Studies On Himalayan Polyextremophilic Microbes and Their Relevance in Agriculture and Industry

THESIS SUBMITTED FOR THE DEGREE OF
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By

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This thesis is dedicated to my Didia

(My late Grandmother), my greatest cheerleader and the source of all inspiration, encouragement and support.

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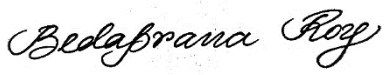
To the one I owe it all,

Bhagavad Gita: Chapter 7, Verse 10

"bījaṁ māṁ sarva-bhūtānāṁ viddhi pārtha sanātanam
buddhir buddhimatām asmi tejas tejasvinām aham"

O Arjun, know that I am the eternal seed of all beings. I am the intellect of the intelligent, and the splendour of the glorious.



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List of Abbreviations

- +: Positive
- -: Negative
- 16S rRNA: 16 Svedberg unit ribosomal ribonucleic acid
- A: Absorbance
- ABA: Abscissic acid
- ACC: Amino cyclopropane 1-carboxylic acid
- ANOVA: Analysis of variance
- $AgNO_3$: Silver Nitrate
- AS30TS: Arsenic 30ppm treated soil
- AR-Grade: Analytical Research Grade
- BLAST: Basic Local Alignment Search Tool
- B.O.D: Biological Oxygen Demand
- B: Boron
- BHI: Brain Heart Infusion
- BOD: Biological oxygen demand
- C: Carbon-oxygen double bonds
- C=O:
- Ca: Calcium
- CAS: Chrome Azurol S
- CAT: Catalase
- C.E.C: Cation exchange capacity
- Cfu: Colony forming unit
- Chl: Chlorophyll
- Cl: Chlorine
- cm: Centimetre
- Co: Cobalt
- CO_2: Carbon dioxide
- CFS: cell free supernatant
- CMC: Carboxymethyl Cellulose

- Cu: Copper
- CUTS: Control Untreated Soils
- CV: Crystal violet
- Da: Dalton
- DF media: Dworkin-Foster media
- Df: degrees of freedom
- DNA: Deoxy ribonucleic acid
- DNS: 3,5-Dinitrosalicylic acid
- DRTS: Drought treated soil
- E.C: Electric Conductivity
- EPS: Exopolysaccharide
- ELISA: Enzyme-Linked Immunosorbent Assay
- ESI MS: Electronic Spray Ionization Mass Spectroscopy
- EtBr: Ethidium bromide
- EDTA: Ethylene diamene tetraacetic acid
- Fe: Iron
- $FeCl_3$: Ferric chloride
- FTIR: Fourier Transform Infrared Spectroscopy
- g acceleration due to gravity, 9.8 m/s'
- GA: Gibberelic acid
- GPX: Guaiacol peroxidase
- H_2O: Water
- H_2O_2: Hydrogen peroxide
- HDTMA: hexadecyltrimethylammonium bromide
- HCI: Hydrochloric acid
- HCN: hydrogen cyanide
- $HgCl_2$: Mercury chloride
- H_2SO_4: Sulphuric acid
- Hydrogen (H)
- I: Iodine

- IAA: Indole acetic acid
- ICAR: Indian Council of Agriculture Research
- ISR: Induced Systemic Resistance
- ITS region : Internal Transcribed Spacer region
- K: Potassium
- KNO_3: Potassium nitrate
- KBr: Potassium bromide
- KOH: Potassium hydroxIde
- KSB: Potassium solubilizing bacteria
- LB broth: Luria Bartani broth
- L.B broth: Luria Bertani broth
- LPS: Lipopolysaccharide
- µg: microgram
- µg/l: microgram per litre
- µM/gm: micromolar per gram
- µg/ml: microgram per millilitre
- mg/kg: milligram per kilogram
- mg/l: milligram per litre
- mM: millimolar
- mm: millimetre
- MDA: Malondialdehyde
- Mg: Manganese
- Mn: Manganese
- Mo: Molybdenum
- M-O: Molecular oxygen bonds
- MEGA: Molecular Evolutionary Genetics Analysis
- MR-VP: Methyl-Red-Voges Proskauer
- MSE: Mean Squared error
- MAE: Mean Absolute error
- N: Nitrogen

- $NaAsO_2$: Sodium Arsenite
- NA: Nutrient agar
- Na: sodium
- NaCI: Sodium chloride
- NB: Nutrient broth
- NGS: Next generation sequencing
- nBLAST: Nucleotide BLAST
- NCBI: National Centre for Biotechnology Information
- NO_3: nitrate
- O_2: Molecular Oxygen
- OD: Optical Density
- OTU: Operational taxonomic unit
- O.C: Organic carbon
- P_2O_5: Phosphorus pentoxide
- PBS: Phosphate Buffered Saline
- PDA : Potato Dextrose Agar
- PDB : Potato Dextrose Broth
- PCR: Polymerase Chain Reaction
- PPGPB: Polyextremophilic plant growth promoting
- PGP: plant growth promoting
- PGPB: Plant Growth Promoting Bacteria
- PGPM: Plant growth-promoting microorganisms
- PGPR: Plant Growth Promoting Rhizobacteria
- PEG: Polyethylene Glycol
- Ppm: Parts per million (mg/L)
- PCR: Polymerase Chain Reaction
- PGPB: Plant Growth Promoting Bacilli
- PGPR: Plant Growth Promoting Rhizobacteria
- pH: Potential of Hydrogen (negative base 10 logarithm of H^+ ion activity)
- PO_4^{3-}: Phosphate ion

- PSB: Phosphate Solubilzing Bacteria
- PMSF: Phenyl methyl sulphonyl chloride
- PGPTS: Plant Growth Promotion treated setup
- PREDS: predicted value
- QC: Quality Control
- RPM: Revolutions per minute
- RMSE: Root mean squared error
- ROS: Reactive Oxygen Species
- R_2: Coefficient of determination
- S: Sulphur
- SA : Salicylic acid
- SMA: Skimmed milk agar
- SRA : Sequence Read Archive
- Sum_sq: sum of squares
- S5TS: Salt 5% treated soil
- TBA: Thiobarbituric acid
- TCP method : Tissue Culture Plate method
- Trp: Tryptophan
- TSB: Trypticase soy broth
- Tyr: Tyrosine
- TOC: Total Organic Carbon
- UV: Ultraviolet
- VOC: Volatile Organic Compound
- v/v: volume by volume
- W.B.: West Bengal
- Zn : Zinc

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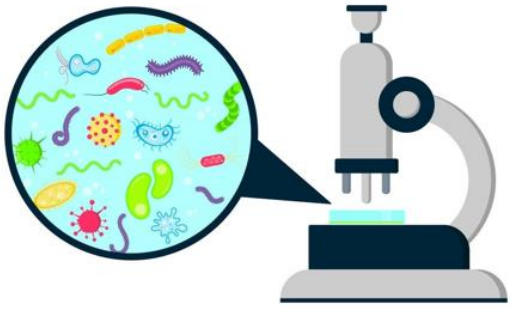
CHAPTER 1: INTRODUCTION



1. Introduction

Polyextremophily in the microbial world came from the Convergence of extremophilic properties in a single microbe. Though scientist have been intrigued the extremophilic properties of microbes, not much has been defined about their evolution, and the underlying mechanisms of survival. Still a lot is unexplored regarding the mystery about these organisms surviving environmental conditions that are incredibly hostile to others. They can relate to life in space, the thought of finding evidences to which has kept generations of scientists motivated. The conflict regarding abiogenesis and the extraterrestrial origin of life is extremely fascinating, that can be explored by studying extremophiles. Thus, the investigation commenced with the prerogative of searching Polyextremophillic microbes along with the exploration of enormous possibilities that lies within.

Extremophiles are organisms that can tolerate certain extreme condition, such as thermophiles, are tolerant to high temperature, halophiles are tolerant to high salt concentration, barophiles or piezophiles are tolerant to high pressure and similarly metallophiles are tolerant to heavy metal concentrations. But Polyextremophiles are those organisms that are not only tolerant to one extreme condition, but multiple extremes of conditions such as temperature (both high and low), pressure, salinity, radiation, pH, heavy metals etc. (Peeples, 2014).

These organisms have various applications in the field of industry, because such polyextremophiles are capable of producing certain enzymes having either industrial (e.g. Amylase, protease, lipase, laccase, lignin peroxidase), pharmaceutical (e.g. lipase, plethora of antibiotics) or agricultural (e.g. urease, nitrogenase, lignin peroxidase, laccase, cellulase, protease etc. biocontrol metabolites, plant hormones) importance, that can be stable at extremes of conditions and thus can be used by the industry with minimal levels of maintenance. Even enzymes used for molecular biological techniques need to be stable at various conditions which can be huge scope to explore in the genome of polyextremophilic microorganisms, as the genes (coding for the required enzymes) will have the necessary modifications needed to survive in such extreme conditions and be active in such a state. (Demirjian et.al, 2001; Van Den Burg, 2003).

Polyextremophiles not only have a promising future in pharmaceutical, agricultural or industrial applications, but they also form a huge link between terrestrial and extraterrestrial life, that is, Astrobiology. As these organisms can survive in multiple stress conditions and proliferate, they might also survive the harsh conditions of space. If we can understand how

these extremophiles can survive and maintain their genomic integrity in the harsh conditions of the Earth, Hoyle and Wickramasinghe Model of Panspermia (1983), that advances the panspermia hypothesis that proposes a part of life on Earth has an extraterrestrial origin and a number of bacteria and viruses, can have some conclusive proofs in its favor.

Moreover, very little literature is available on the evolutionary biology of Polyextremophiles, and their origin. Also, the concept of polyextremophily has contradictions regarding its modus operandi and thus needs further exploration. Not much have been done on this topic specifically be researchers in India, and there are very little or almost no research that has taken place in the Indian sub-continent regarding polyextremophilic microbes. Thus, this topic of investigation is not just thought intriguing but would also provide the researchers with such end products, that will be both commercially and scientifically viable.

The main concept behind the study is the change observed in organisms when subject to different abiotic stresses. The microorganisms that can survive multidimensional stress para meters might have miraculous potentials when it comes to their application in different scientific fields such as industrial and pharmaceutical biotechnology. Studying of the different exudates from polyextremophilic microorganisms might open up new avenues for industrial and pharmaceutical research.

The methodology was designed to meet the respective objectives. Detailed characterization of the bacterial isolates was done to determine their polyextremophilic nature. Different qualitative tests were carried out to understand the potential of the bacteria, for producing various important enzymes and metabolites that could be useful in agriculture and industry.

The Gangotri glacier, situated in Uttarakhand, India, is positioned at an altitude of 3415 metres above sea level. The Gangotri Glacier, located in the Garhwal Himalayan area, is the largest and most significant glacier in the Bhagirathi Valley. The region experiences a daily rainfall of no more than 15 mm. The sample of water studied in this investigation was obtained from the upper course of Ganges, at Gangotri, Uttarakhand the Pilgrimage Ghat of Gangotri (30.9947° N 78.9398° E).

According to Singh et al. (2006), the average highest temperature throughout the year was recorded as 14.6 °C, while the average lowest temperature was reported as 4.1 °C. The bacteria *Bacillus subtilis* BRAM_G1, *Bacillus subtilis* BRAM_G2 and *Bacillus subtilis* BRAM_G3 strains were isolated from the water of the collected from Gangotri. Because of the higher altitude, the UV index is around 34% higher. As a result, organisms in that location are

accustomed to seeing high amounts of UV radiation throughout the day (https://www.worldweatheronline.com/).

Yamunotri, located at coordinates 31.01°N 78.45°E, is the origin of the Yamuna River. It is situated in the Champasar glacier, which is north of Uttarkashi, at a height of 3293 metres above sea level. The river Yamuna originates from a frozen lake situated at an elevation of 4421 metres. However, due to its difficult accessibility, the temple of Yamunotri is situated on the foothills of the glacier. The region is home to several thermal springs, with the Suryakund being the most significant among them. The water sample used for this inquiry was obtained from the Suryakund, a thermal spring in that is a fascinating subject for scientific study due to its geothermal characteristics. Its high temperatures, often reaching 88 °C, indicate a significant geothermal gradient in the region. The hydrogeological setting involves groundwater movement through fractured rock formations, heating the water and emerging as a hot spring. Thermal springs like Suryakund often have unique mineral compositions due to the dissolution of minerals from surrounding rocks as water travels through the Earth's crust. These minerals can include silica, sulfur, calcium, and magnesium etc., thus can serve as a source of potential polyextremophilic bacteria.

Then survival capabilities of these strains were examined at extremes of temperature, UV rays, pressure, pH, salinity, drought and heavy metal concentrations thus proving them to be extremophiles. Furthermore, so as to verify tolerance of multiple extremes, for the justification of the “Polyextremophile” title, the 5 bacterial strains were subjected to 16, two-dimensional and 9 three-dimensional stresses in accordance with the probable combinations that are possible in nature (Mark C. Capece et al., 2013). All the five bacterial species were identified using 16srRNA sequencing. This resulted in 5 new strains of bacteria, which were accepted and acknowledged by GenBank, NCBI. All of them were whole genome sequenced. Formation of biofilm by the 5 strains were studied in detail because of its potential in stress tolerance. Their composition and changes were studied using different experiments. Finally, the increase in biofilm formation was also observed when the bacterial strains were subjected to multiple stress parameters thus signifying its role in protecting the bacteria in hostile conditions.

But, along with all these interesting properties of polyextremophiles, a field they could significantly contribute to is the gradual changing environment due to global warming, that these organisms are already adapted to, the topic addressed in this study. In an investigation by Liverman, 1991, it was explained that future changes in climate will decrease the soil moisture,

making it dry which will severely deplete agricultural productivity of the soil. This dryness will further lead to increased requirement of irrigation. The increase in irrigation rates will in turn increase the soil water table leading to significant increase in the soil salinity severely affecting agriculture as well (Utset. A. et. al, 2001). The reduction in soil organic matter is directly related to global warming in a vicious cycle. Organic matter reduction increases the greenhouse gasses, increasing global warming. The temperature increase due to this global warming can then affect the decomposition pattern of the soil organic matter and the cycle goes on (Miko U.F, 1995). In this alarming situation, polyextremophiles with incredible plant growth promoting and soil enriching qualities can come to the rescue. The microorganisms can thrive in the hostile environment of the soil created by global warming and climate change.

Properties like nitrogen fixation will sustain the soil nitrogen content (DeLuca et al., 1996) production of ligno-cellulytic enzymes will balance the soil organic carbon (Hemati et al., 2021) Production of ACC Deaminase, will help the plants survive the abiotic stresses in the soil (Glick., 2004). Some special properties such as plant hormone production and siderophore production will also have great impacts on the plant and soil health without inflicting any other harm on the general environment (Kraepiel et al., 2009). This would contribute towards the achievement of very effective PPGP bacterial strains which when applied in extreme soils would promote plant growth, survival and yield.

To understand a plethora of proteins and metabolites the bacterial strains are capable of producing, different qualitative and quantitative experiments could be designed, like Electron Spray Ionization -Mass Spectroscopy. The metabolites formed by the 5 bacterial strains in presence and absence of stress parameters can actually be an interesting field of study. The metabolites thus identified, produced by these organisms could therefore be made commercially available for different purposes in industry, pharmaceuticals, agriculture and molecular biology after further study.

Machine learning has recently become an invaluable tool in modeling bacterial properties and plant growth and yield in biological research. By employing various algorithms, researchers can analyze vast datasets to predict bacterial behaviors under different environmental conditions, such as biofilm formation, stress tolerance, and metabolic activities. For instance, machine learning models can predict how bacterial strains like BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 from the Gangotri and Yamunotri glaciers respond

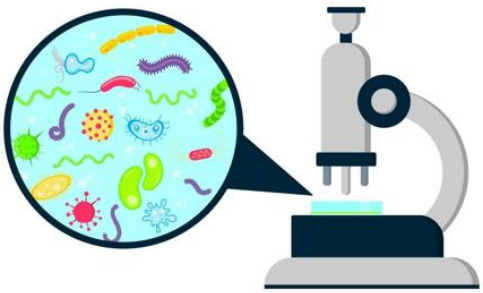
to various stressors, facilitating the identification of robust strains for biotechnological applications.

In conclusion, this topic of investigation will decipher multifarious applications of polyextremophilic microbes in the field of applied microbiology and biotechnology.

The research objectives for the study were thus laid down as follows:

1. Isolation and Characterization of extremophilic bacterial strains.
2. Profiling of their Polyextremophillic nature.
3. Detailing the prospects of the bacteria on agricultural benefits.
4. Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.
5. Construction of a Machine Learning Model for Analysis and Prediction of Plant Growth Promoting Properties and Maize productivity under stress condition.

CHAPTER 2: REVIEW OF LITERATURE



2.1. Introduction to extremophiles

Extremophiles, a term introduced by MacElroy in 1974, refer to organisms capable of thriving in and exhibiting optimal growth under severe and extreme environmental conditions. These environmental circumstances encompass significant variations in temperature, pressure, salt concentrations, pH levels, water availability, and even heavy metal concentrations, all of which are generally regarded as inhospitable. Extremotrophs, on the other hand, are organisms that are capable of growing in these extreme conditions, although not at their optimal level (Mueller et al., 2005). Extremophiles typically pertain to single-celled prokaryotic organisms, primarily found in the Archaea domain. These organisms are single-celled and have no nucleus. Various words are employed to classify distinct extremophiles. For instance, an acidophile, like *Bacillus acidocaldarius*, thrives best at a pH level below 3 or 4. On the other hand, an alkaliphile, such as *Bacillus circulans* or *Bacillus krulwichiae*, thrives best at a pH level above 10. Halophiles, such as *Bacillus* sp., are capable of thriving in salt concentrations of at least 1 M. Thermophiles, such as *Brevibacillus* and *Bacillus* sp., are capable of thriving in temperatures ranging from 60°C to 85°C. Bacteria that have an optimal growth temperature above this range are referred to as hyperthermophiles, for example, *Geobacillus* sp. Psychrophiles, specifically *Bacillus* sp., flourish in environments with temperatures ranging from 10°C to 20°C. Xerophiles are organisms that can flourish in environments with low water activities, while oligotrophs are organisms that survive in conditions of nutrient depletion. Heavy-metal tolerant microorganisms, commonly referred to as metalophiles or metalotolerant organisms, have the ability to thrive in environments that are contaminated with high levels of heavy metals, like copper, arsenic, cadmium, and mercury.

2.2. Taxonomic categorization of extremophiles

Extremophiles have been discovered to thrive in environments that inherently restrict life as we understand it. Typically, the study of individual severe situations includes temperature extremes, salinity extremes, pressure extremes, pH extremes, water limitations, oxygen limitations, and so on. The study of extremophiles provides valuable insights on astrobiology, biotechnology, and biogeography. The capability to reside in such harsh conditions also offers proof regarding the presence of extraterrestrial life. (Dartnell, 2011). Each extremophile living in a specific extreme environment has its own unique appellation.

Psychrophiles are organisms that have the ability to endure extremely low temperatures, typically below 15°C. There are two main groups of psychrophiles: stenopsychrophiles and eurypsychrophiles. Stenopsychrophiles are strictly limited to surviving at temperatures below 15°C, while eurypsychrophiles can develop best at temps below 15°C but can also tolerate higher temperatures. Cavicchioli (2006)

Thermophiles and hyperthermophiles are organisms that thrive at temperatures exceeding 60°C and 80°C, respectively. (Kashefi and Lovely, 2003). Thermophiles are mostly prokaryotic organisms, while hyperthermophiles are primarily archaea that have developed many adaptations to survive and thrive in extremely high temperatures. (Koonin and Martin, 2005).

Extreme temperatures, pH levels, and species that thrive in these extremes are categorised as acidophiles and alkaliphiles. Acidophiles thrive best in an environment with a pH below 3, whereas alkaliphiles thrive best in an environment with a pH over 9. The source cited is (Rothschild and Mancinelli,2001). Acidophiles primarily reside in environments such as acid mine drainage, acidic thermal hot springs, and bioreactors. On the other hand, alkaliphiles have been discovered in alkaline thermal hot springs, hypersaline soda lakes, and similar habitats. The sources used are Reeb and Bhattacharya (2010) and Kanekar et al. (2012).

Halophiles are creatures that can thrive in environments with high salt concentrations, which are often referred to as hypersaline settings. Halophilic organisms are categorised based on their ability to tolerate high levels of salt. Halotolerant species can withstand NaCl concentrations of up to 5%, whereas moderate halophiles are capable of thriving in environments with 20% NaCl concentrations. Lastly, extreme halophiles may thrive in conditions with about 30% NaCl concentrations. (Kanekar et al., 2012).

Living creatures that thrive under high pressure conditions are referred to as barophiles or piezophiles. The Bacterial class of Gama Proteobacteria, Crenarcheota from Archaea, and certain Eukaryotes exhibit a piezophilic tendency. (Abe, 2011). These organisms are primarily located in the deep sea, where the hydrostatic pressure ranges from 38 MPa to as high as 110 MPa. Piezophiles can be classified into two main categories: facultative and obligatory. The categorization is determined by their capacity to endure under standard pressure settings. This characteristic sets them apart from Piezotolerant species, which experience a decrease in growth rates but are not completely stopped from growing at greater pressures (Abe and Horikoshi, 2001).

Exposure to UV or ionising radiation poses a significant risk to live cells. Ultraviolet (UV) radiation causes harm to the genetic material (DNA), proteins, fats (lipids), and other parts of cells, which can result in cell death. The UV radiations with the shortest wavelengths, specifically UV-B and UV-C, which are below 254nm, are highly damaging. They promote thymine dimerization and generate breaks in the DNA backbone. Ionising radiation, such as chest X-rays, beta and gamma rays, can produce reactive oxygen species (ROS) and other free radicals. These substances have the potential to inflict severe harm to cells, perhaps leading to cell death. Microorganisms that are able to withstand such radiations are referred to as radioresistant microorganisms. They have mechanisms that can decrease the development of reactive oxygen species (ROS), creation of free radicals, and mechanisms to shield themselves from the resulting DNA damage. These species typically inhabit hydrothermal vents in the deep sea, where they are exposed to ionising radiation (Jolivet et al., 2004; Cadet and Douki., 2011; Dally ,2009 and 2011).

Water is the primary solvent in all living organisms, making it crucial for their survival. Consequently, situations that restrict water availability are among the most severe challenges an organism may face. In the absence of ATP, all metabolic reactions would cease, causing the cellular components, such as the cell membrane and enzymes, to collapse. Xerotolerant organisms are organisms that have the ability to survive desiccation up to a specific degree. On the contrary, xerophiles are capable of enduring extended durations without any water involvement through a process called anhydrobiosis. Anhydrobiosis is a condition of suspended metabolism, in which organisms can revive when water becomes accessible once more. Desiccating conditions can occur in arid regions such as deserts or evaporating ponds. The organisms endure conditions of limited water availability by employing two types of strategies. The first step involves water retention and prevention of loss, while the second step focuses on restoring the damage once water becomes accessible again. Dehydration causes significant DNA damage, and Xerophiles are recognised for their unique DNA repair systems. (Dose et al., 1992; Wharton and Marshall, 2002; Guidetti and Jönsson, 2002; Schill, 2010).

Metallophiles are organisms capable of thriving in the presence of heavy metals such as lead, mercury, silver, and others. These microorganisms have the ability to break down heavy metals and aid in the process of bioremediation. **Figure 2.1** illustrates the different categories of extremophiles and their respective habitats.

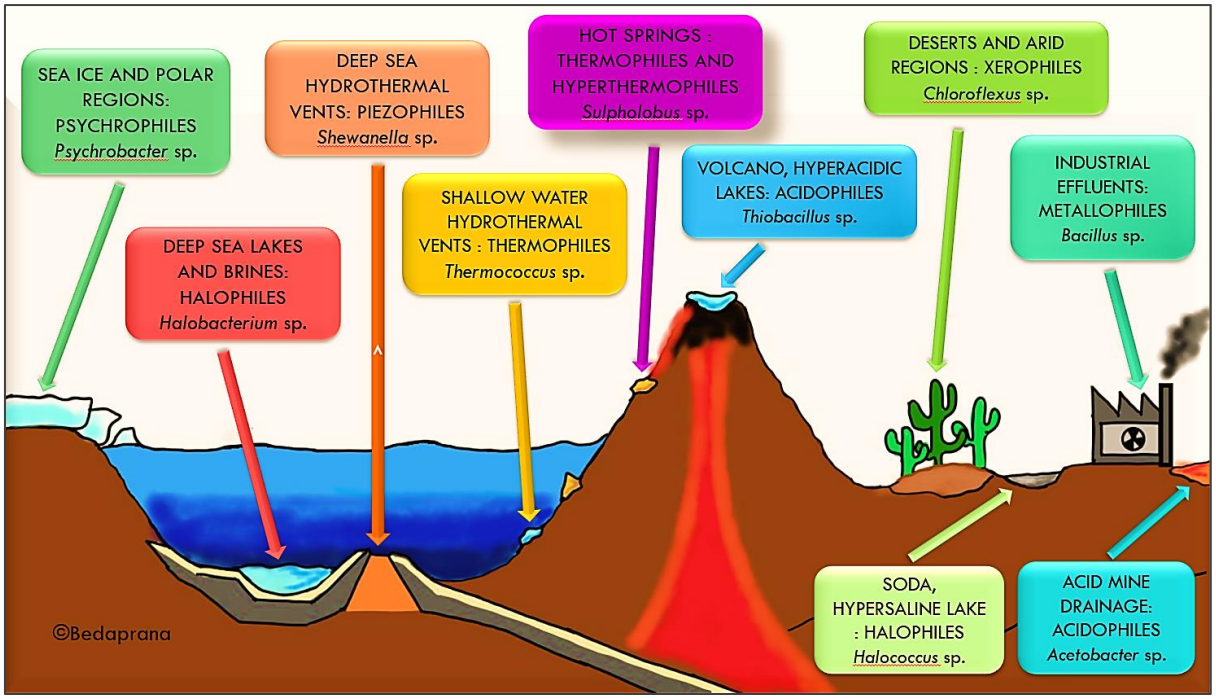


Figure 2.1: Different categories of extremophiles and their habitats with examples.

Defining Polyextremophiles

Polyextremophile, refers to an organism that is capable of thriving in multiple extreme environments. While extremophiles are often characterized by a single extreme condition, there are several natural settings that have two or more extreme conditions. For instance, numerous hot springs exhibit both acidic and alkaline properties, often including a significant number of metals. Additionally, certain hypersaline lakes are highly alkaline (soda lakes). Moreover, the deep ocean is typically characterized by its frigid temperature, poor nutrient content (oligotrophic), and exposure to high pressure. Notably, there has been a growing discovery of species and strains from these ecosystems that are able to withstand and endure various harsh conditions. These organisms are referred to as polyextremophiles and can be found in all three domains of life. Hydrothermal vents support populations of prokaryotes and multicellular creatures, such as tubeworms, clams, and other grazers, in conditions of high pressure, warmth, and acidic pH. There has been extensive research on the effects of individual extremes on microorganisms, but there is a lack of studies that examine the combined implications of these extremes on life as a whole. Our understanding of the evolutionary mechanisms that drive adaptation to multiple extremes is still in its early stages (Seckbach and Pabulo, 2015).

2.4. *Bacillus* as an extremophilic species

Bacillus sp. is the predominant genus that are metalotolerant and even metal remediating (Horikoshi et al., 2011; Orellana et al., 2018; Larkin & Stokes, 1966).

Bacillus subtilis, an extensively researched Gram-positive bacterium, possesses multiple methods to tolerate biotic stress. Pathogens, fungi, and other bacteria engage in competition with the bacterium, yet the bacterium's methods allow it to live and flourish. Bacillus subtilis produces a variety of antibiotics, such as bacitracin, polymyxin, and fengycin, in order to combat other microorganisms (Stein, 2005). This microorganism synthesises siderophores that have a high affinity for iron. *Bacillus subtilis* inhibits the availability of competing microbes to this essential nutrient (Saha et al., 2016). Additionally, they have the ability to create biofilms, which are intricate communities of cells held together by a matrix created by the cells themselves. Biofilms provide bacteria with protection against medicines and other poisons, while also facilitating bacterial collaboration. In addition, they utilise signalling molecules to perceive alterations in population density (Vlamakis et al., 2013). Quorum sensing regulates the growth of biofilms, the formation of spores, and the generation of secondary metabolites that hinder competition (Lopez et al., 2009). *Bacillus subtilis* produces antimicrobial peptides (AMPs) such as subtilin and bacillomycin, which break the cell membranes of competing microorganisms, so inhibiting their growth. They employ efflux pumps and enzymes to degrade or alter antibiotics produced by other microorganisms in order to thrive in competitive environments and prevail in conditions with fluctuating nutrient supply due to its capacity to grow on several substrates (Sonenshein., 2008). These mechanisms enable Bacillus subtilis to endure and flourish in many environments by adjusting to living organisms' stress.

Extremophiles are able to survive in certain severe situations, however, polyextremophiles are capable of optimal growth in numerous extreme settings, which is why they are called polyextremophiles (Saxena et al., 2016). The precise mechanism by which *Bacillus* sp. is able to withstand environmental challenges is through the process of spore production. The spore coat has the ability to shield the bacilli cells from many harsh circumstances, such as UV radiation or desiccation. The resistance to dehydration is attributed to the partial dehydration of the spore core and the protection of the genome by RecA-dependent DNA repair machinery, which binds to the genome. The presence of melanin in *Bacillus subtilis* spores provides them with resilience to UV radiation and even hydrogen peroxide. Melanin neutralizes reactive oxygen species (ROS) and serves as a mediator of DNA damage caused by UV light, providing

protection against both. The spore also provides physical protection against predators in natural environmental settings (Mckenney et al., 2013).

2.5. The origin and evolution of *Bacillus* as an extremophile from Prokaryota

Origin of Extremophiles in prokaryota:

Several hypotheses exist on the origin of life and the most recent shared ancestors. Matin et al. conducted a study in 2007 that regarded hydrothermal vents as potential environments for the emergence of life, thereby leading to the development of extremophiles. These systems are anticipated to be widely dispersed worldwide and have been present since the formation of water on Earth's surface. Black smoker vents are created at the locations where the borders of tectonic plates split into two, directly above the chambers of molten magma. The vents include distinct attributes like intense heat (reaching over 405°C), low pH levels ranging from 2 to 3, and high concentrations of iron, manganese, carbon dioxide, hydrogen sulphide, hydrogen, and methane in the fluids present. In contrast, the carbonate chimneys exhibit contrasting features, including elevated alkalinity (pH 9-11) along with moderate levels of heat, reaching temperatures close to 90°C. Additionally, these vents contain significant quantities of methane, hydrogen, and certain low-molecular-weight hydrocarbons. Serpentinization is a geochemical process that generates a significant quantity of hydrogen in these vents. The hydrogen (H_2) combines with the carbon dioxide (CO_2) present to produce methane, which is a geochemical process. The vents now are predominantly inhabited by anaerobic methanogenic bacteria, which are then replaced by methanotrophic bacteria during periods of lower temperatures and reduced vent activity. Several hyper-thermophilic bacteria or Archaea inhabit black smoker vents. They demonstrate chemoautotrophy, which refers to the generation of energy by the reduction of carbon dioxide or sulphates using hydrogen. Therefore, it can be suggested that the bacterial flora living in these thermal vents of the marine ecosystem exhibit comparable physiological traits to the first microbiological ecosystem on Earth (Martin et al., 2008). Phylogenetic data, which includes rRNA genes from both the small and large subunit, the core protein, cytology, etc., indicate that a substantial group of prokaryotic bacteria likely originated on land during the mid-Archean age. The group referred to as Terrabacteria, also known as this particular group, accounts for over 66% of the prokaryotic species that have been identified thus far. The taxa encompassed by this assemblage comprise Cyanobacteria, Actinobacteria, Chloroflexi, and Firmicutes.

Introduction of Firmicutes in the extremophile category:

The Firmicutes species encompass the class of Bacilli, which consists of individuals that have been discovered to possess extremophilic characteristics. The idea of evolution has been determined to be congruent with the evidence derived from geological markers and biomarkers that have been calibrated in conjunction. Terrabacteria, which includes the Firmicutes and the family of Bacilli, can be found under many extreme environmental circumstances such as those influenced by radiation, high salinity, or desiccation (Horikoshi et al., 2011). In 2007, Pikuta et al. discussed the survival of a species in stress conditions represented by two-dimensional matrices, which include combinations of factors such as pH and temperature, or salinity and pH. Assuming that thermophily was the initial condition, acidophily may have emerged early on. Alkaliphily would only occur if there is a buildup of minerals and a significant concentration of carbon dioxide. The emergence of halophily, however, is only attributed to the presence of arid terrestrial climates.

The Earth has experienced an increase in extreme environmental circumstances due to many human activities and environmental changes, including tectonic plate activity, changes in cryosphere dynamics, and the creation of endorheic basins. Plate borders are formed when two tectonic plates come into contact. The outcome is the production of mountains, profound oceanic trenches, mid-ocean ridges, volcanoes, and the creation of hydrothermal vents and other geothermal phenomena. Tectonic plate movements give rise to regions in the Earth's crust that can exhibit severe environmental conditions, including high temperatures, salinity, acidity, alkalinity, and metal concentrations. Furthermore, a significant reduction in temperature resulted in psychrophily, as reported by Pikuta et al. in 2007. According to Lozupone and Knight (2007), salinity was identified as the primary factor influencing the establishment of microbial communities in an environment, as opposed to factors like severe temperature or pH. While the selection pressures imposed by the severe environmental circumstances may be the primary determinant of extremophily, other characteristics of these settings also significantly contribute to the process of adaptation and development.

A significant portion of the Earth's surface is enveloped by ice, which is water in its solid form, known as the cryosphere (including glaciers, snow-capped mountains, polar ice caps, etc.), and has persisted for millennia. The characteristics and distribution of this frozen part of the Earth's surface are affected by various intricate processes, including precipitation, temperature, oceanic circulations, and so on. These serve as habitats for psychrophiles. Likewise, bodies of

water with a high concentration of salt are frequently located in closed basins and can support a community of salt-loving organisms known as halophiles. The depths of the Earth's ocean provide an extreme environmental situation with a water column reaching up to 3800 m, resulting in immense hydrostatic pressure. This extremely high-pressure habitat is ideal for barophiles, or more specifically, piezophiles. Examples of oligotrophic or nutrient-depleted ecosystems can be found in soils that are deficient in key nutrients like nitrogen, phosphorus, potassium, iron, and others. Oligotrophs are able to thrive in conditions of nutrient deprivation. Extreme settings can also arise due to the existence of chemicals or physical factors that induce toxicity. Instances include the existence of hazardous heavy metals like arsenic, cadmium, copper, chromium, etc. in soils or lakes with elevated levels of radiation. The prevailing harsh environmental conditions encompass the majority of the Earth's ecosystems, which likely evolved throughout time in various locations as a result of natural and geological phenomena. However, in recent times, various severe environmental conditions have emerged as a result of human activities. These include the rise in soil salinity caused by drilling deep wells for irrigation water, the increase in radiation from nuclear power plants, and the excessive pollution from industries, resulting in soils being contaminated with heavy metals and other harmful chemicals (Horikoshi et al., 2011). Hence, the emergence of extreme environmental conditions, whether caused by natural processes or human actions, has a role in the development of extremophilic species. These organisms can adapt to such extreme settings due to their varied molecular systems for tolerating stress. Ongoing exploration and research are uncovering previously unknown extreme environments, such as the subseafloor sediments found at great depths and carbonate chimney vent systems. These environments provide opportunities for the discovery of novel extremophiles, as demonstrated by the work of Kelley et al. (2001).

2.6. Biofilms and its association with extremophily

Environmental extremes occur naturally, as documented by Kaur et. al in 2019. These extremes include high levels of UV radiation, severe temperatures (either high or low), extreme pH levels (both high and low), high salt concentrations, and high exposure to antibiotics. These conditions produce a hostile environment, in which many bacteria have been discovered to not only survive but also grow (Blanco, Y. et al., 2019). These organisms are referred to as extremophiles. The source of this information is Wakai, 2019. The organisms mentioned encompass psychrophiles (able to withstand low temperatures), thermophiles (able to withstand high temperatures), acidophiles (able to withstand low pH levels), alkalophiles (able to withstand high pH levels), halophiles (able to withstand high salinity), barophiles/piezophiles (able to withstand high hydrostatic pressure), metallophiles (able to withstand high concentrations of heavy metals), and radiation-resistant organisms. These extremophilic microorganisms possess specialised methods to withstand stressful environments, with the production of biofilms being a significant mechanism which ultimately serve as a major tool to withstand multiple abiotic stresses (Fleming H.C. et al., 2016; Roy et al., 2022).

2.7. The study of biofilms and their correlation with extreme environments.

Microbial biofilms frequently develop as a result of environmental pressures and offer defence to the microorganisms against these pressures (**Figure 2.2**).

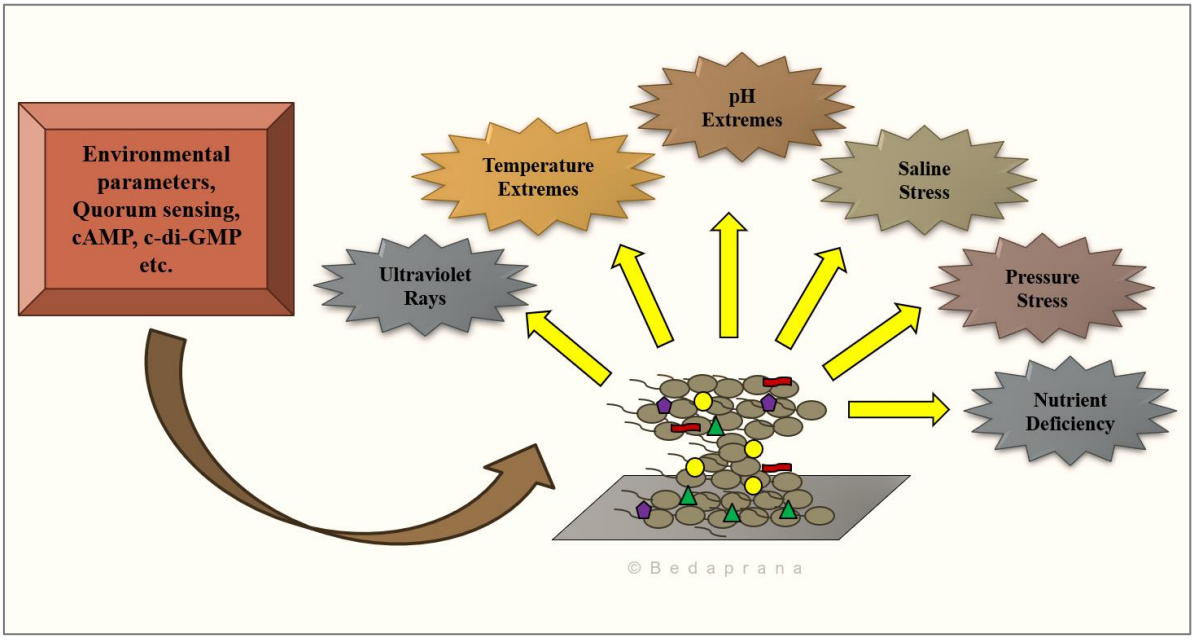


Figure 2.2: The production of biofilm in response to multiple abiotic stresses.

2.7.1. The relationship between UV radiation and biofilms

Solar ultraviolet (UV) radiation can be categorised into three types: UV-A (320-400nm), UV-B (290-320nm), and UV-C (100-290nm), which are distinguished by their specific wavelength ranges (Chatterjee, N et.al, 2017). Fossilised biofilms, dating back 3.2-3.4 billion years, have been discovered in South Africa and Australia (Westall et al., 2000; Rasmussen, 2000). This indicates that the creation of biofilms has aided bacteria in their ability to live in harsh and fluctuating environments, such as those with high levels of UV radiation. Ultraviolet (UV) radiation causes harm to proteins and DNA by producing reactive oxygen species, which can lead to the mutation of nucleotides. The biofilm development of *Listeria monocytogenes* provides protection against UV-C radiation (Bernbom, N et.al, 2011) refers to a source that should be consulted for further information. *Deinococcus geothermalis*, a member of the Deinococcaceae family, exhibits remarkable resistance to radiation. According to a study by Makarova et al. (2007), this bacterium forms biofilms that are significantly more resistant to UV radiation in comparison to individual planktonic cells. The primary mechanism behind this enhanced resistance is the entrapment of reactive oxygen species, which are produced by the breakdown of water molecules under UV irradiation, within the extracellular polymeric

substance (EPS) matrix of the biofilms (Frosler, et al., 2017). Hence, it can be inferred that biofilm functions as a protective barrier that is impervious to radiation, enabling organisms to endure and survive in extremely hostile radiation environments.

2.6.2. Thermal resistance and the formation of biofilms

Several pieces of evidence substantiate the notion that elevated temperatures can stimulate the creation of biofilms, which subsequently serve as a defensive barrier against these high temperatures. The process of biofilm formation was investigated in thermophilic bacteria belonging to the Bacillaceae family. Research has shown that biofilm production is most efficient for thermophilic species such as *Geobacillus*, *Aeribacillus*, and *Thermolongibacillus* at temperatures around 65 °C.

The bacteria belonging to the *Sulpholobus* genus, which are well-known for their ability to thrive at high temperatures and acidic environments, exhibit the highest rate of growth at a temperature of 75 °C (Cihan et.al,2017). However, there is a clear and significant four to five times increase in the development of biofilm in *Sulpholobus acidocaldarius* and *Sulpholobus solfataricus* when the temperatures are raised to 60 °C and 85 °C (Inskeep et.al, 2004 and Macur et.al, 2004).

Archaeoglobus fulgidus, a kind of hyperthermophile, exhibits biofilm development in response to several stressors, including high temperature and antibiotics, as a component of its overall stress response mechanism. Various types of biofilm structures are formed, including sheets, fibres, and even mixtures of these. The density of the biofilm increases as the stress level increases (Lapaglia and Hartzell's, 1997).

2.6.3. Cold tolerance and microbial biofilms

Despite the seemingly impossible conditions at temperatures around -20 °C, certain bacteria are able to survive beneath the snow. They achieve this by maintaining their metabolic processes, lipid membranes, and fluidity through the synthesis of PUFAs using enzymes called polyketide synthases, which remain active even at low temperatures. Several additional psychrophilic enzymes contribute to the preservation of survival under such low temperatures. (Thomas and Dieckman, 2002). However, even in this case, extracellular polymer serves as a sort of protective covering, similar to *Winogradskyella* CAL384, *Colwellia* GW185, and

Shewanella CAL 606. These organisms produce emulsions with the polymer to withstand freeze-thaw cycles (Caruso et al., 2018).

2.6.4. Acidic pH and Bacterial Biofilms

Ferroplasma acidarmanus is an archaeon that is primarily found in biofilm-associated structures, specifically those isolated from an iron mountain mine. Observations have revealed that it generates two unique forms of biofilms with contrasting morphologies, Planar biofilms and three-dimensional biofilms. Planar biofilms typically have a flat morphology and extend over surfaces. They are generally slender and have a greater surface area, enabling more effective exchange of nutrients with the surroundings. Planar biofilms frequently develop on surfaces where spatial and resource distribution are crucial. Three-dimensional biofilms exhibit intricate, multi-layered architectures that can take the shape of towers or mushrooms. Typically, these structures are thicker and have more prominent vertical features, which can offer improved defence against environmental pressures including high metal levels and excessive acidity. The three-dimensional architecture of the biofilm enables the formation of microenvironments that facilitate the growth of various microbial communities and their interactions.

Ferroplasma acidarmanus is an archaeon that thrives in highly acidic environments and has the ability to endure high levels of metals. This is due to its constant presence within a mixed biofilm population, which serves as a protective barrier against the severe environmental conditions (Baker et al., 2010). Under conditions of low pH, the toxicity of heavy metals also increases, leading to considerable alterations in the EPS (extracellular polymeric substances) and thus enhancing its tolerance to these extreme environments (Blanco, Y et al. 2019).

2.6.5. Effects of Elevated pH Levels on Biofilm Formation

Bacterial communities that are alkalophilic or inhabit very alkaline environments also exhibit biofilm development. Research has shown that *Dietzia* and *Alishewanella* are capable of thriving in environments with a pH as high as 11-11.5. However, even in these extreme alkaline settings, the internal pH of these microorganisms remains around 10. This is due to the presence of a thick coating of EPS (extracellular polymeric substances), which acts as a protective barrier. This finding highlights the role of biofilms in facilitating the survival of microbial communities in very alkaline situations (Charles, C.J et al., 2017).

Enterococcus faecalis, *Streptococcus anginosus*, and *Olsenella uli*, which were obtained from infected root canals in teeth, are capable of surviving for a duration of 4 hours at a pH level of 10.5. It has been observed that biofilms, which consist of bacteria, have a greater ability to survive changes in alkalinity compared to when they are in a free-floating form. This is because the biofilm acts as a protective barrier (Chavez de Paz et al., 2007).

2.6.6. Salinity and the Formation of Biofilms

Under extremely saline conditions, bacterial cells experience osmotic lysis and ultimately perish as a result of the escalating osmotic pressure on their cell membrane. Halophiles are able to flourish in saline circumstances because of various properties, one of which is the production of biofilms that are resistant to harm caused by high salt concentrations (Gagliano et.al, 2017). *Halomonas stenophila* HK30, a halophilic strain discovered in Morocco, has been observed to generate biofilm when exposed to a 5% w/v salt concentration in the medium (Amjres et.al, 2015). The halophiles produce an extracellular polymeric substance (EPS) layer that acts as a gel-like matrix, causing the cells to adhere and form aggregates. Microbial aggregates reduce exposure to the very saline environment, providing protection against osmotic stress. The topic of discussion is high pressure and its effects on biofilms. High hydrostatic pressure (HHP) is harmful to cell proteins, causing them to become dysfunctional and affecting cellular functions such as transcription and translation. Barophiles, also known as piezophiles, are microorganisms that have evolved to thrive and propagate in environments with high pressure conditions. Currently, there is limited knowledge on barophiles because of the inability to grow them in laboratory conditions. However, it has been discovered that biofilm formation enhances the resistance of microorganisms to mechanical pressure. Biofilm-producing microorganisms have greater resistance to high pressure compared to their planktonic counterparts. Research has also discovered that biofilms created by Gram-negative bacteria exhibit superior resilience to high hydrostatic pressure (HHP) compared to biofilms formed by Gram-positive bacteria (Masanta et al., 2015).

2.6.7. Oligotrophism and Biofilms

Oligotrophic conditions occur when bacteria experience a scarcity of nutrients, which negatively impacts their growth. Non-tuberculous mycobacteria have the ability to thrive in conditions when carbon concentrations are low, as demonstrated by Falkinham et al. in 2009. During conditions of undernutrition depletion, the biofilm employs many strategies to preserve the normal survival, metabolism, and reproduction of the bacteria. This allows the biofilm to once again serve as a protective barrier against excessive oligotrophism (Mittelman, M.W et.al., 2018).

2.7. Plant Growth Promotion ability of *Bacillus* species

The *Bacillus* genus is a significant component of the plant rhizosphere and has been extensively studied for its ability to promote plant development and its function in controlling plant diseases. They enhance plant development and boost crop output through various ways. These mechanisms encompass the synthesis of plant hormones, such as indole acetic acid (IAA), giberellins, cytokinins, nitrogen fixation, phosphate solubilization, production of siderophores for iron acquisition, and others. These mechanisms enhance the availability of vital nutrients necessary for plant growth. Their ability to manage biological organisms is due to their synthesis of antifungal, antibacterial, and volatile organic compounds. These compounds prevent the invasion of plant pathogens, so safeguarding the plant from harmful living organisms and their associated stresses (Yadav, 2017). Considering the *Bacillus* sp.'s ability to withstand harsh environmental circumstances and promote plant growth, they can be highly beneficial in sustainable agriculture, particularly in regions with challenging agroclimatic conditions. **Figure 2.3** provides a summary of the functions performed by *Bacillus* sp. as PGPB (plant growth promoting bacteria), both in enhancing plant growth and in combating the various environmental and biological stresses faced by the plant.

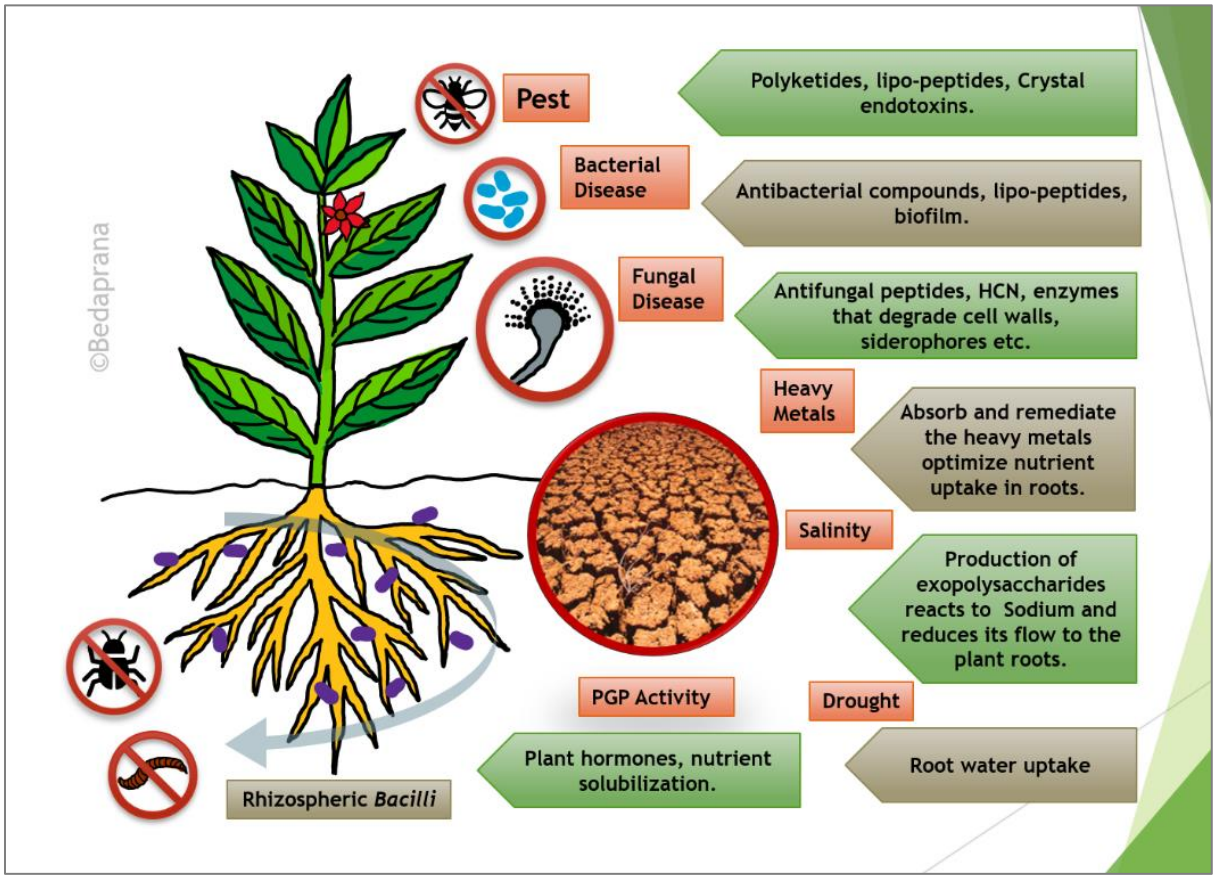


Figure 2.3: Different roles played by *Bacillus* sp. in response to both biotic and abiotic stress and plant growth promotion.

2.8. Investigation of the plant growth-promoting abilities of extremophilic Bacilli in the presence of different abiotic stressors

The extremophilic bacteria can also harness nutrients from nature and the biggest advantage being they can operate in stressful conditions. In the ongoing paragraphs, the role of these bacteria in plant growth promotion, in stressful conditions have been explained.

2.8.1. The role of extremophile *Bacillus* sp. in presence of thermal stress.

Plants face numerous abiotic stress situations, but the most significant obstacle to crop production and productivity is the abrupt fluctuation in temperature, salinity, drought, and other factors. A significant challenge in rainfed agroecosystems is the prevalence of temperature fluctuations. Microorganisms typically react to a fast rise in temperature by producing a distinct group of proteins called heat shock proteins. Research on the response of *Bacillus* sp. to environmental stress stimuli has shown that it is a very complex system governed by

approximately 10 sigma factors, which are influenced by the cellular states of the organism (Hecker et al., 1996; Redder, Hoper, et al., 2012). The sigma factors of *Bacillus* sp. can be categorised into two distinct groups based on their cellular states (Haldeman, 1995). **Figure 2.4** depicts the cellular conditions of the heat shock proteins in *Bacillus*. Several research on the critical thermal threshold for extremophilic *Bacillus* sp. have demonstrated the existence of multiple strains, such as *Bacillus caldolyticus*, with a critical growth range of 72°C to 75°C. *Bacillus stearothermophilus* has a critical growth range of 71°C to 76°C, while *B. coagulas, B. fumarioli, B. infernus, B. methanolicus, B. okuhidrnsis, B. smithii,* and *B. thermoamylovorans* have a critical growth range of 55°C to 65°C (Rainey et al., 2009; Cambi et al., 2001; Ulrich et al., 2018; Souza & Martins, 2001; Budde et al., 2006).

The wide variety of critical temperature thresholds exhibited by thermotolerant *Bacillus* species makes them highly advantageous as plant growth-promoting rhizobacteria. Typically, when *Bacillus* sp. is exposed to higher temperatures after being at its ideal growth temperature, there is a temporary rise in the production of heat shock proteins (HSP) (Redder, Hoper, et al., 2012; D'Amico et al., 2006).

Extremophilic Bacillus species flourish in harsh habitats, such as high-temperature temperatures, and their ability to survive depends on the synthesis of heat shock proteins (HSPs). These species have six recognised categories of Heat Shock Proteins (HSPs) that are essential for safeguarding cellular functioning during stress, namely thermal stress. HSP100, belonging to the Clp family, serves as chaperones by assisting in the reconfiguration of unfolded proteins and the breakdown of impaired proteins. HSP90 has a role in stabilising and properly folding different client proteins, which include proteins involved in signal transmission, cell cycle control, and protein breakdown. HSP70 facilitates the correct folding of newly formed polypeptide chains, repairs proteins that are misfolded or clumped together, and transports proteins across cell membranes. HSP60 assembles into a dimeric structure to facilitate the proper folding of recently produced or damaged proteins. Small Heat Shock Proteins (sHSPs) function as molecular chaperones by attaching to unfolded proteins, inhibiting their aggregation, and aiding their eventual refolding by other chaperones. HSP33 is a chaperone that is regulated by redox reactions. It helps prevent proteins from clumping together when the cells are exposed to oxidative stress (Nystro¨m, 2003).

The thermotolerance effects of *Bacillus cereus* strain SA1 on soybean plants under heat stress were investigated in a study conducted by Khan et al., 2020, yielding promising outcomes. The investigation involved subjecting the participants to heat stress for a period of 5 days in order to enhance the production of HSP. However, after 10 days of heat stress, a noticeable drop in the expression of GmHSP was detected. Strain SA1 was found to enhance the fundamental biomass and chlorophyll levels of soybean plants in both normal and heat stress conditions for a continuous period of 5 and 10 days, respectively. Ultimately, the inoculated plants exhibited an upregulation of stress-responsive genes such as GmLAX3 and GmAKT2, as well as changes in auxin levels, heightened ABA stimuli, potassium gradients, and other heat stress responses. Further analysis of *B. cereus* was conducted to assess its different activities that promote the growth of tomato plants. It was discovered that samples enhanced with bacterial inoculum led to an increase in shoot, root, leaf surface area, and other related factors. Increased production of extracellular polymeric substances (EPS) and the breakdown of 1-aminocyclopropane-1-carboxylic acid (ACC) into alpha-ketobutyrate acid and ammonia were observed under conditions of heat stress. The aforementioned evidence confirms that the chosen strain is a powerful PGPR bacteria for effectively addressing temperature stress circumstances (Mukhtar et al., 2020). **Figure 2.3** depicts the involvement of sigma factors in the sporulation process of *Bacillus* sp. in response to heat shock.

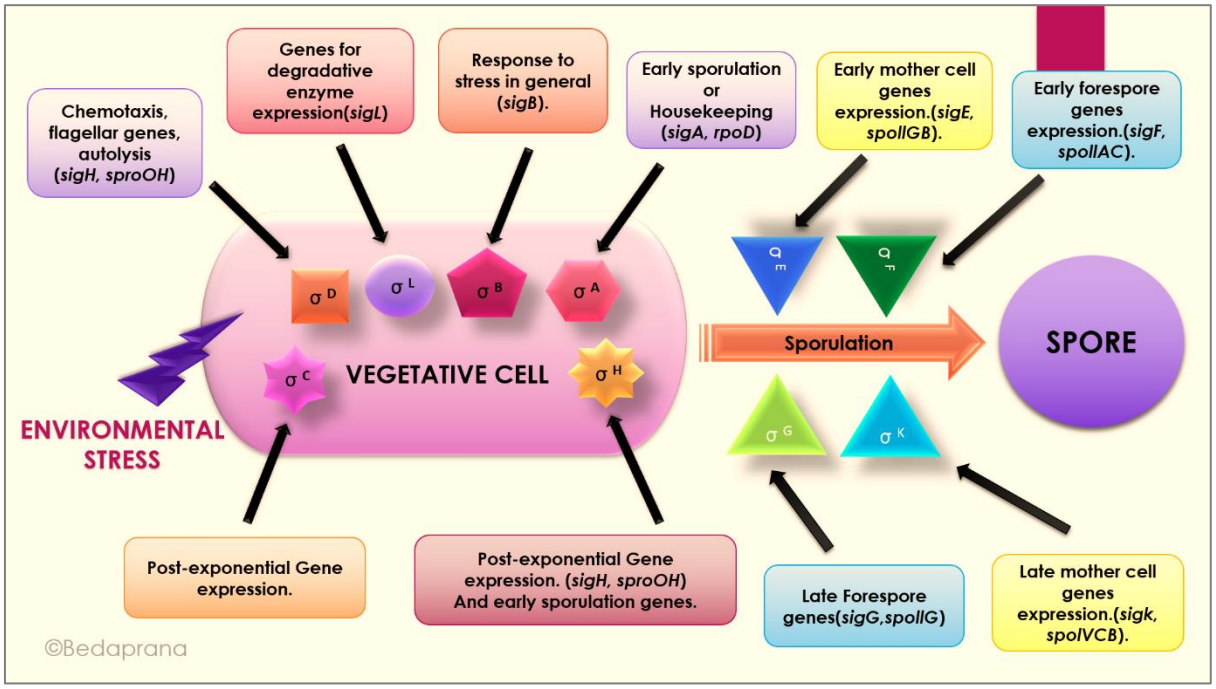


Figure 2.4: The heat shock response and sporulation proteins of *Bacillus* sp.

2.8.2. The role of extremophilic Bacillus sp. in the treatment of salt stress in plants

Salinity is one of the major abiotic factors that significantly affects global agricultural production and productivity. It is widely considered to be the primary factor responsible for desertification, impacting around 25% of the world's arable land (Machado & Serralheiro, 2017). According to reports, salinity is the main cause of degradation of around 10 million hectares of agricultural worldwide each year (Pimentel et al., 2004). It is not only near the coastline due to infiltration of saline water, but also due to creation of salt marshes in higher altitude mainly arising due to technote plate movements. The symbiotic relationship between microorganisms and plant roots has a substantial impact on promoting plant growth. Microorganisms, particularly different species of *Bacillus*, have been discovered to endure and enhance the development of plants in diverse stressful circumstances. Prior studies have demonstrated that in stressful circumstances, the ACC deaminase enzyme decreases the ethylene levels of a plant, hence promoting its growth. The sigma factors of *Bacilli* respond to heat shock and regulate development. A recent study conducted by Yaish et al. (2015) revealed a significant level of ACC deaminase activity in the endophytic bacterium *Paenibacillus xylanexedens* PD-R6, which belongs to the *Bacillus* subfamily and is associated with date palm trees. In addition to the elevated levels of ACC deaminase, many other mechanisms that promote plant growth, such as an enhanced synthesis of IAA, were also observed. Only a few numbers of strains were analysed and shown to possess the capability to chelate ferric iron, solubilize phosphorus and potassium, as well as create ammonia, among other abilities. PD-R6, a specific strain, was observed to enhance the root length of canola plants (*Brassica napus*) in both regular growth conditions and also in case of high salinity stress. It is noteworthy that the degree of ACC activity and IAA synthesis rose significantly with the rising content of NaCl in the growth media which reflects the microorganisms' ability to adapt and assist in the growth of plants in conditions of high salt levels. The microbial activities aid in mitigating the suppressive impact of ethylene and stimulating root development, hence augmenting the plant's overall ability to withstand stress. The mutualistic association between plants and microbes is essential for their survival in hostile situations.

Therefore, it can be inferred that *P. xylanexedens* PD-R6 has the ability to effectively modify the levels of ethylene and IAA in plants, thereby enhancing nutrient absorption and boosting the growth of palm trees in saline environments. Chinnaswamy et al. (2018) conducted experiments that found some strains of *Bacillus megaterium* NMp082, which are found in the

root nodules of *Medicago polymorpha*, had higher levels of IAA and ACC deaminase activity. These strains also have noticeable nifH and nodD genes responsible for nitrogen fixation. Additionally, it was revealed that strain NMp082 enhanced salinity stress tolerance in many plant species such as lucerne (*Medicago sativa*) and *Arabidopsis* sp. Previous research conducted on rhizospheric soils in Iran has demonstrated the existence of a halotolerant strain, K78, of the bacterium *Bacillus mojavensis*. Additional research on the characterisation of these strains has revealed an augmentation in ACC deaminase activity, resulting in an increase in the weight of both the roots and shoots of wheat plants, when exposed to saline stress conditions (Pourbabaee et al., 2016). Pourbabaee also demonstrated that the inclusion of K78 can enhance the ability of wheat plant cells to withstand osmotic stress when exposed to abiotic stress conditions.

When compared to salt-stressed plants that were not infected, the halotolerant strain RS341 *Bacillus aryabhattai* exhibited a 40% increase in both root length and dry weight of canola seedlings (Siddikee et al., 2010). Subsequent research revealed that the strain RS341 exhibited a noteworthy enhancement in seed germination ability when subjected to a NaCl concentration of 120 mM. Additionally, a reduction in seed ACC content and ethylene emissions was observed. In their study, Ghosh et al. (2003) observed an elevated level of ACC deaminase activity in strain *B. circulans* DUC1, which was obtained from the endophytes of several plants such as alfalfa, soybean, tomato, and maize. The strain was observed to enhance plant growth. An analogous finding was observed with the ACC deaminase-producing strain of *Bacillus firmus* DUC2, which was obtained from the rhizospheric areas of canola seedlings. Strain DUC3 of *Bacillus globisporus*, which was obtained from the rhizosphere of maize, tomato, and lucerne, exhibited reduced levels of ethylene and longer root length. In a study conducted by Xu et al. (2014), it was found that tomato seedlings exposed to salt stress conditions showed enhanced activity of ACC deaminase, improved ability to fix nitrogen, and higher plant development when they were inoculated with the endophytic strain HYT-12_1 of *B. subtilis*. In 2005, Han and Lee discovered that *B. subtilis* had the ability to enhance plants' tolerance to salinity by stimulating the synthesis of several plant hormones. The study revealed that plants treated with halophytic strains of *B. subtilis* exhibited enhanced plant growth and yield when exposed to high salt levels. This improvement can be attributed to the higher production of plant hormones such as auxin, cytokinin, gibberellins, etc., as well as reduced levels of ethylene (Bochow et al., 2001; Ashraf et al., 2004). Barnawal et al. (2017) observed similar outcomes where the *B. subtilis* strain LDR2 shown a rise in the levels of IAA in wheat seedlings under

salt stress conditions, leading to enhanced plant tolerance. The rhizospheric bacterial strain *B. subtilis* FZB24 was introduced to experimental plots of brinjal and pepper, and saline groundwater was used for irrigation. Bochow et al. (2001) showed a significant increase in yield of 550% in brinjal and 430% in pepper compared to the control sets that were not inoculated.

Subsequent studies demonstrated a significant increase of around 25% to 50% in the ability of both tested plants to tolerate saline stress. The idea of the bacterial strain's role as a plant growth promoter was examined through a controlled experiment, namely by measuring the levels of auxin and auxin precursors. The experiment involved subjecting the seedlings to millimolar concentrations of auxin precursors, such as tryptophan, indole-3-pyruvic acid, and indole-3-acetic aldehyde. An observed drop of 75% in growth was seen in the uninoculated set under salt stress, which was subsequently compensated within a week. However, similar effects were not observed in pre-applied IAA sets, suggesting that the manner of action of the bacterial strain increased salt stress tolerance. Salinity stress in plants is a major cause of elevated levels of reactive oxygen species (ROS) in plant leaves, which in turn leads to an increase in membrane lipid peroxidation (MDA). When wheat seedlings were exposed to 2% NaCl stress and inoculated with *B. subtilis* strain 10_4, there was a decrease in the amount of MDA or lipid peroxidation stress (Lastochkina et al., 2017). These results provide additional evidence of the protective impact of halophytic bacteria and their capacity to further regulate the activity of oxidative enzymes and the levels of hydrogen peroxide in plants.

2.8.3. The role of extremophilic Bacillus sp. in mitigating drought stress in plants

Drought is a significant and widespread form of stress experienced globally. According to Araus et al. (2008), it hampers the growth of plants and decreases the yield of the crop. The plant immediately adjusts its physiological processes to minimise water use and waste in response to water scarcity. The modifications involve the closure of stomata, decrease in the rate of photosynthesis, buildup of abscisic acid, expression of aquaporins, and adjustments in osmotic pressure to uphold cellular turgidity. Reactive oxygen species (ROS) can cause damage to cellular structure, impair cellular function, and ultimately lead to the death of plants (Araus et al., 2008; Cramer et al., 2011; Ruan et al., 2010; Krasensky & Jonak, 2012). Bacilli, particularly *B. subtilis*, assist plants in combating drought stress by producing phytohormones like indole-3-acetic acid, exopolysaccharides, and volatile organic compounds. It induces systemic resistance, reduces ethylene levels, and enhances nutrient uptake by solubilizing

essential minerals. These actions help plants overcome drought stress and maintain their resilience and productivity.

Various studies (Zhang et al., 2010; Martins et al., 2018; Wang et al., 2012) have demonstrated the existence of mechanisms. A specific strain of *B. subtilis* B26 was discovered to inhabit the internal tissues of *Phleum pratense* L. This strain demonstrated the ability to enhance the growth of the plant when exposed to drought conditions for approximately 8 weeks. The presence of *B. subtilis* B26 resulted in a significant increase in the biomass of both the roots (by 63.8%) and shoots (by 26.6%), as well as an improvement in photosynthetic activity (by 55.2%) and stomatal conductance (by 214.9%) compared to plants that were not inoculated with *B. subtilis* B26 (Gagne´-Bourque et al., 2016). The drought stress tolerance in host plants induced by *B. subtilis* is regulated by various mechanisms, including the production of phytohormones, which are enzymes that eliminate reactive oxygen species (ROS) and prevent ROS-induced damage. Other mechanisms include ACC deaminase activity, which controls the expression of genes related to drought stress response, as well as the accumulation of soluble sugars and osmolytes. These findings were reported by Barnawal et al. in 2017. The production of plant hormones, specifically auxins, by several strains of Bacilli improves the growth of *Zea mays* under situations of limited water availability (Moon et al., 2017). Various drought-tolerant strains of *Bacilli*, including *B. aryabhattai* AB-51, *B. licheniformins* AM-21, *B. toyonensis* AM-51, and *B. pumilus* AB-33, were obtained from Northeastern Pakistan, where the water circumstances were quite challenging. Even in situations of water scarcity, the introduction of a combination of these three specified bacterial strains mentioned above resulted in a remarkable enhancement in the characteristics of both the roots and shoots (Moon et al., 2017). Martins (2018) observed similar results for the *B. subtilis* UFGSI strain when it was employed to mitigate drought stress in soybean plants. Drought-stressed plants have a significant increase in reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), singlet oxygen radicals, hydroxyl ions (OH), superoxide anions, and others. These ROS have harmful effects on plant's well-being and the integrity of their cells. The oxidative damages are reduced by a variety of enzymes, including superoxide dismutase (SOD), phenol oxidase (PO), catalase (CAT), peroxidase, glutathione reductase, and ascorbate peroxidase (APX). Nonenzymatic components, such as cysteine, glutathione, and ascorbic acid, are involved in alleviating oxidative stress (Kaushal and Wani, 2015). Studies have observed that several strains of *B. subtilis* can exhibit CAT, SOD, and APX activity, which helps them to use reactive oxygen species (ROS) generated during drought stress. This ability of *B. subtilis* strains to

relieve plants from oxidative damage caused by ROS accumulation has been observed. The efficacy of *B. subtilis* has been demonstrated in many crops (Saikia et al., 2018; Vardharajula et al., 2011). The plant cells respond to drought stress by increasing the levels of osmolytes such as sugars, polyamines, proline, amino acids, quarternary ammonium compounds, and proteins such dehydrins that are associated with water stress. This helps the plant to adapt to the drought conditions (Chen & Jiang, 2010; Krasensky & Jonak, 2012). Research has shown that *B. subtilis* can create osmolytes in response to drought stress. This helps boost plant growth by increasing the osmotic potential of the plant roots and increasing their turgidity (Paul et al., 2008; Dimkpa et al., 2009). For instance, researchers discovered that a particular strain of *B. subtilis* GB03, which is a bacterium found in soil, enhances the ability of *Arabidopsis* plants to withstand osmotic stress. This is achieved by the production and increased accumulation of osmoprotectants such as glycine betaine (GlyBet) and choline (Cho) (Zhang et al., 2010). The accumulation of soluble sugars in plant cells serves as a defence mechanism against osmotic stress and aids in mitigating drought stress by restoring balance in plant metabolism. This restoration is also facilitated by endophytic *Bacillus* bacteria (Valliyodan & Nguyen, 2006; Gagne´-Bourque et al., 2015). **Figure 2.5** summarises the mechanism of drought and salinity stress management by PGP bacterial strains.

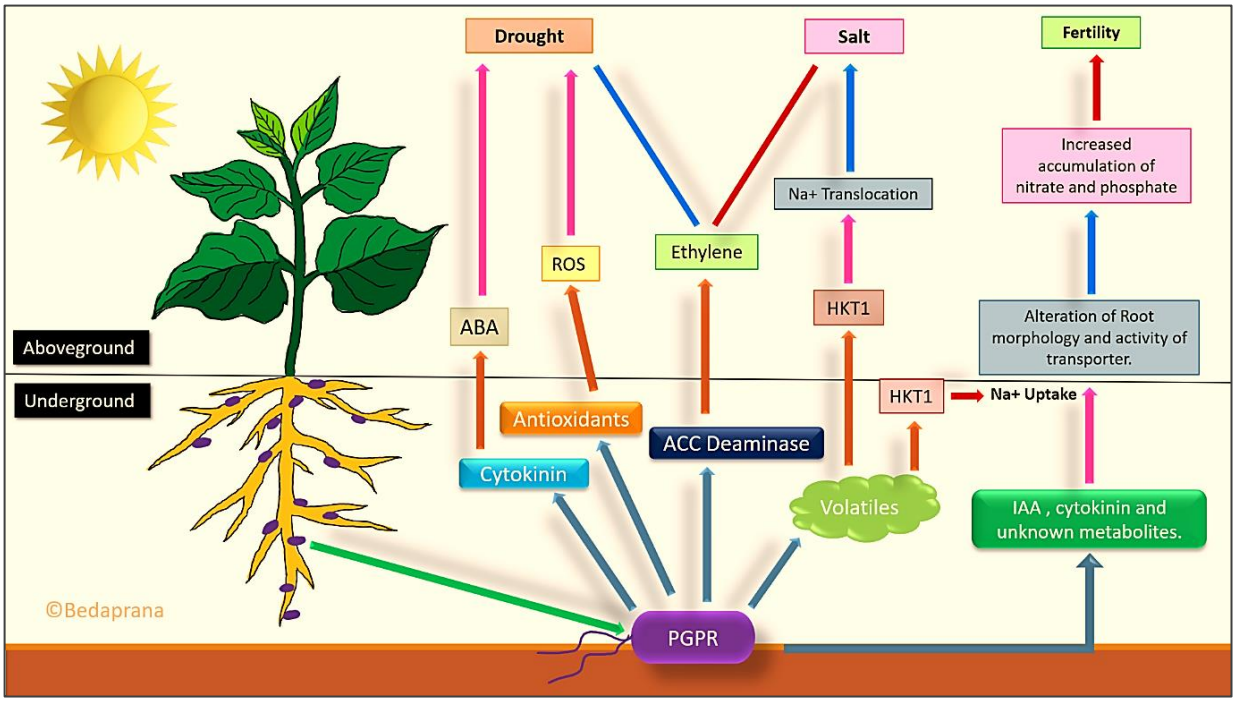


Figure 2.5: PPGP bacteria in response to drought and salinity stress in plant rhizosphere.

2.8.4. The role of extremophilic *Bacillus* sp. in soil contaminated with heavy metals.

Soil is deemed contaminated with heavy metals when the levels of metal deposition, such as mercury, arsenic, cadmium, chromium, lead, etc., surpass the normal background values (Abd-Alla et al., 2012). Because metals are nonbiodegradable, their concentrations increase in the environment, leading to harmful effects on human health (Li et al., 2015). Recently, the rapid growth of industry and other human activities, such as mining, have resulted in the accumulation of large amounts of heavy metals in the soil. The overuse of chemical fertilisers and pesticides is also a contributing factor to the accumulation of heavy metals in soil (Malidareh et al., 2014). The of heavy metals in colour pigment manufacture stems from their brilliant hues and long-lasting properties. Lead, cadmium, chromium, and mercury are frequently utilised in pigments for paints, ceramics, and several other purposes. Lead chromate yields vibrant yellow and orange hues, whilst cadmium sulphide generates intense yellow and red tones. Chromium oxide is renowned for its vibrant green colour, whereas mercury sulphide produces a striking red pigment called vermilion. These pigments, which are composed of metals, are highly valued for their durability and vividness, making them ideal for both artistic and industrial applications. Nevertheless, the production and use of these substances provide notable environmental and health hazards due to the toxicity of heavy metals. These metals have the potential to accumulate in organisms, resulting in a range of health problems including neurological and developmental abnormalities (Ja¨rup, 2003).

The impact of heavy metal concentrations on the soil is manifested in multiple ways. Metals have an impact on the metabolism and respiration of microorganisms, which subsequently affects soil respiration, commonly known as the metabolic entropy response (Blagodatskaya et al., 2006). The level of microbial metabolism entropy is elevated in soils that are polluted with heavy metals. There is a decrease in the amount of carbon that is available for biological processes. These metals have a significant impact on plant growth, causing leaves to turn yellow, chlorosis, leaf spots, senescence induction, reduced photosynthetic activity, and other disruptions to physiological functions. Additionally, these substances have the potential to accumulate in the human body resulting in harmful health effects. For instance, cadmium can lead to weakening of bones, while lead exposure can negatively impact fertility (Bahadir et al., 2007). Various remediation techniques, including physical and chemical methods such as soil cleaning and soil flushing, can be employed. However, these approaches have their own drawbacks, such as the requirement to excavate the contaminated soil when using soil washing.

Soil flushing, on the other hand, entails the injection of a leaching agent directly into the soil, rendering soil excavation unnecessary (Race et al., 2018). Chemical remediation, although effective for removing heavy metals from soil, is not considered environmentally sustainable. That is why the field of microbial heavy metal remediation has promise, as it is both efficient and environmentally benign. Microbial heavy metal cleanup mostly happens through two mechanisms, biosorption and bioleaching.

- *Biosorption* is a process in which microorganisms collect heavy metals by either absorption or adsorption. Absorption refers to the process in which a substance, known as the absorbate or fluid, is dissolved by another substance, referred to as the absorbent, which can be either a solid or a liquid (Jovancicevic et al., 1986). Hence, absorption encompasses the entire volume of the substance, while adsorption is limited to the surface. Adsorption is the process by which heavy metals are attached to the surface of a cell through the development of a complex, which is subsequently taken up by the cell (Danis et al., 2008). The cell wall of bacteria is composed of many functional groups, including oxygen, nitrogen, phosphorus, and sulphur. These groups have the ability to readily interact with heavy metals, forming coordination complexes. As a result, the adsorption and subsequent absorption of heavy metals by bacteria becomes a very straightforward process. One possible contributing component is the ability of carboxyl groups or phosphoric acid and negatively charged ions to readily interact with positively charged surfaces of heavy metals, facilitating adsorption (Brady & Duncan, 1994; Sarret et al., 1998). The adsorption of heavy metals by microorganisms is often a highly efficient process. Bacillus sp. has the ability to adsorb as much as 60% of copper within the first minute at a pH of 7.2. The adsorption equilibrium is achieved within a total of 10 minutes, as reported by He and Tebo in 1998.
- *Bioleaching* involves the secretion of certain chemicals by microorganisms, which have the ability to dissolve heavy metals present in soil contamination. The molecules involved in this process can be classified as organic acids, and the process itself is referred to as bioleaching (Chanmugathas & Bollag, 1988).

In addition to the two above mentioned processes, biomining and bio- oxidation are two others means of mitigating heavy metal stresses. Biomining is a process that involves the mobilisation of heavy metal ions from their insoluble ores utilising biological dissolving agents or complexation techniques. Bio-oxidation, as explained by Brunetti et al. (2012), is a technique that employs microorganisms to facilitate the oxidation of metal ions, resulting in

the creation of stable and less harmful metal compounds. This process is especially important in the context of bioremediation and the synthesis of pigments. Microorganisms, including bacteria and fungi, have the ability to oxidise heavy metals such as iron, manganese, and copper through their metabolic processes. During the process of bio-oxidation, these microorganisms transform metal ions from a state of lower oxidation to a state of higher oxidation, leading to the formation of metal oxides or hydroxides. The stability and brilliant colours of these biogenic oxides and hydroxides make them suitable for harvesting and use as pigments. This technique not only offers a sustainable approach to generate pigments but also aids in the removal of toxic heavy metals, rendering it a valuable procedure for both industrial applications and environmental stewardship. **Figure 2.6** summarises heavy metal stress mitigation and plant growth promotion by HMT-PGP bacteria.

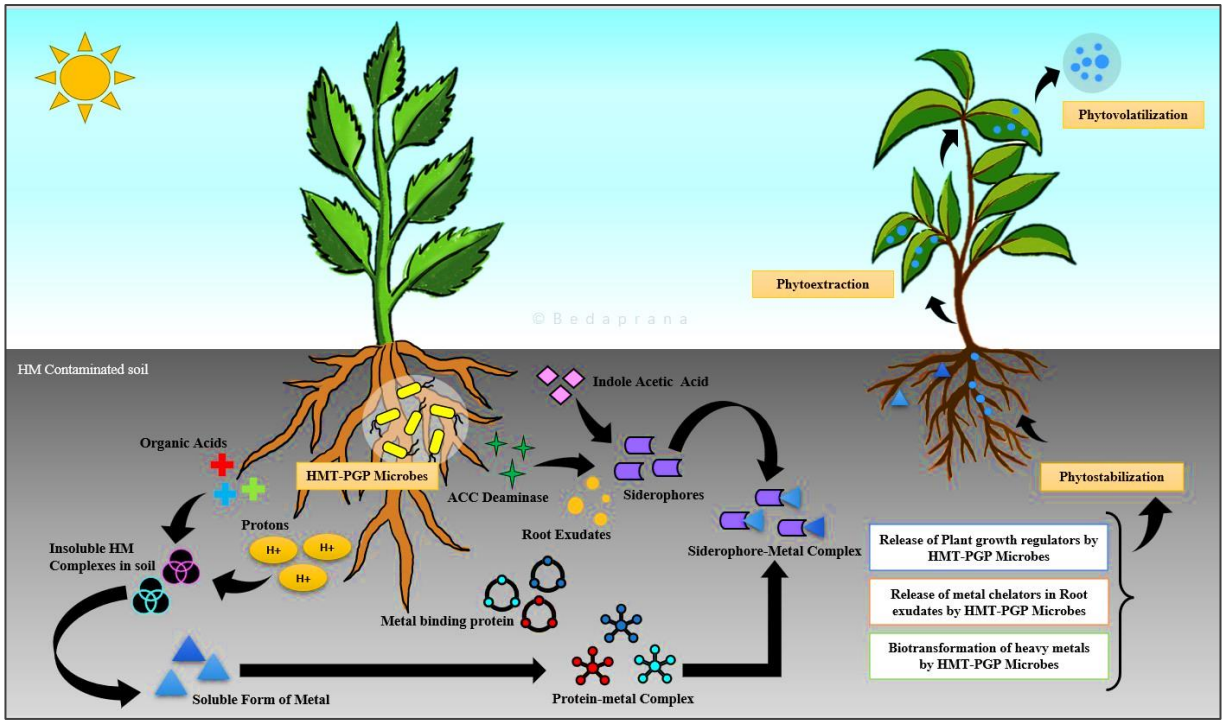


Figure 2.6: Heavy metal tolerant (HMT)-PGP in plant growth promotion and alleviation of HM stress in plants and remediation.

Several bacterial strains have been discovered to perform redox reactions, altering the valence of heavy metals. This can impact the mobility and toxicity of these metals (Gavrilescu, 2004). For example, researchers discovered that deceased *Bacillus licheniformis* R08 can convert Pb^{2+} into Pb^{0} (Goyal and al., 2003). Microbial metal remediation offers several benefits, including cost-effectiveness, preservation of soil structure, and environmental friendliness by preventing pollution. Various microbial species has the capability to carry out microbial heavy metal remediation, such as the *Bacillus* sp., which has been discovered to have significant potential

in the remediation of heavy metals such as copper, zinc, nickel, and others (Wierzba, 2015). *Thiobacillus*, in addition to absorbing heavy metals, may also absorb inorganic ions such as sulphur. When paired with metal ions, this can cause precipitation, making the separation from the soil easy (Leusch et al., 1995). Studies on the chemical kinetics of adsorption have demonstrated that the adsorption of heavy metals by *B. subtilis*, namely mercury (Hg^{2+}), follows second order kinetics more accurately (Jiang et al., 2000). Mullen et al. conducted research and found that B. subtilis exhibited a higher capacity for accumulating copper (Cu^{2+}) compared to *B. cereus* (Mullen et al.,1989). Zouboulis et al. (2004) observed that *B. licheniformis* and *Bacillus laterosporus* shown superior cadmium and chromium absorption capabilities compared to other bacterial species. The absorption of heavy metals is influenced by both pH and temperature. For example, the *Bacillus jeotgali* has the ability to absorb metals at a pH of 7. Deviations from this optimal pH, whether higher or lower, can impact the absorption of metals (Rodrı´guez-Tirado et al., 2012). Similarly, *B. licheniformis* and *B. jeotgali* U3 exhibit the highest absorption of cadmium, chromium, and zinc at their respective ideal temperatures. The *B. jeotgali* U3 strain has been found to exhibit the greatest absorption of cadmium at a temperature of around 35°C, whereas it shows optimal absorption of zinc at a temperature of 30°C (Chanmugathas & Bollag, 1988).

2.8.5. Role of ACC deaminase in combating abiotic stress.

Ethylene, a ubiquitous plant hormone, is synthesised and released by most plants in response to diverse environmental stimuli during their growth and development (Glick, 2014). Optimal quantities of ethylene stimulate seed germination, promote root extension, facilitate the creation of primordia in roots and stems, and contribute to the initiation of flowering and fruit ripening. Ethylene is a component of the volatile compounds that play a crucial role in generating the fragrance in fruits (Choudhary et al., 2017).

Plant ethylene production involves three primary stages, as outlined by Lin et al. (2009):

i. The enzyme S-AdoMet-synthetase catalyses the conversion of methionine to S-adenosyl-methionine.
ii. Followed by the transformation of S-AdoMet into 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of ACS (ACC synthetase).

iii. The production of ethylene occurs through the oxidation of ACC (1-aminocyclopropane-1-carboxylic acid) by ACC-oxidase.

Figure 2.7 summarises the activity of ACC Deaminase. The processes provide a lucid understanding that ACC serves as the precursor for the plant hormone ethylene. Ethylene is present in trace quantities in various plant tissues under non-stressful conditions. The synthesis of ethylene, known as the "hormone of stress," is influenced by various elements including temperature, light, diet, salt, pH, and heavy metals. Plant roots initiate the production of ethylene in response to several stressors, including cold, drought, heavy metals, diseases, and heat (Chen et al., 2002; Glick, 2014). Elevated ethylene production leads to the shedding of leaves, flowers, and petals, as well as the yellowing of leaves. It causes ageing in several parts of plants, ultimately resulting in the plants dying prematurely (Zahir et al.,2009) **(Figure 2.7)**.

Plant growth-promoting bacteria, such as *Bacillus* sp., assist plants in mitigating the effects of harsh climatic conditions by aiding the plant in countering ethylene-induced cellular damage through the use of bacterial ACC deaminases. The ACC deaminase enzyme breaks down ACS, which is responsible for converting S-AdoMet to ACC, the precursor of ethylene. This process results in the production of alpha-ketobutyrate and ammonia (Grichko and Glick, 2001). Therefore, by serving as a reservoir for ACC, it inhibits the production of ethylene, thereby enhancing root growth, shoot growth, and tolerance to abiotic stimuli. It counteracts the excessive production of ethylene, which leads to the suppression of cell division, DNA synthesis, and the growth of roots and shoots (Burg, 1973). Several species of Bacilli have been discovered to possess ACC deaminase activity (Saikia et al., 2018). Research indicates that when a plant is exposed to *B. subtilis* bacteria that produce ACC deaminase, it exhibits enhanced development and increased ability to withstand challenging environmental circumstances (Treesubsuntorn et al., 2018; Saikia et al., 2018). Several studies have indicated that plants exposed to bacteria that produce ACC deaminase exhibit notable enhancements in their health status under various stressful situations, including flooding, salinity, drought, and heavy metal contamination (Saikia et al., 2018). *Bacillus* and *Paenibacillus* strains with ACC deaminase activity cause a notable enhancement in chlorophyll content, as well as the length of roots and shoots in tomato (*Solanum lycopersicum*) plants in dry regions of Arabia, when compared to plants that were not infected. Plants subjected to saline stress conditions could

have yielded comparable outcomes, as demonstrated by Khan et al. (2016).

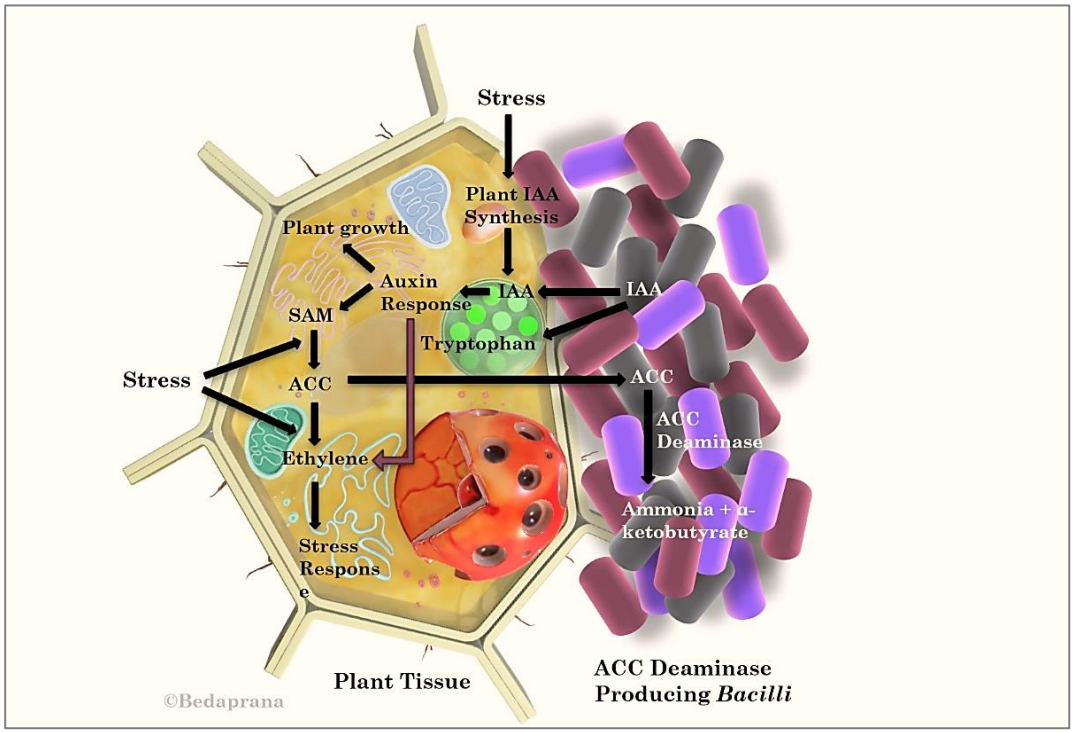


Figure 2.7: Mechanism of ACC deaminase activity.

2.9. Biocontrol activity of extremophilic *Bacillus* sp.

A major peril to agricultural output is the prevalence of plant diseases. The impacts of phytopathogens range from agricultural loss to the widespread dissemination of illnesses on a huge scale. At present, chemical pesticides are considered the primary approach for pest control. While chemical fertilisers and pesticides have been effective in quickly reducing pathogens and increasing agricultural yields, their long-term negative impact on the environment has become a growing worry. Therefore, scientists worldwide have shifted their focus towards using diverse advantageous microorganisms to regulate diseases (Pal & McSpadden Gardener, 2006; Barr & Soila, 1960). Over the years, other researchers have proposed the antagonistic impact of microorganisms on plant diseases, and this interaction is considered to be complex in nature (Cook et al., 2002). A multitude of diverse consequences, such as the synthesis of numerous antipathogenic substances and competition among species for resources, including food, space, mating, or other necessities, result in the activation of defence mechanisms or, in some cases, predation (Cook & Baker, 1983). The Bacillus family, compared to other bacterial antagonists, is highly significant and extensively utilised (Verschuere et al., 2000). The population of *Bacillus* sp. with potential as biocontrol agents is steadily growing. The rapid replication and capacity to endure various harsh climatic conditions

through endospore formation make *Bacillus* a highly effective biocontrol agent. Compant et al. (2005) demonstrated through their experiments that the *Bacillus* family releases a wide variety of volatile chemicals that have a substantial impact on activating the ISR pathway in plants. An in-depth examination of the biocontrol capabilities of Bacillus sp. reveals their capacity to generate a wide array of antibacterial chemicals, which have been discussed in the upcoming sections.

2.9.1. Lipopeptides and antibiotics secreted by Bacillus sp. as a mechanism of Biocontrol of phytopathogens

Bacillus sp. family members are recognised for their ability to excrete a wide variety of lipopeptides. These substances are primarily secondary metabolites that are released due to interactions with particular plant diseases. These metabolites play a significant role in the field of agricultural biotechnology. Approximately 756 lipopeptides have been identified with strong antifungal activities, according to the Microbiology UDoPa database from 2016. Typically, the bacteria release surfactants that cause obstruction, disruption, or the creation of pores in the cell wall and cell membranes of the fungi, resulting in their death. Additional peptides produced by Bacillus sp. have been identified to break down the nucleic acids and mitochondria of fungal infections, leading to their reduction (Zhao et al., 2013). Gong et al. (2015) conducted investigations that revealed *Bacillus* sp. as producers of various surfactant amphiphilic lipopeptides such as bacillomycins, iturins, and mycosubtilins. Modifications in the bacterial culture media can result in the synthesis of substances such as fengycins and plipastatin. The lipopeptides can be classified into two main groups based on their biochemical characteristics related to the presence of L- and D-amino acids: cyclic peptides or iturinics, and macrolactones. The macrolactones group contains molecules such as plipastatins, fengycins, and surfactants (Maget-Dana et al., 1985). The study demonstrated that iturinics induce cell leakage in the fungal cytoplasmic membrane by inserting their hydrophobic tail via the pores formed on the cell membrane. *Bacillus subtilis* is a frequently seen type of *Bacillus* bacteria that produces macrolactones called plipastatins. These macrolactones inhibit the activity of phospholipase A2, resulting in inflammation and increased sensitivity in the targeted fungal pathogen (Hirata & Axelrod, 1980). *Bacillus subtilis* secretes a variety of hemolytic surfactants that have been discovered to be highly effective against mosquitoes. An investigation into the characteristics and makeup of metabolites released by *B. subtilis* that hinder the growth of *Aspergillus flavus* unveiled the existence of four distinct fluids, one of which was identified as Bacillomycin D, a substance capable of inflicting significant harm to both fungal spores and

hyphae. The pure Bacillomycin D was tested in a laboratory setting using in vitro methods. The results showed a substantial decrease of 96.63% in the germination of fungal spores and a decrease of 98.10% in sporulation (Gong et al., 2014).

2.9.2. Enhancing plant growth: indirect mechanism of phytopathogen Biocontrol

In addition to their production of antibiotics and exudates with antagonistic properties, *Bacillus* sp. is recognised for their ability to enhance plant growth and development, so indirectly contributing to the plant's resistance against diseases. Members of the *Bacillus* sp. is known to stimulate the manufacture of plant hormones such as GA3, IAA, Cytokinin, etc. This, in turn, enhances the availability of nutrients to the plants (Chen et al., 2007). Chowdappa et al. (2013) observed a significant improvement in the growth of shoots and roots, as well as an increase in vigour, higher production of plant hormones, and enhanced production of defence enzymes when a spore suspension of the *B. subtilis* strain OTPB1 was applied to potted tomato seeds. Several researchers propose that the reduction in ethylene synthesis by *B. subtilis*, coupled with an increase in hormone production, promotes the colonisation of roots by rhizobacteria (Chen et al., 2007; Harman, 2011). In a comprehensive screening of one hundred bacterial strains of *B. amyloliquefaciens*, Jiang et al. (2015) discovered that strain 54 exhibited a significant ability to enhance both the chlorophyll content and NPK content of the plant. It triggered the activation of plant defence genes such as PR1 and H_2O_2 in plants, resulting in increased resistance to bacterial fruit blotch in cucurbitaceae. Lin et al. (2014) achieved comparable outcomes when they extracted *Bacillus* cultures from vinegar wastes that exhibited a significant level of antagonism towards eight fungal diseases.

2.9.3. Enzymes as a means of biocontrol of phytopathogens

In addition to producing antibiotics and lipopeptides, *Bacillus* species are also capable of producing lytic enzymes such as chitinase and beta-1,3-glucanase. These enzymes have potent abilities to degrade fungal cell walls (Leelasuphakul et al., 2006). Several further investigations conducted by other researchers have demonstrated the effectiveness of *Bacillus* sp. in manufacturing various oxidative enzymes related to defence, such as Poly Phenol Oxidase, or Phenylalanine Ammonia Lyase **(Figure 2.8)** (Jayaraj et al., 2004a, b). Thilagavathi et al. (2007) shown that varied levels of oxidative enzymes stimulate the production of lignin and oxidative phenolics, which are crucial for the formation of defense-related barriers. These compounds promote structural modifications in the cytological defence system of plant pathogens, leading

to their constriction. In addition, Podile and Laxmi demonstrated in 1998 that PAL plays a crucial role in the synthesis of both lignin and flavonoids. PAL is well acknowledged to have a significant impact on the synthesis of plant phenolic chemicals via the phenyl propanoic pathway (Hahlbrock & Scheel, 1989). Polyphenol oxidase (PPO), another crucial plant enzyme, plays a vital function in boosting plant immunity against diseases. It achieves this mostly by catalysing oxidative reactions of phenolic compounds (Li & Steffens, 2002; Thipyapong & Steffens, 1997). Typically, it is hypothesised that enzymes such as chitinase and beta-1,3-glucanase play a significant role in plant defence mechanisms, while enzymes like PO and PAL are more involved in breaking down plant tissues through the phenyl propanoid pathway (Vidhyasekaran et al., 2001). Multiple species of *Bacillus* have been documented for their chitinolytic activity (Das et al., 2010). According to Podile & Prakash (1996), the chitinolytic mechanism of *Bacillus* sp. involves the production of enzymes such as chitinase, glucanase, and chitosanases. These enzymes have the ability to break down the glycosidic bonds in the fungal cell wall, causing damage to its structure. According to Das et al. (2010), chitinase is more effective than glucanase in the biocontrol of fungal diseases because plant cell walls do not contain chitin. Studies have revealed that particular strains of *B. amyloliquefaciens*, such as V656, have the ability to manufacture two distinct forms of chitinase enzymes. These enzymes have been shown to effectively inhibit the growth of *Fusarium oxysporum* (Wang et al., 2004). According to Liu et al. (2010), the chitinase enzymes generated by *Bacillus thuringenesis* sub sp. *colmeri* have been found to significantly inhibit the germination of fungal spores. The application of *B. mycoides* strain BacJ in sugar beets leads to the activation of systemic induced resistance. This activation is caused by an increase in the activity of PO (peroxidase) in the plant. The increased PO activity is crucial for the enhancement of ribozymes such as beta-1,3-glucanase and isozymes of chitinase, which play an important part in the plant's defence mechanism (Bargabus et al., 2002). *Bacillus licheniformis* is capable of producing five distinct forms of chitinolytic enzymes, while *B. circulans* is capable of producing six distinct types of chitinases. However, it is only the type A1 that has been identified to be associated with the process of chitin breakdown (Sanchez et al., 2007). Additional analysis of the three-dimensional structure of the catalytic region of the chitin arms, specifically the tryptophan residues W122 and W134, reveals that hydrolysis in these regions or mutation in these residues will greatly diminish the catalytic function of chitinase (Kim et al., 2000). The bacterial-based chitinases are significantly influenced by a range of environmental parameters, such as the fertility of the field, the composition and type of soil, and the pH level of the soil (Terahara et al., 2009).

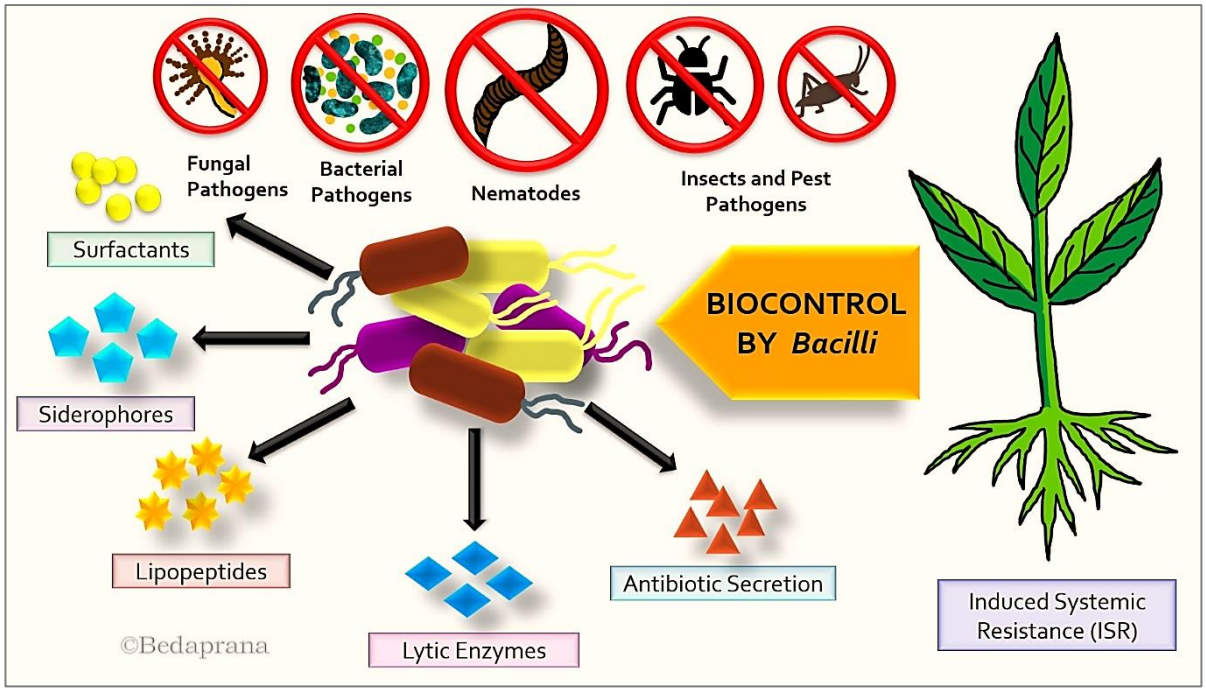


Figure 2.8: Presents a visual representation of the biocontrol characteristics demonstrated by the *Bacilli.*

2.10. Utilisation of Extremophiles in Industry

Extremozymes are enzymes that are naturally produced by organisms and have a high likelihood of tolerating harsh environmental conditions while still maintaining their activity and biocatalytic potential. Extreme conditions, such as high temperatures, benefit industrial processes by enhancing the solubility rates of certain polymeric substances that serve as substrates. This leads to a decrease in the viscosity of the reaction medium, resulting in improved reaction rates and increased productivity. Hence, the utilisation of thermostable polysaccharide degrading enzymes, such as xylanase, amylase, chitinase, pullulanase, pectinases, etc., is extremely important in various industries including food, detergent, textiles, pulp, and paper. Thermophiles employ chaperonins to reestablish the original structure and function of proteins denatured due to high temperatures. Thermostable lipase has a versatile role in several reactions such as trans-esterification, fat hydrolysis (specifically milk fat in the dairy sector), and in the leather industry. Additionally, thermostable amylase, xylanase, and protease enhance the digestibility of poultry and animal feed (Kumar et al., 2001). The bio catalytic potential of psychrophiles is exceptionally high for the manufacture of complex compounds, as well as for applications in the detergent and food sectors (Cavicchioli et al., 2002). They have the ability to generate a wide range of enzymes like as proteinases, lipases, amylases, and more. They possess distinct membrane adaptations characterised by

reduced ionic contacts, little presence of hydrophobic groups, and other modifications that enable them to thrive in extremely freezing temperatures. Psychrophilic membranes have a larger concentration of unsaturated fatty acids in order to enhance membrane fluidity and facilitate nutrition transport in low temperatures (Margesin et al., 2002). These extremophiles have the ability to produce hydrolytic extremozymes that can break down lipids, fats, proteins, carbs, and pitch. This makes them highly beneficial in the detergent industry for cold washing. The utilisation of Psychrophilic keratinase or protease aids in the removal of hair from animal hides in the leather industry and decreases the usage of harmful chemicals in the process (Georlette et al., 2004).

Alkaliphiles and acidophiles can maintain pH neutrality in their membranes in severe conditions through a proton pump mechanism. They have the ability to produce hydrolytic extremozymes like as glucosidases, glucoamylases, pullulanases, and others. Thermoalkaliphilic enzymes have a high level of efficiency as biocatalysts (Serour et al., 2002). Enzymes such as lipases, proteases, and cellulases have excellent performance at elevated pH levels and temperatures. Consequently, they are widely employed as additives in detergents for both laundry and dishwashing purposes. Proteases are employed to enhance the texture of wool and augment its ability to absorb colour (Wiegel and Kevbrin, 2004). Xylanases, which lack cellulase activity, are employed in the paper pulp industry for bioleaching applications. Pectinases aid in the extraction of gum from fibres, a procedure referred to as degumming. Catalase, oxidoreductase, and peroxidases are employed in the textile sector to eliminate any remaining hydrogen peroxide in effluents (Horikoshi, 1999).

The utilisation of thermo-piezophiles, which are microorganisms capable of withstanding high temperatures and pressures, has a significant influence on the thermodynamics of reactions in the industry. The fundamental principles of thermodynamics dictate that a reaction with a negative activation volume change ($\Delta V<0$) will be more favourable with an increase in pressure, while a reaction with a positive activation volume change ($\Delta V>0$) will be more favourable with a reduction in pressure. The rule of change in activation volume (ΔV) can be utilised in industrial enzymatic processes to regulate the specificity of a reaction. α-chymotrypsin catalyses two distinct reactions: the hydrolysis of anilides, which exhibits a negative change in activation volume, and the hydrolysis of esters, which exhibits a positive change in activation volume ($\Delta V>0$). Therefore, raising the pressure of the reaction promoted

the hydrolysis of anilides by the enzyme, whereas reducing the pressure favoured the hydrolysis of esters (Mozhaev et al., 1994).

The biocatalytic characteristics of halophiles and xerophiles have not been extensively investigated. Halophilic enzymes have enhanced activity under extremely high salt concentrations. Occasionally, their limited solubility is investigated in non-aqueous environments. However, the utilisation of organic solvents with halophilic extremozymes was discovered to be restricted to a small number. **Figure 2.9** summarises the industrial applications of extremophilic *Bacillus* sp.

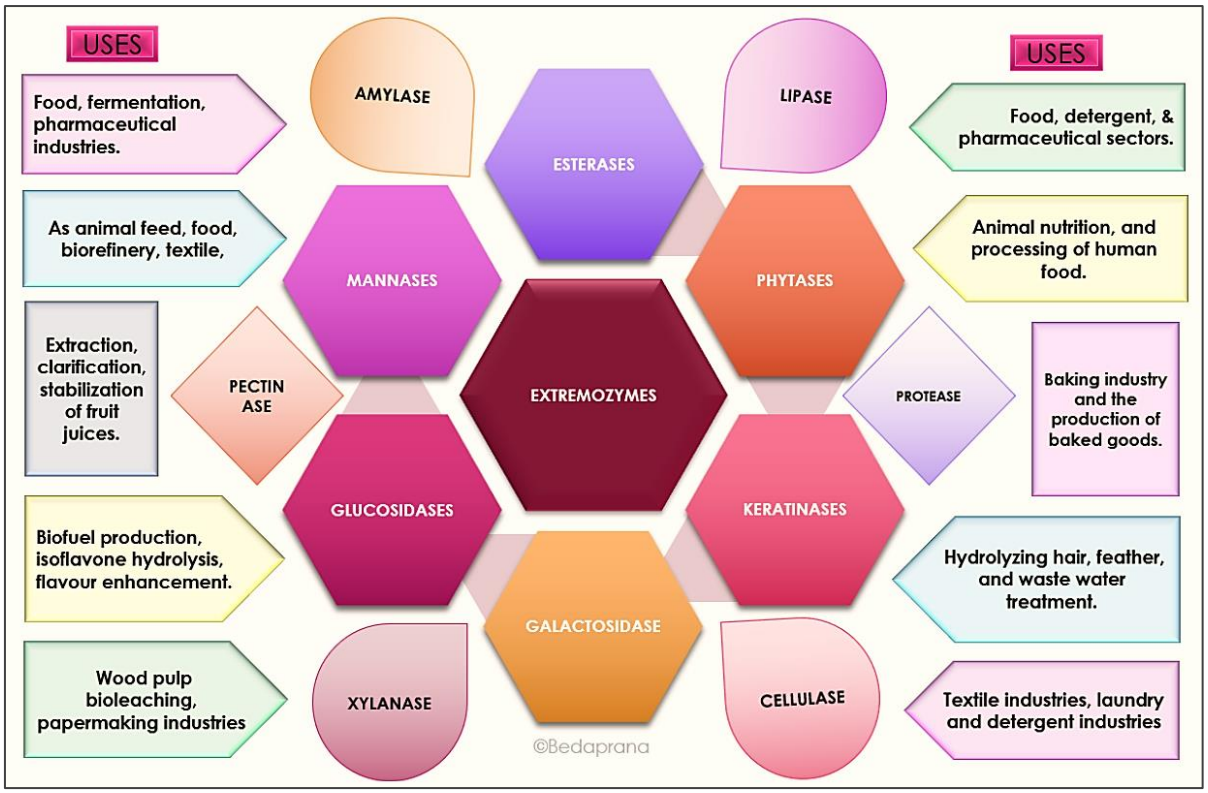


Figure 2.9: Represents certain enzymes (extremozymes) produced by *Bacillus* and their uses in the industry.

2.10.1. Extremophilic microbes: a valuable resource for the pharmaceutical sector

Extremophiles are renowned for their capacity to endure under a diverse range of severe environmental circumstances. The precise technique employed by these bacteria to adapt or modify extremolytes, such as primary or secondary metabolites, is crucial for their survival and growth in unfavourable environments. Extremolytes produced by different types of extremophiles, such as thermophiles, radiation-resistant extremophiles, acidophiles, and halophiles, have been examined for their significant potential in the fields of therapeutics and the medical industry. The primary benefit of utilising extremozymes is in their capacity to serve

as very effective biocatalysts, owing to their remarkable stability across many environmental conditions.

The primary application of radiation-resistant extremophiles is in the treatment of radiation, skin cancer, and premature ageing symptoms. MAA or Mycosporine-like amino acids are commonly found in radiation-resistant extremophiles. These compounds based on MAA have the ability to absorb radiation throughout the range of roughly 310-365nm. As a result, these chemicals hold significant significance in the sunscreen and skin care cosmetic sectors. In 2008, Oyamada et al. demonstrated that MAA has the ability to inhibit the synthesis of DNA dimers when exposed to UV-radiation, hence safeguarding the fibroblast cells on the skin surface from UV-radiation exposure. Various investigations have discovered that distinct strains of extremophiles, which produce chemicals such as bacterioruberin, possess DNA repair pathways. (Shahmohammadi et al., 1998). Ruso et al. (2008) concluded that substances such as pannarin and/or sphaerophorin have the ability to hinder the growth of the M14 human melanoma cell line by triggering apoptosis.

Thermophiles, which are microorganisms with cell membranes that can withstand high temperatures, have become important in the treatment of cancer associated with the production of great heat. One further benefit of utilising thermophiles in medicines is their ability to inhibit the proliferation of numerous pathogenic organisms, as most organisms perish under extreme heat temperatures (Suzuki et al., 2013). An investigation revealed that thermolytes have a substantial impact on the growth pattern of HEK293 cells, providing significant assistance in the management of severe neurodegenerative conditions such as Parkinson's disease. (Jorge et al., 2011). Similarly, it was noted that the transfection of HEK293 cells with Htt-103QEGFP had a significant impact on the production of Huntington's aggregates mediated by Mg-DGP. (Jorge, et al., 2011). The study conducted by Saaranen and Ruddock in 2013 elucidated the function of the cytoplasmic machinery in hyperthermophiles and identified the specific location where protein folding takes place. This process is crucial in the manufacture of antiviral drugs. The extremolytes synthesised by halophiles have a very small molecular mass and are accumulated in response to conditions of salt and temperature stress. (Chakraborty, et al, 2013). The lipids derived from halophilic bacteria are utilised in the production of vaccines and for delivering drugs (Abrevaya, 2013). Neuro-degenerative illnesses such as Machado-Joseph disease, which are caused by the misfolding of proteins, can potentially be treated with halophilic extremozymes that can affect the areas responsible for protein folding. (Furusho et

al., 2005). Ectoine, a low-molecular weight osmoprotectant, has been discovered to have positive effects in the treatment of Alzheimer's disease on a broad scale. The Ectoines were discovered to have a crucial function in suppressing the aggregation of the protein amyloid, therefore reducing the likelihood of amyloid misfolding in the disease (Kumar and et al., 2013) Acidophiles have been employed for a considerable duration to mitigate the risk of stomach and gastric malignancies, particularly in persons who are susceptible to infections. (Foster, 2004). Elmer's comprehensive research conducted in 2001 on different probiotics such as *Saccharomyces boulardii* and *Lactobacillus* sp. demonstrates their efficacy in alleviating mild diarrhoea, stabilising Crohn's disease, and managing other inflammatory bowel disorders. Furthermore, acidophiles are known to develop unique extremozymes that have the potential to be used as medications for treating serious ailments such as stomach ulcers or gastric cancer (Lopez de Saro et al., 2013).

While extremozymes have great potential in medicinal research, optimising their development conditions, particularly in maximising extremolytes, is a challenging task. Nevertheless, the progress in 'omic' investigations has allowed systems biology to reveal other features. These methods can assist in accurately sequencing the genomes of extremophiles, hence limiting down the possible sequences of extremolytes.

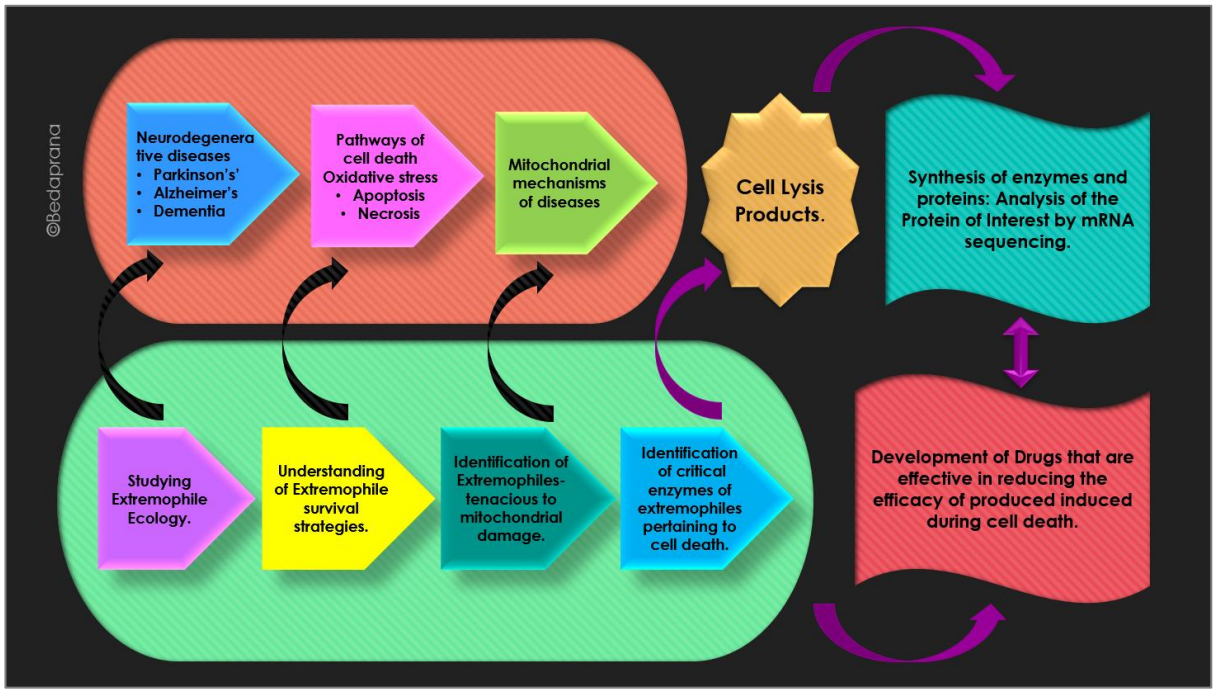
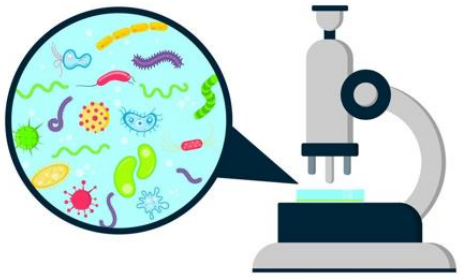


Figure 2.10: Application of extremophiles in pharmaceutical industry.

2.11. Conclusion and Future prospect

The field of extremophily and polyextremophily remains largely unexplored, with significant untapped promise. The field provides irrefutable evidence of the extraterrestrial origins of life and holds great potential in the realm of biotechnology. Throughout the study, we discover that extremophiles, as well as the extremozymes and extremolytes they secrete, are highly significant in several fields such as industry, agriculture, molecular biology, pharmacy, and bioremediation. The utilisation of extremophiles in a bio-refinery holds great potential due to the resilience of these organisms and their ability to thrive in the challenging conditions of the bio-refinery. Their utilisation has demonstrated significant efficiency and cost-effectiveness in all industrial processes, thanks to the simplicity of their survival and the selectivity of their extremozymes. The primary advantage of utilising extremophiles in industrial processes lies in their environmentally sustainable characteristics and enhanced efficiency. Scientists have differing opinions on the difficulty of cultivating extremophiles in laboratory settings. However, a significant advancement in industrial microbiology could be achieved by genetically engineering and inserting extremozyme genes into mesophilic organisms. We are confident that there are still a significant number of extremophiles that have not yet been discovered. Studying these extremophiles will provide valuable insights into various molecular mechanisms of protein folding, protein structure and function, as well as their unique mechanisms of biotransformation and biocatalysis. This research has the potential to revolutionise our understanding of industrial biotechnology. The growing interest in biocatalysis and the pursuit of a Bio-based economy necessitate a thorough examination of extremophiles and their metabolic processes. This research holds the potential to lead us towards a more sustainable future with minimum environmental impact.

CHAPTER 3: MATERIALS AND METHODS



3. "Materials

S. no.	Name of the Media	Manufacturing company
1.	Aleksandro Agar medium	HiMedia
2.	Brain Heart Infusion	HiMedia
3.	Dworkin Foster Medium	HiMedia
4.	Hichrome UTI Agar	HiMedia
5.	Hichrome Bacillus Agar	HiMedia
6.	Jensen's broth	HiMedia
7.	King's B Agar Media	HiMedia
8.	Luria Bertani broth	HiMedia
9.	Minimal salt Media	HiMedia
10.	MRS media	HiMedia
11.	Nutrient broth	HiMedia
12.	Peptone	HiMedia
13.	Pikovskaya Agar	HiMedia
14.	Potato Dextrose Agar	HiMedia
15.	Simmon's Citrate Agar	HiMedia
16.	Stuart's broth	HiMedia
17.	Tributyrin Agar	HiMedia
18.	Tryptone Soy broth	HiMedia
19.	Yeast Extract	HiMedia

Table 3.1: List of Media used.

Sl No.	Name of the Chemical / Reagents	Manufacturing company
1.	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H tetrazolium chloride (INT)	HiMedia (India)
2.	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid	Sigma Aldrich (India)
3.	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide	Sigma Aldrich (India)

4.	Acetone	Merck Millipore (India)
5.	Agarose	Sigma Aldrich (India)
6.	Aminoscyclopropane carboxylic acid (ACC)	Sigma Aldrich (India)
7.	Ammonium metavanadate	Merck (India)
8.	Ammonium sulphate	Merck Millipore (India)
9.	Amonium molybdate tetrahydrate	Merck Millipore (India)
10.	Aluminum chloride	HiMedia (India)
11.	Barton's reagent	HiMedia (India)
12.	Benedict's reagent	SRL (India)
13.	Bradford's reagent	SRL (India)
14.	Bromothymol Blue	Merck Millipore (India)
15.	Boric Acid	HiMedia (India)
16.	Butanol	Merck Millipore (India)
17.	Calcium Chloride	HiMedia (India)
18.	Carboxymethyl cellulose	HiMedia (India)
19.	Casein	HiMedia (India)
20.	Christensen's urea agar	HiMedia (India)
21.	Chrome Azurol S (CAS)	HiMedia (India)
22.	Citric Acid	Merck Millipore (India)
23.	Congo red	Merck Millipore (India)
24.	Cobalt nitrite	HiMedia (India)
25.	Copper sulphate	HiMedia (India)
26.	Crystal Violet	HiMedia (India)
27.	Dinitrosalicylic Acid	Merck Millipore (India)
28.	Diphenyl amine	Merck Millipore (India)
29.	Dipotassium hydrogen phosphate	SRL (India)
30.	DMSO (Dimethyl sulfoxide)	HiMedia (India)
31.	Disodium hydrogen phosphate	SRL (India)
32.	Ethanol	Merck Millipore (India)
33.	Elution buffer	HiMedia (India)
34.	Ethelene-diamine tetraacetic acid (EDTA)	SRL (India)
35.	Ethidium bromide (EtBr)	SRL (India)
36.	Ferric Chloride	SRL (India)

37.	Ferrous sulphate	SRL (India)
38.	Gallic acid	SRL (India)
39.	Folin Ciocalteau reagent	Merck Millipore (India)
40.	Gram's iodine	HiMedia (India)
41.	Gelatin	SRL (India)
42.	Glacial acetic acid	Merck Millipore (India)
43.	Glutaraldehyde	Merck Millipore (India)
44.	Glucose	SRL (India)
45.	Gluconic acid	HiMedia (India)
46.	Glycerol	Merck Millipore (India)
47.	Glycin	HiMedia (India)
48.	Guaiacol	Merck Millipore (India)
49.	Hexadecyl trimethyl ammonium bromide (HDTMA)	HiMedia (India)
50.	Hydrochloric Acid	Merck Millipore (India)
51.	Hydrogen peroxide	Merck Millipore (India)
52.	Laminarin	Sigma Aldrich
53.	Lysis Buffer	HiMedia (India)
54.	Lysate binding buffer	HiMedia (India)
55.	Malachite green	SRL (India)
56.	Magnesium chloride	Merck Millipore (India)
57.	Magnesium sulphate	Merck Millipore (India)
58.	Methanol HPLC grade	HiMedia (India)
59.	Mercuric Chloride	Merck Millipore (India)
60.	Methylene Blue	Merck Millipore (India)
61.	Nesseler's Reagent	Merck Millipore (India)
62.	Ninhydrin	HiMedia (India)
63.	Pectin	HiMedia (India)
64.	Picric acid	HiMedia (India)
65.	Peptone	SRL (India)
66.	Perchloric Acid	Merck Millipore (India)
67.	Phenyl-methyl-sulfonyl-fluoride (PMSF)	Merck Millipore (India)

68.	Piperazine-1,4-bis(2-ethane sulfonic acid) (PIPES)	HiMedia (India)
69.	Potassium hydroxide	HiMedia (India)
70.	Potassium dihydrogen phosphate	Merck Millipore (India)
71.	Potassium permanganate	SRL (India)
72.	Potassium phosphate dibasic	HiMedia (India)
73.	Potassium ferrocyanide	Merck Millipore(India)
74.	Potassium persulfate	HiMedia (India)
75.	Proteinase K	HiMedia (India)
76.	Quercetin dihydrate extrapure	SRL (India)
77.	RNase A solution	HiMedia (India)
78.	Safranin	HiMedia (India)
79.	Skimmed milk powder	HiMedia (India)
80.	Sodium nitrate	Merck Millipore(India)
81.	Sodium acetate	Merck Millipore(India)
82.	Sodium Bicarbonate	Merck Millipore (India)
83.	Sodium chloride	Merck Millipore (India)
84.	Sodium dihydrogen phosphate	Merck Millipore (India)
85.	Sodium hydroxide	Merck Millipore (India)
86.	Sodium molybdate	HiMedia (India)
87.	Sodium potassium tartarate	SRL (India)
88.	Starch	HiMedia (India)
89.	Streptomycin	HiMedia (India)
90.	Stuart's broth	HiMedia (India)
91.	Sulphuric acid	Merck Millipore (India)
92.	Tetra-methyl- p-phenylenediamine dihydrochloride	HiMedia (India)
93.	Thiobarbaturic acid (TBA)	HiMedia (India)
94.	Tricalcium Phosphate	Merck Millipore (India)
95.	Tris	Sigma Aldrich (India)
96.	Trichloroacetic acid (TCA)	HiMedia (India)
97.	Triton X	Merck Millipore (India)
98.	Tryptophan	Merck Millipore (India)

99.	L-Tyrosine	SRL (India)
100.	Urea	Merck Millipore (India)
101.	Zinc acetate	SRL (India)
102.	Zinc sulphate	HiMedia (India)

Table 3.2: List of Chemicals used.

S. No.	Crop	Variety	Procured from
1.	***Oryza sativa***	PB1692	South Kolkata Market (Sealdah Beej patti)
2.	***Zea mays* L**	KOHINOOR 595	South Kolkata Market (Sealdah Beej patti)

Table 3.3: List of Crop varieties used for the experiments

No.	Name of Instrument	Name of manufacturer and model number	Country of manufacture
1.	-80°C freezer	Eppendorf CryoCube	India
2.	B.O.D Incubator	N.R Scientific	India
3.	Bio-safety cabinet	Biocoction Manufacturing pvt ltd.	India
4.	Bright-field compound light microscope	Dewinter, DIG1510, 5.1 MP 1/ 2.5'' CMOS sensor	India
5.	Cooling centrifuge	Eppendorf Centrifuge 5810R	India
6.	Digital colony counter	N.R Scientific	India
7.	Digital weighing balance	Sartorius	India

8.	DNA sequencer	Thermo Fisher, ABI 3730xl Genetic Analyzer, using BDT v3.1 Cycle sequencing kit	United States of America
9.	Electron spray ionization – Mass Spectroscopy (ESI-MS)	Waters, Xevo TQ Absolute IVD system	United States of America
10.	Elisa Reader	Readwell Robonik	India
11.	Gel imaging system	Eppendorf India pvt. ltd. Bio-print	India
12.	Hot water bath	N. R Scientific	India
13.	Hot air oven	Lambda N.R Scientific	India
14.	Incubator	N.R Scientific	India
15.	Lyophiliser	Hahntech corporation 110N	South Korea
16.	Microwave oven	Electrolux	India
17.	Nanodrop	Thermo-Fisher Scientific, Multiscan sky	United States of America
18.	PCR thermal cycler	Bio-Rad T100	India
19.	Transilluminator	N.R Scientific	India
20.	UV-Visible spectrophotometer	Optizen POP	South Korea
21.	Ultra sonicator	QSonica	United States of America
22.	UV-Visible spectrophotometer	Thermo-Fisher Scientific,	United States of America

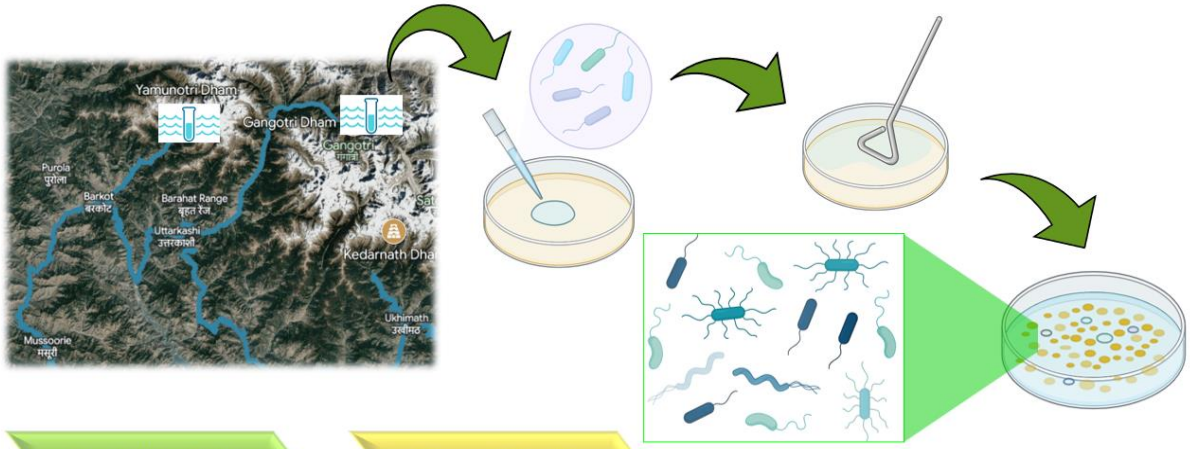
23.	Water Quality Analyzer	Elico PE 138	India
24.	Fourier Transform Infrared Spectrophotometer	Alpha II	Germany
25.	Atomic Absorption Spectrophotometer	PinAAcle 500	Massachusetts, USA.

Table 3.4: List of Instruments used.

Sl No.	Name of the software used	Version and company name	Country of manufacture
1.	Greengenes	Greengenes v.13.8-99 Second genome	United States of America
2.	Whole Genome Sequencing and Metagenome sequencing	Illumina Novaseq 6000 platform	United States of America
3.	Megahit	Megahit v.10.ngs	Open source
4.	Unicycler	KBase predictive biology	Open source
5.	Multiple alignment software	Clustal W, EMBL-EBI	United Kingdom
6.	Circular Map of whole genome sequence	Prokka, Proksee	
7.	Phylogenetic tree construction	MEGA 11	United States of America
8.	Linear Regressor Model	Python 3.1	Open source
9.	AdaBoost Regressor	Python 3.1	Open source

Table 3.5: List of software used.

OBJECTIVE 1:
Isolation and Characterization of Extremophilic Bacterial strains.



Gangotri Water, Water from the Upper course of Ganga, **Altitude: 3100 meters.**

Three Novel Strains of *Bacillus subtilis* Reported and submitted to NCBI.

BRAM_G1: Accession Number MW006633

BRAM_G2 : Accession Numbers MT998278-MT998280

BRAM_G3 : Accession Number MT998617

Yamunotri Water, Water from a Hot Water Spring, **Altitude: 3293 meters.**

Two Novel Strains were reported and submitted to NCBI.

***Mesobacillus subterraneous* BRAM_Y2**

Accession Number : MW002419

***Brevibacillus parabrevis* BRAM_Y3**

Accession Number : MW081864

3.1. Isolation and characterization of extremophilic bacterial strains.

3.1.1. *Sampling and evaluation of the physical characteristics of the Gangotri and Yamunotri, Suryakund water.*

The sample of water studied in this investigation was obtained from the upper course of Ganges, at Gangotri, Uttarakhand the Pilgrimage Ghat of Gangotri, Uttarakhand, at an altitude of 3415 m (30.9947° N 78.9398° E) and a hot water spring situated in the Yamunotri Temple complex, Yamunotri, Uttarakhand, India, at an altitude of 3,293 metres (10,804 ft) (31.01°N, 78.45°E), above sea level and The sampling was done in sterile containers, and gloves were worn to avoid sample contamination. The container was sealed and transported to the laboratory for further analysis. The general physical characterization of the water was done using an Elico PE 138 Water Quality Analyzer (https://www.elico.co/pe-138).

3.1.2. Isolation

The isolation of bacterial strains was carried out via standard serial dilution of the water sample using standard protocols, followed by spread plating on nutrient agar medium. The 5 colonies (BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3) were isolated on the basis of their prevalence in all the dilution plates. The names were conjured with the initials of the researcher and the sample collector along with the first letter of collection site and a numerical indicator of the isolate.

3.1.3. General Characterization of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3

3.1.3.1. Gram staining and colony morphology

The pure cultures of the five strains were first analysed for their colony morphologies. Then, Gram staining was carried out following established procedures (Gram., 1884).

3.1.3.2. IMViC Test

The IMViC reactions are a series of four tests used to identify members of the Enterobacteriaceae family, particularly Escherichia and Enterobacter. These tests are designed to distinguish Gram-negative bacteria in the Enterobacteriaceae family, which consists of numerous species closely related in terms of their biochemical and genetic characteristics. The tests comprise four distinct letters, each representing one of these exams.

The Indole test is a method used to identify certain bacteria that can synthesize indole from the amino acid tryptophan through the enzyme typtophanase. Bacteria are inoculated in peptone water containing tryptophan and incubated overnight at 37°C. A 1% tryptophan broth is prepared and inoculated with *E.coli* as a negative control. Kovac's reagent is added and shaken, and the tubes are allowed to stand for 2 minutes. A red or pink coloured ring at the top is considered positive.

The Methyl Red (MR) test assesses an organism's capacity to generate and sustain stable acidic byproducts through glucose fermentation. The bacterium is inoculated into glucose phosphate broth and incubated at 37°C for 48 hours. The mixed-acid producing organism must produce enough acid to overcome the buffer. The pH is tested with MR reagent, with red colour indicating a positive and a yellow colour indicating a negative result.

The Voges Proskauer (VP) test is designed to specifically detect the presence of butylene glycol producers, in contrast to the MR test which is used to identify mixed acid producers. Bacteria are inoculated into glucose phosphate broth and incubated for 48 hours. Then, alpha-naphthol and 40% KOH are added, shaken, and allowed to stand for 15 minutes. A red colour is taken as a positive test, while negative tubes are held for one hour for maximum colour development. The citrate utilisation test determines an organism's capacity to use citrate as its exclusive source of carbon and energy. Bacteria are introduced into a culture medium containing sodium citrate and a pH indicator called bromothymol blue. The process of using citrate requires the enzyme citritase, which catalyses the breakdown of citrate into oxaloacetate and acetate. Oxaloacetate undergoes further decomposition into pyruvate and carbon dioxide. The utilisation of sodium citrate and ammonium salt leads to the production of Na_2CO_3 and NH_3, respectively, resulting in an alkaline pH. This leads to a transformation in the colour of the medium from green to blue.

1.1.3. Sulphur Indole Motility Agar

The 5 bacterial strains were stably inoculated in SIM medium from Hi-Media and incubated for 24-48 hours at 37 °C. The tubes were observed for black coloration (indicating H_2S production), red ring formation (indicating a positive test for indole), and growth away from the stab line (indicating bacterial motility).

1.1.4. Chromogenic Agar

The five bacterial strains were streaked on both Hi-chrome Bacillus agar and Hi-chrome UTI agar medium from Himedia. The change in the coloration of the colonies and the media plate was observed after an overnight incubation at 37 °C.

1.1.5. Endospore Staining

The endospore staining of the five strains was carried out using the established procedure of the Schaeffer-Fulton method.

1.1.6. Antibiotic sensitivity

The antibiotic sensitivity test was carried out by the Kirby-Bauer disc diffusion method for both BRAM_Y2 and BRAM_Y3, using *Staphylococcus aureus* as a control.

1.1.7. Scanning electron microscopy

The samples of the *Bacillus* sp. and BRAM Y2 and BRAM Y3 strains were prepared for scanning electron microscopy. Using Ammar, 2017, as a guide, the laboratory strain was fixed for electron microscopy using the conventional glutaraldehyde procedure. The strains were imaged using a ZEISS EVO 18 variable pressure scanning electron microscope.

1.1.8. Molecular identification of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3

A portion of the 16S rDNA gene was enlarged utilising the 27F and 1492R primers. Afterward, a distinct PCR amplicon band measuring 1500 base pairs was visualised on an Agarose gel. The PCR product underwent purification to eliminate impurities. Subsequently, both forward and reverse DNA sequencing reactions of the PCR product were executed with the respective primers, utilising the BDT v3.1 Cycle sequencing kit on the ABI 3730xl Genetic Analyzer. The consensus sequence of the 16S rDNA gene was then derived from the aligned forward and reverse sequences using alignment software. This resultant sequence was subjected to a BLAST search against the NCBI GenBank database, and the top ten sequences with the highest identity scores were selected. These sequences were then aligned using the Clustal Omega software to generate a distance matrix. Finally, the MEGA 7 software was utilised to generate a distance matrix, followed by the generation of a phylogenetic tree with regard to the first 10 sequences. (Felsenstein., 1985; Kimura., 1980; Kumar., 2015).

1.2. Biofilm Formation and subsequent characterization

1.2.1. Qualitative Analysis

The qualitative test for analysing biofilm formation by the 5 strains was performed by streaking the bacterial colonies on Brain Heart Infusion (BHI) Congo Red Agar. Biofilm formation on this agar medium is indicated by the development of thick, mucoid, and black colonies on the agar plate. (Roy et al., 2022)

1.2.2. Quantitative determination of the formation of biofilm by the strains

The amount of biofilm formation was measured using the tissue culture plate method with 0.1% crystal violet as the stain. The biofilm attached to the plate was then analysed using a Readwell Robonik Elisa reader at 570 nm (Roy et al., 2020).

1.3. Evaluation of the Biochemical Characteristics of the Biofilms Produced by the 5 Strains

1.3.1. Induction of Primary Biofilm Production

The biofilm produced by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 along with the *Bacillus* sp. control lab strain was harvested with the help of a similar strategy as Raghad and Al Abbasi (2013). The production of the bacterial biofilm was stimulated in a basal salt solution enhanced with 3% glucose, maintaining a pH of 7$\pm$ 0.1. 2 ml of 18-hour cultures of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 strains growing in standard nutrient broth were used to inoculate the aforementioned medium for three days.

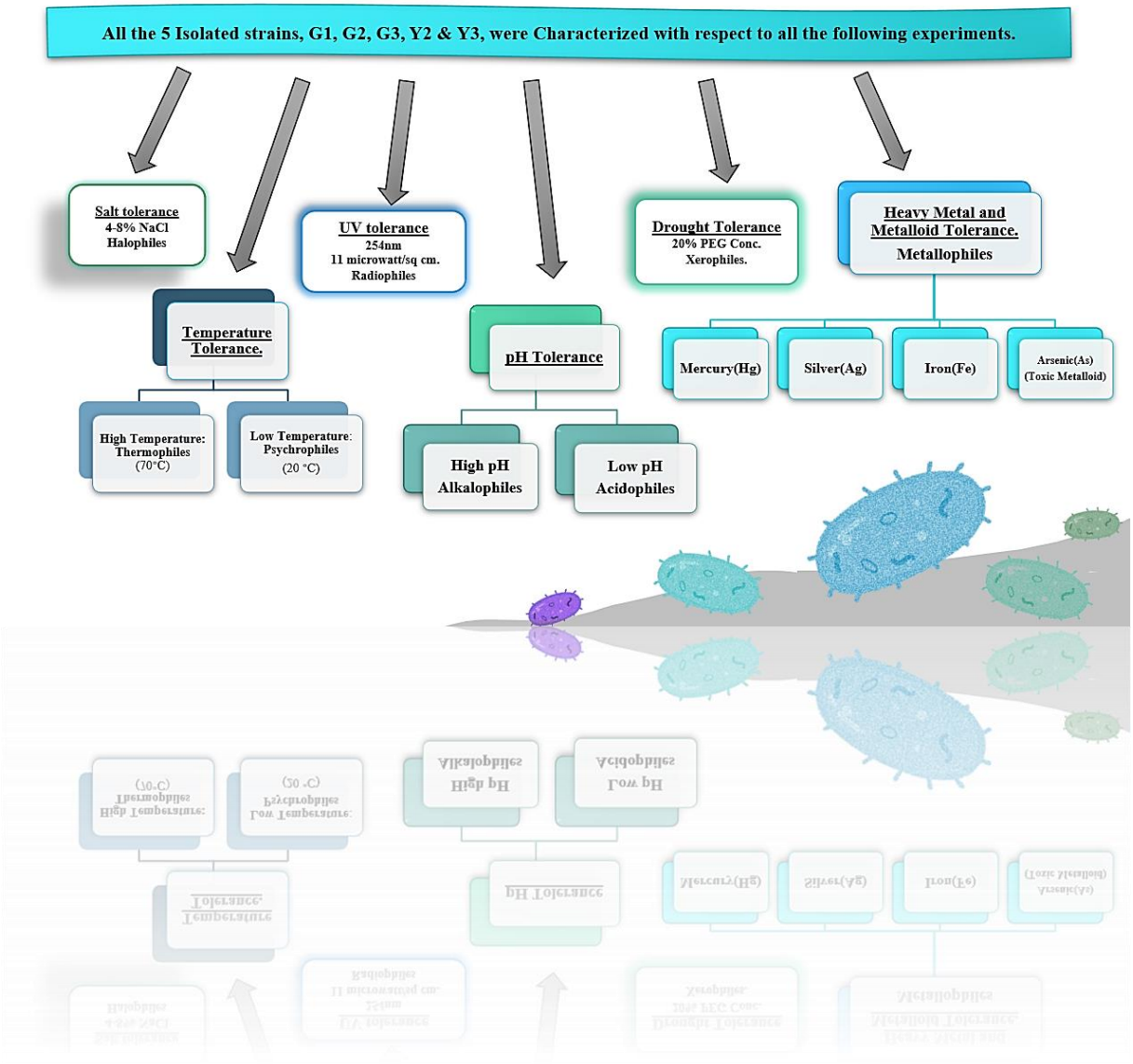
1.3.2. Isolation of the Biofilm Matrix from Culture

The BRAM Y2 and BRAM Y3 and *Bacillus* sp. control were precipitated by centrifugation at 5000 rpm for 10 minutes. The laboratory strain cells were also taken from the 3-day-old culture in a basal salt solution enhanced with glucose. Then, absolute ethanol (stored at low temperatures) was added in a 1:2 volume ratio. Following this, the setup underwent a 24-hour incubation period at 4°C to allow the biofilm matrix to precipitate out of the supernatant. The biofilm matrix that had been precipitated out was recovered by centrifugation at a specific rpm of 6000 for 20 minutes at normal temperature, followed by 24-48 hours of full drying at 70 °C. Scraped and gathered in a centrifuge tube, the EPS powder. (Raghad and Al Abbasi, 2013).

1.3.3. Quantitative estimation of the various biochemical elements of the biofilm

To determine the specific concentration of carbohydrates, DNA, and protein in the biofilm formed by each ml of culture of the biofilms produced by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 and *Bacillus* sp. laboratory strain, three different protocols were adopted. Burton's method utilising diphenyl amine was used to estimate the DNA (Ali et al., 2014). The conventional Lowry method was used to estimate the protein (Lowry et al., 1951). Last but not least, Dubois et al. (1956) evaluated the total content of carbohydrate in the biofilm matrix of the 5 strains using the phenol-sulfuric acid method.

OBJECTIVE 2: Profiling of their Polyextremophillic nature



2. Profiling of their Polyextremophillic nature.

2.1. Single-dimensional stress

2.1.1. Temperature tolerance

The growth of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, and *Bacillus* sp. as the control laboratory strain were tested at three varied temperatures (70°C, 37°C, and 20°C). The 24-hour old culture of each bacterial strain (100µl) were placed in a sterile medium in an erlenmeyer flask and incubated under shaking conditions. The growth was monitored at regular intervals for 8 hours using a colorimeter at a specific wavelength (600 nm), and the results were plotted on a growth curve for each temperature. This method has been described in Roy et al., 2022.

(a) Thermal death time and cold shock treatment.

The thermal death time of the bacterial cultures was determined by incubating them at a high temperature (120 °C) and measuring the number of viable cells over time. For the cold shock treatment, the bacterial cultures were incubated at a low temperature (-20 °C) overnight, and the growth was monitored at regular intervals to observe any reduction.

2.1.2. Salt tolerance

Sodium chloride was added to the bacterial culture medium in concentrations ranging from 1-8%, with 0.5% NaCl serving as the control setup. Along with the bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, *Bacillus* sp., control laboratory strain cultures were then used. The prepared medium was inoculated with the cultures and then incubated for 24 hours at 37 °C. After incubation, optical densities at 600 nm were recorded. (Kumari et al., 2019).

2.1.3. pH tolerance

The six bacterial strains ie., *Bacillus* sp. laboratory strain, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, were grown at different pHs in a buffered liquid nutrient medium to maintain the pH. A total of six setups with pH 1, 3, 5, 7, 10, and 12 were prepared. The pH was adjusted with citrate and carbonate, and bicarbonate buffer was used so

as to maintain the desired pH. The growth was measured after 24 hours spectrophotometrically at 600 nm. (Roy et al., 2022)

2.1.4. Drought tolerance

To mimic the conditions of drought, the growth medium was amended with polyethylene glycol 6000 at different concentrations (0–20%). Along with BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, *Bacillus* sp. control laboratory strain cultures were inoculated in the enhanced medium. After incubating the laboratory strain in the altered medium for five days at 28 °C in a shaker, the optical densities at 600 nm were determined. **(**Kumari *et al.*, 2019)

2.1.5. UV tolerance

The growth of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, and Bacillus sp. control laboratory strain in the presence of ultraviolet rays were measured after inoculating them into fresh nutrient broth and exposing the tubes to UV light (short UV of 254 nm wavelength) of a specific intensity of 11µW/cm^2 for a set amount of time. The optical densities at a specific wavelength were recorded at regular intervals for a set amount of time (1 hour), and the results were plotted on a growth curve showing the optical density over time. (Cayron and Lesterlin, 2019)

2.1.6. Heavy Metal Tolerance

The tolerance to iron, mercury, silver, and arsenic, was tested in specific setups. To adjust the bacterial growth medium and create the necessary amounts of the individual metals, the corresponding metal salts were added. The medium was supplemented with ferrous sulphate ($FeSO_4$) to supply iron. Silver nitrate ($AgNO_3$), Mercuric chloride ($HgCl_2$), sodium arsenite ($NaAsO_2$), and silver (Ag) were used to assess the tolerance to silver, mercury and arsenic respectively. A sterile broth with the required metal amendments was kept for each metal concentration to serve as a control. The *Bacillus* sp. laboratory strain and the bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 were added to the media that had been altered with the four metal salts stated above in accordance with different metal concentrations. The media were incubated at 37 °C with shaking for one night. The optical

density (OD), which serves as a measure of bacterial growth, was then determined at 600 nm using the corresponding sterile medium as a reference. (Roy *et al.*, 2022).

2.2. Two-dimensional stress

The growing ability of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 and *Bacillus* sp. control laboratory strain was tested under 16 different combinations of two-dimensional stresses in broth cultures. The combinations were:

1. Temperature 70°C +20% PEG
2. Temperature 70°C +4% NaCl
3. Temperature 70°C +As 100
4. Temperature 70°C +pH4
5. Temperature 70°C +pH10
6. pH4+4% NaCl
7. pH10+4% NaCl
8. UV+20% PEG
9. UV+ As 100ppm
10. UV+ Fe 200ppm
11. UV+pH4
12. UV+pH10
13. Temperature 20°C + Fe 200ppm
14. T20°C +4% NaCl
15. Temperature 20°C +pH4
16. Temperature 20°C +pH10

The growth in these stress combinations was measured after overnight incubation. The single stress parameter was selected from the data obtained from single dimensional stress experiments. The combinations were designed on the basis of environmental feasibility of the

existence of such stress combinations and in-vivo experimentations (Capece et al., 2013). The standard tissue culture plate method and 0.1% CV stain were then used to measure the biofilm formation of these stressed samples. The results were plotted and compared to a no-stress condition. (Roy et al., 2022)

2.3. Three-dimensional stress

The growing ability of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 and *Bacillus* sp. control laboratory strain was tested in nine combinations of three-dimensional stresses in broth cultures. The combinations were:

1. Temperature 20°C+20% PEG+4% NaCl
2. Temperature 20°C +pH10+4% NaCl
3. Temperature 20°C +pH10+ Fe200ppm
4. pH10+4% NaCl+20% PEG
5. Temperature 70°C +pH10+As100ppm
6. Temperature 70°C +pH10+4%NaCl
7. Temperature 70°C +4% NaCl+20% PEG
8. UV+4% NaCl+20% PEG
9. UV+ Temperature 20°C +20% PEG

The growth in these stress combinations was measured after overnight incubation. The single stress parameter was selected from the data obtained from single dimensional stress experiments. The combinations were designed on the basis of environmental feasibility of the existence of such stress combinations and in-vivo experimentations (Capece et al., 2013). The biofilm formation of these stressed samples was measured using the crystal violet staining method on tissue culture plates. The stain used was 0.1% CV and the samples were left to sit overnight. The results were plotted and compared to the control, which was grown in no-stress condition. (Roy et al., 2022)

2.4. Study of the changes in dynamics of bacterial biofilm with 3d stress using Fourier Transform Infrared Spectroscopy.

The bacterial biofilm was isolated from a 3D stress setup and no stress setups using the methodology described in Section 1.3.2. The study was carried out using KBr as the carrier in FTIR (model: Bruker Alpha II; Made in Germany).

2.5. Whole Genome Sequencing of the 5 Strains

The whole genome sequencing was carried out on the Illumina Novaseq 6000 platform; the assembly was paired-ended and denovo. The bioinformatics analysis pipeline for the de novo genome assembly involved the following steps:

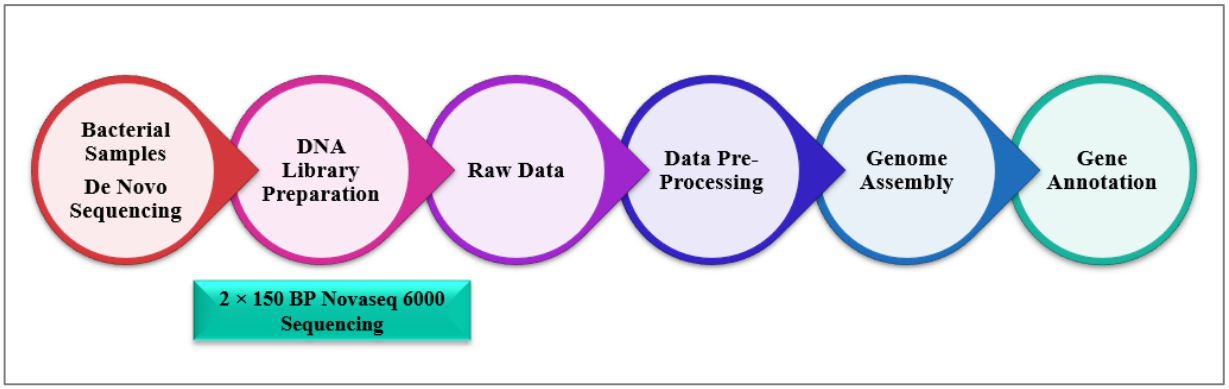


Figure 2.1: The general workflow of the whole genome sequencing of the bacterial samples.

2.5.1. Data QC

The raw data quality was checked using FastQC and MultiQC (Andrews., 2017) software. The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination. All the samples have passed the QC threshold (Q20 > 85%).

Adapter Sequence

P7 adapter read1: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

2.5.2. Data Analysis

Pre-Processing

The raw sequence reads were processed to remove adapter sequences and low-quality bases. using FastP v0.12.4 (Shifu et al., 2018) with default parameters.

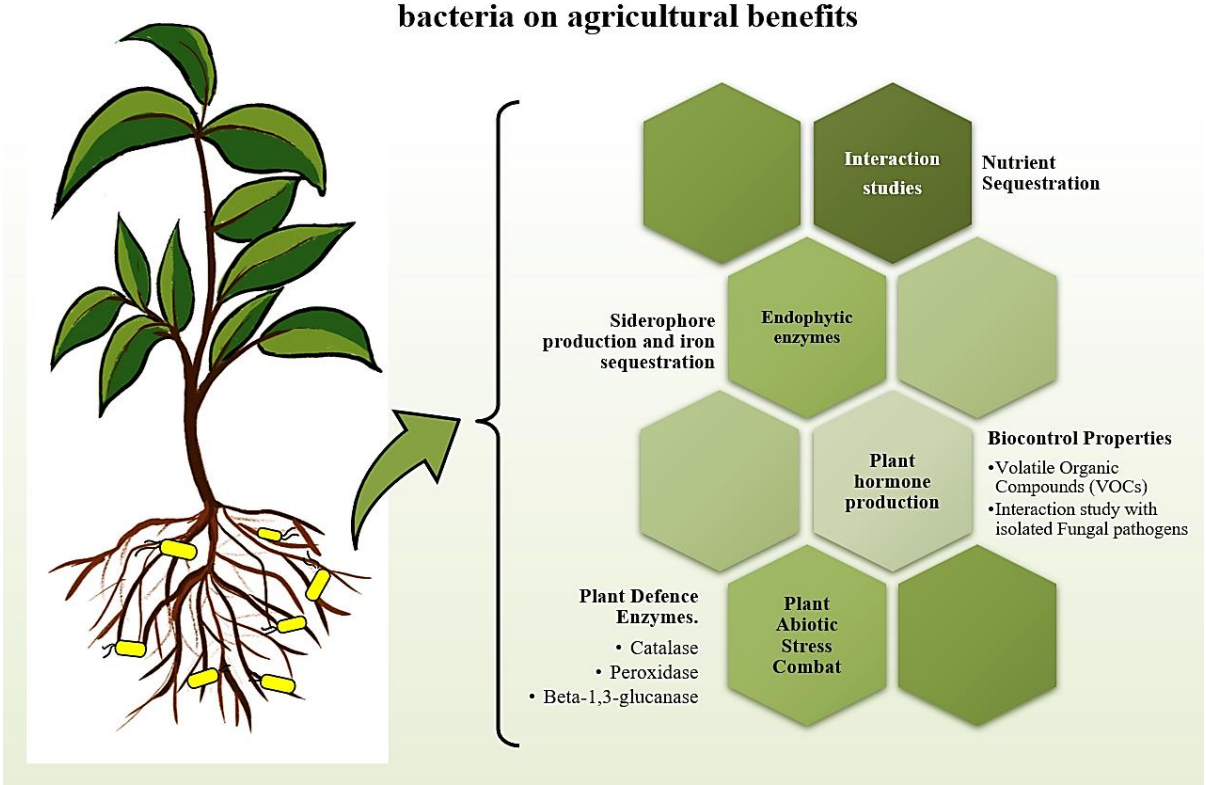
Assembly

The pre-processed reads were assembled into contigs using two assemblers, i.e., Megahit v1.2.9 (Liu et al., 2015) and Unicycler v0.5.0 (Wick et al., 2017). Contigs shorter than 200 bp were removed from the assembly. The assembled genome statistics were assessed using QUAST v5.0.2 (Gurevich et al., 2013). The assembly quality was checked by mapping the reads back on to the assembled contigs using Bowtie2 v2.4.5 (Langmead et al., 2012). At the time ofchecking the assembled genome statistics and the genome completeness, it was found that the results showed unexpected assembly statistics with a high number of contigs.

BUSCO Result

The genome completeness was also checked using BUSCO v5.3.2 (Waterhouse et al., 2018) with bacteria_odb10 as a reference. BUSCO provides a quantitative assessment of the completeness of the expected gene content of a genome assembly**.**

OBJECTIVE 3: Detailing the prospects of the bacteria on agricultural benefits



3. Detailing the prospects of the bacteria on agricultural benefits.

3.1. Plant Growth Promotion Abilities

3.1.1. Macronutrient Sequestration

3.1.1.1.Nitrogen fixation

The nitrogen fixation ability of the 5 strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control Laboratory strain was checked by plating them on modified Jensen's Agar media with indicator Bromothymol blue. The change in the colour of the medium after incubation indicated the different stages of nitrogen fixation. (Sulistiyani & Meliah ., 2017)

3.1.1.2.Phosphate solubilization

In order to determine phosphate solubilising ability of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control Laboratory strain, the strains were plated in Pikovskaya Agar medium (Hi-media) amended with the indicator Bromothymol blue. The change in the colour of the medium after incubation indicated phosphate solubilization. (Maitra et.al, 2022)

Quantification: The phosphate solubilization was carried out using tricalcium phosphate method, where the bacteria was inoculated in Pikovskaya broth medium containing Tricalcium phosphate (5g/L) and incubated at 30°C in a rotary shaker set at 100 rpm for 5 days. The cultures were then centrifuged at 5000 rpm and the supernatant was filtered using a Whatman filter paper. The remaining phosphate in the solution was measured using vanadate molybdate method. The reaction mixture constituted of 35ml of supernatant and 10ml of vanadate molybdate reagent (2.7g of ammonium molybdate tetrahydrate in 30ml of heated distilled water, 0.125g of ammonium metavanadate in 33ml of boiling distilled water, along with 33ml of concentrated HCl, after the solution was cooled, and volume was made up to 100 ml.). The final volume was made up to 50ml and the absorbance of the coloured solutions were measured at 420nm.

3.1.1.3.Potassium solubilization

Potassium solubilization was detected my plating the 5 strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control Laboratory strain on Aleksandro Agar medium modified with acid base indicator Bromothymol blue. The use of the

indicator clearly depicted the production of different acids during the process of solubilization. (Maitra et.al, 2022)

3.1.2. Plant Growth regulator production

3.1.2.1.Indole Acetic Acid

The IAA production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was estimated using the Salkowski reagent. The measurement was done in two set ups, first with the IAA precursor, 0.1% Tryptophan supplemented in the culture medium (Luria-Bertani Broth Medium) and the second without the precursor. The broth was incubated for 48 h at 28 °C at 100 rpm in shaker speed. After incubation, the cell supernatant was taken after centrifugation of the cells at 6720g for 10 min. The IAA production was then determined by using 2 ml of Salkowski reagent (2 ml of 0.5M $FeCl_3$ was prepared, and 49 ml of water and 49 ml of 70% perchloric acid was added. The reagent was freshly prepared and kept in an amber bottle). The IAA production was indicated by appearance of light pink colour of the solution. The optical density of the solution was measured at 540 nm. The standard curve was prepared as per Sarker and Rashid, 2013.

3.1.2.2.Gibberellins

Gibberellic acid production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was done by using modified Holbrook , 1961 method by Sharma and Sharma et.al, 2018. In this process 15 ml of bacterial culture supernatant, was taken after 5 days of incubation in a 50 ml erlenmeyer flask in which 10 ml of absolute alcohol was added. The sample was then diluted to 40 ml of distilled water. 2 ml of zinc acetate reagent (21.9g zinc acetate was added to 1 ml glacial acetic acid, the volume was made up to 100 ml with distilled water). After an incubation time of 2 min, 2 ml of potassium ferrocyanide (10.6% in distilled water) was further added and the volume was adjusted to 50 ml by adding distilled water. The solution was allowed to mix at room temperature with a standing time of 5 min. The contents were filtered using a 0.45 μM milipore syringe filter. 10 ml aliquot of the filtrate of each sample was transferred to 100 ml volumetric flasks with which 8 ml of absolute alcohol was added. Equal volumes of 30% HCl was added to the aliquot and incubated for 75 min at 20 °C. The optical density was measured at 254 nm wavelength. For setting up the blank reading, 5 ml of 5% HCl was used. The same was repeated for the culture set estimated after 7 days of incubation. The concentrations of GA_3 in the two time points were compared.

3.2. Agriculturally Important Metabolites

3.2.1. Stress Response: ACC Deaminase

Qualitative evaluation of ACC Deaminase: ACC Deaminase **(**1-aminocyclopropane-1-carboxylic acid deaminase) production was checked by plating BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain in nitrogen free Dworkin Foster medium, with 3mM ACC supplement (Sigma Aldrich) as the sole nitrogen source. Ammonium chloride was used in one setup as the positive control and minimal Dworkin Foster medium devoid of nitrogen source was used as the negative control. (Kumar et.al, 2012)

Quantitative Estimation of ACC Deaminase: For quantification of ACC deaminase, the standard colorimetric ninhydrin assay was performed. Bacterial colonies were inoculated in 5 ml liquid LB medium, and kept overnight at 28 °C at 200 revolution/minute. 2 ml of culture was harvested by centrifuging at 8000g for 5 min. The cell pellets were washed twice by using 1 ml of DF media. After washing, the cells were resuspended in 2 ml DF media supplemented with ACC, in a 12 ml culture tube. The tubes were incubated for 24 h at a shaker speed of 200 rpm. For the control setup, 2 ml of DF + ACC media was also incubated without any inoculant. After incubation, 1 ml of culture was centrifuged in 1.5 ml centrifuge tubes for 5 min at 8000g. Supernatant (100 µl) was taken and diluted to 1 ml with DF medium. 60 µl of this 10-fold diluted supernatant was pipetted in a 96-well plate with 120 µl of ninhydrin for ninhydrin-ACC estimation. Development of Ruhemann's Purple, and the variation in the colour intensity was measured at 570 nm. For blank only DF medium was used. All isolates were run in triplicates to minimize errors. Standard curve was prepared by following Li . et al.,2011.

3.2.2. Iron Chelation: Siderophore Production

Qualitative estimation of Siderophore production: The CAS agar diffusion assay or CASAD for BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was performed using a modified Schwyn and Neilands, 1987. The modified method was adapted from Shin et al., 2000.

Quantitative evaluation of Siderophore production: Quantification of Siderophore production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was also done by the traditional method of Arora and Verma, 2017. The measurement of siderophore production was done in percent siderophore unit (psu) with the following formula:

$$\text{Siderophore Production (psu)} = \frac{(A_r - A_s) \times 100}{A_r}$$

Where, A_r is the optical density of CAS solution and un-inoculated broth at 630nm and A_s is the optical density of the sample, CAS solution and cell free supernatant of the bacterial; culture. (Payne, 1993)

Typing of the Siderophore produced by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3: The bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain were checked for two types of siderophore ie., hydroxamate and catecholate. The hydroxamate type was detected by the tetrazolium test, in which addition of sodium hydroxide and tetrazolium salt to the cell free supernatant yielded a deep red coloration. The catecholate type was detected by the Arnow's test, in which, addition of nitrous acid, molybdate and an alkali (sodium hydroxide) to the cell free supernatant yielded a pink chromogen that has an absorption maxima at 515nm (Radhakrishnan et al.,2014).

3.3. Biocontrol Properties of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3

3.3.1. Biocontrol Enzymes

3.3.1.1.Peroxidase (total)

To measure the total peroxidase production of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3, and *Bacillus* sp. control laboratory strain, 24hr cultures of the 6 strains were centrifuged at 7000 rpm for 20 minutes to obtains the cell pellets. The pellets were then suspended in a buffer (20 mM sodium phosphate buffer; pH 7.4). The cell suspension was then sonicated for a set amount of time (3 seconds) using a specific output (Amplitude of 50) and strokes (7), and the resulting homogenate was centrifuged under cold conditions. The supernatant was then used as the crude enzyme extract for the peroxidase assay> The reaction mixture consisted of 2mM *n*-Propanol, 10 mM Hydrogen peroxide and citrate buffer (pH 3). One unit enzyme required to oxidize 1 µM of substrate. The method has been described in Kalyani et al. (2011).

3.3.1.2.Catalase

The catalase activity of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control laboratory strain was quantified by plotting a calibration curve with defined unit of catalase activity. Each catalase solution or bacterial suspension (100 µl)

was added in a test tube (13 mm diameter × 100 mm height, borosilicate glass) along with 100 µl of triton X and 100 µl of 30% Hydrogen peroxide solution. The reaction mixture was mixed thoroughly and incubated at room temperature. The Oxygen forming foam that remained constant for 15 minutes was then measured using a ruler following the method of Iwase. et al., 2013.

3.3.1.3.Beta-1,3-glucanase

In order to quantify the production of Beta-1,3-glucanase by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control laboratory strain, the strains were grown in selective media (K_2HPO_4 0.065 g/L, KH_2PO_4 0.24 g/L, $(NH_4)_2SO_4$ 0.05 g/L, NaCl 0.25 g/L, $MgSO_4 \cdot 7H_2O$, 0.012 g/L, yeast extract 0.15 g/L) along with 1% laminarin which was used as the substrate and was incubated for 96 h at 130 rpm. The cell free supernatant thus obtained was used as the crude enzyme extract. The reaction mixture was prepared by adding crude enzyme extract to Dinitro salicylic acid (DNS) reagent in the ratio of 1:2 v/v followed by the measurement of the reducing sugars formed by the enzymatic reaction. The absorbance was measured at 500 nm. (Rais et al., 2017).

3.3.2. Volatile Organic Compounds

3.3.2.1.HCN

HCN production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control laboratory strain was checked by streaking the bacterial colonies on Kings B agar medium with 4.4g/l glycine amendment. A filter paper dipped in Picric acid solution was placed in the upper lid of the petri dish. The dishes were sealed with parafilm and incubated at 28°C for 48 hours and the change in colouration of the filter paper was observed to detect HCN production. (Reetha et al., 2014)

3.3.2.2.Ammonia

The Ammonia production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was detected by first inoculating the bacteria in peptone broth, incubated for 7 days at 30°C followed by the addition of Nesseler's reagent. The brown colour development indicated production of ammonia.

3.3.3. Studies with Phytopathogenic fungi

3.3.3.1.Isolation of phytopathogenic fungi

Four diseased leaves of *Zea mays L*. were collected from the East Kolkata wetlands, following the standard isolation procedure. (Dhara., et al., 2020). The infected leaves were collected in a sterilized, air-tight zip lock bag and were stored at 4°C. Following standard isolation practices, 1x1cm infected pieces were cut randomly from the diseased portions of the leaves with sterilized forceps. The diseased pieces were surface sterilized by dipping them in 70% ethyl alcohol for 2–3s, followed by dipping the samples in $HgCl_2$ solution [0.1% $HgCl_2$(w/v) in autoclaved distilled water] for 30s. Lastly, these pieces were washed in sterile distilled water and blotted dry with sterilized blotting paper to remove excess water. Finally, the surface-sterilized leaf samples were placed on freshly prepared Potato dextrose agar (PDA) plates supplemented with streptomycin (HIMEDIA Streptomycin sulphate (TC035-5G) of 100 ppm concentration) and were incubated at 28°C in a B.O.D incubator for 48 hours for the appearance of colonies.

The subsequent fungal colonies were sub cultured by inoculating on fresh PDA slants supplemented with streptomycin having the same concentration and were incubated at 28 °C until sporulation occurred followed by storage in the refrigerator at 4 °C.

3.3.3.2.In vitro pathogenicity tests

The in vitro pathogenicity was conducted using a single, fresh leaf of *Zea mays L.* for each fungal pathogen. The leaf was surface sterilized and inoculated in a petri dish with fungal conidial suspension. The fungal suspensions were prepared by gently scrapping the sporulating regions from the surface of the media and then adding 10 ml of sterile distilled water. The spore concentration of the inoculants was maintained at 4.1×10^4 spores/ml. The control setup was prepared by using a single fresh leaf of *Zea mays L*. inoculated with 100µl of sterile distilled water. 100µl of fungal suspension was added to the leaves in the form of droplets. The leaves were incubated at sterilized, covered petri plates with adequate moisture supply at room temperature. Dark, blackish, surrounded by chlorophyll degraded regions lesions started to appear on the leaves after 2-3 days of incubation. (Dhara et al. 2020)

3.3.3.3.Identification of phytopathogenic fungi

The pathogens were incubated for 4-5 days at 28°C in a B.O.D. incubator. The colony growth pattern, color, filament shape and size, and spore morphology were examined in a Dewinter compound light microscope. Spore size was estimated by an average of 10 spore sizes. (Dhara et al. 2020).

The phylogenetic identification of the isolates was done using the Internal Transcribed Spacer sequencing region-based method. For isolating the fungal DNA, fresh cultures of fungal isolates were grown on PDA plates. Fungal mycelial mass was taken and was ground on a rough mortal pestle to ensure higher cellular disruption. Ground fungal tissues (100 mg) were taken and 400µl of Lysis Buffer (Hi-Media) was added to it. 2ml of this cellular mixture was taken a centrifuge tube and 20µl of RNase A solution (20mg/ml) is added and the mixture is vortexed vigorously for 5mins. After vortexing the mixture was incubated for 10minutes at 65°C. The contents were mixed thoroughly by inverting the tubes 2-3 times. Precipitation buffer (130µl) was added to the tubes and were incubated for 5mins in an ice bath and was centrifuged for 5mins at 10000 rpm. The lysate was then added to the HiShredder of an uncapped collection tube. The lysate solution was centrifuged at 10000 rpm for 2mins and the flow-through fraction was transferred to another centrifuge tube, without unsettling the cell pellets. To the recovered cell-free lysate, 1.5 vol of binding buffer was added and was mixed thoroughly by pipetting up and down. Lysate-binding buffer (650µl) mixture was added to the Miniprep spin column in a 2ml tube and was centrifuged for 1min at 8000 rpm. The flow-through was discarded and the step was repeated with all residual samples. For washing the sample columns were placed on the same collection tube and 500µl of wash buffer was added. The samples were centrifuged for 1min at 10000 rpm, the flow-through was discarded the same collection tube was centrifuged again for 2mins at 10000 rpm to remove all flow-through and dry the membrane. Finally, the column was placed in fresh centrifuge tubes and 100µl of elution buffer was added to the column. The sample setups were incubated for 1min at room temperature and centrifuged at 10000rpm for eluting the DNA. The step was further repeated with another 100µl of elution buffer for generating higher DNA yield. The quality checking of the eluted DNA was performed on 1.0 % agarose gel, and the appearance of a high-molecular-weight single band of DNA was observed. Further, the ITS region fragment was amplified by PCR. The PCR amplicon was purified to remove any contaminants. Forward and reverse DNA sequencing of the PCR amplicon region was done using two universal ITS region primers: ITS1 and ITS4 primers, employing the BDT v3.1 cycle sequencing kit on an ABI 3730xl Genetic Analyzer. Consensus sequences were generated using aligner software. Finally, the ITS region sequence, thus generated, was used to carry out BLAST with the database of NCBI Genbank. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix and phylogenetic tree were constructed using MEGA 10. (Kimura.,1980; Kumar., 2018). The consensus

sequences thus identified were uploaded to the NCBI GenBank Database and the accession numbers were obtained.

3.3.3.4. Interaction of the 5 bacterial strains with phytopathogenic fungi

For the interaction study between bacterial and fungal strains was carried out following Kumar et al. 2018. 5 mm agar disc from previously grown fungal lawns of the isolated fungal strains were cut and placed on agar plate that has been spotted with 20 µl of bacterial suspension (10^9 cells/ml) at four equidistant corners of the plate, surrounding the centre. The setup was incubated 28°C for a week and regularly monitor the spread of bacteria over the fungal mycelia and were regularly monitored.

3.3.3.5. Volatile interaction of the 5 bacterial strains studies with phytopathogenic fungi

The bacterial strains BRAM_Y2 and BRAM_Y3 were spread plated in a nutrient agar plate, and a phytopathogenic fungus, *Fusarium Fujikuroi* and *Curvularia aeria* discs were placed in a potato dextrose agar plate. The lids of both these plates were discarded and the bottoms were sealed with parafilm facing each other so as to create a physical barrier between the fungus and the bacteria, yet maintain them in the same micro-environment. Thus, ensuring that, any sort of inhibition of fungal growth that takes place after this will be due to the action of the VOCs secreted by the bacteria under experimentation. ((Ruangwong et al., 2021)

3.4.Endophytic enzymes for Recycling of Organic waste

3.4.1. Cellulase

Quantification of cellulase production BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was carried out using 0.5% carboxymethyl cellulose as substrate and cell free supernatant as the crude enzyme extract. The cultures were inoculated in 10 ml of production media containing glucose 0.5 g/L, peptone 0.75 g/L, $FeSO_4$ 0.01 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ 0.5 g/L for 24 h at 37°C in a rotary shaker. The culture medium was centrifuged at 4000 for 15 min to obtain the crude enzyme extract.

The cellulase activity was carried out by following Miller (1959), using 0.2 ml of crude enzyme extract, 1.8 ml of 0.5% carboxymethyl cellulose (CMC) (Hi-media) in 50 mM sodium phosphate buffer pH 7 and incubated at 37 °C for 30 min in a shaking water bath. 3 ml of DNS was added for terminating the reaction. Absorbance of the sample was read at 575 nm (Sethi et al., 2013).

3.4.2. Laccase

The production of laccase by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 AND BRAM_Y3, and *Bacillus* sp. control laboratory strain was quantitatively measured using a specific method described in Fatemeh Sheikhi et al., 2012. The bacterial cultures were centrifuged at 7500 rpm at a temperature of 4°C for 20 minutes. The supernatant was discarded and the pellets were washed with 0.1M phosphate buffer, pH 6.5 containing 10 mM PMSF as bacterial protease inhibitor before sonication. Sonication was carried out 5 times, for 45 s each time, with a gap of 30s between each pulse at 20 MHz. Finally, the cell extract was obtained by centrifuging the sample at 10000 rpm at 4 °C for 20 minutes to use as the crude enzyme extract. 1000u/ml of catalase was added to the crude extract and incubated for 1 h at 37 °C. The enzyme activity was determined by measuring the absorbance at a 465 nm wavelength using a specific substrate (2 mM Guaiacol) in a phosphate buffer at a certain pH (50mM, pH: 6.5). The units of enzyme activity were then calculated and expressed as units per litre, based on the 0.001 unit/min enzyme required to increase the absorbance over time at a certain temperature (55 °C).

3.4.3. Pectinase

Cell free supernatant of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was obtained by centrifuging fresh bacterial cultures at 8500 rpm for 5 minutes. The supernatant thus obtained was utilised as the crude enzyme extract for quantification of pectinase production by the strains. The assay was carried out using 20µl of 1% (w/v) Pectin in 0.1M Phosphate buffer, pH 7 as substrate with 10microlit crude enzyme extract. The reaction mixture was kept in water bath at 50 °C for 10mins. The mixture temperature was lowered to room temperature and 60 µl DNS was added and then kept in boiling water for 5 minutes. The absorbance was measured at 540 nm (Shrestha et al., 2021).

3.4.4. Lignin peroxidase

The extent of lignin peroxidase production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was determined quantitatively by a spectrophotometric assay based on the enzymatic potential of lignin peroxidase to demethylate methylene blue dye. The 6 bacterial strains, were inoculated in a 500 ml erlenmeyer flask with a volume of 100 ml of 0.5% lignin broth (Sigma-aldrich). The inoculates were then incubated at 30 °C with a rotor speed of 120 rpm for 5 days. After day 5, 10 ml of 48hr old bacterial culture was taken and was centrifuged at 3293g at 4 °C in a cooling centrifuge. The tubes were kept in cold ice bath in an undisturbed condition. The enzymatic assay was carried out by preparing the reaction mixture with 1 ml of 50 mM sodium potassium

tartarate buffer at pH 4, 0.1 ml of 0.1 mM hydrogen peroxide as the inducer, 32 µM methylene blue as the substrate, 10 µl of crude enzyme solution. The reaction mixture was then incubated at room temperature for 1 hour. The absorbance was measured at 650 nm. The dye decolourization percent was calculated with respect to the control tube by the following formula (Bholay et al., 2012)

$$\frac{\text{Absorbance 650nm for control} - \text{Absorbance 650nm for test}}{\text{Absorbance 650 for control}} \times 100$$

3.5.In-vivo application of poly extremophilic plant growth promoting strains

3.5.1. Piloting and standardization of treatment mode

The pilot study was designed so as to figure out the best mode of treatment application for the plants. The experiment was conducted on *Zea mays L* (Variety Kohinoor 595).

3.5.1.1. *Interaction amongst the 5 bacterial strains*

This experiment was carried out using the classical method of T-streaking amongst the 5 bacterial strains who were going to be used individually and in consortium.

3.5.1.2. *Treatment preparation*

Three of the bacterial strains were inoculated in LB broth medium previously sterilized by autoclaving. It was then incubated at 37 °C for 48 h under shaking conditions. The dosage for a single plant is as follows: 10 ml of 48-h-old culture half diluted to form 20 ml of treatment.

For the first mode of application or the solid treatment, the bacteria were cultured in Luria–Bertani broth and the diluted culture was then directly mixed with vermicompost; this mixture was then used as treatment for the plants. For the second mode of application or the Luria–Bertani broth treatment, the culture broth was half diluted with water and was directly used in the soil. For the third mode of application or the water suspension treatment, the culture was centrifuged at 10,000 rpm for 30 min. The supernatant was discarded and the pellet was dissolved in sterile mineral water. In this case, the pellet obtained from 20 ml of 48-h culture was dissolved in 40 ml of water to get the half-diluted treatment. The treatment was applied to the plants after 5 weeks of growth.

3.5.1.3. *Application of Bacterial Cultures in the Rhizosphere of Zea mays L. with the three modes*

Mode of Application1 – (Garden soil + Bacterial Treatments) and (Garden soil + Vermicompost + Bacterial Treatments)

In this particular mode of application, the half-diluted bacterial cultures were mixed with a soil-vermicompost mixture (1:1) in 1:1 ratio, that is, 40 ml of culture was mixed with 20 g of garden soil and 20 g of vermicompost to prepare the treatment. Then, with the help of a fork, the upper layer of the soil was loosened without damaging the roots and the mixture was transferred to the following pots circling the stems of the plants:

Table 3.6: Mode of Application 1

Without Compost	**With Vermicompost**
Control	Control
Soil + G1 in L.B	Soil + Compost + G1 in L. B
Soil + G2 in L. B	Soil + Compost + G2 in L. B
Soil + G3 in L. B	Soil + Compost + G3 in L. B
Soil + (G1+G2+G3) in L. B	Soil + Compost + (G1+G2+G3) in L. B

Mode of Application 2 – (Garden soil + Bacterial Treatments) and (Garden soil + Vermicompost + Bacterial Treatments)

As there were 4 plants in each pot, and the dosage for each plant was figured to be 10 ml,40-ml treatment was applied in each pot. With the help of a fork, the upper layer of the soil is loosened without damaging the roots and the mixture was transferred to the following pots circling the stems of the plants.

Table 3.7: Mode of Application 2

Without Compost	**With Vermicompost**
Control	Control
Soil + G1 in L.B	Soil + Compost + G1 in L. B
Soil + G2 in L. B	Soil + Compost + G2 in L. B
Soil + G3 in L. B	Soil + Compost + G3 in L. B
Soil + (G1+G2+G3) in L. B	Soil + Compost + (G1+G2+G3) in L. B

Mode of Application 3 – (Garden soil + Bacterial Treatments) and (Garden soil + Vermicompost + Bacterial Treatments)

Around week 5 bacterial culture were collected. The bacterial consortia were applied by digging the soil around the edge of the pot and pouring 40ml of the respective bacterial culture (in water suspension) in each pot. At the time of applying the consortia it was ensured that the it did not come in direct contact with the roots. The bacterial culture in the respective pots were:

Table 3.8: Mode of Application 3

Without Compost	**With Vermicompost**
Control	Control
Soil + G1 in Water suspension	Soil + G1 in Water suspension
Soil + G2 in Water suspension	Soil + G2 in Water suspension
Soil + G3 in Water suspension	Soil + G3 in Water suspension
Soil + (G1+G2+G3) in Water suspension	Soil + (G1+G2+G3) in Water suspension

After adding the consortia, the edge of the pot was covered again with soil and water was sprayed.

3.5.1.4.*Data collection and determination of the best mode of treatment.*

The data on length of plant, number of leaves, length of leaves and internodal lengths respectively every week after treatment application and noted regularly. The best treatment was selected on the basis of the obtained data which was used in the further set of experiments.

3.5.2. Small Scale field testing on *Zea mays* L. (KOHINOOR 595)

3.5.2.1. Treatment application in the small-scale field study and Data collection

The mode of application was selected from the results obtained from the p`ilot study. The bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 were applied individually and together in a consortium, on test crop *Zea mays* (Variety: KOHINOOR 595). Three plants were taken per setup. The experiments were conducted in the month of September to November for sole crop which is a favourable time for maize harvest as per ICAR guidelines. Cell pellets dissolved in distilled water was used as the mode of treatment. 24 hours old bacterial suspension(7.5ml) (the optical densities of the cultures were 0.7-0.8 at OD 600nm containing 9.8 * 10^9 to 11.2 * 10^9 CFU per ml of bacterial culture), diluted to 15ml with distilled was applied to each plant in the rhizosphere region in a circular pattern. The first treatment was applied at the end of 3rd week and then 2 more treatments were applied at a 2-

week interval, for a total number of three times in the 90 days lifespan of the plant (Roy et.al., 2023). Regular watering was carried out as per the standard parameters of *Zea mays* cultivation by International Maize and Wheat improvement centre (CIMMYT). Physical data was collected at 1week interval with respect to a control where no treatment was applied. The yield parameters were recorded once the fruiting was complete, such as the "total number of fruits", "cob weight", "cob length", "100 seed weight" and "percent grain filling".

The bacterial strains were inoculated in Luria Bertani broth. Cell pellets obtained after centrifugation of the cultures dissolved in distilled water was used as the mode of treatment. The 24 hours old bacterial suspension (7.5ml) , diluted to 15ml with distilled was applied to each plant. The first treatment was applied at the end of 3rd week after the plants got acclamatised in the soil and then 2 more treatments were applied at a 2-week interval, for a total number of three times in the 90 days lifespan of the plant (Roy et.al., 2023). Regular watering was carried out as per the standard parameters of *Zea mays* cultivation by International Maize and Wheat improvement centre (CIMMYT). Physical data was collected at 1week interval with respect to a control where no treatment was applied. The yield parameters were recorded once the fruiting was complete, such as the "total number of fruits", "cob weight", "cob length", "100 seed weight" and "percent grain filling".

3.5.2.2.Plant Pigments

Plant pigments such as chlorophyll and carotenoids were measured from the plant leaves after the completion of the treatment application. Acetone extract of leaves was prepared by crushing 0.1gm leaves in 10ml of 80% acetone, followed by centrifugation. The optical densities were measured at 480nm, 645nm and 663nm. This was followed by the calculation of Chlorophyll A, Chlorophyll B and Total Chlorophyll and Carotenoids using the following formula (Kumari. et al., 2018):

Total Chlorophyll: 20.2*(A645) + 8.02*(A663)

Chlorophyll a: 12.7*(A663) – 2.69*(A645)

Chlorophyll b: 22.9*(A645) – 4.68*(A663)

Carotenoid: [A480 +(0.114*(A663) -(0.638-A645)] ×V/1000×W

3.5.2.3.Statistical analysis of the subsequent data

Analysis of variance was measured for the parameters that were taken into account using python 3.11.

3.5.2.4.Improvement in the Soil Health Parameters after application.

Twenty-two different soil physico-chemical parameters were examined by taking 50g samples from untreated soil initially, followed by another 50g after applying treatments to various setups. These soil samples were gathered from the area surrounding plant roots, specifically around 2cm deep and within the 0-2cm depth range. Subsequently, the soil underwent testing for a range of macro and micro nutrients, as well as other physical characteristics. The Keen-Raczkowski Box method, as detailed by Piper in 1966, was used to determine the soil's physical parameters. Soil pH was assessed by creating a 1:2 mixture of soil and distilled water, then measuring it with a systronic pH meter equipped with glass electrodes, following Jackson's method from 1967. The electrical conductivity of the soil was determined by diluting samples in a 1:2 ratio with distilled water and measuring them using a systronic E.C meter model.

For quantifying the total organic carbon content, the standard wet oxidation method developed by Walkley and Black in 1934 (referenced by Jackson in 1967) was employed. This method involved oxidizing the organic carbon within the soil sample using a solution and gauging the resulting color change, which was then correlated with the organic carbon content. Furthermore, soil samples were analyzed to evaluate the availability of various macro and micro nutrients. Total available nitrogen was determined by distilling the soil with alkaline potassium permanganate solution, followed by measuring the liberated ammonia. The total available phosphorus in the soil was measured using the Bray and Kurtz method (1945) due to the soil's acidic pH. Other nutrients such as available calcium, magnesium, sodium, and potassium were extracted from the soil sample using 1(N) ammonium acetate at pH 7.0. Calcium and magnesium were then extracted via the versene titration method. Sodium and potassium available in the soil were extracted using this process and subsequently tested using a flame photometer (Tandon, HLS, 1993).

3.5.2.5.Metagenome of the Soil before after treatment application.

The workflow obtained for the metagenome sequencing of the soils and the data analysis for the same have been illustrated in **Figure 3.1**.

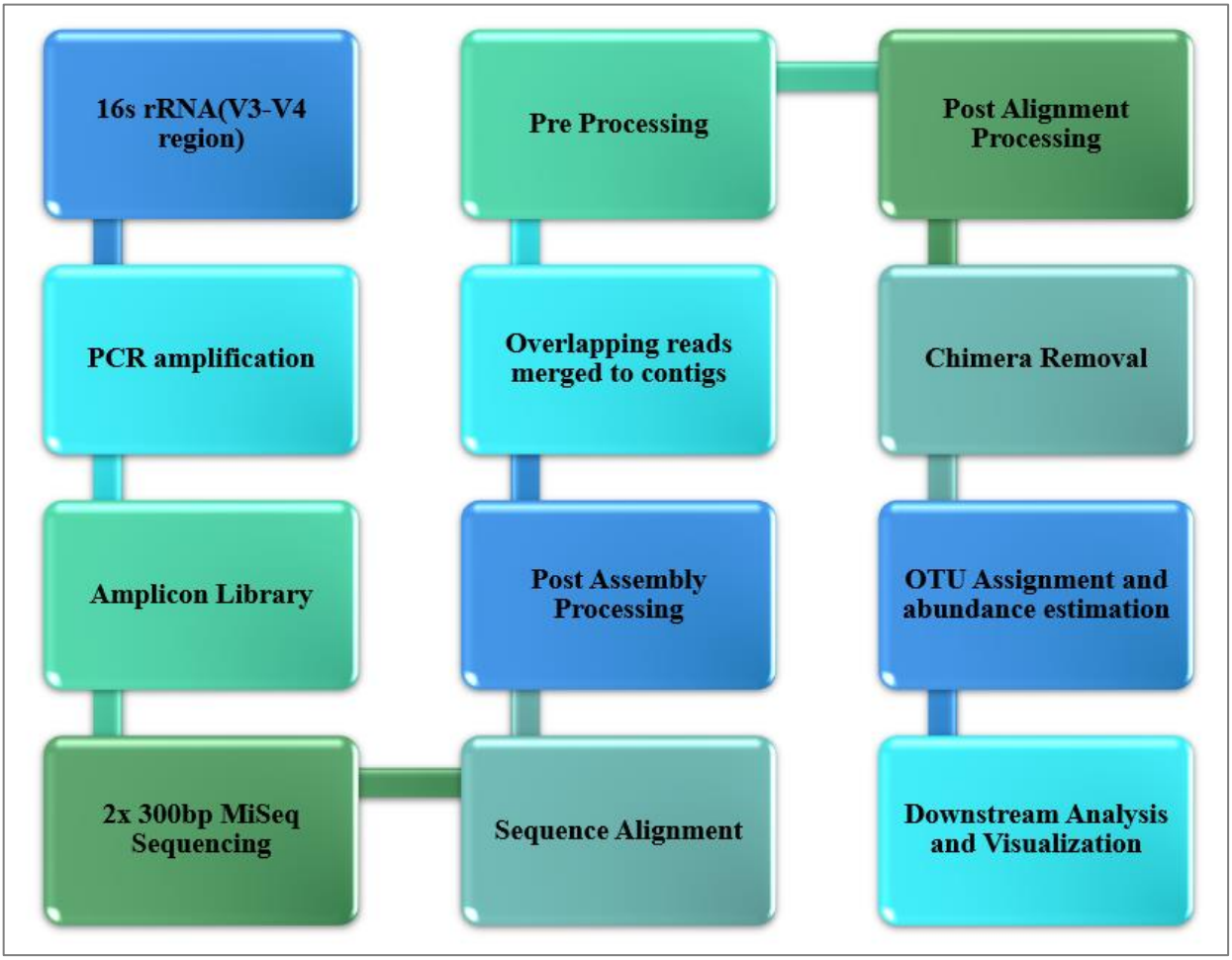


Figure 3.1: The general workflow of Metagenomic sequencing of the test soils.

3.5.2.5.1. Data QC

The raw data quality was checked using FastQC and MultiQC software (Andrews S , 2017). The data was checked for base call quality distribution, % bases above Q30, %GC, and sequencing adapter contamination scores.

Adapter Sequence:

P7 adapter read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

P5 adapter read2 AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3.5.2.5.2. Data Analysis:

- **Pre-Processing**

The raw reads were trimmed (20bp) from 5' end to remove the degenerate primers. The trimmed reads were processed to remove adapter sequences and low-quality bases (Q<20)) using Trimgalore v0.6.7 with default parameters.

- **Overlapping reads merged to contig**

The QC passed reads were imported into mothur v1.46.1 (Schloss et al., 2009) and the pairs were aligned with each other to form contigs.

- **Post Assembly Processing**

The contigs were screened for errors and only those between minimum length (300bp) and maximum length (532bp) was retained. Any contig with ambiguous base calls were rejected. The high quality contigs were checked for identical sequences and duplicates were merged.

- **Sequence Alignment**

Although the primers for the experiment were designed for 16s bacterial rRNA, there are good chances for non-specific amplification of other regions. To correct for this we align the contigs to a known database (GREENGENES v.13.8-99 database (DeSantis et al., 2006)) for 16s rRNA. Depending on the variable region being amplified, most of the contigs would align to its respective region on the database.

- **Post Alignment Processing**

Any ambiguous contigs aligning to other regions on the database were discarded. After this process the gaps and the overhang at the ends from the contigs were removed. Group or merge near-identical sequences together with Pre.cluster.

- **Chimer Removal**

The cleaned contigs are then processed for chimera removal which may have formed due to pcr errors. UCHIME v4.2.40 algorithm (Edgar RC et al., 2011) was used to flag contigs with chimeric regions. A known reference of all the chimeric sequences was used to identify and remove possible chimeric sequences.

- **OTU Assignment and abundance estimation**

The filtered contigs were processed and classified into taxonomical outlines based on the GREENGENES v.13.8-99 database. The contigs were then clustered into OTUs (Operational Taxonomic Unit). After the classification, OTU abundance was estimated.

3.6.Field Application on *Oryza sativa* with Abiotic Stress.

The experimental setups were designed to understand the abiotic stress alleviating potential of the 5 polyextremophilic bacterial strains

3.6.1. Stress application and maintenance

3.6.1.1.Arsenic

The arsenic concentration in the Arsenic stress setups were maintained at 30 parts per million (ppm) Singh et al. (2016). This concentration was chosen to replicate real-world conditions effectively and ensure the relevance of the experiment. Sodium arsenate heptahydrate was used to establish arsenic As (V) stress on the 6 pots designated for arsenic stress setups. The concentration was maintained at 30 ppm periodically by measuring the Arsenic content in the stagnant water and the top soil of the pots using atomic absorption spectroscopy.

3.6.1.2.Salt

Based on a comprehensive review of studies conducted Das et al. (2023), Wahed et al. (2022), it was determined that, for the sake of a standardized and consistent experimental approach, the salt concentration was set at 5% of the total topsoil. The salt concentration was maintained at 5% by the periodical measurement of the electronic conductivity of the top soil and the stagnant using an EC probe from Elico.

3.6.1.3. Drought

The plant in this setup were not watered for the 7 weeks to observe the drought stress mitigative property of the bacteria and the limit to which the treated plant can handle the limited water activity.

3.6.1.4.PGPR setup with stress application

The plants in this setup were watered regularly and was not subjected to any external stress parameters (biotic or abiotic). But they were subjected to bacterial treatments like the stress setups to understand the effectivity of the bacterial strains as plant growth promoters in absence of external stress factors.

3.6.2. Treatment application

The bacterial samples were inoculated in Luria Bertani broth and incubated overnight at 37°C under shaking conditions. The cultures were then (10 ml each) from G1, G2, G3, Y2, and Y3 cultures were centrifuged at 10,000 rpm and individually administered to pots bearing their respective labels. Each bacterial strain was applied to 4 setups, PGP, arsenic stress, salt stress and drought stress.

The bacterial treatment was applied when the plants were 7 weeks old. However, for the negative control pots, which encompasses both the experimental null and the stress control, no bacteria were introduced. This process was consistently applied to all the stress and control samples. Subsequently, the pots were left to foster rhizosphere interactions before any data collection was initiated.

Set ups	Bacterial Treatment	No. of Plants in the setup	Implication
Control	**-**	**3**	**No stress, no Treatment, General Control**
Plant Growth Promotion	**BRAM_G1**	**3**	**Positive control, without stress, to check the PGP abilities of the 5 bacterial strains in *Oryza sativa.***
	BRAM_G2	**3**	
	BRAM_G3	**3**	
	BRAM_Y2	**3**	
	BRAM_Y3	**3**	
Arsenic 30ppm	**-**	**3**	**Negative Control (No bacteria applied)**
	BRAM_G1	**3**	**Test setup for testing of Arsenic stress mitigation abilities of the 5 PPGP bacterial strains in *Oryza sativa.***
	BRAM_G2	**3**	
	BRAM_G3	**3**	
	BRAM_Y2	**3**	
	BRAM_Y3	**3**	
Salt 5%	**-**	**3**	**Negative Control (No bacteria applied)**
	BRAM_G1	**3**	**Test setup for testing of Saline stress mitigation abilities of the 5 PPGP bacterial strains in *Oryza sativa.***
	BRAM_G2	**3**	
	BRAM_G3	**3**	
	BRAM_Y2	**3**	
	BRAM_Y3	**3**	
Drought	**-**	**3**	**Negative Control (No bacteria applied)**
	BRAM_G1	**3**	**Test setup for testing of Drought stress mitigation abilities of the 5 PPGP bacterial strains in *Oryza sativa.***
	BRAM_G2	**3**	
	BRAM_G3	**3**	
	BRAM_Y2	**3**	
	BRAM_Y3	**3**	

Table 3.8: The summarised design of the experiment on *Oryza sativa* setups.

3.6.3. Data Collection

3.6.3.1. Vegetative and reproductive data

In order to understand which treatment of polyextremophilic bacteria is more efficient over others, the vegetative and reproductive characteristics of the rice plant were observed and noted. The data for vegetative characteristics were recorded throughout the growth period of 14 weeks, it is a time series one (time dependent). The reproductive parameters were recorded at the end of 14-week experiment period, it is independent of time.

The vegetative parameters included:

a) Plant Height Measurement- The seven weeks data following the application of the treatment plant height data for all the plants were collected to determine the changes in the treated plant with respect to the stress control and the experimental control.

b) Leaf Count Measurement- The number of leaves of each plant was counted and the data was noted. As it was necessary to determine the influence the extent of vegetative growth upon the application of treatment.

Once vegetative data was collected, it was essential to gather reproductive data to ensure that the treatment, while promoting vegetative growth, did not hinder reproductive growth and promotes the same as well.

a) *Number of Panicles and Spikelets:* The total number of panicles and spikelets was counted during the 7th week period after treatment application (14th week after inoculation).

b) *Measurement of Seed Count and Percent Seed Filling:* The seeds were harvested, and the total number of seeds for all the plants was counted and recorded. This count included both filled and empty seeds. Subsequently, the seeds were screened for filled seeds, and the data was recorded.

c) *Measurement of 100 Seed Weight:* From the total number of seeds with fillings, 100 seeds were collected and weighed for each setup.

3.6.3.2.Study of Plant Pigments

The methodology for this study was adopted from section 3.5.2.2 using the protocol from (Kumari. et al., 2018).

3.6.3.3.Study of Enzymatic and non-enzymatic antioxidants with response to stress.

Both enzymatic and nonenzymatic assays were conducted to assess the impact of rhizosphere interactions on the alleviation of abiotic stress in the plants.

a) Guaiacol Peroxidase (GPX) Assay:

The protocol for guaiacol peroxidase (GPX) activity assay concerning rice plant specimens was adapted from Mukherjee and Chanda (2001). To commence, a fresh rice plant specimen was meticulously cleaned with distilled water. Subsequently, 1 gm of plant tissue was accurately weighed and ground into a fine powder using a pestle and mortar, utilizing 5 mL of 50 mM phosphate buffer at pH 7.0. The resulting homogenate was then transferred to a centrifuge tube and subjected to a 10-minute centrifugation at 10,000 revolutions per minute (rpm). Following this centrifugation, the supernatant, rich in enzyme content, was collected and designated as the enzyme source for further use. The next step involved the preparation of a reaction mixture. This mixture consisted of 1.5 mL of 50 mM phosphate buffer at pH 7.0, 0.5 mL of 20 mM guaiacol, 0.5 mL of 40 mM hydrogen peroxide (H_2O_2), and 0.5 mL of the previously obtained enzyme source. These components were thoroughly mixed in a cyclomixer to ensure a homogeneous mixture, after which the reaction mixture was incubated for 5 minutes at a temperature of 37°C. To quantify the results, the absorbance of the reaction mixture was measured at a wavelength of 470 nm using a UV-VIS spectrophotometer (Optizen). This comprehensive protocol provides a standardized approach for the assessment of GPX activity in rice plant specimens, contributing valuable insights into the enzymatic processes associated with plant stress response.

b) Catalase (CAT) Assay:

The protocol for catalase (CAT) activity assay concerning rice plant specimens, was carried out by following the methodology established by Aebi in 1984. Initially, a fresh rice plant specimen was meticulously cleaned with distilled water to ensure its purity. Subsequently, 1 gm of plant tissue was accurately weighed and finely ground using a pestle and mortar, along with 5 ml of 50 mM phosphate buffer at pH 7.0. The resulting homogenate was then transferred to a centrifuge tube and subjected to a 10 minutes centrifugation at 10,000 revolutions per minute (rpm). Following this centrifugation, the supernatant, which contains the enzyme of interest, was collected and designated as the enzyme source for further use. The next step involves the preparation of a reaction mixture. This mixture comprised 1.5 mL of 50 mM

phosphate buffer at pH 7.0, 0.5 ml of 30 mM hydrogen peroxide (H_2O_2), and 0.5 mL of the previously obtained enzyme source. These components were meticulously mixed, and the timer was immediately started. After precisely 1 minute, the reaction was halted by adding 2 mL of distilled water. To quantify the results, the absorbance of the reaction mixture was measured at a wavelength of 240 nm using a spectrophotometer. This comprehensive protocol provides a standardized approach for the assessment of CAT activity in rice plant specimens, offering valuable insights into the plant's enzymatic processes, particularly its ability to break down hydrogen peroxide and contribute to stress response mechanisms.

c) Malondialdehyde (MDA) Assay:

The protocol for conducting a malondialdehyde (MDA) assay on rice plant specimens, was based on the method described by Heath and Packer in 1992. Initially, a fresh rice plant specimen was thoroughly cleaned with distilled water to ensure its purity. Subsequently, 1 gram of plant tissue is accurately weighed and finely ground in a pestle and mortar, using 5 mL of 0.1% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The resulting homogenate was then transferred to a centrifuge tube and subjected to a 10-minute centrifugation at 10,000 revolutions per minute (rpm). After centrifugation, the supernatant is carefully collected and transferred to a new centrifuge tube. To this supernatant, 2 mL of 5 N HCl was added, and the mixture is thoroughly mixed. The next step involved heating the reaction mixture in a boiling water bath for 15 minutes. After precisely 15 minutes, the mixture was cooled to room temperature. Following the cooling step, the reaction mixture was once again subjected to centrifugation for 10 minutes at 10,000 rpm. The resulting supernatant was then collected and its absorbance was measured at 532 nm using a spectrophotometer. This comprehensive protocol provides a standardized and reliable approach for the assessment of MDA levels in rice plant specimens, offering valuable insights into oxidative stress and lipid peroxidation in plants.

d) Carotenoids:

To extract pigments from one gram of rice plant leaves, the fresh leaf material was meticulously prepared by removing any non-green or damaged portions. The leaves were then finely cut into small pieces and ground to a paste using a mortar and pestle, with the addition of chilled 100% acetone to facilitate the process while keeping the sample cold to prevent pigment degradation. Following grinding, the leaf paste was transferred to a glass vial and fully immersed in ice-cold

100% acetone, ensuring the sample was sealed to protect it from light. This acetone extraction process was allowed to incubate in the dark at 4°C for a minimum of 24 hours, ensuring thorough pigment extraction. After incubation, the resulting acetone solution was then subjected to spectrophotometer measurements at specific wavelengths, 480nm, 645nm and 663nm. The concentration of the pigments was then calculated using the following equations. This method is crucial for assessing chlorophyll content in rice plants and understanding their photosynthetic capacity and response to various treatments or environmental conditions.

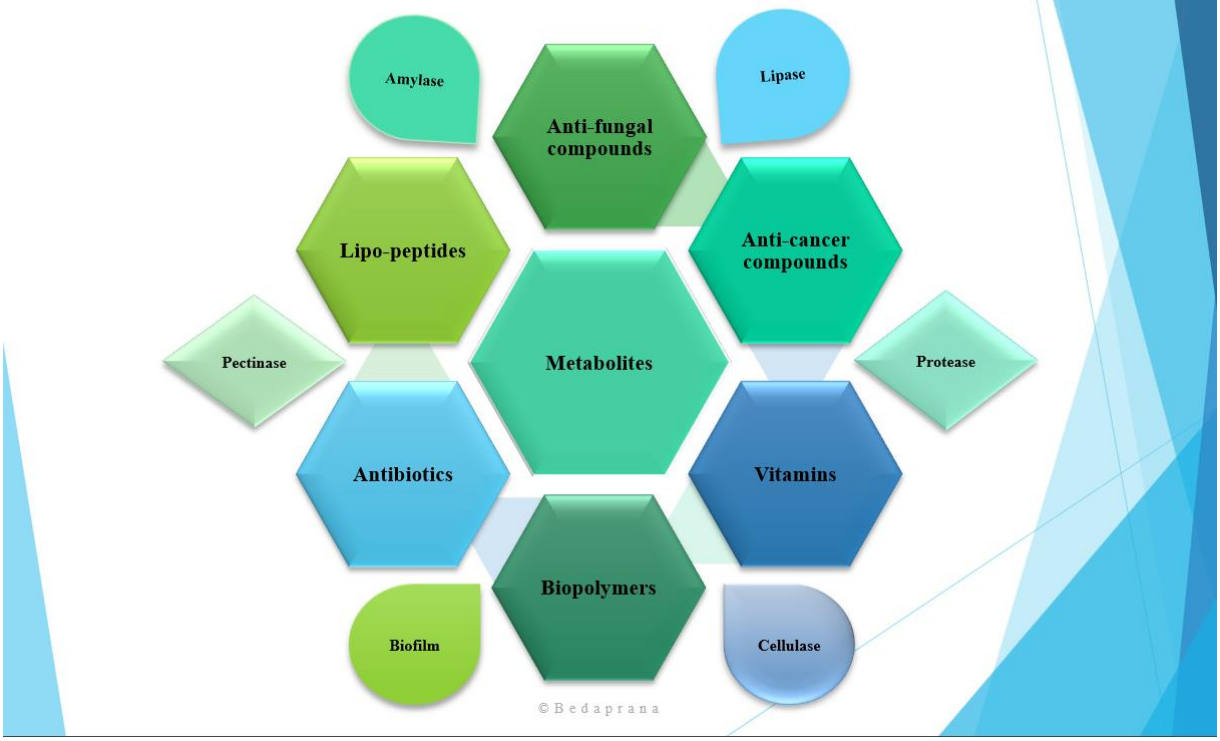
Carotenoid: [A480 +(0.114(A663) -(0.638-A645)] ×V/1000×W

3.6.3.4. Metagenome study of the control and the treated soils

The methodology for this study was adoption from section 3.5.2.5. The 5 sets of soils were subjected to metagenome sequencing for understanding the colonisation of the applied bacterial treatment.

OBJECTIVE 4

Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.



4. Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.

4.1. Industrially and pharmaceutically important enzymes

4.1.1. Pullulanase

Pullulanase activity was assessed by quantifying the release of reducing sugars resulting from the enzyme's action on a pullulan substrate. In a reaction mixture containing 1% pullulan in 0.1M acetate buffer at pH 4.5, along with the enzyme at an appropriate concentration, the Pullulanase activity was measured. This mixture was then incubated at 45°C for 60 minutes. The reaction was halted by rapid cooling in an ice bath and the addition of dinitrosalicic acid (DNS) reagent. The amount of reducing sugars produced was determined using the DNS method. One unit of Pullulanase activity corresponded to the amount of enzyme capable of liberating 1 mM of reducing sugar per minute, using glucose as the standard (Wakso et al., 2011).

4.1.2. Lipase

The cultures were streaked onto plates containing, Tributyrin Agar Media (Hi-media) and incubated at 37ºC for 24 hours-48 hours. Growth and clearing zones around the colonies indicated Lipase Production (Smith, J. A, 2021).

4.1.3. Amylase

The production of amylase by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 AND BRAM_Y3, and *Bacillus* sp. control laboratory strain was measured using the cell-free supernatant from the centrifuged bacterial cultures as the crude enzyme extract and starch as the substrate. The crude enzyme extract was prepared by introducing bacteria into a standard production medium containing soluble starch (10 g/l), peptone (5 g/l), $(NH_4)_2SO_4$ (2 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (2 g/l), and $MgCl_2$ (0.01 g/l), adjusted to pH 7. The cultures were then incubated for 24–48 hours at 37°C. After incubation, the crude enzyme extract was obtained by centrifuging both treated and untreated cultures at 1200 rpm for 20 minutes at 4°C. The resulting supernatant was used as the crude enzyme solution. To quantify enzyme activity, the amount of reducing sugar produced by enzymatic breakdown of the substrate (starch) was measured using the DNS method. The absorbance was measured at 540 nm against a reagent blank (Patel et al., 2019).

4.1.4. Protease

For the quantification of protease production by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. control laboratory strain, the bacterial strains were inoculated in 100 ml MRS broth for 10 days and centrifuged to get the crude enzyme extract. Enzyme quantification was carried out by the method of Tsuchida et al. (1986) using Casein as a substrate.

4.1.5. Urease

The bacterial cultures BRAM_G1, BRAM_G2, BRAM_G3, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 along with *Bacillus* sp. laboratory strain was inoculated in Stuart's broth. A microtitre plate with 96-wells was used for the assay. The cultures, were grown overnight at 150 rpm, and was washed and re-suspended in 0.01M phosphate buffer at pH 7.4. 30 µl of bacterial cultures (O.D600 0.5) was inoculated in 300 µl Stuart's
broth.

The absorbances were measured at 430 nm and 560 nm at 0, 2, 3, 4, 24, 36, and 48-hour time points. The hydrolysis of urea resulted in pink coloration in course of time. The quantification was performed by observing the rate of change of colour over time.

Rate of colour change was measured by the given formula (Okyay and Debora, 2013):

$$\text{Rate of colour change} = \frac{\text{Absorbance at T2} - \text{Absorbance at T1}}{T2 - T1}$$

4.2.ESI-MS Study of metabolites of the 5 bacterial strains under normal conditions

4.2.1. Sample preparation and data acquisition

The metabolome study, or small molecule metabolome study to be more specific involves three main steps a) Quencing, b) Harvesting and then finally c) extraction of the metabolites. The extraction solvent used in this study was cold methanol 99% (v/v) solution (HPLC grade). The bacterial cells were grown in Luria-bertani broth medium for 24 hours at 37°C under shaking conditions. The cultures (in centrifuge tubes) were transferred in ice bath for the process of quenching (a process that stops or slow down bacterial metabolic processes) followed by centrifugation. The supernatant was used for the extraction of extracellular metabolites whereas the cell pellets were stored in -80°C refrigeration conditions for the extraction of the intracellular metabolites (Kamal et al.,2022).

For the intracellular metabolites, the methodology used was the process of controlled leakage where the bacterial cells (cell pellet stored at in -80°C refrigeration conditions) were subjected to multiple freeze thaw cycles (in -80°C refrigeration conditions followed by transfer to ice bath) in the presence of the cold extraction solvent (in this case Cold methanol 50% (v/v) , -30°C) along with vigorous vortexing for 10 seconds. This was followed by centrifugation and the supernatant thus obtained was ready for intracellular metabolite extraction (Villas-Bôas et al., 2007).

For extraction of the metabolites in the biofilm, the biofilm from the 5 bacterial strains were isolated using the methodology described in section 1.3.2. The biofilm was then dissolved in sterile mili Q water, which was used as the extraction solvent for biofilm metabolites.

Thus, as all the three fractions viz., extracellular, intracellular and biofilm were ready for metabolite extraction, the solution was filtered using 0.22 µm pore size, syringe filters, followed by further addition of their respective cold extraction solvents and centrifuged to get particle free, extracted metabolite samples for Electron Spray Ionisation Mass Spectrophotometry. The procedure has been summarised in a schematic representation in Figure 4.1 for easier understanding of the workflow.

For ESI-MS the samples were loaded in using Model: Xevo TQ Absolute IVD System from Waters. The mass spectra were then recorded for all the samples which were then further analysed.

4.2.2. Data Analysis

The interpretation of mass spectral data was done firstly by creating a mass list of probable compounds along with their molecular ionic mass and fragmented ionic masses. The reason behind this being the lack of collated databases for analysis of untargeted metabolome data, from different bacterial species. While creating a personal database or mass list, *Bacillus* species was used as a filter as the study included 5 members of that family itself. The relative heights and the isotopic masses of the peaks were noted and matched with the probable dataset that included the masses of the intact compounds and the fragmented ions as the weak carbon-carbon bonds are most likely to break during the process of ionisation. This process was used to interpret the voluminous mass spectral data of the small molecule metabolome of the 5 bacterial strains in this investigation.

4.3.ESI-MS Study of Extracellular, Intracellular, and Biofilm Metabolites in 3D Stress conditions.

The general procedure followed in this section was exactly the same as discussed in section 4.2. The only difference in this case was the application of a three-dimensional stress combination of 4% Salt+20% PEG and 254nm UV irradiation on the growing cultures of the 5 bacterial strains under investigation to check for the changes in their small molecule metabolome after stress application**.**

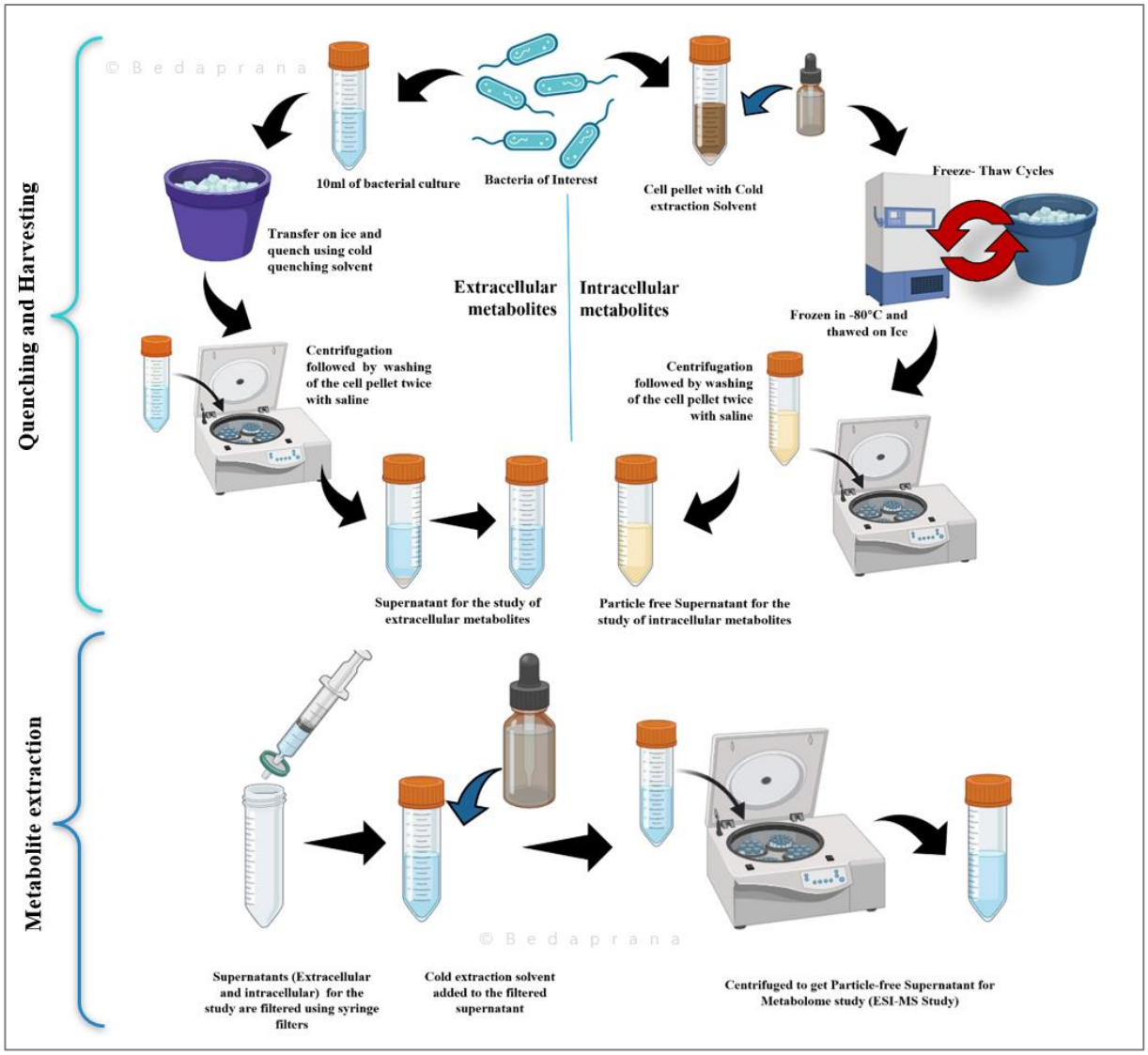
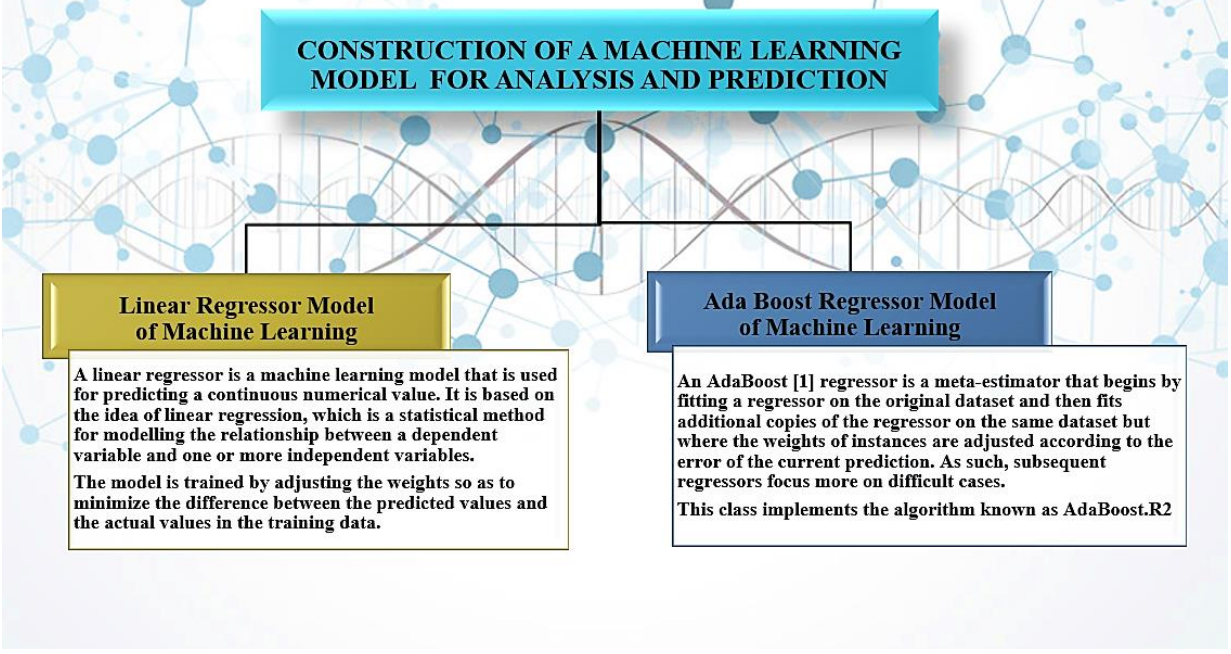


Figure 4.1: The schematic representation of the basic procedure followed for the sample preparation of bacterial metabolome study (extracellular and intracellular metabolites). The process primarily involves 3 steps, Quenching, harvesting and finally extraction.

OBJECTIVE 5

Construction of a machine learning for analysis and prediction of plant growth promoting properties and maize productivity under stressed conditions.



4.4.*Construction of a Machine Learning Model for Analysis and Prediction of Plant Growth Promoting Properties and Maize productivity under stress condition.*

4.5. For the Linear Regressor Model

4.5.1. Processing the Data

The data was first separated into two parts, features and labels. In this case, the data was separated into features and labels, with the features being the data used to make predictions and the labels being the target values that the predictions are trying to approximate.

4.5.2. Encoding the Data

OneHotEncoder was used for the pre-processing of the data. In this case, the experimental setups column of the data was encoded using OneHotEncoder. This involved converting the categorical values in the column into numerical values through the creation of dummy variables. By encoding the data in this way, it becomes possible to use these algorithms to model the data.

4.5.3. Splitting of Data into Training and Testing

In this case, the data was divided into a training set and a testing set, in order to evaluate the performance of the model. The training set was used to fit the model, while the testing set was used to evaluate the model's performance on unseen data. The data was split into 80% for the training set and 20% for the testing set, with the split being performed randomly to ensure that the training and testing sets were representative of the overall dataset. This split is commonly used as it provides a sufficient amount of data for training the model while still allowing for a robust evaluation of the model's performance.

4.5.4. Feature scaling

Feature scaling is a pre-processing step that involves scaling the features of the data so that they have the same scale and are more comparable to each other. In this case, feature scaling was not done on the data because the scales of the features were more or less uniform. This means that the features were already on a similar scale and did not require scaling in order to be comparable to each other.

4.5.5. Training of a Linear regressor model

A linear regressor is a machine learning model that is used for predicting a continuous numerical value. It is based on the idea of linear regression, which is a statistical method for modelling the relationship between a dependent variable and one or more independent variables. In a linear regressor, the prediction made by the model is a linear combination of the input features, with each feature being multiplied by a weight and the results being summed together. The model is trained by adjusting the weights so as to minimize the difference between the predicted values and the actual values in the training data.

The prediction made by the model is given by:

*Prediction = Weight1 * Feature1 + Weight2 * Feature2 + ... + WeightN * FeatureN*

where "Weight1" is the weight associated with "Feature1," "Weight2" is the weight associated with "Feature2," and so on. The weights are learned by the model during training, and they determine the relative importance of each feature in the prediction.

4.5.6. Determination of the Model Performance

The R^2 score is often used as a measure of model performance in regression tasks, with a higher R^2 score indicating a better performing model. The R^2 score, also known as the coefficient of determination, is a measure of the goodness of fit of a regression model. It is calculated as the proportion of the variance in the dependent variable that is explained by the model.

The R2 score can be calculated as:

R2 = 1 - (sum of squared residuals / sum of squared total)

where the sum of squared residuals is the sum of the squared differences between the predicted values and the true values, and the sum of squared total is the sum of the squared differences between the true values and the mean of the true values. The R^2 score ranges from 0 to 1, with a higher value indicating a better fit. A model with an R^2 score of 0 means that it explained none of the variance in the dependent variable, while a model with an R^2 score of 1 meant that it perfectly explained the variance in the dependent variable.

4.5.7. Determination of the model results

Due to the diversity yet shortage of data because of the limitations of wet lab experimentation, the model was tested over the whole dataset instead of just the training dataset and the score for each predicted parameter was calculated as well as the overall score.
(https://scikit-learn.org/stable/modules/generated/sklearn.linear_model.LinearRegression.html)

4.6.For the AdaBoost Regressor

The AdaBoost regressor is an ensemble method that combines multiple weak learners (regression models) to create a strong regression model. Each weak learner is trained on a subset of the training data and assigned a weight that reflects its performance. The final prediction is obtained by combining the predictions of all weak learners, weighted by their performance.

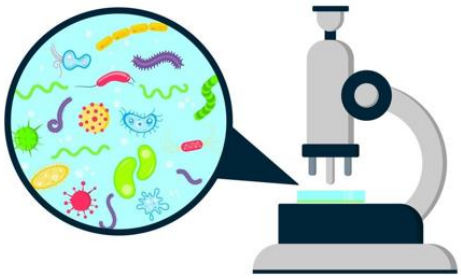
4.6.1. Training AdaBoost Regressor

Initially, all data points were assigned equal weights. Then the weak learners were trained. A weak learner, often a simple regression model such as a decision tree, trained on the training data using the weights assigned in the previous step. The weights assigned in the previous step were used by the weak learner to minimize the error between the predicted and actual target values. The performance of the learner was then calculated. The error of each weak learner was calculated by comparing its predictions with the actual target values. The error was typically represented as the difference between the predicted and actual values, such as the mean squared error. The weights of the data points were updated based on the error of each weak learner. Data points that were predicted accurately by the weak learner are given lower weights, while misclassified points were given higher weights. This process emphasized the misclassified points in the subsequent training iterations. The weak learners were combined into a strong regression model by assigning weights to each weak learner's prediction based on its performance. Typically, more accurate weak learners are given higher weights. Last three steps were repeated for a specified number of iterations or until a desired level of performance was achieved.

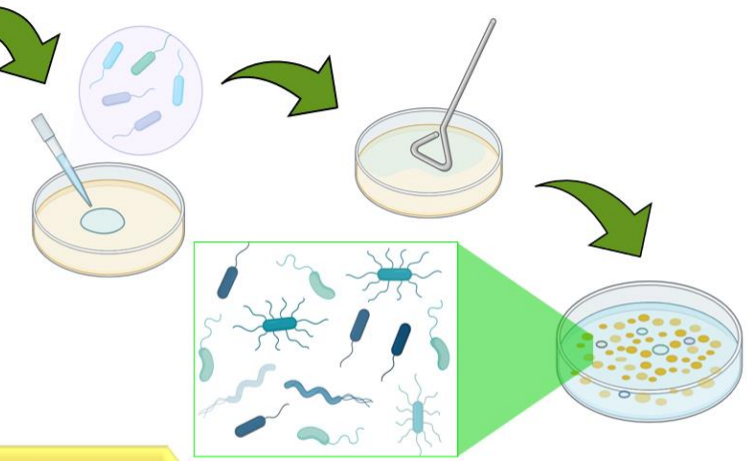
4.6.2. Pointwise evaluation of the AdaBoost Regressor:

To evaluate the AdaBoost regressor pointwise, the dataset was divided into training and testing sets. The training set was used to train the AdaBoost regressor, while the testing set is used to evaluate its performance. The trained AdaBoost regressor is applied to the testing set to obtain predictions for the target variable. Pointwise, evaluation metrics of the AdaBoost regressor were calculated. The predicted values were compared with the actual target values in the testing set using appropriate evaluation metrics for regression tasks. Common metrics included mean squared error (MSE), root mean squared error (RMSE), mean absolute error (MAE), and R-squared (coefficient of determination). These metrics evaluated the performance of the AdaBoost regressor on a pointwise basis, providing insights into the accuracy of predictions for individual data points. By evaluating the AdaBoost regressor pointwise, the ability of the model to make accurate predictions for each specific data point in the testing set can be assessed. This analysis helped in understanding how well the model performs on a case-by-case basis and identify any potential weaknesses or outliers."

CHAPTER 4: RESULTS



OBJECTIVE 1:
Isolation and Characterization of Extremophilic Bacterial strains.



Gangotri Water, Water from the Upper course of Ganga, **Altitude: 3100 meters.**

Three Novel Strains of *Bacillus subtilis* Reported and submitted to NCBI.

BRAM_G1: Accession Number MW006633

BRAM_G2 : Accession Numbers MT998278-MT998280

BRAM_G3 : Accession Number MT998617

Yamunotri Water, Water from a Hot Water Spring, **Altitude: 3293 meters.**

Two Novel Strains were reported and submitted to NCBI.

***Mesobacillus subterraneous* BRAM_Y2**

Accession Number : MW002419

***Brevibacillus parabrevis* BRAM_Y3**

Accession Number : MW081864

1. "Isolation and Characterization of extremophilic bacterial strains.

1.1. Evaluation of the physical characteristics of the Gangotri River water and Yamunotri, Suryakund water.

The 5 bacterial strains, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 were isolated from the waters of Gangotri and Yamunotri, whose physical characterization like, Electric Conductivity, TDS, DO and salinity were carried out to understand the isolation condition **(Table 1.1).**

	Electric Conductivity (µS/cm)	TDS	pH	DO (mg/l)	Salinity (%)
Gangotri Water	93.5	5.1	7.8	0.45	0.017
Yamunotri Water	99.4	6.4	7.9-8.1.	1.5	0.033

Table 1.1: Physical characterization of the waters from which the 5 bacterial strains were isolated.

Key findings from Table 1.1

- The waters of both Gangotri and Yamunotri were very clean with minimal impurities present in them.
- The temperatures were the only extreme condition that the organisms present in the water might have been subjected to, as the water temperatures at Gangotri was 10°C and 88°C at Surya Kund of Yamunotri, which was a thermal spring.

1.2. General Characterization of BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3

The bacterial strains were isolated using spread plate method colonies were selected on the basis of their predominance in the different dilution plates. All the 5 bacterial strains were then characterised according to the Bergey's Manual **(Table 1.2).**

a. Gram staining, colony and cell morphology:

The results indicated that BRAM_G1, BRAM_G2, BRAM_G3 gram positive rods. Whereas, BRAM_Y2 and BRAM_Y3 were found to be gram variable rods.

Bacterial strains	Gram nature	Morphology
G1	Gram positive	Rod shaped, creamy white colony, opaque, margin entire
G2	Gram positive	Rod shaped, creamy white colony with yellowish tinge, opaque, margin entire
G3	Gram positive	Rod shaped, creamy white colony, mucoid, translucent, margin entire

Biochemical tests		Bacterial strains		
		G1	**G2**	**G3**
IMViC Tests	Indole Test	-	-	-
	Methyl Red Test	-	-	-
	Vogues-Proskauer Test	-	-	-
	Citrate Test	++	++	+
Nitrate Utilization Test*		+	+	+
Catalase Test		+	++	+++
Oxidase Test		+	-	-
Motility Test		+	+	+
Sulphate Utilization Test		+	-	+

Bacterial strains	Gram nature	Morphology
Y2	Gram variable	Rod shaped but slightly curved, orange colony, rhizoidal, mucoid, margin entire but with irregular edges
Y3	Gram variable	Rod shaped, creamy white colony, opaque, margin entire, Pearly shine.

Biochemical tests		Bacterial strains	
		Y2	**Y3**
IMViC Tests	Indole Test	-	-
	Methyl Red Test	-	-
	Voges-Proskauer Test	-	-
	Citrate Test	-	-
Nitrate Utilization Test*		+	+
Catalase Test		+	-
Oxidase Test		+	-
Motility Test		+	+
Sulphate Utilization Test		-	-

Table 1.2: General characterization of the bacterial strains.

Key Findings from Table 1.2

- The three bacterial strains from Gangotri were gram positive in nature and the two from Yamunotri were gram variable in nature, i.e., they stained irregularly and appeared as a mixture of pink and purple colonies.
- All 5bacterial strains were rod shaped in nature but had distinctly different colony morphologies.
- All 5 bacterial strains were Indole, Methyl red and Voges Proskauer negative and the 3 bacterial strains from Gangotri were Citrate positive.
- All 5 bacterial strains showed positive results to catalase, nitrate utilization and motility but only G1 showed a positive result for Oxidase and G1 and G3 showed positive results for sulphate utilization.

b. Growth on Chromogenic agar media:

All 5 bacterial strains when streaked on Hichrome UTI agar and Hichrome Bacillus agar showed similarity to the colour indexing found in bacillus strains. The multitudinous variety of the colours visualised after 24hours of incubation at 37°C in the respective chrome mediums (especially Bacillus chrome) indicated a variety of enzyme activities by the strains, which were responsible for the generation of colours (the basic scientific principle of a chromogenic medium) **(Figure 1.1).**

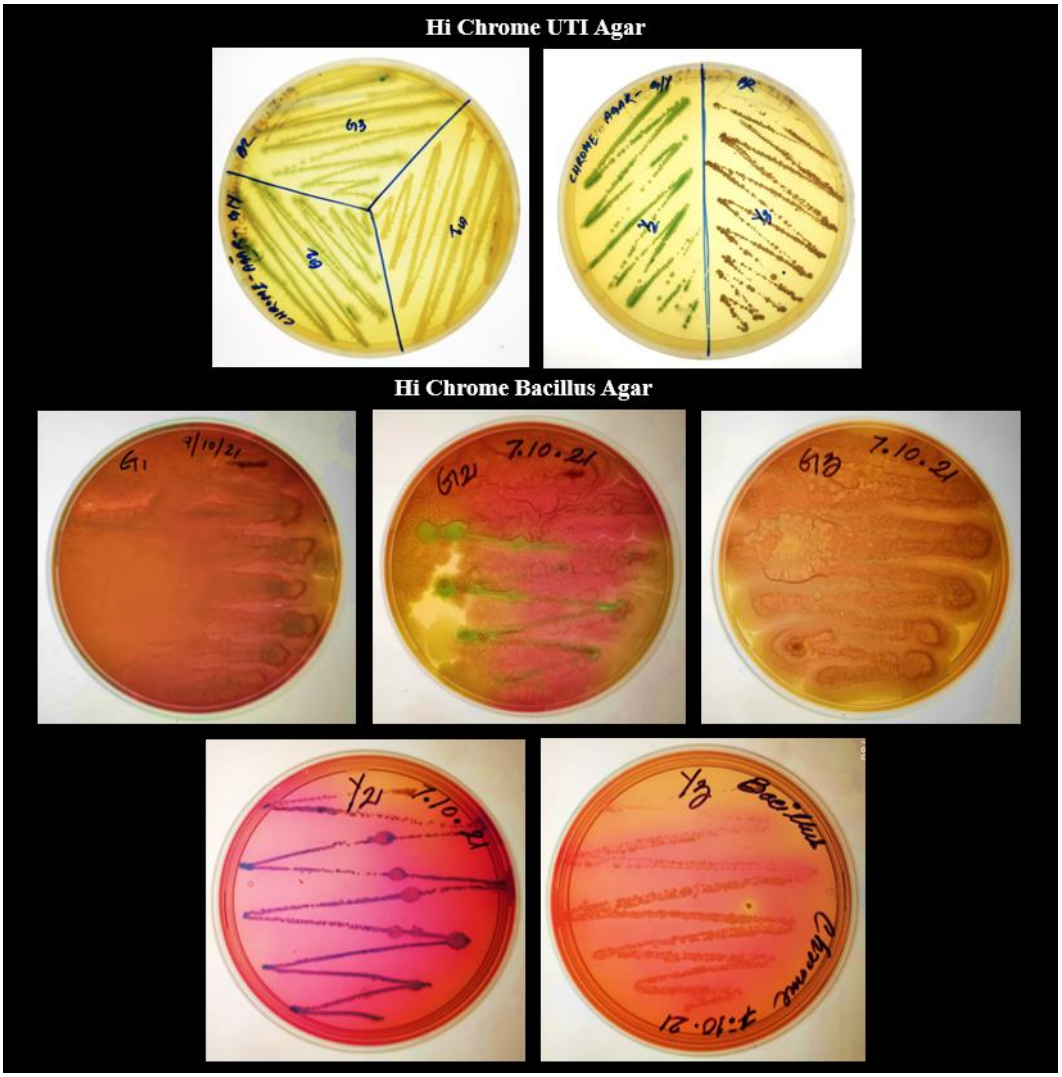


Figure 1.1: The chrome agar plates for the 5 bacterial strains and their colour changes after an incubation period of 24 hours.

Key Findings from Figure 1.1

- The 5 bacterial strains showed peculiar colorations in Hi-chrome UTI agar, due to their abilities to cleave the chromogenic substrates by production of enzymes such as ß-glucosidase and ß-D-galactosidase or both.
- The 5 bacterial strains showed peculiar mixture of colorations in Hi-chrome Bacillus agar plates as well, G1 and G3 showed yellowish mucoid colonies whereas G2 yielded a mixture of green and pink coloration. Y2 showed blue colonies and Y3 pink colonies due to cleavage of various substrates present in the medium.

c. Study of bacterial growth curve:

The bacterial growth curves at normal temperatures were measured only BRAM_Y3 did not show stationary phase, rest 4 showed normal growth curves without much lag phases **(Figure 1.2)**

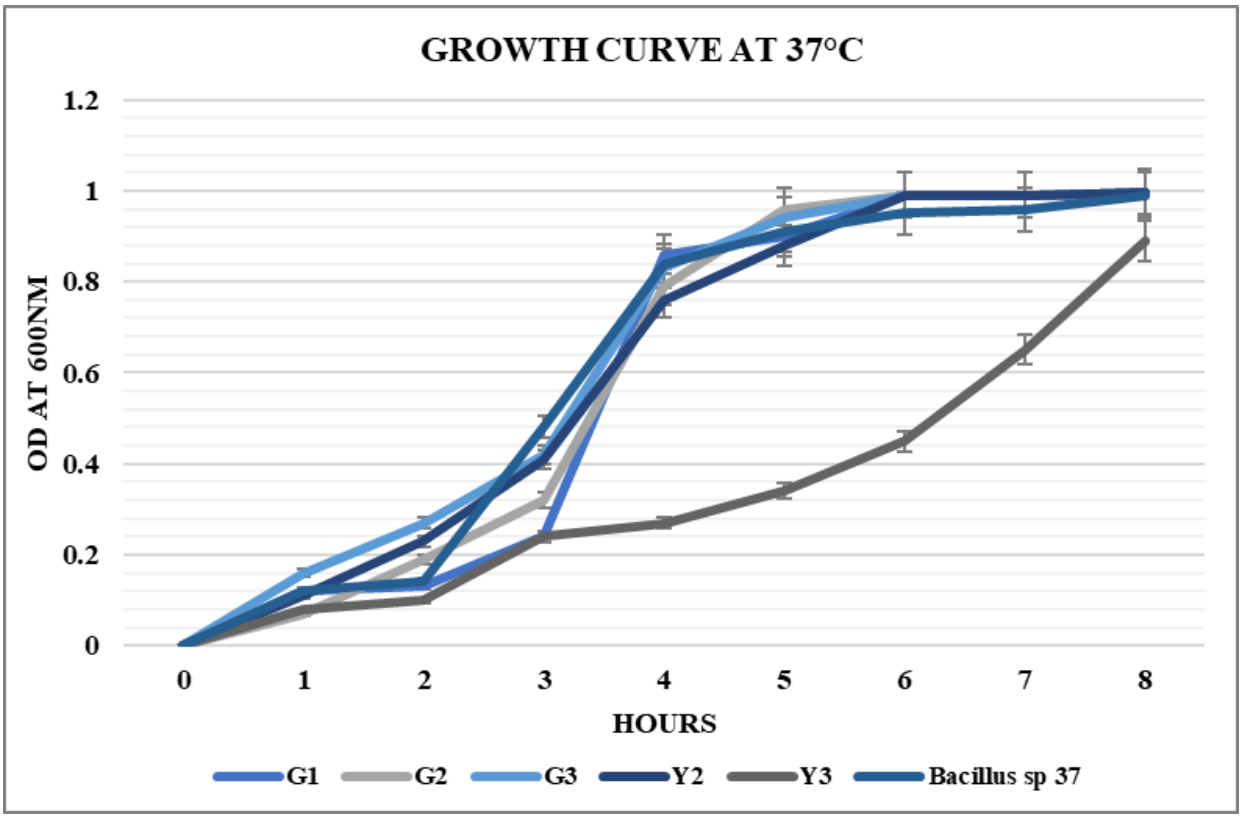


Figure 1.2: The growth curve of the 5 bacterial strains at 37°C.

Key Findings from Figure 1.2

- All 5 bacterial strains have a considerably steep lag phase with a steep log phase followed by a stationary phase after 6 hours except for Y3 which didn't show a stationary phase at all.

d. Antibiotic sensitivity test:

The bacterial strains were subjected to 12 antibiotics to check for their sensitivity towards them. All of them were found to be sensitive to most of the antibiotics and thus do not pose a risk for the environment **(Table 1.2).**

Antibiotics Used	**Zone of Inhibitions (in mm)**					
	Control: *Staphylococcus aureus*.	**G1**	**G2**	**G3**	**Y2**	**Y3**
Oxacillin	22	21	24	21	24	24
Penicillin	24	22	24	18	22	26
Azithromycin	26	26	26	27	26	26
Vancomycin	21	23	25	25	25	26
Teicoplanin	20	24	23	22	24	25
Amoxyclav	26	29	30	25	28	26
Ciprofloxacine	28	30	30	30	29	30
Ceftriaxone	26	28	29	29	30	28
Cefixime	24	14	27	6	26	28
Imipenem	32	31	31	30	29	30
Ampicillin	23	23	23	18	26	25
Cefotaxime	27	30	29	27	27	27

Table 1.3: Antibiotic Sensitivity test of the 5 bacterial strains using the Kirby Bauer Disk Diffusion method.

Key Findings from Table 1.3

- All the 5 bacterial strains were more or less sensitive to all the classes of antibiotics.
- G1 was found to be moderately resistant G3 highly resistant to Cefixime, a cell wall synthesis inhibitor.

e. Scanning electron microscopy of bacterial cells:

The scanning electron microscopy images were in accordance with the Bergey's Manual results, and all the 5 strains were found to be rods with slimy layers of biofilm (**Figure 1.3)**. The assay was carried out CRNN, University of Calcutta. But, unlike most *Bacillus* sp. both the bacterial strains didn't form endospore.

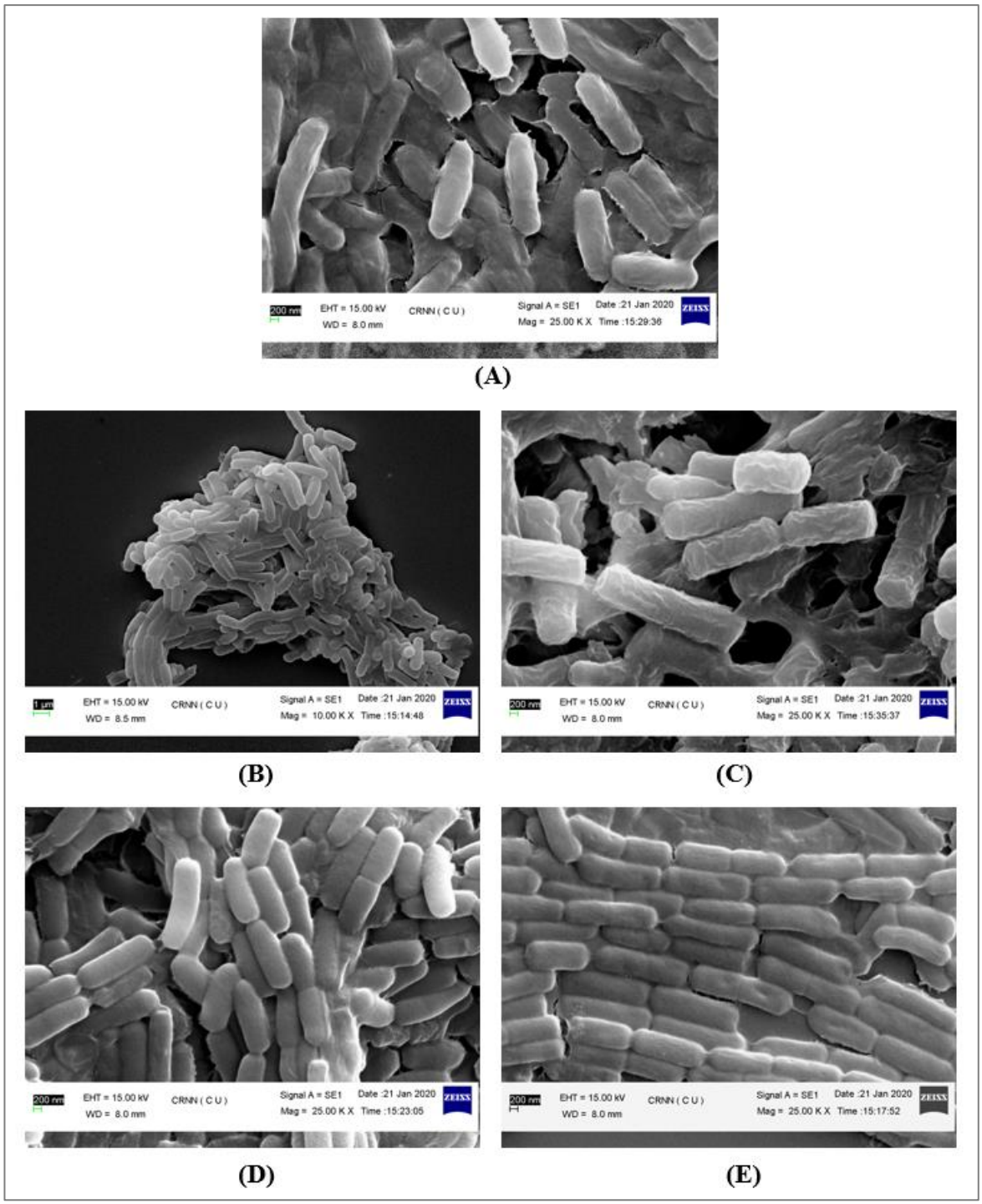


Figure 1.3: Scanning electron Microscopy images of 5 bacterial strains.

Key findings from Figure 1.3

- (A), (B), (C), (D), and (E) are representative images of G1, G2, G3, Y2 and Y3 respectively.
- All 5 bacterial strains were rod shaped and flagellated, clearly visible in (A), (D) and (E) and they seem to be covered in slimy layers, which can be the extracellular polysaccharide.

f. Molecular identification of the bacterial strains using 16s rRNA sequencing:

The 5 bacterial strains were identified with 16s rRNA sequencing. The 5 strains were found to novel strains of *Bacillus*,

Three Novel Strains of *Bacillus subtilis* from waters of Gangotri were reported and submitted to NCBI.

BRAM_G1: Accession Number MW006633

BRAM_G2: Accession Numbers MT998278-MT998280

BRAM_G3: Accession Number MT998617

Two Novel Strains were reported and submitted to NCBI.

Mesobacillus subterraneous BRAM_Y2: Accession Number: MW002419

Brevibacillus parabrevis BRAM_Y3: Accession Number: MW081864

The blast results for the 16srRNA sequences for the 5 bacterial strains and their respective phylogenetic trees have been represented in **Figures 1.4 to 1.8.**

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence	2750	2750	100%	0	100.00%	NR_027552.1
Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence	2745	2745	100%	0	99.93%	NR_112116.2
Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA, partial sequence	2745	2745	100%	0	99.93%	NR_104873.1
Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA, complete sequence	2739	2739	100%	0	99.87%	NR_102783.2
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA, partial sequence	2724	2724	99%	0	100.00%	NR_112629.1
Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	2723	2723	100%	0	99.66%	NR_115063.1
Bacillus vallismortis strain DSM 11031 16S ribosomal RNA, partial sequence	2723	2723	100%	0	99.66%	NR_024696.1
Bacillus mojavensis strain IFO15718 16S ribosomal RNA, partial sequence	2723	2723	100%	0	99.66%	NR_024693.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA, partial sequence	2719	2719	98%	0	100.00%	NR_113265.1
Bacillus nakamurai strain NRRL B-41091 16S ribosomal RNA, partial sequence	2717	2717	100%	0	99.60%	NR_151897.1

Phylogenetic Tree:

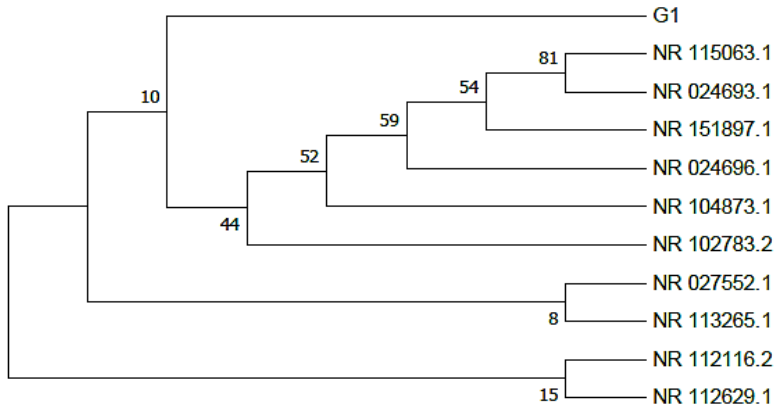


Figure 1.4: The top 10 hits from NCBI Blast results and the phylogenetic tree for BRAM_G1 16s rRNA consensus sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 1.4

- The bacterial strain G1 was confirmed to be *Bacillus subtilis* BRAM_G1 after 16s rRNA sequencing.
- From the phylogenetic tree it can be deduced that, BRAM_G1 strain is closely related to *Bacillus vallismortis*, *Bacillus halotolerans* and *Bacillus mojavensis.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis strain NB-01 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	MF616407.1
Bacillus sp. YBT-003-B-5 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KT223830.1
Paenibacillus polymyxa strain PP17 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	MF001284.1
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: FR1	2728	2728	100%	0	99.60%	AB862127.1
Bacillus vallismortis strain VITSJ-17 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KY003093.1
Bacillus subtilis strain P43 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KU551201.1
Bacillus subtilis strain D43 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KU551149.1
Bacillus subtilis strain IHB B 10201 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KR233775.1
Bacillus tequilensis strain IHBB 9348 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KR085788.1
Bacillus subtilis subsp. inaquosorum strain IHB B 6833 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.60%	KF668463.1

Phylogenetic Tree:

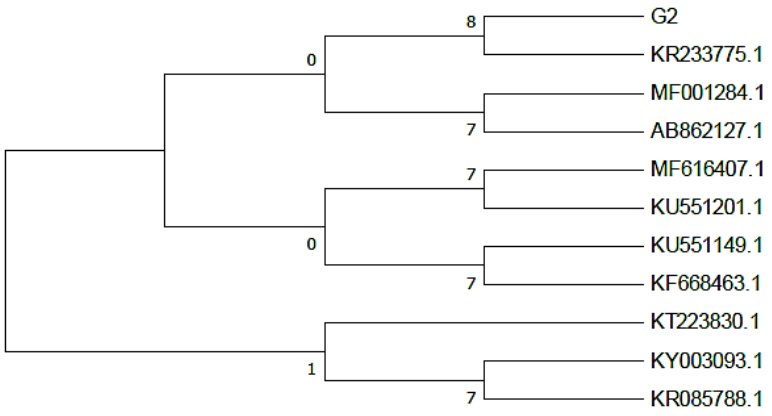


Figure 1.5: The top 10 hits from NCBI Blast results and the phylogenetic tree for BRAM_G2 16srRNA consensus sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 1.5

- The bacterial strain G2 was confirmed to be *Bacillus subtilis* BRAM_G2 after 16s rRNA sequencing.
- From the phylogenetic tree it can be deduced that, BRAM_G1 strain is closely related to *Bacillus vallismortis*, *Bacillus tequilensis* and *Paenibacillus polymyxa.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subtilis strain NB-01 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0	99.47%	MF616407.1
Bacillus sp. YBT-003-B-5 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0	99.47%	KT223830.1
Bacillus vallismortis strain VITSJ-17 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0	99.47%	KY003093.1
Bacillus tequilensis strain IHBB 9348 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0	99.47%	KR085788.1
Bacillus subtilis strain NB-01 16S ribosomal RNA gene, partial sequence	2724	2724	100%	0	99.47%	HM214542.1
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: FR1	2723	2723	99%	0	99.47%	AB862127.1
Bacillus subtilis strain IHB B 10201 16S ribosomal RNA gene, partial sequence	2723	2723	99%	0	99.47%	KR233775.1
Bacillus subtilis subsp. inaquosorum strain IHB B 6833 16S ribosomal RNA gene, partial sequence	2723	2723	99%	0	99.47%	KF668463.1
Bacillus subtilis strain BC18 16S ribosomal RNA gene, partial sequence	2723	2723	99%	0	99.47%	KF636528.1
Bacillus subtilis partial 16S rRNA gene, strain FR1	2723	2723	99%	0	99.47%	HG796154.1

Phylogenetic Tree:

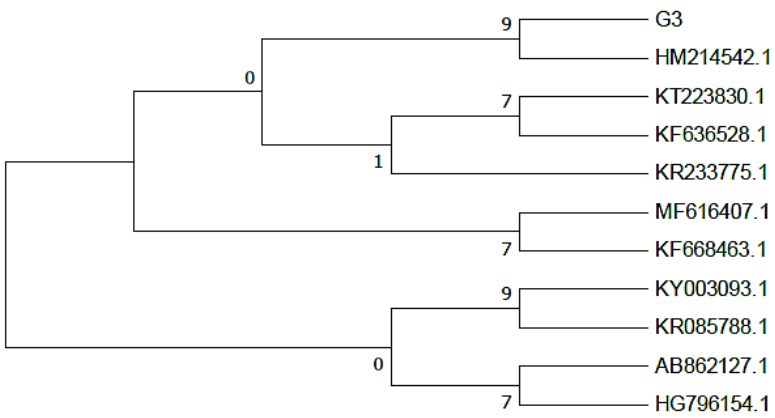


Figure 1.6: The top 10 hits from NCBI Blast results and the phylogenetic tree for BRAM_G3 16srRNA consensus sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 1.6

- The bacterial strain G3 was confirmed to be *Bacillus subtilis* BRAM_G3 after 16s rRNA sequencing.
- From the phylogenetic tree it can be deduced that, BRAM_G1 strain is closely related to *Bacillus vallismortis* and *Bacillus tequilensis.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus subterraneus partial 16S rRNA gene, strain Marseille-P2457	2734	2734	100%	0	99.34%	LT223633.1
Bacillus sp. S22906 16S ribosomal RNA gene, partial sequence	2728	2728	100%	0	99.34%	KF956702.1
Bacillus subterraneus strain A9 16S ribosomal RNA gene, partial sequence	2719	2719	98%	0	99.60%	KY202702.1
Bacillus sp. (in: Bacteria) strain APBSCS98 16S ribosomal RNA gene, partial sequence	2715	2715	100%	0	99.20%	MG733473.1
Bacillus subterraneus strain Lr10/2 16S ribosomal RNA gene, partial sequence	2712	2712	98%	0	99.46%	KJ722433.1
Bacillus subterraneus strain HWG-A11 16S ribosomal RNA gene, partial sequence	2712	2712	100%	0	99.14%	JQ684234.1
Bacillus subterraneus strain A4 16S ribosomal RNA gene, partial sequence	2706	2706	98%	0	99.40%	KY202697.1
Bacillus sp. BWDY-19 16S ribosomal RNA gene, partial sequence	2706	2706	100%	0	99.07%	DQ314538.1
Bacillus subterraneus strain A6 16S ribosomal RNA gene, partial sequence	2704	2704	98%	0	99.40%	KY202699.1
Bacillus boroniphilus strain CM25 16S ribosomal RNA gene, partial sequence	2704	2704	100%	0	99.07%	EU660347.1

Phylogenetic Tree:

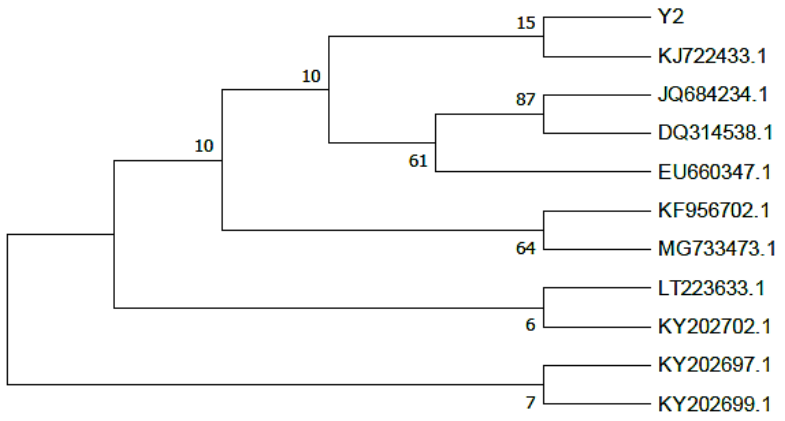


Figure 1.7: The top 10 hits from NCBI Blast results and the phylogenetic tree for BRAM_Y2 16srRNA consensus sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 1.7

- The bacterial strain Y2 was confirmed to be *Bacillus subterraneus* BRAM_Y2 after 16s rRNA sequencing.
- From the phylogenetic tree it can be deduced that, BRAM_Y2 strain is closely related to *Bacillus boroniphilus.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Brevibacillus parabrevis strain IFO 12334 16S ribosomal RNA, partial sequence	2712	2712	100%	0	99.80%	NR_040981.1
Brevibacillus parabrevis strain NBRC 12334 16S ribosomal RNA, partial sequence	2680	2680	98%	0	99.79%	NR_113589.1
Brevibacillus brevis strain DSM 30 16S ribosomal RNA, partial sequence	2627	2627	100%	0	98.65%	NR_112204.1
Brevibacillus agri strain DSM 6348 16S ribosomal RNA, partial sequence	2615	2615	100%	0	98.58%	NR_040983.1
Brevibacillus formosus strain DSM 9885 16S ribosomal RNA, partial sequence	2614	2614	100%	0	98.58%	NR_040979.1
Brevibacillus choshinensis strain DSM 8552 16S ribosomal RNA, partial sequence	2610	2610	100%	0	98.51%	NR_040980.1
Brevibacillus nitrificans strain DA2 16S ribosomal RNA, partial sequence	2603	2603	100%	0	98.44%	NR_112926.1
Brevibacillus brevis strain NBRC 15304 16S ribosomal RNA, partial sequence	2597	2597	98%	0	98.70%	NR_041524.1
Brevibacillus reuszeri strain DSM 9887 16S ribosomal RNA, partial sequence	2597	2597	100%	0	98.38%	NR_040982.1
Brevibacillus antibioticus strain TGS2-1 16S ribosomal RNA, partial sequence	2595	2595	98%	0	98.70%	NR_165725.1

Phylogenetic Tree:

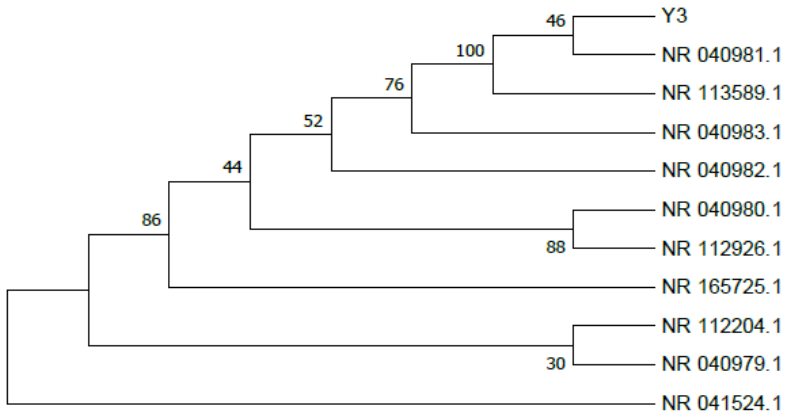


Figure 1.8: The top 10 hits from NCBI Blast results and the phylogenetic tree for BRAM_Y3 16srRNA consensus sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 1.8

- The bacterial strain Y3 was confirmed to be *Brevibacillus parabrevis* BRAM_Y3 after 16s rRNA sequencing.
- From the phylogenetic tree it can be deduced that, BRAM_Y3 strain is closely related to *Brevibacillus brevis, Brevibacillus reuszeri* and *Brevibacillus formosus.*

1.3. Biofilm Formation and subsequent characterization

The 5 bacterial strains were then tested for their biofilm forming capability. After plating on BHI-congo red agar, black colonies were observed for 3 strains of Gangotri which indicated a positive result for biofilm formation, but minute bacterial growth was observed for BRAM_Y2 and BRAM_Y3. Then the Biofilm formation of all 5 strains were quantified using crystal violet method. The observation indicated comparitively higher biofilm fomation in comparison to the control lab strain of *Bacillus.*

The biofilm from the 5 novel bacterial strains were also isolated and their composition were studied with respect to DNA , protein and carbohydrate **(Figure 1.9).**

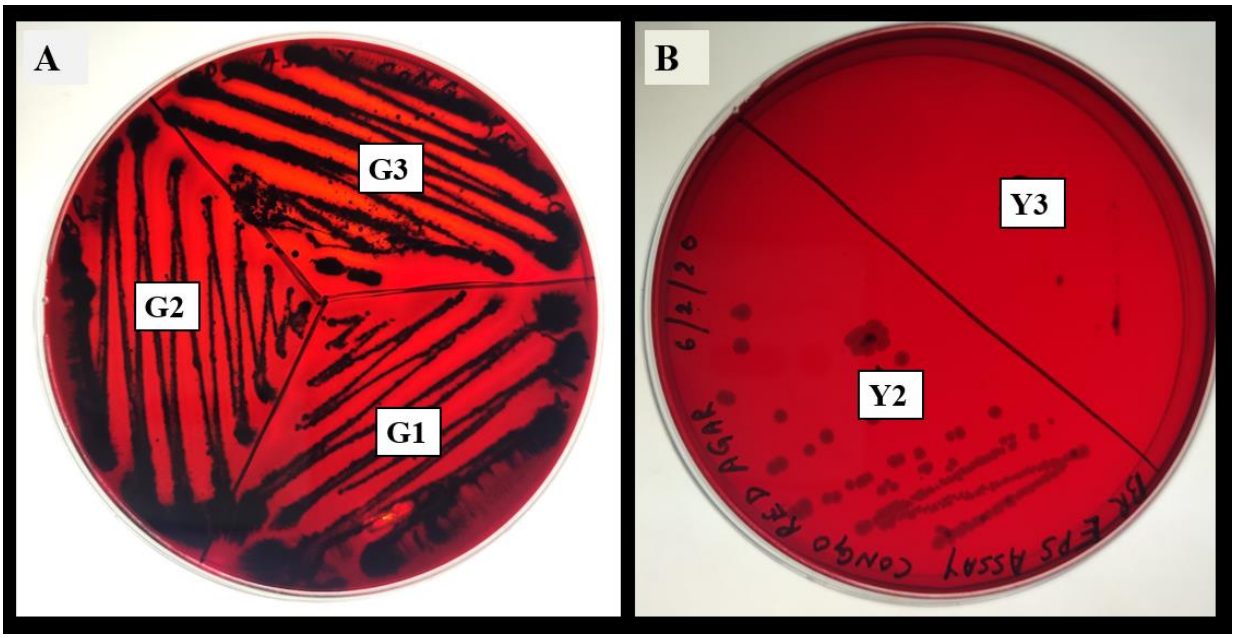
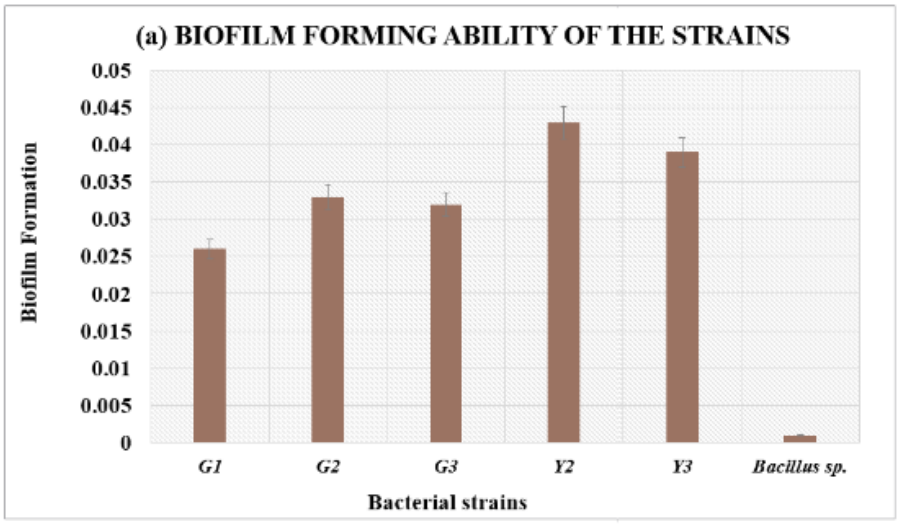


Figure 1.9: The BHI Congo red agar plates for the 5 bacterial strains (A) representing the strains from Gangotri and (B) representing the strains from Yamunotri.

Key findings from Figure 1.9:

- The three strains from Gangotri showed biofilm formation in BHI Congo red agar media plates, by the formation of black mucoid colonies.
- The two strains from Yamunotri did not grow well on the BHI Congo red agar media plates therefore quantification studies were further needed for confirmation of their biofilm formation abilities.



≤ O.D.c / O.D.c < ~ ≤ 2x ODc	Non or weak Biofilm formation
2x O.D.c < ~ ≤ 4x O.D.c	Moderate or medium Biofilm formation.
> 4x O.D.c	Strong Biofilm formation
Optical density cut-off value (O.D.c)	Average O.D. of negative control + 3x standard deviation (S.D.) of the negative control.

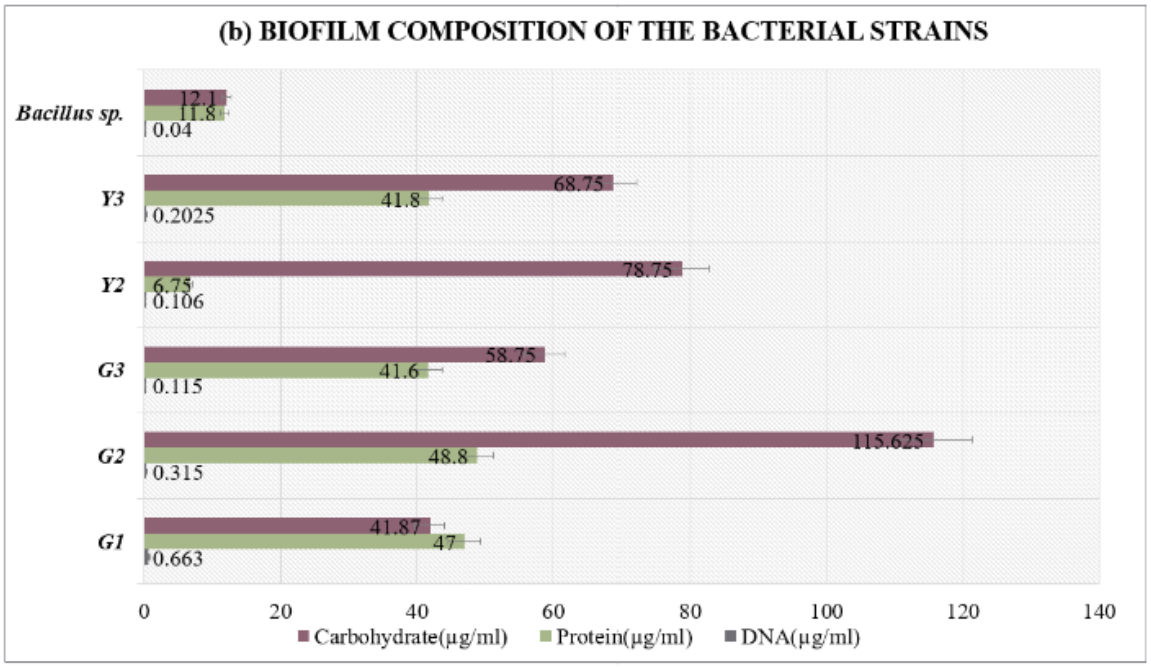


Figure 1.10: Represents the quantified data of the Biofilm formation by the 5 bacterial strains and their composition.

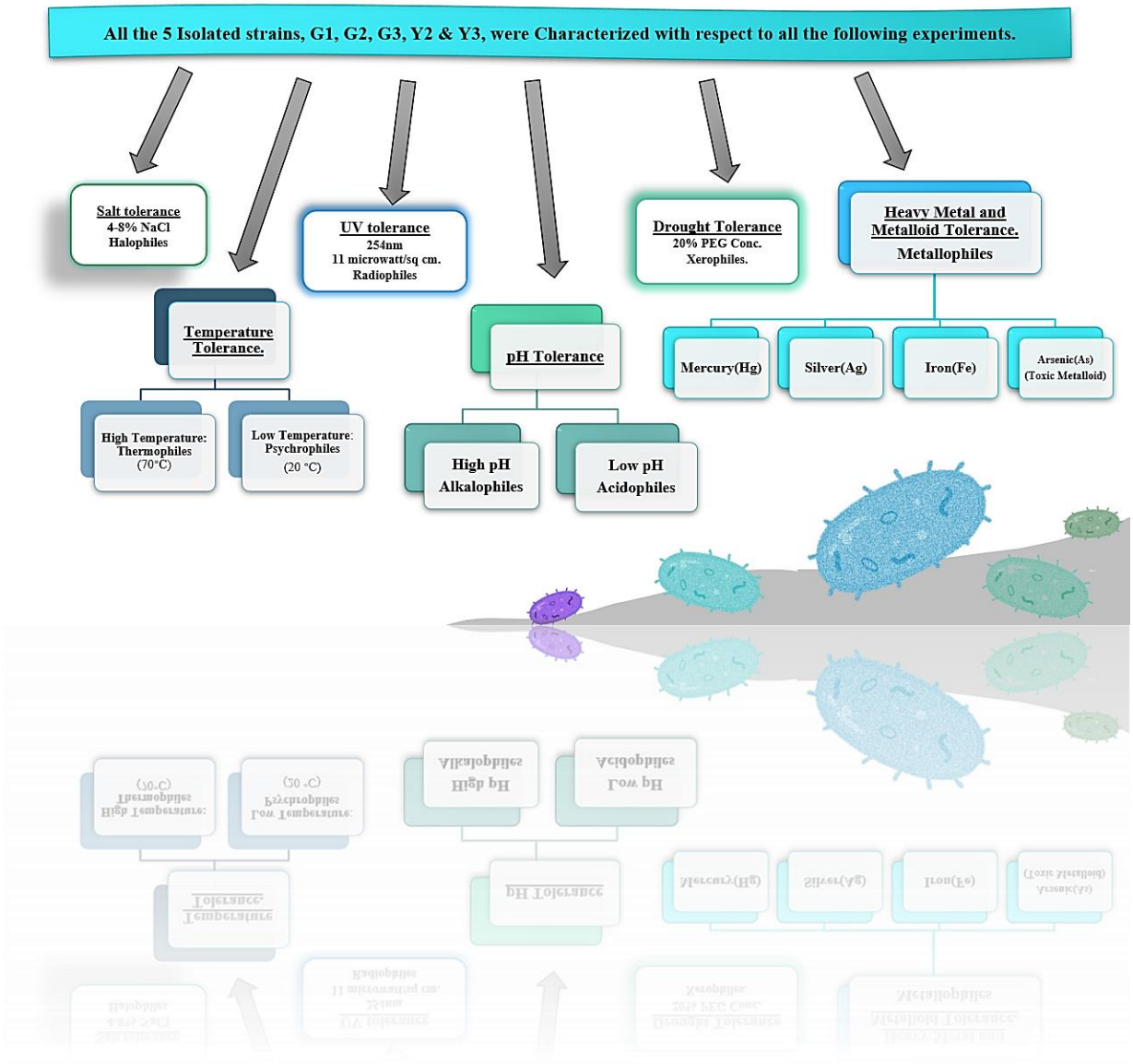
Key Findings from Figure 1.10:

- The 5 bacterial strains produced 20-40 times more biofilm than the control *Bacillus* strain represented in (a).
- The 5 bacterial strains showed humongous increase in the carbohydrate content of the biofilm which is known to be the principal component of EPS, BRAM_G2 being the highest of them all.
- A huge increase was also observed in terms of protein content, highest being BRAM _G2 closely followed by BRAM_G1, BRAM_G3 and BRAM_Y2.
- DNA was found in minimal amounts though much higher than the control strain, with BRAM_G1 being the highest closely followed by BRAM_G2 and BRAM_Y2.

Summarized Findings from Objective 1:

- 5 bacterial strains were isolated from the two water samples of Gangotri and Yamunotri
- All the 5 bacterial strains were characterised according to the Bergeys manual.
- Molecular characterisation with 16srRNA sequencing and microscopic characterization using scanning electron microscopy was carried out for all 5 bacterial strains.
- All the 5 bacterial strains were characterised for their Antibiotic resistance.
- All the 5 bacterial strains were also characterised according to their Biofilm forming abilities.
- All the 5 bacterial strains were studied for their general growth pattern at normal conditions.

OBJECTIVE 2: Profiling of their Polyextremophillic nature



2. *Profiling of their Polyextremophilic nature*

2.1. Single-dimensional stress

The growth of the 5 bacterial strains were then observed at temperatures as high as 70°C and as low as 20°C. The bacterial strains showed diauxic yet luxuriant growth in both the temperatures without any stationary phase. The thermal death was studied at a temperature of time 120 °C and the reduction in the viable cell count was found to be 82-89%. In the cold shock treatment study, an exposure to −20 °C for 48 hours had the viable cell counts reduced by 74-78%. The bacterial cells were also grown in different percentages of salt and PEG 6000 (Drought stress). All 5 bacterial cells showed considerable growth at even 4% salt concentrations and did not grow at 6% and 8% salt concentration. They grew comfortably till 15% PEG concentrations and sparsely at 20% PEG concentrations. The 5 bacterial cells showed impressive growth throughout a wide range of pH 1-12. Though the best growth was observed at pH 10. The bacteria grew luxuriantly in presence of UV rays [the UV Power density: 11microwatt/cm^2, Time of Exposure for the first two hours were 3600s and 7200s. Therefore, UV Dosage Applied on the Bacteria (1hour Exposure) = 39600 µWs/cm^2, (2-hour Exposure) = 79200 µWs/cm^2= 792 J/m^2.] and showed no lag and a steep log phase **(Figure 2.1)**].

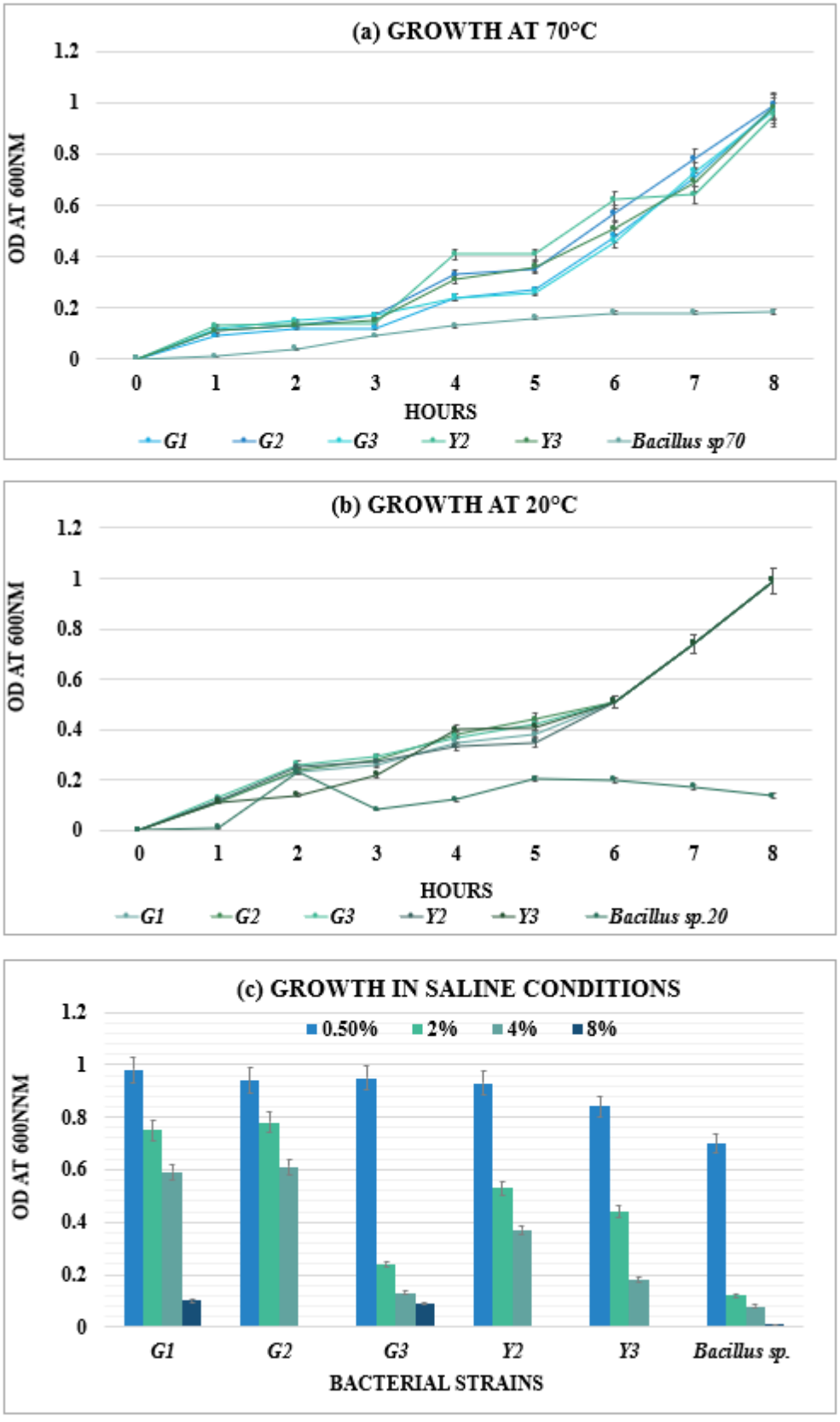


Figure 2.1: The growth of the 5 bacterial strains in different temperatures and salt concentrations.

Key Findings from Figure 2.1

- The 5 bacterial strains showed diauxic growth after the 3rd hour while growing at 70°C but neither of them reached the stationary phase till the 8th hour (Fig 2.1(a)).
- The results provided definitive evidence that the bacterial strains not only exhibited tolerance but also demonstrated the ability to thrive at elevated temperatures, thereby exhibiting the trait of a thermophile.
- The 5 bacterial strains showed diauxic growth after the 4th hour while growing at 20°C, with a considerably long lag phase followed by a steep log phase (Fig 2.1(b)).
- The results provided definitive evidence that the bacterial strains not only exhibited tolerance but also demonstrated the ability to thrive at low temperatures, thereby exhibiting the trait of a psychrophile.
- Amongst the 5 bacterial strains only BRAM_G1 and BRAM_G3 could survive at 8% salt stress, whereas the rest of the strains were tolerant to 4% salt concentrations (Fig 2.1(c)).
- The results provided definitive evidence that the bacterial strains not only exhibited tolerance but also demonstrated the ability to thrive at highly saline conditions, thereby exhibiting the trait of a halophile.

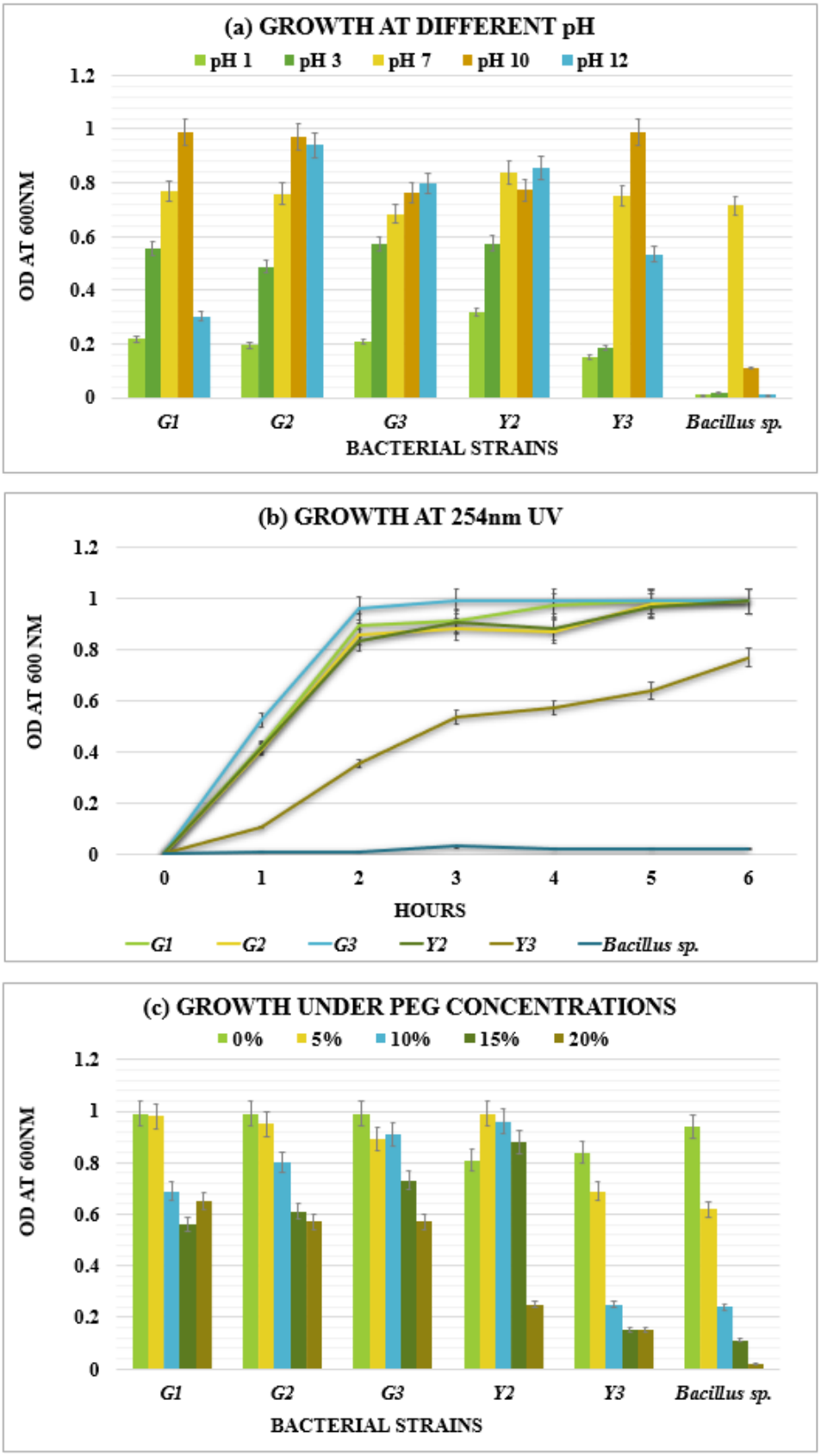


Figure 2.2: The growth of the 5 bacterial strains in different temperatures, pH, UV radiation and Drought conditions.

Key findings from Figure 2.2:

- Amongst the 5 bacterila strains BRAM_G1, BRAM_G2 and BRAM_Y3 showed maximum growth at pH 10 while BRAM_G2 and BRAM_Y2 showed maximum growth at pH 12. (Fig 2.2(a)).
- Therefore the results indicated that the 5 bacterial strains exhibited alakalophilic properties by thriving at a pH range of 10-12 but, also showed considerable tolence and growth at pH as low as, 3 exhibiting the trait of an acidophile as well.
- The bacteria grew luxuriantly in presence of UV rays [the UV Power density: 11microwatt/cm^2, Time of Exposure for the first two hours were 3600s and 7200s. Therefore, UV Dosage Applied on the Bacteria (1hour Exposure) = 39600 µWs/cm^2, (2-hour Exposure) = 79200 µWs/cm^2= 792 J/m^2.] and showed no lag and a steep log phase (Fig 2.2 (b)).
- This hieghtened growth in the presence of such strong radiation could conclusively classify the bacterial strains as radiophiles.
- The bacteria growth was luxuriant till 15% of PEG 6000 concentrations that decreased at 20%. Tolerance to low water activities, are known to be a definitive quality of Xerophiles, and therefore the bacterial strains exhibited xerophilic properties as well (Fig 2.2(c)).

The bacterial cells were also grown in presence of 3 heavy metals, mercury, iron, silver and one toxic metalloid arsenic. They were found tolerant to 10ppm of mercury except BRAM_Y2 which was tolerant to 15ppm of Mercury. The 5 bacterial strains were also tolerant to 200ppm of iron, 300ppm of arsenic and 20ppm of silver. These results proved their extremophilic nature (**Figure 2.2).**

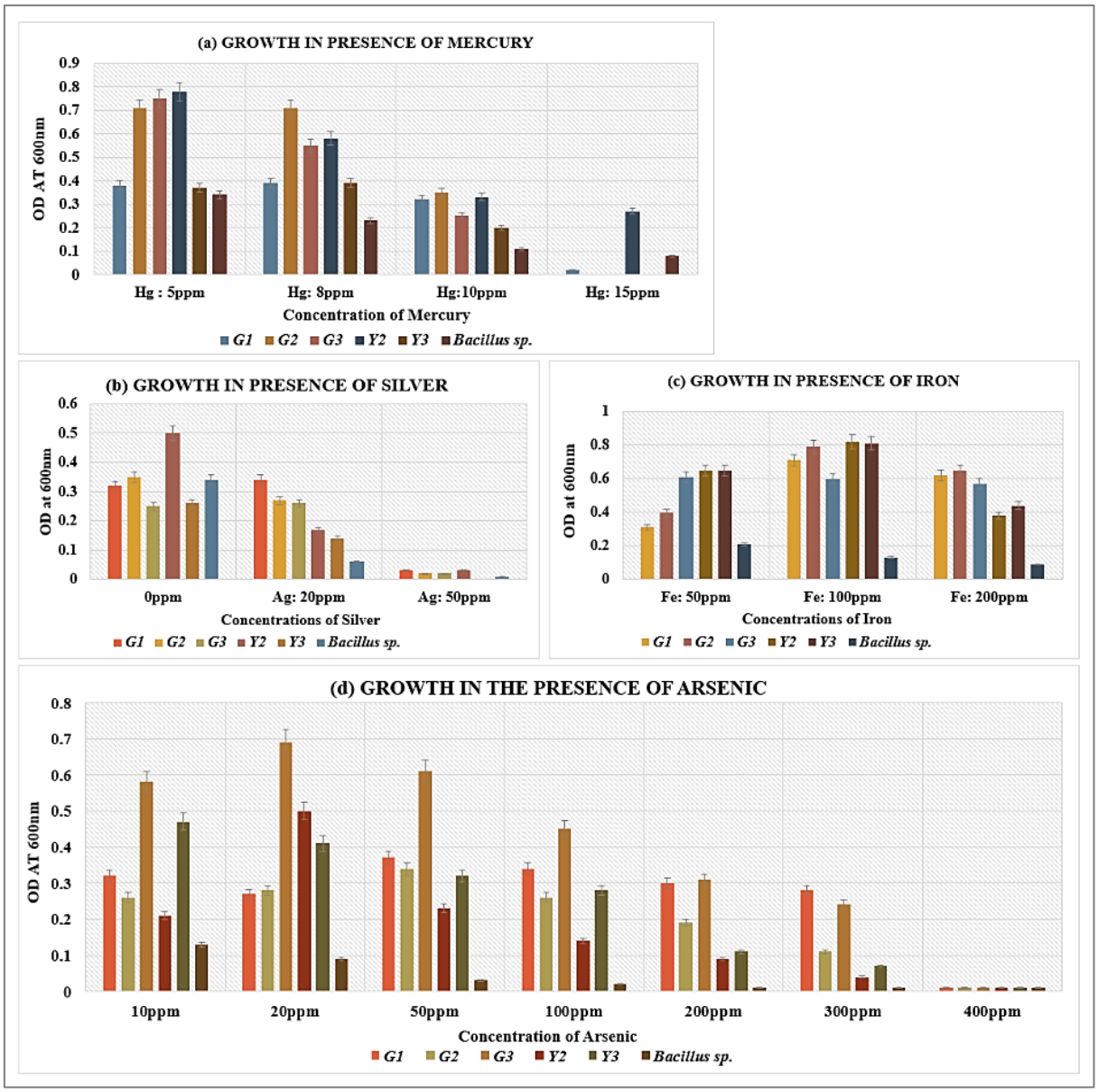


Figure 2.3: The ability of the 5 bacterial strains to tolerate heavy metal concentrations.

Key findings from Figure 2.3:

- The water samples from both Gangotri and Yamunotri did not have heavy metal traces. Therefore 3 heavy metal and one toxic metalloid was selected on the basis of their significance in environment and water contamination.
- Mercury was selected as one of the metals for heavy metal tolerance study because, when discharged into the environment, tends to build in sedimentary deposits in bodies of water, where it undergoes conversion into the poisonous compound methylmercury. This substance then enters the food chain. The presence of mercury in the environment poses a substantial challenge to both human health and the ecosystem due to the ease with which

methylmercury can enter the bloodstream and impact the functioning of the brain. All 5 bacterial strains were tolerant to 10ppm of Mercury, except for BRAM_G1 and BRAM_Y2 who could tolerate 15ppm of Mercury (Fig 2.3(a)).

- Silver was selected because the overutilization of silver nanoparticles has inadvertent repercussions on the environment, including toxicity, which can impair beneficial bacteria, marine species, and soil organisms, as well as pose a threat to human health. The bacterial strains could tolerate up to 20ppm of silver but their growth was severely diminished at 50ppm (Fig 2.3(b)).

- Iron has been found to reduce light transmission in water and thus decrease photosynthetic activity in marine plants. It is beneficial to human health till its concentrations in near about 0.3ppm in water, but excess of iron can cause several physical complications such as hemochromatosis, and same organs such as liver, pancreas and even the heart. The 5 bacterial strains were tolerant to high concentrations of iron (200ppm) and showed luxuriant growth in the same (Fig 2.3(b)).

- Arsenic toxicity can result in many health consequences, such as dermatological lesions, skin, lung, bladder, and kidney malignancies, cardiovascular disorders, and neurological impairments. Arsenic contamination of drinking water is a significant global public health issue in numerous regions. The specific outcomes vary based on the particular species and duration of exposure. The consequences encompass mortality, stunted development, impaired photosynthesis and reproduction, as well as alterations in behaviour. Arsenic-contaminated environments exhibit low species diversity and population sizes within each species. The 5 bacterial strains were tolerant to humongous levels of Arsenic as high as 300ppm BRAM_G1 and BRAM_G3 showing the maximum growth at that point (Fig 2.3(d)).

- The provisions would have permitted the selection of a greater number of metals, but it was limited to four for the sake of better data management. This restriction was intended to clearly demonstrate the metallophilic nature of the five bacterial strains without unnecessarily elaborating on a similar set of experiments while varying a single parameter.

2.2. Two-dimensional and Three-dimensional stress

To prove the Polyextremophilic nature of the 5 bacterial strains they were subjected to 16 combinations of two-dimensional stresses **(Figure 2.4)**:

17. Temperature 70°C +20% PEG

18. Temperature 70°C +4% NaCl

19. Temperature 70°C +As 100

20. Temperature 70°C +pH4

21. Temperature 70°C +pH10

22. pH4+4% NaCl

23. pH10+4% NaCl

24. UV+20% PEG

25. UV+ As 100ppm

26. UV+ Fe 200ppm

27. UV+pH4

28. UV+pH10

29. Temperature 20°C + Fe 200ppm

30. T20°C +4% NaCl

31. Temperature 20°C +pH4

32. Temperature 20°C +pH10

The bacterial strains were also subjected to 9 three-dimensional stress combinations **(Figure 2.5)** along with no stress control and their growth was measured:

10. Temperature 20°C+20% PEG+4% NaCl

11. Temperature 20°C +pH10+4% NaCl

12. Temperature 20°C +pH10+ Fe200ppm

13. pH10+4% NaCl+20% PEG

14. Temperature 70°C +pH10+As100ppm

15. Temperature 70°C +pH10+4%NaCl

16. Temperature 70°C +4% NaCl+20% PEG

17. UV+4% NaCl+20% PEG

18. UV+ Temperature 20°C +20% PEG

All these combinations were selected on the basis of In addition, as in previous experiments it was found that the 5 bacterial strains produced exceedingly high biofilm (almost 20 to 40 times) in comparison to normal lab strain of Bacillus, it was hypothesised that the biofilm might have a significant role in protecting the bacterial strains from the stressed environmental conditions, and thus the increase in biofilm formation when subjected to the 1D, 2D and 3D stresses were also recorded **(Figure 2.4)** to check for some correlation between biofilm formation and extreme stress tolerance. It was found that, in all the setups the bacteria not only grew luxuriantly, they all produced immense amounts of biofilm in presence of stress, with highest biofilm formations in 3-Dimensional stress **(Figure 2.6)** followed by 2 Dimensional **(Figure 2.5)** and 1 Dimensional **(Figure 2.4)**. Plate count was done after each experiment concerning stress so as to keep a record of the bacterial viable cell count.

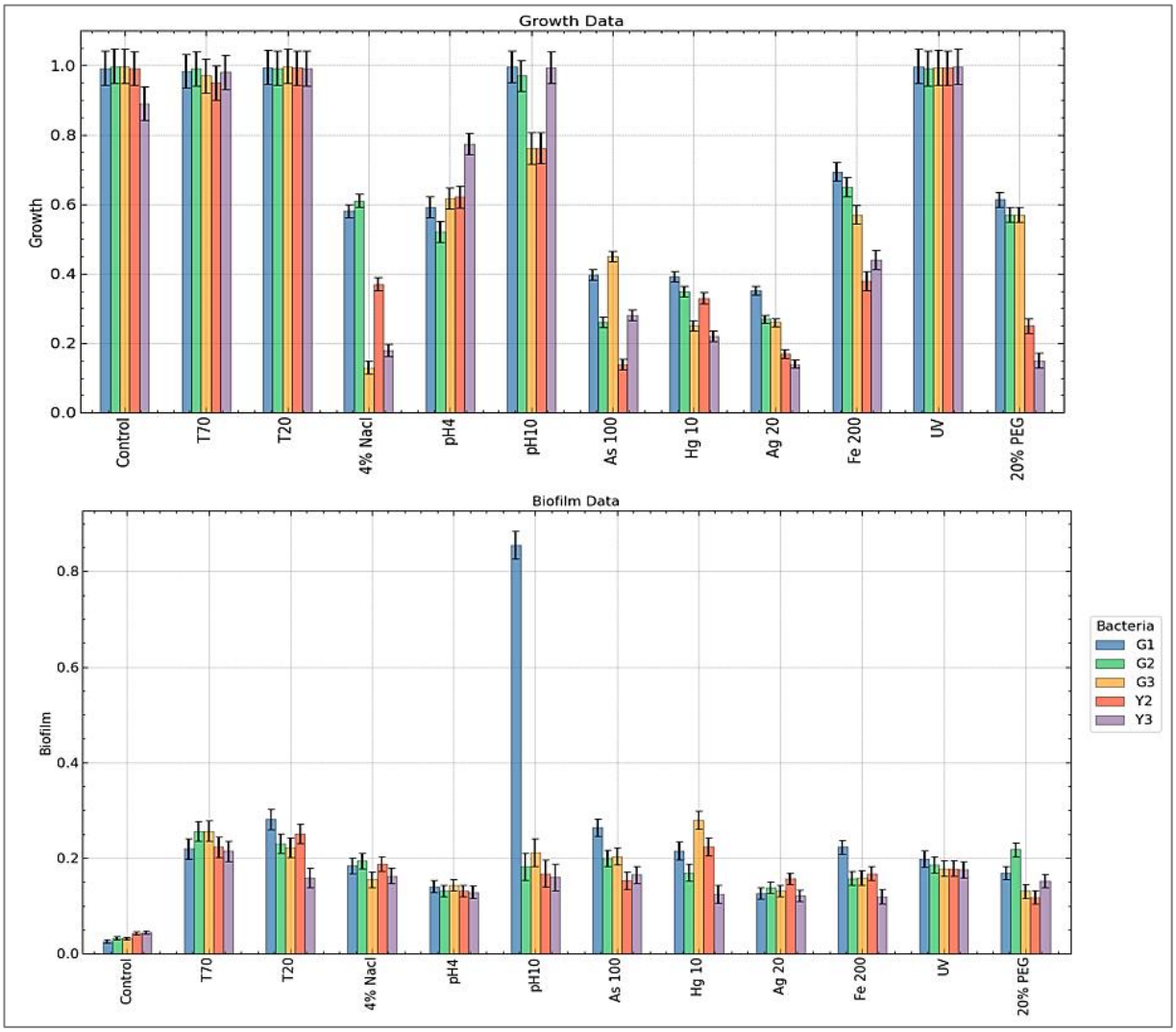


Figure 2.4: The graphical representation of the growth of 5 bacterial strains in Single dimensional stresses with no-stress control and their corresponding increase in Biofilm formation.

Key findings from Figure 2.4:

- Single parameters were selected from the wide range of 1D stress where the bacterial strains exhibited maximum growth and the biofilm formation in the presence of stress was correspondingly recorded.
- It could be inferred from this experiment was that the 5 bacterial strains were extremophiles, as they grew luxuriantly in stresses condition.

- There was also a significant increase in biofilm formation in presence of stress in comparison to no stress control, from which it could be inferred from that biofilm might have played a key role in protecting the bacterial strains against the abiotic stresses.

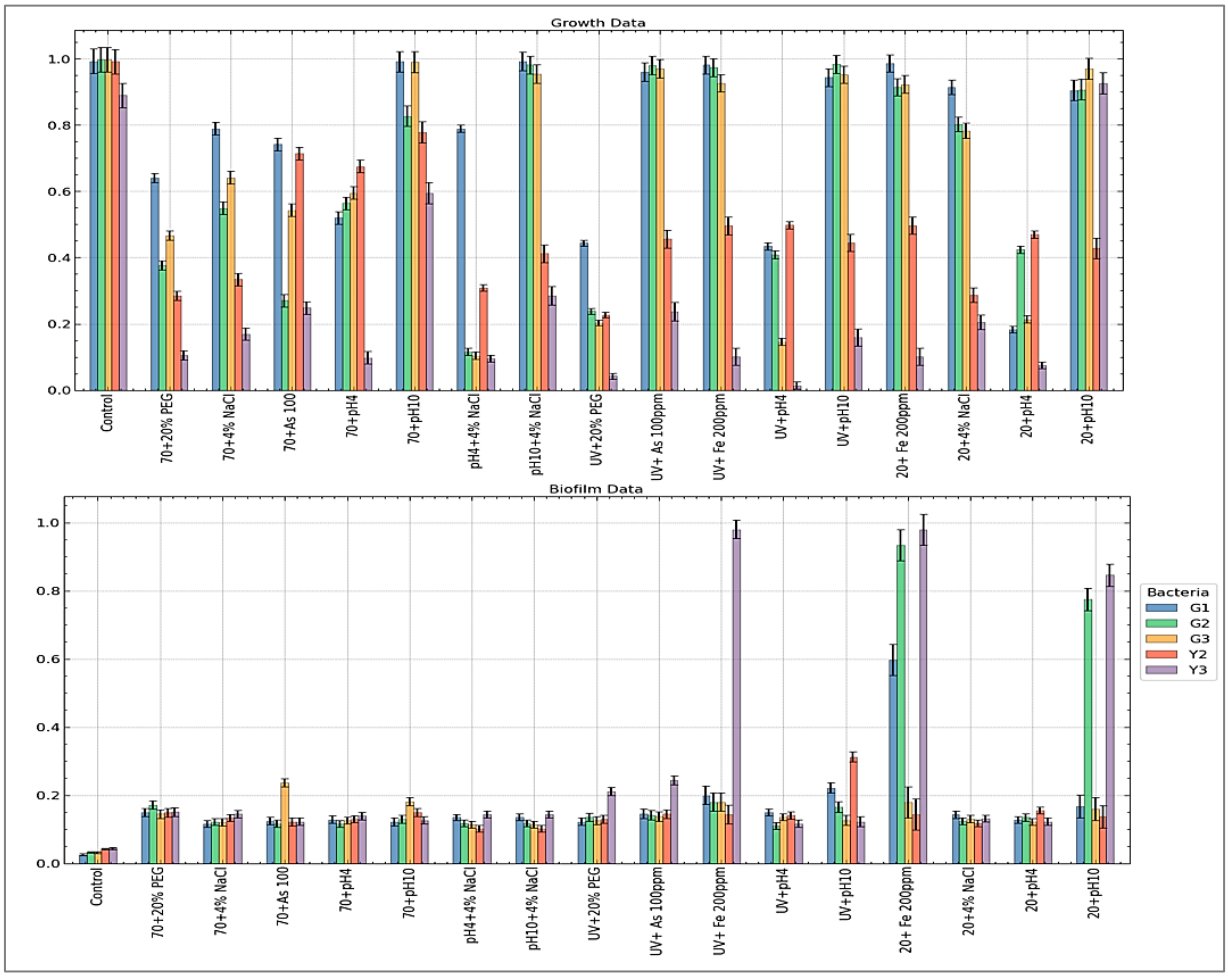


Figure 2.5: The graphical representation of the growth of 5 bacterial strains in 16 Two-dimensional stresses with no-stress control and their corresponding increase in Biofilm formation.

Key findings from Figure 2.5:

- Two-dimensional stress parameters were selected on the basis of probability of existence of the stress combinations in natural systems and the feasibility of the creation of the simulated stress combination in-vitro laboratory setups. The biofilm formation in the presence of these 16 2D-stress combinations was correspondingly recorded.

- It could be inferred from this experiment was that the 5 bacterial strains were polyextremophiles, as they grew luxuriantly in 2D-stress conditions.

- There was also a significant increase in biofilm formation in presence of two-dimensional stresses in comparison to no stress control, from which it could be inferred from that biofilm might have played a key role in protecting the bacterial strains against the abiotic stress combinations.

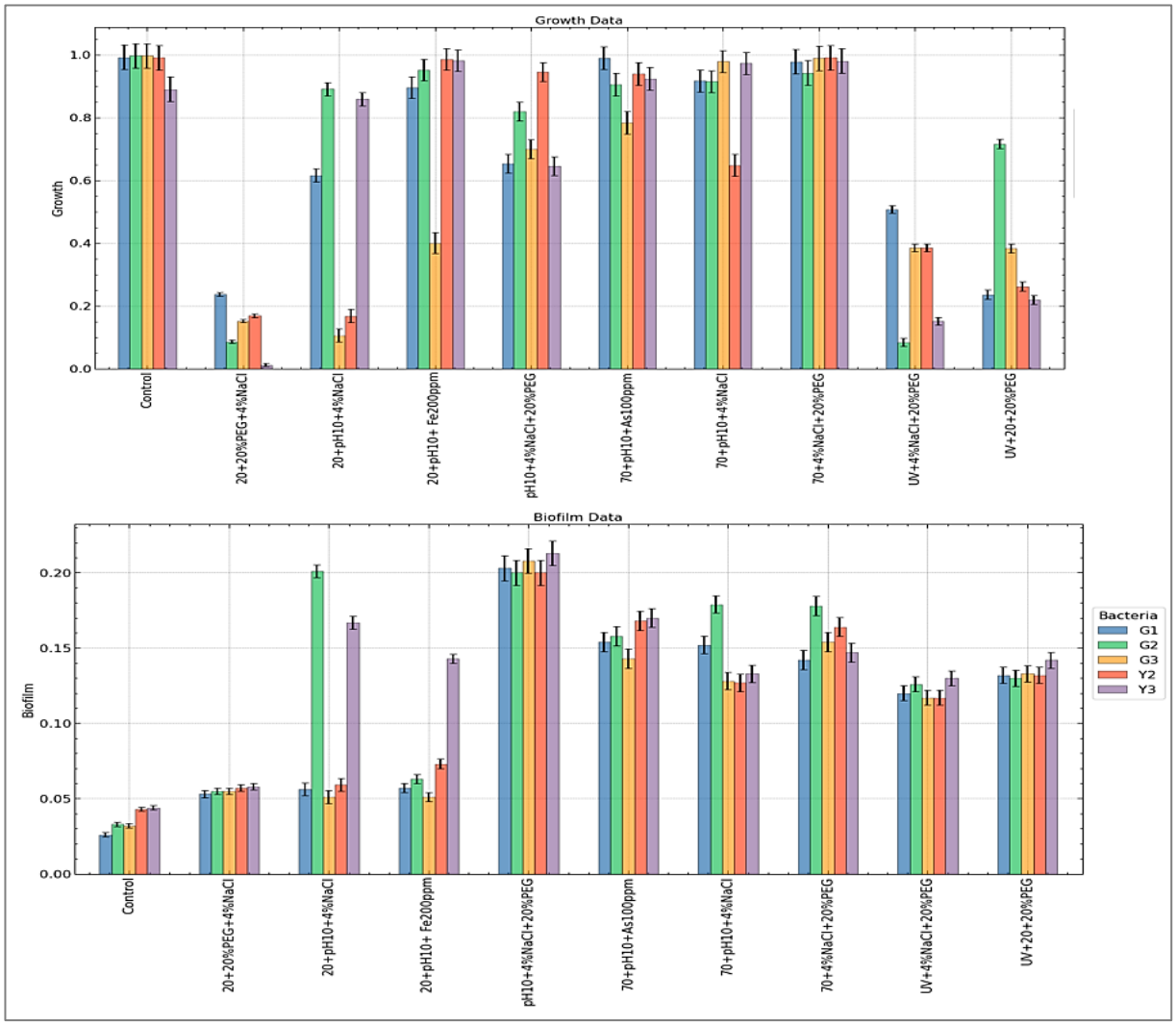


Figure 2.6: The graphical representation of the growth of 5 bacterial strains in 9 Three-dimensional stresses with no-stress control and their corresponding increase in Biofilm formation.

Key findings from figure 2.6:

- Three-dimensional stress parameters were selected on the basis of probability of existence of the stress combinations in natural systems and the feasibility of the creation of the simulated stress combination in-vitro laboratory setups. The biofilm formation in the presence of these nine 3D-stress combinations was correspondingly recorded.
- It could be inferred from this experiment was that the 5 bacterial strains were most certainly polyextremophiles, as they grew luxuriantly in 3D-stress conditions.
- There was also a significant increase in biofilm formation in presence of three-dimensional stresses in comparison to no stress control, from which it could be inferred from that biofilm might have played a key role in protecting the bacterial strains against the abiotic stress combinations.

2.3. Study of the changes in composition of bacterial biofilm with 3D stress using Fourier transform Infrared Spectroscopy (FTIR).

After observing increased biofilm formation after stress application, the biofilm was again isolated from "no stress" control and 3D stress combination (Temperature70 °C + 4% NaCl+20% PEG) that showed best growth, and FTIR study was carried out. The spectra showed presence of lipids, proteins, amides I and II (proteins) and some metal-oxygen bonds, but the M-O peaks differed in all the 5 bacterial strains. The main difference that was observed was in the abundance of the compounds (lipids, proteins etc.). The application of stress increased the abundance of lipids, proteins, amides I and II and tetrahedral metal-oxygen compounds in all 5 bacterial strains **(Figure 2.6).**

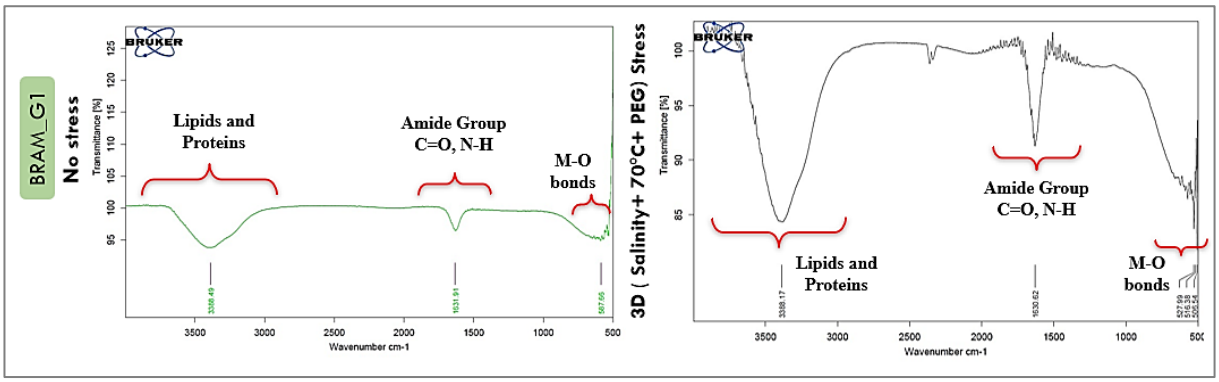


Figure 2.7: FTIR studies of the BRAM_G1 biofilm in presence and absence of 3-Dimensional stress (4% Salinity+ 70˚C+ 20% PEG).

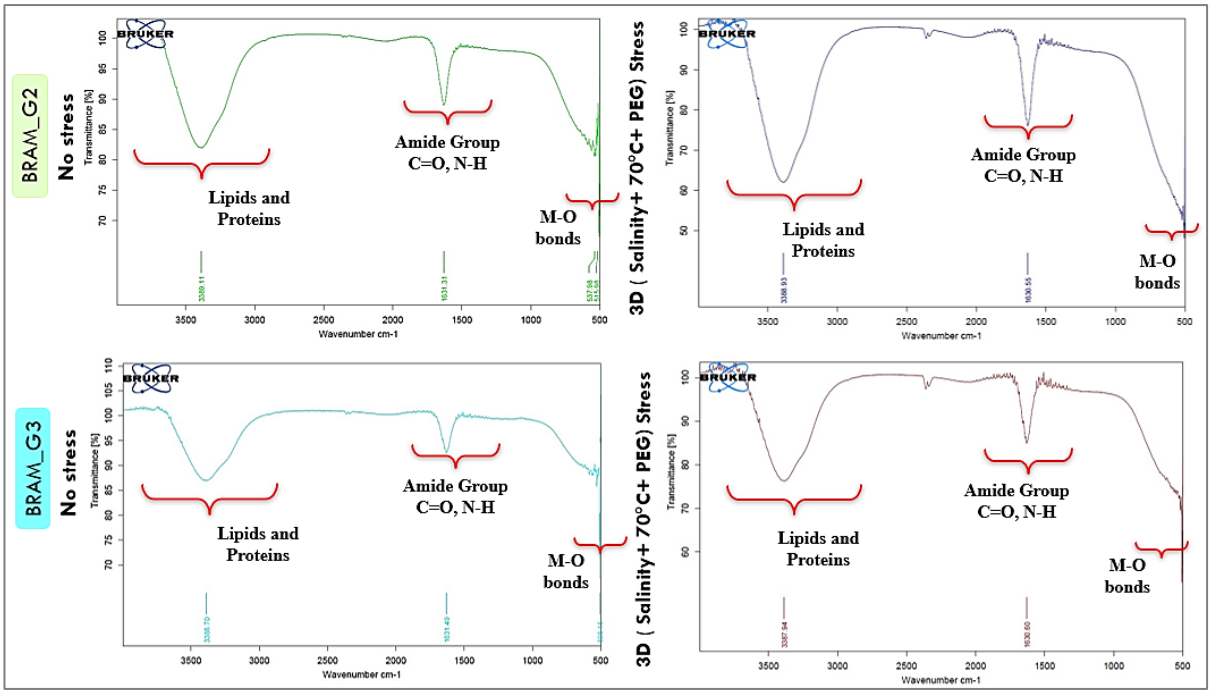


Figure 2.8: FTIR studies of the BRAM_G2 and BRAM_G3 biofilms in presence and absence of 3-Dimensional stress (4% Salinity+ 70˚C+ 20% PEG).

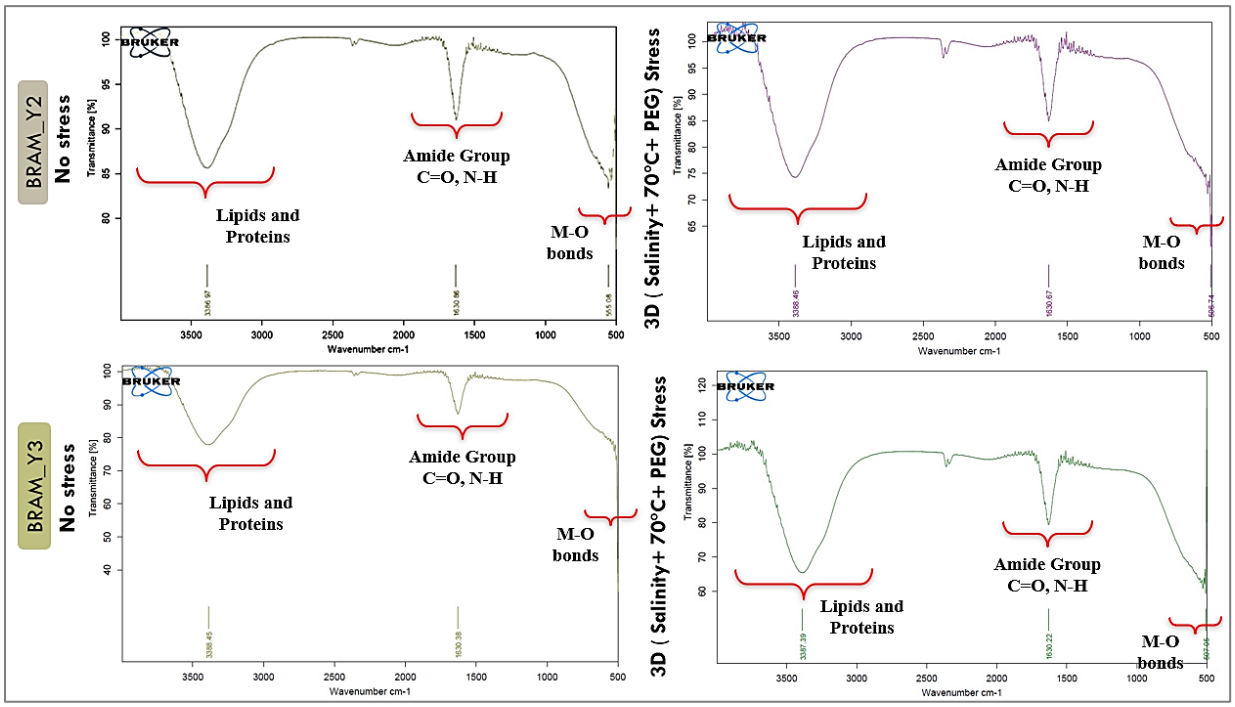


Figure 2.9: FTIR studies of the BRAM_Y2 and BRAM_Y3 biofilms in presence and absence of 3-Dimensional stress (4% Salinity+ 70˚C+ 20% PEG).

Key findings from Figure 2.7-2.9 :

- The fourier transform infrared spectral scans in the images represent the different molecular bonds corresponding to classes of compounds such as lipids, proteins, amino acids, molecular oxygen bonds and so on.
- The decrease in transmittance corresponds to increase in absorbance and therefore increase in the concentrations of certain classes of compounds.
- The fourier transform infrared spectral scans of the extracted biofilms of the 5 bacterial strains in presence and absence of a three dimensional stress exhibit distinct differences with respect to peak hieghts (concentrations) and peak shifts.
- The three-dimensional stress combination used in this experiemnt and the rest of the experiment here onwards was selected on the basis of the overall maximum growth observed by all 5 bacterial strains in the aforesaid combination of Temperature 70 °C + 4% NaCl+ 20% PEG.
- Three categories of peaks were mainly observed in the FTIR scans of the biofilm extracts, 3000-3500cm^{-1}that corresponds to lipids and proteins, 1500-1700 cm^{-1}that corresponds to amide bonds (I and II) and C=C conjugates and lastly 500-600 cm^{-1} which can be molecular oxygen or carbon and halogen bonds (tetrahedral).
- The main observable change that occurred in 3D-stress and no stress conditions was that there was a huge increase in the peak heights and therefore the corresponding concentrations of the various compounds.
- The other change that could be observed was the slight shifts in the peaks though they were present in range of the same class of compounds which might correspond to change in forms of the compounds in stressed conditions.
- These observations clearly indicate a crucial role played by bacterial biofilms in protecting them in times of abiotic stress conditions.

2.4. Whole Genome Sequencing of the 5 Strains

The 5 bacterial strains proved to be Polyextremophiles, that use biofilm as their shields against abiotic stress. But, their 16s rRNA identification revealed BRAM_G1, BRAM_G2, BRAM_G3 as *Bacillus subtilis* which is a well-accepted mesophile. Therefore, to confirm their identities Whole Genome sequencing was carried out for all 5 bacterial strains. BRAM_G2 was found to be *Bacillus thuringiensis*, BRAM_G3 was found to be *Bacillus tequilensis*, BRAM_Y2 was found to be *Mesobacillus thioparans* whereas BRAM_G1 remained *Bacillus subtilis* and, BRAM_Y3 *Brevibacillus parabrevis.* The Genome assembly was done using RAPT pipeline from NCBI and the circular representation was formed using the Proksee server (**Figure 2.7).**

The sequences were submitted to the NCBI server under the BioProject (PRJNA1018306): Polyextremophiles WGS from Gangotri and Yamunotri with the 5 Biosample numbers and the Taxonomic ID as follows:

BioProject (PRJNA1018306): Polyextremophiles WGS from Gangotri and Yamunotri

i. **Biosample: SAMN37432400: BRAM_G1(TaxID: 1423); SRA (Sequence Read Archive): SRR26084659**
ii. **Biosample: SAMN37432401: BRAM_G2(TaxID: 1428); SRA (Sequence Read Archive): SRR26084660**
iii. **Biosample: SAMN37432402: BRAM_G3(TaxID: 227866); SRA (Sequence Read Archive): SRR26084661**
iv. **Biosample: SAMN37432403: BRAM_Y2(TaxID: 370439); SRA (Sequence Read Archive): SRR26084657**
v. **Biosample: SAMN37432404; BRAM_Y3(TaxID: 54914); SRA (Sequence Read Archive): SRR26084658 .**

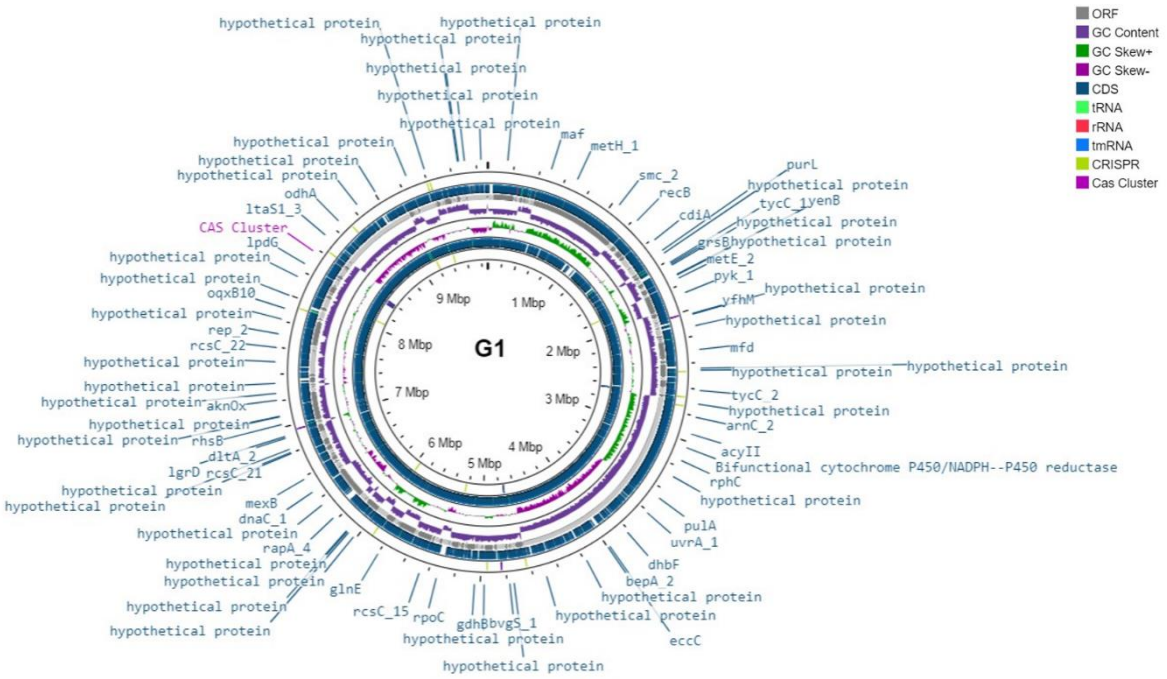


Figure 2.10: The circular representation of the Whole Genome sequence of BRAM_G1

Key findings from Figure 2.10:

- The Wholegenome sequencing of the bacterium was done to reveal their true identities, as it exhibited unique properties that are not innately found in species like *Bacillus subtilis.*
- The Whole genome sequencing results confirmed BRAM_G1 to be a unique strain of *Bacillus subtilis* with highly interesting gene clusters that could be a point of study in future.
- The circular respresentaion of the bacterial whole genome sequence was generataed using Prokka and Proksee servers.

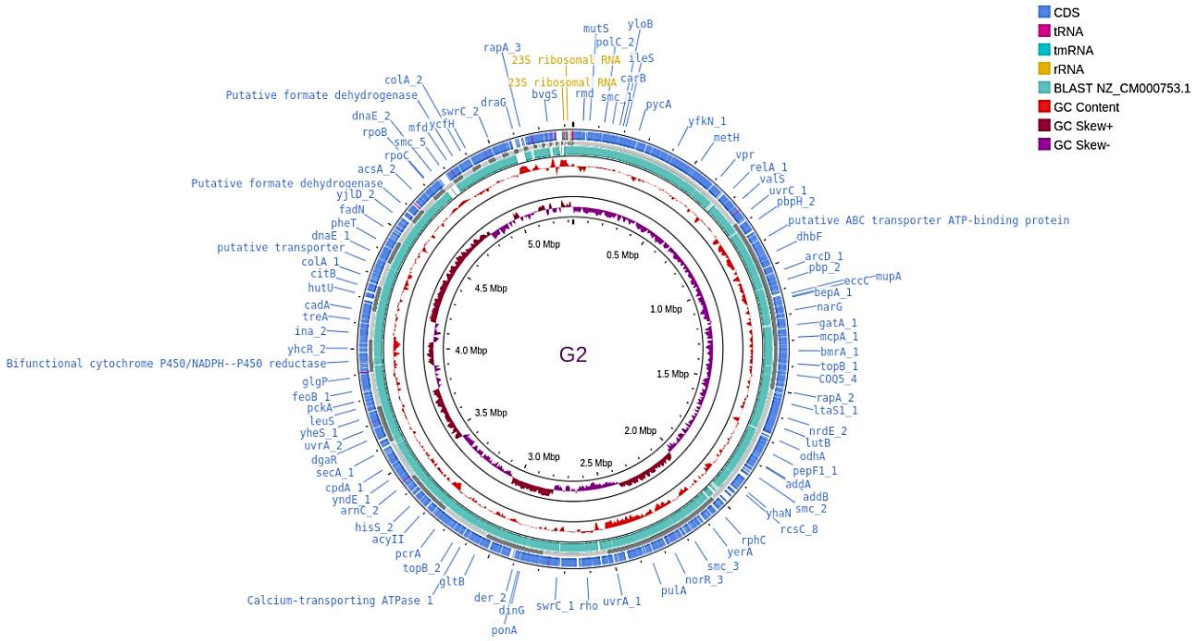


Figure 2.11: The circular representation of the Whole Genome sequence of BRAM_G2

<u>Key findings from Figure 2.11:</u>

- The Wholegenome sequencing of the bacterium was done to reveal their true identities, as it exhibited unique properties that are not innately found in species like *Bacillus subtilis.*
- The Whole genome sequencing results confirmed BRAM_G2 to be a unique strain of *Bacillus thuringiensis* with highly interesting gene clusters that could be a point of study in future.
- The circular respresentaion of the bacterial whole genome sequence was generataed using Prokka and Proksee servers.

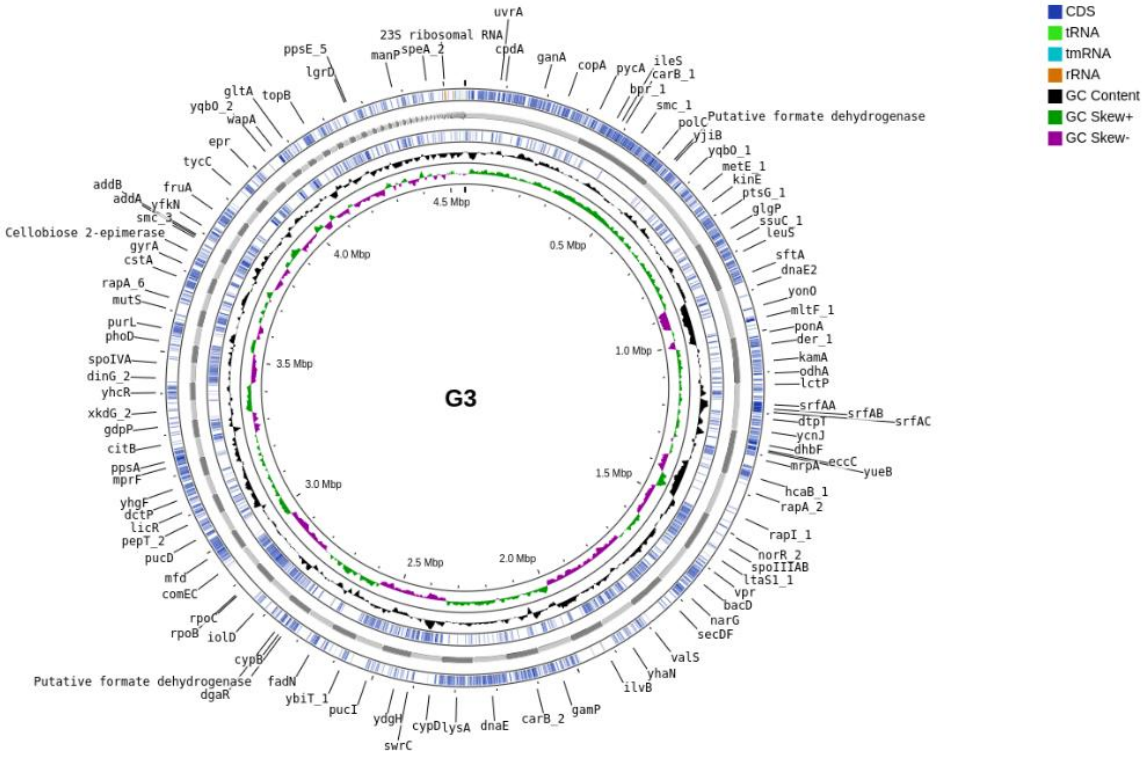


Figure 2.12: The circular representation of the Whole Genome sequence of BRAM_G3

Key findings from Figure 2.12:

- The Wholegenome sequencing of the bacterium was done to reveal their true identities, as it exhibited unique properties that are not innately found in species like *Bacillus subtilis.*
- The Whole genome sequencing results confirmed BRAM_G3 to be a unique strain of *Bacillus tequilensis* with highly interesting gene clusters that could be a point of study in future.
- The circular respresentaion of the bacterial whole genome sequence was generataed using Prokka and Proksee servers.

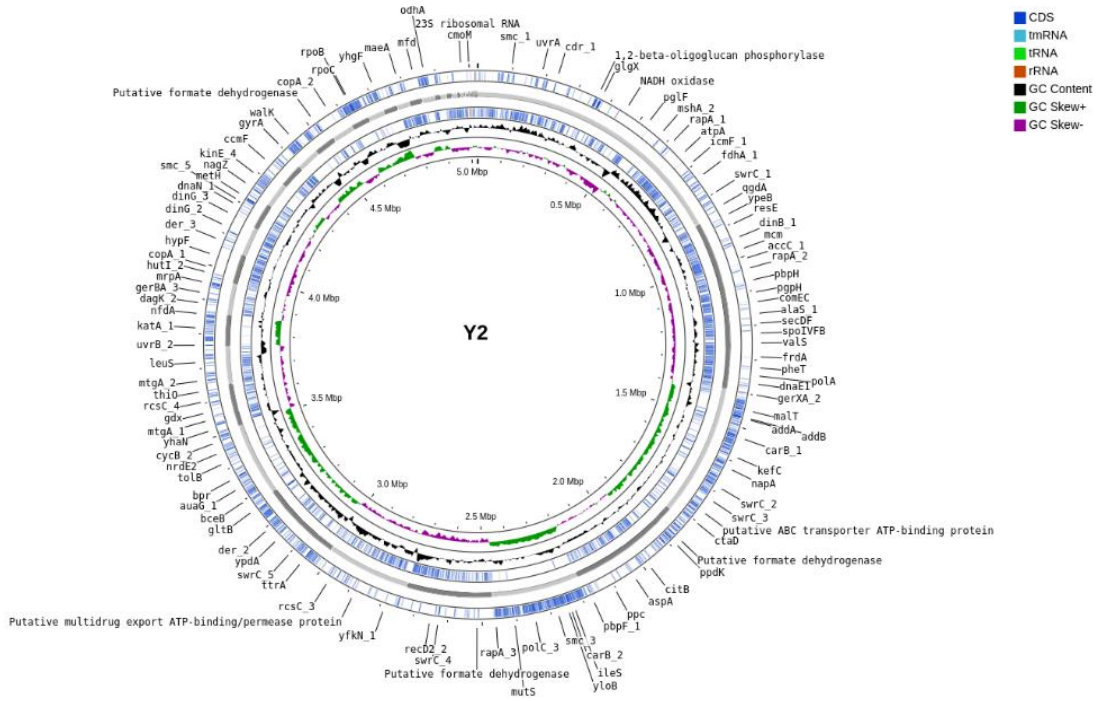


Figure 2.13: The circular representation of the Whole Genome sequence of BRAM_Y2

Key findings from Figure 2.13:

- The wholegenome sequencing of the bacterium was done to reveal their true identities, as it exhibited unique properties that are not innately found in common species of *Bacillus*.
- Though *Mesobacillus subterraneus* was a rare species in itself , the whole genome sequencing results confirmed BRAM_Y2 to be a unique strain of *Mesobacillus thiparans* with highly interesting gene clusters that could be a point of study in future.
- The circular respresentaion of the bacterial whole genome sequence was generataed using Prokka and Proksee servers.

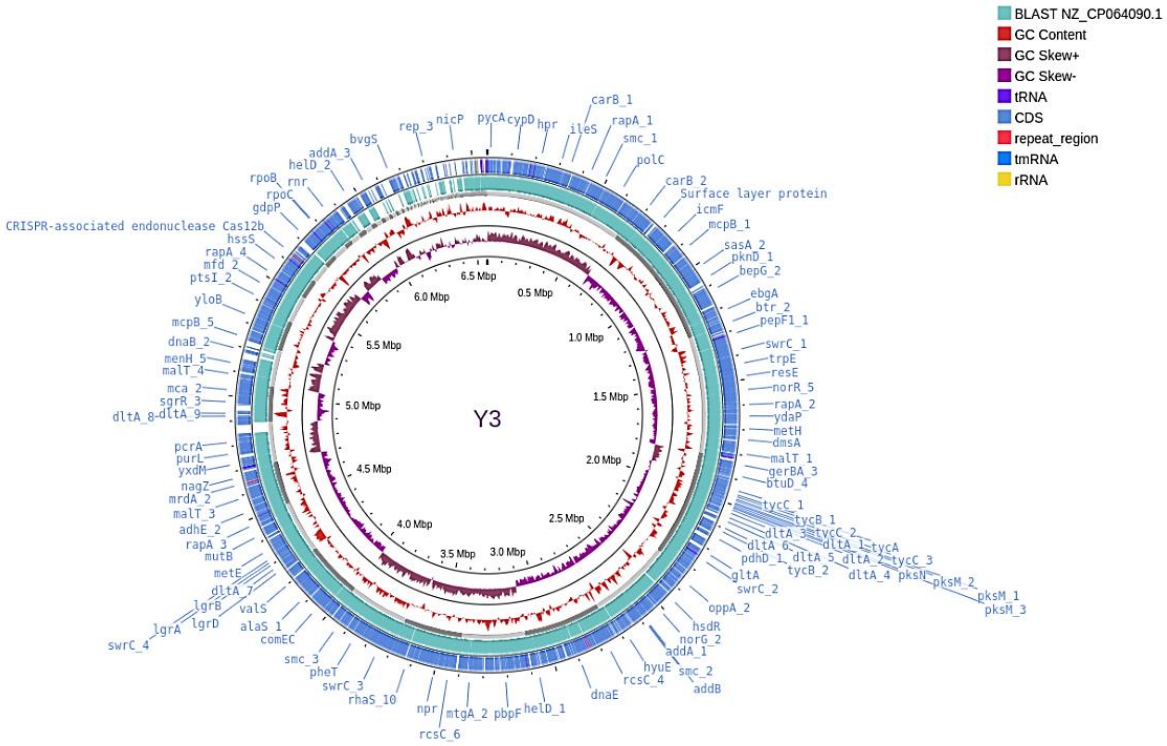


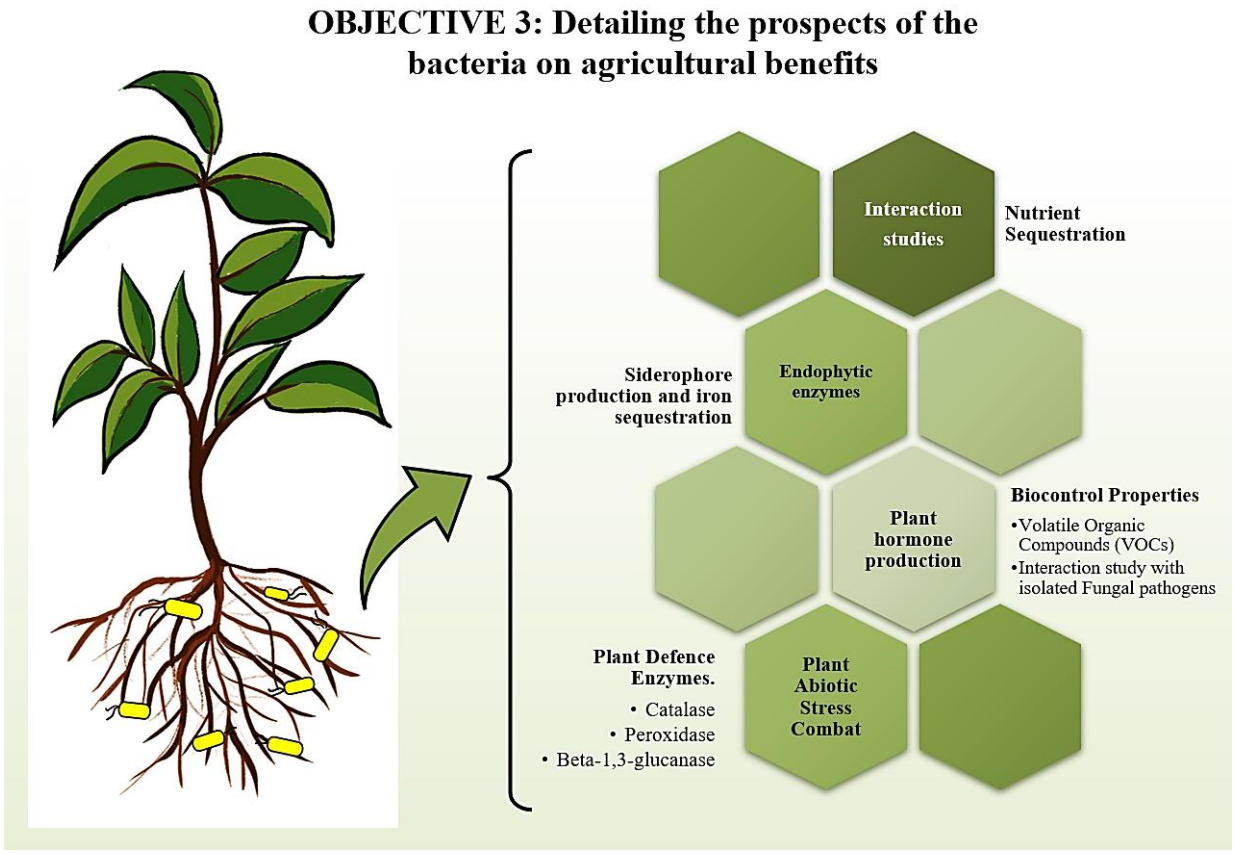
Figure 2.14: The circular representation of the Whole Genome sequence of BRAM_Y3.

Key findings from Figure 2.14:

- The wholegenome sequencing of the bacterium was done to reveal their true identities, as it exhibited unique properties that are not innately found in common species of *Bacillus*.
- Though *Brevibacillus parabrevis* was a rare species in itself , the whole genome sequencing results confirmed BRAM_Y3 to be a unique strain of *Brevibacillus parabrevis* with highly interesting gene clusters that could be a point of study in future.
- The circular respresentaion of the bacterial whole genome sequence was generataed using Prokka and Proksee servers.

Overall Key Findings from Objective 2:

- All 5 bacterial strains were tested for their extremophilic properties such as extremes of temperature, pH, salt, UV, drought and heavy metal stress with respect to four metals.
- Polyextremophilic nature was verified as they were subjected to various two dimensional and three-dimensional stress combinations.
- The role of biofilm in extremophily was studied by the increase in Biofilm formation by the 5 bacterial strains when subjected to different stresses and their combinations.
- The change in Biofilm compositions due to stress was studied using FTIR Spectroscopy.
- All the 5 bacterial strains were subjected to Whole Genome Sequencing for the revelation of their true identities.

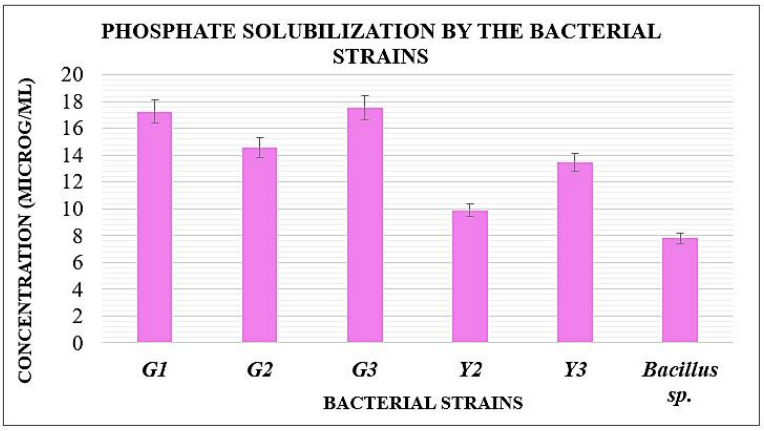

OBJECTIVE 3: Detailing the prospects of the bacteria on agricultural benefits
Interaction studies
Nutrient Sequestration
Siderophore production and iron sequestration
Endophytic enzymes
Biocontrol Properties
•Volatile Organic Compounds (VOCs)
•Interaction study with isolated Fungal pathogens
Plant hormone production
Plant Defence Enzymes.
• Catalase
• Peroxidase
• Beta-1,3-glucanase
Plant Abiotic Stress Combat

3. Detailing the prospects of the bacteria on agricultural benefits.

3.1. Plant Growth Promotion Abilities

The 5 bacterial strains, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 already proved to be polyextremophilic bacterial strains were further examined for their plant growth promoting properties. All the strains grew luxuriantly on Nitrogen deficient medium, thus were nitrogen fixers, solubilized potassium and phosphate. All the strains minimally solubilised phosphate and showed no clearance in the Pikovskaya agar medium and therefore their phosphate solubilization was quantified **(Figure 3.1)**.

Nutrient Sequestration Experiments	G1	G2	G3	Y2	Y3
Phosphate Solubilization	-	-	-	-	-
Zinc solubilization	-	-	-	-	-
Potassium Solubilization	+	+	+	+	+
Nitrogen fixation	+	+	+	+	+



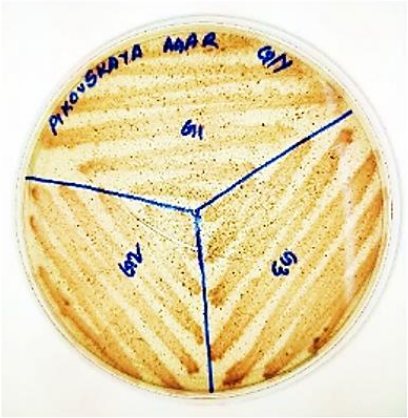


Figure 3.1: Nutrient sequestration by the 5 bacterial strains and the quantification of their phosphate solubilising activity, along with the Pikovskaya plate image for G1, G2 and G3.

<u>Key findings from Figure 3.1:</u>

- In case of phosphate solubilization, though, no clear zones are observed in the Pikovskaya Agar medium, as growth is observed in the phosphate deficient medium, quantification studies were carried out.

- The quantification studies revealed that the 5 bacterial strains solubilised phosphate minimally and slow.
- None of the 5 bacterial strains were able to solubilise zinc.
- All 5 bacterial strains fixed Nitrogen, and showed luxuriant growth on Jensen's Agar medium.
- All 5 of the bacterial strains also solubilised Potassium, and gave clearing zone on Aleksandro agar medium.

All 5 strains also luxuriantly produced the plant hormone IAA both in presence and in absence of a precursor (tryptophan), BRAM_Y3 being exceedingly high. The strains also produced Gibberellin abundantly, a highly sought after plant hormone with BRAM_G2 and BRAM_Y3 being the highest **(Figure 3.2)**

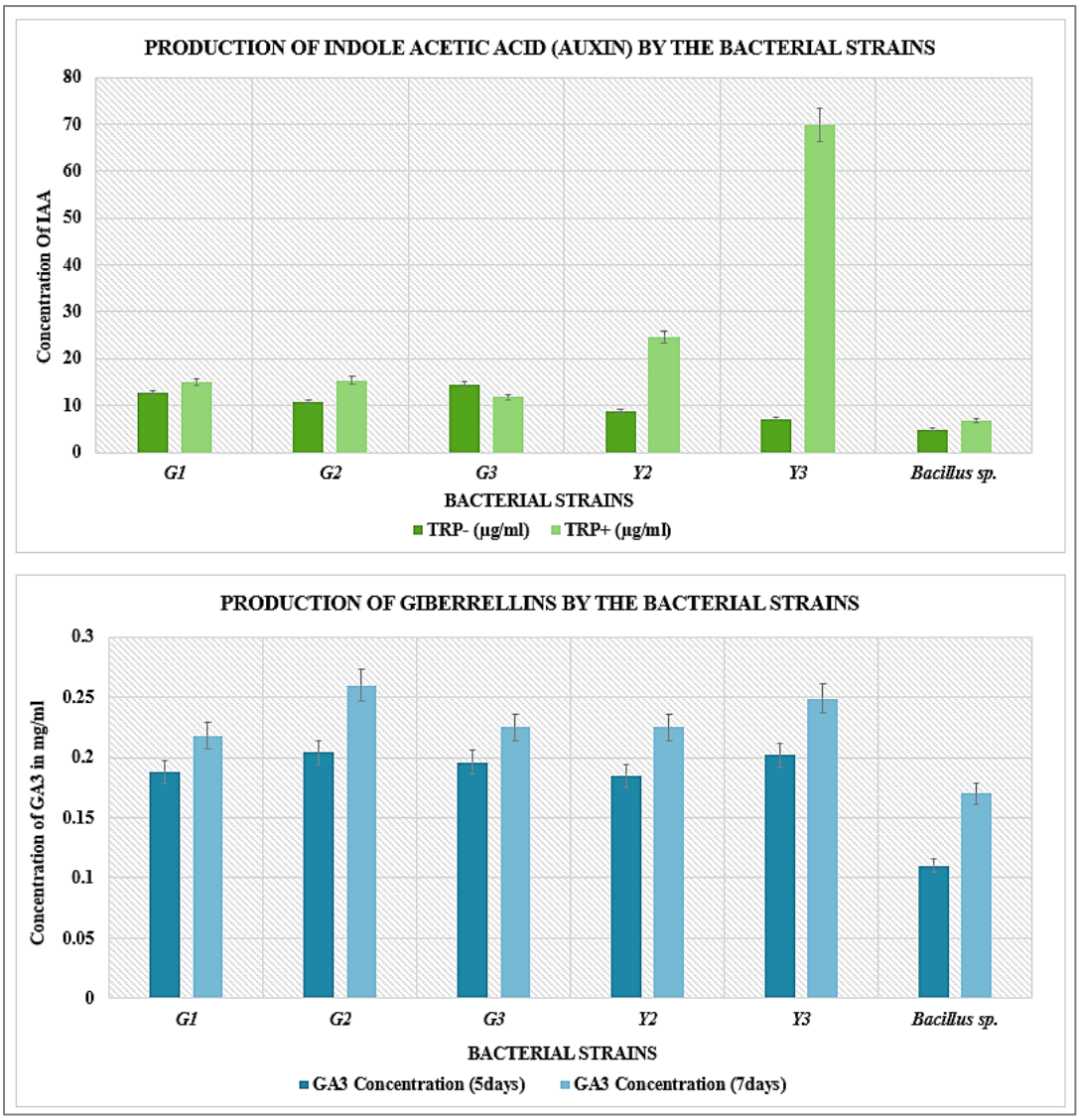


Figure 3.2: Plant Growth regulators (IAA and GA_3) production by the 5 bacterial strains

Key findings from Figure 3.2

- All the 5 bacterial strains could produce Indole Acetic Acid (auxins) in both presence and absence of the precursor tryptophan.
- BRAM_Y3 produced the maximum amounts of IAA in presence of tryptophan compared to the rest.
- All the 5 bacterial strains produced considerable amounts of gibberellins, which was quantified for 5^{th} and 7^{th} day of incubation, which also exhibits the continued and sustained production of the growth regulators, which would promote plant growth for a longer period of time.

3.2. Agriculturally Important Metabolites

ACC Deaminase, the most important enzyme that resists abiotic stress response in plants was also produced by all 5 strains **(Figure 3.3)**.

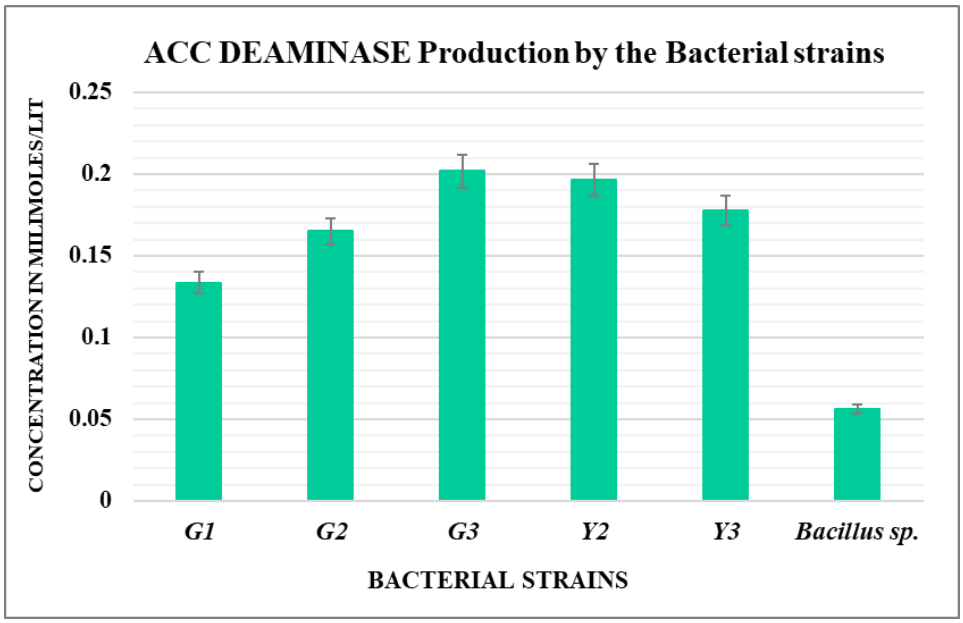


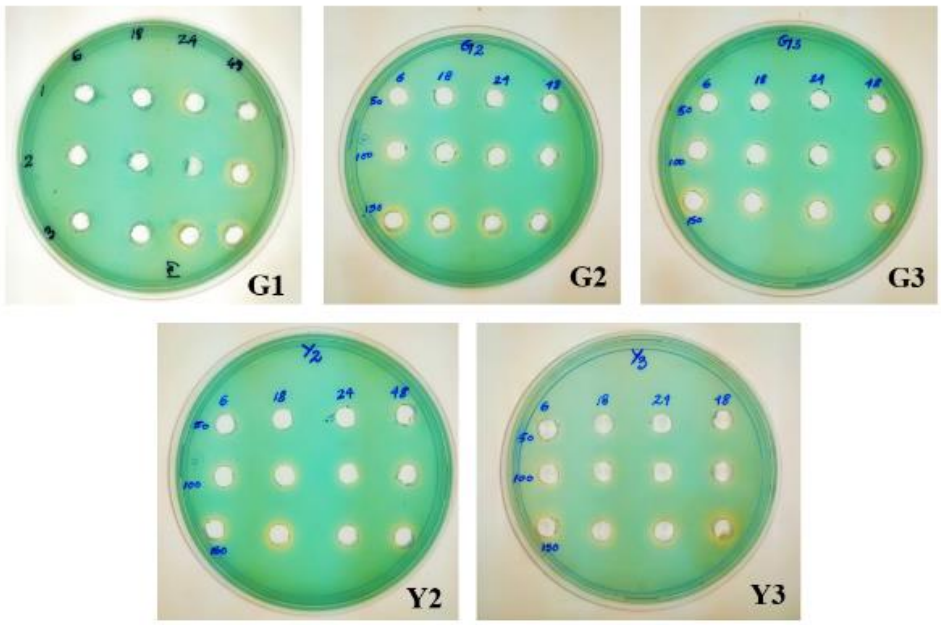
Figure 3.3: Graphical representation of the production of ACC Deaminase by the 5 bacterial strains.

Key findings from Figure 3.3

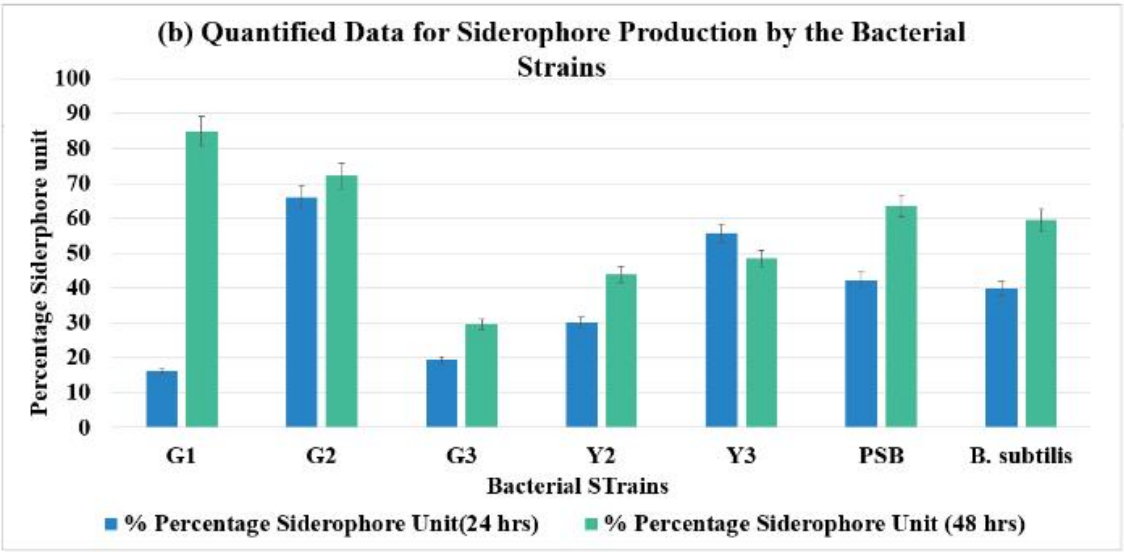
- Ethylene is a plant stress response hormone. It causes senescence in the plant cells, thus causing stress in the plant body and eventual death.
- Ethylene production is triggered by physical stresses like, drought, high temperature.
- 1-aminocyclopropane-1-carboxylic acid is a non-protein amino acid that is the direct precursor of Ethylene.
- Plant growth promoting bacteria can produce the enzyme ACC deaminase by which they can utilise ACC as a Nitrogen source thereby decreasing ethylene production and enhancing plant survival rate in times of physical stress.
- All 5 bacterial strains used in this study could produce ACC deaminase BRAM_G3 being the highest of the lot.

The strains were found to produce iron chelating compounds (Siderophores) detected by the orange halo formation by the cell free supernatants when placed in CAS agar medium. Siderophore also plays an important role in control of phytopathogenic fungi. The siderophore

production by the strains were quantified and typed, and it was found that BRAM_G1, BRAM_G2, BRAM_G3 and BRAM_Y2 produced both the hydroxamate and the catecholate type of siderophores whereas BRAM_Y3 only produced the hydroxamate type **(Figure 3.4)**.



(a) CAS AGAR Diffusion Assay for Siderophore detection.



(c) Siderophore Typing Experiments	G1	G2	G3	Y2	Y3
Hydroxamate Type.	+	+	+	+	+
Catecholate Type	+	+	+	+	-

Figure 3.4: Siderophore production and typing by 5 bacterial strains.

Key findings from Figure 3.4

- Siderophores are iron chelating compounds produced by bacterial cells for the purpose of iron sequestration.
- The Cas agar Diffusion plates show siderophore production and the yellow orange zone formation around the wells in which culture supernatants were introduced.
- Quantification study revealed maximum siderophore production by BRAM_G1 and BRAM_G2 closely followed by BRAM_Y3, BRAM_Y2 and BRAM_G3.
- The siderophores were further characterised for their types based on their chemical structures, and it was found that except for BRAM_Y3 that only produced the hydroxamate type of siderophore, the rest of the four bacterial strains produced both catecholate and hydroxamate types of siderophore that is the mixed type of siderophore.

3.3. Biocontrol Properties of the 5 bacterial strains

These experiments were followed by the investigation of Biocontrol properties exhibited by BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3. **Figure 3.5** shows the quantitative estimation of the biocontrol cluster of enzymes produced by the 5 strains which are Catalase, Beta-1,3-glucanase and peroxidase. The 5 bacteria also produced endophytic enzymes like laccase, pectinase and cellulase except BRAM_Y2, that produced very minimal amounts of cellulase.

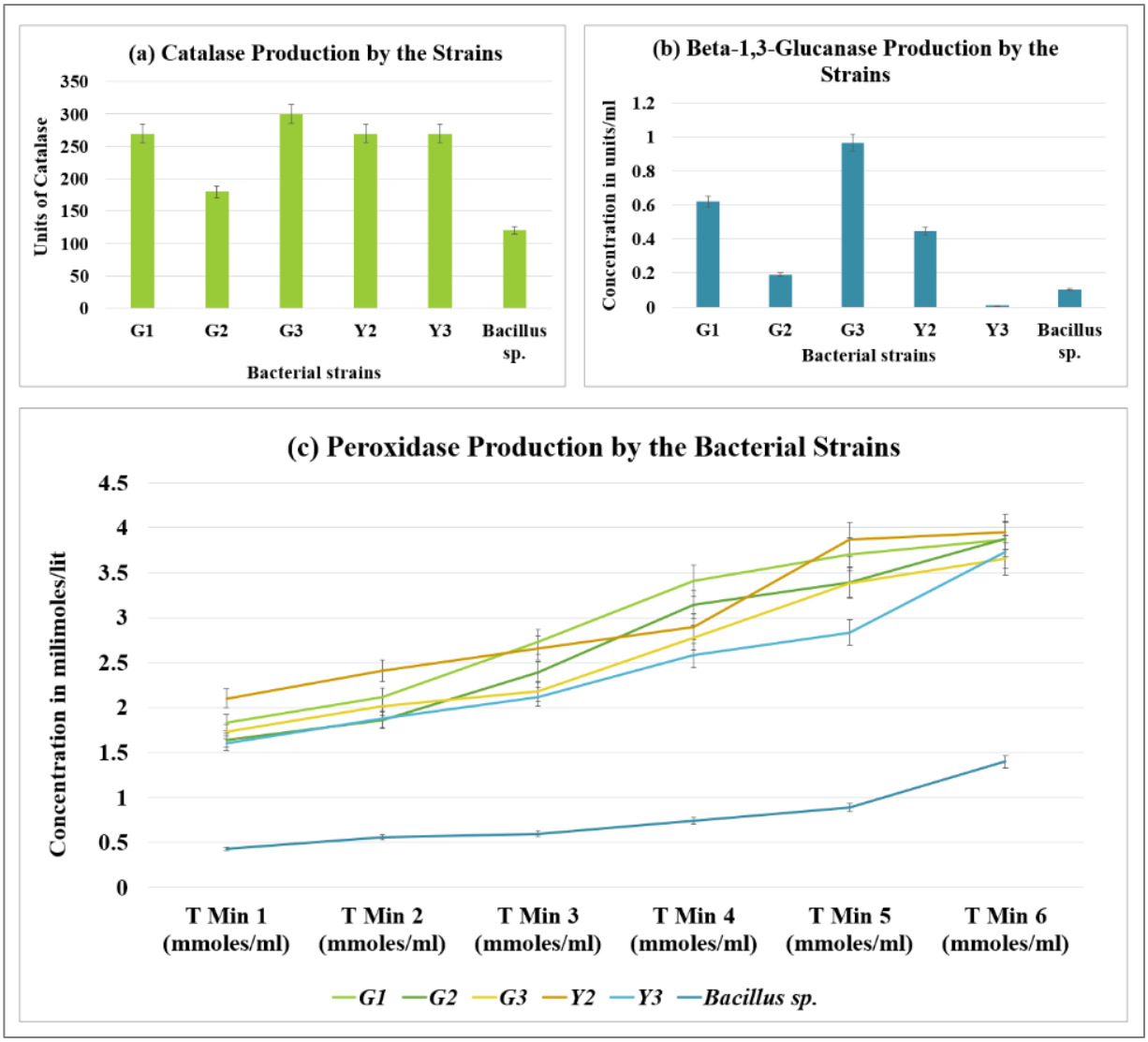


Figure 3.5: Biocontrol enzymes (Catalase, Beta-1,3-glucanase, Peroxidase) production by the 5 bacterial strains.

Key findings from Figure 3.5

- This set of enzymes are responsible for providing the plants resistance to a plethora of phytopathogens, they are also secreted by the plant themselves as a part of their induced systemic resistance.
- The 5 bacterial strains produced catalase, that has significant roles in plant defence, aging and senescence, BRAM_G3 being the highest producer.

- The bacterial strains also produced β-1,3-glucanase, except BRAM_Y3, which produced very minimal quantities of the enzyme.
- Peroxidase was yet again produced in decent quantities by all 5 bacterial strains, making them a potent choice as not on bio-fertilizers but also bio-pesticides.

All the 5 bacterial strains were tested for the production of HCN and Ammonia as volatile organic compounds. BRAM_G1, BRAM_G2 and BRAM_G3 produced both, whereas BRAM_Y3 produced HCN, and BRAM_Y2 produced none.

Two different species of fungi were isolated from the leaves of *Zea mays.* They were named as MC1 and MC2. Their colony morphology was studied in details. MC1 had black mycelia that grew radially whereas MC2 had pinkish white mycelia when grown on Potato Dextrose Agar plates. The microscopic examination of their spore structure revealed curved septate spores for MC1 with bulging in the middle, whereas MC2 had septate spindle shaped spores **(Figure 3.6 and 3.7)**.

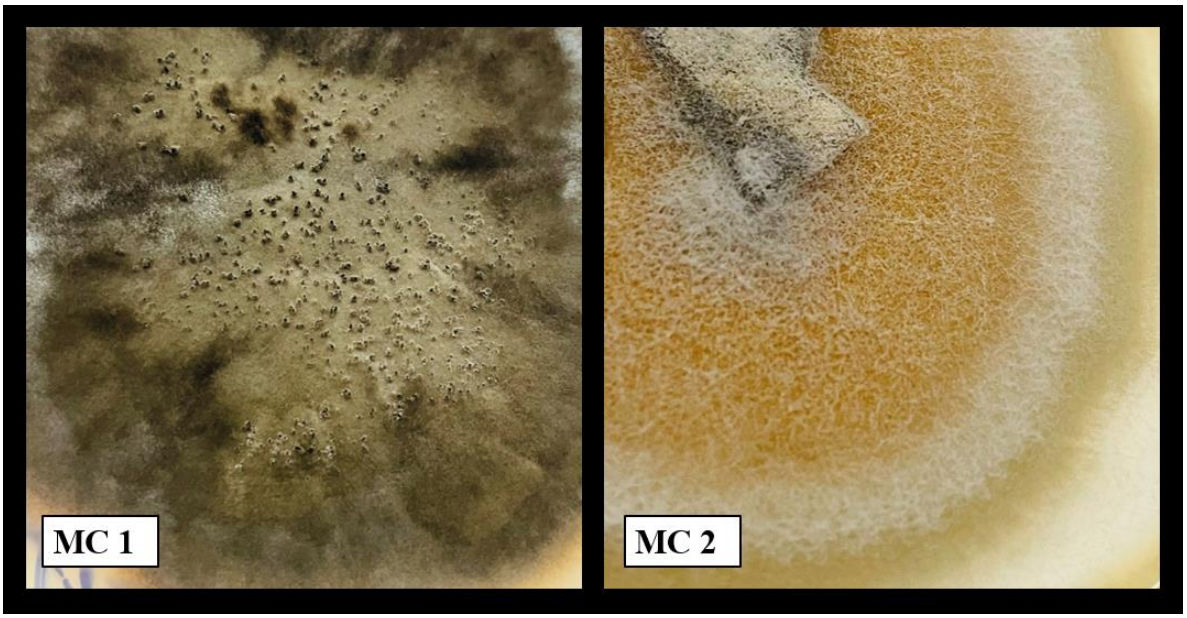


Figure 3.6: Colony morphology of the fungal pathogens of *Zea mays L, Curvularia* sp. MC1 and *Fusarium* sp. MC2.

Key findings from Figure 3.6:

- MC1 and MC2 were isolated from the leaves of *bS.*
- The fungal mycelia of MC 1 had a blackish appearance, that grew radially as well.

- The fungal mycelia of MC2 had a orangish cottony appearance, that grew radially outwards.

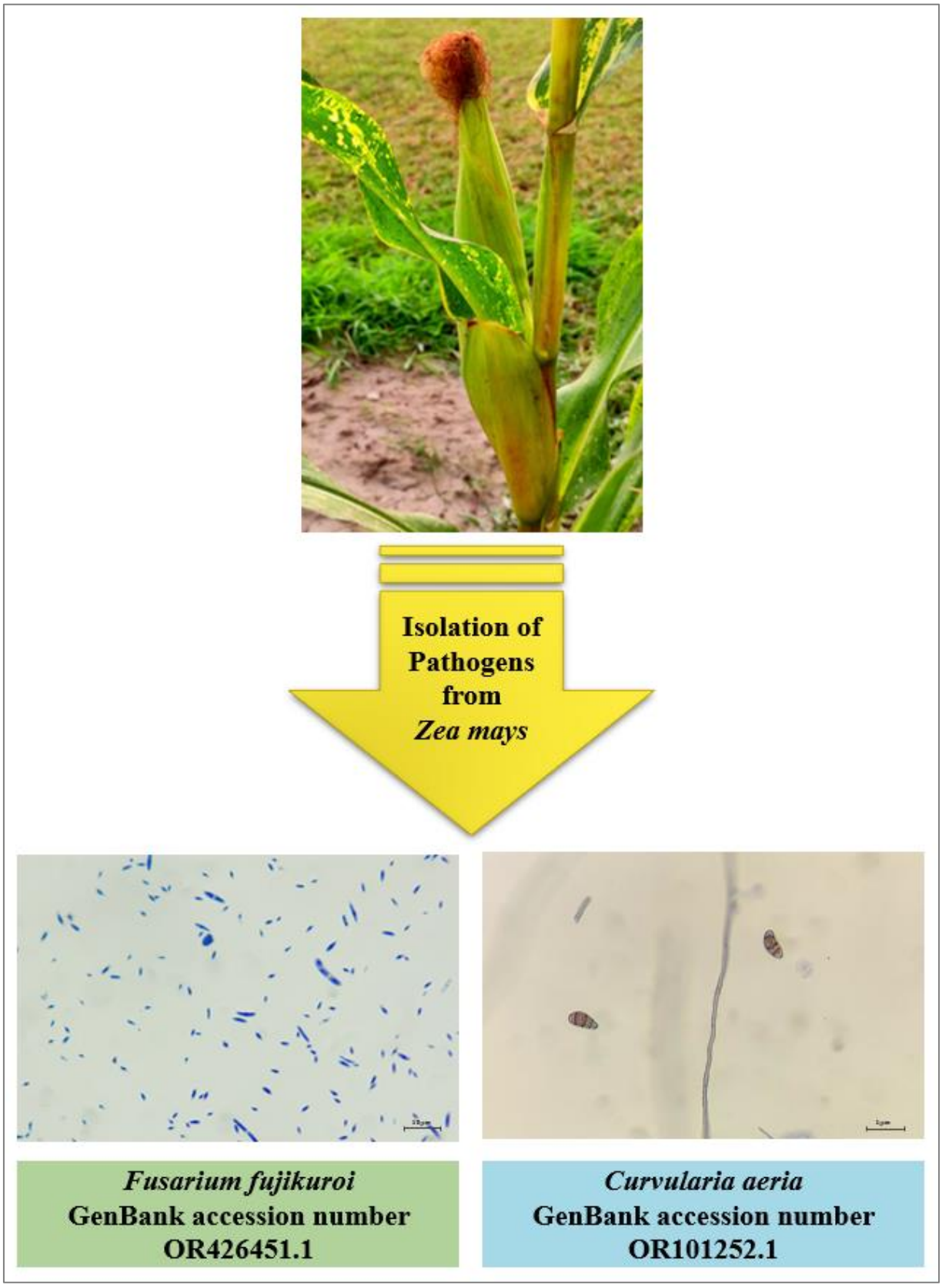


Figure 3.7: Isolation of major fungal pathogens of *Zea mays L* and microscopic imaging of their spores.

Key findings from Figure 3.7:

- MC1 and MC2 were isolated from the leaves of *Zea mays.*
- MC2 showed typical spores like *Fusarium,* spindle shaped, transparent, consisting of two to several cells, formed like a spindle or a sickle, with a longer cell at the top.
- MC1 also showed typical curved septate spores inherent to the *Curvularia* species.

The fungi were inoculated on fresh leaves of *Zea mays* and lesions were observed after 48hours of incubation, as the leaves decayed with chlorosis and eventual browning of the infected spot.

The fungal isolates were sequences for their 18s ITS spacer and it was found that MC1 was *Curvularia aeria* MC1 (GenBank accession number OR101252.1) and MC2 was *Fusarium fujikuroi* MC2 (GenBank accession number OR426451.1). The top ten hits from NCBI blast and the phylogenetic tree of the 2 fungal strains have been represented in **Figures 3. 8 and 3.9.**

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Curvularia aeria isolate B3153	1026	1026	100%	0.0	100.00%	MT043775.1
Curvularia aeria isolate B3144	1026	1026	100%	0.0	100.00%	MT043768.1
Curvularia sp. isolate CCMIBA_R126	1026	1026	100%	0.0	100.00%	MT757840.1
Curvularia aeria isolate 210	1020	1020	100%	0.0	99.82%	KU856633.1
Curvularia aeria strain 1200	1018	1018	99%	0.0	100.00%	KT933659.1
Curvularia aeria voucher LIST 01122022-230-CT3	1018	1018	100%	0.0	99.82%	OQ657945.1
Curvularia aeria strain 816	1016	1016	99%	0.0	100.00%	KT933650.1
Curvularia aeria strain 789	1016	1016	99%	0.0	100.00%	KT933648.1
Curvularia lunata isolate RJ01	1014	1014	100%	0.0	99.64%	MK629001.1
Curvularia aeria strain URM 1534	1013	1013	100%	0.0	99.64%	MH384819.1

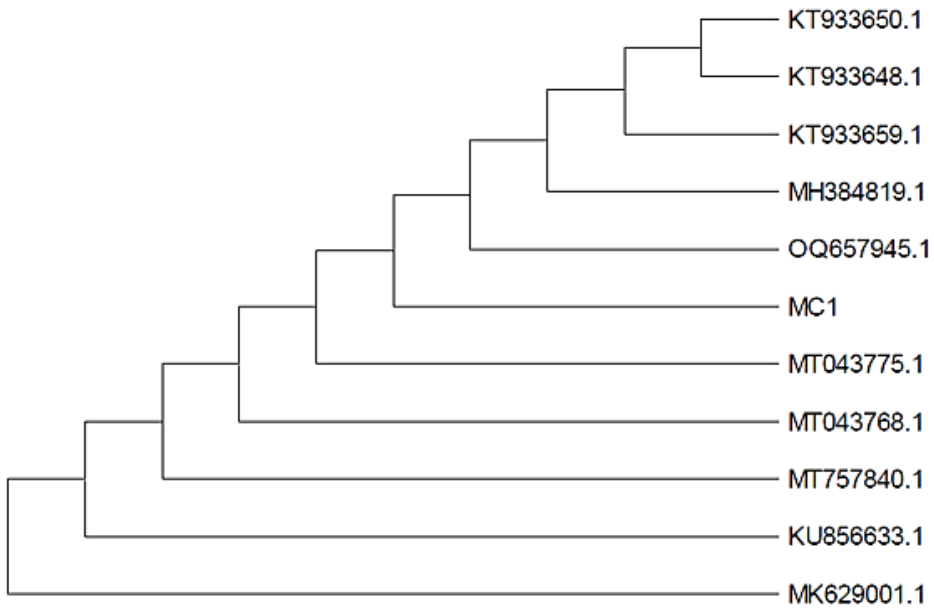


Figure 3.8: The top 10 hits from NCBI Blast results and the phylogenetic tree for *Curvularia aeria* MC1 18S ITS spacer sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 3.7:

- The fungal strain MC1 was confirmed to be *Curvularia aeria* MC1 after 18S ITS spacer sequencing.

- From the phylogenetic tree it can be deduced that, MC1 strain is closely related to *Curvilaria lunata* and other strains of *Curvularia aeria.*

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium fujikuroi isolate FZ04	1013	1013	100%	0.0	100.00%	KJ000432.1
Fusarium verticillioides isolate SYS-qfy41	1007	1007	100%	0.0	99.82%	OM956068.1
Fusarium verticillioides isolate SYS-jb4	1007	1235	100%	0.0	99.82%	OM955986.1
Fusarium sp. isolate ZYY3	1005	1005	100%	0.0	99.82%	OP185344.1
Fusarium fujikuroi strain CF154	1002	1002	100%	0.0	99.64%	OQ629152.1
Fusarium verticillioides isolate SYS-bqfy51	1002	1002	100%	0.0	99.64%	OM956077.1
Fusarium verticillioides isolate SYS-bj22	1002	1002	100%	0.0	99.64%	OM956074.1
Fusarium verticillioides isolate SYS-jz111	1002	1002	100%	0.0	99.64%	OM956061.1
Fusarium verticillioides isolate SH-jx3312	1002	1002	100%	0.0	99.64%	OM956040.1
Fusarium verticillioides isolate QTH-qfz11	1002	1002	100%	0.0	99.64%	OM956034.1

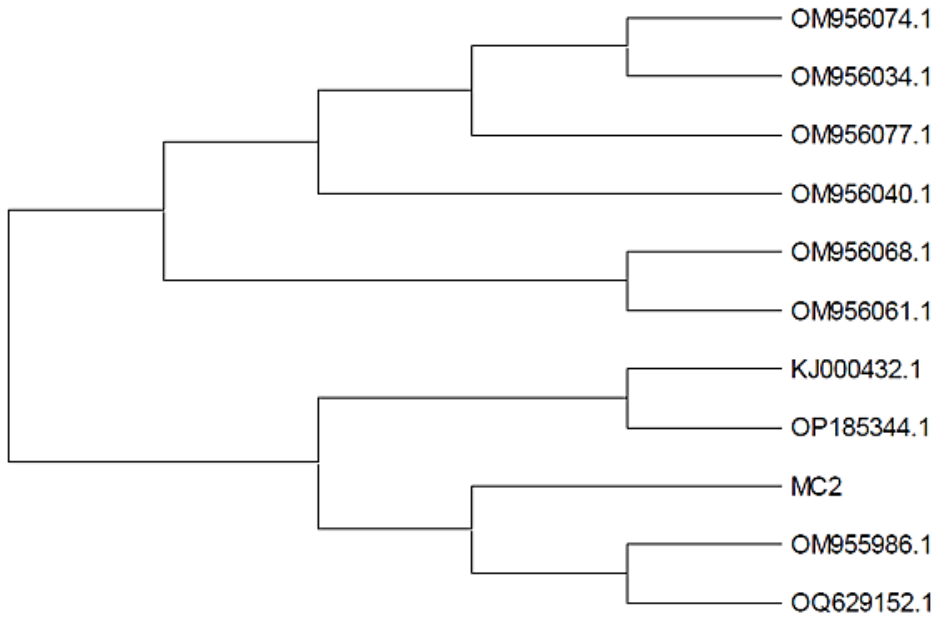


Figure 3.9: The top 10 hits from NCBI Blast results and the phylogenetic tree for *Fusarium fujikuroi* MC2 18S ITS spacer sequence constructed to understand their evolutionary relationships with the same.

Key findings from Figure 3.8:

- The fungal strain MC2 was confirmed to be *Fusarium fujikuroi* MC2 after 18S ITS spacer sequencing.
- From the phylogenetic tree it can be deduced that, MC1 strain is closely related to *Fusarium verticillioides* and other strains of *Fusarium fujikuroi.*

5 bacterial cells excelled in inhibiting the two broad spectrum phytopathogenic fungi isolated from *Zea mays*, *Fusarium fujikuroi* MC2 and *Curvularia aeria* MC1 till day 7, in both VOC sealed plate experiment and also in the direct interaction plates (Table 3.1).

	Fungal Phytopathogen	Bacterial strain	Interaction after 7th Day (Diameter of Fungal Growth in mm)		VOC after 7th Day (Diameter of Fungal Growth in mm)
			Control setup	Test setup (Interaction)	Test setup (VOC)
1.	***Curvularia aeria*** MC1 **GenBank accession number:** OR101252.1	**BRAM_G1**	45mm	11 mm	17 mm
		BRAM_G2	45mm	13 mm	16 mm
		BRAM_G3	45mm	15 mm	16 mm
		BRAM_Y2	45mm	11 mm	13 mm
		BRAM_Y3	45mm	17 mm	11 mm
2.	***Fusarium fujikuroi*** MC2 **GenBank accession number:** OR426451.1	**BRAM_G1**	42 mm	12 mm	15 mm
		BRAM_G2	42 mm	14 mm	16 mm
		BRAM_G3	42 mm	14 mm	14 mm
		BRAM_Y2	42 mm	12 mm	12 mm
		BRAM_Y3	42 mm	18 mm	12 mm

Table 3.1: Plant pathogen inhibition activity by the 5 bacterial strains by direct interaction and VOC sealed plate interaction.

3.4. Endophytic enzymes for Recycling of Organic waste

The 5 bacterial strains were found to produce endophytic enzymes like cellulase, pectinase and laccase. The quantified values for the same have been represented in **Figure 3.10.**

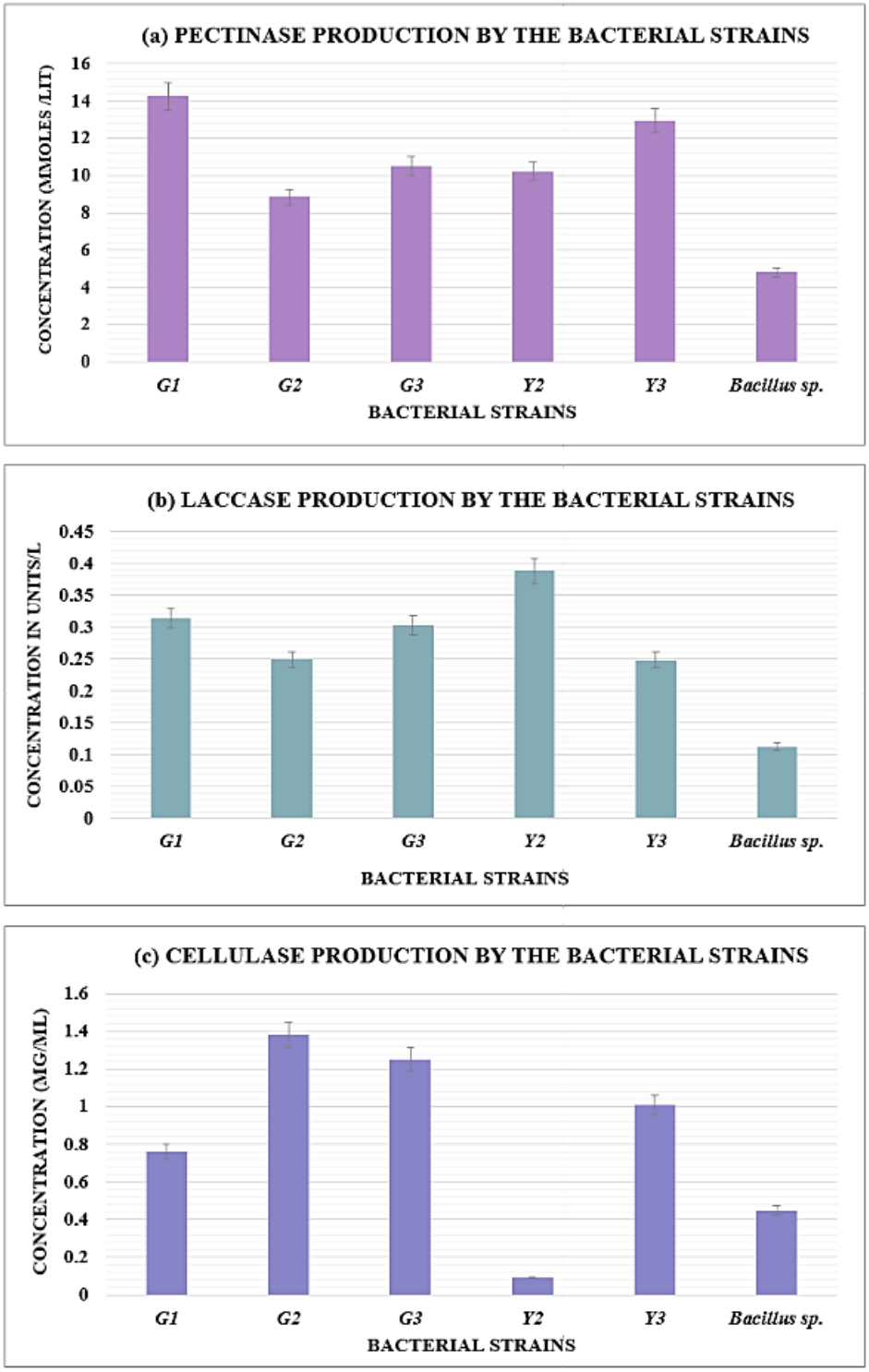


Figure 3.10: Production of the Endophytic enzymes Pectinase, Laccase and Cellulase by all 5 bacterial strains

Key findings from Figure 3.10:

- The three enzymes pectinase, laccase and cellulase help the bacterial cells to penetrate the plant root cell walls and enter the plant system and form a give and take relationship where the bacteria offer plant hormones, sequestered nutrients, defence related enzyme etc. in return for shelter and nutrition.
- These enzymes also known as Ligno-cellulytic enzymes helps in the degradation of plant litter into humus, which contribute to the soil carbon and nitrogen content, in turn upgrading the general soil health.
- All 5 bacterial strains could produce all the three enzymes, except for BRAM_Y3 which produced very minimal levels of cellulase.

3.5. In-vivo application of polyextremophilic plant growth promoting strains

The application was done on *Zea mays L* (Variety: KOHINOOR 595).

The pilot study data, was used to determine the best treatment mode. **Figures 3.11, 3.12 and 3.13** are graphical representations of the vegetative parameters that were measured for all the three modes of treatment application after the treatments were applied. The x-axis represents the time parameter in weeks after application of treatment, whereas the y-axis represents length of plant, number of leaves, length of leaves and internodal lengths respectively. The results indicated that the water suspension treatment was the best mode as it was cost effective and used the concept of whole cell application, thus more scientifically acceptable.

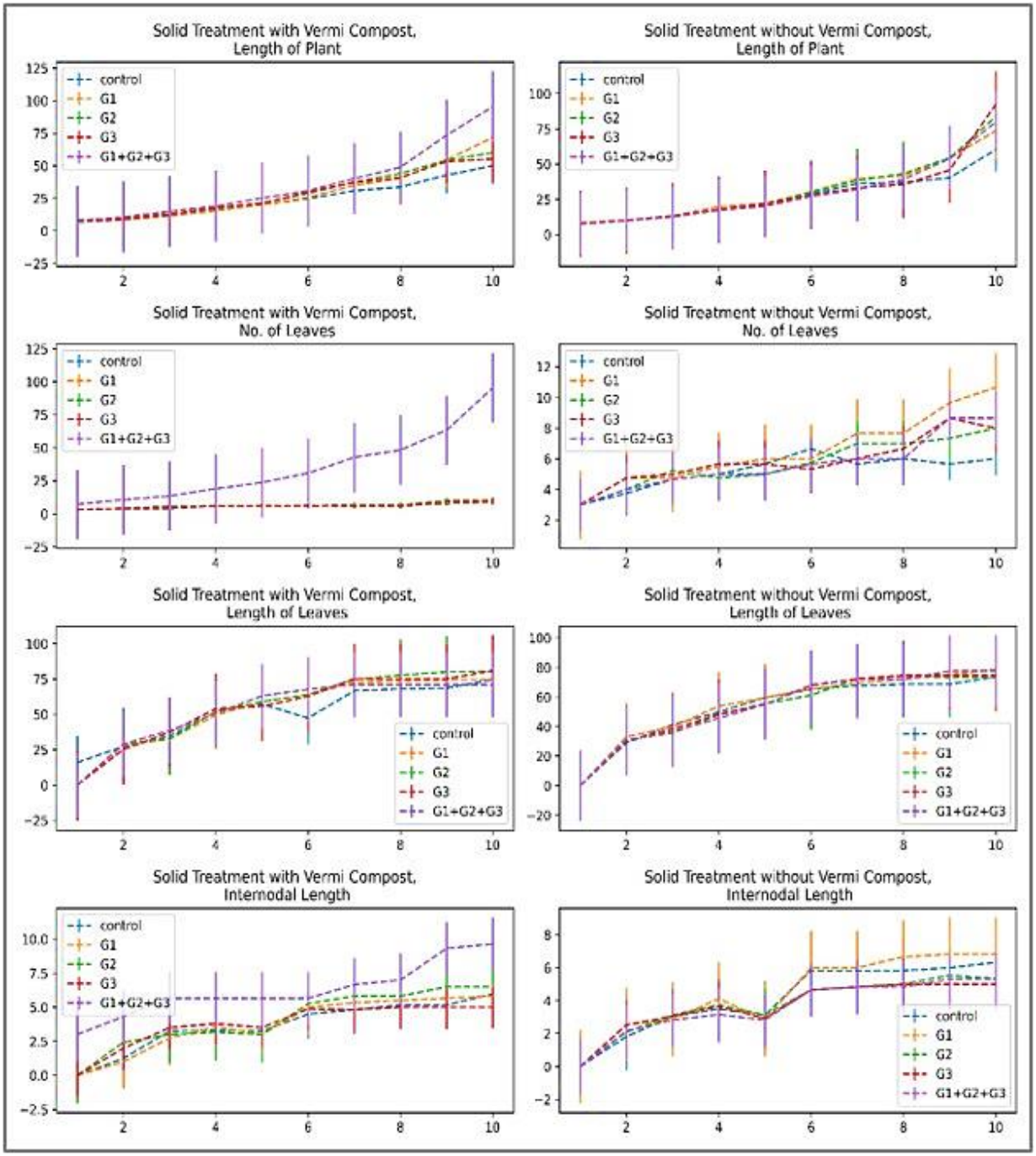


Figure 3.11: The representation of the vegetative parameter data set for mode of application 1, the solid treatment for both sets of soils with and without compost, where the x-axis represents the weeks and the y-axis represents the respective parameters measured.

Key Findings from Figure 3.11

- The experiment was carried out as a part of standardization of the mode of treatment, to be used for experimentation.
- The figure represents the vegetative data set for the Solid treatment (bacterial culture mixed with vermicompost), that includes “length of plant”, “number of leaves”, “length of leaves” and “internodal length”.
- In this case the setup without compost, performed better than the one with compost.
- All the treated setups (treated individually with the three bacterial strains from Gangotri and a consortium of the three) performed better that the control.
- Though the individual bacterial strains performed better than the consortium.

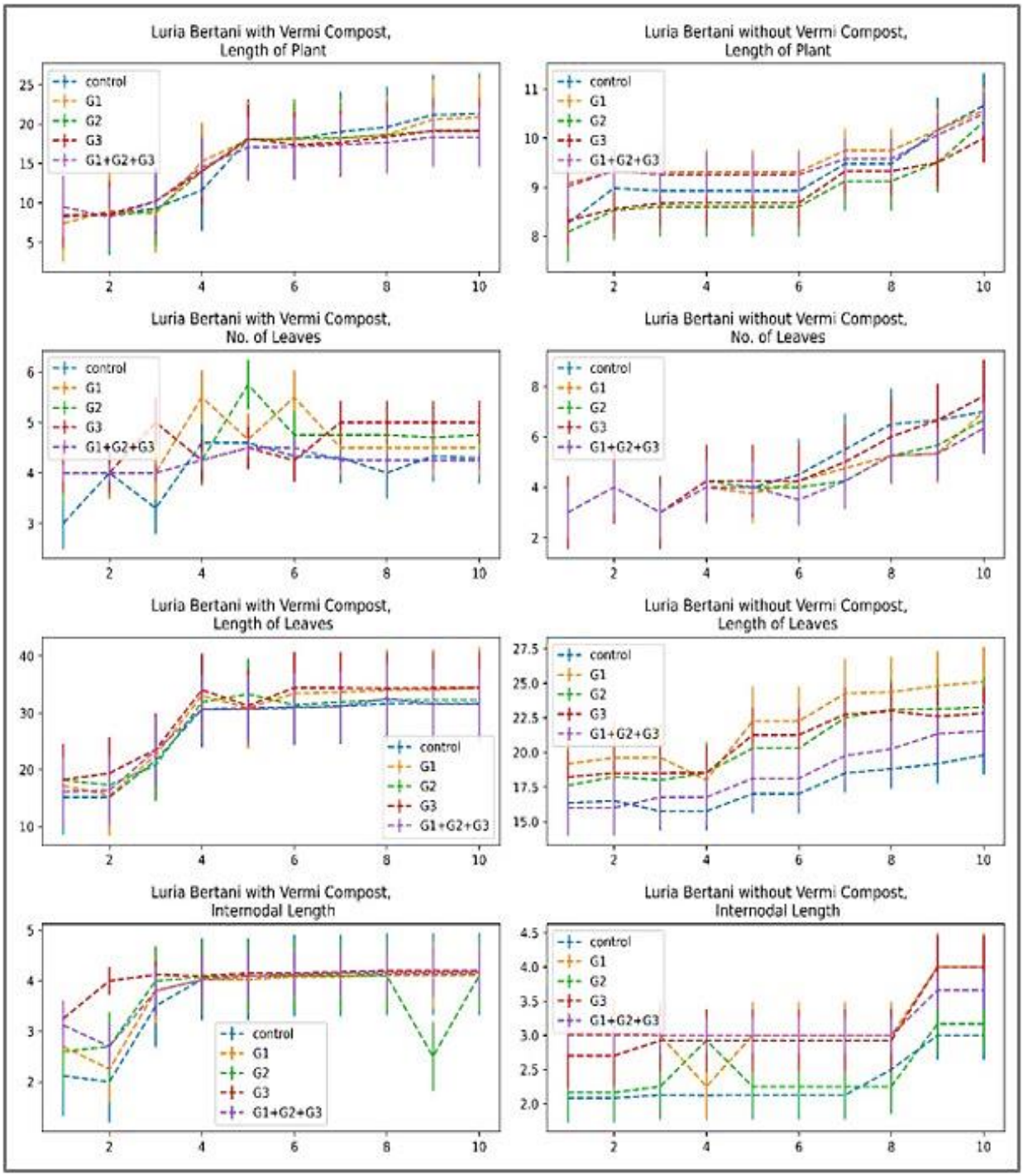


Figure 3.12: The representation of the vegetative parameter data set for mode of application 2, the Luria–Bertani broth treatment for both sets of soils with and without compost, where the x-axis represents the weeks and the y-axis represents the respective parameters measured.

Key Findings from Figure 3.12

- The experiment was carried out as a part of standardization of the mode of treatment, to be used for experimentation.
- The figure represents the vegetative data set for the Luria Bertani broth mode of treatment, that includes “length of plant”, “number of leaves”, “length of leaves” and “internodal length”.
- In this case the setup without compost, performed better than the one with compost.
- All the treated setups (treated individually with the three bacterial strains from Gangotri and a consortium of the three) performed better that the control.
- Though the individual bacterial strains performed better than the consortium.

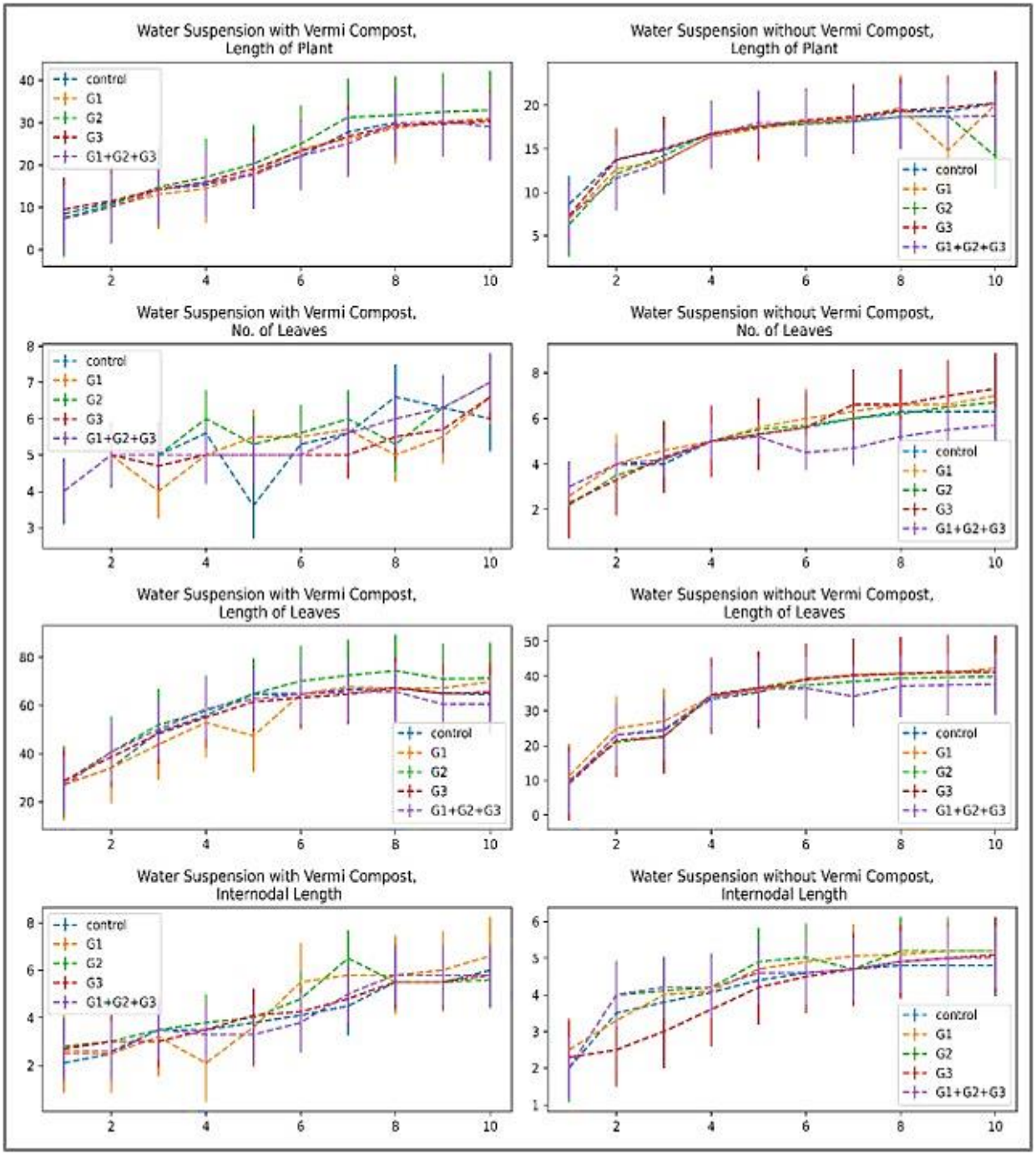


Figure 3.13: The representation of the vegetative parameter data set for mode of application 3, the water suspension treatment for both sets of soils with and without compost, where the x-axis represents the weeks and the y-axis represents the respective parameters measured.

Key Findings from Figure 3.11

- The experiment was carried out as a part of standardization of the mode of treatment, to be used for experimentation.
- The figure represents the vegetative data set for the water suspension (bacterial cell pellet resuspended in water) mode of treatment, that includes “length of plant”, “number of leaves”, “length of leaves” and “internodal length”.
- In this case the setup without compost, performed better than the one with compost.
- All the treated setups (treated individually with the three bacterial strains from Gangotri and a consortium of the three) performed better that the control, except in this mode, the consortium effect was unsatisfactory.
- Though the individual bacterial strains performed better than the consortium.
- This treatment mode was chosen for further investigation because it produced the most favourable outcomes. Additionally, using external components like broth media or vermicompost would have led to inaccurate results when studying the impact of bacterial strains. Therefore, it was determined that using a water suspension with no additional components was the most scientifically sound choice.

Consequently, the field trial was conducted by applying bacteria using water suspension treatment.

Before the field experiment, interaction study amongst the bacterial strains showed negative or neutral interactions amongst each other. The neutral interaction combinations were used in field trials.

During the field experiment, the bacterial strains were administered to the test crop *Zea mays*. All 5 bacterial cells were applied separately as well as in various combinations of consortia. Figure 3.13 and 3.14 depicts a visual representation of the plant's two key vegetative parameters: "Number of leaves" and "Plant Height."

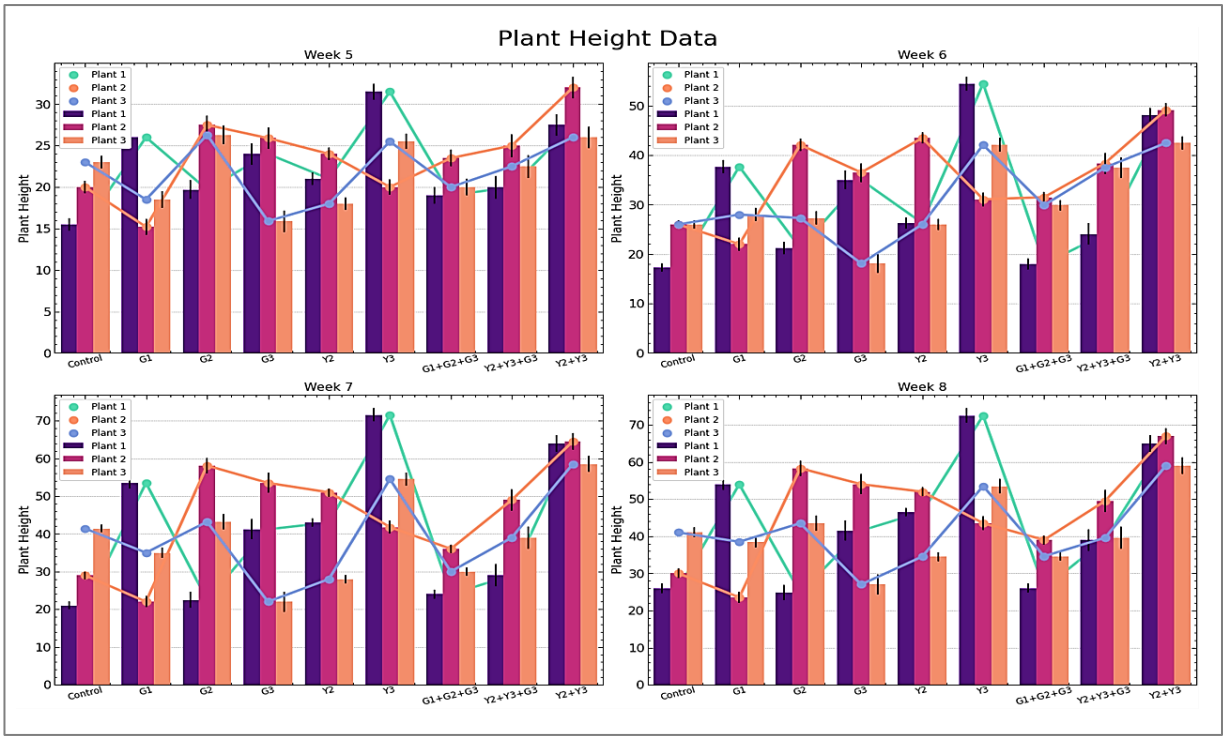


Figure 3.14: Graphical representation of the vegetative parameter "plant height" through the weeks after treatment application with respect to untreated control

Key Findings from the Figure 3.14:

- The plant height was Significantly affected by the application of bacterial treatments in the rhizospheric soil of the *Zea mays*
- All the bacterial treatments including the consortia performed better than the untreated control with respect to plant height.
- Though the best result was observed with BRAM_Y3 and BRAM_Y2+BRAM_Y3 consortia when it comes to plant height.
- This can be interpreted as a consequence of the secretion of auxins by the bacterial strains that has an enormous role to play in increase of plant height and other vegetative parameters.

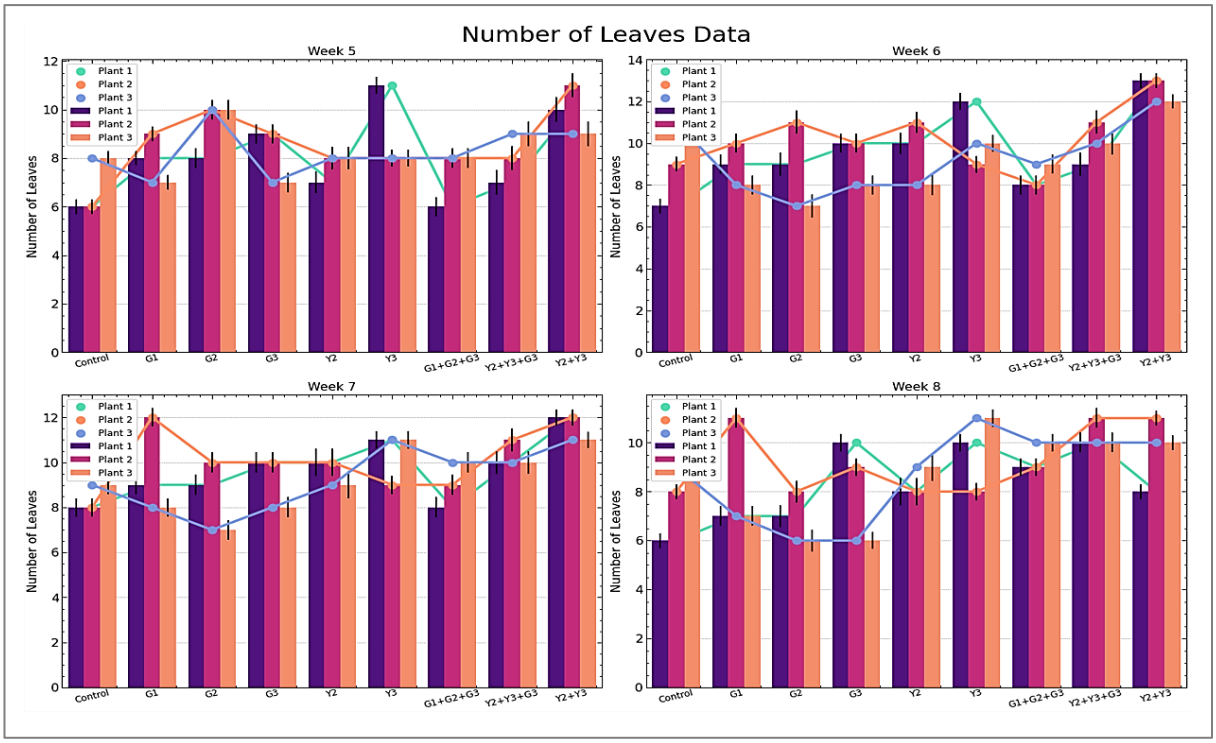


Figure 3.15: Graphical representation of the vegetative parameter "number of leaves" through the weeks after treatment application with respect to untreated control.

Key Findings from the Figure 3.15:

- There was not much variation that could be observed in the untreated and treated plant setups with respect to number of leaves.
- Yet to provide a conclusion once again all the treated setups performed better than the untreated control and specifically in case of number of leaves most of the treatments were at par with each other.

This was followed by the field images of the trial setup (Figure 3.15) and the images of the fruits harvested from the different setups (Figure 3.16).

Figure 3.16: Representative images of field application of the 5 bacterial strains and their combinations on *Zea mays L* (KOHINOOR 595).

Key Findings from the Figure 3.16:

- The set of images is a representative of the field trial of *Zea mays L* (KOHINOOR 595) conducted in the grounds of St. Xavier's College (Autonomous) Kolkata.
- The image shows flowering and fruiting in the setups and also the number of plants which were three per treatment set and the growth from the first month to the last month of the crop lifecycle.

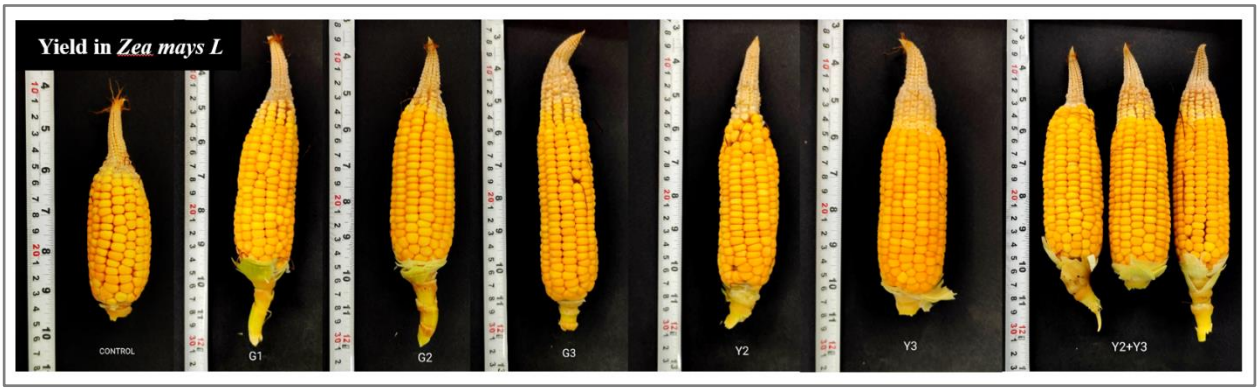


Figure 3.17: Representative images of the yield from the treated plants and the untreated control plants.

Key Findings from the Figure 3.17:

- The above picture is a representative image of the harvest that was collected from all the different treatment setups.
- It is to be noted that two of the consortia, G1+G2+ G3 and Y2+Y3+G3 did not develop fruits and therefore there was no harvest.
- The photograph provides a lucid depiction of the superior performance of the treatment compared to the untreated control. It also illustrates that the fruits appear to be in a healthier state and of higher quality than those in the control group.

The parameters like number of flowers and number of fruits over the weeks in Figures 3.18 and 3.19.

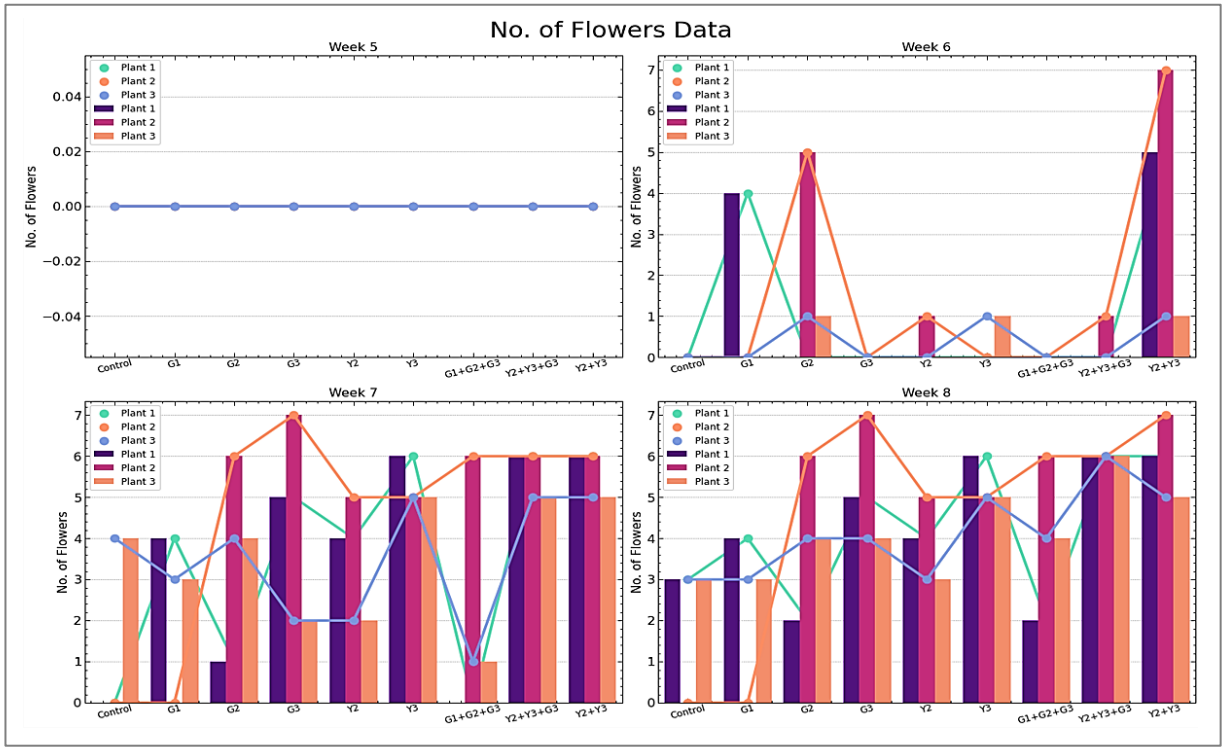


Figure 3.18: Graphical representation of the reproductive parameters "number of flowers" through the weeks after treatment application with respect to untreated control.

Key Findings from the Figure 3.18:

- The parameter represented in this particular graph is the number of flowers present in the treated and untreated plants over the weeks.
- The appearance of flower was observed from the 6th week in the treated plants and in the 7th week in the untreated plants.

- The maximum number of flowers were observed in BRAM_G3 and in the Y2+Y3 consortia.

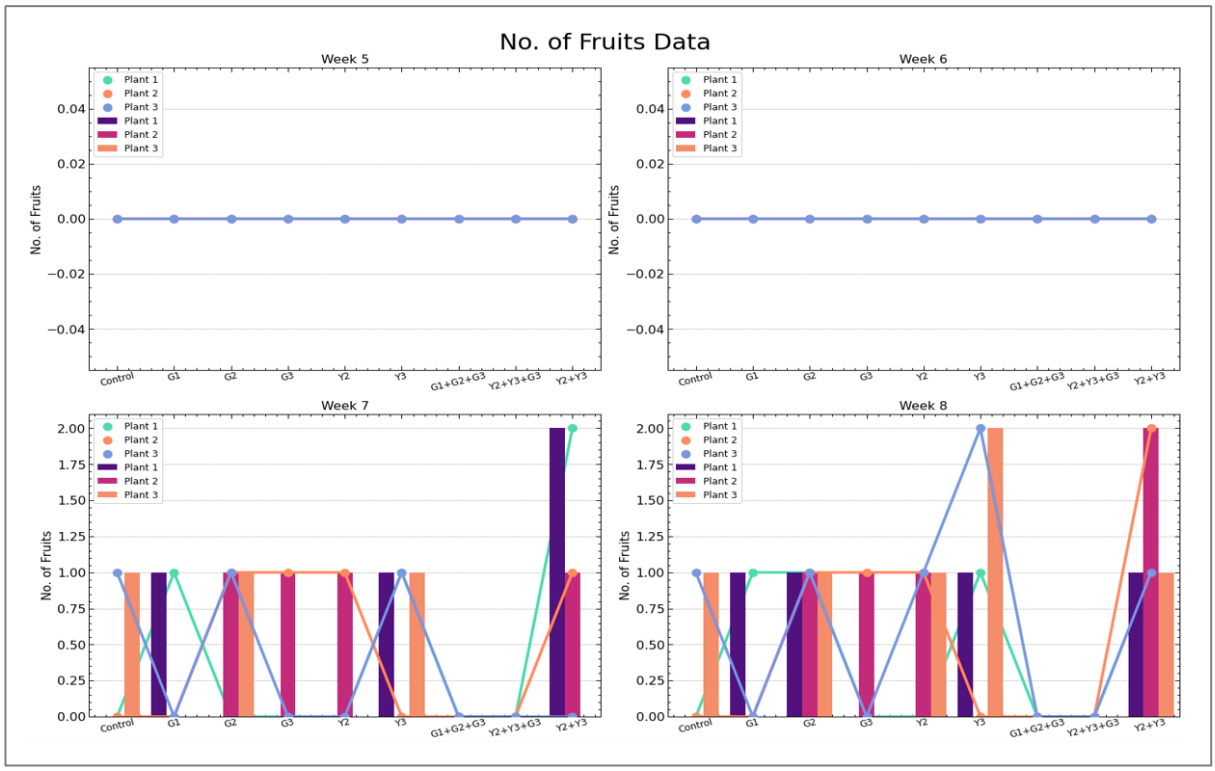


Figure 3.19: Graphical representation of the reproductive parameters "number of fruits" through the weeks after treatment application with respect to untreated control.

Key Findings from the Figure 3.19:

- The parameter represented in this particular graph is the number of fruits present in the treated and untreated plants over the weeks.
- The appearance of fruits was observed from the 6th week in both the treated plants and untreated plants except in two of the consortia, G1+G2+ G3 and Y2+Y3+G3.
- The maximum number of fruits were observed in BRAM_Y3 and in the Y2+Y3 consortia.

The plant was studied for four yield parameters: "Number of fruits," "cob weight," "length of cob," and "Percent Grain filling" of the fruits (Figure 3.19).

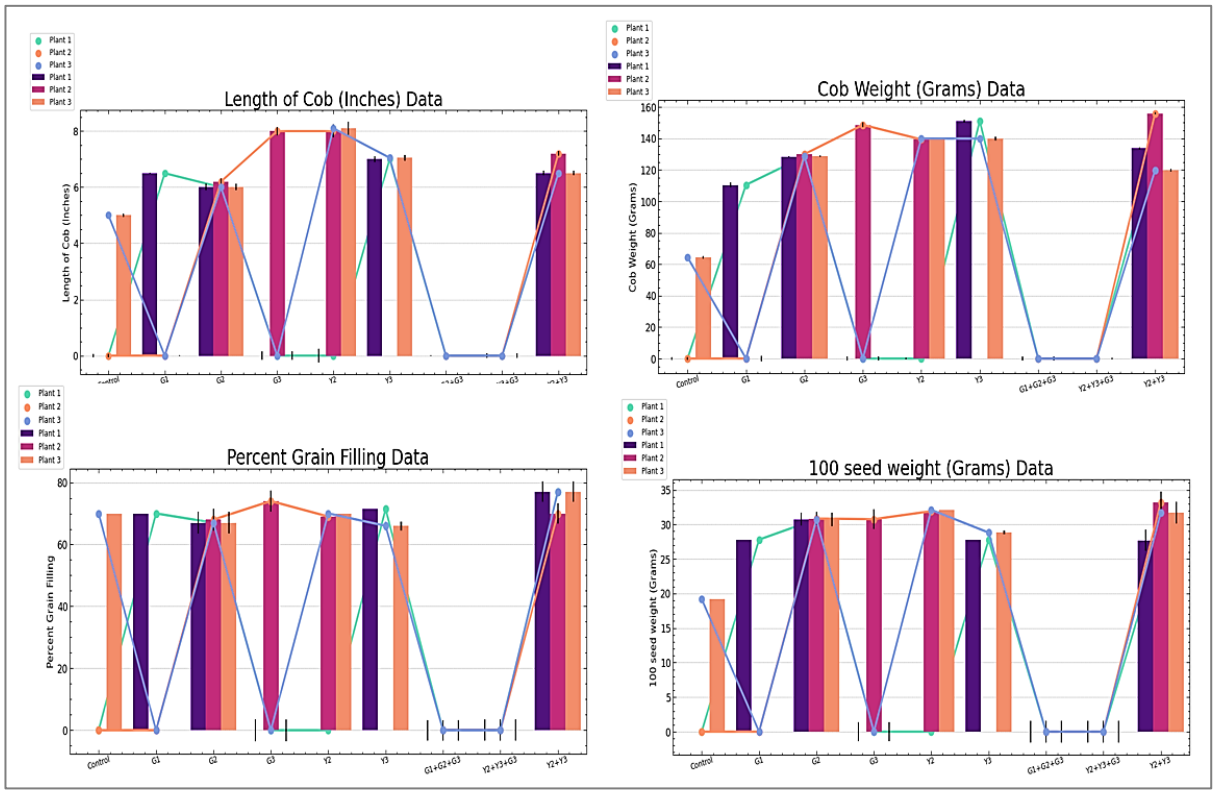


Figure 3.20: Graphical representation of the yield parameters, length of cob, cob weight, percent grain filling, and "100 seed weight" after treatment application with respect to untreated control.

Key Findings from the Figure 3.20:

- The highest length of Cob was observed in BRAM_G3 and BRAM_Y2, with BRAM_Y3 and the Y2+Y3 consortia being somewhat shorter.
- The cob weight was highest in the Y2 and Y3 consortia, closely followed by BRAM_Y3 and BRAM_G3.
- The percentage of grain filling data showed that the Y2+ Y3 consortia had the highest grain filling, followed closely by all 5 individual bacterial strains.
- The 100 seed weight data indicated that the Y2+Y3 consortia had the highest weight, closely followed by all five individual bacterial strains.

Additionally, the plant was also studied for pigments such as Chlorophyll a, Chlorophyll b, total chlorophyll, and carotenoids.

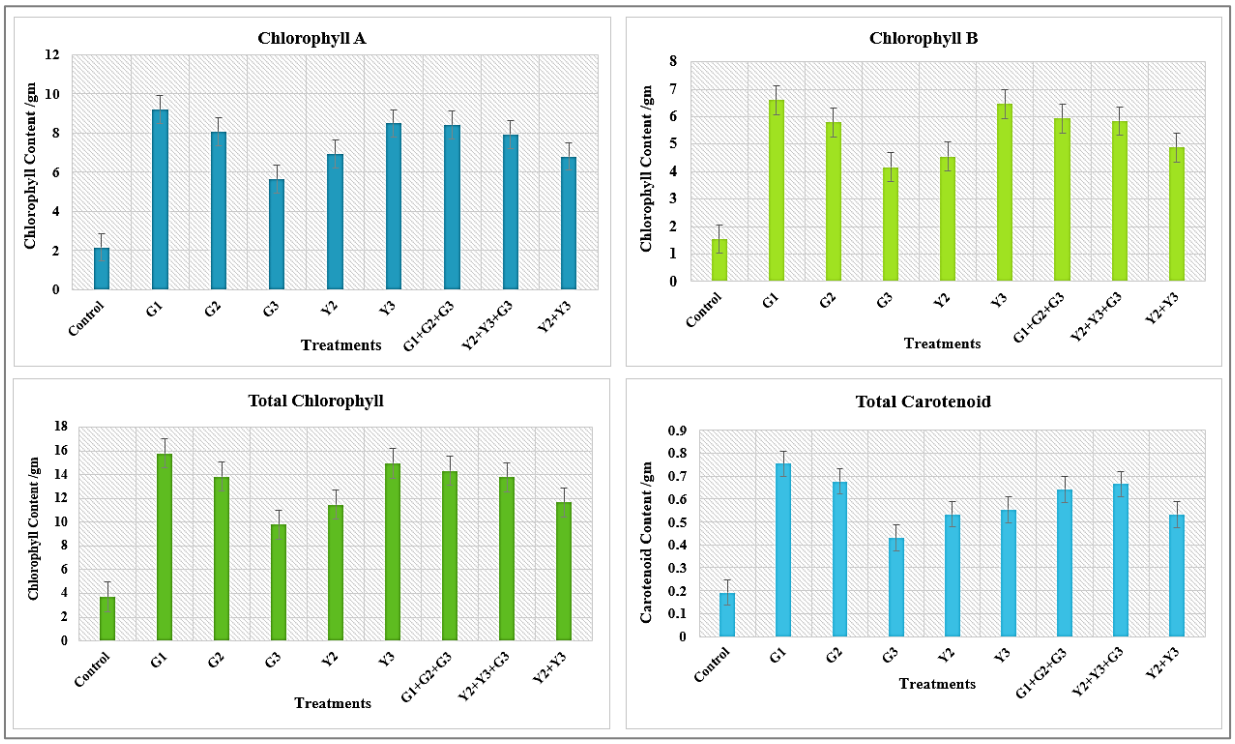


Figure 3.21: Plant Pigment data of *Zea mays L* (Variety: KOHINOOR 595) after treatment application with respect to untreated control.

Key Findings from the Figure 3.21:

- The examination of plant pigments provides us with a distinct understanding of the plant's health.
- All the treated setups performed significantly better than the control when studied for plant pigments.
- The highest chlorophyll content both a and b and total chlorophyll was observed in BRAM_G1 closely followed by BRAM_Y3 and the rest of the treatment setups.
- Carotenoids serve as both plant pigments and non-enzymatic antioxidants. Consequently, a higher concentration of carotenoids in plants leads to enhanced protection against both biotic and abiotic stresses.

- A comparable pattern of carotenoid content rise was seen, with BRAM_G1 ranking the highest, followed by the other treated setups, which were significantly higher than the untreated control.

Table 3.2 displays the physical characteristics of the soil before (initial soil) and after (final soil) the cultivation of maize, using BRAM_Y2 and BRAM_Y3 as treatments. Table 3.3 displays the chemical characteristics of the soil prior to (initial soil) and following (final soil) the cultivation of maize, utilising BRAM_Y2 and BRAM_Y3 as treatments.

	pH	E.C. dsm^{-1}	CEC meq/100gm	Sand (%)	Silt (%)	Clay (%)	Texture of Soil	BD gm/cc	WHC (%)	Pore Space (%)	Moisture (%)	Humic Acid (%)
INITIAL SOIL	7.88	0.16	14.80	44.40	36.00	19.60	Loam	1.39	34.93	43.26	7.60	6.00
FINAL SOIL	8.26	0.11	11.76	46.40	36.00	17.60	Loam	1.32	36.44	44.59	17.40	4.00

Table 3.2: Physical Parameters of the Soil before and after application of the 5 Bacterial strains as treatment during field trail on *Zea mays L*

	O.C. (%)	N Kg/ha	P_2O_5 Kg/ha	K_2O Kg/ha	Cu mg/kg	Zn mg/kg	Fe mg/kg	Mn mg/kg	B mg/kg	Ca mg/kg	Mg mg/kg	S mg/kg
INITIAL SOIL	0.48	439.04	110.74	215.04	5.45	8.00	15.00	10.00	0.624	881.76	607.50	6.32
FINAL SOIL	0.58	426.50	64.90	149.18	4.35	7.00	12.50	7.00	0.412	961.92	510.60	3.20

Table 3.3: **Chemical Parameters of the Soil before and after application of the 5 Bacterial strains as treatment during field trail on *Zea mays* L.**

Key Findings from the Tables 3.1 and 3.2:

- Soil analysis before and after cultivation of *Zea mays* L with the bacterial treatments showed an increase in soil organic carbon, pore space and moisture.
- On the other hand, there was a very slight decrease in soil Nitrogen, because though one of the most utilised nutrients, due to the presence of Nitrogen fixers as treatment, the change was very small.
- A significant decrease in the levels of potassium and phosphate due to solubilization by bacteria and utilization by plants.

- Similarly decrease in the levels of Zinc, Iron, Manganese, Magnesium and sulphur was also observed which may be due to nutrient solubilising ability of the bacterial strains used to treat the *Zea mays* rhizospheric soil.

Table 3.4 and Table 3.5 display the ANOVA tables for the two reproductive parameters, respectively.

ANOVA for Plant height (Zea mays L)				
	sum_sq	df	F	PR(>F)
Intercept	12232.634914	1.0	57.494019	5.824443e-11
C (Treatment)	3100.602003	8.0	7.294548	2.277750e-07
C (week)	158937.355732	3.0	75.166383	5.540294e-18
C (Treatment): C (week)	3183.101469	24.0	1.494482	8.654988e-02
Residual	1832.408590	0	NaN	NaN
Interpretation: Variations are significant in case of treatments.				

Table 3.4: ANOVA Table for the Vegetative parameter: "Plant Height".

Key Findings from the Figure:

- The ANOVA for the vegetative parameters, was carried out to determine the significance of the difference between the treated and untreated setups.

- Treatment (C(Treatment)): The main effect of treatment is significant ($p < 0.05$), indicating that there are differences in plant height among the treatment groups.

- Week (C(Week)): The main effect of week is highly significant ($p < 0.05$), suggesting that plant height changes significantly over time (Weeks 5 to 8).

ANOVA: Yield Parameters (Zea mays L)						
ANOVA on Cob Weight Data	Source of Variation	SS	df	MS	F	P-value
	Between Groups	14438.4839	6	2406.41398	14.7989242	2.00333e-07
	Within Groups	2598.78682	14	185.62763		
	Total	17037.27072	20			
	Interpretation: Means are not same, variations are significant					
ANOVA on 100 seed Weight Data	Source of Variation	SS	df	MS	F	P-value
	Between Groups	119.18166	6	19.86361	4.27849	0.00707
	Within Groups	157.65507	14	11.26108		
	Total	276.83673	20			
	Interpretation: Means are not same, variations are significant					

Table 3.5: ANOVA Table for Yield Parameters: "Cob Weight" and "100 seed weight".

Key Findings from the Figure:

- The ANOVA for the yield parameters, was carried out to determine the significance of the difference between the treated and untreated setups.

- In case of cob weight, Since the p-value (2.00333e-07) is less than the typical significance level of 0.05, we reject the null hypothesis. This suggests that there are statistically significant differences between at least two treatment groups in terms of the cob-seed weight.

- The F-value (14.7989242) being greater than 1 further supports this conclusion, indicating that there is more variability between the treatment groups than within the groups. In conclusion, the ANOVA test indicates that there are significant differences in the cob weight across the treatment groups.

- In case of 100 seed weight, Since the p-value (0.00707) is less than the typical significance level of 0.05, we reject the null hypothesis. This suggests that there are statistically significant differences between at least two treatment groups in terms of the 100-seed weight.

- The F-value (4.27849) being greater than 1 further supports this conclusion, indicating that there is more variability between the treatment groups than within the groups. In conclusion, the ANOVA test indicates that there are significant differences in the 100-seed weight across the treatment groups.

Metagenome study of the Untreated Initial soil and treated final soil to check if the bacterial strains applied for plant growth promotion actually colonise in the treated plant rhizosphere or not. The Krona plot showed that for the maize initial soil the population of the *Bacillus* and associated genera was 4% that increased to 6% **(Figure 3.24)**. The heat maps of both the phylum (Firmicutes) and class (Bacillaece) who percentage increase from the initial soil to the final soil proving the colonisation of the 5 test bacterial strains in the maize rhizosphere **(Figure 3.22 and 3.23).**

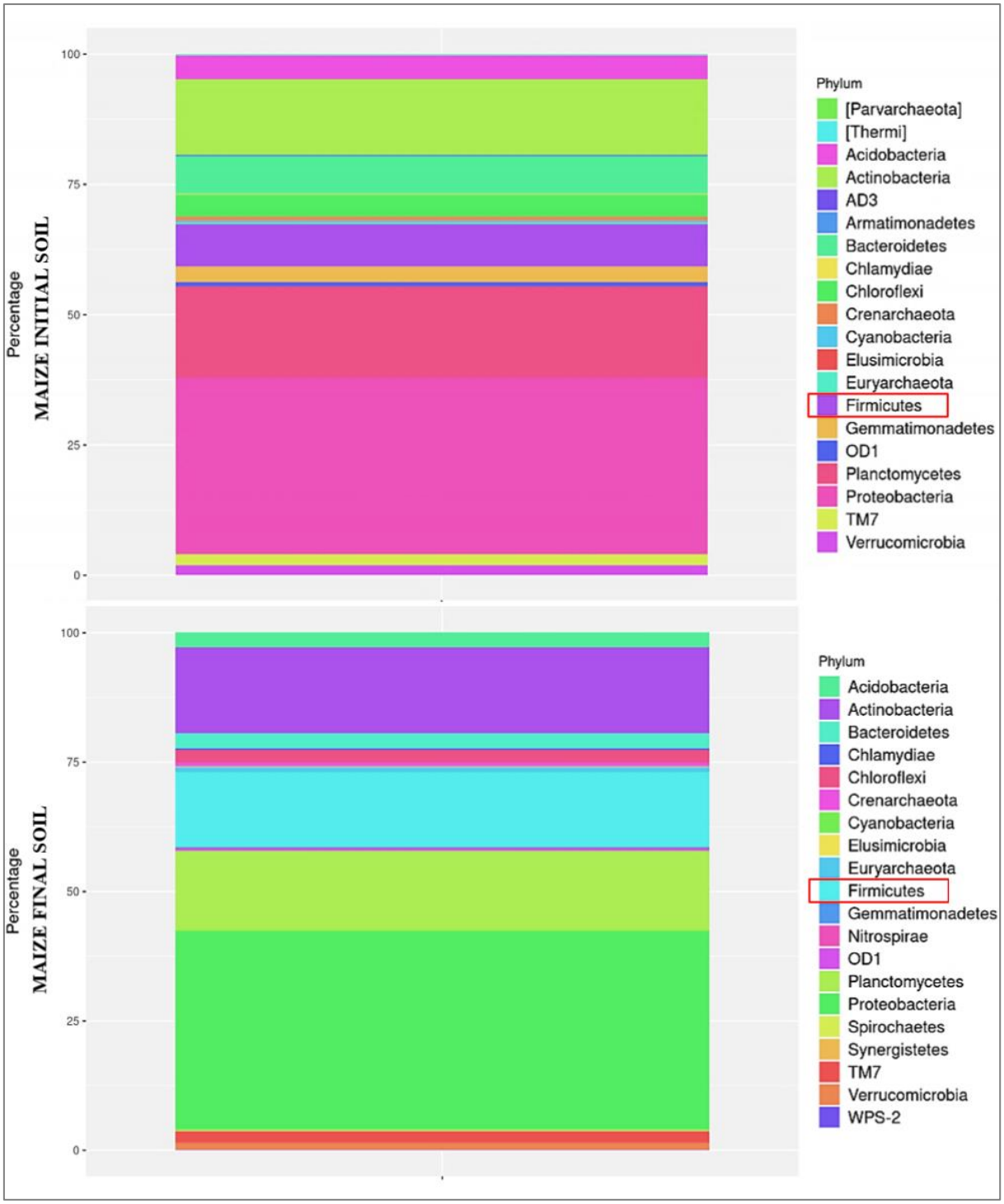


Figure 3.22: Top 20 Phyla abundance distribution, of the initial and final test soil samples in *Zea mays L* respectively.

Key Findings from the Figure 3.22:

- The heat maps in the figure display the distribution of Phylum abundance in the initial (before application of treatment) and final (after completion of treatment application throughout the cropping period) soil samples from the *Zea mays* rhizosphere.
- They reveal a higher prevalence of **Firmicutes** (Phylum) after treatment of the soils compared to the untreated initial soil, which is where all 5 of our bacterial strains belong to.
- This exhibited noteworthy colonisation of the applied bacterial treatment in the treated soil.

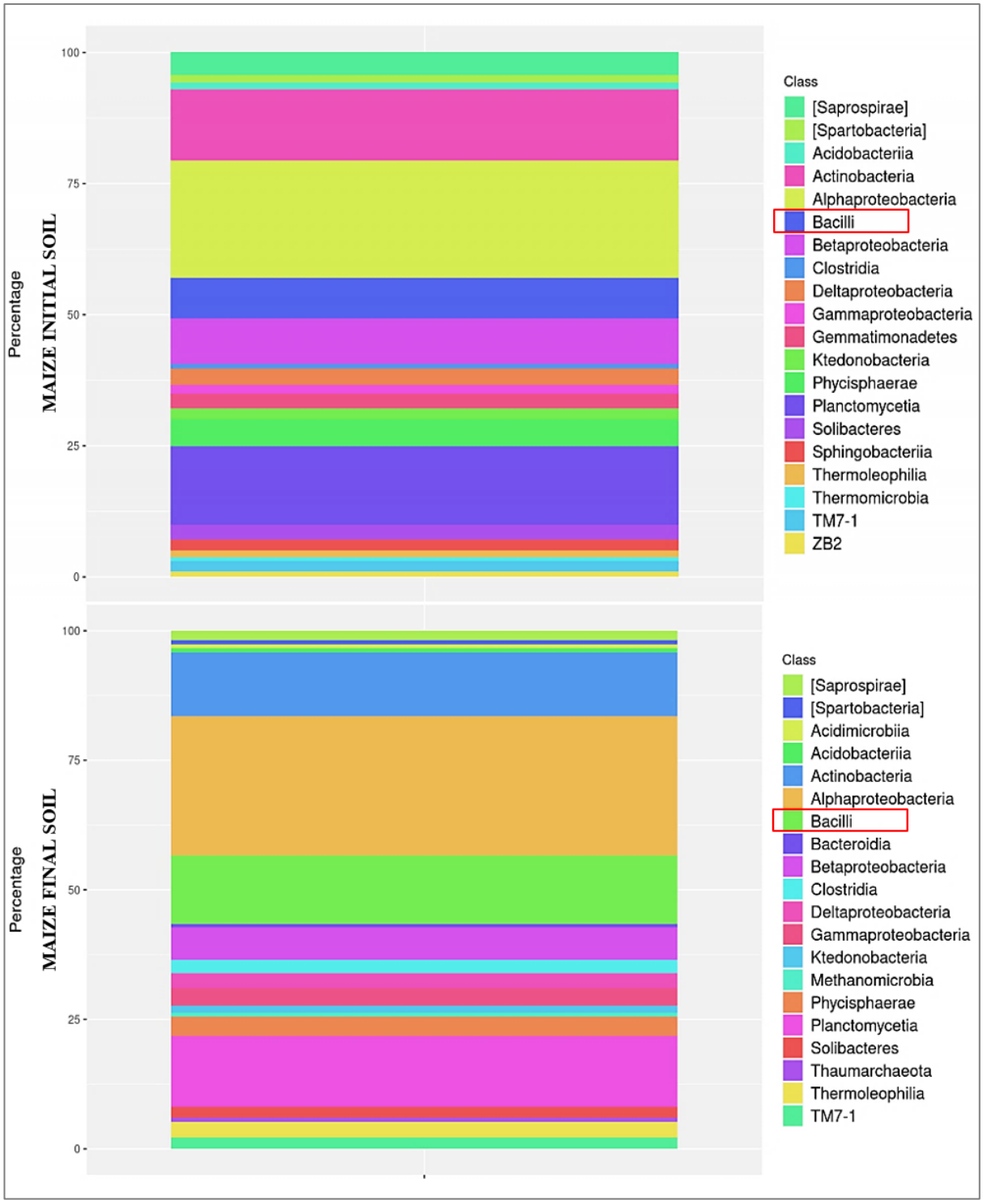


Figure 3.23: Top 20 Class abundance distribution, of the initial and final test soil samples in *Zea mays L* respectively.

Key Findings from the Figure 3.23:

- The heat maps in the figure display the distribution of Phylum abundance in the initial (before application of treatment) and final (after completion of treatment application throughout the cropping period) soil samples from the *Zea mays* rhizosphere.
- They reveal a higher prevalence of **Bacilli** (Class) after treatment of the soils compared to the untreated initial soil, which is where all 5 of our bacterial strains belong to.
- This exhibited noteworthy colonisation of the applied bacterial treatment in the treated soil.

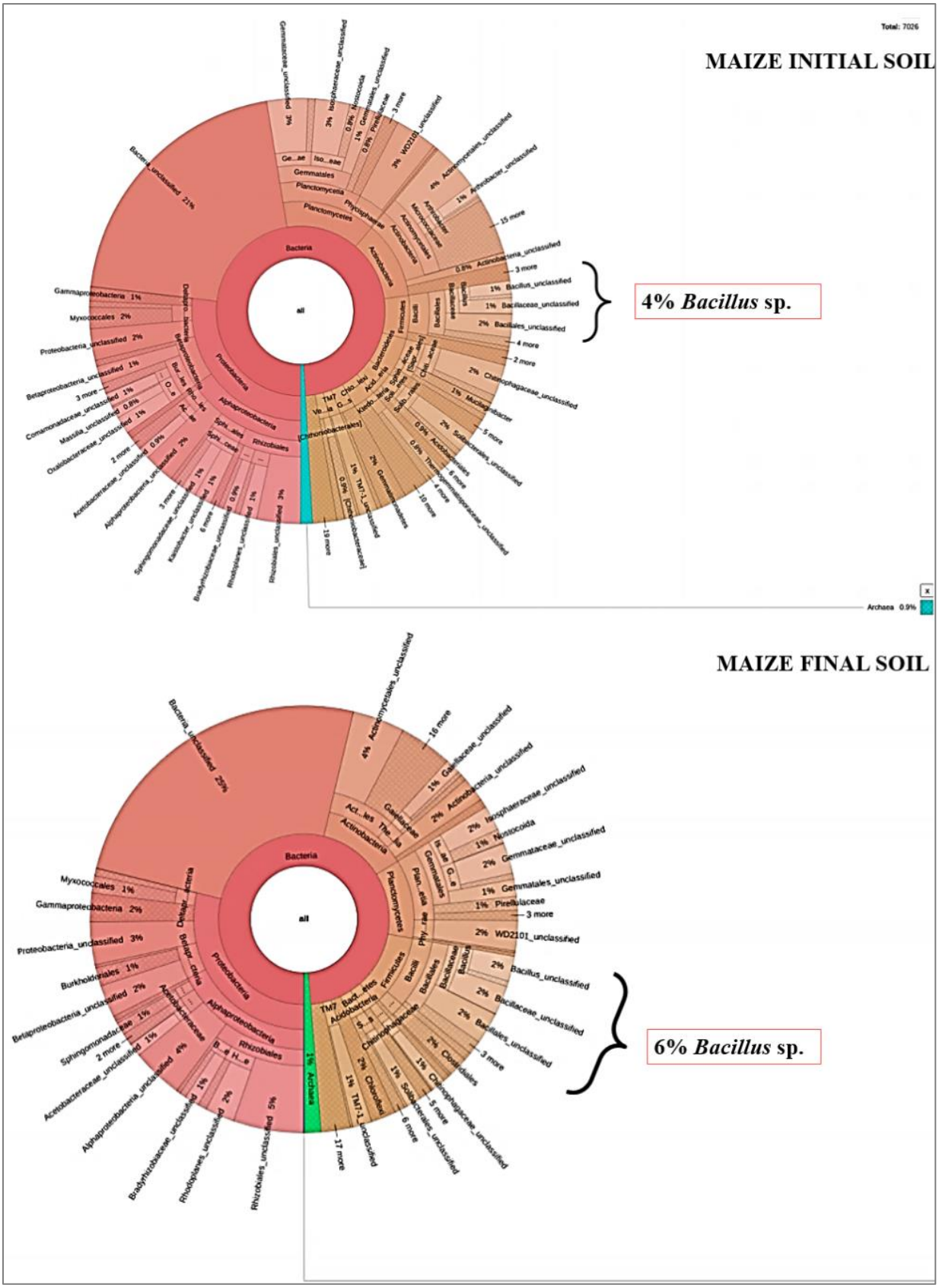


Figure 3.24: Krona graph showing the complete microbial diversity of Initial and Final test soil samples from in *Zea mays L* respectively

Key Findings from the Figures 3.24:

- The microbial diversity of the 5 soil samples was visualised using Krona charts, which displayed the proportion of Firmicutes in each sample.
- The proportion of Firmicutes in the Initial Untreated soil accounted for 4% of the overall bacterial population in the soil, but this proportion rose to 6% in the treated final soil of the *Zea mays L* rhizosphere.
- Thus, the metagenome study definitively demonstrated that the bacterial strains used as treatment in the rice rhizosphere successfully colonised the area and, as a result, alleviated stress.
- This colonisation and stress reduction can be attributed to their role as efficient plant growth promoters.

3.6. Field Application on *Oryza sativa* with Abiotic Stress.

The 5 bacterial strains are effective plant growth promoters, they being extremophiles were also expected to be capable of promoting plant growth during environmental stress. Therefore, the 5 bacteria were applied in the rhizosphere of *Oryza sativa* (Variety: PB1692), first as simple PGPR and then in presence of three stresses, Drought stress, 5% Salt stress and 30ppm Arsenic stress. The setup has been depicted in **Figure 3.25.**

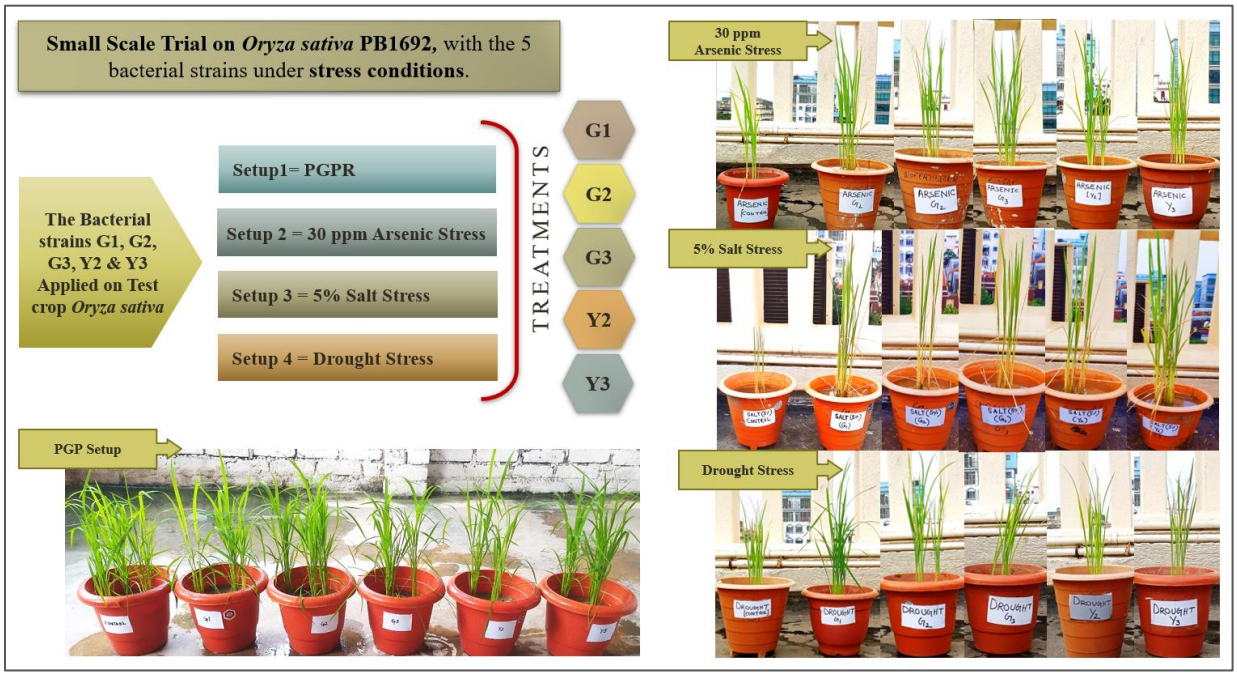


Figure 3.25: The image representing the setup of the small-scale trial on *Oryza sativa* PB1692, in stress and in normal conditions.

Key Findings from the Figure 3.25:

- This is a representative image of the setups that were conjured for the experiment on abiotic stress alleviation by the five bacterial strengths of interest.
- So along with one controlled plant growth promotion set up, there were three stress setups Drought stress, 5% Salt stress and 30ppm Arsenic stress, all of which had one stressed control to which no treatment was applied. That set of plants served as a negative control for each stress.

The vegetative parameters (plant height and number of leaves) were measured till 14th week and after 14th week, as the panicles and spikelets arrived, their numbers were also recorded for all 5 setups including no-treatment-no-stress control. In the case of vegetative parameters like plant height, in the PGP set, BRAM_G3, in case of 30ppm arsenic stress BRAM_Y2, for 5% salt stress and drought stress BRAM_Y3 showed the best results in comparison to the other bacterial treatments and corresponding controls.

On the other hand, with respect to the reproductive parameters BRAM_G3 for PGP set, BRAM_G3 and BRAM_Y2 for Arsenic 30ppm and BRAM_Y3 for 5% Salt performed the best (**Figure 3.27 and Figure 3.28**). But in this data the drought stress could not be included as the plants didn't survive till the onset of reproductive phase, as rice needs water logging conditions to survive and the variety of rice used in this experiment was not an upland rice variety.

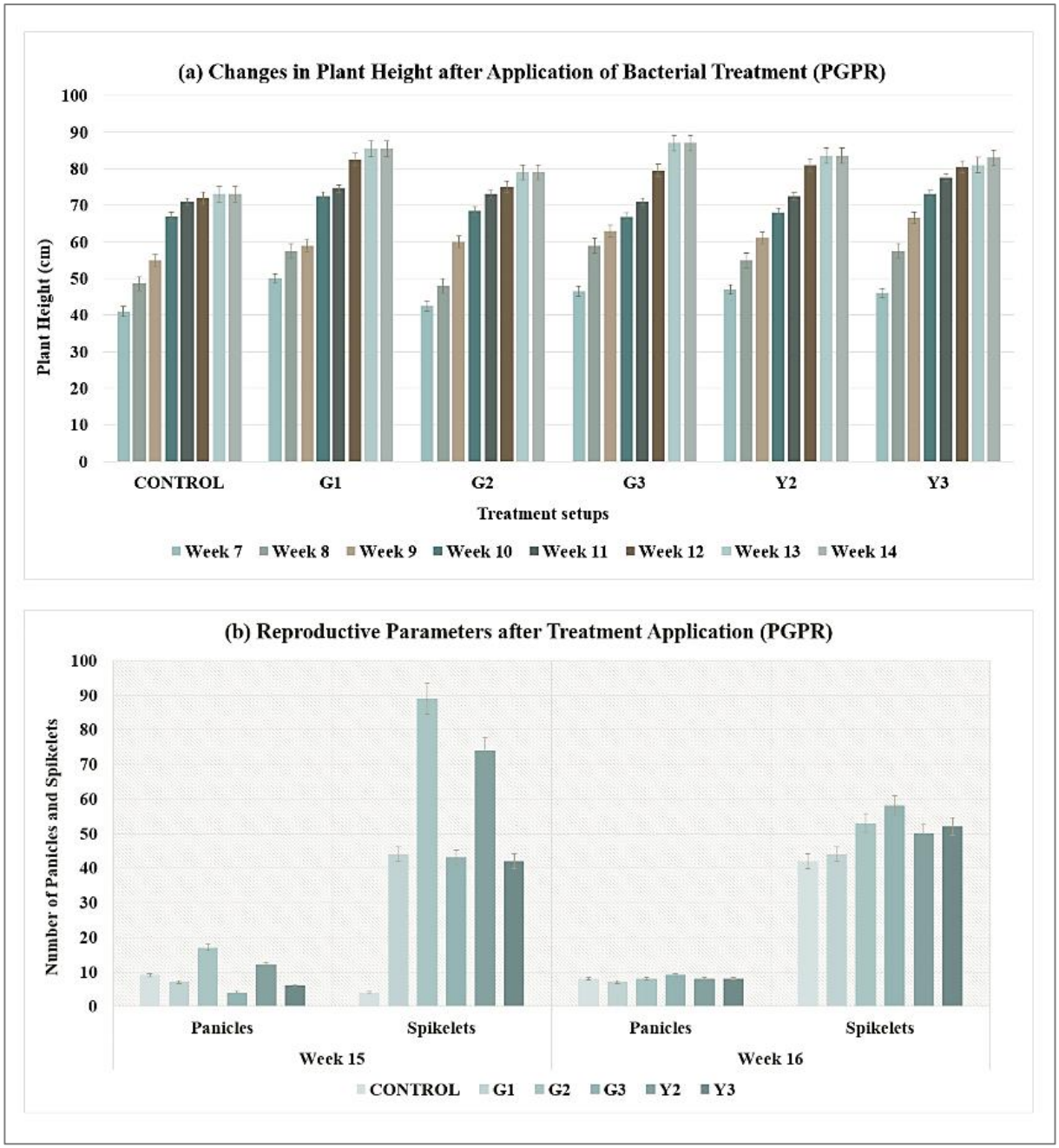


Figure 3.26: Vegetative and Reproductive Growth Parameters of (*Oryza Sativa*, Variety: PB1692) with application of PGP bacteria and no stress (PGP setup).

Key Findings from the Figure 3.26:

- The figure represents the vegetative parameter of plant height across the weeks after application of bacterial treatment in the PGPR setup.
- In case of plant height in the PGPR setup it could be observed that BRAM_G3 performed the best closely followed by BRAM_G1.

- For the reproductive parameters in the PGPR setup such as spikelets and pinnacles again BRAM_G3 showed the best results.

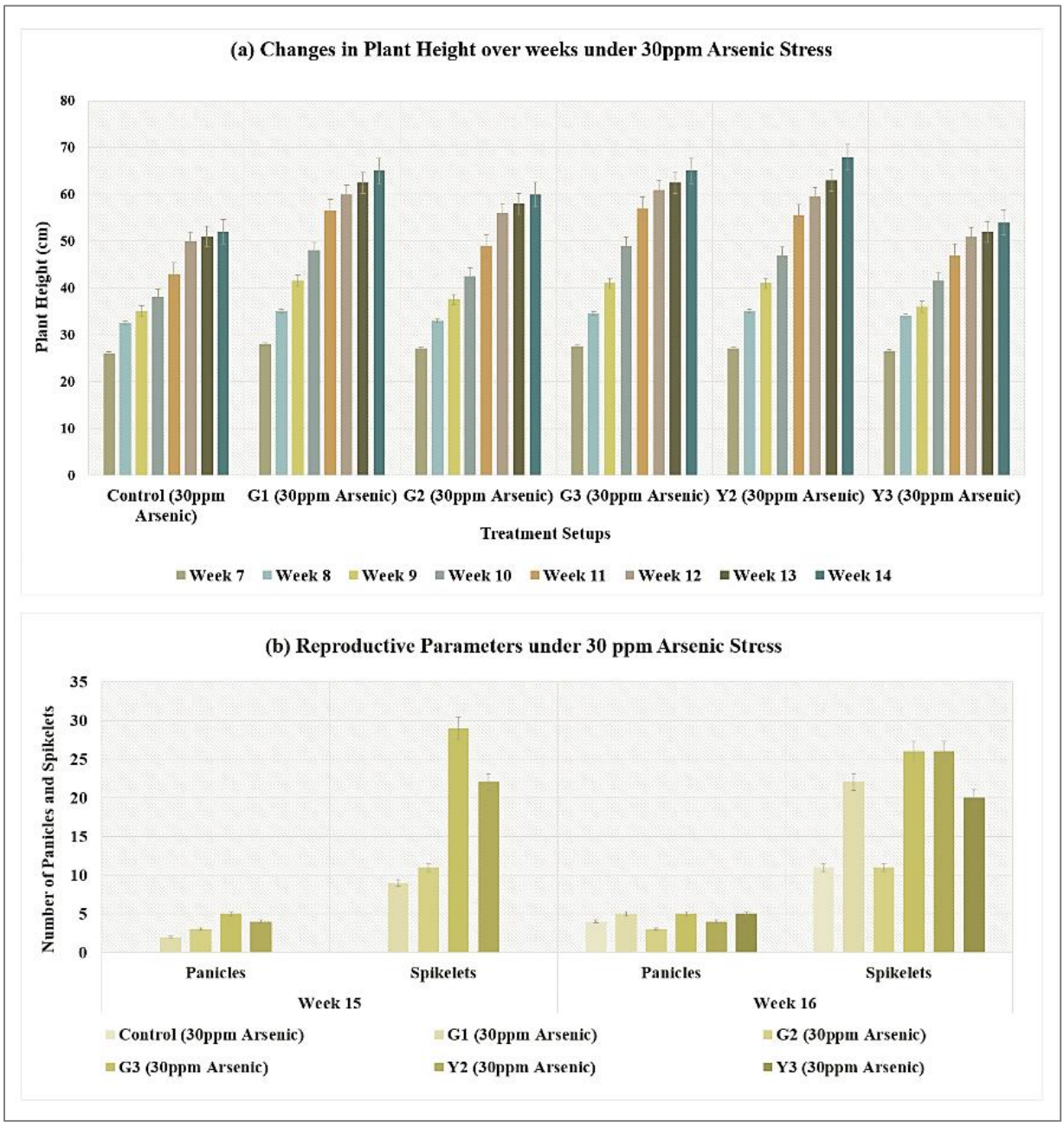


Figure 3.27: Vegetative and Reproductive Growth Parameters of (*Oryza Sativa*, Variety: PB1692) under 30ppm Arsenic Stress setup.

Key Findings from the Figure 3.27:

- The figure represents the vegetative parameter of plant height across the weeks after application of bacterial treatment in the 30ppm arsenic stress setup.
- In case of plant height in the 30ppm arsenic stress setup it could be observed that BRAM_Y2 performed the best closely followed by BRAM_G1.
- For the reproductive parameters in the PGPR setup such as spikelets and pinnacles again BRAM_G3 and BRAM_Y2 showed the best results closely followed by BRAM_G1.

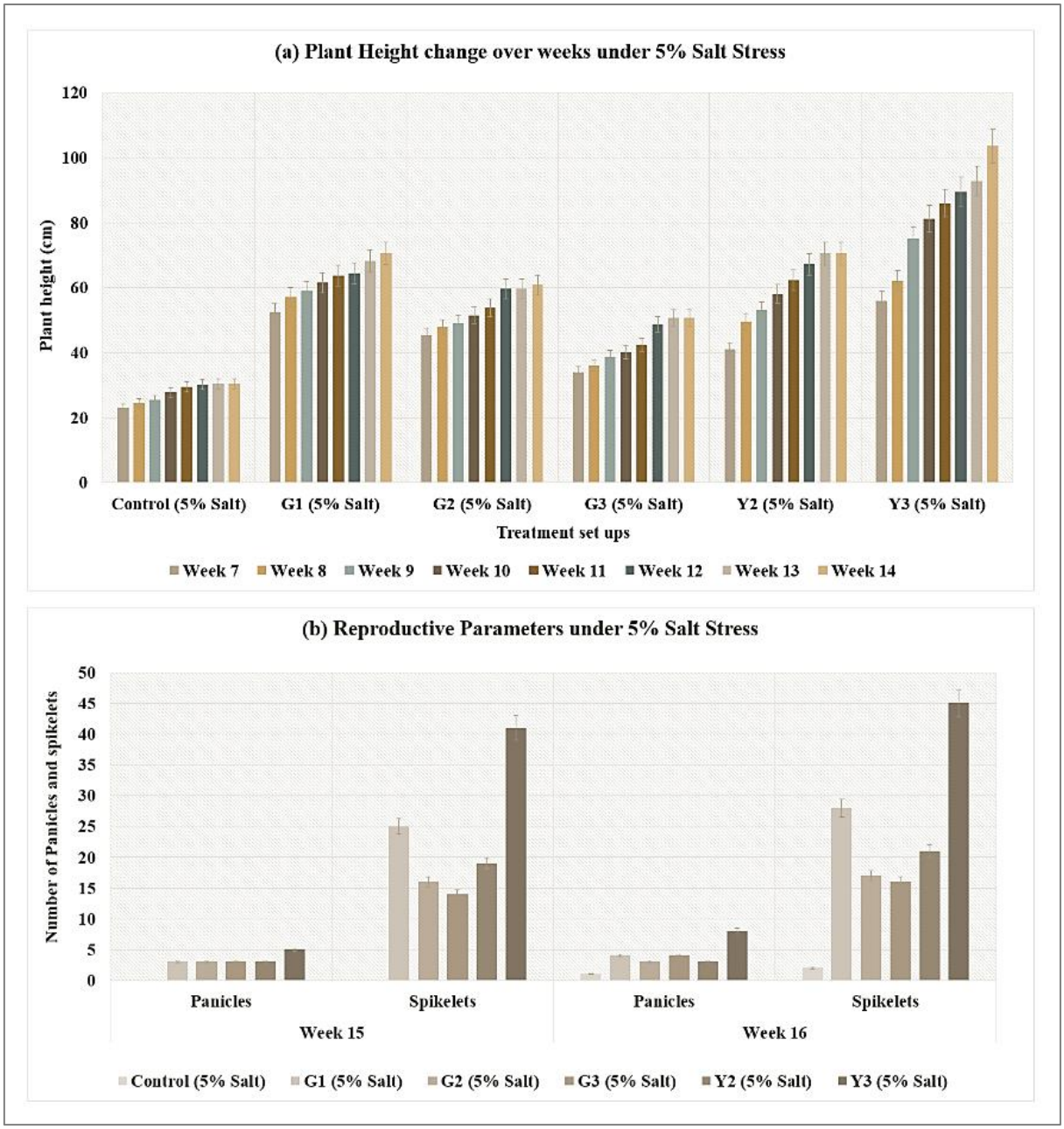


Figure 3.28: Vegetative and Reproductive Growth Parameters of (*Oryza Sativa*, Variety: PB1692) under 5% Salt Stress setup.

Key Findings from the Figure 3.28:

- The figure represents the vegetative parameter of plant height across the weeks after application of bacterial treatment in the 5% salt stress setup.
- In case of plant height in the 5% salt stress setup it could be observed that BRAM_Y3 performed the best.

- For the reproductive parameters in the 5% salt stress setup such as spikelets and pinnacles again BRAM_Y3 showed the best results closely followed by BRAM_G1.

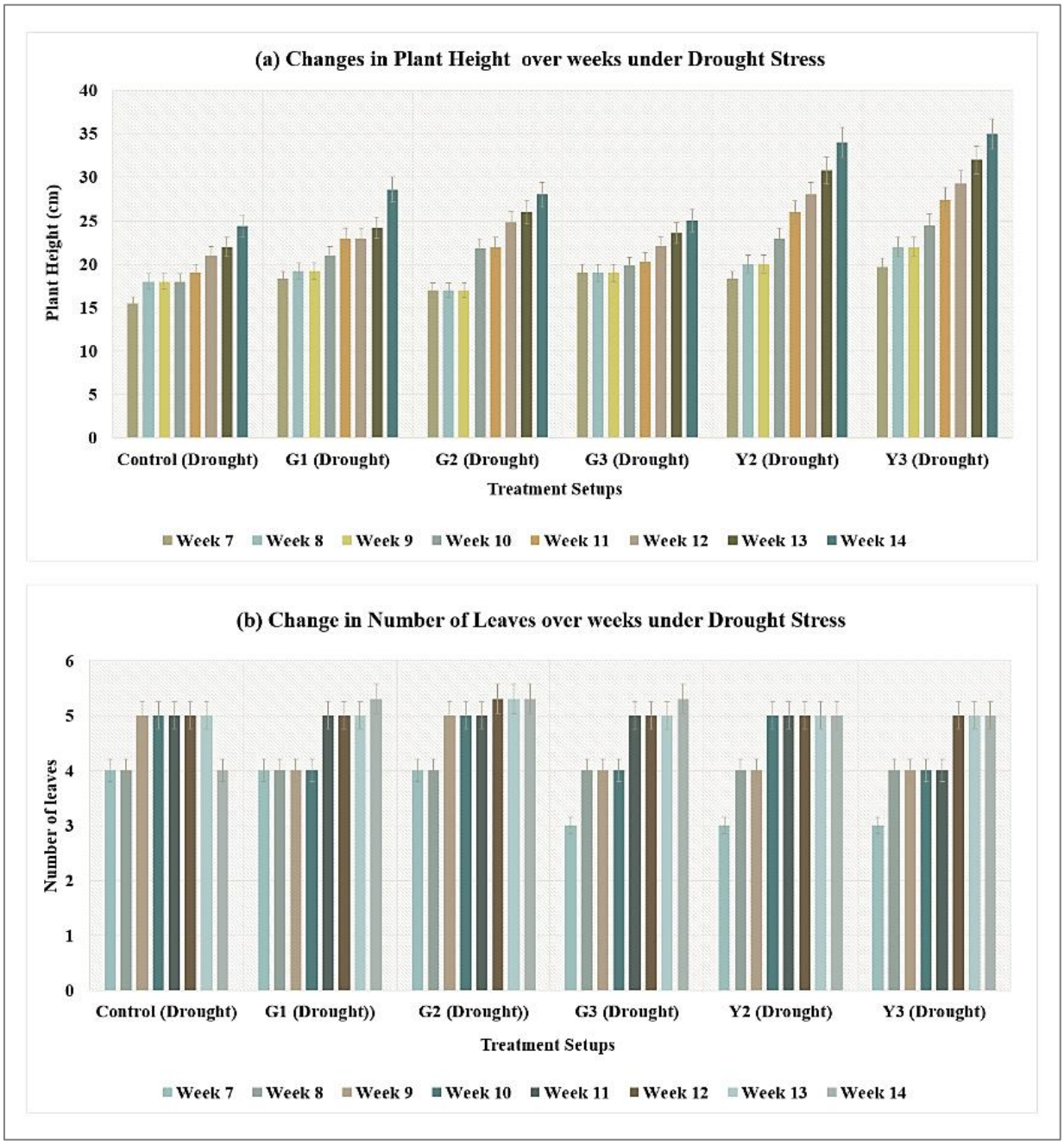


Figure 3.29: Vegetative and Reproductive Growth Parameters of (*Oryza Sativa*, Variety: PB1692) under Drought Stress setup.

Key Findings from the Figure 3.29:

- The figure represents the vegetative parameter of plant height across the weeks after application of bacterial treatment in the drought stress setup.
- In case of plant height in the drought stress setup it could be observed that BRAM_Y2 performed the best closely followed by BRAM_G1.
- For drought stress the plants didn't move to the reproductive stage the number of leaves has been shown in (b) where not much variations were observed amongst the treated and untreated control setups.

Being a staple food crop in India and throughout the world, yield parameters are the most essential for paddy cultivation. The yield parameters, "Total yield", "Percent grain filling" and "100 seed weight" were recorded and have been represented in **Figure 3.**. It can be easily deciphered from the figure, that, the PGP set had a much higher yield in comparison to the stressed setups in accordance to our expectations. The treatment setup showed better results in all stress-setups with respect to their corresponding controls though the total yields were much less than the PGP setup. For PGP set and As30ppm stress, BRAM_G3, and BRAM_G1 and BRAM_Y3 showed the maximum number of seeds. But, the interesting thing to note was that, though the yield quantity differed widely in the stress and PGP setups, parameters like percent grain filling and 100 seed weight showed the true marvel of the bacterial treatments in stressed conditions which were almost at par with the no-stress PGP setups. In case of percent grain filling, BRAM_Y2 in PGP set and 5% salt set, BRAM_G1 in As30ppm set were the highest, whereas for 100 seed weight, BRAM_G2 in PGP set and 5% salt set, BRAM_G1 and BRAM_G3 in As30ppm set showed better results.

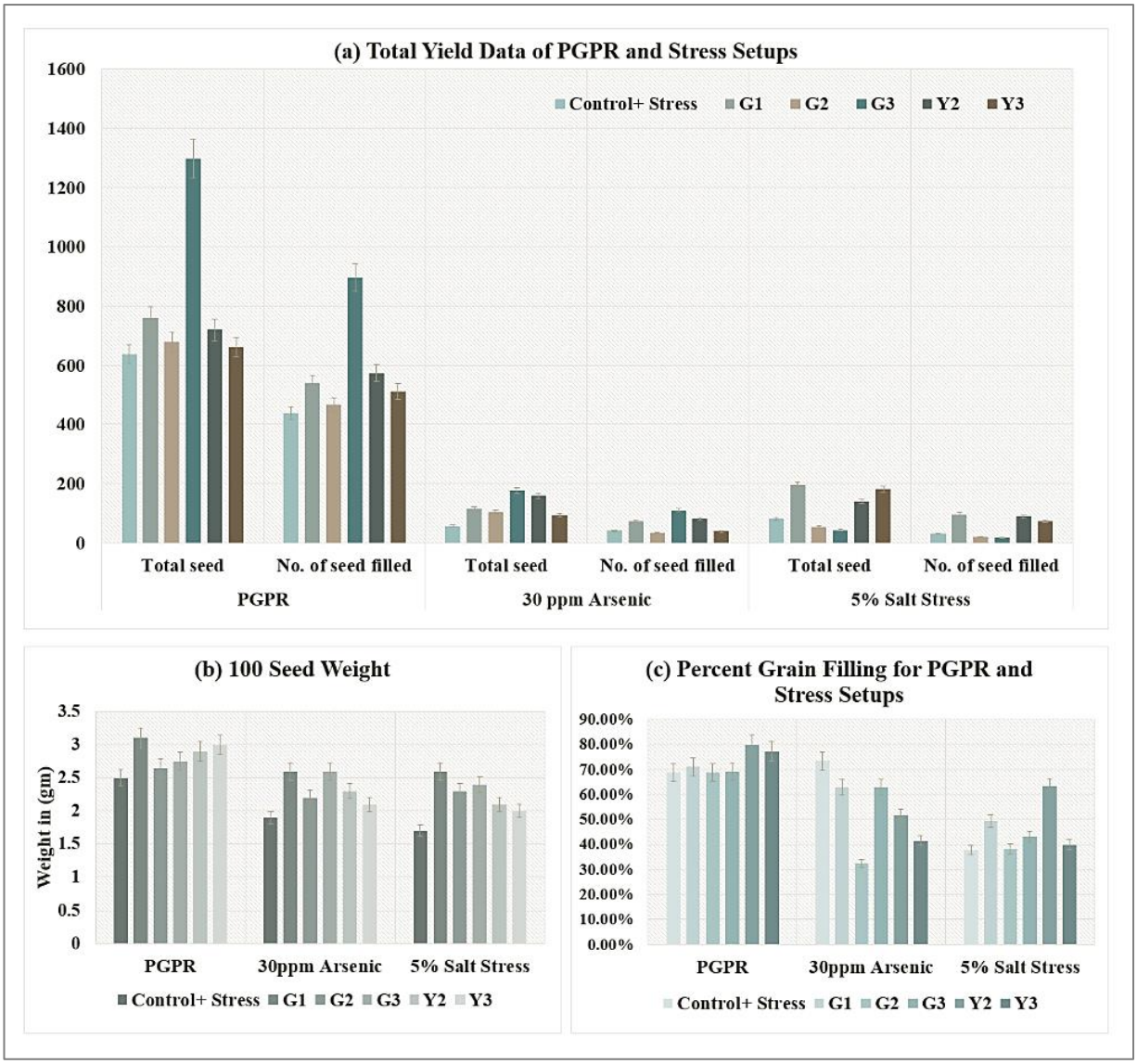


Figure 3.30.: Rice (*Oryza Sativa*, Variety: PB1692) Yield Parameters of PGP, Arsenic (30ppm), Salt (5%) and Drought Stress setups

Key Findings from the Figure 3.30:

- The yield parameters, "Total yield", "Percent grain filling", and "100 seed weight", were measured and are displayed in the figure.
- The figure clearly indicates that the PGP set had a significantly higher yield compared to the stressed setups, as it had been anticipated.
- The treatment setup exhibited superior outcomes in all stress-setups compared to their respective controls, albeit the overall yields were much lower than those of the PGP setup.

- Among the PGP set and As30ppm stress conditions, BRAM_G3, BRAM_G1, and BRAM_Y3 exhibited the highest number of seeds.
- However, it is worth noting that while the yield quantity varied significantly between the stress and PGP setups, certain parameters such as percent grain filling and 100 seed weight demonstrated the remarkable effectiveness of the bacterial treatments under abiotic stress conditions, which were nearly equal to the no-stress PGP setups.
- Regarding percent grain filling, the highest values were seen in BRAM_Y2 in the PGP set and the 5% salt set, while for 100 seed weight, superior results were obtained in BRAM_G2 in the PGP set and the 5% salt set, as well as in BRAM_G1 and BRAM_G3 in the As30ppm set.
- The results indicate significant alleviation by polyextremophilic bacterial treatment intervention.

Further on, the Plant pigments were also analysed, like chlorophyll a, b and total chlorophyll, as because degradation of chlorophyll is one of the primary responses of plants subjected to abiotic stresses **(Figure 3.).** The best chlorophyll concentrations were found to be highest in BRAM_G1 for the PGP, 5% salt and 30ppm Arsenic setups whereas in case of drought setup, BRAM_G2 showed highest chlorophyl concentrations.

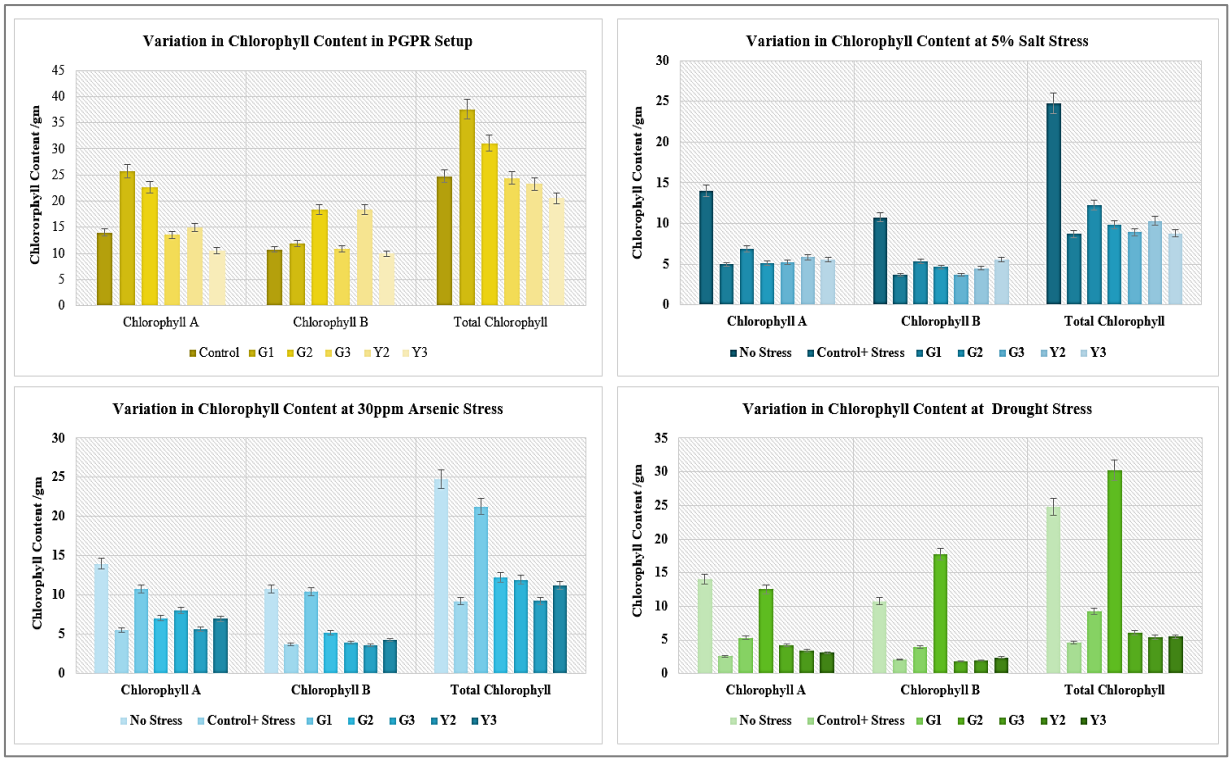


Figure 3.31: Plant Pigment data of (*Oryza Sativa*, Variety: PB1692) under PGP, Arsenic (30ppm), Salt (5%) and Drought Stress setups.

Key Findings from the Figure 3.31:

- In the PGPR setup the best chlorophyll content was observed in BRAM_G1 followed by BRAM_G2.
- In stress setups, the no-stress control consistently had the highest chlorophyll content. This is because abiotic stress significantly damages the chlorophyll content of the plant. However, when compared to the stressed control, in the 5% Salt Stress setups, BRAM_G1 and BRAM_Y2 showed the best results in-terms of chlorophyll content.
- For 30ppm arsenic stress setup, the highest chlorophyll content was seen in BRAM_G1, followed by BRAM_G2 and the other bacterial treatments. However, all of the treated setups performed better than the untreated stress control.
- Finally, in the drought stress setting, the highest chlorophyll content was reported in BRAM_G2, followed by BRAM_G1.

To analyse the effect of bacterial treatments in alleviation of abiotic stress, antioxidant enzymes like, Malondialdehyde (MDA), Guaiacol peroxidase (GPA) and Catalase (CAT) and non-enzymatic antioxidants like Carotenoids were also measured in all the stress setups with bacterial treatment in comparison to both positive control (no stress) and negative control (stress, with no treatment). Decrease in MDA is representative of stress alleviation in terms of decreased lipid peroxidation, a surge of which is unfavourable for plant growth and survival. So, the minimum lipid peroxidation was observed in all the treated setups for the 3 stresses and at par with the no-stress control. For Guaiacol peroxidase (GPX) and Catalase (CAT), their increase helps in ROS scavenging by the plant formed as a response to abiotic stress. So higher the GPX or CAT, greater is stress alleviation. Both these enzymes were found in elevated levels in the treated setups, with respect to both no-stress and stress controls. Carotenoid, an antioxidant, also helps in scavenging of free radicals, but as it is a pigment as well, it is present in much higher concentrations in the no-stress control and is highly depleted in the stress-no-treatment control, and comparatively higher in treated stress setups.

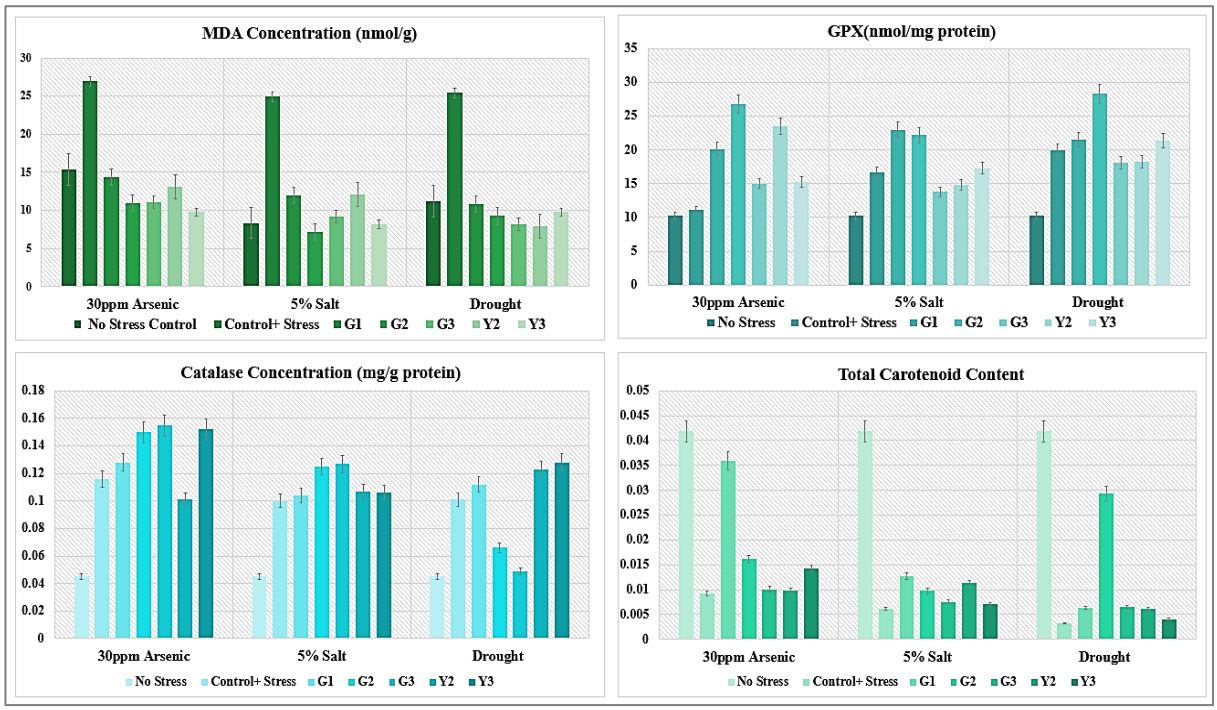


Figure 3.32: Variations in Antioxidant production (both enzymatic and non-enzymatic) of (*Oryza Sativa*, Variety: PB1692) under PGP, Arsenic (30ppm), Salt (5%) and Drought Stress setups

Key Findings from the Figure 3.32:

- The study examined the impact of bacterial treatments on abiotic stress, focusing on antioxidant enzymes like Malondialdehyde (MDA), Guaiacol peroxidase (GPA), and Catalase (CAT) and non-enzymatic antioxidants like Carotenoids.
- The results showed that bacterial treatment led to a decrease in lipid peroxidation, which increases in abiotic stress and is detrimental to plant growth and survival.
- Increases in GPX and CAT enzymes were observed in the bacteria treated stress setups, which helped in scavenging reactive oxygen species (ROS) produced due to abiotic stress and thus contributed to stress alleviation.
- Carotenoids, a non-enzymatic antioxidant, were found to be present in higher concentrations in the no-stress control and depleted in the stress-no-treatment control again exhibiting successful stress alleviation by bacterial treatment intervention.

An analysis of variance (ANOVA), was performed on the reproductive parameter dataset for the four treated setups, the plant growth promotion with no stress, drought, 5% salt and arsenic 30ppm. Since the p-values for all four were less than the significance level (0.05), the null hypothesis was rejected and it could be concluded that there is significant evidence to suggest that at least one group mean is different from the others and thus the differences between treatments were statistically significant. **Tables 3.6 to 3.9** represent the ANOVA tables for all the four setups on their reproductive parameters.

Anova: Single Factor						
	PGPR SET UP (SUMMARY)					
Groups	***Count***	***Sum***	***Average***	***Variance***		
Week 15 Panicles	6	55	9.1666666	22.166667		
Week 15 Spikelets	6	296	49.33333	871.86667		
Week 16 Panicles	6	48	8	0.4		
Week 16 Spikelets	6	299	49.8333333	35.36667		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	10090.83	3	3363.6111	14.470256	3.0439E-05	3.0983912
Within Groups	4649	20	232.45			
Total	14739.833	23				

Table 3.6: ANOVA Table for Yield parameters of Rice PGP Data set.

Key Findings from the Table 3.6:

- The ANOVA was carried out to determine the significance of the difference between the treated and untreated setups.
- For the PGPR set up, the F-value (14.470256) is significantly higher than the critical F-value (3.098391). Additionally, the P-value (3.0439E-05) is much smaller than the commonly used significance level of 0.05.
- Since the P-value is very low and less than 0.05, we reject the null hypothesis that all group means are equal. This means there is strong evidence that there is a significant difference in the means of at least one pair of groups.
- This means that it can be concluded that significant variation is observed between treated and untreated plant sets.

Anova: Single Factor						
DROUGHT SET UP SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
Week 7 Plant Height	6	107.8	17.96666667	2.262666667		
Week 11 Plant Height	6	137.7	22.95	10.527		
Week 14 Plant Height	6	175	29.16666667	19.83066667		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	377.8411111	2	188.9205556	17.37449035	0.000124395	3.682320344
Within Groups	163.1016667	15	10.87344444			
Total	540.9427778	17				

Table 3.7: ANOVA Table for Yield parameters of Rice Drought Stress Data set

Key Findings from the Table 3.7:

- The ANOVA was carried out to determine the significance of the difference between the treated and untreated setups.
- For the drought stress setup, the ANOVA was carried out on plant height. The F-value (17.374) is significantly higher than the critical F-value (3.682320). Additionally, the P-value (0.000124) is much smaller than the commonly used significance level of 0.05.
- Since the P-value is very low and less than 0.05, we reject the null hypothesis that all group means are equal. This means there is strong evidence that there is a significant difference in the means of at least one pair of groups.
- This means that it can be concluded that significant variation is observed between treated and untreated plant sets.

Anova: Single Factor						
SALT (5%) SETUP SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
Week 15 Panicles	6	17	2.833333333	2.566666667		
Week 15 Spikelets	6	115	19.16666667	182.9666667		
Week 16 Panicles	6	23	3.833333333	5.366666667		
Week 16 Spikelets	6	129	21.5	205.1		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1753.3333	3	584.4444	5.9037	0.004684204	3.098391212
Within Groups	1980	20	99			
Total	3733.333333	23				

Table 3.8: ANOVA Table for Yield parameters of Rice Salt Stress Data set

Key Findings from the Table 3.8:

- The ANOVA was carried out to determine the significance of the difference between the treated and untreated setups.
- For the 5% salt stress setup, the F-value (5.9037) is significantly higher than the critical F-value (3.098391). Additionally, the P-value (0.004684204) is much smaller than the commonly used significance level of 0.05.
- Since the P-value is very low and less than 0.05, we reject the null hypothesis that all group means are equal. This means there is strong evidence that there is a significant difference in the means of at least one pair of groups.
- This means that it can be concluded that significant variation is observed between treated and untreated plant sets.

Anova: Single Factor						
ARSENIC (50PPM) SETUP SUMMARY						
Groups	***Count***	***Sum***	***Average***	***Variance***		
Week 15 Panicles	6	14	2.333333333	4.266666667		
Week 15 Spikelets	6	71	11.83333333	137.3666667		
Week 16 Panicles	6	26	4.333333333	0.666666667		
Week 16 Spikelets	6	116	19.33333333	47.06666667		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1081.125	3	360.375	7.61221	0.001380928	3.098391212
Within Groups	946.8333	20	47.34166667			
Total	2027.958333	23				

Table 3.9: ANOVA Table for Yield parameters of Rice Arsenic Stress Data set

Key Findings from the Table 3.9:

- The ANOVA was carried out to determine the significance of the difference between the treated and untreated setups.
- For 30ppn arsenic stress setup, the F-value (7.61221) is significantly higher than the critical F-value (3.098391). Additionally, the P-value (0.001380928) is much smaller than the commonly used significance level of 0.05.
- Since the P-value is very low and less than 0.05, we reject the null hypothesis that all group means are equal. This means there is strong evidence that there is a significant difference in the means of at least one pair of groups.
- This means that it can be concluded that significant variation is observed between treated and untreated plant sets.

The 16s V3-V4 metagenome study of the final soils after 16 weeks showed promising colonisation of the inoculated bacteria. The NCBI submission was processed and the following accession were generated.

BioProject: PRJNA1097495: Rice Rhizosphere Soil Metagenome

- SRA Accession: **SRR28582733**; BioSample: **SAMN40869740**: Drought setup Rice Rhizosphere Soil (TaxID: 410658)
- SRA Accession: **SRR28582734**; BioSample: **SAMN40869718**: Salt (5%) Treated Rice Rhizophere Soil (TaxID: 410658)
- SRA Accession: **SRR28582735**; BioSample: **SAMN40869717**: Arsenic (30ppm) Treated Rice Rhizosphere Soil (TaxID: 410658)
- SRA Accession: **SRR28582736**; BioSample: **SAMN40869700**: PPGP Treated Rice Rhizosphere Soil (TaxID: 410658)
- SRA Accession: **SRR28582737**; BioSample: **SAMN40869697**: Control Untreated Rice Rhizospheric Soil (TaxID: 410658)

The 5 soil samples were of the Control-Untreated-no stress-soil (CUTS), Arsenic 30ppm treated soil (AS30TS), Drought treated soil (DRTS), Salt 5% treated soil (S5TS) and Plant growth promotion setup treated soil (PGPTS). The heat maps of Phylum and class abundance in the 5 soil samples **(Figure 3.33)**, show an increase in the abundance of *Firmicutes* (Phylum) and *Bacilli* (Class), in the treated soils in comparison to the untreated control, to which all our 5 bacterial strains belong. Similar observations are noted in the heatmaps of Order and Family, where there is an increase *Bacillales* (Order) *and Bacillaceae* (Family) **(Figure 3.34).** When the heat map distribution of the top 20 genus in the soil samples were analysed, there was an increase in *Bacillus* and *Brevibacillus* which were the genus, of the inoculated bacterial stains **(Figure 3.5)**. The microbial diversity of the 5 soil samples was represented in Krona charts that showed the percentage of Firmicutes of the soil samples. The percentage of Firmicutes in the Control soil (CUTS) was found to be 2% of the total soil bacterial flora, which increased to 3% in the Plant growth promotion set up (PGPTS). It further increased to 10% in the arsenic 30ppm stress setup, it was found to be 4% in the drought stress setup (DRTS) as well as in the 5% salt stress setup (S5TS) **(Figures 3.36 to 3.38).** This conclusively showed the colonisation of the bacterial strains applied as treatment to the rice rhizosphere and by extension, the stress alleviation, being a result of their effect as polyextremophilic plant growth promoter.

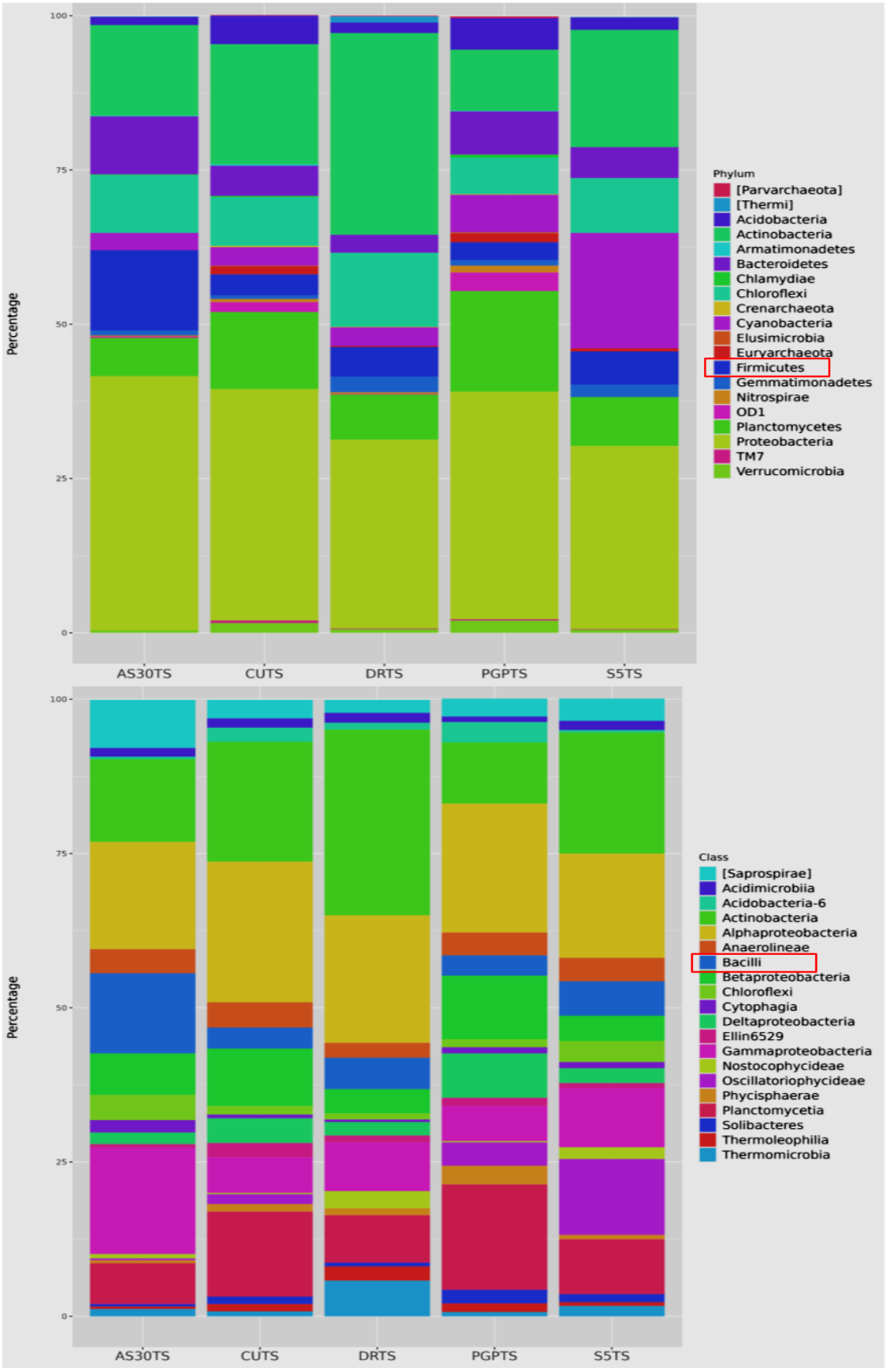


Figure 3.33: Top 20 Phyla and Class abundance distribution, of the 5 soil samples respectively.

Key Findings from the Figure 3.33:

- The heat maps in the figure display the distribution of Phylum and class abundance in the 5 soil samples.
- They reveal a higher prevalence of Firmicutes (Phylum) and Bacilli (Class) in the treated soils compared to the untreated control, which is where all 5 of our bacterial strains belong to.
- This exhibited noteworthy colonisation of the applied bacterial treatment in the treated setups.

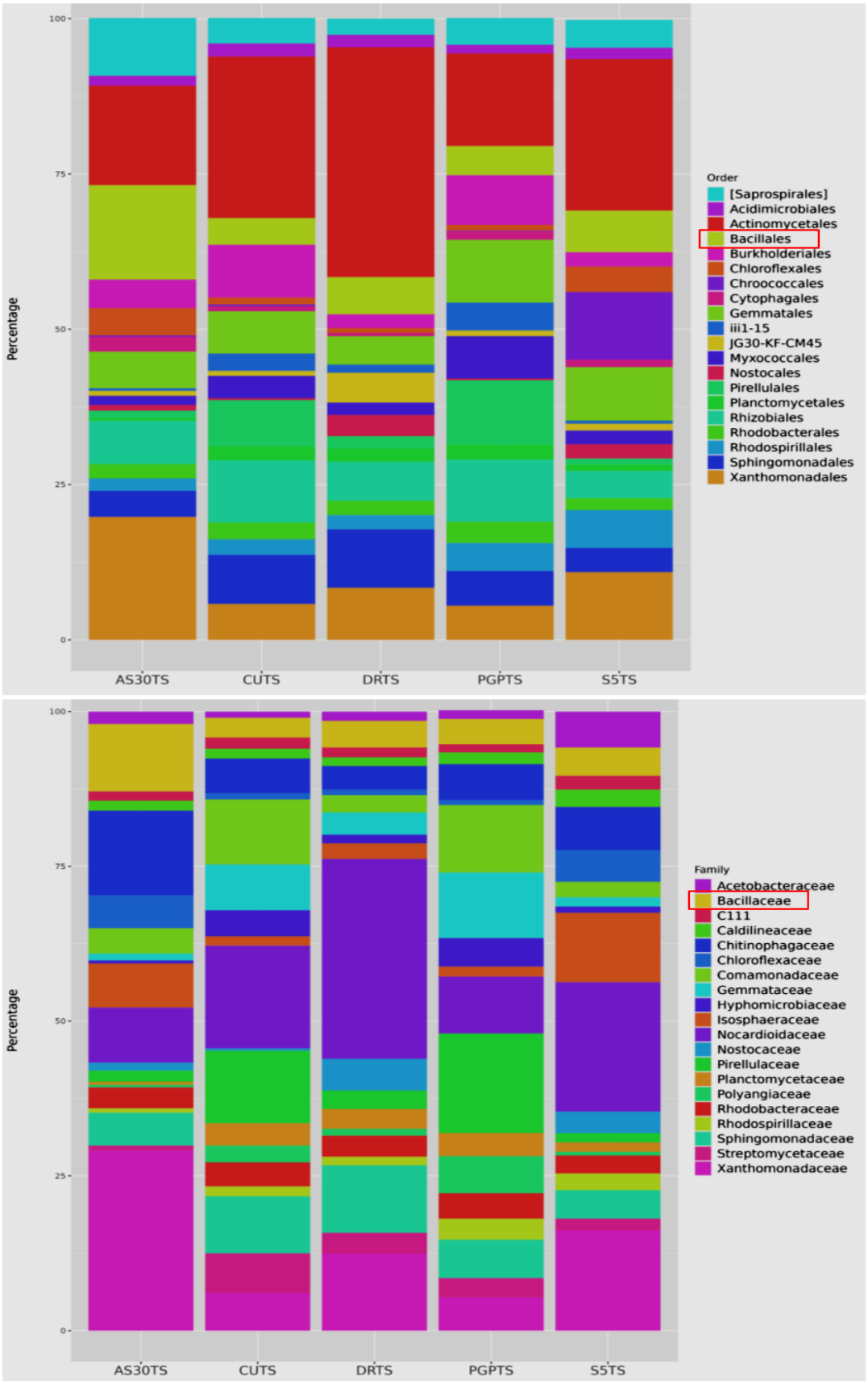


Figure 3.34 Top 20 Order and family abundance distribution of the 5 soil samples respectively.

Key Findings from the Figure 3.34:

- The heat maps in the figure display the distribution of order and family abundance in the 5 soil samples.
- They reveal a higher prevalence of Bacillales (Order) and Bacillaceae (Family) in the treated soils compared to the untreated control, which is where all 5 of our bacterial strains belong to.
- This exhibited noteworthy colonisation of the applied bacterial treatment in the treated setups.

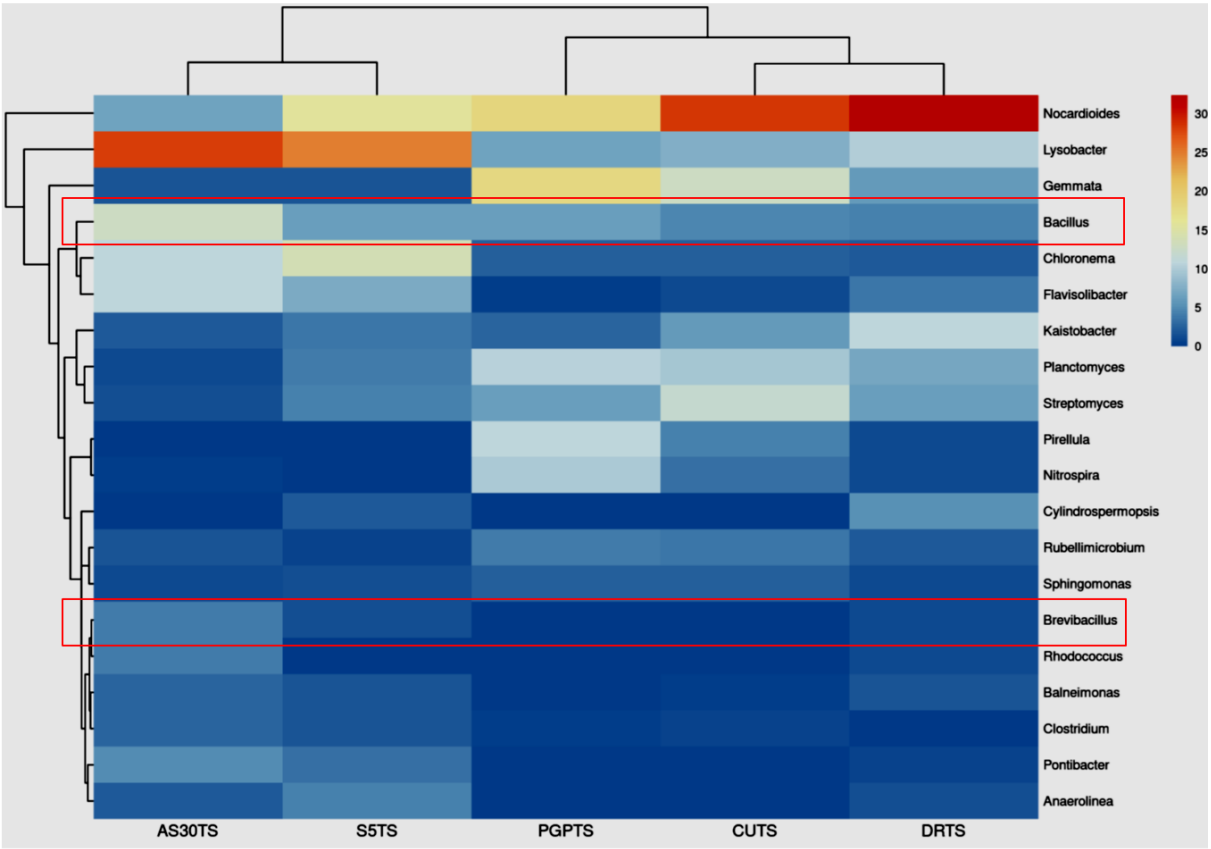


Figure 3.35: Heatmap representing distribution of top 20 Genus present in the 5 soil samples respectively.

Key Findings from the Figure 3.35:

- Upon analysing the heat map distribution of the top 20 genera in the soil samples, it was observed that there was an elevation in the abundance of *Bacillus* and *Brevibacillus*.
- These two genera corresponded to the bacterial strains that were intentionally introduced into the samples.
- This exhibited noteworthy colonisation of the applied bacterial treatment in the treated setups.

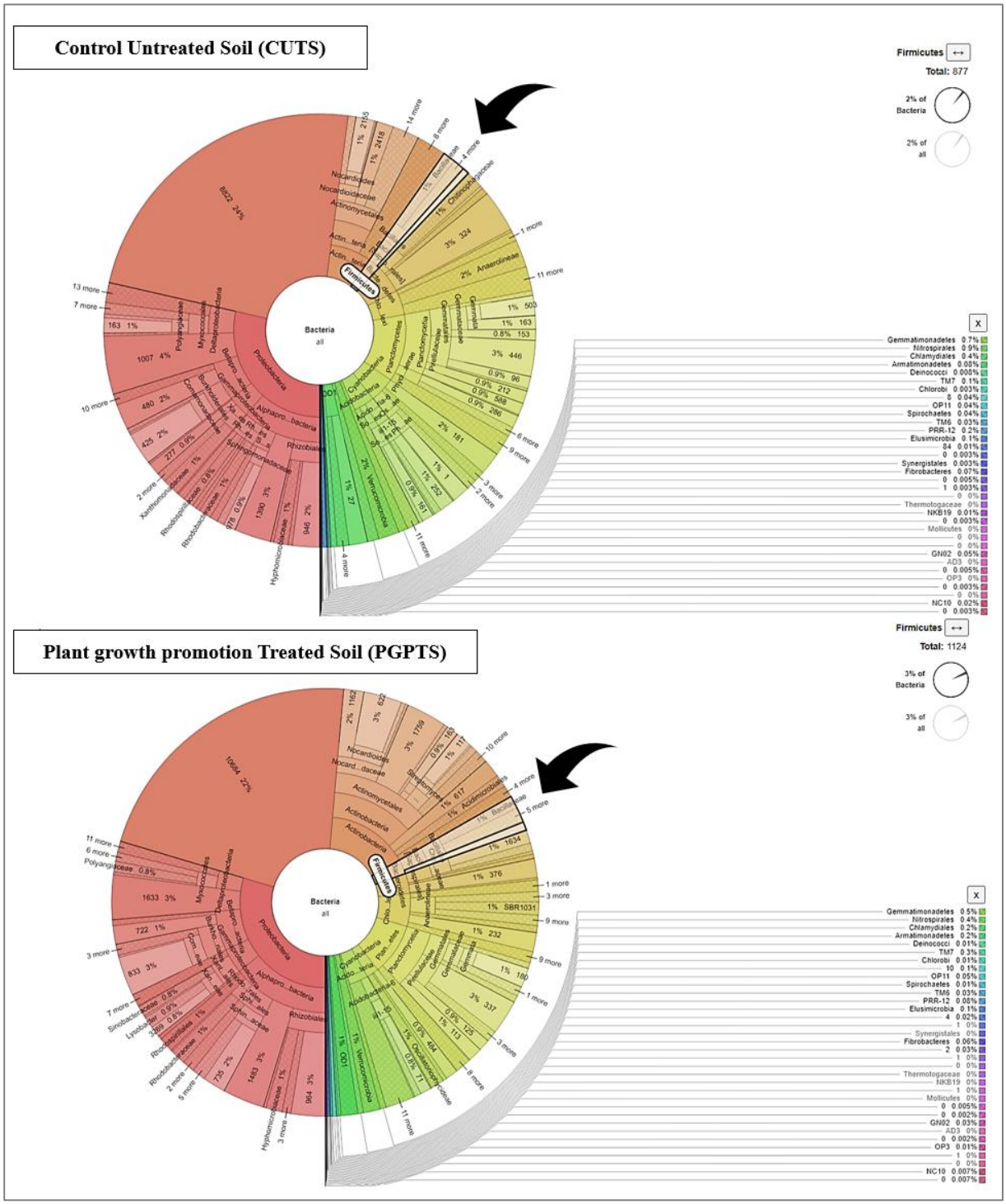


Figure 3.36: Krona graph showing the complete microbial diversity of Samples CUTS and PGPTS.

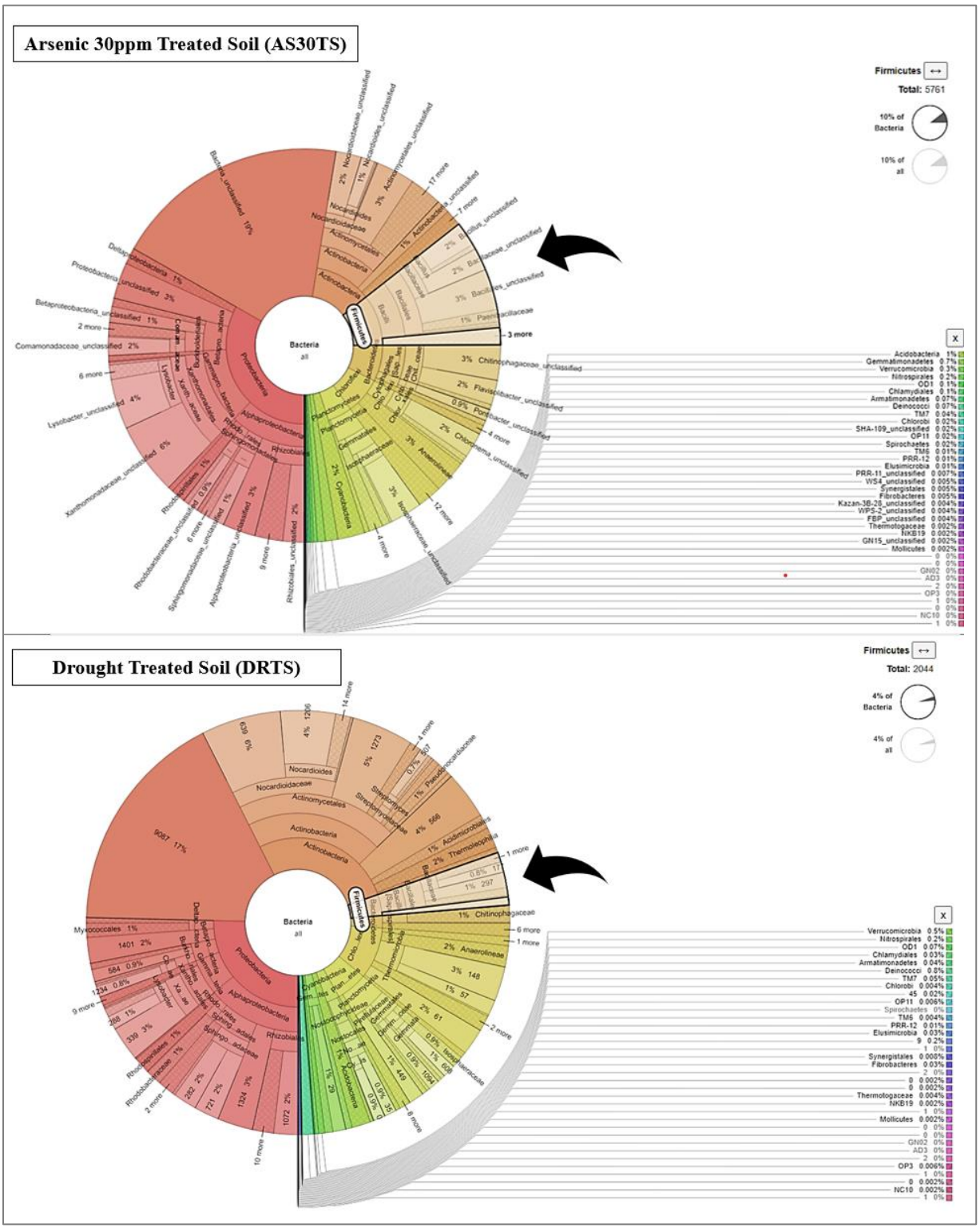


Figure 3.37: Krona graph showing the complete microbial diversity of Samples AS30TS and DRTS.

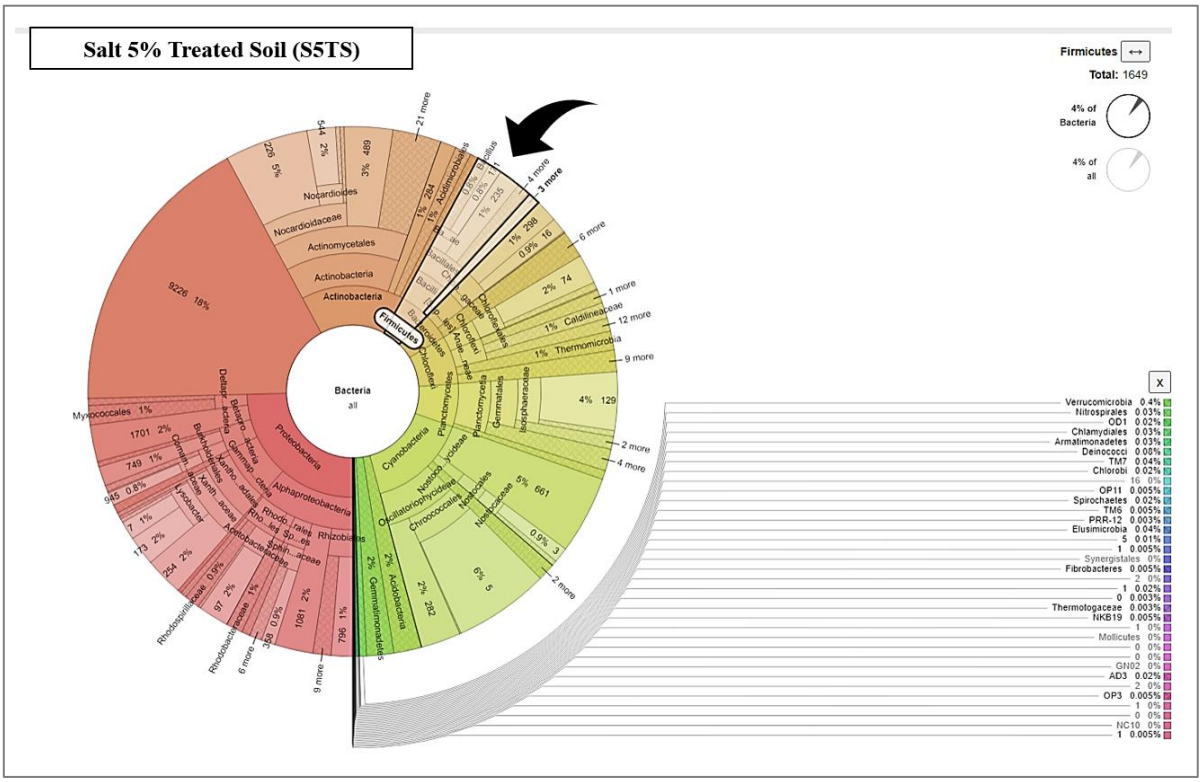


Figure 3.38: Krona graph showing the complete microbial diversity of Samples S5TS.

Key Findings from the Figures 3.36 to 3.38:

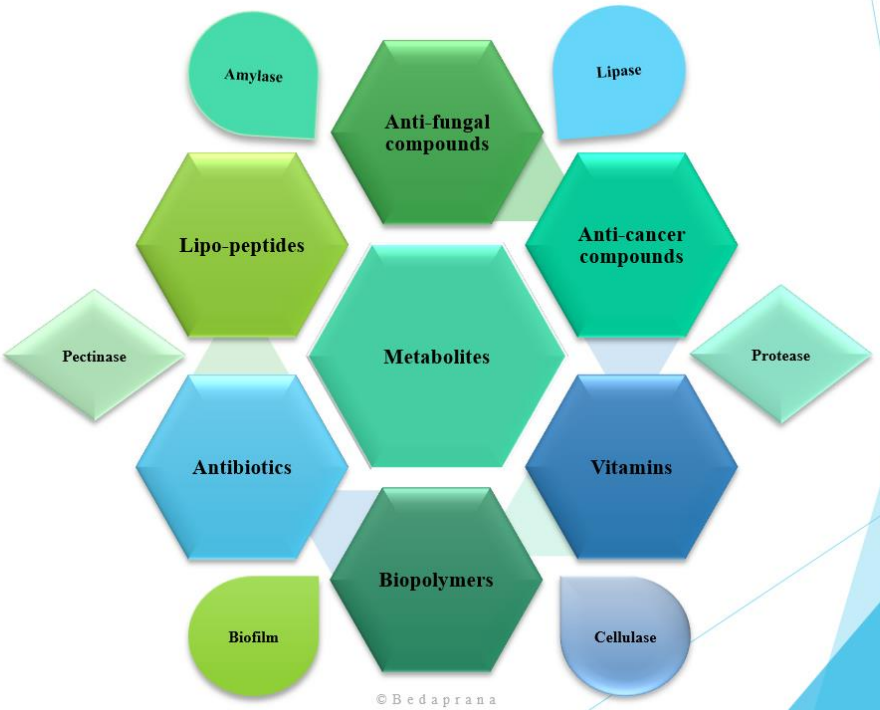
- The microbial diversity of the 5 soil samples was visualised using Krona charts, which displayed the proportion of Firmicutes in each sample.
- The proportion of Firmicutes in the Control soil (CUTS) accounted for 2% of the overall bacterial population in the soil, but this proportion rose to 3% in the Plant growth promotion set up (PGPTS). The percentage further rose to 10% in the arsenic 30ppm stress setting. In both the drought stress setup (DRTS) and the 5% salt stress setup (S5TS), the percentage was determined to be 4%.
- Thus, the metagenome study definitively demonstrated that the bacterial strains used as treatment in the rice rhizosphere successfully colonised the area and, as a result, alleviated stress.
- This colonisation and stress reduction can be attributed to their role as polyextremophilic plant growth promoters.

Key Findings from Objective 3:

- All 5 bacterial strains were tested for their Plant Growth Promoting Properties such as, Nutrient Sequestration, Plant hormone production, Biocontrol Properties, Volatile organic Compounds, Siderophore production, Endophytic enzymes, and Stress Management Properties.
- Dosage Standardization and Mode of treatment selection for application of the bacteria as biofertilizer on the test crop *Zea mays*.
- All the 5 bacterial strains were applied in the field, to the test crop *Zea mays* and the differences in the crop health was determined with respect to the control.
- The soil parameters and the plant pigments were also analysed to check the influence of the bacterial treatments on them.
- Soil Metagenome was also analysed so as to confirm colonisation of the test bacteria in the test crop rhizosphere.
- Bacterial treatment effectivity was also checked on test crop Oryza sativa, with stress application to confirm bacterial PGP activity during abiotic stress.

OBJECTIVE 4

Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.



4. *Production of Industrially Important Enzymes by the Strains the Study of The Small Molecule Metabolome.*

4.1. Industrially and pharmaceutically important enzymes

The 5 bacterial strains, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and BRAM_Y3 now polyextremophilic bacterial strains were studied for their ability to produce industrially and pharmaceutically important enzymes and metabolites. **Figure 4.1** represents production of industrially important enzymes like Pullulanase, amylase and protease production by the 5 bacterial strains. **Figure 4.2** on the other hand represents the production of Urease, a pharmaceutically significant enzyme that also a significant role in removal residual toxicity in soils suffering from over usage of Urea.

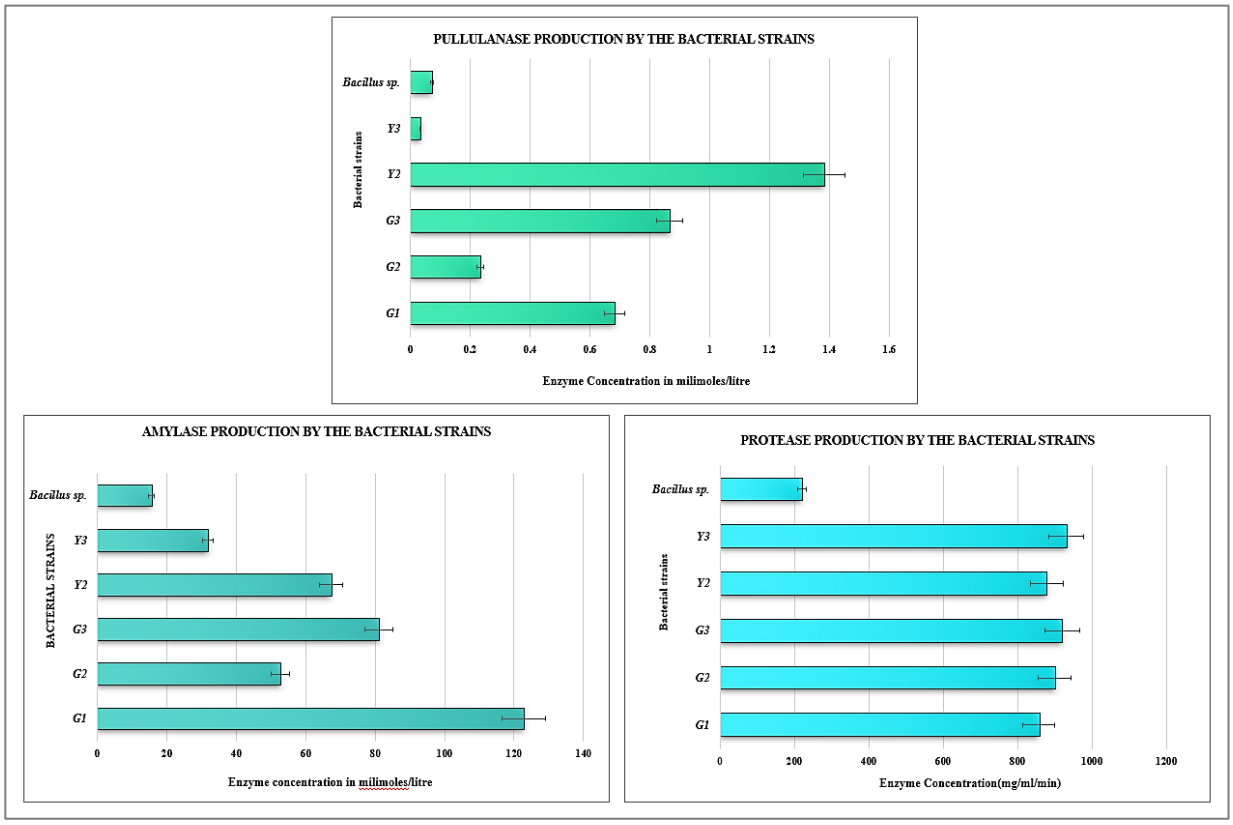


Figure 4.1: Production of industrially important enzymes (Pullulanase, amylase and protease) by all 5 bacterial strains respectively.

Key findings from Figure 4.1

- Pullulanase, a significant debranching enzyme, is commonly employed to break down the α-1,6 glucosidic bonds in starch, amylopectin, pullulan, and similar oligosaccharides. This allows for a thorough and effective transformation of the branched polysaccharides into easily fermentable sugars during the saccharification process.
- All 5 bacterial strains produced Pullulanase, BRAM_Y2 being the highest producer and BRAM_Y3 the lowest.
- Amylases are prominent enzymes utilised extensively in various industries. These enzymes break down the starch molecules into polymers made up of glucose units through hydrolysis. Amylases possess significant potential for utilisation in various industrial sectors, including but not limited to food processing, fermentation, and pharmaceutical production.
- All five bacterial strains produced significant amounts of amylase with BRAM_G1 being the highest closely followed by BRAM_G3 and BRAM_Y2.
- Proteases are highly effective in altering the characteristics of dietary proteins and generating bioactive peptides from proteins. They are extensively utilised in the manufacturing of value-added food ingredients and food processing to enhance the functional, nutritional, and flavour characteristics of proteins.
- All five bacterial strains produced significant amounts of amylase with BRAM_Y3 closely followed by BRAM_G3 and then the rest three bacterial strains.

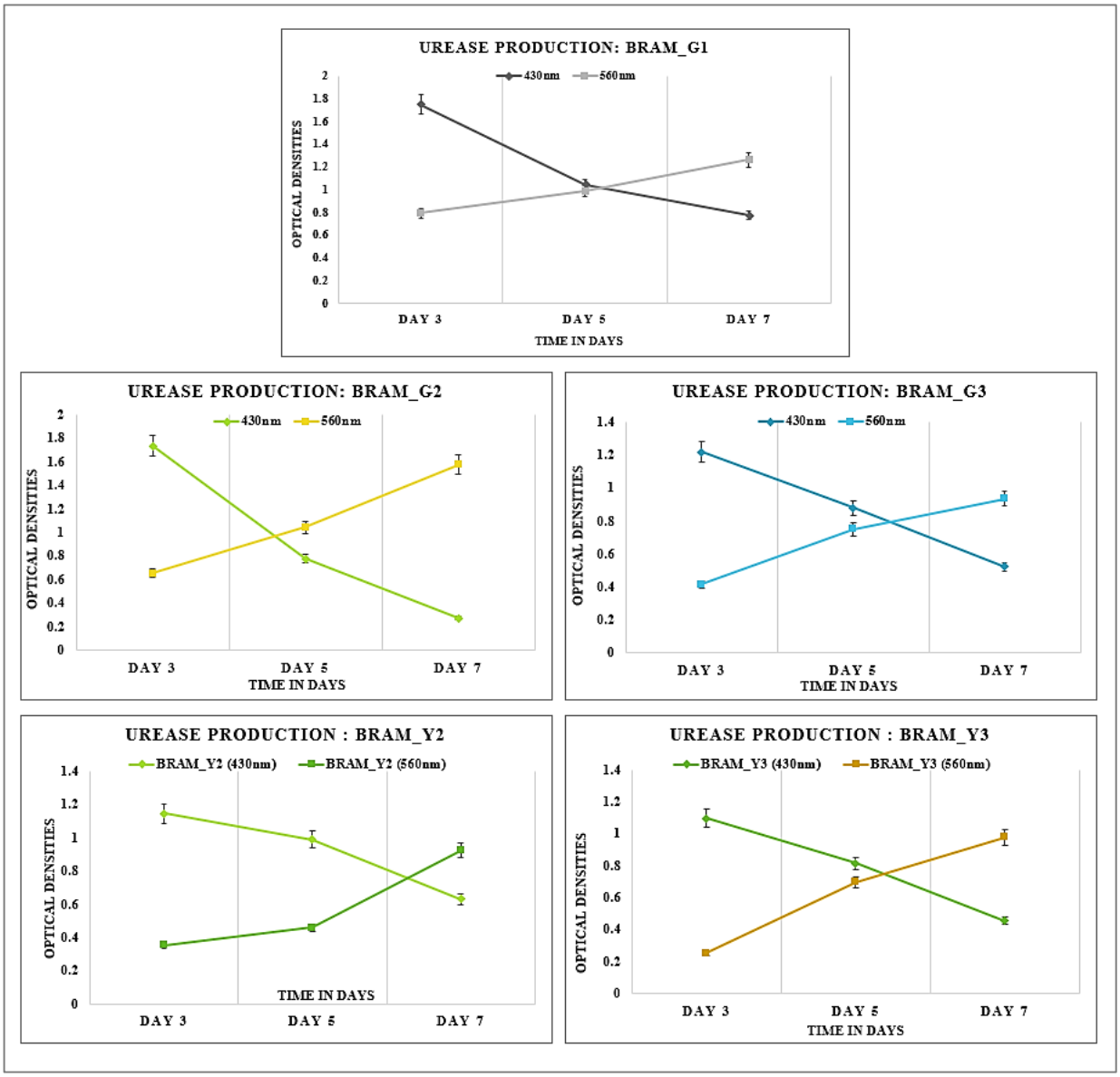


Figure 4.2: Production of Urease (applications in medicinal diagnostics) by all 5 bacterial strains.

<u>Key findings from Figure 4.2</u>

- Urease enzyme has multifaceted applications in terms of both industry, medicine and agriculture. Urease is used for determination of creatinine levels in blood, also used for urea removal in kidney failure patients and also has numerous roles as biosensors for IgG, wastewater detection etc.

- Urease, an enzyme that degrades urea to release carbon dioxide and ammonia is also significant when it comes to sustainable agriculture. Excessive use of Urea in the agricultural fields culminates in a huge residual toxicity as the plants can only utilise a very small percentage of the supplied urea. The bacterial urease removes this excess urea from the soil, in turn reducing its toxicity.

- The 5 bacterial strains produced considerable quantities of urease, that was monitored by the colour change in Stuart' Broth, from yellow to pink. The graph represents the optical densities where with time the intensity if the yellow colour in the broth decreases along with simultaneous increase in the pink colour intensity which corresponds to the increase in the optical density in the absorption wavelength of the pink colour (560nm).

4.2. ESI-MS Study of Extracellular, Intracellular, and Biofilm Metabolites in Normal and Three-dimensional stress conditions.

The bacterial strains were further subjected to Electron Spray Ionisation (Direct-infusion) Mass Spectrophotometry. Three fractions of bacterial cultures were studied, extra cellular, intracellular and extracted Biofilm in two phases, first without application of any stress, and second, after application of a three-dimensional stress condition (Temperature70°C + 4% NaCl+20% PEG). Therefore, for all the fractions there were 2 sets of observation, one with no stress and the other after application of 3D stress. Humongous variation was observed in the peaks observed in all three fractions before and after stress application. In all the 3 fractions, an enormous increase in the number of peaks were observed when stress was applied. **Figures 4.3, 4.4 and 4.5** represent the mass spectra of the extracellular metabolites secreted by the 5bacterial strains. the predicted compounds produced by the 5 strains and their potential uses in medicine and industry have been enlisted in **Table 4.1**. The extracellular metabolites produced after 3D stress application have been enlisted in **Tables 4.2** and **4.3.** The highlighted compounds in the tables not only represent the new compounds produced after stress but also hold immense significance in the field of medicine and industry. The Mass spectra for the same are represented in **Figures 4.6 to 4.11** for all 5 bacterial strains.

Similarly for the intracellular compounds, the mass spectra for the metabolites produced without stress are denoted in **Figures 4.12 to 4.14.** The predicted compounds corresponding to each of the peaks have not only been pointed out in the figures but also been enlisted in **Table 4.4.** Followed by the Intra cellular compound mass spectra representing the peaks found after application of 3D stress have been represented in **Figures 4.15 to 4.20.** The detailed list of compounds has been represented in **Tables 4.5 and 4.6** with highlights on the most significant predicted compounds of interest and their uses in medicine and industry.

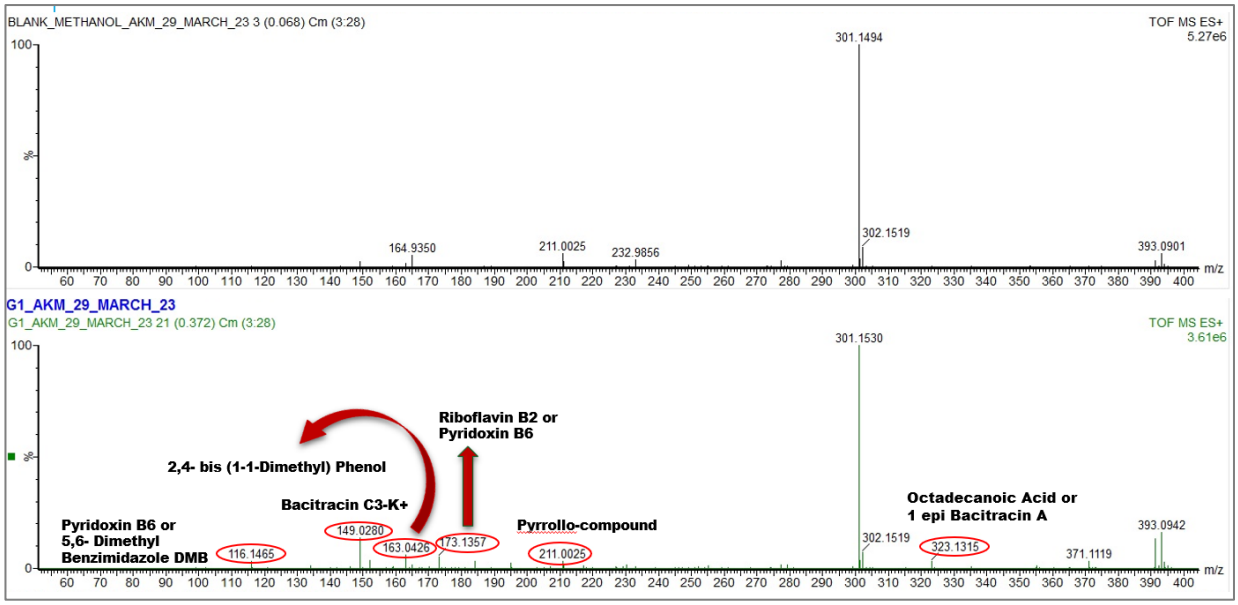


Figure 4.3: Mass spectra of Extracellular metabolite produced by BRAM_G1 with Methanol Blank.

Key findings from Figure 4.3

- The mass spectra in the image depicts the extracellular compounds produced by BRAM_G1 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of $3.61e^{6}$ and included different vitamins, antibiotics, organic acids etc.
- The list of the compounds produced along with their significance in industry have been added later in the section.

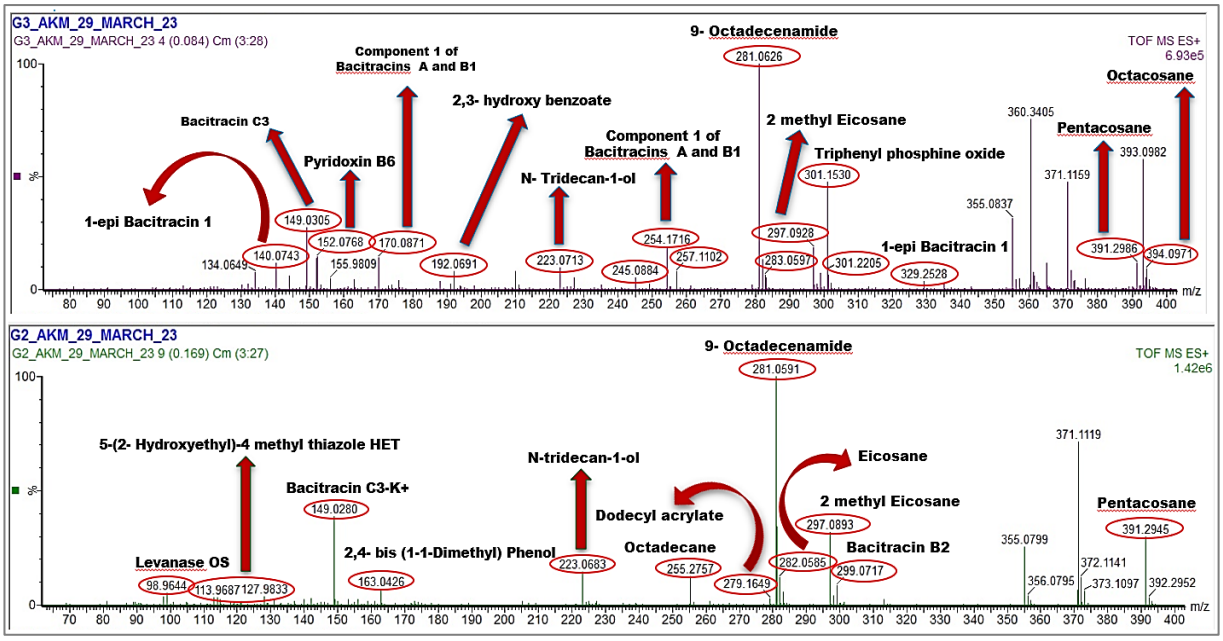


Figure 4.4: Mass spectra of Extracellular metabolite produced by BRAM_G2 and BRAM_G3

Key findings from Figure 4.4

- The mass spectra in the image depicts the extracellular compounds produced by BRAM_G2 and BRAM_G3 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of $6.93e^5$ for BRAM_G2 and $1.42e^6$ for BRAM_G3 and included different vitamins, antibiotics, organic acids and certain alkanoates and pyrollo compounds.
- The list of the compounds produced along with their significance in industry have been added later in the section.

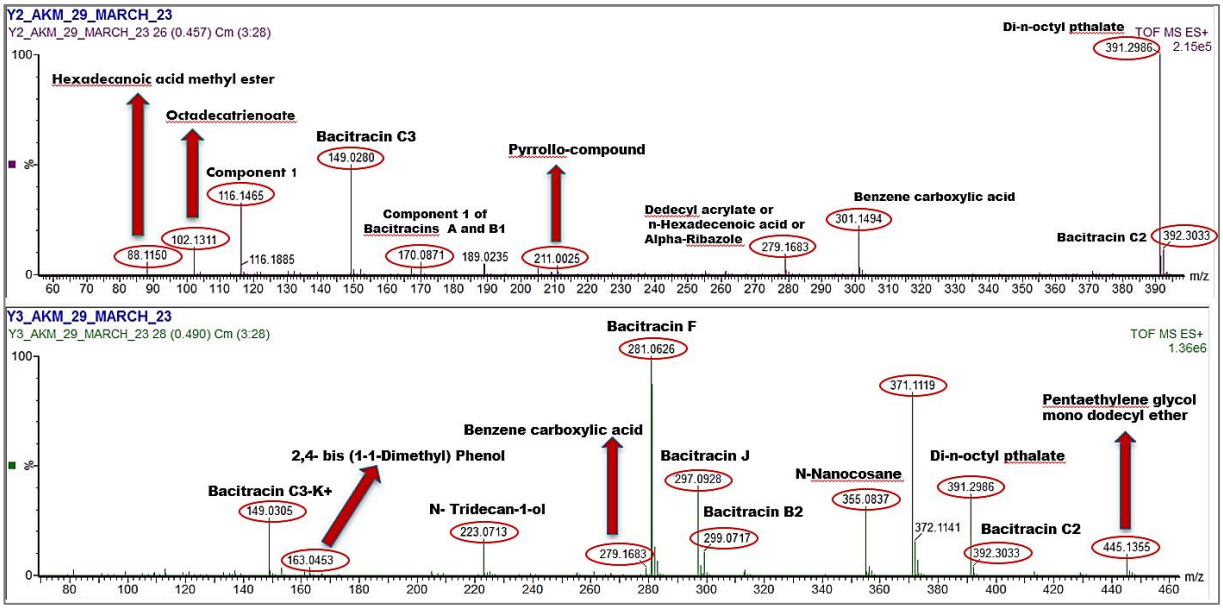


Figure 4.5: Mass spectra of Extracellular metabolite produced by BRAM_Y2 and BRAM_Y3

Key findings from Figure 4.5

- The mass spectra in the image depicts the extracellular compounds produced by BRAM_Y2 and BRAM_Y3 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of $2.15e^5$ for BRAM_G2 and $1.36e^6$ for BRAM_G3 and included different vitamins, vitamin derivatives, antibiotics, organic acids and certain compounds like acrylates, tridecanols and n-cosane compounds.
- The list of the compounds produced along with their significance in industry have been added later in the section.

	Extra –cellular compounds	Bacteria Producing the Compound	Uses in Industry and therapeutics
1.	**Pyridoxin B6**	**BRAM_G1**	Vitamin B-6 (pyridoxine) is important for normal brain development and for keeping the nervous system and immune system healthy.
2.	**5,6- Dimethyl Benzimidazole DMB (Moiety of vitamin B12)**	**BRAM_G1, BRAM_G3**	Keeps body's blood and nerve cells healthy Vitamin B12 also helps prevent megaloblastic anemia, a blood condition that makes people tired and weak.
3.	**Levanase OS**	**BRAM_G2**	An Enzyme which rapidly hydrolyses levan (β-d-fructose polymer) in an endo-type manner to produce a series of levanoligosaccharides
4.	**5-(2- Hydroxyethyl)-4 methyl thiazole HET**	**BRAM_G2**	Thiamine metabolism
5.	**2,4- bis (1-1-Dimethyl) Phenol**	**BRAM_G1, BRAM_Y3**	In medicine, it has the antioxidant, anticancer, antifungal, antibacterial properties and the protection against trimethyltin (TMT) -induced cognitive dysfunction.
6.	**Bacitracin C3-K+,1 epi, A, B2, C2, F, J, C3.**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
7.	**Riboflavin B2**	**BRAM_G1, BRAM_G3**	It reduces oxidative stress and inflammation of nerves
8.	**Pyrrollo-compound**	**BRAM_Y2**	Apoptosis in HeLa cells
9.	**Octadecanoic Acid**	**BRAM_G1**	It is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.

	Extra –cellular compounds	Bacteria Producing the Compound	Uses in Industry and therapeutics.
10.	**N-tridecan-1-ol**	**BRAM_G2, BRAM_G3**	It is used as a lubricant and for the manufacture of surfactants and plasticizers.
11.	**Dodecyl acrylate**	**BRAM_G2, BRAM_G3, BRAM_Y2**	This substance is used in the following products: polymers, adhesives and sealants, coating products and inks and toners.
12.	**Benzene carboxylic acid**	**BRAM_Y2**	Used to make other chemicals, as a food preservative, and for other uses
13.	**n-Hexadecenoic acid**	**BRAM_Y2**	It helps in designing of specific inhibitors of phospholipase A2 as anti-inflammatory agents.
14.	**9- Octadecenamide**	**BRAM_G2, BRAM_G3**	Drugs used to induce drowsiness or sleep or to reduce psychological excitement or anxiety
15.	**2 methyl Eicosane**	**BRAM_G2, BRAM_G3**	Eicosane is a solid n-alkane containing 20 carbon atoms (C20). It is used in cosmetic, lubricants, plasticizers.
16.	**Pentacosane**	**BRAM_G2, BRAM_G3**	It has a role as a semiochemical and a plant metabolite.
17.	**2,3- dihydroxy benzoate**	**BRAM_G3**	An anti-diarrheal and anti-inflammatory agent used for symptomatic treatment of nausea, indigestion, upset stomach, diarrhea, and other temporary discomforts of the stomach and gastrointestinal tract.
18.	**Di-n-octyl-pthalate**	**BRAM_Y3**	It is a colorless, odorless, oily liquid that doesn't evaporate easily. It is a man-made substance used to keep plastics soft or more flexible.

Table 4.1: List of Extra cellular compounds, designated with the bacterial strains that produced them along with their potential uses in medicine and industry.

Key findings from Table 4.1

- This table enlists the compounds that were predicted to be present in the Intra-cellular fraction of the bacterial metabolites.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibiotics, anti-cancer, anti-inflammatory compounds, vitamins and even preservatives, plasticizers, polymers etc.

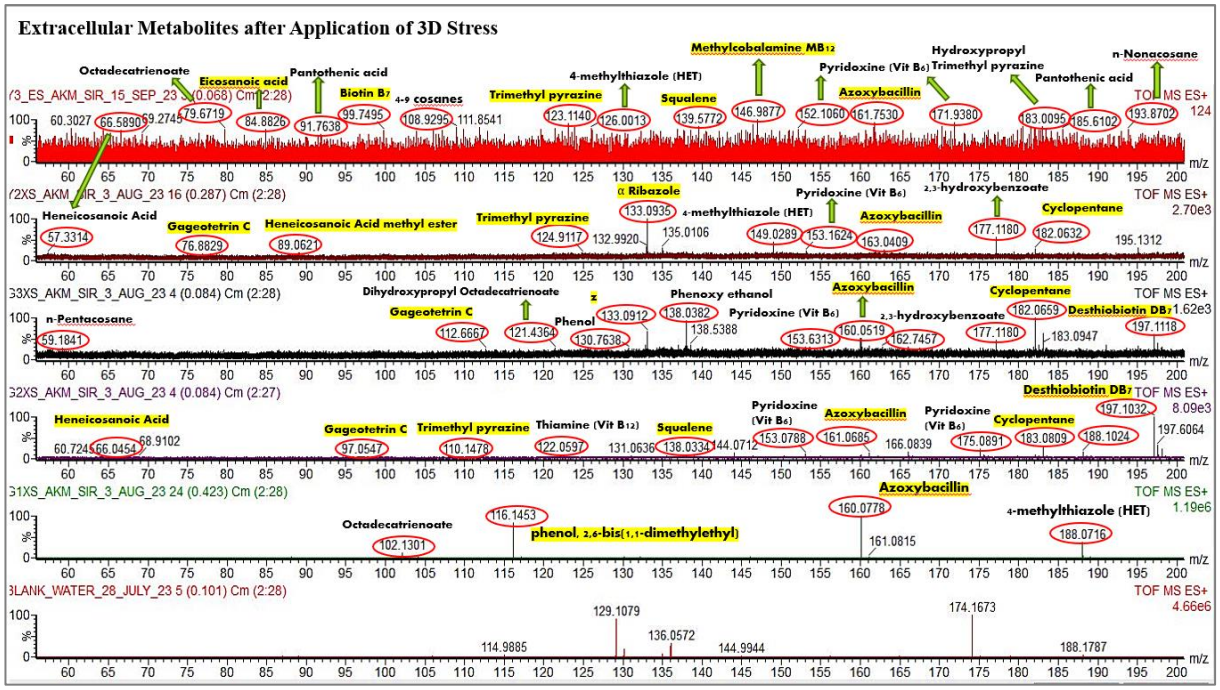


Figure 4.6: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (0-200 Da).

<u>Key findings from Figure 4.6</u>

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(0-200 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

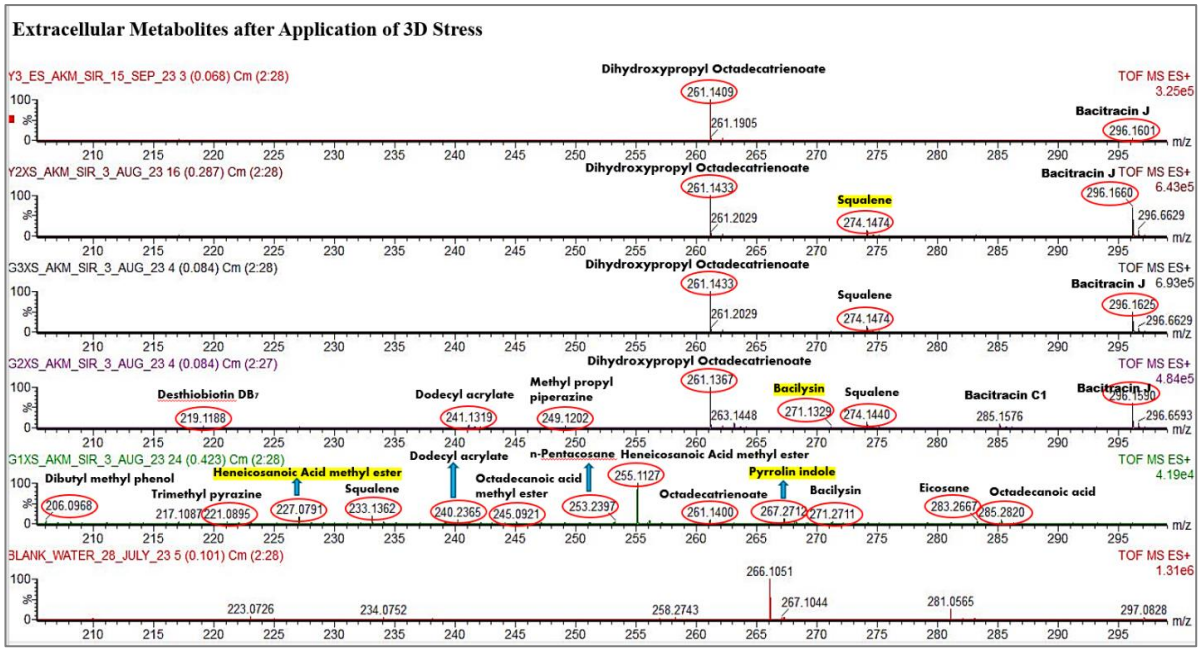


Figure 4.7: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (200-300 Da).

<u>Key findings from Figure 4.7</u>

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(200-300 Da)**.
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

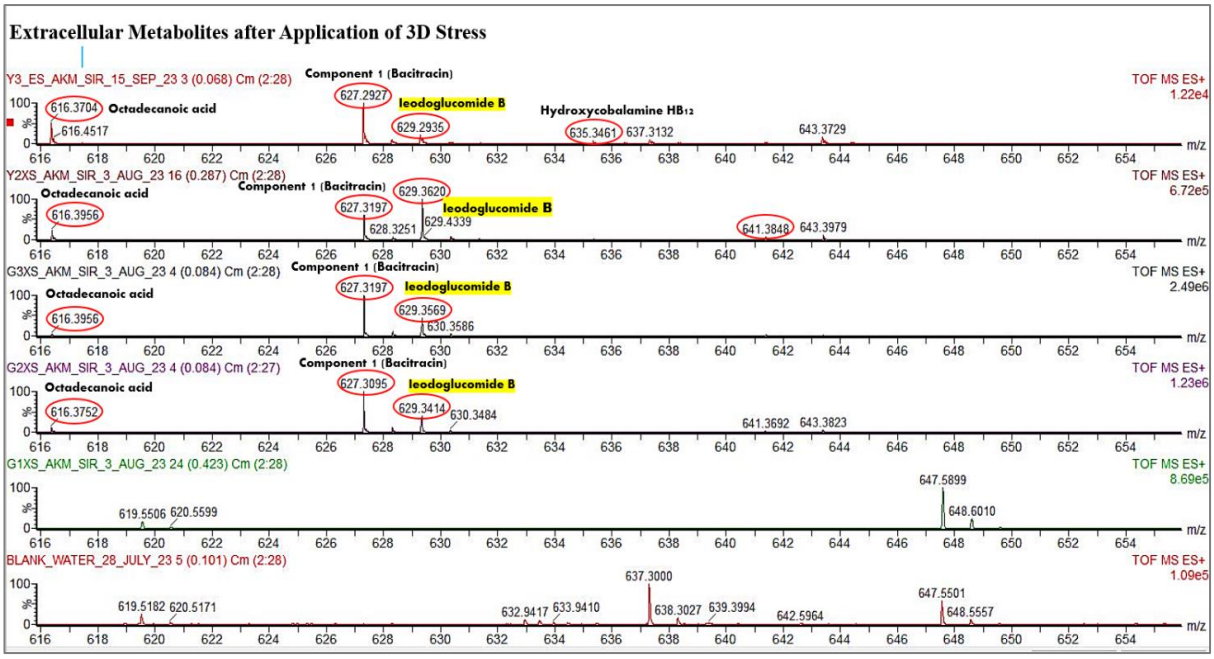


Figure 4.8: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (616-655 Da).

Key findings from Figure 4.8

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(616-655 Da)**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

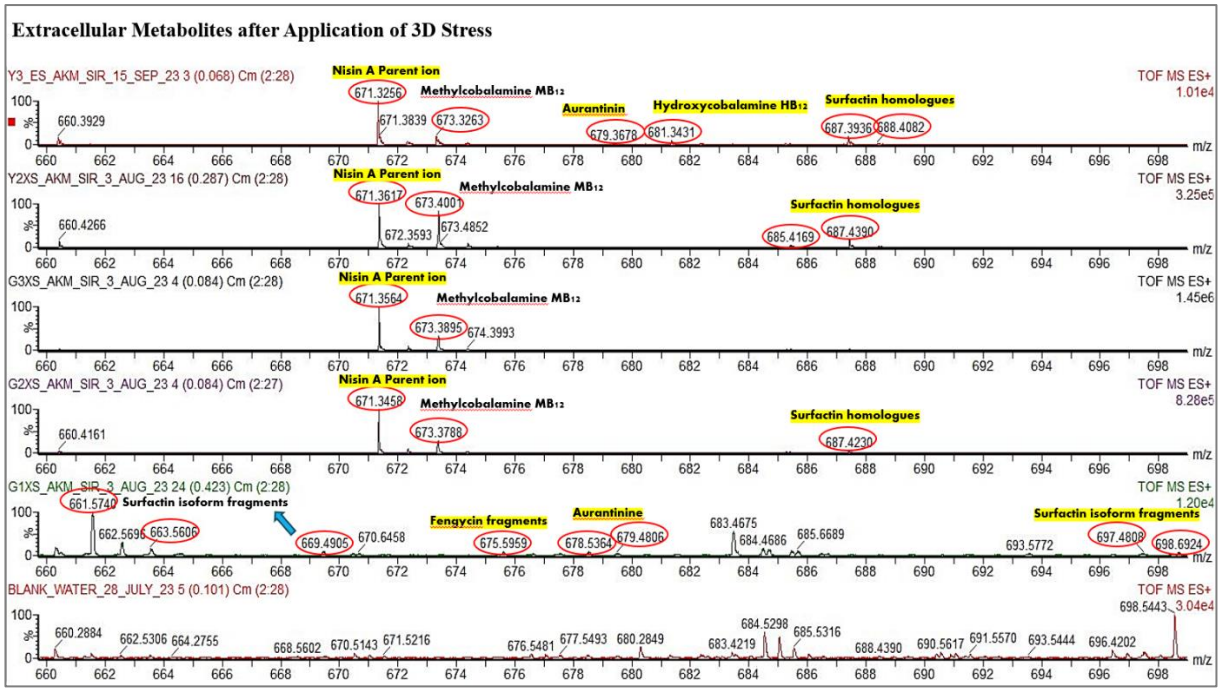


Figure 4.9: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (660-700 Da).

<u>Key findings from Figure 4.9</u>

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(660-700 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

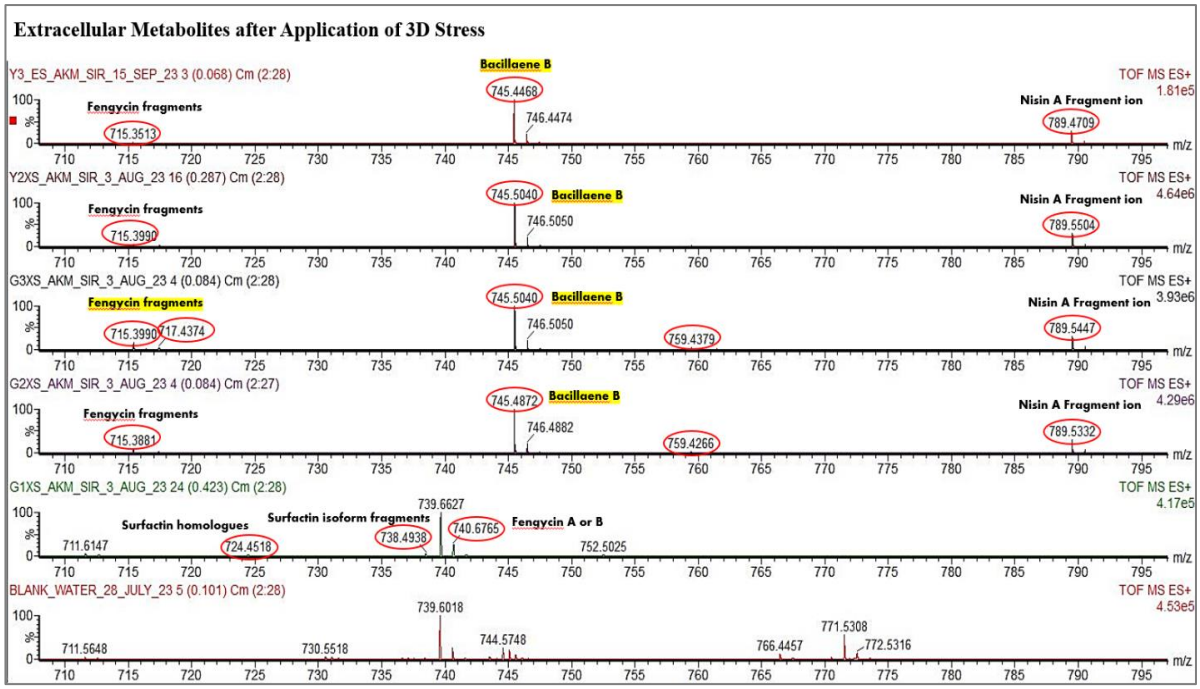


Figure 4.10: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (700-800 Da).

<u>Key findings from Figure 4.10</u>

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(700- 800 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

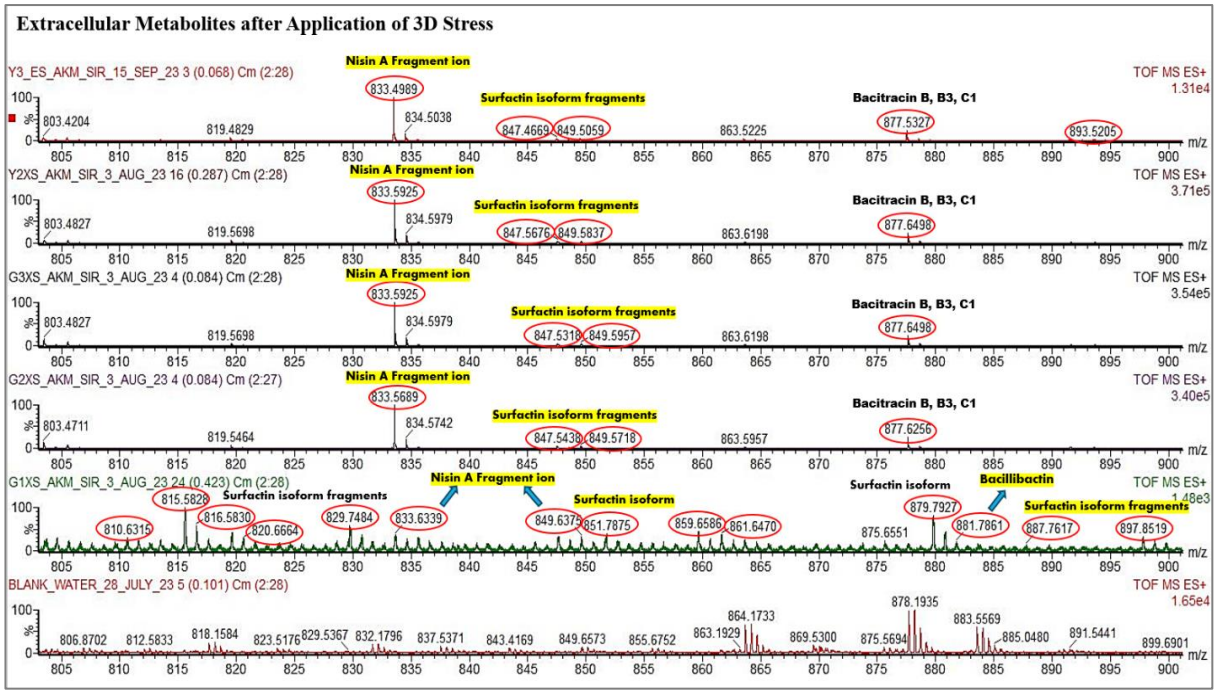


Figure 4.11: Mass Spectra of Extra cellular metabolites produced by the 5 bacterial strains after 3D stress application (800-900 Da).

Key findings from Figure 4.11

- The mass spectra image is a combined representation of the extra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(800-900 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

	Extra –cellular compounds (stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	**Octadecatrienoate**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A Fatty Acid.
2.	**Eicosanoic Acid**	**BRAM_Y3**	Used as an organic thin film in the production of liquid crystals for a wide variety of technical applications.
3.	**Pantothenic Acid**	**BRAM_Y3**	B vitamin and an essential nutrient. All animals need pantothenic acid in order to synthesize coenzyme A (CoA)—essential for metabolizing fatty acid—and to synthesize and metabolize proteins, carbohydrates, and fats.
4.	**Biotin B7**	**BRAM_Y3**	Biotin is necessary for formation of fatty acids and glucose, which are used as fuels by the body.
5.	**Nonacosane**	**BRAM_Y3**	Plays a role in the chemical communication of several insects, including the female Anopheles stephensi (a mosquito).
6.	**Trimethyl pyrazine**	**BRAM_G2, BRAM_Y2, BRAM_Y3**	Induces Apoptosis in HT-29 cells
7.	**4-methyl thiazole (HET)**	**BRAM_G1, BRAM_Y2**	Used in the synthesis of catalytic dendrophanes, as functional mimics of the thiamine-diphosphate-dependent enzyme pyruvate oxidase
8.	**Squalene**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Use of squalene currently is as an adjunctive therapy in a variety of cancers.
9.	**Methylcobalamine**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Methylcobalamin helps treat the deficiency of vitamin B12 by producing a substance known as "myelin"
10.	**Azoxybacillin**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Used to treat bacterial infections, such as chest infections (including pneumonia) and dental abscesses.
11.	**Pyridoxine B6**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Is important for normal brain development and for keeping the nervous system and immune system healthy.
12.	**Gageotetrins**	**BRAM_G2, BRAM_G3, BRAM_Y2,**	A unique class of linear lipopeptides, consisting of di- and tetrapeptides and a new fatty acid, having good antimicrobial activities.

	Extra –cellular compounds (stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	**Octadecatrienoate**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A Fatty Acid.
2.	**Eicosanoic Acid**	**BRAM_Y3**	Used as an organic thin film in the production of liquid crystals for a wide variety of technical applications.
3.	**Pantothenic Acid**	**BRAM_Y3**	B vitamin and an essential nutrient. All animals need pantothenic acid in order to synthesize coenzyme A (CoA)—essential for metabolizing fatty acid—and to synthesize and metabolize proteins, carbohydrates, and fats.
4.	**Biotin B7**	**BRAM_Y3**	Biotin is necessary for formation of fatty acids and glucose, which are used as fuels by the body.
5.	**Nonacosane**	**BRAM_Y3**	Plays a role in the chemical communication of several insects, including the female Anopheles stephensi (a mosquito).
6.	**Trimethyl pyrazine**	**BRAM_G2, BRAM_Y2, BRAM_Y3**	Induces Apoptosis in HT-29 cells
7.	**4-methyl thiazole (HET)**	**BRAM_G1, BRAM_Y2**	Used in the synthesis of catalytic dendrophanes, as functional mimics of the thiamine-diphosphate-dependent enzyme pyruvate oxidase
8.	**Squalene**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Use of squalene currently is as an adjunctive therapy in a variety of cancers.
9.	**Methylcobalamine**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Methylcobalamin helps treat the deficiency of vitamin B12 by producing a substance known as "myelin"
10.	**Azoxybacillin**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Used to treat bacterial infections, such as chest infections (including pneumonia) and dental abscesses.
11.	**Pyridoxine B6**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Is important for normal brain development and for keeping the nervous system and immune system healthy.
12.	**Gageotetrins**	**BRAM_G2, BRAM_G3, BRAM_Y2,**	A unique class of linear lipopeptides, consisting of di- and tetrapeptides and a new fatty acid, having good antimicrobial activities.

	Extra –cellular compounds (stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
24.	**Bacilysin**	**BRAM_G1, BRAM_G2**	a non-ribosomally synthesized dipeptide antibiotic that is active against a wide range of bacteria and some fungi.
25.	**Bacitracin C1, J, B, B3, C1**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
26.	**Octadecanoic Acid**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A straight-chain C18 saturated fatty acid component of many animal and vegetable lipids. As well as in the diet, it is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.
27.	**Pyrrolin indole**	**BRAM_G1**	Apoptosis in HeLa
28.	**Eicosane**	**BRAM_G1**	To form candles and paraffin waxes with solar energy storage capacity. It is also used in cosmetics, lubricants, plasticizers and in the petrochemical industry.
29.	**Ieodoglucomide B**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Ieodoglucomide B exhibited cytotoxic activity against lung cancer and stomach cancer cell lines and moderate in vitro antimicrobial activity.
30.	**Nisin A, Z**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Nisin is used in processed cheese, meats, beverages, etc. during production to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria.
31.	**Aurantinin**	**BRAM_G1**	A novel antibiotic effective against Gram positive Bacteria.
32.	**Hydroxycobalamine HB12**	**BRAM_Y3**	Form of injectable vitamin B12. Clinicians use it in the prevention and treatment of macrocytic anemia associated with vitamin B12 deficiency. It may also be part of therapy to treat Leber optic atrophy (an inherited optic neuropathy associated with a deficiency in vitamin B12).

	Extra –cellular compounds (stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
33.	**Surfactins**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It has been extensively studied for its antibacterial, antiviral, antitumor and hemolytic effects.
34.	**Fengycins**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Fengycin is a cyclic lipopeptide which is used as an agricultural fungicide.
35.	**Bacillaene B**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Bacillaenes play dual roles as antibiotic and biofilm enhancers in a dose-dependent manner, both of which serve in the self-protection of Bacillus.

Table 4.2 : List of Extra-cellular compounds after 3D stress application, designated with the bacterial strains that produced them along with their potential uses in medicine and industry

Key findings from Table 4.2

- This table enlists the Extra-cellular fraction of the bacterial metabolites that were predicted to be present in after three-dimensional stress application.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibiotics, anti-cancer, anti-inflammatory compounds, vitamins and even preservatives, plasticizers, polymers etc.
- The increase in the number of compounds produced after application of the three-dimensional stress, show the change in the bacterial metabolome after stress application.
- The highlighted compounds in the table indicate the compounds that were previously not produced in absence of stress.
- The table also clearly indicated that which of the compounds are produced by which set of bacteria drawing a direct comparison amongst the 5 bacterial strains of interest.

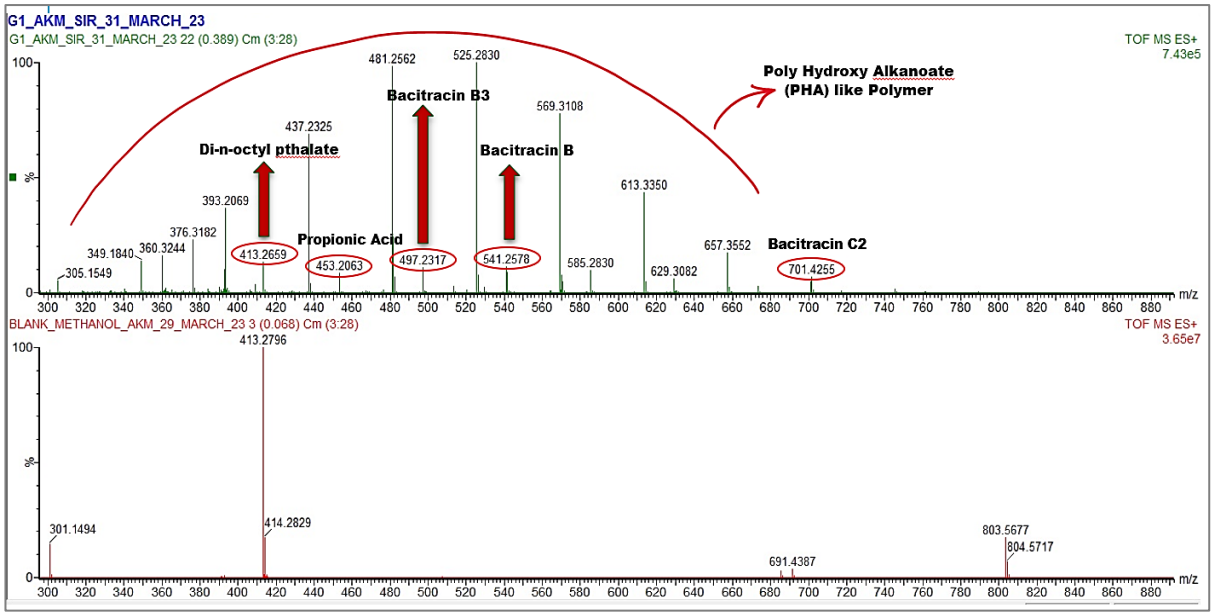


Figure 4.12: Mass spectra of Intracellular metabolite produced by BRAM_G1 with Methanol Blank.

Key findings from Figure 4.12

- The mass spectra in the image depicts the intracellular compounds produced by BRAM_G1 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of 7.43 e^5 for BRAM_G1 and included different vitamins, antibiotics, organic acids and certain alkanoates and pyrollo compounds.
- The list of the compounds produced along with their significance in industry have been added later in the section.

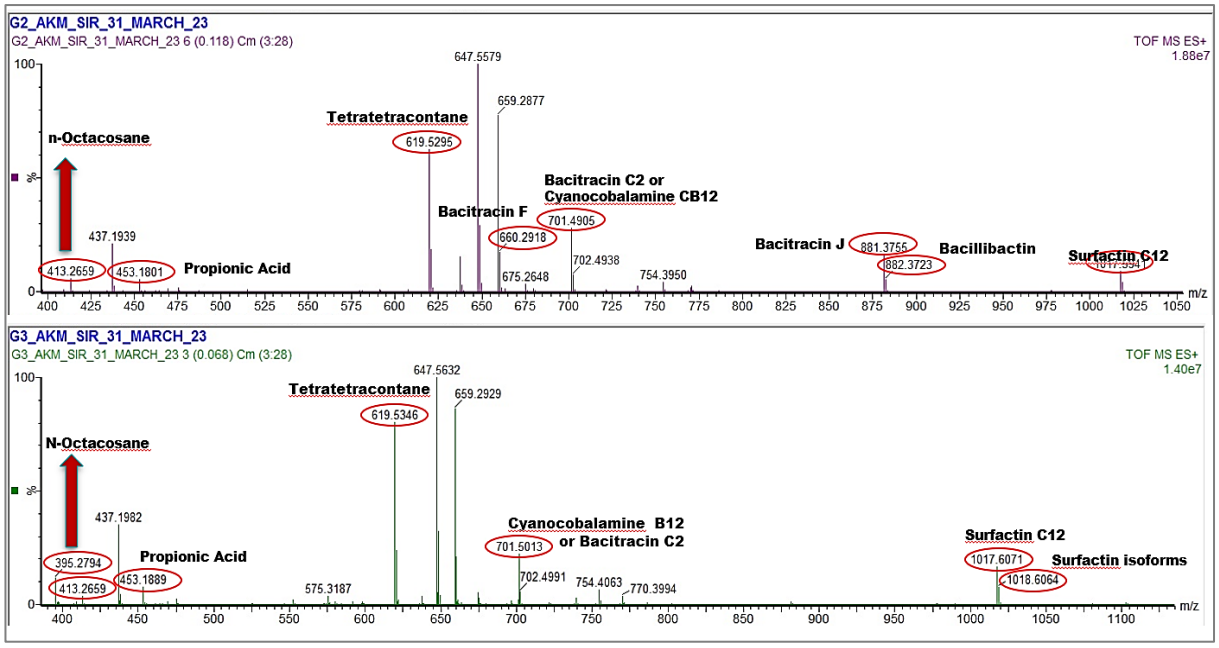


Figure 4.13: Mass spectra of Intracellular metabolite produced by BRAM_G2 and BRAM_G3

Key findings from Figure 4.13

- The mass spectra in the image depicts the intracellular compounds produced by BRAM_G2 and BRAM_G3 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of 1.88 e^7 for BRAM_G2 and 1.40 e^7 for BRAM_G3 and included different vitamins, antibiotics, organic acids and certain alkanoates and pyrollo compounds.
- The list of the compounds produced along with their significance in industry have been added later in the section.

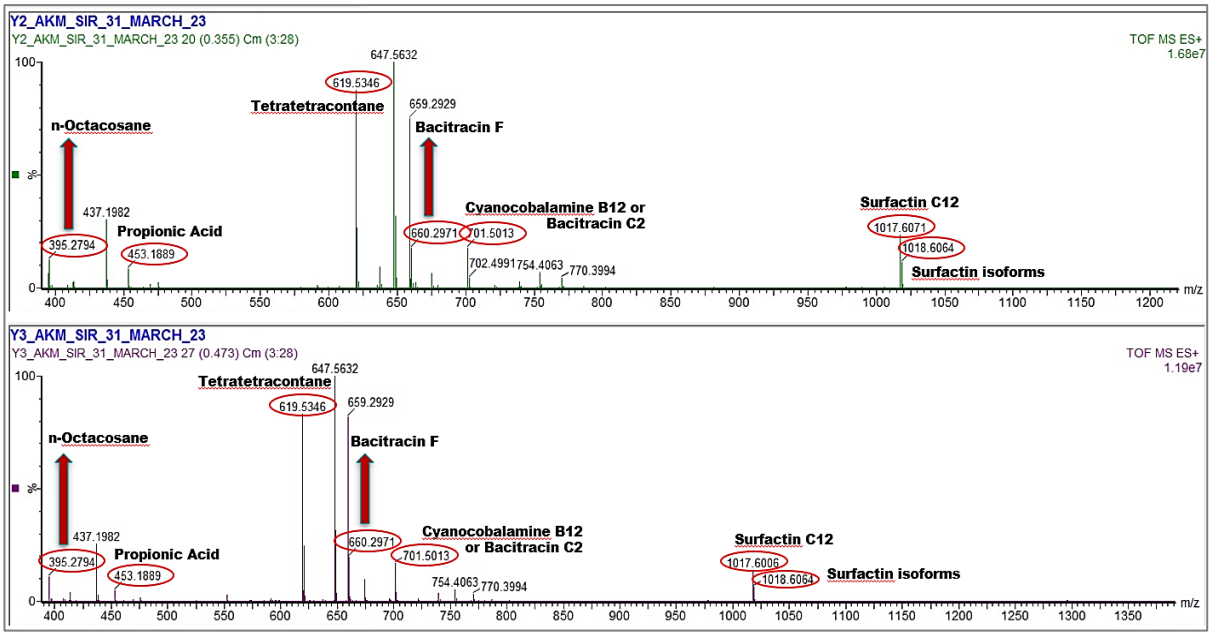


Figure 4.14: Mass spectra of Intracellular metabolite produced by BRAM_Y2 and BRAM_Y3

<u>Key findings from Figure 4.14</u>

- The mass spectra in the image depicts the extracellular compounds produced by BRAM_G2 and BRAM_G3 against a methanol blank that was common to all samples.
- The compounds were found in high intensities of $1.68e^7$ for BRAM_G2 and $1.19e^7$ for BRAM_G3 and included different vitamins, antibiotics, organic acids and certain alkanoates and pyrollo compounds.
- The list of the compounds produced along with their significance in industry have been added later in the section.

	Intra cellular Compounds	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	**Propionic acid**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Used in the manufacture of herbicides, fine chemical intermediates, rubber chemicals, emulsions, and environmentally friendly solvents for coating formulations, artificial fruit flavors, pharmaceuticals, and modified synthetic cellulose fibers.
2.	**Cyanocobalamin Vitamin B12**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Keeps body's blood and nerve cells healthy Vitamin B12 also helps prevent megaloblastic anemia, a blood condition that makes people tired and weak.
3.	**N-Octacosane**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A novel lupene-type triterpenic glucoside
4.	**Tetra-tetracontane**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It is the main constituents of essential oils from Euphorbia macroclada. Tetratetracontane may be used in a passivation layer in copper-phthalocyanine field-effect transistors for improving the charge carrier mobility.
5.	**Bacitracin B3, B2, C2, F, J, C3**	**BRAM_G1**	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
6.	**Di-n-octyl pthalate**	**BRAM_G1**	Used in oil additives as flow improver, in-floor waxes, textile and metal coatings, paint, varnishes, pressure-sensitive adhesives, low temperature caulks, and sealants.
7.	**Surfactins, Surfactin C12**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Surfactin has been extensively studied for its antibacterial, antiviral, antitumor and hemolytic effects.
8.	**Bacillibactin**	**BRAM_G2**	Bacillibactin, one nonribosomal peptide discovered from Bacillus subtilis, serves as a catecholic siderophore in iron acquisition that is essential to the host's life
9.	**Di-n-octyl pthalate**	**BRAM_G1**	It is commonly used as a plasticizer (a substance added to plastics to keep them soft or more flexible)
10.	**Polyhydroxyalkanoate like Polymer**	**BRAM_G1**	Polyhydroxyalkanoates (PHAs) are degradable, biocompatible, thermoplastic polyesters derived from microorganisms, used as a reserve of carbon and energy.

Table 4.3: List of Intra cellular compounds, designated with the bacterial strains that produced them along with their potential uses in medicine and industry.

Key findings from Table 4.3

- This table enlists the compounds that were predicted to be present in the Intra-cellular fraction of the bacterial metabolites.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibiotics, anti-tumour, anti-viral compounds, vitamins and even preservatives, plasticizers, polymers etc.

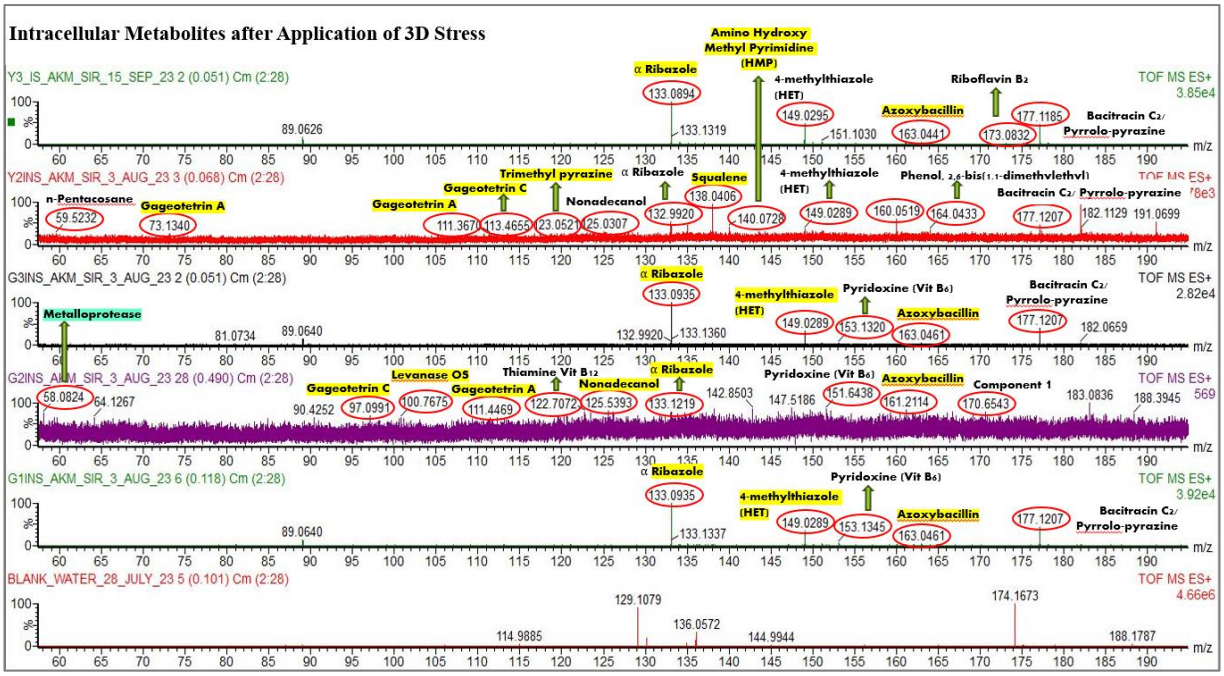


Figure 4.15: Mass Spectra of Intra-cellular metabolites produced by the 5 bacterial strains after 3D stress application (0-200 Da).

Key findings from Figure 4.15

- The mass spectra image is a combined representation of the intra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of (**0-200 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

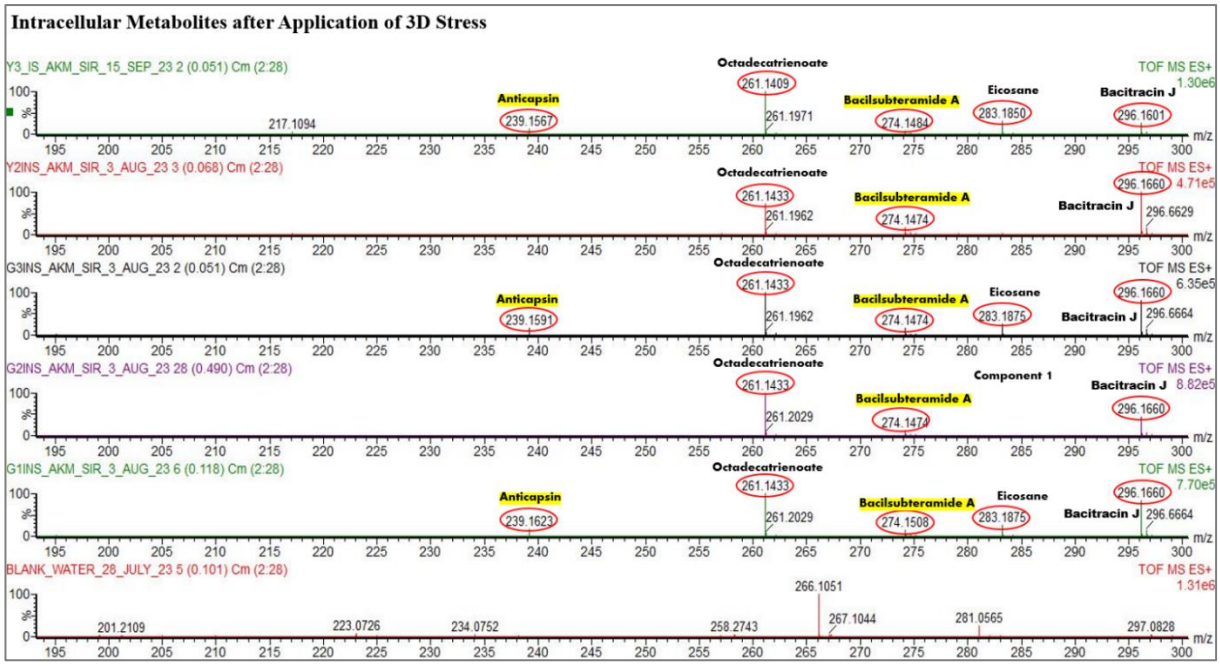


Figure 4.16: Mass Spectra of Intra-cellular metabolites produced by the 5 bacterial strains after 3D stress application (190-300 Da).

Key findings from Figure 4.16

- The mass spectra image is a combined representation of the intra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(190-300 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

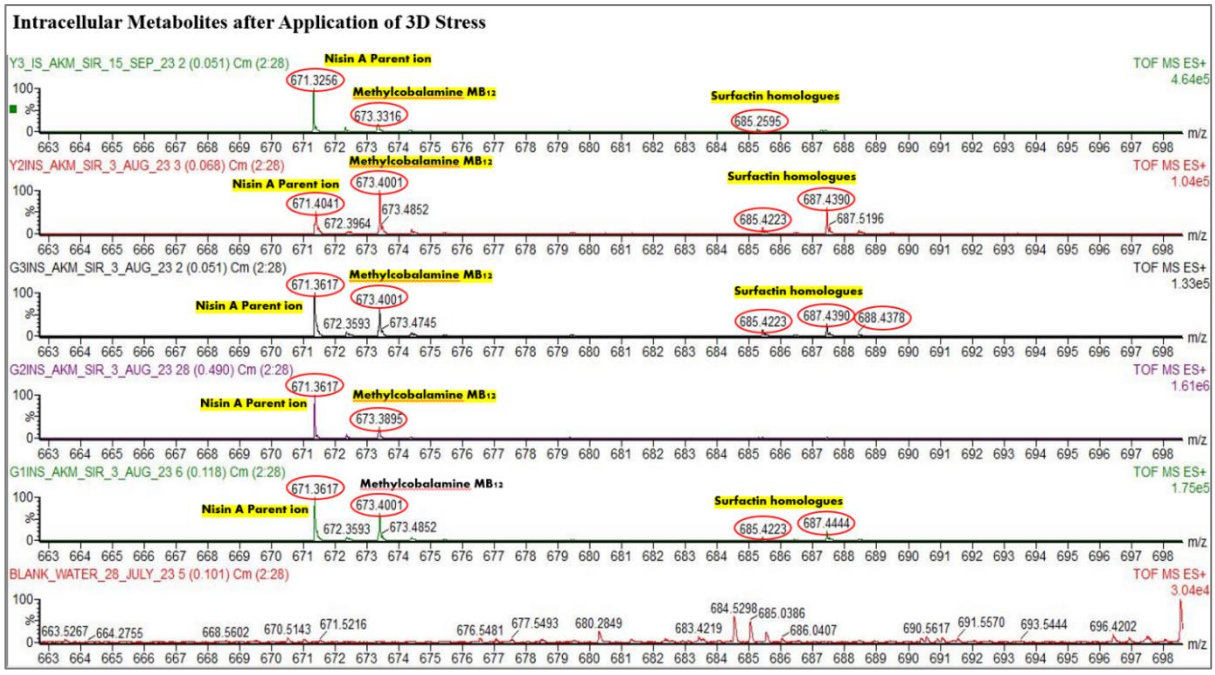


Figure 4.17: Mass Spectra of Intra-cellular metabolites produced by the 5 bacterial strains after 3D stress application (660-700 Da).

Key findings from Figure 4.17

- The mass spectra image is a combined representation of the intra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(660-700 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

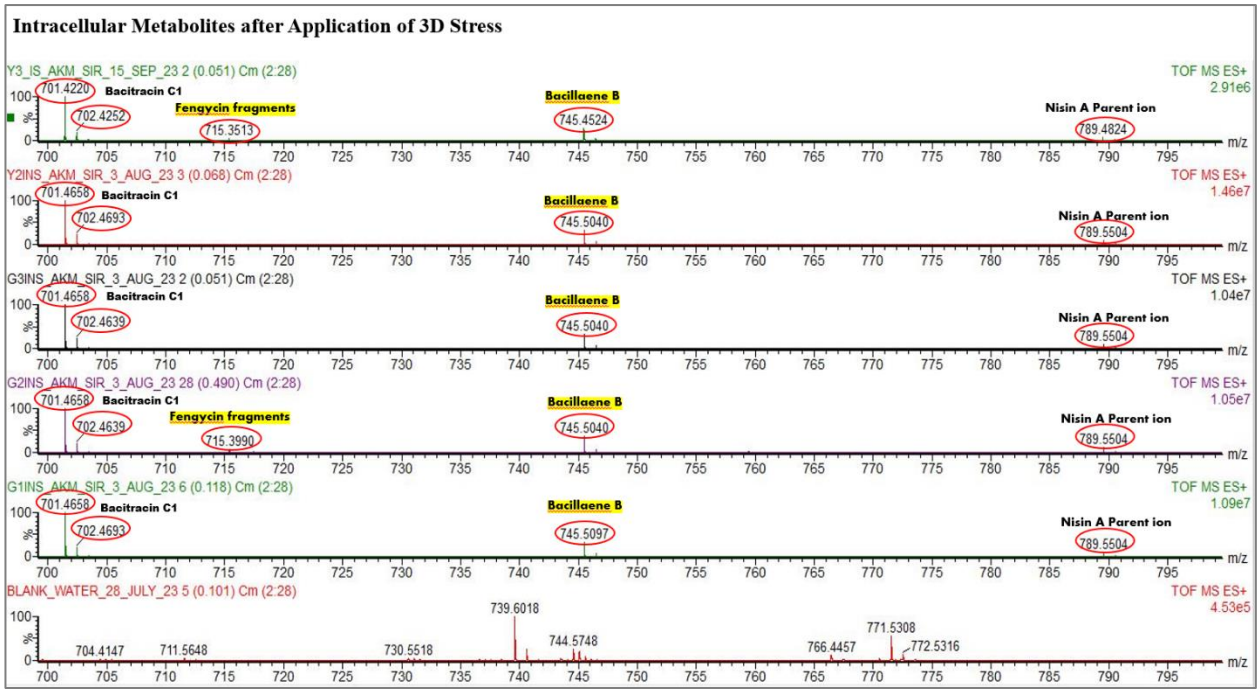


Figure 4.18: Mass Spectra of Intra-cellular metabolites produced by the 5 bacterial strains after 3D stress application (700-800 Da)

<u>Key findings from Figure 4.19</u>

- The mass spectra image is a combined representation of the intra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(700-800 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

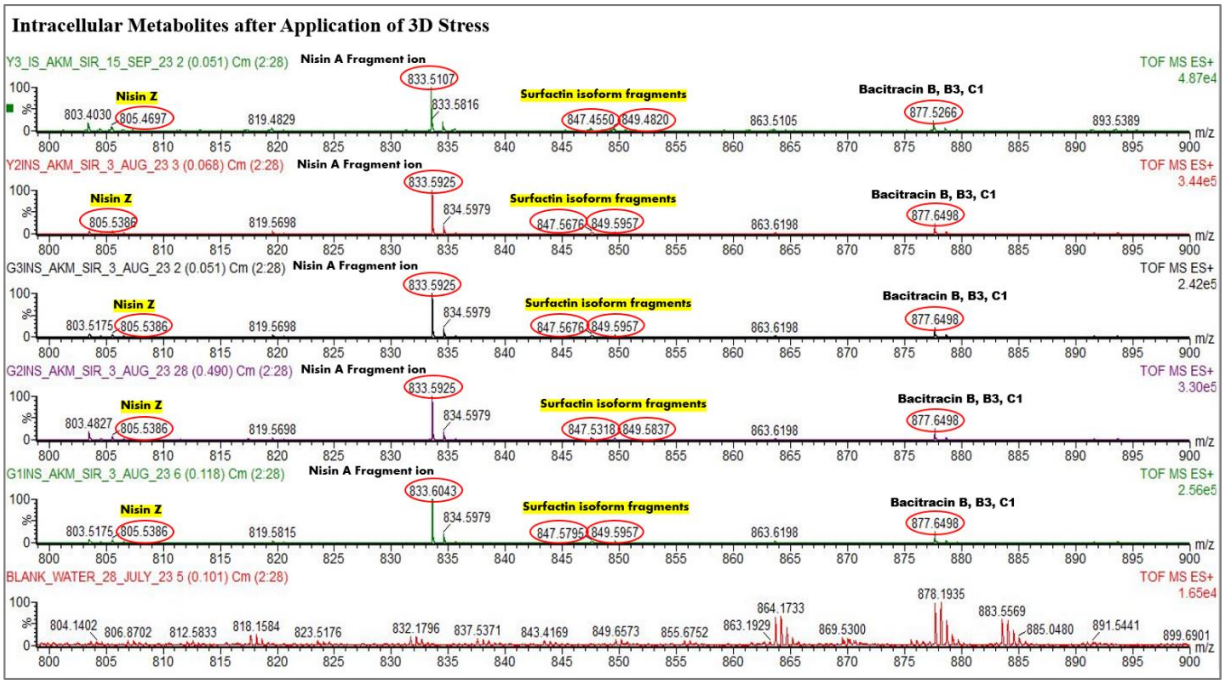


Figure 4.19: Mass Spectra of Intra-cellular metabolites produced by the 5 bacterial strains after 3D stress application (800-900 Da)

Key findings from Figure 4.19

- The mass spectra image is a combined representation of the intra cellular metabolites produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(800-900 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

	Intra cellular Compounds (Stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	Nonadecanol	BRAM_G2, BRAM_Y2	1-Nonadecanol is one of the constituents of supercritical carbon dioxide (SC-CO2) essential oil of freshly collected aerial parts of *Heracleum thomsonii*
2.	Amino hydroxymethyl pyrimidine HMP	BRAM_Y2	Biogenetic precursors to the important biochemical cofactor thiamine pyrophosphate (TPP), a derivative of thiamine (vitamin B1), used to supplement human and animal diets.
3.	Bacitracin C1, J, B, B3, C2	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
4.	Biotin B7		Biotin is necessary for formation of fatty acids and glucose, which are used as fuels by the body.
5.	Anticapsin	BRAM_G1, BRAM_G3, BRAM_Y3	A powerful inhibitor of glucosamine synthetase in extracts of both the bacilysin-sensitive and -resistant strains of Staphylococcus aureus.
6.	Trimethyl pyrazine		Induces Apoptosis in HT-29 cells
7.	4-methyl thiazole (HET)	BRAM_G1, BRAM_G3, BRAM_Y2, BRAM_Y3	Used in the synthesis of catalytic dendrophanes, as functional mimics of the thiamine-diphosphate-dependent enzyme pyruvate oxidase
8.	Squalene		Use of squalene currently is as an adjunctive therapy in a variety of cancers.
9.	Levanase	BRAM_G2	An Enzyme which rapidly hydrolyzes levan (β-d-fructose polymer) in an endo-type manner to produce a series of levanoligosaccharides.
10.	Azoxybacillin	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y3	Used to treat bacterial infections, such as chest infections (including pneumonia) and dental abscesses.
11.	Pyridoxine B6	BRAM_G1, BRAM_G2, BRAM_G3	Is important for normal brain development and for keeping the nervous system and immune system healthy.
12.	Gageotetrins	BRAM_G2, BRAM_Y2	A unique class of linear lipopeptides, consisting of di- and tetrapeptides and a new fatty acid, having good antimicrobial activities.
24.	Bacilsubteramide A	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	A new indole alkaloid From *Bacillus subterraneus*.

	Intra cellular Compounds (Stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
13.	α Ribazole	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	A fluorescent marker for the liquid chromatographic determination of vitamin B12, It has a role as an Escherichia coli metabolite and a mouse metabolite.
14.	Pentacosane	BRAM_Y2	It has a role as a semiochemical and a plant metabolite.
15.	Eicosane	BRAM_G1, BRAM_G3, BRAM_Y3	To form candles and paraffin waxes with solar energy storage capacity. It is also used in cosmetics, lubricants, plasticizers and in the petrochemical industry.
16.	Nisin A, Z	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	Nisin is used in processed cheese, meats, beverages, etc. during production to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria.
17.	Pyrollo-pyrazine	BRAM_G1, BRAM_G3, BRAM_Y2, BRAM_Y3	Pyrrolopyrazine as a biologically active scaffold contains pyrrole and pyrazine rings. Compounds with this scaffold have widely exhibited different biological activities, such as antimicrobial, anti-inflammatory, antiviral, antifungal, antioxidant, antitumor, and kinase inhibitory.
18.	Methylcobalamine HB12	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	Methylcobalamin helps treat the deficiency of vitamin B12 by producing a substance known as "myelin"
19.	Octadecanoic Acid	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	A C18 straight-chain saturated fatty acid component of many animal and vegetable lipids. As well as in the diet, it is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.
20.	Metalloprotease	BRAM_G2	Play an important role in many physiological processes such as embryonic development and growth, tissue remodelling and repair.
21.	Phenol, 2,6-bis(1,1-dimethylethyl)	BRAM_Y2	Widely used phenolic antioxidant. It is used as an ingredient in personal care products and cosmetics, as an additive to petroleum products such as fuels and lubricating oils, in the production of plastics and synthetic rubbers, and as a preservative in food and animal feed.
22.	Fengycins	BRAM_G2, BRAM_Y3	Fengycin is a cyclic lipopeptide which is used as an agricultural fungicide.
23.	Bacillaene B	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	Bacillaenes play dual roles as antibiotic and biofilm enhancers in a dose-dependent manner, both of which serve in the self-protection of Bacillus.
24.	Surfactins	BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3	It has been extensively studied for its antibacterial, antiviral, antitumor and hemolytic effects.

Table 4.4: List of Intra-cellular compounds after 3D stress application, designated with the bacterial strains that produced them along with their potential uses in medicine and industry

Key findings from Table 4.4

- This table enlists the Intra-cellular fraction of the bacterial metabolites that were predicted to be present in after three-dimensional stress application.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibacterial, anti-fungal, anti-tumour, anti-inflammatory compounds, vitamins and even food processing, preservatives, plasticizers, polymers etc.
- The increase in the number of compounds produced after application of the three-dimensional stress, show the change in the bacterial metabolome after stress application.
- The highlighted compounds in the table indicate the compounds that were previously not produced in absence of stress.
- The table also clearly indicated that which of the compounds are produced by which set of bacteria drawing a direct comparison amongst the 5 bacterial strains of interest.

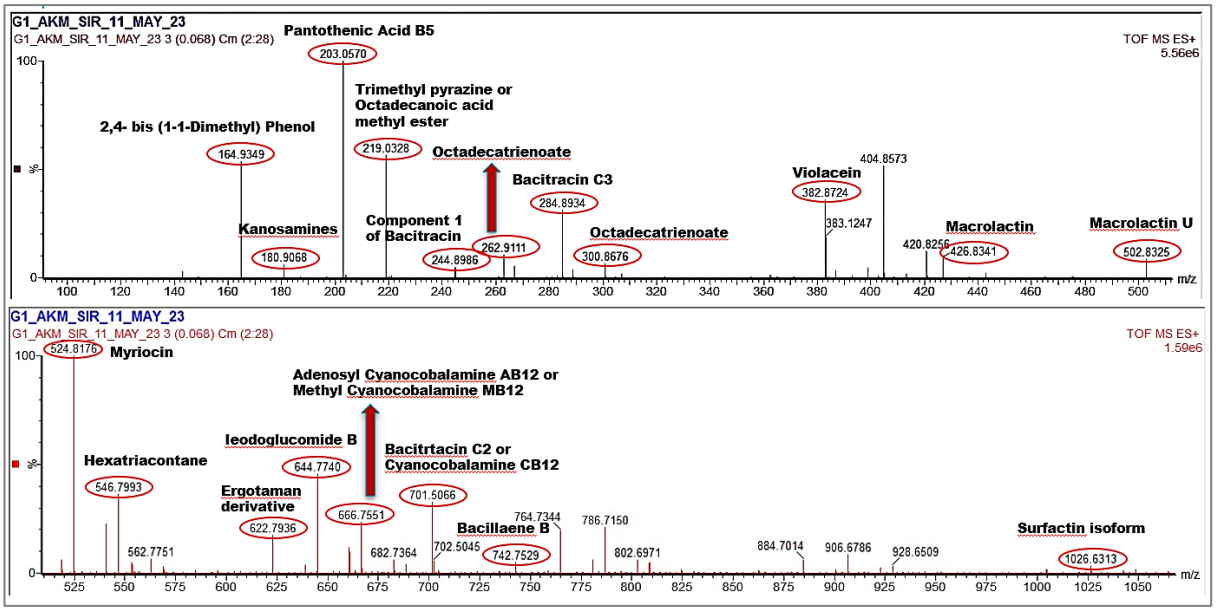


Figure 4.20: Mass spectra of Biofilm metabolite produced by BRAM_G1

Key findings from Figure 4.20

- The mass spectra in the image depicts the compounds present in the extracted Biofilm produced by BRAM_G1 against a water blank that was common to all samples.
- The compounds were found in high intensities of $5.56e^6$ and $1.59e^6$ for BRAM_G1 and included different vitamins, antibiotics, organic acids and certain alkanoates and macrolactins and ergotaman derivatives.
- The list of the compounds produced along with their significance in industry have been added later in the section.

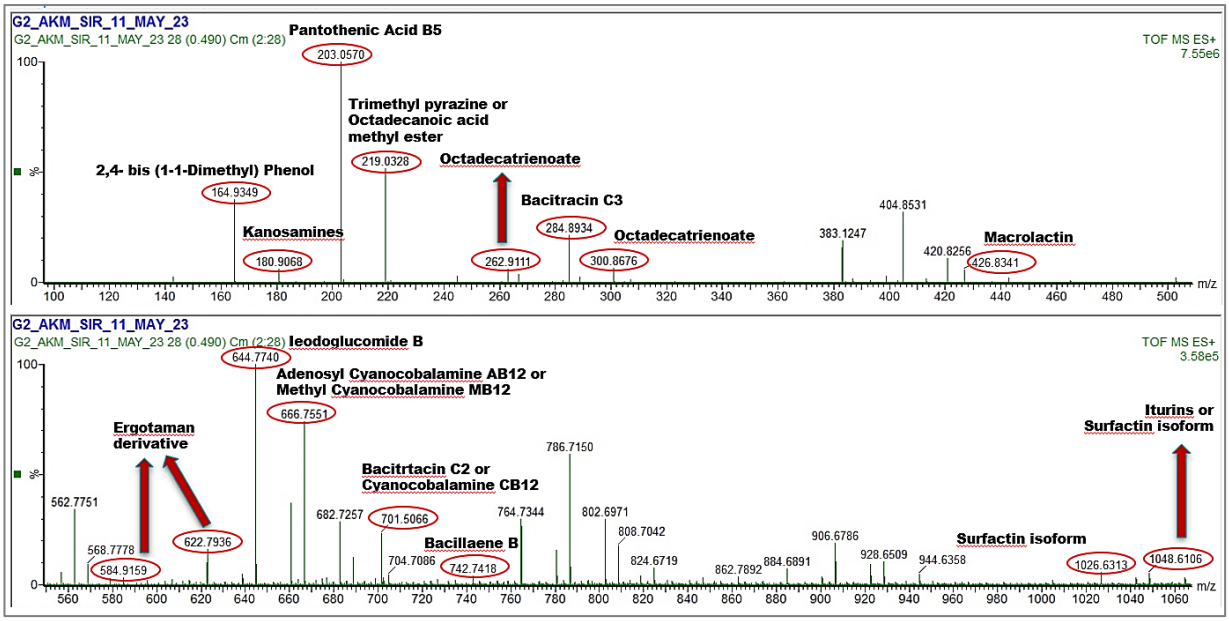


Figure 4.21: Mass spectra of Biofilm metabolites produced by BRAM_G2

Key findings from Figure 4.21

- The mass spectra in the image depicts the compounds present in the extracted Biofilm produced by BRAM_G2 against a water blank that was common to all samples.
- The compounds were found in high intensities of $7.55e^6$ and $3.58e^5$ for BRAM_G2 and included different vitamins, antibiotics, organic acids and certain alkanoates and macrolactins and ergotaman derivatives.
- The list of the compounds produced along with their significance in industry have been added later in the section.

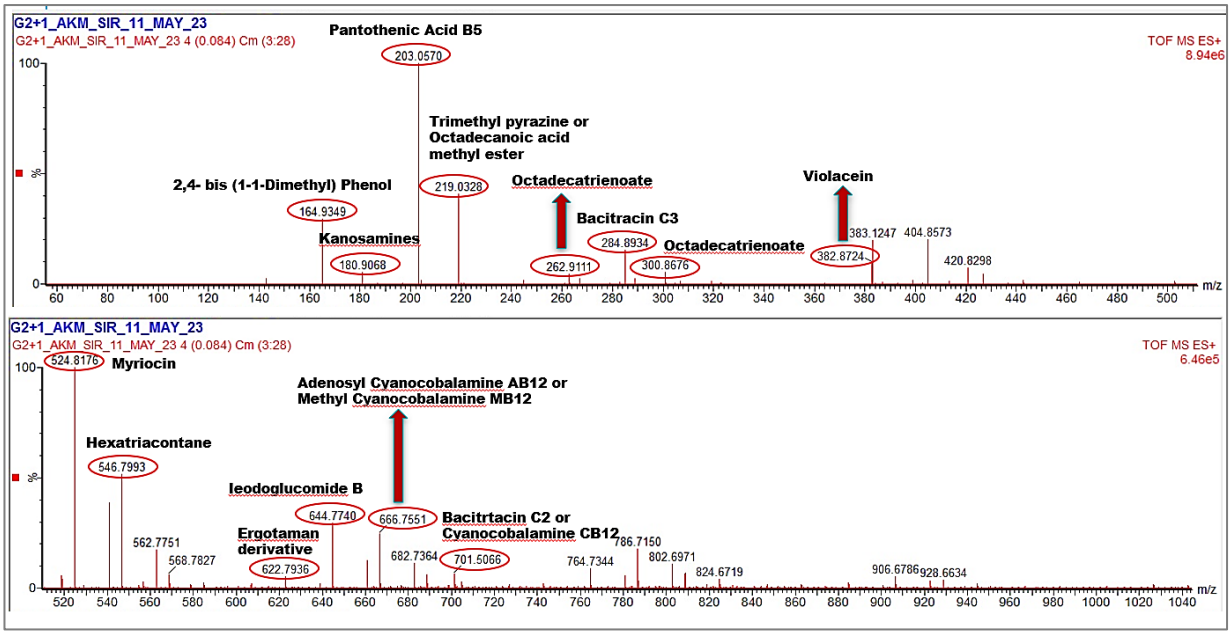


Figure 4.22: Mass spectra of Biofilm metabolites produced by BRAM_G3

Key findings from Figure 4.22

- The mass spectra in the image depicts the compounds present in the extracted Biofilm produced by BRAM_G3 against a water blank that was common to all samples.
- The compounds were found in high intensities of $8.94e^6$ and $6.46e^5$ for BRAM_G3 and included different vitamins, antibiotics, organic acids and certain alkanoates and violacein and ergotaman derivatives.
- The list of the compounds produced along with their significance in industry have been added later in the section.

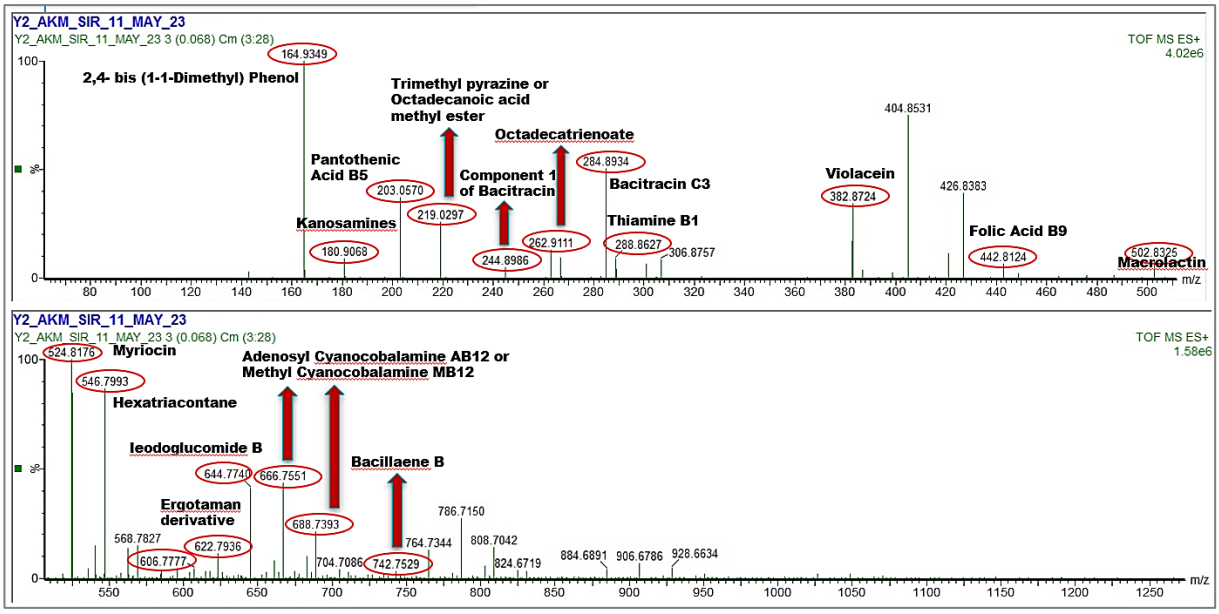


Figure 4.23: Mass spectra of Biofilm metabolites produced by BRAM_Y2

Key findings from Figure 4.23

- The mass spectra in the image depicts the compounds present in the extracted Biofilm produced by BRAM_Y2 against a water blank that was common to all samples.
- The compounds were found in high intensities of $4.02e^6$ and $1.58e^6$ for BRAM_Y2 and included different vitamins, antibiotics, organic acids and certain alkanoates macrolactins, violaceins and ergotaman derivatives.
- The list of the compounds produced along with their significance in industry have been added later in the section.

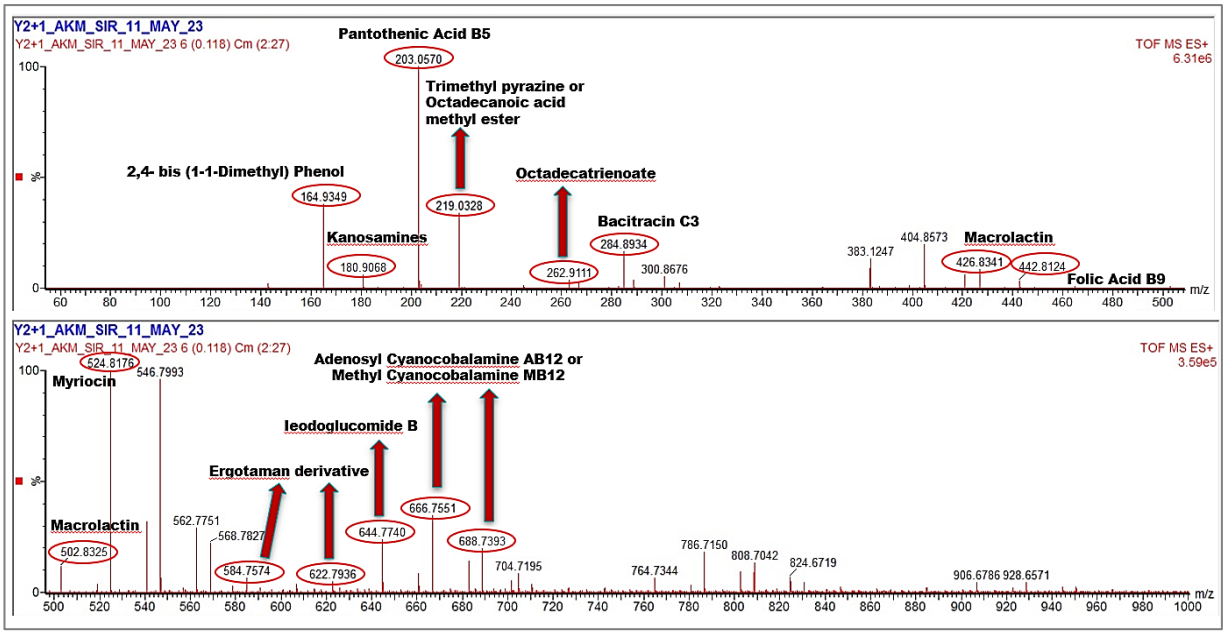


Figure 4.24: Mass spectra of Biofilm metabolites produced by BRAM_Y3

Key findings from Figure 4.24

- The mass spectra in the image depicts the compounds present in the extracted Biofilm produced by BRAM_Y3 against a water blank that was common to all samples.
- The compounds were found in high intensities of $6.31e^6$ and $3.59e^5$ for BRAM_Y3 and included different vitamins, antibiotics, organic acids and certain alkanoates and macrolactins and ergotaman derivatives.
- The list of the compounds produced along with their significance in industry have been added later in the section.

The Biofilm had to be first extracted for both the 3D stress and no stress setups for the ESI-MS study. As it has been seen that the 5 bacterial strains use their Biofilm as protective shields against the environmental stresses, cell to cell communications and many other functions, even without stress application the Biofilm extract showed peaks corresponding to numerous compounds that had significant roles in their survival as well as applications in the medicine and industry (**Figures 4.21 to 4.25) enlisted with their uses in Table 4.7.** After application of a 3-dimensional stress there was a massive change in the plethora of the compounds predictably present in the Biofilm. The **Figures 4.26 to 4.31** represent the mass spectra of the biofilm compounds after application of the 3-dimensional stress and the compounds have been enlisted in **Tables 4.8** along with their uses, with highlights on the most special and interesting members on the list**.**

	Biofilm Compounds	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	**Methylcyanocobalamine HB12**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Methylcobalamine helps treat the deficiency of vitamin B12 by producing a substance known as "myelin"
2.	**Pantothenic acid Vitamin B5**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It is used to make coenzyme A (CoA), a chemical compound that helps enzymes to build and break down fatty acids as well as perform other metabolic functions, and acyl carrier protein, which is also involved in building fats.
3.	**Thiamin Vitamin B1**	**BRAM_Y2**	Thiamine is used to treat beriberi and to treat and prevent Wernicke-Korsakoff syndrome (tingling and numbness in hands and feet, memory loss, confusion caused by a lack of thiamine in the diet).
4.	**Folic Acid Vitamin B9**	**BRAM_Y2, BRAM_Y3**	Folate (folic acid) is necessary for the production of red blood cells and for the synthesis of DNA. Folic acid also helps with tissue growth and cell function.
5.	**Ieodoglucomide B**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Ieodoglucomide B exhibited cytotoxic activity against lung cancer and stomach cancer cell lines and moderate in vitro antimicrobial activity.
6.	**Bacitracin C3, C2, Component 1,**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
7.	**Kanosamines**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	The antibiotic kanosamine, 3-amino-3-deoxy-d-glucose, inhibits the growth of various bacterial species, plant and human pathogenic fungi and oomycetes
8.	**Violacein**	**BRAM_G1, BRAM_G3, BRAM_Y2**	As a compound, violacein is known to have diverse biological activities, including being an anticancer agent and being an antibiotic, with antiproliferative activity against HeLa cells.
9.	**Octadecanoic Acid**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.
10.	**Surfactins**	**BRAM_G1, BRAM_G2**	It has been extensively studied for its antibacterial, antiviral, antitumor and hemolytic effects.

	Biofilm Compounds	Bacteria Producing the Compound	Uses in Industry and therapeutics.
11.	**Macrolactin**	**BRAM_G1, BRAM_G2, BRAM_Y3**	Antibacterial, anti-inflammatory, anti-angiogenic, anticancer, antiviral
12.	**Trimethyl pyrazine**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Apoptosis in HT-29 cells
13.	**Bacillaene B**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2,**	Bacillaene (101) is a polyene antibiotic produced by Bacillus subtilis.
14.	**2,4- bis (1-1-Dimethyl) Phenol**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	In medicine, it has the antioxidant, anticancer, antifungal, antibacterial properties and the protection against trimethyltin (TMT) -induced cognitive dysfunction.
15.	**Ergotaman Derivative**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Treatment of Parkinson's and other disorders
16.	**Hexatriacontane**	**BRAM_G1, BRAM_G3, BRAM_Y2**	Used as a paraffin wax and in candles. It is used as a key starting material for the preparation of lower alkanes by cracking.
17.	**Myriocin**	**BRAM_G1, BRAM_G3, BRAM_Y2, BRAM_Y3**	It is used in biochemical research as a tool for depleting cells of sphingolipids. Myriocin was shown to inhibit the proliferation of an IL-2-dependent mouse cytotoxic T cell line.
18.	**Iturins**	**BRAM_G2**	Iturins are a non-ribosomal cyclic lipopeptide family, has a broad-spectrum antibacterial effect, anticancer, hemolytic and other biological activities .

Table 4.5: **List of Biofilm metabolites designated with the bacterial strains that produced them along with their potential uses in medicine and industry.**

Key findings from Table 4.5

- This table enlists the compounds that were predicted to be present in the extracted biofilm produced by the bacterial strains.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibiotics, anti-tumour, anti-viral compounds, organic acids, vitamins and even preservatives, plasticizers, polymers etc.

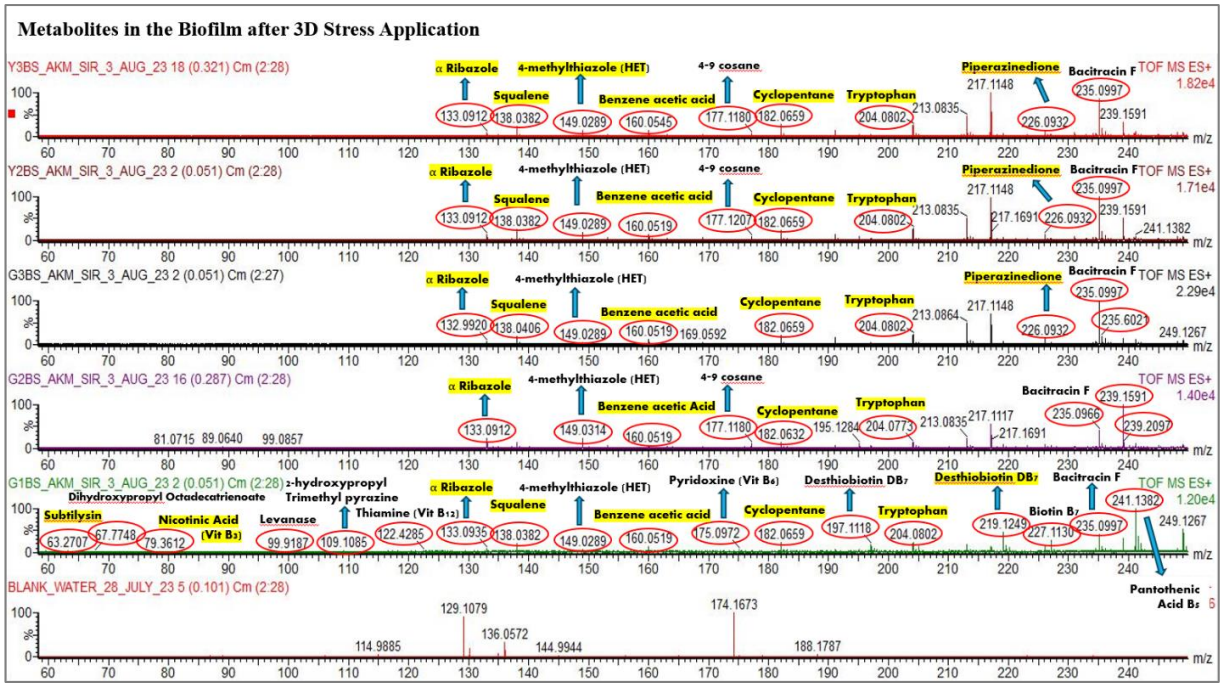


Figure 4.25: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (50-250 Da)

Key findings from Figure 4.25

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(50-250 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

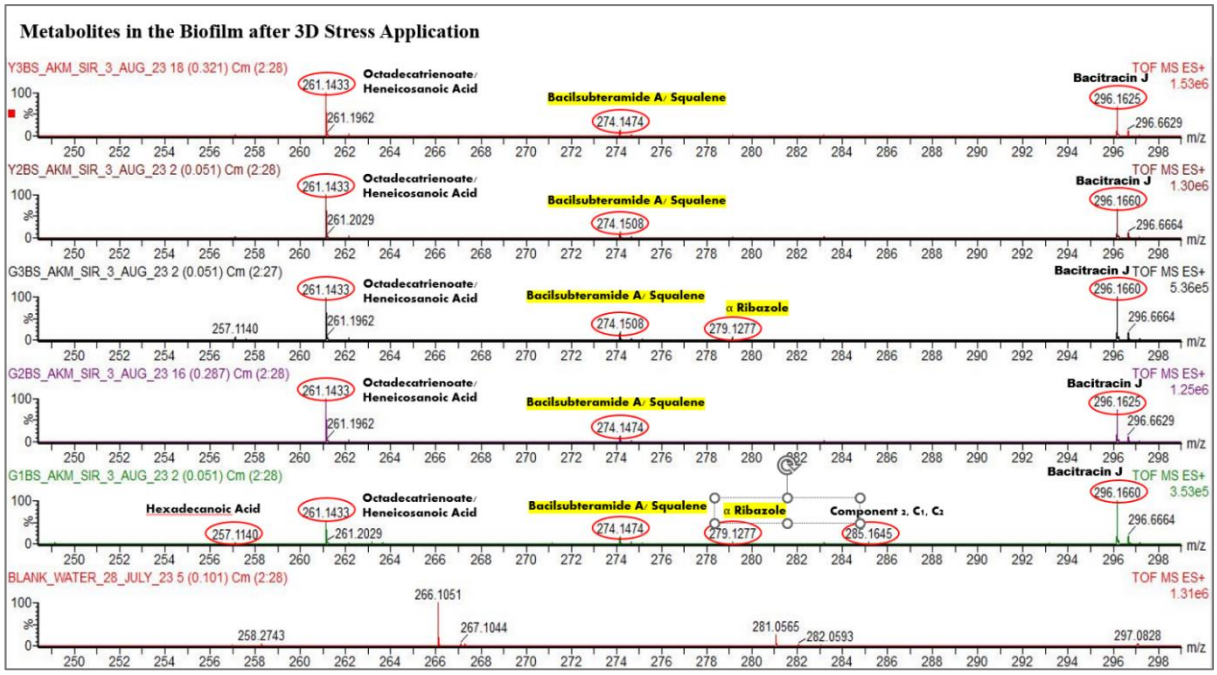


Figure 4.26: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (240-300 Da)

<u>Key findings from Figure 4.26</u>

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(240-300 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, BRAM_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

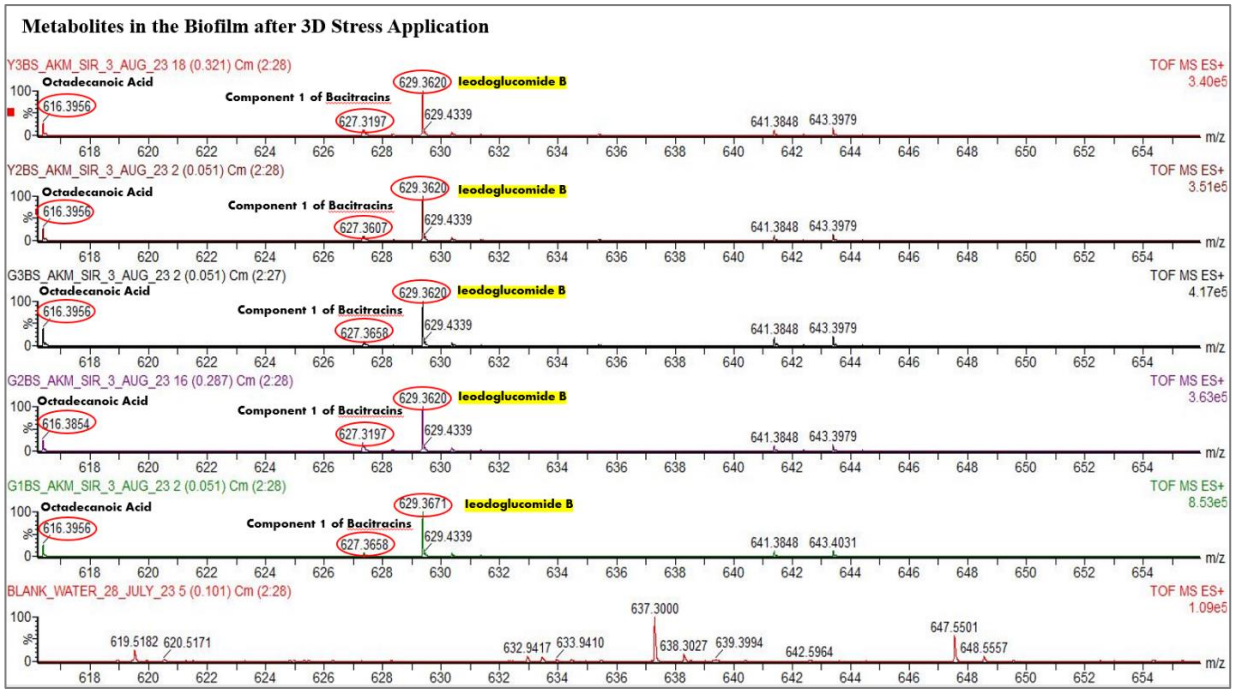


Figure 4.27: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (600-660 Da)

Key findings from Figure 4.27

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(600-660 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

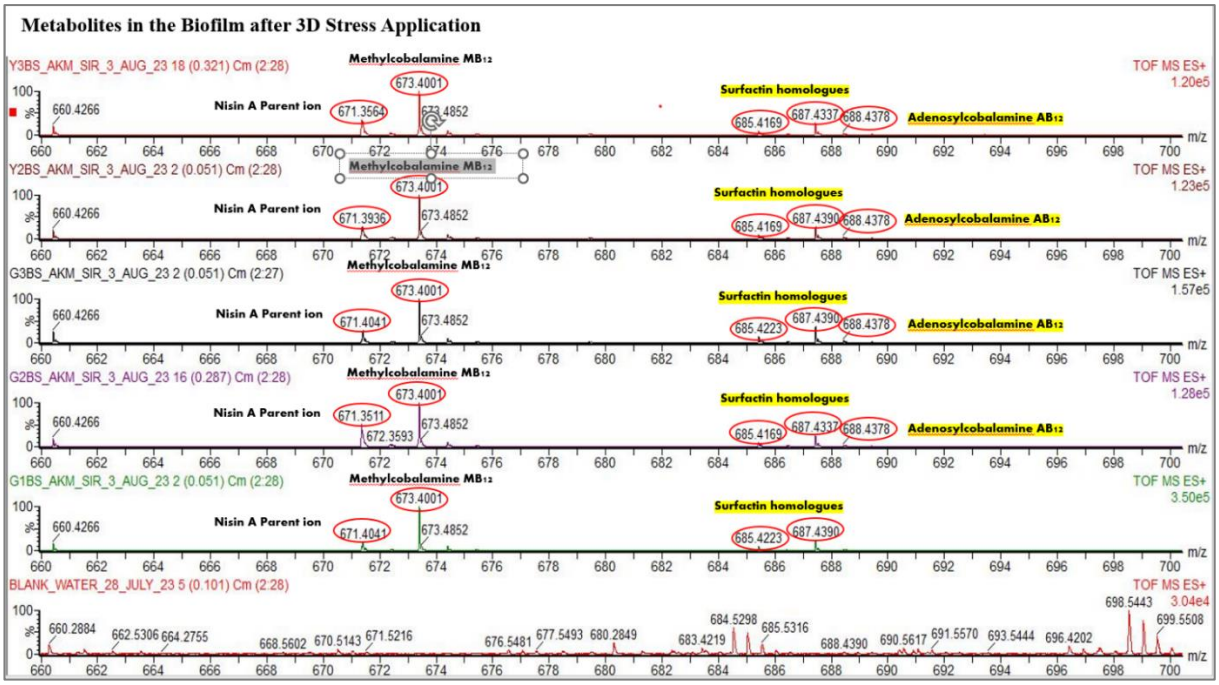


Figure 4.28: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (660-700 Da).

Key findings from Figure 4.28

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(660-700 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

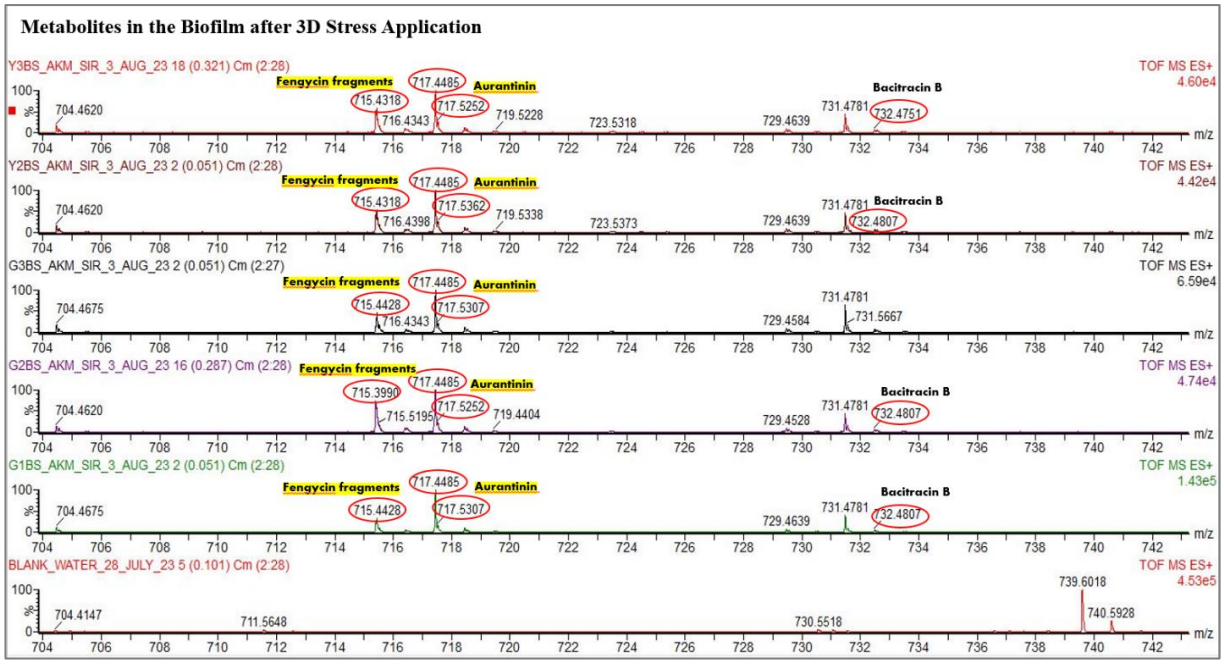


Figure 4.29: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (700-750 Da).

Key findings from Figure 4.29

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(700-750 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_1, BRAM_G2, Bram_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

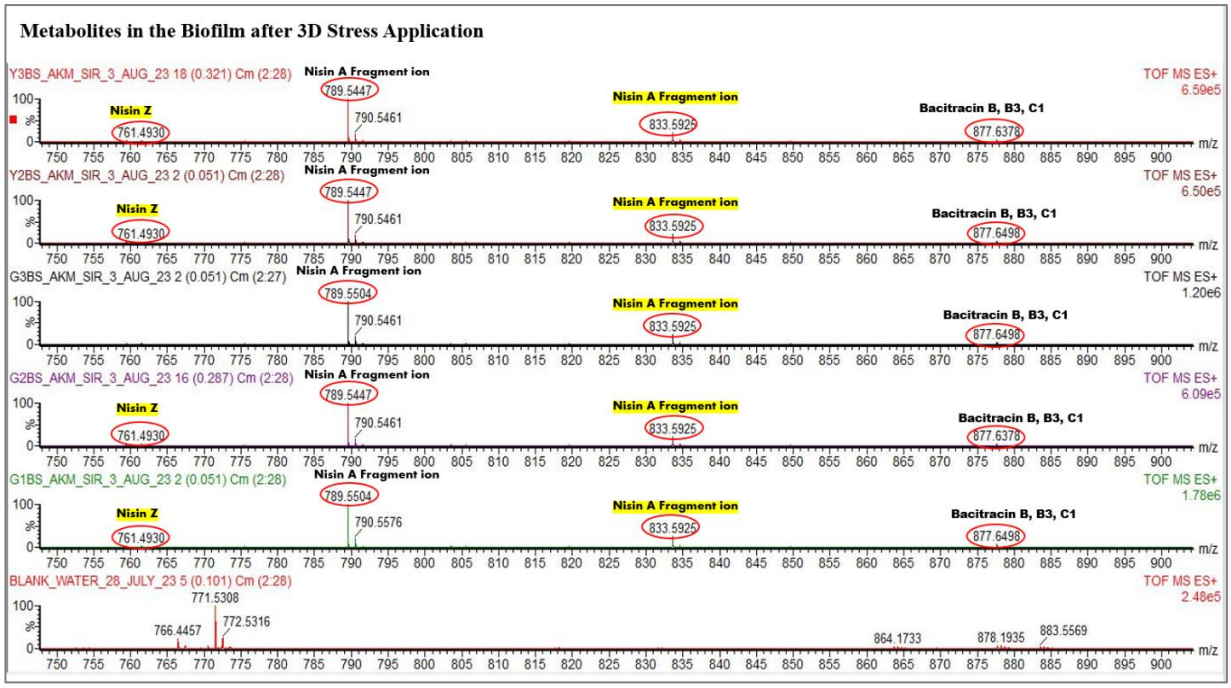


Figure 4.30: Mass spectra of Biofilm metabolites produced by the 5 bacterial strains after 3D stress application (750-900 Da).

Key findings from Figure 4.30

- The mass spectra image is a combined representation of the compounds present in the extracted Biofilm produced by the 5 bacterial strains in the presence of three-dimensional stress (Temperature70°C + 4% NaCl+20% PEG) through the molecular mass range of **(750-900 Da).**
- This image depicts the mass spectra of 5 bacterial strains and the blank in the following order from the bottom: Blank, BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2 and lastly BRAM_Y3.
- The highlighted compounds in the image, indicate the compounds that were previously not produced in absence of stress.
- The peaks are produced by each bacterium in varied intensities (therefore in various concentrations) even though there were a number of compounds that were common to all.
- The intensities are mentioned in the top right corner of each spectrum for better understanding.

	Biofilm Compounds (Stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
1.	**Octadecatrienoate**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Fatty acid present in essential oils.
2.	**Nicotinic Acid B3**	**BRAM_G1**	Plays a role in converting carbohydrates into glucose, metabolizing fats and proteins, and keeping the nervous system working properly.
3.	**Levanase**	**BRAM_G1**	An Enzyme which rapidly hydrolyzes levan (β-d-fructose polymer) in an endo-type manner to produce a series of levanoligosaccharides.
4.	**Biotin B7**	**BRAM_G1**	Biotin is necessary for formation of fatty acids and glucose, which are used as fuels by the body.
5.	**4-9cosanes**	**BRAM_G1, BRAM_G2, BRAM_Y2, BRAM_Y3**	Plays a role in the chemical communication of several insects, including the female Anopheles stephensi (a mosquito).
6.	**Trimethyl pyrazine**	**BRAM_G1**	Induces Apoptosis in HT-29 cells
7.	**4-methyl thiazole (HET)**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Used in the synthesis of catalytic dendrophanes, as functional mimics of the thiamine-diphosphate-dependent enzyme pyruvate oxidase
8.	**Squalene**	**BRAM_G1, BRAM_G3, BRAM_Y2, BRAM_Y3**	Use of squalene currently is as an adjunctive therapy in a variety of cancers.
9.	**Piperazinedione**	**BRAM_G1, BRAM_G3, BRAM_Y2, BRAM_Y3**	Piperazinedione is a crystalline antibiotic fermentation product isolated from the bacterium *Streptomyces griseoluteus* with antineoplastic activity. Piperazinedione alkylates DNA at the N-7 position of guanine, inhibiting DNA replication and inducing cell cycle arrest.
10.	**Pyridoxine B6**	**BRAM_G1**	Vitamin B-6 (pyridoxine) is important for normal brain development and for keeping the nervous system and immune system healthy.

	Biofilm Compounds (Stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
11.	**Benzene Acetic Acid**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Benzene acetic acid is also used to treat type II hyperammonemia to help diminish the amount of ammonia in a patient's blood stream by forming phenylacetyl-CoA, which then reacts with nitrogen-rich glutamine to form phenylacetyl glutamine.
12.	**α Ribazole**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A fluorescent marker for the liquid chromatographic determination of vitamin B12, It has a role as an Escherichia coli metabolite and a mouse metabolite.
13.	**Subtilysin**	**BRAM_G1**	Subtilisin is most known for its role as an additive for detergents in which it is categorized as a serine protease according to MEROPS database.
14.	**Cyclopentane**	**BRAM_G1,BRAM_G2 , BRAM_G3, BRAM_Y2, BRAM_Y3**	Occasionally used as an aerosol propellant in items like spray paint, deodorant, refrigerant and pesticides.
15.	**Tryptophan**	**BRAM_G1,BRAM_G2 , BRAM_G3, BRAM_Y2, BRAM_Y3**	It is a precursor of serotonin (hence its use as an antidepressant and sleep aid).
16.	**Desthiobiotin DB7**	**BRAM_G1**	DB1 labelling is used in malignant cell lines, to lentiviral vector applications to identify RNA-binding proteins.
17.	**Thiamine B12**	**BRAM_G1**	contribute essentially to the maintenance of a healthy nervous system.
18.	**Hexadecanoic Acid**	**BRAM_G1**	Palmitic Acid is a fatty used as a food additive and emollient or surfactant in cosmetics. It may help in designing of specific inhibitors of phospholipase A(2) as anti-inflammatory agents.
19.	**Adenosylcobala mine AB12**	**BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Adenosylcobalamin is an active form of vitamin B12. This essential nutritional supplement works by restoring vitamin deficiency in the body and helps in proper functioning and growth of the body.
20.	**Bacilsubteramide A**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A new indole alkaloid From Bacillus subterraneus.

	Biofilm Compounds (Stress)	Bacteria Producing the Compound	Uses in Industry and therapeutics.
21.	**Bacitracin C1, J, B, B3, C1,F**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Antibiotics to treat minor skin injuries, including cuts, scrapes, burns, pneumonia and empyema in infants, and to treat skin and eye infections.
22.	**Octadecanoic Acid**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A C18 straight-chain saturated fatty acid component of many animal and vegetable lipids. As well as in the diet, it is used in hardening soaps, softening plastics and in making cosmetics, candles and plastics.
23.	**Heneicosanoic Acid**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It is the straight-chain 21-carbon saturated fatty acid. It has shown relevance in the production of foams, paints, and related viscous materials.
24.	**Ieodoglucomide B**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Ieodoglucomide B exhibited cytotoxic activity against lung cancer and stomach cancer cell lines and moderate in vitro antimicrobial activity.
25.	**Nisin A, Z**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Nisin is used in processed cheese, meats, beverages, etc. during production to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria.
26.	**Aurantinin**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	A novel antibiotic effective against Gram posittive Bacteria.
27.	**Methylcobalamine HB12**	**BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Methylcobalamin helps treat the deficiency of vitamin B12 by producing a substance known as "myelin"
28.	**Surfactins**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	It has been extensively studied for its antibacterial, antiviral, antitumor and hemolytic effects.
29.	**Fengycins**	**BRAM_G1,BRAM_G2, BRAM_G3, BRAM_Y2, BRAM_Y3**	Fengycin is a cyclic lipopeptide which is used as an agricultural fungicide.

Table 4.6: List of Biofilm metabolites after 3D stress application, designated with the bacterial strains that produced them along with their potential uses in medicine and industry.

Key findings from Table 4.6

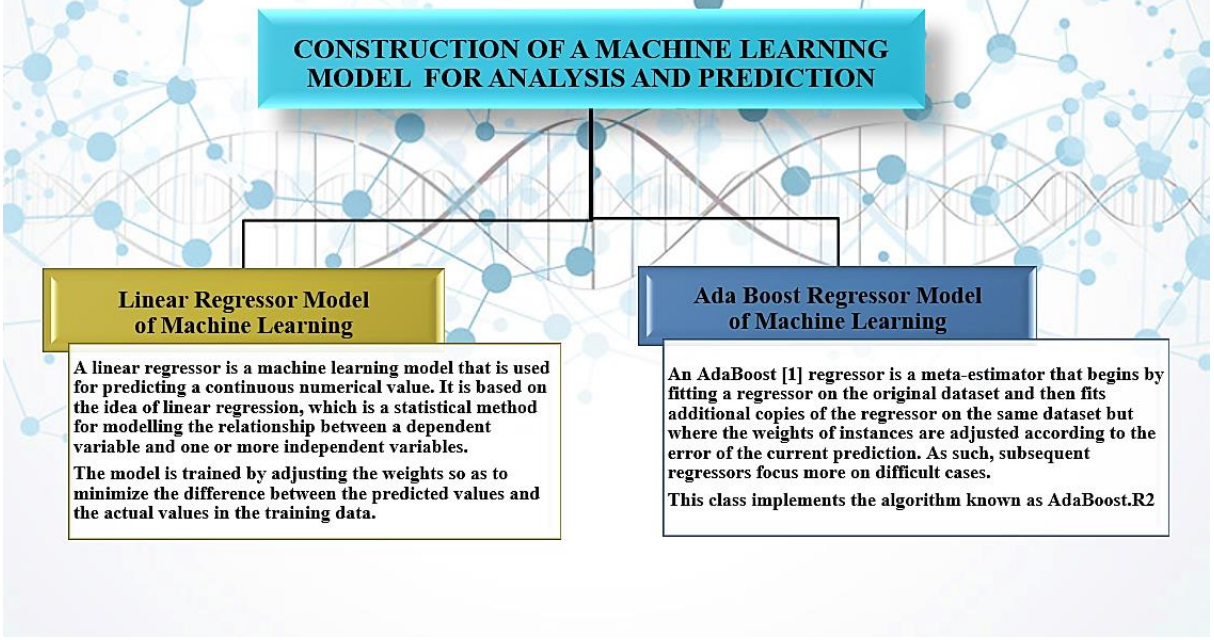
- This table enlists the extracted Biofilm fraction of the bacterial metabolites that were predicted to be present in after three-dimensional stress application.
- The 5 bacterial strains were found to produce numerous industrially and medicinally important compounds including but not limited to include antibacterial, anti-fungal, anti-tumour, anti-inflammatory compounds, vitamins and even food processing, preservatives, plasticizers, polymers etc.
- The increase in the number of compounds produced after application of the three-dimensional stress, show the change in the bacterial metabolome after stress application.
- The highlighted compounds in the table indicate the compounds that were previously not produced in absence of stress.
- The table also clearly indicated that which of the compounds are produced by which set of bacteria drawing a direct comparison amongst the 5 bacterial strains of interest.

Key findings from Objective 4

- It was found that along with certain enzymes the 5 bacterial strains can produced an enormous plethora of compounds and metabolites that can be of use in the field of both pharmaceuticals and industry.
- The validation of the fact that the 5 bacterial strains were Polyextremophiles in nature was the significant increase in their plethora of metabolites after stress application.
- Increase in certain types of compounds such as surfactins and bacillaenes during stress which have significant roles in biofilm formation validate the hypothesis of Biofilm acting as the protective jacket for all 5 bacterial strains in times of stress.
- The role of all 5 bacterial strains as a hub of different metabolites with applications in medicine, industry and agriculture justify their multifaceted potential in all these three aspects and provide the rationale of fulfilling all the objectives previously laid down for this study.

OBJECTIVE 5

Construction of a machine learning for analysis and prediction of plant growth promoting properties and maize productivity under stressed conditions.



5. Construction Of a Machine Learning Model for Analysis and Prediction of Plant Growth Promoting Properties and Maize productivity in stress condition.

The extensive data was used to train machine learning models. As the two models use correlation and regression as their base, all the parameters are first correlated with each other by correlation mapping **(Figure 5.1).**

The trained Linear regressor model of machine learning with all the data previously discussed in this research work, was used to predict, plant growth promoting properties of the 5 bacterial strains at the different stressed conditions. The overall goodness of fit or the R2 score of the trained linear regressor was found to be 0.993371, which was very close to 1, suggesting that it perfectly explains the variance in the dependent variable. Ten parameters were selected for prediction **(Figure 5.2)**, at all the stress parameters. But as, only one parameter can be represented in a 2D Graph, representative 2 single dimension stresses 2 two-dimensional stresses and 2 three-dimensional stresses have been represented in **Figure 5.3**. The graphs show predicted values at stressed conditions vs the actual values in no stress conditions. Only, 3 most crucial parameters could be shown in graphs.

A closer look at the predicted values and the actual values shows that there is not much of a difference in between them. But, the accuracy of the model was found to be more than 99% which, being an excellent number also had the fallacy of being “too good to be true”. The AdaBoost Regressor model uses mutual information scoring for the parameters used in training the machine **(Figure 5.4)**. Thus, the results of AdaBoost regressor model with different parameters like RMSE, MSE and MAE **(Figure 5.5)** (better parameters in comparison to R2 scores for determination of Goodness of fit of a machine learning model) show significant variations in determining the accuracy of the model performance, made the predictions more viable for a biological system (**Figure 5.6)**. Though the variations and the prediction result clearly demonstrate AdaBoost regressor model being a better choice for the modelling of this humongous data. A closer look at the predictions does not show extensive variation in stressed conditions. Therefore, to explain this prediction, the results of increasing biofilm formation with stress are to be referred. The biofilm formation helps the bacteria maintain its PGP activity even in stressed conditions.

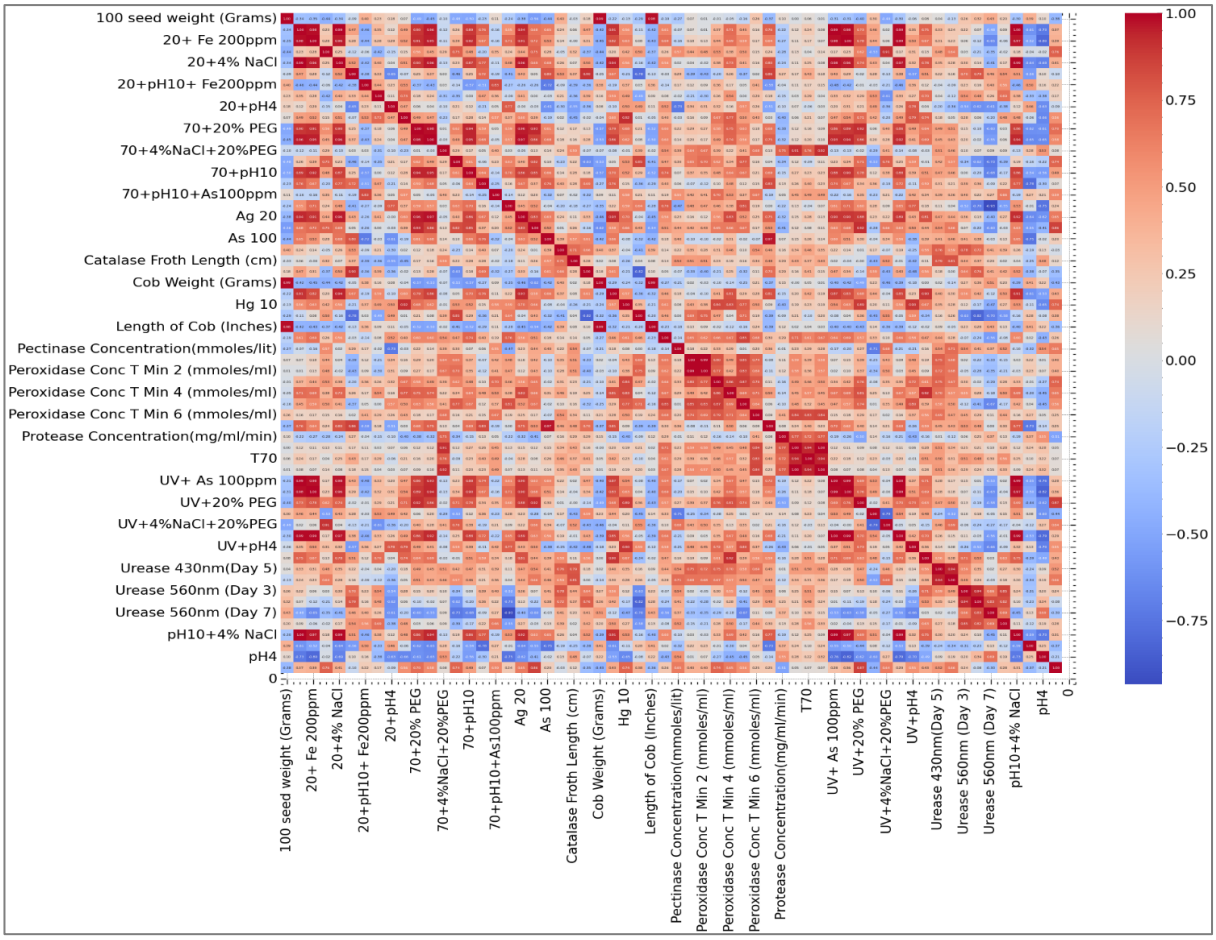


Figure 5.1: **The correlation map of all the parameters used in the Machine Learning Model.**

Key findings from Figure 5.1:

- The data of the investigation was used train two machine learning models that use correlation and regression as their base, therefore all the parameters are first correlated with each other by correlation mapping.
- The correlation between all the parameters, whose data had been entered into the machine learning models, was visually depicted in a graphic known as the correlation map of the parameters

Parameters	R2 scores
Overall	0.993371
ACC DEAMINASE (mmoles/lit)	0.999642
Indole Acetic Production TRP- (µg/ml)	0.999983
Indole Acetic Production TRP+ (µg/ml)	0.999926
Giberrelin production GA3 Concentration (5days)	0.999651
Giberrelin production GA3 Concentration (7days)	0.999631
BETA-1,3-GLUCANASE Concentration(units/ml)	0.999950
% Percentage Siderophore Unit(24 hrs)	0.999933
% Percentage Siderophore Unit(48 hrs)	0.999906
No. of Fruits	1.000000
Percent Grain Filling	0.953539

Table 5.1: Parameters selected for prediction in the Linear Regressor Model of Machine Learning.

Key findings from Table 5.1:

- A linear regressor is a machine learning model that is used for predicting a continuous numerical value. It is based on the idea of linear regression, which is a statistical method for modelling the relationship between a dependent variable and one or more independent variables.
- The model is trained by adjusting the weights so as to minimize the difference between the predicted values and the actual values in the training data.
- The parameters selected for predicted by the Linear regressor was represented in the figure.

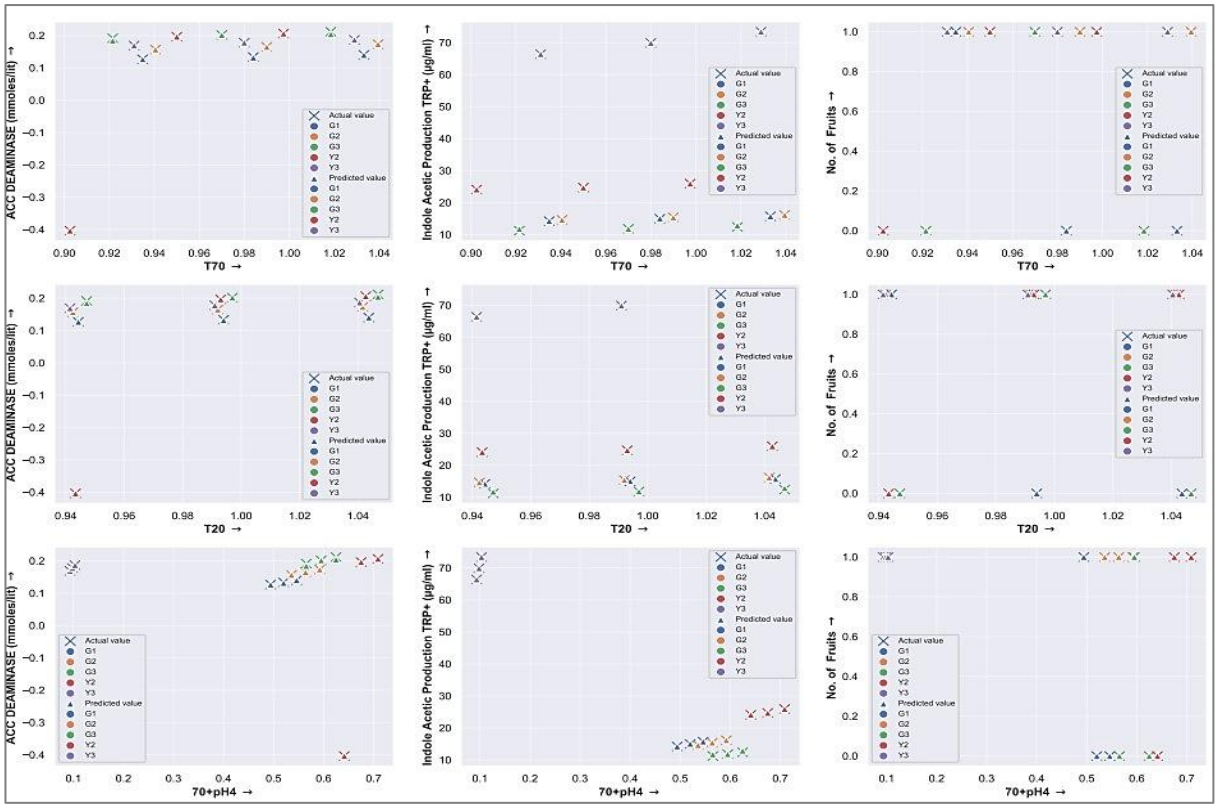


Figure 5.2: **Predicted Data vs the Actual data for three important parameters, ACC Deaminase, Indole acetic acid and Number of Fruits per plant in two of the 1D stresses and two of the 2D stresses (Linear Regressor Model) for all 5 bacterial strains.**

<u>Key findings from Figure 5.2:</u>

- The graphs represent the Actual values verses the predicted values of different parameters.
- The different parameters include ACC Deaminase, Indole Acetic Acid, and No. of fruits when the bacteria is applied to maize.
- The '×' represents actual values whereas '●' represents the predicted values by the machine learning model.
- In linear regressor the actual values and the predicted values almost coincided with each other.
- Though different graphs show different set of stress, the actual prediction was done on the basis of all the stresses including all single-dimensional, two-dimensional and three-dimensional stress combinations. The representation of which was not possible in 2 dimensions and therefore the generation of the graphical representations.

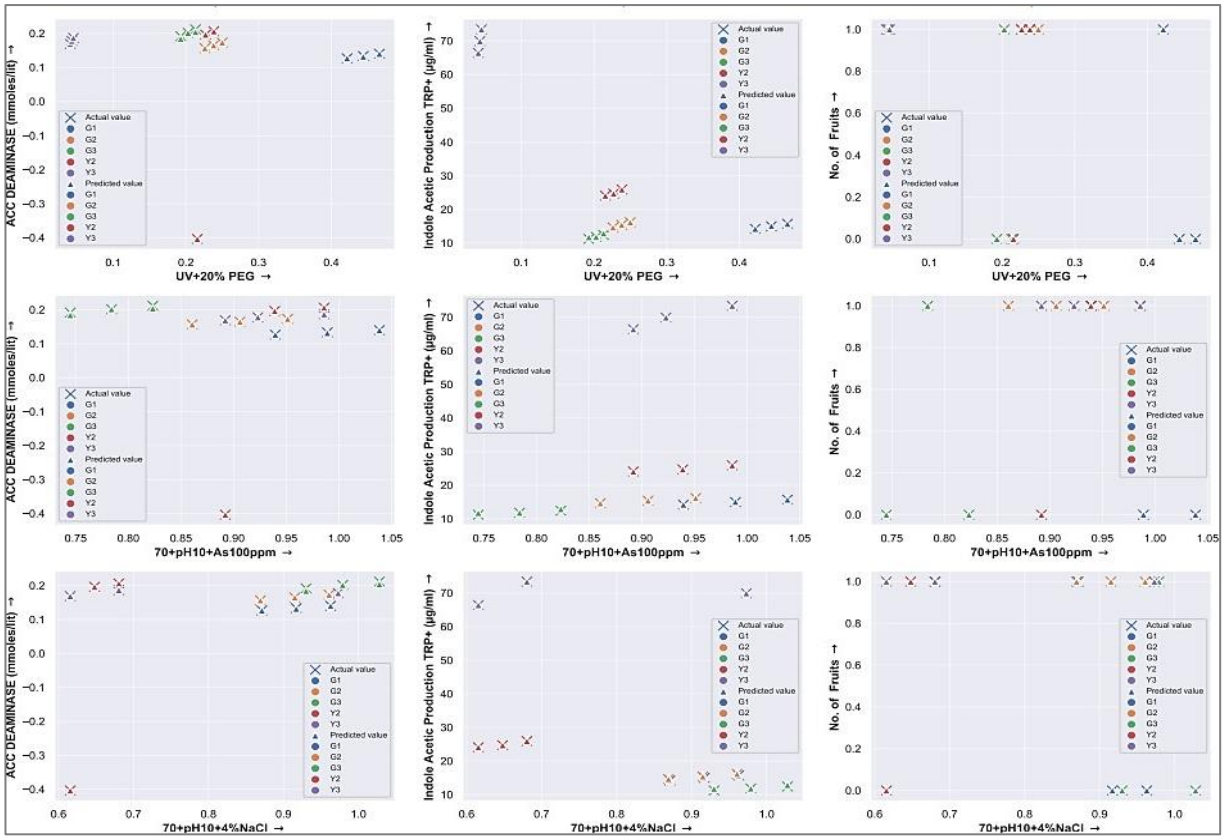


Figure 5.3: Predicted Data vs the Actual data for three important parameters, ACC Deaminase, Indole acetic acid and Number of Fruits per plant in one of the 2D stresses and two of the 3D stresses (Linear Regressor Model) for all 5 bacterial strains.

<u>Key findings from Figure 5.3:</u>

- The graphs represent the Actual values verses the predicted values of different parameters for the linear regressor model trained using the extremophily dataset and PGP dataset of the 5 bacterial strains along with the Maize trial dataset.
- The different parameters include ACC Deaminase, Indole Acetic Acid, and No. of fruits when the bacteria is applied to maize.
- The '×' represents actual values whereas '●' represents the predicted values by the machine learning model.
- In linear regressor the actual values and the predicted values almost coincided with each other.
- Though different graphs show different set of stress, the actual prediction was done on the basis of all the stresses including all single-dimensional, two-dimensional and three-

dimensional stress combinations. The representation of which was not possible in 2 dimensions and therefore the generation of the graphical representations.

- The absence of variation between the actual values and the predicted values was indicative of the appropriateness of the linear regressor model, as linear regression is not a preferred method for biological data sets.

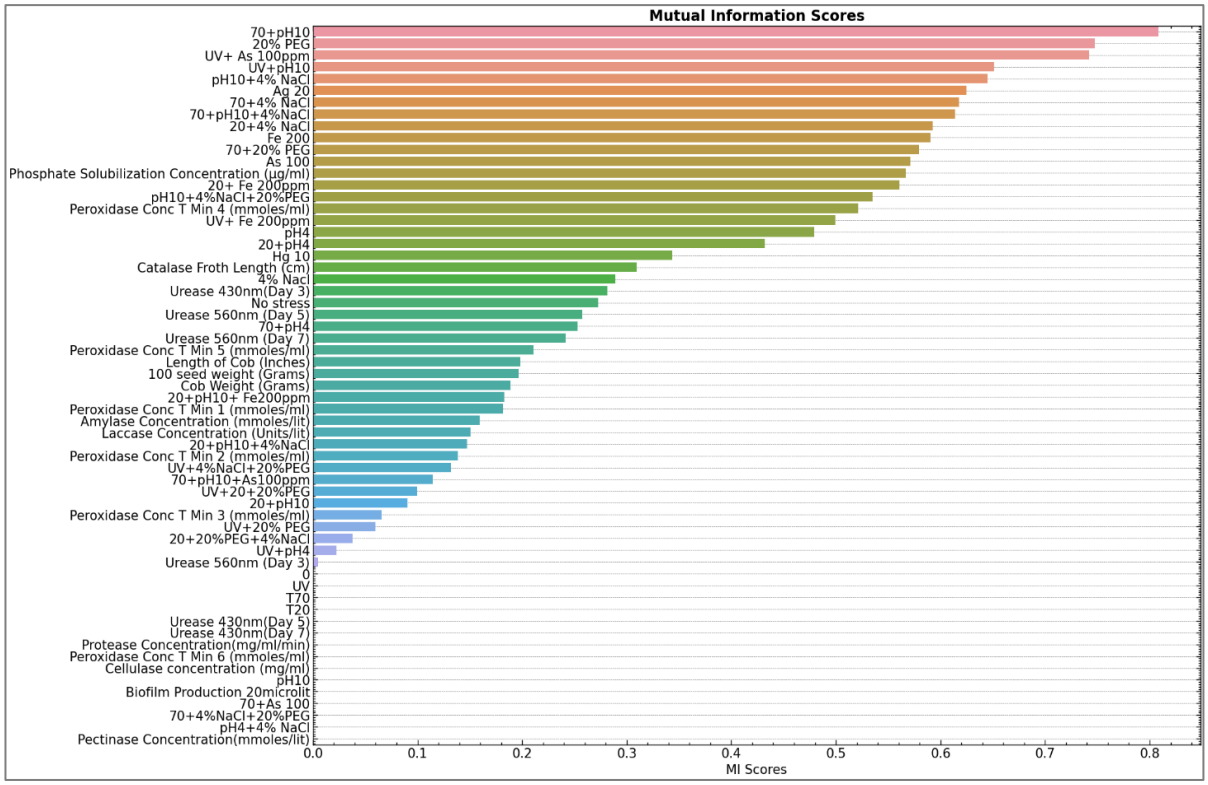


Figure 5.4: **The mutual information scoring plot for all the parameters for the AdaBoost Regressor Model.**

Key findings from Figure 5.4:

- An AdaBoost regressor is a meta-estimator that begins by fitting a regressor on the original dataset and then fits additional copies of the regressor on the same dataset but where the weights of instances are adjusted according to the error of the current prediction. As such, subsequent regressors focus more on difficult cases. This class implements the algorithm known as AdaBoost.R2

- In Probability Theory and information theory, the mutual information (MI) of two random variables is a measure of the mutual dependence between the two variables. More specifically, it quantifies the "amount of information" obtained about one random variable by observing the other random variable.

- AdaBoost regressor computes the mutual information between each feature and the target variable and then uses the mutual information scores to select features that contribute most to predicting the target variable. Higher scores indicate more informative features. The figure represents the mutual information scores for all the parameters.

PREDS	ACTUAL	MSE	RMSE	MAE	LABEL	BACTERIA
0.19915	0.19171	0.000376361	0.019400015	0.016905	ACC DEAMINASE (mmoles/lit)	G3
0.17355	0.1869	0.000376361	0.019400015	0.016905	ACC DEAMINASE (mmoles/lit)	Y3
0.15675	0.126825	0.000376361	0.019400015	0.016905	ACC DEAMINASE (mmoles/lit)	G1
11.3085	10.2315	0.668974898	0.817908857	0.787277778	Indole Acetic Production TRP- (microg/ml)	G2
13.41633333	12.67	0.668974898	0.817908857	0.787277778	Indole Acetic Production TRP- (microg/ml)	G1
11.3085	10.77	0.668974898	0.817908857	0.787277778	Indole Acetic Production TRP- (microg/ml)	G2
24.717	25.95285	1262.003022	35.52468187	29.38845	Indole Acetic Production TRP- (microg/ml)	Y2
24.717	66.4335	1262.003022	35.52468187	29.38845	Indole Acetic Production TRP- (microg/ml)	Y3
24.717	69.93	1262.003022	35.52468187	29.38845	Indole Acetic Production TRP- (microg/ml)	Y3
0.185	0.19425	0.000130965	0.011443994	0.010933333	Giberrelin production GA3 Concentration (5days)	Y2
0.2039	0.196	0.000130965	0.011443994	0.010933333	Giberrelin production GA3 Concentration (5days)	G3
0.20185	0.1862	0.000130965	0.011443994	0.010933333	Giberrelin production GA3 Concentration (5days)	G3
0.2422	0.26	0.000200128	0.014146672	0.013433333	Giberrelin production GA3 Concentration (7days)	G2
0.2289	0.21375	0.000200128	0.014146672	0.013433333	Giberrelin production GA3 Concentration (7days)	G3
0.2289	0.23625	0.000200128	0.014146672	0.013433333	Giberrelin production GA3 Concentration (7days)	Y2
0.192	0.2016	0.000196483	0.014017251	0.010633333	BETA-1,3-GLUCANASE Concentration(units/ml)	G2
0.62	0.62	0.000196483	0.014017251	0.010633333	BETA-1,4-GLUCANASE Concentration(units/ml)	G1
0.4683	0.446	0.000196483	0.014017251	0.010633333	BETA-1,5-GLUCANASE Concentration(units/ml)	Y2
18.354	20.286	2.315293333	1.521608798	1.469333333	% Percentage Siderophore Unit(24 hrs)	G3
18.354	19.32	2.315293333	1.521608798	1.469333333	% Percentage Siderophore Unit(24 hrs)	G3
30.2	31.71	2.315293333	1.521608798	1.469333333	% Percentage Siderophore Unit(24 hrs)	Y2
72.34	68.723	10.83645277	3.291876785	3.248416667	% Percentage Siderophore Unit(48 hrs)	G2
43.59	46.0845	10.83645277	3.291876785	3.248416667	% Percentage Siderophore Unit(48 hrs)	Y2
47.23875	50.8725	10.83645277	3.291876785	3.248416667	% Percentage Siderophore Unit(48 hrs)	Y3
0	1	0.333333333	0.577350269	0.333333333	No. of Fruits	G3
1	1	0.333333333	0.577350269	0.333333333	No. of Fruits	G2
1	1	0.333333333	0.577350269	0.333333333	No. of Fruits	G2
67	70	16.65333333	4.080849585	3.133333333	Percent Grain Filling	G1
67	67	16.65333333	4.080849585	3.133333333	Percent Grain Filling	G2
65	71.4	16.65333333	4.080849585	3.133333333	Percent Grain Filling	Y3

Table 5.2: **Parameters depicting the goodness of fit of the AdaBoost Regressor Model.**

Key findings from Table 5.2:

- When assessing the performance of an AdaBoost regressor (or any regression model), various measures can be employed to evaluate the quality of the fit. Three often used metrics include Mean Squared Error (MSE), Root Mean Squared Error (RMSE), and Mean Absolute Error (MAE).
- The Mean Squared Error (MSE) calculates the average value of the squared differences between the observed actual outcomes and the predicted values.
- RMSE is the mathematical operation of taking the square root of the MSE. It offers a calculation of the standard deviation of the mistakes. The reason it is more interpretable than MSE is because it is measured in the same units as the target variable.

- Mean Absolute Error (MAE) quantifies the average size of errors in a collection of predictions, regardless of their direction. The average of the absolute differences between the predicted and actual observations in the test sample, where each difference is given equal weight.
- The figure demonstrates these above parameters for the Ada boost regressor model generated for this dataset with the corresponding data labels.

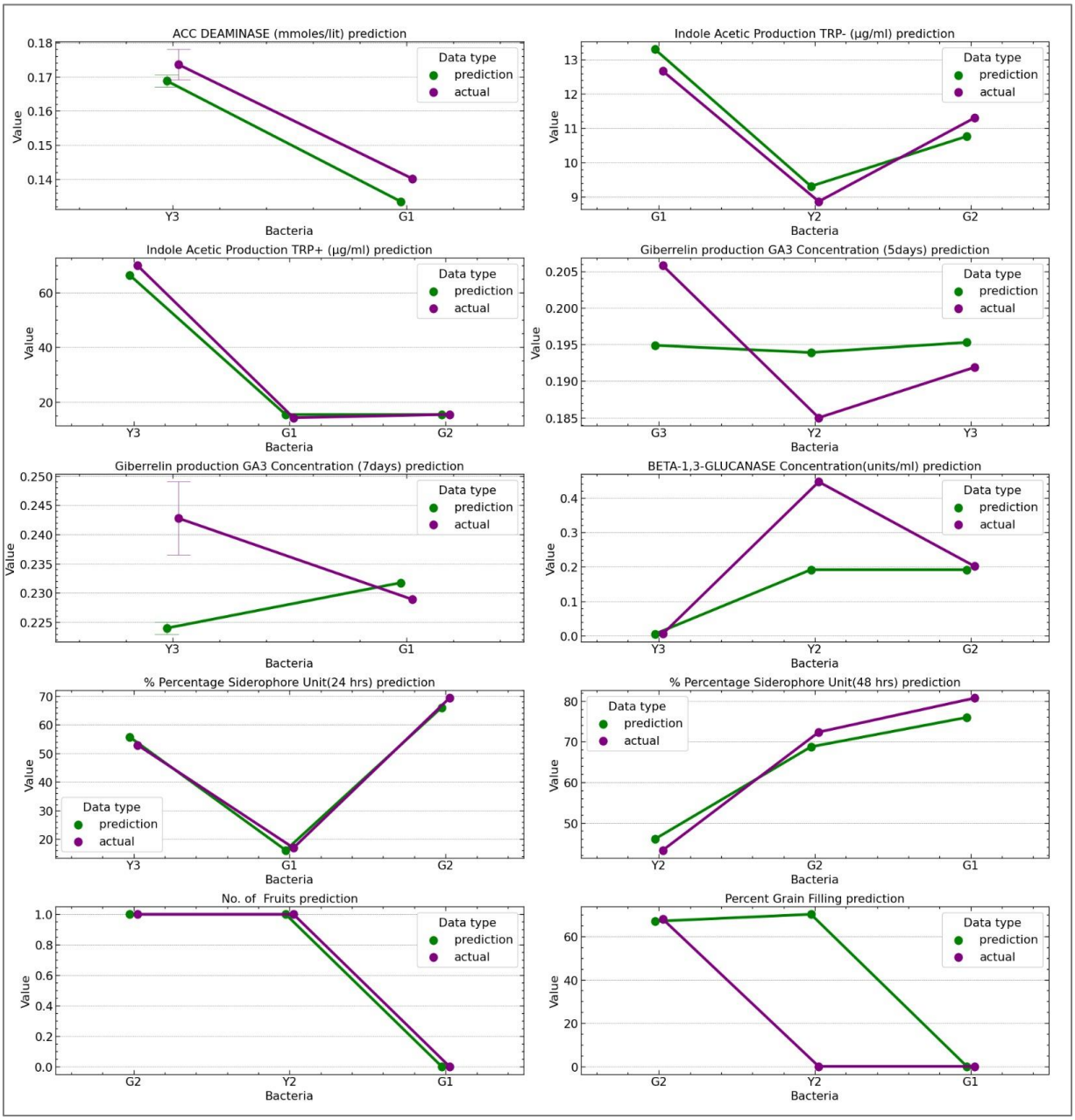


Figure 5.5: Predicted Data vs the Actual data for nine important parameters, ACC Deaminase, Indole acetic acid, Beta-1,3-glucanase, gibberellin, and Number of Fruits per plant, percent grain filling and percent siderophore unit in all the stress parameters, 1D, 2D and 3D combinations. (Ada Boost Regressor Model).

Key findings from Figure 5.5:

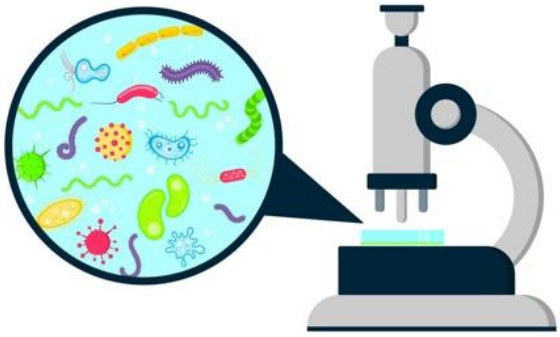
- The stem plots represent the Actual values verses the predicted values of different parameters for the Ada Boost regressor model trained using the extremophily dataset and PGP dataset of the 5 bacterial strains along with the Maize trial dataset.

- The different predicted parameters include ACC Deaminase, Indole Acetic Acid, gibberellins percent grain filling, and no. of fruits, after bacterial treatment application in the maize rhizosphere.
- The predictions are done for 2 or three bacteria at a time as the rest of the dataset is used by the Model as the training dataset.
- Though in case of Ada Boost regressor model the variation in the predicted values vs actual values are higher than the linear regressor, a closer analysis exhibits minimal fluctuation under stressful settings. Thus, in order to elucidate this prediction, we must consider the outcomes of heightened biofilm production under stressful conditions. Biofilm production enables bacteria to sustain its plant growth-promoting (PGP) activities even under stressful situations.

Summarised Key Findings from Objective 5:

- Two machine learning models were designed for the data analyses and their results were compared to designate the best fitting model.
- Though in case of Ada Boost regressor model the variation in the predicted values vs actual values are higher than the linear regressor, a closer analysis exhibits minimal fluctuation under stressful settings. Thus, in order to elucidate this prediction, we must consider the outcomes of heightened biofilm production under stressful conditions. Biofilm production enables bacteria to sustain its plant growth-promoting (PGP) activities even under stressful situations.
- The simulation model predictions that the 5 bacterial strains can produce ample quantities of plant growth promoting metabolites to sustain plant productivity in presence of abiotic stress is validated by the in-vivo studies with *Oryza sativa* in presence of abiotic stress dataset which was not used to train the models, also proving the validity of the machine learning model predictions."

CHAPTER 5: DISCUSSIONS



Objective 1: Isolation and Characterization of extremophilic bacterial strains.

This research involved three strains of Bacilli, that were recovered from the waters of the Gangotri glacier in Uttarakhand, India. These strains, namely *Bacillus subtilis* BRAM_G1, *Bacillus* tequilensis BRAM_G2, and *Bacillus* thuringiensis BRAM_G3, were collected at a height of 3415 m above sea level. *Mesobacillus* thiparans BRAM_Y2 and *Brevibacillus parabrevis* BRAM_Y3 were obtained from a thermal spring in Yamunotri, which is situated in the Champasar glacier, north of Uttarkashi, at an altitude of 3293 metres.

These 5 bacterial strains selected for investigation shared common characteristics, namely belonging to the Bacillaceae family and producing significant quantities of biofilm, unlike typical *Bacillus* strains. Prior research has demonstrated that, the production of biofilms provides a shielding layer for bacterial cells, enhancing their ability to withstand stress. Additionally, extremophilic microbes frequently possess exceptional capacities to create specialised biofilms. Various extremophiles possess distinct properties in their biofilms to adapt to their specific extreme environments (Rathinam and Sani, 2019). Kanso initially documented the existence of the bacterium *Mesobacillus subterraneus*. The microorganisms were identified from a thermal aquifer in Australia (Kanso et al., 2002). Takagi et al conducted the initial investigation of *Brevibacillus parabrevis* in 1993, followed by Hooda, in 2018. Both bacterial species are uncommon in nature and have not been extensively studied in terms of their characterisation and application.

The three bacterial strains, namely Gangotri BRAM_G1, BRAM_G2, and BRAM_G3, were identified as gram-positive rods. BRAM_Y2 and BRAM_Y3 were identified as Gram-variable rods (Gordon et al., 1973). Contrary to the majority of *Bacillus* species none of the five bacterial strains examined in this study were capable of forming endospores. When streaked on Hichrome UTI agar and Hichrome *Bacillus* agar, all 5 bacterial strains exhibited colour indexing similarities to *Bacillus* strains. The diverse range of colours observed after 24 hours of incubation at 37°C in the specific chrome media, particularly in *Bacillus* chrome, indicated a wide array of enzyme activities exhibited by the strains, which were responsible for the production of colours, in the chromogenic medium indicating their genera. Upon further examination (**Chapter 4**: **Figure** 1.1), the bacterial strains displayed several extremophilic characteristics and formed a significantly larger volume of biofilm compared to the control strain. All the five bacterial strains exhibited a notably rapid initial growth phase duration, followed by a rapid log phase duration and then a stable phase after 6 hours, except for strain

Y3, which did not show a stable phase at all (**Chapter 4**: **Figure** 1.2). They exhibited varying degrees of sensitivity to all the types of antibiotics (**Chapter 4**: **Table 1.3**). The scanning electron micrograph displayed elongated and motile bacterial cells coated in viscous layers, maybe consisting of extracellular polymeric substances (EPS) or biofilm (**Chapter 4**: **Figure** 1.3). The 15srRNA sequencing identified three organisms from Gangotri as *Bacillus subtilis* strains BRAM_G1, BRAM_G2, and BRAM_G3, together with *MesoBacillus subterraneus* BRAM_Y2 and *Brevibacillus parabrevis* BRAM_Y3.

Objective 2: Profiling of their Polyextremophillic nature.

Certain bacterial strains have been reported to be polyextremophiles, meaning they can survive in a wide range of severe conditions such as temperature, salinity and pH. This study focuses on five specific bacterial strains, that exhibit this ability (Antranikian et al., 2007). BRAM_G1, BRAM_G2, and BRAM_G3 are newly discovered strains of *Bacillus subtilis*, while *Mesobacillus subterraneus* BRAM_Y2 and *Brevibacillus parabrevis* BRAM_Y3 are also novel strains. These strains not only survive but also exhibit exponential growth within a temperature range of 20°C to 70°C, as shown in **Chapter 4**: **Figure 2.2**. This temperature range indicates that they possess characteristics of both thermophiles, which thrive between 50°C and 80°C, and mesophiles, which have an optimum growth temperature between 20°C and 45°C (Blochl. et al., 1995). However, the ability of these strains to survive has been documented within a broader temperature range, namely from -20 °C to 110 °C. This discovery defies the intrinsic characteristic of a typical *Bacillus subtilis* strain to undergo sporulation, a process in which the bacterial cell converts into a dormant condition called an endospore when exposed to various forms of stress, such as heat, desiccation, or high pressure (Prescott., 1993). Interestingly, these 5 strains of interest do not exhibit endospore formation. This observation is particularly intriguing because it required the deliberate removal of a single gene in *Bacillus subtilis* to create a non-spore forming variant. This modified variant has proven to be highly effective and efficient for industrial purposes. The absence of spores in the staining procedure or during the stress experiments may be attributed to the protective nature of the thick biofilm produced by these strains, which shields the bacteria from stress and prevents sporulation in the well-known sporulating bacterial genus *Bacillus* sp.

The strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 not only exhibited high tolerance to UV doses (254nm; 11 microwatt/cm^2), for 6 hours, but also demonstrated exponential growth under these conditions. Specifically, they were able to withstand doses as high as 792J/m2 (after 2 hours of exposure) and 1584J/m2 (after 4 hours of exposure), as shown in **Chapter 4**: **Figure** 2.2. The possible explanation for this could be that the bacterium strains BRAM_G1, BRAM_G2, and BRAM_G3 were obtained from an altitude of 3415 m above sea level, while BRAM_Y2 and BRAM_Y3 were obtained from an altitude of 3293 m above sea level. At these higher altitudes, the UV irradiation is 34% higher compared to sea level. According to the World Health Organisation (WHO), for every 1000 m increase in altitude above sea level, there is a 10% increase in UV radiation. Consequently, the higher dosage of UV radiation may have acted as a stimulant for the growth of these 5 strains, allowing them to enter the log phase of growth directly without any visible lag phase (**Chapter 4**: **Figure** 2.2). The 5 strains (K. Pullerits et al., 2020; Yang et al., 2008; Budden T et al., 2013) have been demonstrated to be UV-resistant.

BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 all exhibited robust growth across a wide pH range of 1-12, indicating their polyextremophile nature. Polyextremophiles are capable of surviving in both highly acidic conditions (pH less than 3, characteristic of acidophiles) and highly basic conditions (pH greater than 9, characteristic of alkaliphiles). In contrast, the control strain of lab *Bacillus* was able to survive within the pH range of 5-9, with the optimal growth occurring at pH 7 (McGenity et al., 1993; Schleper et al., 1995) (**Chapter 4**: **Figure 2.1).**

BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 exhibited survival rates of 4-8% (1.37M) when exposed to escalating levels of sodium chloride (salt) concentrations. Das Sharma et al. (2006) and Oren 2006 defined a halophile as an organism that can thrive at NaCl concentrations exceeding 0.2M. The bacterial strains identified to flourish in a range of 3-15% NaCl can be classified as moderate halophiles (Barrozzi. et al., 2018). (**Chapter 4**: **Figure 2.2)**

Xerophiles, as defined by Pitt in 1975, are creatures that can survive at water activity (a_w) below 0.85.

$$a_w = exp\left(\frac{-4.2 \times PEG}{1000}\right)$$

Where, a_w is the water activity and PEG is the concentration of PEG 6000 in g/L (Michel., 1983)

Water availability is a crucial necessity for sustaining life. The results for BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 showed that optimal growth was achieved at water activity as low as 0.416. This indicates that the 5 strains have a xerophilic nature, despite being separated from water (Lebre et al., 2017). (**Chapter 4**: **Figure 2.1)**

The BRAM_G1, BRAM_G2, and BRAM_G3 strains have been found to exhibit tolerance to high levels of arsenic (300 ppm), iron (200 ppm), silver (20 ppm) and mercury (10 ppm). Similarly, the BRAM_Y2 and BRAM_Y3 strains have shown tolerance to iron (200 ppm), arsenic (300 ppm), silver (20 ppm), and mercury (10 ppm and 15 ppm). These findings suggest that these strains can be classified as metallophiles. (Golyshina et.al, 2000) (**Chapter 4**: **Figure** 2.3). The high metal tolerance observed may be attributed to the presence of negatively charged functional groups within the biofilm matrix, which have been previously shown to have the ability to bond with heavy metal ions. This could potentially be applied in the process of removing heavy metals from settings that are contaminated with metals (Teitzel and Parsek, 2003; Xie et al., 2015).

There were no detectable levels of heavy metals found in the water samples collected from both Gangotri and Yamunotri. Thus, three heavy metals and one toxic metalloid were chosen based on their importance in environmental and water pollution.

Arsenic toxicity can lead to several health complications, including dermatological lesions, malignancies in the skin, lungs, bladder, and kidneys, cardiovascular diseases, and neurological impairments. Arsenic pollution of drinking water is a prevalent worldwide public health concern in many places. The precise results differ depending on the exact species and length of time of exposure. The repercussions include death, hindered growth, decreased ability to convert sunlight into energy, and changes in behaviour. Arsenic-contaminated habitats display reduced species diversity and smaller population sizes for each species. The 5 bacterial strains

exhibited high tolerance to extremely elevated levels of Arsenic, reaching up to 300ppm. Among these strains, BRAM_G1 and BRAM_G3 demonstrated the highest growth rate at this concentration, as depicted in **Chapter 4**: **Figure** 2.3(d) (Patel et al., 2005).

Iron has been discovered to diminish the passage of light through water, resulting in a decline in photosynthetic activity in marine plants. Iron is good to human health when present in water at concentrations of approximately 0.3ppm. However, an overabundance of iron can lead to many physical issues, including hemochromatosis (accumulation of excess iron in internal organs), which can affect organs such as the liver, pancreas, and even the heart. The 5 bacterial strains exhibited tolerance to elevated levels of iron (200ppm) and demonstrated robust development in the same conditions (**Chapter 4**: Fig 2.3(b)) (Sarkar et al., 2018).

The choice of silver was made due to the unintended consequences of excessive usage of silver nanoparticles on the environment. These consequences include toxicity, which can harm beneficial microorganisms, marine animals, and soil organisms, and even represent a risk to human health. The bacterial strains exhibited tolerance to silver concentrations of up to 20 parts per million (ppm), but their growth was significantly impaired when exposed to 50 ppm of silver (**Chapter 4**: **Figure** 2.3(b)) (Padhye et al., 2023).

Mercury was chosen as a metal for the study on tolerance to heavy metals because, when released into the environment, it tends to accumulate in sedimentary deposits in bodies of water, where it transforms into the toxic chemical methylmercury (Verma et al., 2018). Subsequently, this chemical becomes part of the food chain. The existence of mercury in the environment is a significant obstacle to both human health and the ecosystem due to the ease with which methylmercury can enter the bloodstream and affect brain function. All five bacterial strains exhibited tolerance to 10 parts per million (ppm) of Mercury, with the exception of BRAM_G1 and BRAM_Y2, which shown tolerance to 15ppm of Mercury (**Chapter 4**: **Figure 2.3(a))** (Verma et al., 2018).

The provisions would have allowed for the selection of a larger quantity of metals, but it was restricted to four in order to facilitate more efficient data handling. The purpose of this constraint was to clearly showcase the metallophilic character of the five bacterial strains, without needlessly expanding on a comparable set of studies that involve altering only one parameter.

The high-altitude brackish water of Pangong Lake harbours a multitude of microorganisms, which are known to create significant levels of biofilm. The bacterial communities discovered in the vicinity of Pangong Lake in the Himalayan plateau consisted of Firmicutes, a phylum to which the *Bacillus* spp. Belong which makes the study relevant.

Biofilm development has been observed to be crucial for bacterial survival in harsh environments characterised by high temperature, pressure, or heavy metal pollution. This is because it provides osmotolerance to the cells, as described by Chaudhuri et al. (2017). Halophilic bacteria have been seen to form biofilms as a means of protecting themselves from the osmotic pressure caused by the high salinity of their environment (Kunte., 2006). *Halomonas maura* S30, a type of bacteria that thrives in high salt concentrations, produces a substance called mauran in its extracellular polymeric substance (EPS) that has the ability to capture heavy metals (Llamas et al., 2006). *Sulfobacillus thermosulfidooxidans*, a type of bacteria that can tolerate moderate levels of heat and acidity, has been discovered to generate biofilms that are employed for the leaching of pyrite (Li et al., 2016). High biofilm generation in haloalkaliphilic bacterial populations, such as *Tindallia* sp. and *Desulfonatronum* sp., is observed which can survive and grow well in environments with high alkaline pH levels. (Gorlenko et al., 2004). *Deinococcus geothermalis* has been found to create thin biofilms composed of glycoconjugates. These biofilms have been observed to enhance resistance to harsh conditions, dehydration, and heavy metal tolerance, including uranium, iron, and chromium (Daly., 2000; Peltola et al., 2008).

In terms of one-dimensional stress, the biofilm development of the strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 was assessed compared to a control setup without any stress **(Chapter 4: section 2.1 and 2.2)**. A significant and quick rise in biofilm formation was observed. This was the initial evidence suggesting that biofilm may play a protective role in combating stress, as seen in the **Chapter 4: Figure 2.4**. This observation was further confirmed when the 5 strains were additionally examined in relation to 16 two-dimensional (two stresses applied simultaneously) and 9 three-dimensional (three stresses applied simultaneously) stress configurations, in which the bacterial strain was analysed. In both cases, the production of biofilm was measured and there was a constant increase in its formation compared to the control setup where no stress was applied (**Chapter 4: Figures 2.5 and 2.6**.). This series of experiments addressed two crucial inquiries. Firstly, it investigated the properties of five bacterial strains (BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and

BRAM_Y3) as Polyextremophiles, as demonstrated by their ability to survive and thrive in both two-dimensional and three-dimensional stress environments, comparable to their growth under normal conditions (Zannier et al., 2019; Mark C. Capece et al., 2013). Furthermore, the notable augmentation in biofilm production in relation to each stress configuration confirms the notion of the protective function of biofilm in shielding bacterial cells during intense environmental stress.

Therefore, the results suggest that BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 exhibit all the characteristics of a polyextremophile. One of the mechanisms contributing to their diverse stress tolerance is their capacity to generate robust biofilms, which serve as a protective barrier against various environmental stresses. The control *Bacillus* sp. exhibited a substantial reduction in the formation of biofilm components, including DNA, protein, and glucose. One possible explanation for the strain's inability to survive in various stressful situations, despite the impressive growth and sustenance observed in the five bacterial strains of interest, could be its key contributing factor. While there have been numerous reports on extremophiles and polyextremophiles, *Bacillus subtilis* BRAM_G1, BRAM_G2, BRAM_G3, *MesoBacillus subterraneus* BRAM_Y2, and *Brevibacillus parabrevis* BRAM_Y3 are particularly notable as polyextremophiles. The range of stress factors and their combinations that they can tolerate is exceptionally broad.

The Fourier transform infrared spectrum(FTIR) scans display molecular bonding patterns for lipids, proteins, amino acids, and molecular oxygen bonds, among others. The reduction in transmittance leads to an increase in absorbance and specific chemical concentrations. The FTIR spectral scans of the extracted biofilms of the 5 bacterial strains show variations in peak heights (concentrations) and peak shifts when subjected to a three-dimensional stress. The stress combination employed in this experiment and the subsequent series was selected based on the most significant growth observed by all five bacterial strains at a temperature of 70 °C, with 4% NaCl and 20% PEG. The FTIR scans of biofilm extracts revealed three prominent peaks: 3000-3500 cm-1 corresponding to lipids and proteins, 1500-1700 cm-1 representing amide bonds (I and II) and C=C conjugates, and 500-600 cm-1 indicating the presence of molecular oxygen or carbon and halogen bonds (tetrahedral). The peak heights and compound concentrations significantly increased in both the three-dimensional stress and no stress scenarios. The little deviations in the peaks, despite belonging to the same category of

chemicals, could suggest alterations in compound structures due to stress. The results indicate that bacterial biofilms provide protection against abiotic stress (**Chapter 4**: **Figures 2.7 to 2.9**). These results signify the fact that stress increases the individual components of the biofilm and also works on changing certain components of it. The FTIR scans can't conclusively explain the exact changes in the composition. The changes were observed in later sections where, ESI-MS scans show the exact changes in the composition of biofilm when subjected to stressed conditions.

The amphiphilic protein BslA, previously referred to as YuaB, has been identified as a significant contributor to the surface repellence and colony corrugation of biofilms generated by gram positive *B. subtilis* residing in the soil. BslA collaborates with other matrix components, particularly TasA and exopolysaccharides, during the final phases of biofilm formation. This collaboration results in the development of a distinct hydrophobic layer on the surface of the biofilm, which enhances its repellence. The enhanced repellence seen in biofilm surfaces may augment *B. subtilis* exhibits resilience in its native soil environment by actively resisting environmental contaminants and harmful substances, including heavy metals and antimicrobial agents (Hobley et al., 2013).

Our knowledge of extremophiles on the Earth is based on research conducted on pure cultures. However, in recent decades, our understanding has significantly grown due to advancements in molecular ecology, including the development of advanced DNA sequencing and bioinformatics applications. These technologies have rapidly evolved over the past decade. The advancements in short- and long-read sequencing technology have improved the accuracy and reduced the cost of DNA sequencing. These breakthroughs show potential for conducting cost-effective and precise DNA sequencing investigations on both individual microbial cultures and microbial communities (Slatko et al., 2018). Simultaneously, advancements in processing power according to Moore's law (Moore, 1965) and the emergence of novel bioinformatic tools, machine learning algorithms, and data mining techniques now offer the required resources to handle such a vast volume of data (Gauthier et al., 2019; Goodswen et al., 2021).

The utilization of genomic data from both cultured and uncultured extremophiles is highly beneficial for deducing evolutionary connections and establishing a reliable and precise classification system (Parks et al., 2022). Furthermore, a standardised code of nomenclature, based on genome sequences as nomenclatural types, is expected to be accessible in the near

future (Murray et al., 2020). The majority of known extremophiles have been identified using traditional polyphasic studies, which involve 16S rRNA gene phylogenetic analyses and wet-lab DNA-DNA hybridization approaches. However, these methods are now considered outdated and have limitations in terms of resolution, accuracy, and reproducibility across different laboratories. Phylogenomic research and in silico genome relatedness indexes have surpassed these methodologies (Chun and Rainey, 2014; Jain et al., 2018). Hence, it is imperative to evaluate the existing classification of extremophiles by utilising genomic data. The growing availability of genetic information and the declining cost of next-generation sequencing technology and computation provide a current research topic and provide us with an amazing opportunity to tackle this issue.

Hence, Whole Genome Sequencing (WGS) was conducted on the 5 polyextremophilic bacterial strains to accurately determine their true identities. Additionally, this knowledge will contribute to future research efforts aimed at uncovering further intriguing insights on the subject. The Whole Genome Sequencing (WGS) analysis revealed alterations in the species of three out of the five strains. Specifically, BRAM_G2 was identified as *Bacillus* tequilensis instead of *Bacillus subtilis*, BRAM_G3 was determined to be *Bacillus* thuringiensis, and BRAM_Y2 was identified as *Mesobacillus* thioparans. These results can be found in **Chapter 4**, **Figure**s **2.10 to 2.14**. Thus, the identity of the abovementioned bacteria was considered on the basis of WGS for future research.

Objective 3: Detailing the prospects of the bacteria on agricultural benefits.

The plant growth stimulating abilities of the 5 bacterial strains, which are now more distinguishable as polyextremophilic strains, were investigated. Microbial biofilms facilitate root colonisation and the storage of nutrients (Pandit et al., 2020). They contribute to the nutrient cycle, promote plant growth, enhance plant resistance to specific phytopathogens, and even aid in the purification of degraded soils (Shaikh et al., 2018). The presence of bacterial biofilm at the plant roots aids in the plant's ability to withstand specific abiotic conditions, including drought, salinity, and certain inorganic contaminants. This thus enhances the productivity of crops (Malusa et al. 2012). The biofilms in the rhizosphere play a crucial function in maintaining water stability in the rhizoplane through the utilisation of microbial biomass. This helps regulate the response of root exudates during periods of stress (Kasim et al., 2016). The biofilm structures exhibited atypical characteristics, facilitating their proliferation in challenging environments (Chaudhari et.al, 2020). Hence, the bacterial strains

with a high capacity for biofilm production that were examined in this study were anticipated to possess remarkable potential for boosting plant growth, which was the focus of this investigation.

The five bacterial strains, namely *Bacillus subtilis* BRAM_G1, *Bacillus tequilensis* BRAM_G2, *Bacillus thurigiensis* BRAM_G3, *Mesobacillus subterraneus* BRAM_Y2, and *Brevibacillus parabrevis* BRAM_Y3, exhibited a significant ability to form biofilms compared to the control group. Additionally, these strains demonstrated exceptional proficiency in producing metabolites that enhance plant growth and ensure plant health. In a study, Maitra et al. 2022 confirmed a theory demonstrating the significant impact of biofilm formation on enhancing hormone synthesis and enzymatic activity in bacterial cells. The article also indicates that biofilm enhances the colonisation of bacterial cells on plant roots, hence facilitating cell-to-cell communication within the bacterial community as well as between bacterial cells and plant roots.

The investigation commenced by conducting nutrient sequestration tests. The macronutrients comprise nitrogen, phosphorous, and potassium. All five strains exhibited the ability to dissolve phosphate at low levels of phosphorus, as shown in **Chapter 4**: **Figure** 3.1. In addition, they were able to convert atmospheric nitrogen and thrived on Jensen's agar while also solubilizing potassium, evident by the clearing zones they created on Aleksandro agar upon inoculation.

All five strains produced considerable levels of plant growth regulators, including Indole Acetic Acid (IAA) and gibberellins (GA_3), which play a crucial role in plant growth and development (Gusmiaty et al., 2019). IAA is synthesised by BRAM_G1, BRAM_G2, BRAM_G3 and BRAM_Y2 even in the absence of its precursor, tryptophan, and in very little quantities by BRAM_Y3 in absence of the precursor (**Chapter 4**: **Figure** 3.2).

Specific enzymes and metabolites examined in this study has distinct functions in enhancing plant well-being and stimulating plant development. ACC Deaminase is a crucial enzyme produced by bacteria in the rhizosphere of plants to counteract the effects of abiotic stress on plants. ACC, an abbreviation for 1-aminocyclopropane-1-carboxylic acid, serves as the precursor for the plant stress hormone Ethylene. It is released by the roots into the plant rhizosphere, when the plant experiences abiotic stress conditions such as salt, dehydration, or exposure to heavy metals. Due to its high potency at low concentrations, even a slight increase

in ethylene levels can cause significant and dramatic impacts on the plant. The bacteria secrete ACC deaminase, which breaks down the precursor of the ethylene molecule, ACC (1-aminocyclopropane-1-carboxylate), into ammonia and α-keto butyrate. This process reduces the ethylene-mediated stress response by around 95% (Bernard ., 2005). Hence, by impeding the production of ethylene, a compound that causes significant harm to the physiological processes of plants (Glick, 2013; Mosqueda et al., 2020). The five bacterial strains exhibit significant production of ACC Deaminase, which aids in promoting plant growth under abiotic stress conditions (**Chapter 4**: **Figure 3.3**).

Siderophores have a dual function in promoting plant development. Iron, being a highly desired macronutrient, is crucial for plant health. Its deficiency can have severe consequences as iron is not only essential for chlorophyll production but also serves as a cofactor for other plant enzymes. Consequently, in soils with low levels of iron, bacterial siderophores capture the existing iron and render it usable for plants. This, in turn, prevents other plant-damaging organisms in the soil from accessing the iron, thereby impeding their growth (Pahari et al., 2017). The bacterial strains create two types of siderophores, hydroxamate and catecholate, which aid in acquiring iron and can also rescue plants developing in low iron situations. In addition, they may play a role in the biological control of plant diseases by restricting the availability of iron in their environment (Ahmed et al., 2022). Both bacterial strains exhibited significant siderophore production at both 24 and 48 hours (**Chapter 4**: **Figure** 3.4).

The 5 strains generated enzymes necessary for the biocontrol of plant diseases, including catalase, pectinase, protease, β-1,3-glucanase, and peroxidase, though, BRAM_Y3 strain was unable to produce β-1,3-glucanase. These enzymes are components of the plant's Induced Systemic Resistance pathway. Consequently, they have been found to play significant roles in the sustainable management of plant diseases. These enzymes have the ability to break down the cell wall of the plant pathogens, leading to their eventual demise. The fundamental process involved in the destruction of the cell wall is the disruption of glycosidic connections found within the polysaccharides of fungal cell walls (Reddy, et al. 2022) (Heil and Bostock, 2002) (**Chapter 4**: **Figure** 3.5).

In addition to liquid exudates, the bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 have the ability to produce certain volatile organic compounds (VOCs) that are currently unidentified. It is worth noting that BRAM_Y2 and BRAM_Y3 did

not produce ammonia or hydrogen cyanide gases during experimentation. These VOCs effectively inhibit the growth of two broad spectrum phytopathogenic fungi, *Fusarium fujikuroi* MC2 and *Curvularia aeria* MC1, which were isolated from *Zea mays* and identified on the basis of ITS region sequencing. This information is presented in **Chapter 4**, specifically in **Figure 3.6 to 3.8** and **Table 3.1,** of the study conducted by Ruangwong et al. in 2021.

Urease catalyses the hydrolysis of urea, resulting in the production of ammonia and carbon dioxide. This enzyme is essential for the nitrogen cycle. The indiscriminate application of urea as a nitrogen source in agricultural areas results in the accumulation of residual urea in the soil, which cannot be effectively utilised by plants. This, in turn, leads to residual toxicity. Bacterial urease plays a crucial role in breaking down residual urea and restoring the nitrogen balance in the soil (Mekonnen et al., 2021) (**Chapter 4**: **Figure 4.2).**

Endophytic enzymes, such as laccase, pectinase, and cellulase, are crucial for recycling lignocellulosic wastes in agricultural fields and increasing the organic carbon content of the soil. This, in turn, improves the quality of the soil. The test bacterial strains also secrete substantial quantities of these enzymes, which further enhance soil health. Additionally, these enzymes aid in the breakdown of root cell walls, allowing the bacteria to penetrate plant tissue. Once inside, the bacteria establish a symbiotic relationship with the plant, promoting its growth and providing biocontrol against pathogens. In return, the bacteria receive nutrition and shelter from the plant. (**Chapter 4**: **Figure** 3.10).

All the previously stated investigations confirmed that BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 possess significant potential as agents that promote plant development.

Prior to commencing the field trial trials, three methods of application were explored in this investigation in order to establish a direct comparison and determine the most effective treatment approach for applying PGP bacteria to the plant rhizosphere. Therefore, in future research, when bacterial biofertilizer consortia are used in soil, individuals can make a clear comparison to select the most advantageous method of treatment that is acceptable for their specific objective. For instance, the solid treatment would have a longer shelf life because the vermicompost would supply ample nutrition to support the growth of bacteria, resulting in better performance compared to the control. Conversely, the water suspension treatment would undoubtedly produce the most favourable outcomes and provide optimal conditions for

scientific analysis. Based on the initial assumptions and the outcomes of the preliminary experiments, the field experiments were conducted using water suspension as the method of treatment (**Chapter 4**: **Figure 3.11 to 3.13**).

On the basis of these features, the microorganisms were employed on the experimental crop *Zea mays L*. The five bacterial strains, namely BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3, exhibited remarkable outcomes in terms of both reproduction and vegetative growth compared to the control group. The BRAM_Y2+ BRAM_Y3 consortium demonstrated superior performance in relation to vegetative parameters, as shown in **Chapter 4**: **Figure**s 3.14 to 3.16. BRAM_Y3 and the consortium of BRAM_Y2+ BRAM_Y3 exhibited the highest fruit yield, while BRAM_Y2 outperformed the control but fell short compared to the other varieties. However, the grain filling data indicated that the BRAM_Y2+ BRAM_Y3 consortia had the highest level of grain filling, with the individual bacterial strains closely following. The cob weight was greatest in the BRAM_Y2+ BRAM_Y3 consortia, with BRAM_Y3 and BRAM_G3 closely behind. The analysis of the 100 seed weight data revealed that the BRAM_Y2+ BRAM_Y3 consortia exhibited the greatest weight, with the individual bacterial strains closely trailing behind. This information can be found in **Chapter 4**, specifically in **Figures 3.17 to 3.20**. BRAM_G1 exhibited the highest levels of both chlorophyll a and b, as well as total chlorophyll. Following closely behind was BRAM_Y3, with the remaining treatment settings showing lower levels (**Chapter 4**: **Figure 3.21**).

The soil study conducted before and after cultivating *Zea mays* L with bacterial treatments revealed a significant increase in soil organic carbon, pore space, and moisture. Conversely, there was a minimal decline in soil nitrogen levels. This can be attributed to the presence of Nitrogen fixers in the treatment, which resulted in a negligible change despite Nitrogen being one of the most utilised nutrients. There is a notable reduction in the quantities of potassium and phosphate as a result of bacteria breaking them down into a soluble form and plants using them up. Furthermore, a decline in the concentrations of zinc, iron, manganese, magnesium, and sulphur was also noted, as indicated in **Chapter 4**: **Table 3.2 and Table 3.3**. Soil microorganisms, specifically the bacterial treatment supplied to the plant rhizosphere in this case, have a vital function in producing extracellular enzymes that are necessary for the biogeochemical cycling of nutrients. The pH of the soil is crucial for regulating the activity of these enzymes. The pH of soil affects various processes, such as the release of ammonia,

conversion of ammonia to nitrate, reduction of nitrate to nitrogen gas, breakdown of organic matter, formation and dissolution of organic matter and heavy metals, breakdown of organic pollutants in the soil, and the activity of enzymes in the soil and root zone, among others (Neina., 2019). The soil's pH level is essential for maintaining proper enzyme activity and supporting other key soil processes. Every crop has specific nutrient requirements for ideal growth. Soil carbon is of utmost importance in the field of soil ecology. Soil organic carbon plays a crucial role in enhancing soil fertility and serves as a source of energy for microorganisms. Soil organic carbon improves the soil's capability to retain nutrients, enhance soil aeration, reduce soil compaction, raise soil infiltration rates, and enhance water storage capacity. Oxidisable organic carbon in soil improves soil health by increasing nutrient uptake, improving soil infiltration, and reducing soil evaporation. Furthermore, it has a substantial impact on the global carbon cycle and climate change, in addition to its role in improving soil health (Sithole & Magwaza, 2019). Potassium is the second most crucial nutrient that performs a significant role in maintaining photosynthetic activity and enhancing plant growth. The findings indicate a decrease in the potassium concentration in the soil and its uptake by the plants. Moreover, it has a vital role in controlling the aperture of stomata (Andrés et al., 2014). Sulphur is an essential component for the growth of plants. It plays a crucial role in the physiological processes of the plant, such as photosynthesis and respiration. The uptake of this element also exerts a significant influence on the results.

The colonisation of introduced bacterial strains was additionally verified using the first and final soil metagenomic investigation and analysis. The increased number and distribution of the class and phylum categories in the bacterial treatment compared to the initial control soil indicated successful colonisation. During the brief 3-month cropping period, there was a notable colonisation of the bacteria that were applied, as evidenced in **Chapter 4**: **Figure 3.22 and 3.23**. The Krona charts clearly demonstrate a rise in the proportion of *Bacillus* in the soil, from 4% to 6% (**Chapter 4**: **Figure 3.24)**. This indicates that the enhanced development of plants in the treated soils can be linked to the addition of bacterial treatments to the maize rhizosphere.

The 5 bacterial strains included in this study are polyextremophilic strains that have undergone thorough examination for their abilities to promote plant growth and provide biocontrol, as documented in Roy et al., 2023. Bacteria has the ability to solubilize phosphate to a limited extent, fix atmospheric nitrogen, and solubilize inorganic potassium in the soil. These

capabilities contribute to their ability to acquire nutrients. Additionally, they were shown to generate substantial amounts of plant growth regulators such as Indole acetic acid (auxins) and GA_3 (gibberellins), which play crucial roles in promoting plant growth. Furthermore, the bacterial strains secrete a multitude of enzymes that aid in plant protection against phyto pathogens, ultimately enhancing soil health and structure. The bacterial strains possess other traits such as the ability to sequester iron through siderophores, emit volatile organic compounds that enhance quorum sensing, and exhibit biocontrol of phytopathogens. These characteristics make them highly appealing as polyextremophilic bacteria that promote plant growth. (Roy et al., 2023).

The major purpose of this study was to apply strains of extremophilic agriculture that suppress the synthesis of ethylene, a stress-responsive plant growth regulator, by using the enzyme ACC deaminase during abiotic stress. The crop chosen for investigating its plant growth stimulating capabilities under abiotic stress conditions was paddy (*Oryza sativa*, PB1692). There were two primary reasons for selecting this crop. Paddy is a primary food crop in India and other countries globally. Its cultivation is significantly impacted by arsenic-contaminated soils, infestation of saline water, soil salinization, and severe water scarcity, particularly in South Bengal, India (Bhuyan et al., 2023).

The experimental sets included a control group where no shocks or treatments were administered (**Chapter 4**: **Figure 3.25**). This would provide a benchmark for any alterations in the plant's health that are associated with therapy. Subsequently, a controlled experiment was conducted to assess the capability of these bacteria to thrive in the waterlogged conditions of the paddy rhizosphere, where oxygen availability is a significant concern. This experiment involved a treatment setting without any stress application, known as the PGP setup, to decode the bacteria's ability to function effectively. The difficulty was quickly resolved because the initial source of bacterial isolation was water, which made colonisation unlikely. The remaining three configurations replicated the circumstances of water scarcity or drought, arsenic-contaminated soils (with 30 parts per million of arsenic), and saline soils (with 5% salt). These three initial environmental challenges are encountered by rice farmers worldwide, particularly in the South Bengal region of India.

The concept of the plant rhizospheric microbiome, also known as the phytomicrobiome, to alleviate abiotic stress in plants is captivating. This phenomenon revolves around the remarkable metabolic capabilities of bacterial inoculants (biofertilizers), which not only

enhance plant growth but also generate compounds and stimulate the plant to produce antioxidants and ROS scavenging enzymes. These substances aid the plant in maintaining homeostasis during periods of abiotic stress.

Drought is primarily identified by chlorosis, which is the yellowing of leaves, as well as wilting and the closure of stomata to regulate transpiration. If the conditions persist, it can also result in stunted plant growth. This is supported by the observation of comparable symptoms in the control plants in the drought experiment (**Chapter 4**: **Figure 3.29**). Bacterial inoculants generate many compounds, including osmolytes, phytohormones, and antioxidants, which play crucial roles in alleviating drought stress. The references cited are Iqbal et al. (2022) and Shaffique et al. (2022).

Salinity, on the other hand, adversely affects the soil microbiome, as well as the organic matter and nutrient content of the soil. Additionally, it induces osmotic stress in the plant, leading to the degradation of chlorophyll and a decrease in overall photosynthetic efficiency. All of this indirectly leads to the formation of reactive oxygen species (ROS) in the plant cells, which causes damage to the cells. PGP bacterial inoculants can mitigate the detrimental effects of salinity stress in plants by producing specific osmolytes, enzymes such as proteases, and plant hormones (Chen et al., 2022) (**Chapter 4**: **Figure 3.28).**

Ultimately, the intense pressure caused by heavy metals, such as high levels of mercury and cadmium. Arsenic and other substances are produced as a result of human activities that pollute agricultural soils (Kurniawan et al., 2022). The primary impact on plants is the production of oxidative stress and a reduction in cytoplasmic enzyme activity. PGP Bacterial inoculants have the ability to generate substances like siderophores, which can bind to heavy metals and thereby decrease their availability to plants on one hand, they also immobilise beneficial heavy metals from other competitive pathogenic organisms (**Chapter 4**: **Figure 3.27).**

Plants produce ethylene in response to any abiotic stressors. PGP bacterial inoculants produce ACC Deaminase, which breaks down the precursor of ethylene, ACC. This helps to regulate the ageing process caused by ethylene as a stress reaction. The bacterial inoculants possess a notable attribute of safeguarding plant cells against harm caused by abiotic stresses. This is accomplished through the production of a biofilm, that forms a protective coating on the plant roots. Consequently, the roots are shielded from immediate damage caused by the prevailing

abiotic stress, while also receiving a sufficient supply of stress-alleviating and plant growth-promoting metabolites. (Maitra et al., 2022; Roy at al., 2023).

The treatment configuration had superior results in all stress conditions compared to their respective control groups, however the overall yields were much lower than those of the PGP arrangement. It is important to mention that the yield quantity differed greatly between the stress and PGP setups. However, certain parameters like percent grain filling and 100 seed weight showed the impressive effectiveness of the bacterial treatments under abiotic stress conditions. These results were almost equal to the no-stress PGP setups (**Chapter 4**: **Figure 3.30).**

The reduction of stress in plants can be seen by closely monitoring specific characteristics that elicit a shared set of responses in plants when exposed to the specified abiotic stresses, such as chlorophyll content, ROS scavenging enzymes, and both enzymatic and non-enzymatic antioxidants. Consequently, all the arrangements were observed for these five parameters: chlorophyll concentration, catalase, guaiacol peroxidase, lipid peroxidation, and carotenoid content.

Plants undergo a loss in chlorophyll content when they are under stress. This is caused by the degradation of the structure of their chloroplasts, which leads to a decline in their ability to carry out photosynthesis. The decrease can be ascribed to multiple mechanisms, such as harm to chloroplasts induced by reactive oxygen species, increased catalytic activity of chlorophylls, degradation of photosynthetic pigments, and insufficient ingredients for synthesis. Stress-induced chlorophyll decrease is a reaction to situations like as drought, water stress, and heavy metal stress. Soil and environmental conditions, particularly drought stress, can significantly reduce the amount of chlorophyll present (George et al., 2022). Consequently, the stressed and untreated samples exhibit chlorophyll degradation, whereas the treated samples under stress show less chlorophyll degradation (**Chapter 4**: **Figure 3.31**).

Lipid peroxidation, the process of lipid breakdown, is an important indicator used to evaluate oxidative damage in plants that are subjected to difficult environmental circumstances (Zenk, 1996). Measuring the amounts of malondialdehyde (MDA), which is the final result of lipid peroxidation, is frequently used as a sensitive indication of oxidative stress (Choudhary et al., 2007). Under the influence of stress treatments, the levels of MDA rose due to the excessive

formation of reactive oxygen species (ROS), leading to lipid peroxidation. All plants that were infected with bacteria, showed a reduction in MDA concentration compared to stressed plants that were not inoculated (**Chapter 4: Figure 3.6).** These data indicate that the bacterial strain helps to reduce stress in plants, resulting in different levels of reduction in MDA content compared to arsenic treatment. Several studies have reported increased levels of MDA in response to stress caused by heavy metals, which are significantly reduced, when bacterial inoculation is employed to ameliorate them (Singh et al., 2016).

Catalase (CAT) is found across important locations where hydrogen peroxide (H_2O_2) is produced inside the cellular environment of higher plants. CAT acts as a crucial scavenger of H_2O_2, eliminating reactive oxygen species (ROS) from plant tissues by facilitating the conversion of two H2O2 molecules into water (H_2O) and molecular oxygen (O_2) (Ahmad, 2014). Catalase (CAT) activity is crucial in various aspects of plant biology and resilience. According to Roy et al. (2023), it improves the ability of C4 plants to withstand stress and helps maize adapt to unfavourable conditions. In addition, CAT decreases the accumulation of hydrogen peroxide (H_2O_2) in peroxisomes during photorespiration, as demonstrated by Zeng et al. in 2019. The treatments that involved inoculation exhibited an increase in CAT activity, but this effect was slightly less prominent compared to the treatments without inoculation, when subjected to stressful situations (**Chapter 4**: **Figure 3.32**).

Glutathione peroxidase (GPX) is an enzyme that protects cells from oxidative damage caused by stress (Sobiecka et al., 2022). GPX is present in both the cytosol and mitochondria, where it uses a reducing agent to counteract the effects of hydrogen peroxide and lipid peroxides (Watson, 2014). Furthermore, GPX has a regulatory function in the management of glutathione (GSH), which acts as an electron donor in the GPX redox process (Shao et al., 2020). The study found that the activity of GPX increased in response to stressful settings. After inoculation, the GPX activity showed a significant increase compared to the stress condition alone. This suggests that the presence of bacteria likely played a role in boosting GPX activity, which in turn enhanced the plant's ability to counter the oxidative stress caused by the production of antioxidants under stress (**Chapter 4**: **Figure 3.32).**

The study found that the Carotenoid concentration dropped after stress treatment. This decline was due to the plant's inefficiency in producing sufficient carotenoids, which is also associated with the estimate assay of chlorophyll content. Plants face difficulties in carrying out the process of photosynthesis. This can result in inhibited growth and ultimately mortality, which

can also be associated with the height measurements of the plants under three different conditions. The carotenoid content exhibited an increase in all plants that were inoculated with bacteria, in comparison to plants that were stressed but not inoculated. This outcome suggests that the bacterial strain aids in alleviating stress in plants by enhancing the removal of reactive oxygen species (ROS) through the production of carotenoids, hence aiming to lower ROS levels in plants. **Chapter 4: Figure 3.32** illustrates the different degrees of carotenoid content increase in comparison to the control group treated with arsenic. Several studies have demonstrated a drop in carotenoids during periods of stress, followed by an increase when a stress-alleviating substance is provided. This supports the reasoning mentioned in **Chapter 4**, **Figure 3.32** (Faseela et al., 2018).

The colonisation of the inoculated bacterial strains was also verified through the final soil metagenomic investigation and analysis. The higher abundance of the bacterial treatment categories, including class, phylum, order, and family, compared to the untreated control, indicated colonisation. The distribution of the top 20 genera also demonstrated an elevation in the *Bacillus* and *Brevibacillus* genera, which predominantly comprised the bacterial treatments (**Chapter 4**: **Figure** 3.35). Studies on the impact of bacterial inoculum as biofertilizer have demonstrated that it enhances processes such as nitrogen fixation and phosphorus solubilization, leading to an overall improvement in soil health. Additionally, it also increases the population of other beneficial bacteria in the soil. This phenomenon is also evident in the Krona charts (**Chapter 4**: **Figure**s **3.36 to 3.38**), where it is apparent that the bacterial population is generally greater in the test soils compared to the control soil. Furthermore, it is evident that some bacterial populations, such as actinobacteria and cyanobacteria, exhibit higher levels in the test soils treated with bacterial additions compared to the control. This phenomenon can have advantageous impacts on the soil (**Chapter 4**: **Figure 3.33**). The observations provide conclusive evidence that polyextremophilic plant growth promoting bacteria play a crucial role in alleviating abiotic stress in the plant rhizospheric microbiome. Additionally, these bacteria can positively influence the overall health of the soil (Li et al., 2023).

Objective 4: Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.

Bacillus species are extensively utilised in industrial settings owing to their capacity to synthesise a diverse range of enzymes, antibiotics, and bioactive chemicals. These bacteria play a crucial role in synthesising enzymes such amylases, proteases, and lipases, which are vital in several industries including food processing, detergents, textiles, and biofuels. *Bacillus* species are employed in agriculture as biocontrol agents and biofertilizers, enhancing plant growth and safeguarding crops against pests and illnesses by means of natural insecticidal and antifungal chemicals. Moreover, they have a vital function in bioremediation, decomposing environmental contaminants, and in fermentation procedures for manufacturing fermented foods and medicinal compounds. They are capable of producing biopolymers such as polyhydroxyalkanoates (PHAs), which serve as environmentally-friendly substitutes for traditional plastics. In summary, *Bacillus* species play a crucial role in multiple industries, promoting progress and environmental responsibility in the field of industrial biotechnology.

Pullulanase, a prominent debranching enzyme, is frequently used to hydrolyze the α-1,6 glucosidic linkages in starch, amylopectin, pullulan, and related oligosaccharides. This enables a comprehensive and efficient conversion of the complex polysaccharides with branches into readily fermentable sugars during the saccharification process. All five bacterial strains exhibited the production of Pullulanase, BRAM_Y2 demonstrating the highest production and BRAM_Y3 displaying the lowest production.

Amylases are highly significant enzymes that are widely used in diverse industries. These enzymes catalyse the hydrolysis of starch molecules, resulting in the formation of polymers composed of glucose units. Amylases have considerable potential for use in several industrial sectors, including as food processing, fermentation, and pharmaceutical manufacture. All five bacterial strains exhibited notable amylase production, with BRAM_G1 showing the highest levels, closely followed by BRAM_G3 and BRAM_Y2. Proteases have a strong ability to modify the properties of ingested proteins and produce bioactive peptides from proteins. They are widely used in the production of high-quality food ingredients and in food processing to improve the functional, nutritional, and flavour properties of proteins. All five bacterial strains exhibited substantial amylase production, with BRAM_Y3 demonstrating the highest levels,

followed closely by BRAM_G3, and the remaining three bacterial strains. (**Chapter 4**: **Figure 4.1**)

Urease, an enzyme that breaks down urea into carbon dioxide and ammonia, is also important in the context of sustainable agriculture. The overuse of urea in agricultural fields results in a significant build-up of residual toxicity, as plants are only able to utilise a minimal portion of the provided urea. Bacterial urease enzymatically eliminates the surplus urea from the soil, hence diminishing its toxicity. The 5 bacterial strains exhibited significant urease production, which was measured by the colour change in Stuart's Broth, transitioning from yellow to pink (**Chapter 4**: **Figure 4.2**).

The remarkable repertoire of *B. subtilis* in generating a wide range of bioactive metabolites has been acknowledged in the past decade. Research conducted by Stein (2005) has shown that approximately 5% of the genetic material in a wild-type *B. subtilis* organism is specifically dedicated to producing bioactive chemicals. Previously, the production was limited to cyclic peptides such as iturins, surfactins, and fengycins (Ongena, 2008). However, the discovery of many antimicrobials with linear lipopeptides, PKs, and volatile metabolites has generated significant economic interest. The diverse bioactive metabolites synthesised by the *B. subtilis* group can be categorised according to various criteria, such as their biosynthetic pathways, function, structure, source, physicochemical qualities, molecular targets, or bonding patterns (Wang et al., 2015). *Bacillus subtilis* produces bioactive metabolites that can be categorised into five classes: non-ribosomal peptides (NRPs), polyketides (PKs), ribosomal peptides (RPs), as well as hybrid and volatile metabolites. The five classes are further classified into several subclasses and thoroughly detailed, as indicated by the Electron Spray Ionisation Mass Spectroscopy (ESI-MS) data, which revealed their production by the five bacterial strains **(Chapter 4: Tables 4.1 to 4.6)**.

Natural ribosome-like particles (NRPs) are intricate molecular machinery that utilise multi-modular enzyme complexes instead of relying on a DNA template to synthesise proteins. A module is a component of the non-ribosomal peptide synthetases (NRPS) enzymes that incorporates the amino acid into a specific type of peptide backbone. In addition, it is possible to divide each module into three distinct domains: adenylation (A), thiolation (T), and peptidyl carrier protein (PCP). Furthermore, there is a condensation (C) domain inside each module that facilitates the separate processes of nonribosomal peptide (NRP) synthesis (Drake et al., 2016). The initial product can undergo post-synthetic modifications such as glycosylation,

methylation, hydroxylation, acylation, heterocyclic ring formation, and halogenation to achieve its mature form. Natural product derivatives display extensive structural variability and can exist in linear, branching, and cyclic forms.

Thio-template non-ribosomal peptides (NRPs)

❖ *Cyclic lipopeptides*

Thio-template non-ribosomal cyclic lipopeptides were initially discovered in the 1950s-1960s from *Bacillus* spp. Cyclic lipopeptides are predominantly synthesised through the successive incorporation of residues, either in an iterative or non-iterative fashion. The lipopeptide synthesis pathways exhibit a high degree of flexibility, resulting in the production of peptides that possess a remarkable diversity of characteristics. The cyclic lipopeptides synthesised by *B. subtilis* can be categorised into four distinct classes (Soberón-chávez et al., 2010).

Surfactins were initially discovered in 1968, when they were extracted from the culture supernatant of *B. subtilis*. These surfactins shown a remarkable ability to behave as biosurfactants (Arima et al., 1968). Surfactin was subsequently shown to possess anticancer, antimicrobial, anticoagulant, and hypocholesterolemic properties. These were produced by all 5 bacterial strains in presence of three-dimensional stress **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

Iturins were initially identified from *B. subtilis* in 1949 by Walton and Woodruff. These compounds have antifungal properties. In 1950, another chemical called iturin was discovered. It was named after Ituri, the location in Congo where the soil sample was obtained. Iturins are recognised for their strong antifungal properties and can be utilised as an active component in many solutions for controlling plant pathogens. The closely similar cyclic lipopeptides can be categorised as iturin, including bacillomycin L, mycosubtilin, bacillomycin D, bacillomycin F, mojavensin A, and subtulene A (Besson et al., 1977; Thasana et al., 2010).

Fengycins were discovered in 1986 by scientists from Japan and Germany, who concurrently identified them in *B. subtilis*. At first, it was found that fengycin hinders the growth of filamentous fungi but had no effect on non-filamentous fungi and bacteria. Subsequently, its ability to combat viruses, germs, and cancer was documented. Furthermore, it demonstrated a beneficial characteristic of stimulating plant growth, which is much sought after in the field of

agriculture (Zhao et al., 2017). These were produced by all 5 bacterial strains in presence of three-dimensional stress **(Chapter 4: Sections 2.1 to 2.3).**

❖ *Linear lipopeptides*

Various strains of *B. subtilis* have synthesised linear lipopeptides. They can be categorised into the subsequent two subclasses

Gageopeptides: A number of linear lipopeptides have recently been identified and examined in detail from the bacterium *B. subtilis*. For example, the compound Gageostatin was discovered in a strain of marine-derived *B. subtilis*, as described by Treq and al. in 2014. Gageostatin is composed of a 3-beta hydroxyl fatty acid that is linked to a heptapeptide. It consists of identical residues as those documented for surfactin. Nevertheless, disparities were observed in their configurations and molecular weights. Gageostatins were discovered in a linear configuration containing only L-leucine, whereas surfactins are cyclic lipopeptides that contain both L-leucine and D-leucine. Gageotetrins A–C and gageopeptides A–D are linear antimicrobial peptides that are similar to gageostatins. Unlike gageostatins, these compounds do not have any harmful effects on cells and effectively inhibit the growth of bacteria and fungus. It is likely that they are produced through a combination of biological synthesis methods, and gageotetrin A has the potential to be a precursor for gageotetrin B. Gageotetrins were recently discovered from the *B. subtilis* strain 109GGC020 and demonstrated significant antibacterial and antifungal effects in a time-dependent manner (Chakraborty et al., 2020). These were produced by all 5 bacterial strains in presence of three-dimensional stress **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

Siderophores are tiny molecules that have a strong attraction to ferric iron. They are utilised to scavenge iron and create stable complexes with significant metals. Siderophores can be classified into hydroxamate, catecholate, and carboxylate categories. *Bacillus* sp. Bacteria synthesise catecholate siderophores, such as Bacillibactin (Produced by BRAM_G2) **(Chapter 4: Table 4.1, 4.3 and 4.5)**, which exhibit potent antibacterial effects and moderate cytotoxicity. Siderophores were produced by all the 5 bacterial strains, when studied in the plant growth promotion section of this investigation, Bacillibactin was found in ESI-MS data. **(**Zhou et al., 2018; Khan et al., 2020).

❖ *Non-Thio-Template Non-ribosomal Peptides (NRPs)*

Bacillus subtilis has the ability to produce antimicrobial non-ribosomal peptides (NRPs) by a method that does not include thio-template. Rhizocticins are a type of non-thio-template peptides composed of an arginine molecule connected to L-2-amino-5-phosphono-3-cis-pentanoic acid. Occasionally, leucine, isoleucine, and valine are added as supplements (Kugler et al., 1990).

Bacillus subtilis has the ability to produce dipeptide nonribosomal peptides (NRPs) such as bacilysin (tetain) and chlorotetain, which possess potent antibacterial and antifungal properties. The antibacterial effect is achieved by the action of L-anticapsine, which hinders the production of glucosamine-6 phosphate and peptidoglycan, a key component of the bacterial cell wall. Anticapsine has the ability to inhibit the production of chitin and fungal cell membrane mannoproteins, therefore exhibiting antifungal action. Tetain and chlorotetain exert inhibitory effects on the growth of pathogenic *Aspergillus fumigatus* and *Candida albicans*. Mycobacillin and bacitracin are non-thio-template polypeptides synthesised by *B. subtilis*. They hinder the growth of *Aspergillus niger* by modifying its cell membrane causing disruption of membrane integrity and function leading to the leakage of vital cellular components and a breakdown of essential cellular processes. The production of Mycobacillin is facilitated by a Nonribosomal Peptide Synthetase (NRPS) complex, which separates it into three components: A, B, and C. Bacitracin, a seven-amino acid peptide, counteracts the growth of Gram-positive bacteria by impeding the production of peptidoglycan, a key component of their cell walls (Mahlstedt et al., 2010; Milewski et al., 1986). All these NRPs were produced by the 5 bacterial strains in both presence and absence of three-dimensional stress **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

❖ *Ribosomal peptides (RPs)*

Ribosomal peptides (RPs), often referred to as ribosomally synthesised and post-translationally modified peptides (RiPPs), are produced from a relatively brief precursor peptide and undergo maturation by post-translational modification [Oman et al., 2010]. Multiple enzymes participate in these changes, resulting in the production of peptides with varied structures. Therefore, the *B. subtilis* strains that are known to produce RiPPs can be categorised into three primary classes and multiple subclasses.

Polyketides, (PK)

Polyketides are diverse bioactive metabolites with methylene and carbonyl groups. They are widely used as medicinal agents for various disorders, including antibacterials like tetracycline and erythromycin, antifungal agents like amphotericin, and anticancer medications like anthracyclin. Polyketides are produced by a multi-domain enzyme, including ketosynthase, acyltransferase, and thioesterase. The process starts with the acyl CoA attached to the acyl carrier protein, followed by elongation through ketoreductase, enoyl reductase, and dehydratase. The thioesterase domain then cyclizes or hydrolyzes the PK chain, releasing a fully formed peptide. (Fang et al., 2018; Hertweck et al., 2009).

Polyketide synthases (PKs) are divided into three subclasses based on their structural arrangement: type I PKs, type II PKs, and type III PKs. Type I PKs have multiple functions, type II PKs have a single function, and type III PKs are similar to chalcone synthases. PKs can have an iterative or non-iterative biosynthetic process, with bacteria using non-iterative type I PKS enzymes. PKs can also be combined with other PKSs or linked with NRPSs or fatty acid synthetases.
Nevertheless, the PKs generated by *B. subtilis* can be categorised into two primary classifications, namely Polyenes and enediynes.

Polyenes

Difficidins are unsaturated macrocyclic polyene compounds synthesised by the type 1 polyketide synthase (PKS) enzyme. Oxydifficidin, a variant of difficidin, has antibacterial properties and hinders protein production in *E. coli*. The *B. amyloliquefaciens* strain FZB42 showed biocontrol efficacy against *Xanthomonas oryzae* through difficidin production. Scanning electron microscope results showed difficidin inhibits phytopathogen growth by rupturing the bacterial cell wall it targets. It also suppresses genes related to *Xanthomonas* cell wall formation, cell growth, and disease-causing ability. These studies explore the potential of these strains as biological control agents against plant infections like bacterial spot, bacterial blight, citrus canker, bacterial leaf streak etc. caused by *Xanthomonas* sp. (Zimmerman et al. 1987, Zweerink et al., 1987).

Aurantinin, a polyketide compound, was first isolated from *Bacillus* aurantinus and has been found to exhibit antibacterial properties (Nakagawa et al., 1988). Recently, the aurantinins C and D were reisolated in combination with the genome mining technique. Aurantinin and its

analogues has a highly atypical structure, consisting of 5, 6, 7, and 8-membered rings, along with a remarkably varied tail. However, the complete arrangement of the structure has not been fully explained, resulting in many unsolved issues regarding its conformation. Aurantinin B, C, and D demonstrate potent antibacterial action against *Clostridium sporogenes* and *S. aureus*. The antibacterial mechanism of aurantinin B-D was investigated and found to include the disruption of bacterial cell membranes, resulting in cytoplasmic rupture and subsequent cell death (Yang et al., 2016). These are produced by the 5 bacterial strains in presence of three-dimensional stress **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

Macrolactins are a class of fungicides that belong to type 1 polyketide synthases (PKS). They have the ability to suppress the growth of bacteria and have been obtained from different species. The compound is composed of 24 lactone rings and three diene moieties in its carbon skeleton. The biosynthetic gene cluster mln consists of nine operons and 11 KS domains that utilise acetate and malonate as the sole building blocks. The *B. subtilis* group primarily synthesises macrolactin A derivatives, which have been utilised as biocontrol agents against phytopathogenic bacteria and fungi in conjunction with bioorganic fertilisers. Macrolactin B, which was initially discovered in 1989, demonstrates strong antifungal properties without causing harm to cells. Macrolactin W is the sole instance of macrolactin that possesses both 7-O glycosylation and esterification. It exhibits antibacterial capabilities akin to macrolactin A and B, but lacks any cytotoxic effect. The cytotoxic, antiviral, and anti-inflammatory properties of the primary metabolite macrolactin A have not been established yet (Li et al., 2016). These are produced by 3 of the bacterial strains in absence of stress parameters **(Chapter 4: Table 4.1, 4.3 and 4.3).**

Enediynes

Polyketide enediyne is the most potent natural substance for killing cancer cells and has been proven in clinical trials. Despite its limited applicability due to cytotoxicity, its use in antibody-drug conjugates and polymer-based drug delivery systems has yielded significant achievements. *Streptomyces* spp. are common sources of enediynes, but recent reports from *Bacillus* sp. have been reported using genome mining. The full biosynthetic apparatus and structure need to be clarified.

Metabolites with hybrid characteristics

Hybrid metabolites are the end results of biosynthetic pathways that involve the combined action of both NRPS (nonribosomal peptide synthetase) and PKS (polyketide synthase) kinds of modular enzymes. Queries pertaining to the amalgamation of hybrid products are currently of significant interest, as their resolution pertains to genetic engineering endeavours. Both processes rely on thio-template for the elongation of acyl chains and the activation of monomers. Currently, the mechanisms by which molecular processes facilitate the integration of different chemical components in a functional assembly line pathway are still not fully understood. According to current understanding, the hybrid metabolites generated by *B. subtilis* can be categorised as bacillaene and isocoumarins.

Bacillaene is a compound with a linear structure that was initially discovered in *B. subtilis* strains 55,422 and 3610 (Patel et al., 1995). The bacillaene PksX synthase is responsible for encoding the hybrid PKS-NRPS biosynthetic gene cluster. The pksX mega gene cluster in the *B. subtilis* 168 genome is composed of 5 open reading frames, specifically pksJ, pksL, pksM, pksN, and pksR. The initial two adenylation domains of pksJ assimilate glycine and α-hydroxy-isocaproic acid. The third adenylation domain, known as pksN, is accountable for the assimilation of alanine. The open reading frames pksC, baeD, and baeE encode three distinct AT domains that are responsible for integrating malonyl-CoA (Calderone et al., 2006). Bacillaene exerts inhibitory effects on the growth of many bacteria and fungi, including *Myxococcus xanthus* and *Trichoderma* spp. (Müller et al.,2014). Bacillaene exhibits selective inhibition of protein production in bacteria, suggesting its ability to selectively inhibit other strains in their environment (Patel et al., 1995). These are produced by the 5 bacterial strains in presence of three-dimensional stress **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

Isocoumarins, a group of over 200 compounds, are produced by *Bacillus* subtilis. Amicoumacin A-C and amicoumacin F are dihydroisocoumarins with potent antibacterial effects against *Helicobacter pylori*. Bacilosarcin A and B, obtained from the marine-derived *B. subtilis* strain TP-B0611, have distinct heterocyclic core structures. Although not suitable for agriculture or human health, their potent anti-MRSA action is promising for therapeutic development. Further research is needed to establish the correlation between amicoumacin structure and activity (Itoh et al., 1981).

Some of these metabolites are produced only in presence of the three-dimensional stress or show significant rise in the presence of stress, and therefore might have potential roles in abiotic and biotic stress combat and biofilm formation, which has been proven till now in this study as one of the most significant stress combat mechanisms of the 5 polyextremophilic bacterial strains of interest **(Chapter 4: Table 4.2, 4.4 and 4.6)**.

Those metabolites and their role in biofilm formation and stress combat has been discussed in this section.

- Gageotetrins as discussed earlier are linear lipopeptides with antibacterial properties that are synthesised by various species of *Bacillus*. These chemicals improve the ability of *Bacillus* to withstand stress by offering antimicrobial qualities, which aid in the establishment of *Bacillus* in novel environments by limiting the growth of other microorganisms. In addition, they contribute to the production of biofilms by strengthening the structural integrity and durability of the biofilm matrix (Chakraborty et al., 2020).
- Azoxybacillin is a bactericidal substance synthesised by many types of *Bacillus* bacteria. It serves to safeguard the bacterial colony within the biofilm from external microbial assaults. Azoxybacillin promotes the survival and structural integrity of *Bacillus* biofilms by suppressing the growth of other bacteria that could compete with them, especially in stressful setting (He etal., 2018).
- Ieodoglucomide B is a bioactive compound that helps *Bacillus* species by inhibiting the growth of competing microbes, thus facilitating biofilm formation and maintenance. It also enhances stress resistance by acting as an antimicrobial agent (Chen, et al., 2017)
- Nisin is a well-known antimicrobial peptide produced by *Bacillus* species that helps in inhibiting the growth of Gram-positive bacteria. Its role in biofilm formation includes maintaining a microbial balance by reducing competition from other bacterial species (Field et al., 2015)
- Aurantinin is an antibiotic produced by *Bacillus aurantinus*. It helps in protecting the *Bacillus* biofilm from microbial threats, thereby aiding in the survival of *Bacillus* under stressful conditions and supporting biofilm integrity (Nakagawa et al., 1988).
- Anticapsin is a peptide that is synthesised by *Bacillus* species and does not contain a thio-template. It demonstrates potent antibacterial and antifungal properties, which aid in safeguarding the biofilm against microbial rivalry. Anticapsin, found in the biofilm matrix, enhances the resilience and durability of the biofilm when exposed to stressful situations

(Mahlstedt etal.,2010) All the compounds that are antimicrobials in nature have similar mode of action in promoting biofilm and reducing competitions.

- Bacillaene is a compound that is a combination of polyketide and nonribosomal peptide metabolites. The substance possesses antibacterial characteristics that hinder the process of protein synthesis in other microbes it is in competition with. Bacillaene plays a crucial role in preserving the dominance and stability of *Bacillus* biofilms in challenging conditions by inhibiting the growth of competing organisms (Calderone et al., 2006)
- Surfactin is a potent biosurfactant synthesised by *Bacillus subtilis*. It reduces the force that holds molecules together on the surface, making it easier for bacteria to spread and build biofilms on surfaces. Surfactin possesses antibacterial capabilities that aid in protecting the biofilm from pathogenic incursions, hence increasing the biofilm's overall ability to withstand stress (Ongena et al., 2008). While promoting biofilm formation in its producer Bacillus subtilis, generally inhibits biofilm formation in other bacterial species through mechanisms such as membrane disruption and interference with cell signalling pathways. These properties make surfactin a potential candidate for controlling harmful biofilms in various industrial and medical applications (Lopez et al., 2009; Rivardo et al., 2009).
- Trimethylpyrazine is a volatile organic compound (VOC) that is synthesised by *Bacillus* species. This chemical functions as a signalling molecule in quorum sensing, a process that is essential for the creation and upkeep of biofilms. Biofilm coordination facilitates the synchronisation of bacterial cell activity, hence improving their collective resilience against environmental pressures (Zhang et al., 2018).
- *Bacillus* species create another volatile organic compound called methylthiazole. Methylthiazole, like trimethylpyrazine, contributes to quorum sensing and intercellular communication in the biofilm. By facilitating synchronised reactions to alterations in the environment, this process improves the biofilm's ability to withstand stress and maintain its structural integrity (Ryu et al., 2003).
- Piperazinedione is a cyclic dipeptide that is synthesised by bacteria of the *Bacillus* genus. This chemical facilitates biofilm formation by augmenting the structural characteristics of the biofilm matrix. Additionally, it possesses antibacterial characteristics that aid in safeguarding the biofilm against external dangers, hence enhancing its ability to withstand stress (Chatterjee and Biswas., 2019).
- Benzeneacetic acid (Phenylacetic acid), also referred to as phenylacetic acid, is a secondary metabolite synthesised by *Bacillus* species. It functions as an antibacterial agent, safeguarding the biofilm from harmful microbes and contributing to the stress response.

Additionally, it plays a role in maintaining the general integrity of the biofilm structure, hence improving its capacity to endure environmental shocks (Castro and Galvez.,2011)

- Biotin, a carboxylase enzyme cofactor, aids in metabolic processes and supports *Bacillus* biofilm formation by enhancing metabolic activity and stress resistance. It also aids in stress survival by mitigating oxidative stress and promoting biofilm stability and growth (Beckett., 2007).
- Alpha-Ribazole, a precursor to cobalamin (Vitamin B12) and methyl-cobalamine itself, supports *Bacillus* survival under stress by synthesis of essential cofactors for enzymatic reactions. (Fedosov and Fedosova., 2010; Hazra and Banerjee., 2010)

These compounds collectively improve the capacity of *Bacillus* species to endure stressful circumstances and create more robust biofilms. They accomplish this by utilising a mix of antibacterial properties, promoting quorum sensing, and strengthening the structure of the biofilm matrix. These chemicals improve the survival of *Bacillus* biofilms in challenging conditions by impeding competition, enhancing intercellular communication, and strengthening the biofilm structure.

In addition to the role of these aforementioned compounds, in biofilm formation and stress tolerance, they have significant roles in industry and medicine, which mean that the 5 polyextremophilic strains produce industrially important compounds that are not only rare but also highly desirable and fascinating. **(Chapter 4: Tables 4.2, 4.4 and 4.6)**

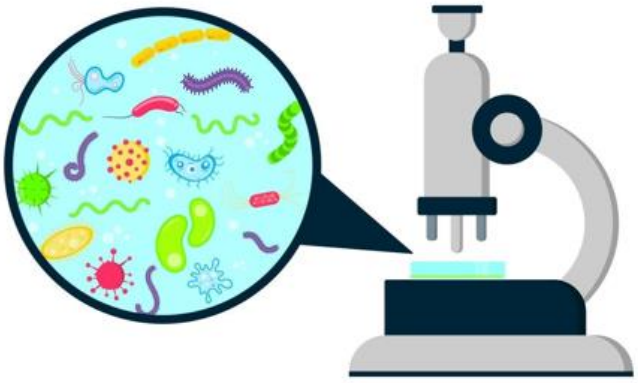
Objective 5: Construction of a Machine Learning Model for Analysis and Prediction of Plant Growth Promoting Properties and Maize productivity under stress condition.

In agronomy, machine learning techniques are utilized to model plant growth patterns and predict crop yields based on environmental factors, soil properties, and genetic data. These models help optimize agricultural practices, enhance crop productivity, and improve plant breeding programs by identifying key growth-promoting traits. Studies, such as those by Jha et al. (2020), have demonstrated the efficacy of machine learning in integrating complex biological data, thereby providing a comprehensive understanding of the interactions between bacteria and plants, and enabling more precise and effective interventions in agriculture and biotechnology.

The machine learning model, specifically the Linear regressor, was trained using the entire dataset that was previously described in this inquiry. The training data set comprised growth data obtained under stressed settings, a dataset on plant growth promotion, and field experiment data specifically related to *Zea mays*. The model was utilised to forecast the plant growth stimulating characteristics of the bacterial strains BRAM_G1, BRAM_G2, BRAM_G3, BRAM_Y2, and BRAM_Y3 under various stressful situations. The linear regressor's R^2 score, which measures the goodness of fit, was determined to be 0.993371. This value, being close to 1, indicates that the regressor effectively explains the variability in the dependant variable. A total of ten stress parameters were used for prediction. However, because to the limitations of a 2D graph, only one parameter may be depicted. Therefore, (**Chapter 4**: **Figure 5.2)** shows two single dimension stresses and one two-dimensional stress, while (**Chapter 4**: **Figure 5.3)** shows one two-dimensional stress and two three-dimensional stresses. The graphs depict the projected values under strained conditions compared to the actual values under no stress situations. Graphs can only display a maximum of three parameters. Consequently, the forecasts for all 10 specified parameters have been displayed in the table **(Chapter 4: Table 5.1).**

Upon closer examination of the projected values and the actual values, it becomes evident that there is minimal disparity between them. However, the model's accuracy was determined to be over 99%, which, although an impressive figure, also raised suspicions of being excessively perfect. The results of the AdaBoost regressor with different parameters, such as RMSE, MSE, and MAE, exhibit substantial variations in accurately assessing the performance of the model. These parameters are considered more reliable than R^2 scores for evaluating the goodness of fit of a machine learning model. Consequently, the predictions made by the model become more applicable for a biological system. The variations and prediction results clearly indicate that the AdaBoost regressor model is the superior choice for modelling this large dataset. Upon closer examination, the forecasts do not exhibit significant variance under conditions of stress. Thus, in order to elucidate this forecast, we must consider the outcomes of heightened biofilm development under stressful conditions. Maitra et al. (2022) assert that biofilm is crucial in the bacteria's ability to promote plant growth. The greater the biofilm formation, the more effective the root colonisation of the rhizospheric bacteria, resulting in enhanced synthesis of plant growth promoting compounds. Therefore, as stress levels rise, the biofilm continues to maintain its plant growth promoting qualities without any hindrance.

CHAPTER 6: SUMMARY



Objective 1: Isolation and Characterization of extremophilic bacterial strains.

"Major Findings from Objective 1:

- 5 bacterial strains were isolated from the two water samples of Gangotri and Yamunotri
- All the 5 bacterial strains were characterised according to the Bergeys manual.
- The three bacterial strains from Gangotri were gram positive in nature and the two from Yamunotri were gram variable in nature, i.e., they stained irregularly and appeared as a mixture of pink and purple colonies.
- All 5bacterial strains were rod shaped in nature but had distinctly different colony morphologies.
- Molecular characterisation with 16srRNA sequencing and microscopic characterization using scanning electron microscopy was carried out for all 5 bacterial strains.
- The bacterial strain G1 was confirmed to be *Bacillus subtilis* BRAM_G1, G2 was confirmed to be *Bacillus subtilis,* G3 was confirmed to be *Bacillus subtilis* BRAM_G3 , Y2 was confirmed to be *Bacillus subterraneus* BRAM_Y2 and Y3 was confirmed to be *Brevibacillus parabrevis* BRAM_Y3 after 16s rRNA sequencing.
- All the 5 bacterial strains were characterised for their Antibiotic resistance and they were found to be more or less sensitive to all the classes of antibiotics.
- The 5 bacterial strains 20-40 times more biofilm than the control *Bacillus* strain showed humongous increase in the carbohydrate content of the biofilm which is known to be the principal component of EPS, BRAM_G2 being the highest of them all.
- All 5 bacterial strains have a considerably steep lag phase with a steep log phase followed by a stationary phase after 6 hours except for Y3 which didn't show a stationary phase at all.

Objective 2: Profiling of their Polyextremophillic nature.

<u>Major Findings from Objective 2:</u>

- All 5 bacterial strains were tested for their extremophilic properties such as extremes of temperature, pH, salt, UV, drought and heavy metal stress with respect to four metals.

- The results provided definitive evidence that the bacterial strains not only exhibited tolerance but also demonstrated the ability to thrive at elevated temperatures, thereby exhibiting the trait of a thermophile.
- The results provided definitive evidence that the bacterial strains not only exhibited tolerance but also demonstrated the ability to thrive at low temperatures, thereby exhibiting the trait of a psychrophile.
- Therefore the results indicated that the 5 bacterial strains exhibited alakalophilic properties by thriving at a pH range of 10-12 but, also showed considerable tolence and growth at pH as low as, 3 exhibiting the trait of an acidophile as well
- The bacteria grew luxuriantly in presence of UV rays [the UV Power density: 11microwatt/cm^2, Time of Exposure for the first two hours were 3600s and 7200s. Therefore, UV Dosage Applied on the Bacteria (1hour Exposure) = 39600 µWs/cm^2, (2-hour Exposure) = 79200 µWs/cm^2= 792 J/m^2.] and showed no lag and a steep log phase (Fig 2.2 (b)). This hieghtened growth in the presence of such strong radiation could conclusively classify the bacterial strains as radiophiles.
- The bacteria growth was luxuriant till 15% of PEG 6000 concentrations that decreased at 20%. Tolerance to low water activities, are known to be a definitive quality of Xerophiles, and therefore the bacterial strains exhibited xerophilic properties as well (
- The presence of mercury in the environment poses a substantial challenge to both human health and the ecosystem due to the ease with which methylmercury can enter the bloodstream and impact the functioning of the brain. All 5 bacterial strains were tolerant to 10ppm of Mercury, except for BRAM_G1 and BRAM_Y2 who could tolerate 15ppm of Mercury
- The bacterial strains could tolerate up to 20ppm of silver but their growth was severely diminished at 50ppm.
- Excess of iron can cause several physical complications such as hemochromatosis, and same organs such as liver, pancreas and even the heart. The 5 bacterial strains were tolerant to high concentrations of iron (200ppm) and showed luxuriant growth in the same
- Arsenic-contaminated environments exhibit low species diversity and population sizes within each species. The 5 bacterial strains were tolerant to humongous levels of Arsenic as high as 300ppm BRAM_G1 and BRAM_G3 showing the maximum growth at that point.

- Polyextremophilic nature was verified as they were subjected to various two dimensional and three-dimensional stress combinations.
- Two-dimensional stress parameters were selected on the basis of probability of existence of the stress combinations in natural systems and the feasibility of the creation of the simulated stress combination in-vitro laboratory setups. The biofilm formation in the presence of these 16 2D-stress combinations was correspondingly recorded.
- It could be inferred from this experiment was that the 5 bacterial strains were polyextremophiles, as they grew luxuriantly in 2D-stress conditions.
- There was also a significant increase in biofilm formation in presence of two-dimensional stresses in comparison to no stress control, from which it could be inferred from that biofilm might have played a key role in protecting the bacterial strains against the abiotic stress combinations
- Three-dimensional stress parameters were selected on the basis of probability of existence of the stress combinations in natural systems and the feasibility of the creation of the simulated stress combination in-vitro laboratory setups. The biofilm formation in the presence of these nine 3D-stress combinations was correspondingly recorded.
- It could be inferred from this experiment was that the 5 bacterial strains were most certainly polyextremophiles, as they grew luxuriantly in 3D-stress conditions.
- There was also a significant increase in biofilm formation in presence of three-dimensional stresses in comparison to no stress control, from which it could be inferred from that biofilm might have played a key role in protecting the bacterial strains against the abiotic stress combinations.
- The change in Biofilm compositions due to stress was studied using FTIR Spectroscopy. Three categories of peaks were mainly observed in the FTIR scans of the biofilm extracts, 3000-3500cm^{-1}that corresponds to lipids and proteins, 1500-1700 cm^{-1}that corresponds to amide bonds (I and II) and C=C conjugates and lastly 500-600 cm^{-1} which can be molecular oxygen or carbon and halogen bonds (tetrahedral).
- The main observable change that occurred in 3D-stress and no stress conditions was that there was a huge increase in the peak heights and therefore the corresponding concentrations of the various compounds.

- The other change that could be observed was the slight shifts in the peaks though they were present in range of the same class of compounds which might correspond to change in forms of the compounds in stressed conditions.
- These observations clearly indicate a crucial role played by bacterial biofilms in protecting them in times of abiotic stress conditions.
- All the 5 bacterial strains were subjected to Whole Genome Sequencing for the revelation of their true identities. The Whole genome sequencing results confirmed BRAM_G1 to be a unique strain of *Bacillus subtilis* , BRAM_G2 to be a unique strain of *Bacillus thuringiensis*, BRAM_G3 to be a unique strain of *Bacillus tequilensis*, BRAM_Y2 to be a unique strain of *Mesobacillus thiparans* and BRAM_Y3 to be a unique strain of *Brevibacillus parabrevis* with highly interesting gene clusters that could be a point of study in future.

Objective 3: Detailing the prospects of the bacteria on agricultural benefits.

<u>Major Findings from Objective 3:</u>

- All 5 bacterial strains were tested for their Plant Growth Promoting Properties such as, Nutrient Sequestration, Plant hormone production, Biocontrol Properties, Volatile organic Compounds, Siderophore production, Endophytic enzymes, and Stress Management Properties.
- All 5 bacterial strains used in this study could produce the enzyme ACC deaminase by which they can utilise ACC as a Nitrogen source thereby decreasing ethylene production and enhancing plant survival rate in times of physical stress.
- The three enzymes pectinase, laccase and cellulase help the bacterial cells to penetrate the plant root cell walls and enter the plant system and form a give and take relationship where the bacteria offer plant hormones, sequestered nutrients, defence related enzyme etc. in return for shelter and nutrition.
- Dosage Standardization and Mode of treatment selection for application of the bacteria as biofertilizer on the test crop *Zea mays.* This treatment mode "Water suspension" was chosen for further investigation because it produced the most favourable outcomes. Additionally, using external components like broth media or vermicompost would have

led to inaccurate results when studying the impact of bacterial strains. Therefore, it was determined that using a water suspension with no additional components was the most scientifically sound choice.

- All the 5 bacterial strains were applied in the field, to the test crop *Zea mays* and the differences in the crop health was determined with respect to the control. For all the studied parameters the individual bacteria performed better than that of the consortia and all the treated setups performed better in comparison to the untreated control.
- The soil parameters and the plant pigments were also analysed to check the influence of the bacterial treatments on them. Soil Metagenome was also analysed so as to confirm colonisation of the test bacteria in the test crop rhizosphere the results of which gave a positive conclusion of better plant and soil heath, and nutrient sequestration during cropping period with significant colonisation of the bacterial treatment.
- Bacterial treatment effectivity was also checked on test crop Oryza sativa, with stress application to confirm bacterial PGP activity during abiotic stress. The reduction of stress in plants can be seen by closely monitoring specific characteristics that elicit a shared set of responses in plants when exposed to the specified abiotic stresses, such as chlorophyll content, ROS scavenging enzymes, and both enzymatic and non-enzymatic antioxidants. Consequently, all the arrangements were observed for these five parameters: chlorophyll concentration, Catalase, Guaiacol peroxidase, Lipid peroxidation, and carotenoid content.
- Consequently, the stressed and untreated samples exhibit chlorophyll degradation, whereas the treated samples under stress show less chlorophyll degradation. All plants that were infected with bacteria showed a reduction in MDA concentration compared to stressed plants that were not inoculated. The treatments that involved inoculation exhibited an increase in CAT activity, but this effect was slightly less prominent compared to the treatments without inoculation, when subjected to stressful situations. This suggests that the presence of bacteria likely played a role in boosting GPX activity, which in turn enhanced the plant's ability to counter the oxidative stress caused by the production of antioxidants under stress. The carotenoid content exhibited an increase in all plants that were inoculated with bacteria, in comparison to plants that were stressed but not inoculated. This outcome suggests that the bacterial strain aids in

alleviating stress in plants by enhancing the removal of reactive oxygen species (ROS) through the production of carotenoids, hence aiming to lower ROS levels in plants.

- The colonisation of the inoculated bacterial strains was also verified through the final soil metagenomic investigation and analysis. The higher abundance of the bacterial treatment categories, including class, phylum, order, and family, compared to the untreated control, indicated colonisation. The distribution of the top 20 genera also demonstrated an elevation in the *Bacillus* and *Brevibacillus* genus, which predominantly comprised the bacterial treatments.

Objective 4: Characterization of industrially and pharmaceutically important enzymes and metabolites and detailed study of the small molecule metabolome.

<u>Major Findings from Objective 4:</u>

- It was found that along with certain enzymes the 5 bacterial strains can produced an enormous plethora of compounds and metabolites that can be of use in the field of both pharmaceuticals and industry.
- The validation of the fact that the 5 bacterial strains were Polyextremophiles in nature was the significant increase in their plethora of metabolites after stress application.
- Increase in certain types of compounds such as surfactins, bacillaenes different antimicrobial peptides during stress which have significant roles in biofilm formation validate the hypothesis of biofilm acting as the protective jacket for all 5 bacterial strains in times of stress.
- These compounds are known collectively improve the capacity of *Bacillus* species to endure stressful circumstances and create more robust biofilms. They accomplish this by utilising a mix of antibacterial properties, promoting quorum sensing, and strengthening the structure of the biofilm matrix. These chemicals improve the survival of *Bacillus* biofilms in challenging conditions by impeding competition, enhancing intercellular communication, and strengthening the biofilm structure.
- In addition to the role of these aforementioned compounds, in biofilm formation and stress tolerance, they have significant roles in industry and medicine, which mean that the 5 polyextremophilic strains produce industrially important compounds that are not only rare but also highly desirable and fascinating.

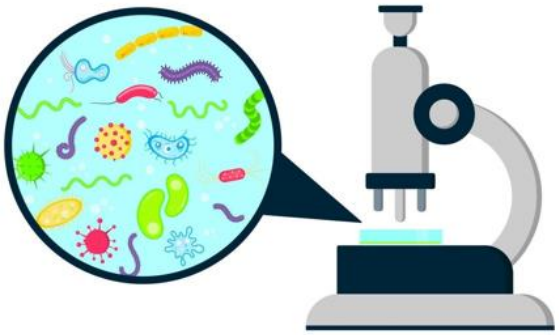
- Thus, the role of all 5 bacterial strains as a hub of different metabolites with applications in medicine, industry and agriculture justify their multifaceted potential in all these three aspects and provide the rationale of fulfilling all the objectives previously laid down for this study.

Objective 5: Construction of a Machine Learning Model for Analysis and Prediction of Plant Growth Promoting Properties and Maize productivity under stress condition.

<u>Major Findings from Objective 5:</u>

- Two machine learning models were designed for the data analyses and their results were compared to designate the best fitting model. These models help optimize agricultural practices, enhance crop productivity, and improve plant breeding programs by identifying key growth-promoting traits. Studies, such as those by Jha et al. (2020), have demonstrated the efficacy of machine learning in integrating complex biological data, thereby providing a comprehensive understanding of the interactions between bacteria and plants, and enabling more precise and effective interventions in agriculture and biotechnology.
- Though in case of Ada Boost regressor model the variation in the predicted values vs actual values are higher than the linear regressor, a closer analysis exhibits minimal fluctuation under stressful settings. Thus, in order to elucidate this prediction, we must consider the outcomes of heightened biofilm production under stressful conditions. Biofilm production enables bacteria to sustain its plant growth-promoting (PGP) activities even under stressful situations.
- The simulation model predictions that the 5 bacterial strains can produce ample quantities of plant growth promoting metabolites to sustain plant productivity in presence of abiotic stress is validated by the in-vivo studies with *Oryza sativa* in presence of abiotic stress dataset which was not used to train the models, also proving the validity of the machine learning model predictions."

CONCLUSION
&
FUTURE PROSPECTS



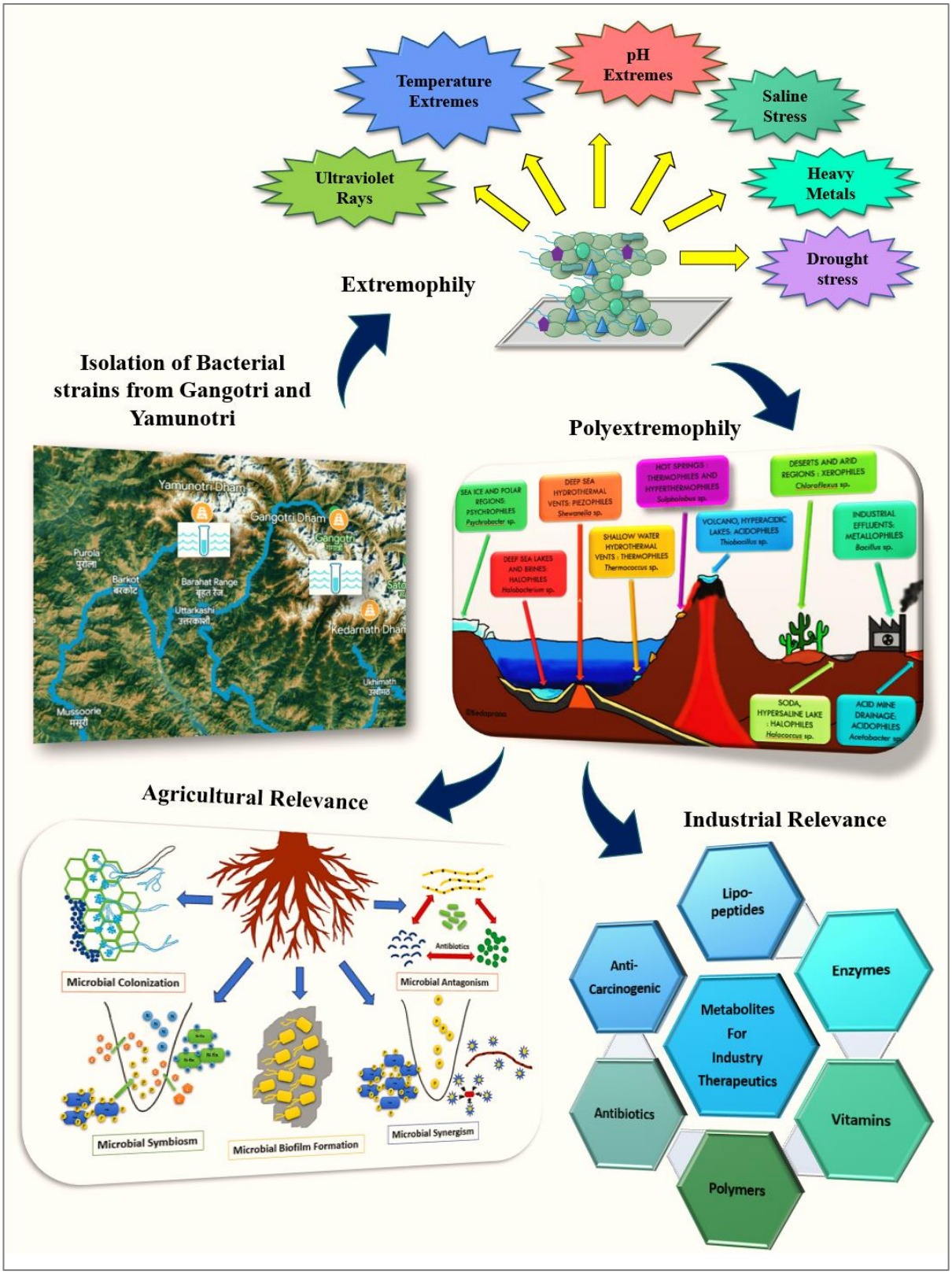
Conclusion and Future prospects

This investigation has clearly demonstrated that India being a land of biodiversity the upper tracts of Himalayas are no exceptions. In that particular condition we have actually identified organisms which apparently would have been psycrophiles, but were found to have, thermophily, alkalophily, halophily, xerophily, acidophily etc as a part of their characteristics.

The thorough examination of the five polyextremophilic bacterial strains demonstrates their exceptional ability to adapt and their promise for many uses in agriculture, industry, and medicine. Their whole genome sequences offer a tremendous asset for comprehending the genetic foundation of extremophilic nature and for investigating novel biotechnological uses.

Further investigation of these genomes has the potential to unveil new routes and processes involved in hydrocarbon breakdown and the synthesis of extremozymes. The implications of these results can be applied practically, for instance, by devising more efficient methods for bioremediation, designing resilient industrial biocatalysts, and identifying novel medicinal compounds.

In summary, the findings from this research highlight the immense capacity of extremophiles to contribute to sustainable agriculture, cutting-edge industrial processes, and improved medicinal therapies. Future research should prioritize the functional identification of genes and pathways found in these extremophiles, as well as the creation of biotechnological applications that take use of their distinctive features.



Schematic Representation of the work.

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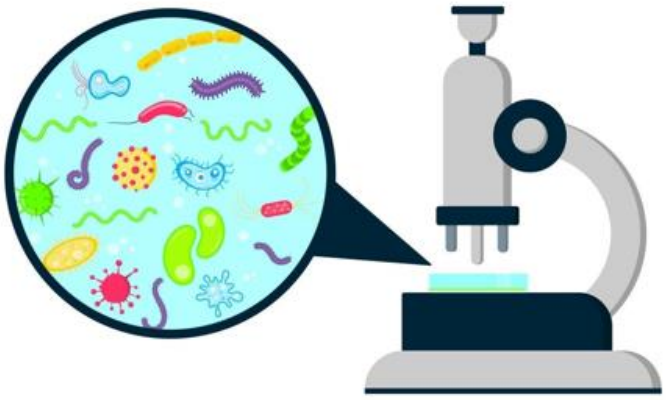

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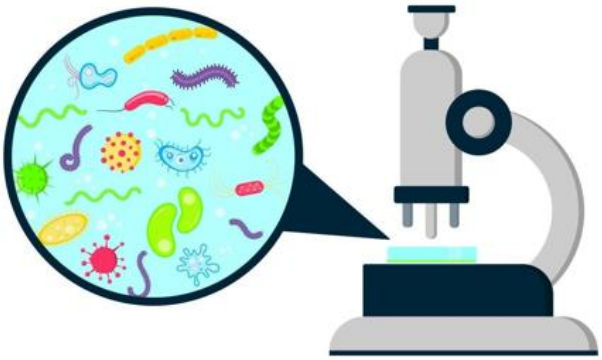
PUBLICATIONS



Journal Publications

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Seminar Presentations

- Paper entitled "Himalayan Polyextremophiles: The untapped resource for Agriculture and Industry" in 1st Botanical Congress organised (An International Meet) organised by Botanical Society of Bengal in Collaboration with Dept. Of Botany, University of Calcutta, in the Young scientist category held on 23-25th March, 2023.
- Paper entitled "Himalayas: The abode of Multifaceted Polyextremophiles' in Bilateral International Conference on Ecotoxicology and Environmental Sciences (ICEES-2022) in the Young scientist category, held during 19th-20th October, 2022 at Khulna University, Khulna, Bangladesh.
- Paper entitled, "A Novel Biofilm producing Polyextremophillic Bacillus: A marvel of Agriculture" at Bio Nexus: A new axis for advanced biological sciences, held at Department of Biotechnology, School of Science & Technology, The Neotia University, April 27,2022.

Laurels Achieved

- Won the **Best Oral Presentation (1st Position)** in the 6th International Conference on Strategies and Challenges in Agriculture and Life Science for food security and sustainable Environment (SCALFE-2023), for presenting the paper titled, " Suryakund: The abode of divine Polyextremophiles par excellence", Himachal Pradesh University, Summer hill, Shimla, HP.
- Won the **Best Interdisciplinary Poster** in the International Symposium on modern perspectives of Chemistry in Biology, for presenting the paper titled, "Synthesis, Molecular structure, and Antibacterial efficacies of Transition Metal Azo-oximates of Fe(II) and Mn(II), a Sustainable Solution to Multi Drug Resistance", organised by Department of Chemistry, St. Xavier's College, Kolkata, 6th January , 2024.

Patents Published

A patent has been published titled, “Design of a Novel Bacterial Bio-fertilizer for Enhancing crop productivity at varying Agro-climatic conditions”, using the bacterial strains under investigation. **Application no.: 202431043574.**

Office of the Controller General of Patents, Designs & Trade Marks
Department for Promotion of Industry and Internal Trade
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सत्यमेव जयते

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Alleviation of abiotic stress in *Oryza sativa* by the application of novel polyextremophilic plant growth promoting *Bacillus*

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ABSTRACT

Oryza sativa (rice or paddy) is a primary food crop that provides 21% of global human per capita energy and 15% of per capita protein. Yet, paddy cultivation faces numerous challenges like water scarcity, inappropriate use of fertilizers, soil salinization, heavy metal contamination, etc. which affects both its quality and yield. Recent changes in climate, demands the agricultural practices to cope with aforementioned environmental adversities without hampering yield. Therefore, the use of polyextremophilic plant growth promoting bacteria (PPGPB) in paddy cultivation can be a sustainable solution that would not only enhance productivity but also alleviate the effects of these environmental stresses in the plants.

With this background, this study investigates the role of 5 polyextremophilic PGPB strains Bacillus subtilis BRAM_G1, Bacillus subtilis BRAM_G2, Bacillus subtilis BRAM_G3 isolated from high-altitude waters of Ganges at Gangotri and *Mesobacillus subterraneus* BRAM_Y2 and *Brevibacillus parabrevis* BRAM_Y3 in not only stimulating the growth of *Oryza sativa* PB1692, in normal conditions and in presence of abiotic stress factors like 5% salt, 30 ppm Arsenic and drought, but also in the alleviation of the stress responses in the plants when subjected to these stresses. It was observed while in presence of stress parameters, the plants showed stunted growth, degraded chlorophyll and little to no yield, the PPGPB treated stress setups showed remarkable improvements in vegetative, biochemical as well as reproduction. The metagenome studies showed colonization of the bacterial inoculants, in the treated soils proving that the PPGPB treatment can enhancing growth and alleviate abiotic stress in paddy.

1. Introduction

In the vast tapestry of agriculture, the rice plant (*Oryza sativa* L.) emerges as a revered cereal crop, serving as the cornerstone of sustenance for over half the global population. With more than 50% of the world's rice production originating in the fertile fields of Asia, it is an agricultural drive. However, the path to rice's full potential is fraught with challenges, with abiotic stresses such as

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ORIGINAL ARTICLE

Efficacy of High-Altitude Biofilm-Forming Novel *Bacillus subtilis* Species as Plant Growth-Promoting Rhizobacteria on *Zea mays* L

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Abstract

With the global population explosion, the need for increasing crop productivity is reaching its peak. The significance of organic means of cultivation including biofertilizers and biopesticides is undeniable in this context. Over the last few decades, the use of rhizobacteria to induce crop productivity has gained particular interest of researchers. Of these, several *Bacillus* spp. have been known for their potential plant growth-promoting and phyto-pathogenic actions. Keeping this background in mind, this study was formulated with an aim to unravel the PGPR and phyto-pathogenic potency of *Bacillus* sp. isolated from extreme environmental conditions, viz. high-altitude waters of Ganges at Gangotri (Basin Extent Longitude Latitude—73° 2′ to 89° 5′ E 21° 6′ to 31° 21′ N). Based on recent studies showing the impact of biofilm on bacterial PGPR potency, three novel strains of *Bacillus subtilis* were isolated on basis of their extremely high biofilm-producing abilities (BRAM_G1: Accession Number MW006633; BRAM_G2: Accession Numbers MT998278-MT998280; BRAM_G3: Accession Number MT998617), and were tested for their PGPR properties like nutrient sequestration, growth hormone production (IAA, GA_3), stress-responsive enzyme production (ACC deaminase) and lignocellulolytic and agriculturally important enzyme productions. The strains were further tested for the plethora of metabolites (liquid and VOCs) exuded by them. Finally, the strains both in individually and in an association, i.e. consortium was tested on a test crop, viz. *Zea mays* L., and the data were collected at regular intervals and the results were statistically analysed. In the present study, the role of high-altitude novel *Bacillus subtilis* strains as potent PGPR has been analysed statistically.

Keywords Biofilm · Agriculture · Plant growth promotion · Treatment

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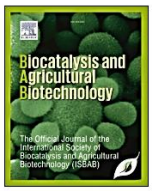

Biofilm production in a novel polyextremophilic *Bacillus subtilis*: A strategic maneuver for survival

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Keywords:
Extremophiles
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ABSTRACT

Extremophiles are well-known to flourish in hostile extreme habitats. For instance, extremes of temperatures, acidic or alkaline environments, high pressure, UV irradiation, salinity and even presence of heavy metal concentrations. While extremophiles can survive in an individual extreme, polyextremophiles can survive in combinations of such extreme environmental niches. Polyextremophily mainly exists in two dimensional matrices of extreme conditions such as temperature and pH, temperature and salinity etc. It provides the potential to delineate from the habitability envelope by putting constraints on biological processes, and dislocating them from their natural niche. Microbial biofilm, which is an assemblage of microbes in extracellular polymeric substances, secreted by the microbes themselves not only play a huge role in microbial colonization, nutrient sequestration and quorum sensing but also protects the microbes from the aforementioned array of environmental hostilities.

This paper deals with, a novel polyextremophilic strain of *Bacillus* isolated from the waters of The Ganges, at Gangotri situated in Uttarakhand, at an altitude of 3,415 m from sea level, on the Greater Himalayan range. The strain *Bacillus subtilis* BRAM_G1 (Accession Number: MW006633), was found to be tolerant to a huge plethora of extreme conditions ranging from temperature (from −20 °C to 110 °C), ultraviolet radiation (79200 μW/cm^2), pH (1–12), salinity (8%) to heavy metal concentrations (arsenic, silver, iron etc.). On further investigation, the strains were found to produce enormous amounts of biofilm and a control laboratory strain of *Bacillus* sp. which did not produce biofilm was also found to be sensitive to the array of extreme conditions the novel strains survived. Thus, providing a conclusive proof about the role played by microbial biofilm formation as one of the survival strategies for inhabiting such extreme niches.

Authorship contributions

Conception and design of study: **Bedaprana Roy, Debapriya Maitra, Arup Kumar Mitra,** acquisition of data: **Bedaprana Roy, Debapriya Maitra,** analysis and/or interpretation of data: **Bedaprana Roy, Debapriya Maitra, Ayan Chandra,** Drafting the manuscript: **Bedaprana Roy,** revising the manuscript critically for important intellectual content: **Bedaprana Roy, Debapriya Maitra, Jaydip Ghosh, Arup Kumar Mitra,** Approval of the version of the manuscript to be published (the names of all authors must be listed), Bedaprana Roy, Debapriya Maitra, Ayan Chandra, Jaydip Ghosh and Arup Kumar Mitra.

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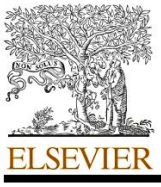

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Biofilm producing *Bacillus vallismortis* TR01K from tea rhizosphere acting as plant growth promoting agent

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ARTICLE INFO

Keywords:
Biofilm
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Lignin

ABSTRACT

Microbial biofilms are an aggregation of single or multi-species bacteria that acquires the capacity to adhere to any surface where they can act like a wholesome system chemically "speaking with" each other through quorum sensing. This synergism is very prominently noticed in the rhizospheric regions of plant roots subsequently forming a dome which can protect the root-rhizospheric niche from various biotic and abiotic stress. The bacterial EPS have a number of roles like adhesion, cohesion and aggregation of soil particles, retaining water molecules, acts as a potential barrier on the rhizospheric regions, facilitating ionic and genetic information exchange within the matrix component, enhanced production of plant readily available nutrients etc. Keeping this scenario in mind, this study was formulated on to isolate and explore novel rhizobacteria with colossal biofilm forming ability showing great potential as a source of lignocellulolytic plant growth promoting agent.

The crop chosen for this study was tea or *Camellia sinensis,* a quintessential beverage that is consumed across the globe. The rhizospheric region of a woody plant like tea, acts as a hub for lignocellulosic enzyme producing bacteria. The novel rhizobacteria isolated from cultivated tea soil, *Bacillus vallismortis* TR01K [NCBI Genbank Accession Number MT672714], was found to have an immense biofilm forming potential that ranged approximately 40x times higher than normal standard bacterial biofilm forming potentials when tested under laboratory conditions. Further *in vitro* characterizations of the novel strain showed it's immense potential to make plant nutrient available, to produce plant growth hormones (IAA, GA3, Cytokinin) and produce plant stress mitigating hormone (ACC deaminase). Lignocellulolytic enzymes are a vital part of lignocellulosic biomass degradation-a sustainable biotechnological approach for enzymes, organic acids, feed and biofuel production. The selected bacteria was tested elaborately for the family of lignocellulolytic enzymes (cellulase, laccase, lignin peroxidase, pectinase, amylase, chitinase, beta glucanase etc.) which showed promising results. Thus, proving the entire set of experiments in compliance with the aforementioned hypothesis that the novel bacterial isolate from tea rhizosphere has a significant biofilm forming potential with a colossal potency for being lignocellulolytic plant growth promoting agent.

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REVIEW ARTICLE

WILEY

Biofilm and metallothioneins: A dual approach to bioremediate the heavy metal menace

Bedaprana Roy[1] | Debapriya Maitra[1] | Subham Sarkar[2] | Rajeshwari Podder[2] | Tannishtha Das[2] | Jaydip Ghosh[1] | Arup Kumar Mitra[1]

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Abstract

Metallothioneins are a class of proteins produced by both prokaryotes and eukaryotes, having low molecular weight and abundant cysteine residues. These proteins play a humongous role in binding, sequestration, and even buffering of the intracellular metal ions. Though they are a wide class of proteins, much of them are yet to be explored. Their metal binding attribute is unique and they form distinctive metal-thiolate clusters. They have also been known to have ROS scavenging activities due to the presence of the cysteine residues. Phytochelatins also play major roles in metal sequestration pathways. Biofilms on the other hand are clusters of bacterial cells surrounded by an extracellular matrix of polymeric substances secreted by the bacteria themselves. Biofilms play multiple roles, from nutrient sequestration, stress resistance to surface adherence. But lesser explored arenas include assistance in heavy metal trapping and bio-remediation. Researchers have conducted studies that have demonstrated increased metal trapping, resistance and uptake in biofilm forming strains than non-biofilm forming mutants. Therefore, this study would explore the dual role of metallothionein and biofilm in their activity of metal sequestration and heavy metal remediation and provide certain insights so as to keenly understand the correlations between the two.

KEYWORDS
bacteria, biofilm, bio-remediation, heavy metal, metallothioneins, phytochelatins

1 | INTRODUCTION

Current concerns revolving around heavy metal pollution are due to the gradual increase in levels of toxic heavy metals in the face of rapid industrialization and a lack of effective methods of management and removal of these from the environment. The increment in heavy metal accumulation is attributed to the exponential increase in the usage of toxic heavy metals in several industrial, agricultural, domestic, and technological sectors (Gautam et al., 2016). These heavy metals are difficult to eliminate from nature and eventually make their way into the food chain, resulting in bio magnification at various trophic levels. The release of untreated wastewater from industries like textiles, tanneries, and mining is rich in acids, alkalis, heavy metals, and toxic dyes. The consumption of such polluted water causes long-term health concerns. As per the "Status of trace and toxic metals in Indian rivers." 57 out of 414 Indian River stations have reported heavy metal accumulation beyond acceptable limits. There are more than five million sites where heavy metal accumulation has been noted (Li et al., 2019). As per the 2019 report of the United Nations, 1.8 billion individuals are exposed to heavy metals that are above the permissible level, thereby increasing their risk of developing serious health issues (United Nations).

In terms of chemistry, heavy metals are a class of metals and metalloids with atomic numbers greater than 20, such as mercury (Hg),

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RESEARCH ARTICLE

WILEY

Organic farming in the improvement of soil health and productivity of tea cultivation: A pilot study

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Abstract

The sub-mountainous tea gardens of the Dooars region of West Bengal, which contribute approximately 25% of the national tea yield, are constantly fighting with diminishing soil fertility. Inorganic alternatives like chemical fertilizers can provide easier yet short-term solutions, as their prolonged and indiscriminate usage leaches the soil, devouring its productivity, increasing the soil's heavy metal contents, and subsequently accumulating those heavy metals in leaves. A plausible substitution in this scenario could be the use of organic alternatives like composting or biofertilizer. Although references to such alternative means are found in the literature, a holistic approach targeting plant growth promotion along with mitigating soil metal toxicity is lacking. Keeping this background in mind, this pilot study was designed to optimize the dosage of novel biofertilizers (using resident and alien flora) that can reduce heavy metal loads and residual toxicity in soil, thereby improving overall soil health and tea production. Two potential metallophilic plant growth-promoting strains of Bacillus sp. (previously reported) were selected and applied to potted tea plants of two different varieties of tea: TV9 and TV25. Among the two modes of treatment tested: solid treatment (compost amended with bacterial culture) and liquid treatment (cell pellets mixed in water suspension), the water suspension-based direct application of resident soil bacteria showed the highest physiological growth with reduced metal toxicity. Based on physiological data and physico-chemical data collected, it was observed that direct application of bacteria showed better results in both plant and soil health improvement in comparison to regular compost amended with beneficial microflora. Therefore, this small-scale pilot study aimed to optimize the dosage and mode of application of novel biofertilizers for improved soil and plant health.

KEYWORDS
pedology, soil quality, tea rhizosphere

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Molecular and Electronic Structures, Spectra, Electrochemistry and Anti-bacterial Efficacy of Novel Heterocyclic Hydrazones of Phenanthrenequinone and Their Nickel(II) Complexes

Soumitra Dinda[+],[a] Debapriya Maitra[+],[b] Bedaprana Roy,[b] Prattusha Khan,[b] Aratrika Samajdar,[b] Arup Kumar Mitra,[b] Subhadip Roy,[c] Arpan Mondal,[d] Kausikisankar Pramanik,*[e] and Sanjib Ganguly*[a]

A pair of tridentate ligands incorporating heterocyclic hydrazones of 9,10-phenanthrenequinone *viz*,. pyridyl-hydrazino-phenathrenequinone HL^{Py} **1a** and benzothiazolyl-hydrazino-phenathrenequinone HL^{Benz} **1b** have been synthesized and both behave as monoanionic towards nickel(II), forming meridional octahedral complexes of type $[Ni(L^{Py})_2]$ **2a** and $[Ni(L^{Benz})_2]$ **2b** as evident from single crystal X-ray diffraction studies. The complexes are electro-active in solution and nature of redox orbitals has been analysed by theoretical means. They display two oxidative and two reductive responses that have been ascribed to the redox processes of coordinated ligands. The electronic absorption spectral patterns of two complexes are analogous barring the fact that lower energy transition for **2b** is marginally bathochromically shifted relative to that of **2a** and it has been clarified by TD-DFT studies. Anti-bacterial efficacy of the ligands HL^{Py} **1a**, HL^{Benz} **1b** and complexes $[Ni(L^{Py})_2]$ **2a**, $[Ni(L^{Benz})_2]$ **2b** against four Gram-positive and four Gram-negative strains has been explored. $[Ni(L^{Py})_2]$ **2a** exhibits more pronounced efficacy than $[Ni(L^{Benz})_2]$ **2b** and these are greater than those of the corresponding ligands. The mode of action of **2a** is essential *via* DNA damage while protein leakage and membrane lipid damage were observed upon treatment with **2b**.

Introduction

A major concern to worldwide public health is associated with extensive usage of common antibiotics like tetracyclines, cephalosporins, aminoglycosides, macrolides. Presently, its effect is significant since there are failures in treatment associated with multidrug-resistant bacteria, thereby compromising with quality of health care.[1] It is reported that nearly seven lakhs of yearly global deaths are attributed to antibiotic resistance and with the outbreak of COVID-19 pandemic, the situation has even worsened since irrational usage of common antibiotics has further enhanced, in spite of constant warnings from WHO and CDC.[2] Under the current crisis, it is indeed a challenge for scientific community to synthesize and explore the efficacy of novel antimicrobials for sustenance of human life. It has been described that bivalent nickel may act as enzyme cofactors in a variety of organisms since they have the aptitude to catalyse several types of remarkable biochemical reactions.[3] Furthermore, aqueous solution of nickel(II) fails to exhibit any redox chemistry of biologically relevance since water will be able to oxidize and reduce at potentials less extreme than that of metal ion. Therefore, ligand environment is often vital for fine-tuning of redox potential of Ni(II) into a biologically accessible range.[4] It has also been found that certain complexes of nickel(II) can exhibit diverse *in vitro* biological activities, ranging from antimicrobial and antiinflammatory to antiproliferative as well as enzyme inhibitory and it has also been emphasized that complexes with redox-active ligands are probably responsible for the biological activities.[5]

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A Study on the Application and Efficiency of Novel Biofertilizer On Paddy: A Small-Scale Study

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Globally farmers use around 115 million tonnes of chemical fertilizer out of which only 35% is used by plants. The remaining 65% is redundant and is therefore one of the major soil pollutants. Due to their disadvantages, chemical fertilizers are being progressively substituted by bio-fertilizers. The extraordinary advantages and sustainability of bio-fertilizers make them propitious candidates for application in agriculture. Keeping this background, a novel bio-fertilizer was designed and applied to paddy (test crop) under *in-vivo* conditions and its various modes of application were assiduously scrutinized to standardize the quintessential means of treatment implementation for the crop. The selected novel plant growth-promoting bacterial strains (with standardized dosages) were parameterized and investigated in the various modes of application. Different economically suitable modes like the application of bio-fertilizer in suspension or by mixing it in compost are known to provide more victual and nourishment to the plants. Therefore, in this study, a comparative analysis was drawn to standardize the best mode of treatment application. Apart from the known and popular means of application of bio-fertilizer, a new technique of utilization of a proportionate mixture of soil, bio-fertilizer, and bio-synthetic capsules was also tested to ascertain the viability of such setups with synthetic compounds. Treatment was given after 30 days and meticulous observations were taken at a regular interval (7 days). Statistical tools were used for analysis and interpretation of the results of each treatment.

Keywords: Bio-fertilizer, compost, Plant growth promoting bacteria, suspension, rice plants

INTRODUCTION

Rice (*Oryza sativa*), a cereal grain and a monocot, is one of the primary food crops in the world (Mallick *et al*. 2013). It is a complex carbohydrate and acts as a primary source of energy and a staple diet for almost half of the world's population. About more than 500 million metric tons of milled rice were produced an average in the last few harvesting seasons throughout the world. Whilst rice farms are present globally, it's concentrated mainly in Asian developing countries Apart from providing the world with a good nutrient source, the remaining parts of the plant can be re-used as cooking fuel, used for feeding livestock, and reprocessed to manufacture paper (Kaur *et al*. 2017), furniture and upholstery (Sumarno *et al*. 2020). According to crop cultivation, consumption, and export statistics, Asian countries have the most prodigious share of the world's rice production. According to recent official data, with a production quantity of over 212 million metric tons in 2021, China was the world's foremost rice producer, followed by India and Bangladesh (Fig.1). This makes developing countries like India and Bangladesh important contributors to world's food requirements (Shahbandeh, 2023).

The use of chemical fertilizers for the production of rice has been a tradition followed for ages but the detrimental and pernicious effects of chemical fertilizers on the soil, the plant, humans, and the ecosystem have given rise to perturbation and apprehension among agriculturists and environmentalists (Thorat and More, 2022). It has long been recognized that excessive and

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Unveiling the potential of Halotolerant bacteria as PGPR with an ANOVA and Time-Series enhanced study for sustainable agriculture

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This study explores harnessing the potential of halotolerant plant growth-promoting bacteria (PGPR) from Ghoramara Island, addressing challenges of salinity and cyclone-induced soil runoff in the ecologically vulnerable region. *Bacillus altitudinis* (OS-1) and *Priestia megaterium* (TDS-3) were chosen as initial bio-fertilizer consortia to enhance okra (*Abelmoschus esculentus*) growth, a crucial dietary crop. After salt tolerance and nutrient content analysis, bacterial suspensions were applied to the okra plants, confirming the growth-promoting abilities of native halotolerant strains. OS-1 exhibited the highest growth promotion, with TDS-3 showing intermediate effects. However, the combined application of both strains had an antagonistic effect, emphasizing the necessity for further analysis and gene sequencing to understand the underlying molecular dynamics. This study pioneers the design of a preliminary biofertilizer for optimal agricultural benefits in challenging environments, highlighting the vital role of PGPR in sustainable agriculture. The inclusion of Time and Series analysis along with ANOVA testing enhances the comprehensiveness, providing rigorous statistical analysis of halotolerant PGPR effects on okra growth, ultimately offering a promising avenue to boost crop yields and alleviate environmental stressors in vulnerable regions.

Keywords: Antagonism,bio-fertilizer, biological nitrogen fixation, bioremediation, Ghoramara island, plant growth promoting rhizobacteria.

INTRODUCTION

Ghoramara Island, situated in the Bay of Bengal, stands as a poignant testament to the relentless forces of nature. This fragile landmass is exceedingly vulnerable to the devastating impacts of cyclones and rising sea levels, which perpetually threaten its very existence. The recurrent onslaught of cyclones not only disrupts the normal lives of the island's inhabitants but also wreaks havoc on its precious soil content and fertility through runoff, leading to the gradual disintegration of this once-thriving island ecosystem (Hajraand Ghosh, 2018).

In light of these dire circumstances, our research endeavors to address the multifaceted challenges faced by the Ghoramara Island community. We have embarked on a mission to explore sustainable agricultural practices that can not only enhance the livelihoods of the island's residents but also contribute to the conservation of this unique ecological haven. Central to our investigation is the exploration of halotolerant Plant Growth-Promoting Rhizobacteria (PGPR) indigenous to Ghoramara Island.

The rhizosphere, teeming with diverse microbes, supports plant growth through interactions, particularly with Plant Growth-Promoting Rhizobacteria (PGPR). These bacteria, including *Azospirillum, Rhizobium, Azotobacter, Pseudomonas, and Bacillus*, can fertilize a variety of crops like rice, beans, strawberries, potatoes, maize, tomatoes, and cucumbers. They stimulate plant development through nutrient acquisition, phytohormone production, biological nitrogen fixation (BNF), disease control via induced systemic resistance (ISR), antagonism, and the release of volatile organic compounds (VOCs) (Lee *et al.* 2021). These helpful bacteria colonize

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ISOLATION AND CHARACTERIZATION OF PROSPECTIVE SALT TOLERANT BACTERIA WITH PLANT GROWTH PROMOTING PROPERTIES FROM MANGROVES OF SUNDARBAN, WEST BENGAL, INDIA

Shivashis Bikram Banerjee[1], Debapriya Maitra[2], Bedaprana Roy[2], Bikram Dhara[2*], Ramalakshmi Datta[3], Sanjay Haldar[3] and Arup Kumar Mitra[2]
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ABSTRACT

Increasing soil salinity acts as a major abiotic stress for crop plants. Increasing global temperatures are leading to greater evaporation from soil, along with change in rainfall patterns, which is resulting in reduced soil water availability for crop plants and increased soil salinity. Consequently, crop plants face water and nutrient shortage leading to yield losses. In fact, crop plants cannot be grown easily on such saline soil without some form of remediation. Plant Growth Promoting Rhizobacteria (PGPR) have shown prospective results in this regard. Halotolerant PGPRs have the ability to grow in such saline soils, while providing plant roots in the vicinity with growth nutrients and hormones. In the present study, we obtained six bacterial isolates from mangrove pneumatophores of *Aegialitis rotundifolia* Roxb. and *Ceriops tagal* C. B. Rob. with associated rhizobial soil from Kshetra Mohanpur site in the Sundarbans of West Bengal. They were screened for salt tolerance, nitrogen fixation, phosphate solubilizing, potassium solubilizing and auxin synthesizing ability. Two of the six isolates showed all these properties. Hence, we propose their use as halotolerant PGPR biofertilizers for soil bioremediation.

Keywords: *Plant growth promoting rhizobacteria, Biofertilizer, Soil salinity, Climate change*

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ISOLATION OF SALINE TOLERANT PGPR (ST-PGPR) FROM SINKING ISLAND OF GHORAMARA

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ABSTRACT

Soil salinity, a global issue, is a threat to the agricultural crops. The deposition of excess salt in the soil reduces the growth, development and yield of crops. Initially techniques like leaching and flushing were applied to reclaim such salt infested soil. However, the success rate was low and it greatly affected the agroecosystem. This had led to the search for saline tolerant and salt resistant crops. The sinking island of Ghoramara is also facing the problem of soil salinity. In this investigation, soil samples were collected from coastal, transition and agricultural region of the island and the physico-chemical parameters of the soil sample were analyzed to understand its quality. The parameters pH and EC were carefully analyzed since they are of utmost importance for elucidating soil salinity. Saline tolerant bacteria were isolated using increasing concentrations of salt in the growth medium. The isolated bacteria were grown on chrome agar followed by gram staining to identify the genus of the isolated bacteria. This was followed by the study of NPK fixation and solubilization analysis, zinc solubilizing efficiency and siderophore production i.e., iron chelating ability of the isolated strains. Based on the results obtained from the above set of experiments five bacteria were selected. These five bacteria would be identified using 16S rRNA. Further experiments for analyzing plant growth promoting hormone production and other plant growth promoting traits will be carried out with these five bacterial strains in the future. Depending on the result a consortium can be developed, specifically designed for the saline infested soil of Ghoramara to improve their crop productivity.

Key words: *Salt tolerance, PGPR, Physico-chemical parameter, Soil microflora.*

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ROLE OF RESIDUAL MICROFLORA FROM INDIAN SPICES IN INCREASING THEIR SHELF LIFE

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ABSTRACT

Spices impart flavor, taste and aroma to food. Spices have inherent microflora which may have varied roles and may interact variously amongst each other. Different spice samples were analyzed to isolate the indigenous microflora (bacteria and fungi). These isolates were purified. The colony characteristics and morphology of the isolates were studied and specific staining was performed to identify some selected isolates. Enzyme production ability of the selected bacterial isolates were assayed, and based on the absence of degradative enzymes, three harmless bacteria were tested against one of the fungal isolates, which was identified as *Aspergillus flavus* by partial sequencing. The antagonistic relationship between the fungi and the bacteria were carried out using plate assay method and three of the bacterial isolates were observed to be effective in controlling *Aspergillus flavus*. They were identified by partial sequencing and was found to be *Bacillus australimaris*, *Bacillus subtilis* and *Bacillus cereus*. The microbial enrichment may prove to be useful in terms of nutritive value addition to the spices and increase its shelf life.

Key words: *Aspergillus flavus, Bacillus australimaris, Bacillus subtilis, Bacillus cereus*

INTRODUCTION

Spices are an essential element of the global food tradition and have been used in colouring, flavouring, preservation of food as well therapeutic intentions since time immemorial (Jiang, 2019). The term 'spices' has been defined by the US-Food and

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The Need for Auto-Tailored Wetlands for the Treatment of Untampered Wastes of Wineries and Breweries

Chapter | First Online: 06 July 2023

pp 197–212 | Cite this chapter

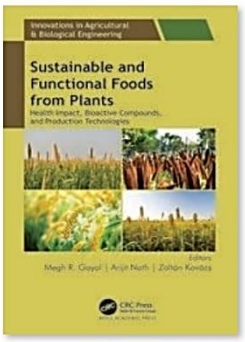
Recent Trends in Constructed Wetlands for Industrial Wastewater...

Bedaprana Roy, Debapriya Maitra, Bidisha Chatterjee, Pallab Ghosh, Jaydip Ghosh & Arup Kumar Mitra Show fewer authors

Abstract

The wastewater generated from the wineries and breweries contains a large number of organic compounds such as carbohydrates, sugars, organic acids, phenolic compounds, etc., that are highly biodegradable in nature. And thus, the disposal and treatment of these wastes become

Chapter



Health Impacts of Common Flavoring Agents in Indian Cuisine

By Bedaprana Roy, Bidisha Chatterjee, Tamanna Sultana, Arup Kumar Mitra

Book Sustainable and Functional Foods from Plants

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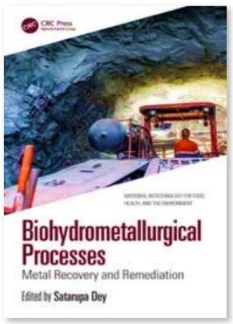
Chapter



Metagenomic Analysis of Acid Mine Drainage, Presence of Acidometallophiles, and Their Possible Role in Biomining

By Bedaprana Roy, Riddhi Chakraborty, Niti Choudhury, Aindri Ghosh, Rajeswari Chakraborty, Jaydip Ghosh, Arup Kumar Mitra

Book Biohydrometallurgical Processes

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Pages	16
eBook ISBN	9781003451457

Biotechnological applications extremophiles: the golden epoch ahead

From the book Extremophiles

Bedaprana Roy, Debapriya Maitra, Rajeshwari Podder, Jaydip Ghosh and Arup Kumar Mitra

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Abstract

Extreme environments act as habitats that nurture a number of magnificent organisms that are tolerant to such harsh environments. These environments include areas with extreme temperatures, pH, salinity, pressure, and heavy metal concentrations. Biological and chemical processes encounter numerous such stressed conditions, control of which makes these processes delicate, prolonged, and expensive. For example,

Microbes and Microbial Biotechnology for Green Remediation

2022, Pages 287-304

Chapter 15 - Unique extremophilic *Bacillus*: their application in plant growth promotion and sustainable agriculture

Bedaprana Roy, Debapriya Maitra, Jaydip Ghosh, Arup Kumar Mitra

https://doi.org/10.1016/B978-0-323-90452-0.00021-9

Abstract

A number of extreme soil environments exist due to variation in soil temperature, salinity, pH, etc. Extremophile microbes, specifically the *Bacillus* sp. residing in these soils or when added externally can adapt to such extreme environments, can enrich such soil with both macronutrients (nitrogen, phosphorus, potassium) and micronutrients (zinc, iron, magnesium, etc.). A number of plant-rhizospheric *Bacillus* sp. are capable of fixing these nutrients, such as *B. subtilis. B. mycoides* are shown to survive in temperature range of 0°C–5°C with a rate of 3.9×10^6 and 10^7 cells/g of rhizosphere. Extremophilic strains have been found, and they can carry out plant growth-promoting activities in soils where there is drought stress, temperature stress (both high and low), pH stress, or salinity issues. Several cyclic lipopeptides, iturin A analogs secreted as volatile exudates and metabolites from species like *B. vallismortis*, *B. licheniformis*, *B. subtilis*, and *B. megaterium* hav

Dynamics of Soil Microbiome and Its Role in Sustainable Agriculture

Chapter | First Online: 21 August 2022

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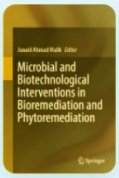


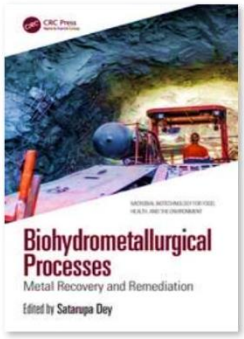
Microbial and Biotechnological Interventions in...

Debapriya Maitra, Bedaprana Roy, Sudeshna Shyam Choudhury & Arup Kumar Mitra Show fewer authors

Abstract

Soil is believed to be one of the greatest reserves of microbial population. Certain studies on spatial ecology show that 1 g of soil can contain as much as 10^{10} bacterial cells, with a population diversity of approximately 4.10^{3}. The impact of these microbes including bacteria, fungi, and other organisms on overall soil health, fertility, and crop

Chapter



Metagenomic Analysis of Acid Mine Drainage, Presence of Acidometallophiles, and Their Possible Role in Biomining

By Bedaprana Roy, Riddhi Chakraborty, Niti Choudhury, Aindri Ghosh, Rajeswari Chakraborty, Jaydip Ghosh, Arup Kumar Mitra

Book Biohydrometallurgical Processes

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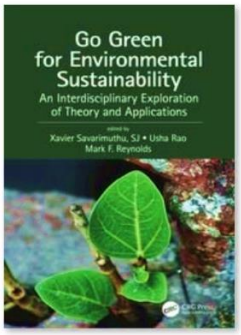
Chapter



Dynamic Role of Specific Microbes in Bioremediation of Heavy Metals and Dyes from The Textile Industry

By Tamanna Sultana, Debapriya Maitra, Bedaprana Roy, Arup Kumar Mitra, Xavier Savarimuthu

Book Go Green for Environmental Sustainability

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Methods of Sample Preparation and Assay of Bacterial Biofilms with Special Reference to Their Significance in Agriculture and Extreme Environments

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Analytical Methodologies for Biofilm Research

Bedaprana Roy, Debapriya Maitra & Arup Kumar Mitra

Part of the book series: Springer Protocols Handbooks ((SPH))

Abstract

Microbes tend to exist in polymicrobial communities embedded in a matrix of several compounds produced by themselves such as polysaccharides, proteins, extracellular nucleic acids, such as DNA or RNA, humic substances, signalling molecules, etc. These matrices are