Study on Phosphate Solubilizing Bacteria Isolated from Ghulkin Glacier, Hunza Valley

Submitted

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DEDICATION

I dedicate the outcome of my efforts to:

- My father, who is an ideal to me and his suggestions and motivations remained hope for me throughout my research work.
- My mother (an amazing lady), who always encouraged me to do the best, my supervisor.
- My beloved and dear in my life, my brothers and sisters.

Declaration

I, Syed Khalid Shah declares that the material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Syed Khalid Shah

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled **"Study on Phosphate Solubilizing Bacteria Isolated from Ghulkin Glacier, Hunza Valley"** was conducted by **Mr. Syed Khalid Shah** under the supervision of **Prof. Dr. Fariha Hasan.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-I-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Master of Philosophy in field of Microbiology.

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List of abbreviations

UV	Ultra Violet
NASA	National Aeronautics and Space Administration
ISE	International Society for Extremophiles
M	Molar
h	Hours
N	Nitrogen
ACC	1-Amino-Cyclopropane-1-Carboylic acid
mg	Milligram
%	Percentage
^{o}C	Degree Celsius
min	Minutes
PGPR	Plant Growth Promoting Rhizobacteria
P	Phosphorus
NA	Nutrient agar
PSB	Phosphate Solubilizing Bacteria
PSF	Phosphate Solubilizing Fungi
OAs	Organic Acids
PGPB	Plant Growth Promoting Bacteria
PSMs	Phosphate Solubilizing Microorganisms
AFPs	Anti-Freez Proteins
IRI	Isoamylase Reverse Interference
TH	Thermal HyMrolysis
HSP	Heat Shock Protein
CSP	Cold Shock Proteins
PPIase	Peptidyl-Prolyl Isomerase
SCP	Stress Combating Proteins
AAA	ATPases Associated with diverse cellular Activities
CS	ChonMroitin Sulfate
ADP	Adenosine Di-Phosphate
ATP	Adenosine Tri-Phosphate

PS	Phosphate Solubilization
NSAPs	Non-Specific Acid Phosphatase
RPM	Revolutions Per Minute
PVK	Pikovskaya
mM	Millimolar
IU	International Unit
CP	Carbon Phosphorus
HCL	HyMrochloric Acid
NH4	Ammonium
NH3	Ammonia
NO3	Nitrate
PQQ	Pyrroloquinoline Quinone
SI	Solubilizing Index
TLC	Thin Layer Chromatography
FTIR	Fourier-transform infrared
HPLC	High Performance Liquid Chromatography

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Abstract

Phosphorus (P) stands out as a vital macronutrient essential for the growth and reproductive processes of plants. Plants absorb P from the soil solution as phosphate anions. However, due to their high reactivity, phosphate anions can precipitate with cations such as Fe3+, Al3+, Mg2+, and Ca2+, contingent on the particular properties of the soil. since of this, plants cannot use the phosphate since it is so extremely insoluble. To promote plant development and P uptake, phosphate-solubilizing bacteria (PSB) have been used as fertilizer. Improving soil fertility is a commonly used strategy to increase agricultural productivity. Maintaining high levels of available phosphorus (P) and nitrogen (N), the two nutrients that limit soil fertility the most, is still a major undertaking. For the first time, we isolated and characterized psychrophilic PSB from Ghulkhin Glacier that had strong solubilizing capacities for calcium phosphate (Ca-P), hydroxyapatite (H-Ap), and tricalcium phosphate (TCP). Optimizing for the solubilization of phosphate (P) by the isolates *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 was done. Phosphate solubilizing efficiency (PSE) capabilities were assessed using a variety of insoluble P sources, including TCP, Ca-P, H-Ap, Al-P, and Fe-P. Temperature of 5 degrees Celsius, pH values of 3 for *Pseudomonas frederiksbergensis* GA23 and 15 for *Acinetobacter johnsonii* GB30, fructose for *Pseudomonas frederiksbergensis* GA23 and xylose for *Acinetobacter johnsonii* GB30, ammonium sulphate for *Pseudomonas frederiksbergensis* GA23 and ammonium nitrate for *Acinetobacter johnsonii* GB30, potassium nitrate for *Pseudomonas frederiksbergensis* GA23 and potassium chloride for *Acinetobacter johnsonii* GB30, and 10% salt stress for both *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were found to be the ideal conditions. Nonetheless, the highest increase in Drought stress was 6% for *Pseudomonas frederiksbergensis* GA23 and 5% for *Acinetobacter johnsonii* GB30. The release of organic acids in culture was observed to be correlated with phosphate solubilization (PS). The presence of organic acids is confirmed by the TLC plate when an orange, yellow, blue background appears. For additional assurance, FTIR analysis was carried out. Three known acids (oxalic, acetic, and formic acid) and ten unknown acids were found by HPLC analysis of the culture broth after 48 hours of incubation, whereas five known organic acids (citric, oxalic, acetic, gluconic, and formic acids) were found by HPLC analysis of

Acinetobacter johnsonii GB30 after 96 hours of incubation. These organic acids help to promote plant growth and solubilize inorganic phosphate to soluble phosphate. Glacier samples exhibited high productivity of organic acids, which could be utilized as biofertilizers to enhance agricultural growth in the cold regions of Pakistan.

1.Introduction

1.1. Extremophiles

Extremophilic microbial communities thrive in diverse habitats, encompassing both typical natural conditions and exceptionally challenging environmental factors such as severe temperatures, salt, water scarceness, and levels of ph. In order to endure in such environments, these resilient microorganisms have acquired adaptive characteristics to grow, reproduce, and in the face of severe conditions generate bioactive substances and secondary metabolites (A. N. Yadav, Verma, *et al*., 2015). During the 1980s and 1990s, biologists made the discovery that bacteria life possesses remarkable adaptability to thrive in highly challenging conditions. Microorganism communities in various extreme microbes have developed physiological adaptations to endure severe environmental challenges, including extreme temperatures, high salinity or hyper salinity, drought, acidic or alkaline conditions, UV radiation, and various chemical stressors (A. N. Yadav, Gulati, *et al*., 2019). According to NASA in 2003, certain scientists have reached the conclusion that lifecycle might have originated on Earth within hydrothermal vents located deep beneath the surface of the ocean(Gupta *et al*., 2014). In 1974 McElroy was initially coined the word "extremophile". The majority of extremophiles are classified into the kingdoms of archaea, bacteria, and eukarya (Rothschild & Mancinelli, 2001). Before ten years, extremophiles were considered rare creatures, explored by only a few research teams worldwide. Enzymologists have identified these bacteria as a promising resource for exploitation in several sectors (Van Den Burg, 2003). Over the past 10 years, research on extremophiles has advanced to the point that the First International Congress on Extremophiles was held in Portugal in 1996, and the scholarly magazine "Extremophiles" was founded in 1997. The International Society for Extremophiles (ISE), established in 2002, serves as a platform for global exchange of knowledge and expertise in the rapidly expanding field of extremophile research. The enormous range of unique features exhibited by extremophiles is what makes them highly valuable in different industrial and other applications. The primary motivation behind research on extremophiles is the biotechnological practices linked to the bacteria and their biological products. The metabolic processes and specialized biological functions of these microorganisms are regulated by enzymes and proteins, which are accountable for the

unique characteristics of these organisms. Given the concept that extremophiles may survive in harsh environments, it is presumed that their enzymes are specifically altered to perform at their best in such situations. Undoubtedly, the data gathered for the enzymes that have lately been extracted from these unusual bacteria substantiate this presumption. These enzymes possess distinctive characteristics, including exceptional thermal stability, resistance to chemical denaturants such as chaotropic agents, detergents, organic solvents, and severe pH conditions (R. Gaur *et al*., 2010; Karan *et al*., 2011).

Microbial communities in extreme adverse settings, including archaeal, bacterial, and fungal communities, have garnered attention for their potential uses in several fields such as medicine, white and green biotechnology, processing business and food production (A. N. Yadav, Kour, *et al*., 2019). Extremophiles and stress-Adaptive microbes are living microorganisms that are able to adapt in environments with stressful environments such as varying pH levels (acidic or alkaline), high pressure (piezophiles), radiation exposure (radiation resistance), different redox potentials (xerophiles), high salinity (halophiles), and extreme temperatures (psychrophilic or thermophilic) (A. N. Yadav, Sachan, Verma, & Saxena, 2015; A. N. Yadav, Sachan, Verma, Tyagi, *et al*., 2015). Extremophiles are capable of surviving in highly hostile settings on Earth, characterized by extreme salinity levels (2-5 M NaCl; halophiles), pH values below 4 (acidophiles) or over 9 (alkaliphiles), and temperatures ranging from -20 °C to 20 °C (psychrophiles/psychotropic) or from 60 ºC to 115 ºC (thermophiles/hyperthermophiles) (Sahay *et al*., 2017; Saxena *et al*., 2016). Polyextremophilic microbiomes possess the ability to thrive and proliferate in multiple stressful and severe environmental situations. The extremophilic microbiomes, which are capable of adapting to stressful conditions, play a significant role in promoting agro-environmental sustainability. These microbes possess the ability to produce bioactive compounds that are important for agro-industrial purposes, such as indole acetic acids (IAA), 1-aminocyclopropane-1-carboxylate deaminase, gibberellic acids (GA), cytokinin, siderophores, hydrogen cyanides, ammonia, and extracellular microbial hydrolytic enzymes (amylase, pectinase, xylanase, chitinase, cellulase). Microbes exhibit diverse applications across several domains, such as agriculture, the dairy industry, biodegradable production, chemical processing, molecular biology, hemicellulose bioconversion, composting, the food industry, detergent industry, feed

additives, leather industry, paper and cellulose industry, pharmaceutical industry, peptide synthesis, and the development of therapeutic agents (Barcelos *et al*., 2020; Dumorné *et al*., 2017; M. Kumar *et al*., 2021). Extremophilic microbiomes are employed in the food and food processing sector due to their potential to generate a diverse array of secondary metabolites, bioactive compounds, and value-added products, including tastes, food components, and vitamins (Rasmussen & Morrissey, 2007; Saxena *et al*., 2020). Based on the preceding discussion, we will now proceed with the examination of psychrophiles.

Psychrophilic microorganisms, which can grow at temperatures of 15°C or lower are psychrotolerant microorganisms, which can grow at temperatures near to the freezing point of water but have their maximum growth rate at temperatures above 20°C, are commonly found in low temperature environments on Earth. These environments include glaciers, refrigerated appliances, polar regions, ocean deeps, upper atmosphere, shallow subterranean regions, and on and in plants and animals that live in cold environment (Cavicchioli & Siddiqui, 2006). Even in the exorbitant conditions of space, such as severe radiation, temperatures, changing gravity, salinity, and nutrition levels, the growth of other species is limited, but these microorganisms are able to thrive. Approximately 70% of the Earth's biosphere, including regions such as the Arctic, Antarctic, and moderately cold areas, experience temperatures below 5ºC (De Maayer *et al*., 2014; Feller & Gerday, 1997; Margesin *et al*., 2007; Salwan & Kasana, 2013). The frigid conditions are inhabited by microorganisms classified as psychrophiles and psychrotrophs. Psychrophiles have optimal growth at or below 15°C, however they are capable of displaying both maximum and lowest growth within the temperature range of 0-20ºC. Psychrotrophs exhibit optimal growth at temperatures equal to or greater than 20ºC, but are also capable of tolerating temperatures below 5ºC (Joshi & Satyanarayana, 2013; Kasana, 2010; R. Y. Morita, 1975). Psychrophilic microorganisms are found in consistently cold habitats such as mountains, deep seas, and Polar Regions. On the other hand, psychrotrophs inhabit environments where the temperature changes (D'Amico *et al*., 2002; Pulicherla *et al*., 2011; Russell, 1990; X. Zhang *et al*., 2008). According to Price and Sowers (2004), the microorganisms are capable of surviving in their natural environment at temperatures as low as -30°C. Additionally, there is evidence to suggest that their metabolism may function at even

colder temperatures, such as -40°C. The survival of microorganisms in such environments is achievable through adaptations to the specific conditions. Psychrophiles, for instance, have developed certain adaptations in their cellular structures. These adaptations include the production of antifreeze proteins, maintaining membrane fluidity, and the synthesis of stress-related proteins (M. V. Brown *et al*., 2012). The microorganisms primarily consist of bacteria, including *Pseudoalteromonas*, *Vibrio*, *Pseudomonas*, *Arthrobacter*, and *Bacillus* (Collins *et al*., 2002; Okuda *et al*., 2004; Zeng *et al*., 2004), as well as archaea, such as *Methanogenium* and *Halorubrum*. Additionally, fungi like *Penicillium* and *Cladosporium* (Sakamoto *et al*., 2003), and yeast such as *Candida* and *Cryptococcus* (Nakagawa *et al*., 2004) are also present. The cold-adapted microbes produce psychrophilic enzymes that exhibit a remarkable catalytic efficacy at extreme temperatures. These enzymes have significant applications in various industries such as pharmaceutical, detergent, leather, brewing and wine, food, paper, textile, and pulp (L. Kumar *et al*., 2011). Psychrophiles and associated enzymes have been suggested as a potential alternative to physiochemical approaches for the bioremediation of waste waters and soils (S. K. Singh *et al*., 2011). The ability of enzymes to function at extreme temperatures is attributed to the flexible structures of cold-active enzymes. These structures are obtained by a combination of specific properties, such as reduced hydrophobicity in the core, fewer ionic contacts, heightened surface charge, and longer surface loops. The alterations result in a decrease in the rigidity of psychrophilic proteins and an increase in their structural flexibility (Gerday *et al*., 2000).

It is widely recognized that a significant number of microbial species, particularly those found in the plant roots, have the ability to positively influence plant growth. Consequently, researchers have been extensively studying the utilization of these microorganisms as biofertilizers or control agents to enhance agricultural practices (Davison, 1988; Glick, 1995a; Lemanceau, 1992; Okon, 1993; Suslow, 1982). The bacteria in this group are referred to as 'plant growth promoting rhizobacteria' (PGPR) (J. W. Kloepper, 1978). They include strains from several genera such as *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, and *Flavobacterium*. The efficacy of PGPR in promoting the growth of various crops has been proven through experiments

conducted in both controlled laboratory settings and real-world field trials. *Pseudomonas putida* and *Pseudomonas fluorescens* strains have been found to enhance the growth of roots and shoots in lettuce, canola, and tomato plants (Glick *et al*., 1997; Hall *et al*., 1996). Additionally, these strains have been shown to boost crop yields in many plants including sugar beet, potato, radishes, ornamental plants, rice, tomato, apple, citrus, lettuce, beans, and wheat (J. Kloepper *et al*., 1988; Lemanceau, 1992; Okon, 1993; Suslow, 1982). The application of *Azotobacter* inoculation resulted in a maximum wheat yield increase of 30%, while the use of *Bacillus* inoculants led to a maximum yield increase of 43% (J. W. Kloepper *et al*., 1989). Furthermore, field trials combining *Bacillus megaterium* and *Azotobacter chroococcum* indicated a yield increase of 10-20% in the same crop (M. E. Brown, 1974). *Azospirillum* spp. have demonstrated enhanced crop productivity in maize, sorghum, and wheat (Baldani *et al*., 1987; Kapulnik *et al*., 1985; SARIG *et al*., 1990), whereas *Bacillus* spp. have shown higher yield in sorghum, peanut, wheat and potato, (Broadbent, 1977; Burr *et al*., 1978; Capper & Campbell, 1986). Bacterial inoculants have been employed in various nations to enhance plant productivity, and they are presently accessible as commercial items. In Cuba, many biofertilizers are manufactured and utilized commercially in conjunction with diverse crops, primarily utilizing strains of *Azotobacter*, *Rhizobium*, *Azospirillum*, and *Burkholderia*. PGPR can enhance plant development through two distinct mechanisms: direct and indirect (Sivan & Chet, 1992). Indirect growth promotion refers to the reduction of harmful effects caused by pathogenic microorganisms, primarily through the production of antibiotics (Glick, 1995a) or siderophores (Leong, 1986) by the bacteria. Promoting growth can be directly achieved through various mechanisms. These mechanisms encompass the synthesis of phytohormones (Xie *et al*., 1996), nitrogen fixation (Christiansen-Weniger, 1992), reduction of root membrane potential (Bashan & Levanony, 1991), and the synthesis of enzymes (such as ACC deaminase) that regulate plant hormone levels (Glick *et al*., 1998). Additionally, growth promotion involves the mineralization of organic phosphate and the solubilization of inorganic phosphate, ultimately enhancing the availability of phosphorus to plants (A. Gaur & KP, 1972; Krasilnikov, 1961; Rao, 2016).

Phosphorus is the second most prevalent mineral nutrient that limits the growth of crops, behind nitrogen. P is a crucial component for the growth and maturation of

plants, constituting around 0.2% of the plant's dry weight. It has an essential biochemical function in respiration and photosynthesis, energy storage and transfer, cell enlargement, cell division and various other functions in the live plant. Sufficient phosphorus availability during the initial phases of plant development enhances physiological processes such as early root establishment and plays a significant role in the initiation of reproductive structures in plants. Additionally, it is recognized for enhancing the quality of vegetables, numerous fruits, and cereal crops. It occurs naturally in a diverse range of both organic and inorganic forms. The availability of phosphorus in soils is limited due to its fixation as insoluble P of iron, aluminum, and calcium. Chemical phosphatic fertilizers are extensively employed to attain optimal yields due to the crucial role of phosphorus deficiency in limiting the growth of plant. The soluble forms of phosphorus (P) fertilizer that are often used tend to quickly form insoluble compounds, resulting in the need for excessive and recurrent application of P fertilizer to agricultural fields. The primary objective of soil phosphorus management is to maximize crop yield while minimizing phosphorus loss from the soil. The PGPB are rhizospheric bacteria found in the soil and that enhance growths of plant through many processes (Glick, 1995b). Among these mechanisms, the capacity of these microbes to solubilize P regarded as a crucial characteristic for improving plant P nutrition. Utilizing PGPB in agriculture sustainability methods which is beneficial due to the detrimental effects and rising expenses associated with artificial fertilizers. In recent times, agriculturists have shown interest in phosphate solubilizing microbes (PSB) as soil inoculums for enhancing plant development and yield. This has been highlighted in studies conducted by (C.-C. Young, 1994), (C. Young *et al*., 1998), (A. H. Goldstein *et al*., 1999) and (Fasim *et al*., 2002). Rock phosphates are the primary sources of phosphate fertilizers (Van Kauwenbergh, 2006). They are significantly more affordable than soluble phosphorus fertilizers. Phosphate rocks typically consist of apatite minerals, which contain different amounts of P_2O_5 within a calcium framework. The largest repositories of phosphorus are found in mineral forms, notably in rocks and deposits that have evolved throughout geological time. The major minerals, namely oxyapatite, hydroxyapatite, and apatite, are primarily characterized by their insolubility. After being applied as chemical fertilizer to agricultural soil, a significant amount of soluble inorganic phosphate quickly becomes immobilized and hence cannot

be used by plants (K. Yadav & Dadarwal, 1997). Nevertheless, a significant part of chemical phosphatic fertilizers undergo precipitation in the soil. These substances consist of various toxic metals (Azzi *et al*., 2017), which leads to the accumulation of these metals in the soil. This accumulation has negative consequences for soil fertility, as well as the health of animals and consumers. Additionally, it contributes to eutrophication and an increase in carbon emissions (Huang *et al*., 2017). The immediate availability of phosphorus upon application is reduced, however, sufficient rainfall in acidic soils the process of rock phosphate dissolution can sustain the necessary amounts of phosphorus over a period of time (Anderson, 1980; Bolan & Hedley, 1990). The manufacturing of chemical phosphatic fertilizers is a significantly energy-intensive procedure, necessitating an annual expenditure of US $4 billion in energy to fulfill the worldwide demand (A. Goldstein *et al*., 1993). The condition is exacerbated by the circumstance that a significant portion, ranging from 75 to 90%, of the phosphatic fertilizer that is applied gets converted into solid precipitates due to the presence of metal cation complexes in the soils (Kuma Megersa, 2019). Hence, the dependence on fossil fuel energy for fertilizer production and the possible shortage of costly fertilizer inputs in the future have made the matter of mineral PS a significant concern. PSB strains facilitate mineral PS by the release of low molecular weight organic acids (OAs) (A. H. Goldstein, 1995; H. J. Kim *et al*., 2017). The carboxyl and hydroxyl groups of these acids combine with the cations that are attached to phosphate, resulting in the formation of complexes. Consequently, the phosphate undergoes a conversion into soluble forms (Kpomblekou-a & Tabatabai, 1994). Nevertheless, the process of P-solubilization is a multifaceted occurrence that is influenced by various parameters, including the nutritional, physiological, and growth circumstances of the culture (Reyes *et al*., 1999). Phosphate solubilizing bacteria (PSB), phosphate solubilizing fungi (PSF), and *actinomycetes* are known to be effective in converting insoluble phosphate into soluble phosphate (Chabot *et al*., 1993; Pal, 1998). The microbial system can extract significant quantities of nutrients from the natural reservoir and enhance the soil with essential yet limited nutrients. Stimulating the crop microbial ecosystem in sustainable agriculture can lead to notable improvements in ecological stability and environmental quality. Phosphate-solubilizing organisms have the potential to improve the availability of soluble phosphate, thereby promoting plant growth by increasing the

efficiency of biological nitrogen fixation and improving the accessibility of additional trace elements like iron and zinc. Furthermore, these organisms can produce regulators that stimulate plant growth, as documented by Kucey *et al*. (1989), Ponmurugan and Gopi (2006), and Sattar and Gaur (1987). In phosphorus-deficient settings, this relationship could lead to either enhanced absorption of the existing phosphates or the ability to obtain previously unavailable sources of phosphorus for the plant. The study conducted by (Son *et al*., 2006) revealed that *Pseudomonas* spp. had a positive impact on various aspects of soybean crop growth and productivity. This included an increase in the number and weight of nodules, as well as improvements in grain yield, yield components, nutrient accessibility, and nutrient uptake. Furthermore, these biofertilizers are both environmentally benign and non-toxic. Plant growth-promoting microorganisms (PSMs) release various OAs such as gluconic acids, citric acid, oxalic acid, succinic acids and malic acids. They also produce enzymes like phosphatases and phytases, as well as ion chelators like siderophores. These compounds efficiently improve the accessibility of P for plants (Tomer *et al*., 2016). PSMs also possess the ability to enhance plant development. They promote the holistic expansion and maturation of plants in both challenging and non-challenging conditions. Multiple studies have documented that PSMs possess the ability to dissolve potassium, improve nitrogen fixation, and generate plant growth regulators such as auxins, cytokinins, and gibberellins. Additionally, they defense plants from harmful microorganisms by secreting antibiotics and antifungals, making them highly effective biocontrol agents. Additionally, they can also assist in the process of bioremediating soil that has been contaminated with heavy metals. Several PGPR produce the enzyme ACC deaminase, which mitigates the adverse effects of ethylene stress on plants under stressful conditions. These minuscule creatures can thus maintain ecological equilibrium, generate secure food, and meet the requirements of a sustainable agricultural policy (Rawat *et al*., 2020).

1.2. Aims of the Study

To study screening, optimization, production and characterization of organic acids

produced by bacteria isolated from Ghulkin glacier.

1.3. Objectives of the Study

1. Screening of Organic Acids producing psychrophilic bacteria.
2. Optimization of fermentation conditions for maximum production of organic acids by the selected bacterial isolates.
3. Production and Purification of Organic acids by Lyophilization method.
4. Characterization of Organic acids produced by selected bacterial isolates.

2. Literature Review

2.1. Extremophiles

Extremophiles are microorganisms that inhabit or thrive in physically or geochemically harsh environments that are damaging to the most life forms on Earth. The word "extremophiles" is derived from the Latin word "extremus," meaning "extreme," and the Greek word "philia," which denotes "love." In contrast, organisms that inhabit more suitable habitats can be classified as neutrophiles or mesophiles. In the 1980s and 1990s, biologists discovered that microbes have a remarkable ability to adapt to extreme settings, including those that are extremely hot, acidic, or cold, which would normally be unsuitable for most species. Hydrothermal vents are widely considered by scientists to be the main environments supporting life beneath the ocean's surface (Gupta *et al*., 2014).

The word "extremophile" was 1st introduced in 1974 by MacElroy (MacElroy, 1974). These organisms were subsequently classified into three groups: archaea, bacteria, and eukarya, as described by Rothschild and Mancinelli in 2001(Rothschild & Mancinelli, 2001). However, three decades ago, extremophiles were relatively unknown and only a few researchers worldwide were studying them. They have become the most valuable organisms for researchers investigating enzymology and assisting in numerous sectors (Van Den Burg, 2003). Over the past two or three decades, significant progress has been made in these studies. This progress was evident when the first conference on extremophiles was held in Portugal in 1996, and a journal called "extremophiles" was established in 1997. In 2002, the "International Society of Extremophiles" was established to facilitate the exchange of knowledge and expertise in the rapidly growing subject of extremophiles.

The efficacy of extremophiles in numerous industries and diverse applications stems from their array of distinctive characteristics. There is a significant motivation to gather research on the likelihood of biotechnological applications and the metabolites of extremophiles, which are microorganisms that thrive in extreme environments. The creation of these metabolites and other products is closely linked to the participation of enzymes and proteins in the pathways they utilize for manufacture. It can be deduced that the enzymes of these creatures are likewise adapted to function effectively in harsh

environments, based on the theory that these organisms can survive in such conditions. This theory is substantiated by the data collected and acquired through the examination of enzyme and microbe characteristics in recent years. Organisms exhibit resistance to several environmental stressors such as high temperatures, chemically denaturing detergents, agents that disrupt hydrogen bonding (chaotropic agents), extreme pH levels, and organic solvents (R. Gaur *et al*., 2010). Referred to as "extremozymes," these enzymes provide remarkable catalytic capabilities for industries operating in harsh settings, when enzymes from typical microbes have lost their inherent abilities and structures (Hough & Danson, 1999).

Extremophiles can be classified into various categories based on the extreme environments they are capable of inhabiting or tolerating. Acidophiles, for example, thrive in highly acidic environments with a pH range of 1 to 5. Alkaliphiles, on the other hand, prefer alkaline environments with a pH above 9. Halophiles are organisms that can survive in highly salty environments with high salt concentrations. Thermophiles thrive in temperatures ranging from 60°C to 80°C, while hyperthermophiles can withstand temperatures above 80°C. Psychrophiles, on the other hand, have an optimal temperature range below 15°C and can even tolerate temperatures as low as 20°C. There are also other types of organisms that may thrive in extreme conditions, such as barophilic (able to withstand high pressure), oligotrophic (able to survive in nutrient-poor environments), endoliths (able to live inside rocks), and xerophilic (able to tolerate dry conditions). Some organisms are even capable of surviving in several extreme conditions, and they are referred to as polyextremophiles. Based on the preceding discussion, we will now proceed with the examination of psychrophiles.

2.2. Psychrophiles

Psychrophiles are organisms that thrive at cold temperatures. Schmidt-Nielsen (Schmidt-Nielsen, 1902) first identified these microorganisms as being capable of not only surviving at 0℃, but also reproducing. In contrast, Horowitz-Wlassowa and Grinberg (R. Y. Morita, 1975) referred to actual psychrophiles as psychrobes, and used the term psychrophiles for organisms that can grow at 0℃ as well as higher temperatures. In 1957, a dictionary called "Dictionary for Microbiology" was created

after gathering enough data about these microorganisms. It classified psychrophiles as microorganisms that had an optimal development temperature of 15℃ (Lasztity, 2009). Psychrophiles are microorganisms that have an optimal growth temperature at or below 0°C, 15°C, and 20°C, respectively. On the other hand, psychrotolerant microorganisms are able to tolerate temperatures of up to or above 25°C (Helmke & Weyland, 2004). The sustainability of metabolic activities and normal growth rate in such environments is challenging due to the significant amount of adaptation required to withstand extreme temperatures and survive. This presents an intriguing opportunity for researchers to study and understand the genetic behavior, acclimation processes, and adaptive abilities of organisms in these environments (Feller & Gerday, 2003; Junge *et al*., 2004; Zakhia *et al*., 2008).

When observing the Earth's surface, it is evident that 14% is comprised of polar regions, while the remaining 71% is covered by oceans. Within these oceans, 90% of the water has a temperature below 5°C. Interestingly, the oceans are able to maintain a consistent temperature of 4-5°C, regardless of differences in latitude. This unique characteristic is the reason why the majority of organisms that thrive in such environments are either psychotropic or psychrophilic in nature (Yayanos, 1986). Psychrotrophs are capable of thriving in the same environment as psychrophiles, but they are able to maintain a higher cell count. This is due to their ability to withstand the fluctuating temperatures on ice surfaces, whether they are in the northern or southern polar regions, which can reach temperatures as high as 28°C. Psychrophiles are unable to survive in high or fluctuating temperatures. If the idea that life evolved from mesophiles or thermophiles is correct, it is also possible that psychrophiles evolved from psychrotrophs (R. Morita & Moyer, 2000). The study indicates that residents of cold settings ensure that these surroundings have an adequate number of vital nutrients and an optimal nutrient regeneration process (Deming, 2002). Furthermore, it has been discovered that certain microorganisms have successfully survived for millions of years under the permafrost by cryobiosis (Vorobyova *et al*., 1997). The arctic sea ice serves as a habitat for microorganisms, where they are able to survive at extremely cold temperatures of -20°C. This demonstrates the remarkable adaptability of microorganisms to exist in an environment that combines freezing temperatures with a liquid surface (Junge *et al*., 2004). Although the development of these microorganisms at a temperature of -12°C

has not been documented (Breezee *et al*., 2004), they have been shown to live in their natural environment at -30°C and there are predictions that their metabolism can function at -40°C as well (Price & Sowers, 2004).

2.3. Physiological adaptations of psychrophiles

The rapid proliferation of psychrophiles is linked to their ability to adapt to low temperature environments. However, this adaptation is particularly effective in environments that provide an ample supply of energy sources for the cells. Interestingly, some obligate psychrophiles outperform psychrotrophs in this regard, indicating their superior ability to mineralize in cold conditions (Harder & Veldkamp, 1971). Several of these adaptations are discussed below.

2.3.1. Antifreeze Proteins

The creation of antifreeze proteins is a highly effective adaptation that psychrophiles have developed. These proteins allow them to thrive in cold conditions and enhance their survival. These proteins are non-disruptive to the solution's melting property, but they facilitate microorganisms in adjusting their temperature and thriving in a colligative manner (Davies *et al*., 2002). Thermal hysteresis refers to the process in which psychrophiles modify the shape of ice by binding proteins to it (H. J. Kim *et al*., 2017). Additionally, antifreeze proteins prevent the formation and growth of ice crystals, a phenomenon known as ice recrystallization inhibition (Kawahara, 2013). The proteins were initially identified in arctic fish and subsequently found to be present in plants, other fish species, diatoms, and microorganisms as well. The study conducted by Hashim, Bharudin *et al*. in 2013 found that G. Antarctica is the first microorganism known to secrete these proteins when cultivated in a frigid climate (Bharudin *et al*., 2018; Hashim *et al*., 2013). The genome research of yeast revealed the presence of nine distinct genes responsible for encoding the GaAFP proteins, which contribute to the formation of ice crystals with varying forms (Turchetti *et al*., 2011). Each of the genomes in these fungi exhibited a low TH activity in a temperature range of 0.05-0.08°C and much greater values for the IRI activity (Firdaus-Raih *et al*., 2018). The category of antifreeze proteins (AFPs) has significant structural diversity; however, they share a common function: preventing cellular frosting or freezing. The technique of horizontal gene transfer is believed to play a role in the evolution of this gene in

animals that encounter unusual settings (Davies, 2014). According to reports, they undergo folding into β-helices, however this folding occurs in three distinct manners (Hashim *et al*., 2014).

2.3.2. Membrane fluidity

Under any circumstances of stress, the cell consistently acts as the primary barrier for the flow of nutrients, signaling, and energy transduction between the environment and the cell, or vice versa (Siliakus *et al*., 2017). At low freezing temperatures, the cell membrane undergoes a transformation into a rigid state, resulting in the inactivation of many proteins involved in the transfer of carrier and transport proteins within the membrane (Los & Murata, 2004). Delta-9 and delta-12 are examples of fatty acid desaturases that are suited to cold temperatures. These enzymes are reported to be increased in activity at approximately -12℃. If both the first and second double bond are present in a cell, they are believed to be accountable for introducing these double bonds into the fatty acid structure (Bharudin *et al*., 2018). The fatty acid profile indicates that the majority of the fatty acids are in the form of unsaturated fatty acids, with many double bonds, except for oleic acid, which consists of chains with a single double bond. Remarkably, the quantity of these polyunsaturated fatty acids is augmented by around 1-2% under freezing temperatures, such as -12℃, which enhances the fluidity of the cell membrane (Bharudin *et al*., 2018; Firdaus-Raih *et al*., 2018). Similar behavior has been observed in other bacteria, such as *Shewanella* sp. (GA-22) (Gentile *et al*., 2003), and archaea, specifically *Methanococcoides burtonii* (Nichols *et al*., 2004).

2.3.3. Stress-induced Proteins

Environmental stressors can induce high levels of stress, such as significant temperature decreases, reduced food availability, radiation exposure, excessive UV radiation, or elevated osmotic pressure (De Maayer *et al*., 2014). In order to survive in stressful environments, both internally and externally, psychrophiles employ various proteins to protect themselves. These proteins include heat shock proteins (HSPs), cold shock proteins (CSPs), cold active enzymes, and molecular chaperones. The role of these proteins is to counteract the effects of stress by restoring the natural structure of abnormal or denatured proteins. Several studies have investigated the presence and

function of these proteins in psychrophiles (Baeza *et al*., 2021; Feller, 2013; Keto-Timonen *et al*., 2016; Santiago *et al*., 2016; Turchetti *et al*., 2020). G. antarctica exhibits the presence of stress proteins, specifically chaperones, heat shock proteins, and peptidyl-prolyl isomerases (PPIase). Upon comparing the genomes of psychrophilic yeasts with non-psychrophilic yeast, it is evident that the former lacks any genome sequence encoding stress-combating proteins (SCPs). There are four genes responsible for encoding CSPs, PPIase, and six HSPs. It has been observed that these genes are exclusively found in the psychrophilic P. destructants. Therefore, it can be demonstrated that these proteins have been modified by the psychrophile in order to ensure their survival in such conditions (Firdaus-Raih *et al*., 2018). The microorganisms are examined for a total of eighty-nine potential molecular chaperones, including TRiC chaperone, HSPs, HSP70, HSP40, HSP20, HSP90, CSP, AAA proteins, CS-domain proteins, tetra tricopeptide repeat domain proteins, and ubiquitins (Nur Athirah *et al*., 2015). Furthermore, a noteworthy discovery has been made in the study, revealing that G. Antarctica synthesizes a distinctive protein known as the expansion protein. This protein is the first of its type and plays a crucial role in facilitating the softening and loosening of the cell wall during cell growth. The expansion of the cell wall is a result of the expansion occurring in the non-covalent linkages of the glucan's matrix and the microfibrils of the cellulose (Nor *et al*., 2020).

2.4. Psychrophiles of Pakistan

Glaciers provide ideal conditions for psychrophiles, as the temperature ranges within the glaciers consistently remain at optimal levels with minimal volatility that does not pose a threat to their survival. The Tibetan plateau, located in the Hindukush-Karakoram-Himalayas Mountain ranges, is often referred to as the third pole due to its vast coverage of approximately 104,850km. This area includes 49,873km in China, as well as around 40,000km in both Pakistan and India. The plateau is renowned for having the highest number of glaciers and the most concentrated amount of ice and snow on them (D'Amico *et al*., 2002). These ranges are rarely examined for the presence of microbial communities. (Rafiq *et al*., 2017) have successfully identified many bacterial strains obtained from the Siachen glaciers in Pakistan. The identified genera were *Pseudomonas*, *Alcaligenes*, *Jonthenobacterium*, *Rhodococcus*, *Carnobacterium*, *Arthrobacter*, *Bacillus*, *Lysinibacillus*, *Staphylococcus*, and

Planomicrobium. (Rafiq *et al*., 2019) have identified and separated many members of the microbe groups *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The researchers Shen, (Shen *et al*., 2012) examined the Rongbuk glacier and identified four primary categories of bacteria: *actinobacteria*, *firmicutes*, *alpha-proteobacteria*, and *gamma-proteobacteria*. (Shivaji *et al*., 2011) have identified multiple bacterial phyla from the Pindari glacier in the Himalayas by the analysis of 16S rRNA sequencing gene libraries. The paucity and limited abundance of bacterial isolates in such environments can be attributed to the excessive disposal of non-biodegradable garbage (Rafiq *et al*., 2017). Deep below the depths of the glaciers, the troops have deposited a far larger quantity of discarded ammo. Approximately 40% of the total garbage polluting the Shyok River, which is connected to the Indus River at Skardu, consists of plastics, cadmium, cobalt, and chromium. The Indus River serves as a common supply for both drinking water and irrigation (Kemkar, 2006; Rafiq *et al*., 2017).

2.5. Phosphorus (P)

Phosphorus is a vital macronutrient that is essential for crucial metabolic processes in plants, including cell division, energy production, synthesis of large molecules, maintaining the integrity of membranes, transmitting signals, and carrying out photosynthesis. In addition, it contributes to the process of respiration in plants (M. S. Khan *et al*., 2010) and the fixation of nitrogen (N) in leguminous crops (Qu *et al*., 2019). The P content in top soil typically ranges from 50 to 3000 mg kg−1. However, only 0.1% of the total P is accessible for plant absorption. This limited availability is attributed to the precipitation of phosphorus with soil cations, its immobilization, adsorption, and conversion into organic forms. As a result, there has been a gradual rise in the need for and utilization of phosphatic fertilizers (PF) to provide a consistent provision of P in plants. Nevertheless, a significant part of PF undergoes precipitation in the soil. These substances consist of many toxic elements (Azzi *et al*., 2017), which leads to the accumulation of these elements in the soil. This accumulation has negative consequences on soil fertility, as well as the health of animals and consumers. Additionally, it contributes to eutrophication and an increase in carbon emissions (Huang *et al*., 2017). Given the current situation, there is a pressing need for an environmentally benign method that may accomplish the same objective as synthetic fertilizers, without the drawbacks. PSMs are bioinoculants that have the potential to

replace agrochemicals. They use various methods to convert inorganic phosphorus into a soluble organic form, thereby reducing the need for phosphate fertilizer in agricultural areas. This has been demonstrated in the research conducted by Hussain (Hussain *et al*., 2019). The researchers noted that the combination of PSMs (Pseudomonas, *Mycobacterium*, *Bacillus*, *Pantoea*, *Rhizobia*, and *Burkholderia*) and phosphate fertilizer had a positive effect on wheat grain production, increasing it by 22%. Additionally, phosphorus uptake was enhanced by 26%. This enhancement was accomplished by decreasing the fertilizer application by 30%, specifically reducing it from 120 to 90 kg of P2O5. Furthermore, these biofertilizers are environmentally benign and devoid of any toxicity. PSMs release various OAs such as gluconic acids, citric acid, oxalic acid, succinic acids and malic acids. They also produce enzymes like phosphatases and phytases, as well as ion chelators like siderophores. These substances effectively enhance the accessibility of phosphorus for plants (Tomer *et al*., 2016). PSMs also possess the ability to enhance plant development. They promote the holistic expansion and maturation of plants in both challenging and favorable conditions. Multiple studies have documented that PSMs possess the ability to dissolve potassium, improve nitrogen fixation, and generate plant growth regulators such as auxins, cytokinins, and gibberellins. Additionally, they safeguard plants from pathogens by secreting antibiotics and antifungals, making them highly effective biocontrol agents. Furthermore, they can also assist in the process of bioremediation for soil that is contaminated with heavy metals. Several PGPR produce the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which mitigates the adverse effects of ethylene stress on plants under stressful conditions. These minuscule organisms can thereby maintain ecological equilibrium, generate safe food, and meet the requirements of a sustainable agricultural policy (Rawat *et al*., 2020).

2.6. Soil Phosphorus Pool

P is existing in soil in both organic and inorganic forms due to different soil processes. More than 20% of the total P in the soil is made up of organic phosphorus. This organic phosphorus comes from biological metabolic processes such as the assimilation of orthophosphate and the leftovers of microbes, plants, or animals. These can be classified into three categories based on type of P bond: (a) phosphate esters (such as sugar phosphates, phospholipids, nucleic acids, and inositol phosphates); (b)

phosphonatases (involving C–P bonds); and (c) phosphoric acid anhydrides (including ADP and ATP) (Huang *et al*., 2017). The soil contains inorganic phosphorus in the form of phosphates that are attached to minerals such as calcium phosphate, aluminum phosphate, and iron phosphate. These minerals serve as the primary supply of P for young plants (Boitt *et al*., 2018).

The rapid progress of technology in agricultural operations, along with the increasing population pressure, has led to sudden alterations in the P dynamics within soil. Approximately 67% of the global agricultural area exhibits phosphorus deficiency, as reported by (Dhillon *et al*., 2017). The use of agrochemicals in soil poses a significant risk, whereas the crops' P recovery efficiency is about 10-20% (Rose *et al*., 2010). Typically, the highest concentration of phosphorus (ranging from 0.1 to 5mg P l−1) is found near the top layer of soil as a result of fertilizer application. In contrast, subsoils have a low concentration of dissolved phosphorus, measuring less than 0.1 mg P l−1 (Bol *et al*., 2016). The soil's strong ability to retain nutrients and the process of rendering the applied phosphorus inactive through immobilization, precipitation, and complexation lead to a limited availability of phosphorus for plants. Aluminum (Al) and iron (Fe) phosphates dominate as the primary types of P found in acidic soil, while calcium phosphates are prevalent in alkaline soil (R. Kumar & Shastri, 2017). The determination of soil pH plays a crucial role in understanding the process of P immobilization in soil (J. Zhu *et al*., 2018). Additionally, labile organic carbon, which serves as an energy source for microorganisms, is a significant factor that enhances the mobilization of P in paddy soil through microbial iron (III) reduction (I. Khan *et al*., 2019). In order to ensure the healthy growth and productivity of crops, it is crucial to maintain an optimal concentration of P in the soil. Due to the present condition of phosphorus levels in the soil, it is necessary to find different approach to make P available. The use of chemicals is inadequate in meeting the phosphorus requirements of crops and also has detrimental effects on soil quality and microbiome features.

2.7. Phosphate-Solubilizing Microorganisms (PSMs)

Soil harbors a rich and varied microbiome. PSMs are organisms capable of transforming insoluble P into a soluble form that plants can use. The abundance of these microorganisms in soil allows for the analysis of their ability to solubilize phosphorus

using both qualitative and quantitative approaches (S. Mehta & Nautiyal, 2001). PSMs have a wide range of diversity. Bacteria from the genera *Pseudomonas*, *Enterobacter*, *Bacillus*, *Serratia*, *Pantoea*, *Rhizobium*, *Arthrobacter*, *Burkholderia*, and *Rahnella aquatilis HX2*, as well as the fungus *Penicillium brevicompactum*, *Aspergillus niger*, *Acremonium*, *Hymenella*, and *Neosartorya*, have been identified as potent producers of PSMs (Biswas *et al*., 2018; Buch *et al*., 2008; Ichriani *et al*., 2018; Liu *et al*., 2019; Perea Rojas *et al*., 2019; Sulbarán *et al*., 2009; Teng *et al*., 2019; Whitelaw, 1999; L. Zhang *et al*., 2019). Soil microbes play a crucial role in soil through their metabolic activities and are an important component of integrated nutrient management. They enhance the plant's ability to acquire nutrients from the soil. These microorganisms act as powerful biofertilizers that enhance agricultural productivity while also addressing ecological considerations (Rajwar *et al*., 2018) PSMs exhibit a wide range of functions. Additionally, they are recognized for their ability to enhance plant development by producing chemicals that promote growth. Several investigations have been carried out to examine the effect of PSMs on promoting plant development, both in settings of stress and normalcy.

PSM release growth-promoting hormones such as auxins, cytokinins, and gibberellins. These substances trigger various processes such as cell division, cell differentiation, shoot growth, root development, flowering, germination, and xylem differentiation (Puri *et al*., 2020). A recently identified variant, *Bacillus tequilensis*, has been shown to generate plant growth regulators, including abscisic acid, auxin, and gibberellins (GA1, GA3, GA5, GA18, and GA19). Application of this strain to soybean plants has demonstrated enhancements in shoot biomass, improved leaf structure, and increased levels of photosynthetic pigments, particularly under heat stress conditions (Kang *et al*., 2019) noted a decrease in stress abscisic acid concentration and an elevation in rhizosphere jasmonic acid and salicylic acid levels. Several newly discovered microbes, as mentioned earlier, possess the capability to solubilize phosphorus across a broad spectrum and produce phytohormones. Bacterial hormone IAA inhibits the growth of fungal infections by interacting with the host defense system (Linu *et al*., 2019). PSM are recognized for their capacity to produce the ACC deaminase enzyme, aiding in reducing ethylene levels in plants exposed to stressful environments. This decrease in ethylene levels retards overall plant growth (Gamalero & Glick, 2015).

PGPM control plant diseases caused by pathogens through various mechanisms, including competitive root colonization, production of lytic enzymes and allelochemicals, and neutralization of virulence factors. Furthermore, they induce systemic resistance against plant pathogens (Compant *et al*., 2005; Paul & Sinha, 2017). Several taxa, such as *Pseudomonas*, *Serratia*, *Bacillus*, and *Streptomyces*, release antifungal compounds like viscosinamide, peptaibols, daucans, gliovirin, terpenoids, polyketides, pyrrolnitrin, and phenazines, mitigating the harmful effects of pathogenic organisms in the host plant (Myo *et al*., 2019). Certain microbial strains possess the ability to eliminate harmful substances from the environment, including xenobiotics, pesticides, heavy metals, herbicides, and organic solvents, all of which pose risks to soil, plants, humans, and animals (El-Nahrawy *et al*., 2019). In combination of PSMs *P. mallei* and *P. cepaceae* along with nano-phosphorus (0.1gL−1) enhanced the growth, yield, photosynthetic efficiency, chlorophyll content, and antioxidant enzyme activity of *Phaseolus vulgaris* in calcareous soil. Notably, specific antioxidant enzymes like catalase, glutathione reductase, proline dehydrogenase, glutathione-S-transferase, superoxide dismutase, and ascorbate peroxidase exhibited increased activity, as reported by Rady *et al*. (2020). Boroumand *et al*. (2020) observed improved development of land cress plants with the addition of PSB P. stutzeri and *Mesorhizobium* sp., along with nano-silica at concentrations of 0.05 and 0.07 ppm. This enhancement was accompanied by increased nitrogen and phosphorus levels in the soil. The recently discovered *Serratia plymuthic* BMA1 strain dissolved 450 mg L−1 of P and increased the dry weight of Vicia faba L. plants by 76% compared to non-inoculated plants, according to Borgi *et al*. (2020). Phosphorus concentration in the roots and shoots of treated plants was three times higher than in control plants. A group of *phosphobacteria* (*Klebsiella* sp. RC3, *Stenotrophomonas* sp. RC5, *Klebsiella* sp. RCJ4, *Serratia* sp. RCJ6, and *Enterobacter* sp. RJAL6), combined with P fertilization, raised the P concentration in the shoot of Lolium perenne by 29.8% compared to the non-inoculated control group in P-deficient soil (Barra *et al*., 2019).

2.7.1 Phosphorus Solubilization Mechanism by PSMs

The behavior of P in soil is influenced by three main processes: dissolution and precipitation, sorption and desorption, and the alteration between organic and inorganic forms of phosphorus (Thomas Sims & Pierzynski, 2005). The primary methods by

which PSMs disperse P in soil are mineralization, solubilization, and immobilization. These processes are controlled by the presence of accessible inorganic minerals in the soil. PSMs employ many key mechanisms to dissolve phosphates, including (a) releasing OAs, protons, and siderophores through exudation; (b) secreting extracellular enzymes; and (c) breaking down the substrate through mineralization.

2.7.1.1. Organic Phosphate Solubilization (PS)

The phosphorus content in soil is composed of organic phosphates, which account for 20-30% of the total. Enzymes expedite the mineralization process, resulting in the decomposition of organic phosphates (R. Kumar & Shastri, 2017).

2.7.1.2. Non-Specific Acid Phosphatases (NSAPs)

NSAPs, or phosphomonoesterases, can be classified into two types: acid and alkaline phosphatases. These enzymes are released by PSMs (Nannipieri *et al*., 2011). Enzymes are classified according to their optimal pH levels. Acid phosphatases are more abundant in acidic soil, while alkaline phosphatases are more prevalent in alkaline to neutral soil (Eivazi & Tabatabai, 1977; Renella *et al*., 2006). Phosphatases catalyze the dephosphorylation of phosphoesters or phosphoanhydride linkages in organic molecules. Research has shown that microorganisms in soil secrete both acid and alkaline phosphatases, with a stronger preference for organic phosphatases (Sharma *et al*., 2013a; Tarafdar *et al*., 2001). Moreover, alkaline phosphatases facilitate the breakdown of approximately 90% of the overall organic phosphorus present in soil, so rendering P accessible to plants (Jarosch *et al*., 2015). The study conducted by (P. Singh & Banik, 2019) observed that the application of pure alkaline phosphatases derived from *B. licheniformis* MTCC 2312 to soil resulted in a significant increase in the P content of the root and stem of Zea mays L. Specifically, the P content in the root and stem of Zea mays L. increased by 2.35-fold and 1.76-fold, respectively. The simultaneous introduction of the PSF *Talaromyces helices L7B* and the AMF into soil. *Rhizophagus irregularis* is the scientific name for a certain organism. The soil's alkaline phosphatase activity was significantly increased to 459.38 EU compared to the uninoculated soil's activity of 47.86 EU. Additionally, the soil's soluble phosphorus content was enhanced by 50% compared to the control group, as reported by (Della Mónica *et al*., 2020).

2.7.1.3. Phytases

Phytases facilitate the extraction of P from the phytate compound, which is a prevalent organic P found in soil. Phytate is the primary supplier of inositol and stored P in seeds and pollen (Sharma *et al*., 2013a). The capacity of plants to acquire P from phytate is highly restricted. Studies have demonstrated that microorganisms have a substantial impact on the process of mineralizing phytate. In an experiment, the Arabidopsis plant was genetically modified by introducing the phyA gene from *Aspergillus niger*. As a result, the changed plant exhibited enhanced phosphorus feeding and growth ((Richardson, 2001). The overview of phytase-producing bacteria into cereal crops led to increased P absorption without the need for fertilizer (Martínez *et al*., 2015). Fungi that are capable of solubilizing phosphate During a 48-hour solid-state fermentation, *Aspergillus niger* achieved the highest levels of phytase (133 IU) and phosphatase (170 IU) production, while also solubilizing P up to 835 ppm. Field trials demonstrated that the introduction of the fungal strain resulted in improved height of plant, leaf length, and fruit yield per plant in Lagenaria siceraria and Abelmoschus esculentus, as compared to plants that were not inoculated (Din *et al*., 2019). The high-performance strains *P. corrugata* SP77 and *Serratia liquefaciens* LR88 were examined to assess their ability to produce phytase. Both strains exhibited phytase activity levels of up to 23.02 and 24.84U/mL, respectively. Additionally, they demonstrated enhanced efficiency in solubilizing phosphorus, with levels reaching up to 714.96 and 306.74µg/mL, respectively (Ben Zineb *et al*., 2020).

2.7.2. Inorganic Phosphate Solubilization (PS)

Inorganic phosphates like Fe–P, Al–P, and Ca–P in the soil is solubilized in the following ways

2.7.2.1. Indirect Mechanism

The rhizospheric microorganisms acquire a significant quantity of P from the soil by an indirect process, where insoluble phosphorus are dissolve (Halvorson *et al*., 1990). Under stressful situations, the breakdown of microbial cells results in the release of P into the soil. This phosphorus is then absorbed by plants and other microorganisms in the soil (Butterly *et al*., 2009). An investigation revealed that subjecting soil to drying

followed by fumigation for different durations, specifically 2 and 14 days at a temperature of 40°C, led to a reduction in microbial biomass P by 61% and 70% respectively. The discovery suggested a connection between soil dehydration, the death of bacterial cells, and the amount of P in microbial biomass. This implies that microbes indirectly affect the availability of P in soil (S. U. Khan *et al*., 2019).

2.7.2.2. Organic Acids Productions

PSMs release organic acids such as citric acid, gluconic acid, oxalic acid, and tartaric acid. These acids dissolve inorganic phosphates through several mechanisms: (a) binding to cations that are attached to phosphates, (b) lowering the pH, (c) forming complexes with metal ions that are attached to phosphates, and (d) competing with phosphates for adsorption sites (Kishore *et al*., 2015). Organic acids are small molecules that bind to the cation attached to phosphorus by using their hydroxyl (OH) and carboxyl (COOH) groups. They also reduce the pH in the rhizosphere by exchanging gases (O2/CO2) and balancing protons and bicarbonate. This process helps to release the phosphorus that was previously bound (K. Y. Kim *et al*., 1997). Organic acid production by PSMs is primarily achieved through fermentation or direct oxidation. These metabolic pathways lead to acidification in the surrounding area of the microbes. Acidification occurs through the substitution of protons for cations such as Fe+3 and Al+3, which liberates phosphates from complexes. Alternatively, acidic anions can replace phosphate ($PO_4 2-$) through an exchange process (A. Goldstein, 1994). PSMs (Deubel *et al*., 2000) excrete significant amounts of 2-Ketogluconic acid and gluconic acid. Gluconic acid secretion by PSMs is facilitated by the enzyme glucose dehydrogenase, which is a Quino protein, in the direct glucose oxidation route. The glucose dehydrogenase enzyme is produced by the glucose dehydrogenase (gcd) gene and utilizes pyrroloquinoline quinone (PQQ) as a cofactor. PQQ is a chemical with redox activity that is expressed by the pqq operon. The pqq operon includes the core genes pqq A, B, C, D, E, and F, which are important for the activity of dehydrogenase and the solubilization of mineral phosphate in microorganisms (Wan *et al*., 2020). A prior investigation found that the pqqA gene disruption in gram-negative *Rahnella aquatilis* HX2 resulted in a substantial decrease in gluconic acid concentration in the culture media, reducing it from 9.68 to 0.65 g/l. The mutation of the pqqA gene led to a decrease in the concentration of soluble phosphorus (99.7mg/l) compared to the

phosphorus concentration (465.9mg/l) of the wild-type strain (Li *et al*. 2014). The ability of bacteria and fungi to solubilize inorganic phosphate has been found to be dependent on the presence of the pqq gene, as demonstrated by several research (Chen *et al*., 2016; Oteino *et al*., 2015; Suleman *et al*., 2018). The primary method by which PSMs dissolve phosphorus is by the secretion of dominating organic acids. The *Pseudomonas* sp. strain AZ15, which has the ability to solubilize phosphorus, produced various organic acids including oxalic acid, gluconic acid, acetic acid, lactic acid, and citric acid. Additionally, it was able to solubilize phosphorus up to a concentration of 109.4µg/ml. In addition, this particular strain also increased the many characteristics related to crop productivity, such as the accumulation of dry matter, the amount of grain produced, the number of nodules, and the weight of these nodules in chickpea (Zaheer *et al*., 2019). The data clearly showed that the phosphorus-solubilizing *Trichoderma* strains (AMS 34.39, AMS 31.15, and AMS 1.43) produced various organic acids, such as ascorbic acid, citric acid, malic acid, gluconic acid, and phytic acid, which were the main means of solubilizing phosphorus in soybean. Furthermore, these newly discovered variants enhanced plant growth by a range of 2.1 to 41.4% when compared to plants that were not treated, as reported by (Bononi *et al*., 2020).

2.8. How P-solubilizing bacteria promote plant growth?

Utilizing PS bacteria as inoculants enhances both phosphorus uptake by the plant and agricultural productivity. The strains belonging to the genera *Pseudomonas*, *Bacillus*, and *Rhizobium* have high efficacy in solubilizing phosphorus. In addition to supplying plants with soluble phosphorus, these organisms enhance plant growth and development by generating or altering the concentration of plant hormones, such as IAA (Ahmad *et al*., 2008; P. A. Wani *et al*., 2007a, 2007b). (ii) Nitrogen fixation that occurs either without a symbiotic relationship or with a symbiotic relationship (Zaidi, 1999; Zaidi & Khan, 2007) (iii) Inhibition of phytopathogenic bacteria such as *Fusarium* spp. and *Macrophomina* (M. Khan *et al*., 2002) The synthesis of siderophores (P. Wani *et al*., 2007), antibiotics, and cyanide (P. A. Wani *et al*., 2008) is seen. The organism possesses the capacity to produce a crucial enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which breaks down ACC (a direct precursor of the plant hormone ethylene) into NH3 and a-ketobutyrate. This enzymatic activity effectively mitigates the inhibitory impact of ethylene on plant

growth and also aids in the solubilization of metals or the mitigation of toxicity caused by specific heavy metals in soils under stress. The generation of siderophores by PS bacterial strains has been identified as a potential method to enhance plant growth in the presence of iron deficiency (Ahmad *et al*., 2008; P. A. Wani *et al*., 2007a).

Conversely, there is a hypothesis suggesting that some bacteria that solubilize phosphate can operate as helper bacteria for mycorrhizal fungi (Frey-Klett *et al*., 1997; Garbaye, 1994). Multiple studies have demonstrated that PSB engage in a symbiotic relationship with VAM by releasing phosphate ions into the soil. This interaction creates a synergistic effect, enabling more efficient utilization of P sources that are not easily soluble (Azcon-Aguilar *et al*., 1986; Piccini & Azcon, 1987; Valverde *et al*., 2007). The bacteria's ability to solubilize phosphate is expected to enhance the plant's uptake of nutrients through a mycorrhizae-mediated connection between the roots and the surrounding soil, facilitating the transport of nutrients from the soil to the plants (Gianinazzi & Schüepp, 1994). (Toro *et al*., 1997) demonstrated that PSB, which are linked with VAM, enhance the accumulation of minerals (N and P) in plant tissues. This was achieved by the use of radioactive 32P labeling. According to these authors, the rhizobacteria that were introduced might have released phosphate ions from rock phosphate (RP) that is not soluble, as well as other sources of phosphorus. These ions would subsequently be absorbed by the mycelium of the VAM fungus that is outside the plant. The effectiveness of the product is likely influenced significantly by the soil's phosphorus concentration, which is considered a crucial factor.

3. Materials and Methods

3.1. Strains Isolation

In the current study the strains used were already isolated from the sample collected from Ghulkin Glacier, Gilgit Baltistan. These strains were then preserved at -20℃ and were used for further studies. The whole research was conducted in the Applied Environmental and Geomicrobiology lab (AEG), Department of Microbiology, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad.

3.2. Culturing and Inoculum Preparation

All the available strains were refreshed on nutrient agar plates having composition 20g/L and number of media prepared were 25ml/plate incubated for 48hours. After completion of the incubation time a loop full of colonies picked from the nutrient plate, shifted to nutrient broth and then incubated for 48hours giving strain enough time to grow at a temperature of 15℃ and agitation speed of 140rpm.

3.3. Optimizing the Media and Growth Conditions for Phosphate Solubilization.

P solubilizing ability of microbial strains were tested in Pikovskaya liquid media. Pikovskaya (RI, 1948) broth containing the following components per liter: glucose 10g, TCP 5g, (NH4)2SO4 1g, KCl 1g, MgSO4.7H2O 0.1g, yeast extract 0.5g, $MgSO_4$.7H2O, MnSO4.7H2O 0.002g, FeSO4 0.002g and PH 7.0, agar 2g/l was used for qualitative analysis supplemented bromothymol blue 2mg/50ml as color indicator. The experiment was conducted using PVK media containing 0.5% tricalcium phosphate. Flasks were added with a 10% (v/v) suspension of inoculum and placed on a shaker at a temperature of 15ºC for a duration of 6 days.

3.4. Quantification of Soluble Phosphate

The cultures were collected at various stages of growth to measure the amount of soluble phosphorus released in the media. Following centrifugation at a speed of 10,000 revolutions per minute (rpm) for a duration of 15 minutes, 500µl of the liquid portion of the culture were extracted and combined with an equal volume of a solution containing 10% (weight/volume) trichloroacetic acid in a test tube. Next, a mixture containing 4 milliliters of color reagent consisting of 3 molar sulfuric acid (H_2SO_4), 2.5% weight/volume ammonium molybdate, 10% weight/volume ascorbic acid, and

distilled water in a ratio of 1:1:1:2 was added. The mixture was then incubated at room temperature for 15 minutes. The measurement of the absorbance of the developing blue was conducted at a wavelength of 820nm.

3.5. Soluble Phosphate Standard Curve

To make a standard curve for the soluble phosphate, 10ml of stock solution was prepared and then six dilutions of different concentrations were made. The quantification of soluble phosphorus was conducted using the molybdenum method developed by Bray and Kurtz in 1945, with KH2PO4 serving as the standard. The concentration of soluble P was quantified as the amount of milligrams of phosphorus released per liter of culture media. This measurement was used to determine the phosphate solubilizing efficiency (PSE) of the isolates in the culture broth. (Gothwal et al., 2006). After preparing dilutions these were studied for the absorption at a wavelength of 820nm and then standard curve chart was made in the Microsoft Excel sheet.

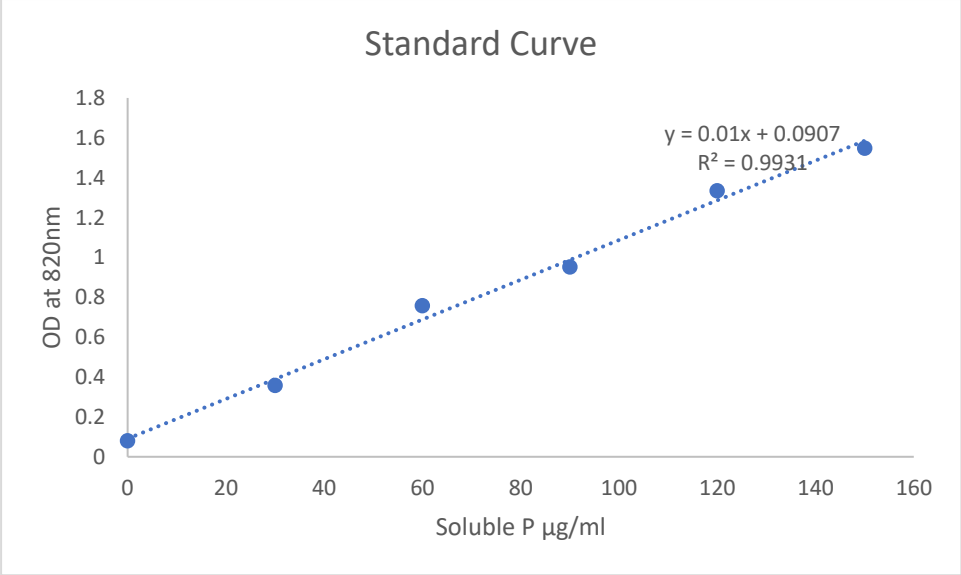


Figure 3.5.1. Standard curve of soluble Phosphate

3.6. Optimization of Phosphate Solubilization

In order to optimize the media conditions, all tests were conducted using tri-calcium phosphate (5.0 g/l) as the only source of phosphorus in the PVK media. Strain *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were

inoculated into NB broth at a temperature of 15ºC and a speed of 140 rpm. After 48 hours of incubation, the optical density (OD) of the culture broth was adjusted to 820 nm. This adjusted broth was then utilized for various optimization and analytical tests. Sampling occurred at 24-hour intervals to measure the PSE up to 96 hours. The most favorable outcomes of each trial were preserved and subsequently utilized in subsequent studies. Ultimately, the process of characterizing mineral PS was carried out under the most favorable conditions for growth.

3.6.1. Optimization of Incubation Time

Pikovskaya media was studied for soluble phosphate production for 0-120hours at 15℃ and agitation speed of 140rpm in shaking incubator. Samples at different times were taken from inoculation time, Day1, Day2, Day3, and Day4 to Day5 of incubation. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.2. Temperature Optimization

Pikovskaya media was studied for soluble phosphate production at different temperatures like 5℃, 15℃, 25℃ and 35℃ with agitation speed of 140rpm in shaking incubator. Samples at different optimum times were taken as on 48hours for *Pseudomonas frederiksbergensis* GA23 and 96hours incubation for *Acinetobacter johnsonii* GB30. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.3. Effect of pH

Soluble phosphate production at different pH levels ranging 3,4,5 to 9 at a temperature of 5℃ for *Pseudomonas frederiksbergensis* GA23 and 15℃ for *Acinetobacter johnsonii* GB30 with agitation speed of 140rpm in shaking incubator was studied in Pikovskaya media. Samples at 48hours for *Pseudomonas frederiksbergensis* GA23 and 96hours for *Acinetobacter johnsonii* GB30. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.4. Optimization of Carbon Source

Strain *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were grown in different carbon sources (0.5g/50ml) to observe PSE in PVK medium. The carbon sources used were glucose, xylose, glycerol, mannitol, sucrose, maltose, dextrose, galactose, lactose and fructose. In this experiment fructose in *Pseudomonas frederiksbergensis* GA23 and xylose in *Acinetobacter johnsonii* GB30 was found to show maximum PSE therefore, its different concentrations (1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5% and 6%) were confirmed to find out optimal fructose and xylose concentration in PVK media. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.5. Nitrogen Source Effect

In order to investigate the impact of nitrogen source on PSE, *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were inoculated into various media containing different nitrogen sources, namely ammonium oxalate, ammonium nitrate, ammonium chloride, calcium nitrate, ammonium tartrate, sodium nitrate, potassium nitrate, urea, and ammonium acetate. These nitrogen sources were substituted for ammonium sulfate in the PVK medium at a concentration of 50mg/50ml of equivalent nitrogen weight. The experiment aimed to determine the optimal concentration of ammonium sulfate in PVK media by testing several concentrations ranging from 10mg/50ml to 100mg/50ml. Ammonium sulfate exhibited the highest PSE in this experiment. The Molybdenum blue test method was subsequently utilized to ascertain the level of soluble phosphate generation.

3.6.6. Potassium Source Optimization

The impact of different potassium sources on the bacterial strain was assessed by cultivating it on various potassium compounds, including potassium sulfate, potassium nitrate, potassium nitrite, potassium iodide, and potassium gluconate. These compounds were used as substitutes for potassium chloride in the PVK medium, with a concentration of 50mg/50ml of potassium weight equivalent. In this experiment potassium nitrate for both strains was found to show maximum PSE. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.7. Effect of Phosphorus Source

To assess the strains' capacity to dissolve various P sources, diverse types of phosphorus sources such as hydroxyapatite, aluminum phosphate, ferric phosphate, calcium phosphate, and tri-calcium phosphate were used. The isolates were examined for their ability to solubilize in PVK broth supplemented with various concentrations (0.5%, 1%, 1.5%, and 2%) of different phosphorus sources. Molybdenum blue assay method was then performed to determine the soluble phosphate production.

3.6.8. Tests of Salt

NaCl tolerance was determined in PVK media and nutrient agar plate containing 0%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% 10%, 12.5%, 15%, 20% and 25% (w/v) total salts. Molybdenum blue assay method was then performed for broth to determine the soluble phosphate production.

3.6.9. Drought Tolerance

Drought tolerance was determined in PVK media and nutrient agar plate containing 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8%, (w/v) total polyethylene glycol (PEG-6000). Molybdenum blue assay method was then performed for broth to determine the soluble phosphate production.

3.7. Qualitative Measurement of Phosphate Solubilization

The bacterial isolates were tested for their ability to solubilize TCP on PVK plates using bromothymol blue as a greenish color indicator. Before agar plating, sterile cork borers were used to create wells with a diameter of 10 mm. 10 microliters from each bacterial strain, aged for 3 days, was added to each well. Subsequently, the plates were incubated at a temperature of 15 ± 2^{o}C for a duration of 2 days until a yellow zone becomes visible around the wells. The phosphate solubilizing efficiency was assessed using the formula proposed by Nguyen et al. in 1992. The presence of a yellow color surrounding a developing group of organisms indicated the process of PS and was quantified as the PS index (SI). The SI was determined by dividing the sum of the colony diameter and the yellow zone diameter by the colony diameter.

PSE = halo zone diameter/ colony diameter×100

SI = (colony diameter + halo zone diameter) / colony diameter.

Diameter of the colony the data was recorded three times for each observation. PSB can be easily identified by the presence of yellow color zones around their colonies.

3.8. Production and Extraction of Organic acids

To produce organic acids in higher amount all of the optimized fermentation conditions were applied and the same procedure was adapted, as adapted for screening and optimization process but the media concentration was kept much higher than that for the screening amounting 500ml for each strain in Erlenmeyer's flasks and were kept in the shaking incubator for the incubation at their respective optimized conditions with the agitation speed of 140rpm.After the completion of incubation, extraction was done in the following steps:

3.8.1. Centrifugation

After the completion if the incubation the soluble phosphate production PVK media was centrifuged at a speed of 10,000rpm for 15 minutes so that the cell debris and the high molecular weight substance in the media settle down to the supernatant at the top for extraction.

3.8.2. Separation of Bacterial Cell Debris

The process of centrifugation has enabled us to separate the pellet and supernatant of the media after passing the media through the centrifuge cycle. So, after centrifugation, supernatants were collected in separate labeled flask for each strain.

3.8.3. Extraction of the Organic acids Through Lyophilization Method

To extract the organic acids from supernatant transfer it to sterilized petri plate and freeze at -50^{o}C temperature. Then transfer the freeze supernatant to vacuum machine to vaporize the water from it, the lyophilization process was done at -50^{o}C temperature. After the lyophilization process the extract was preserved for further analyses at 20°C in labelled vial for extract of each strain. The extract was measured by measuring the vials before and after the transfer of the extract into it. The net weight was calculated for the weight of the extract.

3.9. Characterization of Phosphate Solubilizing Bacteria

For the confirmation of the production of organic acids by *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 the extract was characterized by the following processes.

3.9.1. Thin Layer Chromatography

Thin layer chromatography (TLC) is a purification method for crude proteins. It involves running the extract over a layer coated with silica gel or aluminum oxide, using a solvent. This allows for the distinct observation of bands created by different components in the extract. The identical principle was applied to segregate the constituents of the extract. The stationary phase utilized in the experiment was a commercially available silica gel covered on an aluminum plate, given by the university supplier. The sample was sliced to a size of 2µl from each extract, and a standard organic acid of the same size was also included. Certain organic acids are accessible in both solid and liquid forms. In order to match the dilution of our extract, the solid form should be transformed into a liquid condition by adding distilled water. To achieve this, a 1.56mg standard of organic acids was mixed with 1ml of distilled water. The same dilution was then applied to the extract samples, resulting in a concentration of 1.56mg/ml. The plate that was collected had a height of 12cm, with sample loading and solvent cutoff lines measuring 1.5cm each. The samples were loaded with a spacing of 1cm apiece. Additionally, two samples were included, and the width of the plate used was 5cm. The mobile phase utilized in this method consisted of benzene, methanol, and acetic acid in a ratio of 90:16:8. After completing all the necessary preparations, the solvent mixture for the mobile phase was transferred to a beaker. The plate was then positioned vertically and carefully to ensure that the mobile phase initiated a uniform movement, carrying the sample at a consistent speed from all locations. The beaker was covered with aluminum foil to prevent the solvent from evaporating. The chamber was left for approximately 50 minutes to allow the mobile phase to rise above the stationary phase until the cutoff line was reached. The cutoff line was then immersed in a solution of 0.4%(w/v) bromocresol green in ethanol to detect the presence of organic acid (Kraiker & Burch, 1973). The organic acids were observed as yellow dots against a greenish blue background. Subsequently, the plate was let to desiccate prior to visualization.

3.9.2. Fourier Transform Infra-Red Spectroscopy

The Fourier transform infra-red (FT-IR) analysis was conducted on the culture supernatants of PVK broth in order to determine the specific type of organic acids formed during P solubilization. The Pikovskaya culture broth was subjected to centrifugation at a speed of 10,000rpm for a duration of 15 min. The resulting clear liquid above the sediment was then frozen and dried using a process called lyophilization at a temperature of -50℃. This dried material was preserved for future use. FT-IR analysis for the samples processed through TLC was done with (Perkin Elmer Spectrum 64) in the general lab of Department of Microbiology, Quaid-I-Azam University Islamabad to confirm the presence of organic acids in the extract by the absorbance of the spectrum. The extract preserved in the distilled water were loaded and the analysis was done by adjusting the transmission mode of the instrument from 400-4000cm-1(Model-Shimadzu iraffinity-1, Japan).

3.9.3. Organic acid Production

High-performance liquid chromatography (HPLC) was used for the analyses of organic acids produced by particularly psychrophilic PSB in a liquid culture medium. After optimum incubation time, a psychrophilic PSB culture was grown in 50 milliliters of PVK broth that was supplemented with calcium phosphate, hydroxyapatite, and tricalcium phosphate. After that, the culture was centrifuged for 20 minutes at 10,000 rpm. The resultant culture supernatant was first run through nylon filters measuring 0.2 mm (Millipore, USA). Following this, 20 mL aliquots of every supernatant were analyzed using an HPLC system fitted with a C18 column (150 mm in length, 4 mm in diameter, and 120 Å pore size) and Turbochrom software (Perkin Elmer, USA). A solution with a pH of 2.4 that contained 1 millimolar H_2SO_4 and 8 millimolar Na_2SO_4 made up the mobile phase. A flow rate of 0.6 ml/min was selected. Sigma (USA) provided the organic acids that were utilized as standards. Using spectrometry set at 210 nm, the following acids were found in the supernatant: fumaric acid, gluconic acid, acetic acid, citric acid, succinic acid, formic acid, malic acid, and citric acid. Each organic acid's concentration was given in milligrams per milliliter, or mg/ml.

4. Results

4.1. Optimization of Fermentation Condition for Soluble Phosphate and Organic acids Production

The fermentation conditions optimized were incubation time, temperature, pH, carbon source, nitrogen source, potassium source, phosphate source, salts stress and drought stress. *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were cultured separately in Erlenmeyer flaks at each of these conditions and were incubated in the shacking incubators. Samples were collected from each of the flasks and were processed for further estimation. The maximum productions detected in each phase are discussed below.

4.1.1. Time Incubation Optimization

The incubation period is the most important factor for the estimation of maximum quantity of metabolites production. The maximum production of soluble phosphate and organic acids was detected at 48hrs of incubation for *Pseudomonas frederiksbergensis* GA23 with an amount of about 257.56µg/ml and showed the decrease of about 15.89µg/ml, 153.43µg/ml, 225.16µg/ml, 204.26µg/ml and 179.26µg/ml respectively in production at 0hrs, 24hrs, 72hrs, 96hrs and 120hrs. While for *Acinetobacter johnsonii* GB30 incubation time the maximum production with amount of about 371.26µg/ml of the soluble phosphate and organic acids was detected at 72hrs of incubation and the decrease of about 11.73µg/ml, 102.86µg/ml, 152.59µg/ml, 208.73µg/ml and 94.19µg/ml respectively at 0hrs, 24hrs, 48hrs, 72hrs and 120hrs was detected.

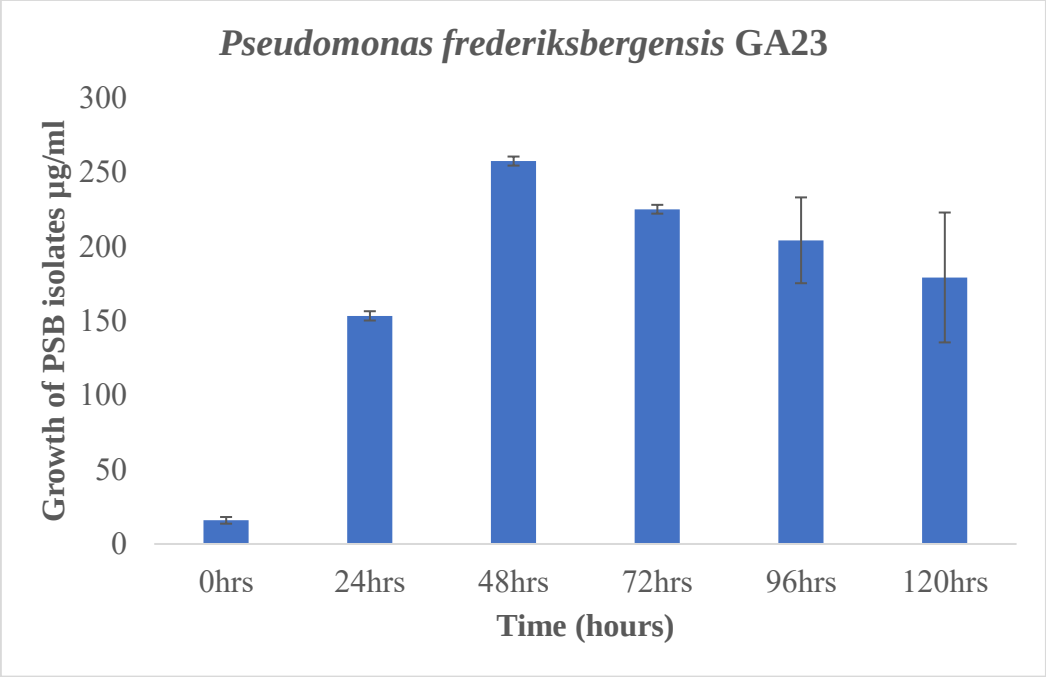


Figure 4.1.2. Optimization of Incubation Time for *Pseudomonas frederiksbergensis* GA23

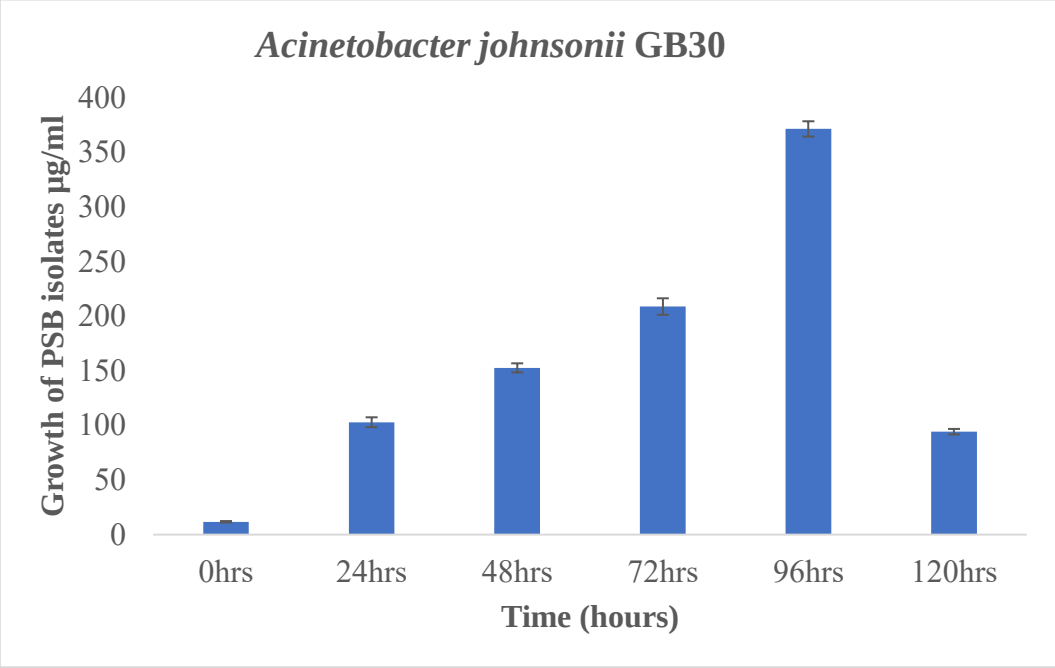


Figure 4.1.3. Optimization of Incubation Time for *Acinetobacter johnsonii* GB30

4.1.2. Effect of Temperature

Temperature can affect the production of plant hormone and that is why growth can also retard. For this purpose, the optimum temperature detected to be 5℃ for strain *Pseudomonas frederiksbergensis* GA23. The highest amount of 353.39µg/ml organic

acids production was estimated with a decrease of about 247.29µg/ml, 79.73µg/ml and 20.13µg/ml in production at 15℃, 25℃ and 35℃ respectively when incubated for 48hours. Similarly, for *Acinetobacter johnsonii* GB30 showed the highest amount of production at 15℃ with an amount of 362.09µg/ml having a decrease of about 145.83µg/ml, 257.03µg/ml and 199.09µg/ml at 5℃, 25℃ and 35℃ respectively.

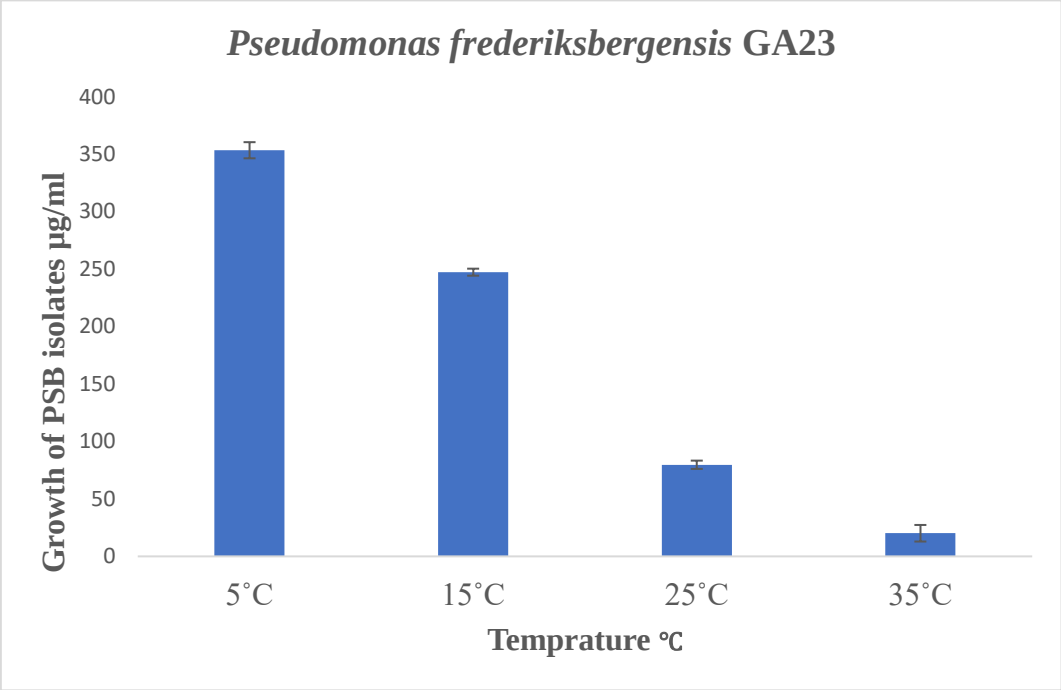


Figure 4.1.4. Effect of Temperature on *Pseudomonas frederiksbergensis* GA23

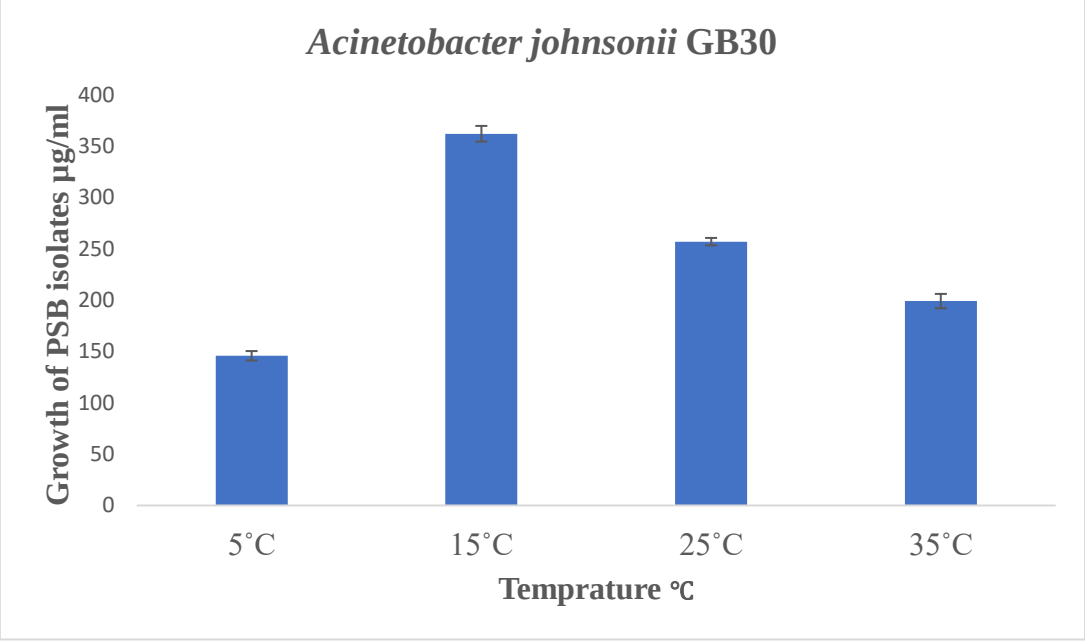


Figure 4.1.5. Effect of Temperature on *Acinetobacter johnsonii* GB30

4.1.3. Effect of pH

Both acidic and basic environments drastically affect the growth of plants by disturbing their metabolic pathways. That is why optimization was done to find out the best production pH of media for isolates. At optimum time and temperature *Pseudomonas frederiksbergensis* GA23 has shown the highest production with an amount of 299.86µg/ml at pH range of 3 with 93.73µg/ml and 76.26µg/ml decrease in production at pH 7 and 9, respectively see fig 6. While for *Acinetobacter johnsonii* GB30 the highest amount of production was 367.09µg/ml at 7 pH and the decrease of production at 5 and 9 pH was estimated to be 258.06µg/ml and 277.83µg/ml respectively see fig 6 and 7.

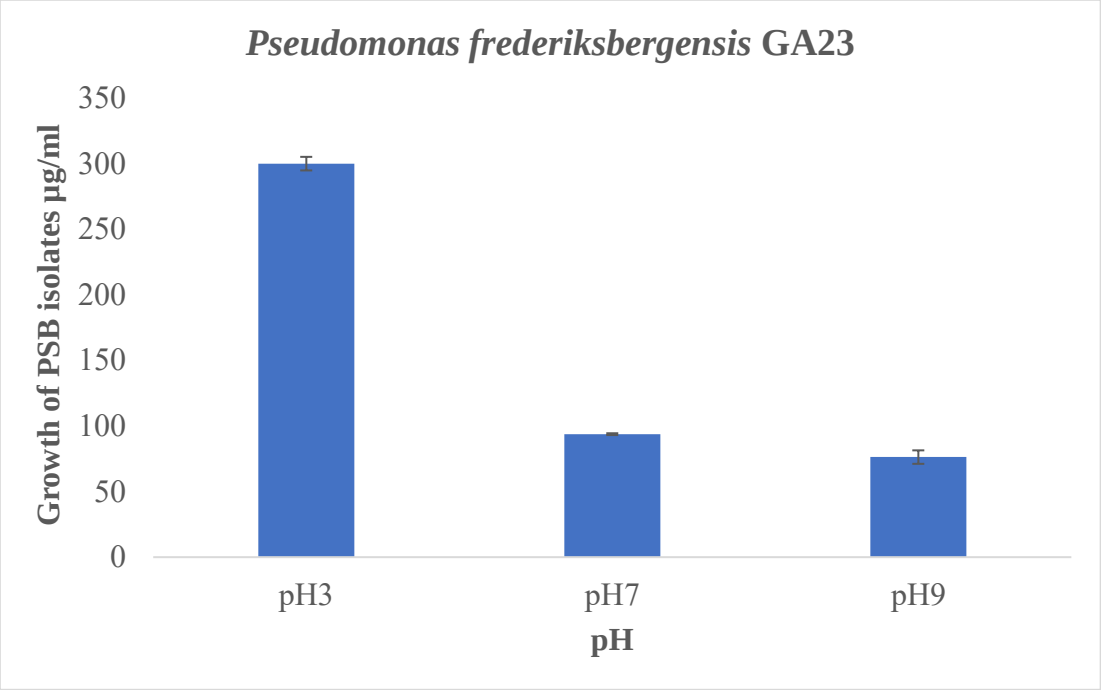


Figure 4.1.6. Optimization of pH for *Pseudomonas frederiksbergensis* GA23

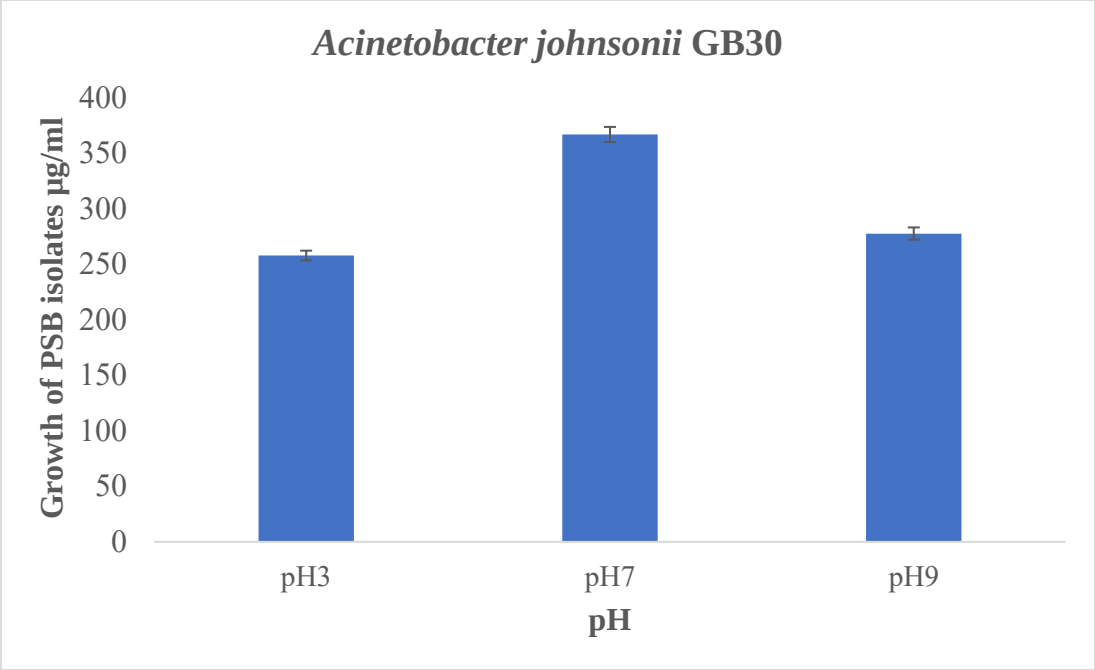


Figure 4.1.7. Optimization of pH *Acinetobacter johnsonii* GB30

4.1.4. Carbon Sources Optimization

The culture of each strain was cultivated in PVK media, utilizing optimized time, pH, and temperature, while employing various carbon sources. A concentration of 10g/l was added to investigate its impact on PSE, and the outcomes are depicted in Fig. 8 and 9.The maximum PSE in *Pseudomonas frederiksbergensis* GA23 (271.66µg/ml) was reported when fructose was used as a carbon source in the growth medium followed by glucose (266.53µg/ml), sucrose (265.16µg/ml), lactose (255.29µg/ml), maltose (241.19µg/ml), glycerol (237.06µg/ml), galactose (235.23µg/ml), dextrose (233.29µg/ml), xylose (230.49µg/ml), mannitol (224.69µg/ml) and blank (212.26µg/ml). The P solubilization for *Pseudomonas frederiksbergensis* GA23 was achieved higher with fructose than other carbon sources except mannitol.

While in *Acinetobacter johnsonii* GB30 xylose show maximum growth on PVK media (303.86µg/ml) followed by maltose (284.39µg/ml), dextrose (273.49µg/ml), glucose (272.39µg/ml), galactose (236.03µg/ml), glycerol (226.56µg/ml), sucrose (217.23µg/ml), blank (141.06µg/ml), lactose (56.89µg/ml), fructose (32.06µg/ml) and mannitol (18.33µg/ml). The P solubilization in *Acinetobacter johnsonii* GB30 was accomplished higher with xylose than other carbon sources except mannitol.

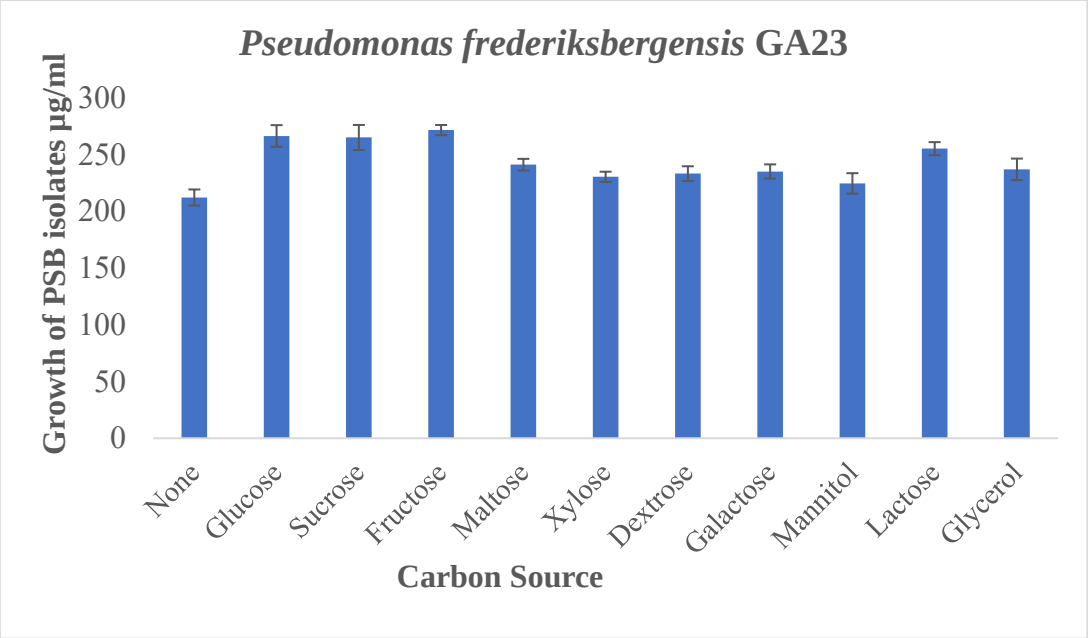


Figure 4.1.8. Optimization of Carbon Sources for *Pseudomonas frederiksbergensis* GA23

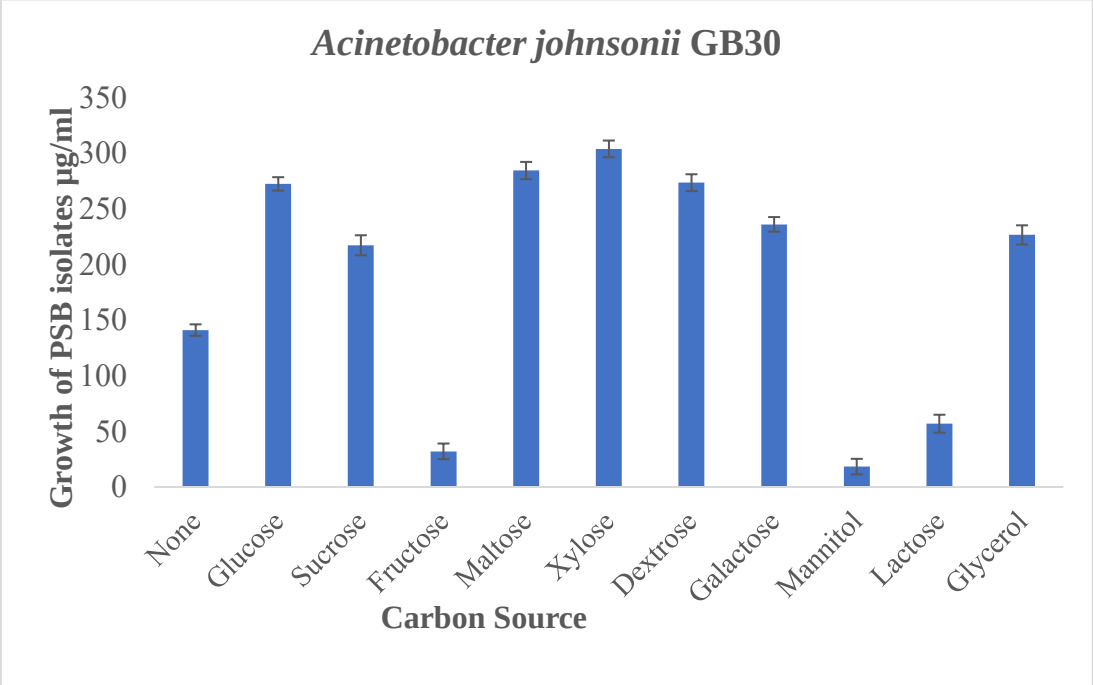


Figure 4.1.9. Optimization of Carbon Sources for *Acinetobacter johnsonii* GB30

In addition, the concentrations of fructose and xylose were manipulated to determine the most favorable concentration instead of glucose. This was done by incubating for 48 hours at a temperature of 5°C and a pH of 3 for *Pseudomonas frederiksbergensis* GA23 and for 72 hours at a temperature of 15°C and a pH of 7 for *Acinetobacter johnsonii* GB30. In the *Pseudomonas frederiksbergensis* GA23 case, PSE exhibited an

increase with an elevation in fructose concentration from 1% to 1.5% g/l, followed by a subsequent decrease at higher fructose concentrations up to 6%. The optimum concentration was observed at 1.5% g/l (344.09µg/ml), succeeded by values of (272.33µg/ml), (262.73µg/ml), (246.39µg/ml), (227.56µg/ml), (206.03µg/ml), (168.89µg/ml), (134.96µg/ml), (117.79µg/ml), and (100.79 µg/ml) at concentrations of 1%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, and 6%, respectively see fig 10.

Similarly, in the case of *Acinetobacter johnsonii* GB30, PSE increased with a 1% xylose concentration and then decreased at higher xylose concentrations up to 6%. The optimal concentration was found at 1% (311.19µg/ml), followed by values of (289.86µg/ml), (259.53µg/ml), (240.06µg/ml), (216.16µg/ml), (196.36µg/ml), (165.93µg/ml), (131.96µg/ml), (112.83µg/ml), and (98.56µg/ml) at concentrations of 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, and 6%, respectively see fig 11.

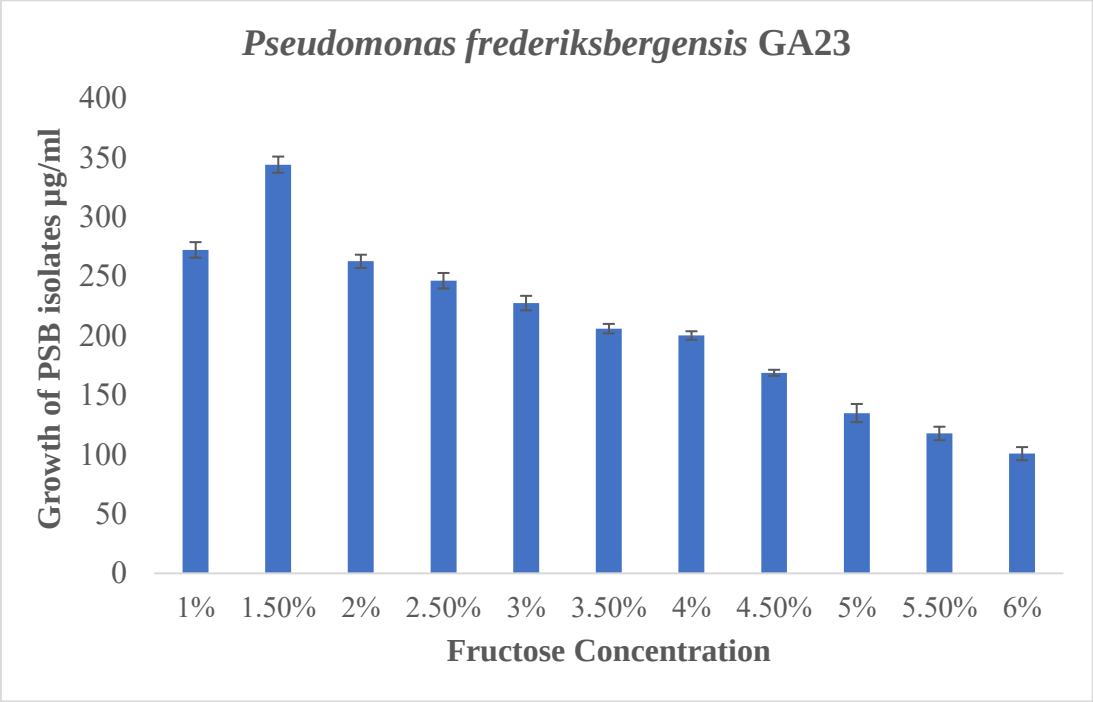


Figure 4.1.10. Optimization of Fructose Concentration for *Pseudomonas frederiksbergensis* GA23

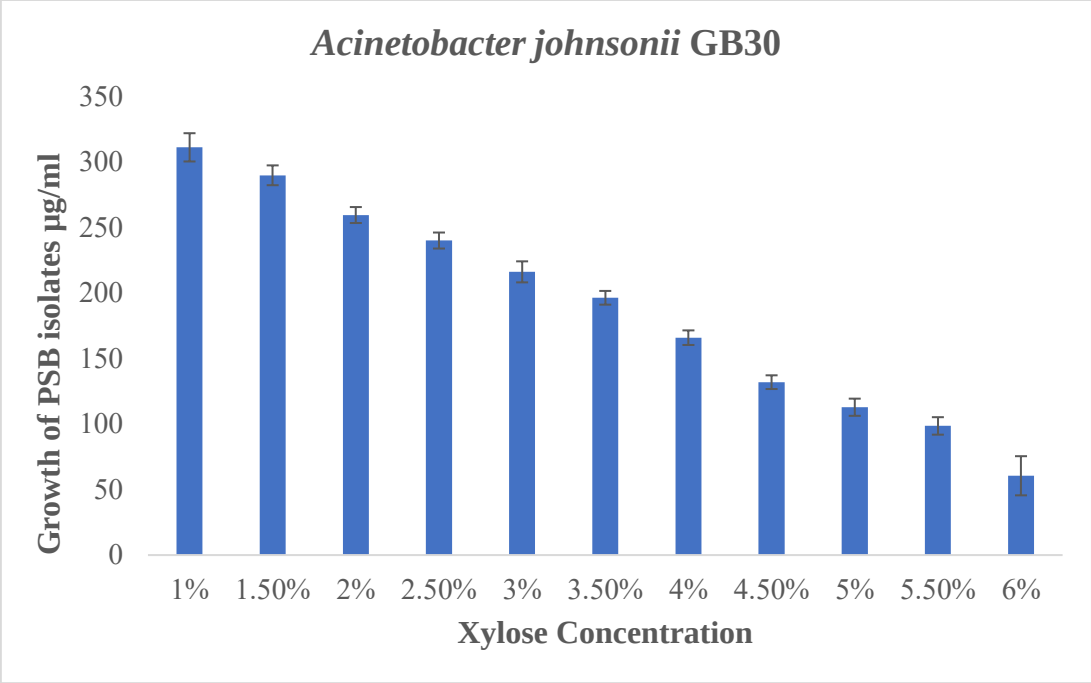


Figure 4.1.11. Optimization of Fructose Concentration for *Acinetobacter johnsonii* GB30

4.1.5. Nitrogen Source Optimization

The study investigated the impact of various nitrogen (N) sources on PVK media. This was done by substituting ammonium sulfate in the PVK culture media with different sources of N, each containing 0.1g equivalent in 50ml. In the case of *Pseudomonas frederiksbergensis* GA23, Tri ammonium citrate was found to show minimum PSE (218.19µg/ml) whereas, with ammonium sulfate maximum PSE was detected (352.73µg/ml) which was higher than other N sources followed by sodium nitrate (257.86µg/ml), ammonium tartrate (256.79µg/ml), ammonium nitrate (248.19µg/ml), diammonium phosphate (243.89µg/ml) ammonium iron sulfate (241.83µg/ml), ammonium acetate (241.13µg/ml), and blank (224.16µg/ml) (as shown in Fig. 12. Ammonium sulfate was determined to be the most effective nitrogen source.

While in case of *Acinetobacter johnsonii* GB30, blank was found to show minimum PSE (217.63µg/ml) whereas, maximum PSE was detected with ammonium sulfate (339.96µg/ml) which was higher than other N sources followed by sodium nitrate (334.26µg/ml), diammonium phosphate (312.13µg/ml), ammonium nitrate (310.66µg/ml), ammonium iron sulfate (300.83µg/ml), ammonium acetate (269.46µg/ml), ammonium tartrate (245.03µg/ml) and tri ammonium citrate

(225.09µg/ml), as shown in Fig. 3a. In *Acinetobacter johnsonii* GB30 Ammonium sulfate was also determined to be the best N source see fig 13.

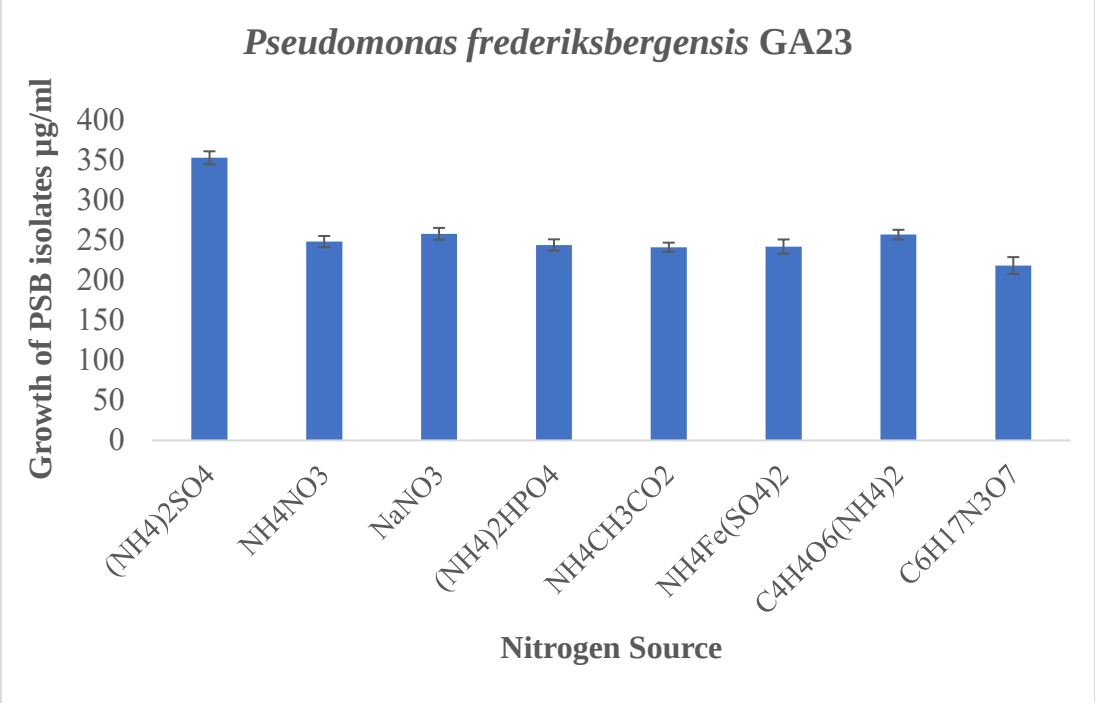


Figure 4.1.12. Optimization of Nitrogen Source for *Pseudomonas frederiksbergensis* GA23

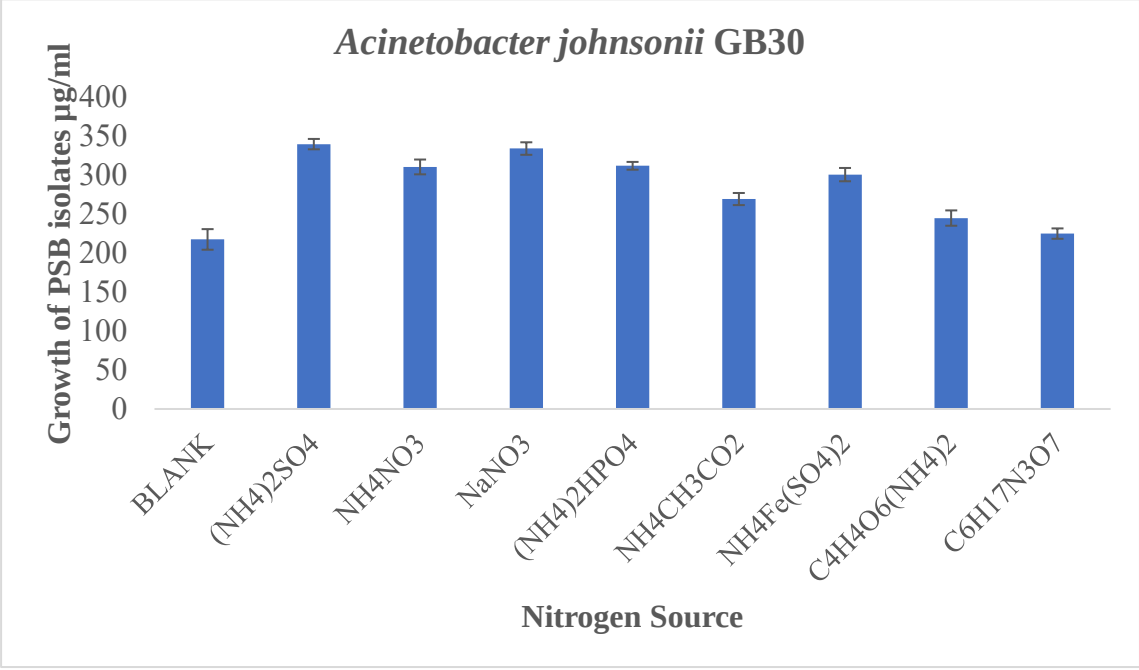


Figure 4.1.13. Optimization of Nitrogen Source for *Acinetobacter johnsonii* GB30

Furthermore, variations in ammonium sulfate concentrations were explored to determine the optimal concentration in PVK media. The incubation period was set at 48 hours, with a temperature of 5°C and pH 3 for *Pseudomonas frederiksbergensis* GA23 and 72 hours at 15°C with pH 7 for *Acinetobacter johnsonii* GB30. For *Pseudomonas frederiksbergensis* GA23, PSE exhibited an increase at an ammonium sulfate concentration of 10mg/50ml, followed by a decline at higher concentrations, reaching its peak at 10mg/50ml (275.19µg/ml). Subsequently, the values decreased to (262.46 µg/ml), (249.76 µg/ml), (233.79 µg/ml), (223.83 µg/ml), (204.46 µg/ml), (179.39 µg/ml), (141.93 µg/ml), (123.09 µg/ml), and (96.39 µg/ml) within the concentration range of 20mg to 100mg see fig 14.

Similarly, for *Acinetobacter johnsonii* GB30, PSE increased with an ammonium sulfate concentration from 10mg/50ml to 20mg/50ml, reaching its optimum at 20mg/50ml (281.09µg/ml). Subsequent concentrations, ranging from 10mg to 100mg, exhibited a decline with values of (168.03µg/ml), (257.83µg/ml), (244.46µg/ml), (230.89µg/ml), (204.76µg/ml), (180.16µg/ml), (145.33µg/ml), (120.86µg/ml) and (98.76µg/ml) see fig 15.

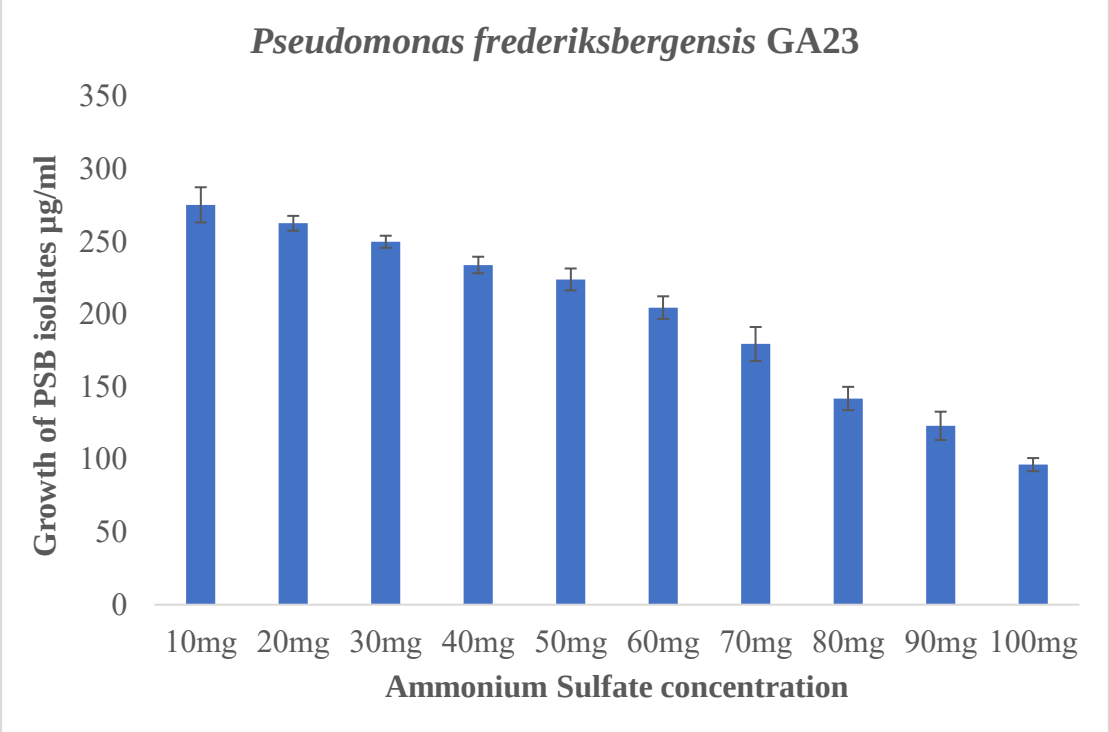


Figure 4.1.14. Optimization of Ammonium Sulfate concentration for *Pseudomonas frederiksbergensis* GA23

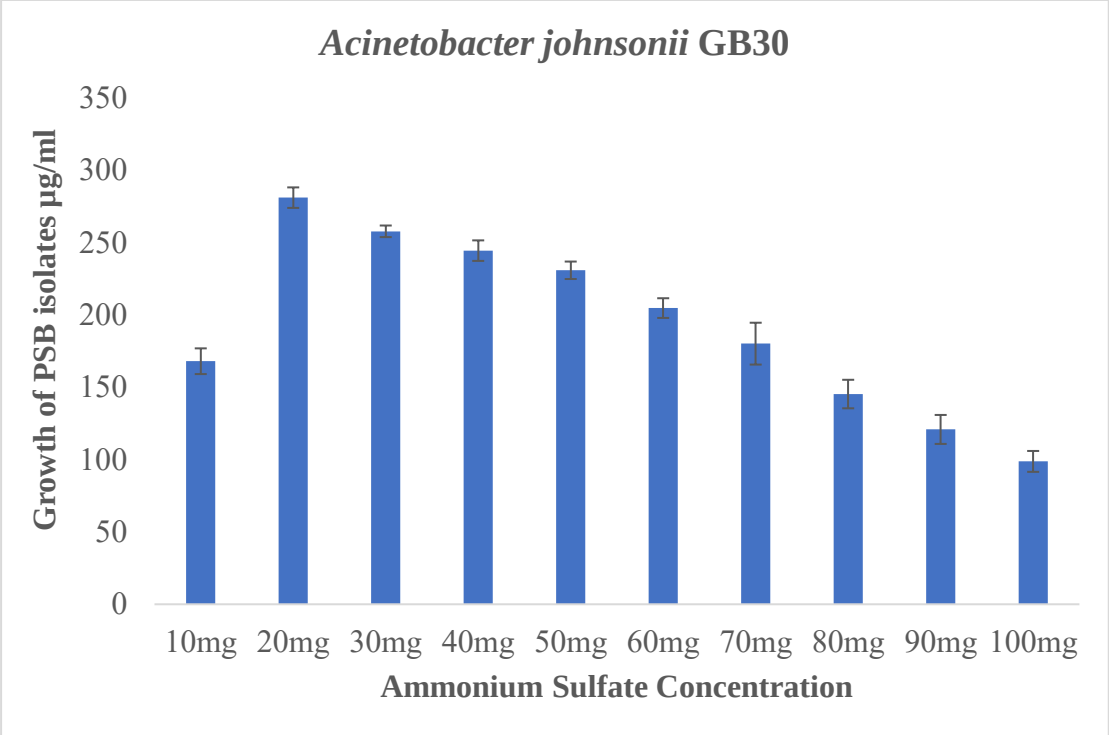


Figure 4.1.15. Optimization of Ammonium Sulfate concentration for *Acinetobacter johnsonii* GB30

4.1.6. Potassium Source Optimization

The impact of different potassium (K) sources on PSE was evaluated by modifying the K sources in the PVK media, each containing 0.1 g equivalent K in 50 ml fluid. The results are depicted in Figure 16 and 17. In *Pseudomonas frederiksbergensis* GA23, potassium nitrate proved the highest PSE (294.03µg/ml) when utilized in the growth medium, followed by dipotassium phosphate (279.06µg/ml), potassium nitrite (273.69µg/ml), blank (271.43µg/ml), potassium sulfate (257.56µg/ml), potassium chloride (257.13µg/ml), potassium carbonate (253.26µg/ml), potassium acetate (242.36µg/ml), and potassium iodide (208.93µg/ml) after 48 hours of incubation at pH 3. PSE with potassium nitrate was found maximum than rest of the K sources.

While in *Acinetobacter johnsonii* GB30 strain potassium nitrate was also found to give the highest PSE (369.53µg/ml) when employed in a growth medium subsequent to the addition of potassium chloride (338.29µg/ml), blank (334.76µg/ml), dipotassium phosphate (322.73µg/ml), potassium acetate (300.33µg/ml), potassium iodide (269.96µg/ml), potassium carbonate (230.69µg/ml), potassium sulfate (216.89µg/ml) and potassium nitrite (102.03µg/ml) after 96 h of incubation at ph7. The highest growth

was observed while using potassium chloride as the source of potassium, compared to other potassium sources.

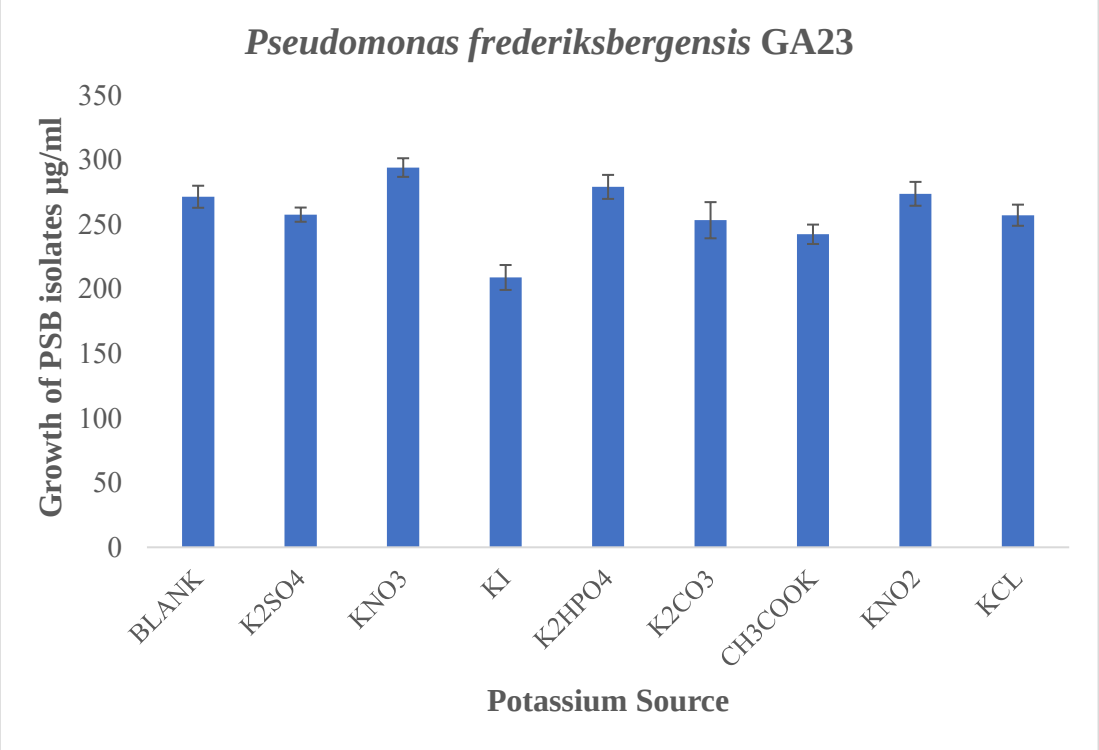
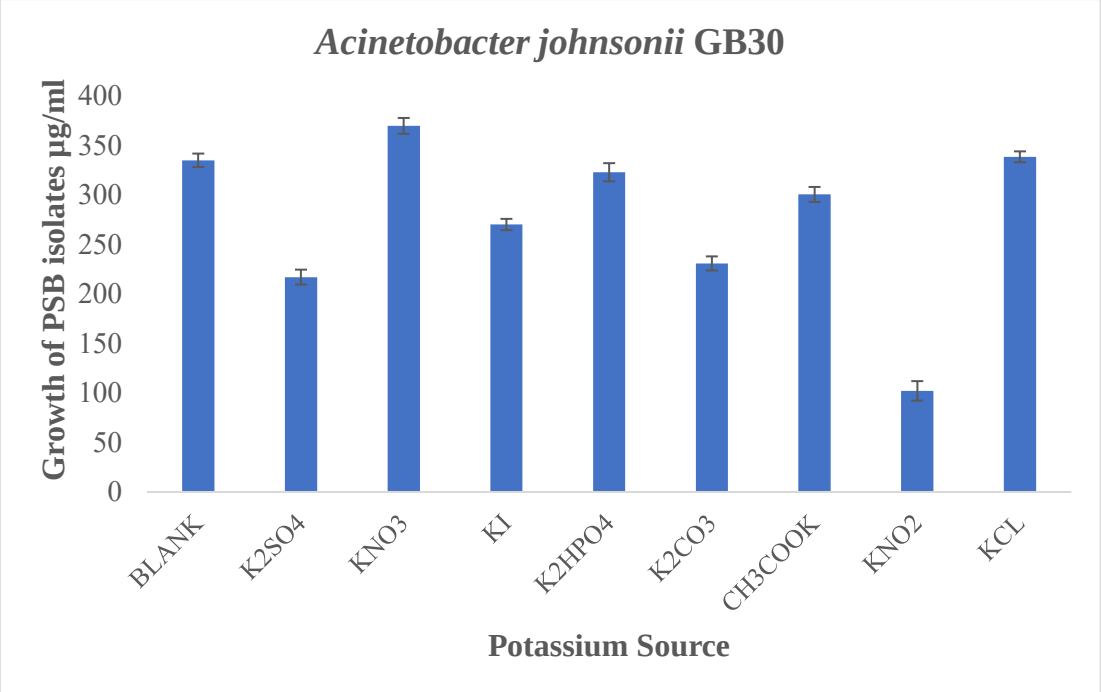


Figure 4.1.16. Optimization of Potassium Source for *Pseudomonas frederiksbergensis* GA23



4.1.17. Optimization of Potassium Source for *Acinetobacter johnsonii* GB30

4.1.7. Effect of Phosphorus Source

In optimization of P source various amounts of each insoluble phosphate, ranging from 0.5% to 2% (w/v), were incorporated into the optimal medium to investigate the influence of different insoluble phosphate sources on the solubilization process. Table 1 demonstrates the increased production of soluble phosphorus by *Pseudomonas frederiksbergensis* GA23 and table 2 demonstrates *Acinetobacter johnsonii* GB30 when utilizing Ca3(PO4)2, CaHPO4, and hydroxyapatite compared to FePO4 and AlPO4. As the quantities of these insoluble phosphates were augmented, there was a corresponding rise in the generation of soluble phosphorus. Following two and four days of incubation, *Pseudomonas frederiksbergensis* GA23 exhibited maximum concentrations of soluble phosphorus from Ca3(PO4)2, CaHPO4, and hydroxyapatite at 311.58, 314.03, and 313.89 mg/l, respectively. In the case of *Acinetobacter johnsonii* GB30, the maximum concentrations were 314.79, 361.89, and 361.13 mg/l for the same insoluble phosphate sources.

Table no. 4.1.1. Shows optimization of Phosphate Source for *Pseudomonas frederiksbergensis* GA23

Phosphate source	Concentration (%)	Soluble P	Phosphate source	Concentration (%)	Soluble P
$Ca_3(PO_4)_2$	0.5	253.56±10.7	$FePO_4$	0.5	0.26±0.32
	1	308.09±6.16		1	0.76±0.3
	1.5	311.98±6.2		1.5	0.66±0.51
	2	311.59±6.02		2	0.36±0.32
$CaHPO_4$	0.5	303.06±6.61	$AlPO_4$	0.5	0.59±0.4
	1	314.03±6.08		1	1.33±0.98
	1.5	300.26±10.40		1.5	5.16±3.15
	2	306.03±3.65		2	203.26±4.1
Hydroxyapatite	0.5	251.03±12.6			
	1	270.59±6.8			

	1.5	306.66±5.35			
	2	313.89±3.05			

Table no. 4.1.2. Shows optimization of Phosphate Source for *Acinetobacter johnsonii* GB30

Phosphate source	Concentration (%)	Soluble P	Phosphate source	Concentration (%)	Soluble P
$Ca_3(PO_4)_2$	0.5	252.76±2.00	$FePO_4$	0.5	0.36±0.35
	1	296.16±5.5		1	1.26±1.01
	1.5	312.13±1.5		1.5	1.43±1.38
	2	314.79±1.05		2	0.39±0.35
$CaHPO_4$	0.5	361.86±3.41	$AlPO_4$	0.5	0.13±0.26
	1	354.76±1.7		1	1.89±1.84
	1.5	253.96±4.91		1.5	3.43±0.43
	2	267.03±4.85		2	3.56±0.40
Hydroxyapatite	0.5	289.39±1.55			
	1	359.69±4.3			
	1.5	361.13±8.26			
	2	206.63±0.72			

4.1.8. Effect of Various Salt Source

In order to assess the impact of different levels of salt (NaCl) on the insoluble phosphate-solubilizing activity of *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30, the optimum medium was supplemented with varying concentrations of salt, ranging from 1% to 10%, 12%, 15%, 18%, 20%, and 25% (w/v). Figure 18 demonstrates that in *Pseudomonas frederiksbergensis* GA23, the synthesis of soluble phosphorus remained consistent even when the concentration of NaCl increased up to 6% (w/v). The production of soluble P decreased as the concentration of NaCl increased. However, even at a concentration of 6% (w/v) of NaCl, 229.83µg/ml of soluble P was still produced while in nutrient agar plate the minimum growth was appeared at 8%. This was followed by concentrations of 2% (215.46µg/ml), 4%

(222.96µg/ml), 8% (204.86µg/ml), 10% (188.03µg/ml), 12% (113.56µg/ml), 15% (92.0µg/ml), 18% (26.73µg/ml), 20% (20.49µg/ml), and 25% (9.23µg/ml) of NaCl. In contrast, there was a remarkable decline in the synthesis of soluble phosphorus when the NaCl concentration exceeded 10% to 25% in PVK broth. The generation of soluble P in *Acinetobacter johnsonii* GB30 was sustained even when the concentration of NaCl increased up to 8% (w/v) the same result was recorded in nutrient agar plate see fig 19. The production of soluble P decreased as the concentration of NaCl increased. However, even at a concentration of 8% (w/v) of NaCl, 207.06µg/ml of soluble P was still produced. The production of soluble P then followed a decreasing trend with concentrations of 2% (256.19µg/ml), 4% (234.99µg/ml), 6% (219.13µg/ml), 10% (161.49µg/ml), 12% (105.19µg/ml), 15% (92.19µg/ml), 18% (24.23µg/ml), 20% (11.06µg/ml), and 25% (2.86µg/ml). In contrast, there was a remarkable reduction in the synthesis of soluble phosphorus when the NaCl concentration exceeded 10% to 25% in PVK broth.

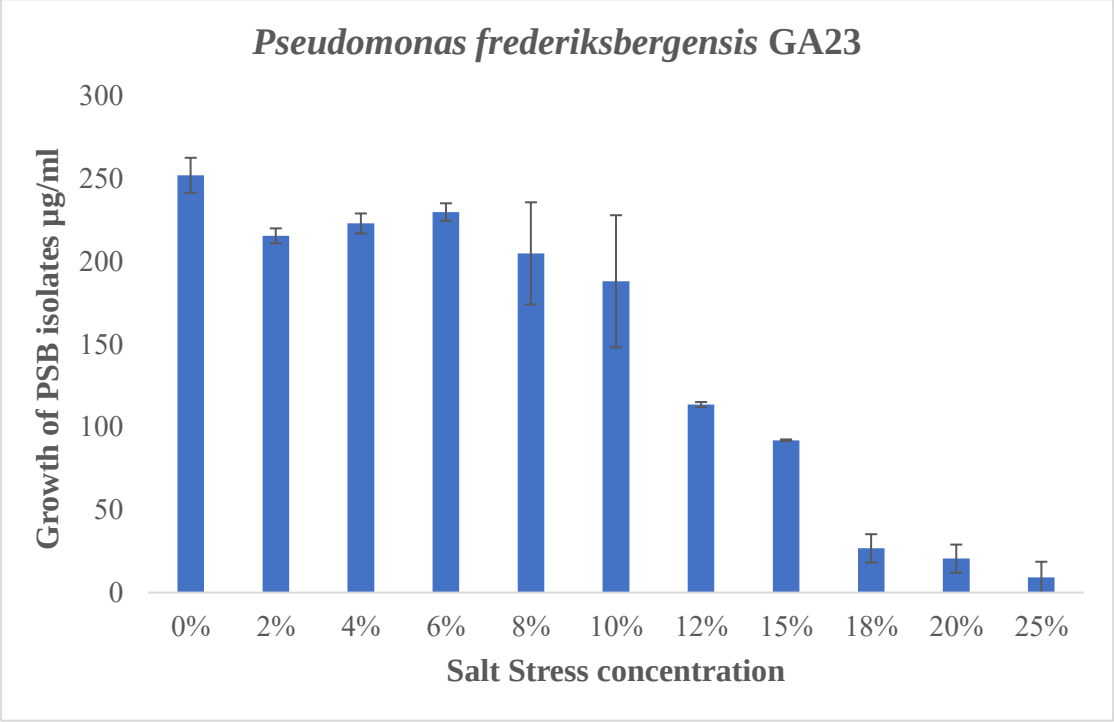


Figure.4.1.18. Optimization of Salt Stress Concentration for *Pseudomonas frederiksbergensis* GA23

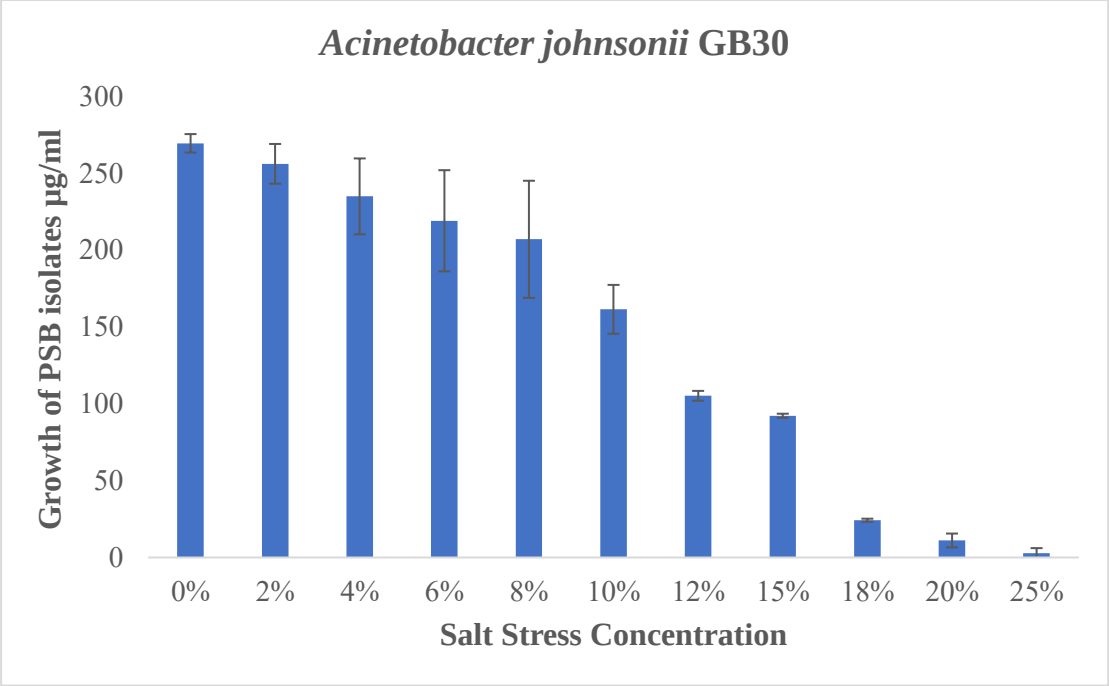


Figure.4.1.19. Optimization of Salt Stress Concentration for *Acinetobacter johnsonii* GB30

4.1.9. Effect of Drought Stress Using Polyethylene Glycol (PEG-6000) Source

The objective was to examine the impact of drought stress on the insoluble phosphate-solubilizing activity of *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 by employing different concentrations of polyethylene glycol (PEG-6000) ranging from 0% to 8% (w/v). These concentrations were added to the ideal PVK broth medium and nutrient agar plates. Figure 20 demonstrates that the synthesis of soluble phosphorus in *Pseudomonas frederiksbergensis* GA23 remained consistent even when the concentration of PEG-6000 increased up to 6% (w/v) the result was recorded in nutrient agar plate. The presence of maximum PEG-6000 adversely impacted the generation of soluble P. However, even at a concentration of 6% (w/v) of PEG-6000, a remarkable amount of soluble P was still produced, measuring 200.26µg/ml. As the concentration of PEG-6000 increased, there was a decline in the production of soluble phosphorus, with values of 0% (333.09µg/ml), 1% (301.89µg/ml), 2% (279.06µg/ml), 3% (247.99µg/ml), 4% (230.63µg/ml), 5% (216.46µg/ml), 7% (102.03µg/ml), and 8% (72.86µg/ml) in PVK broth. In contrast, there was a remarkable reduction in the formation of soluble phosphorus at a concentration of 6% PEG-6000.

With regards to *Acinetobacter johnsonii* GB30, the generation of soluble phosphorus remained consistent even when the concentration of PEG-6000 increased up to 5% (w/v). The concentration of PEG-6000 had a negative effect on the production of soluble P see fig 21. However, even at a concentration of 5% (w/v) of PEG-6000, a remarkable amount of soluble P (225.76µg/ml) was still produced, the same result was recorded in nutrient agar plate. The production of soluble P decreased as the concentration of PEG-6000 increased, with the following amounts observed 0% (375.09µg/ml), 1% (334.76µg/ml), 2% (304.76µg/ml), 3% (270.89µg/ml), 4% (249.03µg/ml), 6% (102.76µg/ml), 7% (82.93µg/ml), and 8% (43.93µg/ml) in PVK broth. In contrast, there was a remarkable reduction in the synthesis of soluble phosphorus when the concentration of PEG-6000 exceeded 5%.

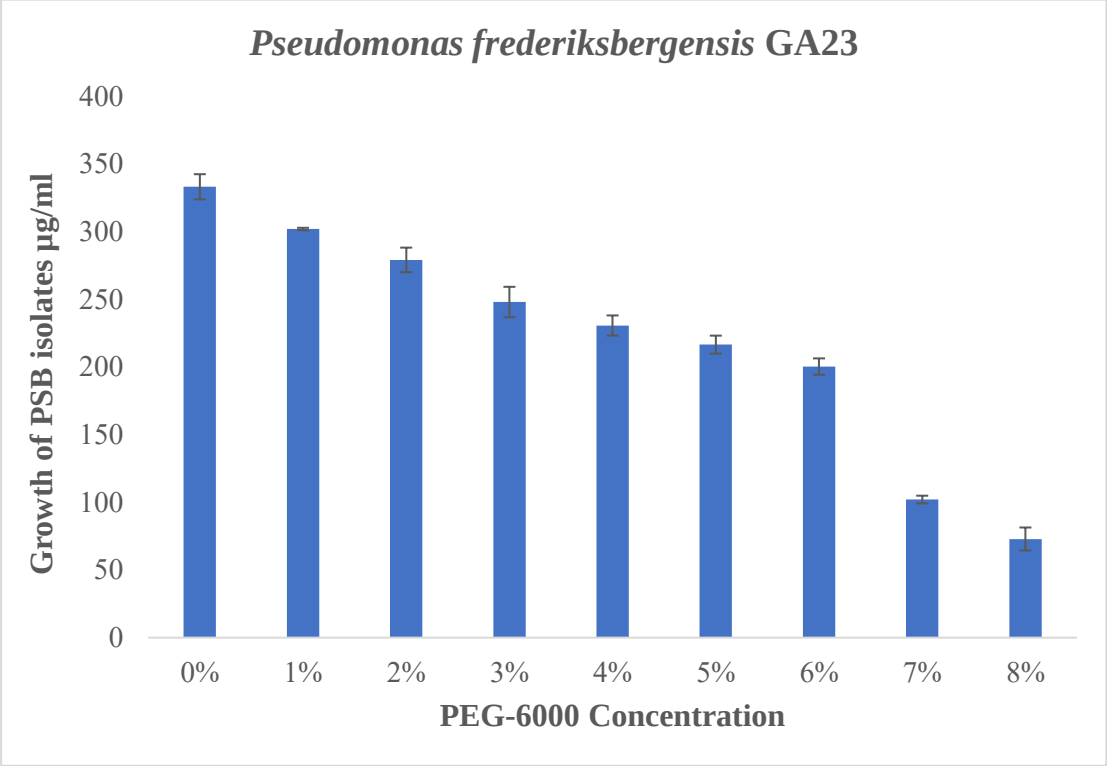


Figure.4.1.20. Optimization of Drought Stress for *Pseudomonas frederiksbergensis* GA23

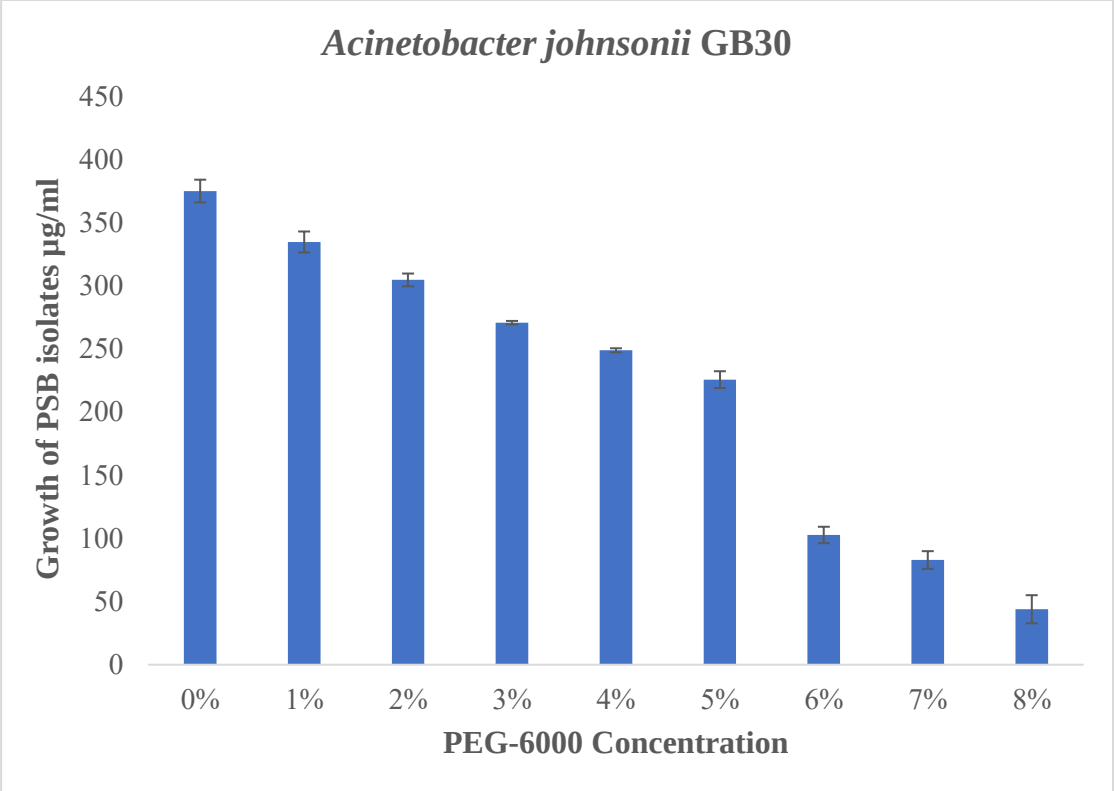


Figure.4.1.21. Optimization of Drought Stress for *Acinetobacter johnsonii* GB30

4.2. Qualitative Method

The ability to solubilize phosphate was assessed by observing the formation of a distinct yellow zone around the well on PVK agar after 48 hours of incubation at 15°C. Both bacterial isolates *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 had distinct yellow zones on PVK agar. Additionally, *Pseudomonas frederiksbergensis* GA23 isolates demonstrated the largest clearance zones with variable diameters. The diameter of the halo zone generated by the *Pseudomonas frederiksbergensis* GA23 was 43mm and *Acinetobacter johnsonii* GB30 was 42mm. The *Pseudomonas frederiksbergensis* GA23 strain SI had the highest level 5.3mm when compared to the *Acinetobacter johnsonii* GB30 strain 5.2mm. The phosphate solubilizing efficiency (PSE) was determined for the chosen isolates, with values ranging from 420 to 430mm. The greatest PSE value of 430mm was observed in the case of *Pseudomonas frederiksbergensis* GA23 followed by *Acinetobacter johnsonii* GB30 with a value of 420mm (Fig. 22).

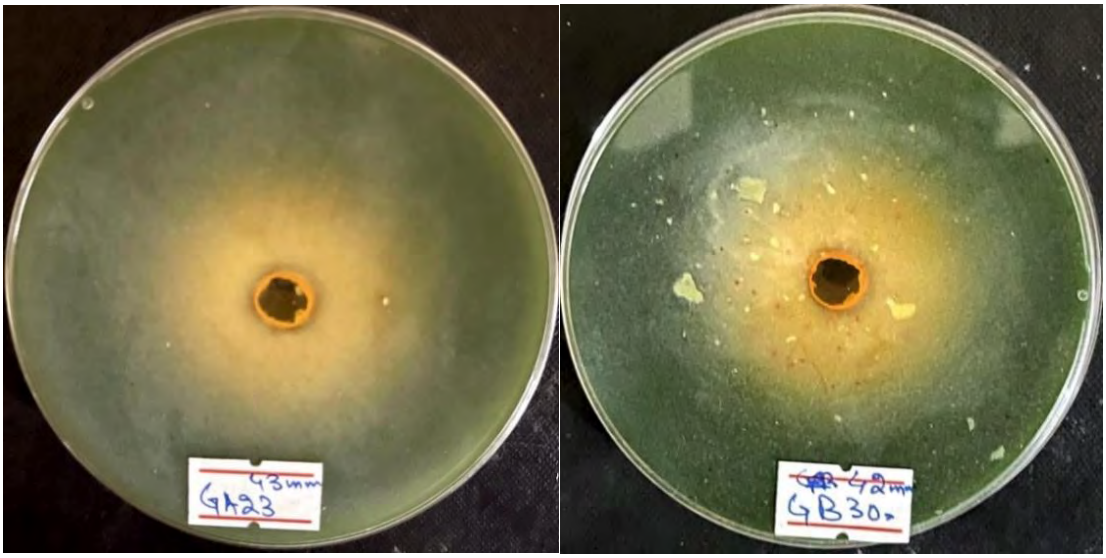


Figure 4.2.22. Shows the Halo Zone diameter of *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30

4.3. Thin Layer Chromatography

Organic acids are bacterial metabolite that can be produced by bacteria along with many other metabolites. Thin layer chromatography was done to separate organic acids from the rest of the compounds in the crude extract from the strain by loading 2µl of the solution from standards (malic, formic, oxalic, gluconic and citric acid) and both samples i.e., (*Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30). The plate was then dipped in 0.4% bromocresol green (ethanol) the presence of organic acids in samples were confirmed through visualizing the yellow spots on a greenish blue background see fig. 23. *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 which were almost the same as that for the standard confirming the presence of the OAs which were further confirmed by FTIR.

Figure 4.3.23. TLC of Organic acids Produced by *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30

4.4. Fourier Transform Infra-Red Spectroscopy

A FT-IR analysis of the psychrophilic phosphate solubilizing bacteria grown in PVK liquid medium were obtained (as shown in Fig.24 and 25). In the present study first, we run 7 different standards on FT-IR which give different peaks given below on the table no 3.

Table no 4.3.3. Shows Peaks Values of the Standards and Samples

S.no			Standards			Samples	
	Citric acid	Formic acid	Oxalic acid	Malic acid	Gluconic acid	*Pseudomonas frederiksbergensis* GA23	*Acinetobacter johnsonii* GB30
1	3259cm−1	3265cm−1	3257cm−1	3259cm−1	3262cm−1	3250 cm−1	3241cm−1
2	2147cm−1	2151cm−1	2151cm−1	2153cm−1	2152cm−1	1635cm−1	1626cm−1
3	1635cm−1	1637cm−1	1635cm−1	1638cm−1	1637cm−1	1419cm−1	1418cm−1
4	578cm−1	603cm−1	619cm−1	607cm−1	584cm−1	1237cm−1	1363cm−1
5	562cm−1	579cm−1	583cm−1	584cm−1	570cm−1	1074cm−1	1074cm−1
6	550cm−1	572cm−1	571cm−1	571cm−1	560cm−1	1027cm−1	1027cm−1
7	539cm−1	565cm−1	557cm−1	579cm−1	548cm−1	591cm−1	602cm−1
8	524cm−1	535cm−1	542cm−1	556cm−1	543cm−1	575cm−1	591cm−1
9		527cm−1	531cm−1	550cm−1	535cm−1	550cm−1	575cm−1
10		520cm−1	525cm−1	543cm−1	531cm−1	531cm−1	556cm−1
11				527cm−1	527cm−1	521cm−1	551cm−1
12					522cm−1		

Comparison with standards for *Pseudomonas frederiksbergensis* GA23 is

Citric Acid:

3259 cm-1, 1635 cm-1, 550 cm-1 (Match for peaks 1,2 and 6)

Formic Acid:

3265 cm-1, 1637 cm-1, 579 cm-1 (Match for peaks 1, 2, 6, and 7)

Oxalic Acid:

3257 cm-1, 1635 cm-1, 571 cm-1,531 cm-1, 525 cm-1 (Match for peaks 1, 2, 6,9 and 10)

Malic Acid:

3259 cm-1, 1638 cm-1, 579 cm-1, 550 cm-1 (Match for peaks 1, 2, 7, and 9)

Gluconic Acid:

3262 cm-1, 1637 cm-1, 584 cm-1, 560 cm-1, 527 cm-1 (Match for peaks 1, 2, 4, 6, and 11)

While Comparison with Standards for *Acinetobacter johnsonii* GB30:

Citric Acid:

3241 cm-1, 1626 cm-1 (Partial match for peaks 1 and 2)

Formic Acid:

3241 cm-1, 1626 cm-1, 603 cm-1, 572 cm-1 (Match for peaks 1, 2, 4 and 6)

Oxalic Acid:

3241 cm-1,1626 cm-1, 571 cm-1, 557 cm-1 (Partial match for peaks 1, 2, 6 and 7)

Malic Acid:

3241 cm-1, 1626 cm-1, 607 cm-1, 571 cm-1, 556 cm-1, 550 cm-1 (Match for peaks 1, 2, 4, 16, 8 and 9)

Gluconic Acid:

3241 cm-1, 1626 cm-1, 570 cm-1, 560 cm-1 (Match for peaks 1, 2, 5 and 6)

Based on this comparison, it appears that the *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 samples contain peaks aligning with various organic acids, such as citric acid, formic acid, oxalic acid, fumaric acid, succinic acid, malic acid, and gluconic acid. In terms of functional groups, the hydroxyl group exhibited a broad band in the range of 3000 to 3500 cm−1, with peaks at 3250.55 cm−1 and 3241.07 cm−1 indicating the presence of the –OH group. Bands at 1635.02 cm−1 and 1626.88 cm−1 signified the C = O group (Carboxyl group), and those at 1419.97 cm−1 and 1418.64 cm−1 represented the C = C group (Carbonyl group). Peaks at 1237.52 cm−1 and 1363.47 cm−1 indicated C-O-P, while 1074.64 cm−1 and 1074.83 cm−1 were indicative of organic phosphate (P-O). The band within the 900 to 500 cm−1 range could not be attributed to any specific group. The presence of these functional groups suggests the existence of organic acid molecules in our extract. However, for a more precise identification and quantification, we conducted an analysis using HPLC.

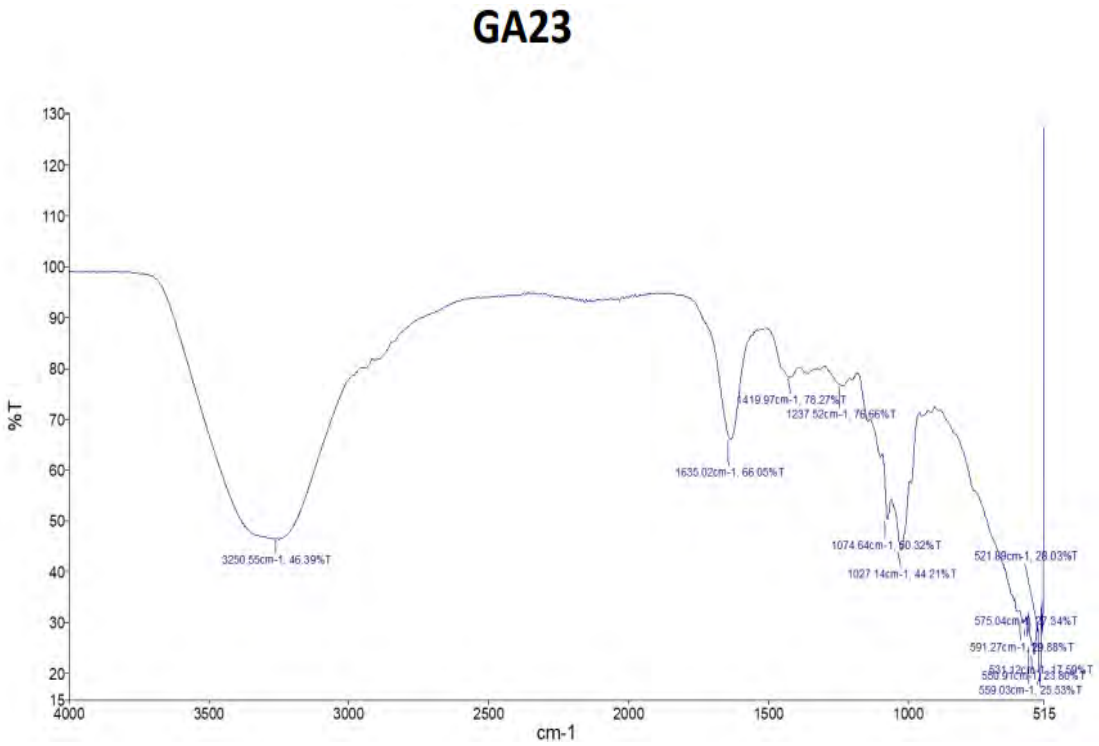


Figure 4.3.24. FTIR analysis of Organic acids Produced by *Pseudomonas frederiksbergensis* GA23

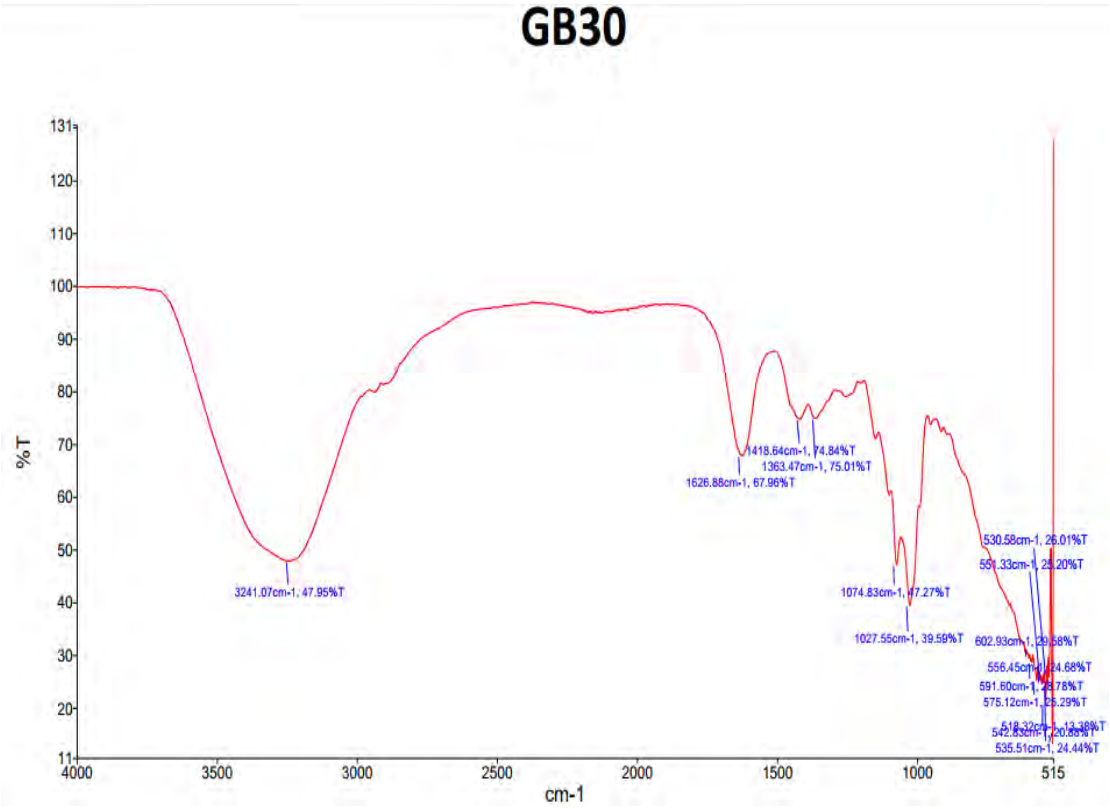


Figure 4.3.25. FTIR analysis of Organic acids produced by *Acinetobacter johnsonii* GB30

4.5. High Performance Liquid Chromatography (HPLC)

Following the optimal 48 hours of incubation *Pseudomonas frederiksbergensis* GA23, we do an HPLC analysis following the confirmation of organic acids on FTIR. The results are shown in Fig. 26 and 27. In PVK medium, strain *Pseudomonas frederiksbergensis* GA23 generated thirteen distinct organic acids, of which three were known (acetic, oxalic, and formic acid) and ten were unknown. Ten unknown organic acids were kept for the following lengths of time: 0.46, 4.24, 4.85, 6.41, 8.04, 8.44, 9.95, 11.04, 15.19, and 18.89 for unknowns 1 through 10. According to the investigation, throughout the incubation time, this generates a number of organic acids that help solubilize TCP that is distinctly soluble; the maximal solubilization was reached after 48 hours. The strain *Pseudomonas frederiksbergensis* GA23 produced the highest amount of acetic acid of all known organic acids, followed by oxalic acid and formic acid. At 48 hours of growth, strain *Pseudomonas frederiksbergensis* GA23 produced roughly 146.73 ppm of acetic acid, 129.25 ppm of oxalic acid, and 44.98 ppm of formic acid in the media, according to estimates of the secreted acids and soluble P

content. Following a 96-hour incubation period, the findings are shown in Fig. 6 while in *Acinetobacter johnsonii* GB30 HPLC. In PVK medium, strain *Acinetobacter johnsonii* GB30 produced fourteen distinct organic acids, of which five were known (acetic, oxalic, gluconic, citric, and formic acid) and nine were novel. Nine unidentified organic acids were retained for the following lengths of time: 0.10, 0.98, 1.89, 5.18, 5.91, 8.45, 9.29, 11.31, and 18.48 for unidentified 1 through unidentified 10. According to the investigation, during the incubation time, this generates a number of organic acids that help solubilize TCP that is distinctly soluble; the maximal solubilization was reached after 96 hours. The strain *Acinetobacter johnsonii* GB30 produced the highest amount of gluconic acid among all known organic acids, followed by citric, formic, acetic and oxalic acids. Strain *Acinetobacter johnsonii* GB30 produced around 1161.72 ppm of gluconic acid, 320.39 ppm of oxalic acid, 68.15 ppm of formic acid, 58.40 ppm of oxalic acid, and 4.57 ppm of acetic acid in the media, according to estimates of the secreted acids and soluble P content at 96 hours of culture.

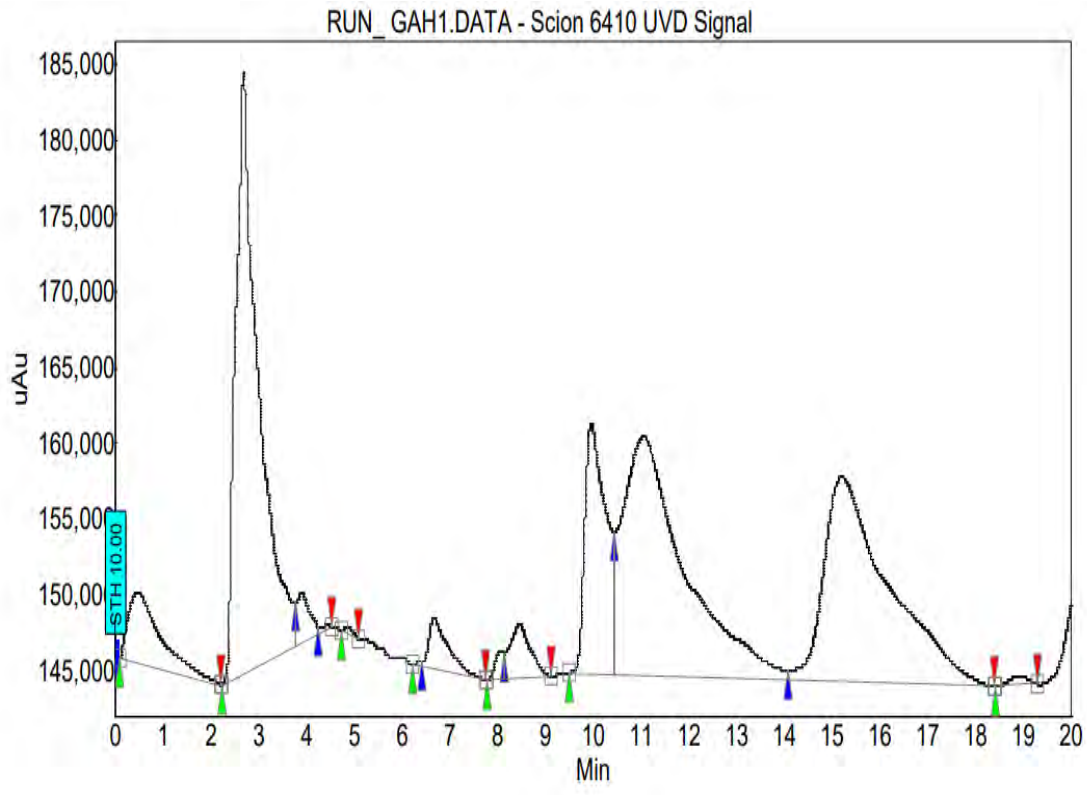


Figure 4.4.26. HPLC analysis of Organic acids produced by *Pseudomonas frederiksbergensis* GA23

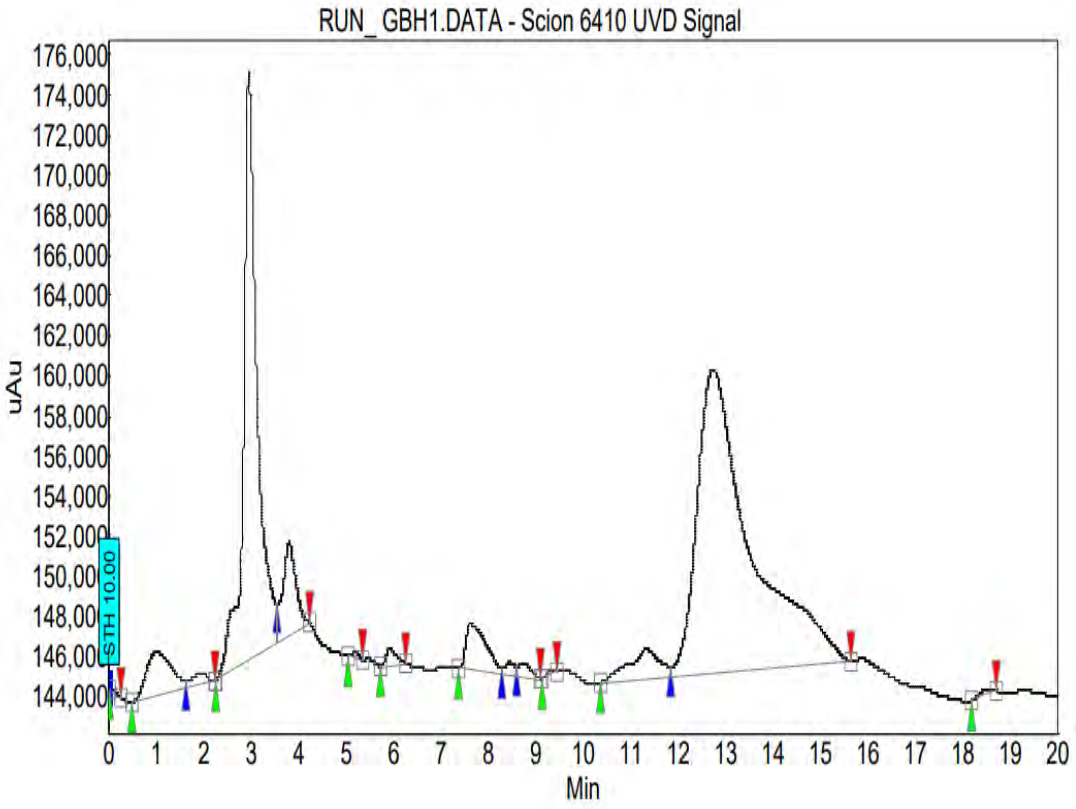


Figure 4.4.27. HPLC analysis of Organic acids produced by *Acinetobacter johnsonii* GB30

5. Discussion

In the present study two PSB designated as *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were isolated from Ghulkin Glacier. The *Pseudomonas frederiksbergensis* GA23 were found to belong to the genus *Pseudomonas* and *Acinetobacter johnsonii* GB30 were belong to *Bacilli*.

This conclusion is corroborated by a previous study which states that the most effective and commonly seen bacteria that can solubilize phosphate belong to the genus *Pseudomonas* or the genus *Bacillus* (Sundram, 1994). Haile (1999) and Keneni *et al.,* (2010) both identified PSB, all of which were classified as members of the genus Pseudomonas.

When cultivated in culture media containing Tri-calcium phosphate, all of the isolates exhibited the formation of a halo zone surrounding the colonies, indicating the dissolution of the phosphate source utilized. The presence of phosphate solubilizing microorganisms is identified by the development of transparent halos surrounding their colonies. The halo is formed as a result of the solubilization of insoluble phosphates, which occurs through the creation of organic acid in the surrounding environment (Gaur, 1990). The isolate *Pseudomonas frederiksbergensis* GA23 exhibited the highest solubilization index, with *Acinetobacter johnsonii* GB30 ranking second (Table 2). The P solubilization ability of all isolates was validated using the phospho-molybdate test. The phospho-molybdate test was used to quantitatively determine the presence of accessible phosphorous. The results showed that the isolates *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 were able to solubilize phosphate. Gupta *et al*., 2022 conducted an identical study. According to the study conducted by Saleemi *et al*. in 2017, PS4 exhibited the highest level of PS, followed by PS3, PS2, and PS1. Additionally, PSM-202 shown greater output than PSM-305. Bray and Kurtz (1945) conducted a study where they quantified soluble phosphorus using the molybdenum method, with KH2PO4 serving as the standard.

The P-solubilizing activity is assessed based on the microbial metabolic capacity to generate and discharge organic acids. These acids, with their carboxylic groups, form chelates with cations (mostly Ca) that are attached to phosphate, thereby transforming

them into soluble forms (Kpomblekou and Tabatabai, 1994).

Screening for soluble Phosphorus and Organic acids

Screening of strain to select those that can produce organic was done by following the conventional method of culturing on nutrient agar, making inoculum in nutrient broth and incubation in the Pikovskaya production media to produce organic acids. At the end of the completion of the incubation period molybdenum assay was done by following the (Bray and Kurtz, 1945) after the 15min incubation supernatant turns dark blue color showing the presence of soluble P because of its ability to change its color in the presence of organic acids. The soluble P was utilized as the PSE of the isolates in culture broth and was expressed in milligrams per liter of P released in the culture medium, per a work by Gothwal *et al*. from 2006. An additional approach that the (Jackson, 1973) Orthophosphate that is readily available or soluble, measured in milligrams per milliliter in supernatant and calibrated using KH2PO4 at various time intervals using the Spectro vanadomolybdate phosphoric yellow color method (Jackson, 1973). After vigorous shaking, 5 milliliters of ammonium molybdate reagent were added to 10 milliliters of supernatant. The working solution was then supplemented with 1 ml of chlorostannous acid, bringing the total volume of the reaction mixture to 50 ml with distilled water. 600 nm was used to calculate the O.D. The potassium dihydrogen phosphate curve was used to determine the presence of phosphorus. We therefore use the first procedure since ammonium molybdate is readily available in our lab and produces accurate findings.

5.3 Optimization of Parameters for Maximum soluble phosphate and Organic acids Production

Optimizing culture conditions is crucial for achieving the highest possible yield of soluble P. The production of Soluble P relies on the bacteria's growth rate, which reaches its greatest potential under optimal conditions such as incubation time, temperature, pH, and so on. Consequently, a range of circumstances were supplied to facilitate the growth and optimal production of soluble P by bacteria. The primary objective of this stage was to facilitate bacterial development under optimal conditions and boost their production capacity. Traditional techniques were employed for this optimization. The production capacity of organisms relies on the careful selection of

suitable substrates and optimal growing conditions (Bessai, Bensidhoum *et al*., 2022). In this study, we cultivated our strain under various temperature, pH, carbon supply, nitrogen source, potassium source, and phosphate source conditions. Samples were collected at intervals of 24 hours from 0 to 120 hours to analyze the strain's behavior throughout a 48-hour period. The highest production achieved by *Pseudomonas frederiksbergensis* GA23 occurred at a temperature of 5℃, pH of 3, with 1.5% fructose as the carbon source, nitrogen source, potassium source, and the presence of TCP as the phosphate source. The incubation period lasted for 48 hours. The maximum generation of soluble phosphorus by *Acinetobacter johnsonii* GB30 occurred at a temperature of 15℃, a pH of 7, with xylose as the carbon source, a nitrogen source, a potassium source, and the presence of TCP as the phosphate source. The incubation period was 72 hours. A study conducted by Yadav *et al*. in 2013 investigated the optimal conditions for TCP phosphate supply, ammonium oxalate as a nitrogen source, potassium sulphate as a potassium source, a pH of 7.5, and xylose as a carbon source for the incubation period of 48 hours in BISR-HY65. (Son *et al*. 2006) has modified the ideal circumstances for *P. agglomerans* R-42 strains. The study revealed that the optimal conditions for production were a temperature of 30℃, pH of 7.5, glucose as the carbon source, ammonium nitrate as the nitrogen source, and the presence of TCP as the phosphate source. The incubation period lasted for 5 days. In our investigation on salt optimization, the concentration of *Pseudomonas frederiksbergensis* GA23 was optimized at 6%, whereas *Acinetobacter johnsonii* GB30 was optimized at 8%. This similar optimization was also carried out by Zhu *et al*. in 2011. *Kushneria sinocarnis* exhibited optimal growth at a concentration of 6%, as observed in the study conducted by Reang *et al*. in 2022However, the most substantial increase in growth rate occurred when the concentration reached 10%. Despite this, when investigating the impact of drought stress using PEG-6000, PVK broth revealed the peak growth rates of 6% for *Pseudomonas frederiksbergensis* GA23 and 5% for *Acinetobacter johnsonii* GB30. This investigation aligns with previous studies conducted by Yadav *et al*. in 2015 and Kour *et al*. in 2020, both reporting a maximum growth rate of 5% on nutrient agar-infused plates. Consequently, these inquiries contributed to the formulation of a simplified chemically defined medium. This medium included 1.5% (w/v) fructose, 0.1% (w/v) NH4NO3, 0.02% (w/v) MgSO4.7H2O, and 0.02% (w/v) FeSO4.7H2O,

with an initial pH of 3 for *Pseudomonas frederiksbergensis* GA23. For GA30, the medium comprised 1.5% (w/v) xylose, 0.1% (w/v) NH4NO3, 0.02% (w/v) MgSO4.7H2O, and 0.02% (w/v) FeSO4.7H2O, with an initial pH of 7. Monitoring the growth of *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30, pH fluctuations, and soluble P production over time under optimal cultural conditions revealed that cells, cultivated in a medium with Ca3(PO4)2 as the sole phosphorus source, entered the stationary phase after 6 days of development. The highest degree of solubilization occurred after 2 and 4 days of incubation, corresponding to the logarithmic phase of cell growth. Prolonged incubation periods led to a decrease in soluble phosphorus concentration. Nevertheless, these findings were in line with studies conducted by Son *et al*. in 2006 and Vazquez *et al*. in 2000.

5.4 Extraction of crude Organic acids

To extract the organic acids from supernatant transfer it to sterilized petri plate and freeze at -50^{o}C temperature. Then transfer the freeze supernatant to vacuum machine to vaporize the water from it, the lyophilization process was done at -50^{o}C temperature. After the lyophilization process the extract was preserved for further analyses at -20℃ in labelled vial for extract of each strain. The extract was measured by measuring the vials before and after the transfer of the extract into it. The net weight was calculated for the weight of the extract. The process is also followed by almost all the researchers and used the lyophilization method to evaporate the solvent. Rodriguez-Sanchez *et al*., 2017 have centrifuged the media it at 3000rpm for 10 minutes and then lyophilized the supernatant to extract OAs and after the extract was preserved at 4℃ in 3ml water for further study. In the research conducted by Saranya *et al*. in 2018 and Rajasankar *et al*. in 2013, the sample was also subjected to lyophilization. Meanwhile, Sowmya *et al*. (2014) grew the two chosen PSB (*YU-SS-SB-25* and *YU-SS-SB-29*) in 25ml of Pikovskaya's broth for a duration of 5 days. The culture supernatants were acquired through centrifugation (5000 rpm, 20 min) and subsequently lyophilized, and the resulting extract was utilized for further analysis.

5.5 Characterization by TLC

Thin layer chromatography is a separating process of components in a mixture on the basis of retention ability while moving with the mobile phase with capillary movement.

The mobile phase used in this process was a mixture of chloroform, ethyl acetate and formic acid with benzene, methanol, and acetic acid in a ratio of 90:16:8 v/v and the stationary phase use were silica gel pasted on aluminum plate. The same mobile phase was used by Swetha *et al*., 2016 followed Kraiker and Burch 1973. The organic acids were observed as yellow white dots against a greenish blue background. By comparing with the above-mentioned studies, it can be confirmed that the results obtained from our technique were complete enough for the confirmation of OAs presence.

5.6 Fourier Transform Infra-Red Spectroscopy

The psychrophilic phosphate solubilizing bacteria cultivated on PVK liquid medium were subjected to FT-IR analysis, as depicted in Figure 7a. The FT-IR spectra exhibits a peak at 3250.55cm−1 and 3241.07cm−1 which corresponds to the presence of hydroxyl functional groups. The bands located at 1635.02cm−1 and 1626.88cm−1 indicate C = O group and the band at located at 1419.97cm−1 and 1418.64cm−1 represents the C = C group. Peak at 1237.52cm−1 and 1363.47cm−1 represent C-O-P. The peak at 1074.64cm−1 and 1074.83cm−1 was indicative of organic phosphate (P-O), while the band between 900 and 500 cm−1 could not be ascertained to any group. The same results were recorded by Saranya *et al*., 2018. The presence of stretching peaks at 3000 and 2800 cm−1 suggests the stretching of C−H bonds in −CH2 and −CH3 groups. The peak seen at 1626.80 cm−1 corresponds to the stretching of the C=O bond in the amide I group (CO−NH) of peptides. The peak at 1533.26 cm−1 represents the amide II group (Doshi *et al*., 2007). The spectral regions ranging from 1450.33 to 1413.69 cm−1 are associated with the presence of −C−H and COO− functional groups, as stated by Bai *et al*. in 2014. The spectral range from 1200 to 900 cm−1 corresponds to the stretching of C−O−P bonds in polysaccharides, whereas the range from 965 to 850 cm−1 corresponds to the C−C(O) stretching of acyl halides (Zhou *et al*., 2014; Park and Chon, 2016). Rajasankar *et al*., 2013 has also reported organic acids by using the COOH group had an IR spectral range of 1,608 cm-1 in the control treatment. The shift in the spectrum was detected in the broth containing monocrotophos (1,627 cm-1) and imidacloprid (1,610 cm-1).

High Performance Liquid Chromatography (HPLC)

P solubilization is linked to the synthesis of several organic acids, according to our HPLC investigation, which we used to identify the kind of organic acid the strain produced. Ten new acids and three distinct kinds of known organic acids have been found in the P solubilization process of *Pseudomonas frederiksbergensis* GA23. In contrast, seven unknown acids and five different kinds of known organic acids have been found in the P solubilization process of *Acinetobacter johnsonii* GB30. Previous research has primarily reported on gluconic, citric, succinic, lactic, and propionic acids, which are known to aid in the process of solubilizing P (Chen *et al*., 2006; Farhat *et al*., 2009). The strain *Pseudomonas frederiksbergensis* GA23 was observed to secrete acetic, oxalic, and formic acids in our instance as well. Meanwhile, the *Acinetobacter johnsonii* GB30 strain produced gluconic, acetic, oxalic, formic, and citric acids during its growth phase in PVK media, which is consistent with the research that have been published. We also discovered peaks of 10 unidentified acids in the culture supernatant, which may be related to this strain's ability to solubilize minerals. The same investigation revealed that the predominant acid among all acids was gluconic, as reported by Rajasankar *et al*., 2013; Yadav *et al*., 2013; and Rfaki *et al*., 2020.

Conclusion

Pseudomonas frederiksbergensis GA23 and *Acinetobacter johnsonii* GB30 isolated form the Ghulkin glacier of the Gilgit Baltistan, Pakistan is investigated in the current study to be the competent producing psychrophiles of soluble phosphate, organic acids and other PGPRs. Incubating *Pseudomonas frederiksbergensis* GA23 for 48hrs and *Acinetobacter johnsonii* GB30 for 96hrs produce the highest amount of soluble P and OAs. *Pseudomonas frederiksbergensis* GA23 at 3 pH at 5°C and *Acinetobacter johnsonii* GB30 at 7 pH and at 15°C temperature produces its highest amount of soluble P and OAs at the above-mentioned incubation time. Keeping fructose as the carbon source, ammonium nitrate as nitrogen source, potassium nitrate as potassium source and TCP as phosphate source for *Pseudomonas frederiksbergensis* GA23 and xylose as carbon source, ammonium sulfate as nitrogen source, potassium chloride as potassium source and TCP as phosphate source for *Acinetobacter johnsonii* GB30 in the PVK production media. Crude OAs extracted by lyophilization method can be purified by thin layer chromatography and can be confirmed by finding its orange yellow blue color background and then processing through FTIR spectroscopy. The bonds comparison of organic acids produced by selected strains and sample can be compared for confirmation and can also be compared with the studies done by past research. Bond wise confirmation of the organic acids presence was done by performing HPLC that has given us different organic acids such as citric acids, oxalic acids, gluconic acids and formic acids and all other metabolites, unknown organic acids present in the extract. These organic acids help to promote plant growth and solubilize inorganic phosphate to soluble phosphate.

Future Prospects

- The use of PSB and their organic acid production can be further optimized for efficient nutrient management in agriculture.
- the use of microbial consortia, where different strains of bacteria work together synergistically to enhance nutrient availability and plant growth.
- Advances in biotechnology may lead to the development of genetically modified PSB with enhanced phosphate-solubilizing capabilities.
- Different properties of organic acids produced by *Pseudomonas frederiksbergensis* GA23 and *Acinetobacter johnsonii* GB30 strains can also be explored for many other applications, like its anti-pathogenic activity.
- Future applications may involve integrating PSB and organic acid production into precision agriculture practices. This could include the development of bio-fertilizers that release nutrients in response to specific plant needs or environmental conditions
- Future research could focus on understanding the long-term effects on soil microbiota, ecosystems, and potential unintended consequences.
- How these bacteria can help plants adapt to changing climatic conditions.

References

Abbaszadeh-Dahaji, P., Masalehi, F., & Akhgar, A. (2020). Improved growth and nutrition of sorghum (Sorghum bicolor) plants in a low-fertility calcareous soil treated with plant growth–promoting rhizobacteria and Fe-EDTA. Journal of Soil Science and Plant Nutrition, 20, 31–42.

Ahmad, F., Ahmad, I., & Khan, M. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research, 163(2), 173–181.

Anderson, G. (1980). Assessing organic phosphorus in soils. The Role of Phosphorus in Agriculture, 411–431.

Azcon-Aguilar, C., Gianinazzi-Pearson, V., Fardeau, J., & Gianinazzi, S. (1986). Effect of vesicular-arbuscular mycorrhizal fungi and phosphate-solubilizing bacteria on growth and nutrition of soybean in a neutral-calcareous soil amended with 32 P-45 Ca-tricalcium phosphate. Plant and Soil, 96, 3–15.

Azzi, V., Kanso, A., Kazpard, V., Kobeissi, A., Lartiges, B., & El Samrani, A. (2017). Lactuca sativa growth in compacted and non-compacted semi-arid alkaline soil under phosphate fertilizer treatment and cadmium contamination. Soil and Tillage Research, 165, 1–10.

Babu-Khan, S., Yeo, T. C., Martin, W. L., Duron, M. R., Rogers, R. D., & Goldstein, A. H. (1995). Cloning of a mineral phosphate-solubilizing gene from Pseudomonas cepacia. Applied and Environmental Microbiology, 61(3), 972–978.

Baeza, M., Zúñiga, S., Peragallo, V., Barahona, S., Alcaino, J., & Cifuentes, V. (2021). Identification of stress-related genes and a comparative analysis of the amino acid compositions of translated coding sequences based on draft genome sequences of Antarctic yeasts. Frontiers in Microbiology, 12, 623171.

Baldani, V., Baldani, J., & Döbereiner, J. (1987). Inoculation of field-grown wheat (Triticum aestivum) with Azospirillum spp. In Brazil. Biology and Fertility of Soils, 4(1–2), 37–40.

Barcelos, M. C., Ramos, C. L., Kuddus, M., Rodriguez-Couto, S., Srivastava, N., Ramteke, P. W., Mishra, P. K., & Molina, G. (2020). Enzymatic potential for the valorization of agro-industrial by-products. Biotechnology Letters, 42, 1799–1827.

Barra, P. J., Pontigo, S., Delgado, M., Parra–Almuna, L., Duran, P., Valentine, A. J., Jorquera, M. A., & de la Luz Mora, M. (2019). Phosphobacteria inoculation enhances the benefit of P–fertilization on Lolium perenne in soils contrasting in P–availability. Soil Biology and Biochemistry, 136, 107516.

Bashan, Y., & Levanony, H. (1991). Alterations in membrane potential and in proton efflux in plant roots induced by Azospirillum brasilense. Plant and Soil, 137, 99–103.

Ben Farhat, M., Farhat, A., Bejar, W., Kammoun, R., Bouchaala, K., Fourati, A., Antoun, H., Bejar, S. and Chouayekh, H., 2009. Characterization of the mineral

phosphate solubilizing activity of Serratia marcescens CTM 50650 isolated from the phosphate mine of Gafsa. Archives of microbiology, 191, pp.815-824.

Ben Zineb, A., Trabelsi, D., Ayachi, I., Barhoumi, F., Aroca, R., & Mhamdi, R. (2020). Inoculation with elite strains of phosphate-solubilizing bacteria enhances the effectiveness of fertilization with rock phosphates. Geomicrobiology Journal, 37(1), 22–30.

Bergkemper, F., Kublik, S., Lang, F., Krüger, J., Vestergaard, G., Schloter, M., & Schulz, S. (2016). Novel oligonucleotide primers reveal a high diversity of microbes which drive phosphorous turnover in soil. Journal of Microbiological Methods, 125, 91–97.

Bergkemper, F., Schöler, A., Engel, M., Lang, F., Krüger, J., Schloter, M., & Schulz, S. (2016). Phosphorus depletion in forest soils shapes bacterial communities towards phosphorus recycling systems. Environmental Microbiology, 18(6), 1988–2000.

Bharudin, I., Bakar, M. F. A., Hashim, N. H. F., Isa, M. N. M., Alias, H., Firdaus-Raih, M., Illias, R. M., Najimudin, N., Mahadi, N. M., & Bakar, F. D. A. (2018). Unravelling the adaptation strategies employed by Glaciozyma antarctica PI12 on Antarctic sea ice. Marine Environmental Research, 137, 169–176.

Biswas, J. K., Banerjee, A., Rai, M., Naidu, R., Biswas, B., Vithanage, M., Dash, M. C., Sarkar, S. K., & Meers, E. (2018). Potential application of selected metal resistant phosphate solubilizing bacteria isolated from the gut of earthworm (Metaphire posthuma) in plant growth promotion. Geoderma, 330, 117–124.

Boitt, G., Simpson, Z. P., Tian, J., Black, A., Wakelin, S. A., & Condron, L. M. (2018). Plant biomass management impacts on short-term soil phosphorus dynamics in a temperate grassland. Biology and Fertility of Soils, 54, 397–409.

Bol, R., Julich, D., Brödlin, D., Siemens, J., Kaiser, K., Dippold, M. A., Spielvogel, S., Zilla, T., Mewes, D., & von Blanckenburg, F. (2016). Dissolved and colloidal phosphorus fluxes in forest ecosystems—An almost blind spot in ecosystem research. Journal of Plant Nutrition and Soil Science, 179(4), 425–438.

Bolan, N., & Hedley, M. (1990). Dissolution of phosphate rocks in soils. 2. Effect of pH on the dissolution and plant availability of phosphate rock in soil with pH dependent charge. Fertilizer Research, 24, 125–134.

Bononi, L., Chiaramonte, J. B., Pansa, C. C., Moitinho, M. A., & Melo, I. S. (2020). Phosphorus-solubilizing Trichoderma spp. From Amazon soils improve soybean plant growth. Scientific Reports, 10(1), 2858.

Borgi, M. A., Saidi, I., Moula, A., Rhimi, S., & Rhimi, M. (2020). The attractive Serratia plymuthica BMA1 strain with high rock phosphate-solubilizing activity and its effect on the growth and phosphorus uptake by Vicia faba L. plants. Geomicrobiology Journal, 37(5), 437–445.

Boroumand, N., Behbahani, M., & Dini, G. (2020). Combined effects of phosphate solubilizing bacteria and nanosilica on the growth of land cress plant. Journal of Soil Science and Plant Nutrition, 20, 232–243.

Breezee, J., Cady, N., & Staley, J. (2004). Subfreezing growth of the sea ice bacterium "Psychromonas ingrahamii." Microbial Ecology, 47, 300–304.

Broadbent, P. (1977). Effect of Bacillus sp on increased growth of seedlings in steamed and non-treated soil. Phytopathology, 67, 1027–1034.

Brown, M. E. (1974). Seed and root bacterization. Annual Review of Phytopathology, 12(1), 181–197.

Brown, M. V., Lauro, F. M., DeMaere, M. Z., Muir, L., Wilkins, D., Thomas, T., Riddle, M. J., Fuhrman, J. A., Andrews-Pfannkoch, C., & Hoffman, J. M. (2012). Global biogeography of SAR11 marine bacteria. Molecular Systems Biology, 8(1), 595.

Buch, A., Archana, G., & Kumar, G. N. (2008). Metabolic channeling of glucose towards gluconate in phosphate-solubilizing Pseudomonas aeruginosa P4 under phosphorus deficiency. Research in Microbiology, 159(9–10), 635–642.

Burr, T., Schroth, M., & Suslow, T. (1978). Increased potato yields by treatment of seed pieces with specific strains of Pseudomonas fluorescens and P. putida. Phytopathology, 68(9), 1377–1383.

Butterly, C. R., Bünemann, E. K., McNeill, A. M., Baldock, J. A., & Marschner, P. (2009). Carbon pulses but not phosphorus pulses are related to decreases in microbial biomass during repeated drying and rewetting of soils. Soil Biology and Biochemistry, 41(7), 1406–1416.

Capper, A. L., & Campbell, R. (1986). The effect of artificially inoculated antagonistic bacteria on the prevalence of take-all disease of wheat in field experiments. Journal of Applied Microbiology, 60(2), 155–160.

Cavicchioli, R., & Siddiqui, K. S. (2006). Cold-adapted enzymes. Enzyme Technology, 615–638.

Chabot, R., Antoun, H., & Cescas, M. (1993). Stimulation of growth of maize and lettuce by inorganic phosphorus solubilizing microorganisms. Canadian Journal of Microbiology, 39, 941–947.

Chen, W., Yang, F., Zhang, L., & Wang, J. (2016). Organic acid secretion and phosphate solubilizing efficiency of Pseudomonas sp. PSB12: Effects of phosphorus forms and carbon sources. Geomicrobiology Journal, 33(10), 870–877.

Chen, Y.P., Rekha, P.D., Arun, A.B., Shen, F.T., Lai, W.A. and Young, C.C., 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Applied soil ecology, 34(1), pp.33-41.

Christiansen-Weniger, C. (1992). N 2-fixation by ammonium-excreting Azospirillum brasilense in auxine-induced root tumours of wheat (Triticum aestivum L.). Biology and Fertility of Soils, 13, 165–172.

Collins, T., Meuwis, M.-A., Stals, I., Claeyssens, M., Feller, G., & Gerday, C. (2002). A novel family 8 xylanase, functional and physicochemical characterization. Journal of Biological Chemistry, 277(38), 35133–35139.

Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology, 71(9), 4951–4959.

D'Amico, S., Claverie, P., Collins, T., Georlette, D., Gratia, E., Hoyoux, A., Meuwis, M.-A., Feller, G., & Gerday, C. (2002). Molecular basis of cold adaptation. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 357(1423), 917–925.

Davies, P. L. (2014). Ice-binding proteins: A remarkable diversity of structures for stopping and starting ice growth. Trends in Biochemical Sciences, 39(11), 548–555.

Davies, P. L., Baardsnes, J., Kuiper, M. J., & Walker, V. K. (2002). Structure and function of antifreeze proteins. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 357(1423), 927–935.

Davison, J. (1988). Plant beneficial bacteria. Bio/Technology, 6(3), 282–286.

De Maayer, P., Anderson, D., Cary, C., & Cowan, D. A. (2014). Some like it cold: Understanding the survival strategies of psychrophiles. EMBO Reports, 15(5), 508–517.

Della Mónica, I. F., Godeas, A. M., & Scervino, J. M. (2020). In vivo modulation of arbuscular mycorrhizal symbiosis and soil quality by fungal P solubilizers. Microbial Ecology, 79(1), 21–29.

Deming, J. W. (2002). Psychrophiles and polar regions. Current Opinion in Microbiology, 5(3), 301–309.

Deubel, A., Gransee, A., & Merbach, W. (2000). Transformation of organic rhizodepositions by rhizosphere bacteria and its influence on the availability of tertiary calcium phosphate. Journal of Plant Nutrition and Soil Science, 163(4), 387–392.

Dhillon, J., Torres, G., Driver, E., Figueiredo, B., & Raun, W. R. (2017). World phosphorus use efficiency in cereal crops. Agronomy Journal, 109(4), 1670–1677.

Din, M., Nelofer, R., Salman, M., Khan, F. H., Khan, A., Ahmad, M., Jalil, F., Din, J. U., & Khan, M. (2019). Production of nitrogen fixing Azotobacter (SR-4) and phosphorus solubilizing Aspergillus niger and their evaluation on Lagenaria siceraria and Abelmoschus esculentus. Biotechnology Reports, 22, e00323.

Doshi, H., Ray, A. and Kothari, I.L., 2007. Biosorption of cadmium by live and dead Spirulina: IR spectroscopic, kinetics, and SEM studies. Current Microbiology, 54, pp.213-218.

Dumorné, K., Córdova, D. C., Astorga-Eló, M., & Renganathan, P. (2017). Extremozymes: A potential source for industrial applications.

Eivazi, F., & Tabatabai, M. (1977). Phosphatases in soils. Soil Biology and Biochemistry, 9(3), 167–172.

El-Nahrawy, S., Elhawat, N., & Alshaal, T. (2019). Biochemical traits of Bacillus subtilis MF497446: Its implications on the development of cowpea under cadmium stress and ensuring food safety. Ecotoxicology and Environmental Safety, 180, 384–395.

Fasim, F., Ahmed, N., Parsons, R., & Gadd, G. M. (2002). Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiology Letters, 213(1), 1–6.

Feller, G. (2013). Psychrophilic enzymes: From folding to function and biotechnology. Scientifica, 2013.

Feller, G., & Gerday, C. (1997). Psychrophilic enzymes: Molecular basis of cold adaptation. Cellular and Molecular Life Sciences CMLS, 53, 830–841.

Feller, G., & Gerday, C. (2003). Psychrophilic enzymes: Hot topics in cold adaptation. Nature Reviews Microbiology, 1(3), 200–208.

Firdaus-Raih, M., Hashim, N. H. F., Bharudin, I., Abu Bakar, M. F., Huang, K. K., Alias, H., Lee, B. K., Mat Isa, M. N., Mat-Sharani, S., & Sulaiman, S. (2018). The Glaciozyma antarctica genome reveals an array of systems that provide sustained responses towards temperature variations in a persistently cold habitat. PLoS One, 13(1), e0189947.

Florentino, A. P., Weijma, J., Stams, A. J., & Sánchez-Andrea, I. (2016). Ecophysiology and application of acidophilic sulfur-reducing microorganisms. Biotechnology of Extremophiles: Advances and Challenges, 141–175.

Fraser, T. D., Lynch, D. H., Gaiero, J., Khosla, K., & Dunfield, K. E. (2017). Quantification of bacterial non-specific acid (phoC) and alkaline (phoD) phosphatase genes in bulk and rhizosphere soil from organically managed soybean fields. Applied Soil Ecology, 111, 48–56.

Frey-Klett, P., Pierrat, J. C., & Garbaye, J. (1997). Location and survival of mycorrhiza helper Pseudomonas fluorescens during establishment of ectomycorrhizal symbiosis between Laccaria bicolor and Douglas fir. Applied and Environmental Microbiology, 63(1), 139–144.

Gaind, S.A. and Gaur, A.C., 1990. Influence of temperature on the efficiency of phosphate solubilizing microorganisms. Indian journal of microbiology. New Delhi, 30(3), pp.305-310.

Gaind, S. (2016). Phosphate dissolving fungi: Mechanism and application in alleviation of salt stress in wheat. Microbiological Research, 193, 94–102.

Gamalero, E., & Glick, B. R. (2015). Bacterial modulation of plant ethylene levels. Plant Physiology, 169(1), 13–22.

Garbaye, J. (1994). Mycorrhization helper bacteria: A new dimension to the mycorrhizal symbiosis [interaction, specificity]. Acta Botanica Gallica (France).

Gaur, A., & KP, O. (1972). Influence of phosphate dissolving bacilli on yield & phosphate uptake of wheat crop.

Gaur, R., Grover, T., Sharma, R., Kapoor, S., & Khare, S. K. (2010). Purification and characterization of a solvent stable aminopeptidase from Pseudomonas aeruginosa: Cloning and analysis of aminopeptidase gene conferring solvent stability. Process Biochemistry, 45(5), 757–764.

Gentile, G., Bonasera, V., Amico, C., Giuliano, L., & Yakimov, M. (2003). Shewanella sp. GA-22, a psychrophilic hydrocarbonoclastic antarctic bacterium producing polyunsaturated fatty acids. Journal of Applied Microbiology, 95(5), 1124–1133.

Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.-P., Claverie, P., Collins, T., D'Amico, S., Dumont, J., Garsoux, G., & Georlette, D. (2000). Cold-adapted enzymes: From fundamentals to biotechnology. Trends in Biotechnology, 18(3), 103–107.

Gianinazzi, S., & Schüepp, H. (1994). Impact of Arbuscular Mycorrhizas on Substainable Agriculture and Natural Ecosystems. Springer Science & Business Media.

Glick, B. R. (1995a). The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology, 41(2), 109–117.

Glick, B. R. (1995b). The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology, 41(2), 109–117.

Glick, B. R., Liu, C., Ghosh, S., & Dumbroff, E. B. (1997). Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium Pseudomonas putida GR12-2. Soil Biology and Biochemistry, 29(8), 1233–1239.

Glick, B. R., Penrose, D. M., & Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. Journal of Theoretical Biology, 190(1), 63–68.

Goldstein, A. (1994). Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by gram-negative bacteria. Phosphate in Microorganisms: Cellular and Molecular Biology. ASM Press, Washington, DC, 197–203.

Goldstein, A. H. (1995). Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biological Agriculture & Horticulture, 12(2), 185–193.

Goldstein, A. H., Braverman, K., & Osorio, N. (1999). Evidence for mutualism between a plant growing in a phosphate-limited desert environment and a mineral phosphate solubilizing (MPS) rhizobacterium. FEMS Microbiology Ecology, 30(4), 295–300.

Goldstein, A., Rogers, R., & Mead, G. (1993). Separating phosphate from ores via bioprocessing. Biotechnology, 11, 1250–1254.

Gothwal, R., Nigam, V., Mohan, M., Sasmal, D., & Ghosh, P. (2006). Phosphate solubilization by rhizospheric bacterial isolates from economically important desert plants. Indian Journal of Microbiology, 46(4), 355.

Gupta, G., Srivastava, S., Khare, S., & Prakash, Vjij. (2014). Extremophiles: An overview of microorganism from extreme environment. International Journal of Agriculture, Environment and Biotechnology, 7(2), 371–380.

Gupta, R., Kumari, A., Sharma, S., Alzahrani, O.M., Noureldeen, A. and Darwish, H., 2022. Identification, characterization and optimization of phosphate solubilizing rhizobacteria (PSRB) from rice rhizosphere. Saudi Journal of Biological Sciences, 29(1), pp.35-42.

Haile, W., Fassil, A. and Asfaw, H., 1999, May. Studies on phosphate solubilizing ability of bacteria isolated from some Ethiopia soils. In Proc. of the 9th Annual Conference of the Biological Society of Ethiopia (p. 31).

Hall, J. A., Peirson, D., Ghosh, S., & Glick, B. (1996). Root elongation in various agronomic crops by the plant growth promoting rhizobacterium Pseudomonas putida GR12–2. Israel Journal of Plant Sciences, 44(1), 37–42.

Halvorson, H., Keynan, A., & Kornberg, H. (1990). Utilization of calcium phosphates for microbial growth at alkaline pH. Soil Biology and Biochemistry, 22(7), 887–890.

Hao, X., Zhu, Y.-G., Nybroe, O., & Nicolaisen, M. H. (2020). The composition and phosphorus cycling potential of bacterial communities associated with hyphae of Penicillium in soil are strongly affected by soil origin. Frontiers in Microbiology, 10, 2951.

Harder, W., & Veldkamp, H. (1971). Competition of marine psychrophilic bacteria at low temperatures. Antonie van Leeuwenhoek, 37, 51–63.

Hashim, N. H. F., Bharudin, I., Nguong, D. L. S., Higa, S., Bakar, F. D. A., Nathan, S., Rabu, A., Kawahara, H., Illias, R. M., & Najimudin, N. (2013). Characterization of Afp1, an antifreeze protein from the psychrophilic yeast Glaciozyma antarctica PI12. Extremophiles, 17, 63–73.

Hashim, N. H. F., Sulaiman, S., Abu Bakar, F. D., Illias, R. M., Kawahara, H., Najimudin, N., Mahadi, N. M., & Murad, A. M. A. (2014). Molecular cloning, expression and characterisation of Afp4, an antifreeze protein from Glaciozyma antarctica. Polar Biology, 37, 1495–1505.

Helmke, E., & Weyland, H. (2004). Psychrophilic versus psychrotolerant bacteria-occurrence and significance in polar and temperate marine habitats. Cellular and Molecular Biology, 50(5), 553–561.

Hough, D. W., & Danson, M. J. (1999). Extremozymes. Current Opinion in Chemical Biology, 3(1), 39–46.

Huang, L.-M., Jia, X.-X., Zhang, G.-L., & Shao, M.-A. (2017). Soil organic phosphorus transformation during ecosystem development: A review. Plant and Soil, 417, 17–42.

Hussain, A., Adnan, M., Hajira, S. I., Fahad, S., Saeed, M., Mian, I. A., Muhammad, M. W., Romman, M., Perveez, R., & Wahid, F. (2019). Combining phosphorus (P) with phosphate solubilizing bacteria (PSB) improved wheat yield and P uptake in alkaline soil. Pure and Applied Biology. Vol. 8, Issue 2, pp1809-1817.

Ichriani, G. I., Syehfani, -, Nuraini, Y., & Handayanto, E. (2018). Formulation of biochar-compost and phosphate solubilizing fungi from oil palm empty fruit bunch to improve growth of maize in an ultisol of central kalimantan. Journal of Ecological Engineering, 19(6), 45–55.

Illmer, P., & Schinner, F. (1995). Solubilization of inorganic calcium phosphates—Solubilization mechanisms. Soil Biology and Biochemistry, 27(3), 257–263.

Jackson, M., 1973. Soil chemical analysis prentice hall of India. Pvt. Ltd., New Delhi, p. 498.

Jarosch, K. A., Doolette, A. L., Smernik, R. J., Tamburini, F., Frossard, E., & Bünemann, E. K. (2015). Characterisation of soil organic phosphorus in NaOH-EDTA extracts: A comparison of 31P NMR spectroscopy and enzyme addition assays. Soil Biology and Biochemistry, 91, 298–309.

Joshi, S., & Satyanarayana, T. (2013). Biotechnology of cold-active proteases. Biology, 2(2), 755–783.

Junge, K., Eicken, H., & Deming, J. W. (2004). Bacterial activity at− 2 to− 20 C in Arctic wintertime sea ice. Applied and Environmental Microbiology, 70(1), 550–557.

Kang, S.-M., Khan, A. L., Waqas, M., Asaf, S., Lee, K.-E., Park, Y.-G., Kim, A.-Y., Khan, M. A., You, Y.-H., & Lee, I.-J. (2019). Integrated phytohormone production by the plant growth-promoting rhizobacterium Bacillus tequilensis SSB07 induced thermotolerance in soybean. Journal of Plant Interactions, 14(1), 416–423.

Kapulnik, Y., Gafny, R., & Okon, Y. (1985). Effect of Azospirillum spp. Inoculation on root development and uptake in wheat (Triticum aestivum cv. Miriam) in hydroponic systems. Canadian Journal of Botany, 63(3), 627–631.

Karan, R., Singh, R., Kapoor, S., & Khare, S. K. (2011). Gene identification and molecular characterization of solvent stable protease from a moderately haloalkaliphilic bacterium, Geomicrobium sp. EMB2. J Microbiol Biotechnol, 21(2), 129–135.

Kasana, R. C. (2010). Proteases from psychrotrophs: An overview. Critical Reviews in Microbiology, 36(2), 134–145.

Kawahara, H. (2013). Characterizations of functions of biological materials having controlling-ability against ice crystal growth. In Advanced Topics on Crystal Growth (pp. 119–143). InTech Rijeka, Croatia.

Kemkar, N. A. (2006). Environmental peacemaking: Ending conflict between India and Pakistan on the Siachen Glacier through the creation of a transboundary peace park. Stan. Envtl. LJ, 25, 67.

Keneni, A., Assefa, F. and Prabu, P.C., 2010. Isolation of phosphate solubilizing bacteria from the rhizosphere of faba bean of Ethiopia and their abilities on solubilizing insoluble phosphates. Journal of Agricultural Science and Technology, 12(1), pp.79-89.

Keto-Timonen, R., Hietala, N., Palonen, E., Hakakorpi, A., Lindström, M., & Korkeala, H. (2016). Cold shock proteins: A minireview with special emphasis on Csp-family of enteropathogenic Yersinia. Frontiers in Microbiology, 7, 1151.

Khan, I., Fahad, S., Wu, L., Zhou, W., Xu, P., Sun, Z., Salam, A., Imran, M., Jiang, M., & Kuzyakov, Y. (2019). Labile organic matter intensifies phosphorous mobilization in paddy soils by microbial iron (III) reduction. Geoderma, 352, 185–196.

Khan, M. S., Zaidi, A., Ahemad, M., Oves, M., & Wani, P. A. (2010). Plant growth promotion by phosphate solubilizing fungi–current perspective. Archives of Agronomy and Soil Science, 56(1), 73–98.

Khan, M., Zaidi, A., & Aamil, M. (2002). Biocontrol of fungal pathogens by the use of plant growth promoting rhizobacteria and nitrogen fixing microorganisms. Ind J Bot Soc, 81, 255–263.

Khan, S. U., Hooda, P. S., Blackwell, M. S., & Busquets, R. (2019). Microbial biomass responses to soil drying-rewetting and phosphorus leaching. Frontiers in Environmental Science, 7, 133.

Kim, H. J., Lee, J. H., Hur, Y. B., Lee, C. W., Park, S.-H., & Koo, B.-W. (2017). Marine antifreeze proteins: Structure, function, and application to cryopreservation as a potential cryoprotectant. Marine Drugs, 15(2), 27.

Kim, K. Y., Jordan, D., & Krishnan, H. B. (1997). Rahnella aquatilis, a bacterium isolated from soybean rhizosphere, can solubilize hydroxyapatite. FEMS Microbiology Letters, 153(2), 273–277.

Kishore, N., Pindi, P. K., & Ram Reddy, S. (2015). Phosphate-solubilizing microorganisms: A critical review. Plant Biology and Biotechnology: Volume I: Plant Diversity, Organization, Function and Improvement, 307–333.

Kloepper, J., Lifshitz, R., & Schroth, M. (1988). Pseudomonas inoculants to benefit plant production. ISI ATLAS SCI: ANIM. PLANT SCI., 1(1), 60–64.

Kloepper, J. W. (1978). Plant growth-promoting rhizobacteria on radishes. 2, 879–882.

Kloepper, J. W., Lifshitz, R., & Zablotowicz, R. M. (1989). Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology, 7(2), 39–44.

Kour, D., Rana, K.L., Kaur, T., Sheikh, I., Yadav, A.N., Kumar, V., Dhaliwal, H.S. and Saxena, A.K., 2020. Microbe-mediated alleviation of drought stress and acquisition of phosphorus in great millet (Sorghum bicolour L.) by drought-adaptive and phosphorus-solubilizing microbes. Biocatalysis and Agricultural Biotechnology, 23, p.101501.

Kpomblekou-a, K., & Tabatabai, M. (1994). Effect of organic acids on release of phosphorus from phosphate rocks1. Soil Science, 158(6), 442–453.

Kraiker, H., & Burch, R. (1973). Thin layer chromatography of Krebs cycle acids.

Krasilnikov, N. (1961). On the role of soil bacteria in plant nutrition. The Journal of General and Applied Microbiology, 7(2), 128–144.

Krishnaraj, P., & Dahale, S. (2014). Mineral phosphate solubilization: Concepts and prospects in sustainable agriculture. 80(2), 389–405.

Krishnaraj, P., & Goldstein, A. (2001). Cloning of a Serratia marcescens DNA fragment that induces quinoprotein glucose dehydrogenase-mediated gluconic acid production in Escherichia coli in the presence of stationary phase Serratia marcescens. FEMS Microbiology Letters, 205(2), 215–220.

Kucey, R. M. N., Janzen, H., & Leggett, M. (1989). Microbially mediated increases in plant-available phosphorus. Advances in Agronomy, 42, 199–228.

Kuma Megersa, M. (2019). Effects of lime and phosphorus levels on selected soil chemical properties and bread wheat (tritium aestivum L.) yield on acidic soil of Banja district, North Western Ethiopia.

Kumar, L., Awasthi, G., & Singh, B. (2011). Extremophiles: A novel source of industrially important enzymes. Biotechnology, 10(2), 121–135.

Kumar, M., Yadav, A. N., Saxena, R., Rai, P. K., Paul, D., & Tomar, R. S. (2021). Novel methanotrophic and methanogenic bacterial communities from diverse ecosystems and their impact on environment. Biocatalysis and Agricultural Biotechnology, 33, 102005.

Kumar, R., & Shastri, B. (2017). Role of phosphate-solubilising microorganisms in sustainable agricultural development. In Agro-Environmental Sustainability: Volume 1: Managing Crop Health (pp. 271–303). Springer.

Lasztity, R. (2009). Micro-organisms important in food microbiology. Food Quality and Standards, 3, 26–40.

Lemanceau, P. (1992). Effets bénéfiques de rhizobactéries sur les plantes: Exemple des Pseudomonas spp fluorescents. Agronomie, 12(6), 413–437.

Leong, J. (1986). Siderophores: Their biochemistry and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology, 24(1), 187–209.

Linu, M., Asok, A. K., Thampi, M., Sreekumar, J., & Jisha, M. (2019). Plant growth promoting traits of indigenous phosphate solubilizing Pseudomonas aeruginosa isolates from Chilli (Capsicum annuum L.) Rhizosphere. Communications in Soil Science and Plant Analysis, 50(4), 444–457.

Liu, C., Mou, L., Yi, J., Wang, J., Liu, A., & Yu, J. (2019). The eno gene of Burkholderia cenocepacia strain 71-2 is involved in phosphate solubilization. Current Microbiology, 76, 495–502.

Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1666(1–2), 142–157.

Ludueña, L. M., Anzuay, M. S., Angelini, J. G., Barros, G., Luna, M. F., Monge, M. del P., Fabra, A., & Taurian, T. (2017). Role of bacterial pyrroloquinoline quinone in phosphate solubilizing ability and in plant growth promotion on strain Serratia sp. S119. Symbiosis, 72, 31–43.

MacElroy, R. D. (1974). Some comments on the evolution of extremophiles. Biosystems, 6(1), 74–75.

Margesin, R., Neuner, G., & Storey, K. (2007). Cold-loving microbes, plants, and animals—Fundamental and applied aspects. Naturwissenschaften, 94, 77–99.

Martínez, O., Crowley, D., Mora, M., & Jorquera, M. A. (2015). Short-term study shows that phytate-mineralizing rhizobacteria inoculation affects the biomass, phosphorus (P) uptake and rhizosphere properties of cereal plants. Journal of Soil Science and Plant Nutrition, 15(1), 153–166.

Mehta, P., Sharma, R., Putatunda, C., & Walia, A. (2019). Endophytic fungi: Role in phosphate solubilization. Advances in Endophytic Fungal Research: Present Status and Future Challenges, 183–209.

Mehta, S., & Nautiyal, C. S. (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology, 43, 51–56.

Morita, R., & Moyer, C. (2000). Origin of psychrophiles. Schulze, and GD Tilman (Ed.), Encyclopedia of Biodiversity, 4, 917–924.

Morita, R. Y. (1975). Psychrophilic bacteria. Bacteriological Reviews, 39(2), 144–167.

Murgese, P., Santamaria, P., Leoni, B., & Crecchio, C. (2020). Ameliorative effects of PGPB on yield, physiological parameters, and nutrient transporter genes expression in barattiere (Cucumis melo L.). Journal of Soil Science and Plant Nutrition, 20, 784–793.

Myo, E. M., Liu, B., Ma, J., Shi, L., Jiang, M., Zhang, K., & Ge, B. (2019). Evaluation of Bacillus velezensis NKG-2 for bio-control activities against fungal diseases and potential plant growth promotion. Biological Control, 134, 23–31.

Nakagawa, T., Nagaoka, T., Taniguchi, S., Miyaji, T., & Tomizuka, N. (2004). Isolation and characterization of psychrophilic yeasts producing cold-adapted pectinolytic enzymes. Letters in Applied Microbiology, 38(5), 383–387.

Nannipieri, P., Giagnoni, L., Landi, L., & Renella, G. (2011). Role of phosphatase enzymes in soil. Phosphorus in Action: Biological Processes in Soil Phosphorus Cycling, 215–243.

Nichols, D. S., Miller, M. R., Davies, N. W., Goodchild, A., Raftery, M., & Cavicchioli, R. (2004). Cold adaptation in the Antarctic archaeon Methanococcoides burtonii involves membrane lipid unsaturation. Journal of Bacteriology, 186(24), 8508–8515.

Nor, N. M., Hashim, N. H. F., Quay, D. H. X., Mahadi, N. M., Illias, R. M., Bakar, F. D. A., & Murad, A. M. A. (2020). Functional and structural analyses of an expansin-like protein from the antarctic yeast Glaciozyma antarctica PI12 reveal strategies of nutrient scavenging in the sea ice environment. International Journal of Biological Macromolecules, 144, 231–241.

Nur Athirah, Y., Abu Bakar, F., Illias, R., Mahadi, N., & Murad, A. A. (2015). IN SILICO CHARACTERISATION OF THE Glaciozyma antarctica GENOME: MINING THE MOLECULAR CHAPERONES. Malaysian Applied Biology, 44(1).

Okon, Y. (1993). Azospirillum/plant associations. CRC Press.

Okuda, M., Sumitomo, N., Takimura, Y., Ogawa, A., Saeki, K., Kawai, S., Kobayashi, T., & Ito, S. (2004). A new subtilisin family: Nucleotide and deduced amino acid sequences of new high-molecular-mass alkaline proteases from Bacillus spp. Extremophiles, 8, 229–235.

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., & Dowling, D. N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Frontiers in Microbiology, 6, 745.

Pal, S. S. (1998). Interactions of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. Plant and Soil, 198, 169–177.

Park, K., Lee, C., & Son, H. (2009). Mechanism of insoluble phosphate solubilization by Pseudomonas fluorescens RAF15 isolated from ginseng rhizosphere and its plant growth-promoting activities. Letters in Applied Microbiology, 49(2), 222–228.

Parks, E. J., Olson, G. J., Brinckman, F. E., & Baldi, F. (1990). Characterization by high performance liquid chromatography (HPLC) of the solubilization of phosphorus in iron ore by a fungus. Journal of Industrial Microbiology and Biotechnology, 5(2–3), 183–189.

Paul, D., & Sinha, S. N. (2017). Isolation and characterization of phosphate solubilizing bacterium Pseudomonas aeruginosa KUPSB12 with antibacterial potential from river Ganga, India. Annals of Agrarian Science, 15(1), 130–136.

Perea Rojas, Y. del C., Arias, R. M., Medel Ortiz, R., Trejo Aguilar, D., Heredia, G., & Rodríguez Yon, Y. (2019). Effects of native arbuscular mycorrhizal and phosphate-solubilizing fungi on coffee plants. Agroforestry Systems, 93, 961–972.

Piccini, D., & Azcon, R. (1987). Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization of Bayovar rock phosphate by alfalfa plants using a sand-vermiculite medium. Plant and Soil, 101, 45–50.

Ponmurugan, P., & Gopi, C. (2006). Distribution pattern and screening of phosphate solubilizing bacteria isolated from different food and forage crops. Journal of Agronomy, 5(4), 600–604.

Prabhu, N., Borkar, S., & Garg, S. (2018). Phosphate solubilization mechanisms in alkaliphilic bacterium Bacillus marisflavi FA7. Current Science, 845–853.

Price, P. B., & Sowers, T. (2004). Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. Proceedings of the National Academy of Sciences, 101(13), 4631–4636.

Pulicherla, K., Ghosh, M., Kumar, P., & Rao, K. (2011). Psychrozymes-the next generation industrial enzymes. J Marine Sci Res Development, 1(2).

Puri, A., Padda, K. P., & Chanway, C. P. (2020). In vitro and in vivo analyses of plant-growth-promoting potential of bacteria naturally associated with spruce trees growing on nutrient-poor soils. Applied Soil Ecology, 149, 103538.

Qu, P., Fu, F.-X., Kling, J. D., Huh, M., Wang, X., & Hutchins, D. A. (2019). Distinct responses of the nitrogen-Fixing marine cyanobacterium Trichodesmium to a thermally variable environment as a function of phosphorus availability. Frontiers in Microbiology, 10, 1282.

Rady, M. M., El-Shewy, A. A., Seif El-Yazal, M. A., & Abd El-Gawwad, I. F. (2020). Integrative application of soil P-solubilizing bacteria and foliar nano P improves Phaseolus vulgaris plant performance and antioxidative defense system components under calcareous soil conditions. Journal of Soil Science and Plant Nutrition, 20, 820–839.

Rafiq, M., Hayat, M., Anesio, A. M., Jamil, S. U. U., Hassan, N., Shah, A. A., & Hasan, F. (2017). Recovery of metallo-tolerant and antibiotic resistant psychrophilic bacteria from Siachen glacier, Pakistan. PloS One, 12(7), e0178180.

Rafiq, M., Hayat, M., Zada, S., Sajjad, W., Hassan, N., & Hasan, F. (2019). Geochemistry and bacterial recovery from Hindu Kush Range glacier and their potential for metal resistance and antibiotic production. Geomicrobiology Journal, 36(4), 326–338.

Rajasankar, R., Manju Gayathry, G., Sathiavelu, A., Ramalingam, C. and Saravanan, V.S., 2013. Pesticide tolerant and phosphorus solubilizing Pseudomonas sp. strain SGRAJ09 isolated from pesticides treated Achillea clavennae rhizosphere soil. Ecotoxicology, 22, pp.707-717.

Rajwar, J., Chandra, R., Suyal, D. C., Tomer, S., Kumar, S., & Goel, R. (2018). Comparative phosphate solubilizing efficiency of psychrotolerant Pseudomonas jesenii MP1 and Acinetobacter sp. ST02 against chickpea for sustainable hill agriculture. Biologia, 73, 793–802.

Rao, N. S. (2016). Advances in agricultural microbiology. Elsevier.

Rasmussen, R. S., & Morrissey, M. T. (2007). Marine biotechnology for production of food ingredients. Advances in Food and Nutrition Research, 52, 237–292.

Rasul, M., Yasmin, S., Suleman, M., Zaheer, A., Reitz, T., Tarkka, M. T., Islam, E., & Mirza, M. S. (2019). Glucose dehydrogenase gene containing phosphobacteria for biofortification of Phosphorus with growth promotion of rice. Microbiological Research, 223, 1–12.

Rawat, P., Shankhdhar, D., & Shankhdhar, S. (2020). Plant growth-promoting rhizobacteria: A booster for ameliorating soil health and agriculture production. Soil Health, 47–68.

Reang, L., Bhatt, S., Tomar, R.S., Joshi, K., Padhiyar, S., Vyas, U.M. and Kheni, J.K., 2022. Plant growth promoting characteristics of halophilic and halotolerant bacteria isolated from coastal regions of Saurashtra Gujarat. Scientific reports, 12(1), p.4699.

Renella, G., Egamberdiyeva, D., Landi, L., Mench, M., & Nannipieri, P. (2006). Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biology and Biochemistry, 38(4), 702–708.

Reyes, I., Bernier, L., Simard, R. R., & Antoun, H. (1999). Effect of nitrogen source on the solubilization of different inorganic phosphates by an isolate of Penicillium rugulosum and two UV-induced mutants. FEMS Microbiology Ecology, 28(3), 281–290.

Rfaki, A., Zennouhi, O., Aliyat, F.Z., Nassiri, L. and Ibijbijen, J., 2020. Isolation, selection and characterization of root-associated rock phosphate solubilizing bacteria in moroccan wheat (Triticum aestivum L.). Geomicrobiology Journal, 37(3), pp.230-241.

RI, P. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiologiya, 17, 362–370.

Richardson, A. E. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Functional Plant Biology, 28(9), 897–906.

Rodríguez, H., Fraga, R., Gonzalez, T., & Bashan, Y. (2006a). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and Soil, 287, 15–21.

Rodríguez, H., Fraga, R., Gonzalez, T., & Bashan, Y. (2006b). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and Soil, 287, 15–21.

Rodriguez-Sanchez, V., Guzman-Moreno, J., Rodriguez-Gonzalez, V., Flores-de la Torre, J.A., Ramirez-Santoyo, R.M., Vidales-Rodriguez, L.E., 2017. Biosorption of lead phosphates by lead-tolerant bacteria as a mechanism for lead immobilization. World J. Microbiol. Biotechnol. 33 (8), 150

Rose, T. J., Hardiputra, B., & Rengel, Z. (2010). Wheat, canola and grain legume access to soil phosphorus fractions differs in soils with contrasting phosphorus dynamics. Plant and Soil, 326, 159–170.

Rothschild, L. J., & Mancinelli, R. L. (2001). Life in extreme environments. Nature, 409(6823), 1092–1101.

Roy, S., & Roy, M. (2019). Characterization of plant growth promoting feature of a neutromesophilic, facultatively chemolithoautotrophic, sulphur oxidizing bacterium Delftia sp. Strain SR4 isolated from coal mine spoil. International Journal of Phytoremediation, 21(6), 531–540.

Russell, N. (1990). Cold adaptation of microorganisms. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 326(1237), 595–611.

Sahay, H., Yadav, A. N., Singh, A. K., Singh, S., Kaushik, R., & Saxena, A. K. (2017). Hot springs of Indian Himalayas: Potential sources of microbial diversity and thermostable hydrolytic enzymes. 3 Biotech, 7, 1–11.

Sakamoto, T., Ihara, H., Kozaki, S., & Kawasaki, H. (2003). A cold-adapted endo-arabinanase from Penicillium chrysogenum. Biochimica et Biophysica Acta (BBA)-General Subjects, 1624(1–3), 70–75.

Saleemi, M., Kiani, M.Z., Sultan, T., Khalid, A. and Mahmood, S., 2017. Integrated effect of plant growth-promoting rhizobacteria and phosphate-solubilizing microorganisms on growth of wheat (Triticum aestivum L.) under rainfed condition. Agriculture & Food Security, 6(1), pp.1-8.

Salwan, R., & Kasana, R. C. (2013). Purification and characterization of an extracellular low temperature-active and alkaline stable peptidase from psychrotrophic Acinetobacter sp. MN 12 MTCC (10786). Indian Journal of Microbiology, 53, 63–69.

Santiago, M., Ramírez-Sarmiento, C. A., Zamora, R. A., & Parra, L. P. (2016). Discovery, molecular mechanisms, and industrial applications of cold-active enzymes. Frontiers in Microbiology, 7, 1408.

Saranya, K., Sundaramanickam, A., Shekhar, S., Meena, M., Sathishkumar, R.S. and Balasubramanian, T., 2018. Biosorption of multi-heavy metals by coral associated phosphate solubilising bacteria Cronobacter muytjensii KSCAS2. Journal of environmental management, 222, pp.396-401.

SARIG, S., OKON, Y., & Blum, A. (1990). Promotion of leaf area development and yield in Sorghum bicolor inoculated with Azospirillum brasilense. Symbiosis.

Sattar, M., & Gaur, A. (1987). Production of auxins and gibberellins by phosphate-dissolving microorganisms. Zentralblatt Für Mikrobiologie, 142(5), 393–395.

Saxena, A., Padaria, J., Gurjar, G., Yadav, A., Lone, S., & Tripathi, M. (2020). Insecticidal formulation of novel strain of Bacillus thuringiensis AK 47. Indian Patent, 340541, 388.

Saxena, A., Yadav, A. N., Rajawat, M., Kaushik, R., Kumar, R., Kumar, M., Prasanna, R., & Shukla, L. (2016). Microbial diversity of extreme regions: An unseen heritage and wealth. Indian Journal of Plant Genetic Resources, 29(3), 246–248.

Schmidt-Nielsen, S. (1902). Ueber einige psychrophile Mikroorganismen und ihr Vorkommen. Zentr. Bakteriol. Parasitenkd. Infektionsk. Hyg. Abt., 9, 145–147.

Selvapandiyan, A., & Bhatnagar, R. K. (1994). Isolation of a glyphosate-metabolising Pseudomonas: Detection, partial purification and localisation of carbon-phosphorus lyase. Applied Microbiology and Biotechnology, 40, 876–882.

Sharan, A., Shikha, & Darmwal, N. S. (2008). Efficient phosphorus solubilization by mutant strain of Xanthomonas campestris using different carbon, nitrogen and phosphorus sources. World Journal of Microbiology and Biotechnology, 24, 3087–3090.

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013a). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus, 2, 1–14.

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013b). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus, 2, 1–14.

Shen, L., Yao, T., Xu, B., Wang, H., Jiao, N., Kang, S., Liu, X., & Liu, Y. (2012). Variation of culturable bacteria along depth in the East Rongbuk ice core, Mt. Everest. Geoscience Frontiers, 3(3), 327–334.

Shivaji, S., Pratibha, M., Sailaja, B., Hara Kishore, K., Singh, A. K., Begum, Z., Anarasi, U., Prabagaran, S., Reddy, G., & Srinivas, T. (2011). Bacterial diversity of soil in the vicinity of Pindari glacier, Himalayan mountain ranges, India, using culturable bacteria and soil 16S rRNA gene clones. Extremophiles, 15, 1–22.

Shrivastava, M., Srivastava, P., & D'souza, S. (2018). Phosphate-solubilizing microbes: Diversity and phosphates solubilization mechanism. Role of Rhizospheric Microbes in Soil: Volume 2: Nutrient Management and Crop Improvement, 137–165.

Shulse, C. N., Chovatia, M., Agosto, C., Wang, G., Hamilton, M., Deutsch, S., Yoshikuni, Y., & Blow, M. J. (2019). Engineered root bacteria release plant-available phosphate from phytate. Applied and Environmental Microbiology, 85(18), e01210-19.

Siliakus, M. F., van der Oost, J., & Kengen, S. W. (2017). Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. Extremophiles, 21, 651–670.

Singh, P., & Banik, R. M. (2019). Effect of purified alkaline phosphatase from Bacillus licheniformis on growth of Zea mays L. Plant Science Today, 6(sp1), 583–589.

Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K., & Garg, S. K. (2011). A novel psychrotrophic, solvent tolerant Pseudomonas putida SKG-1 and solvent stability of its psychro-thermoalkalistable protease. Process Biochemistry, 46(7), 1430–1435.

Sivan, A., & Chet, I. (1992). Microbial control of plant diseases. Environmental Microbiology, 335–354.

Son, T. T. N., Diep, C. N., & Giang, T. T. M. (2006). Effect of Bradyrhizobia and phosphate solubilizing bacteria application on soybean in rotational system in the Mekong delta. Omonrice, 14(2), 48–57.

Song, O.-R., Lee, S.-J., Lee, Y.-S., Lee, S.-C., Kim, K.-K., & Choi, Y.-L. (2008). Solubilization of insoluble inorganic phosphate by Burkholderia cepacia DA23 isolated from cultivated soil. Brazilian Journal of Microbiology, 39, 151–156.

Sowmya, S., Rekha, P.D. and Arun, A.B., 2014. Uranium (VI) bioprecipitation mediated by a phosphate solubilizing Acinetobacter sp. YU-SS-SB-29 isolated from a high natural background radiation site. International Biodeterioration & Biodegradation, 94, pp.134-140.

Sulbarán, M., Pérez, E., Ball, M. M., Bahsas, A., & Yarzábal, L. A. (2009). Characterization of the mineral phosphate-solubilizing activity of Pantoea aglomerans MMB051 isolated from an iron-rich soil in southeastern Venezuela (Bolivar state). Current Microbiology, 58, 378–383.

Suleman, M., Yasmin, S., Rasul, M., Yahya, M., Atta, B. M., & Mirza, M. S. (2018). Phosphate solubilizing bacteria with glucose dehydrogenase gene for phosphorus uptake and beneficial effects on wheat. PloS One, 13(9), e0204408.

Sundram, K.P., 1994. Fertilizers News, Fertilizer Association of India. New Delhi.

Suslow, T. (1982). Role of root-colonizing bacteria in plant growth. Phytopathogenic Prokaryotes, 187–223.

Swetha, S. and Padmavathi, T., 2016. Study of Acid Phosphatase in Solubilization of Inorganic Phosphates by. Polish Journal of Microbiology, 65(4), pp.407-412.

Tarafdar, J. C., Yadav, R. S., & Meena, S. C. (2001). Comparative efficiency of acid phosphatase originated from plant and fungal sources. Journal of Plant Nutrition and Soil Science, 164(3), 279–282.

Teng, Z., Chen, Z., Zhang, Q., Yao, Y., Song, M., & Li, M. (2019). Isolation and characterization of phosphate solubilizing bacteria from rhizosphere soils of the Yeyahu Wetland in Beijing, China. Environmental Science and Pollution Research, 26, 33976–33987.

Thaller, M. C., Berlutti, F., Schippa, S., Lombardi, G., & Rossolini, G. M. (1994). Characterization and sequence of PhoC, the principal phosphate-irrepressible acid phosphatase of Morganella morganii. Microbiology, 140(6), 1341–1350.

Thomas Sims, J., & Pierzynski, G. M. (2005). Chemistry of phosphorus in soils. Chemical Processes in Soils, 8, 151–192.

Tomer, S., Suyal, D. C., & Goel, R. (2016). Biofertilizers: A timely approach for sustainable agriculture. Plant-Microbe Interaction: An Approach to Sustainable Agriculture, 375–395.

Toro, M., Azcon, R., & Barea, J. (1997). Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability ((sup32) P) and nutrient cycling. Applied and Environmental Microbiology, 63(11), 4408–4412.

Turchetti, B., Marconi, G., Sannino, C., Buzzini, P., & Albertini, E. (2020). DNA methylation changes induced by cold in psychrophilic and psychrotolerant Naganishia yeast species. Microorganisms, 8(2), 296.

Turchetti, B., Thomas Hall, S. R., Connell, L. B., Branda, E., Buzzini, P., Theelen, B., Müller, W. H., & Boekhout, T. (2011). Psychrophilic yeasts from Antarctica and European glaciers: Description of Glaciozyma gen. Nov., Glaciozyma martinii sp. Nov. And Glaciozyma watsonii sp. Nov. Extremophiles, 15, 573–586.

Valverde, A., Burgos, A., Fiscella, T., Rivas, R., Velazquez, E., Rodríguez-Barrueco, C., Cervantes, E., Chamber, M., & Igual, J.-M. (2007). Differential effects of coinoculations with Pseudomonas jessenii PS06 (a phosphate-solubilizing bacterium) and Mesorhizobium ciceri C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions. 43–50.

Van Den Burg, B. (2003). Extremophiles as a source for novel enzymes. Current Opinion in Microbiology, 6(3), 213–218.

Van Kauwenbergh, S. (2006). Fertilizer Mineral Resources of Africa. IFDC Publication R-16, IFDC–An International Center for Soil Fertility and Agricultural Development, Muscle Shoals, Alabama.

Vazquez, P., Holguin, G., Puente, M., Lopez-Cortes, A., & Bashan, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biology and Fertility of Soils, 30, 460–468.

Vorobyova, E., Soina, V., Gorlenko, M., Minkovskaya, N., Zalinova, N., Mamukelashvili, A., Gilichinsky, D., Rivkina, E., & Vishnivetskaya, T. (1997). The deep cold biosphere: Facts and hypothesis. FEMS Microbiology Reviews, 20(3–4), 277–290.

Wan, W., Qin, Y., Wu, H., Zuo, W., He, H., Tan, J., Wang, Y., & He, D. (2020). Isolation and characterization of phosphorus solubilizing bacteria with multiple phosphorus sources utilizing capability and their potential for lead immobilization in soil. Frontiers in Microbiology, 11, 752.

Wang, Y.-Y., Li, P.-S., Zhang, B.-X., Wang, Y.-P., Meng, J., Gao, Y.-F., He, X.-M., & Hu, X.-M. (2020). Identification of phosphate-solubilizing microorganisms and

determination of their phosphate-solubilizing activity and growth-promoting capability. BioResources, 15(2), 2560–2578.

Wani, P. A., Khan, M. S., & Zaidi, A. (2007a). Chromium reduction, plant growth–promoting potentials, and metal solubilizatrion by Bacillus sp. Isolated from alluvial soil. Current Microbiology, 54, 237–243.

Wani, P. A., Khan, M. S., & Zaidi, A. (2007b). Synergistic effects of the inoculation with nitrogen-fixing and phosphate-solubilizing rhizobacteria on the performance of field-grown chickpea. Journal of Plant Nutrition and Soil Science, 170(2), 283–287.

Wani, P. A., Khan, M. S., & Zaidi, A. (2008). Chromium-reducing and plant growth-promoting Mesorhizobium improves chickpea growth in chromium-amended soil. Biotechnology Letters, 30, 159–163.

Wani, P., Khan, M., & Zaidi, A. (2007). Co-inoculation of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea. Acta Agronomica Hungarica, 55(3), 315–323.

Whitelaw, M. A. (1999). Growth promotion of plants inoculated with phosphate-solubilizing fungi. Advances in Agronomy, 69, 99–151.

Xie, H., Pasternak, J., & Glick, B. R. (1996). Isolation and characterization of mutants of the plant growth-promoting rhizobacterium Pseudomonas putida GR12-2 that overproduce indoleacetic acid. Current Microbiology, 32, 67–71.

Yadav, A. N., Gulati, S., Sharma, D., Singh, R. N., Rajawat, M. V. S., Kumar, R., Dey, R., Pal, K. K., Kaushik, R., & Saxena, A. K. (2019). Seasonal variations in culturable archaea and their plant growth promoting attributes to predict their role in establishment of vegetation in Rann of Kutch. Biologia, 74, 1031–1043.

Yadav, A. N., Kour, D., Rana, K. L., Yadav, N., Singh, B., Chauhan, V. S., Rastegari, A. A., Hesham, A. E.-L., & Gupta, V. K. (2019). Metabolic engineering to synthetic biology of secondary metabolites production. In New and future developments in microbial biotechnology and bioengineering (pp. 279–320). Elsevier.

Yadav, A. N., Sachan, S. G., Verma, P., & Saxena, A. K. (2015). Prospecting cold deserts of north western Himalayas for microbial diversity and plant growth promoting attributes. Journal of Bioscience and Bioengineering, 119(6), 683–693.

Yadav, A. N., Sachan, S. G., Verma, P., Tyagi, S. P., Kaushik, R., & Saxena, A. K. (2015). Culturable diversity and functional annotation of psychrotrophic bacteria from cold desert of Leh Ladakh (India). World Journal of Microbiology and Biotechnology, 31, 95–108.

Yadav, A. N., Verma, P., Kumar, M., Pal, K. K., Dey, R., Gupta, A., Padaria, J. C., Gujar, G. T., Kumar, S., & Suman, A. (2015). Diversity and phylogenetic profiling of niche-specific Bacilli from extreme environments of India. Annals of Microbiology, 65(2), 611–629.

Yadav, H., Gothwal, R.K., Nigam, V.K., Sinha-Roy, S. and Ghosh, P., 2013. Optimization of culture conditions for phosphate solubilization by a thermo-tolerant phosphate-solubilizing bacterium Brevibacillus sp. BISR-HY65 isolated from phosphate mines. Biocatalysis and Agricultural Biotechnology, 2(3), pp.217-225.

Yadav, K., & Dadarwal, K. (1997). Phosphate slubilization and mobilization through soil microorganisms. Biotechnological Approaches in Soil Microorganisms for Sustainable Crop Production., 293–308.

Yayanos, A. A. (1986). Evolutional and ecological implications of the properties of deep-sea barophilic bacteria. Proceedings of the National Academy of Sciences, 83(24), 9542–9546.

Young, C., Chang, C., Chen, L., & Chao, C. (1998). Characterization of the nitrogen fixation and ferric phosphate solubilizing bacteria isolated from a Taiwan soil. JOURNAL-CHINESE AGRICULTURAL CHEMICAL SOCIETY, 36, 201–210.

Young, C.-C. (1994). Selection and application of biofertilizers in Taiwan. ASPAC Food & Fertilizer Technology Center.

Zaheer, A., Malik, A., Sher, A., Qaisrani, M. M., Mehmood, A., Khan, S. U., Ashraf, M., Mirza, Z., Karim, S., & Rasool, M. (2019). Isolation, characterization, and effect of phosphate-zinc-solubilizing bacterial strains on chickpea (Cicer arietinum L.) growth. Saudi Journal of Biological Sciences, 26(5), 1061–1067.

Zaidi, A. (1999). Synergistic interactions of nitrogen fixing microorganisms with phosphate mobilizing microorganisms.

Zaidi, A., & Khan, M. S. (2007). Stimulatory effects of dual inoculation with phosphate solubilising microorganisms and arbuscular mycorrhizal fungus on chickpea. Australian Journal of Experimental Agriculture, 47(8), 1016–1022.

Zakhia, F., Jungblut, A.-D., Taton, A., Vincent, W. F., & Wilmotte, A. (2008). Cyanobacteria in cold ecosystems. Psychrophiles: From Biodiversity to Biotechnology, 121–135.

Zeng, X., Xiao, X., Wang, P., & Wang, R. (2004). Screening and characterization of psychrotrophic, lipolytic bacteria from deep-sea sediments. Journal of Microbiology and Biotechnology, 14(5), 952–958.

Zhang, L., Fan, J., Feng, G., & Declerck, S. (2019). The arbuscular mycorrhizal fungus Rhizophagus irregularis MUCL 43194 induces the gene expression of citrate synthase in the tricarboxylic acid cycle of the phosphate-solubilizing bacterium Rahnella aquatilis HX2. Mycorrhiza, 29, 69–75.

Zhang, L., Feng, G., & Declerck, S. (2018). Signal beyond nutrient, fructose, exuded by an arbuscular mycorrhizal fungus triggers phytate mineralization by a phosphate solubilizing bacterium. The ISME Journal, 12(10), 2339–2351.

Zhang, X., Yao, T., Tian, L., Xu, S., & An, L. (2008). Phylogenetic and physiological diversity of bacteria isolated from Puruogangri ice core. Microbial Ecology, 55, 476–488.

Zhao, K., Penttinen, P., Zhang, X., Ao, X., Liu, M., Yu, X., & Chen, Q. (2014). Maize rhizosphere in Sichuan, China, hosts plant growth promoting Burkholderia cepacia with phosphate solubilizing and antifungal abilities. Microbiological Research, 169(1), 76–82.

Zhou, W., Liu, D., Kong, W. and Zhang, Y., 2014. Bioremoval and recovery of Cd (II) by Pseudoalteromonas sp. SCSE709-6: comparative study on growing and grown cells. Bioresource technology, 165, pp.145-151

Zhu, F., Qu, L., Hong, X. and Sun, X., 2011. Isolation and characterization of a phosphate-solubilizing halophilic bacterium Kushneria sp. YCWA18 from Daqiao Saltern on the coast of Yellow Sea of China. Evidence-Based Complementary and Alternative Medicine, 2011.

Zhu, J., Li, M., & Whelan, M. (2018). Phosphorus activators contribute to legacy phosphorus availability in agricultural soils: A review. Science of the Total Environment, 612, 522–537.

Zhu, X., Lee, S. Y., Yang, W. T., Lee, S.-W., Baek, D., Li, M., & Kim, D.-H. (2019). The Burholderia pyrrocinia purple acid phosphatase Pap9 mediates phosphate acquisition in plants. Journal of Plant Biology, 62, 342–350.