MINING METAGENOME OF NORTHWESTERN HIMALAYAN HOT SPRINGS FOR HYDROLASES

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Biotechnology

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2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Mining metagenome of Northwestern Himalayan hot spring for hydrolases**" in fulfilment of degree of **Doctor of Philosophy** (**Ph. D**.) is outcome of research work carried out by me under the supervision Dr. Reena Singh, working as Associate Professor in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India, and Co-supervision of Dr. Juliana Heloisa Pinê Américo Pinheiro, Assistant Professor, Department of Forest Science, Soils and Environment, São Paulo State University, Brazil. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or 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 "**Mining metagenome of Northwestern Himalayan hot spring for hydrolases**" submitted in fulfillment of the requirement for the reward of degree of Doctor of Philosophy (Ph.D.) in the Department of Biotechnology of School of Bioengineering and Biosciences, is a research work carried out by Atif Khurshid Wani, 12009032, is bonafide record of his original work carried out under our supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Microorganisms are fundamental drivers of environmental dynamics, orchestrating key processes that shape ecosystem function and sustainability. Microorganisms profoundly influence the health and resilience of ecosystems across diverse habitats. They mediate the cycling of nutrients, break down organic matter, form symbiotic associations with plants and animals, remediate pollutants, regulate greenhouse gas emissions, contribute to soil fertility, and modulate disease dynamics. Understanding the complex interactions and functions of microorganisms in ecosystems is crucial for preserving biodiversity and maintaining ecosystem services. Culture methods in microbiology have evolved since the late 19th century, starting with Louis Pasteur's work on liquid media and fermentation, and Robert Koch's development of techniques to isolate disease-causing microorganisms. While culturable methods have been instrumental in microbiology, their limitations in capturing the full diversity and functionality of microbial communities necessitate the adoption of metagenomics as an unculturable method for studying microbes in their natural habitats. Metagenomics offers a powerful approach to unraveling the complexities of microbial ecosystems and advancing our understanding of microbial ecology, evolution, and biotechnological potential.

Metagenomics is a field of study that focuses on analyzing the collective genetic material recovered directly from environmental samples, without the need for isolating and culturing individual organisms. This approach encompasses several key steps: sample collection, DNA extraction, library preparation, sequencing using advanced technologies like next-generation sequencing, and bioinformatic analysis. By sequencing DNA from diverse habitats such as soil, water, and the human body, metagenomics unveils the genetic composition and functional potential of entire microbial communities. It enables taxonomic profiling, identifying diverse microbial taxa, and functional annotation, revealing metabolic pathways and ecological roles. Metagenomics finds applications in environmental microbiology, human health, biotechnology, and beyond, offering insights into microbial community dynamics, biogeochemical cycles, and biotechnological innovations. Overall, metagenomics serves as a powerful tool to study the hidden microbial diversity and understand its implications for ecosystems, human health, and biotechnological advancements.

The current study implies the exploration of the Rajouri hot spring (RHS), an extreme high-altitude environment situated in the Pir Panjal range of the North-Western Himalayan region in Jammu and Kashmir, India, for the bioprospecting of hydrolases using both function-based and sequence-based metagenomic approaches. The physiochemical parameters, including temperature, pH, electrical conductivity, alkalinity, concentrations of sulphates, bicarbonates, sodium, potassium, silica, and calcium, were comprehensively analyzed. Metagenomic DNA (mDNA) was extracted from both water and sediment samples and subjected to screening using degenerate primers. The amplification of proteases and amylases was achieved from sediment mDNA. This sediment mDNA was sequenced using Next Generation Sequencing (NGS) to elucidate the biome diversity and gene presence within this ecologically significant niche. The microbial diversity analysis involved a thorough examination utilizing the SILVA database, a comprehensive resource for ribosomal RNA sequences, to characterize the taxonomic composition of the microbial community. Additionally, gene diversity was investigated using Prodigal, a microbial gene prediction tool, to identify potential hydrolase genes. Computational parameters viz mismatches, e-value, bit score, and the number of gaps were employed to select two specific hydrolase genes: Carboxypeptidase (*CarP*), a protease, and Trehalose synthase (*TreS*), an amylase. These genes, with respective sizes of 1510 bp and 515 bp, were cloned into the pJET1.2 cloning vector and subsequently transformed into *DH5-α* competent cells. Expression of *CarP* and *TreS* was facilitated using the pET28 expression vector, followed by transformation into *BL21-DE3* cells. The resulting metagenomic libraries were then screened for enzymatic activity against suitable substrates. The positive clones of *CarP* and *TreS* were sequenced, and *In-silico* analysis ensued. The *In-silico* analyses encompassed the prediction of phylogeny, physicochemical parameters, amino acid composition in secondary structures, 3D structure modeling, and validation through Ramachandran plot analysis. Furthermore, purification of both proteins was achieved using Ni-His tag columns, followed by biochemical characterization to assess the effects of temperature, pH, metal ions, surfactants, and inhibitors on enzyme activity and stability.

The findings revealed that the RHS water exhibited a temperature range of 65-68 ℃, a pH range of 7.3-8.0, alkalinity levels ranging from 480 to 495 ppm, and sulphate concentrations ranging from 70 to 76 ppm. The microbial diversity analysis of the microbial community indicated the prevalence of *Proteobacteria* (41.03%) in the amplicon library, followed by *Actinobacteria* (16.7 %), *Firmicutes* (16.7%), *Bacteroidetes* (7.42%), and *Planctomycetes* (7.01%). Prediction of the

RHS metagenome revealed an estimate of 452217 genes with a total size of 212.142064 mb, averaging a gene length of 469 bp. The promising candidate genes, *CarP* and *TreS*, were successfully identified, cloned, and expressed in suitable vectors, and their molecular weights were determined to be approximately 58.6 kDa and 19.2 kDa, respectively. Screening of positive clones revealed intracellular enzyme activity, as evidenced by hydrolytic activity localized in the cell-free extract. Bioinformatics analysis indicated the hydrophilic nature of *CarP* and *TreS*, with a negative GRAVY score, and their localization within the cytoplasm demonstrated a globular topology. Furthermore, *CarP* and *TreS* exhibited isoelectric points (pI) of 4.81 and 5.78, respectively, and their concentrations were estimated to be 9.19 mg and 7.14 mg, respectively. *CarP* protease displayed an activity of 29.01 U/mL, with an optimum temperature of 70°C and broad operational stability from 10°C to 80°C, while *TreS* amylase exhibited maximum activity at 80°C with similar thermal stability. Both enzymes displayed activity and stability in alkaline pH, with increased activity in the presence of Zn^{2+} for *CarP* and CO^{2+} for *TreS*, highlighting their potential for biotechnological applications in extreme environments. The sequences of *CarP* and *TreS* were submitted to the National Center for Biotechnology Information (NCBI) and have been published under the accession numbers OR906153 and OR906319, respectively.

This research marks a significant milestone as the first report on microbial diversity and enzyme bioprospecting from the high-altitude RHS in the Pir Panjal range of the North-Western Himalayas. By exploring an uncharted ecological niche, this study pioneers the understanding of microbial communities thriving in extreme environments. The novelty lies in uncovering previously unknown microbial taxa, and enzymes uniquely adapted to the harsh conditions of RHS. Furthermore, the identification and characterization of hydrolases like *CarP* and *TreS* represent a novel biotechnological resource with potential applications in various industries.

Acknowledgements

I express my deepest gratitude towards Allah for His divine support and guidance throughout the journey of this research endeavor. May Allah's blessings continue to shower upon me as I strive to utilize the knowledge gained for the betterment of humanity.

I extend my heartfelt gratitude to my esteemed supervisor, ***Dr. Reena Singh***, and co-supervisor, ***Dr. Juliana Heloisa Pinê Américo Pinheiro***, for their invaluable guidance and unwavering support throughout the course of this research. Their profound insights, expertise, and encouragement have been instrumental in shaping the direction of my study and enhancing the quality of this work. I am deeply grateful for their mentorship, patience, and dedication, which have inspired and motivated us to overcome challenges and strive for excellence.

I extend my sincere gratitude to the Senior Dean & Head of the School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, ***Dr. Neeta Raj Sharma***, and to the Head of the Department of Molecular Biology and Genetic Engineering, ***Dr. Umesh Gautam***, for their unwavering encouragement and steadfast support throughout the duration of my research endeavors. Their guidance and mentorship have played a pivotal role in facilitating the smooth progression of my research activities.

I extend my heartfelt gratitude to ***Dr. Aruna Bhatia***, a distinguished member of my research committee, for her instrumental role in shaping my research and for her invaluable suggestions. I wish to extend my heartfelt gratitude to all the esteemed faculty members ***Dr. Showkeen Muzamil Bashir***, ***Dr. Chirag Chopra***, and ***Dr. Vijay Kumar*** for invaluable guidance throughout the course of my research endeavors. Their profound expertise, unwavering commitment, and insightful suggestions have been pivotal in shaping the quality and direction of my work.

With a heart full of gratitude, I extend my deepest thanks to my beloved friends Nahid Akhtar, Tahir ul Gani Mir, Marwa Formuly, Nafiaah Naqash, Saikat Sena, Nirdesh Ranjan, Zaheer Ahmad, Aftab Ahmad, Daljeet Singh Dhanjal, Aftab Ahamd Salroo, and Rustum Karimi. You have been my pillars of strength, my confidants, and my source of unwavering support, and for that, I am eternally grateful. May our bond continue to grow stronger with each passing day, and may we always find solace and joy in each other's company. Thank you, from the bottom of my heart, for being the incredible friends that you are.

In the journey of my academic pursuits, there are no words sufficient to express my deepest gratitude to my beloved parents, Father ***Khurshid Ahmad Wani*** and Mother ***Fatima Akhtar***. Your sacrifices, both seen and unseen, have laid the foundation upon which my dreams have been built, and for that, I am eternally grateful. You have been my pillars of strength, my guiding stars, and my greatest source of love and inspiration. Your love will forever be the greatest gift I have ever received.

Atif Khurshid Wani

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List of abbreviations

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

mDNA: Metagenomic deoxyribonucleic acid

TFs: Transcription factors

TRN: Transcription regulatory network

HGT: Horizontal gene transfer

UV: Ultraviolet

DTM: DNA transport module

IMG: Integrated Microbial Genome

CRESS DNA: circular Rep-encoding single-stranded DNA

GC: Guanine Cytosine

WoS: Web of Science

SCP: Single country publications

MCP: Multiple country publications

TLS: Total Link Strength

RHS: Riniae Hot Spring

$CaCl_2$: Calcium chloride

$CaCo_3$: Calcium carbonate

PVP: Polyvinylpyrrolidone

CsCl: Cesium chloride

PES: Polyethersulfone

NGS: Next generation sequencing

PCR: Polymerase chain reaction

CarP: Carboxypeptidase

TreS: Trehalose synthase

LB: Luria broth

DDW: Double distilled water

IPTG: Isopropyl β-d-1-thiogalactopyranoside

SDS: Sodium dodecyl sulfate

TEMED: Tetramethylethylenediamine

CFE: Cell free extract

BSA: Bovine serum albumin

NaOH: Sodium hydroxide

TCA: Trichloroacetic acid

PMSF: Phenylmethanesulfonyl fluoride

DTT: Dithiothreitol

H_20_2: Hydrogen peroxide

EDTA: Ethylenediaminetetraacetic acid

ME: Mercaptoethanol

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Chapter 1

INTRODUCTION

1.1. Microbial Dynamics

Microbes, the invisible architects of life, exhibit a remarkable omnipresence across an astonishing array of environments (Gupta *et al.*, 2017). From the depths of oceans to the highest peaks (Sunagawa, Coelho, Chaffron, Kultima, Bork, *et al.*, 2015, Ciccazzo *et al.*, 2016), from scorching deserts to freezing polar regions (Clark *et al.*, 2023, Li *et al.*, 2023), and from the human body to the far reaches of outer space (Rackaityte and Lynch, 2020, Deshevaya *et al.*, 2024), microbes persist and thrive in conditions once thought uninhabitable. This pervasive presence underscores their extraordinary adaptability and underscores their fundamental role in shaping ecosystems and sustaining life on Earth (Hartmann and Six, 2023). In aquatic ecosystems, microbes are the unseen drivers of nutrient cycles, playing critical roles in carbon and nitrogen fixation, as well as in the breakdown of organic matter (Tran *et al.*, 2021). In soil, they form intricate networks that facilitate plant growth, nutrient uptake, and soil fertility (Balestrini *et al.*, 2024). Even extreme environments such as hot springs (Kaur *et al.*, 2018), acid mines (Méndez-García *et al.*, 2015), and deep-sea vents (Ding *et al.*, 2017) harbor microbial communities uniquely adapted to harsh conditions, pushing the boundaries of life as we know it. The human body, too, is a vast ecosystem teeming with microbial life. The gut microbiome, in particular, influences digestion, immune function, and even mood, highlighting the intricate interplay between microbes and human health (Gomaa, 2020a). Furthermore, microbes play crucial roles in industrial processes, agriculture, and bioremediation, harnessing their metabolic capabilities for human benefit (Demain and Adrio, 2008, Abatenh *et al.*, 2017, Gomaa, 2020b, Akinola *et al.*, 2021).

Life's ability to persist and thrive in diverse environments is a testament to the remarkable adaptability of organisms (Ma *et al.*, 2019a). Across ecosystems ranging from the scorching deserts to the freezing polar regions, organisms have evolved an array of ingenious adaptation mechanisms to cope with environmental challenges. Understanding these strategies not only sheds light on the intricacies of evolution but also holds implications for conservation, biotechnology, and human health. One of the fundamental mechanisms of adaptation is phenotypic plasticity (Kalirad and Sommer, 2024), wherein organisms alter their traits in response to environmental cues. This

flexibility allows them to adjust their physiology, morphology, and behavior to match changing conditions. Another crucial adaptation mechanism is genetic diversity, which provides the raw material for evolutionary change. Through genetic mutations, recombination, and gene flow, populations can generate novel genetic variants that confer advantages in specific environments (Tokuda and Shintani, 2024). Examples abound, from antibiotic resistance in bacteria to coloration patterns in camouflage (Yan *et al.*, 2024, Zvonareva *et al.*, 2024). Beyond individual organisms, cooperative behaviors and symbiotic relationships play vital roles in adaptation. Mutualistic interactions, where two or more species benefit from each other's presence, can enhance resilience to environmental stressors. For instance, microbial communities in the gut provide essential functions for host organisms, such as digestion and immunity (Almeida *et al.*, 2019). In extreme environments, specialized adaptations enable organisms to thrive where others cannot. Extremophiles, for example, inhabit habitats with extreme temperatures (Burkhardt *et al.*, 2024a), pH levels, salinity, or pressure (Holden, 2009). These organisms have evolved unique biochemical pathways, membrane structures, and protective mechanisms to withstand harsh conditions. Their study not only informs astrobiology but also holds promise for biotechnological applications. As human-induced environmental changes accelerate, understanding adaptation mechanisms becomes increasingly urgent. By deciphering the strategies that enable organisms to survive and flourish in varying environments, we can better predict and mitigate the impacts of climate change, habitat loss, and pollution. Moreover, harnessing nature's adaptive prowess may inspire innovative solutions for sustainable agriculture, medicine, and conservation. Hence, the study of adaptation mechanisms serves as a window into the resilience and ingenuity of life on Earth (Gerrish, 2001, Voolstra and Ziegler, 2020).

1.2. Constraints in culturable techniques

Microbial culture techniques are essential for studying microorganisms in controlled laboratory environments, enabling researchers to isolate, grow, and study microbes. These techniques involve sample collection, media preparation, inoculation, incubation, and maintenance. Microbial culture techniques, while indispensable for studying microorganisms, encounter various constraints that influence the breadth and accuracy of our understanding (Lewis et al., 2021). One significant limitation is culturability bias, where certain organisms are favored for cultivation, leaving many uncultured due to slow growth rates or specialized requirements (Prakash *et al.*, 2021).

Contamination poses a persistent challenge, necessitating rigorous sterilization protocols to maintain culture purity (Abatenh *et al.*, 2018). Moreover, the selectivity of culture media can skew microbial community profiles, limiting the detection of less abundant species (Bridson and Brecker, 1970). Viability issues, including dormancy and low metabolic activity, further complicate cultivation efforts. Additionally, the ecological relevance of laboratory-cultured microorganisms compared to their natural counterparts raises questions about the extrapolation of findings (Alonso, 2016).

1.3. Metagenomics: An unculturable method for microbial analysis

Metagenomics stands as an innovative alternative to traditional culturing methods, effectively surmounting their inherent limitations. Directly analyzing the genetic material of microbial communities present in environmental samples, metagenomics circumvents biases towards cultivable organisms, thereby providing a more comprehensive understanding of microbial diversity (Handelsman, 2004). This approach unlocks the potential to explore unculturable microbes, unveiling novel genes, metabolic pathways, and bioactive compounds with applications across biotechnology, medicine, and environmental science. Moreover, metagenomics enables ecological and environmental studies, illuminating microbial roles in ecosystem functioning and responses to environmental changes (Nagar *et al.*, 2023). In clinical microbiology, metagenomics aids in diagnosing infectious diseases, detecting antimicrobial resistance, and profiling the human microbiome for personalized healthcare strategies (Cibulski et al., 2021; Schmieder and Edwards, 2012). Metagenomics thus represents a powerful tool for unraveling the complexities of microbial communities and their interactions, heralding new avenues for scientific exploration and practical applications.

Metagenomics is indeed a fascinating field that revolutionized microbial analysis. Traditional methods for studying microbes often involve culturing them in the lab, which can be quite limiting because many microbes cannot be cultured using standard techniques (Berini *et al.*, 2017). However, metagenomics allows researchers to study the collective genetic material from entire microbial communities directly from environmental samples, without the need for culturing (Adjeroud *et al.*, 2020). Researchers collect samples from various environments, such as soil, water, or the human gut. These samples contain a diverse array of microorganisms, including bacteria, archaea, viruses, and fungi (Bexfield and Kellam, 2011, Jung *et al.*, 2016). Instead of

trying to isolate and culture each microbe individually, which may be impossible for many of them, researchers extract the genetic material from the entire microbial community present in the sample. Once the genetic material is extracted, it is sequenced using high-throughput sequencing technologies. This produces vast amounts of DNA or RNA sequence data representing all the organisms present in the sample. Bioinformatics tools are then used to analyze this data, including assembly of genomes, identification of genes and pathways, and taxonomic classification of the organisms (Kunin *et al.*, 2008, Dudhagara *et al.*, 2015).

1.4. Metagenomic Approaches

Metagenomic strategies encompass a range of approaches for analyzing the genetic material of microbial communities directly from environmental samples. Shotgun metagenomics involves sequencing the entire DNA or RNA content of a sample, providing a comprehensive snapshot of microbial diversity and functional potential. Assembly-based methods reconstruct individual microbial genomes from metagenomic data, facilitating the study of specific organisms and their metabolic pathways (Aragão *et al.*, 2023). Targeted metagenomics employs primers or probes to amplify and sequence specific genetic markers, allowing for the selective detection of taxa or functional genes of interest. Additionally, functional metagenomics focuses on identifying genes and pathways with particular biological activities, such as antibiotic resistance or carbohydrate metabolism, through functional screening of metagenomic libraries (dos Santos *et al.*, 2015, Kanokratana *et al.*, 2015). These diverse metagenomic strategies enable researchers to explore microbial communities in various environments, uncovering insights into their composition, function, and ecological roles. Metagenomic strategies broadly fall into two main categories: sequence-based and function-based approaches (Figure 1.1).

1.4.1. Sequence based approaches

This approach involves the direct sequencing of DNA or RNA extracted from environmental samples. In shotgun metagenomics, the entire genetic material present in the sample is sequenced, providing a comprehensive view of microbial diversity without prior knowledge of the community composition (Loman *et al.*, 2013). Assembly-based methods then reconstruct individual genomes from metagenomic data, allowing for the study of specific organisms and their genomic features. Taxonomic profiling and functional annotation techniques further characterize the microbial community structure and metabolic potential, respectively. This approach is particularly useful for

exploring microbial diversity, identifying novel taxa, and understanding the genetic repertoire of microbial communities (Mangrola *et al.*, 2015, Babalola, Fadiji, *et al.*, 2020).

1.4.2. Function based approaches

Function-based metagenomics focuses on identifying genes and pathways with specific biological activities within microbial communities. This approach involves cloning environmental DNA into host organisms, such as *E. coli or yeast*, and screening for phenotypic traits of interest, such as antibiotic resistance, enzyme activity, or biodegradation capabilities (Sharma *et al.*, 2010, Sun *et al.*, 2015). By expressing metagenomic DNA fragments in a heterologous host, researchers can identify functional genes and pathways without prior knowledge of their sequences. Function-based metagenomics provides valuable insights into the functional potential of microbial communities and facilitates the discovery of novel biocatalysts, bioactive compounds, and other biotechnologically relevant molecules (Itoh *et al.*, 2014, Bharwad *et al.*, 2023). Both sequence-based and function-based metagenomic approaches offer unique advantages and insights into microbial communities, and their combined use provides a comprehensive understanding of microbial diversity, function, and ecological roles in various environments.

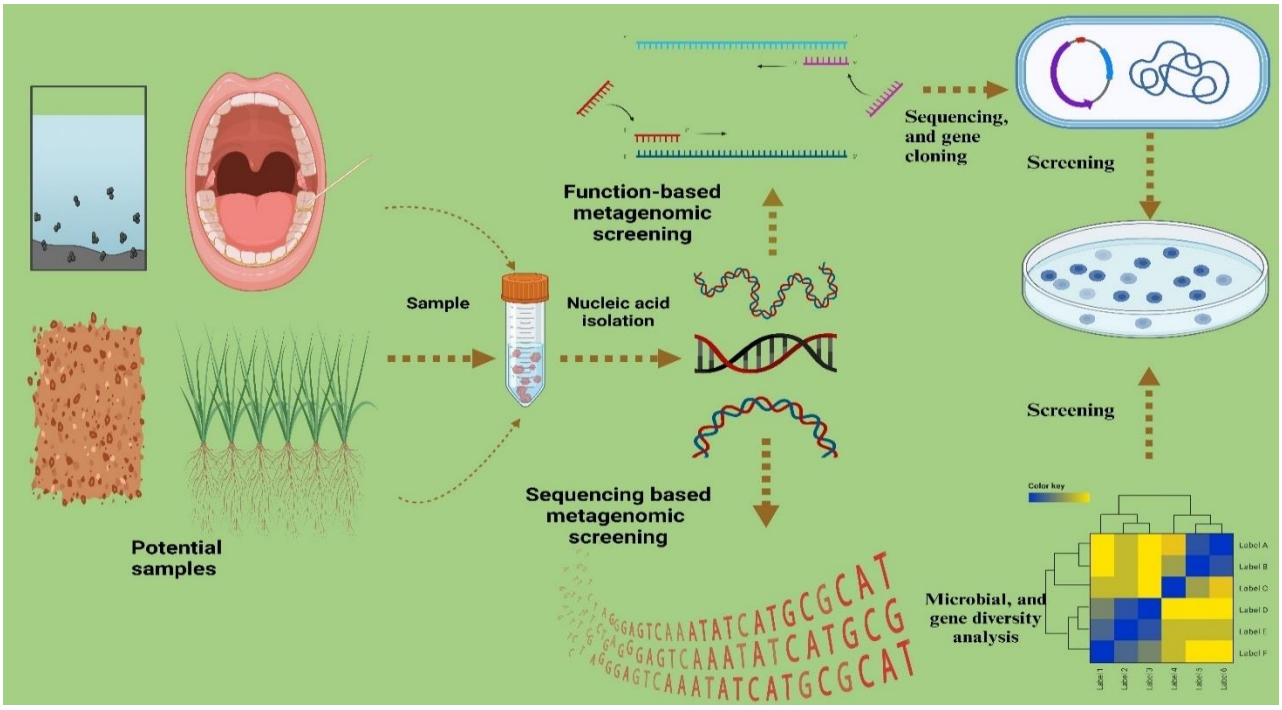

Figure 1.1: Function and sequence based metagenomic approaches for bioprospecting different environmental samples.

1.5. Hot Springs: Reservoir of thermophiles and thermostable enzymes

Hot springs serve as remarkable reservoirs of thermophiles, organisms adapted to thrive in high-temperature environments (Adjeroud *et al.*, 2020, Burkhardt *et al.*, 2024a). These extreme habitats, often found in geothermally active regions such as volcanic areas or deep-sea hydrothermal vents, offer unique ecological niches characterized by elevated temperatures, high mineral content, and sometimes extreme pH levels (Damer and Deamer, 2020). Thermophiles have evolved specialized molecular mechanisms to withstand these harsh conditions, making them of particular interest in biotechnology, especially for their production of thermostable enzymes (Zeldovich *et al.*, 2007). Thermostable enzymes are enzymes that remain active and functional even at high temperatures, a property that is advantageous for industrial processes requiring high temperatures or harsh conditions (Hamdan *et al.*, 2021). Hot springs harbor a rich diversity of thermophilic microorganisms, including bacteria, archaea, and some eukaryotes, which produce a wide array of thermostable enzymes adapted to their extreme habitats (Hou *et al.*, 2013, Bennett *et al.*, 2022). One of the most extensively studied groups of thermostable enzymes from hot springs is thermophilic polymerases, such as DNA polymerases, RNA polymerases, and reverse transcriptases (Prangishvilli *et al.*, 1982, Vellore *et al.*, 2004, Akram *et al.*, 2023). These enzymes are essential for DNA amplification techniques like PCR and reverse transcription PCR, which are widely used in molecular biology and diagnostics. Thermostable polymerases sourced from hot springs, such as those from *Thermus aquaticus* (Taq polymerase) or *Thermococcus kodakarensis*, have revolutionized molecular biology by enabling rapid and efficient amplification of DNA even at high temperatures, thus eliminating the need for frequent enzyme replenishment during PCR (Kim *et al.*, 1995, Takashima *et al.*, 2019). Beyond polymerases, hot springs are also rich sources of other thermostable enzymes with industrial applications, including proteases (Kahled *et al.*, 2022), lipases (Johri *et al.*, 2012), amylases (Kikani and Singh, 2011), cellulases (Khalid *et al.*, 2019), and xylanases (Sun *et al.*, 2015). These enzymes have diverse functions and can be used in various industrial processes, such as food processing, biofuel production, textile manufacturing, and waste treatment. Their thermostability allows for more efficient and cost-effective processes, as they can withstand high temperatures, harsh chemicals, and prolonged reaction times without losing activity or denaturing. Moreover, the study of thermophiles and their enzymes from hot springs has broader implications beyond industrial applications. Understanding the molecular mechanisms underlying thermostability can provide insights into protein folding, stability, and

evolution, with potential implications for biotechnology, medicine, and astrobiology (Müller *et al.*, 2005, Bååth *et al.*, 2020, Boersma, 2024). Additionally, hot springs are considered analogs for early Earth environments and potentially for extraterrestrial habitats, making them valuable sites for studying the origins and evolution of life on Earth and the potential for life elsewhere in the universe (Segerer *et al.*, 1993, Russell, 1996).

1.6. Hydrolases as essential industrial enzymes

Hydrolases represent a crucial class of industrial enzymes with diverse applications across various sectors (Kimura, 2000). These enzymes catalyze the hydrolysis of chemical bonds through the addition of water molecules, resulting in the breakdown of substrates into smaller molecules (Gotor, 1999). Their versatility and efficiency make them indispensable in numerous industrial processes, ranging from food and beverage production to pharmaceutical manufacturing and environmental remediation (Flores-Gallegos *et al.*, 2019). Hydrolases play a vital role in food processing and beverage production. For instance, carbohydrases, including amylases and cellulases, break down complex carbohydrates into simpler sugars, facilitating the production of sweeteners, such as glucose, fructose, and maltose (Ejaz *et al.*, 2021, Rao *et al.*, 2023). These enzymes are used in the production of various food products, including bread, beer, wine, and fruit juices. Additionally, lipases are employed in cheese ripening and flavor development, as well as in the production of margarine and other lipid-based food products (Goswami, 2020). Proteases and lipases are key components in laundry and dishwashing detergents. Proteases degrade protein-based stains, such as blood and grass, into smaller peptides and amino acids, improving the efficacy of detergent formulations (dos Santos Aguilar and Sato, 2018). Lipases, on the other hand, break down lipid-based stains, such as oils and grease, into fatty acids and glycerol. These enzymes enhance the cleaning performance of detergents under various temperature and pH conditions, resulting in more efficient stain removal. Enzymes, including cellulases and proteases, are widely used in the textile industry for fabric finishing and garment processing. Cellulases help soften and improve the feel of cotton-based fabrics by removing surface fuzz and pilling through the hydrolysis of cellulose fibers. Proteases aid in fabric biopolishing, where they selectively remove protruding fibers and surface irregularities, resulting in smoother and more uniform textiles (Araujo *et al.*, 1996, Mojsov, 2014). Hydrolases play critical roles in pharmaceutical manufacturing, particularly in the production of pharmaceutical intermediates and active

pharmaceutical ingredients. For instance, chiral hydrolases, such as lipases and esterases, catalyze enantioselective reactions, enabling the synthesis of optically pure compounds used in drug formulations (Goli *et al.*, 2011). Moreover, glucuronidases and sulfatases are involved in drug metabolism and the biotransformation of drug conjugates in the body. Hydrolases are employed in environmental remediation efforts, including wastewater treatment and bioremediation of contaminated soil and water (Schmitz *et al.*, 1990, Masuno and Molinski, 2018). Enzymes, such as lipases and esterases, assist in the degradation of organic pollutants, such as fats, oils, and hydrocarbons, into simpler and less toxic compounds. Additionally, cellulases and hemicellulases are used in the bioconversion of lignocellulosic biomass into biofuels and other value-added products, contributing to sustainable energy production (Juturu and Wu, 2014).

This study focused on extracting mDNA from a high-altitude hot spring located in the Pir Panjal range of the Northwestern Himalayas, followed by screening for hydrolytic enzyme production and cloning of relevant bioactivity-related genes. The mDNA was analyzed through next-generation sequencing (NGS) to assess microbial and gene diversity. The primary aim was to explore thermostable hydrolases i.e. proteases, amylases, cellulases, ureases, lipases etc. A protease enzyme Carboxypeptidase (*CarP*) and an amylase Trehalose Synthase (*TreS*) were amplified, cloned, expressed, purified, and analyzed in silico. The wet lab work involved the extraction of mDNA from soil samples, screening for hydrolytic enzymes through PCR amplification, and cloning the target genes into expression vectors. The proteins were overexpressed in *E. coli* and purified using affinity chromatography, with their purity confirmed through SDS-PAGE. Characterization of *CarP* and *TreS* involved determining temperature and pH optima, stability, and the effects of metal ions, surfactants, and inhibitors. The study concluded with in silico analysis of the genes and proteins, providing valuable insights into their structure and function.

Chapter 2

REVIEW OF LITERATURE

2.1. Microbial omnipresence in diverse environments

The ubiquity of microbes in diverse environments is a fascinating topic that underscores the resilience and adaptability of microbial life forms (Whitfield, 2005). From the depths of the ocean to the highest peaks , and from extreme temperatures to seemingly inhospitable habitats, microbes thrive in virtually every corner of our planet (Bergo *et al.*, 2021, Sood *et al.*, 2022). Microbes, including bacteria, archaea, fungi, protists, and viruses, are the oldest and most abundant forms of life on Earth (Morono *et al.*, 2020). They inhabit a vast array of environments, ranging from the human body (Gomaa, 2020a) to extreme environments such as hot springs (Wani *et al.*, 2023), polar ice caps (Segawa *et al.*, 2010), and deep-sea hydrothermal vents (Ding *et al.*, 2017). Their ability to colonize such diverse habitats is attributed to their metabolic versatility, genetic plasticity, and ability to form symbiotic relationships with other organisms. One of the most remarkable aspects of microbial omnipresence is their adaptation to extreme conditions (Wani *et al.*, 2022).

Extremophiles, microbes that thrive in environments with extreme temperatures, pH levels, salinity, or pressure, challenge our understanding of the limits of life (Holden, 2009). For example, thermophiles flourish in hot springs with temperatures exceeding 70°C (Burkhardt *et al.*, 2024b), while psychrophiles thrive in icy environments where temperatures hover around freezing (De Maayer *et al.*, 2014). These extremophiles possess specialized enzymes and membrane structures that enable them to withstand and even thrive in such harsh conditions (Wani *et al.*, 2022). Microbes play a crucial role in biogeochemical cycles, cycling nutrients such as carbon, nitrogen, sulfur, and phosphorus between living organisms and the environment. For instance, nitrogen-fixing bacteria convert atmospheric nitrogen into a form that plants can use, contributing to plant growth and productivity (Zhang *et al.*, 2022). Methane-producing archaea are essential for decomposing organic matter in anaerobic environments, such as wetlands and the digestive tracts of animals (Amin *et al.*, 2021). The widespread distribution of microbes in diverse environments has profound implications for astrobiology, the study of life beyond Earth. The discovery of extremophiles on Earth has led scientists to speculate about the possibility of microbial life existing in similarly extreme environments on other planets or moons within our solar system, such as Mars

or Europa (Bashir *et al.*, 2021). Studying terrestrial extremophiles provides valuable insights into the potential habitability of extraterrestrial environments and informs the search for life beyond Earth (Carré *et al.*, 2022, Coleine and Delgado-Baquerizo, 2022). Microbes' ability to thrive in diverse environments has practical applications in biotechnology (Candido da Silva *et al.*, 2017), agriculture (Vishwakarma *et al.*, 2020), and environmental remediation (Leal *et al.*, 2017). Extremophiles produce enzymes and metabolites with unique properties that are valuable for industrial processes, such as the production of enzymes for laundry detergents or the synthesis of biofuels from organic waste (Burkhardt *et al.*, 2024b). Additionally, microbial communities are increasingly being used to remediate contaminated soil and water by breaking down pollutants and toxins (Chandran *et al.*, 2020).

2.2. Microbial adaptations in different environmental conditions

Microorganisms are adjusted in natural environments for optimum and normal functioning. Any fluctuation in abiotic factors from the normal operating range induces stress on microorganisms (Jordan and Tomberlin, 2017). The degree of shift level regulates the survival chances of a particular microorganism (Hibbing *et al.*, 2010). The changes in abiotic environmental factors can initiate stress responses as changes are more survival-oriented than growth (Meena *et al.*, 2017). The maximum microbial populations withstand the little environmental changes by adapting over a certain time duration. They do so by inducing cellular, genetic, and morphological modulations and thus resist the stress (Brooks *et al.*, 2011). Often, the degree of tolerance increases by providing cells with an extended time for acclimatizing in dynamic environments. Microorganisms incessantly customize in fluctuating environmental conditions. In the changing environment where maximum organisms experience maladaptive coping, the microbial lineages exploit other microcosm opportunities by modulating physiological states, thereby adapt to the new environments via positive selection (Wadsworth, 2015). The existence of microorganisms as acidophiles, halophiles, and psychrophiles is testimony of the regulated lifestyle of the microorganisms (Rampelotto, 2013). Bacteria, in general, have a morphological make-up in such a way that it prepares well for the transforming environment. Their membranes, along with genetic products, can undergo alterations with a particular stimulus that emerges as a result of varying abiotic conditions like temperature, pH, pressure, and salinity (Bartlett *et al.*, 2007, Ranawat and

Rawat, 2017, Zuñiga *et al.*, 2017). These changes are well regulated and can be passed on to the next generations as well.

The emergence of resistance against several anti-microbial drugs in numerous microorganisms is a good example of microbial adaptation. The resistance to penicillin by *Staphylococcus aureus* was reported soon after its introduction and by 1990, 80% of *S. aureus* were reported as resistant to this drug (Lowy, 2003). The adaptation to the anti-microbial agent is either driven by an inherent character like the presence of outer lipid membrane in gram-negative bacteria or it is driven by acquired (genetic) character (Brooks *et al.*, 2011, Munita and Arias, 2016). Once adaptability is mediated by genetic make-up, it is passed on to the generations that follow. The microbes can also adapt to the changing environmental condition by transforming to different cell types that help the microbes to maintain optimum growth in new environment (Freitas *et al.*, 2020). An example of this changing environment adaptability towards the changing surrounding support is reported in *Vibrio parahaemolyticus* (Freitas *et al.*, 2020). In a highly viscous vicinity, the bacteria form a swarmer cell that ensures motility for the process of chemotaxis (Jose and Singh, 2020). The adaptation is well regulated and involves the expression or repression of many genes. This mechanism transforms intracellular, extracellular, and surface properties of the microorganism (Niño-Martínez *et al.*, 2019). The microbes adapt to high temperature damage (unfolding of cellular proteins and production of aggregates of misfolded proteins) by activating heat shock response (Rodríguez-Verdugo *et al.*, 2020). The heat shock response of *Escherichia coli* is a very familiar example of this kind of adaptation (Arsène *et al.*, 2000). The bacteria sense the increase in temperature by the help of sensory biomolecules called thermosensors which then activates the heat shock response where the expression of two types of proteins namely chaperons and proteases is increased (Roncarati and Scarlato, 2017, Rodríguez-Verdugo *et al.*, 2020). Furthermore, in *E. coli* and *Zymomonas mobilis* the genes responsible for membrane transport, energy metabolism, DNA repair, tRNA modification, membrane stabilization, and cell division have been found to be important for growth under high temperature (Murata *et al.*, 2011, Charoensuk *et al.*, 2017). Recently, the recombinant transfer of cold shock protein, named CspL, among different bacteria has also been reported to confer resistance to high temperature (Zhou *et al.*, 2021). Furthermore, the formation of solid surface adherent populations popularly known as biofilms have also been reported to play important role in microbial ressitance to extreme environmental conditions such as UV irradiation, high pH, salinity and temperature (Yin *et al.*, 2019, Penesyan *et al.*, 2021). *V.*

parahaemolyticus and many other bacterial species also show adaptability of environmental changes by forming biofilms (Yildiz and Visick, 2009).

Biofilms also provide protective environment where the cells with reduced fitness to the impacts of the environmental changes can accumulate mutations that will offset the impact and help the microbe to adapt and evolve (Penesyan *et al.*, 2021). Adapting to the mode of biofilm formation involves a sequence of changes that involves the expression of unexpressed genes or even deactivation of hyperactive genes (Wolska *et al.*, 2016). Chemotaxis by *Bacillus subtilis* and other bacteria is the widely studied adaptation mechanism of the bacteria whereby bacterium senses chemical make-up and either move towards a particular compound or away from it. This is a highly regulated system and is controlled by 40 or more genes that mediate flagella synthesis (Mukherjee and Kearns, 2014). There have been several comprehensive studies on evaluation of the microbial adaptation (Katarína *et al.*, 2018, Merino *et al.*, 2019, Ji and Wei, 2020, Manriquez *et al.*, 2021) .

2.2.1. Temperature

The submarine, subterranean and certain terrestrial areas of high-temperature harbour diverse hyperthermophiles. These are found in hydrothermal land sources heated up by volcanic exhalations forming sulfur-rich alkaline hot springs (Stetter, 2002). More than 50 hyperthermophilic bacterial and archaeal species are known so far, and most of them are classified using 16S rRNA gene-based classification (Miyazaki and Tomariguchi, 2019). Hyperthermophiles generally adapt to high-temperature environments by their nutritional requirements and physiological state, making their genome, cellular components stable and functional (Figure 2.1) (Vieille and Zeikus, 2001). The cellular ingredients like nucleic acids, proteins and lipids usually known to be heat sensitive (Casares *et al.*, 2019); however, *Thermotoga maritima*, a bacterial hyperthermophile, contains a membrane lipid known as 15,16-dimethyl-30-glyceryloxy-triacontanedioic acid instead of the esterlipids that increases the membrane stability and prevents hydrolysis at higher temperatures (Siliakus *et al.*, 2017). In *Archaea* domain, both mesophiles and hyperthermophiles contain di(biphytanyl)-diglycerol and diphytanyl-glycerol that exhibit resistance at an acidic pH and high temperature against hydrolysis (Jaenicke and Sterner, 2006, Siliakus *et al.*, 2017). For example, *Pyrolobus fumarii*, chemolithoautotrophic *Archaea*, survives at a temperature up to 113 °C, thereby representing the life of upper border temperature. The mode of nutrition in hyperthermophiles is generally chemolithoautotrophic, whereby inorganic redox

reaction acts as energy sources and CO_2 is the carbon source (Stetter, 2002). Besides hyperthermophiles on marine and terrestrial ecosystems, many psychrophiles are well adapted to cold environments (Barria *et al.*, 2013). They adjust to such cold temperatures by maintaining the fluidity of membranes, structural adjustment through enzymes, cold shock protein expression and storage of cold-resistant solutes (Tribelli and López, 2018). The carbon metabolism reconstruction in *Psychrobacter arcticus*, a permafrost bacterium, survives at -10 °C and it is devoid of phosphotransferase system and glycolysis genes but expresses the gluconeogenic enzymes like phosphoenolpyruvate synthase and fructose-1,6-bisphosphatase which indicates that it is unable to metabolize sugars but prefers other sources of oxidised carbon (Ayala-del-Río *et al.*, 2010). One of the studies carried out to evaluate the adaptive behaviour of two strains of *Pseudoalteromonas* in Antarctica showed glutathionylspermidine and glutathione as the main agents responsible for their adaptation in such cold regions (Tribelli and López, 2018). Table 2.1 provides the list of some extremophiles at varying environmental conditions.

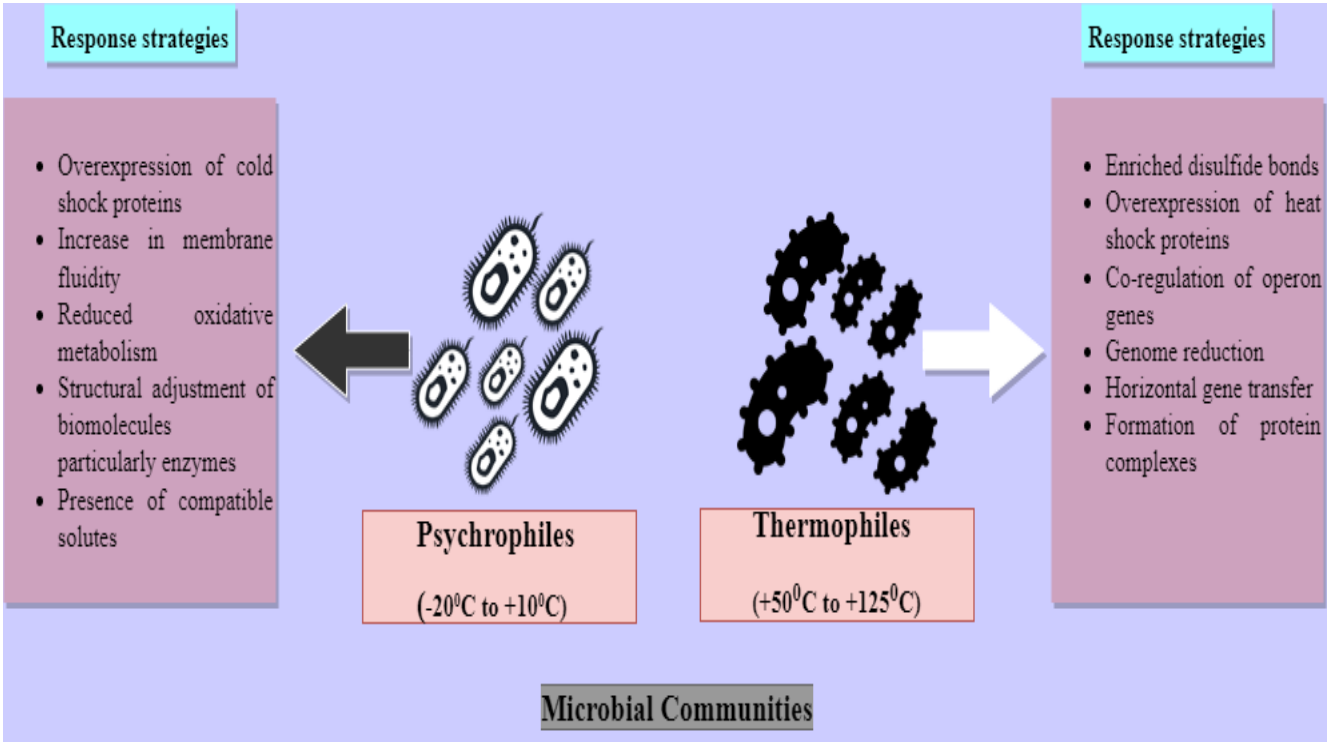

Figure 2.1: Response strategies of psychrophiles and thermophiles towards cold and heat respectively (Wani *et al.*, 2022).

2.2.2. Salinity

The diversity of microbes in saline environments is very well known (Gan *et al.*, 2020, Chen *et al.*, 2021, Xing *et al.*, 2021, Xue *et al.*, 2021). The hypersaline microcosms are inhabited by halotolerant and halophilic bacteria and archaea (Oren, 2006). The strategies followed by microbes to adapt in high salt concentration environments continue to gain the interest of many scientists. One attribute associated with halophiles is the isosmotic nature of the intracellular domain with the outside area (Ventosa *et al.*, 1998). To maintain increased osmotic pressure intracellularly, the salt-in strategy is followed by maintaining osmotic balance by accumulating inorganic salts of higher concentrations (Oren, 1999). Low-salt-in compatible solute strategy is also an adaptive feature of microorganisms in which a defined solute concentration allows the cellular processes to function effectively (Weinisch *et al.*, 2018). The compounds that have been evaluated and found compatible in different microorganisms include sugars, amino acids, polyols, ectoines, and betaines (Roberts, 2005). Microorganisms using organic solutes are usually more dynamic, and they easily adapt to the increasing salinity or dilution stress than those that utilise the salt-in strategy (Ma *et al*., 2010). In *Haloarcula marismortui, Halobacterium salinarum* and some related organisms, the intracellular ionic concentrations have been analyzed. It is reported that cells contain sufficient salt concentration for osmotically balancing the external medium-salinity (Poidevin and MacNeill, 2006). In halophilic archaea such as *H. salinarum* and *H. marismortui*, Na^+ is found in low concentration, and K^+ is present in higher concentration, whereas chloride ion balances the intracellular cations to balance the salinity (Gunde-Cimerman *et al.*, 2018, Szatmári *et al.*, 2020). Another attribute associated with the halophilic archaea is the acidic proteome having increased excess to amino acids of negative charge over positively charged amino acids (Reed *et al.*, 2013). Microorganisms encounter fluctuating pH stress during various bioprocesses (Guan and Liu, 2020). Microbial species have thus developed adaptive strategies to resist both alkaline and acidic environments (Padan *et al.*, 2005). The damage caused in high acidic environments is mitigated by membrane fluidity and integrity, pH homeostasis, DNA repair and metabolic regulation (Yang *et al.*, 2019). The proton influx in acid-tolerant microbes is reduced by chemiosmotic strategy (Baker-Austin and Dopson, 2007). Biofilm formation is another adaptive strategy that microorganisms use to resist acid stresses (Singh *et al.*, 2017). The pH homeostasis maintenance for acid stress resistance results from multiple transport system interactions (Padan *et al.*, 2005). Protons are expelled from the cell by electrogenic proton pumps resulting in the

generation of pH gradient and membrane potential (Palmgren and Morsomme, 2019). In *Helicobacter pyroli,* the urease system neutralises H^+ by producing NH_3 (ammonia) which helps to resist the acidic environment during its culturing (Ansari and Yamaoka, 2017). In alkaliphilic microorganism, pH maintenance is driven by active and passive strategies (Salwan and Sharma, 2020). The low permeability of membranes and cytoplasmic polyamine pools drive passive regulation while ion channels of Na^+ mediate active regulation (Yumoto, 2002). Besides, alkaliphilic compensate pH gradient mode by causing the coupling of Na^+ expulsion to transport electrons for homeostasis of pH and maintaining higher membrane potential (Krulwich *et al.*, 1998).

Table 2.1: List of psychrophilic, thermophilic, halophilic, and acidophilic microorganisms with optimum pH and temperatures ranges for metabolism.

Microorganism	**Temperature (°C) range**	**pH**	**Metabolism**	**References**
Acidianus infernus	60-95	1.5-5	Lithoautotrophs (aerobic-sulfur metabolizing)	(Plumb *et al.*, 2007)
Aeropyrum pernix	70-100	5-9	Autotrophic (aerobic)	(Balestrieri *et al.*, 2011)
Aquifex pyrophilus	85-95	5.4-7.5	Autotrophs (Typically uses oxygen for respiration but can also grow anaerobically by reducing nitrogen instead of oxygen)	(Wäber and Hartmann, 2019)
Archaeoglobus fulgidus	60-95	5.5-7.5	Chemolithoautotrophs (anaerobic sulfate reducing archaea)	(Hocking *et al.*, 2014)

Bacillus psychrosaccharolyticus	-2 – 20	5.2 – 7	Autotrophic (facultative anaerobe)	(Fresco-Taboada *et al.*, 2013)
Cryobacterium flavum	-6 – 28	5.5-10.5	Chemoautotrophic (aerobic)	(Liu *et al.*, 2012)
Cryobacterium roopkundense	-10 – 20	5- 10	Autotrophic (aerobic)	(Liu *et al.*, 2020)
Exiguobacterium sibiricum	-12 to 55	5-11	chemolithoautotrophs (facultative anaerobes or aerobes)	(Rodrigues *et al.*, 2008)
Halorhodospira halophile	55-70	7.5-10.6	Exhibits autotrophic growth	(Antón *et al.*, 2000)
Hyperthermus butylicus	80-108	7	Heterotrophic (anaerobic)-sulfur reducing	(Zillig *et al.*, 1990)
Metallosphaera sedula	65-85	2-5	Autotrophic (fixation of carbon dioxide in the presence of H2) and heterotrophic (uses organic molecules except for sugars)-obligate aerobic	(Maezato *et al.*, 2012, Terrado *et al.*, 2017)
Methanococcus jannaschii	50-86	5.5-6.5	Hydrogenotrophic (anaerobic)	(Jones *et al.*, 1983)
Natronomonas pharaonic	20-45	7.5-9.0	Utilizes amino acids as carbon source with higher degree of self-sufficiency in nutrition.	(Falb *et al.*, 2005)

Planococcus halocryophilus	-16 to 10	7-8	Chemolithoautotrphs (aerobe)	(Raymond-Bouchard *et al.*, 2017)
P. arcticus	-10 to 40	5-7	Autotrophs (aerobic)	(Hinsa-Leasure *et al.*, 2013)
Pseudomonas fluorescens	4-37	5-7	Versatile metabolism (obligate aerobe-Some strains use nitrate instead of oxygen)	(Rainey, 1999)
Pyrobaculum islandicum	65-98	4.5-8.5	Heterotrophic (anaerobic)	(Huber *et al.*, 1987)
Staphylothermus marinus	65-98	4.5-8.5	Heterotrophic (anaerobic)-sulfur respiring	(Jung *et al.*, 2012)
Thermoanaerobacter mathranii	50-75	4.7-8.8	Heterotroph (anaerobic)	(Larsen *et al.*, 1997)
Thermococcus celer	75- 93	4-7	Chemolithoautotrophic (anaerobic)	(Wong *et al.*, 2003)
Thiohalospira alkaliphile	50-60	6.5-8.3	Chemolithoautotrophic Sulphur oxidizing bacteria	(Sorokin *et al.*, 2008)

2.3. Molecular perspective of microbial adaptation

Since the environment is extremely heterogeneous at different levels, microorganisms face changing biotic and abiotic components. Table 2.2 illustrates the different cellular adaptive

strategies of microorganisms in fluctuating environments. The variables in their microcosms are generally temperature, pH, competition, and nutrient availability (Hibbing *et al.*, 2010). One of the examples is the *E. coli* population found in the mammalian gastrointestinal tract. Within the digestive tract, cells adapt to a series of stresses like fluctuating oxygen levels and increasing temperature, changing carbon sources (Freter *et al.*, 1983). One of the major factors that ensure the active adaptive mechanism in a rapidly fluctuating environment is its predictability (Bleuven and Landry, 2016a). The adaptation in such scenarios prefers the evolution of molecular networks that can predict the coming fluctuations like gene regulation by upregulating or downregulating an involved gene or maintaining a memory of previous encounters (Mitchell *et al.*, 2009). An example is the prediction of seasonal changes before the variation with the help of certain signals generated from the environment (Brunke and Hube, 2014). However, in unpredictable changes, the adaptability favours a bet-hedging strategy in which genotype visits phenotypes randomly and thus increases the adaptation probability (Tal and Tran, 2020). Experimental studies have presented a more in-depth view of underlying molecular mechanisms in different laboratory setups under controlled conditions. *S. cerevisiae* evolved under carbon limiting conditions for more than 200 generations, leading to multiple mutations and increased HXT6/7 gene copy number that codes for a transporter protein (hexose) (Payen *et al.*, 2016). The strains of yeast carrying amplified versions of this gene show enhanced fitness compared to the ancestral forms (Broach, 2012). In the absence of a drug, the drug resisting gene may cause fitness issues that are compensated via gene amplification of that particular gene (Depardieu *et al.*, 2007). Another potential trade-off source is the over-expressing genes (Kim *et al.*, 2020). This strategy may also result in loss of response which can be devastating for those genes where downregulation is required (Karasov *et al.*, 2017). (Kvitek and Sherlock, 2013) evolved some yeast strains in a chemostat deficient of nutrients and examined the evolution of adaptive response. The key to enhanced fitness as a stress response was the loss of the cAMP/PKA pathway and environmental sensing. Still, maladaptive nature was found during starvation when nutrient availability was not constant (Kvitek and Sherlock, 2013). When environmental fluctuations follow a regular pattern, the anticipation mechanism guides the adaptive strategies. The adaptability within such environment has been reported in *Candida albicans, E. coli* and *V. cholera* (Siavoshi and Saniee, 2014). Several cells express essential genes to protect from oxidative stress during the heat shock, allowing the cells to acclimatize to the stressful ECs (Morano *et al.*, 2012).

Table 2.2: Summary of adaptation strategies of microorganisms in fluctuating environments.

Microorganisms	**Microorganism in extreme environments**	**Mechanism of Adaptation**	**References**
Acidithiobacillus ferrooxidans, Halarchaeum acidiphilum, Metallosphaera sedula, Thiobacillus prosperous, Acetobacter aceti	Acidophile (pH 0.5 to 5)	Efficient efflux system, Acid tolerant membrane, proton exclusion and secondary transporters driven by protons.	(Krulwich and Guffanti, 1983, Baker-Austin and Dopson, 2007)
Chloroflexus aurantiacus,	Xerotolerant/xerophile	Organic and inorganic osmolyte accumulation, synthesis of extracellular polysaccharides and regulated differentiation of cells into desiccation resistant cells like spores.	(Bachar *et al.*, 2007, Lebre *et al.*, 2017)
Deinococcus radiodurans.	Radiation tolerant/resistant	Strong pigmentation and efficient DNA repair system	(Krisko and Radman, 2013)
Ferroplasma acidarmanus	Metalo-tolerant/ resistant	Detoxification and/or sequestering of metals by alkylation and/or reduction	(Mangold *et al.*, 2013)

Haloarcula amylolytica, H. argentinensis, H. hispanica, H. japonica, H. Quadrata	Halotolerant/Halophile (2-6 M NaCl and -1.5 to -40 Mpa water potential)	Accumulation of osmolytes and synthesis of salt-tolerant/dependent enzymes.	(González-Hernández and Peña, 2002, Siglioccolo *et al.*, 2011, Hänelt and Müller, 2013)
Halorhodospira halochloris, Thiohalospira alkaliphila, Bacillus firmus, Natronomonas pharaonic	Alkaliphile (pH 8.5 to 11)	Efficient proton uptake system mediated by antiporters of the membrane, OH- ion membrane impermeability.	(Krulwich and Guffanti, 1983, Kulkarni *et al.*, 2019)
Leptospirillum ferriphilum	Acidophile	Active pH regulating pumps	(Patel *et al.*, 2012)
Pseudomonas putida, Ralstonia solanacearum	Toxin tolerant/ resistant, Efficient xenobiotic decomposer	Effective efflux pump	(Chen *et al.*, 2006, Fernández *et al.*, 2009, Colburn-Clifford *et al.*, 2010)
Psychrobacter adeliensis, P. aestuarii, P. alimentarius, P aquimaris, P. luti, P glacincola	Psychrophile (-10 to 40 °C)	Synthesis of unsaturated fatty acids to prevent the decrease of membrane fluidity reduces the size of the cell and elevates cellular water in an ordered fashion.	(Feller *et al.*, 1997, Ayala-del-Río *et al.*, 2010, Q. Liu *et al.*, 2019)

P. fumarii, thermobaculum terrenum, Picrophilus torridus	Hyperthermophile (60 – 100 °C)	Synthesis of saturated long-chain fatty acids and cyclic lipids, and heat-stable proteins, lipids and, nucleic acids.	(Stetter, 1999, Soo *et al.*, 2009)
Serratia ureilytica	Thermophile (20-54 °C)	Modified vegetative cells into resistant structures.	(Filippidou *et al.*, 2019)
Streptomyces atacamensis	Xerotolerant	Dormancy and sporulation response to desiccation, and up regulation of proteins that are functional during xeric stress.	(Lebre *et al.*, 2017, Orellana *et al.*, 2018)
Thermococcus litoris	Hyperthermophile (55-100 °C)	Facultatively sulphur dependent and produces exopolysacchrides	(Price and Giovannelli, 2017)
Thermopiezophile Pyrococcus	Piezophile	Presence of higher proportion of fatty acids in cytoplasmic membranes.	(Siliakus *et al.*, 2017)

2.3.1. Adapting via transcriptional regulation

Microbial adaptation to different environments is largely attributed to their genetic make-up and its regulation. Figure 2.2 illustrates the fundamentals of microbial adaptation via changing genetic and community structure and composition. It is well known that microbial genome particularly of *Bacteria* are diverse in size, and content with few conserved genes among the sequenced genomes. Cases and his team investigated the genome fraction that is associated with metabolism, transport,

and transcription and reported that larger genomes harbor more transcription factors (TFs) per gene than the smaller ones (Cases *et al.*, 2003). This is a strong indication that in complex conditions signal integration and gene expression regulation enable rapid adaptation to environmental signals (Table 2.3). Transcription regulatory network (TRN) builds a response to a particular condition despite being highly diversified. While the dynamics and topology of these networks have been center of attention for research groups, the evolution of the topological features which continues to drive adaptive nature of microorganisms is still lesser known. One of the research groups studied the evolution of TRNs from reductive perspective and found that the action exerted by TFs correlate with conservation degree with dual regulators found to be more conserved than the activators and repressors in extreme reduction conditions. Besides they found that preponderant conservation of these dual regulators may be attributed to their action as nucleoid-associated proteins and global regulators (Galán-Vásquez *et al.*, 2016). This suggests that TRNs play a pivotal role in understanding the basic principles underlying microbial adaptability. Scientists studied chalcopyrite bioleaching by 2,4, and 6 acidophilic strains with similar inoculation density and found that 6-strain community adapted first to bioleaching conditions and maintained suitable function until late stage. The comparative transcriptomic analysis showed upregulation of 226 and 737 genes at early and later stage respectively (Ma *et al.*, 2019b). This strongly indicates that microbes adapt to oligotrophic environment by improving catalytic activation, cell proliferation, and binding action to maintain different life activities. Human microbiome also produces metabolites that influence the resistance and susceptibility. (Becattini *et al.*, 2021) colonized mice with anaerobic symbionts and show that the immune responses lead to dramatic transcriptional reprogramming with negligible alterations in their abundance. The transcriptomic alterations include downregulation of carbohydrate degrading factors and induction of stress-response mediators (Becattini *et al.*, 2021). *Mycobacterium tuberculosis*, a human pathogen, resists the unfavorable environment of phagosomes and alters dendritic cells and macrophages to prolong its survival, thus making transcriptome capture slightly difficult during infection (Rienksma *et al.*, 2015). Extreme thermophilic bacteria belonging to the genus *Caldicelulosiruptor* degrade the plant cell wall carbohydrates and then subsequently utilize them. (Rodionov *et al.*, 2021) evaluated the transcriptional regulation mechanism of carbohydrate using genes and investigated the TF binding sites and regulons with transcriptomic analysis for *C. bescii* grown on glucose, xylan, cellulose, and cellobiose.

2.3.2. Adapting via translational regulation

One of the vital molecular mechanisms capable of detecting and adapting to changing ECs is gene expression regulation at the translational level. The microbial communities use environmental stimuli to tune the protein concentration and translational rate (Tollerson and Ibba, 2020). The fluctuations in nutrient concentration leads to translational responses like riboswitch folding, and alarmone (*ppGpp*) synthesis causing changes in cellular physiology (Wurch *et al.*, 2019). Alarmone synthesis starts in response to stimuli like ribosome pausing during elongation phase of translation process. When cells have lesser availability of amino acids, the uncharged tRNA fraction increases that raises the chances of uncharged tRNA entering ribosomal A-site. This induces the Rel A binding of *ppGpp* synthase and alarmone synthesis begins thereby regulating wide range of cellular processes (Traxler *et al.*, 2008, Brown *et al.*, 2016). Cyanobacterial heat shock proteins preserve the integrity of thylakoid membranes under stress conditions like heat, light, or salt stress (Cimdins *et al.*, 2014, Wani *et al.*, 2021). Psychrophiles adapt to low temperature ranges through a series of structural, physiological, metabolic, and sequence adjustments. Aspartic acid, alanine, serine, and threonine are overrepresented in the coiled regions of secondary structures pertaining to psychrophilic bacteria, while as leucine and glutamic acid in helical regions are underrepresented (Metpally and Reddy, 2009). Thermophilic bacteria show heat resistance through tRNA and tRNA associated enzymes mediating cascade of translational events. The combination of tRNA enzymes and modified nucleosides like, dihydrouridine, 4- thiouridine, N^2-methyl guanosine, form a network that regulates tRNA modifications at higher temperature (Hori, 2019). Halophilic *Methanohalophilus portucalensis*, a methanoarchaeon, synthesizes osmolyte betaine in response to salt stress making it as an essential osmoadaptive strategy (Lai and Lai, 2011). Translational regulation in *E. coli* during temperature shift from 37 °C (Optimum temperature) to 10 °C (Cold shock) has been well illustrated through the expression of thermosensing RNAs. Cell growth and protein synthesis stops during acclimation phase and thereafter grows at slower rate. Importantly, the mRNAs encoding these cold shock proteins make certain structures that ensures their regulartion at lower temperatures (Lindquist and Mertens, 2018, Zhang *et al.*, 2018).

2.3.3. Adapting via horizontal gene transfer

Horizontal gene transfer (HGT) entails the movement of genetic material, either DNA or RNA, between organisms via non-sexual methods where the transferred genetic material can either replace the existing genes or add new genes to the genome (Keeling and Palmer, 2008). HGT is more common in prokaryotes where they play an important role in microbial evolution and adaptation to new environment (Wagner *et al.*, 2017, Hall *et al.*, 2020). Canonically, the HGT occurs in prokaryotes through mechanism such as conjugation, transduction and transformation (García-Aljaro *et al.*, 2017). However, the HGT in prokaryotes can occur via other ways such as through mobile genetic elements, integrating conjugative element, membrane vesicles, and gene transfer elements (García-Aljaro *et al.*, 2017). In archaea also other ways of horizontal gene transfer occurs such as cell fusion and a specific DNA transport system which plays an important role during DNA damage (Wagner *et al.*, 2017). The role of HGT in helping the microbes to adapt to extreme environmental conditions such as UV irradiation has been explored in *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* (Ajon *et al.*, 2011). *S. acidocaldarius* and *S. solfataricus*, the hyperthermophilic archaea, when exposed to DNA damaging factors like bleomycin, UV irradiation or mitomycin C, there is an upsurge in the induction of species-specific cellular aggregation (Götz *et al.*, 2007). In *S. acidocaldarius*, cellular aggregation induced by UV has been reported to mediate chromosomal exchange with a higher frequency. The recombination rates well exceeds the uninduced cultures by three times (Ajon *et al.*, 2011). Scientists have reported that the cellular aggregation improves species specific transfer of DNA among the *Sulfolobus* cells to provide enhanced DNA damage repair system through homologous recombination (Wagner *et al.*, 2017). This was also supported by the findings of (van Wolferen *et al.*, 2013), suggesting DNA exchange under extreme conditions in hyperthermophiles which plays an essential role in DNA repair through homologous recombination. Accurate DNA repair is important for maintaining gene functions. *Chalamydomonas sp. ICE-L*, a unicellular alga, thrives in cold polar sea where it withstands high salinity, low temperature and seasonal changes. (Zhang *et al.*, 2020) assembled genome sequence of ~542 Mb and reported that retrotransposon proliferation largely contributes to the genome size of ICE-L, which was hypothesized that it may support fatty acid synthesis, ionic homeostasis, ROS detoxification, DNA repair, and photoreception. The acquisition of several ice binding proteins via putative HGT likely supports the lifestyle of psychrophiles (Raymond and Remias, 2019). The lesser genomic content limits the

adaptive nature of thermophiles in fluctuating environment (Hickey and Singer, 2004). The active HGT allows the fast spread of strain specific adaptive gene networks in the entire population. The constitutive expression of an efficient DNA transport module (DTM) is at the middle of HGT-mediated improved adaptability (Sun *et al.*, 2019). The function of DTM is highly integrity and longevity dependent of the transformed extracellular DNA (eDNA) improved by extracellular vesicles. (Blesa and Berenguer, 2015) studied the contribution of vesicle protected eDNA to HGT in *Thermus spp*. and reported protection against DNAse was a resultant of eDNA association to vesicles. Figure 2.2 illustrates the changes in genetic composition while adapting to varying environments.

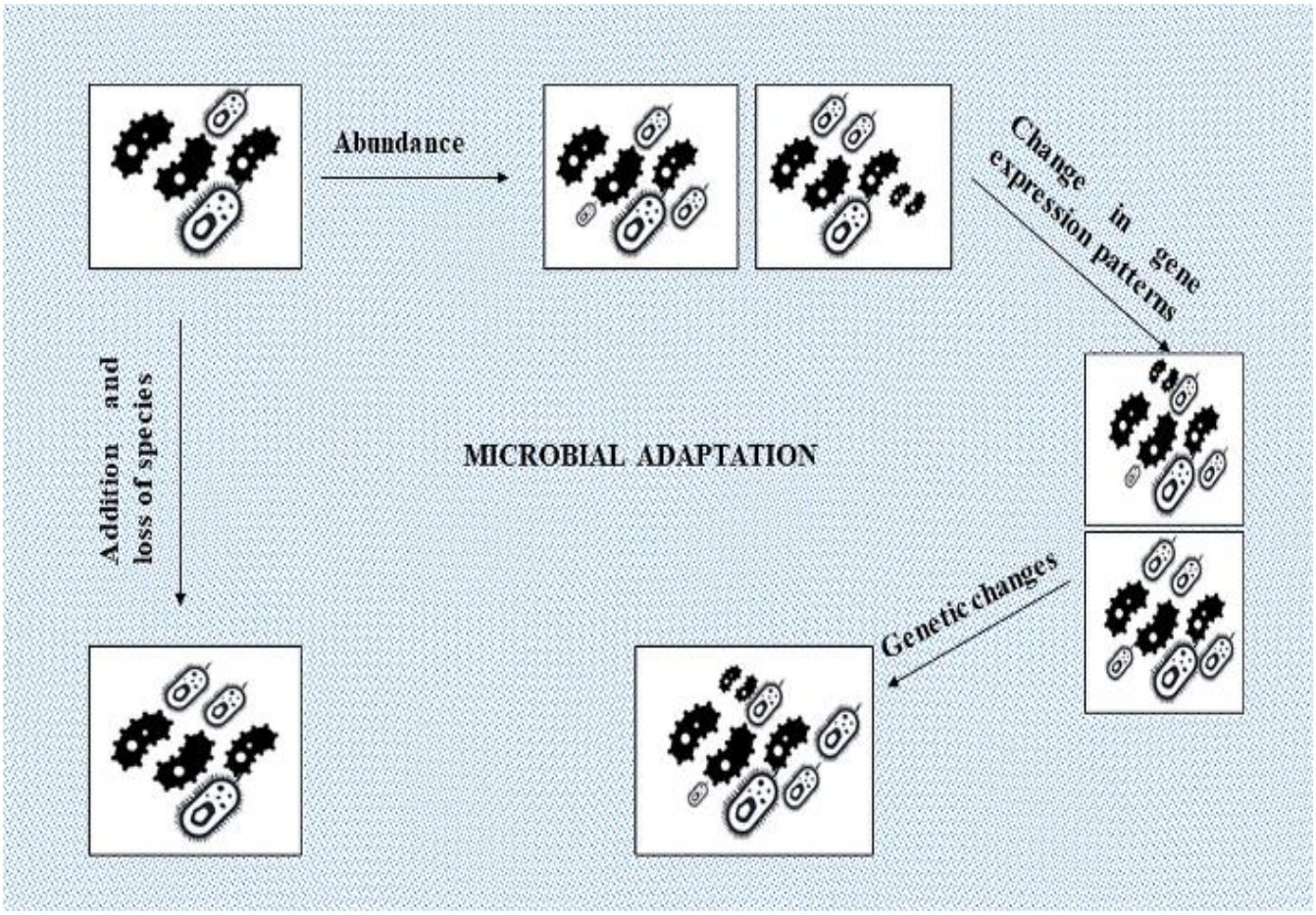

Figure 2.2: Changes in genetic and community structure and composition during microbial adaptation to extreme environments (Wani *et al.*, 2022).

Table 2.3: List of extremophiles and their adaptive strategies involving genes and gene products.

Extremophiles	Examples	Adaptive strategy	References
Hyperthermophile	M*ethanopyrus kandleri*	The membrane consists of terpenoid lipids. 1B DNA topoisomerase V and the two-subunit reverse gyrase are two notable enzymes	(Slesarev *et al.*, 2002)
Psychrophiles	*Arthrobacter cryoconiti* sp. nov., *Chryseobacterium greenlandensis, Arthrobacter* sp., *Psychrobacter* sp.	Downregulation of genes translating into charged surface protein. Reduced hydrophobic core	(Margesin *et al.*, 2012, Fresco-Taboada *et al.*, 2013, Koh *et al.*, 2019)
Thermophiles	*Alicyclobacillus acidocaldarius, A. tengchongensis, A. ferrooxydans*	Upregulation of genes giving rise to proteins forming prominent hydrophobic core and least charged protein surface.	(Reed *et al.*, 2013, van Wolferen *et al.*, 2013)
Piezophiles	*Pyrococcus yayanosii, Shewanella* sp., *Moritella* sp.,	Smaller amino acids form hydrophobic core. Presence of pressure-sensing operon system with their growth	(Peoples *et al.*, 2020)

		regulated by both temperature and pressure	
Barophiles	*Halomonas salaria*	Tight membrane lipid packing. Homoviscous adaptation through elevation of unsaturated fatty acids and robust DNA repair system.	(Michoud and Jebbar, 2016, Hamsanathan *et al.*, 2021)
Hyperalkaliphile	*Bacillus pseudofirmus, Alkaliphilus metalliredigens*	Intracellular pH homoeostasis. High Level of Monovalent Cation/Proton Antiporters. Effective Proton Capturing by ATP Synthase	(Preiss *et al.*, 2015, Mamo, 2020)
Radiation resistant	*Deinococcus radiodurans, Ignicoccus hospitalis, Methylobacterium radiodurans*	Efficient repair system for disintegrated DNA. Multiple antioxidant systems. Increased genomes copy number	(Krisko and Radman, 2013, Koschnitzki *et al.*, 2021, Maeng *et al.*, 2021)

2.4. Microbial studies through culturable methods

Microbial studies through culturable methods have played a foundational role in microbiology, allowing scientists to isolate, cultivate, and characterize microorganisms under controlled laboratory conditions (Martiny, 2019). These methods involve providing suitable growth conditions, such as temperature, pH, and nutrient availability, to encourage the proliferation of specific microorganisms in culture media (Lagier *et al.*, 2015). By observing their growth patterns, morphology, and biochemical activities, researchers can gain valuable insights into the physiology, metabolism, and ecological roles of these microbes (Jackman, 2011). One of the primary advantages of culturable methods is the ability to obtain pure cultures of microorganisms, which are essential for conducting detailed studies on their biology and behavior (Bonnet *et al.*, 2019). Pure cultures consist of a single species or strain of microbe, enabling researchers to investigate its characteristics without interference from other organisms. This is particularly important for studying microbial physiology, genetics, and interactions with other organisms, as well as for applications in biotechnology and medicine (Lewis *et al.*, 2021). Culturable methods have also been instrumental in the discovery and isolation of novel microorganisms with unique properties and functionalities. By screening diverse environmental samples, such as soil (Gaete *et al.*, 2020), water (Bhumbla *et al.*, 2020), and sediments (Hu *et al.*, 2021), researchers have uncovered a wealth of previously unknown microbial species capable of producing antibiotics (Tran *et al.*, 2022), enzymes (Masi *et al.*, 2021), and other bioactive compounds (Tabacchioni *et al.*, 2021). These discoveries have contributed to the development of new drugs, biocatalysts, and bioproducts with applications in pharmaceuticals, agriculture, and industrial processes.

However, it's important to recognize the limitations of culturable methods, particularly in capturing the full extent of microbial diversity. Many microorganisms, especially those from extreme environments or in complex microbial communities, resist cultivation using traditional techniques. These "unculturable" microbes represent a significant portion of microbial diversity and are often overlooked in culture-based studies (Wani *et al.*, 2022). To address this limitation, researchers have developed alternative approaches, such as metagenomics and single-cell genomics, which enable the study of microbial communities without the need for cultivation (Handelsman, 2004, Stepanauskas, 2012). Despite these challenges, culturable methods remain indispensable tools for microbiological research, providing valuable insights into the biology, ecology, and

biotechnological potential of microorganisms. Integrating culturable techniques with high-throughput sequencing and bioinformatics approaches allows researchers to bridge the gap between cultivable and non-cultivable microorganisms, leading to a more comprehensive understanding of microbial communities and their functions in natural and engineered ecosystems. By leveraging the strengths of both culture-dependent and culture-independent methods, scientists can unlock the full potential of microbial diversity for applications in biotechnology, environmental sustainability, and human health (Venkatachalam *et al.*, 2015, Qaisrani *et al.*, 2019).

2.5. Metagenomics: a gateway to exploration microbial world

Metagenomics represents a transformative approach to exploring the microbial world, offering unprecedented insights into the diversity, ecology, and functional potential of microbial communities (Bashir *et al.*, 2014). Unlike traditional microbiological methods that rely on isolating and culturing individual microorganisms, metagenomics allows researchers to study microbial communities directly from environmental samples, without the need for cultivation. By analyzing the collective DNA extracted from these samples, metagenomics reveals the genetic composition of entire microbial communities, providing a comprehensive view of their taxonomic diversity and metabolic capabilities (Azli *et al.*, 2022, Fan *et al.*, 2022). One of the key advantages of metagenomics is its ability to uncover the "unculturable" fraction of microbial diversity that eludes traditional cultivation-based methods (Handelsman, 2004). Many microorganisms, particularly those from extreme environments or in symbiotic relationships, are difficult or impossible to culture in the laboratory. Metagenomic approaches bypass this limitation by capturing the collective genetic information of all microorganisms present in a sample, regardless of their cultivability. This enables researchers to discover novel microbial taxa, genes, and metabolic pathways that may have important ecological, evolutionary, or biotechnological significance. Metagenomics also provides insights into the functional potential of microbial communities, revealing the diverse metabolic activities and interactions that drive ecosystem processes (Achudhan *et al.*, 2023). By analyzing the genetic repertoire of microbial communities, researchers can infer the biochemical pathways involved in nutrient cycling, energy metabolism, and the degradation of complex organic compounds (Gu *et al.*, 2021, Liu *et al.*, 2023). This information is invaluable for understanding the ecological roles of microorganisms in natural environments, as well as for predicting their responses to environmental changes, such as pollution, climate change,

or habitat disturbance. Furthermore, metagenomics has revolutionized the field of biotechnology by enabling the discovery of novel enzymes, biosynthetic gene clusters, and bioactive compounds with potential applications in medicine, agriculture, and industrial processes (Biver *et al.*, 2014, Apolinar-Hernández *et al.*, 2016). By mining metagenomic data for genes encoding enzymes with desirable properties, such as thermostability, substrate specificity, or novel catalytic activities, researchers can identify biocatalysts for a wide range of biotechnological applications (Singh *et al.*, 2015a). Metagenomic approaches have already led to the discovery of new antibiotics, enzymes for biomass degradation, and biofuels production, among other innovations (Campanaro *et al.*, 2016, Berini *et al.*, 2017). Figure 2.3 gives an insight about the workflow of metagenomics for bioprospecting.

2.5.1. Sequence based metagenomic approach

Sequence-based metagenomic approaches have revolutionized our understanding of microbial communities by enabling the direct analysis of DNA extracted from environmental samples (Jeffries *et al.*, 2016). These methods leverage high-throughput sequencing technologies to generate vast amounts of sequence data, which can then be analyzed to characterize the taxonomic composition, functional potential, and ecological roles of microbial communities without the need for cultivation (Lizarazo *et al.*, 2019, Babalola, Alawiye, *et al.*, 2020). The workflow of sequence-based metagenomics typically involves several key steps. First, DNA is extracted from environmental samples, such as soil (Liu *et al.*, 2023), water (D'Auria *et al.*, 2018), or the human gut (Turnbaugh *et al.*, 2009), preserving the genetic material of all microorganisms present in the sample. Next, the extracted DNA is fragmented into smaller pieces, which are then sequenced using platforms like Illumina (Vo and Jedlicka, 2014), PacBio (Xie *et al.*, 2020), or Oxford Nanopore technologies (Buytaers *et al.*, 2021). The resulting sequence data, known as metagenomic reads, are then processed and analyzed bioinformatically to infer various aspects of microbial community structure and function (Ladoukakis *et al.*, 2014). One of the primary applications of sequence-based metagenomics is taxonomic profiling, which involves identifying and quantifying the microbial taxa present in a sample (Alotaibi *et al.*, 2020, Hesami Zokaei *et al.*, 2021). This is typically done by comparing metagenomic reads to reference databases of microbial genomes or marker genes, such as the 16S rRNA gene for bacteria and archaea or the 18S rRNA gene for eukaryotes (Segawa *et al.*, 2010, Banos *et al.*, 2018). By mapping metagenomic reads to

these reference sequences, researchers can estimate the relative abundance of different taxa within the microbial community and infer their phylogenetic relationships. In addition to taxonomic profiling, sequence-based metagenomics enables the prediction of functional potential within microbial communities. This is achieved by annotating metagenomic reads with functional information, such as protein-coding genes, metabolic pathways, and functional modules. Tools like MG-RAST (Meyer *et al.*, 2019), MetaPhlAn (Truong *et al.*, 2015), and QIIME (Fung *et al.*, 2021) have been developed to automate this process and provide insights into the metabolic capabilities of microbial communities. By reconstructing metabolic pathways and gene networks from metagenomic data, researchers can gain a deeper understanding of the ecological roles and biotechnological potential of microorganisms in their natural habitats.

2.5.2. Function based metagenomic approach

Function-directed metagenomic approaches represent a targeted strategy for exploring the genetic and functional diversity of microbial communities based on specific biological activities or functions of interest (Ngara and Zhang, 2018). Unlike traditional shotgun metagenomics, which aims to sequence all DNA present in a sample, function-directed metagenomics focuses on identifying and characterizing genes or gene clusters associated with particular biochemical pathways, metabolic activities, or biotechnological applications (Prakash and Taylor, 2012). The workflow of function-directed metagenomics typically begins with the selection or enrichment of environmental samples that exhibit the desired function or activity of interest. This could involve screening samples based on physiological assays, biochemical tests, or functional assays targeting specific enzymatic activities (Sharon *et al.*, 2011). Once the target samples are identified, DNA is extracted, and metagenomic libraries are constructed using methods such as fosmid cloning (Felczykowska *et al.*, 2014), cosmid cloning (Lam *et al.*, 2014), or PCR amplification of target genes (Suenaga, 2012). Next, the metagenomic libraries are screened or screened to identify clones containing genes or gene clusters of interest. This can be achieved through functional screening assays, where clones are tested for specific activities, such as enzyme catalysis, substrate utilization, or bioactivity against pathogens (Suenaga *et al.*, 2007). Positive clones are then subjected to DNA sequencing to determine the nucleotide sequences of the genes or gene clusters responsible for the observed function. Once the sequences of interest are obtained, bioinformatic analyses are performed to annotate and characterize the genes or gene clusters based on their

predicted functions, structural features, and evolutionary relationships. This may involve comparing the sequences to databases of known genes and proteins, predicting protein structures and domains, and reconstructing metabolic pathways or biochemical networks associated with the target function (Iwai *et al.*, 2010).

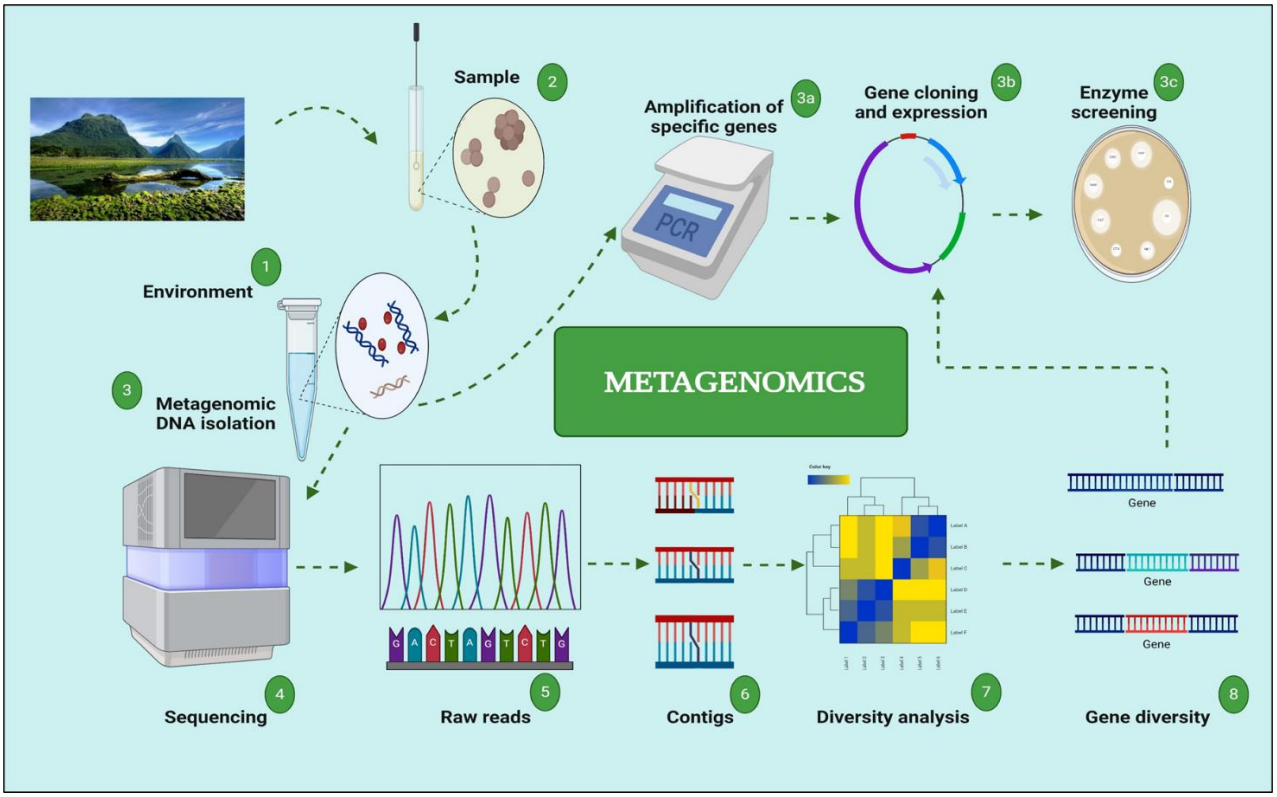

Figure 2.3: Illustration depicting the metagenomic approach for the study of microbial diversity and characterization of different gene clusters.

2.6: Metagenomics and its applications

2.6.1. Bioremediation

It is impossible to replicate the exact environment of the microorganisms in the laboratory setup. Thus, the limitations of culture-dependent approaches have led to the demand for culture-independent approaches (Phulpoto *et al.*, 2020). Handelsman provided a novel gateway in the form of Metagenomics for exploring and characterizing hidden microbial diversity through a culture-independent technique (Handelsman, 2004). This approach is efficient in understanding genome-centric processes, like bioremediation (Costa *et al.*, 2020). Table 2.4 gives an account of culture-dependent and culture-independent applications in environmental management. Metagenomics

approach is used to identify and evaluate specific microbial genes that are involved in bioremediation processes like accumulation, absorption, filtration, and detoxification (Jaiswal and Shukla, 2020). The isolation of XMcr-6 strain of *B. cereus* from the chromium contaminated site based on the *16S rRNA* was done and its reduction potential under aerobic conditions was reported (Duong *et al.*, 2013). Studies have characterized *Acidovorax* (Cai *et al.*, 2018), *Geobacter* (Liu *et al.*, 2021), *Geothro*x (Demanèche *et al.*, 2009), and *Desulfosporosinus spp.* (Liu *et al.*, 2018) through unculturable methods mediating denitrification and dehalogenation processes. Haldar and Nazareth characterized *Dothideomycetes, Saccharomyces, Sordariomycetes*, and *Agariocomycetes* from the Zuari inlets of Goa, India having contaminant-degrading potential using the metagenomic approach (Haldar and Nazareth, 2019). A similar kind of research on the mangrove forest sediments highlighting the importance of microorganisms in methanogenesis and nitrogen fixation has also been conducted (Li *et al.*, 2019). Another study has reported *Desulfuromonadales* as a potential naphthalene degrading microorganism (Toth *et al.*, 2018). The culture-independent metagenomics approach has helped in the identification of several *Proteobacteria, Bacteriode,* and *Actinobacteria* as potential sources for vinyl chloride degradation (Liu *et al.*, 2018). The ammonia-oxidizing archaea have been isolated via metagenomics having potential to remediate nitrogen. The metagenomics isolated from the estuary were studied, the comparative analysis of *Nirosopumilus maritimus* strains and *Nitrosomatrinus catalina* strains showed genomic similarity with each other (Zou *et al.*, 2019). The combined studies of metagenomics and meta-omics have been employed to evaluate and examine the microorganisms involved in the biodegradation of bisphenol-A (BPA). The *Pseudomonas* and *Sphingomonas* species coculture demonstrated increased biodegradation potential of BPA in comparison to the separate culture of *Sphingomomas* species (Yu *et al.*, 2019). Through culture-independent strategy the microbial community involved in the degradation of toxic hydrocarbons (Tarballs) including, *Halomonas* and, *Marinobacter* have been explored (Fernandes *et al.*, 2019). Metagenomics in combination with metatranscriptomics studies has been extended in studying the bioaugmentation effect while carrying out the removal of dichloroethylene and/or trichloroethylene from the contaminated sites. Scientists reported the presence of trichloro pyridinol encoding gene, screened by using a metagenomic library, utilized for chlorpyrifos degradation which is harmful to animals including humans (Watahiki *et al.*, 2019). Bioremediation mediated by microorganisms identified through a culture-independent approach has increased the degree of degradation of various xenobiotics by either in situ or ex situ treatment

(Haiser and Turnbaugh, 2013). Metagenomics driven microbial studies also demonstrated the five phyla involved in the degradation of antimony and arsenic such as *Nitrspirae, Firmicutes, Actinobacteria, Terericutes,* and *Gemmatimonadetes* (Luo *et al.*, 2014). The isolation of the XMcr-6 strain of *B. cereus* from the chromium-contaminated site and based on the *16S rRNA* was done and biochemical attributes the *Bacillus* strain showed reduction potential under aerobic conditions (Duong *et al.*, 2013). Metagenomics offers limited clarification about the gene activity, however metatranscriptomics helps in predicting and exploring the functional aspect of the microorganisms. This is of enormous interest for understanding bioremediation processes. The approach has been applied to an herbicide degrading the *tdfA* gene, coding for dioxygenase (Nicolaisen *et al.*, 2008). Metatranscriptomic investigation reported that extracellular electron transfer activity is performed by nine *Pelobacter/Geobacter* microbes depending upon their surface potential and preferable substrates (Ishii *et al.*, 2018). Other *meta-omics* studies have led to the discovery of microorganisms with the potential of ethyl-benzene anaerobic degradation, and carbon stimulation (Kühner *et al.*, 2005). Microorganisms are rich reservoirs of secondary metabolites that are useful for environmental cleaning, medical, and domestic purposes. Metabolomics is among the recent omics approaches used for the expansion of microbial studies through their metabolite profiling (Peng *et al.*, 2020). The degradation of dementon –S- methyl by *Corynebacterium glutamicum* was analyzed by NMR method (Girbal *et al.*, 2000). Moreover, bioremediation kinetics can be directly studied utilizing 1 H ^{1}D NMR while metabolite identification can be done by ^{1}H-^{15}N NMR and 2D ^{1}H-^{13}C (Emwas *et al.*, 2019). MG and omics approaches boost the analytical understanding of the degradation abilities of microorganisms and their gene products. However, to predict the organism function in composite environments, it is imperative to advance metabolism models that help to understand multiple reactions occurring in the microbial cells. Metagenomics and *in silico* approach to study the bacterial characteristics in response to the hydrocarbon pollution in different contaminated sites has been employed. More than 50 sets of 16s rRNA from 12 polluted sites to decode metagenomic features of the resident microbial communities were analyzed (Mukherjee *et al.*, 2017). The common computational tools that have been developed specifically for analyzing the metagenome include: MEtaGenome Analyzer/MEGAN **(**http://www-ab.informatik.uni-tuebingen.de/software/megan),Metagenomic Rapid Annotation using Subsystems Technology/MG-RAST (http://metagenomics.anl.gov/), Simple Metagenomics Analysis Shell for Microbial Communities/ SmashCommunity (http://www.bork.embl.de/software/smash),

Integrated Microbial Genomes and Metagenome/IMG/M (http://img.jgi.doe.gov/), MetaVelvet (http://metavelvet.dna.bio.keio.ac.jp/), METAGENassist (http://i.cs.hku.hk/~alse/MetaCluster), MetaclusterTA (http://i.cs.hku.hk/~alse/MetaCluster), and MetaPhlAn2 (http://segatalab.cibio.unitn.it/tools/metaphlan2/). The tools help in the assembly of contigs, phenotypic mapping, 16s rRNA meta-profiling, and taxonomic analysis.

Table 2.4: Summary of gene product/enzyme-mediated decontamination of environmental pollutants isolated through culturable and non-culturable approaches.

Microorganisms	**Technique used**	**Gene product**	**Contaminant/**	**References**
Sphingobium francense, and *Pseudomonas pseudoalcaigenes*	Culture dependent	Biphenyl-2,3-dioxygenase (*bphA1*)	Organochlorinated pesticide	(Lovecka *et al.*, 2015)
Bacillus subtilis	Culture dependent	Surfactin	Polyethylene	(Vimala and Mathew, 2016)
Rhodococcus erythropolis, and *Pseudomonas* sp.	Culture independent	Acetophenone carboxylase	Petroleum hydrocarbons	(Perdigão *et al.*, 2021)
Agrobacterium radiobacter	Culture dependent	Organophosphohydrlase (*OpDA*)	Polyester	(Blatchford *et al.*, 2012)
Pseudomonas stutzeri	Culture dependent	Catechol 1,2 dioxygenases (*catA)*	Petroleum n-alkanes	(Medić *et al.*, 2020)
Cellulosimicrobium sp.	Culture dependent	NA	Chromium	(Bharagava and Mishra, 2018)
Pseudomonas sp.	Culture independent	Acetyl xylan esterase (*AcXE*)	Xylan	(Adesioye *et al.*, 2016)
Planctomyces, and *Marinobacter*	Culture independent	Dehydrogenase	Polycyclic aromatic hydrocarbon	(Zhang *et al.*, 2019)

Polymorphum gilvum, Ralstonia, Mycobacterium, Pseudomonas, and Burkholderia	Culture independent	Hydroxylating Dioxygenases (*HDα*)	Polycyclic aromatic hydrocarbon	(Liang, Huang, Wang, *et al.*, 2019)
Bacillus subtilis, Candida krusei, and Schizophyllum sp.	Both	Peroxidases	Dyes	(Bansal and Kanwar, 2013)
Proteobacteria, Firmicutes, and *Bacteroidetes*	Both	Dioxygenase	Hydrocarbon	(Lee *et al.*, 2018)
Sphingomonas, Rhodococcus, and *Thioclava dalianensis*	Culture independent	Hydratase-aldolase and dioxygenase (*pahAc* and *pahE*)	Hydrocarbon	(Liang, Huang and Wang, 2019)
Shewanella oneidensis	Culture dependent	Cytochrome oxidases	Chromium and Uranium degradation	(Kouzuma *et al.*, 2015)
Loligo vulgaris	Culture dependent	Diisopropylfluorophosphatase	Organophosphate	(Jain *et al.*, 2019)
Cytophaga, Cohnella, Paludisphaera, Devosia, and *Pseudorhodoplanes*	Culture independent	NA	Xenobiotcics	(Jayaramaiah *et al.*, 2022)
Sulfuricella, Thiobacillus, and *Proteobacteria*	Culture independent	NA	Cadmium	(Feng *et al.*, 2018)

Pelobacter seleniigenes, Salinisphaera shabanensis, and *Salinibacter*	Culture independent	Laccase and peroxidase	Malachite green	(Qu *et al.*, 2018)
Rhinopithecus bieti, and *Ailuropoda melanoleucaa*	Culture independent	Hydrolase	Lignocellulose	(Bai *et al.*, 2021)
Aspergillus species	Culture dependent	Tannasea and protease	Tannasea and protease	(Deshmukh *et al.*, 2016)
Actinobacter, Bacteroidetes, and *Proteobacter*	Culture independent	Hydrolase	Polyethylene terephthalate	(Danso, Schmeisser, Chow, Zimmermann, Wei, Leggewie, Li, Hazen and Wolfgang R Streit, 2018)
Pseudomonas entomophila	Culture dependent	Reductase	Chromium	(Wani *et al.*, 2019)
Thermophilic bacteria (Actinomycetes)	Both	Cutinase	PET	(Oda *et al.*, 2021)

2.6.1.1. Plastic degradation

Several other research groups have identified novel bacteria from different sites including extreme environments like hot springs, deserts, and deep-sea sediments for bioprospecting using a metagenomic approach (Tang *et al.*, 2018, Alotaibi *et al.*, 2020, Najar *et al.*, 2020, Zhu *et al.*, 2022). Global ocean sampling revealed about 40 million non-redundant novel genes from more than 30,000 species, whereas over 97% of the 150 million genes reported in topsoil globally cannot be found in the existing gene catalogue. This is a strong indicator that microbiomes carry huge functional potential, with unculturable microorganisms as acting enzyme reservoir (Sunagawa, Coelho, Chaffron, Kultima, Labadie, *et al.*, 2015, Bahram *et al.*, 2018). In a study, hidden Markov models were constructed from experimentally verified enzymes and mined soil and ocean metagenomes to assess the ability of microorganisms in degrading plastics. They compiled almost 30,000 non-redundant enzymes that were homologues with known enzymes having plastic degrading potential (Zrimec *et al.*, 2021). A study presents a sequence-based *in silico* strategy for screening and characterization of PETases from MG datasets. The MG screening of a novel PET esterase through in vitro expression system has also been developed using next generation sequencing (Chow *et al.*, 2023). In a recent study, distinct microbial communities have been unveiled through metagenomicss that degrade hydrocarbon chains, which are units of plastic polymers (Hauptfeld *et al.*, 2022). Using 16S rRNA datasets obtained through metagenomics, the taxonomic and functional characteristics of PE-degrading microorganisms have been analyzed from one of the waste recycling sites in Tehran, Iran (Hesami Zokaei *et al.*, 2021).

Integrated Microbial Genome (IMG) helps to identify candidate genes from different metagenomes (Zaidi *et al.*, 2021). In a metagenomic study, two heat stable enzymes with application in plastic degradation were partially characterized (Danso *et al.,* 2018). Shotgun metagenomics has revealed the microbial community response to plastic contamination in coastal environments (Pinnell and Turner, 2019). Shotgun metagenomics generated 3,314,688 contigs (DNA sequences that overlap providing contiguous representation of a genomic region) and 120 microbial genomes. This was followed by the functional gene annotation to identify microbiomes that harbor genes encoding esterases, lipases, and monooxygenases that are known to degrade different types of plastics (Radwan *et al.*, 2020). Scientists reported hydrolysis of PET by a metalloprotease and a serine protease. The study provided intrinsic insight into PET degradation and opened a gateway for hunting more plastic-degrading enzymes (Hu *et al.*, 2021). Scientists

also characterized a novel polyester hydrolase from *P. aestusingri* for the degradation of synthetic PET (Bollinger *et al.*, 2020). Table 2.5 highlights some of the abundant microbes and enzymes isolated and characterized from microorganisms through culture-based and sequence- and function-based metagenomic approaches having MP-degrading potential. Even though the MP-degradation by microorganisms and their gene products is effective, the rate of degradation has always been a matter of concern. Metagenomic investigation allows upscaling the degradation rate by modifying the microbial composition and genome engineering.

Table 2.5: Summary of sequence and function-based metagenomic approaches for the identification of abundant microbes and /or enzymes useful in targeting different plastic substrates.

Microbes/Enzymes	Metagenome source	Metagenome strategy	Target plastic substrate	References
Bryozoa, Cyanobacteria, Alphaproteobacteria, and *Bacteroidetes*	Sea water	Sequence based	Mixed plastic debris	(Bryant *et al.*, 2016)
Flavobacteriaceae, Methylophilaceae, Rhodobacteraceae, Planctomycetaceae, Nocardiaceae, and *Verrucomicrobiaceae*	Surface sea water	Sequence based	PS	(Sekiguchi *et al.*, 2009, Kirstein *et al.*, 2019)
Rhodococcus sp. (YC-SY1, YC-BJ1, and YC-GZ1)	Soil	Sequence based	Triphenyl phosphate (Plasticizer)	(Wang *et al.*, 2019)
PET hydrolase	Marine water	Function based	PET	(Danso, Schmeisser, Chow, Zimmermann, Wei, Leggewie, Li, Hazen and

				Wolfgang R. Streit, 2018)
Thalassospiracea, Alteromonadaceae, Alcanivoraceae, and *Vibrionaceae*	Beach sediment	Sequence, and function based	PET	(Wright *et al.*, 2021)
Proteobacteria, Firmicutes, Actinobacteria, and *Firmicutes*	Landfill soil	Sequence based	PE and PS	(Kumar *et al.*, 2021)
Polyurethane esterase	Landfill	Function based	PU	(Gaytán *et al.*, 2019)
Cutinase	Compost	Function based	PET	(Sulaiman *et al.*, 2012)
Esterase	Seawater	Function based	Polyhydroxybutyrate, and polylactic acid	(Tchigvintsev *et al.*, 2015)
Esterase	Compost	Sequence and function based	PU	(Kang *et al.*, 2011)
Protease	Marine sediment	Function based	Polyester	(Lim *et al.*, 2005, J. Sun *et al.*, 2020)

2.6.2. Metagenomics, and phytomicrobiome

The study of the phytomicrobiome using metagenomics represents a powerful approach to unraveling the complex interactions between plants and their associated microbial communities. This figure highlights the metagenomic workflow employed to investigate the phytomicrobiome, from sample collection to data analysis. Environmental samples, such as plant roots, rhizosphere soil, or leaf surfaces, are collected and processed to extract microbial DNA. Metagenomic libraries are then constructed and subjected to high-throughput sequencing to generate vast amounts of

sequence data. Bioinformatic analyses are employed to identify and characterize microbial taxa, functional genes, and metabolic pathways associated with plant-microbe interactions.

The metagenomic sequencing pipeline involves the random sequencing of DNA fragments from a complex microbial sample. This method captures the genetic material of all microorganisms present. The key steps include sample collection, DNA extraction, library preparation, high-throughput sequencing, quality control, taxonomic and functional profiling, data analysis, visualization, statistical analysis, and biological interpretation (Bovo *et al.*, 2020, Beck *et al.*, 2021). The pipeline provides a detailed molecular perspective on microbial communities, allowing for the identification of taxa, functional potential, and insights into the roles of microorganisms in a given environment (Figure 2.4). The application of shotgun metagenomics has streamlined the taxonomic and functional classification of non-culturable microbial populations, thereby enhancing our comprehension of microbial behaviors and processes within their environments (Table 2.6). This methodology has been instrumental in acquiring comprehensive insights into the specific microorganisms inhabiting the rhizosphere, elucidating their respective roles, abundance, and compositional dynamics. Researchers examined the functional genes within the microbial community inhabiting the rhizosphere of maize through shotgun metagenomic analysis. The findings revealed the presence of microbial genes engaged in processes such as nitrogen fixation, phosphate solubilization, synthesis of quorum sensing molecules, trehalose metabolism, siderophore production, phenazine biosynthesis protein activity, and resistance to daunorubicin (Akinola *et al.*, 2021). The rhizosphere microbiomes of *Deschampsia* antarctica and *Colobanthus quitensis* were scrutinized with shotgun metagenomic DNA sequencing approach and using eggNOG for comparative and functional metagenomics. The taxonomic and functional annotation showed that microorganisms from rhizospheric soil samples have significant differences in diversity and gene abundance (Molina-Montenegro *et al.*, 2019). According to shotgun metagenomic analysis conducted on the Illumina HiSeq platform, the predominant genera identified within the rhizosphere of sunflowers are documented to be *Conexibacter* (17%) and *Nocardioides* (8%) (Babalola, Alawiye, *et al.*, 2020). Through shotgun metagenomic analysis, *Acinetobacter* (4.85%) and *Pseudomonas* (3.41%) were identified as prevailing microbial genera within the rhizosphere of *Lactuca sativa* (Babalola *et al.*, 2023). Employing a shotgun metagenomics methodology, an investigation systematically evaluated the functional diversity and metabolic capabilities inherent in microbial communities thriving within the rhizosphere of the

soybean genotype designated as link 678. Predominant genera identified encompass *Geobacter*, *Nitrobacter*, *Burkholderia*, *Candidatus*, *Bradyrhizobium*, and *Streptomyces*. The analysis revealed the presence of twenty-one functional categories, with fourteen of them consistently dominating across all samples. Noteworthy functions include those related to carbohydrates, fatty acids, lipids, isoprenoids, amino acids and derivatives, sulfur metabolism, as well as nitrogen metabolism (Ajiboye *et al.*, 2022). While shotgun metagenomics has significantly advanced microbial molecular studies, it is essential to acknowledge its limitations, which span various aspects of experimental design, data analysis, and interpretation. The accuracy of taxonomic and functional assignments heavily relies on the comprehensiveness and accuracy of reference databases. Incomplete or biased databases can lead to misinterpretations and limitations in identifying novel taxa or functions. Shotgun metagenomics may not always provide sufficient resolution at the strain level. Distinguishing closely related strains or understanding strain-specific functions can be challenging, hindering a comprehensive understanding of microbial diversity. The concerns about environmental contamination and the high economic cost of shotgun metagenomics further add to its limitations.

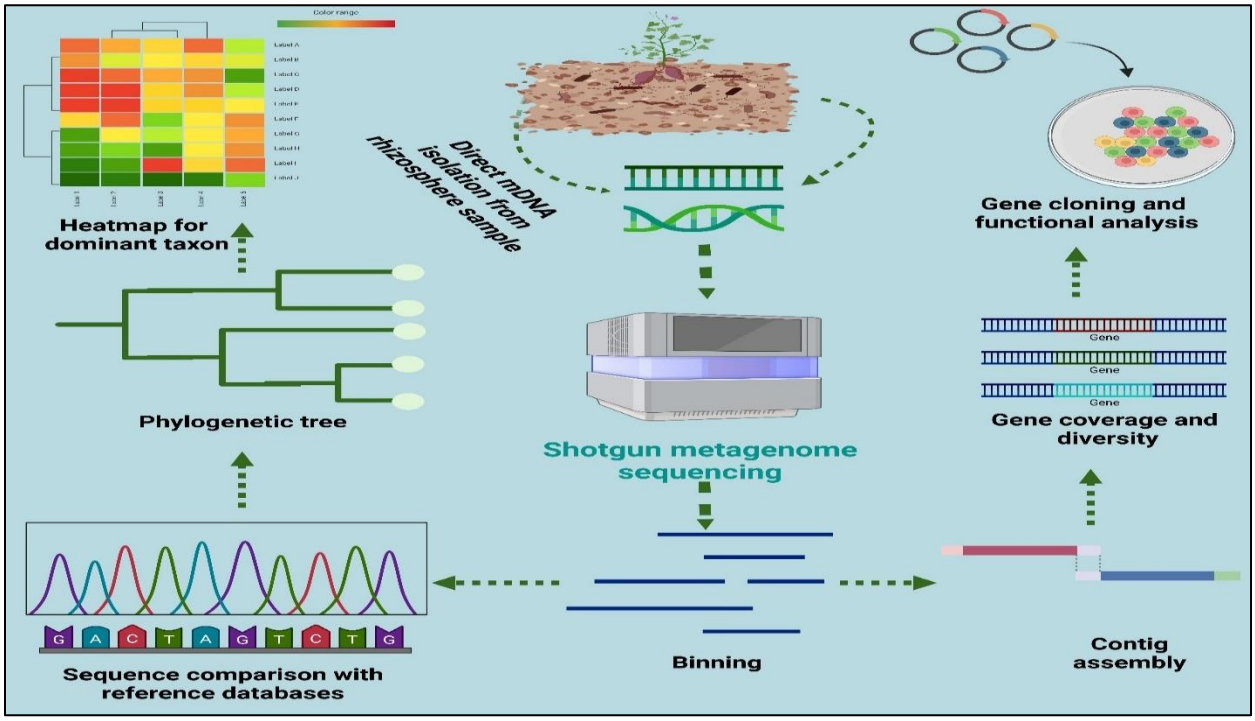

Figure 2.4: Microbial diversity analysis and gene annotation of rhizosphere/soil metagenome using shotgun metagenomic sequencing.

Table 2.6: Characterization of abundant microbial genera in the plant systems using metagenomics.

Plant system	Dominant genera	Shotgun sequencing platform	Databases used	References
Solanum lycopersicum	*Planctomycetes*, and *Proteobacteria*	Illumina NovaSeq 6000	MG-RAST, and SolexaQA	(Adedayo *et al.*, 2023)
Brachypodium distachyon	*Proteobacteria*, and *Actinobacteriota*	Illumina 150	MetaBAT, DESeq2, and GTDB	(Acharya *et al.*, 2023)
Zea mays	Myoviridae, and Podoviridae	Illumina NovaSeq 6000	MG-RAST, and M5NR	(Nwokolo and Enebe, 2022)
Z. mays	*Proteobacteria,* and *Dienococcus-Thermus*	Illumina NovaSeq 6000	SEED	(Dlamini *et al.*, 2023)
S. lycopersicum	*Proteobacteria* and, *Actinobacteria*	Illumina NovaSeq 6000	SEED	(Babalola *et al.*, 2022)
Dipterygium glaucum	*Actinobacteria*, and *Proteobacteria*	Illumina HiSeq 2500	UPARSE, DIAMOND, and MEGAN	(Shami *et al.*, 2022)
Carya illinoinensis	*Proteobacteria,* and *Actinobacteria*	Illumina HiSeq	DIAMOND, and DESeq2	(Shi *et al.*, 2023)
Panax notoginseng	*Proteobacteria*, and *Actinobacteriota*	Illumina NovaSeq 6000	MG-RAST	(Wei *et al.*, 2022)

Glycine max	*Actinobacteria,* and *Proteobacteria*	Illumina TruSeq	STAMP	(Ren *et al.*, 2023)
Phaseolus vulgaris	*Streptomyces* and, *Nocardiodes*	Illumina NovaSeq	PlusPFP database	(Gonzalez *et al.*, 2023)
Abutilon fruticosum	*Mycobacterium, and Vibrio*	Illumina HiSeq 2500	MEGAHIT, and MEGAN	(Alshehri *et al.*, 2023)

2.6.2.1: Rhizosphere/soil microbiome through metagenomics for enzyme mining

Numerous investigations have established that the metagenome serves as an exceptional reservoir for the identification of unprecedented microbial enzymes and antibiotics (Berini *et al.*, 2017). Thus far, a diverse array of enzymes, including lipases (Sahoo *et al.*, 2020), cellulases (Lee *et al.*, 2014), amylases (Sharma *et al.*, 2010), chitinases (Berini *et al.*, 2019), proteases (Faheem *et al.*, 2016), polyphenol oxidases (Nilewski *et al.*, 2021), and nitrilases (Achudhan *et al.*, 2023), has been sourced from the metagenomic milieu. Enzymes play a key role in nutrient cycling and other biochemical processes. The rhizospheric environment of plants embodies an intricately nuanced ecosystem characterized by a myriad of interactions, encompassing both symbiotic and parasitic relationships between plants and microorganisms. Lipids constitute vital constituents of the epidermal cell wall in plant roots, with various lipid compounds derived from both plant and bacterial sources serving as pivotal signaling molecules. Bacteria-produced lipolytic enzymes play a significant role in mediating bacterial-plant interactions (Kulminskaya and Oberer, 2020). Consequently, the plant rhizosphere ecosystem emerges as a distinctive microbial milieu, offering a promising avenue for the exploration of novel enzymes through the application of metagenomic methodologies. By studying the metagenome, researchers can identify and understand the functional potential of the microbial community, offering practical applications in agriculture for optimizing plant-microbe interactions and enhancing nutrient availability (Table 2.7). The impact of soil fertilization on the spatial distribution and biodiversity of phosphorus cycling genes and microbial communities in the rhizosphere of maize has been investigated through shotgun metagenomics (Liao *et al.*, 2023). Notably, genes such as *ppk*, associated with polyphosphate

formation, and *pstSABC*, responsible for phosphate transportation, exhibit significant enrichment under these fertilization treatments. These genes play a pivotal role in the immobilization of phosphorus. Conversely, under conditions of high inorganic fertilizer application or low compost manure treatment, there is observed repression of phosphorus cycling genes, leading to a decrease in their abundance (Enebe and Babalola, 2021). The exploration of metagenomic analyses pertaining to plant growth-promoting and carbon-cycling genes within maize rhizosphere soils characterized by distinct land-use and management histories has been undertaken. Notably, genes associated with nutrient mobilization, such as *nifA, fixJ, norB, pstA*, *kefA*, and *ktrB*, exhibit significantly higher abundance in Ventersdorp rhizosphere soils. Concerning carbon-cycling genes, 12 genes, encompassing those participating in methane degradation, demonstrate more pronounced abundance in grassland soils, whereas only five genes exhibit significantly higher abundance in Mafikeng soils (Chukwuneme *et al.*, 2021). The genetic elements residing within the rhizospheric milieu, along with their associated protein products, bestow prospective advantageous attributes upon the sunflower, encompassing capabilities such as the synthesis of siderophores, nitrogen fixation, phosphate solubilization, and 1-aminocyclopropane-1-carboxylate deaminase activity (Alawiye and Babalola, 2021). Additionally, a discernible prevalence of genes responsible for exopolysaccharide production, high-temperature stress response, as well as heat and cold shock response has been observed within the rhizospheric context across diverse plant species. These genetic determinants collectively contribute to the plants' capacity to endure and mitigate environmental stresses (Pramanik *et al.*, 2020, Guo *et al.*, 2022, Liu *et al.*, 2023). Researchers also employed shotgun DNA sequencing methodologies to elucidate the correlation between bacterial community composition and functional capacity within the rhizospheric microbiomes associated with invasive *Acacia dealbata* populations in South Africa. The comprehensive analysis revealed a noteworthy overrepresentation of several genes associated with traits conducive to plant growth in the rhizospheric metagenomes, as compared to adjacent bulk soils. Predominantly, these genes are implicated in the metabolic processes related to nitrogen, carbohydrates, and vitamins, as well as diverse membrane transport systems. The overrepresented genes exhibited a discernible association with a restricted set of bacterial taxa, predominantly comprising *Bradyrhizobium* species, the favored nitrogen-fixing rhizobial symbiont of Australian acacias (Kamutando *et al.*, 2019). An investigation posits that the microbiome within the rhizosphere potentially plays a role in facilitating associative nitrogen fixation through the unique diazotrophic properties of

Janthinobacterium in switchgrass (White *et al.*, 2023). Thus, rhizosphere microbiome plays a pivotal role in enzyme mining through metagenomics, offering a rich source of diverse microbial communities that actively engage in intricate interactions with plant roots. This specialized ecosystem represents a dynamic interface where plants and microorganisms establish symbiotic relationships, influencing the production and secretion of enzymes. Advances in sequencing technologies and bioinformatics offer the potential to unlock the genetic richness of rhizospheric microorganisms. This can lead to the discovery of novel enzymes with applications in diverse industries, contributing to sustainable practices and eco-friendly solutions. The exploration aligns with the trend toward innovative biotechnological applications, including enhanced crop productivity, waste remediation, and bioenergy production. However, the complexity and diversity of the rhizosphere environment pose difficulties in data analysis and interpretation. Functional screening methods that mimic rhizospheric conditions are needed to link genetic information to enzyme function efficiently. Understanding the intricate metabolic interactions within the rhizosphere microbiome is crucial, requiring efforts to unravel crosstalk between microorganisms and plants.

Table 2.7: Summary of enzyme mining from plants using metagenomic-based approaches.

Plants	**Enzyme/gene**	**Application**	**Metagenomic approach**	**References**
Capsicum annuum	Esterase	Plastic degradation	Function-based	(Lee *et al.*, 2010, Gil-Rivas *et al.*, 2023)
Grassland	Endo-β-1,4-glucanase (Cellulase)	Brewing, and Biofuel production	Function-based	(Wierzbicka-Woś *et al.*, 2019, Bhardwaj *et al.*, 2021)
Colobanthus quitensis	Exoribonuclease	Improves stress tolerance	Function-based	(de Francisco Martínez *et al.*, 2022)
Jerusalem artichoke	Catalase	Protection against pathogens	Sequence-based (16S rDNA)	(Yue *et al.*, 2020)

Z. mays	Dehydrogenase, and alkaline phosphatase	Improves stress tolerance	Illumina sequencing	(Chaudhary *et al.*, 2021)
O. sativa	Osmotin-like proteins	Drought tolerance	Illumina Hiseq	(GeethaThanuja *et al.*, 2023, Yan *et al.*, 2023)
Saccharum officinarum	*AprAB* and *DsrAB*	Sulfate reduction	Illumina Miseq	(Pang *et al.*, 2021)
Z. mays	*nifH, nifA, groES, and cspA*	Siderophore production, and nitrogen production	Shotgun metagenome sequencing	(Omotayo *et al.*, 2022)
Nicotiana tabacum	α-amylase	Ethanol production	Sequence-based (Illumina HiSeq)	(Lu *et al.*, 2023)
Paspalum scrobiculatum	Amidohydrolase	Nitrogen fixation	Sequence-based (Illumina HiSeq)	(Prabha *et al.*, 2019)
Rhazya stricta	Laccase	Phytoremediation	Function based	(Noor *et al.*, 2021)
Zanthoxylum bungeanum	Carbohydrate-active enzymes	Exopolysaccharide production	Shotgun sequencing	(Liao *et al.*, 2022)
Elymus nutans	Hydrolase	Water retention	Sequence based	(Wei *et al.*, 2022)

2.6.3. Metagenomics, and livestock microbiome

In animal-microbe interaction, the obtained information through metagenomics has been an important advancement in decoding the interplay between microbes and hosts (Singh *et al.*, 2008). Metagenomic studies through function-based and/or sequence-based approaches unveil the impact of complete metagenome in establishing pathogenicity of microbes, susceptibility of the host and

infection outcomes (Bashir *et al.*, 2014). The role of microorganisms and their secreted biomolecules in digestion, excretion, pregnancy, and fitness of livestock is a well-established fact (Hanning and Diaz-Sanchez, 2015). The secreted molecules can be easily characterized with the help of metatranscriptomics and metabolomics (Aguiar-Pulido *et al.*, 2016). A study that used both culturable and non-culturable techniques to evaluate the microbial diversity of t the teat skin of cows and identified 29 and 27 microbial species using culture-dependent and culture-independent approaches, respectively (Verdier-Metz *et al.*, 2012). Microbes isolated from the rumen and gastrointestinal parts of the farm animals using various metagenomics approaches have been reported to show beneficial effects on the performance and health of farm animals. They have improved feed efficiency, amino acid and carbohydrate metabolism, fatty acid oxidation, and rumen fermentation (Table 2.8). A metagenomic study revealed the presence of similar microbial communities in Fleckvieh, Holstein, and Nellore, which comprised *Proteobacteria*, *Firmicutes,* and *Bacteroidetes* microbial species (Thoetkiattikul *et al.*, 2013). The microbial community in reproductive tract parts identified by different metagenomics approaches has been reported to have beneficial and harmful effects on farm animals. Microbes identified in these parts by metagenomics approaches have been reported to improve immune response in gilts.

Table 2.8: List of beneficial and pathogenic microorganisms isolated from digestive system of farm animals using culture and non-culturable techniques.

Beneficial Microorganisms					
Microorganisms	**Animal**	**Niche**	**Action**	**Isolation technique**	**References**
Neocallimastix californiae	Dairy cows	Rumen	Fatty acid oxidation	Sequence driven metagenomics	(Neelakanta and Sultana, 2013, Peng *et al.*, 2021)
Methanobrevibacter spp., Prevotella spp., Bacteroidetes	Cattle	Rumen	Improve feed efficiency and rumen fermentation	Sequence driven metagenomics	(Li et al., 2019)

Prevotella ruminocola	Buffalo	Rumen	Identification of BT-01 protein which can be used as animal feed supplement	Function driven metagenomics	(Nguyen *et al.*, 2012)
Methanobrevibacter spp., Prevotella spp., Bacteroidetes spp., Ruminococcus spp., Eubacterium spp., Roseburia spp.	Black Bengal Goats	Rumen	Identification of enzymes that can be used in food and pharmaceutical industries	Sequence driven metagenomics	(Suryawanshi *et al.*, 2019)
Prevotella spp.	Pigs	Gut, and oral cavity	Lipopolysaccharide production useful for mucus and cytokine synthesis	Culture driven	(Amat *et al.*, 2020)
Lactobacillus reuteri	Cattle	Ruminal fluid	Suppresses *E. coli* to improve food safety	Function driven metagenomics	(Bertin *et al.*, 2017)
Selenomonas ruminantium	Ruminants	Rumen	Fermentation of lactate and succinate	Sequence driven metagenomics	(Nathani *et al.*, 2013)
Lachnospira multipara	Cow and camel	Intestine	Help in absorption	Culture-based	(Cornick and Stanton, 2015, Redington *et al.*, 2016)
Megasphaera elsdenii	Sheep	Gut	Lactate degradation	Culture-based	(Chen *et al.*, 2019)
Butyrivibrio spp, Ruminococcus spp.,	Cattle	Gastrointestinal tract	Amino acid and carbohydrate metabolism	Function-driven metagenomics	(Mao *et al.*, 2015)

Campylobacter spp, Desulfobulbus spp					
Pathogenic Microorganisms					
Actinobacillus lignieressi	Cattle	Gastrointestinal tracts	Lymph-node swelling	Function-driven metagenomics	(Caffarena *et al.*, 2018)
Arcanobacterium bovis	Sheep and Goats	Oral cavity	Mandibular lesions	Culture-based	(Delano *et al.*, 2002)
Brucella melitensis	Goats	Rumen	Brucellosis (Inflammation of epididymis and abortion)	Sequence driven metagenomics	(Samadi *et al.*, 2010)
Campylobacter intestinalis and C. jejuni	Goats	Intestinal tract	Diarrhoea	Culture driven	(Underwood *et al.*, 2015)
Clostridium perfringens	Ruminants	Gastrointestinal tracts	Produces toxins leading to enterocolitis	Culture driven	(Uzal *et al.*, 2010)
Pseudomonas aeruginosa and *Fusobacterium spp.*	Bovine	Esophagus, omasum and tongue	Esophagitis, omasitis, and glossitis	Culture driven	(Tosaki *et al.*, 2018)

2.6.4. Metagenomics, and viruses

Numerous techniques are employed to identify viruses, primarily relying on comparisons with known viral counterparts. These methods encompass electron microscopy, serology, and cell culture, among others (Metzger *et al.*, 2018). However, it has been observed that these techniques are beset by certain inherent limitations. For instance, a significant proportion of viruses cannot be cultured, restricting the applicability of cell culture and serological methods to a subset of viruses.

Undeniably, these techniques have significantly contributed to the discovery of various viruses, yet their effectiveness hinges on prior knowledge of similar viral agents. In contrast, contemporary metagenomic approaches have emerged as a promising alternative. Viral metagenomics is a molecular method used to monitor and detect evolving zoonotic viruses, which can jump from animals to humans and pose health risks (Bai *et al.*, 2022, Santiago-Rodriguez and Hollister, 2022). It involves collecting samples from potential hosts, extracting genetic material, and using high-throughput sequencing and bioinformatics to identify known and novel viruses. Phylogenetic and functional analysis helps understand viral evolution and potential threats (Zhao *et al.*, 2022). These zoonotic viruses exhibit the potential for human transmission through either direct means, such as direct contact with animals, or indirect routes involving exposure to an infected animal's urine, feces, or bites from blood-feeding insects (McLean and Graham, 2022). Metagenomics has profound implications for virology, especially in the context of preventing and controlling zoonotic diseases. It allows for the discovery of new zoonotic viruses, understanding viral diversity, rapid identification of emerging viruses, and insights into viral evolution and host switching. The One Health approach is enhanced as it examines viral dynamics in both human and animal populations. Metagenomics supports surveillance, vaccine development, public health preparedness, and epidemiological investigations for zoonotic outbreaks (Santiago-Rodriguez and Hollister, 2023).

The systematic identification and documentation of viruses harbored by pangolins represents a logical and systematic approach to assess the potential pathogen reservoirs within this species, thereby contributing to conservation efforts. A recent research investigation furnished valuable insights into the virome inhabiting Malayan Pangolins (*Manis javanica*) and shed light on the molecular epidemiology of predominant pathogenic viruses shared between Malayan Pangolins and other host organisms. In this study, a comprehensive dataset of 62,508 de novo assembled contigs was meticulously constructed. Subsequent BLAST searches unveiled a subset of 3,600 contigs (≥300 nucleotides) exhibiting associations with viral sequences. Among these, 68 contigs demonstrated a high degree of sequence homology with established viruses, with notable representation from Sendai virus and Coronavirus (Liu *et al.*, 2019). Investigations utilizing viral metagenomics were undertaken on beef, pork, and poultry products retailed in Southern Brazil. Within the poultry samples, six discrete gyroviruses (GyV) were identified, notably encompassing GyV3 and GyV6, marking their inaugural identification in avian specimens. Additionally, a previously uncharacterized smacovirus species and two profoundly divergent circular Rep-

encoding single-stranded DNA (CRESS-DNA) viruses were discovered (Cibulski *et al.*, 2021). Cibulski and colleagues conducted an analysis of the viral population present in oropharyngeal samples collected from *T. brasiliensis*, employing a viral metagenomic approach. Within these samples, they identified genomic sequences corresponding to various viral families, including *Circoviridae, Genomoviridae, Herpesviridae, Paramyxoviridae, Coronaviridae,* and *Astroviridae*. This research represents an initial step toward comprehending the oropharyngeal virome of *T. brasiliensis*, potentially serving as a foundation for the future discovery and isolation of novel viruses. It also underscores the imperative for ongoing investigations in this domain (Cibulski *et al.*, 2021). Researchers successfully identified and characterized 11 contiguous DNA sequences representing previously undiscovered *cressdnaviruses*. These sequences were derived from a rectal swab specimen collected from a Cynopterus bat in Yunnan Province, China, in the year 2011. Among these, full genomes of two cressdnaviruses (designated as OQ267680, comprising 2069 nucleotides, and OQ351951, comprising 2382 nucleotides) were fully elucidated, while a nearly complete genome for a third cressdnavirus (OQ267683, spanning 2361 nucleotides) was partially resolved. Through comprehensive phylogenetic analyses and an examination of the distinctive features of these viral genomes, this investigation has unveiled a remarkable degree of diversity within the single-stranded DNA virus category (Kane *et al.*, 2023). Metagenomics offers several advantages over the traditional culture dependent strategies (Figure 2.5). Viral metagenomics, a technique for studying viral communities, has limitations including its sensitivity to low-concentration viruses, dependence on reference databases, difficulty in detecting entirely novel viruses, challenges in identifying host organisms, potential biases and contamination, computational complexity, and the need to account for seasonal and temporal variations. Proper sample preservation and handling are also crucial. Despite these challenges, viral metagenomics is a valuable tool for exploring viral diversity and ecosystem dynamics.

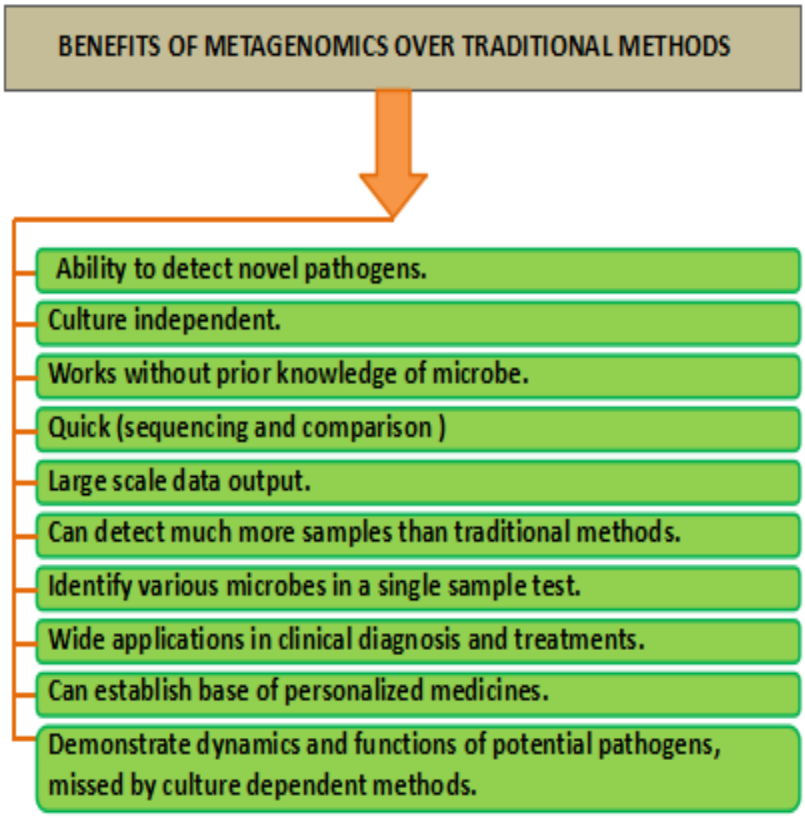

Figure 2.5: Benefits of metagenomics over traditional culture dependent methods (Wani *et al.*, 2023).

2.6.4.1. Metagenomics and SARS-CoV-2 dynamics

Metagenomics has been crucial in the fight against SARS-CoV-2 by providing molecular insights into the virus. It has helped identify, characterize, and monitor the virus, its mutations, and its interactions with the host. Metagenomics has been vital for diagnostics, vaccine development, and public health efforts during the COVID-19 pandemic. An investigation examined the potential correlation between SARS-CoV-2 and the metagenomic composition of specimens collected from the Huanan Seafood Market. The findings indicate that while analyzing the metagenomic composition of environmental samples is a valuable tool for detecting the presence of animals or animal-derived products traded at the market, it is improbable that the amalgamation of genetic material from animals and the virus can consistently serve as a reliable indicator of whether any animals had been infected with SARS-CoV-2 (Bloom, 2023). Metagenomic signatures specific to SARS-CoV-2 infection were investigated in a research study. Notably, there was a substantial increase in the relative abundance of *Candidatus_Saccharibacteria*, accompanied by a marked reduction in the levels of *Fibrobacteres* among individuals infected with SARS-CoV-2.

Furthermore, a discernible distinction was observed in the prevalence of Spirochaetes between patients exhibiting symptoms and asymptomatic cases. At the genus level, *Tyzzerella* emerged as a pivotal taxon, displaying a significant increase in both symptomatic and asymptomatic individuals (Liu *et al.*, 2021). A comprehensive meta-metagenomic exploration reveals the presence of sequences closely resembling SARS-CoV-2 in the viromes of pangolin lung samples (Wahba *et al.*, 2020). Lawal and colleagues conducted metagenomic sequencing analyses on SARS-CoV-2 RNA present in composite wastewater influent samples collected from ten distinct regions within Ontario, Canada. This investigation took place during the transitional period between the emergence of the Delta and Omicron variants of concern. The study revealed the presence of mutations defining the Delta and Omicron variants, specifically the BA.1/BA.1.1 and BA.2 mutations, which were observed at varying frequencies within both the consensus and subconsensus sequences of the composite samples (Lawal *et al.*, 2022). The occurrence of simultaneous infection or co-infection involving disparate SARS-CoV-2 genotypes has been documented as an integral component of the epidemiological monitoring of the COVID-19 pandemic. Particularly, in light of the widespread transmission of highly transmissible variants throughout 2021, co-infections have assumed significance not only in terms of their potential impact on clinical manifestations but also as opportunities for the generation of novel genotypes through genetic recombination events. Nevertheless, only a limited number of computational methods have been devised to establish bioinformatic pipelines capable of detecting instances of co-infections (Molina-Mora *et al.*, 2022). Investigators conducted a comprehensive examination of the temporal transcriptional dynamics of SARS-CoV-2 and its correlation with longitudinal alterations in the fecal microbiome of COVID-19 patients. Fecal specimens exhibiting a distinct profile indicative of elevated SARS-CoV-2 infectivity demonstrated increased prevalence of specific bacterial species, namely *Collinsella aerofaciens, Collinsella tanakaei, Streptococcus infantis, Morganella morganii,* along with heightened functional capacity for de novo biosynthesis of nucleotides, amino acids, and glycolysis. Conversely, fecal samples exhibiting a signature suggestive of low-to-negligible SARS-CoV-2 infectivity displayed elevated quantities of short-chain fatty acid-producing microorganisms such as *Parabacteroides merdae, Bacteroides stercoris, Alistipes onderdonki*i, and members of the *Lachnospiraceae* family (Zuo *et al.*, 2021). The eukaryotic component of the respiratory virome in a cohort of 120 COVID-19 patients was comprehensively analyzed using whole metagenomic sequencing. The findings underscore the

potential of metagenomics as a valuable tool for profiling the extended peripheral virological milieu within SARS-CoV-2-infected individuals, particularly those with varying disease prognoses (Iša *et al.*, 2022). Metagenomics is a powerful approach for investigating SARS-CoV-2 dynamics, yet it exhibits notable limitations. These include susceptibility to sampling biases, challenges in accurately quantifying viral variants, difficulties in assembling complete genomes from fragmented sequences, limited sensitivity in detecting low-frequency mutations, complexity in dealing with mixed clinical samples, potential inaccuracies in capturing host responses, temporal resolution constraints, data volume and computational complexity, incomplete functional insights, and ethical concerns related to privacy and data sharing. Table 2.9 gives an account of virus metagenomics applications.

Table 2.9: Summary of zoonotic virus detection using metagenomic-based strategy.

Sample type	Virus	Findings	Reference
Nasopharyngeal specimens of Brazilian pediatric patients	*Malawi polyomavirus* MWPyV	First molecular and genomic characterization of MWPyV	(Silva *et al.*, 2023)
Fecal sample of Domestic Dogs	Virome	*Protoparvovirus, Inovirus*, and Chlamydiamicrovirus were dominant genus	(Wang *et al.*, 2023)
Serum samples	Dengue, Zika, and Chikungunya	Identification of metavirome of the emerging arboviruses	(Souza *et al.*, 2022)
Fecal specimens	Virome	60.3% of the previously unknown viruses	(G. Wang *et al.*, 2023)
Fecal sample of children with diarrhea	Virome	*Caliciviridae* as the dominant family	(Yang, He, *et al.*, 2022)
Nasopharyngeal swabs of Brazilian patients	Chikungunya	Identification of unsuspected viral agents	(Quintão *et al.*, 2022)
Respiratory samples	Virus Pathogen Detection	Sensitivity for divergent viruses and variants	(Carbo *et al.*, 2022)
Respiratory samples from adult patients of Kuala Lumpur	Rhinovirus and Influenza virus	49.1% and, 7.4% prevalence	(Chong *et al.*, 2022)

Tissue samples of diseased pigs	Virome	*Siphoviridae*, and Myoviridae as dominant families	(Yang, Zhang, *et al.*, 2022)
Fecal sample of bats	Virome	*Circoviridae* and *Genomoviridae* as the dominant families	(Bolatti *et al.*, 2022)
Wastewater	SARS-CoV-2 (B.1.1.7)	Detection of B.1.1.7 variant	(Landgraff *et al.*, 2021)
Southern California wastewater	SARS-CoV-2	Detection of single nucleotide variants	(Rothman *et al.*, 2021)

2.6.5. Hot spring metagenomics

The existence of microorganisms in hot springs ranges from simple thermophiles (50-65 ℃) to hyperthermophiles (above 70℃) (Holden, 2009). The genetic network involved in the survival of microorganisms in hot temperatures has drawn substantial attention owing to its relevance in producing stable biomolecules like proteins and enzymes (Vieille and Zeikus, 2001). Generally, emphasis has been laid on the features of derived biomolecules like protein stability, enzyme activity, and their structures. The genome of thermophiles is often reported to have more stability than mesophiles. This is generally reported based on guanine (G) – cytosine (C) content which is considered to be a vital DNA stability indicator (Hurst and Merchant, 2001, Wu *et al.*, 2012). *Thermus thermophilus* has been reported to have 69.41% GC content and *Geobacillus kaustophilus* has 52.1 % (Doi *et al.*, 2013, Napper and Culver, 2015). However, some investigators have argued that higher GC content is not universal to all thermophiles (Hurst and Merchant, 2001, Wang *et al.*, 2015). Certain microorganisms like *Caldicellulosiruptor hydrothermalis* has a GC content of only 35% with 70℃ as the optimal growth temperature (Basak *et al.*, 2010). Moreover, a substantially high AG content in transcribed DNA (mRNA) is regarded as a selective response approach for the survival of thermophiles (Paz *et al.*, 2004). There is also purine and purine cluster enrichment with lesser pyrimidine/purine ratios in thermophiles (Lao and Forsdyke, 2000). However, a clear correlation of the thermophiles and purine content lacks advanced confirmation and needs further evaluation about other attributes of both gram-positive and gram-negative bacteria. Since base bias contributes significantly to the amino acid variation and their usage in proteins (Kreil and Ouzounis, 2001). Comparative studies between mesophiles and thermophiles

suggest a wide proteome variation. The variations include, increase in charged amino acids (Arg, Lys, and Glu), decrease in polar uncharged amino acids (Ser, Gln, Thr, and Asn), increase in the ratio of Lys+ Glu / His+Gln, and decrease in thermo-labile amino acids (Thr, Gln, and His) (Haney *et al.*, 1999, Goldstein, 2007). One of the studies has claimed IVYWREL amino acid sequence as a hallmark of thermophilic microorganisms (Zeldovich *et al.*, 2007). The operon assembly is also an important aspect in microbial adaptations to fluctuating temperatures (Bleuven and Landry, 2016b). Mesophiles have 60% of the operon density whereas thermophiles have around 50% (Zeldes *et al.*, 2015). However, the operon system is believed to be significantly stable in thermophiles than non-thermophiles (Wang *et al.*, 2015). A transcriptomic study revealed differential expression of more than 10 genes in *Thermotoga maritima* in high temperatures. The temperature shifts induce genomic changes during evolution which thereby offers microorganisms the ability to withstand high temperatures (Kim *et al.*, 2020).

2.6.5.1 Bioprospecting hot spring metagenomes

The groundwater gets heated up geothermally by molten rocks or by circulation via hot rocks of Earth's crust and emerges as hot water. Hot springs have long been associated with great therapeutic potential. South-Asian countries have been taking baths in hot springs for the last thousand years for both relaxation and cleanliness. Because of folklore and designated medicinal value hot springs have become the center of attention for many researchers. The hot bath was commonly used in the case of lead poisoning in the 19th century which may have been due to the over-production of urine thus increasing lead excretion. Besides, a significant boost was achieved by ankylosing spondylitis and rheumatoid arthritis patients by hot bath therapy. Taking the advancements in science into account molecular studies about microorganisms have become imperative. While conventional genomics and genome sequencing rely upon the cultivation of microorganisms to develop a microbial profile specific to the sample. However, the majority of microbial diversity has remained obscure by culture-based methods. Metagenomics is thus vital in revealing the hidden microbial diversity besides bioprospecting them. Saxena has carried out a metagenomic analysis of several hot springs in Central India and revealed the presence of thermophiles with an ability to degrade hydrocarbon and their survival strategies in extreme environments (Saxena *et al.*, 2017). Chan and his team studied the thermophilic diversity of Sungai Klah hot spring Malaysia using 16S rRNA and shotgun metagenomics. They revealed that 57% of

the microbiome was dominated by *Proteobacteria* and *Firmicutes* with *Hydrogenobacter spp*., as one of the major phyla (Chan *et al.*, 2015). Scientists performed metagenomic characterization of a proterozoic-analogue hot spring known Jinata Onsen in Japan and revealed thermophilic lithotrophs, iron-tolerant phototrophs and some previously unknown microorganisms. They reported the presence of previously some of the uncharacterized *Calditrichaeota* and *Chloroflexi* members (Ward *et al.*, 2019). Another research group performed metagenomic studies on Reshi and Polok hot springs of Sikkim India and revealed the molecular profile and metabolic pathways of its resident microbial communities (Sharma *et al*., 2020). Since South Asia is considered to be ecologically pristine and due to geo-tectonic activities, several hot springs have evolved with diverse microbial populations. Since abundant genomic resources from South-Asian hot springs remain unexplored thus require in-depth exploration for bioprospecting. Table 2.10 gives an account of metagenomic studies carried out on hot springs worldwide.

Table 2.10: Summary of metagenomic studies on hot springs worldwide.

Hot spring	Country	Metagenomic Findings	References
Shi-Huang-Ping	Taiwan	Identification of carbon assimilatory pathways. Identification of dominant microbes capable of metabolizing sulfur-related compounds	(Lin *et al.*, 2015)
Yumthang and Reshi	India (Sikkim)	The dominance of Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria. Detection of metal resistant and antibiotic-resistant genes.	(Najar *et al.*, 2020)
Chillas and Hunza	Pakistan	The presence of sulfur-reducing bacteria. Identification of uncharacterized and unclassified Operational Taxonomic Units (OTUs).	(Amin *et al.*, 2017)
Eryuan	China	Decrease in microbial diversity with increasing temperature.	(Menzel *et al.*, 2015)

		Presence of thermophilic *Acidithiobacillus sp.*	
Deulajhari	India (Odhisa)	Identification of 28 phyla with Proteobacteria (88.12%) being the dominant and Chloroflexi (0.35%) being least dominant.	(Singh and Subudhi, 2016)
Tatta Pani	Pakistan (Muzzafarabad)	Isolation of thermophilic bacteria and thermostable enzymes.	(Zahoor *et al.*, 2016)
Yamunotri Tapt Kund, Surya Kund and Draupadi Kund	India (Uttrakhand)	Identification of 21, 22 and 22 uncharacterized microbial strains were identified from these hot springs respectively.	(Kumar and Sharma, 2020)
Jakrem	India (Meghalaya)	Identification of novel bacterial communities dominated by the members of Firmicutes (61%), Chloroflexi (21.37%) and unclassified bacteria (1.2%).	(Panda *et al.*, 2015)
Mahapelessa	Sri Lanka	Characterization of growth-promoting bacteria like *Bradyrhizobium liaoningense* and *Azospirillum halopraeferens*	(Samarasinghe *et al.*, 2021)
Sungai Klah	Malaysia	Detection of thermophilic photosynthetic microorganisms and acidothermophiles.	(Chan *et al.*, 2015)
Pali	India (Maharashtra)	Characterization of thermostable bile salt hydrolase with an optimum temperature of 60℃	(Sridevi *et al.*, 2009)
North-West Himalayas	India	Identification and characterization of lipase from thermophilic *Bacillus circulans* IIIB 153 having biofilm disruptive properties.	(Johri *et al.*, 2012)
Manikaran	India (Himachal Pradesh)	Characterization of β-D-galactosidase with 65℃ as its optimum temperature.	(Batra *et al.*, 2002)

Tulsi Shyam	India (Gujrat)	Identification of thermophilic *Anoxybacillus beppuensis* TSSC-1 and subsequent characterization of amylase with an optimum temperature of 80℃	(Kikani and Singh, 2011)
Tatapani	India (Himachal Pradesh)	Characterization and purification of thermos-active amidase.	(Sharma *et al*., 2013)
Western Sichuan	China	Distinct microbial communities within 14 hot springs with *Aquificae, Proteobacteria* and *Cyanobacteria* as dominant phyla	(Tang *et al.*, 2018)
Kirishima area	Japan	Identification *Crenarchaeota* and *Aquificae* as dominant phyla	(Nishiyama *et al.*, 2018)
Chumthang	India (Jammu and Kashmir)	Exploration and characterization of thermotolerant oxidation-resistant and alkali stable protease.	(Singh *et al*., 2015)
Tatapani	India (Odisha)	Exploration of metagenome-derived lipase	(Sahoo *et al.*, 2020)
Transhimalaya	China	High microbial diversity along the hydrothermal gradients dominated by *Deinococcus-thermus, Chloroflexi*, Proteobacteria*, Firmicutes* and Cyanobacteria.	(Roy *et al.*, 2020)

2.7: Bibliometric analysis on Hot Spring metagenomics

Bibliometric analysis helps to map the research trends and priorities by analyzing the frequency of certain keywords or phrases in publications like metagenomics, researchers can gain insight into the topics and areas of research that are currently receiving the most attention (Yue *et al.*, 2020, Stasi *et al.*, 2023). This helps in understanding collaborations between researchers and institutions. By analyzing co-authorship patterns and affiliations, researchers can gain insights into the networks and collaborations that exist within the field. Bibliometric analysis can be used to evaluate the impact of research in any field including metagenomics. By analyzing citation patterns, researchers can identify the most influential publications and researchers in the field (Huang *et al.*, 2022, Wu *et al.*, 2023). It can help to monitor the growth of the field of metagenomics over the period. By analyzing the number of publications and citations, researchers can track the growth and development of the field, as well as identify emerging areas of research (Xu *et al.*, 2022). In this research we have studied the recent trends in hot spring metagenomics from 2005-2022 using Scopus and WoS databases.

2.7.1: Search strategy

A bibliometric analysis was conducted to study articles related to hot spring metagenomics. The analysis involved examining papers published in journals indexed in Scopus and WoS databases from January 2005 to December 2022. The Scopus and WoS indexing systems were used to locate all relevant papers, and their bibliographic details were extracted using the databases' export functions. They provide access to a vast amount of scholarly literature and offer a range of tools for measuring research impact and tracking trends in a particular field of study. The researchers can use these databases to identify and analyze papers from different fields, which can be particularly useful for interdisciplinary research (Wang *et al.*, 2014, Sweileh, 2018). To analyze publication trends in the field of hot spring metagenomics, a literature survey was conducted. Only research articles published in scientific journals were considered for the analysis, as the aim was to focus on the scientific information available in this field. The comprehensive coverage, high-quality data, citation analysis, and advanced search functionality make Scopus and WoS databases preferred for bibliometric analysis. On January 11th, 2023, data from both databases was obtained. TITLE-ABS-KEY ("Metagenome" OR "Metagenomics" AND "Hot spring" OR " Geothermal spring" OR " Thermal spring") AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (

DOCTYPE , "re") OR LIMIT-TO (DOCTYPE , "cp")) AND (LIMIT-TO (LANGUAGE , "English")) AND (LIMIT-TO (SRCTYPE , "j")), search query was run in both the databases resulting in 564 databases. The search query is designed for a bibliographic database and employs a combination of Boolean operators and field qualifiers. It begins with the specification "TITLE-ABS-KEY," indicating that the search terms are to be found in the title, abstract, and keywords of documents. In the above search query, the primary search query focuses on metagenome or metagenomics-related content and includes hot spring, geothermal spring, or thermal spring as additional criteria. This combination of terms is connected by logical operators (OR and AND) to refine the search. Furthermore, the query includes limitations on document types, specifically targeting articles ("ar"), reviews ("re"), and conference papers ("cp"). This ensures that the search results comprise scholarly articles, reviews, or conference contributions. Another constraint is imposed on the language of the documents, restricting them to English. Lastly, the search is narrowed down to documents sourced from journals ("SRCTYPE" limited to "j"). The data obtained was exported and scrutinized for duplicates with Zotero (v.6.0.7).

To conduct bibliometric analysis, we utilized various software tools including VOSviewer (version 1.6.18), open-source Biblioshiny (RStudio- version 2022.02.2), MS Access, and MS Excel. Biblioshiny employs the primary functions of the bibliometrix package to conduct scientific mapping analysis. VOSviewer software was used to generate maps that provide an easy-to-understand representation of bibliographic data, including co-authorship, citation, bibliographic coupling, keyword co-occurrence, and co-citation maps. The study also employed Biblioshiny to prepare a Three-field plot analysis that represents the relationship between author, country, and source.

2.7.2: Annual publication pattern of Hot spring metagenomics

Examining the increase in the publications in hot spring metagenomics can provide valuable insights into the current patterns and advancements in metagenomics. By scrutinizing this growth, one can gain knowledge about future research areas and potential strategies for addressing obstacles confronted in microbiology. We analyzed publications over time to understand how research on traceability in hot spring metagenomics has developed (Figure 2.6). Our analysis showed that the earliest article on hot spring metagenomics was published in 2005. In both 2007 and 2008, there were five articles that were published. There has been a continuous increase in the

number of publications from 2008-2013. The number of cumulative publications has increased exponentially from 2005. The maximum number of articles on hot spring metagenomics have been published in 2021 (51) followed by publications in 2018 and 2022 (44 each).

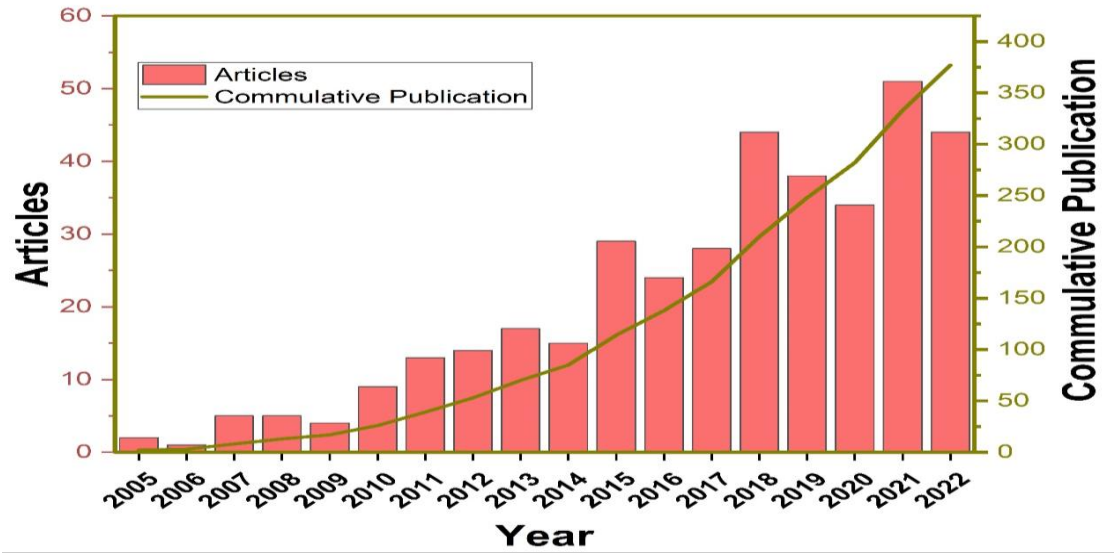

Figure 2.6: Annual distribution and publication trend of hot spring metagenomics (Wani *et al.*, 2023).

2.7.3: Publication distribution by countries and organizations

The total number of 564 publications came from 24 countries, out of which the United States (US) has the highest number of 167 (29.60 %) publications, followed by India (12.58 %), China (8.15 %), Japan (6.73 %), and Germany (6.56 %). Besides, top five countries, France, Spain, Italy, Russian federation, and Canada have published articles ≥15 (Figure 2.7). The findings showed that articles were predominantly published by economically developed nations and that research collaboration was primarily happening among those developed nations. India, China, and Japan are the three Asian countries in top 5 showing significant interest and outcome in hot spring metagenomic research. India's investment in research, diverse ecosystems, academic excellence, international collaborations, and government initiatives contribute to its prominence. India's diverse ecosystems, ranging from Himalayan glaciers to coastal regions, provide rich environments for metagenomic exploration. Researchers can study a wide array of microbial communities in different climatic and geographical settings. This can be attributed to the fact these Asian countries have large number of hot spring sources (Kubota *et al.*, 2013, Zhu *et al.*, 2015,

Sahay *et al.*, 2017). Furthermore, the study examined both single country publications (SCPs) and multiple country publications (MCPs) to assess the collaboration patterns within and between different countries in publishing articles related to hot spring metagenomics. Until 2022, the US had the highest MCPs and SCPs. India had lower MCP and higher SCP than China. South Africa and Australia had zero MCP and SCP in hot spring metagenomic research respectively (Figure 2.8).

The Total Link Strength (TLS) was utilized as a metric to gauge collaborative research conducted among multiple countries (Table 2.11). Based on the TLS analysis data, it was found that the United States had the highest level of collaborative research, with a TLS score of 931 for all 564 articles that were published. Most countries had their collaborative research on hot spring metagenomics with the US. Denmark had a TLS score of 281, placing them in the second position, while Japan was in third place with a TLS score of 263. Most countries consider the US, India, Japan, and Denmark as the most significant partners for hot spring metagenomic research, according to the Country Cooperation Network Map and the TLS score (Figure 2.9).

According to the data, articles originating from the US received the highest number of citations at 5803 (with an average of 37.34 citations per document). China followed with 1061 citations (average of 23.06), then India with 1047 citations (average of 14.74), and Germany with 805 citations (average of 21.75). It is noteworthy that Denmark ranks twelfth in terms of the quantity of articles produced on hot spring metagenomics. However, Denmark stands out as the top performer in terms of the average number of citations received per document, with an impressive score of 54.14. This is based on a total of 14 documents that have collectively received 758 citations. In the field of hot spring metagenomics, Denmark has the highest average citations per document at 54.14, followed by Malaysia at 42.85, Australia at 39.35, and the Netherlands at 36.28. The United States ranks fifth with an average of 34.74 citations per document. In addition, we examined organizations that had published at least three articles in the field of hot spring metagenomics. The study found that out of 580 organizations, only 27 met the criteria of having published at least three articles. Among these 27 organizations, only one had published over 50 articles, while three had published nine or more articles. The study discovered that Montana State University Bozeman in the US had the most publications, a total of 56, and also had the highest number of citations with 2360 (the average number of citations per publication was 42.14).

University of Nevada Las Vegas and Pennsylvania State University of US hold the second and third place respectively in terms of number of publications from 2005-2022. Wesleyan University, located in Middletown, USA, achieved the highest ranking with an average of 97.33 citations across three documents (292 citations and 69 TLS). In second place was the University of Tennessee, Knoxville, USA, with an average of 73 citations across three documents (TLS=3). Table 2.11 presents the organization that has the greatest quantity of articles published and citations.

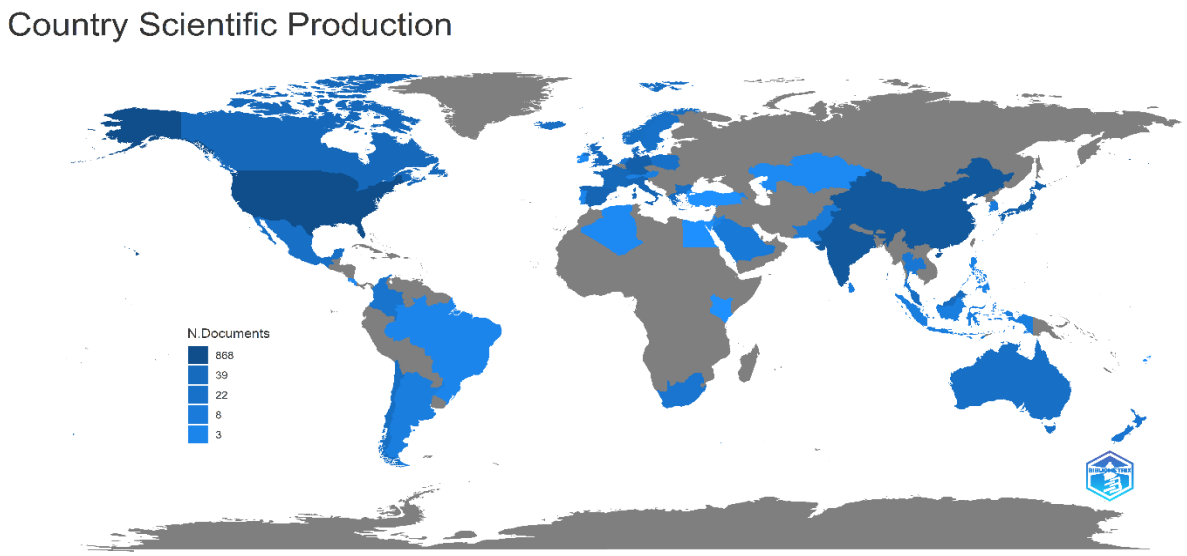

Figure 2.7: Hot spring metagenomic country wise publication distributions (Wani *et al.*, 2023).

Table 2.11: Countries with ≥ 5 published articles on hot spring metagenomics.

Country	Documents	Percentage (%) of Documents	citations	Average citations	Total link strength
United States	167	29.60	5803	34.74	931
India	71	12.58	1047	14.74	245
China	46	8.15	1061	23.06	209
Japan	38	6.73	708	18.63	263

Germany	37	6.56	805	21.75	221
France	20	3.54	452	22.6	127
Spain	20	3.54	351	17.55	218
Italy	18	3.19	364	20.22	175
Russian Federation	16	2.83	324	20.25	116
Canada	15	2.65	272	18.13	44
Australia	14	2.48	551	39.35	174
Denmark	14	2.48	758	54.14	281
United kingdom	13	2.30	314	24.15	80
Norway	9	1.59	148	16.44	82
Chile	8	1.41	90	11.25	131
Mexico	8	1.41	142	17.75	62
New Zealand	8	1.41	111	13.87	33
Greece	7	1.24	103	14.71	77
Malaysia	7	1.24	300	42.85	90
Netherlands	7	1.24	254	36.28	74
Sweden	6	1.06	143	23.83	64
Bulgaria	5	0.88	6	1.2	2
South Africa	5	0.88	155	31	35
Taiwan	5	0.88	86	17.2	24
Total	564		14348		

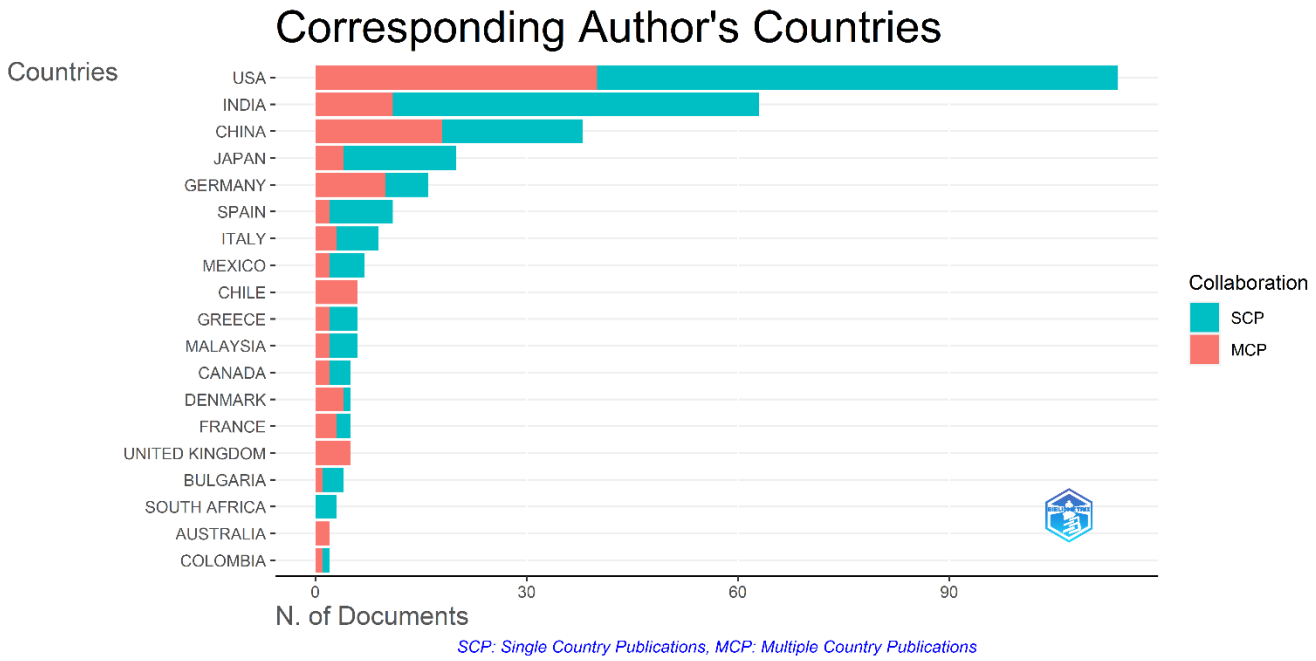

Figure 2.8: Collaboration between different countries and within countries using MCP and SCP indicators. MCP indicates collaboration among different countries, while SCP indicates research produced by a single country. The selection of countries for analysis was based on the country of the corresponding author (Wani *et al.*, 2023).

Table 2.12: Top organizations with ≥ 3 published articles related to hot spring metagenomics.

Organization	Documents	Citations	Average citations	Total link strength
Montana state university, Bozeman, US	56	2360	42.14	611
University of Nevada Las Vegas, US	10	376	37.60	85
Pennsylvania State University, PA, US	9	492	54.67	172
Bigelow laboratory for ocean sciences, East Boothbay, United States	6	154	25.67	36
Guangdong provincial key laboratory of plant resources and southern marine science and engineering Guangdong laboratory (Zhuhai), school sun yat-sen university, China	6	60	10.00	23

China University of Geosciences, Wuhan, China	5	48	9.60	26
Panjab university, Chandigarh, India	5	45	9.00	4
Xinjiang institute of ecology and geography, Chinese Academy of sciences, Urumqi, China	5	55	11.00	23
Arizona state university, Tempe, US	4	98	24.50	14
University of Chinese academy of sciences, Beijing, China	4	43	10.75	0
Academy of scientific and innovative research (ACSIR), New Delhi, India	3	52	17.33	0

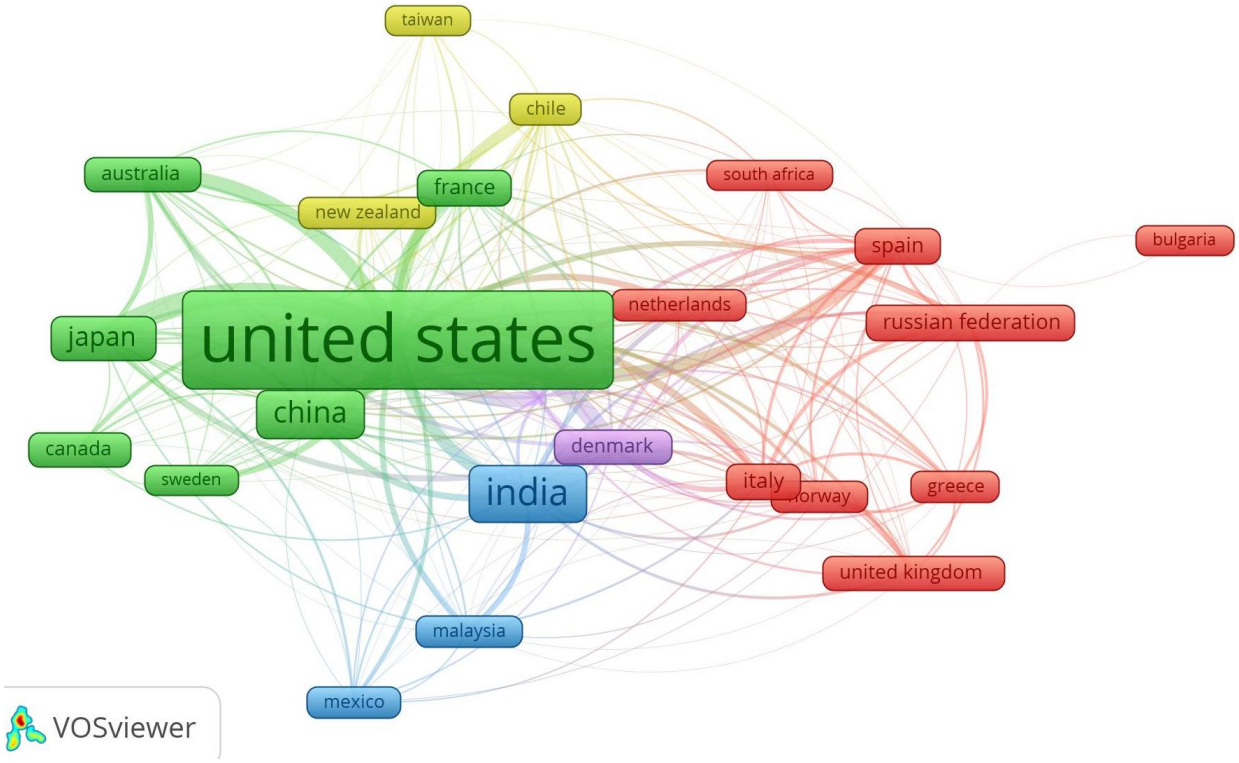

Figure 2.9: The representation of collaborative research on hot spring metagenomics across countries has been visualized through a network. The connecting lines depict the collaborations among the countries (Wani *et al.*, 2023).

2.7.4: Author and co-author relationship

The authors who have published the most in the field of hot spring metagenomics were determined by looking at both the number of publications and the number of citations they received. Out of 1774 authors, only 60 of them had published five or more than five articles on hot spring metagenomics. According to the data that was analyzed, Bryant A. had the most articles published, with a total of 17, and had the highest number of citations, with a total of 1173. Boyd S. had the second highest number of articles published, with 16, and a total of 388 citations. Ward M. had 15 articles published with 1202 citations, while Hedlund P. had 14 articles published with 591 citations**.** Bateson M. has published 6 articles, which ranks twelfth in terms of quantity. However, Bateson M. has the highest average citation count of 116, putting them at the top in terms of citation impact till 2022. Klatt G., with 8 documents and sixteenth in the table stands third with 847 citations and 432 TLS (Table 2.13). The citation count difference between Scopus and WOS databases was unified by employing a systematic approach to standardize and normalize the data. We utilized data collected from both Scopus and WOS, and the merging process was facilitated using Zotero software. Zotero automatically identifies and merges similar articles based on shared information. Consequently, duplicate citations were harmonized to present a unified count, while any unique citations from both databases were retained and normalized to ensure accurate representation.

To comprehend how hot spring metagenomics research has evolved globally in terms of space and time, the publication production of authors was examined and analyzed over a period (Figure 2.10). The most prominent author in terms of the quantity of articles published from 2005 to 2022 was Bryant A., who had the highest number of publications (17 in total). Boyd S. published the highest number of 5 articles in the year 2019 in a total of 16 publications. In 2006, Ward M. commenced hot spring metagenomics research and published four articles but was unable to maintain his involvement in this field of metagenomics. Li J. and Singh P. who started working on hot spring metagenomics in 2016 and 2017 respectively, have been actively working in the field in addition to Hedlund P. and Colman R. The level of collaboration among authors was assessed using the TLS metric (Figure 2.11). The results revealed that Bryant A. had the highest TLS score of 653, followed by Ward M., Klatt G., Rusch B., and Bateson M. with TLS scores of 643, 432, 323, and 262 respectively. Despite having only six publications as of 2022, Bateson M. ranks 25th in terms

of the number of publications, but still manages to make it to the top 5 researchers based on their TLS score. This implies that these authors are highly productive researchers in the domain of hot spring metagenomics and possess impressive networking and collaboration abilities.

Table 2.13: List of top 30 authors with a minimum of five publications on hot spring metagenomics. The overall strength of an author's co-authorship connections with other researchers is represented by the Total link strength in the table.

Author	Documents	Citations	Average citations	Total link strength
Bryant A.	17	1173	69	653
Boyd S.	16	388	24	168
Ward M.	15	1202	80	643
Hedlund P.	14	591	42	188
Colman R.	11	137	12	63
Singh P.	11	152	14	69
Jay J.	10	533	53	163
Li J.	10	256	26	79
Rusch B.	10	690	69	323
Subudhi E.	10	86	9	33
González-siso I.	9	95	11	78
Inskeep P.	9	510	57	157
Woyke T.	9	220	24	78
Young J.	9	254	28	100
Hua S.	8	231	29	57
Klatt G.	8	847	106	432
Mcglynn E.	8	133	17	74
Peng x.	8	379	47	92
Sahoo K.	8	68	9	32
Becerra M.	7	43	6	62
Dodsworth A.	7	364	52	129
Murugapiran K.	7	218	31	133

Ward M.	7	117	17	71
Antranikian G.	6	59	10	13
Bateson M.	6	698	116	262
Dong H.	6	253	42	79
Escuder-rodríguez J.	6	14	2	58
Liu z.	6	336	56	219
Quake R.	6	267	45	70
Sharma A.	6	90	15	53

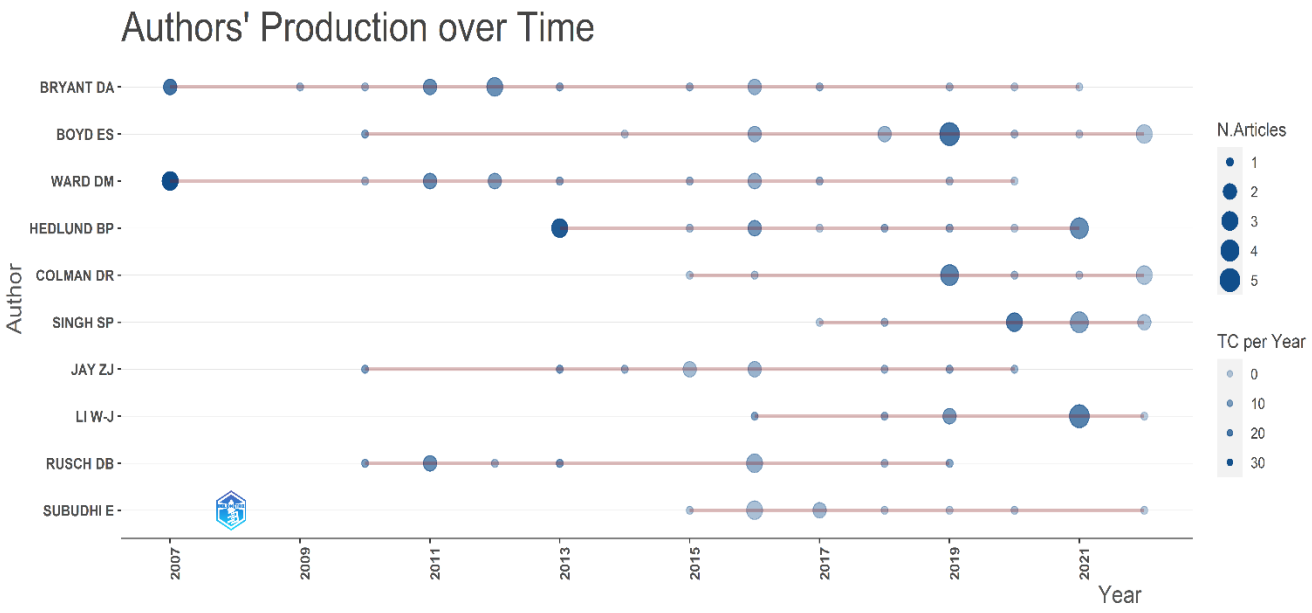

Figure 2.10: The impact of the top 10 authors in various years is shown through red lines. The quantity of publications during different years is represented by the size of the dots, while the color of the dots (ranging from light to dark) indicates the total number of citations per year (TC) (Wani *et al.*, 2023).

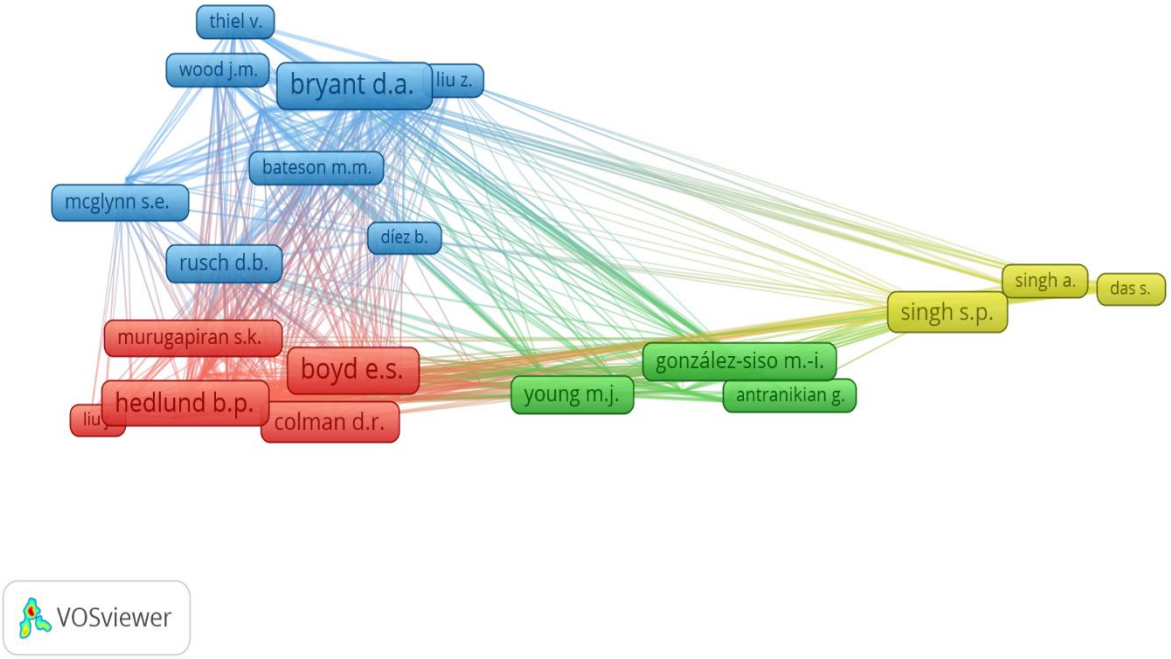

Figure 2.11: Network visualization of collaborative research among authors on hot spring metagenomics (Authors with a minimum of five publications were taken into consideration). The different colors in the figure represent clusters (Wani *et al.*, 2023).

2.7.5: Publication sources

The origin of the publication provides insight into the journals that esteemed researchers selected for disseminating their scientific discoveries on hot spring metagenomics. Table 2.14 presents an overview of the preeminent journals that have published research papers concerning hot spring metagenomics. Based on the data, Frontiers in Microbiology has been the most favored journal for publishing investigations on hot spring metagenomics, with the largest count of papers published (43) with 1038 citations. Even though ISME Journal comes in third place for the quantity of papers published in the field of hot spring metagenomics, it has received the most citations, with a total of 1118 from 2005 to 2022. In terms of citations received, PLOS One (734), Applied and Environmental Microbiology (661), Environmental Microbiology (487), Scientific Reports (226), and mSystems (205) hold the 3rd, 4th, 5th, 6th, and 7th positions, respectively. The information implies that there is no notable prevalence of a specific publisher in disseminating papers regarding hot spring metagenomics. This may be attributed to the fact that this field encompasses both microbiology and environmental science, and depending on a researcher's area of expertise, they choose a journal irrespective of the publisher. Nature Microbiology (3 articles) and Nature

Communications (7 articles), both of which possess the highest impact factors of 30.96 and 17.69, respectively, are published by Nature Portfolio.

Table 2.14: List of journals with ≥ 3 publications in the domain of hot spring metagenomics from 2005-2022.

Journal	Publisher	Impact factor	Documents	Citations	Average citation
Frontiers in Microbiology	Frontiers	6.064	43	1038	161
Applied and Environmental Microbiology	American Society for Microbiology	5.005	25	661	70
ISME journal	Nature Portfolio	10.3	20	1118	126
PLOS One	Public Library of Sciences	3.752	14	734	42
Environmental Microbiology	Wiley-Blackwell	4.933	13	487	51
Microbiology Resource Announcements	American Society for Microbiology	0.89	13	94	13
Genomics Data	Elsevier	1.99	10	199	37
Scientific Reports	Nature Portfolio	4.996	8	226	18
Applied Microbiology and Biotechnology	Springer	3.53	7	182	4
Extremophiles	Springer	3.035	7	117	12
Msystems	American Society for Microbiology	7.324	7	205	12
Nature Communications	Nature Portfolio	17.69	7	374	29
Geomicrobiology Journal	Taylor & Francis	2.412	6	46	26
Archives of Microbiology	Springer	2.552	5	54	27

International Journal of Biological Macromolecules	Elsevier	8.025	5	71	23
Microbes and Environments	Japanese Society of Microbial ecology	2.45	5	59	26
BMC Genomics	Springer	4.56	4	83	11
Frontiers in Bioengineering and Biotechnology	Frontiers	6.064	4	37	7
Microbiology spectrum	American Society for Microbiology	9.043	4	8	25
Science of Total Environment	Elsevier	10.75	4	57	8
Systematic and Applied Microbiology	Elsevier	3.283	4	78	6
Current Microbiology	Springer	2.29	3	3	1
FEMS Microbiology Ecology	Oxford University Press	4.519	3	150	9
FEMS Microbiology Letters	Oxford University Press	2.742	3	24	0
Frontiers in Genetics	Frontiers	4.772	3	42	14
International Journal of Molecular Sciences	MDPI	6.208	3	1	7
Journal of Virology	American society for microbiology	6.208	3	119	16
Microbiology	Springer	2.956	3	7	3
Microorganisms	MDPI	4.926	3	12	16
Nature Microbiology	Nature Portfolio	30.96	3	80	6

2.7.6: Document and citation relationship

The correlation between journal articles and the number of citations they receive is essential for determining the caliber and extent of the publication. A higher quantity of citations received by a particular article indicates that it has been acknowledged by other scholars in the same field. For this study, articles with at least 70 citations were chosen, and the results showed that out of the total 2945 articles, only 72 have obtained a minimum of 70 citations. According to the data, Bryant's article which was published in Science in 2008 has obtained the highest number of citations, totaling 292 till 2022 (Table 2.15). The investigation conducted on the metagenomic data of phototrophic microbial mats from alkaline siliceous hot springs in Yellowstone National Park has exposed the presence of a unique phototrophic bacterium that can synthesize bacteriochlorophyll (BChl). The bacterium was able to grow in a photoheterotrophic manner, produce both BChl a and c even in the presence of oxygen, and exhibited chlorosomes and type 1 reaction centers when cultured in a highly enriched environment (Bryant *et al.*, 2007).

The number of citations a research paper receives indicates how often other researchers have referenced and used that paper in their own work. Therefore, a higher number of citations generally indicates that the paper has had a greater impact on the field and is considered more influential. This can also reflect the popularity of the research field, as more researchers are likely to be working and publishing in a field that is receiving a lot of attention and citations. However, it's important to note that citation count alone should not be used as the sole measure of a research field's popularity, as there are many other factors that can influence the level of interest and activity in a particular field (Sarli *et al.*, 2010, Wang, 2013). Petersen 's and Lloyd 's research papers were ranked second and third, respectively, among the most cited papers due to their citation scores of 196 and 179 (Petersen *et al.*, 2011, Lloyd *et al.*, 2018). Among the top 30 papers in the field of hot spring metagenomics 17 papers have more than 100 citations. Out of the 30 most prominent papers in hot spring metagenomics, 17 of them have over 100 citations.

Table 2.15: List of top 30 articles in the domain of hot spring metagenomics with ≥ 70 citations.

Document	Citations	Average citations per Year	Links	Reference
Candidatus Chloracidobacterium thermophilum: an aerobic phototrophic acidobacterium	292	17.18	9	(Bryant *et al.*, 2007)
Hydrogen is an energy source for hydrothermal vent symbioses	196	15.08	0	(Petersen *et al.*, 2011)
Phylogenetically novel uncultured microbial cells dominate earth microbiomes	179	29.83	0	(Lloyd *et al.*, 2018)
Metagenomes from high-temperature chemotrophic systems reveal geochemical controls on microbial community structure and function	157	11.21	11	(Inskeep *et al.*, 2010)
Population level functional diversity in a microbial community revealed by comparative genomic and metagenomic analyses	154	9.06	7	(Bhaya *et al.*, 2007)
Assembly of viral metagenomes from Yellowstone hot springs	148	9.25	7	(Schoenfeld *et al.*, 2008)
A comprehensive census of microbial diversity in hot springs of Tengchong, Yunnan Province China using 16S rRNA gene pyrosequencing	146	13.27	4	(Hou *et al.*, 2013)
Single-cell and metagenomic analyses indicate a fermentative and saccharolytic lifestyle for members of the OP9 lineage	139	12.64	4	(Dodsworth et al. 2013)
Phylogenetic and functional analysis of metagenome sequence from high-	137	12.45	4	(Inskeep *et al.*, 2013)

temperature archaeal habitats demonstrate linkages between metabolic potential and geochemistry				
Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential	128	9.85	19	(Klatt *et al.*, 2011)
Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches	121	8.07	0	(Wang *et al.*, 2009)
A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses	119	9.92	1	(Diemer and Stedman, 2012)
Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing	115	12.78	3	(Chan *et al.*, 2015)
Novel thermophilic and thermostable lipolytic enzymes from a Thailand hot spring metagenomic library	111	6.94	4	(Tirawongsaroj *et al.*, 2008)
Life in hot spring microbial mats located in the trans-Mexican volcanic belt: a 16S/18S rRNA gene and metagenomic analysis	104	17.33	4	(Prieto-Barajas *et al.*, 2018)
Community structure and function of high temperature chlorophototrophic microbial mats inhabiting diverse geothermal environments	103	9.36	11	(Klatt *et al.*, 2013)
ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases	102	8.5	0	(Zargar *et al.*, 2012)

Thermophiles in the genomic era: Biodiversity, science, and applications	91	10.11	11	(Urbieta *et al.*, 2015)
Abundant transposases encoded by the metagenome of a hydrothermal chimney biofilm	91	6.07	3	(Brazelton and Baross, 2009)
Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated Yellowstone hot springs	88	7.33	6	(Bolduc *et al.*, 2012)
Metagenomics uncovers gaps in amplicon-based detection of microbial diversity	87	10.88	3	(Eloe-Fadrosh *et al.*, 2016)
The Sorcerer II Global Ocean Sampling Expedition: metagenomic characterization of viruses within aquatic microbial samples	87	5.44	1	(Williamson *et al.*, 2008)
Complete Genome Sequence of the Aerobic CO-Oxidizing Thermophile *Thermomicrobium roseum*	84	5.6	5	(Wu *et al.*, 2009)
Using CRISPRs as a metagenomic tool to identify microbial hosts of a diffuse flow hydrothermal vent viral assemblage	83	6.38	4	(Anderson *et al.*, 2011)
Comparative genomics provides evidence for the 3-hydroxypropionate autotrophic pathway in filamentous anoxygenic phototrophic bacteria and in hot spring microbial mats	83	4.88	5	(Klatt *et al.*, 2007)
Comparative Metagenomics of Eight Geographically Remote Terrestrial Hot Springs	82	9.11	2	(Menzel *et al.*, 2015)
Microbial communities evolve faster in extreme environments	82	8.2	2	(Li *et al.*, 2014)
Geochemical and Metagenomic Characterization of Jinata Onsen, a	77	12.83	2	(Ward *et al.*, 2019)

Proterozoic-Analog Hot Spring, Reveals Novel Microbial Diversity including Iron-Tolerant Phototrophs and Thermophilic Lithotrophs				
Diverse capacity for 2-methylhopanoid production correlates with a specific ecological niche	73	7.3	0	(Ricci *et al.*, 2014)
Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat	70	5.31	12	(Liu *et al.*, 2011)

2.7.7: Co-occurrence of author keywords

Through keyword co-occurrence, the research identified central themes and temporal trends, unveiling shifts in research focus. Keywords are important in research articles as they help to identify and communicate the main themes, topics, and areas of focus of the study. They serve as a guide for readers who are searching for information on a particular topic and help to ensure that relevant articles are identified and included in literature reviews (Adegoke *et al.*, 2023, Giwa *et al.*, 2023). Additionally, keywords can improve the discoverability of research articles through online search engines and databases, making it easier for other researchers to find and build upon the work. Therefore, choosing the right keywords is crucial for researchers to ensure their work is widely read, cited, and recognized within their field (Choi *et al.*, 2011).

Our study focused on keywords that appeared at least 90 times. Out of the 864 keywords, only 10 met this criterion. The most used keyword across all articles was “Metagenomics” with 533 occurrences, followed by "Hot spring", " Phylogeny", " Bacterium", " Genetics", "Microbiology", and "Metabolism " (Figure 2.12). According to our research findings, there has been a greater emphasis on the investigation of "Phylogeny," "Bacterium," and "Genetics" in the field of hot spring metagenomics, as opposed to the exploration of "Archaea" and "16s rRNA." The major trends and hot topics in hot spring metagenomic research include the exploration of microbial diversity and novel species, functional genomics to understand metabolic pathways, adaptation of microorganisms to extreme conditions, biotechnological applications focusing on enzymes,

investigation of virus-microbe interactions, analysis of microbial community dynamics, metagenomic mining for natural products, examination of ecological and environmental impacts, study of microbial evolution and evolutionary processes, integration of multi-omics data, comparative metagenomics across different locations, and cultivation strategies for previously unculturable microorganisms (Bodor *et al.*, 2020, Lewis *et al.*, 2021). These trends collectively contribute to a deeper understanding of extremophiles and their potential applications in various fields. Understanding which areas of research receive more attention can help guide future investigations and potentially lead to the discovery of new insights into the microbial ecology of hot springs. Additionally, identifying areas that may be underexplored can bring attention to potential knowledge gaps that need to be addressed in order to further advance our understanding of hot spring microbial communities.

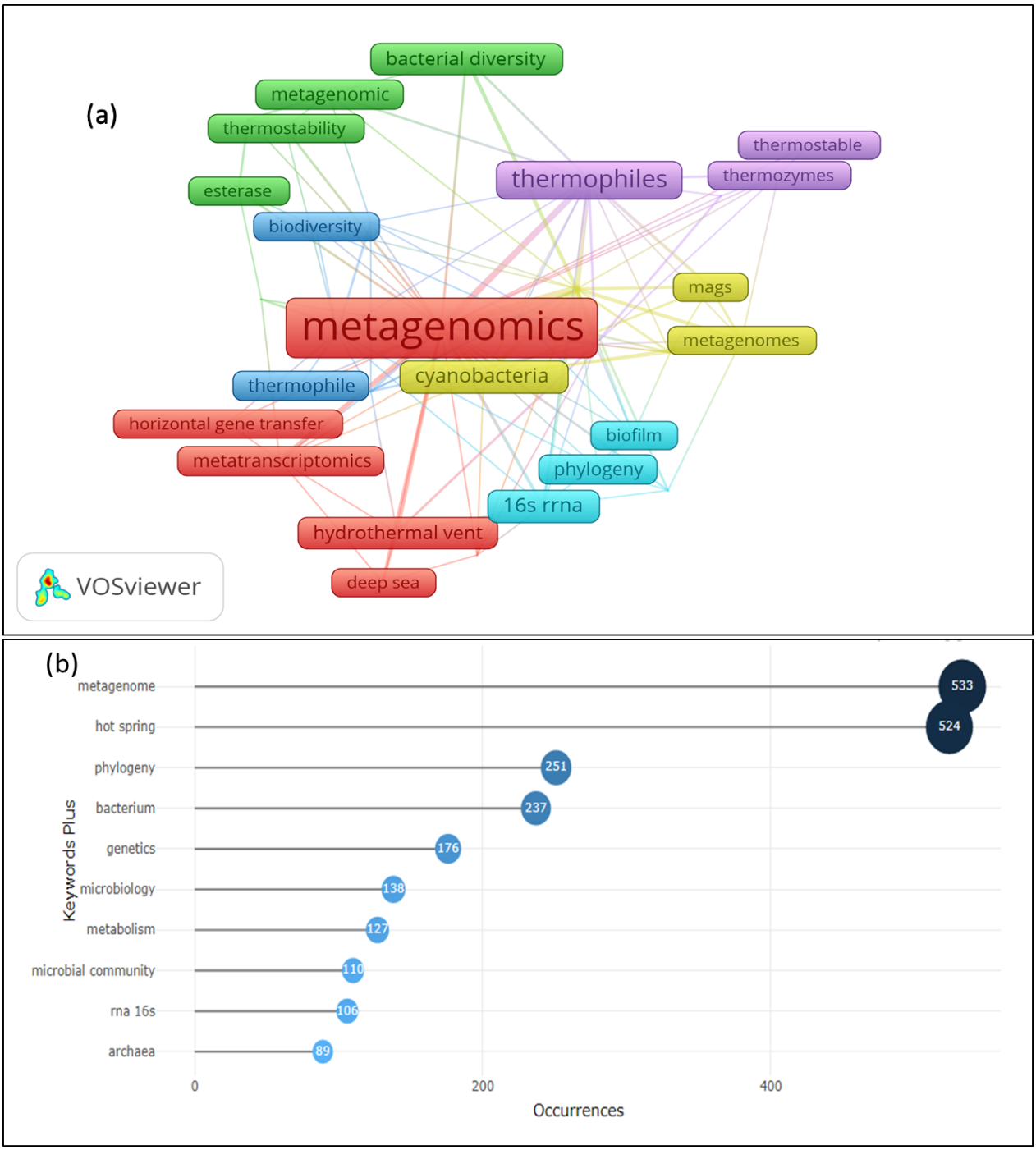

Figure 2.12: Network visualization (a) Keywords generally used in hot spring metagenomic research (b) Most relevant keywords in the field of hot spring metagenomics (Wani *et al.*, 2023).

2.8: Hypothesis of the study

The hot springs are characterized by extreme conditions, including high temperatures, high salinity, and often acidic or alkaline pH levels. These harsh environments present unique challenges for microbial life, leading to the evolution of specialized enzymes to survive and thrive in such conditions. Given the extreme conditions of the Northwestern Himalayan hot springs, it was hypothesized that the metagenomic analysis will reveal a diverse array of hydrolase enzymes adapted to function in high-temperature environments. Based on rich organic matter and biomass present in hot spring ecosystems, it was hypothesized that a diverse array of hydrolases could potentially be present in these environments. Furthermore, it was predicted that these hydrolases will exhibit unique structural and functional characteristics, potentially suitable for biotechnological applications.

Chapter 3

AIMS AND OBJECTIVES

Owing to the extreme environment of hot springs harboring unique microbial life and thermostable enzymes, the hydrolases were chosen as a target due to their market demand. The broad aim of the study was to explore the microbial diversity and enzymatic potential of hot springs located in the Northwestern Himalayas using a metagenomic approach. The objectives framed are as follows:

1. **Construction of metagenomic library from hot spring samples of Northwestern Himalayas.**
2. **Screening the metagenomic library for hydrolytic enzymes.**
3. **DNA Sequencing and analysis of the gene encoding the enzyme from positive metagenomic clone.**
4. **Biochemical characterization of selected hydrolytic enzymes.**

Chapter 4

MATERIAL AND METHODS

Objective 1: Construction of metagenomic library from hot spring samples of Northwestern Himalayas

4.1: Sampling site

Rajouri hot spring (RHS) is a natural wonder located in the picturesque region of Jammu and Kashmir in India. Surrounded by lush greenery and towering trees, this hidden gem offers visitors a peaceful oasis where they can relax and unwind in the healing waters of its geothermal pools. RHS is located at approximately 33.1829° N, 75.1441° E at an elevation of approximately 1050 m (3445 feet) above sea level (Figure 4.1). RHS is fed by an underground aquifer that is recharged by rainwater and snowmelts from the surrounding mountains. The aquifer is located within a fractured rock formation that allows water to flow through the rock and reach the surface at the hot spring. The hot spring is located within a dense forest area that is dominated by coniferous trees such as pine, spruce, and fir. The surrounding mountains are also covered in forests, with occasional meadows and grasslands. RHS is accessible by road, with a well-maintained paved road leading to the site. The nearest town is Kalakote, which is approximately 10 km away (Singh *et al.*, 2022).

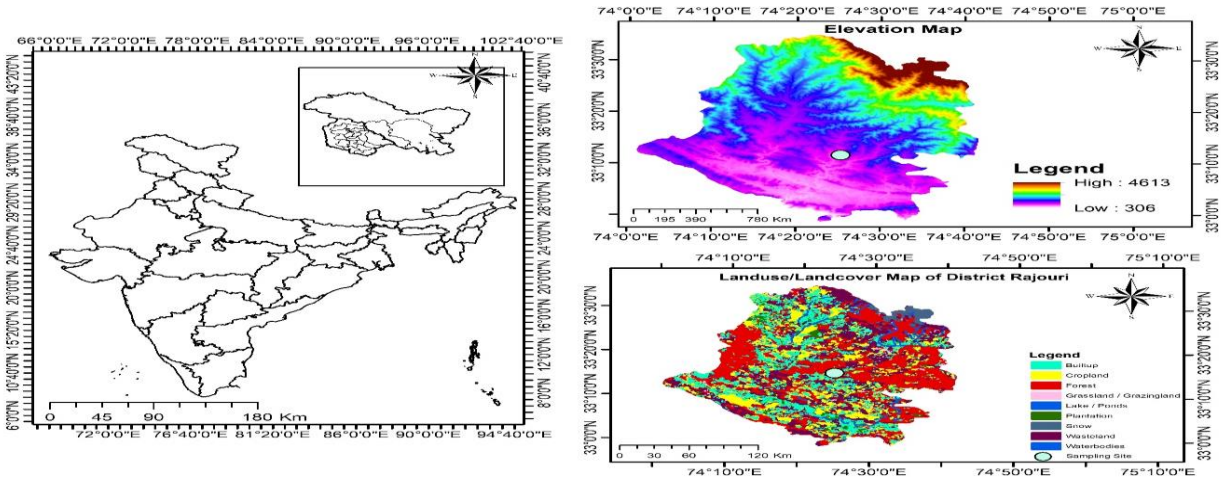

Figure 4.1: Maps depicting the geographic location and land cover of the RHS.

4.2: Sample collection

During the collection of soil and water samples from RHS careful attention to safety protocols and a specific methodology to obtain representative samples was followed (Daniel, 2005). Before collecting samples, it was ensured that the RHS site is safe to access. Hot springs in general can be dangerous due to high temperatures, and toxic gases. The samples were collected from the area where water was bubbling because it is likely to have a large microbial population of thermophiles and a higher chance of finding thermostable enzymes (Figure 4.2). The samples were collected using sterile trowel and sterile collection bags. The samples were collected from different areas within RHS. The samples were collected from the deeper layers because upper layers may contain contaminants of anthropogenic origin. Immediately after collecting the samples, the samples were placed in sterile sample containers, and zip lock bags and labelled with the location, and date. The samples were stored at 4 ℃ till further analysis (Bharti and Grimm, 2019, Tsuji *et al.*, 2019). The processing of the samples started 24 hours after the sample collection.

Figure 4.2: RHS sample site (A) Temperature check, (B) Measurement of electrical conductivity, (C) Origin of RHS water, (D) Sulphur deposition on RHS drainage.

4.3: Physiochemical analysis

The Beckmann thermometer (BeckZee, USA) was used to measure the temperature of the water (Beckmann, 1905). Multiple measurements at different depths were taken to obtain a temperature profile of the RHS. Labnox® pH meter (LabBox labware, Spain) and pH strips were used to measure the pH of the water. Electrical conductivity meter of METTLER TOLEDO (MT, India) and nephelometer (PhoenixSpec, India) was used to check the electrical conductivity and turbidity of the RHS water. The alkalinity of water was measured by titration method (Millero *et al.*, 1993). This was done by the addition of standardized solution of sulfuric acid to a water sample containing phenolphthalein indicator and recording the volume of acid used. The alkalinity was calculated and reported in terms of parts per million (ppm). The concentrations of sulphates, bicarbonates, sodium, potassium, silica, and calcium were measured in accordance with the prescribed procedures (White *et al.*, 1956, Stumm *et al.*, 1996, Nickson *et al.*, 2005). The quantification of moisture content involved subjecting 1g of soil sample to desiccation at 103º C for a duration of 30 minutes. Subsequently, the percentage moisture content of the soil was measured utilizing the following formula:

(Initial weight – dry weight) / Initial weight × 100

4.4: Optimization of metagenomic DNA (mDNA) isolation protocols

Several methodologies were implemented for the extraction of mDNA from soil and water samples, including specific protocols tailored for mitigating the presence of humic acids. The various extraction techniques employed for metagenomic DNA are illustrated in Table 4.1. The good quality mDNA from soil was achieved through the use of MO Bio's PowerSoil DNA Isolation kit (Catalog No. 12888-50) (Xiong *et al.*, 2023), and MO Bio Laboratories PowerWater® DNA Isolation Kit (Catalog No. 87321-12).

Table 4.1: Optimization of mDNA isolation protocols.

Isolation method	Humic acid removal	Reference
CTAB extraction with 1 M Nacl, and 50 % PEG	2 % $CaCl_2$ post treatment	(Singh *et al.*, 2014)
Glass bead mediated lysis	$CaCO_3$ pre-treatment	(Moré *et al.*, 1994)

Extraction with CTAB	10 % PVP	(Zhou *et al.*, 1996)
Cold lysozyme- and SDS-assisted lysis	2 % CsCl	(Smalla *et al.*, 1993)
MO BIO's PowerSoil DNA Isolation	-	https://www.qiagen.com/us
MO Bio PowerWater® DNA Isolation Kit	-	https://us.vwr.com/store/

4.5: Metagenomic DNA isolation from soil and water

Metagenomic DNA isolation from the RHS soil was conducted using a standardized kit protocol (Figure 4.3). The PowerBead tubes were employed as the primary vessel for processing, into which 2 grams of soil sample were added and homogenous mixing was done by vortexing. Subsequently, 10 mL of lysis solution was added, and the mixture was briefly vortexed. The tubes were then centrifuged at 10,000 rpm for 30 seconds at room temperature. The resultant supernatant was carefully transferred to a 2 ml collection tube. To this, 500 µl of protein denaturing solution was added, followed by a 5-second vortexing. The tubes were incubated at 4°C for 5 minutes, followed by centrifugation at room temperature for 1 minute at 10,000 rpm. A volume of 600 µl of the supernatant was carefully transferred to a fresh 2 ml collection tube, ensuring that the pellet remained undisturbed. The next step involved the addition of 500 µl of humic acid degrading solution, with subsequent brief vortexing and a 5-minute incubation at 4°C. After centrifugation at room temperature for 1 minute at 10,000 rpm, up to 750 µl of supernatant (excluding the pellet) was transferred to a clean 2 ml collection tube. For the washing step, the washing solution was prepared and added to the supernatant, with thorough mixing by shaking. To this mixture, 1200 µl of DNA precipitating solution was added, followed by a 5-second vortexing. Further, centrifugation at room temperature for 30 seconds at 10,000 rpm led to the discarding of the flow-through. DNA harvesting was accomplished through a 30-minute centrifugation at 12,000 rpm at room temperature. The harvested DNA was then washed with 70% ice-cold ethanol dissolved in 1X TE buffer. Several research studies focusing on soil metagenomics have employed MO Bio's PowerSoil DNA Isolation kit for their analyses. These studies have utilized MO BIO kits to examine the genetic material present in soil, offering a comprehensive understanding of the

microbial communities and their functions within this environment (Walden *et al.*, 2017, Videnska *et al.*, 2019, Calderon-Franco *et al.*, 2023, Sung *et al.*, 2023).

2 liters of RHS water sample was subjected to filtration utilizing a sterile polyvinylidene fluoride (PVDF) membrane filter with a bore diameter of 0.45µm and a polyethersulfone (PES) membrane filter with a bore diameter of 0.22µm. This filtration process was conducted using a vacuum manifold. The filter, containing the retained materials on the membrane, was meticulously sectioned into eight equivalent pieces. These sections were then transferred into a sterile 50 mL polypropylene tube, pre-filled with 5 mL of DNA extraction buffer. Subsequent the steps of MO Bio PowerWater® DNA Isolation Kit were similar to the protocol employed for metagenomic DNA isolation from soil sample (Bramucci *et al.*, 2021, Raza *et al.*, 2021, Byrne *et al.*, 2022).

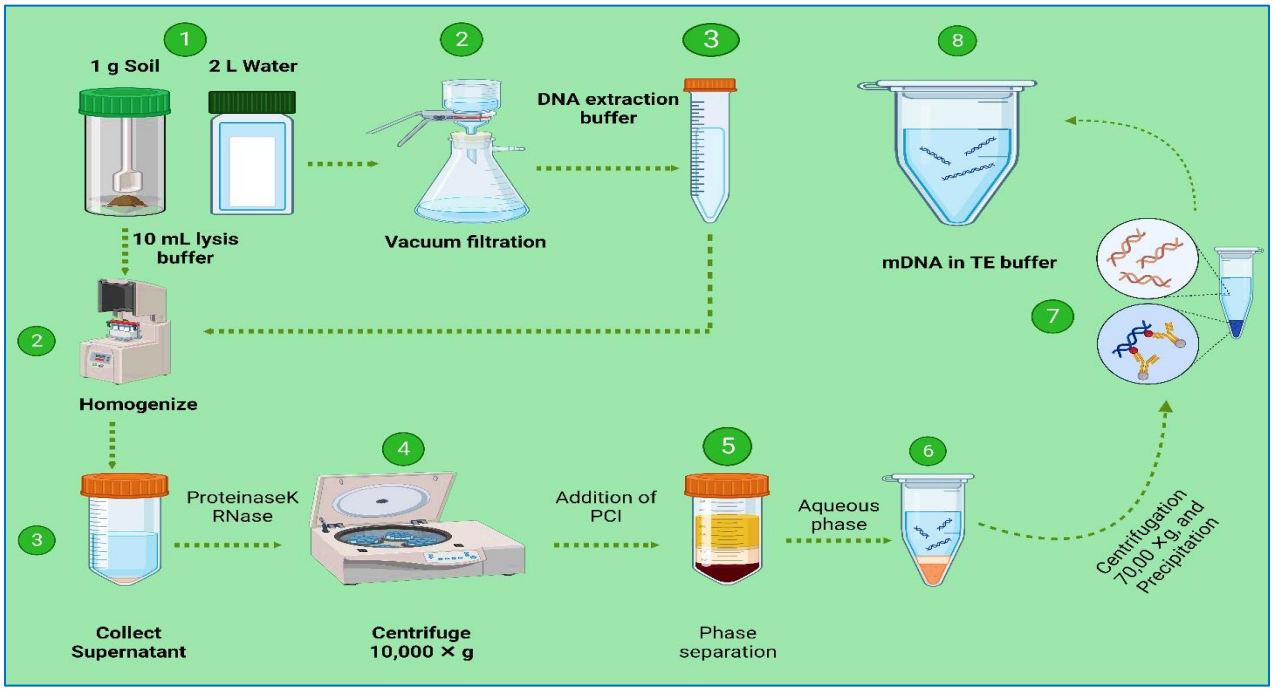

Figure 4.3: Graphical representation of the major steps involved in the extraction of mDNA from soil and water samples.

4.6: Quality check and quantification of isolated mDNA

Agarose powder (0.8%) was dissolved in 1X TAE buffer, 2 µl ethidium bromide as intercalating agent was added, and the mixture was poured into a gel mold to solidify. The gel tank was filled with 1X TAE buffer. The 5µL of the isolated mDNA samples were mixed with 1µL gel loading dye and loaded into wells along with the Uncut Lamda DNA (48.5 kb). The gel tank was connected

to a power supply, and electrophoresis was run at constant voltage (100V) until the dye front reached the bottom of the gel. The gel was placed on a UV transilluminator of gel documentation system and DNA bands were visualized under UV light. The gel image was documented using a gel documentation system (Syngene, USA) (Armstrong and Schulz, 2015).

4.7: Spectrophotometric analysis of mDNA

The spectrophotometric analysis of mDNA, isolated from soil and water, was subjected to quality check. The mDNA samples were meticulously prepared, ensuring appropriate dilution (1:100) within the spectrophotometer's linear range, with nuclease free water serving as the blank reference. The UV-Vis spectrophotometer was then set to a wavelength of 230 nm, 260 nm, and 280 nm for DNA analysis. A blank measurement was conducted by pipetting distilled water into a quartz cuvette, setting the absorbance to zero. Subsequently, DNA absorbance measurements were taken at 230 nm, and 260 nm and 280 nm to measure humic acid and protein contamination respectively. Calculations were performed to determine DNA concentration and the 230/260, and 280/260 ratio, which serves as a potential indicator for purity of DNA. The resulting data were carefully documented, including absorbance values and purity ratios.

$$\text{DNA concentration (µg/µl)} = \text{Absorbance at 260 nm} \times 50\ \text{µg/mL} \times \text{Dilution factor}$$

The total yield was obtained by multiplying the mDNA concentration by the total purified sample volume.

$$\text{DNA yield (µg)} = \text{DNA concentration} \times \text{total sample volume (mL)}$$

4.8: PCR amplification of extracted mDNA using degenerate primers for NGS

After analyzing the mDNA quality, degenerate primers targeting hydrolases (proteases, amylases, cellulases and lipases) were used for detecting the presence of hydrolases. The PCR reaction, using these primers, amplified the targeted mDNA fragments. The PCR products were then verified on 1.5 % agarose gel for further analysis. The resulting data provided insights into the presence and diversity of hydrolase genes in the RHS soil and water microbial communities. The use of degenerate primer sets in the context of mDNA analysis provided a scientifically robust strategy for selecting the most suitable mDNA samples for NGS (Smits *et al.*, 1999, Wilson and Piel, 2013).

The PCR reaction was set up at the analytical scale and scale up as mentioned in Table 4.2 (reactions conditions), and Table 4.3 (reaction mixture).

Table 4.2: Reaction conditions for PCR amplifications.

Stage	Temperature (°C)	Time
Initial denaturation	94	3 minutes
Denaturation	94	30 seconds
Annealing	55-60	45-55 seconds
Extension	72	90 seconds
Final extension	72	5 minutes
Hold	4	∞

Table 4.3: Reaction mixture for PCR amplification.

Components	Amount (µL)
Milli Q-grade water	6
PCR master mix (Buffer, DNTPs, Taq polymerase)	10
Degenerate forward primer (10pm/ µl)	1
Degenerate reverse primer (10pm/ µl)	1
Template DNA (1:10)	2

4.9: Next-Generation sequencing of RHS soil mDNA

The soil mDNA derived from RHS soil was chosen for NGS analysis following the results of PCR. The mDNA sample was sent to Biologia Pvt. Ltd., located in Karnal, Haryana, India, for NGS processing.

4.10: NGS library preparation

The paired-end sequencing library was constructed utilizing the Illumina MiSeq DNA Library preparation kit. The library construction proceeded with 200 ng of mDNA. Ultrasonication was employed to mechanically fragment the mDNA into smaller fragments, resulting in a fragment size of approximately 200 bp. Subsequently, continuous step of end-repair was executed, wherein a nucleotide 'A' was appended to the 3' ends of the DNA fragments, rendering them primed for adapter ligation. Following this step, platform-specific adapters were ligated to both ends of the DNA fragments. These adapters carries the sequences crucial for facilitating the binding of dual-barcoded libraries to a flow cell for sequencing, thereby enabling the binding of standard Illumina sequencing primers (Bragg and Tyson, 2014, Vo and Jedlicka, 2014). To ensure optimal yields from limited quantities of initial material, a high-fidelity amplification step was carried out using HiFi PCR Master Mix. The construction of metagenomic DNA library was followed by the de novo assembly of top-tier paired-end reads, which was accomplished utilizing the metaSPADES (v3.11.1) software package with default settings. Further, adapter trimming and the exclusion of reads exhibiting low quality scores below a phred score threshold of 30, scaffolds were generated. The sequencing data output files (FASTQ format) from the sequencing platform were collected for further analysis.

4.11: Trimming and filtering of sequences

FastQC (0.11.9) for trimming metagenomic sequence reads was used. The low-quality bases, adapter sequences, and other artifacts were removed from the sequencing reads, ensuring that the resulting data is of higher quality and more suitable for downstream analysis. FASTQC was used for quality control and trimming of the metagenomic data, with a Phred score threshold of 20 and a minimum read length of 50 base pairs. A sliding window of 4 bases was applied, trimming reads where the average quality fell below 20. Trimming is an essential step to improve the accuracy and reliability of the subsequent metagenomic analysis, such as taxonomic profiling and functional annotation (Schmieder and Edwards, 2011).

Short reads that fall below a certain length threshold are filtered out (<15bps). FastP (0.23.2), a versatile tool, for preprocessing and filtering high-throughput sequencing data was used. FastP allows us to filter out reads that fall below a specified length threshold. Short reads can be discarded, as they are often associated with sequencing artifacts or low-quality data. After filtering

the metagenomic sequences, which involved removal of low-quality reads, adapter sequences, and host DNA contamination, the remaining high-quality reads are typically short fragments of the original genomes or genetic material (Chen *et al.*, 2018). These short reads were assembled into longer contiguous sequences, known as contigs or scaffolds using MEGAHIT (1.2.9) with k-mer sizes ranging from 21 to 141, a minimum contig length of 200 bp, and a minimum k-mer frequency of 2. A memory limit of 60 GB was set to optimize performance for large datasets. MEGAHIT was used to reconstruct the genetic information and understand the structure and functional potential of the microbial community (Li *et al.*, 2016).

4.12: GC content estimation

The sequences were uploaded, and analysis parameters were set, including selecting the analysis type and adjusting parameters such as window size and step size for GC content calculation using GC-Profile 2.0 with default parameters. The analysis was then initiated, and upon completion, results were reviewed and interpreted. The GC content was calculated as the percentage of guanine and cytosine bases in the read (Lai and Gao, 2022).

4.13: Taxonomic analysis

The taxonomic classification algorithm i.e. r to assign taxonomic identities to the 16S rRNA gene sequences was used. The algorithm compares the sequences against a reference database i.e., SILVA v138 (https://www.arb-silva.de/) and assigns the sequences to the most likely taxonomic group based on the level of sequence similarity (Quast *et al.*, 2012). The alpha diversity metrics such as Shannon index and Chao1 index were calculated to assess the diversity of microbial communities within the RHS. The diversity and diversity indices were calculated using QIIME2 version 2022.8 (https://qiime2.org/). This version provides comprehensive tools for microbial community analysis, including diversity metrics.

The results were interpreted by identifying the most abundant taxa, the diversity of microbial communities, and the differences in community composition in RHS (Kim *et al.*, 2017).

4.14: Gene prediction using Prodigal v2.6.3

The entirety of scaffolds produced from RHS soil mDNA was subjected to gene prediction using Prodigal v2.6.3. Prodigal was used in meta mode with automatic model training and the closed-

ends option to optimize gene prediction from metagenomic data. Prodigal is an efficient software which serves as a prominent gene prediction tool, proficient in discerning genes within brief, unattributed coding sequences with remarkable precision (Hyatt *et al.*, 2010).

4.15: Construction of metagenomic library

4.15.1: Primer design

Primer3 (4.1.0) with default settings (https://primer3.org/) was used for designing of specific primers for *CarP*, and *TreS.* SnapGene (https://www.snapgene.com/) tool of latest version 7.2.1 with default settings was utilized to analyze primer properties such as Tm, GC content, and secondary structures. The primer sequences were then submitted to Integrated DNA Technologies (IDT) for primer synthesis (Table 4.4).

Table 4.4: Forward and reverse primer sequences of *CarP*, and *TreS*.

Gene	Forward primer	Reverse primer
CarP	CGC CAT ATG ATG AAC CAC GAC GCC AAG	CCG GAA TTC TCA AAC GCG AAC GCC GTA GAC
TreS	CGC CAT ATG ATG GGC GAC AAC GTC TAC CT	CCG GAA TTC TCA CGC ATC CGG CTC CTC CTC

4.15.2: Amplification of CarP and TreS

Table 4.5: PCR reaction mixture for *CarP*, and *TreS* amplification.

Components	Volume
2X MasterMix	10µL
Forward primer	1 µL
Reverse primer	1 µL
Template (1:10 v/v)	1 µL
Milli Q	7 µL
Total volume	20 µL

Table 4.6: PCR reaction conditions for *CarP*, and *TreS* amplification.

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	94	1 minute	30
Final denaturation	98	10 sec	
Annealing	66 (*CarP*), and 60 (*TreS*)	15 sec	
Initial extension	68	15 sec	
Final extension	68	3 minutes	
Hold	12	∞	

4.15.3: Purification of amplicons

After PCR amplification, the resulting amplicons (*CarP*, and *TreS*) were purified using the QIAquick Gel/PCR Extraction Kit (Qiagen). The purified amplicons were stored at -20°C for downstream applications such as sequencing, and cloning.

4.15.4. Ligation in pJET1.2 vector

Purified amplicons were used for carrying the ligation reaction with the pJET1.2 blunt end cloning vector (Figure 4.4), following the protocol outlined in Table 4.7. The ligation reaction was incubated at a temperature of 16°C for a duration of 2 hours (Wu and Wallace, 1989). The pJET1.2 vector is a derivative of the pBluescript II SK (+) plasmid featuring a unique characteristic that eliminates the need for restriction digestion. This vector contains a T/A cloning site, which means that after PCR amplification, the PCR product can be directly ligated into the vector without the need for prior restriction digestion. This is facilitated by the presence of single adenine (A) overhangs at both ends of the PCR product, which can easily hybridize with the thymine (T) overhangs present in the vector's cloning site. As a result, the PCR product can be seamlessly inserted into the vector using standard ligation techniques, simplifying the cloning process and bypassing the need for restriction digestion (Nawawi *et al.*, 2022).

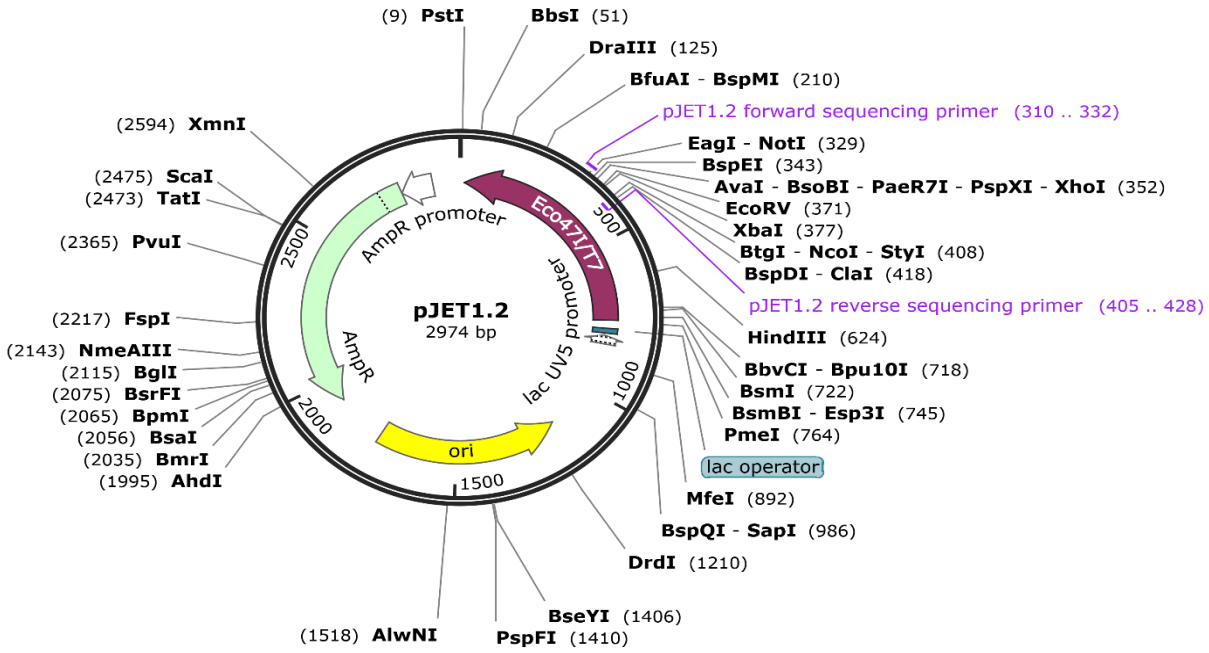

Figure 4.4: Graphical representation of the pJET.1.2 vector map.

Table 4.7: Ligation reaction.

Component	Volume
2X reaction buffer	10 µl
Purified PCR product	1 µl
pJET1.2 vector	1 µl
Nuclease free water	7 µl
T4 DNA ligase enzyme	1 µl
Total volume	20 µl

4.15.5: Competent cell preparation

The *E. coli* (DH-5α) culture with an optical density of 0.4 was inoculated into 10 ml of Luria broth. Following inoculation, the culture was kept on ice for a period of 10 minutes to induce cellular cooling. The centrifugation was performed at 12000 rpm for 10 minutes at 4 ℃, resulting in the separation of the supernatant from the cellular pellet. The obtained pellet was then gently resuspended in 1.6 ml of ice-cold 100 mM $CaCl_2$ solution by swirling on ice. The resuspended pellet was allowed to incubate on ice for a duration of 30 minutes. Further centrifugation of the

pellet was carried out at 12000 rpm for 10 minutes after resuspension in ice-cold 1.6 ml of $CaCl_2$ solution. The resulting pellet was carefully removed and stored in 0.5 ml of ice-cold 80% glycerol for further use (Chang *et al.*, 2017).

4.15.6: Transformation of competent cells

The ligated products resulting from the fusion of the *CarP*, and *TreS* amplicons with the pJET vector were subjected to transformation into *E. coli* DH5α cells for cloning purposes. *E. coli* competent cells were retrieved from a -80℃ deep freezer and allowed to thaw on ice for a duration of 5 minutes. Upon thawing, the ligation mixture was gently added to the cells and mixed through slight tapping, followed by incubation on ice for 30 minutes. A heat shock treatment lasting 90 seconds was administered to the cells at 42℃, followed by immediate cooling on ice. The cells were then incubated at 37℃ with agitation at 200 rpm in LB media. The cells were spread onto LB agar plates supplemented with ampicillin (10mg/mL) antibiotic to facilitate the selection of positive colonies. The cells were allowed to incubate overnight at 37℃ to promote the growth of recombinants (Sambrook and Russell, 2006).

4.15.7: Colony PCR for screening of recombinants

Upon observation of colony growth on the agar plate, random clones were selected and resuspended in molecular-grade water for confirming the gene of interest. For colony PCR, *CarP* and *TreS* specific primers as detailed in Table 4.4 were utilized, and reaction mixtures were prepared according to the specifications outlined in Table 4.5. Subsequently, the thermal cycling conditions were set as per the parameters provided in Table 4.6. The resultant colony PCR amplicons were subjected to analysis on a 1.5% (w/v) agarose gel. Amplicons were visualized under UV light to confirm successful amplification.

4.15.8: Plasmid isolation

A primary culture derived from the positive clone was initiated in LB media containing ampicillin antibiotic (10 mg/mL) to ensure selective growth of the positive clone. The culture was then incubated overnight at 37℃ with agitation at 200 rpm. The culture was centrifuged to pellet down cells from a 5 ml aliquot, followed by plasmid isolation utilizing the Favorgen plasmid isolation kit (Catalog # FAPDE100) in accordance with the manufacturer's protocol (Trevors, 1985). The

purified plasmid DNA obtained after elution was sent to Biologia Research India Pvt. Ltd (Haryana, India) for sanger sequencing.

4.16: Cloning in pET28a expression vector

For expression into the pET28a vector having T7lac promoter (Figure 4.5), the final positive clones were expressed and subjected to quality assessment on 1.5% agarose gel. Digestion checks of the pJET clone for all genes were conducted using restriction enzymes (*NdeI* and *EcoRI*), followed by 1.5% agarose gel electrophoresis. The final digested bands were excised from the agarose gel and purified using the QIAquick gel extraction kit (Qiagen), followed by another round of quality assessment on an 1.5 % agarose gel. The purified digested bands were then employed to set up ligation reactions with pET28a vector, which had been digested with *NdeI* and *EcoRI* as per the specifications outlined in Table 4.7. The ligation reaction mixtures were incubated at 22°C for 1 hour, followed by storage at 4°C. It was followed by transformation of ligated products in *E. coli* (*BL21-DE3*) strain as mentioned in 4.15.6.

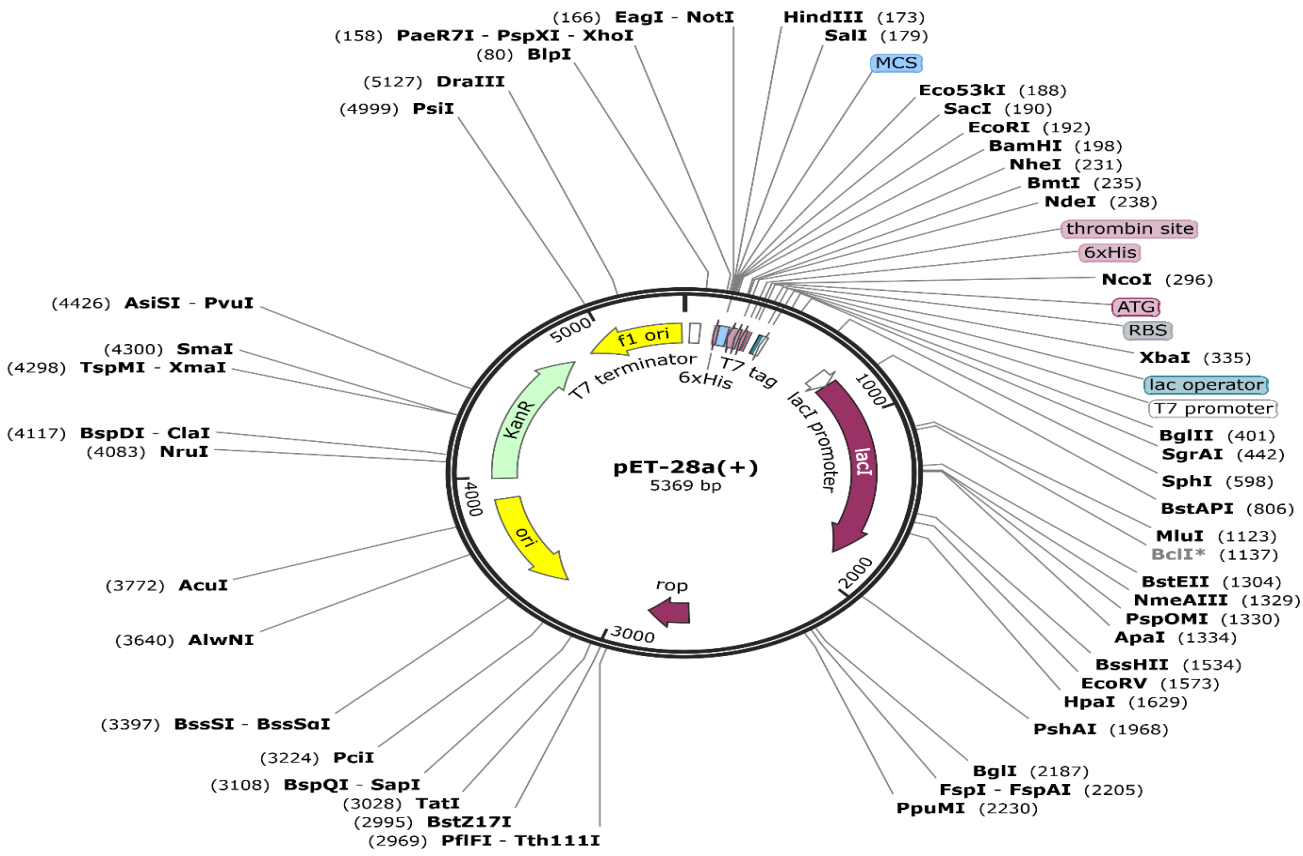

Figure 4.5: Graphical representation of the pET-28a (+) vector map.

4.17: Colony PCR of transformants

After 16 hours of incubation at 37°C, the clones were grown on LB agar plate. These clones were meticulously selected and transferred to a fresh plate, designated as the master plate, for further analysis. Colony PCR was conducted utilizing gene-specific forward and reverse primers to discern positive colonies of *CarP*, and *TreS*. The PCR products were then subjected to 1.5% agarose gel electrophoresis for visualization and subsequent confirmation of the desired *CarP*, and *TreS* inserts. Digestion checks of the pET28a clone harboring *CarP* and *TreS* genes were performed using *NdeI* and *EcoRI* restriction enzymes. The digested samples were subjected to 1.5 % agarose gel electrophoresis to assess the presence of *CarP*, and *TreS*. Following cellular transformation, a subset of cells was chosen at random and applied to LB agar plates supplemented with ampicillin (10 mg/mL), followed by 24-hour incubation period at 28°C. Further, the cellular patches were replicated onto fresh plates utilizing velveteen cloth and preserved at -20°C for subsequent analytical procedures (Sanders, 2012).

Objective 2: Screening the metagenomic library for hydrolytic enzymes.

4.18: Induction of *CarP*, and *TreS* expression

The *CarP* and *TreS* transformants were inoculated into 5 mL of LB broth supplemented with 10 mg/mL kanamycin. The culture was subjected to an overnight incubation period at 37°C under agitation at 200 rpm to facilitate optimal growth. After achieving an appropriate optical density reading at OD_{600} from the primary culture, the requisite volume of the primary culture was carefully transferred into the secondary culture medium. This was followed by the addition of 1 mM IPTG to the secondary culture to initiate the induction of protein expression. Under controlled conditions of 37°C and 200 rpm agitation, the secondary culture monitored over an incubation period of 18 hours, with periodic measurements of OD_{600} to gauge cellular growth dynamics (Larentis *et al.*, 2014, Kielkopf *et al.*, 2020). On completion of the induction phase, the bacterial cultures were subjected to harvesting process through centrifugation at 6000 rpm at 4°C for a duration of 10 minutes. The resulting cellular pellets were subsequently resuspended in a protein isolation buffer characterized by a composition of 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 5% glycerol, and 0.5% NP-40, ensuring optimal conditions for subsequent protein extraction and downstream analysis.

4.19: Molecular weight determination using SDS-PAGE

For the SDS-PAGE analysis, the resolving gel was meticulously prepared by blending deionized water, 10% (w/v) SDS, Tris-HCl buffer (pH 8.8), and TEMED to compose a 40 mL separating gel with a concentration of 12%. Following the amalgamation, the mixture underwent a 10-minute deaeration period. Post deaeration, 10% (w/v) ammonium persulfate (APS) was swiftly incorporated, and the resultant solution was swiftly poured to initiate the gel polymerization. The stacking gel was prepared using a spacer gel with a concentration of 5%. This involved the preparation of a stock gel solution comprising 1M Tris-HCl buffer (pH 6.8), DDW, 10% (w/v) SDS, 10% (w/v) APS, and TEMED. The resultant spacer gel was overlaid onto the polymerized separating gel, with an immediate insertion of a comb between the glass plates to facilitate polymerization. Prior to loading, the protein samples underwent denaturation by subjecting them to a 15-minute incubation at 100°C in SDS-containing protein loading dye. The gel slab was carefully positioned into the electrophoresis apparatus, and the protein samples were loaded into their designated wells along with the protein marker (Bis-Tris 10%). Electrophoresis was executed at 100 volts until the front indicator dye, Bromophenol blue, traversed the gel and reached the bottom. Upon completion of electrophoresis, the process was arrested, and the gel slab was extracted from the electrophoresis apparatus. The gel, confined between the glass plates, was gently removed, and subjected to staining with Coomassie Brilliant Blue R-250 for a duration of one hour. After staining, destaining was carried out using methanol, water, and acetic acid solution in a volumetric ratio of 45:45:10 (v/v%) until the gel attained transparency, thereby revealing discernible blue bands indicative of separated protein components (Harithpriya *et al.*, 2024).

4.20: Primary Screening of clones

4.20.1: CarP

The primary screening of *CarP* protease clone was done using 1% (w/v) skimmed milk as substrate. A single line was streaked on LB agar media, and incubated overnight at 37 °C. After incubation, the plates were examined for zone hydrolysis along the streaked line, indicating protease activity manifested by hydrolysis of protein in the skimmed milk (Tennalli *et al.*, 2022).

4.20.2: TreS

The primary screening of the *TreS* amylase clone was conducted utilizing 1% (w/v) starch as the substrate. A singular line was streaked onto LB agar media, followed by a 28 hours of incubation period at 37 °C. After the incubation phase, the plates were inspected for zone of hydrolysis along the streaked line after staining with Gram's iodine solution indicative of amylase activity facilitating the hydrolysis of starch molecules (Schepper *et al.*, 2021).

4.21: Secondary Screening for enzyme localization

Following the completion of primary screening for *CarP* and *TreS* enzymes, secondary screening procedure was initiated to further assess their activity. The supernatant and cell-free extract obtained from the cultures were subjected to evaluation for enzyme localization using the standard agar diffusion method (Yassin *et al.*, 2021, Aguilera-Toro *et al.*, 2023). The samples were examined for the presence of zones of clearance, indicative of enzymatic activity, to ascertain the distribution and localization of the enzymes within the culture medium.

4.21.1: CarP

2% (w/v) LB agar medium was prepared, and 1% (w/v) skimmed milk was added and thoroughly dissolved. 20 mL of the media was dispensed into sterile plastic petri plates and allowed to solidify. Upon solidification, 5 mm diameter holes were bored into the agar using a sterile tool. Following this, 10µL aliquots of CFE, supernatant, lysis buffer (50 mM Tris, 1% glycerol, 0.1% Triton X-100, and 50 mg/mL lysozyme), positive control (proteinase K), and broth (negative control) were carefully loaded into their respective wells. The plates were then incubated at 37°C overnight to allow for the visualization of clear zone of hydrolysis formed due to hydrolysis activity. This assay serves to evaluate the enzymatic hydrolysis capability of the *CarP* clone.

4.21.2: TreS

2% (w/v) LB agar medium was meticulously prepared, and 1% (w/v) starch was incorporated and thoroughly dissolved. 25 mL of the resulting media was dispensed into sterile plastic petri plates and allowed to solidify. Once solidified, 5 mm diameter holes were precisely bored into the agar using a sterile tool. Following this, 10µL aliquots of CFE, supernatant, lysis buffer (50 mM Tris, 1% glycerol, 0.1% Triton X-100, and 50 mg/mL lysozyme), positive control (amylase), and broth (negative control) were meticulously loaded into their respective wells. The plates were then incubated at 37°C overnight to facilitate the visualization of zone of hydrolysis resulting from

enzymatic hydrolysis activity. The clear zones were made visible post-incubation through the addition of Gram's iodine solution. This assay serves to assess the enzymatic hydrolysis capability of the *TreS* clone and controls, aiding in the characterization of their enzymatic properties.

Objectives 3: DNA Sequencing and analysis of the gene encoding the enzyme from positive metagenomic clone.

4.22. Sequencing

The purified pET28a containing *CarP* and *TreS* gene fragments were sent to Biologia Pvt. Ltd, Haryana, India, for Sanger sequencing. The sequencing was conducted using the iSeq 100 System, an advanced platform developed by Illumina. This system involves library preparation, adapter ligation, and cluster generation. The fluorescently labeled nucleotides are added one at a time to DNA clusters, and the emitted signals are used to determine the sequence (Kim *et al.*, 2021).

4.23. Sequence translation

The nucleotide sequence of *CarP* and *TreS* was translated and resulted in the conversion of these sequences into their respective protein primary sequences. This was achieved using the TranslatorX 15.0 (2) (Abascal *et al.*, 2010), and GEMBASSY (Itaya *et al.*, 2013) under default settings.

4.24. Phyogenetic relationships

The Identification of closely related *CarP*, and *TreS* sequences and preservation of domains were executed through NCBI BLASTp and CD tools (Giraldo-Forero *et al.*, 2012). The evolutionary relationship of *CarP*, and *TreS* gene extracted from RHS mDNA in diverse organisms was investigated using MEGA11 (Tamura *et al.*, 2021) , and iTOL (https://itol.embl.de/) with default parameters.

4.25. Structural analysis

The ExPASy was employed to predict amino acid quantity, molecular weight, isoelectric point, atomic composition, estimated half-life, instability index, and aliphatic index with default settings (Artimo *et al.*, 2012). The Grand average of hydropathicity (GRAVY) was assessed to determine the overall hydrophobicity or hydrophilicity of the protein sequence. The dihedral angles φ (phi) and ψ (psi) of *CarP*'s and *TreS* amino acid residues in its polypeptide chain were represented

through a Ramachandran plot using PROCHECK (Laskowski *et al.*, 1993) and PDBsum (Laskowski *et al.*, 1997). Phyre2, a web-based tool, was used for predicting and analyzing protein structures. It employs homology modeling and ab initio modeling techniques to generate 3D structures based on input protein sequences (Kelley *et al.*, 2015). BUSCA (http://busca.biocomp.unibo.it) was utilized to discern the subcellular localization of the protein, and DeepTMHMM (Teufel *et al.*, 2022) predicted the topology and transmembranes. PSIPRED was employed for predicting the secondary structure, encompassing alpha helices, beta strands, and random coil regions, from the amino acid sequences (Buchan and Jones, 2019). The 3D structures of *CarP* and *TreS* were analyzed to identify important structural features such as domains, active sites, and binding pockets (Chakrabarti *et al.*, 2005). Table 4.8 provides the list of web tools and softwares used for computational analysis of *CarP*, and *TreS*.

Table 4.8: In silico analysis of *CarP*, and *TreS* using different computational software/web tools.

Software/Web tool	Version	Analysis	Reference/Link
Expasy ProtPram	Standalone	Evaluation of physical and chemical protein parameters	https://web.expasy.org/protparam/
FastTree	2.1	Phylogenetic relationship	(Price *et al.*, 2010)
PSIPRED	4.0	Secondary structure analysis	http://bioinf.cs.ucl.ac.uk/psipred/
Phyre2	2.0	3D structure prediction	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
PDBsum	Standalone	3D structure prediction	https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/
PyMoL	3.0	3D visualization	(DeLano, 2002)
PROCHECK	v.3.5.4	Stereochemical quality of a protein structure	https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/
DeepTMHMM	1.0	Prediction of transmembrane helices	https://dtu.biolib.com/DeepTMHMM
BUSCA	3.0	Sub cellular localization	https://busca.biocomp.unibo.it/
ProSA	Standalone	Z-score assessment	https://prosa.services.came.sbg.ac.at/prosa.php

Objective 4: Biochemical characterization of selected hydrolytic enzymes

4.26: Purification of *CarP*, and *TreS*

The His-Tagged Bacterial Protein Purification Kit by HiMedia (HiGenoMB, MBPP005SP) was used to purify *CarP* and *TreS* proteins. This kit comprises a Ni-NTA agarose spin column, 10X Buffer solution, and 3M Imidazole. Specifically, the kit provided pre-packed columns filled with 0.2 ml of Ni-NTA Agarose resin in a 30% ethanol solution, which were conveniently ready for immediate use.

The bottom cap of the column was taken off and positioned into the 2 ml collection tube. It was then centrifuged at 500 rpm for 1 minute to facilitate the removal of the preservative. The spin column underwent equilibration with 0.4 ml of equilibration Buffer (consisting of 10X Buffer and 3M Imidazole) and was manually agitated. Subsequently, centrifugation at 500 rpm for 1 minute ensued, and the resulting flow-through was discarded. Particular attention was paid to prevent drying of the resin bed. The outlet of the spin column was sealed with a cap, and the sample (CFE) containing the HIS-tagged *CarP* and *TreS* was introduced from the top of the spin column for purification. The lid was closed, allowing the samples and resin to interact for a minimum of 30 minutes before the lower cap was removed. Following this, centrifugation at 500 rpm for 1 minute was conducted, and the resulting flow-through was collected. The spin column was relocated to a fresh collection tube, and 0.4 ml of Wash Buffer (10X Buffer and 3M Imidazole) was introduced from the top to eliminate any proteins not bound to the column. After, centrifugation at 500 rpm for 1 minute, the resulting flow-through was discarded. This washing procedure was iterated twice, amounting to a total of three wash cycles. The spin column was relocated to a fresh collection tube, and the column outlet was sealed with a cap. Subsequently, 0.4 ml of elution buffer was introduced, and the lid was tightly secured. The contents were then thoroughly mixed for 10 minutes before removing the bottom cap. Following this, centrifugation at 500 rpm was performed for 1 minute, and the eluate was collected. This elution process was reiterated twice. The eluates were assessed using protein estimation assays and SDS-PAGE as described in section 4.19.

4.27: Protein estimation using BSA standard curve.

The CFE of the positive clones containing the candidate genes for *CarP* and *TreS* was prepared as described in section 4.21. The concentration of the CFE was determined using a standard curve established with BSA.

A stock solution of BSA at a concentration of 1 mg/mL was prepared, and different dilutions of the protein were made. Then, 1 mL of Bradford reagent was added to each mixture and mixed well. The tubes were allowed to sit at room temperature for 5 minutes before measuring the absorbance at 595 nm. A graph was plotted correlating the absorbance values with the concentrations of BSA (Kruger, 2009).

4.27.1: Protease assay

Narendra 's method was modified and employed for protease assay, using the CFE as the enzyme source. A tyrosine calibration curve was generated by measuring the absorbance of free tyrosine that reacts with Folin's reagent under alkaline conditions (Figure 4.6). Dilutions of pure tyrosine were prepared from a 1.1mM stock solution. To each dilution, 1 mL of Tris buffer was added, followed by 2 mL of NaOH and 500 µL of a 1:4 diluted Folin's reagent. Absorbance was recorded at the end point of 620 nm, and the curve was plotted to relate absorbance to tyrosine concentration (Narendra *et al.*, 2012).

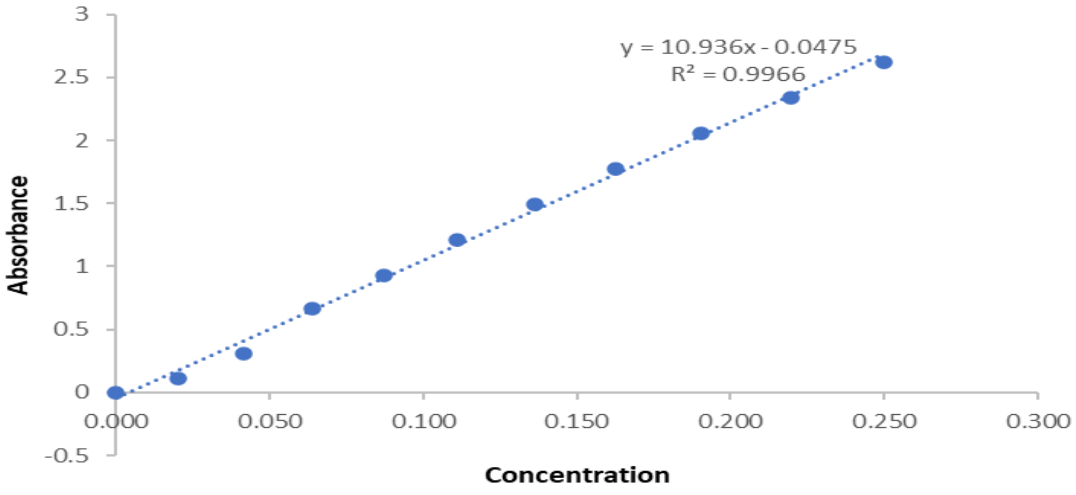

Figure 4.6: Tyrosine standard curve for determination of *CarP* activity.

4.27.2: Estimation of protease (CarP) activity

A test tube containing 1 mL of 1% casein (substrate) mixed with 50 mM Tris buffer (pH 7.5) was prepared, and 50 µL of culture supernatant (enzyme) was added. The mixture was incubated for 30 minutes at room temperature. To stop the reaction, 2 mL of TCA was added, and the precipitates were removed via centrifugation at 2000 rpm. Next, 0.5 mL of the reaction supernatant was combined with 0.5 mL of 50 mM Tris-HCl (pH 7.5), followed by the addition of 2 mL of NaOH and 0.5 mL of Folin's reagent. The mixture was then incubated in the dark for 20 minutes before measuring the absorbance at 620 nm. The amount of tyrosine released during casein hydrolysis was quantified using a tyrosine standard curve.

One international unit (IU) of protease activity was defined as the amount of enzyme required to liberate 1 µM of tyrosine from casein at room temperature within 30 minutes.

$$\text{Enzyme activity} = \frac{\text{Product concentration (mg)} \times \text{Total reaction volume (ml)}}{\text{Molecular weight} \times \text{Enzyme added} \times \text{incubation time}}$$

4.27.3: Amylase assay

McCleary's method was employed for amylase assay, using the CFE as the enzyme source. A maltose calibration curve was generated by measuring the absorbance of free maltose that reacts with DNS reagent under alkaline conditions (Figure 4.7). Dilutions of pure maltose were prepared from a 1.1mM stock solution. To each dilution, 1 mL of Tris buffer was added, followed 1:5 DNS. Absorbance was recorded at the end point of 540 nm, and the curve was plotted to relate absorbance to tyrosine concentration (McCleary and Sheehan, 1987).

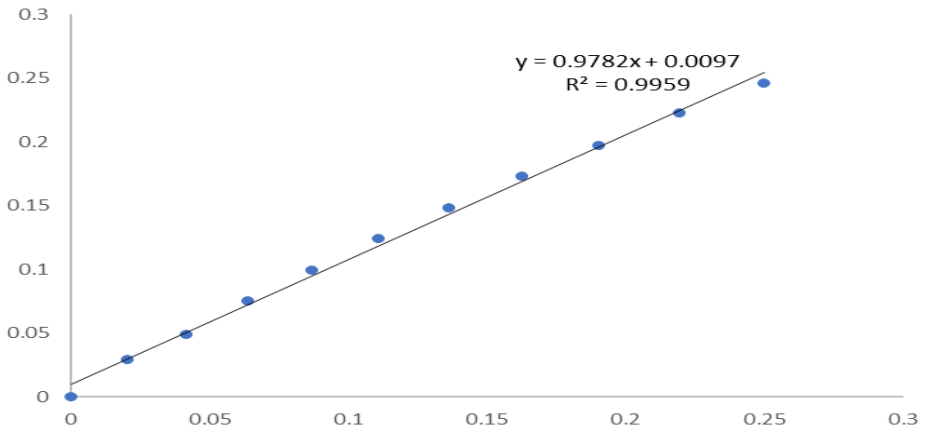

Figure 4.7: Maltose standard curve for determination of *TreS* activity

4.27.4: Estimation of amylase (TreS) activity

A test tube containing 1 mL of 1% starch (substrate) mixed with 50 mM Tris buffer (pH 7.5) was prepared, and 50 µL of culture CFE (enzyme) was added. The mixture was incubated for 30 minutes at room temperature before measuring the absorbance at 540 nm. The amount of maltose released during starch hydrolysis was quantified using a maltose standard curve.

4.28. Biochemical characterization of *CarP*, and *TreS*

The purified *CarP* and *TreS* enzymes were characterized in terms of their optimal and stable temperature and pH ranges, as well as their interactions with metal ions, surfactants, substrate specificity, and inhibitors. The assays for these experiments were conducted using 1% casein as the substrate for *CarP* and 1% starch for *TreS*, with tyrosine and maltose release measured at 620 nm and 540 nm, respectively. All experiments were performed in triplicate and repeated three times to ensure the reliability of the results.

4.28.1. Effect of temperature

The effect of temperature on *CarP* enzyme activity was measured spectrophotometrically by incubating the enzyme with casein for 30 minutes at a range of temperatures from 10- 110 °C. *CarP*'s thermal stability was evaluated by first incubating the enzyme with Tris buffer for 30 minutes at various temperatures, followed by an incubation with casein.

The effect of temperature on *TreS* enzyme activity was measured spectrophotometrically by incubating the enzyme with starch for 3 hours at a temperature range of 10- 110 °C. The thermal stability of *TreS* was evaluated by first incubating the enzyme with Tris buffer for 30 minutes at various temperatures, followed by an incubation with starch.

4.28.2. Effect of pH

The influence of pH on the activities of *CarP* and *TreS* was quantified by measuring the release of tyrosine and maltose, respectively, after incubating *CarP* with casein and *TreS* with starch for 30 minutes at varying pH levels. The pH values were adjusted using different buffers at a concentration of 50 mM: trisodium citrate (pH 3-5), potassium dihydrogen phosphate (pH 6-7.7), Tris buffer (pH 7.5-9), and glycine-NaOH (pH 10-12).

The pH stability of the *CarP* and *TreS* enzymes was assessed by pre-incubating the enzymes with 50 mM buffers across a pH range of 3-12 for 3 hours, followed by incubation with their respective substrates for an additional 30 minutes.

4.28.3: Effect of metal ions

This involved incubating *CarP* and *TreS* enzymes with their respective substrates, casein and starch, in the presence of different divalent metal ions (Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Na^{2+}, CO^{2+}, Cu^{2+}, and Ni^{2+}) at concentrations of 5 mM and 10 mM for 30 minutes (*CarP*) and 3 hours (*TreS*). The total activity of the enzymes was quantified by measuring the release of tyrosine and maltose. Statistical analyses, including standard deviation, standard error, and *p*-value calculations, were performed to assess the data reliability and significance.

4.28.4: Effect of surfactants

To assess the impact of surfactants on the activity of *CarP* and *TreS*, the enzymes were tested under standard assay conditions in the presence of Tween-20, Tween-80, Triton X-100, and SDS at concentrations of 5% and 10%. The enzyme activity was evaluated while using these surfactants to observe how they influence the performance of *CarP* and *TreS*. Statistical analyses, including standard deviation, standard error, and *p*-value calculations, were performed to assess the data reliability and significance.

4.28.5: Effect of inhibitors

The effect of inhibitors on *CarP* activity was assessed by PMSF, DTT, EDTA, β-ME, and H_2O_2 at concentrations of 5 mM and 10 mM. The effect of inhibitors on *TreS* was assessed by acarbose, miglitol, sulfonylurea, amylase inhibitor (chickpea), and amylase inhibitor (papaya seeds) at concentrations of 5 mM and 10 mM (Hao *et al.*, 2009, Agada *et al.*, 2020). Statistical analyses, including calculations of standard deviation, standard error, and p-value, were performed to assess the reliability and significance of the data obtained.

4.28.6. Substrate specificity

The purified protease enzyme *CarP* was evaluated for its activity on five different substrates: casein, azocasein, BSA, gelatin, and fibrin, each at a concentration of 1% (w/v). All reactions were conducted at 70°C, as *CarP* exhibits maximum activity at this temperature. Statistical analyses,

including calculations of standard deviation, standard error, and p-value, were performed to assess the reliability and significance of the data obtained.

The purified amylase enzyme *TreS* was evaluated for its activity on five different substrates: starch, dextrin, amylose, amylopectin, and glycogen, each at a concentration of 1% (w/v). All reactions were conducted at 80°C, as *TreS* exhibits maximum activity at this temperature. Statistical analyses, including calculations of standard deviation, standard error, and p-value, were performed to assess the reliability and significance of the data obtained.

Chapter 5

RESULT AND DISCUSSION

Objective 1: Construction of metagenomic library from hot spring samples of Northwestern Himalayas.

5.1: Physiochemical parameters

The physiochemical properties that were measured for water and soil samples collected from RHS site included temperature, pH, electrical conductivity, total dissolved solids, and concentrations of various minerals such as calcium, magnesium, sodium, and potassium (Table 5.1). The alkalinity of water (480-494 ppm) was found to be significantly higher than the permissible level (200 ppm) as per WHO guidelines for drinking water. Since alkalinity is determined by measuring the concentration of bicarbonates and carbonates so HCO_3^- concentration (240-244 ppm) was found to be near to permissible value (250 ppm). This suggests that the water may not be safe for consumption and may require treatment or purification before it can be used for drinking. The results obtained from analyzing the physiochemical properties of RHS provided insights into the potential health benefits of RHS water and that can determine its suitability for various applications, such as drinking, bathing, or agricultural use. It also provided information about the geological and hydrological characteristics of the area surrounding the RHS. The alkalinity of RHS water, which is an important parameter to consider when assessing its suitability for various uses, including drinking, was found to be higher than the permissible level. Excess alkalinity in water can lead to a bitter taste, deposition of scale in pipes and other surfaces, and can also affect the efficacy of water treatment processes. Furthermore, high levels of alkalinity in drinking water can also have negative health effects, such as increasing the risk of dental caries (Jafar and Aldafaai, 2022). Thus, appropriate measures should be taken to ensure that it is safe for consumption.

Table 5.1: Physiochemical condition of the RHS.

Parameter	Value	Permissible level for drinking water (WHO)
Temperature air (° C)	23	-
Temperature water (° C)	65-68 °C	-
pH	7.3-8.0	6.5-8.5

Turbidity (NTU)	1.2	< 5
Alkalinity (ppm)	480-495	200
EC (µS/cm)	475-485	-
Hardness (ppm)	217	500
DO (ppm)	5.1	6.5-8
Na^+ (ppm)	30-55	200
SO_4^{2-} (ppm)	70-76	200
HCO_3^- (ppm)	240-244	250
Ca^{2+} (ppm)	70-72	100-200
K^+ (ppm)	4.2-5.4	12

5.2: Metagenomic DNA (mDNA) extraction

In this study, the mDNA extraction methodology employed exhibited a high yield of high molecular weight metagenomic DNA. The kits used showed proficient performance across diverse soil and water sources. The extraction procedure was characterized by its efficiency, temporal economy, and, notably, the consistent generation of replicable outcomes. The extracted mDNA displayed reasonably high quality across a spectrum of sample types, a crucial prerequisite for delving into microbial diversity and the establishment of a metagenomic gene repository (Zhou *et al.*, 2014). The total metagenomic DNA was directly isolated from environmental samples and subsequently subjected to analysis via 0.8 % (w/v) agarose gel electrophoresis (Figure 5.1). This methodological approach ensures the preservation of DNA integrity and facilitates accurate examination of the genetic material's composition and characteristics.

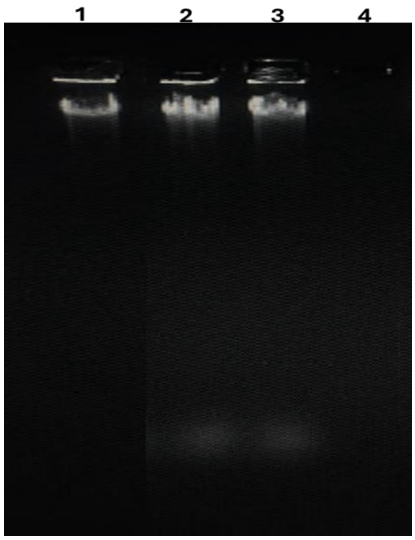

Figure 5.1: 0.8 % Agarose gel electrophoresis

of RHS mDNA; (1) Uncut λ DNA, (2) RHS soil mDNA, (3) RHS water mDNA, and (4) Empty well

5.3: Spectrophotometric analysis

The extracted high molecular weight mDNA was quantified for both yield and purity by spectrophotometric measurements based on absorbance ratios at 260/230 nm (DNA/humic acids) and 260/280 nm (DNA/protein). The purity of the extracted mDNA concerning humic acid contamination was reflected by a high 260/230 ratio, while a high 260/280 nm ratio indicated purity in relation to protein contamination (Dwivedi and Yadav, 2023).

A 260/280 nm ratio close to 1.8 indicates the presence of pure DNA, while a ratio greater than 1.8 suggested potential RNA contamination. Ratios below 1.8 for 260/280 and above 0.5 for 230/260 indicated contamination with protein and/or phenol. The ratios exceeding 1.8 for both 260/280 and 230/260 suggested the possibility of RNA and protein/phenol contamination, respectively (Olson and Morrow, 2012). The spectrophotometrically calculated DNA yield and purity are presented in Table 5.2.

Table 5.2: Spectrophotometric analysis of isolated mDNA from different samples.

Sample	A260/280	A260/230	Concentration (µg/mL)	Purified Volume (mL)	Yield (µg)
RHS soil	1.758	1.802	87.9	0.03	175.8
RHS water	1.489	1.234	74.45	0.03	148.9

5.4: PCR amplification

The RHS soil sample exhibited successful amplification when amplified with the protease and amylase degenerate primer sets; however, there was an absence of amplification with other degenerate primer sets (cellulase, and lipase). The mDNA derived from the RHS water sample did not yield positive results with any of the employed degenerate primer sets (Table 5.3). The observed differences in PCR amplification results between the mDNA of RHS soil and water samples using degenerate primer sets can be attributed to a range of factors. The degenerate primers may not cover the entire spectrum of genetic diversity within a targeted gene family. Some sequences with mismatches to the primers may be poorly or not amplified, leading to incomplete coverage. PCR primer sequences are frequently derived from amino acid sequences when the precise nucleotide sequence of the target is not known. Due to the degeneracy of the genetic code, the derived sequences may differ at one or more positions. To address this, a common approach is to employ a degenerate primer, which consists of a blend of similar primers featuring distinct bases at the variable positions (Linhart and Shamir, 2005, Keeley *et al.*, 2020).

Table 5.3: Amplification of hydrolases from soil and water extracted mDNA using degenerate primer sets.

Primer Sets (Label)	Forward primer sequence	Reverse primer sequence	mDNA soil	mDNA water
Protease				
Set (1)	AAR GCN GTN CGN GAR AAR CCN CTN	NGG NGD NGC CAT NGW NGT NCC	-	-
Set (2)	GTT GCT GCC GGT AAC GAC AAC	GTG GCC ATG GAG GTA CCG GAG	-	-
Set (3)	GCN GTN ATY GAC ACC GGC GTA TA	NGG NGT NGC CAT NGA TGT ACC GCT	+	-
Amylase				
Set (4)	CGS GTS GAY GCB GTK GCB TC	TGD ACC ACT TCR TCR TGV GA	+	-

Set (5)	GGN GGN GAY TGG CAR GGN	CAT RTC RTG RTT RTC DAT RAA	-	-
Set (6)	GAR TTY GGN CAN ATG GAY GAY TTY G	AAN CCR TCD ATN CCY TTR TCN ARC CAC C	-	-
Cellulase				
Set (7)	CGH AAY TTH GGC RAT GAC TG	GCR AAN CCY AGH TAR ACG GT	-	-
Set (8)	GGA TCA ATT TGO CAN CCC C	GGA TCA ATT TGC CAN CCC C	-	-
Set (9)	ACT TTT TGC TGY AAT GGG	GAC ATG GCC ZAY GAH CCG	-	-
Lipase				
Set (10)	ATG GGT ITN GAC TAY AAI AAC	GCG TAR TGR TCG TTG AAG ITG ACG ATR	-	-
Set (11)	GGT TIN YTA GGA CTG AAG CC	ACG TGR ITG AAY TGO AAG GC	-	-
Set (12)	GAC TGC AAT IGH TCA AAG	GGC TGR ICT YGA GAA TGC	-	-

5.5: NGS data analysis

The NGS data revealed that the aggregate sequence data amassed for subsequent bioinformatics analysis amounted to 14.2 GB (Table 5.4).

Table 5.4: NGS data

Sample	Read 1	Read 2	Total Reads	Total data
RHS soil mDNA	31692830	31692830	63385660	14.2 GB

5.6: FastQC (Trimming) and FastP (Filtering) results

The results indicated that after trimming read 1 and read 2 sequences, no sequences were identified as low quality. Moreover, the majority of sequences possessed a length of 151 bp, indicating uniformity in sequence length (Table 5.5). Additionally, the GC content of the trimmed sequences averaged at 66%, suggesting a moderate to high GC richness within the analyzed DNA fragments.

This consistent GC content across the sequences implied stability in the genomic regions under investigation and may have implications for various downstream analyses, such as gene prediction or taxonomic classification. The absence of leftover unwanted sequences or adaptors post-trimming was crucial for ensuring the purity and accuracy of the sequencing data (Figure 5.2). The removal of these artifacts minimized background noise and enhanced the reliability of downstream analyses.

Table 5.5: Trimming of read 1 and read 2 sequences.

Read_1 before trimming	
Measure	**Value**
Total sequences	31692830
Sequences flagged as poor quality	10
Sequence length	151
% GC	66
Read_2 before trimming	
Total sequences	31692830
Sequences flagged as poor quality	10
Average sequence length	151
% GC	66
Read_1 after trimming	
Total sequences	31411972
Sequences flagged as poor quality	0
Average sequence length	150
% GC	66
Read_2 after trimming	
Total sequences	31411972
Sequences flagged as poor quality	0
Average sequence length	150
% GC	66

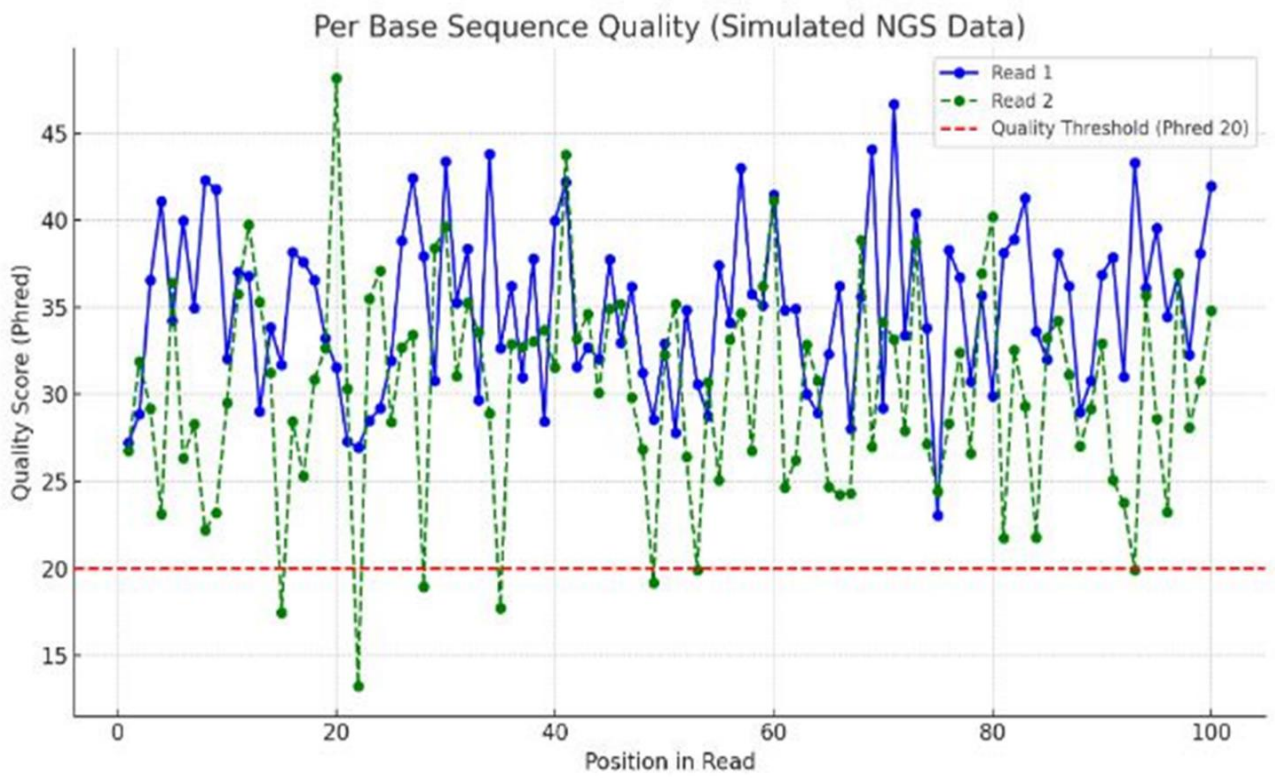

Figure 5.2: Simulated chart representing the per-base sequence quality for both Read 1 and Read 2. Each point shows the quality score (Phred score) at different base positions, with a threshold line indicating a Phred score of 20, commonly used to assess sequence quality.

5.7: Sequence assembly using MEGAHIT

After filtering the metagenomic sequences to remove low-quality reads, adapter sequences, and host DNA contamination, the resulting dataset consists of high-quality reads that are typically short fragments of the original genomes or genetic material. These short reads serve as the foundation for downstream analysis (Table 5.6). To reconstruct the genetic information and understand the structure and functional potential of the microbial community, these short reads were assembled into longer contiguous sequences, referred to as contigs or scaffolds. This assembly process allows for genome reconstruction, taxonomic assignment, functional annotation, and comparative analysis (Li *et al.*, 2016). By assembling short reads into longer sequences, it becomes possible to unlock the genetic information contained within metagenomic datasets.

Table 5.6: Assembly metrics

Metric	Value
Contigs	822682
Total contig length (bp)	4.99×10^8
Average contig size (bp)	915
Largest Contig size (bp)	56153
Contig N50 (bp)	871
GC (%)	68.54

5.8: Taxonomic analysis

The results for phylum abundance indicated that *Proteobacteria (41.03%)* were prevalent in the amplicon library. The phyla dominance was followed by *Actinobacteria* (16.7%), *Firmicutes* (16.7%), *Bacteroidetes* (7. 42%), *Planctomycetes* (7. 01%), *Chloroflexi (2. 47%),* and *Verrucomicrobia* (2.47%) (Figure 5.3). The class is dominated by *Alphaproteobacteria* (18.67%), Actinobacteria (15.02%), *Gammaproteobacteria* (10.73%), *Bacilli* (10.3%), *Betaproteobacteria* (7.3%), Clostridia (6.44%), *Deltaproteobacteria* (4.29%), and *Planctomycetia* (3.43%) (Figure 5.4). The taxonomic order is dominated by *Bacillales* (18.04%), *Rhizobiales* (14. 55%), *Clostridiales* (6.52%), and *Burkholderiales* (5.22%) (Figure 5.5). Based on the taxonomic analysis, the most dominant family found in RHS was *Bacillaceae* (13.61%) (Figure 5.6). These family belonged to the genus of *Acidothiobaccillus* (Figure 5.7). These results are consistent with previous studies on geothermal springs that have also identified these families as dominant members of the microbial community (Gebbie *et al.*, 2020, Podar *et al.*, 2020, Thakur, 2021). Most of the dominant genus and family were either unidentified or uncultured, indicating that there is still much to be learned about the microbial communities. This highlights the importance of continued research and analysis to gain a deeper understanding of the diversity and function of the microbial community in extreme environments. The Shannon index, which considers both species richness and evenness, was calculated to be 3.28, indicating high diversity within the microbial community. The Simpson index, which is a measure of dominance, was calculated to be 0.053, indicating low dominance in the community. The Chao1 index, which estimates species richness, was calculated to be 198.5, suggesting that the actual species richness may be higher than the

number of observed species. Diversity indices are essential tools for quantifying and understanding the diversity of species within ecological communities. They help measure biodiversity, assess ecological health, reveal community composition, guide conservation efforts, and provide insights into ecosystem functioning and species interactions. These indices influence conservation decisions, scientific research, and public awareness, offering a standardized and meaningful way to evaluate the richness and balance of ecosystems.

The results of the metagenome sequencing revealed the taxonomic composition of the microbial community in the RHS. The taxonomic profile of the RHS was consistent with previous studies on hot spring microbial communities (Inskeep *et al.*, 2010, Ghai *et al.*, 2013). The dominance of *Proteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* suggested that these microbial groups play important roles in the adaptation and function of the hot spring microbial community (Wani *et al.*, 2022). The presence of *Firmicutes* and *Bacteroidetes* also suggests the potential for carbohydrate metabolism and fermentation in the hot spring environment. The dominance of *Bacillaceae* and *Sphingomonadaceae* in RHS was consistent with previous studies on hot springs, which have also identified these families as important members of microbial communities (Figure 5.5) with dominant species belonging to uncultured group of bacteria (Figure 5.6). *Bacillaceae* are known to be spore-forming bacteria that can tolerate extreme conditions, making them well-suited for survival in hot springs (Harirchi *et al.*, 2022). *Sphingomonadaceae,* on the other hand, are known to be involved in the degradation of organic compounds and have been found in a variety of environments (Sharma *et al.*, 2021). Figure 4.6 represents the dominant bacterial species studied though the NGS. The results of the diversity indices suggested that the RHS microbial community is highly diverse, stable, and may contain more species than have been observed (Kim *et al.*, 2017). Further, studies using more advanced metagenome sequencing technologies may be able to shed more light on the full extent of the microbial diversity present in hot springs. Hot springs host a rich array of extremophilic microorganisms, including diverse bacteria and archaea adapted to extreme temperatures, pH levels, and chemical compositions (Burkhardt *et al.*, 2024b). These microbes contribute to biogeochemical cycles, form intricate biofilms, and offer novel genes and enzymes of industrial interest (Ma *et al.*, 2024). The varied temperature gradients within hot springs foster distinct microbial communities, shedding light on adaptation strategies to extreme conditions. The study of hot spring microbial diversity informs ecological understanding and has practical implications in biotechnology and environmental science.

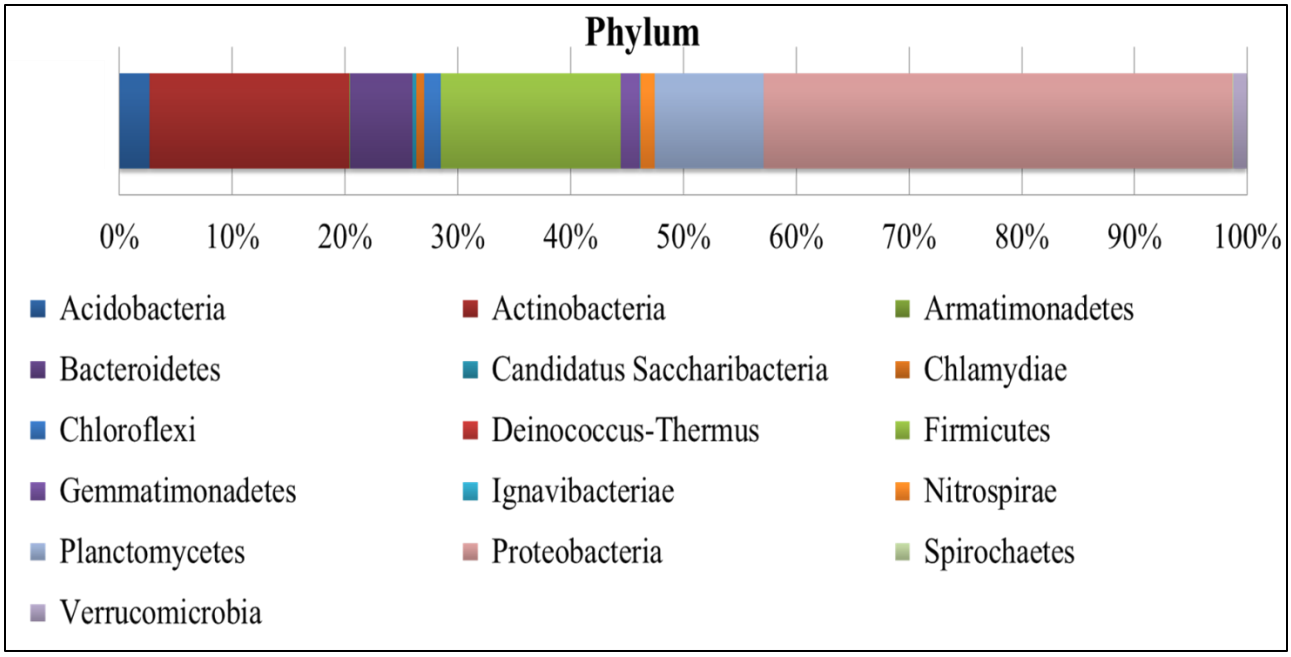

Figure 5.3: Stacked graph illustrating the distribution of major phyla, with Proteobacteria, Actinobacteria, and Firmicutes as the dominant groups

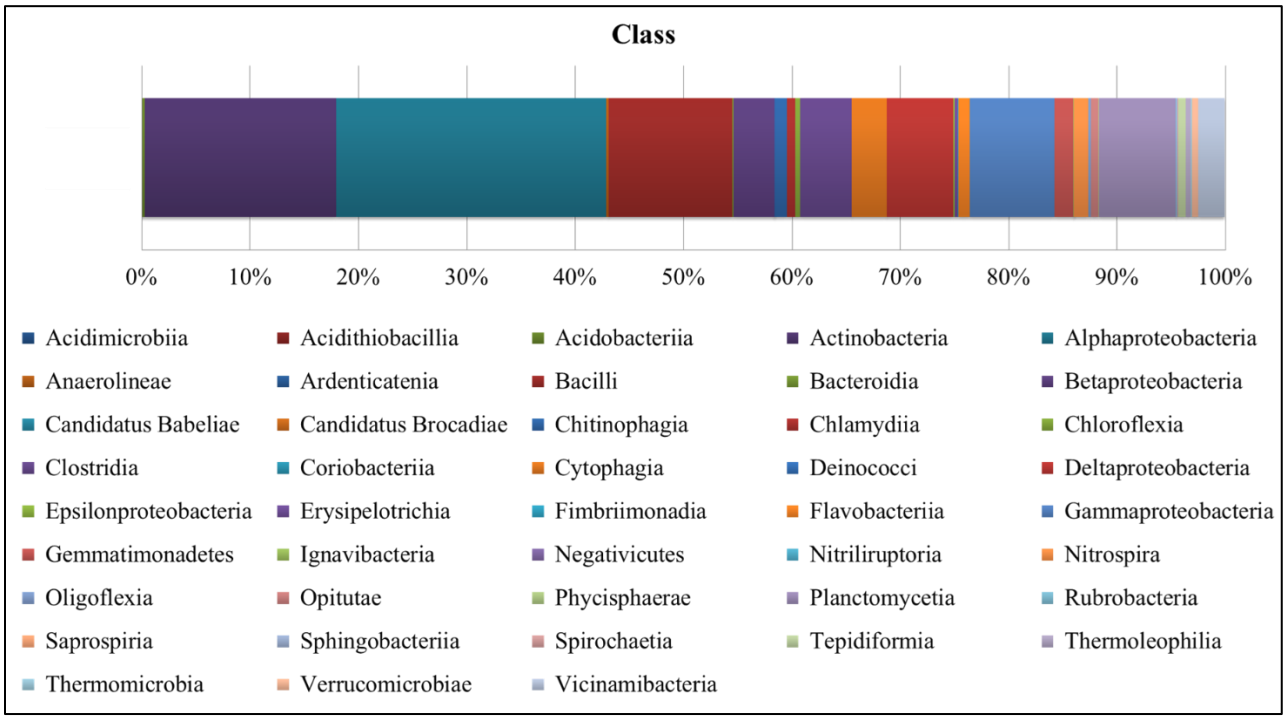

Figure 5.4: Stacked graph depicting the major classes, highlighting Alphaproteobacteria and Actinobacteria as the dominant groups.

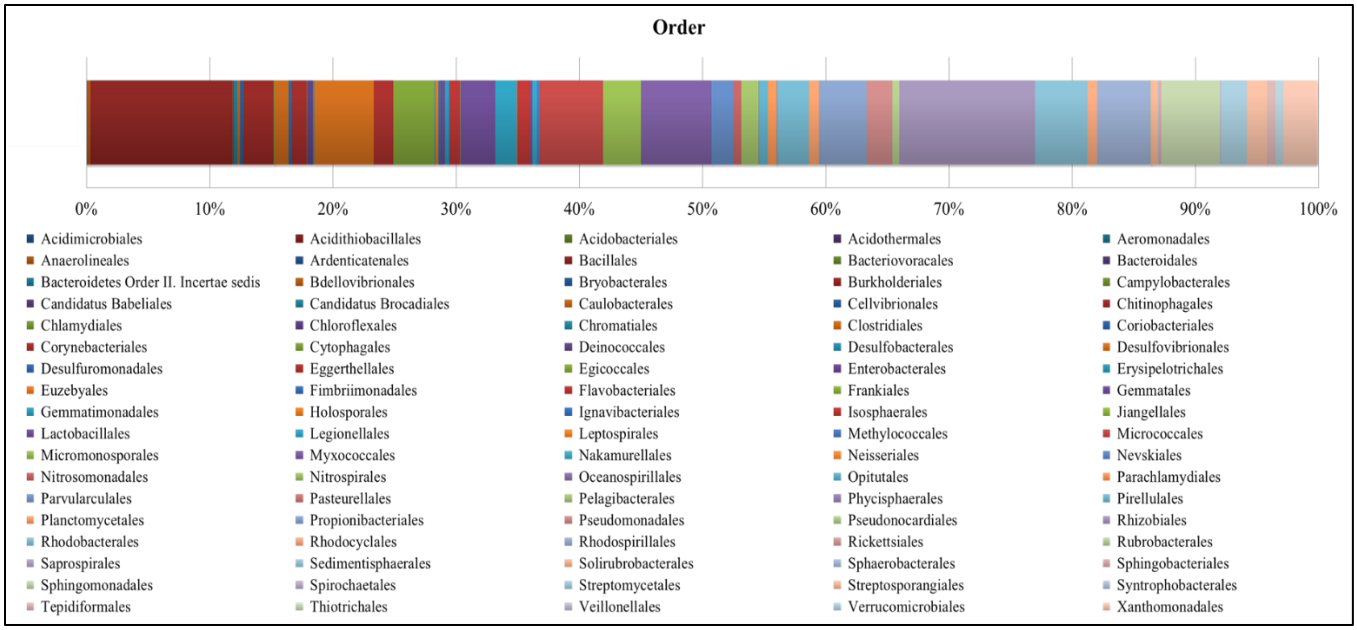

Figure 5.5: Stacked graph showing the major orders, with Bacillales and Rhizobiales identified as the dominant groups

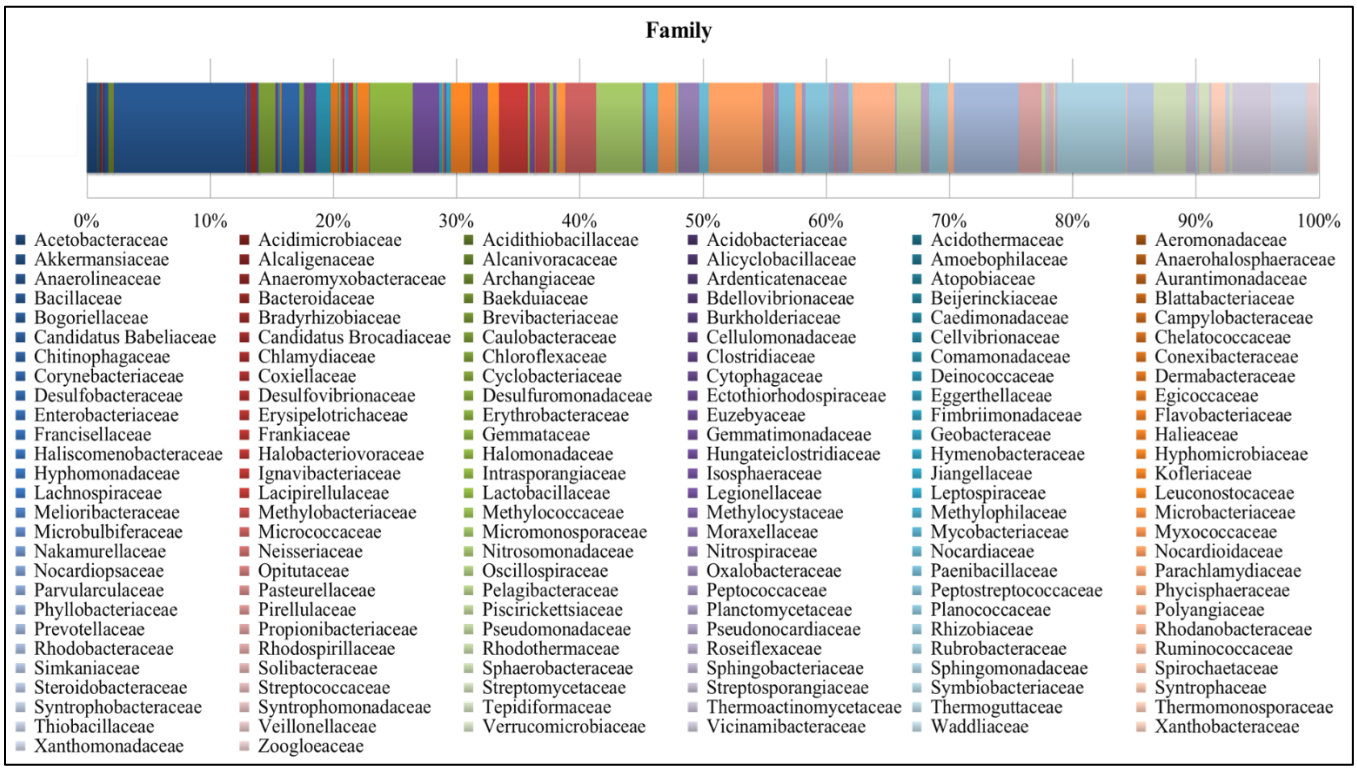

Figure 5.6: Stacked graph illustrating the major families, with Bacillaceae as dominant group

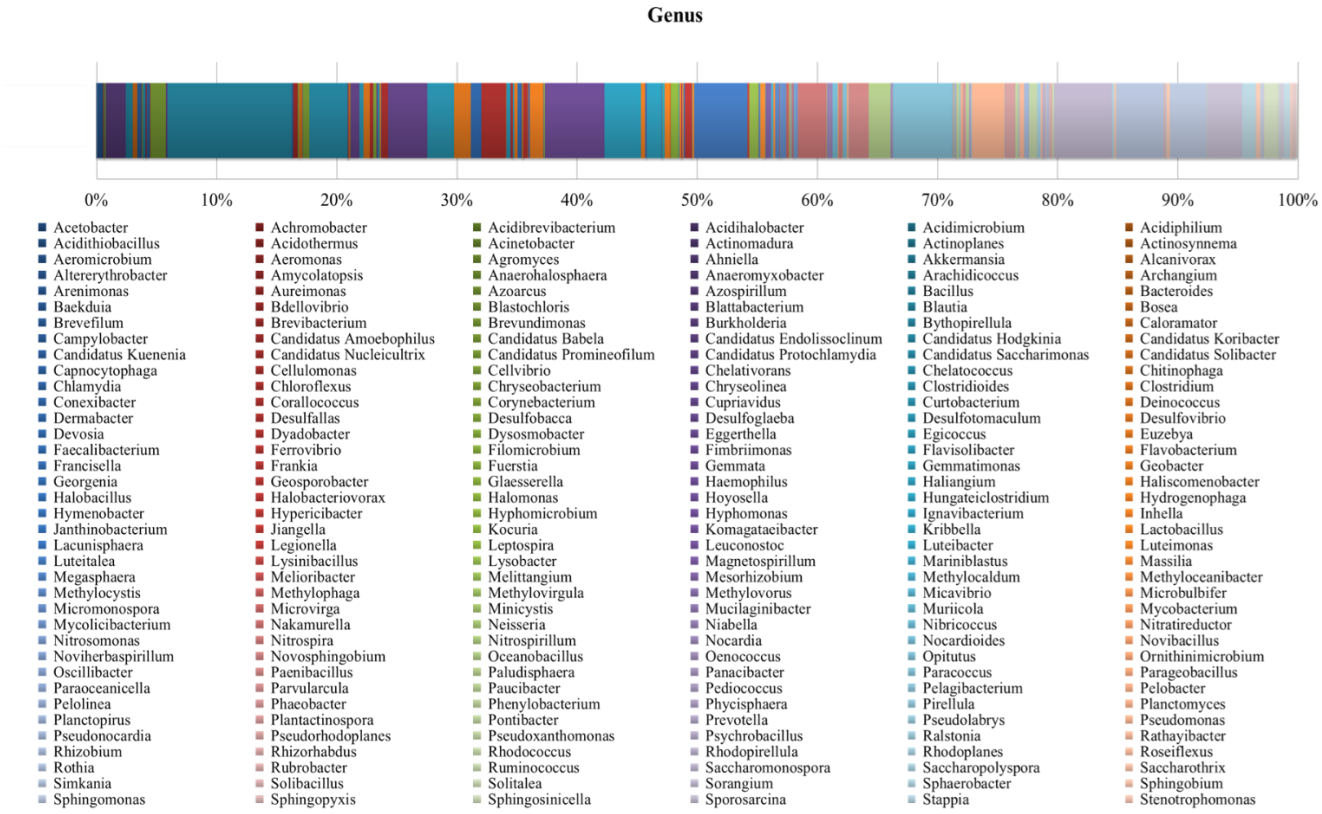

Figure 5.7: Stacked graph displaying the major genera, with Acidothiobacillus identified as the dominant genus

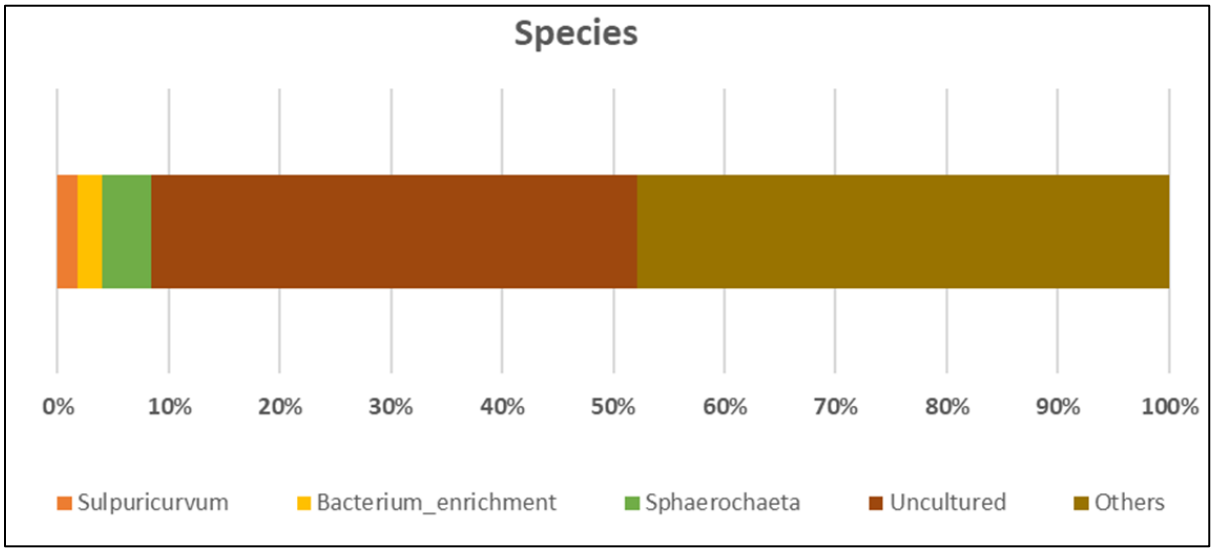

Figure 5.8: Stacked graph representing the major species, with the majority consisting of uncultured bacteria and other unidentified species.

5.9: Gene prediction, and selection

The results indicated the prediction of 452217 genes in a RHS metagenome, with a total size of 212.142064 mb. The average gene length is calculated at 469 bp. Higher gene density suggested a compact genome with genes packed closely together, whereas lower gene density implied a more spacious arrangement (Roy *et al.*, 2024). Understanding the predicted genes allows researchers to further investigate their functions, structures, and potential roles in biological processes. This can involve comparing them with known genes in other organisms, conducting functional assays, or analyzing gene expression patterns. Comparing gene predictions across different species or within related organisms can provide insights into evolutionary relationships, gene conservation, and the emergence of novel genes or gene families (Kelley *et al.*, 2012).

The genes *TreS* and *CarP* were identified as promising candidates for further investigation based on computational parameters such as bit score, e-value, number of hits, and number of gaps (Table 5.7) (Pearson, 2013). The bit score reflects the statistical significance of the alignment between sequences, while the e-value represents the expected number of similar alignments occurring by chance (Pagni and Jongeneel, 2001). Higher bit scores and lower e-values indicate stronger sequence similarity and greater confidence in the match. Selecting genes with high bit scores and low e-values ensures that the chosen sequences are closely related to known genes of interest. For gene selection, an e-value threshold typically falls in the range of 1e-5 to 1e-50 (VanderWeele and Ding, 2017). The bit score measures the similarity between sequences and is calculated based on the alignment of the query sequence with sequences in the database. Higher bit scores indicate stronger sequence similarity (Srivastava *et al.*, 2021).

Table 5.7: Gene selection for downstream analysis using computational analysis.

	Computational parameters			
Gene	**E-value**	**Bit score**	**Number of gaps**	**Number of hits**
CarP	1e-6	92	0	17
TreS	1e-7	86	0	32

5.10: Sequences and accession numbers

***5.10.1: Carp*: >OR906153.1: Uncultured bacterium clone carboxypeptidase-like gene**

GTG AAC CAC GAC GCC AAG CTG ACC GAG CTG CGG GAG CGG CTC GGT GTG ATC CTC GAC CTC AAC CGC GCG AGC GCC CTG CTC GGC TGG GAC CAG CAG ACG ATG ATG CCG CCC AAC GGC ACC GAG AGC CGG TCG TAC CAG CTG TCC ACG ATC GAC CGC TTC TCG CAC GAG CTCT TCG TCT CCG ACG AGG TTG GCC GGC TCC TGG AGG AGC TCG CGC CCT ACG AGG CCG AGC TCG ATC CCG AGT CGA TCG ACG CGG CGT TGA TCC GCG TCA CGC GGC GCG ACT ACG AGA AGG CCC GGC GCA TCC CGG CCG AGC TGC GGT CGG AGA TCA CGA AGG CCG AGG TGA TCG GGA TCC CGG CCT GGG CGG AGG CCA GGC AGA ACT CCGA CTT CGA GGC CTT CCT TCC CTA CCT GGA GAA GAA CGT GGA CCT GAA GCG CCG CTA CAT CGA GTG CTT CAA GGA CAC GGG GAA GTC GGA CTA CGA CAT CCT GCT TGA CGA CTA CCA GGA GGA CGC ACG CAG CGA GGA CAT CAC GGC GAT CTT TGA GCG GAT CAA GGA GGT CGT GAT CCC GCT CAT CCG CGA GGT CCG CGC CGC CGA TCC GGT CGA GAG CGA GTT CGC GCA CGG CCA CTA CCC GG TGG AGA AGC AGG AGC TGA TCG GGC GCG AGA TCC TCG AGC GCC TCG GCT ACT CGT CCG AGG CCT GGC GGC TCG ATC CGA CGG TAC ACC CGT TCG CGA CCT CGA TCT CCG TCG ACG ACA TCC GCC TCA CGA CGC GCT ACT CGG AGG CGG ACG CCG AGT CGG TGT TCC ACA CCG CGC ACG AGT TCG GGC ACG GGA TCT ACG AGC ACG GCA TCG ACC GGG AGC TCG AGC GGA CCC CGC TCG TGG AGT TGA ACT CGA TGG TGC TGC ACG AGT CGC AGA GCC GGC TGT GGG AGA ACC TCG TCT GCC GCT CGA AGC CGT TCT GGC GCT TCT TCT TCC CGC GGC TCC AGG AGG TCT TCC CGG AGC AGC TGA ACG GCG TGA CGG ACG AGA TGT ACT GGC GGT ACG TCA ACC GTG TCC AGC CCG ACT TCA TCC GCG TCG AGG CGG ACG AGG TCA CCT ACG GGA TGC ACA TCA TCC TGC GCT ACG AGC TGG AGC AGG ACA TCA TCG CCG GGC GGC TCG AGC CGC GCG ACC TGC CGC GCG TCT GGA ACG AGA AGA TGC AGG AGT ACC TCG GGCT CGA CGT CCC CGA CGA CGC CCG CGG CGT CCT GCA GGA CGT CCA CTG GTC GGG CGG ATC GTT CGG GTA CTT CCC GAC GTA CCT GCT CGG GAC GAT CGC CTC GGT GCA GAT CTG GGA GCG GCT GCG CGG CGA CCT GAC GGA CCT CGA CGC GCA GAT GGA GGC GGG CGA GTT CGG GGC GCT GCG CGA ATG GCT CGG CGA GCA CAT GTA CCG CTG GGG GAG GCG CTT CGC GCC GGA CGA GAT GCT CGC GCG

GAT CGT CGG CGG GCC GCT GGA CGT CGA GCC GTA TCT CGC GTA TCT GCG GTC GAA GGT CGA GGC GGT CTA CGG CGT TCG CGT TGA

5.10.2: TreS: **>OR906319.1 Uncultured bacterium clone trehalose synthase/amylase-like gene**

ATG GGC GAC AAC GTC TAC CTC GGC GAC CGC GAC GGG GTG CGC ACC CCG ATG CAG TGG ACG GGC GAC CGG AAC GGC GGC TTT TCG CGT GCG GAC TTC GCG CAG CTC TAC CTG CCG CCG CTG ATG GAC CCG GTG TTC GGC TTC CAG GCC GTC AAC GTC GAG GCG CAG CTG CGC TCC CCG ACC TCG CTG CTG CGC TGG CTG CAG CGC TTC GTC GCG CTT CGC AAG GAG CAC CCG GTC TTC GGG CTC GGG ACG TAC GAG GCG CTC GAG ACG TCG AAC CCG CGC ATC TTC GCG CAC GTG CGG CAA CAC GAC CGG GAC ACG GTG CTC TGC GTC CAC AAC CTC GCG CGG TCG GCG CAG GCG GTC GAG CTC GAC CTC GCC CGC TTC CAG GGA CGG ACG CCG ATC GAG ATG ACG GGG AGG ACC CCG TTC CCG TCGA TCG GCG ACC TGC CGT ACC TGC TCA CGT TCG GGC CGC GTG GCT TCT TCT GGT TCCT GCT GCC GGA GGA GGA GCC GGA TGC TGA

5.11: Amplification of *CarP*, and *TreS*

The amplification successfully yielded a prominent band of *CarP*, and *TreS* at the expected size of 1515 bp and 510 bp respectively on the 1.5 % agarose gel (Figure 5.9). The successful amplification of *CarP* and *TreS* genes with the RHS metagenome indicated their wide distribution within the microbial communities. The consistent presence of these genes suggested their importance and prevalence within the studied ecosystems (Suenaga, 2012).

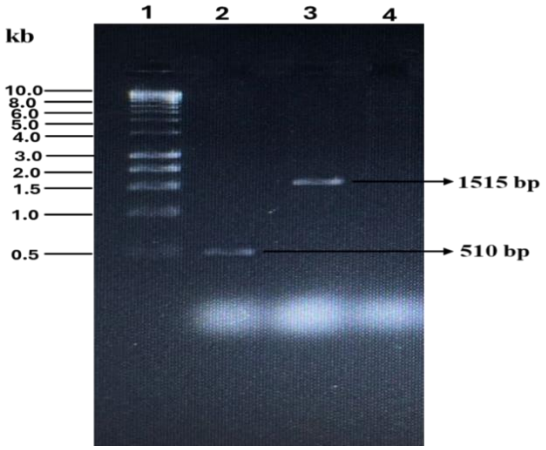

Figure 5.9: Agarose (1.5%) gel electrophoresis of purified amplicons. Lane 1: 1 kb Ladder, Lane 2: *CarP*, Lane 3: *TreS,* and Lane 4: Blank.

5.12: Ligation, and transformation

CarP and *TreS* genes were successfully ligated into the pJET1.2 vector which is pivotal in downstream processes. These recombinant clones were ligated in DH5-α, facilitated by the presence of an ampicillin-resistant gene in the vector. Upon transformation, the use of LB agar plates supplemented with ampicillin (10mg/mL) provided a robust indicator of successful cloning and transformation, as only transformed cells carrying the recombinant plasmids containing the ampicillin resistance gene were grown under selective condition (Figure 5.10). This transformative process enabled the generation of substantial quantities of the *Carp*, and *TreS* sequences, facilitating downstream applications such as sequencing, protein expression, and functional studies. Notably, the utilization of DH5- α cells as the host strain ensured a heightened efficiency in pJET1.2 uptake, thus augmenting the probability of successful transformation and subsequent gene expression (Balasubramani *et al.*, 2011). Importantly, the method yielded a higher percentage of recombinant clones compared to conventional techniques and TA cloning. This achievement lays a robust foundation for forthcoming investigations aimed at unraveling the roles and functionalities of *CarP* and *TreS* genes.

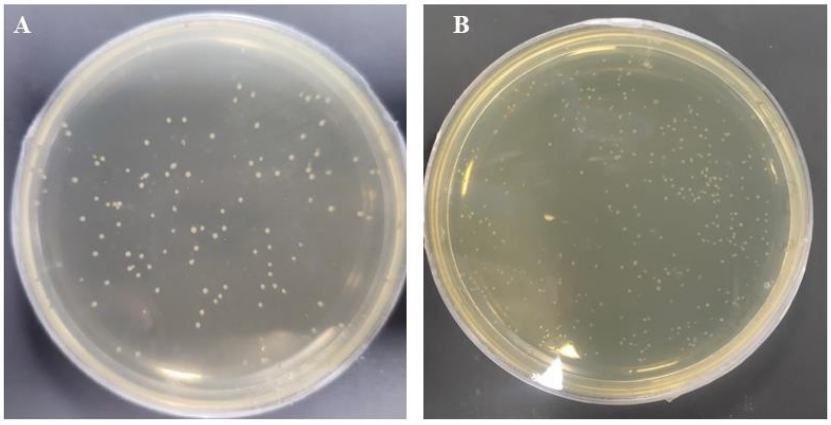

Figure 5.10: Transformation of DH5- α with pJET1.2 vector containing *CarP* and *TreS* (A) *CarP* recombinants (B) *TreS* recombinants.

5.13: Plasmid isolation, and colony PCR

The pJET1.2 plasmid DNA, with a length of 2979 bp, was successfully isolated from recombinant clones. Three clones were selected for the amplification of *CarP* (1515 bp) and *TreS* (510 bp) genes. The successful isolation of the pJET1.2 plasmid from random clones demonstrates the

stability and presence of the vector within the transformed DH5- α population. Further, the equal selection of clones for *CarP* and *TreS* gene amplification ensured consistency in the experimental approach, reducing potential biases. The observation of bands corresponding to the expected sizes of *CarP* and *TreS* genes on the 1.5 % agarose gel confirmed the successful amplification of the target genes (Figure 5.11). The sizes of the PCR products aligned with the expected lengths based on their respective gene sequences, indicating accurate amplification. Overall, these results indicate that the pJET1.2 vector effectively harbors the *CarP* and *TreS* gene inserts. This sets the stage for further downstream applications (Naufal *et al.*, 2019).

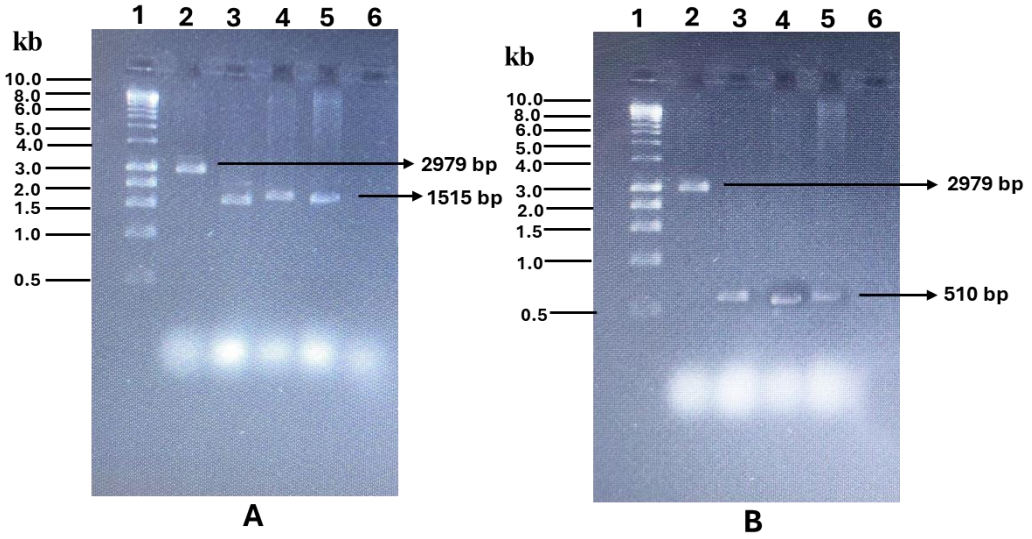

Figure 5.11: Agarose (1.5 %) gel electrophoresis of recombinants. In A: Lane 1 is ladder, Lane 2 is plasmid DNA, Lane 3-5 are recombinants of *CarP*, and Lane 6 is blank. In B: lane L is ladder, Lane 2 is plasmid DNA, Lane 3-5 are random recombinants of *TreS*, and Lane 6 is blank.

5.14: Cloning in pET-28a (+) expression vector

The cloning of *CarP* (1515 bp) and *TreS* (510 bp) into pET-28a (+) expression vector (5369 bp) was successfully achieved. Restriction digestion of pET-28a (+) with *NdeI* and *EcoRI* resulted in the release of the insert fragments (*CarP* and *TreS*) (Figure 5.12). The appearance of distinct bands corresponding to the *CarP* and *TreS* inserts confirmed the successful release of these fragments from the plasmid backbone upon restriction digestion. The sizes of the inserted fragments were within the range compatible with the pET-28a (+) vector, ensuring stability and proper functioning of the recombinant plasmid. The successful digestion of the vector with *NdeI* and *EcoRI* enzymes

demonstrated the specificity of the restriction enzymes in cutting at their respective recognition sites within the vector sequence.

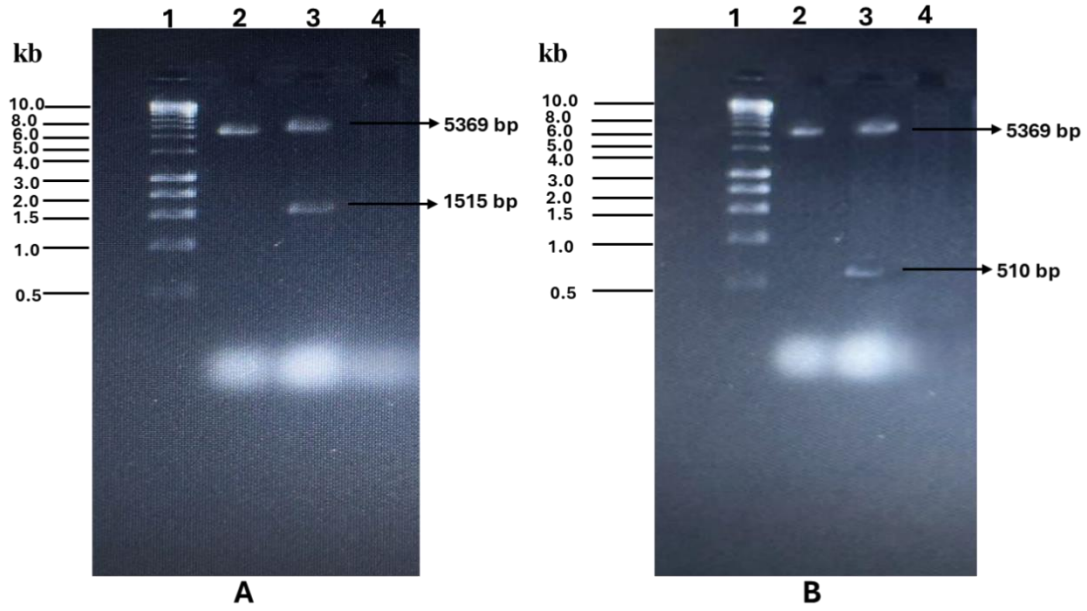

Figure 5.12: Digestion check of recombinants on 1.5 % agarose gel using *EcoRI* and *NdeI.* In A: Lane 1 is the ladder, Lane 2 is plasmid DNA, Lane 3 is *CarP* release from pET-28a (+), and Lane 4 is blank. In B: Lane 1 is ladder, Lane 2 is plasmid DNA, Lane 3 is the *TreS* release from pET-28a (+) and Lane 4 is blank.

5.15: Ligation and transformation in *BL21-DE2*

The successful ligation of the *CarP*, and *TreS* fragments into the digested vector was followed by transformation into *BL21-DE3* cells which indicated the formation of recombinants. *BL21-DE3* cells used for recombinant protein expression, ensured the expression of *CarP* and *TreS* proteins for further analysis. The use of ampicillin supplemented agar plates ensured the growth of only those cells that showed the expression of genes. *BL21-DE3* cells contains the T7 RNA polymerase necessary for the expression of proteins under the control of the T7 promoter present in the pET vector series (Li *et al.*, 2022). The ampicillin resistant gene in the plasmid backbone allowed the selection of cells harboring the recombinants. The process of selecting a subset of transformed cells and culturing them on LB agar plates supplemented with ampicillin provided a means to ensure that only cells harboring the recombinants. Replicating cellular patches onto fresh plates using velveteen cloth allowed for the preservation of transformed colonies (Sanders, 2012) (Figure

5.13). This step ensured that the transformed cells could be stored for subsequent analytical procedures, such as protein expression analysis, enzyme assays, or further genetic manipulation.

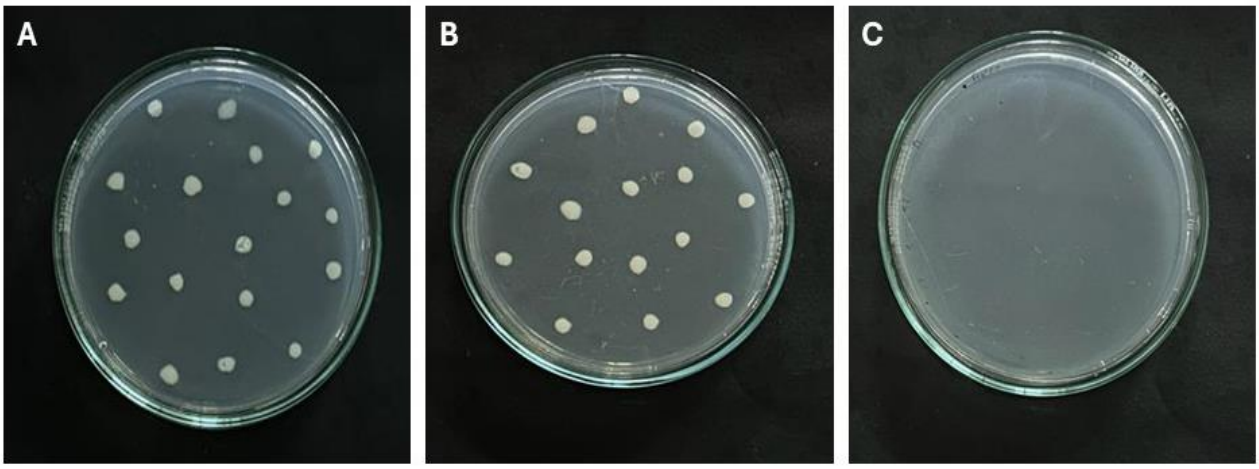

Figure 5.13: *BL21-DE3* recombinants cells on nutrient agar media (A) *CarP*, (B) *TreS,* and (C) Control.

Objective 2: Screening the metagenomic library for hydrolytic enzymes.

5.16: *CarP*, and *TreS* expression

Inducible protein expression systems play a crucial role in molecular biology, enabling the controlled production of specific proteins for various applications. IPTG was used as a chemical inducer for the activation of lac operon, leading to the expression of target proteins in *E. coli* (BL21-DE3) expression systems (Gomes *et al.*, 2020). SDS-PAGE analysis revealed distinct bands corresponding to *CarP* and *TreS* proteins in the induced cell lysates. The molecular weights of *CarP* and *TreS* were determined to be approximately 58.6 kDa and 19.2 kDa, respectively (Figure 5.14). These findings were consistent with the expected sizes based on the amino acid sequences of the proteins. Additionally, *in silico* analysis further confirmed the molecular weights of *CarP* and *TreS*, validating the experimental results.

The successful expression of *CarP* and *TreS* proteins induced by IPTG in the *BL21-DE3* expression system validates the versatility and effectiveness of the bacterial expression host. *BL21-DE3* is widely used in recombinant protein expression due to its high transformation efficiency, rapid growth rate, and well-characterized genetics. Moreover, the T7 RNA polymerase-based expression system in *BL21-DE3* allows tight regulation of target gene expression, enabling high-

level production of heterologous proteins (Pan and Malcolm, 2000, Du *et al.*, 2021). Previous studies have utilized the *BL21-DE3* expression system to express various hydrolases, including proteases (Ramchuran *et al.*, 2002), amylases (Shahhoseini *et al.*, 2003), lipases (Pulido *et al.*, 2020), ureases (Liu *et al.*, 2017), and cellulases (Khalid *et al.*, 2019), demonstrating its utility in diverse biochemical applications.

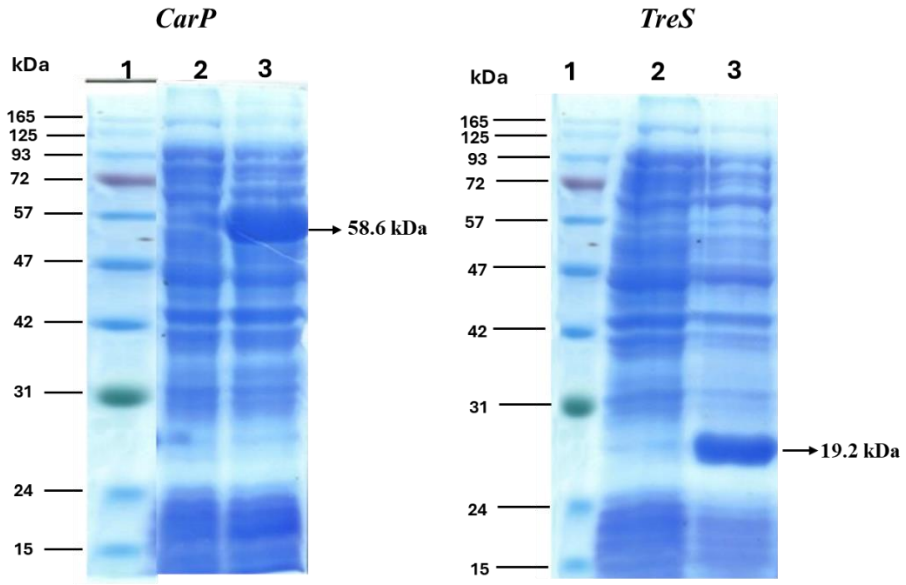

Figure 5.14: SDS-PAGE (12%) of expressed *CarP*, and *TreS* induced by IPTG in *BL21-DE3* expression system; Lane 1: Marker, Lane 2: Uninduced, and Lane 3: Induced with IPTG (50µg/mL).

5.17: Primary screening

5.17.1. CarP

The primary screening experiments revealed the presence of a zone of hydrolysis around the streaked *CarP* clone on 1 % skimmed milk LB agar plates (Figure 5.15A). This observation indicates the proteolytic activity of the *CarP* clone. The experiments conducted in triplicates demonstrated consistency in the results. Notably, the pattern of protease activity observed in the study aligned with previously reported findings obtained through both culturable and unculturable methods (Singh *et al.*, 2015a, Yang *et al.*, 2021). This discovery holds promise for various industrial applications, including food processing, detergent manufacturing, pharmaceuticals, bioremediation, and enzyme engineering, highlighting the potential of *CarP* clone in contributing

to efficient and sustainable processes across diverse industries (Matkawala *et al.*, 2021, Solanki *et al.*, 2021).

5.17.2. TreS

The primary screening experiments revealed the presence of a zone of hydrolysis around the marked area of *TreS* clone growth on starch-supplemented LB agar plates after iodine staining (Figure 5.15C). This observation indicated enzymatic breakdown of starch in the media by the *TreS* clone isolates. The consistency in the results was demonstrated through triplicate experiments, further supported by their reproducibility upon repetition. Notably, the pattern of starch breakdown observed in this study aligned with previously reported findings obtained through culturable and unculturable methods (Motahar *et al.*, 2020, Shofiyah *et al.*, 2020, Ariaeenejad *et al.*, 2021). The findings hold significant implications for industrial applications requiring starch hydrolysis, such as biofuel production, starch processing, and food manufacturing (Msarah *et al.*, 2020, Movahedpour *et al.*, 2022).

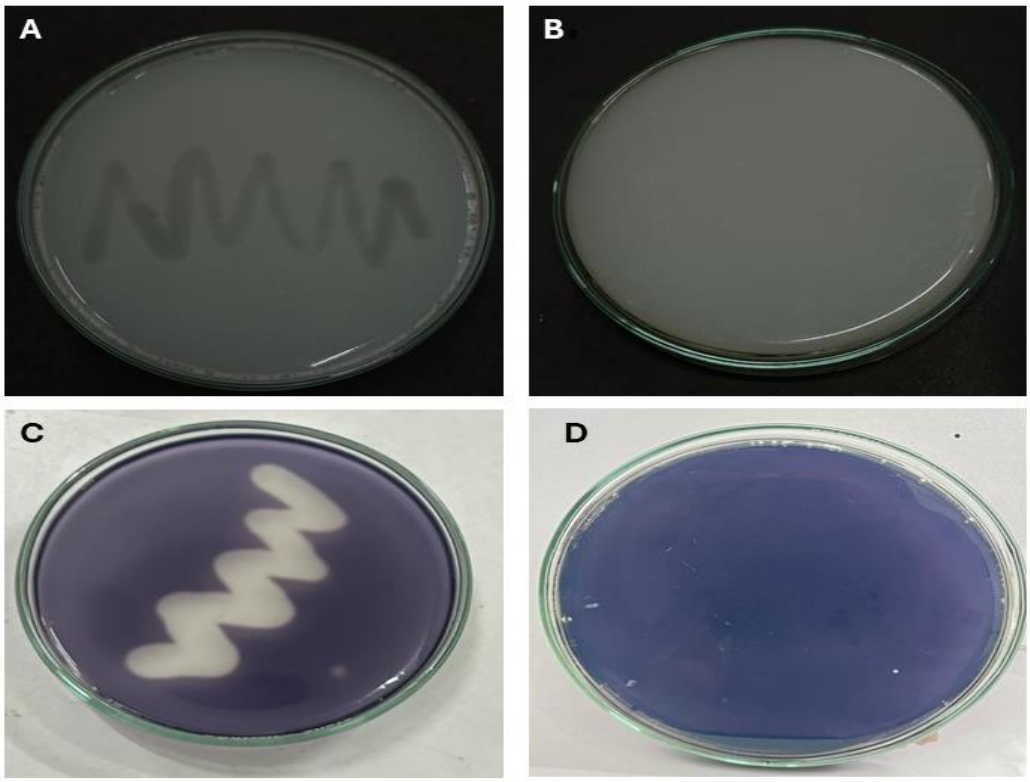

Figure 5.15: Primary screening of hydrolases on LB agar plates supplemented with their specific substrates. (A) *CarP* protease activity (B) Control (C) *TreS* amylase activity (D) Control.

5.18: Secondary screening of *CarP*, and *TreS*

The enzyme localization (extracellular or intracellular) of the protease (*CarP*) and amylase (*TreS*) positive clone was investigated using agar diffusion assay with the supernatant and cell-free extract. After incubation at 37°C, the appearance of a zone of hydrolysis around the cell free extract confirmed the hydrolytic activity of the selected clones from the constructed RHS metagenomic library (Figure 5.16). Predominantly, the hydrolytic activity was localized in the cell-free extract, in both suggesting the intracellular enzyme activity of clones (Hamza, 2017). This finding has significant implications for the purification of these enzymes. Intracellular enzymes typically require cell disruption methods, such as sonication or enzymatic lysis, to release them from the cells before purification (Siepen *et al.*, 1975, Chung *et al.*, 1995, Satoh *et al.*, 1997, Setyorini *et al.*, 2006). Understanding the intracellular localization of the enzymes allows researchers to tailor purification protocols, accordingly, optimizing the yield and purity of the target proteins. Many microbial proteases and amylases are known to be intracellularly produced, requiring extraction from the cells for purification. However, there are exceptions, as some microorganisms secrete these enzymes extracellularly for immediate substrate hydrolysis (Deb *et al.*, 2013, Christensen *et al.*, 2022). Therefore, as the intracellular localization was observed in the present study and found to be consistent with previous studies, it is essential to consider the specific characteristics of each enzyme. The confirmation of enzyme localization through *in silico* analysis enhances the reliability of the findings. *In silico* analysis provided insights into the presence of signal peptides and other localization signals in the enzyme sequences, supporting the experimental observations.

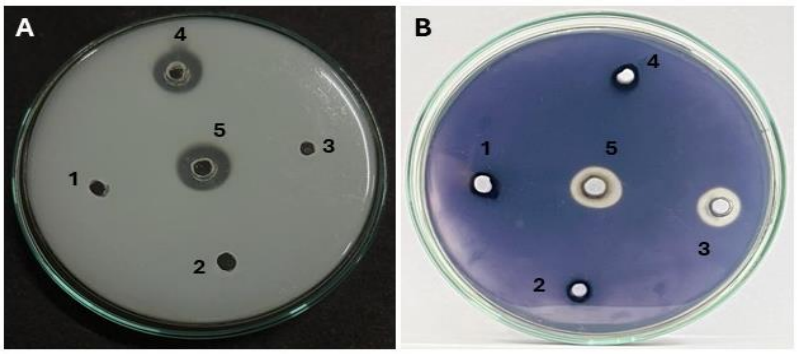

Figure 5.16: Secondary screening of *Carp*, and *TreS* for localization of enzymes. (A) *CarP*: 1: Supernatant, 2: Lysis buffer, 3: Broth, 4: Cell free extract, and 5: Positive control (proteinase K 10 mg/mL). (B) *TreS*: 1: Supernatant, 2: Lysis buffer, 3: Cell free extract, 4: Broth, and 5. Positive control (amylase 10 mg/mL).

Objective 3: DNA Sequencing and analysis of the gene encoding the enzyme from positive metagenomic clone.

5.19: Sequencing, and translation

The nucleotide sequence of *CarP* and *TreS* genes was determined with high accuracy. No unexpected mutations or sequence errors were detected in the *CarP* and *TreS* gene sequences. The nucleotide sequences of *CarP* and *TreS* were successfully translated into their respective protein primary sequences using the TranslatorX tool, followed by validation through GEMBASSY. The translation process resulted in the generation of a 504-amino acid-long *CarP* protein sequence and a 169-amino acid-long *TreS* protein sequence.

5.19.1. CarP

MNH DAK LTE LRE RLG VIL DLN RAS ALL GWD QQT MMP PNG TES RSY QLS TID RFS HEL FVS DEV GRL LEE LAP YEA ELD PES IDA ALI RVT RRD YEK ARR IPA ELR SEI TKA EVI GIP AWA EAR QNS DFE AFL PYL EKN VDL KRR YIE CFK DTG KSD YDI LLD DYQ EDA RSE DIT AIF ERI KEV VIP LIR EVR AAD PVE SEF AHG HYP VEK QEL IGR EIL ERL GYS SEA WRL DPT VHP FAT SIS VDD IRL TTR YSE ADA ESV FHT AHE FGH GIY EHG IDR ELE RTP LVE LNS MVL HES QSR LWE NLV CRS KPF WRF FFP RLQ EVF PEQ LNG VTD EMY WRY VNR VQP DFI RVE ADE VTY GMH IIL RYE LEQ DII AGR LEP RDL PRV WNE KMQ EYL GLD VPD DAR GVL QDV HWS GGS FGY FPT YLL GTI ASV QIW ERL RGD LTD LDA QME AGE FGA LRE WLG EHM YRW GRR FAP DEM LAR IVG GPL DVE PYL AYL RSK VEA VYG VRV

5.19.2: TreS

MGD NVY LGD RDG VRT PMQ WTG DRN GGF SRA DFA QLY LPP LMD PVF GFQ AVN VEA QLR SPT SLL RWL QRF VAL RKE HPV FGL GTY EAL ETS NPR IFA HVR QHD RDT VLC VHN LAR SAQ AVE LDL ARF QGR TPI EMT GRT PFP SIG DLP YLL TFG PRG FFW FLL PEE EPD A

5.20: Phylogenetic relationship of *CarP*, and *TreS*

The phylogenetic analysis of *CarP* (OR906153) revealed its highest sequence identity, reaching 84.92%, with Carboxypeptidase Taq from the *Gaiellaceae bacterium*. Subsequently,

Carboxypeptidase M32 from the *Actinomycetes bacterium* showed a sequence identity of 75.79%. Considering the source of thermostable microorganisms, the high identity percentage with Carboxypeptidase Taq is of great industrial importance owing to its thermotolerant nature (Table 5.8). *Gaiellaceae bacterium* is known for its thermophilic characteristics, thriving in high-temperature environments. The observed sequence similarity may imply that *CarP* shares some thermostable properties, suggesting potential adaptation to heat-resistant conditions (Motoshima and Kaminogawa, 2004). This correlation is noteworthy, especially if the host organism is a thermophile or resides in extreme environments. Figure 5.17 gives the evolutionary relationship between *CarP,* and its other similar matches.

Similarly, the phylogenetic analysis of *TreS* reveals a noteworthy 92.31% sequence identity between the *TreS* gene and Trehalose synthase (maltose alpha-D-glucosyltransferase) proteins sourced from *Gaiella* sp. and various other bacterial species and strains cataloged in the NCBI GenBank (Table 5.9). This high level of sequence similarity underscores the intimate genetic connection between the *TreS* gene and maltose alpha-D-glucosyltransferase proteins across a broad spectrum of bacterial origins. The constructed phylogenetic tree demonstrated a close clustering of the *TreS* gene (OR906319) with maltose alpha-D-glucosyltransferase (MCZ7589705), indicating a potential evolutionary link and shared ancestry between these genes. This relationship implies potential functional similarities, particularly in carbohydrate metabolism pathways, suggesting a conserved role among thermophiles in utilizing carbohydrates for energy production and cellular processes. Figure 5.18 shows the evolutionary relationship between *TreS,* and its other similar matches.

Table 5.8: Sequence similarity of *CarP* with other protein sequences for establishing an evolutionary relationship.

Matches gene/organism	**Max score**	**Total score**	**Query cover**	**E-value**	**Identity**	**Accession number**
Carboxypeptidase Taq [*Gaiellaceae bacterium*]	899	899	100%	0.0	84.92%	MDF2753195.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	822	822	100%	0.0	75.79%	MBA2361749.1

carboxypeptidase M32 [*Actinomycetota bacterium*]	595	595	98%	0.0	57.52%	TMK80227.1
carboxypeptidase M32 [*Actinomycetota bacterium*]	573	573	98%	0.0	55%	TML14220.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	573	573	96%	0.0	56.47%	MBA2474331.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	569	569	97%	0.0	55.08%	MBA3733832.1
Carboxypeptidase M32 [*Actinomycetota bacterium*]	569	569	97%	0.0	55.28%	TML60668.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	569	569	98%	0.0	55.53%	MBA3412342.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	563	563	98%	0.0	54.33%	MBA3716788.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	563	563	98%	0.0	54.33%	MBA3365167.1
Carboxypeptidase M32 [*Thermoleophilia bacterium*]	561	561	98%	0.0	54.42%	MCL4290322.1
Carboxypeptidase M32 [*Actinomycetes bacterium*]	558	558	94%	0.0	55.14%	MBA2569322.1

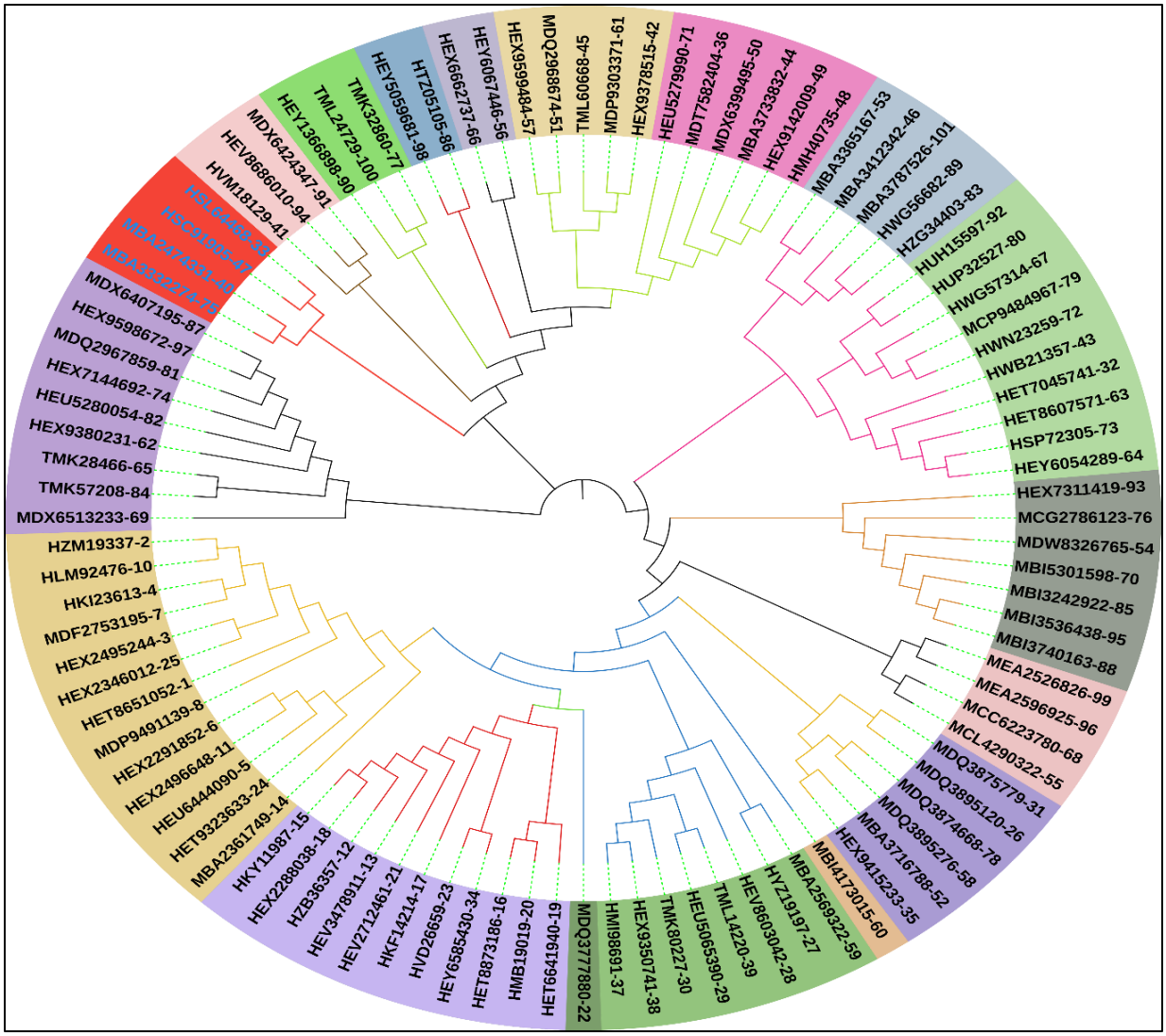

Figure 5.17: Representation of phylogeny of *CarP* in the form cladogram. The range of different colors indicates the resemblance range with that protein group.

Table 5.9: Sequence similarity of *TreS* with other protein sequences for establishing the evolutionary relationship.

Matches gene/organism	Max score	Total score	Query cover	E-value	Identity	Accession number
Maltose alpha-D-glucosyltransferase [*Gaiella* sp.]	322	322	100%	6e-105	92.31%	MCZ7589705.1
maltose alpha-D-glucosyltransferase [*Actinomycetia bacterium*]	315	315	100%	3e-102	88.17%	MBA2294167.1

Maltose alpha-D-glucosyltransferase [*Solirubrobacterales bacterium*]	291	291	99%	5e-98	80.36%	MCW2990814.1
Alpha-glucosidase C-terminal domain-containing protein [*Actinomycetia bacterium*]	284	284	100%	1e-94	80.47%	MCA1689178.1
Maltose alpha-D-glucosyltransferase [*Gaiella occulta*]	295	295	99%	1e-94	80.95%	WP_114794579.1
Alpha-glucosidase C-terminal domain-containing protein [*Solirubrobacterales bacterium*]	277	277	98%	4e-92	78.31%	MBV9682978.1
Alpha-glucosidase C-terminal domain-containing protein [*Actinomycetia bacterium*]	283	283	100%	8e-92	79.88%	MCA1689272.1
Alpha-amylase [*Actinomycetia bacterium*]	275	275	95%	1e-91	79.01%	MSW53131.1
GH13_16 [uncultured *Solirubrobacteraceae bacterium*]	277	277	98%	1e-91	77.71%	CAA9495017.1
TreS1 [*Solirubrobacterales bacterium*]	275	275	99%	2e-91	77.98%	MCW3048113.1

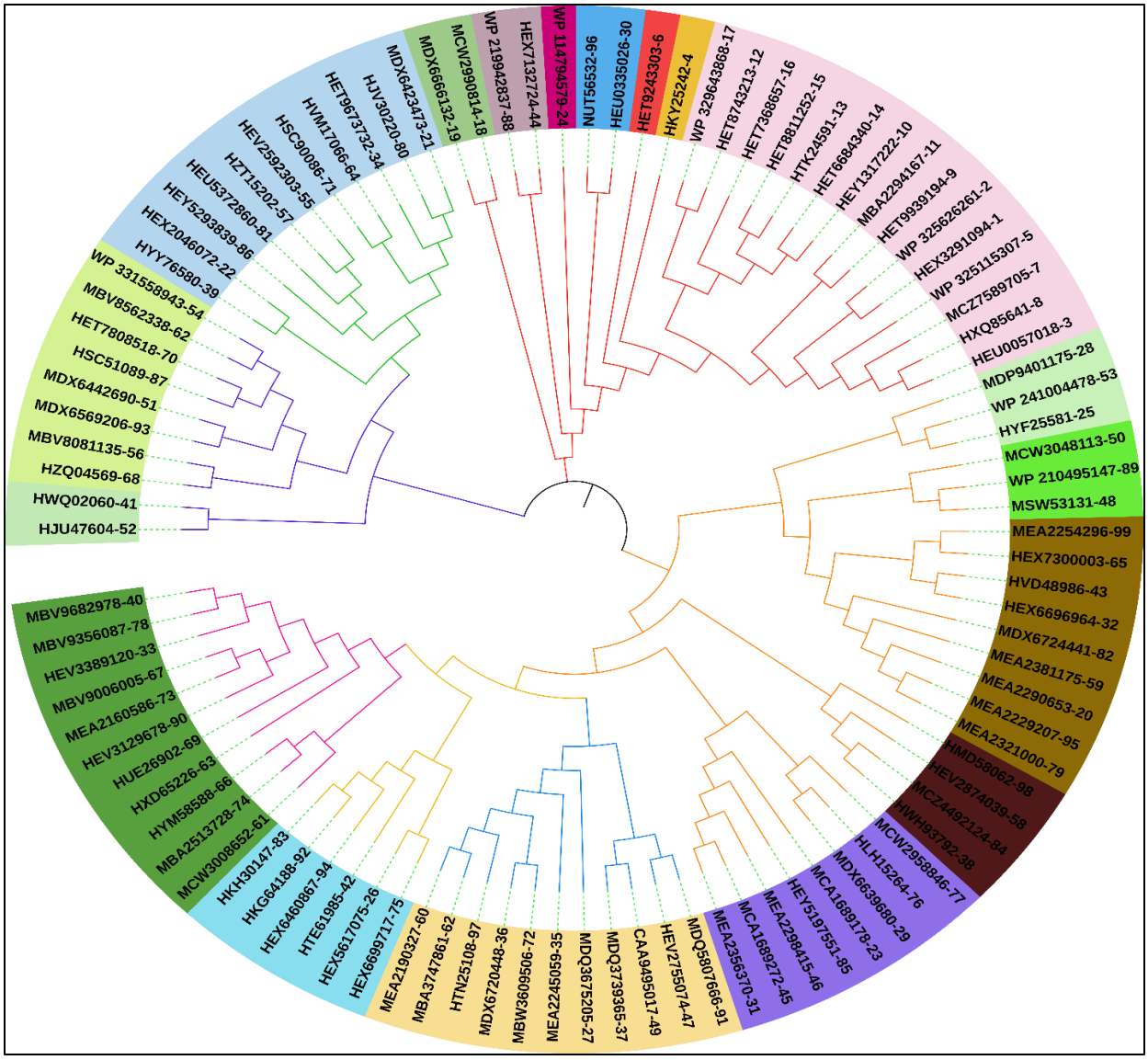

Figure 5.18: Representation of *TreS* phylogeny through cladogram. The range of different colors indicates the resemblance range with that protein group.

5.21: Computation of physical, and chemical parameters of *Carp*, and *TreS*

The bioinformatics analysis indicated the hydrophilic nature of *CarP* and *TreS*, as evidenced by a negative GRAVY score. The genes were found to be localized within the cytoplasm, demonstrating a globular topology. *CarP*, and *TreS* exhibit a pI of 4.81 and 5.78 besides possessing a molecular weight of 58.65 and 19.2 kDa respectively. These findings provided valuable insights into the physicochemical characteristics of *CarP* and *TreS* contributing to a comprehensive understanding of their functional attributes within cellular processes. The other parameters included the number

of atoms, aliphatic index, instability index, and estimated half-life are mentioned in Table 5.10. The instability index of *CarP*, and *TreS* was calculated and found to be 43.43 and 39.97 suggesting a moderate degree of instability. This index is commonly employed to assess the stability of a protein, with higher values indicating increased instability. While an instability index above 40 may imply that the protein could be labile, additional factors such as the presence of stabilizing elements and functional requirements must be considered for a comprehensive evaluation (Gamage *et al.*, 2019). The aliphatic index, determined as 89.19 for *CarP*, and 79.05 for *TreS* is indicative of the protein's thermostability. Higher aliphatic indices are generally associated with increased thermal stability, suggesting that both proteins may possess robust structural characteristics that can allow them to withstand elevated temperatures. This information is particularly relevant when considering potential applications in industrial processes requiring enzymes with thermal resilience (Ikai, 1980).

Table 5.10: Predicted physiochemical parameters of *CarP*, and *TreS* using Expasy Protpram.

Property	***CarP***	***TreS***
Number of amino acids	504	169
Molecular weight	58. 65 kDa	19.20 kDa
Theoretical pI	4.81	5.78
Total number of negatively charged residues (Asp + Glu)	94	20
Total number of positively charged residues (Arg + Lys)	59	17
Formula	$C_{2628}H_{4043}N_{719}O_{785}S_{12}$	$C_{869}H_{1322}N_{242}O_{243}S_5$
Total number of atoms	8187	2681
Estimated half-life	>10 hours (*E. coli*, in vivo)	>10 hours (*E. coli*, in vivo)
Instability index	43.43	39.97
Aliphatic index	89.19	79.05
Grand average of hydropathicity (GRAVY)	-0.447	-0.285

5.22: Secondary structural analysis

5.22.1: CarP

The predictive analysis of *CarP's* secondary structure revealed the presence of alpha helices, beta strands, and random coil regions. The examination of amino acid distribution, as depicted in the annotation grid, suggested that the helical structure is supported by the highest percentage of amino acids (Figure 5.19). The observation that the majority of amino acids in the *CarP* protein favor helical structure suggested that the protein is rich in alpha-helices. Alpha-helices are often crucial for the structural integrity and stability of proteins and may play key roles in the function of *CarP* protein (Jabalia *et al.*, 2015). The presence of metal-binding sites in the *CarP* protein may play an essential role in the protein's function, such as catalyzing chemical reactions, aiding in structural stability, or mediating interactions with other molecules.

The secondary structure analysis of the *CarP* protein reveals an amino acid composition that suggested potential for thermostability. The presence of 28% smaller nonpolar amino acids and 26% hydrophobic amino acids indicated a compact and stable core, enhancing structural integrity and resistance to thermal denaturation. Meanwhile, the 36% polar amino acids on the protein's surface contributed to solubility and facilitated interactions with other molecules. The 10% aromatic amino acids can provide further stability stacking interactions and hydrogen bonding (Figure 5.20).

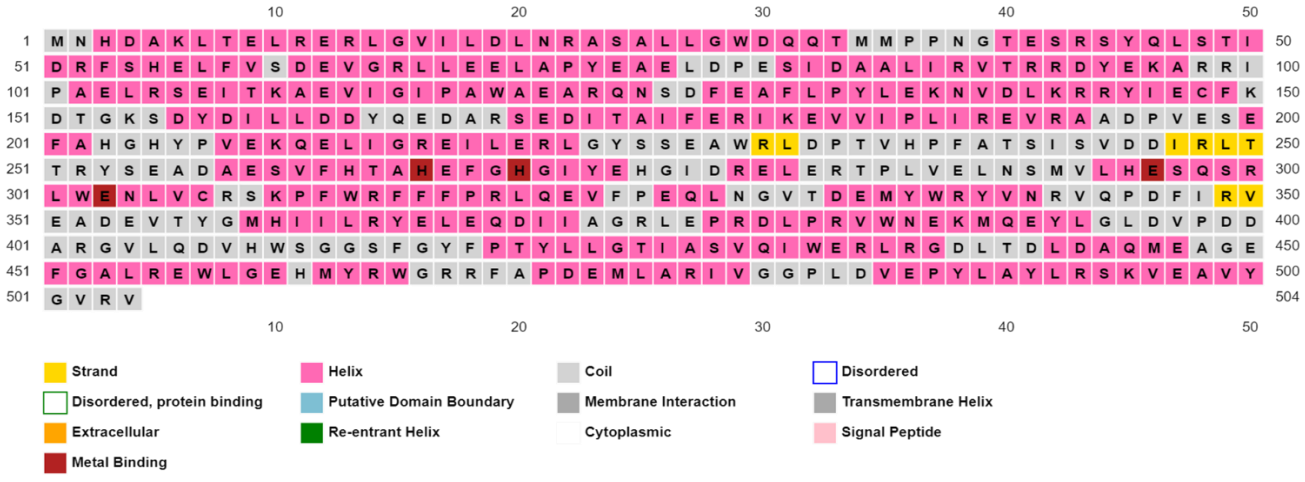

Figure 5.19: Secondary structural elements of a *CarP* amino acid sequence.

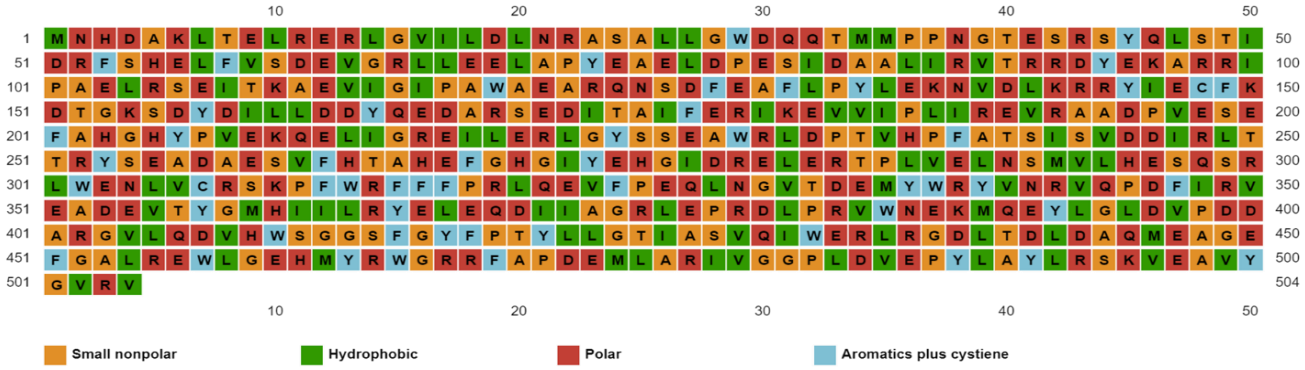

Figure 5.20: Polar, small nonplar, hydrophobic, and aromatic plus cysteine amino acids of the *CarP.*

5.22.2: TreS

The secondary structure of *TreS* provides a prediction of alpha helices, beta strands, and random coil regions (5.21A). The amino acid distribution analysis revealed that leucine (L) exhibited the highest percentage at 11.8%, followed by arginine (R) at 9.5%, glycine (G) at 8.3%, phenylalanine (F) at 7.7%, and alanine (A) at 7.1% (Figure 5.21B). The notable prevalence of leucine suggested its potential involvement in ligand binding, transmembrane domains, and the formation of specific structural domains within the protein.

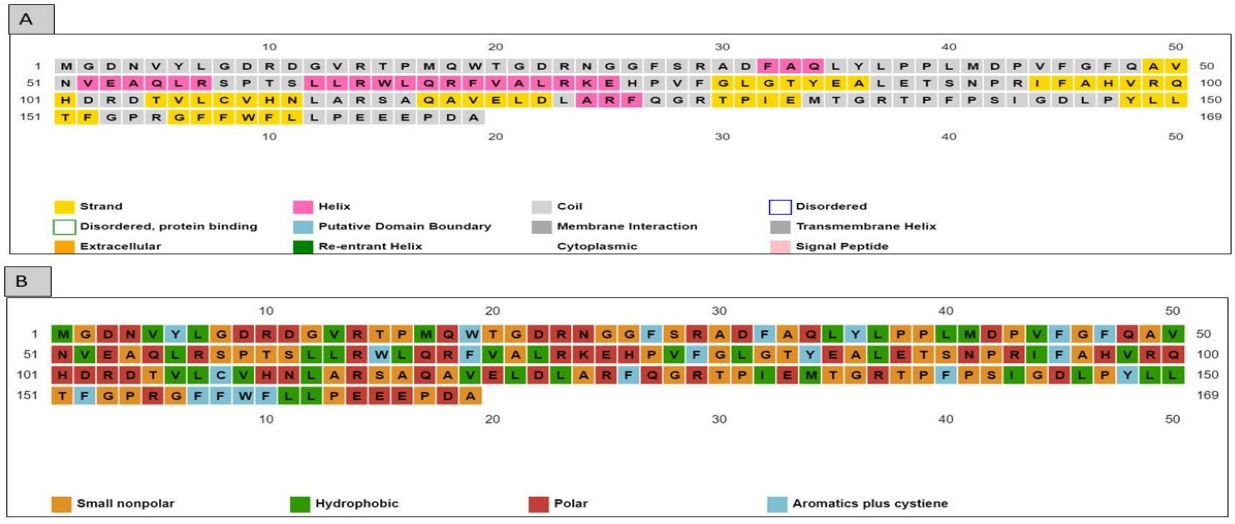

Figure 5.21: Secondary structure of *TreS* (A) Secondary structural elements of a *TreS* (B) Polar, small nonplar, hydrophobic, and aromatic plus cysteine amino acids of the *TreS.*

5.23: 3D structural analysis of *CarP*, and *TreS*

The 3D structural analysis of the proteins *CarP* and *TreS* provided insights into their conformations and potential functions. *CarP's* structure revealed specific domains and motifs, including alpha-helical and beta-sheet elements that formed a stable conformation, along with binding sites for cofactors and substrates, suggesting roles in enzymatic activity or regulatory pathways (Figure 5.22A). *TreS* displayed well-defined secondary structures such as alpha-helices and beta-strands, as well as active sites or binding pockets, indicating its enzymatic activity and potential functions (Figure 5.22B).

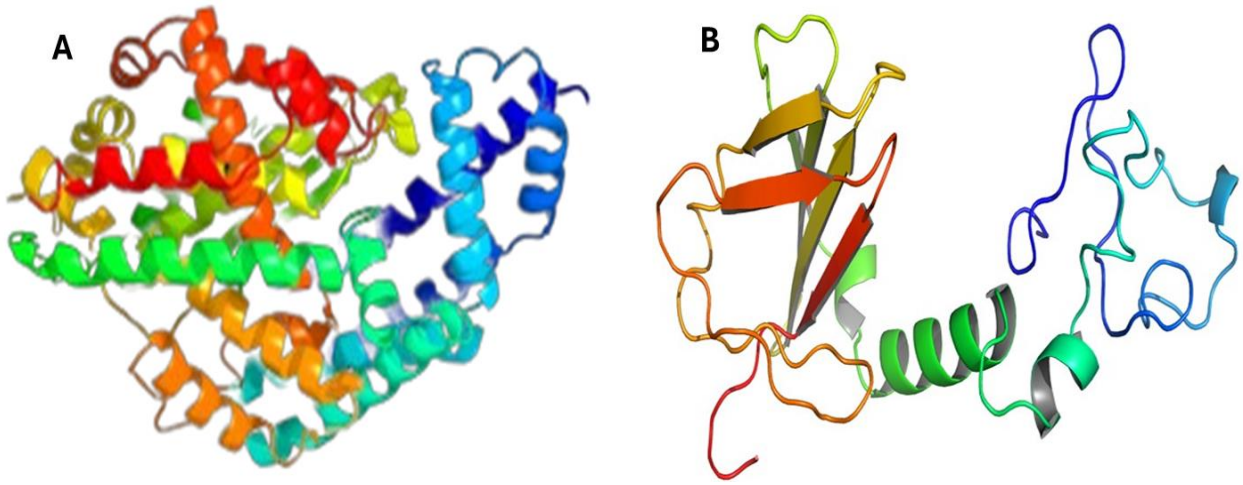

Figure 5.22: 3D cartoon structure of proteins using homology modeling showing blue as N-terminal, red as C-terminal, green as hydrophobic regions, yellow as charged regions, and cyan as binding regions (A) *CarP*, and (B) *TreS*.

5.24: Ramachandran plot

The structural integrity of *CarP,* and *TreS* was assessed utilizing a Ramachandran plot, a graphical representation illustrating the distribution of phi-psi angles of its constituent amino acid residues. The analysis of *CarP* revealed that an overwhelming majority of 407 residues (91%) resided within the most favored regions, attesting to the robust conformational characteristics of the protein. Furthermore, 8.3% of residues were situated in additional allowed regions, denoting a permissible yet less optimal conformational space. A minimal fraction of 0.4% occupied generously allowed regions, with specific attention to 29 glycine and 24 proline residues (Figure 5.23). The majority of residues of *TreS* (91.3%) fell within the most favored regions, followed by 8% in additional

allowed regions, and 0.7% in generously allowed regions, with 9 % residues each of glycine and proline (Figure 5.24). The Ramachandran plot serves as a pivotal analytical tool for scrutinizing the accuracy and fidelity of protein structures. The prevalence of residues within the most favored regions suggests a high degree of structural reliability and adherence to energetically favorable conformations. The presence of residues in additional and generously allowed regions, particularly those rich in glycine and proline, warrants consideration, as deviations from optimal conformations may influence protein stability and folding dynamics (Hooft *et al.*, 1997, Gore *et al.*, 2017).

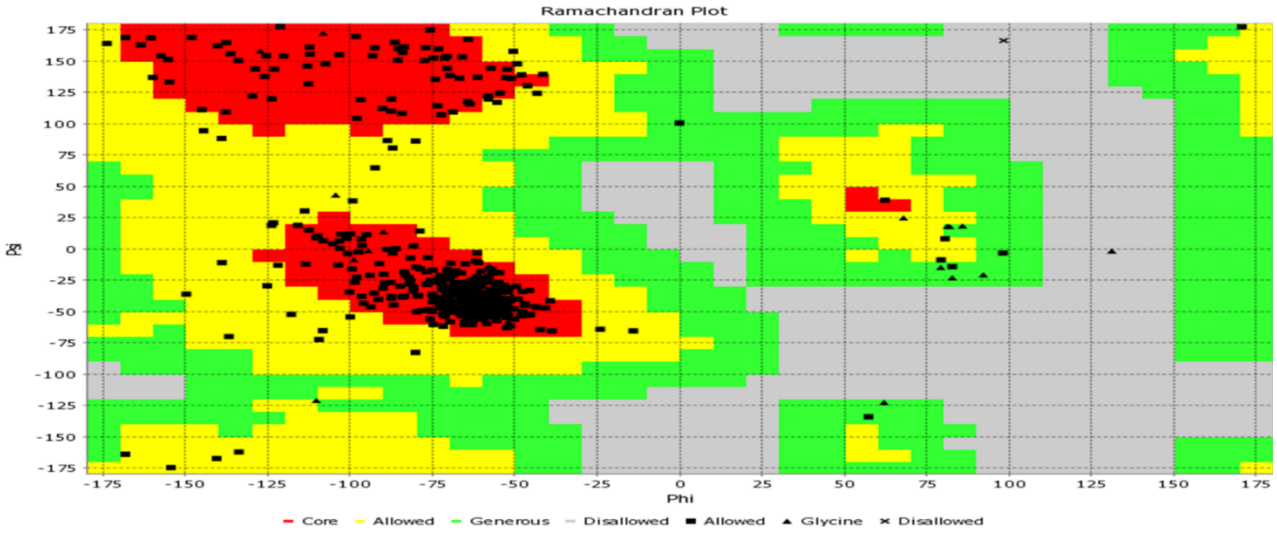

Figure 5.23: Pictorial representation of the Ramachandran plot of *CarP*.

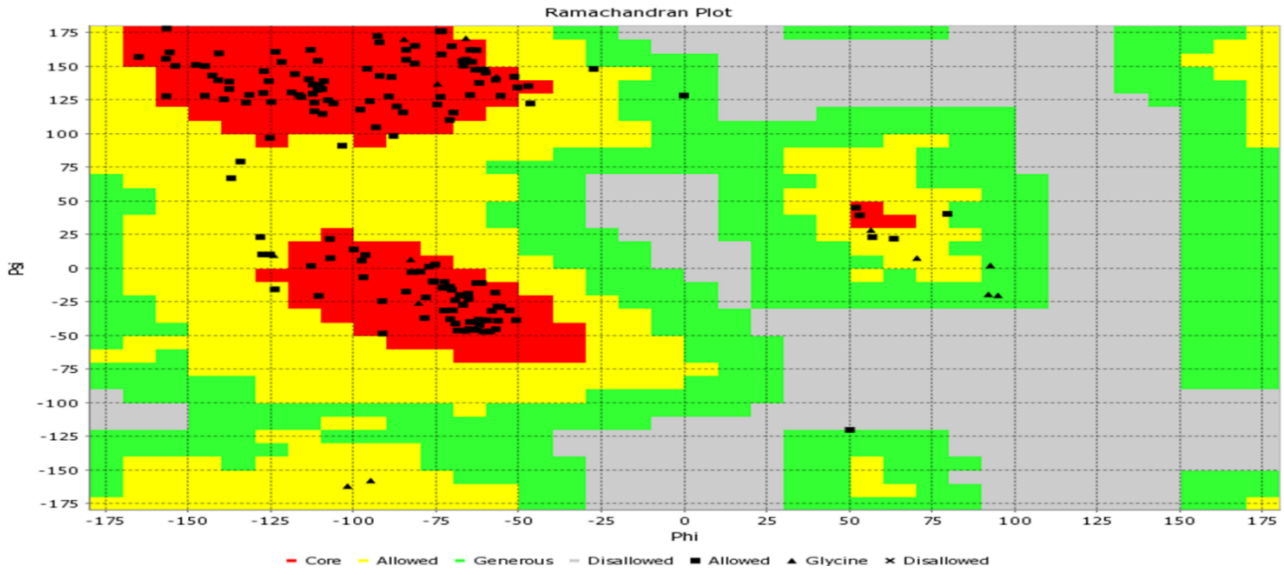

Figure 5.24: Pictorial representation of the Ramachandran plot of *TreS*.

4.25: Z-score assessment

The protein *CarP*, consisting of 504 amino acid residues, exhibits a Z-score of -9, whereas *TreS*, composed of 169 amino acid residues, displayed a Z-score of -4.09. The Z-score is a value that indicates how a protein's structure compares to the average of a database of protein structures (Figure 5.25). Typically, a Z-score greater than zero suggests a structure is more similar to the average than different, while a negative Z-score suggests the structure is less similar to the average. Both proteins exhibited lower-than-average Z-scores, which suggested the potential structural uniqueness (Zhang and Skolnick, 1998).

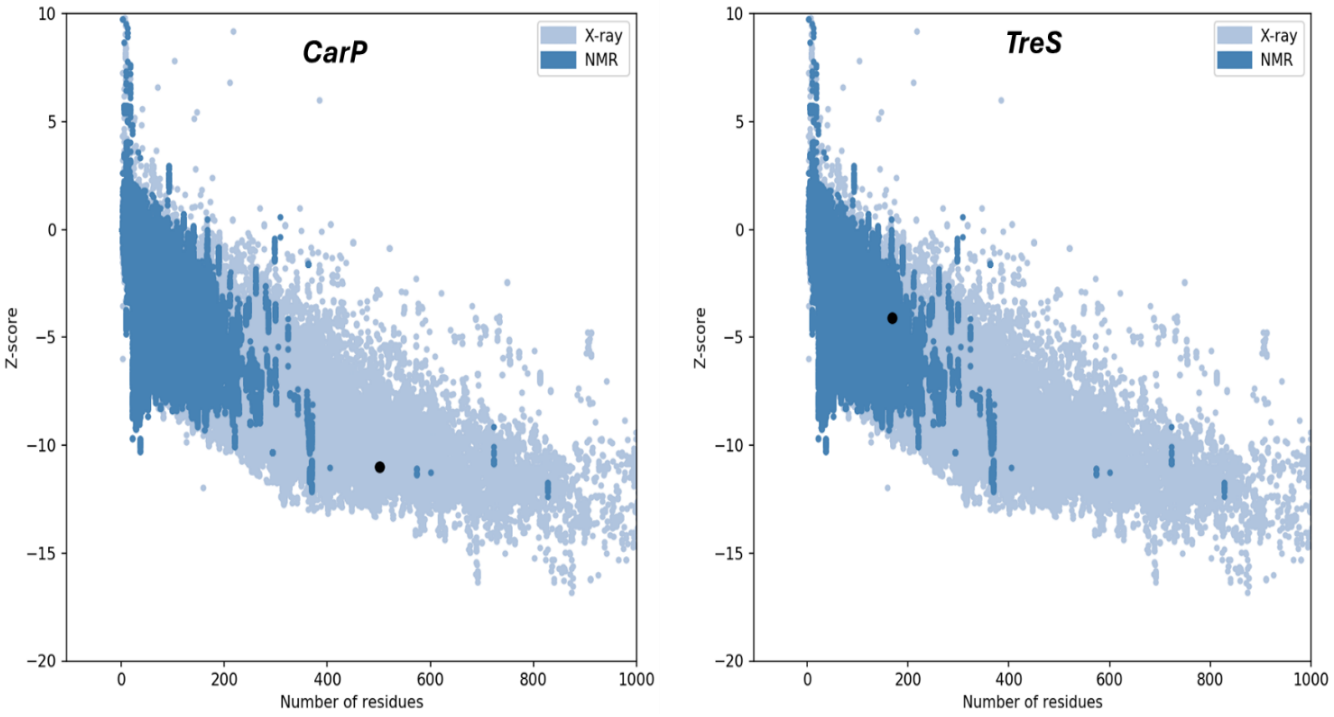

Figure 5.25: Z-score assessment of *CarP*, and *TreS* for determining structural uniqueness.

Objective 4: Biochemical characterization of selected hydrolytic enzymes

5.26: *CarP*, and *TreS* purification.

The purification of *CarP* and *TreS* proteins using the HIS-Tagged Bacterial Protein Purification Kit with Ni-NTA column yielded positive results. SDS-PAGE analysis confirmed the successful purification of both proteins, with observed molecular weights of 58.6 kDa for *CarP* and 19.2 kDa for *TreS* (Figure 5.26). These molecular weights aligned closely with the predicted values obtained through computational tools. Furthermore, the induction of protein expression by IPTG supported the successful isolation of *CarP* and *TreS*, further corroborating their identity and purity. The results highlighted the efficiency and reliability of the Ni-NTA column for purifying target proteins in biotechnological applications.

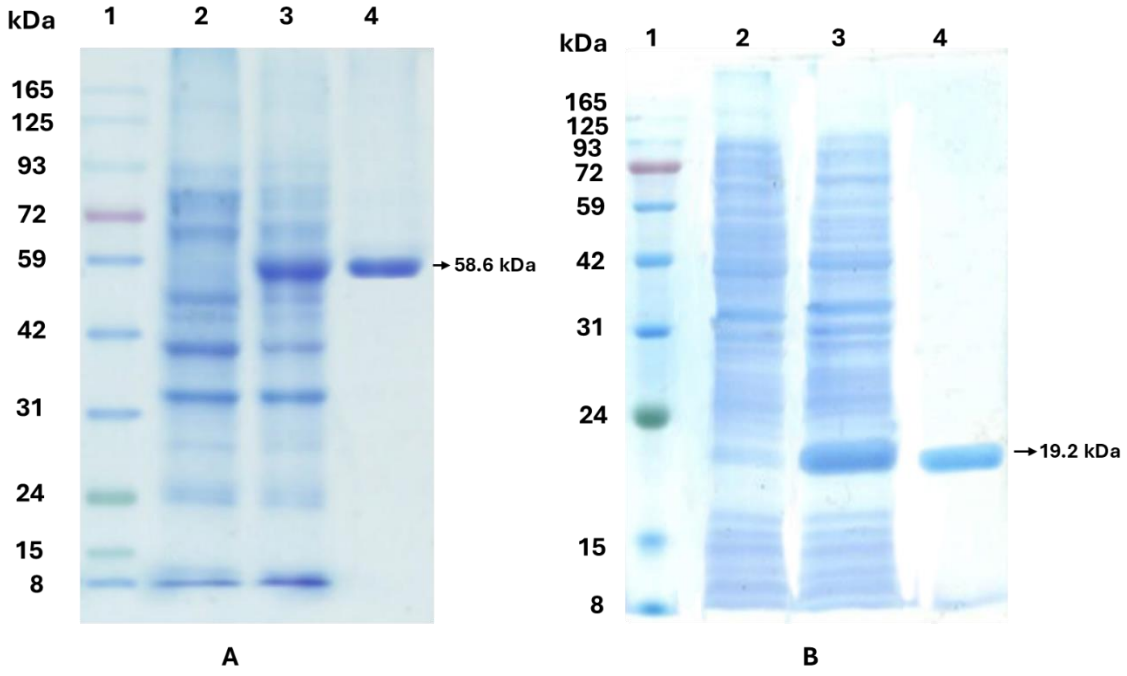

Figure 5.26: SDS-PAGE (12 %) of purified *CarP*, and *TreS*. A is *CarP*: Lane 1 is the ladder, Lane 2 is uninduced *CarP* clone, Lane 3 is induced, and Lane 4 is purified *CarP*. B is *TreS*: Lane 1 is ladder, Lane 2 is uninduced *TreS*, Lane 3 is induced *TreS* clone, and Lane 4 is purified *TreS*.

5.27: Protein estimation, and enzyme activity

The estimation of the *CarP* and *TreS* proteins was conducted using a standard curve based on BSA. The concentrations of the proteins were measured to be 9.19 mg for *CarP* and 7.14 mg for *TreS*.

The activities of *CarP* protease and *TreS* amylase were determined spectrophotometrically. The *CarP* protease exhibited an activity of 29.01 U/mL, while the *TreS* amylase displayed an activity of 13.70 U/mL (Table 5.11). The determination of enzyme concentration and studying enzyme activity are important for understanding enzyme function and regulation in biological systems. These measurements provided insights into enzyme kinetics and catalytic efficiency, helping researchers analyze metabolic pathways (Choi *et al.*, 2017).

Table 5.11: Protein estimation, and enzymatic activity of *CarP*, and *TreS*.

Protein	Estimated protein (mg)	Total activity (U/mL)	Specific activity (U/mg)
CarP	9.19	29.01	3.16
TreS	7.14	13.70	1.91

5.28. Effect of temperature

5.28.1. Effect of temperature on CarP activity, and stability

The *CarP* protease enzyme displayed an optimum temperature of 70°C, where its enzymatic activity reached its peak (Table 5.12, Figure 5.27A). In terms of enzyme stability, *CarP* demonstrated a broad operational range from 10°C to 80°C (Table 5.13, Figure 5.27B), maintaining stability throughout this temperature interval. This range highlights the enzyme's adaptability to different environments, allowing for versatility in various applications. The *CarP* activity started to decline beyond 80°C, indicating a reduction in its efficiency at higher temperatures. There was a significant decrease in stability beyond 90°C, suggesting a risk of denaturation or conformational changes that could impair its functionality. Compared to previously reported thermostable enzymes from hot spring sources, such as those from the genera *Thermus* and *Pyrococcus*, *CarP* protease showed similar characteristics in terms of its temperature profile and stability (Yokoyama and Matsui, 2005, Valenzuela *et al.*, 2023). Thermostable enzymes from hot springs often have optimal activities at elevated temperatures and exhibit stability over a wide range of high temperatures. This enzyme stability at high temperatures is usually due to structural adaptations, such as enhanced hydrophobic interactions, ionic bonds, and hydrogen bonds that stabilize their tertiary and quaternary structures (Kambourova, 2018, Wani *et al.*, 2022). Similar

structural adaptations may also be present in *CarP* protease, contributing to its high-temperature activity and stability.

Table 5.12: Effect of temperature on the activity of *CarP*.

Temperature (°C)	Mean A_{620}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
10	0.30	4.01	0.17	0.09	0.00
20	0.37	4.77	0.22	0.13	0.057
30	0.61	7.52	0.06	0.03	0.002
40	0.75	9.14	0.06	0.03	0.0005
50	0.92	11.07	0.17	0.09	0.004
60	1.25	14.73	0.11	0.06	0.0001
70	2.02	23.48	0.17	0.09	0.0002
80	1.75	20.12	0.23	0.13	0.0005
90	1.62	18.95	0.06	0.03	0.016
100	1.45	16.99	0.11	0.06	0.0003

Table 5.13: Effect of temperature on the *CarP* stability.

Temperature (°C)	Mean A_{620}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
10	2.14	24.80	0.11	0.06	0.00
20	2.13	24.68	0.11	0.06	0.422
30	2.11	24.42	0.06	0.03	0.072
40	2.04	23.67	0.11	0.06	0.017
50	1.96	22.80	0.06	0.03	0.001
60	1.92	22.35	0.06	0.03	0.020
70	1.88	21.89	0.17	0.09	0.057
80	1.63	19.06	0.17	0.09	0.003
90	1.04	12.35	0.11	0.06	0.0004
100	0.88	10.54	0.11	0.06	0.0013

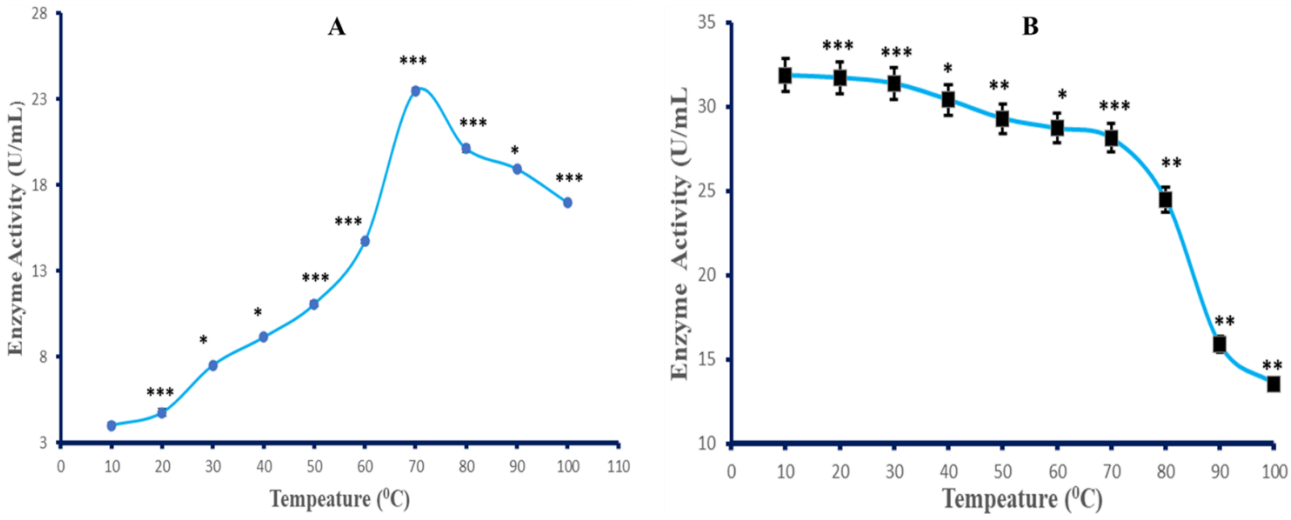

Figure 5.27: Line graph showing the temperature-dependent dynamics of *CarP* enzyme: (A) impact on catalytic activity and (B) assessment of thermal stability (*p<0.05; **p<0.01, ***p>0.05).

5.28.2. Effect of temperature on TreS activity, and stability

The *TreS* amylase enzyme exhibited its maximum activity at an optimum temperature of 80°C (Table 5.14, Figure 5.28A). The enzyme-maintained stability within a temperature range of 10°C to 80°C (Table 5.15, Figure 5.28B). However, beyond 80°C, both enzyme activity and stability began to decrease. Comparatively, other thermostable amylases from hot spring sources often demonstrate similar temperature profiles and stability (Kiran *et al.*, 2018, Sudan *et al.*, 2018). The decline in enzyme activity and stability after 80°C could be attributed to structural changes in the enzyme at higher temperatures. The elevated temperatures can lead to denaturation or alterations in the enzyme's conformation, affecting its catalytic efficiency and structural integrity. This is a common characteristic observed in many enzymes, including other thermostable amylases from thermophilic sources (Daniel and Danson, 2010, Arcus *et al.*, 2020).

Table 5.14: Effect of temperature on the activity of *TreS*.

Temperature (°C)	Mean A_{540}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
10	0.071	4.15	0.046	0.02	0.00
20	0.074	4.33	0.066	0.03	0.013
30	0.076	4.50	0.096	0.05	0.013

40	0.093	5.60	0.066	0.03	0.007
50	0.128	7.94	0.066	0.03	0.00
60	0.153	9.62	0.066	0.03	0.002
70	0.197	12.56	0.066	0.03	0.0006
80	0.231	14.83	0.066	0.03	0.0002
90	0.180	11.44	0.102	0.05	0.0005
100	0.146	9.15	0.066	0.03	0.0001

Table 5.15: Effect of temperature on the stability of *TreS*.

Temperature (℃)	**Mean A_{620}**	**Total activity (U/mL)**	**Standard deviation**	**Standard error**	***p*-value**
10	0.222	14.28	0.102	0.059	0.00
20	0.218	14.01	0.038	0.022	0.0202
30	0.200	12.80	0.102	0.059	0.001
40	0.201	12.83	0.133	0.077	0.807
50	0.206	13.21	0.168	0.097	0.135
60	0.201	12.83	0.133	0.077	0.042
70	0.202	12.89	0.066	0.038	0.422
80	0.199	12.72	0.102	0.059	0.207
90	0.143	8.99	0.102	0.059	0.00057
100	0.117	7.23	0.102	0.059	0.0025

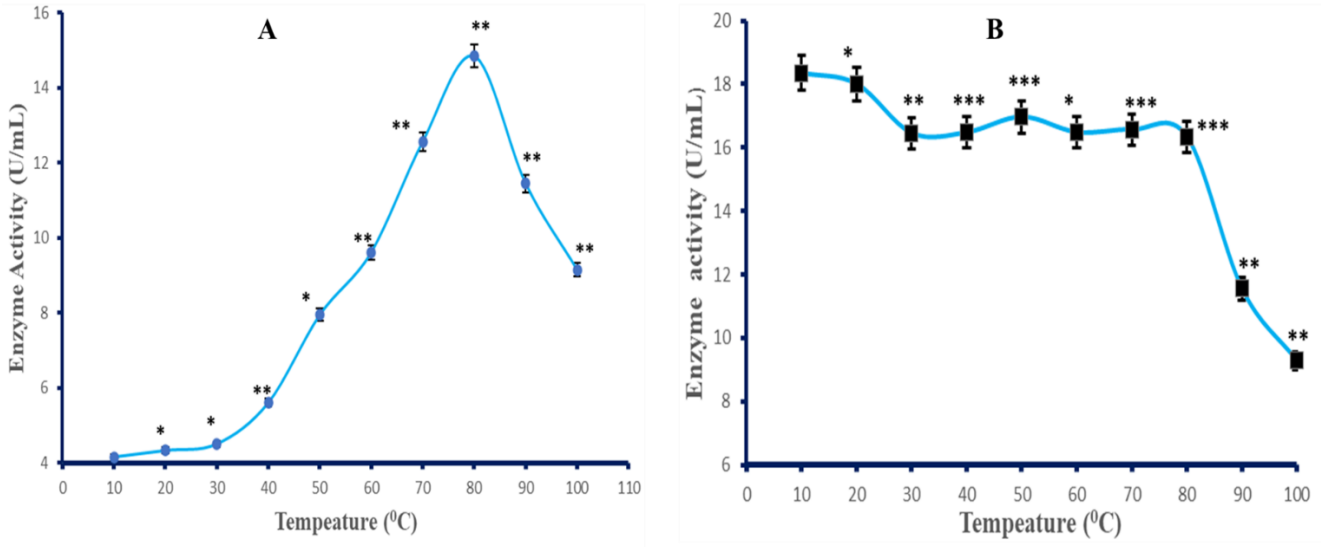

Figure 5.28: Line graph showing temperature-dependent dynamics of *TreS* enzyme: (A) impact on catalytic activity and (B) assessment of thermal stability (*p<0.05; **p<0.01, ***p>0.05).

5.29: Effect of pH

4.29.1. Effect of pH on CarP activity and stability

The purified *CarP* protease exhibited maximum activity at pH 10.0 (Table 5.16, Figure 5.29A). The enzyme demonstrated stability within the pH range of 8.0 to 11.0 (Table 5.17, Figure 5.29B). However, the enzyme's stability decreased beyond pH 11.0. The observed maximum activity of the *CarP* enzyme at pH 10.0 aligns with the alkaline nature of the sample site, suggesting that the enzyme is well-adapted to operate under alkaline conditions. The *CarP*'s stability across a relatively wide pH range indicates its adaptability to varying alkaline conditions, which can be advantageous for industrial processes that may experience fluctuations in pH levels. This characteristic is important for potential applications in industries that require enzymatic activity in alkaline environments, such as in detergent formulations or wastewater treatment. The stability and activity of *CarP* at high pH are consistent with the previous findings, supporting the fact that the enzyme possesses structural features that enable it to maintain function in alkaline conditions (Manni *et al.*, 2020, Sarkar and Suthindhiran, 2020, Farooq *et al.*, 2021, Masi *et al.*, 2021).

Table 5.16: Effect of pH on *CarP* activity.

pH	Mean A_{620}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
3	0.08	1.48	0.112	0.065	0.00
4	0.086	1.56	0.172	0.099	0.004
5	0.106	1.78	0.065	0.037	0.003
6	0.120	12.93	0.112	0.065	0.002
7	0.503	6.25	0.172	0.099	0.001
8	0.736	8.88	0.172	0.099	0.003
9	1.52	17.79	0.130	0.075	0.020
10	2.08	24.02	0.112	0.065	0.0004
11	1.273	14.93	0.130	0.075	0.0001
12	0.85	10.16	0.112	0.065	0.0008

Table 5.17: Effect of pH on *CarP* stability.

pH	Mean A_{620}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
3	0.08	1.37	0.065	0.037	0.00
4	0.083	1.63	0.130	0.075	0.004
5	0.09	1.67	0.065	0.0375	0.001
6	0.12	1.86	0.065	0.0375	0.002
7	0.53	6.59	0.130	0.112	0.0005
8	1.39	16.25	0.195	0.0375	0.0001
9	1.92	22.30	0.065	0.0375	0.0011
10	2.08	24.06	0.065	0.0375	0.0013
11	1.96	22.63	0.065	0.0375	0.009
12	1.04	12.38	0.260	0.150	0.0001

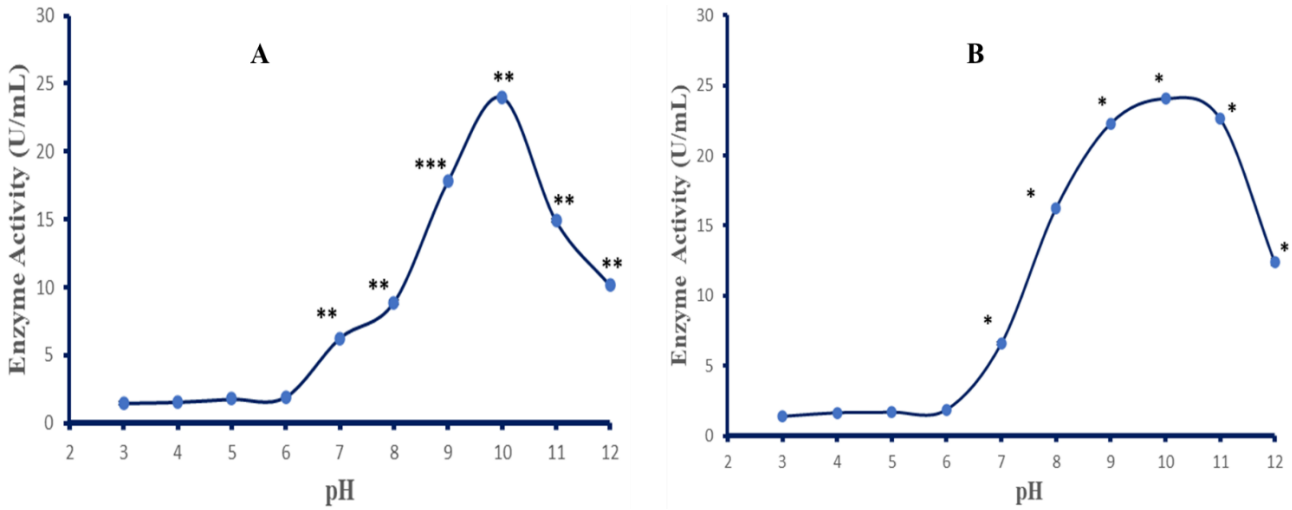

Figure 5.29: Line graph representing pH-dependent dynamics of *CarP* enzyme: (A) impact on catalytic activity and (B) stability (*p<0.05; **p<0.01, ***p>0.05).

5.29.2. Effect of pH on TreS activity and stability.

The purified *TreS* amylase exhibited its peak activity at an alkaline pH of 9.0 (Table 5.18, Figure 30A). The enzyme demonstrated stability within a pH range from 8.0 to 10.0. Beyond pH 10.0, the stability of the enzyme decreased significantly (Table 5.19, Figure 5.30B). The performance of the purified *TreS* amylase under different pH conditions suggested its potential utility in various industrial applications, provided that the pH range is carefully controlled to optimize enzyme activity and stability. Previous studies on amylases isolated from hot spring metagenomes have often reported enzymes with optimal activity at elevated temperatures and alkaline pH levels (Thakur *et al.*, 2021, Chauhan *et al.*, 2023).

Table 5.18: Effect of pH on *TreS* activity.

pH	Mean A_{540}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
3	0.078	4.60	0.066	0.038	0.001
4	0.083	4.98	0.1022	0.059	0.0051
5	0.103	6.29	0.1022	0.059	0.001
6	0.115	7.09	0.077	0.044	0.002
7	0.135	8.41	0.066	0.038	0.0004

8	0.183	11.62	0.066	0.038	0.001
9	0.214	13.70	0.066	0.038	0.0011
10	0.192	12.27	0.038	0.022	0.0002
11	0.152	9.57	0.102	0.059	0.0008
12	0.131	8.19	0.038	0.022	0.0018

Table 5.19: Effect of pH on *TreS* stability.

pH	Mean A_{540}	Total activity (U/mL)	Standard deviation	Standard error	*p*-value
3	0.09	5.54	0.066	0.038	0.021
4	0.10	5.74	0.066	0.038	0.0031
5	0.10	5.87	0.066	0.038	0.0001
6	0.10	6.05	0.139	0.080	0.0003
7	0.14	9.00	0.102	0.059	0.002
8	0.19	12.32	0.139	0.080	0.001
9	0.20	12.99	0.139	0.080	0.000
10	0.20	12.56	0.066	0.038	0.069
11	0.16	10.57	0.139	0.080	0.001
12	0.14	8.93	0.139	0.080	0.000

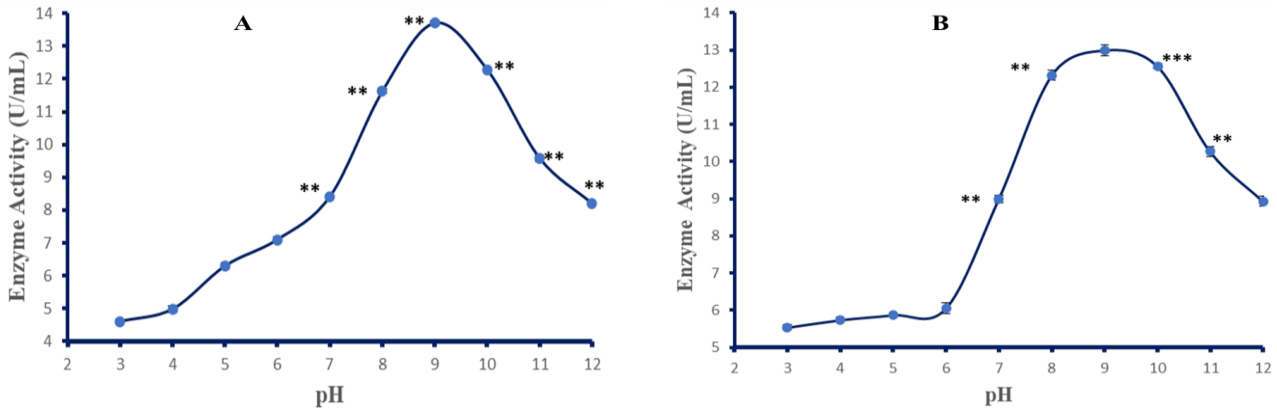

Figure 5.30: Line graph representing pH-dependent dynamics of *TreS* enzyme: (A) impact on catalytic activity and (B) stability (*p<0.05; **p<0.01, ***p>0.05).

5.30: Effect of metal ions

5.30.1. Effect of metal ions CarP activity

The impact of diverse divalent cations on the protease activity of *CarP* was systematically evaluated, with particular focus on metal ion concentrations of 5 and 10 mmol/L (Table 5.20, Figure 5.31). Zn^{2+} exhibited the highest efficacy at both the concentrations. Co^{2+} and copper ions Cu^{2+} demonstrated slightly elevated effectiveness at the 10 mmol/L concentration. Fe^{2+} and Na^{2+} exhibited enhanced *CarP* activity at both concentrations. In contrast, Mn^{2+}, Mg^{2+}, Ca^{2+}, and Ni^{2+} resulted in a reduction in *CarP* activity compared to other divalent metal ions. Zinc is known to play a crucial role as a cofactor in many enzymes, including proteases. Zn^{2+} likely interacts with specific binding sites on *CarP*, promoting proper enzyme conformation and enhancing its catalytic activity (Sarkar *et al.*, 2022). The slightly higher efficacy observed at 10 mmol/L suggests a concentration-dependent effect, possibly involving more optimal saturation of metal ion binding sites (Farooq *et al.*, 2021). The decrease in *CarP* activity with Mn^{2+}, Mg^{2+}, Ca^{2+}, and Ni^{2+} could be attributed to several factors. These ions may competitively inhibit the binding of essential cofactors or disrupt the proper folding of the enzyme, leading to decreased catalytic efficiency. Additionally, they may interfere with substrate binding or alter the electrostatic environment required for optimal enzyme activity (Poulson *et al.*, 2020).

Table 4.20: Effect of metal ions on *CarP* activity at 5mM and 10mM concentration.

Metal ion	A_{620}	Total activity (U/mL)	Standard deviation	*p*-value
5mM				
Control	1.103	13.075	0.234	0.001
Mn^{2+}	0.361	4.662	0.113	0.00038
Mg^{2+}	0.226	3.153	0.173	0.0005
Ca^{2+}	0.551	6.81	0.114	0.0006
Zn^{2+}	1.626	18.99	0.132	0.0012
Fe^{2+}	1.262	14.48	0.115	0.0125
Na^{2+}	1.151	13.60	0.113	0.0035
Co^{2+}	1.367	16.16	0.170	0.0027

Cu^{2+}	1.416	16.62	0.170	0.0001
NI^{2+}	0.423	5.37	0.173	0.0008
10 mM				
Control	1.086	12.833	0.0651	0.0001
Mn^{2+}	0.426	5.394	0.234	0.0005
Mg^{2+}	0.363	4.680	0.1722	0.0001
Ca^{2+}	0.476	5.957	0.1722	0.0002
Zn^{2+}	1.610	18.731	0.2254	0.0005
Fe^{2+}	1.346	15.763	0.1722	0.0019
Na^{2+}	1.226	14.411	0.1722	0.0016
Co^{2+}	1.461	17.041	0.1127	0.0001
Cu^{2+}	1.473	17.191	0.1722	0.0002
NI^{2+}	0.351	4.529	0.1127	0.0001

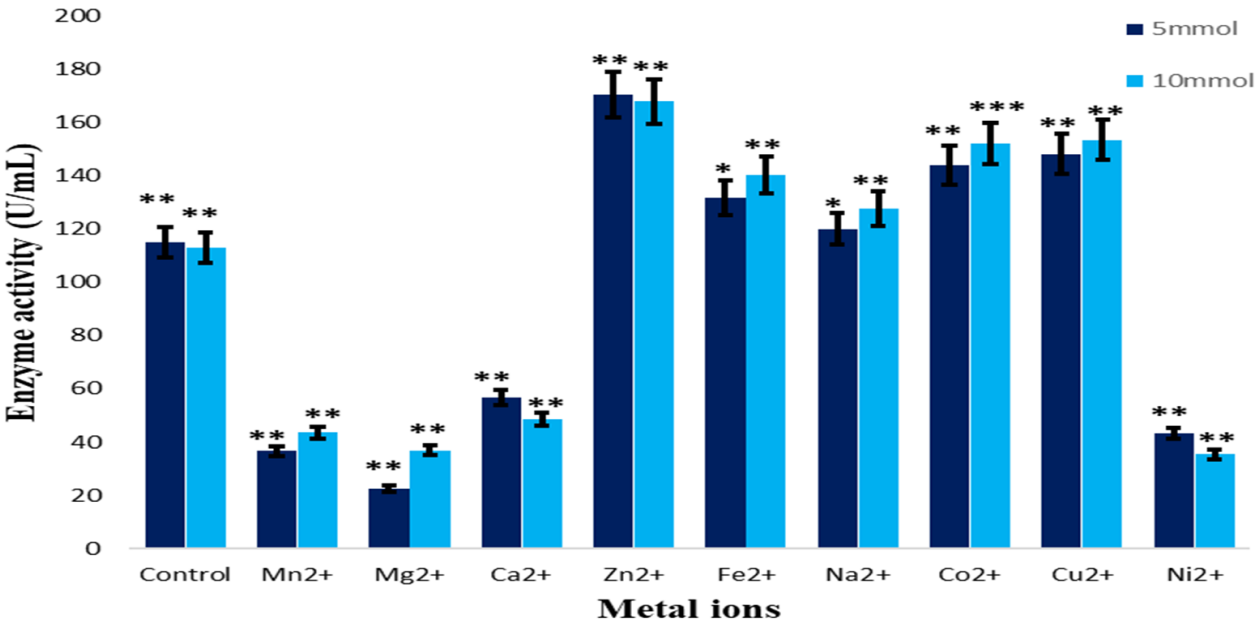

Figure 5.31: Bar graph depicting modulation of *CarP* protease activity in response to metal ions (*p<0.05; **p<0.01, ***p>0.05).

5.30.2. Effect of metal ions TreS activity

The assessment of the effect of various divalent metal ions on *TreS* amylase activity provided insightful results at concentrations of 5 mM and 10 mM (Table 5.21). The data revealed that the presence of divalent cations such as $CO^{2+,}$ Ca^{2+}, and Mg^{2+} enhanced *TreS* amylase activity, suggesting that these cations may play a role in enzyme stabilization or catalysis. CO^{2+} demonstrated a modest increase in enzyme activity, with measurements of 13.47 U/mL and 13.70 U/mL at 5 mM and 10 mM concentrations, respectively (Figure 5.32). This represents a notable increase compared to the control group, which showed enzyme activities of 11.69 U/mL and 11.96 U/mL at the respective concentrations. The presence of Ni^{2+} had a marked inhibitory effect on *TreS* amylase activity. At 5 mM and 10 mM concentrations, enzyme activity dropped significantly to 4.28 U/mL and 4.40 U/mL, respectively. This significant reduction highlights the sensitivity of *TreS* amylase to this specific metal ion. Comparing these results with prior studies on the effect of metal ions on amylases, it becomes evident that metal ions generally play a pivotal role in modulating enzyme activity. For instance, previous studies have shown that Ca^{2+} acts as a cofactor for many amylases, promoting enzyme stability and activity (Silva-Salinas *et al.*, 2021). Similarly, Mg^{2+} has been found to positively influence the function of amylases, possibly by aiding in substrate binding and transition state stabilization (Fatoki and Onilude, 2022). However, the detrimental impact of Ni^{2+} on *TreS* amylase activity is noteworthy and consistent with earlier findings on the inhibitory effects of certain metal ions on amylase function (Abd-Elaziz *et al.*, 2020).

Table 5.21: Effect of metal ions on *TreS* activity at 5mM and 10mM concentration.

Metal ion	A_{540}	Total activity (U/mL)	Standard deviation	*p*-value
5mM				
Control	0.184	11.69	0.066	0.0001
Mn^{2+}	0.1113	6.83	0.077	0.001
Mg^{2+}	0.174	11.07	0.102	0.005
Ca^{2+}	0.193	12.34	0.102	0.015
Zn^{2+}	0.093	5.64	0.102	0.0001
Fe^{2+}	0.106	6.49	0.102	0.0021

Na^{2+}	0.150	9.41	0.066	0.000
Co^{2+}	0.210	13.47	0.139	0.002
Cu^{2+}	0.132	8.25	0.102	0.0006
NI^{2+}	0.073	4.28	0.102	0.010
10 mM				
Control	0.188	11.96	0.066	0.0001
Mn^{2+}	0.116	7.14	0.066	0.000
Mg^{2+}	0.179	11.36	0.066	0.0121
Ca^{2+}	0.196	12.49	0.066	0.0202
Zn^{2+}	0.096	5.80	0.066	0.0001
Fe^{2+}	0.110	6.76	0.102	0.0002
Na^{2+}	0.154	9.68	0.066	0.0008
Co^{2+}	0.214	13.70	0.066	0.0014
Cu^{2+}	0.133	8.28	0.066	0.0004
NI^{2+}	0.075	4.40	0.066	0.0032

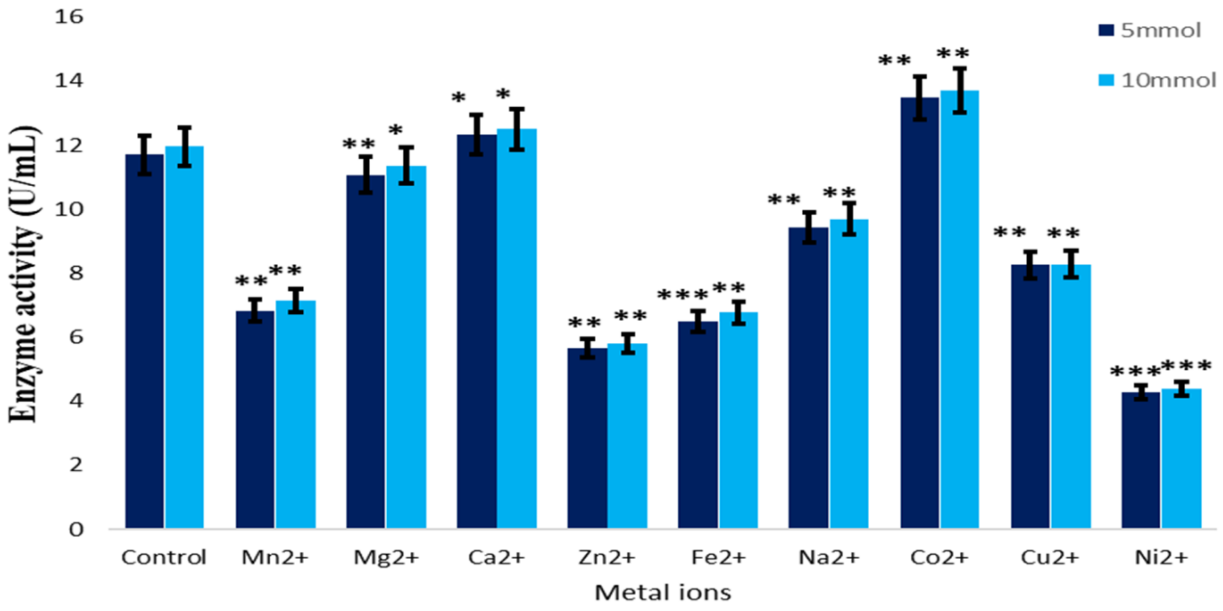

Figure 5.32: Bar graph depicting modulation of *TreS* amylase activity in response to metal ions (*p<0.05; **p<0.01, ***p>0.05).

5.31: Effect of surfactants

5.31.1. Effect of surfactants on CarP activity

The impact of surfactants on *CarP* protease activity is depicted in Table 5.22, and Figure 5.33. The non-ionic surfactant Tween-20 elicited a 21.03% enhancement in activity at a 5% concentration and a corresponding 18.26% increase at a 10% concentration. At a 5% concentration, Tween 80 exhibited a decline of 16% in *CarP* activity, while at a 10% concentration, a marginal augmentation in *CarP* activity was observed. The 18.26% increase at a 10% concentration suggested that there might be an optimal concentration range where Tween-20 positively influences the conformation and catalytic efficiency of *CarP* (Zhang and Zhang, 2016). Triton X and SDS, both at 5% and 10% concentrations, demonstrated a reduction in protease activity when compared to the control group. The increase in protease activity may result from improved solubilization of *CarP*, leading to enhanced enzymatic activity. The decline in *CarP* activity at a 5% concentration of Tween 80 could be due to potential denaturation or inhibition effects, possibly associated with the surfactant concentration surpassing an optimum level (Holmberg, 2018).

Table 5.22: Effect of surfactants on *CarP* activity at 5 % and 10% concentrations.

Surfactants	**A_{620}**	**Total activity (U/mL)**	**Standard deviation**	***p*-value**
5%				
Control	0.96	11.52	0.172	0.0002
Tween-20	1.48	17.33	0.113	0.004
Tween-80	0.7	8.50	0.637	0.012
Triton X-100	0.75	9.07	0.113	0.0044
SDS	0.326	4.28	0.172	0.0005
10%				
Control	0.94	11.22	0.113	0.0001
Tween-20	1.36	16.01	0.172	0.0032
Tween-80	0.95	11.33	0.113	0.225
Triton X-100	0.64	7.90	0.065	0.0005
SDS	0.25	3.49	0.284	0.0008

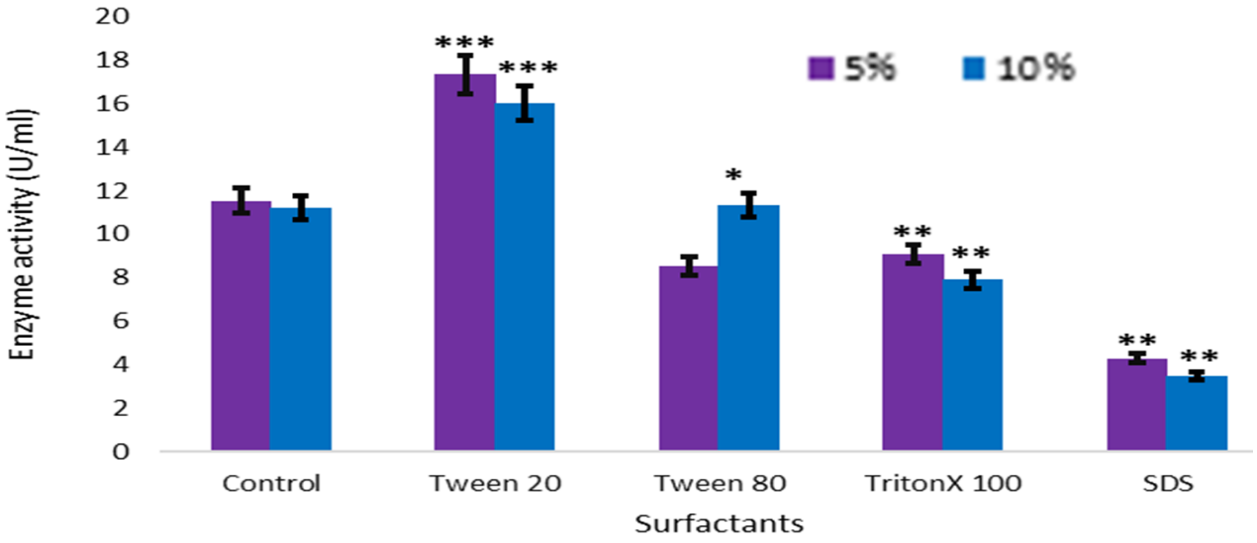

Figure 5.33. Bar graph depicting modulation of *CarP* protease activity in response surfactants (*p<0.05; **p<0.01, ***p>0.05).

5.31.2. Effect of surfactants on TreS activity

The effect of various surfactants on the activity of *TreS* amylase at concentrations of 5% and 10% was assessed (Table 5.23). The results indicate that the presence of surfactants can either enhance or inhibit enzyme activity depending on the specific type of surfactant. Among the surfactants tested, Triton X-100 and SDS both enhanced the enzyme activity compared to the control group at both concentrations. Specifically, Triton X-100 demonstrated enzyme activity of 12.16 U/mL and 12.49 U/mL at 5% and 10% concentrations, respectively. Likewise, SDS increased *TreS* amylase activity to 10.95 U/mL at 5% concentration and 11.4 U/mL at 10% concentration. In contrast, nonionic surfactants such as Tween-20 and Tween were found to reduce *TreS* amylase activity compared to the control group, suggesting that these surfactants may negatively interact with the enzyme or its substrate (Figure 5.34).

Studying the effect of surfactants on enzymes is important for several reasons. Surfactants are commonly used in industrial and laboratory applications to enhance the solubility and dispersion of proteins and other biomolecules. Understanding their impact on enzyme activity is crucial to optimize enzymatic reactions in various biotechnological processes. Additionally, surfactants can play a role in stabilizing enzymes and preventing their denaturation, which is important for the

development of robust and efficient enzyme-based systems. Moreover, insights into the specific interactions between surfactants and enzymes can guide the design of enzyme formulations for pharmaceuticals, food products, and other industries where enzyme activity is essential (Bento *et al.*, 2020, Goswami, 2020, Dao *et al.*, 2022).

Table 5.23: Effect of surfactants on *TreS* activity at 5 % and 10% concentrations.

Surfactants	**A_{540}**	**Total activity (U/mL)**	**Standard deviation**	***p*-value**
5%				
Control	0.134	8.28	0.331	0.0001
Tween-20	0.079	4.66	0.230	0.054
Tween-80	0.113	6.94	0.354	0.013
Triton X-100	0.191	12.1	0.644	0.051
SDS	0.173	10.95	0.571	0.048
10%				
Control	0.134	8.37	0.139	0.023
Tween-20	0.085	5.06	0.066	0.0001
Tween-80	0.115	7.12	0.102	0.001
Triton X-100	0.196	12.49	0.133	0.0002
SDS	0.177	11.24	0.102	0.0023

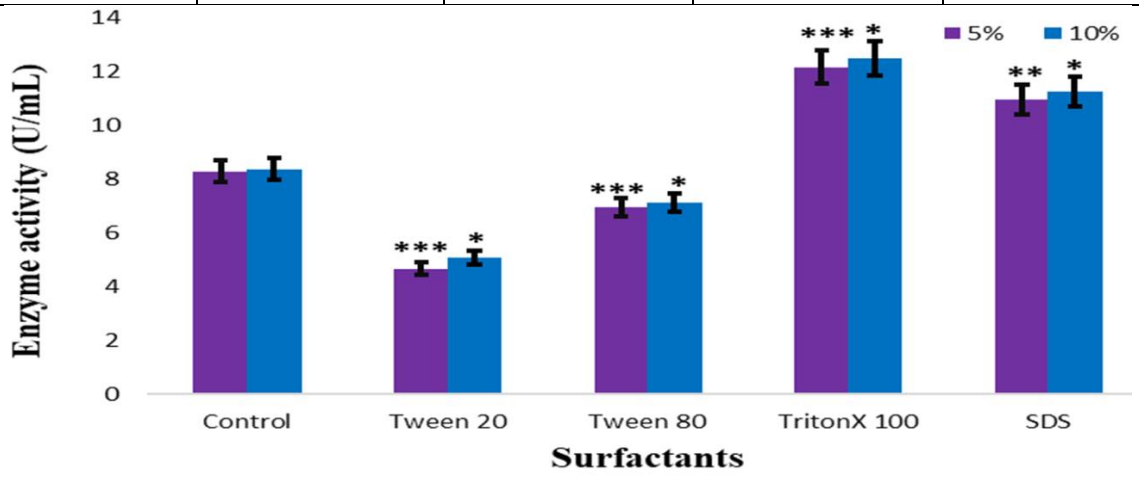

Figure 5.34: Bar graph depicting the effect of *TreS* amylase activity in response surfactants (*p<0.05; **p<0.01, ***p>0.05).

5.32: Substrate specificity

5.32.1: Substrate specificity of CarP protease

The observed substrate specificity of *CarP*, with higher activity on casein, BSA, and azocasein (Table 5.24, Figure 5.35), holds promising implications for its potential application in the food industry, particularly in processes involving casein utilization. Casein is a major protein component in milk, and its hydrolysis by proteolytic enzymes, such as *CarP*, is a crucial step in various dairy-related applications. The observed lower preference for fibrin and gelatin proteins may indicate a more selective substrate profile for *CarP* (Solanki *et al.*, 2021).

Table 5.24: Substrate specificity of *CarP* protease.

Substrate	A_{620}	Total activity	Standard deviation	*p*-value
Casein	1.826	21.257	0.172	0.0001
Azocasein	1.326	15.599	0.172	0.001
BSA	1.636	19.107	0.172	0.000
Gelatin	0.536	6.660	0.172	0.0001
Fibrin	0.421	5.339	0.113	0.007

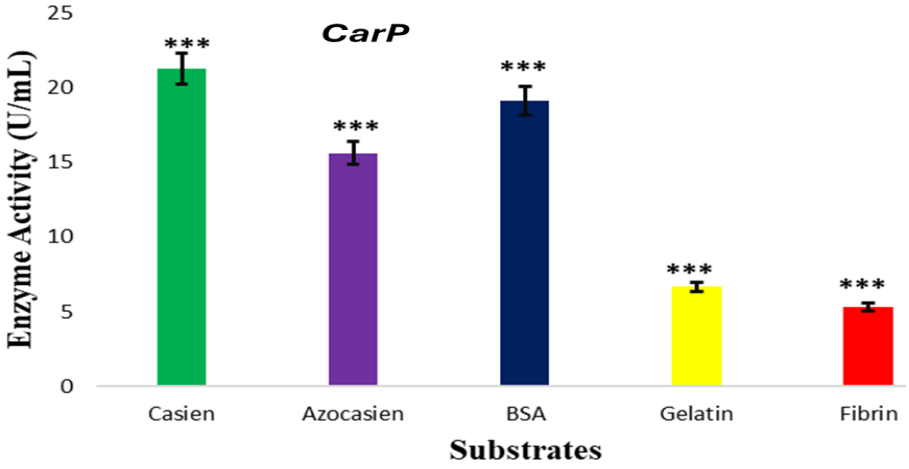

Figure 5.35: Bar graph depicting the substrate preferences of *CarP* protease (*p<0.05; **p<0.01, ***p>0.05).

5.32.2: Substrate specificity of TreS amylase

The enzyme amylase *TreS* exhibited varying substrate specificities when tested against five major amylase substrates: starch, dextrin, amylose, amylopectin, and glycogen (Table 5.25). Notably, *TreS* demonstrated its highest enzymatic activity of 12.94 U/mL against starch, indicating a strong affinity for this substrate. This suggested that *TreS* may be particularly effective in hydrolyzing the α-1,4 glycosidic linkages prevalent in starch, contributing to its pronounced activity with this substrate. Conversely, *TreS* showed its lowest activity of 4.11 U/mL against glycogen (Figure 5.36). This reduced activity implies a lower affinity or efficacy in cleaving the α-1,6 glycosidic bonds present in glycogen, compared to other substrates. Such findings highlight the enzyme's substrate selectivity and provide insights into its potential applications, particularly in processes involving starch hydrolysis.

Table 5.25: Substrate specificity of *TreS* amylase.

Substrate	A_{540}	Total activity	Standard deviation	*p*-value
Starch	0.202	12.94	0.139	0.0001
Dextrin	0.183	11.67	0.168	0.0009
Amylose	0.152	9.57	0.102	0.005
Amylopectin	0.119	7.34	0.066	0.0004
Glycogen	0.070	4.11	0.102	0.0001

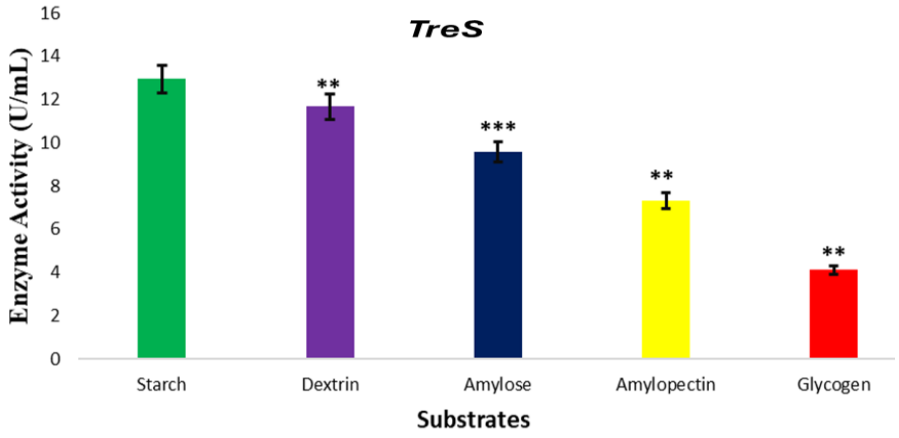

Figure 5.36: Bar graph depicting the substrate preferences of *TreS* amylase (*p<0.05; **p<0.01, ***p>0.05).

5.33: Enzyme inhibitors

5.33.1. CarP protease inhibitors

The impact of inhibitors on *CarP* activity is mentioned in Table 5.26. PMSF demonstrated significant inhibitory effects against *CarP*, resulting in a pronounced decrease in activity by 74.82% and 79.31% at concentrations of 5 mmol/L and 10 mmol/L, respectively. PMSF is a serine protease inhibitor, known for its specificity towards serine proteases like *CarP* (Figure 5.37). This suggests that the active site of *CarP* likely contains serine residues crucial for its catalytic activity, and their modification by PMSF results in a dose-dependent reduction in enzymatic function (Khan *et al.*, 2021). The β-ME is a reducing agent that can impact protein structure by breaking disulfide bonds. The observed inhibitory effect on *CarP* activity at both concentrations implies a redox sensitivity of *CarP.* The reduction in activity may be attributed to alterations in the enzyme's conformation or disruption of essential disulfide bonds critical for its catalytic efficiency (Bajaj *et al.*, 2023). The impact of EDTA on *CarP* activity revealed a relatively modest effect on the *CarP.* EDTA exhibited a reduction in *CarP* activity by 19.86% and 27.21% at concentrations of 5 $mmolL^{-1}$, and 10 $mmolL^{-1}$, respectively. EDTA is a well-known chelating agent that forms stable complexes with metal ions. The observed reduction in *CarP* activity suggested that metal ions may play a role in supporting the enzyme's catalytic activity. By chelating metal ions, particularly divalent cations, EDTA may interfere with essential cofactors or metal-dependent interactions crucial for *CarP* function (F. Sun *et al.*, 2020).

Table 5.26: Effect of protease inhibitors on *CarP* activity at 5mmol/L, and 10mmol/L concentrations.

Inhibitors	A_{620}	Total activity (U/mL)	Standard deviation	*p*-value
5mmol/L				
Control	1.153	13.71	0.001	0.0001
PMSF	0.293	3.98	0.195	0.0001
DTT	0.531	6.62	0.065	0.289
EDTA	0.941	11.11	0.195	0.0018
B-ME	0.421	5.30	0.065	0.201
H_20_2	0.761	9.14	0.065	0.068

10mmol/L				
Control	0.153	13.60	0.113	0.001
PMSF	0.246	3.37	0.653	0.0136
DTT	0.453	5.71	0.172	0.0002
EDTA	0.861	10.31	0.113	0.0003
B-ME	0.300	3.98	0.113	0.000
H_20_2	0.681	8.28	0.113	0.004

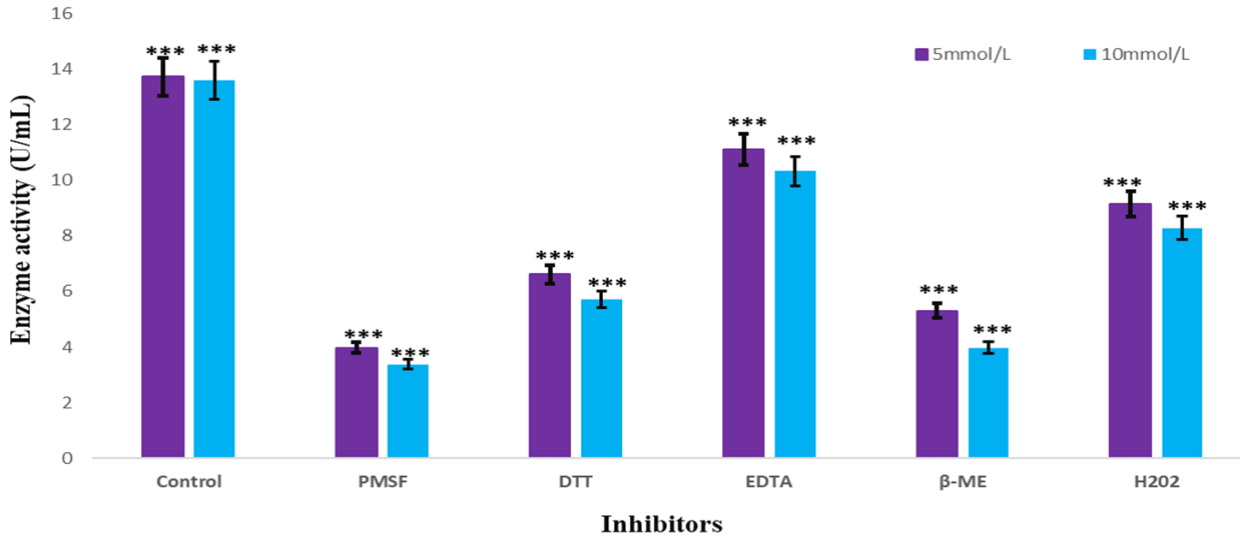

Figure 5.37: Bar graph depicting the effect of protease inhibitors on *CarP* activity (*p<0.05; **p<0.01, ***p>0.05).

5.33.2: TreS amylase inhibitors

The impact of amylase inhibitors on the activity of *TreS* was assessed in the context of their ability to diminish enzymatic function (Figure 5.38). As shown in Table 5.27, the inhibitor acarbose exhibited a significant inhibitory effect on *TreS*, reducing amylase activity by 82.91% at a concentration of 5 mmol/L and by 87.94% at 10 mmol/L. These substantial reductions suggested that acarbose effectively blocks the enzyme's active site or interferes with substrate binding, thereby greatly diminishing its activity. Similarly, the inhibitor miglitol also led to notable decreases in *TreS* activity, with reductions of 58.22% and 61.05% at concentrations of 5 mmol/L

and 10 mmol/L, respectively. Although miglitol's inhibitory effects are somewhat less pronounced than those of acarbose, they still represent a significant impact on the enzyme's function.

Table 5.27: Effect of amylase inhibitors on *TreS* activity at 5mmol/L, and 10mmol/L concentrations.

Inhibitors	A_{540}	Total activity (U/mL)	Standard deviation	*p*-value
5mmol/L				
Control	0.210	13.479	0.102	0.004
Acarbose	0.045	2.303	0.102	0.087
Miglitol	0.093	5.627	0.102	0.0002
Sulfonylurea	0.135	8.415	0.066	0.0001
Amylase inhibitor (Chickpea)	0.184	11.761	0.066	0.001
Amylase inhibitor (Papaya seeds)	0.172	10.891	0.133	0.0018
10mmol/L				
Control	0.210	13.367	0.066	0.0001
Acarbose	0.033	1.612	0.102	0.0188
Miglitol	0.087	5.203	0.066	0.0121
Sulfonylurea	0.126	7.813	0.066	0.0038
Amylase inhibitor (Chickpea)	0.165	10.467	0.139	0.0038
Amylase inhibitor (Papaya seeds)	0.143	8.973	0.102	0.0009

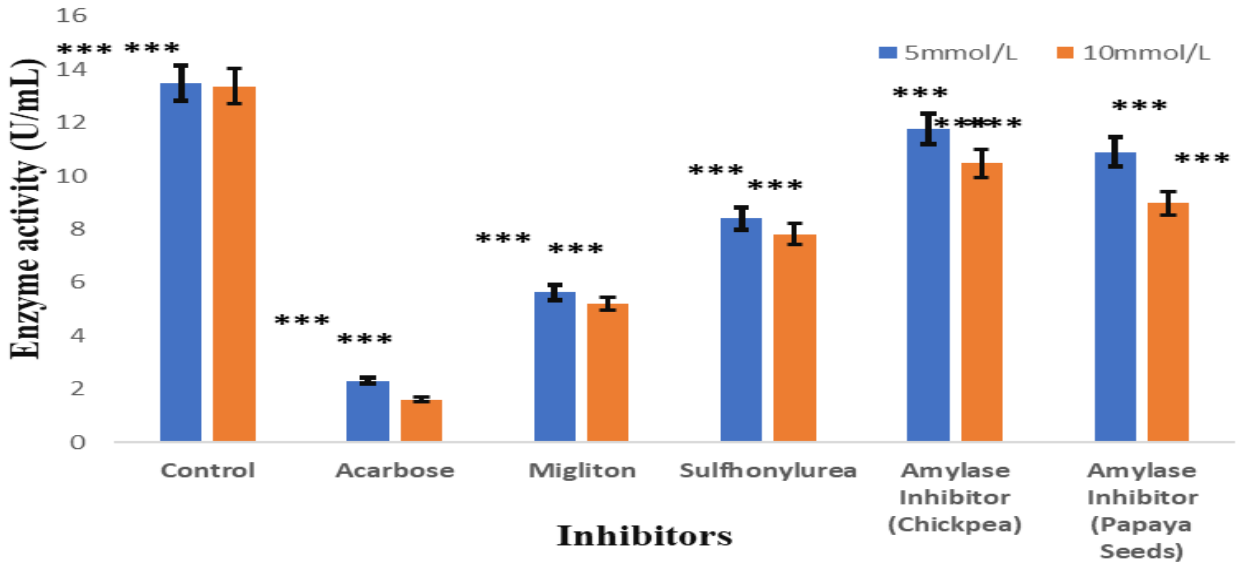

Figure 5.38: Bar graph depicting the effect of amylase inhibitors on *TreS* activity (*p<0.05; **p<0.01, ***p>0.05).

Chapter 6

SUMMARY AND CONCLUSION

Understanding the vast array of microbial diversity and its ecological significance is a central pursuit in modern microbiology. Metagenomics, a powerful approach that involves the collective analysis of microbial DNA extracted directly from environmental samples, has revolutionized our ability to explore and decipher the hidden microbial world. Metagenomics allows researchers to study entire microbial communities in their natural habitats without the need for laboratory culturing. By sequencing and analyzing DNA fragments obtained from environmental samples, metagenomic studies provide insights into the taxonomic composition, functional potential, and ecological roles of microbial communities. This approach has unveiled a staggering diversity of microorganisms, many of which were previously unknown or unculturable using traditional methods.

RHS, nestled within the picturesque Pir Panjal range of the North-Western Himalayas, stands as an intriguing ecosystem for metagenomic exploration. This unique thermal spring, characterized by its elevated altitude and distinct geochemical features, presented an ideal setting to study microbial communities thriving in extreme environments. The metagenomic study conducted in RHS holds significant promise for unraveling the genetic diversity, metabolic potential, and ecological significance of microorganisms inhabiting this geothermal site. The extreme conditions prevailing in RHS, including high temperatures, elevated mineral content, and varying pH levels, posed challenges and opportunities for microbial life. Through metagenomic analyses, we delved into the genomic makeup of microbial populations inhabiting RHS, uncovering novel genus, and genes tailored to survive and thrive in this specialized habitat. This metagenomic study facilitated the discovery of novel microbial taxa and functional genes with potential applications in biotechnology and environmental remediation.

In the study, industrially important genes encoded *CarP* and *TreS* were retrieved from NGS data of soil mDNA. These enzymes play vital roles in various biochemical pathways and are of interest due to their potential industrial and biotechnological applications. The target genes were identified and amplified from the soil mDNA. The amplified gene fragments were then cloned into two different expression vectors: pJET1.2 cloning vector and pET28 expression vector. The pJET1.2 vector facilitated the initial cloning step, while the pET28 vector enabled protein expression in a

heterologous host system. After successful cloning, the recombinant proteins were expressed in a *BL21-DE3* host organism. Metagenomic libraries containing genes encoding *CarP* and *TreS* were subjected to primary and secondary screening processes. Positive clones were identified and sequenced, followed by thorough computational analysis to investigate their phylogenetic relationships and structural properties. The proteins were subsequently purified using Ni-His tag columns based on affinity chromatography. The His-tag fused to the proteins allowed for efficient purification, exploiting the specific interaction between histidine residues and nickel ions immobilized on the column matrix. The purification process yielded highly pure samples of *CarP* and *TreS* enzymes, suitable for downstream biochemical and structural studies. The purified enzymes were then characterized through a series of enzymatic assays to assess their catalytic activities. *CarP* was evaluated for protease activity, while *TreS* was examined for amylase activity. Subsequently, the purified proteins underwent extensive characterization studies to elucidate their biochemical properties. The effects of various environmental factors, including temperature, pH, metal ions, surfactants, and inhibitors, on the enzymatic activity of *CarP* and *TreS* were investigated. Remarkably, both enzymes exhibited high thermostability and alkalophilicity, retaining their catalytic activities under extreme conditions. The detailed structural analysis of *CarP* and *TreS*, combined with their robust enzymatic activities and stability profiles, underscore their potential for diverse biotechnological applications. These findings expand our understanding of microbial enzyme diversity and provide valuable insights into the adaptation strategies of microorganisms in complex environments. The findings of the study are summarized below:

- The physiochemical parameters of the RHS site were meticulously examined, revealing a temperature range of 65-68°C and a pH range of 7.3-8.0.
- mDNA was successfully isolated from both water and soil samples collected from RHS. Subsequently, hydrolase genes were amplified via PCR using degenerate primers.
- Following the amplification process, the mDNA extracted from RHS soil was subjected to NGS analysis. Taxonomic analysis revealed *Proteobacteria* (41.03%), and *Actinobacteria* (16.7%) as the dominant phyla, with *CarP* and *TreS* selected for further investigation.
- Both *CarP* and *TreS* genes were cloned into pJET1.2 cloning vector and pET28 expression vector within the *BL21-DE3* host system.
- Positive clones were subjected to primary and secondary screening, and both were found to be show intracellular enzyme activity.

- Sequencing of positive clones was conducted, and subsequent sequence analysis was performed utilizing computational tools.
- *CarP* and *TreS* demonstrated distinct pI of 4.81 and 5.78, respectively, underscoring their differing biochemical properties.
- The purified *CarP* and *TreS* proteins were isolated using Ni-His affinity column chromatography. The concentrations were accurately determined to be 9.19 mg and 7.14 mg, respectively, which provided crucial quantitative data for further experimental analyses.
- *CarP* exhibited robust protease activity, reaching 29.01 U/mL, with an optimal temperature of 70°C and remarkable operational stability ranging from 10°C to 80°C. Similarly, *TreS* displayed maximum amylase activity at 80°C, showcasing similar thermal stability.
- Biochemical characterization involved studying the effects of temperature, pH, and metal ions on enzyme activity. Both enzymes demonstrated activity and stability across a broad pH range, with enhanced activity observed in the presence of Zn^{2+} for *CarP* and Co^{2+} for *TreS*,

6.1: Future scope

In the realm of metagenomics, the exploration of extreme environments presents both challenges and opportunities for scientific inquiry and technological advancement. Moving forward, research efforts should focus on developing innovative sampling techniques tailored to the unique conditions of extreme habitats, including deep-sea hydrothermal vents, polar regions, and high-altitude ecosystems (Ghiotto *et al.*, 2024, Zheng *et al.*, 2024). Additionally, there is a dire need for the advancement of bioinformatic tools capable of handling large-scale metagenomic datasets, enabling more robust analysis and interpretation of microbial communities in extreme environments. Functional metagenomics holds promise for identifying novel enzymes and metabolic pathways with biotechnological relevance, necessitating the implementation of high-throughput screening assays and bioinformatics pipelines for functional annotation (Dixit *et al.*, 2024, Middha *et al.*, 2024). Understanding the adaptive mechanisms of extremophiles is crucial for elucidating their ecological significance and evolutionary dynamics, requiring the integration of multi-omics approaches to decipher gene expression patterns, regulatory networks, and metabolic pathways involved in stress response and community interactions (Coleine *et al.*, 2024).

Moreover, leveraging extremophile-derived enzymes and biomolecules for biotechnological applications, such as biocatalysis, bioremediation, and pharmaceutical discovery, holds immense potential for addressing global challenges in sustainability and environmental stewardship (Santos-Pereira *et al.*, 2024). Synthetic biology and genome editing technologies offer new avenues for engineering microbial communities and designing custom-tailored functions for specific applications in extreme environments, while also enabling the exploration of climate change impacts on microbial biodiversity and ecosystem resilience (Chen *et al.*, 2024, Srivastava and Verma, 2024). By prioritizing interdisciplinary collaboration, technological innovation, and sustainable practices, researchers can unlock the untapped biotechnological treasures hidden within Earth's most extreme habitats and pave the way for transformative discoveries in metagenomics.

Chapter 7

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List of publications

S. No	Title	Journal
1	Characterization of thermostable carboxypeptidase from high-altitude hot spring metagenome	International Journal of Biological Macromolecules
2	Mining microbial tapestry using high-throughput sequencing and In silico analysis of Trehalose synthase (TreS) derived from hot spring metagenome	Biocatalysis and Agricultural Biotechnology
3	Building the taxonomic profile of the Riniaie Marwah hot spring of Kishtwar in Jammu and Kashmir: the first high-throughput sequencing-based metagenome study	Iranian Journal of Microbiology
4	Exploring the world hot springs: A bibliometric analysis of global trends in metagenomics research	Current Research in Biotechnology
5	Metagenomic screening strategies for bioprospecting enzymes from environmental samples	IOP Conference Series: Earth and Environmental Science
6	Discovering untapped microbial communities through metagenomics for microplastic remediation: recent advances, challenges, and way forward	Environmental Science and Pollution Research
7	Prospects of advanced metagenomics and meta-omics in the investigation of phytomicrobiome to forecast beneficial and pathogenic response	Molecular Biology Reports
8	Metagenomics in the fight against zoonotic viral infections: A focus on SARS-CoV-2 analogues	Journal of Virological Methods
9	Microbial adaptation to different environmental conditions: molecular perspective of evolved genetic and cellular systems	Archives of Microbiology
10	Metagenomic profiling of rhizosphere microbiota: Unraveling the plant-soil dynamics	Physiological and Molecular Plant Pathology
11	Revisiting metagenome of South-Asian hot springs for exploration of biomolecules	Book chapter
12	Role of Genomics, Metagenomics, and Other Meta-Omics Approaches for Expunging the Environmental Contaminants by Bioremediation	Book chapter

List of conferences and workshops

S. No	Conference name	Title	Host country
1.	International Conference on Bioengineering and Biotechnology (IConBET 2021)	Metagenomics for bioprospecting extreme environments	Malaysia
2.	The 2nd International Conference on Sustainable Plantation (ICSP)	Metagenomic screening strategies for bioprospecting enzymes from environmental samples	Indonesia
3.	6th International Conference: Strategies and Challenges in Agricultural and Life Science for Food Security and Sustainable Environment (SCALFE 2023)	Unraveling the metagenome based taxonomic profile of two Himalayan hot springs: a comparative study	India
4.	JK AGRI-MED Science Congress 2024	Hotspots of biocatalysis: Metagenomic analysis of thermostable hydrolases in extreme environments	India
5	International Conference on Plant Physiology, and Biotechnology 2021	Investigation of plant microbial networks using metagenomics and meta-omics for sustainable agriculture and ecosystem management	India
6.	Short term course on gene cloning and genome editing	Hands on training	India