{Eelec} + w4_{EDARS})}$$

$\mathbf{E_{rep}}$ - Van der Waals interaction energy's repulsive contributions
$\mathbf{E_{attr}}$ - Van der Waals interaction energy's attractive contributions
$\mathbf{E_{elec}}$ - Electrostatic energy
$\mathbf{E_{DARS}}$ - Pairwise structure-based potential
w1, w2, w3, and w4 - The coefficients of weights of the corresponding terms

Before submitting the target proteins to ClusPro software, the 3-D structure of all the receptor and ligand proteins was generated through Phyre 2.0 software and was checked for the removal of solvent (H_2O) molecules and missing atoms if any. On the homepage of ClusPro 2.0 software Dock option was selected and the 3-D structure of receptors and ligands were uploaded in their respective columns and clicked on the Dock option. All bacterial and viral toxin proteins were uploaded as receptor molecules including *Clostridium difficile* toxin B (PDB ID 6OQ5), *E. coli* Heat stable toxin A (PDB ID 1EHS), *E. coli* Heat stable toxin B (PDB ID 1EHS), *E. coli* Heat labile toxin A (PDB ID 1LTA), *E. coli* Heat labile toxin B (PDB ID 1LTA), *Vibrio cholera* toxin A (PDB ID 1S5B), *Vibrio cholera* toxin B (PDB ID 1S5B), *Salmonella enterica* (PDB ID 7EE4), *Shigella dysenteriae* toxin A (PDB ID 1DM0), *Shigella dysenteriae* toxin B (PDB ID 1DM0), *Shiga*-like toxin *E. coli* subunit A (PDB ID 1R4P), *Shiga*-like toxin, E. *coli* subunit B (PDB ID 1R4P), Rotavirus NSP (PDB ID 3MIW), Norovirus GII.4 VP1 (PDB ID 5IYN). These proteins were docked against all three peptide molecules, which were uploaded as ligand molecules on Cluspro 2.0.

3.2.5 Visualization of Docked Complexes Using PyMOL

The protein-protein complexes generated after docking were visualized using Open-Source PyMOL (https://pymol.org) **(Martinez et al., 2019).** The homepage of PyMOL has two windows. The window on the top of the screen is called External GUI which has a menu bar with multiple commands in it. The second window below the first one is called the viewer window where all docked 3-dimensional structures are visualized and modified accordingly. The object control panel is present on the right side of the homepage, which has the commands to modify the docked structures. Some important options include A (Action), S (Show), H (Hide), L (Label), and C (Colour). After obtaining the most stable protein-protein complexes among all the toxin proteins the docked structures were opened in PyMOL software to study the interacting amino acids of receptor and ligand molecules within 5Å diameter of the ligand. To do this the docked complex of protein protein molecules was opened in PyMOL. The docked complex is highlighted as Rec in the control panel. Selected the ligand-protein structure and renamed it Protein 1 by entering a new name in the command line situated above the viewer window. A new tab with the name Protein 1 is opened on the right side of the screen in the control panel. The receptor was hidden using the Hide command. To visualize the interaction of ligand to the closest amino acid residues of receptor type **> show sticks, byres all within 5 of Protein 1**, press **Enter**. The neighboring amino acids appeared surrounding the ligand molecule. Also, the amino acid residues of both protein structures appeared on the top of the viewer's window. To observe the polar interactions of ligand to the receptor molecule, select the ligand molecule structure, click on the **Action** tab, click on **Find**, and select **Polar contacts** > **to any other atom**. A yellow dotted line will appear showing the polar contacts within protein protein complexes. Molecules not making any polar contacts were hidden by clicking on the residues and using the **Hide** command. From here the interacting amino acids of both toxin proteins and ligand proteins were observed and compared for all complexes. PyMOL analysis was performed only with *Clostridium difficile* toxin B (TcdB). TcdB is ~270 kDa protein, having four functional domains: an N-terminal glucosyltransferase domain (GTD), a cysteine protease domain (CPD), a central delivery and receptor-binding domain

(DRBD), and a combined repetitive oligopeptides (CROPs) domain **(Figure 16)**. Ligand binding sites exist in the amino-terminal GTD domain, theactivation by phosphorylation of the GTD is done by the cysteine protease domain. The N-terminal glucosyltransferase domain is the enzymatic activity domain as it regulates the activation of toxins inside host cells, CPD is involved in the attachment of toxins to host membranes, the receptor-binding domain is involved in thedelivery and attachment of toxins, CROP domains are considered to be involved in thetranslocation of the toxin **(Aktories et al., 2017).**

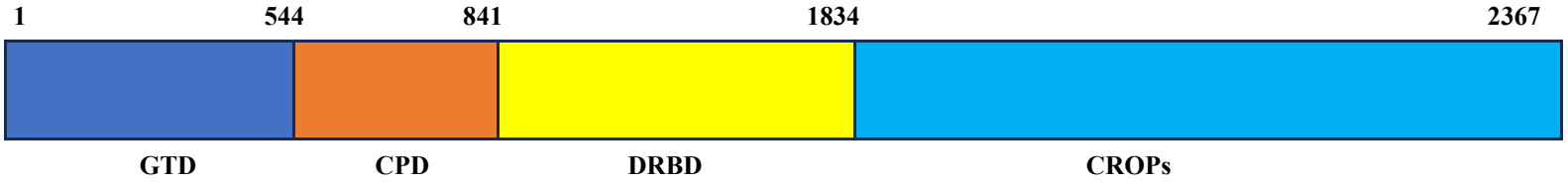


Figure 16. Detailed structure of TcdB holotoxin. N-terminal glucosyltransferase domain from 1-544 amino acids (Navy blue), cysteine protease domain (CPD) lies between 545 to 841 amino acids (orange), receptor-binding domain (DRBD) is between 842 to 1834amino acids (yellow), combined repetitive oligopeptides domain (CROPs) (sky blue).

3.2.6 Molecular Dynamics (MD) Simulation

The docking complexes were further studied using Molecular Dynamics (MD) Simulations. The purpose of simulation software was to study the protein-protein complex interaction under simulation of the biological environment to confirm the changes in the stability of the complex **(Sinha et al., 2023), (Zheng et al., 2024).** This was performed by using Desmond software **(Figure 17).**

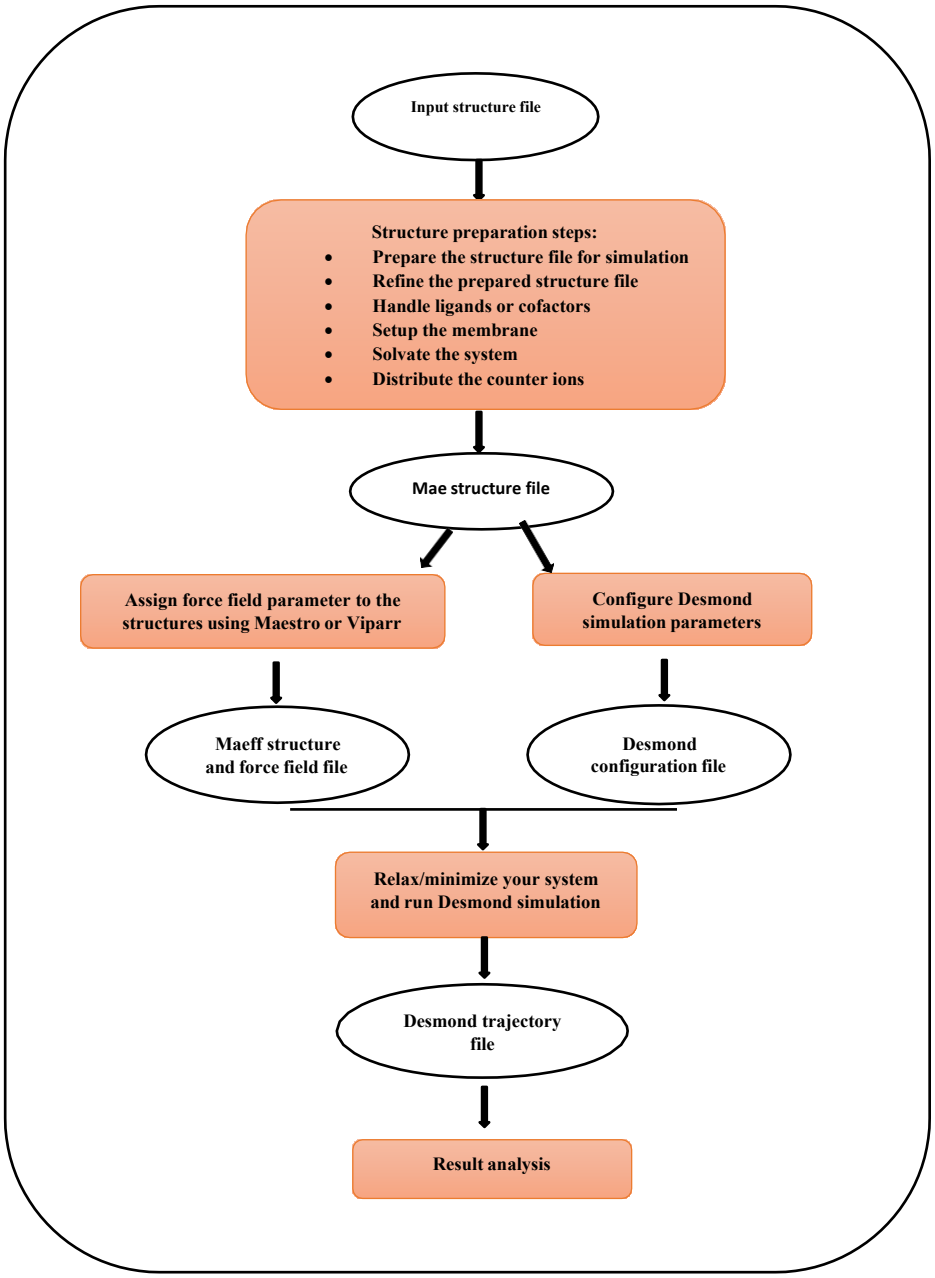


Figure 17. Simulation process on Desmond.

Desmond is a Graphics Processing Unit-powered high-performance molecular dynamics (MD) simulation engine (https://www.schrodinger.com/platform/products/desmond/), that explores the different aspects of protein-protein complex stability. The system is designed to perform simulations from picoseconds to microseconds. The system is designed to work at a speed 100X faster than a single CPU as it is powered by GPU. To upload a docking file of the protein-protein complex to Desmond's homepage, open the Terminal, type "cd/opt/Schrodinger 2020-1/", press Enter, type, "maestro", and press Enter. Clicked on File, selected Import structure, selected the protein complex, and clicked on Open. To choose the simulation environment, click on Protein Preparation Wizard> Import and Process. Assigned bond orders, added hydrogen bonds, created disulfide bonds, and deleted weavers beyond 5Å from hit groups. From the Refine option, more settingswere added to the H-bond assignment including pH 7.0, and water removal from 3Å distance. To create the simulation environment, clicked on Taskbar, and the solvation parameters were adjusted. It included, Predefined: SPC, Box shape: Orthorhombic, Boxsize calculation method: Buffer, Distance: 10x10x10. Clicked on the Show boundary box followed by Minimize Volume. Neutralize ions, Add Salts, and click on Run. The monitor showed, Stage 1 completed, Stage 2 completed, and Stage 3 completed. To runthe molecular dynamics, select the Browse option, click on Load, choose the Simulation Time which was 100 nanoseconds, enter the Job name, and click on Run. The structureswere then exported outside the Desmond software for further analysis.

3.1 RESULTS

3.1.1 Sequence Retrieval and Creation of 3-D Structures

The clausin peptide sequence showed similarity to several sequences, but sorting of best alignment was done based on the lowest E-value score. The top two peptides from pBLAST were selected with the lowest E-value of 0.01 and they had a percentage similarity above 75% in the homology search with clausin sequence were chosen for study (**Figure 18**).

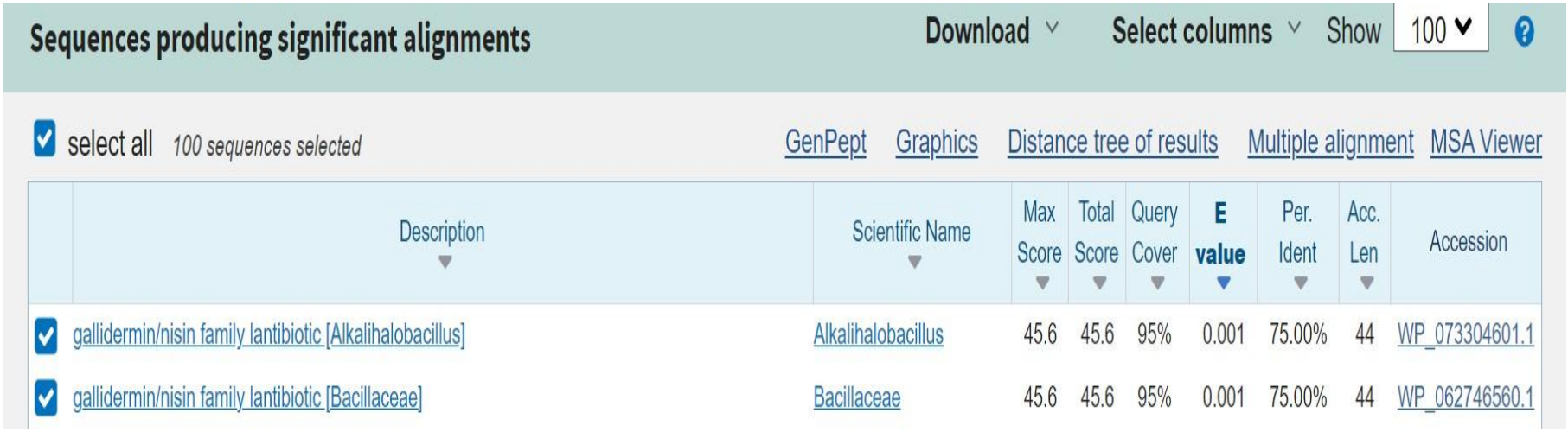


Figure 18. Screenshot of pBLAST homology search for peptide sequences.

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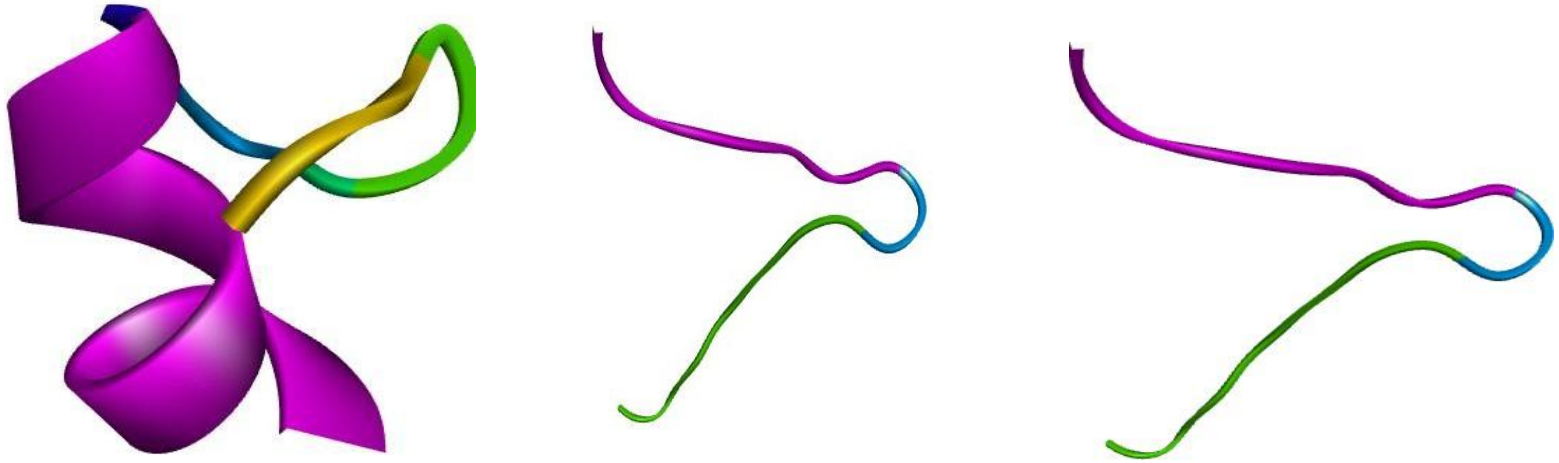
The ligand sequence data was retrieved and stored on a personal computer. Further sequences of bacterial toxins were obtained from the protein data bank. 3D structures were created using the Phyre^2 tool **(Figure 19, 20).**



a **b** **c**

Figure 19. (a) 3D structures of clausin, (b) Gallidermin 1, (c) Gallidermin 2.

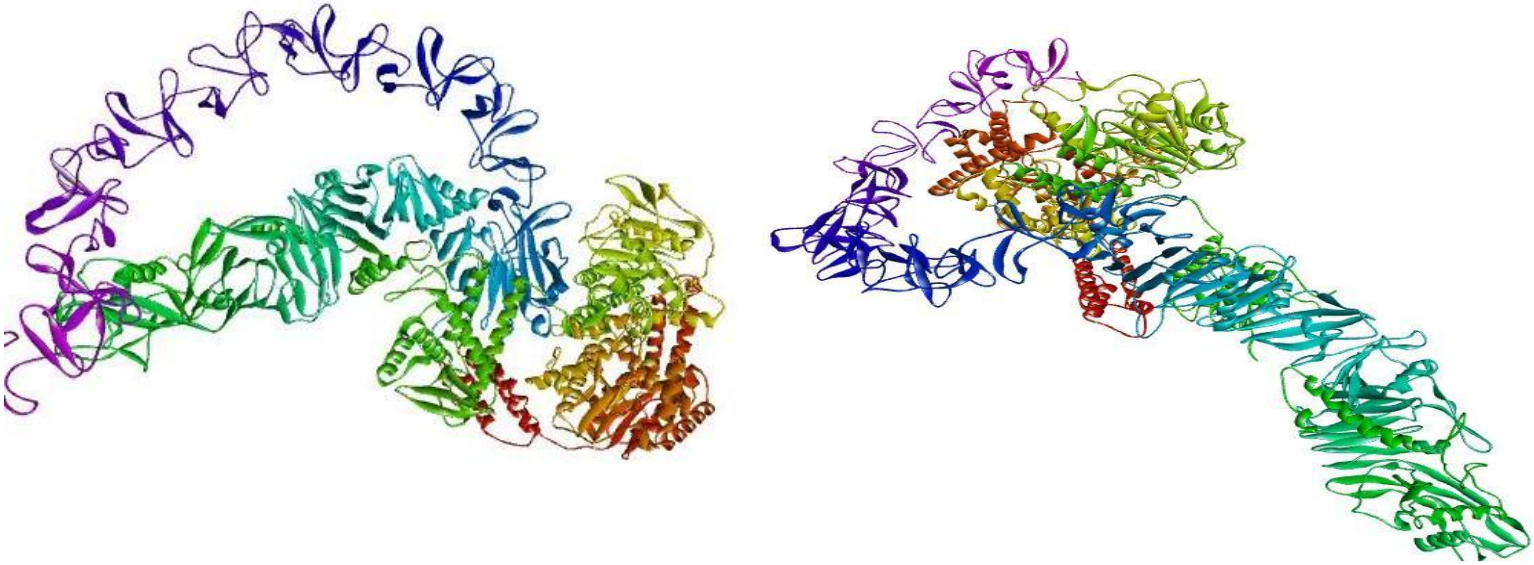
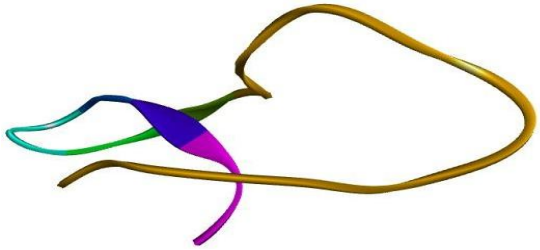
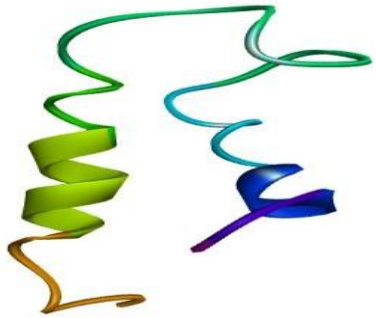


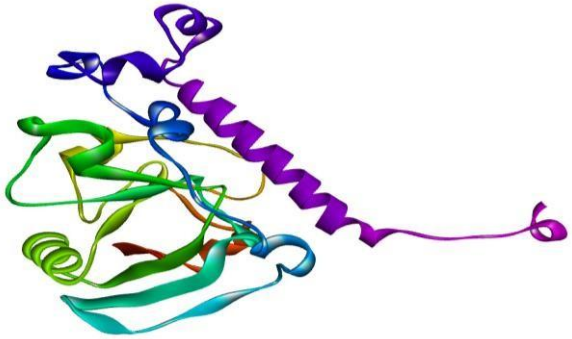
Figure 20 a) *Clostridium difficile* toxin A **b)** *Clostridium difficile* toxin B



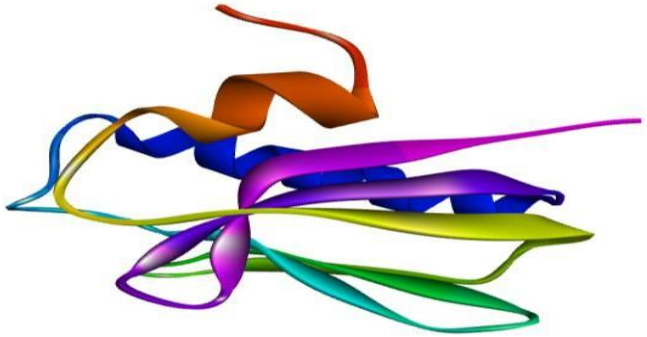
c) *E. coli* Heat stable toxin A



d) *E. coli* Heat stable toxin B

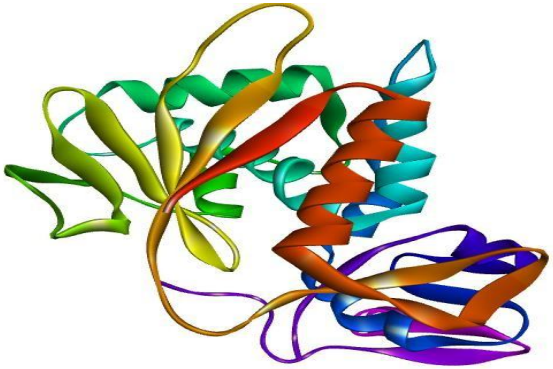


e) *E. coli* Heat labile toxin A

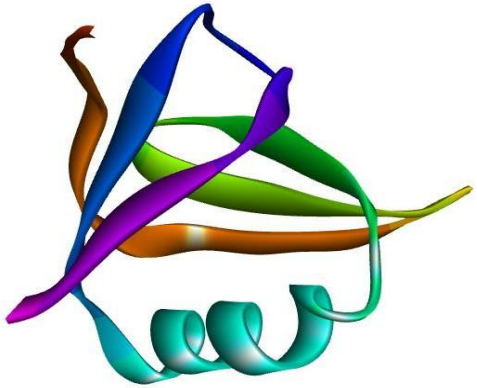


f) *E. coli* Heat labile toxin B

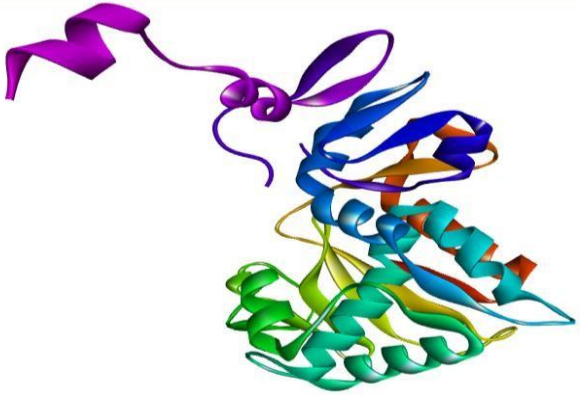
g) *Salmonella enterica*



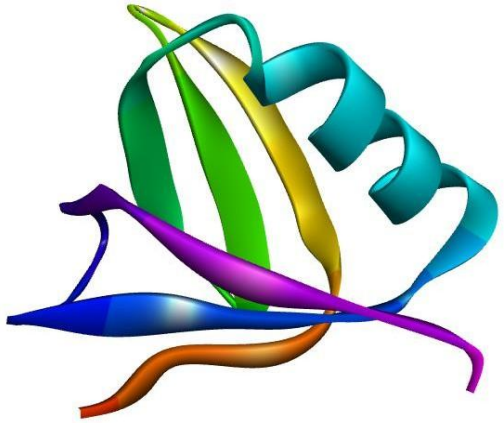
h) *Shigella dysenteriae* toxin A



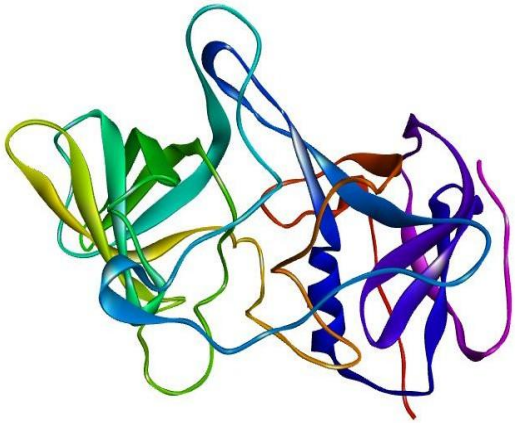
i) *Shigella dysenteriae* toxin B



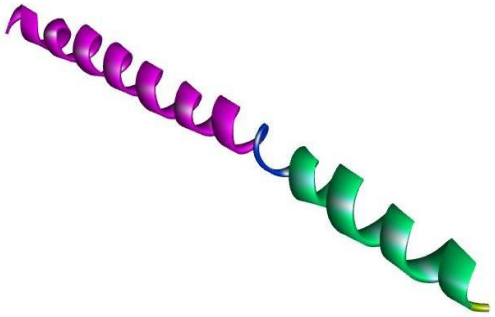
j) *Shiga*-like toxin *E. coli* subunit A



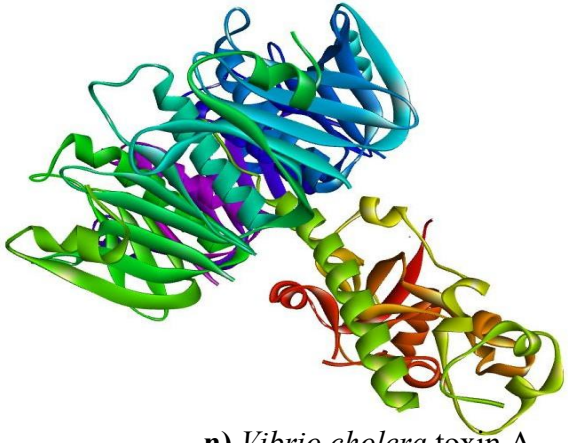
k) *Shiga*-like toxin *E. coli* subunit B



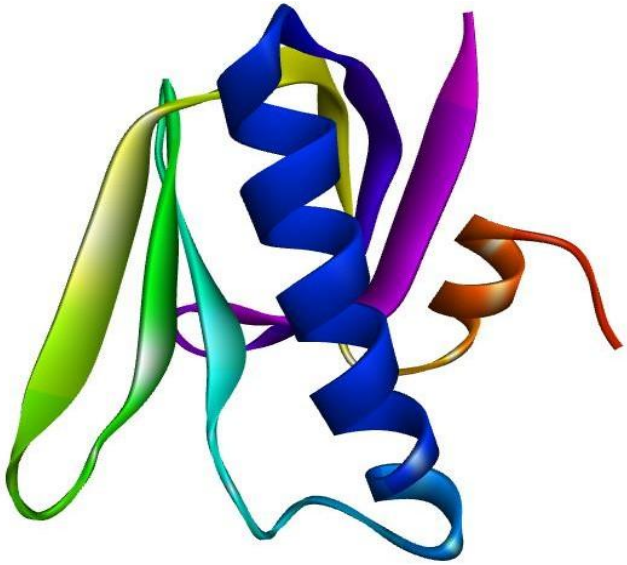
l) Norovirus GII.4 VP1



m) Rotavirus NSP



n) *Vibrio cholera* toxin A



o) *Vibrio cholera* toxin B

Figure 20. 3D structures of bacterial toxin proteins.

3.3.2 Protein-Protein Docking in ClusPro 2.0

After creating 3D structures of bacterial toxin and ligand proteins, protein-protein docking was performed using the ClusPro 2.0 server. A list of the most favored modelsappeared on the result page, and the one with the lowest binding energy was chosen asthe most stable configuration of protein-protein complexes **(Table 8)**. The lowest binding energy was the total of energy scores ($E = w1_{Erep} + w2_{Eattr} + w3_{Eelec} + w4_{EDARS}$). Among all the bacterial toxins, the most stable protein-protein complex was formed by*Clostridium difficile* toxin B, with all three ligand protein molecules, which were - 908.8, -116.6, and -1093.6 for clausin, gallidermin 1, and gallidermin 2 respectively. Hence further analysis of complex stability was performed only with *Clostridium difficile* toxin B, for all three ligand proteins.

Table 8. List of the peptides with protein (toxins) with the lowest balanced energy

S.NO.	Diarrhea causing pathogenic bacteria	Clausin Lowest Balanced Energy	Gallidermin 1 Lowest Balanced Energy	Gallidermin 2 Lowest Balanced Energy
1	*Clostridium difficile* toxin A	-750.9	-962.9	-916.1
2	*Clostridium difficile* toxin B	-908.8	-1116.6	-1093.6
3	*E. coli* Heat stable toxin A	-765.7	-824.3	-821.1
4	*E. coli* Heat stable toxin B	-604.2	-819.7	-817.5
5	*E. coli* Heat labile toxin A	-721.1	-885.6	-876.2
6	*E. coli* Heat labile toxin B	-743.9	-728.1	-726.4
7	*Vibrio cholera* toxin A	-671.2	-869.8	-900.0
8	*Vibrio cholera* toxin B	-780.7	-848.2	-840.5
9	*Salmonella enterica*	-752.9	-703.0	-705.7
10	*Shigella dysenteriae* toxin A	-785.2	-917.6	-889.8
11	*Shigella dysenteriae* toxin B	-644.9	-583.7	-569.8
12	*Shiga like toxin E. coli* subunit A	-738.9	-889.6	-887.5
13	*Shiga like toxin E. coli* subunit B	-667.3	-660.8	-656.0
14	Rotavirus NSP	-644.1	-703.5	-715.9
15	Norovirus GII.4 VP1	-783.9	-810.0	-804.6
	Best lowest binding energy match	*Clostridium difficile* toxin B	*Clostridium difficile* toxin B	*Clostridium difficile* toxin B

3.3.3 PyMOL Analysis

The Protein-protein complexes of *Clostridium difficile* toxin B, with all three ligand protein molecules, obtained from the ClusPro 2.0 database were visualized using PyMOL software **(Figure 21).** The interacting amino acid residues of receptor and ligand proteins were determined using PyMOL. Though all peptides were interacting in the vicinity of the catalytic domain, none of the peptides occupied the absolute ligand binding site of the toxin protein. Polar bond formation between protein-protein molecules within the 5Å region was determined. The first ligand molecule i.e. clausin had polar bond interaction with 300 to 785 amino acid residues of *Clostridium* protein, which was partially part of both activity and receptor domains. TcdB interacted from 936 to 1627 amino acid residues with gallidermin 1 molecule, which were the parts of attachment and delivery domains **(Figure 22).** Gallidermin 2 formed polar bond interactions with amino acids 938 to 1629 **(Figure 23)**. As none of the ligand-protein originally attached itself to ligand binding pockets, one can consider it to be non-competitive inhibition.

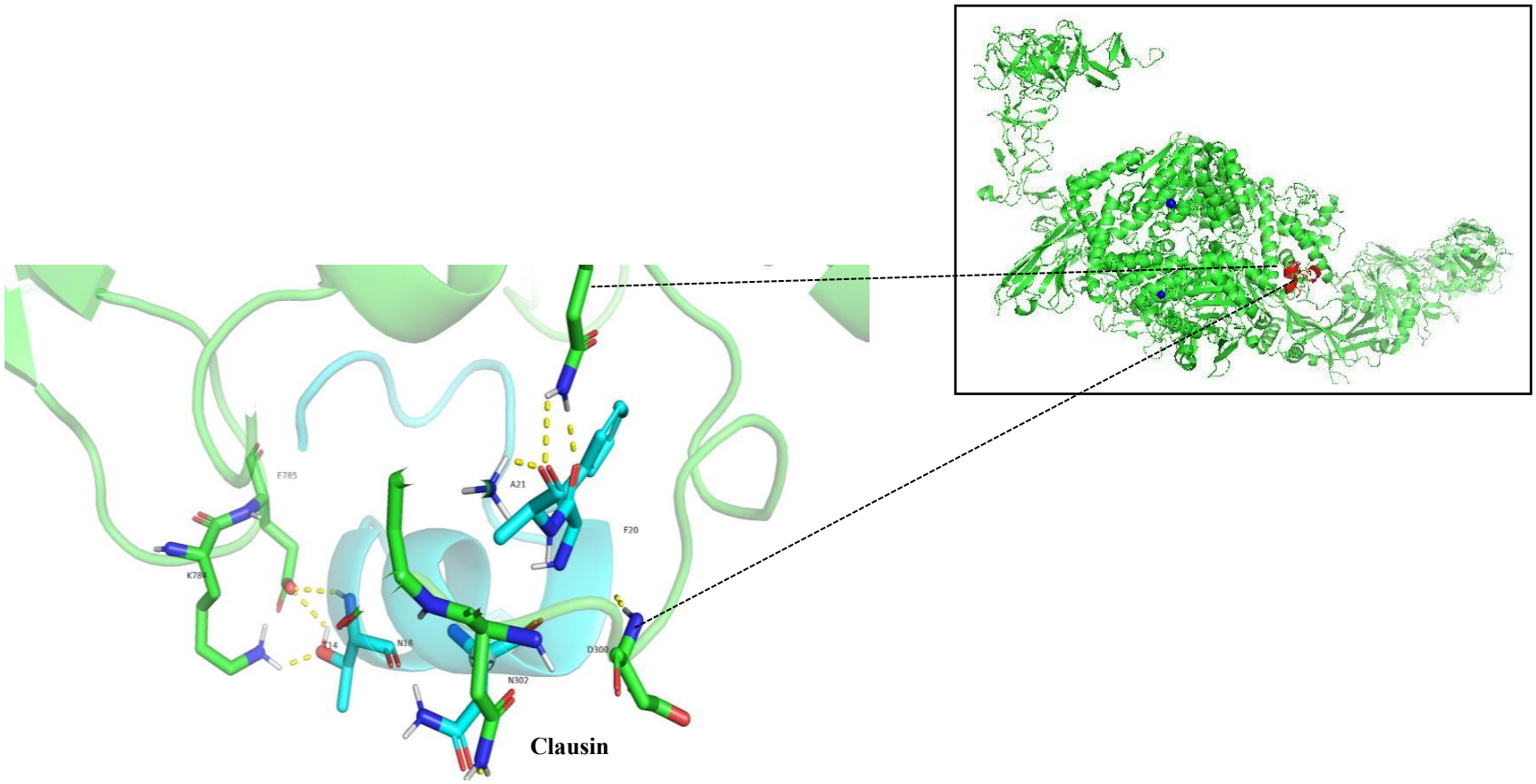


Figure 21. Protein-protein docking complex of TcdB with Clausin. Polar bond interaction within the 5Å region lies between amino acids D300 to E785.

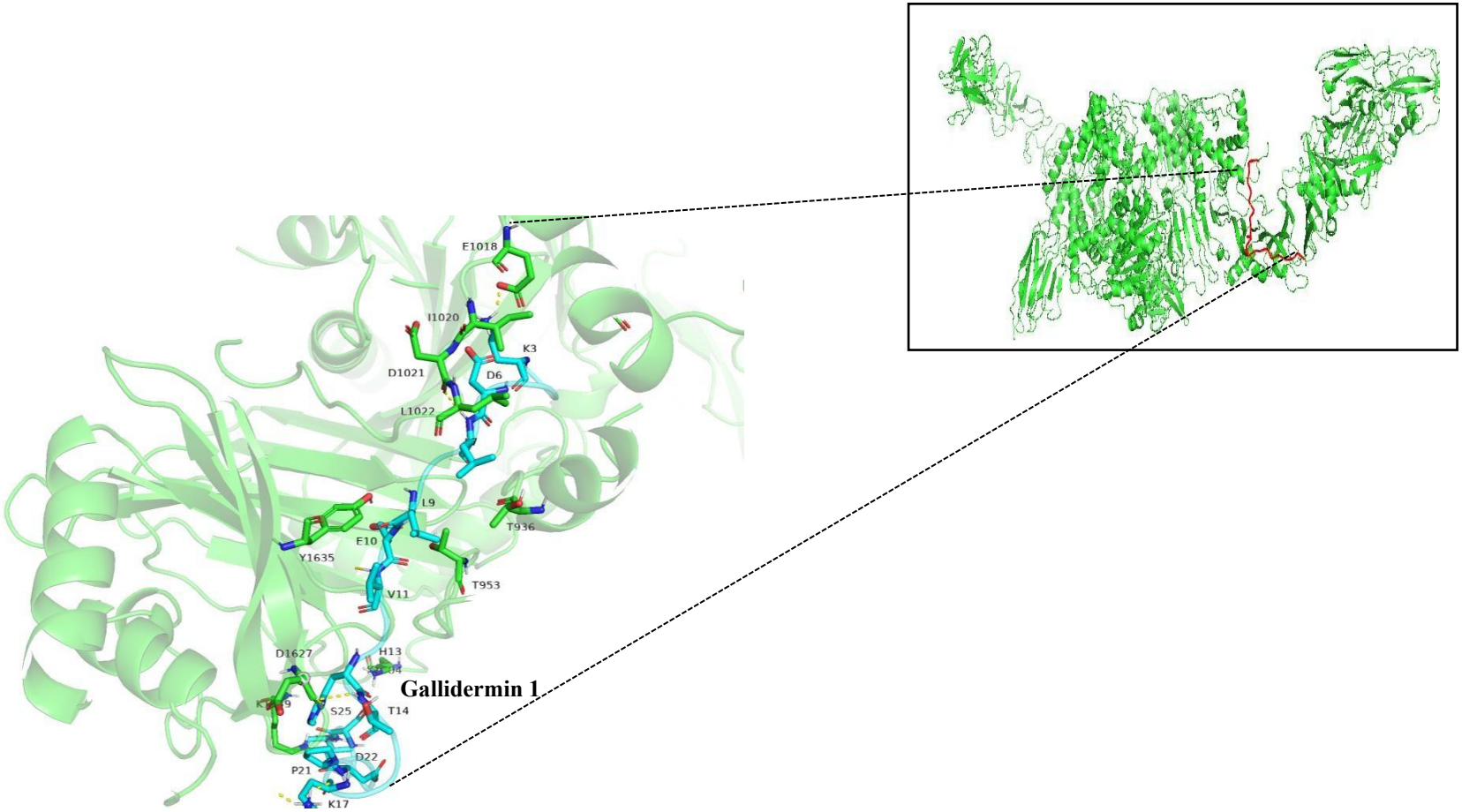


Figure 22. Protein-protein docking complex of TcdB with gallidermin 1**.** H-bond interaction within the 5Å region lies between amino acids T936 to D1627.

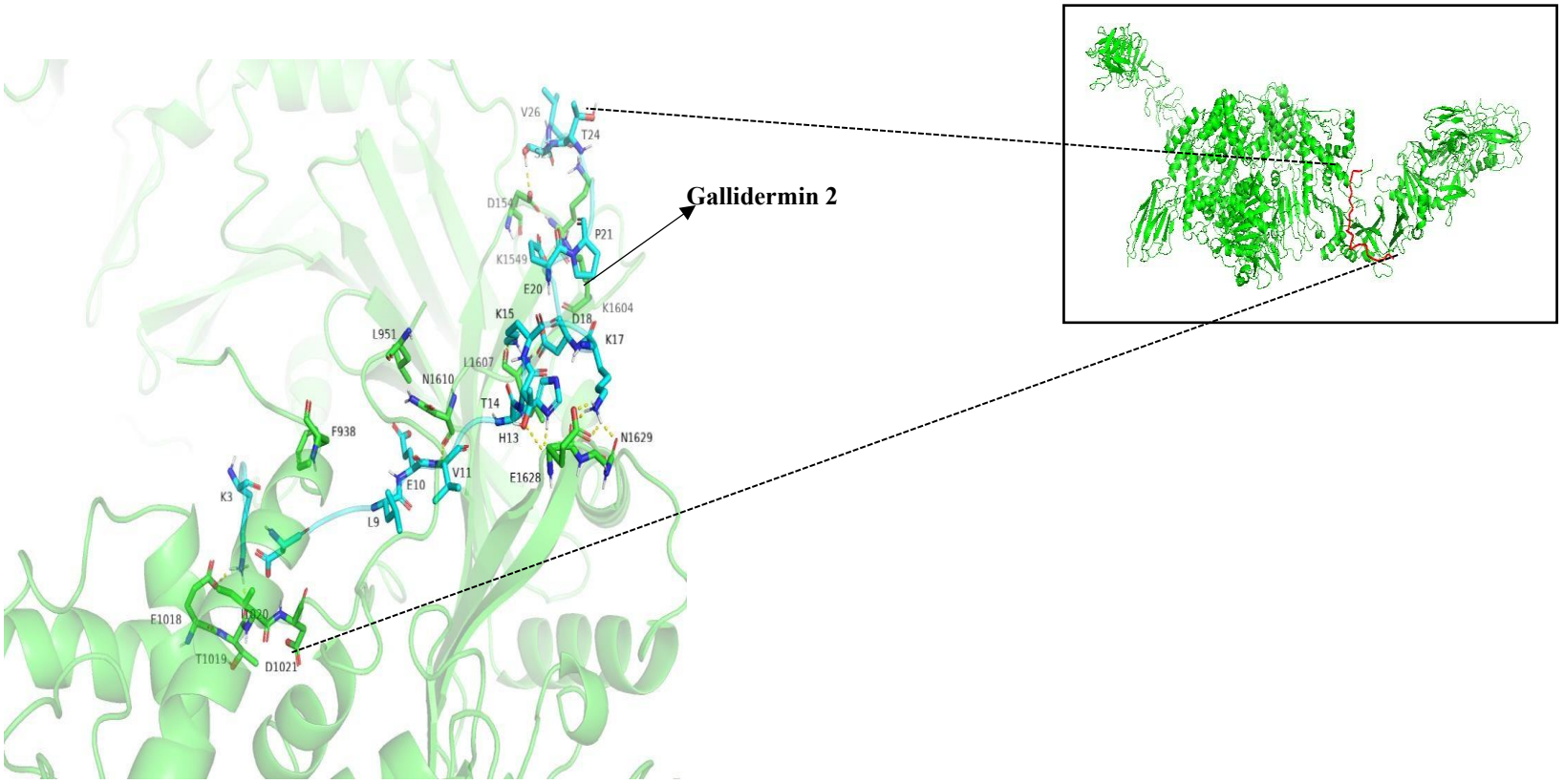
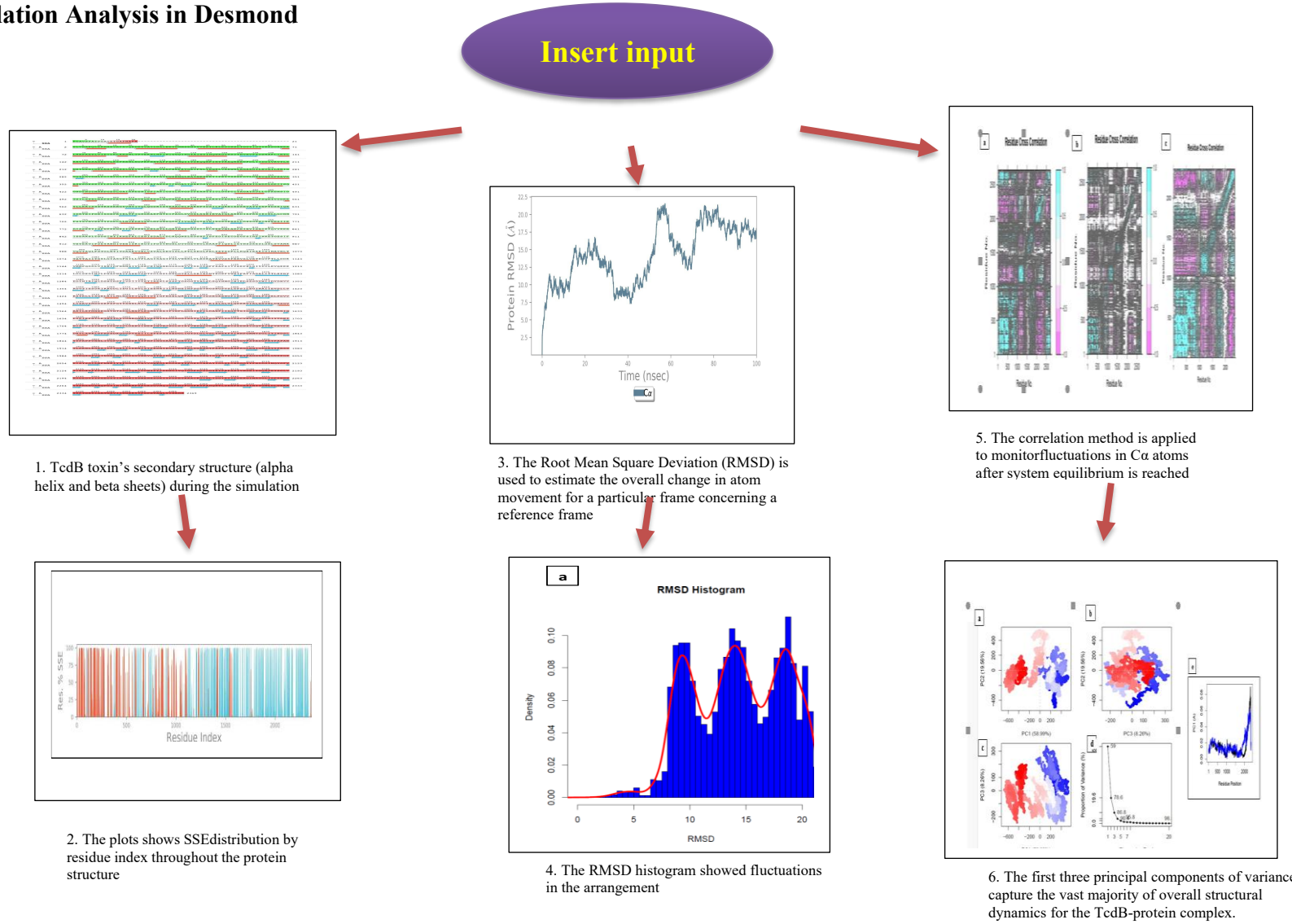


Figure 23. Protein-protein docking complex of TcdB with gallidermin 2**.** H-bond interaction within the 5Å region lies between amino acids F938 to N1629.

3.3.4 MD Simulation Analysis in Desmond



1. TcdB toxin's secondary structure (alpha helix and beta sheets) during the simulation

2. The plots shows SSEdistribution by residue index throughout the protein structure

3. The Root Mean Square Deviation (RMSD) is used to estimate the overall change in atom movement for a particular frame concerning a reference frame

4. The RMSD histogram showed fluctuations in the arrangement

5. The correlation method is applied to monitorfluctuations in Cα atoms after system equilibrium is reached

6. The first three principal components of variance capture the vast majority of overall structural dynamics for the TcdB-protein complex.

Figure 24 a. Flowchart of MD Simulation Analysis in Desmond.

3.3.4.1 Simulation Report for *Clostridium difficile* Toxin B (TcdB) with Clausin

Jobname: desmond_md_job_1

Entry title: CDB PEP1 – preprocessed

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
1	mdsim	NPT	300.0	100.030	507348	156312	0

Protein information:

Tot. Residues	Protein Chain(s) Residues in Chain(s)	# Atoms	# Heavy Atoms	Charge
2346	'A' ict values [2346]	37356	19007	-184

Counter Ion/Salt Information

Type	Num	Concentration [mM]	Total Charge
Na	620	72.117	+620
Cl	436	50.714	-436

3.3.4.2 Simulation Report for *Clostridium difficile* Toxin B with Gallidermin 1

Jobname: desmond_md_job_1

Entry title: CDB PEP2 - preprocessed

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
1	mdsim	NPT	300.0	100.030	506416	154214	0

Protein information

Tot. Residues	Prot. Chain(s) Res. in Chain(s) # Atoms	# Heavy Atoms	Charge
2719	**A','B','icdD','vaElu','eFs' ([2346, 24, 126, 107, 427643])**	**21717**	-171

Counter Ion/Salt Information			
Type	**Num.**	**Concentration [mM]**	**Total Charge**
Na	601	70.858	+601
Cl	430	50.697	-430

3.3.4.3 Simulation Report for *Clostridium difficile* Toxin B with Gallidermin 2

CPU #	Job Type	Ensemble	Temp. [K]	Sim. Time [ns]	# Atoms	# Waters	Charge
1	mdsim	NPT	300.0	100.030	506388	154205	0

Protein Information

Tot. Residues	Prot. Chain(s) Res. in Chain(s)	# Atoms	# Heavy Atoms	Charge
2719	**'A','B','i c d D','v a E l u','e F s' ([2346, 24, 126, 107,4121764]**	**1**	**21717**	-172

Counter Ion/Salt Information			
Type	**Num.**	**Concentration [mM]**	**Total Charge**
Na	602	70.980	+602
Cl	430	50.700	-430

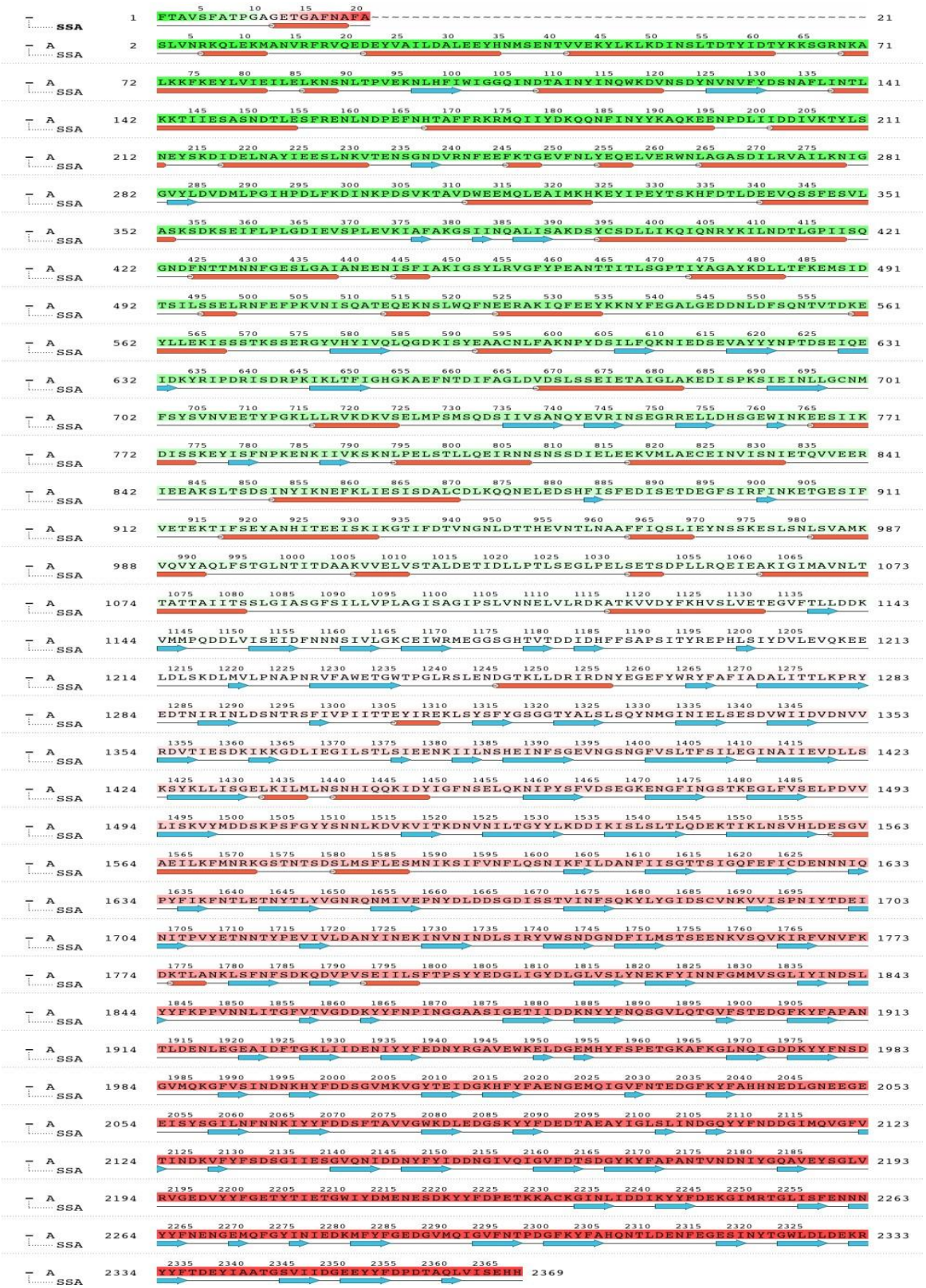


Figure 24 b. TcdB toxin's secondary structure (alpha helix and beta sheets) during the simulation.

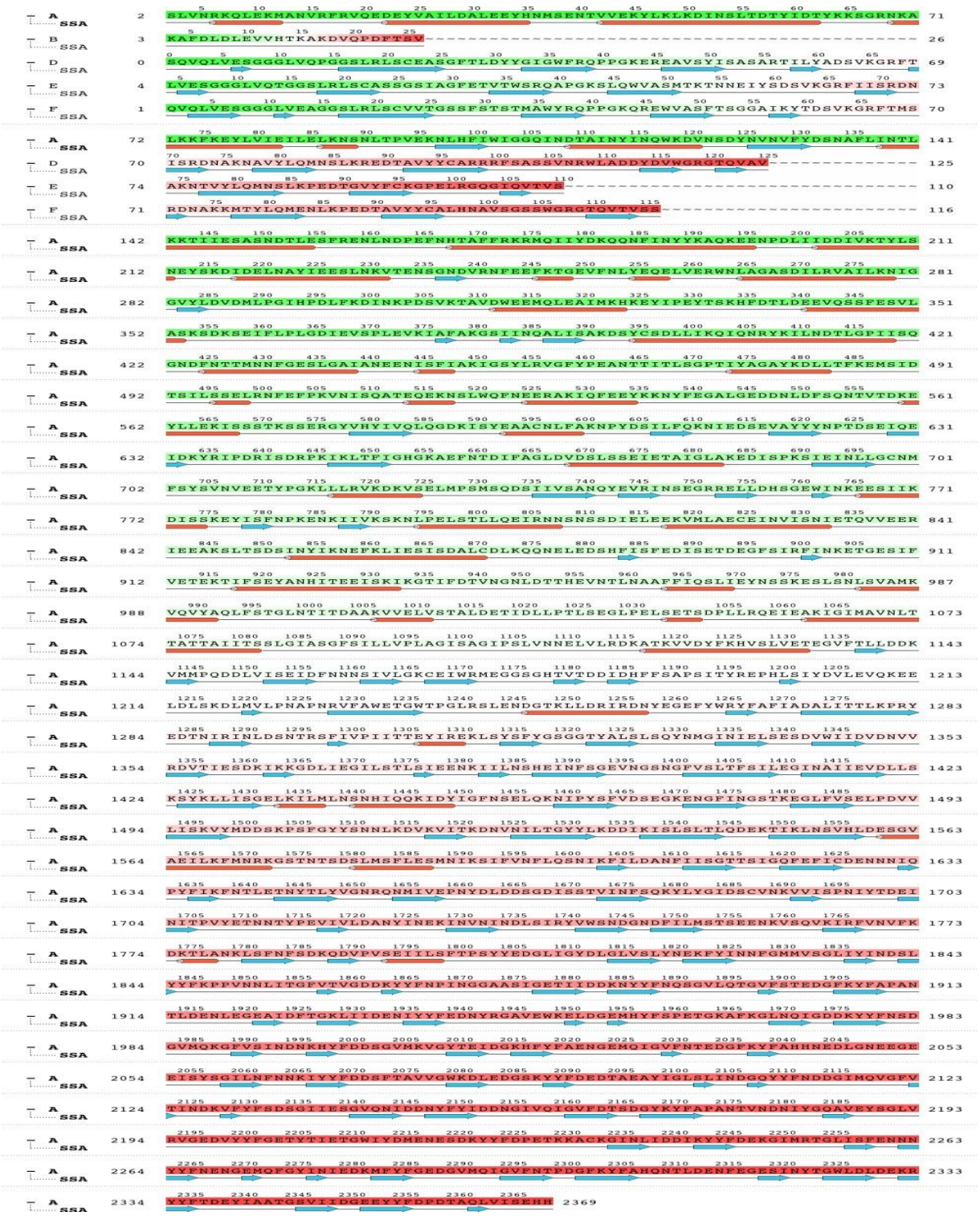


Figure 25. TcdB toxin's secondary structure (alpha helix and beta sheets) during the simulation.

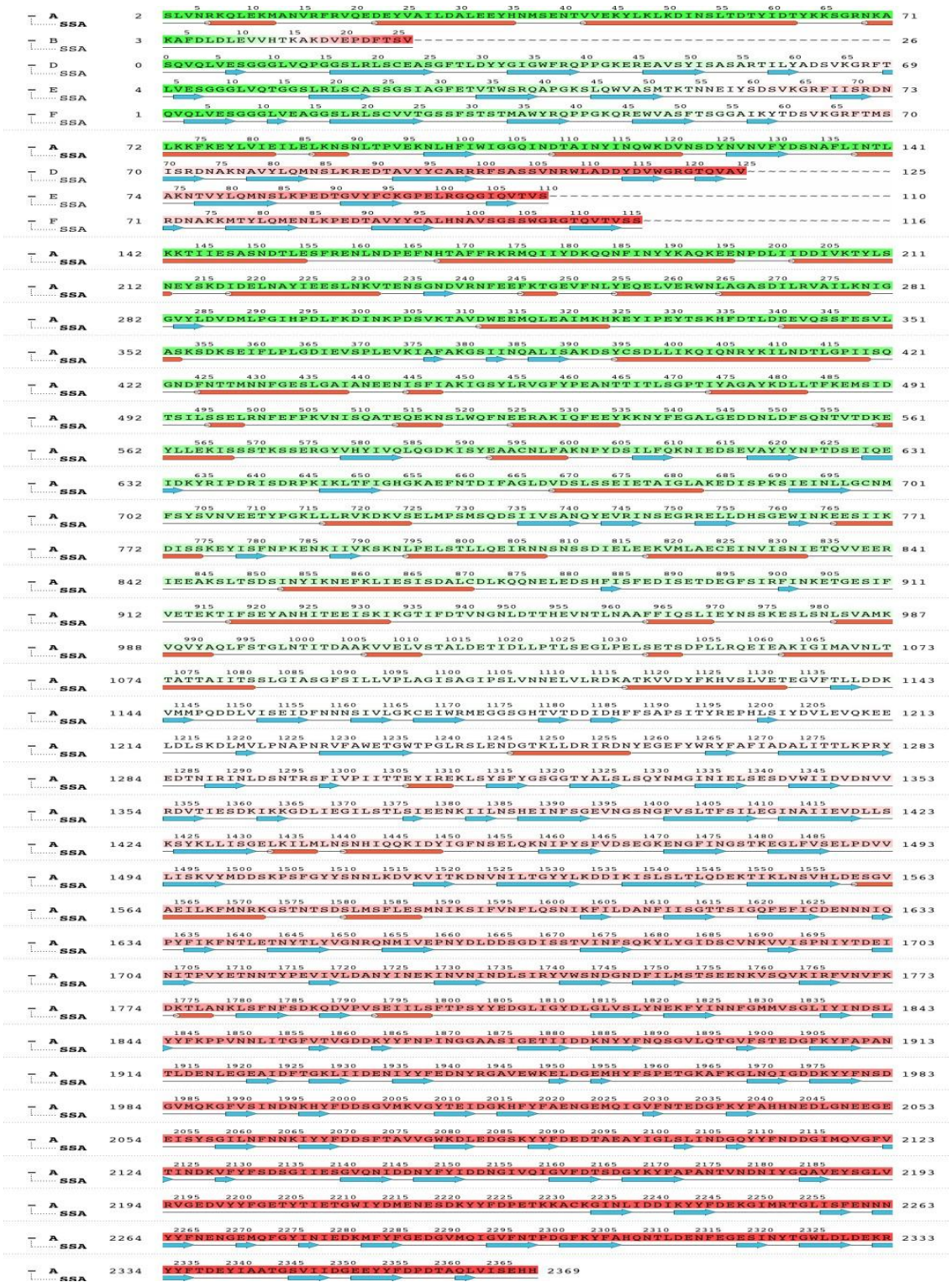


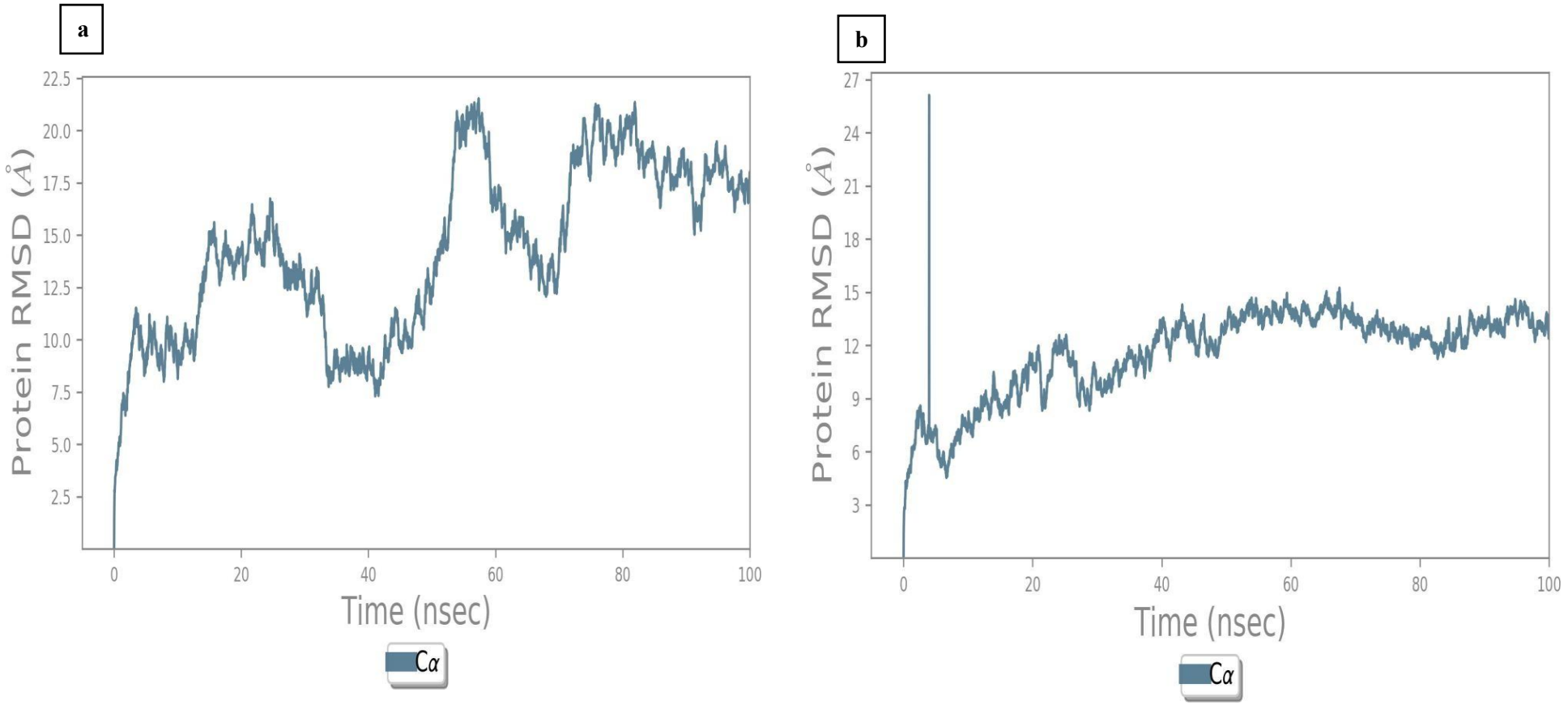
Figure 26. TcdB toxin's secondary structure (alpha helix and beta sheets) during the simulation.

3.3.4.4 Protein-Ligand RMSD

The Root Mean Square Deviation (RMSD) is used to estimate the overall change in atom movement for a particular frame concerning a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame *x* is calculated as:

$$RMSD_x = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(r_i'(t_x)) - r_i(t_{ref}))^2}$$

where N is the number of atoms in the atom selection; t_{ref} is the reference time, (typically the first frame is used as the reference and it is regarded as time t=0); and r' is the position of the selected atoms in frame *x* after superimposing on the reference frame, where frame *x* is recorded at time t_x. The methodology is repeated for all frames in the simulation trajectory.


a
b
Protein RMSD (Å)
Time (nsec)
Cα
Protein RMSD (Å)
Time (nsec)
Cα

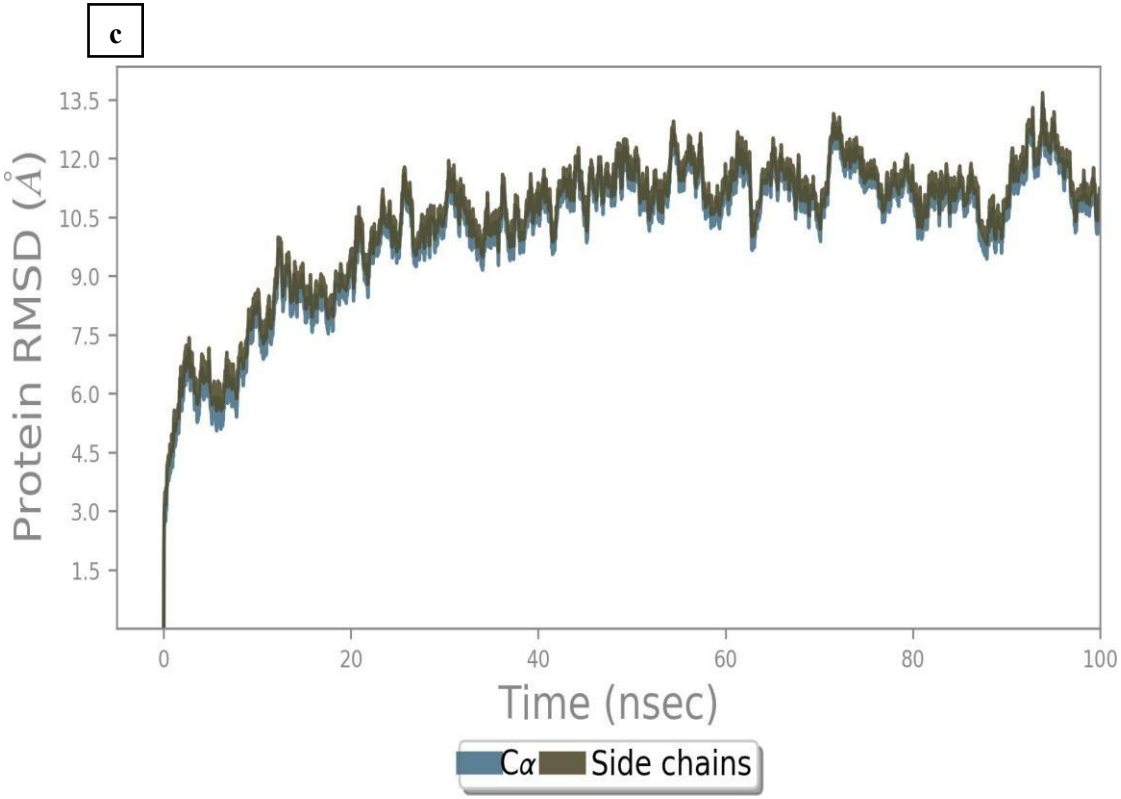


Figure 27. Docked protein simulation graph showing stability at 100 nanoseconds.

Protein RMSD

Figure 28a shows the RMSD changes of a protein (left *Y*-axis). A reference frame was used to arrange the protein frames and then theRMSD was estimated regarding the chosen atom. Protein RMSD analysis provided insights into its structural configuration during the simulation. RMSD exploration indicated if the simulation has evened up its fluctuations towards the end of the simulation around some thermal average structure. For small proteins like globular proteins, changes between the range of 1-3 Å were accepted. Above the order of 1-3 Å, large changes in protein configuration represented large changes during the simulation studies. The simulation required connecting the RMSD values to obtain a single stable value. For example, if the overall RMSD of the protein was still varying at the end of the simulation, then this situation represented that the system has not achieved a balance, and the simulation would be dissatisfying for extensive stability analysis.

The second plot **(Figure 28b)** showed the RMSD curve of TcdB concerning Gallidermin 1 (left Y-axis). An ideal, well-suited, stable protein frame was used to arrange the remaining protein frames and RMSD was calculated for the chosen target atom. RMSD analysis had given an insight into the structural configuration throughout the simulation. RMSD analysis concluded whether the simulation has balanced its fluctuations around some thermal average structure towards the end of the simulation.

The third plot **(Figure 28c)** showed protein-peptide binding stability during 100 nanoseconds of simulation time. A stable curve between 25 nanoseconds to 100 nanoseconds proved the interaction to be stable.

3.3.4.5 Protein RMSF

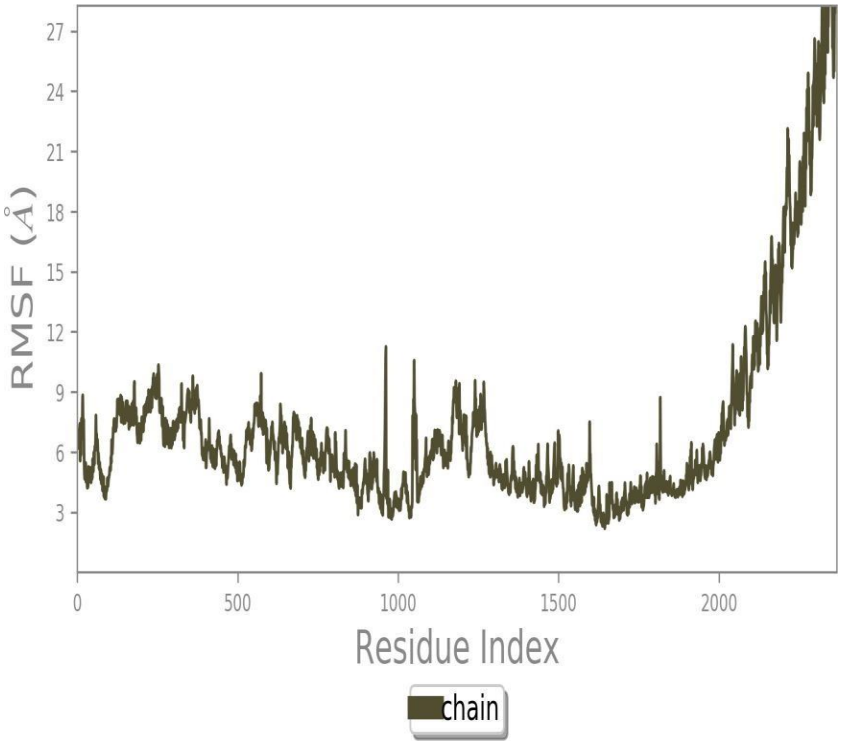
The Root Mean Square Fluctuation (RMSF) is useful for recognizing the basic fluctuations and changes in the protein structure. The RMSF for residue *i* is:

$$RMSF_i = \sqrt{\frac{1}{T}\sum_{t=1}^{T} < (r_i'(t)) - r_i(t_{ref}))^2 >}$$

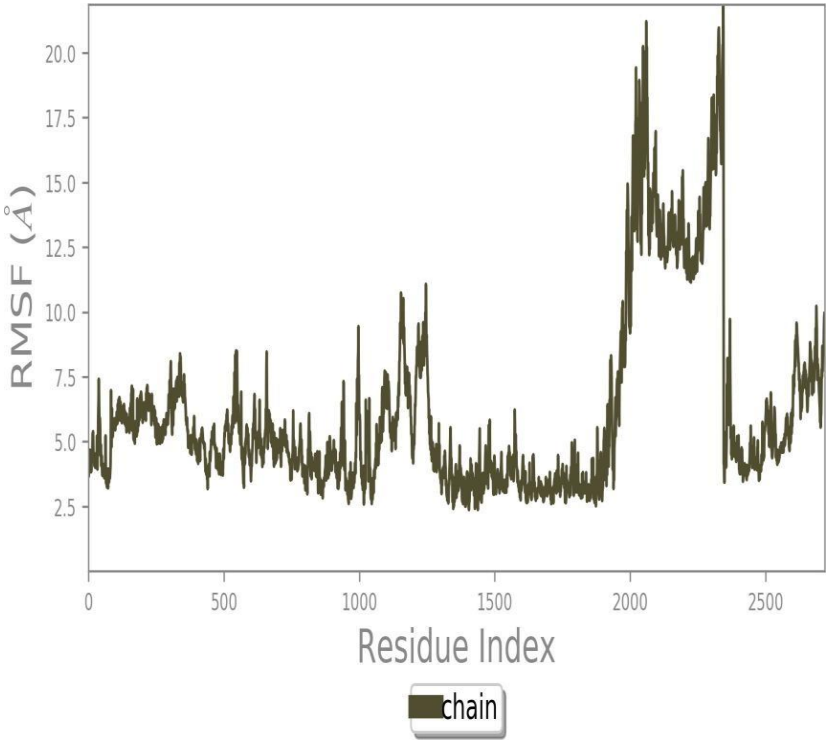
where T is the trajectory time over which the RMSF is calculated, *tref* is the reference time, *ri* is the position of residue *i*; *r′* is the position of atoms in residue *i* after superposition on the reference, and the angle brackets indicate that the average of the square distance is taken over the selection of atoms in the residue.

Protein RMSF

A



B



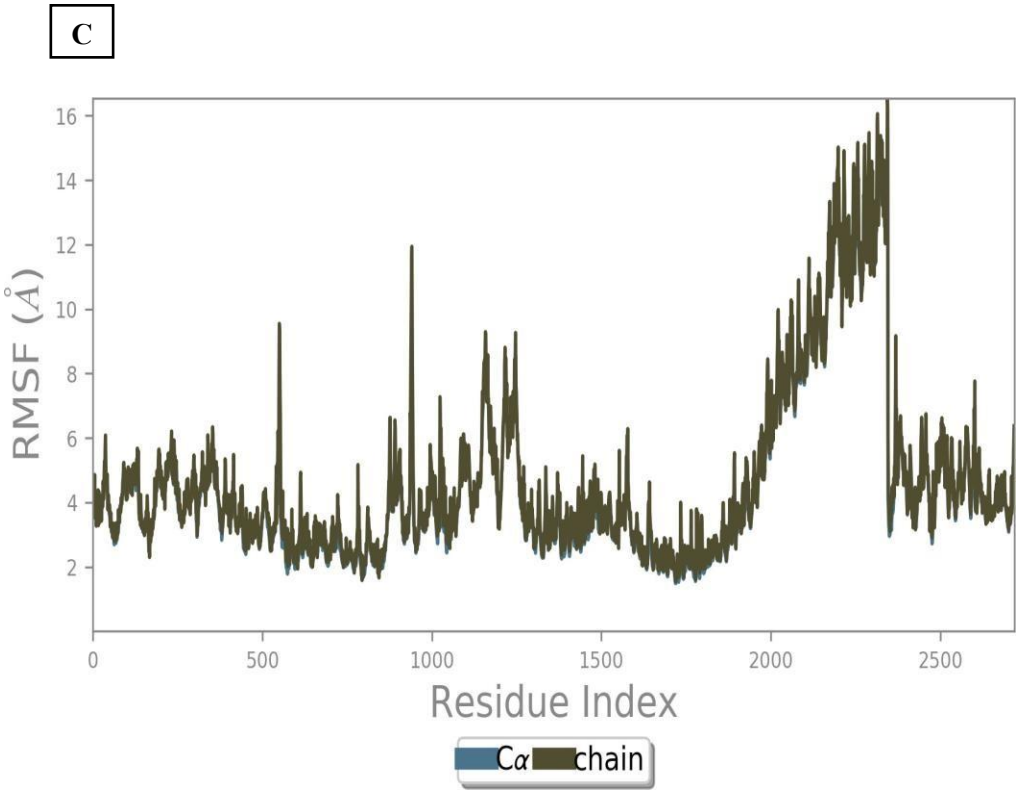


Figure 28. The root mean square fluctuation graph shows the complex stability of up to 2000 amino acids of TcdB, with minimum fluctuations.

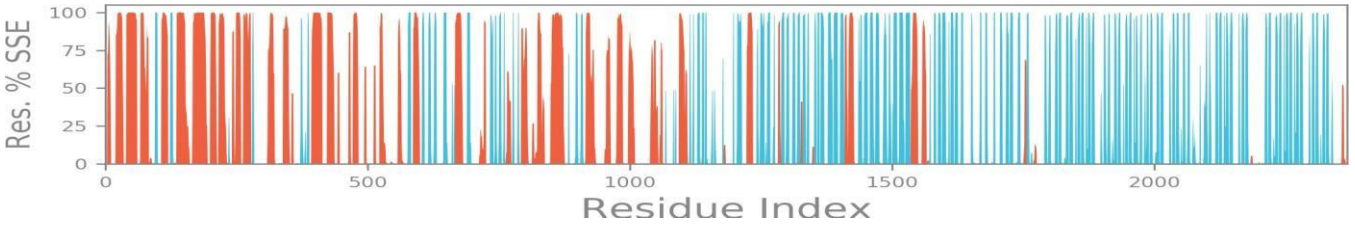
The first RMSF plot showed the interaction between TcdB and peptide clausin **(Figure 29 a).** The image showed those portions of protein, while in simulation that fluctuated the most. Typically, the amino and carboxyl-terminal endings fluctuated more than other part of the protein. Alpha helices and beta strands, the basic secondary structure elements showed more rigidity than the unshaped part of the protein and thus fluctuated less than the loop regions. Similarly, on the second and third plots **(Figure 29 b & c)** respectively, protein interaction showed overall stability of the protein-peptide complex. Also, plot c showed more fluctuations compared to plot b.

3.3.4.6 Protein Secondary Structure

During simulation protein's secondary structure elements (SSE) (alpha-helices and beta-strands) were analyzed (**Figure 30 a, b, c)**. The plots below reported SSE distribution by residue index throughout the protein structure.

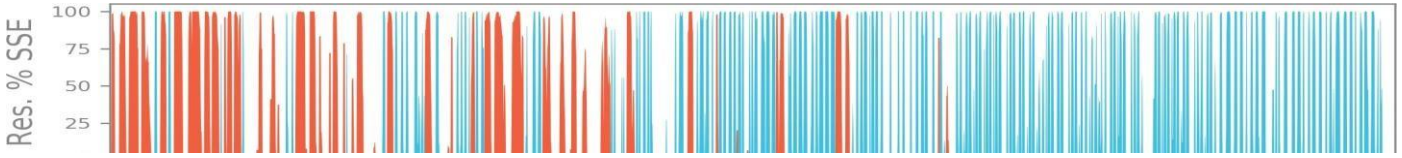
a

% Helix	% Strand	% Total SSE
19.36	24.88	44.23



b

% Helix	% Strand	% Total SSE
17.25	27.98	45.23



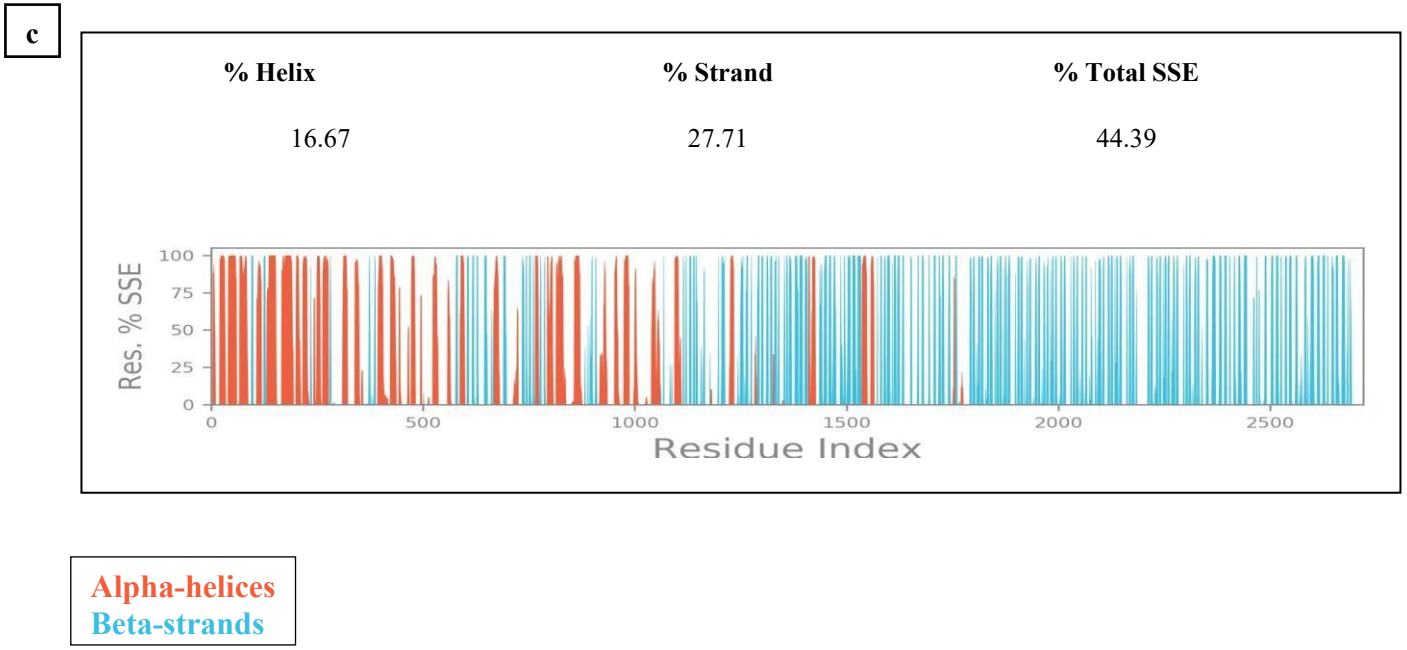


Figure 29. Protein secondary structure.

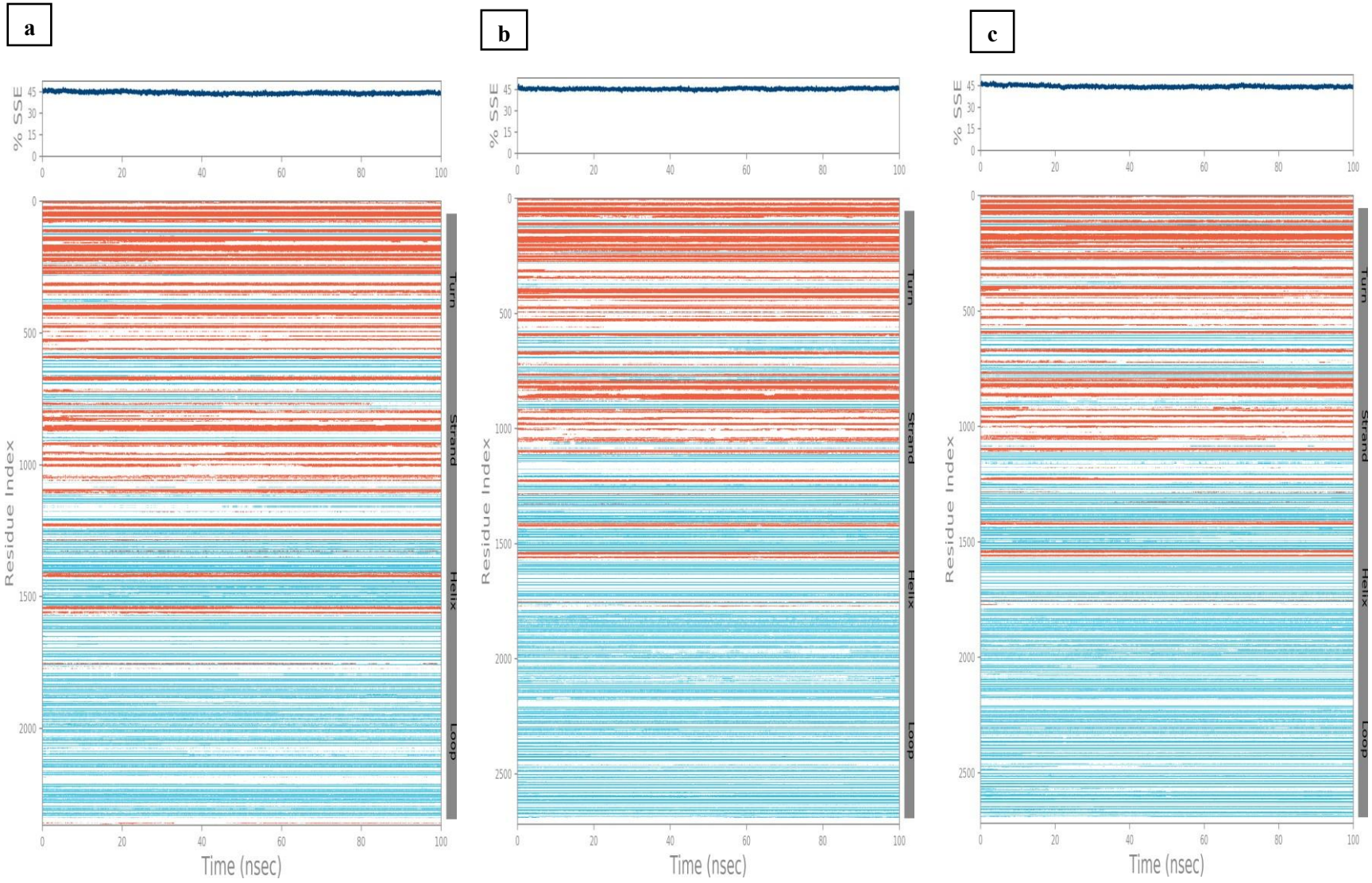


Figure 30. The plot represents the SSE composition for each trajectory frame throughout the simulation.

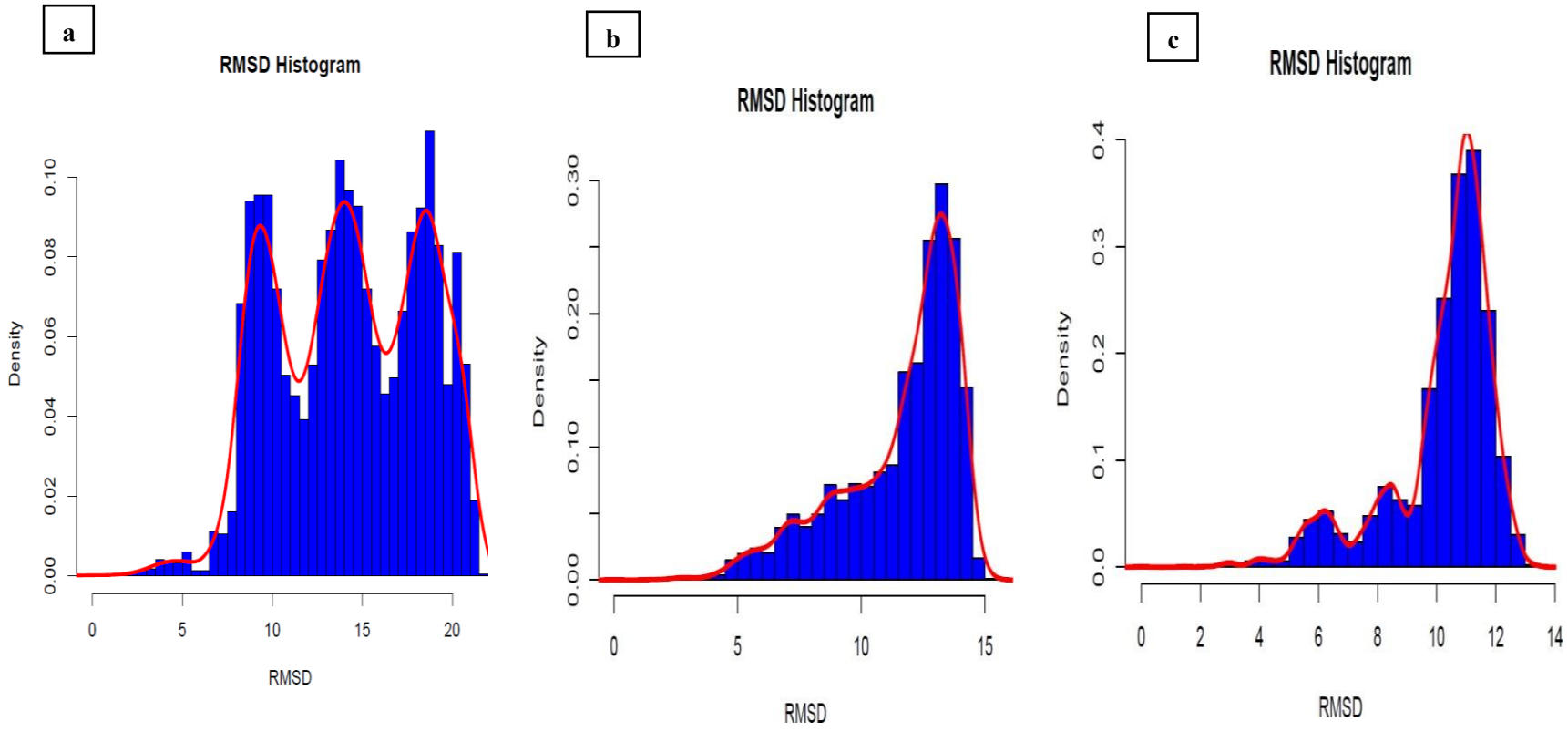


Figure 31. (a) The RMSD histogram showed fluctuations in the arrangement, with stable confirmation. (b) The RMSD histogram showed fluctuations in the arrangement, with average stable confirmation at 10, 15, and 20 Å. (c) The RMSD histogram showed fluctuations in the arrangement, with average stable confirmation at 10 to 14 Å.

3.3.4.7 Cross-Correlation Analysis

The analyses of the molecular dynamics (MD) trajectories, which were generated in the investigation, were checked for the mutual relation of the atoms, which is the observation of their welfare (measured in terms of the intensity of coordinated movement) within the system. The correlation method was applied to monitor fluctuations in Cα atoms after system equilibrium was reached **(Figure 32a)**. The correlation coefficients were studied in the following manner:

- If the score is 1, the atoms are completely correlated and therefore move together.
- A score of -1 describes when the atoms go against each other and, therefore, are not correlated.
- A value of 0 is indicative of the cases in which no correlation is going on; i.e., the atoms all move independently.

The proteins detected in cells of the TcdB complex interact with each other, as seen in the blue-colored area of this map **(Figure 32a)**, while the proteins at positions 1 to1000 are significantly correlated with each other, that is, they are in one phase of theirmotion, which causes most of them to move together in the same way. Moreover, thereddish area displayed anti-correlating cognition, which was manifested mainly by those entities falling into the range of 1000 to 1500 residues, which represents reverse momentum. The examination of this cross-correlation brings more light to thediscussion of the dynamics. The peptide that binds to the TcdB complex has shown dynamic coordination in its mobility that was particularly correlated with Gallidermin 1 in the region between residues 1 to 500 (denoted by the sky-blue area). However, an anti-correlated motion occurred in residues ranging from 500 to 1500 away from the peptide, denoted by thepink area **(Figure 32b)**. The unique patterns of correlations indicate the peptide underwent distinct interaction dynamics with different regions of the TcdB complex. Cross-correlation analysis was carried out to investigate the movement of Cα atoms after equilibrating the system in the course of the molecular dynamics simulation **(Figure 32c)**. The analysis revealed positively correlated motion (sky-blue area) for the **Clostridium difficile** Toxin B in complex with Gallidermin 2 for

residues 1 to 500, indicating movement sharing in this region of the protein. In contrast, the pink area indicates anti-correlated motion within the protein for residues 500 to 1500, indicating motion in the opposite direction for protein residues in this region. This type of analysis provides insight into the dynamic behavior and intermolecular coordination of the TcdB-Gallidermin 2 complex.

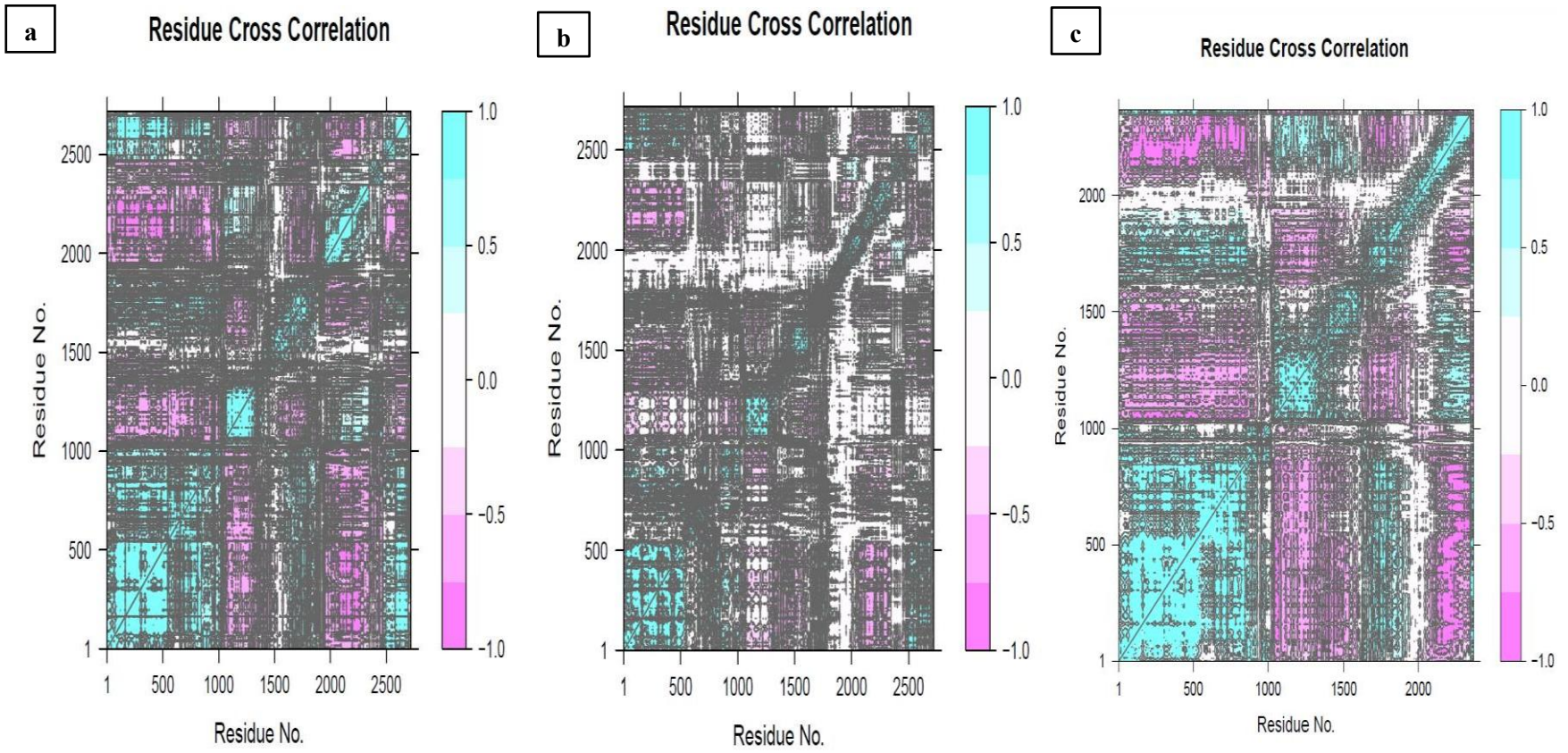


Figure 32. Cross-correlation of protein-protein complex residues.

3.3.4.8 Principal Component Analysis

To investigate the conformational states that the TcdB molecular complex could access, principal component analysis (PCA) was utilized. This method involved analyzing the molecular dynamics (MD) trajectory of the system and assessing the coordinated motions of the protein across the first three principal components (PCs), thus obtaining a clearer perspective of how the system was behaving in three-dimensional space **(Figure 33)**. To establish the degree of structural variability and collective motion within the complex, we calculated the eigenvalues associated with these principal components, revealing the principal conformational changes that occurred throughout the simulation.

To evaluate the coordinated movement of the TcdB-Gallidermin 1 protein complex, principal component analysis (PCA) was executed on the MD trajectory. The first three principal components were analyzed to visualize the system's conformational behavior in three-dimensional space **(Figure 34)**. Eigenvalues were also used to quantify the overall structural variability and collective movement of spatially and temporally correlated eigenvalues, which contributed to a holistic measure of the dynamics of the complex.

The Principal Component Analysis was utilized to analyze the combined motion of the TcdB-Gallidermin 1 molecule complex in the molecular dynamics (MD) trajectory in the first three principal components to visualize the system in three dimensions in the 3D plot **(Figure 35).** The other measure of the extensive combined structural variation was determined by examining the eigenvalues to understand the principal modes of motion in the complex.

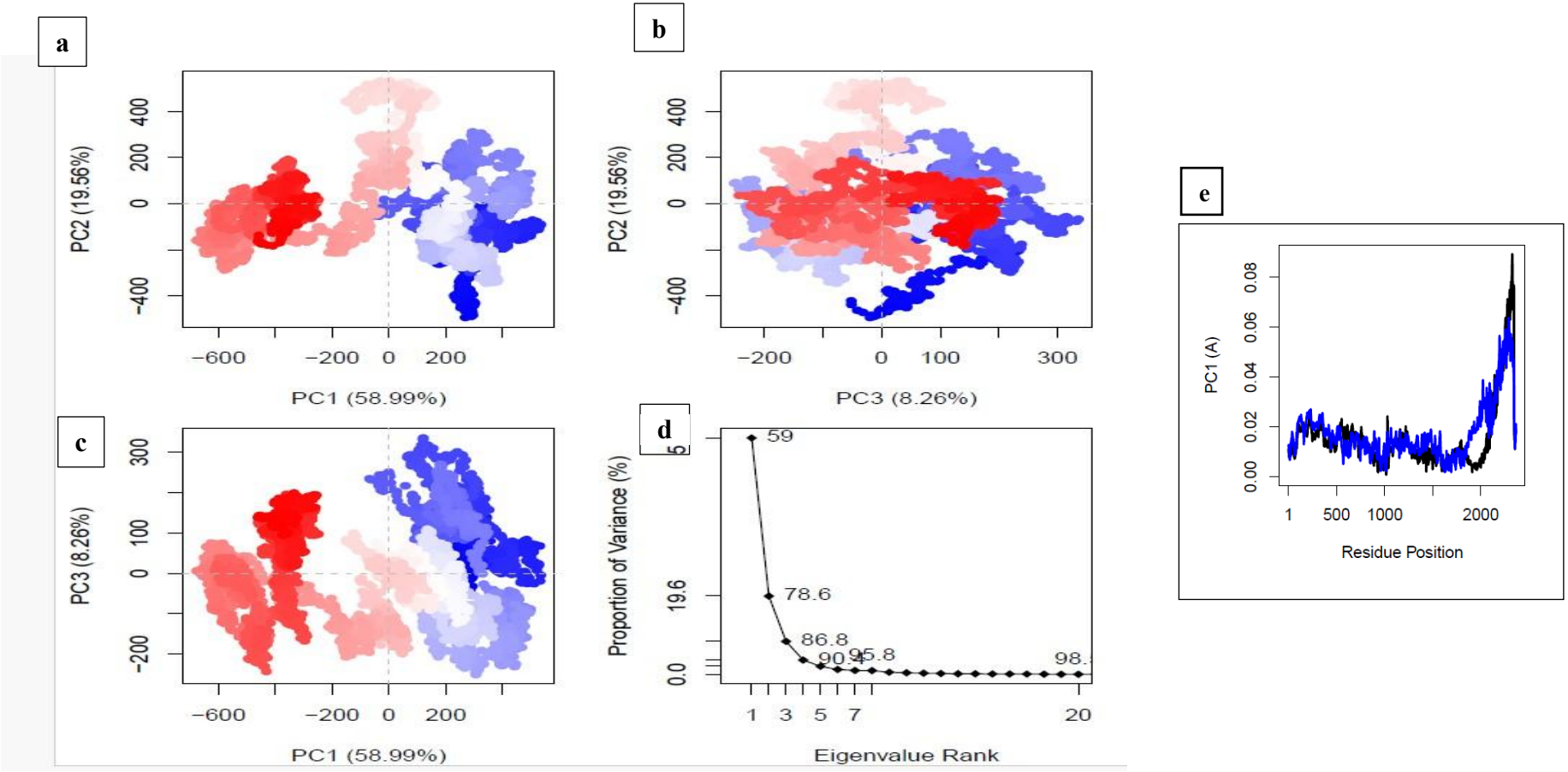


Figure 33. Principal component analysis (PCA) of the TcdB-clausin complex. (a) The first two principal components (PC1 and PC2) account for 78.55% of the total structural variance which highlights the primary conformational changes. (b) Combined PC2 and PC3 account for 27.82% of the total variance to address any secondary structural variance. (c) PC1 and PC3 together account for 67.25% of the total structural variance to show additional conformational changes. (d) Scree plot of eigenvalues of TcdB-clausin complex, with a sharp decrease in eigenvalues after the first few components. To summarize, the first three principal components of variance capture the vast majority of overall structural dynamics for the TcdB-clausin complex.

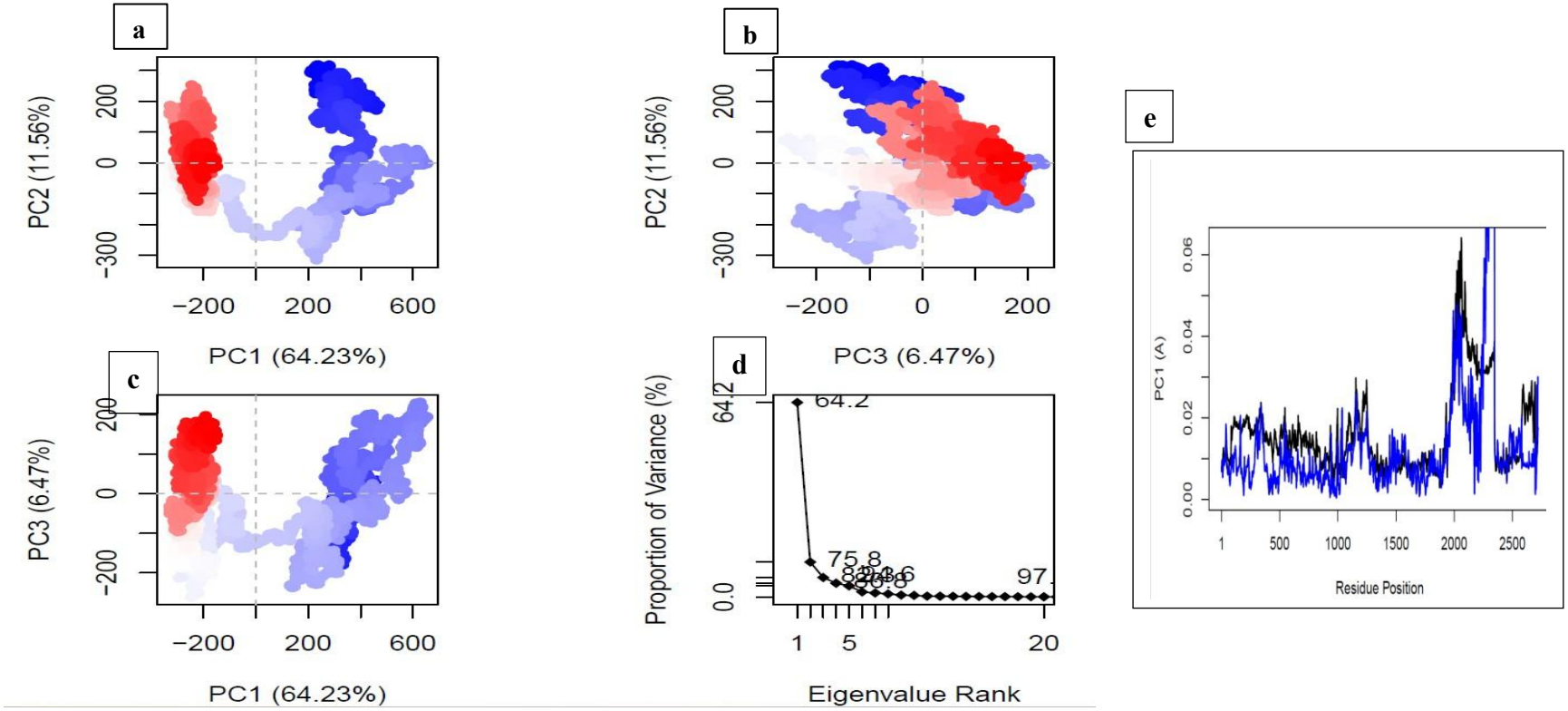


Figure 34. Principal component analysis of TcdB complex with Gallidermin 1. (a) The first two PCs i.e. PC1 & PC2 displayed 75.79% of the total structural variance. (b) PC2 and PC3 showed 18.03% of the total structural variance. (c) PC1 & PC3 contributed to 70.7% of the total variance in the structure. (d) The scree plot of eigenvalues of the TcdB-Gallidermin 1s complex showed a decline after the first value.

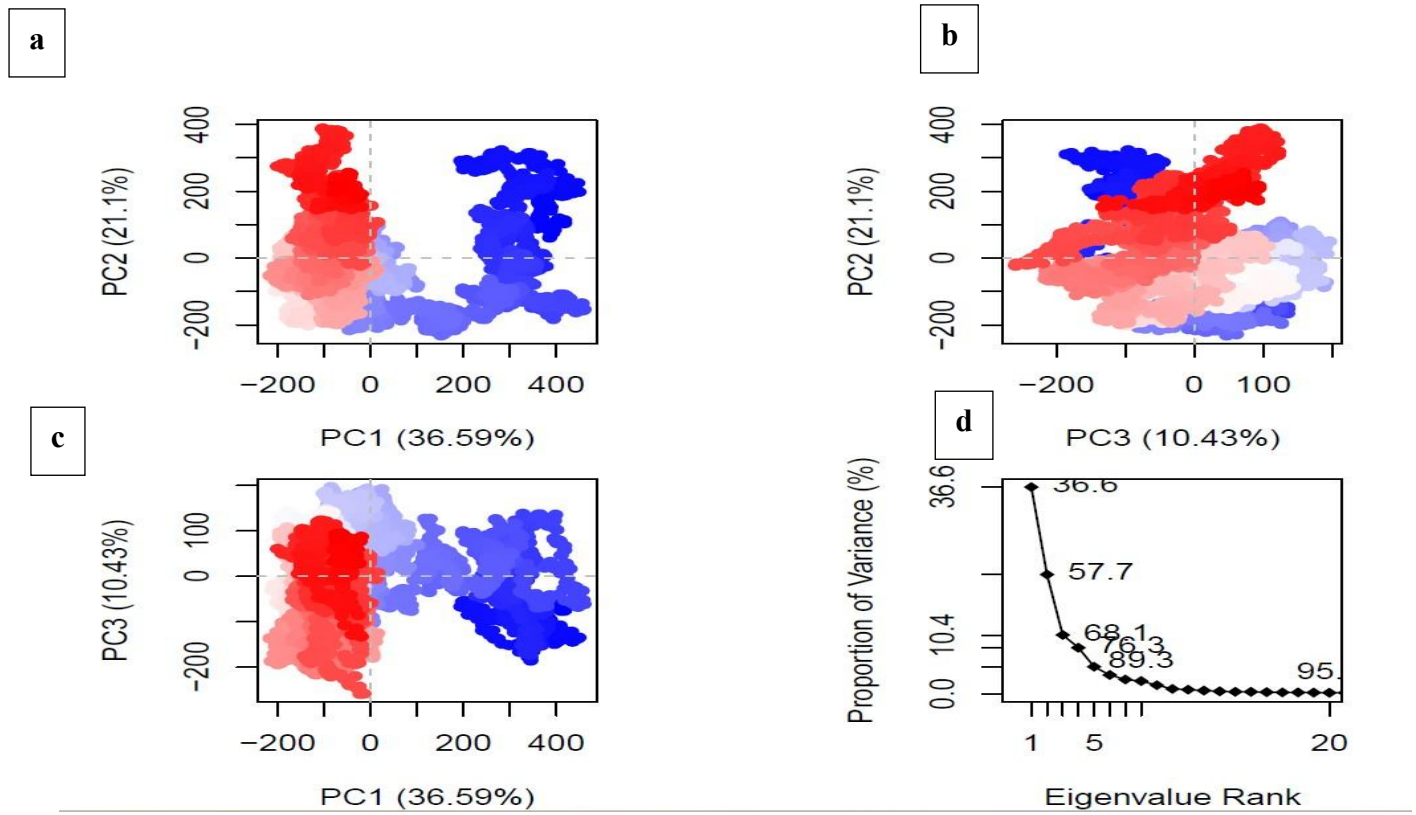


Figure 35. Principal component analysis (PCA) of the TcdB-Gallidermin 2 complex. (a) The first two principal components (PC1 and PC2) account for 57.69% of the total structural variance which highlights the primary conformational changes. (b) Combined PC2 and PC3 account for 31.53% of the total variance to address any secondary structural variance. (c) PC1 and PC3 together account for 47.02% of the total structural variance to show additional conformational changes. (d) Scree plot of eigenvalues of TcdB-Gallidermin 2 complex, with a sharp decrease in eigenvalues after the first few components. To summarize, the first three principal components of variance capture the vast majority of overall structural dynamics for the TcdB-Gallidermin 2 complex.

3.4 DISCUSSION

This exploratory study was focused on peptides that could be therapeutically derived from *B. clausii*, a bacterium with favorable probiotic properties. Given the critical involvement of protein-protein interactions in the pathogenesis of various infections, they represent an important target area for the development of therapeutic approaches. In this respect, computer-aided drug discovery (CADD) has emerged as an important tool in (but not limited to) determining novel drug targets and forming a foundation for empirical research. Individually isolated peptides and proteins from different species of *Bacillus clausii* have shown potential bactericidal activity against diarrhea-causing pathogens and their toxins. This study aimed to screen all available peptides from the former bacteria to obtain structural and functional clarity. This could be further implemented to identify and compare novel proteins and peptides for their anti-diarrheal potential. The objective utilized *in silico* techniques, protein databases, and software including Basic Local Alignment Tool (BLAST), Phyre, ClusPro, BIOVIA Discovery Studio, and Desmond software. Only three peptides with complete sequences (and high sequence similarity) were identified from the literature, limiting the depth of the study with regard to the diverse possibilities of therapeutic peptide interaction.

To add further depth to the study, sequences from a broad range of bacterial and viral toxins related to diarrheal disease were compiled from protein data bank. All protein, and peptide sequences were turned into three-dimensional structures using Phyre software. Protein-protein docking simulations were conducted using ClusPro software, where the structures of the toxins were docked against the three identified *B. clausii* peptides. Protein-protein docking shows the interaction of different proteins in biological systems, which can be used to explore the structural stability of the interacting molecules. Using ClusPro software multiple structures related to each protein-peptide complex were obtained. ClusPro software provides separate information about the Vander Waal, covalent, and hydrophobic forces of interacting complexes, which made the analysis more precise. From the various energy models created, the lowest binding energy modeled was chosen for further analysis as those predicted to exhibit the most favorable interactions.

Further analysis of protein interactions in three dimensions was performed using PyMol software. In the comparative assessment of the protein-protein docking results, every identified peptide exhibited the most stable interaction against TcdB, which is a toxin produced by *Clostridium difficile*. Sequence analysis of interacting molecules in PyMol software depicted that the interacting sites for all three peptides with TcdB molecules lay in the cysteine binding domain of the toxin molecule, concluding the inhibitory interaction to be allosteric.

To further assess these findings, MD simulations were conducted using Desmond for 100 nanoseconds. Molecular dynamics simulations are used to study the characteristics of molecules in a predefined system/ environment for a given period. Our study focused on exploring the target toxin molecule (TcdB) behavior in association with ligand molecules. The results indicated that the peptide-toxin justifications were stable throughout the simulation period and change in structural conformation did not hampered the structure stability with all three peptide molecules.

In summary, CADD provides a viable approach in this exploratory study that indicatespeptides from *B. clausii* have stable binding affinities towards pathogenic toxins such as TcdB. These results suggest that peptides (or proteins) with structural similarities tothose peptides identified for *B. clausii* building blocks could serve as potential anti-diarrheal agents. Further investigations into structurally related proteins could further increase the possibility of novel therapeutic peptide identification.

Chapter 4

To study the anti-diarrheal activity of newly isolated protease using *Drosophila*

4.1 Introduction

New treatments for human ailments have been developed by studying animal models in multiple-phase trials. Though many prokaryotic and eukaryotic organisms are used as animal models, preference is always given to vertebrate models based on the basic relatedness of humans to animal models. However, a preference has been shown for the invertebrate model in recent years. *Drosophila* offers a cost-effective substitute for other vertebrate and invertebrate mammalian models, providing an option for animal welfare. Choosing *Drosophila* as a gut study model comes from the presence of a gut system like humans but with a more simplified structure. The *Drosophila* flies have a short life span, huge egg-laying capacity, easy maintenance, and availability, and no special ethical approvals are needed for their use. 75% of *D. melanogaster's* disease-causing genes are functional homologs to human genes, followed by many conserved elements of the innate immune system including, transcription regulators, and signaling pathways. The fruit flies have a gut system anatomically similar to that of humans, *Drosophila* and humans share common gut microbes, thus making it an excellent study model to study gut disorders and observe the activity with their cures. Like the human gut, the gut of *D. melanogaster* acts as home to many infectious bacteria, viruses, and fungi. For pathogens like *Candida albicans, Enterococcus faecalis, Mycobacterium, Pseudomonas aeruginosa*, *Staphylococcus aureus, Serratia marcescens, Vibrio cholerae*, and there exist well-established infection models in *D. melanogaster* that have been explored to study host-pathogen interactions. Studies with these pathogens in *Drosophila* have reported a correlation between the infection and the metabolic adaptations in the host gut leading to gut dysbiosis state. *Drosophila* is a magnificent model to explore the impact of gut microflora in gut dysbiosis studies, where the impact of infectious agents can be analyzed based on the host's activity and their metabolic functions (nutrient content, rate of release, or depletion of certain compounds). *Drosophila* has been widely studied as a gut infection model and ample research is reportedrelated to host pathogens interactions which further makes it suitable to perform comparative analysis.

Hence our present study focused on studying the curative potential of purified protease against diarrhea in *Drosophila melanogaster.* The pathogen used

to induce diarrhea was *Salmonella enterica,* a Gram-negative, typhoid-causing bacteria**(Ayres et al., 2009).** Hydrolytic metalloprotease belonging to Dinb protein family was isolated from the *Bacillus clausii* UBBC07 strain, which is indigenous to India, and aseries of *in vivo* assays were performed on *Drosophila* to explore the antimicrobialpotential of purified protease. The curative action of protease was determined based on reduced bacterial load, increased survivability, and reduced oxidative stress.

Statement of the problem and hypothesis of the present study

- If protease uptake via food uptake is possible in *Drosophila* or not
- If so, then what amount of protease will help in the recovery of *Drosophila* fliesfrom the infection?
- What will be the impact on intracellular pathways of *Drosophila,* alongside recovery?

4.2 MATERIAL AND METHODS

4.2.1 Chemicals and Reagents

All the reagents and chemicals of molecular grade were purchased from Thermo Fisher Scientific.

A) Standard fly food

Maize 35 g

Sugar 30 g

Yeast 12 g

Agar 4 g

Sodium benzoate 2 g

Propionic acid 1 ml (Dissolve in 8 ml of 80% ethanol),

Distilled water 1000 ml

B) Phosphate buffer saline (0.1M, pH 7.2)

137mM NaCl 8 g

2.7 mM KCl 0.2 g

10 mM Na_2HPO_4 1.44 g

1.8 mM KH_2PO_4 0.24 g

Distilled water 1000 ml

C) Sucrose solution

Sucrose 5 g

Distilled water 100 ml

D) Potassium Phosphate Buffer (0.1M, pH 7.4)

Potassium phosphate dibasic 162.8 g

Potassium phosphate monobasic 8878 mg

Distilled water 1000 ml

E) 5,5′-dithiobis (2-nitro-benzoic acid) (DTNB) 10 mM

DTNB 4 g

Dimethylsulfoxide (DMSO) 1000 ml

Store at 4 ºC for 3 months

F) 100 mM Glutathione reduced

GSH 30 g

Distilled water 1000 ml

Store at -20 ºC for 3 months

G) 0.25 M potassium phosphate buffer (pH7.0) 1000 ml

Potassium phosphate dibasic 40 g

Potassium phosphate monobasic 2.2 mg

2.5 mM EDTA 0.7 g

H) 1-chloro-2, 4- dinitrobenzene (CDNB) 25mM

CDNB 3 g

95% Ethanol 1000 ml

I) 300 mM H_2O_2

H_2O_2 30.0 ml

Distilled water 969 ml

J) Loperamide hydrochloride tablets I.P.

K) Pestle and mortar

L) Catalase (150U/ml)

Catalase 1.5 mg

Potassium phosphate buffer (0.1 M) 1000 ml

4.2.2 Protease Isolation and Purification

Protein purification was done as previously described by Liu et al., **(2020), (Sakuma et al., 2021).** A pure culture of *Bacillus clausii* UBBC07 strain was grown in two-litre Muller Hinton broth for four days at 30 ºC. The bacterial culture was centrifuged and the supernatant was acid-precipitated with 1N HCl. Partially purified protein was run on 15% Tricine SDS PAGE gel and tested for antimicrobial compound presence using a zymogram. The target protein band was excised. The protein was manually purified with acetonitrile: ammonium bicarbonate (1:1) treatment. The concentration of purified protein was estimated using the Folin-Lowry assay. The protein was stored at 4 ºC till further use.

4.2.3 *Drosophila melanogaster* Stock and Culture

The flies were cultured and maintained under standard environmental conditions, as described by Iorjiim et al., (2020). *Drosophila melanogaster* wild-type strain Oregon^{R} was received from Stock Centre, Animal Tissue Culture laboratory, Lovely Professional University, India. Flies were maintained on standard fly food unless specified for treatments, at 25 ºC, with 60 % humidity, 12:12 hour day: light cycle, throughout the experiment.

4.2.4 Bacterial Infection Dosage Size

Infection dosages were prepared as described previously by Harnish et al., **(2020)**. The bacterial strain *Salmonella enterica* (MTCC 1164) was obtained from the CSIR- Institute of Microbial Technology. Flies were fed the infection bacteria orally. Flies were first exposed to *Salmonella enterica* infection using $O.D._{600\ nm}$ = 100. For this *Salmonella enterica* was grown in Luria Bertani broth from a single colony in bulk amount and centrifuged to obtain the pellet with $O.D._{600\ nm}$ = 100. The pellet was dissolved in a 5% sucrose solution. A sterile cotton was treated with the target bacterial cell pellet and placed inside the test vials containing *Drosophila* food. Once the cotton had dried up, young flies were put inside the test vials. Control flies were given the cotton soaked in 5% sucrose only. The flies were observed for infection every 24-hour interval.

4.2.5 Experiment Design

4.2.5.1 Preliminary Survival Assay

To conclude the appropriate protease dosage for *Drosophila* treatment a preliminary study was performed **(Iorjiim et al., 2020),** where bacterial load, bacterial shedding, and surviving flies were recorded for 15 days during the treatment. For this, after infecting the flies with *Salmonella enterica*, they were shifted to new vials (height 7 cm, diameter 2.5 cm) having fly food mixed with protease at variable concentrations i.e. 2 mg/ 40 ml food, 4 mg/ 40 ml food, 10 mg/ 40 ml food. The positive control was flies treated with Loperamide drug (4 mg/ 40 ml food), and the control was healthy flies. After determining the appropriate protease concentration for the treatment, the final assay was performed with a single protease dosage.

4.2.5.2 Final Survival Assay

After determining the appropriate protease dosage, a final assay was performed in two replicates followed by duplicates each time. The experiment set included the followinggroups:

Group I: Control flies with no infection or drug dosage.

Group II: Treatment flies with protease concentration of 10 mg/ 40 ml food.

4.2.5.3 Survival Assays

The five assays included bacterial load determination, bacterial shedding determination, survivorship curve, negative geotaxis, and reproduction. 15 flies were distributed in each vial, flies were shifted to new respective food vials every 5-day interval.

4.2.5.3.1 Bacterial Load Determination

Bacterial load calculation was performed as described previously by Siva-Jothy et al., **(2018)**. To confirm the oral infection, a live fly from the test vials (control and infection) was removed and shifted to a small-sized microcentrifuge tube. The fly was surface sterilized by placing them in 70% ethanol solution for 30 seconds. Ethanol was removed and the fly was washed with distilled water, three times. To the microcentrifuge tube, 100 µl of 1X phosphate buffer saline and fly was homogenized. The homogenate was transferred to the top well of 96 well microtitre

plate and dilutions up to 10^{-5} were made. 10 µl of each dilution was spread on Luria Bertani plates and colony-forming units were calculated using the Quebec colony counter (Thermo Scientific). The random bacterial colonies from the control plate were always subtracted from treatment plates and the experiment was performed with two independent replicates each having a duplicate.

4.2.5.3.2 Bacterial Shedding

Bacterial shedding was calculated as described by Siva et al., **(2018)**. Bacterial shedding is the release of infected bacteria from the fly body into the external environment. Flies from the control, treatment, and positive control vial were transferred to a 1.5 ml microcentrifuge tube containing 100 µl of 1X PBS, for 16 hours at 4 ºC. After the completion of incubation, the fly was removed and the tube was heavily vortexed. The solution of the tube was plated on an LB agar plate and colony-forming units were calculated.

4.2.5.3.3 Negative Geotaxis

Negative geotaxis was performed in the same manner as described previously by Lopez et al., **(2022)**. 15 treatment and 15 control flies were transferred without anesthesia to a clean, 15 cm tall glass vial, once every afternoon in light at room temperature. After 10 minutes of adaptation of flies, the glass vials were gently tapped three times, and fly climbing was observed for 30 seconds. The number of flies crossing a 10 cm mark was recorded until the movement of control and treatment flies became similar.

4.2.5.3.4 Lifespan Assay

The survivorship curve was plotted as previously described by Lopez et al., **(2022)**. Sixty flies from two replicates were fed protease-supplemented (10 mg/ 40 ml food) fly food, while the control flies were fed standard fly food only. The dead flies from treatment and control vials were counted every day till day 30 of the treatment, and themean survival rate was calculated.

4.2.5.3.5 Fecundity Rate

The reproductive abilities of the treatment flies were examined based on the protocol described by Iorjiim et al., (**2020).** A total number of 15 flies were placed in treatmentand control vials in replicates, where they laid eggs. The flies were

shifted to a new vialevery 48-hour interval and previous containers were monitored for 14 days for the appearance of new flies. The cumulative number was recorded and survivorship as an average mean was determined.

4.2.5.4 Biochemical Assays

4.2.5.4.1 Enzymatic Assays

Enzymatic assays were followed as previously executed by Iorjiim et al., **(2020)**. Sixty flies from Group I, and Group II were collected every 5 days, anesthetized using deep freeze treatment, and homogenized on ice in 1X phosphate buffer saline (0.1 M). The homogenate was tested for protein estimation using the Folin-Lowry method. The assays were performed for 25 days until all the treatment flies showed restoredenzymatic activity similar to the control flies. Tests were performed in two replicates followed by two duplicates each time. Flies were shifted to fresh food vials every 5 days.

4.2.5.4.2 Determination of Total Thiol Content

The Ellman method was utilized to estimate the total thiol content of flies **(Iorjiim et al., 2020)**. The constituents of the reaction mixture included 25 µl of homogenized protein sample, 30 µl of 5,5′-dithiobis (2-nitro-benzoic acid) (Ellman reagent) (10 mM), and 510 µl of 0.1M potassium phosphate buffer. The reaction mix was incubated for 30 minutes at room temperature and absorbance was calculated using a spectrophotometer at 412 nm. The standard curve of reduced glutathione (100 mM GSH) was used to calculate the thiol content and expressed as (µmol/mg of protein). The standard curve of reduced glutathione (100 mM GSH) was prepared by using dilutions from 0-1000 micromole/ml of reduced glutathione. 100 mM GSH wasprepared in 1X potassium phosphate buffer, pH 7.2. All working solutions were mixed with Ellman reagent (30 µl) and potassium phosphate buffer to make a reaction mixture of 565 µl. For all concentrations, a 30-minute incubation of the reaction mixture at room temperature followed by an absorbance at 412 nm was performed. A standard curve of absorbance was plotted against reduced glutathione concentrations. From the slope of the curve thiol content for respective biological samples was calculated.

4.2.5.4.3 Determination of Glutathione- S-Transferase Activity

Glutathione- S-transferase (EC 2.5.1.18) activity was determined by the methodology described by Adedara et al., **(2022).**

Assay Principle

GST in the target sample will catalyze the conjugation of L-glutathione to CDNB through the thiol group of the glutathione.

GST GSH + CDNB ⟶ GS-DNB Conjugate + HCl

The reaction product, GS-DNB Conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample**.**

Solution A was prepared by adding 20 ml potassium phosphate buffer, 10.5 mL of distilled water, 500 µl GSH 500 ml. The enzyme cocktail consisted of 270 µl of solution A, and 10 µl CDNB. To this mixture 20 µl test sample (1:5 dilution) was added. The increased absorbance was observed immediately at 340 nm for 2 minutes at 10-second intervals. The data was expressed as µmol/ml/min of protein utilized in making conjugates. The molar extinction coefficient for CDNB conjugate was 9.6 $mM^{-1}cm^{-1}$. As the absorbance goes on to increase to a certain extent average absorbance is calculated as:

Calculation:

(ΔA340)/min = A 340(Final read) - A 340 (Initial read)/ Reaction time (minutes)

GST specific activity:

$$\frac{(\Delta\ \mathbf{A\ 340)\ /\ min \times V\ (ml) \times Dilution\ factor}}{\varepsilon_{mM} \times \mathbf{V\ enzyme\ (ml)}} = \mu\ \mathbf{mol\ /\ ml\ /\ min}$$

Where:

Dilution factor = The dilution factor of the original sample (5)

ε_{mM} (mM-1cm-1)- 9.6 mM for CDNB conjugate at 340 nm. For test in 1 ml cuvette = 1

V– The reaction volume (0.69 ml)

V_{enz} – The volume of the enzyme sample tested (0.06 ml)Time- 2 minutes

4.2.5.4.4 Determination of Catalase Activity

Catalase (EC 1.11.1.6) activity was evaluated as previously reported by Adedara et al., **(2022).** with some changes in the protocol. Solution A was prepared by adding 1800 µL of potassium phosphate buffer, pH 7.0 to 180 µl of 300 mM H_2O_2. 10 µl of the test sample and 990 µL of solution A were mixed well and absorbance was visualized at 240 nm for 2 minutes with a 10-second interval. The results were expressed as µmol of H_2O_2 consumed/min/mg protein.

Catalase enzyme was used to plot a standard graph. A stock solution of catalase enzyme150 U/ml was prepared in potassium phosphate buffer by adding 1.5 mg in 1 ml buffer. From the standard solution working stocks of range 0-150 U/ml were prepared. For the catalase assay, hydrogen peroxide was used as a substrate, which was prepared in potassium phosphate buffer to a final concentration of 300 millimolars. The reaction mixture was prepared by adding 590 µl of substrate solution to 10 µl of working stocks of catalase. Absorbance was immediately captured at 240 nm for two minutes at 10-second intervals. The standard curve of catalase was plotted as $Absorbance_{240nm}$ against catalase (U/ml). The absorbance decreases with time representing the hydrogen peroxide degradation hence the total absorbance is calculated as:

$$\mathbf{A_{240nm} = Absorbance\ _{Initial} - Absorbance\ _{Final} / Time\ interval}$$

The further slope of the standard curve was used to calculate catalase units in all biological samples.

4.2.6 Statistical Analysis

The statistical analysis of data was done by using Sigma plot 11.0 and the results were calculated as mean ± SE (standard error). One-way analysis of variance (ANOVA) was done and all data with $p < 0$ and $p < 0.8$ was considered significant.

4.3 RESULTS

4.3.1 Preliminary Survival Assays

To evaluate the effective protease dosage involved in the treatment of infected flies three variable protease concentrations i.e. 2mg/ 40ml meal, 4mg/ 40ml meal, and 10mg/ 40ml meal were fed to the flies for 15 days. Loperamide, a synthetic molecule-based anti-diarrheal drug was used as a positive control in concentration 4mg/ 40ml meal. Flies fed on the protease concentration of 10mg/ 40ml meal, showed significant recovery, inthe form of reduced internal bacterial load, reduced bacterial shedding, and increased survivability **(Table 9), (Table 10), (Table 11).** Data was collected by performing two independent sets of experiments for all biological samples i.e. control, positive control, and treatment **(Figure 36), (Figure 37), (Figure 38)**. Data was analyzed as Mean±SEM (SE-standard error) for all observations, the data was found to be highly significant with $p<0$ for all three assays. All remaining analysis protease concentration of 10mg/ 40ml meal was used to treatthe infected flies.

Table 9. Preliminary analysis of protease effectiveness showing reduced bacterial shedding in treatment flies

S. No.	Time interval (Days)	Treatment 1		Treatment 2		Treatment 3		Positive control	
		Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction
1	0	830000000	8.91	780000000	8.89	1700000000	9.23	7650000000	9.39
2	2	750000000	8.87	690000000	8.83	821000000	8.61	811000000	8.59
3	4	552000000	8.74	600000000	8.77	19500000	6.99	34000000	7.21
4	6					3105000	6.19	1400000	5.86
5	8					92250	4.96	94250	4.96
6	10					3400	3.51	3400	3.57
7	12					89.5	1.68	87	1.67
8	14					15.5	0.93	12	0.84

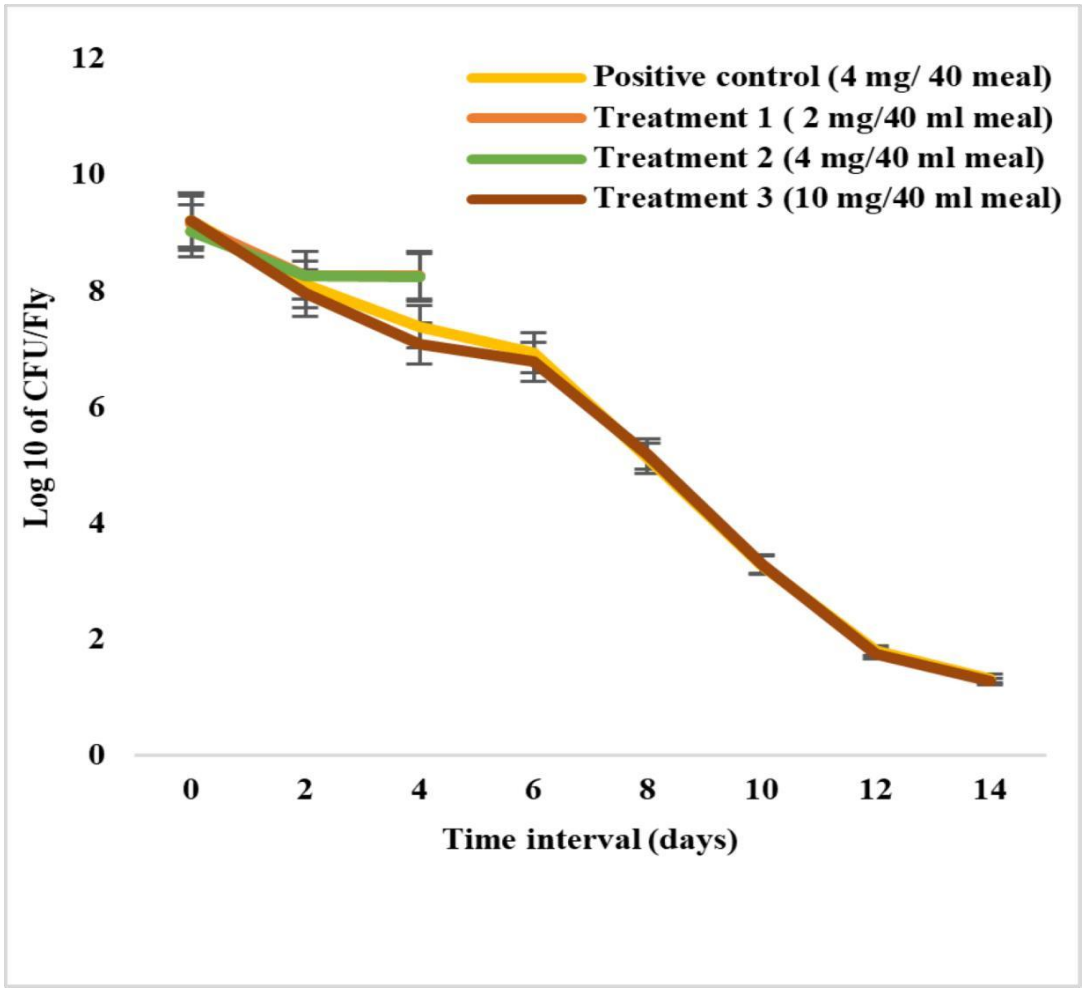


Figure 36. Preliminary analysis of protease effectiveness showing reduced bacterial shedding in treatment flies in a dose-dependent manner with the protease concentration of 10mg/ 40 ml food.

Table 10. Preliminary analysis of protease effectiveness showing reduced internal bacterial load in treatment flies

S. No.	Time interval (Days)	Treatment 1		Treatment 2		Treatment 3		Positive control	
		Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction	Average CFU/ ml	Log 10 reduction
1	0	1500000000	9.17	1100000000	9.04	1700000000	9.23	1700000000	9.23
2	2	189000000	8.27	190000000	8.27	188500000	7.97	187000000	8.12
3	4	189000000	8.27	177500000	8.24	24850000	7.09	34700000	7.38
4	6					12250000	6.78	12400000	6.94
5	8					154500	5.18	133000	5.12
6	10					1995	3.30	1890	3.27
7	12					115	1.75	90	1.80
8	14					35	1.27	30	1.32

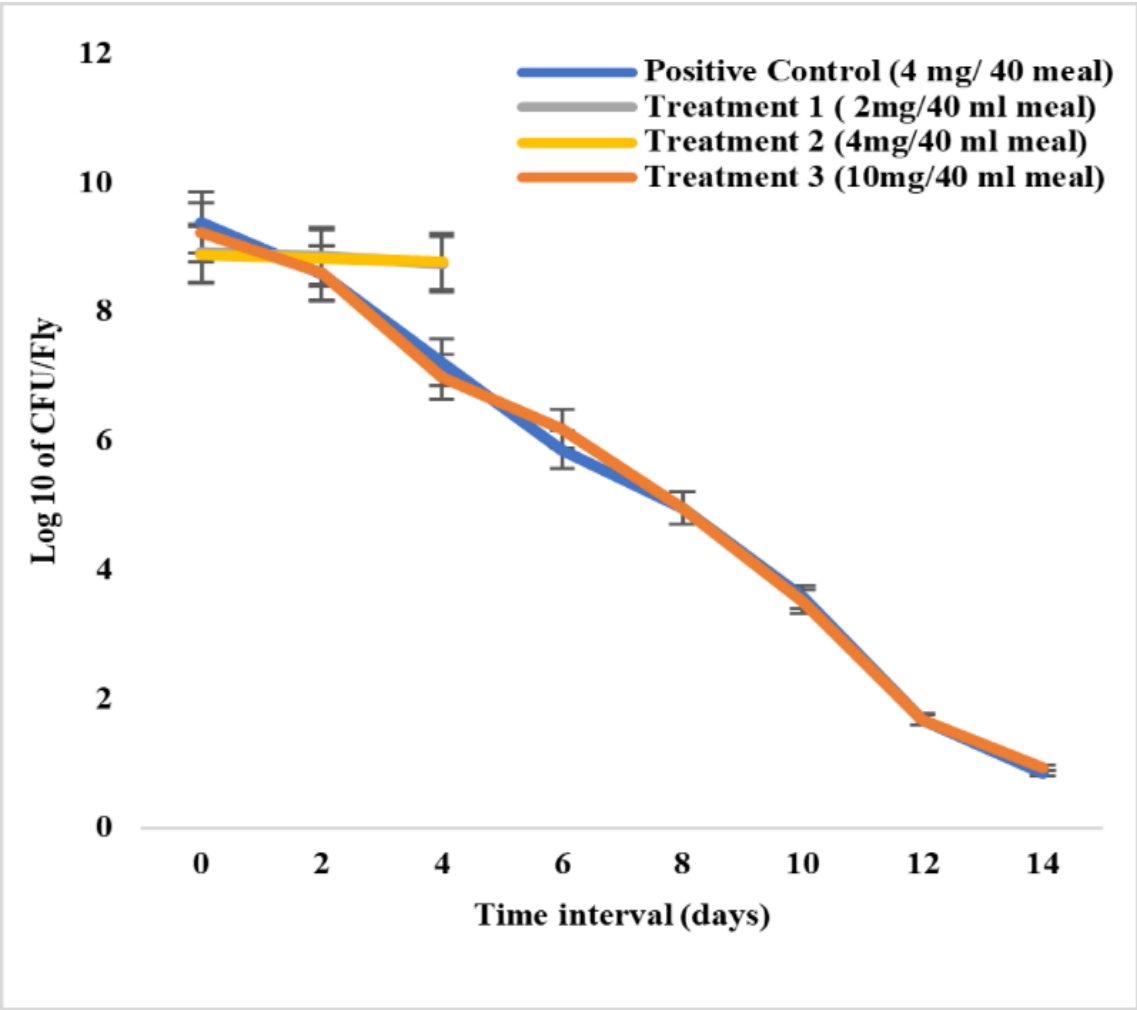


Figure 37. Preliminary analysis of protease effectiveness showing, reduced internal bacterial load in a dose-dependent manner with the protease concentration of 10 mg.

Table 11. Preliminary analysis of protease effectiveness based on the survival rate

S. No.	Time interval (Days)	Treatment 1		Treatment 2		Treatment 3		Positive control	
		Mean survival	Percentage	Mean survival	Percentage	Mean survival	Percentage	Mean survival	Percentage
1	0	15	100	15	100	15	100	15	100
2	2	6	40	5.5	36.66	14	93.33	15	100
3	4	1.5	10	1.5	10	13	91.22	14.25	95
4	6	0	0	0	0	12	87.27	13.75	91.66
5	8					10.5	76.36	13.75	91.66
6	10					10.5	76.36	13.75	91.66
7	12					11	80	13.75	91.66
8	14					10.5	76.36	13.75	91.66
9	16					9.5	73.07	13	86.66
10	18					9.5	73.07	13	86.66
11	20					9.5	73.07	13	86.66

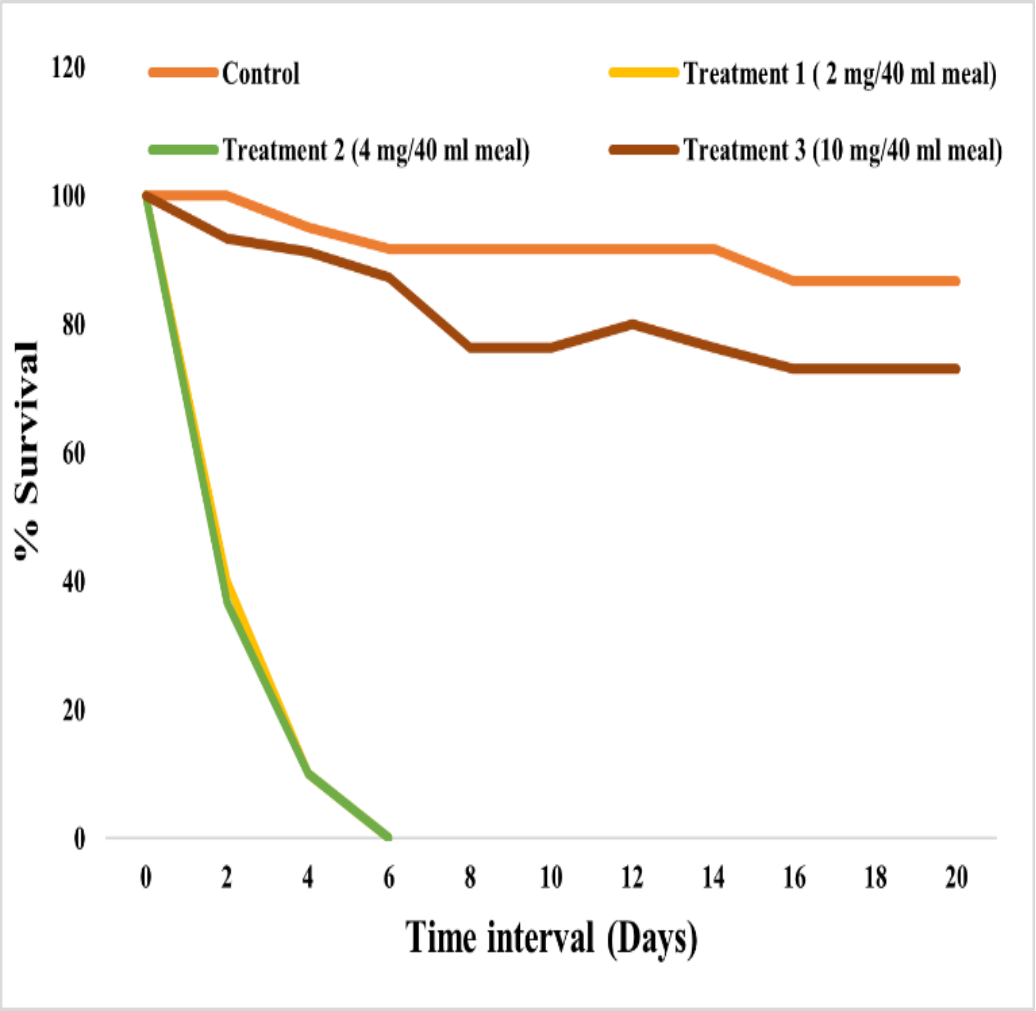


Figure 38. Improved survival rate in flies treated with 10 mg protease dosage.

4.3.2 Final Survival Assays

4.3.2.1 Bacterial Load and Bacterial Shedding Estimation

Flies were left to consume the bacterial infection until the colony-forming units reached approx. 1×10^9 CFU/ml. Day zero was taken as the day when the infection had reached the required amount and flies were shifted to the treatment vial (supplemented with protease). Flies were regularly analyzed for internal bacterial load with 48-hour intervals until no colony appeared on LB media plates **(Figure 39).** The average mean of *Salmonella enterica* colonies as cfu/ml was calculated **(Table 12).** Data was significant with $p<0$, for treatment and control.

Table 12. Reduction in internal bacterial load and bacterial shedding in treatment fly

S. No.	Time interval (Days)	Internal bacterial load		Bacterial shedding	
		Average mean	Log 10 reduction	Average mean	Log 10 reduction
1	0	1700000000	9.23	1700000000	9.23
2	2	188500000	7.97	815500000	8.61
3	4	25050000	7.09	19750000	6.99
4	6	12250000	6.78	3102500	6.19
5	8	154750	5.18	91375	4.96
6	10	2000	3.30	3250	3.51
7	12	112.5	1.75	97.25	1.68
8	14	37.5	1.27	17.25	0.93
9	16	6.75	0.52	5.5	0.43
10	18	0	0	0	0
11	20	0	0	0	0

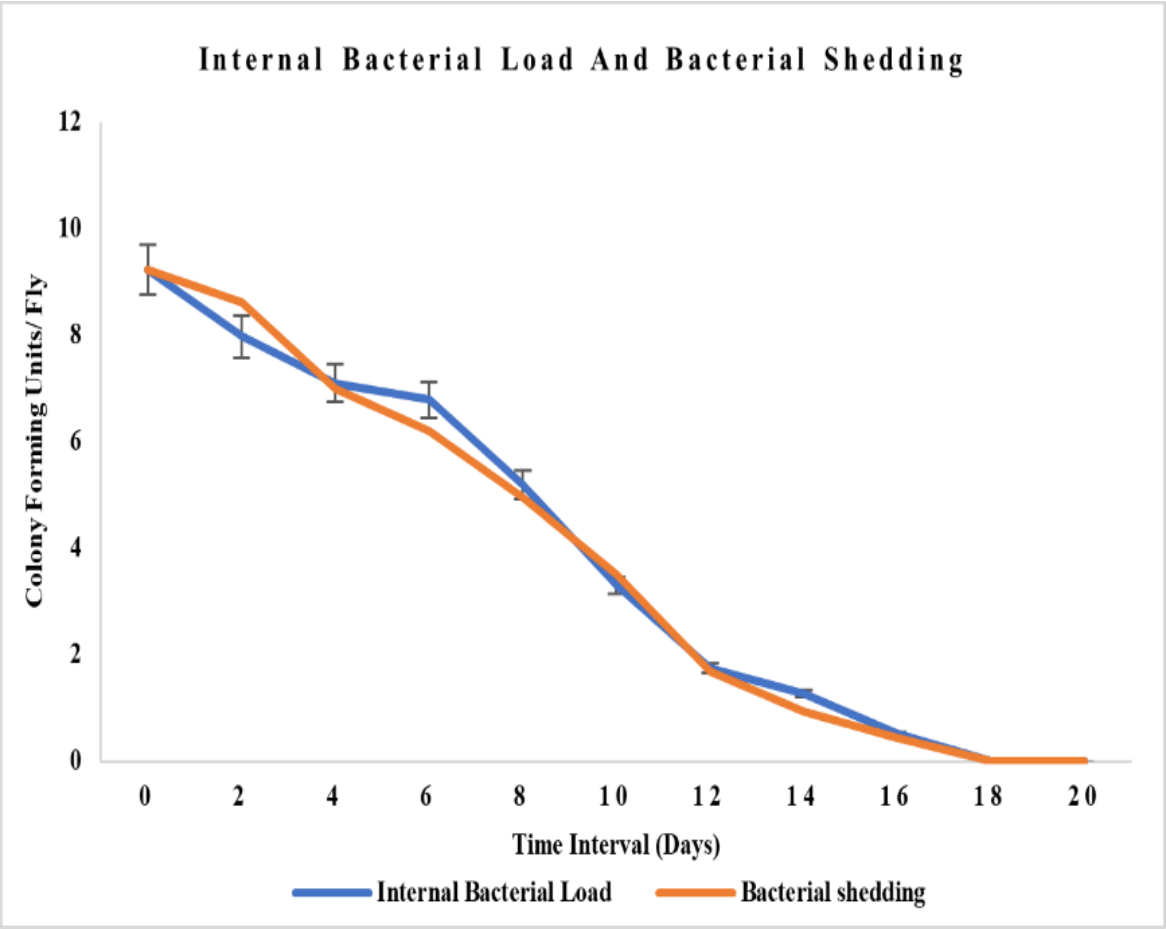


Figure 39. Bacterial load and bacterial shedding reduction in treatment fly were dosage-dependent, using a single final Dinb proteaseconcentration for 20 days. Random bacterial colony appearance in control samples was subtracted from treatment, and data from treatment flies was presented.

4.3.2.2 Climbing Assay

Fly health was estimated as a means of their climbing activity. The results were documented every 24-hour interval. The climbing ability of treatment flies showed 80 % recovery after 10 days of treatment, and the climbing ability of up to 96.6 % was restored after 20 days of treatment, similar to control flies **(Table 13)**. The climbing abilities of flies were stabilized after 15 days during the treatment **(Figure 40).** Data was significant with $p < 0$ for treatment and $p < 0.8$ for control.

Table 13. The average climbing ability of *Drosophila* flies

S. No.	Time interval	Control	Control flies	Mean	Treatment flies
1	1	15	100	0	0
2	2	14.7	98.3	3	20
3	3	14.2	95	7	31.66
4	4	14.5	96.6	7.75	46.66
5	5	14.25	95	8.25	50
6	6	13.75	91.6	11.25	65
7	7	14	93.3	12	80
8	8	14.5	96.6	12	80
9	9	14.5	96.6	12.5	80
10	10	14	93.3	12.75	80
11	11	14.5	96.6	12.75	80
12	12	14.25	95	12.75	85
13	13	14.5	96.6	12.75	85
14	14	14.25	95	13.25	88.33
15	15	14.75	98.3	13.25	88.33
16	16	14.75	98.3	13	86.6
17	17	14.5	96.6	14.25	95
18	18	14.75	98.3	15.75	96.6
19	19	14.5	96.6	17.5	98.3
20	20	14.5	96.6	18.75	96.67

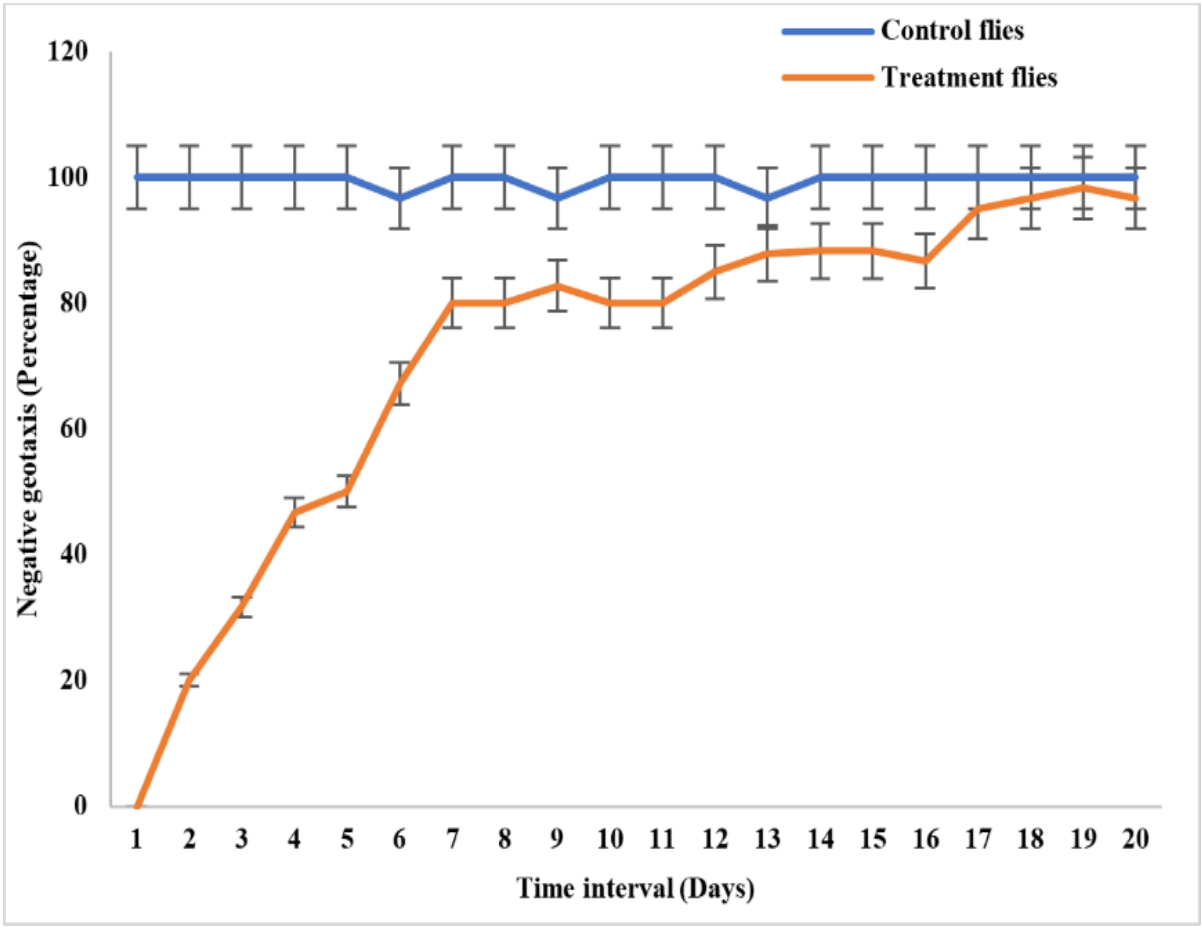


Figure 40. Dinb protease supplementation (10mg/ 40 ml meal) significantly improved the climbing abilities in *S. enterica-infected* flies in a dose-dependent manner. In the 20-day treatment duration, the climbing ability of the treatment flies appeared similar to those of the control flies.

4.3.2.3 Survivorship Curve

The dead flies from control and treatment vials were counted every 48-hour interval for 30 days. The fly count initially decreased from 100 % to 71% in treatment vials within10 days during the treatment while the survival rate in control flies decreased to 93% **(Figure 41)**. After 15 days of treatment, the flies retained 69 % survival when observed for 30 days **(Table 14)**. Control flies retained an 87 % survival rate till 30 days of observation.

Table 14. Rate of survival in control and treatment *Drosophila* flies

Time interval (Days)	Control flies	% Control	Treatment flies	%Treatment = (Treatment/Control*100)
0	15	100	15	100
2	15	100	13.75	93.3
4	14.25	95	13	91.2
6	13.75	91.6	11.25	87.2
8	13.75	91.6	10.25	76.3
10	13.75	91.6	10.25	76.3
12	13.75	91.6	10.25	80
14	13.75	91.6	9.75	76.36
16	13	86.6	9.25	73.07
18	13	86.6	9.25	73.07
20	13	86.6	9.25	73.07
22	13	86.6	9.25	73.07
24	13	86.6	9.25	73.07
26	13	86.6	9.25	73.07
28	13	86.6	9.25	73.07
30	13	86.6	9.25	73.07

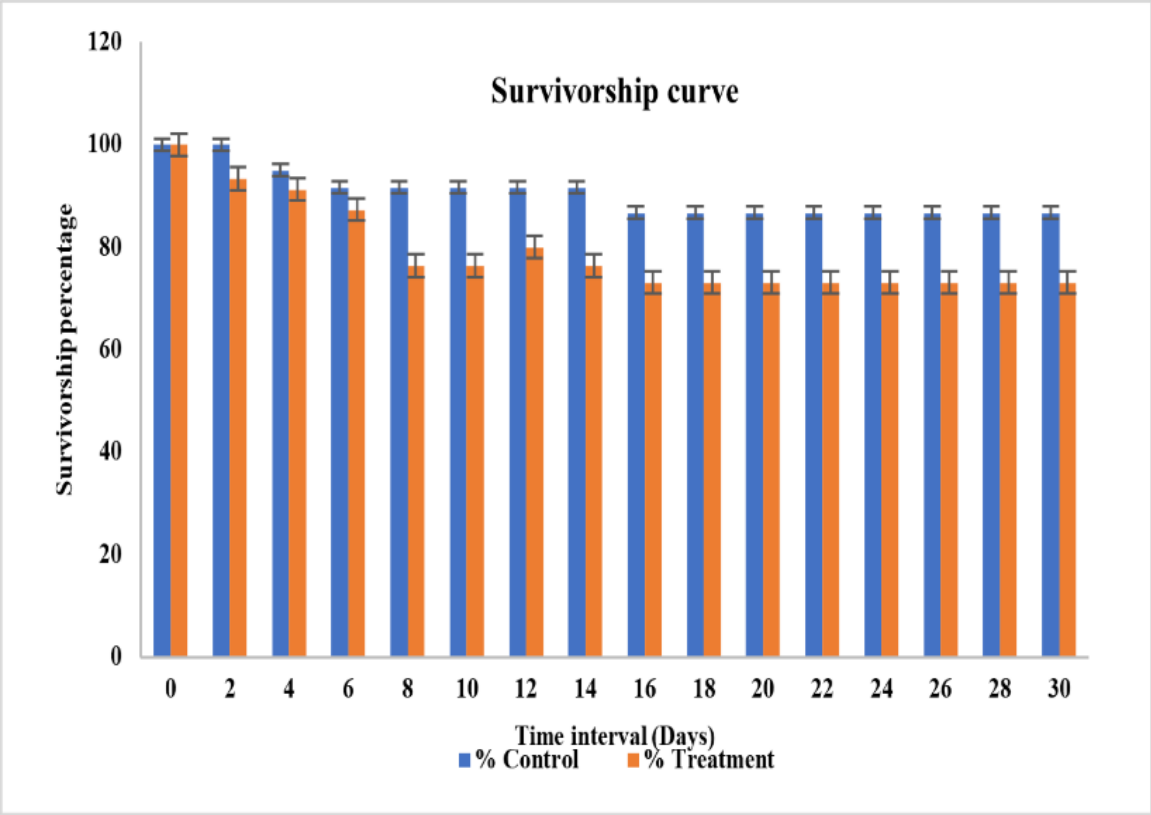


Figure 41. Dinb protease supplementation (10 mg/ 40 ml meal) rescued the flies from the *Salmonella enterica* induced bacterial infection in a dose-dependent manner, stabilizing the fly number between 15 to 30 days of treatment. Fly survival was calculated from day 1 of the treatment. Data was presented as Mean ± SEM of two independent experiments with duplicates each time, with a significance level of 0 for control and treatment.

4.3.2.4 Fecundity Rate

The reproduction rate of infected flies was low compared to the healthy control flies. During the first 10 days of the treatment, the fecundity rate increased from 38 % to 67.5 % and reached 98 % concerning the control set during the 20 days **(Table 15).** The reproduction rate in control flies increased from 100 % to 125 % in 20 days of observation with $p < 0.00071$ for treatment and $p < 0$ for control **(Figure 42).**

Table 15. Fecundity curve representing the average reproduction in *Drosophila* flies

S. No.	Time interval (Days)	Average control mean	% Control	Average treatment mean	% Treatment
1	0	20	100	7.5	37.5
2	2	21.5	107.5	9	45
3	4	20	100	10.5	52.5
4	6	22.5	112.5	13	65
5	8	22	110	13.5	67.5
6	10	23.5	117.5	13.5	67.5
7	12	23	115	16	80
8	14	24	120	16	80
9	16	23.5	117.5	18.5	92.5
10	18	25	125	20	100
11	20	25	125	19.5	97.5

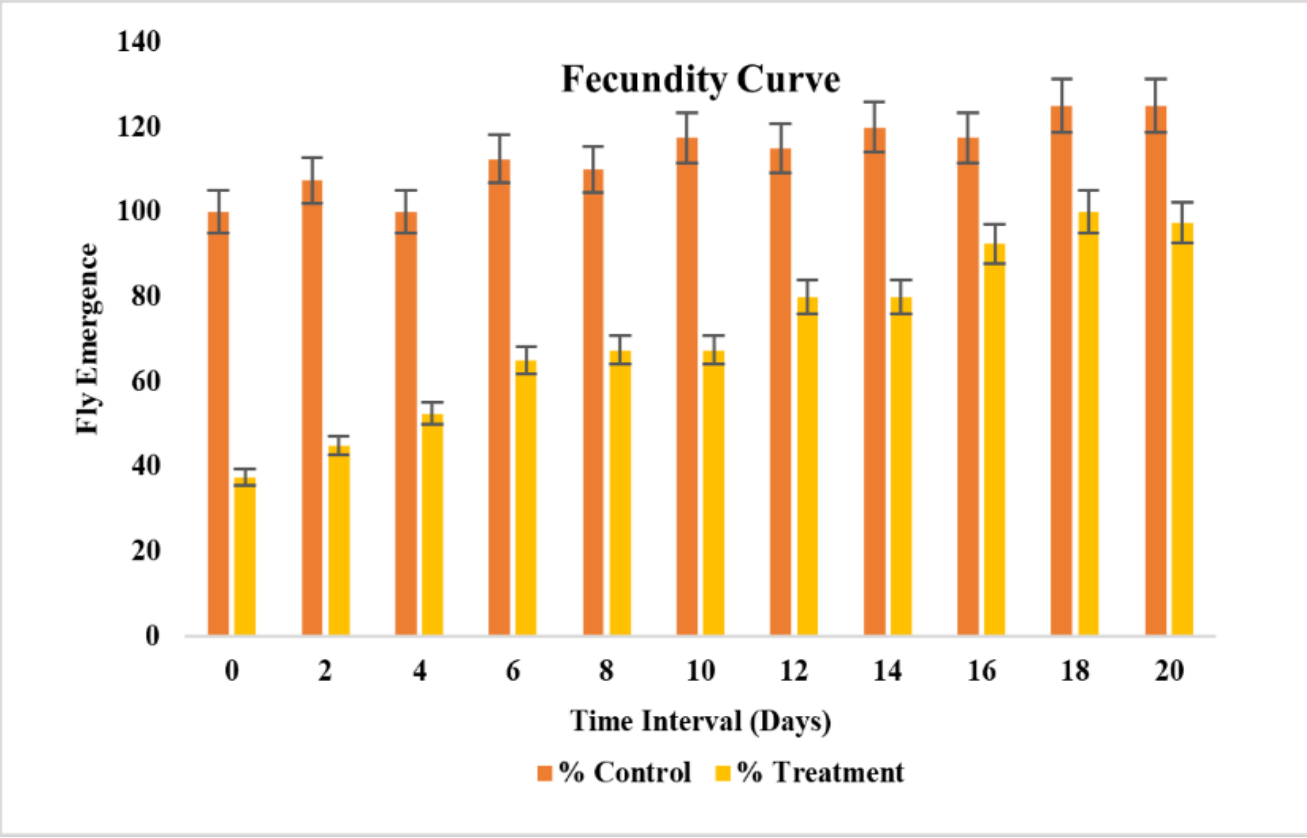


Figure 42. Dinb protease (10 mg/ 40 ml meal) treatment restored the reproduction abilities of infected flies, leading to an increased fly emergence during the 20-day duration of treatment, $p < 0.00071$ for control and significance 0 for treatment were observed.

4.3.3 Biochemical Assays

4.3.3.1 Total Thiol Content

Total thiol content was calculated from the standard curve of reduced glutathione **(Figure 43)** by using the slope, **y = 0.001x + 0.1831** and concentration was expressed as µmole/mg of protein.

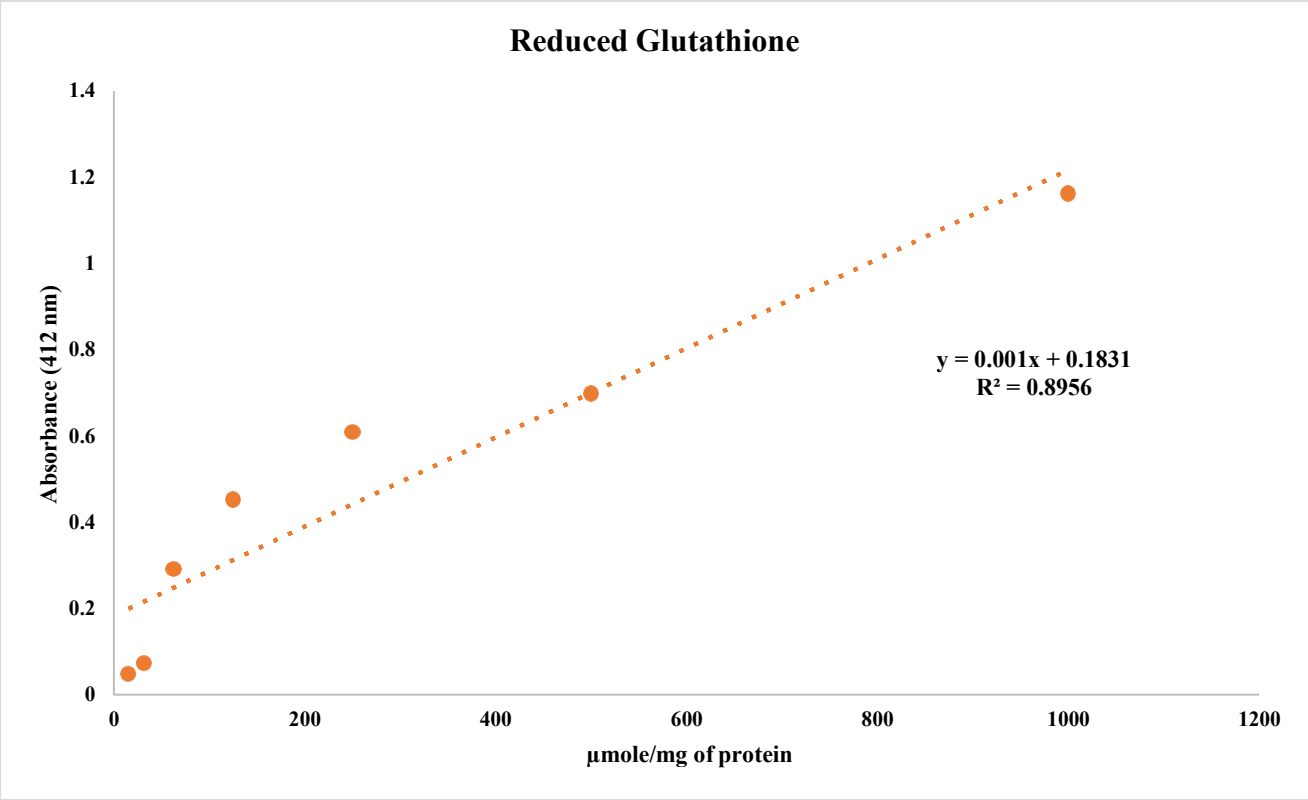


Figure 43. Standard curve of reduced glutathione.

The results of total thiol content estimation showed a significant increase in thiol content in all the protease-fed flies during the experiment **(Table 16)**. The protease supplementation (4mg/ 40 ml food) significantly increased the thiol content from 9 µmol to 55 µmol when compared to control flies whose thiol content remained between64 µmol to 62 µmol during 25 days of treatment **(Figure 44).**

Table 16. Estimation of Total thiol content

S. No.	Day	Average control (Protein concentrationin µmol)	% Control	Average treatment (Protein concentration in µmol)	% Treatment
1	0	64.32	100	9.03	14.04
2	5	64.97	101.003	24.74	38.46
3	10	63.68	98.99	38.51	59.87
4	15	60.67	94.31	45.18	70.24
5	20	57.44	89.29	54.21	84.29
6	25	61.96	96.321	55.29	85.96

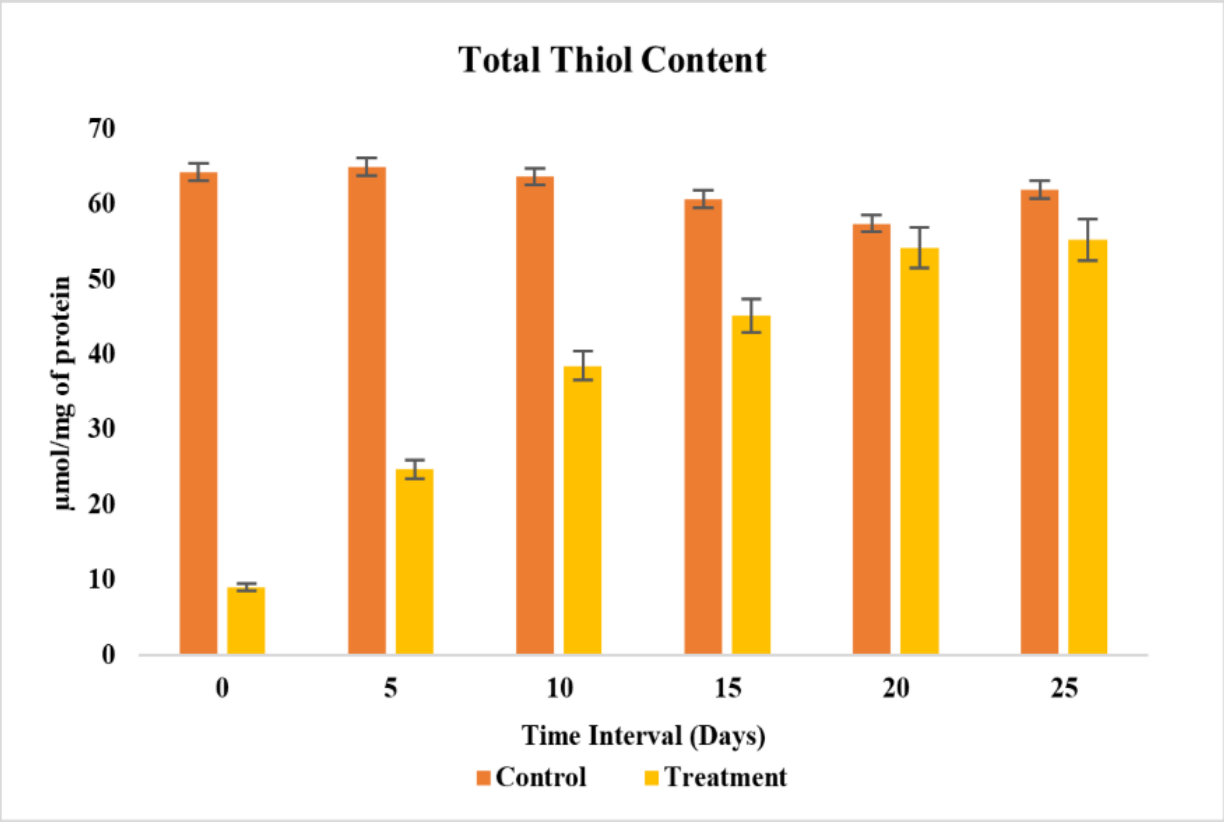


Figure 44. Total thiol content from Day 0 to Day 25 of the treatment duration. Thiol content increased in treatment samples in a dose (10 mg/ 40 ml meal) dependent manner from 14% to 86%, where for treatment $p < 0.00002$ obtained and for control data $p < 0.1$ obtained.

4.3.3.2 Glutathione-S-Transferase Activity

Treatment groups that were exposed to protease (4mg/ 40 ml) showed a significant ($p<0$) increase in Glutathione-S-transferase activity from 0.74 U/ml to 3.67 U/ml within 25 days of the treatment **(Table 17)**, while the control flies showed the enzyme activitybetween 3.7 to 4.4 U/ml with $p < 0.02$ **(Figure 45).**

Table 17. Estimation of Glutathione-S-transferase activity

S. No.	Day	Average control GST-specific activity (µ mol/ml/min)	% Control	Average treatment GST-specific activity (µ mol/ml/min)	% Treatment
1	0	3.78	100.1	0.74	19.73
2	5	3.86	102.17	1.48	39.41
3	10	4.07	107.8	1.77	47.0
4	15	4.31	114.1	2.77	73.45
5	20	4.05	107.1	3.50	92.66
6	25	3.65	96.69	3.67	97.21

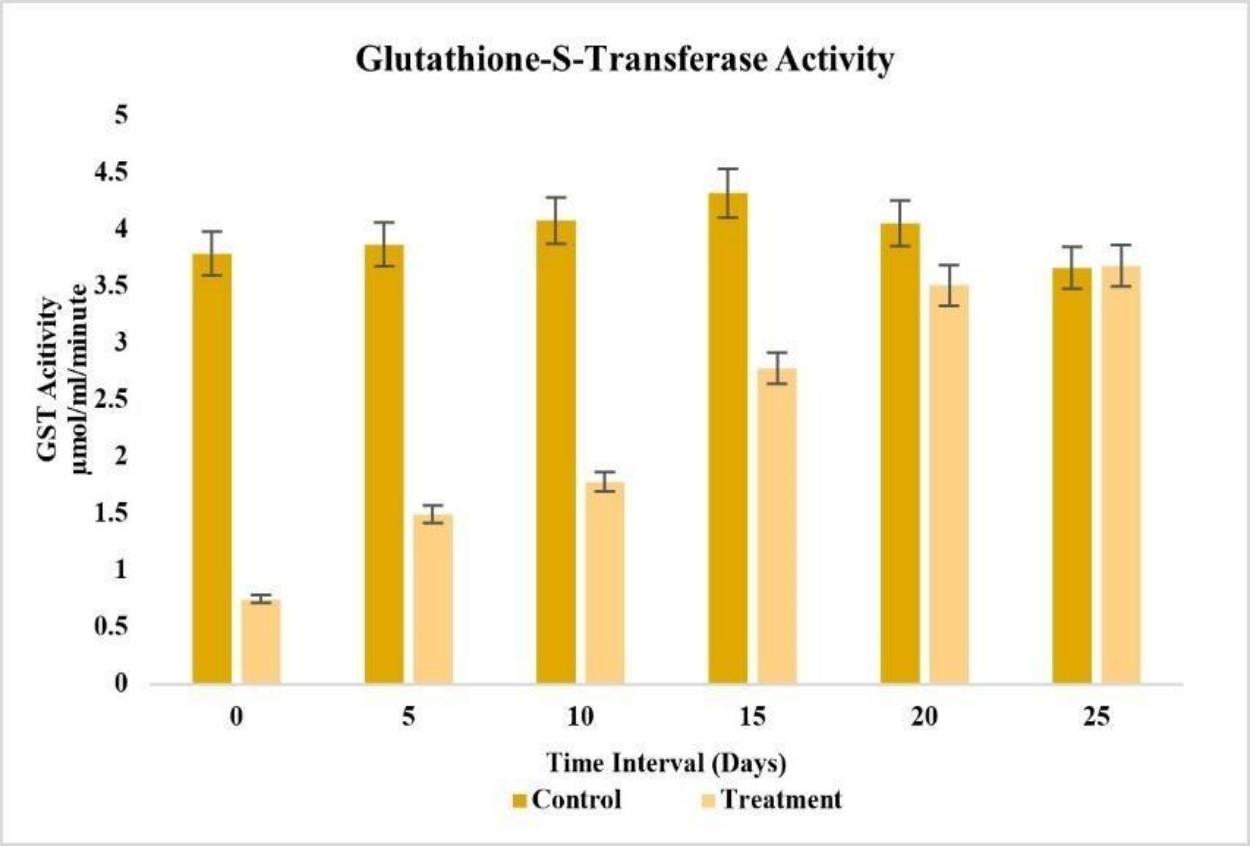


Figure 45. Glutathione-S-transferase levels were restored, with an increase from 19.7% to 97% when compared to the control flies. The p-value for control was $p < 0.02$, and for treatment significance was 0.

4.3.3.3 Catalase Activity

One of the important indicators of oxidative stress is the catalase enzyme. The slope of the standard catalase curve i.e. **y = 0.0023x + 0.0136** was used to calculate the catalase units in biological samples **(Figure 46)**. The enzyme activity was significantly reduced ($p < 0.00001$) in the treatment flies from 188 U/ml to 97.5 U/ml, indicating the effect of protease supplementation in restoring normal cellular functions in flies **(Table 18)**. In control flies the catalase concentration remained majorly between 95 U/ml to 100 U/ml with $p < 0.001$, during the 25-day of treatment **(Figure 47).**

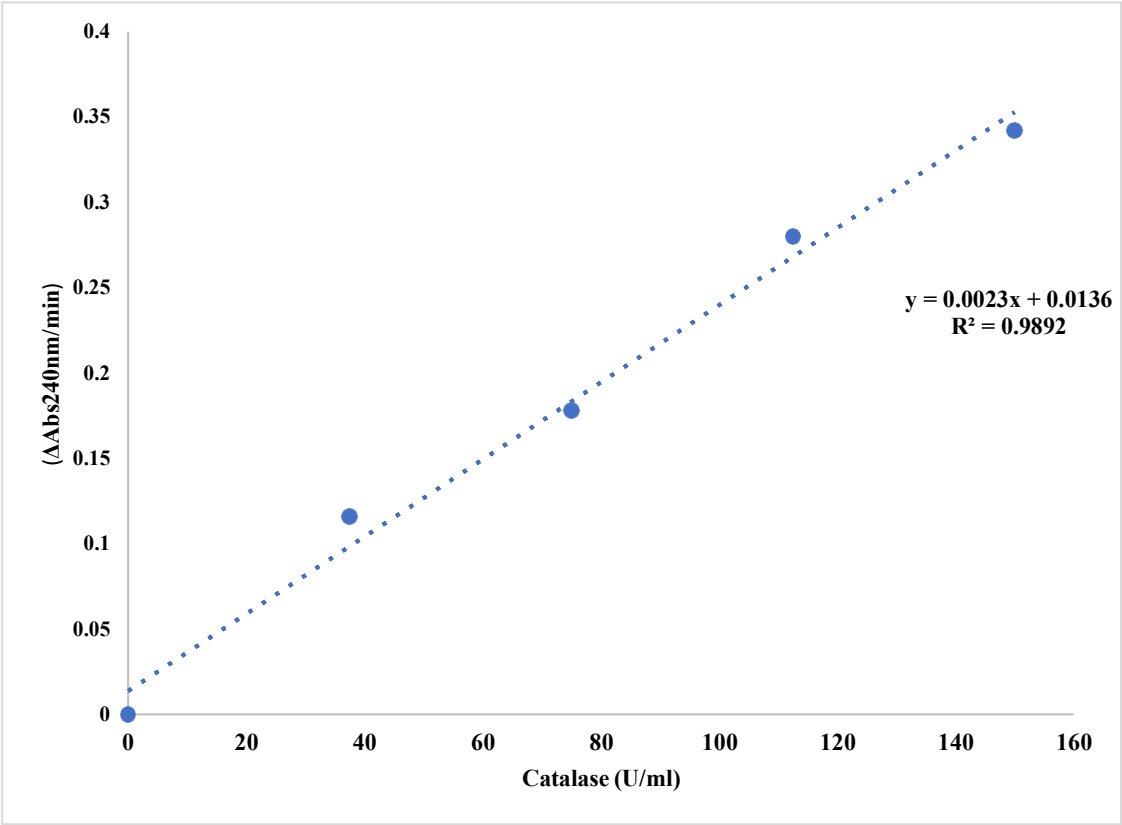


Figure 46. Standard curve of catalase.

Table 18. Estimation of catalase activity

S. No.	Day	Average control (µmol of H_2O_2 consumed/min/mg protein)	% Control	Average treatment (µmol of H_2O_2 consumed/min/mg protein)	% Treatment
1	0	94.95	100	188.86	198.91
2	5	97.99	103.20	186.26	196.16
3	10	102.78	108.24	181.47	191.13
4	15	101.04	106.41	153.65	161.82
5	20	100.17	105.50	99.73	105.04
6	25	100.17	105.50	97.56	102.75

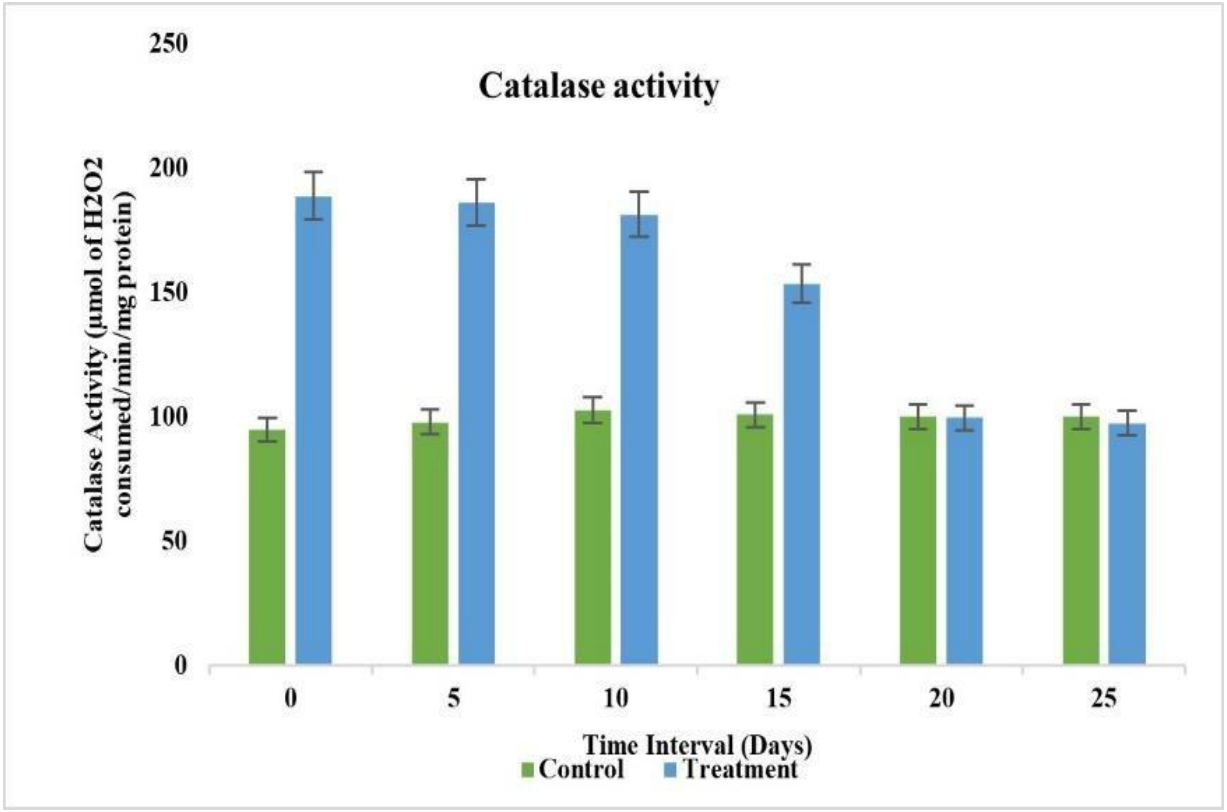


Figure 50. Dinb protease supplementation (10mg/ 40ml meal) restored the catalase activity to normal in infected flies, and the catalase levels of fly homogenate from 20-day treatment showed a significant similarity to the control flies (healthy fly homogenate), where $p < 0.00001$ for treatment vs control $p < 0.001$.

4.4 DISCUSSION

Researchers have reported the protective action of serine proteases isolated from *Bacillus clausii* in Vero and Caco-2 cell lines against *Bacillus cereus* and *Clostridium difficile* pathogens **(Ripert et al., 2016).** So far, no direct application of any protease in *Drosophila* concerning diarrhea treatment has been reported. However, the impact of many drugs and antibiotic administration on diarrhea infection in *Drosophila* has been reported **(Li et al., 2014).** In the present study, we have evaluated the impact of protease-based supplementation against diarrhea pathogen in *Drosophila melanogaster.* Researchers have already reported *Drosophila melanogaster* as a model to study entericinfections **(Najjar et al., 2022)** where the flies were infected orally. Following a similar methodology, we infected our test organism and observed for the presence of bacterial infection using a colony forming assay. The infection reached 1×10^9 CFU/ml after 96 hours of incubation. *Salmonella enterica* infected flies showed increased fly death after1×10^9 CFU/ml of the bacterial load was attained. Brandt et al., have reported fly death from *Salmonella enterica* serovar *typhimurium (S. typhimurium)* after they had a bacterial load of 1×10^5 CFU/ml **(Ayres et al., 2009), (Harnish et al., 2020)**. As *S. typhimurium* is more pathogenic than *S. enterica,* fly mortality must require a higher bacterial load. The purified protease concentration for the treatment was initially optimized under preliminary studies where protease was in 2 mg/ 40 ml fly food, 4 mg/40 ml fly food, and 10 mg/ 40 ml fly food. The initial treatments were exclusively observed for the reduced bacterial load and the total number of surviving flies. From the preliminary assays performed for 15 days, a final protease concentration of 10 mg was chosen for the final experiments.

From day 0 to the whole duration of treatment (30 days), protease treatment with 10 mg/ 40 ml standard fly food, in *Salmonella*-infected flies showed reduced bacterial loadand bacterial shedding. Impaired climbing ability in infected flies was observed duringthe onset of infection. On the zeroth day of treatment, no flying in infected flies was observed, but the movement improved during the treatment, and by the end of the 20-day treatment climbing rates of treatment flies became similar to the control flies. Theresults were supported by Li et al., **(2024)** where the protease

administration helped flies recover. Treatment flies were rescued and mortality rates were stabilized after 15 days during the treatment, showing an increase in survival. The reproductive abilities of treatment flies also showed recovery and an increase after 12 days of the treatment.

The etiology of enteric infections involves the disruption of the epithelial barrier, Microbiota dysbiosis, imbalance in intestinal homeostasis, and elevated oxidative stress leading to gut inflammation. Researchers have reported the association of oxidative stress with gut inflammation during diarrhea **(Xiu et al., 2022)** leading to macromolecule disruption. Xiu et al., have reported the formation of reactive oxygen species in the *Drosophila* intestine during acute, chronic diarrhea, and in inflammatory bowel disease (IBD) **(Xiu et al., 2022)**. IBD patients are reported to show an increased level of reactive oxygen species (ROS), leukocytes, and cytokines within the inflamed intestinal segments **(Zeng et al., 2024).** The release of reactive oxygen species and free radicles boosted the development of IBD by disrupting the mucosal lining of the gut, inducing signal transduction pathways, and damaging macromolecules, proteins, and lipids. ROS proliferation results in the release of oxidative molecules like malonaldehyde (MDA), thiol compounds, enzymes like glutathione peroxidase (GPX), Superoxide dismutase (SOD), catalase (CAT), and nonenzymatic compounds like vitamin, cysteine, etc. **(Fu et al., 2024)**. Hence studying the pathways related to oxidative stress molecule release can give insights into the disease pathology.

Similar observations were made during our study whenbiomarkers of oxidative stress were analyzed using enzymatic assay from zeroth day to day 25 of treatment. Initially, the total thiol content of treatment flies was 14 % and it increased to 86 % when compared to healthy control flies during the treatment. Another marker enzyme of gut epithelial injury is Glutathione-S-transferase, which is involvedin the function of cell protection, detoxification, and removal of reactive oxygen species **(Keshav et al., 2020)**. These enzymes are prevalent in the kidney, liver, and intestine. Analysis of Glutathione-S- transferase activity in infected flies exhibited a significant drop in overall enzyme activity, but protease supplementation in infected flies showed an increase in the activity of Glutathione-S- transferase (EC 2.5.1.18) from 19 % to 97 % was observed when compared to the control flies.

Another enzyme that is a part of an antioxidant defense system is catalase. The enzyme concentration increases in the cells to protect the cells from stress injuries **(Ramond et al., 2021)**. The analysis of catalase (EC 1.11.1.6) in infected flies showed a 198% increase in catalase activity compared to the control cells. The enzyme levels of treatment flies were normalized during the protease treatment within 25 days. The analysis concluded that the protease supplementation reduced bacterial infection and restored the overall activities of oxidative stress markers.

Even with all the above-mentioned observations, *Drosophila* alone should not be considered entirely as an alternative to other model systems. The reason lies in the distant phylogenetic relationship between humans and fruit flies. Physiologically *Drosophila* lacks an adaptive immune response, which restricts its use in the exploration of signaling pathways concerning host-pathogen interactions. *Drosophila* gut cannot rear all kind of complex microbes that prevails in the human gut and are related to disease pathogenesis, which makes it not suitable for the humanized microbiota model. The study on *Drosophila* is limited since mice are mammals with well-defined physiological systems, giving validations to studies related to complex human diseases. This concludes that, especially for biomedical research, *Drosophila* can be used in conjunction with rodent and other mammalian models.

Chapter 5

Summary & Conclusion

5.1 Result Briefing and Conclusion

A commercially available probiotic strain named *Bacillus clausii* UBBC07 was used for the isolation of a new metalloprotease. The highest synthesis of antimicrobial protease was observed in the late static period of bacterial growth. The antimicrobial property of clear supernatant was observed against various diarrhea pathogens, using a well diffusion assay. The metalloprotease was found to be effective against both Gram-positive and Gram-negative bacteria. The metalloprotease was purified through a series of methods including, centrifugation, acid precipitation, SDS-PAGE run, protein band excision, and chemical treatment to elute out the protein. The purified protease was identified by MALDI-TOFF mass spectroscopy and molecular mass was also confirmed which matched the molecular mass of protease in SDS-PAGE i.e. 23.4 kDa. Mass spectrometry analysis revealed the protein to be a metalloprotease with strong hydrolytic activity, a member of the damage-inducible protein family (Dinb family). The protein did not get digested with the Glu-C enzyme, leaving it partially sequenced. This observation concluded the protein to be novel with some unique amino acid sequences, which were not digested by common proteases. Purified protease showed a firm activity at a range of temperatures, pH, and several detergents.

In vitro studies on purified protease were performed using two diarrhea pathogens i.e. *Bacillus cereus* and *Salmonella enterica.* Time-kill kinetics concluded the bacterial death time to be 4 hours with the treatment of metalloprotease. Protease-treated bacterial cells when observed under a compound microscope after Gram staining showed debris formation around healthy cells. Scanning electron microscopy (FESEM) showed overall bacterial cell disruption in the case of both test bacteria (*Bacillus cereus* and *Salmonella enterica).*

In silico analysis of diarrhea toxins and pathogens was performed against already existing peptides and protein molecules reported from *Bacillus clausii.* Pathogens used in the study were, *Clostridium difficile, Salmonella enterica, Shiga*-like toxin of *E. coli,* rotavirus, norovirus, etc. A total of 17 different diarrheal toxins were used in the study. For peptide, clausin was used, and similar peptides were obtained from a BLAST search. Molecular docking showed TcdB binding to all three peptides to be

highly stable among all the toxins, complex stability was further confirmed by molecular simulation using Desmond software. The complexes were stable for 100 nanosecond-long simulations. The peptides were binding to the middle domain of the toxin,predicting an allosteric inhibition of the toxin molecule.

In vivo studies of protease efficacy were performed using *Drosophila melanogaster*. Healthy *Drosophila* 5 days old were infected with *Salmonella enterica* and fed on protease protease-supplemented diet for one month to observe the changes in the overallhealth of flies. The recovery was calculated based on reduced bacterial load inside the flies, increased survival, reproduction, climbing ability, and biochemical assays. Oxidative stress markers which are expressed concerning gut disorders in *Drosophila* flies were examined. Enzyme activities for glutathione-s-transferase, catalase, and thiol content of flies were restored during protease treatment. In conclusion, the metalloprotease of *Bacillus clausii* UBBC07 inhibited the growth of *Bacillus cereus* and *Salmonella enterica*. Such secreted proteins/ proteases of *Bacillus clausii* can be a strong tool in treating diarrhea infection. The area of protease-based drug therapies in diarrhea treatment requires more research.

The following conclusions were made based on the research outcome:

A) Purified metalloprotease possesses a broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria.

B) Protease is firm against broad temperatures, pH range, and detergents. Also, optimal activity at pH 7.2 concluded that the protease is a neutral protease.

C) Based on sequence determination the metalloprotease concluded to be a novel one in the Dinb protein family.

D) The Successful recovery of Drosophila from bacterial infection confirmed the antimicrobial effect of metalloprotease, which can be a target for future medicines.

Therefore, this study investigates the therapeutic potential of a protease isolated from *Bacillus clausii* UBBC07 in mitigating diarrhea in *Drosophila* models. Our findings demonstrate that the protease exhibits significant anti-diarrheal activity, evidenced by a marked reduction in the occurrence and severity of diarrhea signs in treated fliescompared to controls. The underlying mechanisms appear to involve the modulation of gut microbiota, as the protease facilitates a more balanced microbial composition, promoting the growth of beneficial bacteria while inhibiting pathogenic strains. Furthermore, histological analysis of the intestinal tissues indicated enhanced integrity and reduced inflammatory markers in treated flies, suggesting that the protease contributes to gut health and stability. These results highlight the potential of *Bacillus clausii* UBBC07-derived protease as a promising biotherapeutic agent for managing diarrhea, possibly paving the way for novel treatments in both invertebrate and vertebrate systems.

5.2 Future Scope of Work

1. To study the protease on molecular and cellular levels. Further structural characterization by Nuclear magnetic resonance, and circulardichroism.
2. *In vivo* studies of protease efficacy in mice models for drug safety data analysis.
3. To perform the computational analysis to explore the possible therapeutic targets of the protease.
4. Genetic modification to enhance the temperature and pH stability in purified metalloprotease and also to increase the anti-diarrheal spectrum of the protease.

Future studies should focus on elucidating the precise molecular mechanisms at play and exploring the efficacy of this protease in more complex biological models. Overall, this research underscores the importance of microbial-derived compounds in addressing gastrointestinal disorders and their potential applications in health management.

5.3 Bibliography

1. Adedara, A.O., Babalola, A.D., Stephano, F., Awogbindin, I.O., Olopade, J.O., Rocha, J.B.T., Whitworth, A.J., Abolaji, A.O., An assessment of the rescue action of resveratrol in parkin loss of function-induced oxidative stress in Drosophila melanogaster. Science Reports, (2022), Mar 10;12(1):3922. doi: 10.1038/s41598-022-07909-7. PMID: 35273283; PMCID: PMC8913626.

2. Ahire Jayesh J., Kashikar Megha S., Survival and Germination of Bacillus clausii UBBC07 Spores in vitro Human Gastrointestinal Tract Simulation Model and Evaluation of Clausin Production, Frontiers in Microbiology, (2020), VOLUME11, DOI:10.3389/fmicb.2020.01010.

3. Alekseenko, A., Ignatov, M., Jones, G., Sabitova, M., Kozakov, D., Protein-Protein and Protein–Peptide Docking with ClusPro Server. In: Kihara, D. (eds) Protein Structure Prediction. Methods in Molecular Biology, (2020), vol 2165. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-0708-49.

4. Alsoufi M.A, Raghad A., Production of Aspartame by Immobilized Thermolysin. Iraqi Journal of Science, (2019), Vol. 60, No.6, pp: 1232-1239 DOI: 10.24996/ijs.2019.60.6.6.

5. Aquaro S, Borrajo A, Pellegrino M, Svicher V., Mechanisms underlying antiretroviral drugs in different cellular reservoirs with a focus on macrophages. Virulence, (2020), Dec;11(1):400-413. doi: 10.1080/21505594.2020.1760443. PMID:32375558; PMCID: PMC7219522.

6. Avila G., Sommer B., Daniel A., Posada M., Ramos C., Hernandez A.G., Valencia R.F., Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. Critical Reviews in Oncology/Hematology, (2019), Volume 137, Pages 57-83, ISSN 1040-8428, https://doi.org/10.1016/j.critrevonc.2019.02.010.

7. Ayres, J.S., and Schneider, D.S., The role of anorexia in resistance and tolerance to infections in *Drosophila.* PLOS Biology, (2009), https://doi.org/10.1371/journal.pbio.1000150.

8. Barzkar N., Babich O., Sukhikh S., Maran B.A., Jahromi S.T., Luwor R.B., Sorsa T., Das R., Exploring the sources and potential applications of marine

collagenases, Biocatalysis and Agricultural Biotechnology, (2024), Volume 58103150,ISSN 1878-8181, https://doi.org/10.1016/j.bcab.2024.103150.

9. Basile, F. W., Fedele, M. C., & Lo Vecchio, A., Gastrointestinal Diseases in Children Living with HIV. Microorganisms, (2021), *9*(8), 1572. https://doi.org/10.3390/microorganisms9081572.

10. Baskaran D., Chinnappan K., Manivasagan R., Mahadevan D.K., Partitioning of crude protein from aqua waste using PEG 600-inorganic salt aqueous two-phase systems. Chemical Data Collections, (2018), 15-16:143–52.

11. Batool, M.; Ahmad, B.; Choi, S., A Structure-Based Drug Discovery Paradigm. *Int. J. Mol. Sci.* (2019), *20*, 2783. https://doi.org/10.3390/ijms20112783.

12. Benevides G. R., & Billington C., Bacteriophages as Alternatives to Antibiotics in Clinical Care. Antibiotics, (2019), 8(3), 138. https://doi.org/10.3390/antibiotics8030138.

13. Bharatam, P.V. Computer-Aided Drug Design. In: Poduri, R. (eds) Drug Discovery and Development. Springer, Singapore. (2021), https://doi.org/10.1007/978-981-15-5534-3_6.

14. Boon, L., Ugarte-Berzal E., Vandooren J., Opdenakker G., Protease propeptide structures, mechanisms of activation, and functions. Critical Reviews in Biochemistry and Molecular Biology, (2020), 55(2),111–165.https://doi.org/10.1080/10409238.2020.1742090.

15. Bond J.S., Proteases: History, discovery, and roles in health and disease. Journal of Biological Chemistry, (2020), DOI 10.1074/jbc.TM118.004156

16. Bordenave G. Review: on the shoulders of giants Louis Pasteur (1822–1895) Microbes and Infection, (2003), 553–560.

17. Bueno A., Abreu A., Guarner F., *Bacillus clausii* for Gastrointestinal Disorders: A Narrative Literature Review. Advanced Therapeutics, (2022), 39, 4854–4874, https://doi.org/10.1007/s12325-022-02285-0.

18. Camacho D.M., Collins K.M., Powers R.K., Costello J.C., Collins J.J., Next-generation machine learning for biological networks. Cell, (2018),173:1581–92.

19. Caminero A., Guzman M., Libertucci J., Lomax A.E. The emerging roles of bacterial proteases in intestinal diseases. Gut Microbes, (2023), 15:1, 2181922, DOI: 10.1080/19490976.2023.2181922.

20. Capasso C, Supuran C.T. Chapter 3.3 - Bacterial zinc proteases, Editor(s): Claudiu T. Supuran, William A. Donald, Metalloenzymes, Academic Press, (2024), Pages 185-195, ISBN 9780128239742, https://doi.org/10.1016/B978-0-12-823974-2.00019-X.

21. Chapman J., Ismail A.E., Dinu C.Z., Industrial Applications of Enzymes: Recent Advances, Techniques, and Outlooks. Catalysts, (2018), 8, 238. https://doi.org/10.3390/catal8060238..

22. Chellappan S., Chekidhenkuzhiyil J., Basheer S.M., Kuzhiyil E.K., Bhat S.G., Muthusamy C., Production, purification, and partial characterization of a novel protease from marine Engyodontium album BTMFS10 under solid-state fermentation, Process Biochemistry, (2006), Volume 41, Issue 4, Pages 956-961, ISSN 1359-5113, https://doi.org/10.1016/j.procbio.2005.10.017.

23. Cohen M.B., Bacterial, viral, and toxic causes of diarrhea, gastroenteritis, and anorectal infections. 2947-3005. https://doi.org/10.1002/9781119600206.ch144, 18 February Chapter in Book/Report/Conference proceeding › Chapter › peer-review, (2022).

24. Cuesta S. A., Mora J. R., Zambrano, C. H., Torres F. J., & Rincón L., Comparative study of the nucleophilic attack step in the proteases catalytic activity: Atheoretical study. Molecular Physics, (2019), 118 (14). https://doi.org/10.1080/00268976.2019.1705412.

25. Dadgostar P., Antimicrobial Resistance: Implications and Costs. Infectious Drug Resistance, (2019), 12:3903-3910. doi: 10.2147/IDR.S234610.

26. Devi S.C., Keziah M., Naine J., Mohanasrinivasan V., Actinomycetes: Microbiology to Systems Biology. In: Karthik, L. (eds) Actinobacteria. Springer, Singapore, (2022), https://doi.org/10.1007/978-981-16-5835-8_1.

27. Dixit A., Pandey H., Rana R., Kumar A., Herojeet R., Lata R., Ecological and human health risk assessment of pharmaceutical compounds in the Sirsa River of Indian Himalayas, Environmental Pollution, (2024), Volume 347, 123668, ISSN 0269-7491, https://doi.org/10.1016/j.envpol.2024.123668.

28. Dubey S., Sharma N., Thakur S., Patel, Reddy B.M., *Bacillus cereus* food poisoning in Indian perspective: A review, The Pharma Innovation Journal, (2021), 10(9): 970-975.

29. Espinosa-Gongora C., Jessen L.R., Kieler I.N., Damborg P., Bjørnvad C.R., Gudeta D.D., Impact of oral amoxicillin and amoxicillin/clavulanic acid treatment on bacterial diversity and β-lactam resistance in the canine fecal microbiota. Journal of Antimicrobial Chemotherapy, (2020), Feb 01; 75(2):351-361.

30. Frederick R. E., Bearden R., Jovanovic A., Jacobson N., Sood R., & Dhall S., *Clostridium* Collagenase Impact on Zone of Stasis Stabilization and Transition to Healthy Tissue in Burns. International Journal of Molecular Sciences, (2020), 22(16), 8643. https://doi.org/10.3390/ijms22168643.

31. Fu Z., Yang Y., Ma L., Malmuthuge N., Guan L., & Bu D. Dynamics of oxidative stress and immune responses in neonatal calves during diarrhea. Journal of Dairy Science, (2024). 107(2), 1286-1298. https://doi.org/10.3168/jds.2023-23630.

32. Galeotti N., Hackemann E., Jirasek F, Hasse H., Prediction of the elution profiles of proteins in mixed salt systems in hydrophobic interaction chromatography. Separation and Purification Technology, (2020), 233.

33. Gioia M., Ciaccio C., Calligari P., De Simone G., Sbardella D., Tundo G., Role of proteolytic enzymes in the COVID-19 infection and promising therapeutic approaches, Biochemical Pharmacology, (2020), Volume 182,2020,114225, ISSN 0006-2952, https://doi.org/10.1016/j.bcp.2020.114225.

34. Gómez-Escudero O., and Remes-Troche J.M., Approach to the adult patient with chronic diarrhea: A literature review. Revista de Gastroenterología de México, (2021), Volume 86, Issue 4, , Pages 387-402, ISSN 2255-534X, https://doi.org/10.1016/j.rgmxen.2021.08.007.

35. Grandinetti T., Biedermann L., Bussmann C., Eosinophilic Gastroenteritis: Clinical Manifestation, Natural Course, and Evaluation of Treatment with Corticosteroids and Vedolizumab. Digestive Diseases and Sciences, (2019), 64, 2231–2241, https://doi.org/10.1007/s10620-019-05617-3.

36. Gurumallesh P., Alagu K., Ramakrishnan B., Muthusamy S., A systematic reconsideration on proteases. International Journal of Biological Macromolecules, (2019), Volume 128, Pages 254-267, ISSN 0141-8130,https://doi.org/10.1016/j.ijbiomac.2019.01.081.

37. Habicher T., John A., Scholl N., Daub A., Klein T., Philip P., & Büchs J., Introducing substrate limitations to overcome catabolite repression in a protease producing *Bacillus licheniformis* strain using membrane-based fed-batch shake flasks. Biotechnology and Bioengineering, (2019), 116(6), 1326-1340. https://doi.org/10.1002/bit.26948.

38. Hailemichael F., Production and Industrial Application of Microbial Aspartic Protease: A Review. International Journal of Food Engineering and Technology, (2021), Vol. 5, No. 2, pp. 85-90. doi: 10.11648/j.ijfet.20210502.17.

39. Hartman R.M., Cohen A.L., Antoni S., Mwenda J., Weldegebriel G., Biey J., Risk Factors for Mortality among children younger than age 5 years with severe diarrhea in low- and middle-income countries: Findings From the World Health Organization-coordinated Global Rotavirus and Pediatric Diarrhea Surveillance Networks. Clinical Infectious Diseases, (2023), Feb 8;76(3):e1047-e1053. doi: 10.1093/cid/ciac561.

40. Harnish, J.M., Link, N., & Yamamoto, S., *Drosophila* as a Model for Infectious Diseases. International Journal of Molecular Sciences, (2020), *22*(5), 2724. https://doi.org/10.3390/ijms22052724.

41. Harwood C.R., Kikuchi Y., The ins and outs of *Bacillus* proteases: activities, functions, and commercial significance, FEMS Microbiology Reviews, (2022), Volume46, Issue 1, https://doi.org/10.1093/femsre/fuab046.

42. Hasan M.J., Haque P., Rahman M.M., Protease enzyme based cleaner leather processing: A review, Journal of Cleaner Production, (2022), Volume 365, 132826, ISSN 0959-6526, https://doi.org/10.1016/j.jclepro.2022.132826.

43. Hasan R., Rony M.N., Ahmed R., *In silico* characterization and structural modeling of bacterial metalloprotease of family M4, Journal of Genetic Engineering and Biotechnology, (2021), Volume 19, Issue 1, 25, ISSN 1687-157X, https://doi.org/10.1186/s43141-020-00105-y.

44. He F., Chao J., Yang D., Zhang X., Yang C., Xu Z., Yongqiang T., Optimization of fermentation conditions for the production of neutral metalloprotease by *Bacillus subtilis* SCK6 and its application in goatskin-dehairing. Preparative Biochemistry & Biotechnology, (2021), 52(7), 789–799. https://doi.org/10.1080/10826068.2021.1995413.

45. Hernandez-Valdes J.A., aan de Stegge M., Hermans J., Teunis J., van Tatenhove-Pel R.J., Teusink B., Bachmann H., Kuipers O.P., Enhancement of amino acid production and secretion by *Lactococcus lactis* using a droplet-based biosensing and selection system. Metabolic Engineering Communications, (2020).

46. Hitch TCA., and Clavel T., A proposed update for the classification and description of bacterial lipolytic enzymes. Peer J, (2019), https://doi.org/10.7717/peerj.7249.

47. Hioki T., Yamashita D., Tohata M., Endo K., Kawahara A., Okuda M., Heterologous production of the active form of beta-lytic protease *Bacillus subtilis* and improvement of staphylolytic activity by protein engineering. Microbial Cell Factories,(2021), 20, 231 https://doi.org/10.1186/s12934-021-01724-x.

48. Hofer F., Kraml J., Ursula Kahler U., Kamenik A.S., and Liedl K.R., Catalytic Site pK_a Values of Aspartic, Cysteine, and Serine Proteases: Constant pH MD Simulations. Journal of Chemical Information and Modeling**,** (2020), *60* (6), 3030-3042DOI: 10.1021/acs.jcim.0c00190.

49. Hou Y., Wu Z., Dai Z., Wang G., Wu G., Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. J. Anim. Sci. Biotechnol. (2017), 8, 24.

50. https://www.downtoearth.org.in/blog/health/what-we-can-do-to-reduce-the-economic-cost-of-antimicrobial-resistance-74262.

51. Iorjiim W.M., Omale S., Bagu G.D., Gyang S.S., Alemika E.E. *Moringa oleifera* Leaf Extract Extends Lifespan and Ameliorate HAART Drug-Induced Locomotor, Reproductive, and Antioxidant Deficits in *Drosophila melanogaster*, Journal of Complementary and Alternative Medical Research, (2020), 11(4): 33-46, ISSN: 2456-6276.

52. Jangra M., Kaur M., Tambat R., Rana R., Mauryaet S.K, Tridecaptin M., A new variant discovered in mud bacterium, shows activity against colistin and extremely drug-resistant *Enterobacteriaceae*. Antimicrobial Agents Chemotherapy, (2019), 63:6,https://doi.org/10.1128/AAC.00338 -19.

53. Jayakumar J., Kumar V. A., Biswas L., & Biswas R., Therapeutic applications of lysostaphin against *Staphylococcus aureus*. Journal of Applied Microbiology, (2021), 131(3), 1072-1082, https://doi.org/10.1111/jam.14985.

54. Ji D., Ma J., Xu M., and Agyei D., Cell-envelope proteinases from lactic acid bacteria: Biochemical features and biotechnological applications. Comprehensive Reviews in Food Science and Food Safety, (2021), 20, 369–400.

55. Jian Z., Zeng L., Xu T., Sun S., Yan S., Yang L., Huang Y., Jia J., & Dou T. Antibiotic resistance genes in bacteria: Occurrence, spread, and control. Journal of Basic Microbiology, (2021), *61*(12), 1049-1070. https://doi.org/10.1002/jobm.202100201.

55. Kalyani A.L.T., Maha Lakshmi M., Ahalya M.N., Anusha P., Seema S.D., Maneesha T., Classification of different antibiotics and their adverse effects and uses from origin to present, International Journal of Pharmaceutical Sciences, (2024), Vol 2,Issue 2, 98-121.

56. Karas J.A., Carter G.P., Howden B.P., Turner A.M., Paulin O.K.A., Swarbrick J.D., Baker M.A., Structure-Activity Relationships of Daptomycin Lipopeptides. Journal of Medicinal Chemistry, (2020), 63, 22, 13266–13290, https://doi.org/10.1021/acs.jmedchem.0c00780.

57. Katti A.K.S., S., Mudgulkar, S.B., Diversity and Classification of Rare Actinomycetes. In: Yaradoddi, J.S., Kontro, M.H., Ganachari, S.V. (eds) Actinobacteria. Rhizosphere Biology. Springer, Singapore, (2021), https://doi.org/10.1007/978-981-16-3353-9_7.

58. Keshav, N., Ammankallu, R., Shashidhar, Paithankar, J.G., Baliga, M.S., Patil, R.K., Kudva, A.K., Raghu, S.V. (2022). Dextran sodium sulfate alters antioxidant status in the gut affecting the survival of *Drosophila melanogaster*. *3 Biotech*, 12, 280https://doi.org/10.1007/s13205-022-03349-2.

59. Kermasha S., Eskin M.N.A., Chapter Nine - Selected industrial enzymes, Editor(s): Kermasha S., Eskin M.N.A., Enzymes, Academic Press, (2021), Pages 259- 305, ISBN 9780128002179, https://doi.org/10.1016/B978-0-12-800217-9.00009-5

60. Khambhaty Y., Applications of enzymes in leather processing. Environment Chemistry Letters, (2020), 18, 747–769, https://doi.org/10.1007/s10311-020-00971-5.

61. Khan I., Bai Y., Zha L., Ullah N., Ullah H., Shah S. R. H., Sun H., and Zhang C., Mechanism of the Gut Microbiota Colonization Resistance and Enteric

Pathogen Infection. Frontiers in Cellular and Infection Microbiology, (2021), 11:716299. doi: 10.3389/fcimb.2021.716299.

62. Kieliszek M., Pobiega K., Piwowarek K., Kot A.M., Characteristics of the Proteolytic Enzymes Produced by Lactic Acid Bacteria. *Molecules* (2021), *26*, 1858. https://doi.org/10.3390/molecules26071858.

63. Kinugasa T., Okabe K., Jinno K., Uchida K., Nishii Y., Improvement of lysozyme recovery method from the precipitate of protein-anionic surfactant complexes. Separation Science and Technology, (2017), 52(18):2918–25.

64. Klaus A., Carsten S., Thomas J., *Clostridium difficile* toxin biology, annual review of microbiology, (2017), Vol. 71:281-307, https://doi.org/10.1146/annurev-micro-090816-093458.

65. Köckerling E., Karrasch L., Aparna Schweitzer A., Razum O., Krause G., Public Health Research Resulting from One of the World's Largest Outbreaks Caused by Entero-Hemorrhagic *Escherichia coli* in Germany 2011: A Review Front. Public Health, Sec. Infectious Diseases: Epidemiology and Prevention, (2017), Volume 5 – 2017, https://doi.org/10.3389/fpubh.2017.00332.

66. Kryštůfek R., Šácha P., Starková J., Brynda J., Hradilek M., Tloušťová E., Grzymska J., Re-emerging Aspartic Protease Targets: Examining *Cryptococcus neoformans* Major Aspartyl Peptidase 1 as a Target for Antifungal Drug Discovery. *Journal of Medicinal Chemistry* 2021 *64* (10), 6706-6719, DOI: 10.1021/acs.jmedchem.0c02177.

67. Kühn D., Dürrschmidt P., Mansfeld J., Ulbrich-Hofmann R. Boilysin and thermolysin in dipeptide synthesis: a comparative study. Biotechnology and Applied Biochemistry, (2002), 36:71–76.

68. Lakshmi SG., Jayanthi N., Saravanan M., and Ratna MS., Safety assessment of *Bacillus clausii* UBBC07, a spore-forming probiotic. Toxicology Reports, (2017), Jan 5;4:62-71. doi 10.1016/j.toxrep.

69. Lambré, C., Barat Baviera J. M., Bolognesi C., Cocconcelli P. S., Crebelli R., Gott D. M., et al., Safety evaluation of the food enzyme thermolysin from the non-genetically modified *Anoxybacillus caldiproteolyticus* strain. EFSA Journal, (2024), 22(2), e8634. https://doi.org/10.2903/j.efsa.2024.8634.

70. Lee K.H., Lee P.M., Siaw Y.S., Morihara K., Aspartame precursor synthesis in

water-miscible cosolvent catalyzed by thermolysin. Biotechnology Letters, (1992), 14:1159–1164.

71. Lensink M.F., Velankar S., Wodak S.J., Modeling protein-protein and protein-peptide complexes: CAPRI 6th edition. Proteins, (2017), 85:359–377.

72. Lerminiaux N.A., and Cameron A.D.S., Horizontal transfer of antibiotic resistance genes in clinical environments. Canadian Journal of Microbiology, (2018), https://doi.org/10.1139/cjm-2018-0275.

73. Linda C., Marco F., Claudio L., Mechanism and Inhibition of Matrix Metalloproteinases, Current Medicinal Chemistry, (2019), Volume 26, Number 15, pp. 2609-2633(25) DOI: https://doi.org/10.2174/0929867325666180326163523.

74. Li B., Xiu M., He L., Zhou S., Yi S., Wang X., Cao W., Liu Y., He J. Protective effect of San Huang Pill and its bioactive compounds against ulcerative colitis in *Drosophila* via modulation of JAK/STAT, apoptosis, Toll, and Nrf2/Keap1 pathways. Journal of Ethnopharmacology, (2024), Volume 322, 117578, ISSN 0378-8741, https://doi.org/10.1016/j.jep.2023.117578.

75. Liu S., Li Z., Yu B., Wang S., Shen Y., Cong H., Recent advances on protein separation and purification methods, Advances in Colloid and Interface Science, (2020), https://doi.org/10.1016/j.cis.2020.102254.

76. Liu X., Shi D., Zhou S., Liu H., Liu H., & Yao X., Molecular dynamics simulations and novel drug discovery. *Expert Opinion on Drug Discovery*, (2018), *13*(1), 23–37, https://doi.org/10.1080/17460441.2018.1403419.

77. Liu Z, Guan X, Zhong X, Zhou X, Yang F., *Bacillus velezensis* DP-2 isolated from Douchi and its application in soybean meal fermentation. Journal of The Scienceof Food and Agriculture, (2020), https://doi.org/10.1002/jsfa.10801.

78. López C.C., Villafán C.P., Aluja M., Garcia H.S., Aaron F., Cordova G., Cordoba B.V., Mendoza A.H. Safety Assessment of the Potential Probiotic Bacterium *Limosilactobacillus fermentum* J23 Using the Mexican Fruit Fly (Anastrepha ludens Loew, Diptera: Tephritidae) as a Novel *In Vivo* Model. Probiotics and Antimicrobial Proteins, (2022), DOI: 10.1007/s12602-022-10034-6.

79. Lunde C., Gjermansen M., Use of M4 metalloprotease in wort production. US Patent No US 10, 450, 539 B2, (2019).

80. Mahnashi Mater H., Muddapur, Uday M., Bhagya T., Ibrahim AhmedS.,

A., Alshahrani, Merae M., A Review on Versatile Eco-Friendly Applications of Microbial Proteases in Biomedical and Industrial Applications. Science of Advanced Materials, (2022), Volume 14, pp. 622-637(16),https://doi.org/10.1166/sam.2022.4264.

81. Majerová T., & Novotný P., Precursors of Viral Proteases as Distinct Drug Targets. Viruses, (2021), *13*(10), 1981, https://doi.org/10.3390/v13101981.

82. Maqbool H., Visnuvinayagam S., Zynudheen A., Safeena M.P., Kumar S., Antibacterial Activity of Beetroot Peel and Whole Radish Extract by Modified Well Diffusion Assay. International Journal of Current Microbiology and Applied Sciences,(2020), DOI: 10.20546/ijcmas.2020.901.135.

83. Martinez X., Krone M., Alharbi N., Rose A.S., Laramee R.S., Molecular graphics: bridging structural biologists and computer scientists. Structure, (2019).

84. Mestrovic T., Aguilar G.R., Swetschinski L.R., Ikuta K.S., Gray A.P., Weaver N.D., The burden of bacterial antimicrobial resistance in the WHO European region in 2019: a cross-country systematic analysis. European Antimicrobial Resistance Collaborators (2022), DOI:https://doi.org/10.1016/S2468-2667(22)00225-0.

85. Moghri S., Kiadeh S.G., Rahaiee S., *In silico* investigation of lysostaphin-producing novel strains as an antibiotic against methicillin-resistant *Staphylococcus aureus*. Informatics in Medicine Unlocked, (2021), Volume 24, 100623, ISSN 2352- 9148, https://doi.org/10.1016/j.imu.2021.100623.

86. Motamedi H., Fathollahi M., Abiri R., Kadivarian S., Rostamian M., Alvandi A., A worldwide systematic review and meta-analysis of bacteria related to antibiotic-associated diarrhea in hospitalized patients. PLoS ONE, (2021), 16(12): e0260667. https://doi.org/10.1371/journal.pone.0260667.

87. Nair I.C., Jayachandran K., Aspartic Proteases in Food Industry. In: Parameswaran B., Varjani S., Raveendran S. (eds) Green Bio-processes. Energy, Environment, and Sustainability. Springer, Singapore, (2019), https://doi.org/10.1007/978-981-13-3263-0_3.

88. Najjar H., Al-Ashmar S., Qush A., Asmar J., Rashwan S., Elgamal A., Zeidan A., Kamareddine L. Enteric pathogens modulate metabolic homeostasis in the *Drosophila melanogaster* host. Microbes and Infection, (2022), 104946, https://doi.org/10.1016/j.micinf.2022.104946.

89. Nalezinková M., Loskot J., & Fučíková M.A., The use of scanning electron microscopy and fixation methods to evaluate the interaction of blood with the surfacesof medical devices. Scientific Reports, (2024), *14*(1), 1-13. https://doi.org/10.1038/s41598-024-55136-z

90. Nargotra P., Sharma V., Sharma S., Bangotra R., & Bajaj B.K., Purification of an ionic liquid stable cellulase from Aspergillus aculeatus PN14 with potential for biomass refining. Environmental Sustainability, (2022), 5, 313–323, https://doi.org/10.1007/s42398-022-00232-x.

91. Naveed M., Nadeem F., Mehmood T., Protease—A Versatile and Ecofriendly Biocatalyst with Multi-Industrial Applications: An Updated Review. Catalysis Letters, (2021), 151, 307–323 https://doi.org/10.1007/s10562-020-03316-7

92. Nicoloff H., Hjort K., Levin B.R., & Andersson D.I., The high prevalence of antibiotic hetero resistance in pathogenic bacteria is mainly caused by gene amplification. Nature Microbiology, (2019), 4(3), 504-514. https://doi.org/10.1038/s41564-018-0342-0

93. Noor Z., Ahn S.B., Baker M.S., Ranganathan S., Mohamedali A., Mass spectrometry-based protein identification in proteomics - a review. Briefings in Bioinformatics, (2021), Volume 22, Issue 2, Pages 1620–1638, https://doi.org/10.1093/bib/bbz163.

94. O'Rourke A., Beyhan S., Choi Y., Morales P., Chan A., Espinoza J.L., Mechanism-of-Action Classification of Antibiotics by Global Transcriptome Profiling. Antimicrobial Agents in Chemotherapy, (2020), 64:10.1128/aac.01207-19, https://doi.org/10.1128/aac.01207-19.

95. Oyewole O., Kareem S., Adeleye T., Biotechnologies/Fermentation Technologies for Large-Scale Industrial Enzyme Production for the Food and Beverage Industry Book: Fermentation and Algal Biotechnologies for the Food, Beverage and Other Bioproduct Industries, Edition1st Edition, First Published (2022), ImprintCRC Press, Pages27, eBook ISBN9781003178378.

96. Ozen K., Prachanronarong A.N., Matthew D.I., Soumana C.A., Schiffer, Resistance outside the substrate envelope: hepatitis C NS3/4A protease inhibitors, Critical Reviews in Biochemistry and Molecular Biology, (2019), 11–26.

97. Pasquale R.D., Vaccaro S., Caputo M., Cuppari C., Caruso S., Catania A., &

Messina L., Collagenase-assisted wound bed preparation: An in vitro comparison between Vibrio alginolyticus and *Clostridium histolyticum* collagenases on substrate specificity. International Wound Journal, (2019), *16*(4), 1013-1023. https://doi.org/10.1111/iwj.13148

98. Patel A., and Kevin M., Macrolides in children: judicious use, avoiding resistance and reducing adverse effects, volume 106, number 4, pages 216-219, doi 10.1136/archdischild-2020-320357, Royal College of Paediatrics and Child Health, (2021), ISSN 1743-0585, https://ep.bmj.com/content/106/4/216.

99. Patel H.A., Rawat M., Patel A.L., & Bedekar A.V. Celite-Polyaniline supported palladium catalyst for chemoselective hydrogenation reactions. Applied Organometallic Chemistry, (2019), 33(4), e4767. https://doi.org/10.1002/aoc.4767

100. Pavelques i S.L., Almeida A.C., Rodrigues A.R., Maria C., Orsi D.C., & Da Silva I.C., Presence of tetracycline and sulfonamide resistance genes in *salmonella* spp.:literature review. Antibiotics, (2021), *10*(11), 1314. https://doi.org/10.3390/antibiotics10111314

101. Pecoraro A., Nappi L., Crescenzi L., et al., Chronic Diarrhea in Common Variable Immunodeficiency: a Case Series and Review of the Literature. Journal of Clinical Immunology, (2018), 38, 67–76 https://doi.org/10.1007/s10875-017-0461-z

102. Peng Q., Wu Y., Cong H., Shen Y., Mahmood K., Yu B., Preparation of monodisperse porous polymeric ionic liquid microspheres and their application as stationary phases for HPLC. Talanta, (2020), 208:120462.

102. Poli G., Granchi C., Rizzolio F., & Tuccinardi T. Application of MM-PBSA Methods in Virtual Screening. *Molecules*, (2019), 25(8), 1971. https://doi.org/10.3390/molecules25081971.

103. Priya A., and Pandian S.K., Chapter: Bacteriology of Ophthalmic Infections, Book: Pocket Guide to Bacterial Infections, Edition 1st Edition First Published (2019),Imprint CRC Press, Pages 45, eBook ISBN9781315166377.

104. Qiu J., Wilkens C., Barrett K., Meyer A.S., Microbial enzymes catalyzing keratin degradation: Classification, structure, function. Biotechnology Advances, (2020), Volume 44, 15 November, 107607.

105. Rajkishan T., Rachana A., Shruti S., Bhumi P., Patel D., Computer-Aided Drug Designing. In: Singh, V., Kumar, A. (eds) Advances in Bioinformatics. Springer, Singapore. (2021), https://doi.org/10.1007/978-981-33-6191-1_9.

106. Ramond, E., Jamet, A., Ding, X., Euphrasie, D., Bouvier, C., Lallemant, L., He, X., Arbibe, L., Coureuil, M., Charbit, A., Reactive oxygen species-dependent innate immune mechanisms control methicillin-resistant *Staphylococcus aureus* virulence in the Drosophila larval model. mBio, (2021), 12:e00276-21, https://doi.org/10.1128/mBio.00276-21.

107. Rana N., Verma N., Vaidya D., Dipta B., Application of Bacterial Amylase in Clarification of Juices and Bun Making. Journal of Pharmacognosy Phytochemistry, (2017), 6, 859–864.

108. Razzaq A., Shamsi S., Ali A., Ali Q., Sajjad M., Malik A., Microbial Proteases Applications. Frontiers in Bioengineering and Biotechnology, (2019), Volume 7 - 2019https://doi.org/10.3389/fbioe.2019.00110

109. Rawlings N.D., Twenty-five years of nomenclature and classification of proteolytic enzymes, Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, (2020), Volume 1868, Issue 2, 140345, ISSN 1570-9639, https://doi.org/10.1016/j.bbapap.2019.140345

110. Razew A., Schwarz J.N., Mitkowski P., Sabala I., Drobek M.K., One fold, many functions—M23 family of peptidoglycan hydrolases. Frontiers of Microbiology,(2022).

110. Ripert G., Racedo SM., Elie A., Jacquot C., Bressollier P., and Urdaci MC., Secreted Compounds of the Probiotic *Bacillus clausii* Strain O/C Inhibit the Cytotoxic Effects Induced by *Clostridium difficile* and *Bacillus cereus* Toxins. Antimicrobial Agents and Chemotherapy, (2016), DOI:.https://doi.org/10.1128/aac.02815-15

111. Rival S., Saulner J., Wallach J., On the mechanism of action of pseudolysin: kinetic study of the enzymatic condensation of Zala with Phe-NH2. Biocatalysis and Biotransformation, (2000), 17:417–429.

112. Sachdeva V., Roy A., Bharadvaja N., Current Prospects of Nutraceuticals: A Review. Current Pharmaceutical Biotechnology, (2020), Volume 21, Number 10, pp. 884-896(13) DOI: https://doi.org/10.2174/1389201021666200130113441

113. Sakuma C., Tomioka Y., Li C., Shibata T., Nakagawa M., Kurosawa Y., Analysisof protein denaturation, aggregation, and post-translational modification by agarose native gel electrophoresis. International Journal of Biological Macromolecules, (2021),Volume 172, Pages 589-596, ISSN 0141-8130,https://doi.org/10.1016/j.ijbiomac.2021.01.075.

114. São Pedro M. N., Azevedo A. M., Aires-Barros M. R., & Soares R., Minimizing the influence of fluorescent tags on igg partition in peg–salt aqueous two-phase systemsfor rapid screening applications. Biotechnology Journal, (2019), 14 (8), 1800640, https://doi.org/10.1002/biot.201800640.

115. Scarcella M., d'Angelo D., Ciampa M., Tafuri S., Avallone L., Pavone L.M., De Pasquale V., The Key Role of Lysosomal Protease Cathepsins in Viral Infections. International Journal of Molecular Sciences., (2022), 23, 9089. https://doi.org/10.3390/ijms23169089.

116. Shaddeau A.W., Schneck N.A., Li Y., Ivleva V.B., Arnold F.J., Cooper J.W., Development of a new tandem ion exchange and size exclusion chromatography method to monitor vaccine particle titer in cell culture media. Analytical Chemistry, (2019), 91(10): 6430–4 .

117. Sharma A., Gupta G., Ahmad T., Mansoor S., & Kaur B., Enzyme Engineering: Current Trends and Future Perspectives. Food Reviews International, (2019), 37(2), 121–154. https://doi.org/10.1080/87559129.2019.1695835.

118. Sharma M., Gat Y., Arya S., Kumar V., Panghal A., and Kumar A., A Review on Microbial Alkaline Protease: An Essential Tool for Various Industrial Approaches. Industrial Biotechnology, (2019), https://doi.org/10.1089/ind.2018.0032.

119. Solanki P., Putatunda C., Kumar A., et al., Microbial proteases: ubiquitous enzymes with innumerable uses. 3 Biotech, (2021), 11, 428
https://doi.org/10.1007/s13205-021-02928-z.

120. Shen S., Chahal B., Majumder K., You S.J., Wu J., Identification of novel antioxidative peptides derived from a thermolytic hydrolysate of ovotransferrin by LC-MS/MS. Journal of Agriculture and Food Chemistry, (2010), 58:7664–7672.

121. Sharma B., Srivastava M.K., Parul Gosvami M., and Jain U., Superbugs: The Nightmare Bacteria. Journal of Animal Research, (2021), 11(05): 765-773.

122. Sharma P., Rani L., Grewal A.S., Srivastav A.L., Chapter 2 - Impact of pharmaceuticals and antibiotics waste on the river ecosystem: a growing threat, Editor(s): Madhav S., Kanhaiya S., Srivastav A., et al., Ecological Significance of River Ecosystems, Elsevier, (2022), Pages 15-36, ISBN 9780323850452, https://doi.org/10.1016/B978-0-323-85045-2.00015-7.

123. Singh R., Kumar M., Mittal A., Mehta P. K., Microbial Enzymes: Industrial Progress in 21st Century. 3 Biotech, (2016), 6, 174. DOI: 10.1007/s13205-016-0485- 8.

124. Sinha P., & Yadav A.K. *In silico* identification of cyclosporin derivatives as potential inhibitors for RdRp of rotavirus by molecular docking and molecular dynamic studies. Journal of Biomolecular Structure and Dynamics, (2023), *42*(10), 5001–5014.https://doi.org/10.1080/07391102.2023.2239918.

125. Siva-Jothy J.A., Prakash A., Vasanthakrishnan R.B., Monteith K.M., Vale P.F. Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. Journal of Visualized Experiments, (2018), (135), e57676, doi:10.3791/57676.

126. Souza P.M., Bittencourt M.L.A., Caprara C.C., Freitas M.D., Almeida R.P., and Silveira D., A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology, (2015), https://doi.org/10.1590/S1517-838246220140359.

127. Stokes, J. M., Yang, K., Swanson, K., Jin, W., Cubillos-Ruiz, A., Donghia, N. M., A deep learning approach to antibiotic discovery. Cell, (2020), 180, 688-702.e13. doi: 10.1016/j.cell.2020.01.021.

128. Suberu Y., Akande I., Samuel T., Lawal A., and Olaniran A., Optimization of protease production in Indigenous *Bacillus* species isolated from soil samples in Lagos, Nigeria using response surface methodology. Biocatalysis and Agricultural Biotechnology, (2019), Volume 18, 10, 11, ISSN 1878-8181, https://doi.org/10.1016/j.bcab.2019.01.049.

129. Taggar R, Jangra M, Dwivedi A, Bansal K, Patil P., Bacteriocin isolated from the natural inhabitant of Allium cepa against Staphylococcus aureus. World Journal of Microbiology and Biotechnology, (2021), 37:20, https://doi.org/10.1007/s11274-020- 02989-x.

130. Tochukwu O.C., David A., Kelley L.A., Mezulis S., Islam S.A., Mersmann S.F.,PhyreRisk: A Dynamic Web Application to Bridge Genomics, Proteomics and 3D Structural Data to Guide Interpretation of Human Genetic Variants, Journal of Molecular Biology, (2019), Volume 431, Issue 13, Pages 2460-2466, ISSN 0022-2836,https://doi.org/10.1016/j.jmb.2019.04.043.

131. Troncoso F.D., Sánchez D.A., Ferreira M.L., Production of Plant Proteases and New Biotechnological Applications: An Updated Review. Chemistry Open, (2022), https://doi.org/10.1002/open.202200017.

132. Tuccinardi T., What is the current value of MM/PBSA and MM/GBSA methods in drug discovery? Expert Opinion on Drug Discovery, (2021), 16 (11), 1233–1237. https://doi.org/10.1080/17460441.2021.1942836.

133. Tuyen Do T., Thang Le V.T., Ngo C.C., Hong Do T.T., and Dang T.H.P., Biological characteristics and classification of thermophilic actinomycetes showed extracellular hydrolytic enzymes producing ability isolated from compost. E3S Web ofConferences, (2021), Volume 265,https://doi.org/10.1051/e3sconf/202126504008

134. Uddin T.M., Chakraborty A.J., Khusro A., Zidan B.M., Mitra S., Talha, et al., Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and prospects, Journal of Infection and Public Health, (2021), Volume 14, Issue 12, Pages 1750-1766, ISSN 1876-0341, https://doi.org/10.1016/j.jiph.2021.10.020.

135. Urdaci MC., Lefevre M., Lafforgue G., Cartier C., Rodriguez B., and Fioramonti J., Antidiarrheal Action of *Bacillus subtilis* CU1 CNCM I-2745 and Lactobacillus plantarum CNCMI-4547 in Mice. Frontiers in Microbiology, (2018), 9:1537. doi: 10.3389/fmicb.2018.01537.

136. Vandenberghe L., Karp S.G., Pagnoncelli M., Tavares M., Junior N., Diestra K., Viesser J., Soccol C., Chapter 2 - Classification of enzymes and catalytic properties, In; Singh S.P., Pandey A., Singhania R.R., Larroche C., Li Z., Biomass, Biofuels, Biochemicals, Elsevier, (2020), Pages 11-30, ISBN 9780128198209, https://doi.org/10.1016/B978-0-12-819820-9.00002-8.

137. Vedant S., Arpita R., Bharadvaja, and Navneeta B., Current Pharmaceutical Biotechnology, Volume 21, Number 10, (2020), pp. 884-896(13), https://doi.org/10.2174/1389201021666200130113441.

138. Wei G., Darwish G., Oppenheim F.G., Schuppan D., Helmerhorst E.J., Commensal bacterium Rothia aeria degrades and detoxifies gluten via a highly effective subtilisin enzyme. Nutrients, (2020), 12:3724. doi: 10. 3390/nu12123724.49.

139. Wei Y., Bu J., Long H., Zhang X., Cai X., Huang A., Ren W., Xie Z., Community structure of protease-producing bacteria cultivated from aquaculture systems: potential impact of a tropical environment. Frontiers in Microbiology, (2021),https://doi.org/10.3389/fmicb.2021.638129.

140. Wilson C., Lukowicz R., Merchant S., Flynn V.H., Caballero J., Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. Research and Review. Journal of Engineering and Technology, (2017), http://www.rroij.com/open- access/quantitative-and-qualitative-assessment-methods-for-biofilm-growth-a- minireview.

141. Worsztynowicz P., Białas W., and Grajek W., Integrated approach for obtainingbioactive peptides from whey proteins hydrolyzed using a new proteolytic lactic acid bacteria. Food Chemistry, (2020), 312, 126035.

142. Xiu M., Wang Y., Yang D., Zhang X., Dai Y., Liu Y., Lin X., Li B., He J., Using *Drosophila melanogaster* as a suitable platform for drug discovery from naturalproducts in inflammatory bowel disease. Frontiers of Pharmacology, (2022), 13:1072715. Doi: 10.3389/fphar.2022.1072715.

143.Yi L, Luo L, Lu X., Efficient exploitation of multiple novel bacteriocins by a combination of complete genome and peptidome. Frontier of Microbiology, (2018), 9:1567. https ://doi.org/10.3389/fmicb .2018.01567.

144.Zeng C., Liu F., Huang Y., Liang Q., He X., Li L., Xie Y., *Drosophila*: An Important Model for Exploring the Pathways of Inflammatory Bowel Disease (IBD) in the Intestinal Tract. *Int. J. Mol. Sci.* (2024), *25*, 12742. https://doi.org/10.3390/ijms252312742

145. Zephyr J., Yilmaz N.K., and Schiffer C.A., Viral proteases: Structure, mechanism and inhibition. The Enzymes, (2021), Volume 50 ISSN 1874-6047 301 https://doi.org/10.1016/bs.enz.2021.09.004.

146. Zhao G., Ding L.L., Pan Z.H., Kong D.H., Hadiatullah H., Fan Z.C, Proteinase and glycoside hydrolase production is enhanced in solid-state fermentation by

manipulating the carbon and nitrogen fluxes in *Aspergillus oryzae*, Food Chemistry,(2019), Volume 271, Pages 606-613, ISSN 0308-8146, https://doi.org/10.1016/j.foodchem.2018.07.199.

147. Zhang J., Peng Z., Chen K., Zhan Z., Shen H., Genomic Characterization of *Salmonella enterica* serovar Weltevreden Associated with Human Diarrhea. Microbiology Spectrum, (2023), 11: e03542-22, https://doi.org/10.1128/spectrum.03542-22.

148. Zheng J., Haseeb A., Wang Z., & Wang H., Network pharmacology, computational biology integrated surface plasmon resonance technology reveals the mechanism of ellagic acid against rotavirus. Scientific Reports, (2024), 14(1), 1-17. https://doi.org/10.1038/s41598-024-58301-6.

5.4 List of publications in Science Citation Indexed (SCI) journals related to thisPh.D. thesis

1. Jyoti Guleria[1], Mohammad Rashid Khan[2], Minhaj Ahmad Khan[2*] (2024). A novel protease from *Bacillus clausii* UBBC07, a healthful solution against diarrhea-causing pathogens. Journal of Applied Biology & Biotechnology, Vol. 13(1), pp. 112-118, Jan-Feb, 2025, DOI: 10.7324/JABB.2024.200074.

2. Jyoti Guleria[1], Mohammad Rashid Khan[2], Minhaj Ahmad Khan[2*] (2024). The Protective Action of a Novel Dinb Protease Against Diarrhea Infection in *Drosophila Melanogaster.* Journal of Applied Biology & Biotechnology, Volume 13, Supplement 1, July, 2025, DOI: 10.7324/JABB.2025.210947.

Review papers:

1. Jyoti Guleria[1] and Minhaj Ahmad Khan[2] (2024), Biomaterials and biopolymers in the circular economy: Latest trends and applications. *AIP Conf. Proc.* 2986, 030102, https://doi.org/10.1063/5.0192970.

2. Guleria Jyoti[1], Khan Ahmad Minhaj[2], Mechanistic Insight into the Role of Peptides Secreted from Bacillus clausii and Future Opportunities, Current Reviews in Clinical and Experimental Pharmacology; Volume 19, Issue 4, Year 2024, e190224227110.DOI: 10.2174/0127724328273252240201071756.

Other publication:

1. Gaur D, Kumar N, Ghosh A, Singh P, Kumar P, **Guleria J**, Kaur S, Malik N, Saha S, Nystrom T, Sharma D. Ydj1 interaction at nucleotide-binding-domain of yeast Ssa1 impacts Hsp90 collaboration and client maturation. PLoS Genet. 2022 Nov9;18(11):e1010442. doi: 10.1371/journal.pgen.1010442.

2. Gaur D, Singh P, **Guleria J**, Gupta A, Kaur S, Sharma D. The Yeast Hsp70 Cochaperone Ydj1 Regulates Functional Distinction of Ssa Hsp70s in the Hsp90 Chaperoning Pathway. Genetics. 2020 Jul;215(3):683-698. doi: 10.1534/genetics.120.303190.

3. Sharma M, Sharma NR, Bansal A, Khan MA, **Guleria J**, Roy A, Kaushik S, Singh G. Global Impact of Mosquito-borne Alphaviruses on Humans: Their spread and Rehabilitation. J Commun Dis. 2023;55(3):93-110. DOI: https://doi.org/10.24321/0019.5138.202330.

5.5 Conferences attended and Posters Presented:

Jyoti Guleria[1] and Minhaj Ahmad Khan[2] (2022), Biomaterials and biopolymers in the circular economy: latest trends and applications in the "3rd International Conference on Functional Materials, Manufacturing, and Performances (ICFMMP-2022)" held onJuly29-30th, 2022.

Jyoti Guleria[1] and Minhaj Ahmad Khan[2] (2024), The antidiarrheal properties of a newly isolated protease from *Bacillus clausii* strain UBBC07 in the "3rd International Conference on Clinical and Applied Microbiology" held on July 26-27th, 2024.

5.6 Workshops

Participated in IP Awareness/ Training program under National Intellectual Property Awareness Mission on 31 Aug 2023.