Evaluation of Phosphate Solubilizing Pattern of Selected Bacterial Strains and their Agrobiotechnological Potential

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By

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بسم الله الرحمن الرحيم

Declaration

I certify that research work titled “Evaluation of phosphate solubilizing pattern of selected bacterial strains and their agrobiotechnological potential” is my own work. The work has not been presented elsewhere for assessment. Where material has been used from other sources it has been properly acknowledge/ referred.

Saba Talib

Certificate

This thesis, submitted by Ms. Saba Talib to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the degree of Master of Philosophy in Microbiology.

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List of Abbreviations

P	Phosphorus
ATP	Adenosine triphosphate
PSB	Phosphate solubilizing bacteria
PSI	Phosphate solubilization index
PSE	Phosphate solubilization efficiency
PGPR	Plant growth promoting bacteria
NBRIP	National Botanical Research Institute's Phosphate
OD	Optical density
$Ca_3(PO_4)_2$	Tricalcium phosphate
$AlPO_4$	Aluminium phosphate
G	Gram
$HClO_4$	Perchloric acid
$FeCl_3$	Iron chloride

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Abstract

Phosphorus (P) is the second most essential macronutrient, after nitrogen, required for plant growth and development. Despite its abundance in the soil, most of P remains inaccessible due to fixation with metal ions in the soil. The utilization of chemical fertilizers to meet P requirements and enhance crop yield has resulted in negative consequences for the ecosystem, soil health, and balance of soil microbiota. To address this challenge, there is a need to develop a cost-effective, environmentally friendly, and sustainable approach. The present study involves the use of phosphate solubilizing bacteria (PSB) along with inorganic phosphate sources, which can convert unavailable phosphorus into soluble (available form) soil. In the current study, *Bacillu*s strains were isolated from soil and screened for their phosphate solubilizing ability. Selected bacterial strains, S5 and S32 were optimized at different pH (5, 7, 9, and 11) with two different inorganic phosphate sources i.e. $Ca_3 (PO_4)_2$ and $AlPO_4$. *Bacillus* strains S5 and S32 showed the highest PSI value i.e. 2.714cm and 2.785cm at pH 7 and pH 5 respectively. In both quantitative and qualitative assessments, S5 and S32 showed maximum phosphate solubilizing ability for $Ca_3 (PO_4)_2$ as compared to $AlPO_4$ due to the presence of acidic phosphatases enzymes at different pH. Furthermore, the in-vivo experiment on *Zea mays* showed an increase in plant growth in the presence of *Bacillus* strains S5 and S32 and inorganic phosphate source ascompared to positive control. Based on these results, it can be concluded that *Bacillus* strains S5 and S32 have the potential to solubilize precipitated phosphate and make it available for the plant. Thus, *Bacillus* strains S5 and S32 could be effective as alternative biofertilizers for agriculture sustainability.

1. Introduction

Globally, food security, water scarcity and environmental pollution are becoming prominent issues considering the increasing population and industrialization. Agriculture sector is facing serious challenges owing to the changing climate, soil and water contamination and perturbation of soil properties. One of the most important issue emerged is the restricted mobility and bioavailability of soil nutrients. Inadequate soil nutrients supply poses a substantial technical problem that influences agricultural productivity, leading to reduced crop yield. Phosphorus availability to the plants is limited due to soil complexity, less moisture content, continuous cultivation of high-yielding crops, and fixation with metal ions at acidic and basic pH, root growth limitation, and soil erosion.

In order to overcome phosphorus limitation, farmers use different phosphate fertilizers. Although applying phosphatic fertilizers can improve plant growth and yield, most phosphate fertilizers remain in precipitated form and cause eutrophication in water bodies. These fertilizers contain heavy metals, leading to their accumulation in the soil, which negatively impacts soil fertility, and animal and consumer health.

Existing environmental challenges have stimulated the investigation of sustainable approaches to providing nutrients. Considering these situations, there is a need to use bio-based products. Currently, there is a widespread global focus on ecological farming. In this regard, Plant growth promoting rhizobacteria (PGPR) are used instead of using chemical phosphatic fertilizers. Nowadays, researchers are using phosphate-solubilizing bacteria (PSB) as bio-inoculants to increase soil quality and crop yield (Hussain *et al*., 2019). PPSBs are environmentally friendly, less cost-effective, and can convert fixed phosphorus into soluble phosphorus form so that plant roots can easily absorb soluble phosphorus.

Phosphate solubilizing bacteria adopt different mechanisms for the conversion of fixed phosphorus into soluble form. One of the main mechanisms is the organic acids production that are secreted by microorganisms and released into surroundings that lower the pH of the surroundings. These organic acids cause the chelation of ions and drop pH. Due to decreased pH, phosphate ions are released from phosphate minerals. This phenomenon occurs by the substitution of H^+ with Ca^{+2}. In sustainable agricultural practices, phosphate-solubilizing bacteria

have great importance and these organisms are used as biofertilizers (Rajwar*et al*., 2018; Rawat *et al*., 2020).

Pakistan is situated within the arid to semi-arid climatic region. Pakistan's soil is rich in P content but it is not available to plants due to precipitation reaction with Ca^{+2} and other metal ions, alkaline nature, and less moisture content.

Phosphorus (P) is the second most important macronutrient after nitrogen as it is needed by the plants in DNA replication for the formation of phosphorous diester bonds, in the plasma membrane as a component of phospholipids as well as cellular respiration as energy currency i.e., ATP. During the initial stages of plant growth, a sufficient level of phosphorus is applied to promote early root formation and development of reproductive plant parts. Additionally, phosphorus has been recognized for its positive impact on the quality of numerous fruits, vegetables, and grain crops. Deficiency of P in plants may lead to inhibition of shoot growth, darkening of leaves, or yellowishness in severe causes.

In soil, phosphorus exists in both organic and inorganic forms. Organic forms of phosphorus include phosphodiesters, nucleic acids, and phospholipids phosphomonoesters. In inorganic form, phosphorus may combine with calcium, aluminum, and iron, and form insoluble complexes that can't be absorbed by plants (Boitt*et al*., 2018). Due to the precipitated form of phosphorus, it becomes unavailable to plants. Soil pH has a great influence on phosphorus adsorption in plants (Zhu *et al*., 2018). In alkaline soils, it forms bound with calcium while in acidic soil, phosphorus may combine with aluminum and iron (Kumar & Shastri, 2017). Due to the fixation of phosphorus with calcium, iron, and aluminum, its availability to plants is reduced.

In order to overcome phosphorus limitation, farmers use different phosphate fertilizers (Kishore *et al*., 2015). Although applying phosphatic fertilizers can improve plant growth and yield, most phosphate fertilizers remain in precipitated form and cause eutrophication in water bodies (Azzi *et al*., 2017; J. Huang *et al*., 2017). These fertilizers contain heavy metals, leading to their accumulation in the soil, which negatively impacts soil fertility, and animal and consumer health, and contributes to eutrophication (J. Huang *et al*., 2017). P fertilizers are also expensive and all people cannot afford these types of fertilizers to improve the crop yield.

The existing environmental challenges have stimulated the investigation of sustainable approaches to provide nutrients to plants. Considering these situations, there is a need to use bio-based products. Currently, there is a widespread global focus on ecological farming. In this regard, PGPR is used instead of using chemical phosphatic fertilizers. Nowadays, researchers are using PSBs as bio-inoculants to increase soil quality and crop yield (Hussain *et al*., 2019). PPSBs are environmentally friendly and less cost-effective and are a means of increasing phosphorus availability to plants. PSB can convert fixed phosphorus into soluble phosphorus form so that the roots of plants can easily absorb it. In sustainable agricultural practices, phosphate-solubilizing bacteria have great importance and these organisms are used as a biofertilizers (Rajwar*et al*., 2018; (Rawat *et al*., 2020).

In plant rhizospheric regions, plant growth-promoting bacteria are present that can transform, mobilize, and solubilize nutrients. The rhizospheric region of crops plays a vital role in facilitating interaction between microorganisms, soil, and plants. The specific associations that are formed are influenced by the type of microorganisms present, the soil's nutrient level and environment, and the plant's defense mechanism. Bacteria increase plant growth due to direct and indirect mechanisms. In indirect mechanism, bacteria produce different phytohormones (ethylene or jasmonic in stress conditions) and to compete in the rhizosphere while indirect mechanisms, siderophores production, atmospheric nitrogen fixation, phosphate solubilization, and phytohormones (auxins, gibberellins, cytokinins) are produced (Puri *et al*., 2020).

Phosphate solubilizing bacteria adopt different mechanisms for the conversion of fixed phosphorus into soluble form. It includes the production of different organic acids and lowering of pH and the production of siderophores and phosphatases enzymes ((Kishore *et al*., 2015; Tomer *et al*., 2016). One of the main mechanisms is the organic acid production that is secreted by microorganisms and released into surroundings that lower the pH of the surroundings. These organic acids cause the elation of ions and a drop in pH value. Due to decreased pH, $P^-$is released from P-minerals. This phenomenon occurs by the substitution of H^+ with Ca^{+2}. H^+ ions release phosphate ions. The nature and strength of organic acids that are released by PSB play an important role in phosphate solubilizing efficiency.

Certain microorganisms produce inorganic acids like HNO_3 and H_2SO_4 to solubilize phosphate, although their efficiency is lower as compared to organic acids. For instance, *Nitrobacter*

produces nitric acid while *Thiobacillus* spp. utilizes sulfuric acid to dissolve phosphorus from various sources (Abbaszadeh-Dahaji *et al*., 2020). Acidophilic and sulfur-oxidizing bacteria are capable of producing hydrogen sulfide (H_2S) as a metabolic byproduct through various processes such as organic matter decomposition, reduction of sulfate, and other biochemical reactions. Ferrous sulfate is formed by reacting H_2S with ferric phosphate and subsequent release of bound phosphorus (Florentino *et al*., 2016).

Proton release is an alternative mechanism employed by microorganisms to dissolve phosphorus in soil (Parks *et al*., 1990). In the soil, phosphate-solubilizing microorganisms (PSMs) assimilate ammonium (NH_4^+) for amino acid synthesis. Within the microbial cell, the excess proton (H^+) is released into the cytoplasm by converting ammonium into NH_3, leading to the acidification of the surrounding medium. So, insoluble phosphates are solubilized in this acidic environment, making phosphorus more readily available for uptake by plants and other organisms (Gaind, 2016). pH of soil becomes low by releasing proton. The efficiency of proton excretion and phosphorus solubilization depends on the nitrogen source used. Studies have shown that phosphorus solubilization is more effective when NH^{4+} is the nitrogen source, compared to NO^{3-} (Sharan *et al*., 2008). Siderophores are small, high-affinity iron-chelating compounds released by microorganisms and plants in iron-stressed environments. They are known for their strong ability to complex with ferric ions (Birch & Bachofen, 1990). Siderophores are also released by Phosphate-solubilizing microorganisms as a strategy to chelate iron from Fe–P complexes in the soil (Collavino*et al*., 2010).

In soil, organic phosphorus can be hydrolyzed into inorganic phosphorus form due to the presence of phosphatases and phytases. Phosphatases are classified into two categories based on their optimal activities, namely acid phosphatases and alkaline phosphatases. Acid phosphatases exhibit their maximum activity at low pH levels, typically around pH 6.5, while alkaline phosphatases show their highest activity at high pH levels, typically around pH 11. This classification is based on their distinct pH preferences. In soil, organic material that is stored in phytate form, phosphorus is released from this due to phytase enzymes (Sharma *et al*., 2013). Carbon-Phosphorus lyase enzymes play a role in breaking down the Carbon-Phosphorus bond found in organophosphates, which ultimately enhances the availability of phosphorus for plants. However, the effectiveness of lyases in promoting significant solubilization of organic

phosphorus is limited due to their substrate's scarcity in the soil. Carbon-Phosphorus lyases have been observed to exhibit activity in diverse phosphate-solubilizing bacteria, including *Bacillus* and *Pseudomonas,* and fungi like *Aspergillus*, and *Penicillium*(Mehta *et al*., 2019; Kishore *et al*., 2015).

In the lab, Phosphate solubilizing bacteria are isolated by using two media that are Pikovskayas media and NBRIP media. The efficiency of phosphate solubilizing bacteria to form clear colony zones and holozones is higher by using NBRIP media as compared to Pikovskayas media. Phosphate solubilizing bacteria can solubilize inorganic phosphates in both solid and liquid media. By preparing NBRIP media, different inorganic phosphates such as $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$ are used. On solid NBRIP media, PSB can form clear colony diameters and holozones. By measuring these zones, the phosphate solubilizing index can be met based on PSI value, efficient PSBs can be isolated.

Aim and Objectives

Aim

The aim of the present study was to "Evaluation of phosphate solubilizing pattern of selected bacterial strains and their agro-biotechnological potential".

Objectives

1. Isolation and screening of phosphate solubilizing bacteria from agriculture soil sample.
2. Quantification of phosphate solubilization potential of bacterial strains by using $Ca_3(PO_4)_2$ and Al_3PO_4 as phosphate sources at varying pH.
3. Evaluation of plant growth-promoting traits and phosphatases detection of phosphate solubilizing bacteria.
4. Effect of selected bacterial strains on *Zea mays* growth.

2. Literature Review

Phosphorus (P) is momentous as a cardinal nutrient for plants. Phosphorus (P) ranks as the second most significant plant nutrient that poses a limitation to plant growth(Shrivastava *et al*., 2018).Globally, phosphorus (P) presents a substantial limitation for crop growth due to its predominant precipitation and strong adsorption to alkaline and weathered acidic soils(Kishore *et al*., 2015). In contrast to nitrogen (N), which plants can acquire from the atmosphere, phosphorus (P) is not readily soluble in the atmosphere. Instead, phosphorus is predominantly found in the soil, but it mostly exists in a precipitated form that cannot be easily absorbed by plants. Phosphorus content in the upper layer of soil typically ranges from 50 to 3000 mg kg^{-1}, but the portion available for plant absorption is merely 0.1% of the overall phosphorus content (Zhu *et al*., 2018). This limited availability results from processes such as soil cation precipitation, adsorption, and transformation into organic forms (Kishore *et al*. 2015).

2.1. Role of phosphorus in plant growth

2.1.1. Structural Role of Phosphorus

Inorganic orthophosphate form and organic phosphate are the two conformations of P in which they reside in the plant tissues. The total concentration of phosphorus is very crucial, as it sectionalizes phosphorus within a variety of cells. The surplus of P is stockpiled in the vacuole and consigned to cytoplasm under penury while giving the buffering capacity to the whole cell. Pi is metabolically active and stationed in the cytoplasm (Malhotra *et al*., 2018).

2.1.1.1. Nucleic Acids: Genetic Transfer

The indispensable part of the genetic material of all life is Phosphorus which constitutes 0.3 to 2.0 mg P g^{-1} DW in innumerable crops (Malhotra *et al*., 2018). The increased pool of rRNA is approximately 85% of the total content of nucleic acid and hence leads to gene expression and protein synthesis with an increase in P consignment(Suzuki *et al*., 2010).

2.1.1.2. Phosphorylated sugar

Phosphorylated monosaccharide's are the building blocks of sugar phosphates which are formed after their reaction with ATP and are classified as the Pi ester group of P. These phosphorylated

compounds constitute phytic acid, glucose-6-phosphate, and di-hydroxy-acetone phosphate and have a crucial role in important processes of life including photosynthesis, starch synthesis, and breakdown. Moreover, they are also involved in glycolysis and respiratory reactions (Malhotra *et al*., 2018).

2.1.1.3. Phospholipids: Membrane Component

P also constitutes the membrane phospholipids and consists of polar and non-polar regions. P deficiency leads to an increase in leakage of electrolytes due to sulpholipids and galactolipids accumulation in membranes(Byrne *et al*., 2011; Gaude *et al*., 2008).

2.1.2. Significance of phosphorus in plants' Growth and Development

P has enormous applications in life processes outlined below:

2.1.2.1 Germination of seed

Seed P is crucial for young seedlings in comparison to mature plants as it plays a crucial role in early seedling growth. Later on, P necessity for the plant is provided through roots from media. It was noticed that plants provided with plenty of P are better in root development(Zhu & Smith, 2001). However, few studies reported that no variations in seedlings were seen with low seed P so, it can be estimated that for proper seed germination, an optimum concentration is needed. Moreover, we can predict that high P concentration is of no use (Pariasca-Tanaka *et al*., 2015; Rose *et al*., 2012).

2.1.2.2. Increasing Root and Shoot Strength

P is vital for cell division and cell enlargement of the whole e plant from the cellular level focusing on plant height, leaf width, and shoot development etc., It is noticed that plant leaves having penury of P seemed small in size and overall reduction in shoot biomass(Assuero *et al*., 2004). However, leaf dry weight relies on the overall content of cellulose, hemicellulose, and starch in comparison to loss in leaf expansion due to P penury. It is predicted that plant growth parameters mostly depend on P availability rather than the vital process of photosynthesis. Prolong P deficiency leads a decrease in a plant's overall growth rate because of the scarcity of ATP in the root zone (Gniazdowska *et al*., 1998).

Nevertheless, the genotype with greater PUE under P penury can manage phenomena like greater root biomass and lower root respiration in comparison with the genotype with lower PUE indicating that these genotypes without incurring the additional carbon cost for the development of roots adopted a good strategy (Malhotra *et al*., 2018).

2.1.2.3. Flower and Seed Formation

Reproductive growth of plants such as seed, and flower formation also relies upon P availability. It is indicated that under phosphorus deprivation the concentration of anthocyanin in flower stalk is reduced due to reduced activities of phenylalanine ammonia-lyase and chalconeisomerase. A few parameters like seed number, seed size, and viability in the case of cereal plants are affected under P penury as it supplies P to plants stored in seed. Moreover, the optimum concentration of P can have a positive influence on all parameters. P is stored in various plants in the form of phytin and its concentration varies from species to species. For example, in rice, wheat, and maize approximately 75% of total P is phytin. These crops are deprived of inorganic phosphate and cellular phosphate in quantities of 4–9% to 15–25%, respectively as mentioned.(Chen *et al*., 2013; White & Veneklaas, 2012)

2.1.3. Energy transfer process involving phosphorus

2.1.3.1. Phosphates rich in energy:

Phosphorus is crucial for cellular metabolism as it accompanies the high energy bonds. Phosphorus is the vital component of the major energy currency of the cell that is responsible for innumerable processes including nutrient transport against the concentration gradient, and synthesis of macromolecules (Malhotra *et al*., 2018).

2.1.4. Physiological significance of phosphorus

2.1.4.1. Photosynthetic activity and utilization of carbon

Photophosphorylation is the foremost step in the process of photosynthesis and relies on the availability of Pi. The inner membrane of chloroplast also accompanied Pi translocators for the transport of 3-PGA as explained by Heber and Heldt 1981 and Flugge and Heldt 1984.

Phosphorus also regulates the availability of phosphorylated metabolites in the cytoplasm. Moreover, PCR cycle intermediates are also dependent on the availability of P. Under a P-deficient environment vacuolar pool is found to be decreased because phosphorus is consigned to the cytoplasm hence, the cytosolic concentration remains stable. More drastic effects of P-deficiency can also be seen such as photo-inhibition of PS-II, reduction in CO2 fixation as more diffusion of electrons, antenna becoming dephosphorylated, and many more.

Non-phosphorylating respiratory pathways including the cyanide resistance pathways accompanied by plants under P penury, lead to ATP deficiency which in turn affects energy-dependent processes of plants (Rychter *et al*., 1992).

2.1.4.2. Nitrogen Fixation

The leguminous plant's roots are the residents of rhizo-bacterium carrying out nitrogen fixation by converting unusable forms of nitrogen to usable forms. This bacterium needed the energy for this conversion which is supplied by the ATP degradation. Moreover, legumes are good candidates for maintaining soil fertility and the human diet. It is predicted that P deficiency also affects important life processes including nitrogen fixation (Bonetti *et al*., 1984). In various crops like pea plants, root biomass increased by increasing P demand (Jakobsen, 1985).

2.2. Phosphorus crosstalk with micro and macro nutrients

Phosphorus in the soil also accompanies both synergistic and antagonistic relationships with other nutrients and affects their bioavailability. Before sowing, a complete analysis of the site can give a proper and good yield of crops. The P availability can be increased by enhancing the ammonical-N source. 93 bu/ac increased in sorghum is seen due to the synergistic effect of P and N(Schlegel & Bond, 2018). Another important macronutrient K has a synergistic effect with P. For proper plant yield, there must be a proper ratio of both nutrients for example 64 bu/ac grain yield is enhanced as compared to alone (Usherwood & Segars, 2001). Another type of interaction, antagonistic, was also observed with many of the nutrients like S and P in moong seeds. Micro nutrients interactions with P gained more attention due to the availability of good analytical techniques and these nutrients carry a crucial process for uptake and utilization of

nutrients by plants (Chowdhury *et al*., 2015). An antagonistic interaction is observed between P and Zn (Malhotra *et al*., 2018).

2.3. Phosphorous deficiency

P-deficient soil is characterized by erosion weathering and $CaCO_3$ enrichment. Phosphorus availability to the plants is limited due to soil complexity, less moisture content, continuous cultivation of high-yielding crops, and fixation with metal ions at acidic and basic pH, root growth limitation, and soil erosion. Deficiency of P in plants may lead to inhibition of shoot growth, darkening of leaves, or yellowishness in severe causes. Limitations have drastic effects on crop yield and quality. Phosphorus deficiency also causes oxidative stress in plants.

2.4. Phosphorus uptake, transport, and sectionalization

Uptake marks the initial stage of the pathway through which elements move from the soil into roots and other parts of plants. Various factors that affect phosphorus (P) presence in the soil are the pH of the soil, texture, P concentration, metals (Hinsinger, 2001), etc. The robust interaction between P and these soil components promotes its movement to plant roots primarily through the process of diffusion. In the soil, P exists in both organic and inorganic forms. Around 20-80% of soil phosphorus (P) exists in an organic state, and a significant constituent of this form is phytic acid, also known as inositol hexaphosphate. The rest of the P is found in its inorganic form. Soil micro-organisms play a pivotal role in releasing insoluble forms of P into the soil, making it available for plants.

The optimal uptake of P occurs within a soil pH range of 5.0 to 6.0, where it largely exists in the $H_2PO_4^-$ form (Furihata *et al*., 1992). The concentration of various inorganic P forms in the soil ranges from 0.1 to 10 μM, which is less concentration as compared to plant tissues (5-20 mM) (Shen *et al*., 2011). To overcome this concentration disparity, plants use transporters that work against natural flow. Roots membranes contain these transporters. With the help of transporters, plants take P. Various organic acids and extracellular phosphatases are secreted from plants and microorganisms, leading to acidification of the rhizosphere, which facilitates P movement within the root system (Hinsinger, 2001).

Upon entry into the root surface, P reaches the xylem through a symplastic path. From there, it moves from the xylem to the aboveground parts of the plant. The movement of P within the cytoplasm and its eventual storage in the vacuole, both inter- and intracellularly, involves an energy-dependent mechanism (Malhotra *et al*., 2018; Ullrich & Novacky, 1990).

2.5. Phosphatic fertilizers effects

Phosphorus (P) in soil exhibits low solubility due to strong binding to particle surfaces, resulting in limited concentrations in the soil solution. This scarcity of available P makes it a limiting factor for optimal crop growth, necessitating the use of P fertilization to ensure profitable crop production (Azzi *et al*., 2017). Phosphorus fertilizers are primarily sourced from sedimentary phosphorite deposits. However, these P sources are expected to be depleted within the next few centuries, leading to global P scarcity. Addressing this issue and implementing effective P management strategies have become crucial to tackling agricultural and environmental challenges.

When P fertilizers are added to soil, various reactions take place, including attachment to soil particles and precipitation. Characteristics of the soil such as chemical composition, moisture levels, and texture, alongside the selection and placement of fertilizers, influence the P fertilizer transformation rate in the soil. Heavy P fertilization can prevent micronutrient deficiencies like Fe etc. and also hinder the uptake of toxic trace elements. High concentrations of P in soils and plants, especially with elevated pH levels and iron oxide content, disturb the uptake and metabolism of Zn.

In soil, nutrient deficiency is a major global issue that affects agricultural production, leading to reduced crop quantity and quality. To boost crop yields, chemical fertilizers have been widely used, but this practice has adverse effects on indigenous organisms, deteriorates agro ecosystems, and affects aquatic resources. Most phosphate fertilizers remain accumulate in the soil and cause eutrophication(J. Huang *et al*., 2017). These fertilizers contain heavy metals, leading to their accumulation in the soil, which negatively impacts soil fertility and animal and consumer health (Huang *et al*., 2017a).

2.6. Forms of phosphorus in soil

In soil, phosphorus exists in organic and inorganic forms. Organic phosphorus is derived from different biological metabolic processes : (a) Phosphate esters (b) Phosphonatases and (c) Phosphoric acid anhydrides (L.-M. Huang *et al*., 2017). Inorganic phosphorus is present in a combined form with calcium, aluminum, and iron (Boitt *et al*., 2018). In acidic soil, phosphorus is tightly bound with Aluminum and iron, whereas in alkaline soil, phosphorus reacts with calcium. Soil pH also influences the solubilization of phosphorus in the soil. The pH level of the soil determines the availability and solubility of phosphorus for plants. Therefore, by maintaining appropriate soil pH, phosphorus availability to plants can be increased.

2.7. Phosphate solubilizing microorganisms (PSMs)

In soil, microbes play an important role in plant nutrient acquisition. In soil, diverse types of microorganisms are present in the rhizospheric portion that promotes plant growth(Biswas *et al*., 2018). These microorganisms are known as PGPR bacteria. Phosphorus in the soil is mostly present in a fixed or insoluble form that plants can't be used. So, phosphate solubilizing bacteria are used to convert inaccessible phosphorus to available form through solubilization and mineralization. Instead of using chemical fertilizers, phosphate-solubilizing microorganisms are used that are eco-friendly. In soil, most phosphate solubilizing bacteria are present in the rhizospheric region. Root exudates released by plants can promote the population and activity of phosphate-solubilizing biofertilizers in the soil. These exudates are compounds secreted by plant roots in the rhizospheric region. Varieties of organic compounds exist in root exudates, such as amino acids, organic acids, and enzymes. A symbiotic relationship is created between bacteria and plants. By increasing phosphate solubilizing bacteria, the phosphorus availability of plants is improved. *Bacillus* sp. is commonly recognized as rhizobacteria that contributes to the growth of plants by aiding in the solubilization of phosphorus.

Bacillus spp. offer several advantages as they are commonly found in the rhizospheric region of various crops (Abd-Allah *et al*. 2018), eco-friendly, control phytopathogens (Alori *et al*., 2017), and have the ability to thrive under stressful conditions. By combining the use of Bacillus spp. with multiple capabilities, including phosphate solubilization, alongside phosphate fertilizers, it becomes possible to enhance plant growth parameters and preserve the fertility of soil even in biotic and abiotic stresses.

2.8. Utilization of phosphate solubilizing microorganisms as a biological fertilizer

In agriculture, through the inoculation of phosphate solubilizing bacteria, phosphorus availability can be improved. In soil, diverse types of microorganisms are present in the rhizospheric portion that promote the growth of PSMs and are considered beneficial bio-inoculants that offer potential alternatives to agrochemicals. This reduction in chemical usage can have positive effects on the environment and provide economic benefits. By using PSBs, the need for chemical fertilizers decreased. Phosphate solubilizing bacteria use different mechanisms for the conversion of precipitated phosphorus form to available phosphorus form and promote the overall growth of plants by producing plant growth promoting hormones, phosphate solubilizing enzymes, organic acids, and low pH. The use of PSB as biofertilizers is safe and environmentally friendly (Rajwar *et al*., 2018). In the lab, by using quantitative and qualitative methods, the efficiency of PSBs can be measured, and then select the efficient phosphate solubilizing bacterial strains (Mehta & Nautiyal, 2001). Biofertilizers have a distinct advantage over chemical fertilizers in that they release nutrients slowly, responding to the specific needs of the crop while also complementing other essential minerals and growth factors. Unlike chemical fertilizers, which often release nutrients rapidly and lack personalized nutrient supply, biofertilizers' gradual nutrient release promotes sustainable agricultural practices and reduces nutrient losses to the environment. Furthermore, their natural ability to fix nitrogen and enhance soil fertility makes them a viable and eco-friendly alternative to chemical fertilizers. Chemical fertilizers can contribute to soil degradation and environmental pollution if misused. Embracing biofertilizers can support environmentally friendly and economically viable farming practices while decreasing reliance on synthetic fertilizers.

2.9. Advantages and roles of phosphate solubilizing microorganisms that enhance phosphorus solubility

In soil, phosphorus exists in large proportions, but plants can absorb this accumulated phosphorus because of the presence of insoluble or fixed phosphorus form. Therefore, for the conversion of fixed phosphorus form into soluble phosphorus form, phosphate solubilizing bacteria are used as biofertilizers. In agriculture, the quality of agro-ecosystem and food

production is increased by using phosphate solubilizing bacteria. *Zea mays* growth was improved when using phosphate solubilizing bacteria as a bioinoculant as compared to the control that was not inoculated with phosphate solubilizing bacteria. For sustainable agriculture production, by enhancing phosphate availability to plant growth, phosphate solubilizing bacteria enhance root and shoot development, nitrogen fixation, photosynthesis, wet and dry weight of shoots, zinc and iron bioavailability, and yields.

The utilization of phosphate solubilizing microorganism's technology offers a promising solution to enhance agricultural productivity in sodium-rich-alkaline soil, without the associated environmental risks linked to the continuous use of synthetic fertilizers.

On crop growth and development, phosphate solubilizing microorganisms have been observed to have a significant positive impact. Against plant pathogens, phosphate solubilizing bacteria exhibit potential as biocontrol. Due to the production of various antifungal compounds such as siderophores, antibiotics, HCN, and lytic enzymes, phosphate solubilizing bacteria can combat pathogens and work collectively to inhibit the growth of plant pathogens and protect crops. Phosphate solubilizing bacteria also produce growth-promoting hormones, IAA (Linu *et al*., 2019), and ACC deaminase (Puri *et al*., 2020).

2.10. Phosphate solubilization mechanism

2.10.1. Inorganic Phosphate Solubilization

In soil, phosphate combines with iron, aluminum, and calcium and forms insoluble complexes. These inorganic phosphates are solubilized through the following methods.

2.10.1.1. Organic acid production

Phosphate solubilizing microorganisms adopted different mechanisms for phosphate solubilization. One of the strategies is the production of organic acids that are secreted by microbes and released into the surroundings (Kishore *et al*., 2015). Gluconic acid plays a crucial role in phosphate solubilization (Duebel *et al*., 2000). Through the glucometabolic pathway, organic acids are released into the surroundings by dropping the pH level. These organic acids then form chelation with mineral ions and will drop the pH of the surroundings. P-ions are

released from P-minerals due to the acidification of microbial cells and their adjacent environment. This phenomenon occurs by the substitution of H^+ with Ca^{+2}. Phosphate solubilizing efficiency mainly depends upon the nature, strength, and concentration of organic acids. PSMs produce acids that maybe organic and inorganic. Organic acids produced by PSMs have both OH^- and carboxyl groups. Both these groups chelate the cations that are bound to phosphate and convert insoluble phosphorus form to soluble form. A powerful chelator of calcium is 2- 2-ketogluconic acid. Inorganic acids such as H_2SO_4, HNO_3, and carbonic acid are also produced. Calcium phosphate is converted into soluble form by reacting with nitric and sulphuric acids.

For phosphorus solubilization, the quality of acids produced by PSMs plays a significant role. Different PSMs produce different kinds of organic acids depending upon insoluble phosphorus sources and affect the phosphate solubilizing activity of phosphate solubilizing bacteria. The activity of phosphate solubilization (PS) is inversely correlated with the alterations in pH values induced by organic acids. Hydrogen ions (H^+) liberated by organic acids can displace metal ions from tricalcium phosphate, leading to the liberation of soluble phosphate ions (P-ions). Organic acids are not secreted by phosphate-solubilizing bacterial strains, including Pseudomonas sp., Rather, they release hydrogen ions (H^+) as a byproduct of respiration or during NH_4^+ assimilation but the soluble P-ions are released by chelating organic acids with metal ion.

2.10.1.2. Inorganic acids and H2S production

Certain microorganisms, such as Nitrobacter and Thiobacillus spp., are known to produce inorganic acids like HNO_3 and $H_2SO_4$to solubilize phosphate, although their efficiency is lower compared to organic acids. For instance, Nitrobacter employs nitric acid production, while Thiobacillus spp. utilizes sulfuric acid to dissolve phosphorus from various sources (Abbaszadeh-Dahaji *et al*., 2020).

Acidophilic and sulfur-oxidizing bacteria are capable of producing hydrogen sulfide (H_2S) as a metabolic byproduct through various processes. These processes include the decomposition of organic matter, reduction of sulfate, and other biochemical reactions. Ferrous sulfate is formed by reacting H_2S with ferric phosphate and releasing bounded P (Florentino *et al*., 2016). Studies

have demonstrated that certain sulfur-oxidizing bacteria can significantly increase PSE when inoculated in plants, such as *Brassica juncea*.

2.10.1.3. Proton release from NH_4(Assimilation /Respiration)

Proton release, also known as proton extrusion, is an alternative mechanism employed by microorganisms to dissolve phosphorus in soil (Parks *et al*., 1990). Some microorganisms, such as Pseudomonas sp., exhibit the ability to dissolve phosphorus without producing organic acids (Illmer & Schinner, 1995). In the soil, phosphate-solubilizing microorganisms (PSMs) assimilate ammonium (NH_4^+) for amino acid synthesis. Within the bacterial cell, ammonium is converted to NH_3, and H^+ is released into the cytoplasm, leading to the acidification of the surrounding medium. The acidic conditions created around the microbial cell help in the solubilization of insoluble phosphates, making phosphorus more readily available for uptake by plants and other organisms (Gaind, 2016). pH of soil becomes low by releasing proton. The efficiency of proton excretion and phosphorus solubilization depends on the nitrogen source used. Studies have shown that phosphorus solubilization is more effective when NH_4^+ is the nitrogen source, compared to NO^{3-}(Sharan *et al*., 2008). Proton extrusion is just one of several mechanisms employed by microorganisms for phosphorus solubilization, and its prevalence varies among different microbes. Other modes of phosphorus solubilization have been reported by researchers, such as those investigated by Park *et al*. (2009). For example, an alkalophilic strain of *Bacillus* marisflavi FA7 demonstrated the highest tricalcium phosphate dissolution when supplied with NH_4Cl (Prabhu *et al*., 2018). Similarly, the multifunctional *Bacillus* subtilis BPM12 strain exhibited the highest phosphorus solubilization (272.02 μg/mL) in vitro when $(NH_4)_2SO_4$ is added to the medium as the N source (Wang *et al*., 2020).

2.10.1.4. Indirect mechanism

Microbes present in rhizospheric regions are essential for indirectly assimilating a significant quantity of phosphorus from the soil through the dissolution of insoluble forms. In situations of stress, these microbes undergo cell lysis, which results in the release of stored phosphorus into the surrounding soil. As a consequence, plants and other soil microbes can take up this released phosphorus (Butterly *et al*., 2009).

2.10.1.5. Direct oxidation pathway

Goldstein (1995) proposed an extracellular oxidation pathway used by specific microbes to dissolve precipitated phosphates in the soil (Goldstein, 1995). This pathway involves converting glucose into gluconic acid through the action of glucose dehydrogenase. These acids function as chelator, facilitating the release of minerals such as Ca^{2+} and Fe^{2+} from their phosphate-bound form. The effectiveness of this mechanism was demonstrated by Song *et al*. (2008) during their research on insoluble phosphorus solubilization by *Burkholderia cepacia* DA23 (Song *et al*., 2008). This particular phosphorus solubilization mechanism is chiefly identified in gram-negative bacteria. In this bacterial category, through alternate glucose oxidation pathways, dominant organic acids such as gluconic acid are produced. This mode of phosphorus solubilization is primarily found in gram-negative bacteria, where dominant organic acids like gluconic acid are produced through alternative glucose oxidation pathways. These organic acids diffuse through the bacterial periplasm into the surrounding environment (Krishnaraj & Dahale, 2014). Another example of phosphorus solubilization through the direct oxidation pathway can be observed in Pseudomonas aeruginosa KR270346, which secretes gluconic acid for PS (Linu *et al*., 2019).

2.10.1.6. Exopolysaccharide Production

EPS are carbohydrate polymers with an organic or inorganic component, excreted by microbes outside their cell wall (Sutherland, 2001). Microbes synthesize EPS in response to various stressors or during biofilm formation. They possess the capability to form complexes with metal ions found in the soil, with the preference order being Al^{3+}> Cu^{2+}> Zn^{2+}> Fe^{3+}> Mg^{2+}> K^{+} (Ochoa-Loza *et al*., 2001). This indicates that EPS-producing microorganisms might utilize a similar mechanism for phosphorus (P) solubilization.

2.10.1.7. Siderophores production

Siderophores are small, high-affinity iron-chelating compounds released by microbes and plants in iron-stressed environments. They are known for their strong ability to complex with ferric ions (Birch & Bachofen, 1990). PSM employing siderophores represents a valuable approach to chelate iron from Fe–P complexes in soil(Collavino *et al*., 2010). However, the precise role of

siderophores in phosphate dissolution requires further investigation. Studies have shown that certain phosphate solubilizers, such as Bacillus megaterium, Bacillus subtilis, Rhizobium, and Pantoeaallii, produce siderophores in the range of 80 to 140 µmol L^{-1} under alkaline conditions (Ferreira *et al*., 2019). This not only supports the survival of organisms in stressful environments but also enhances phosphorus solubilization.

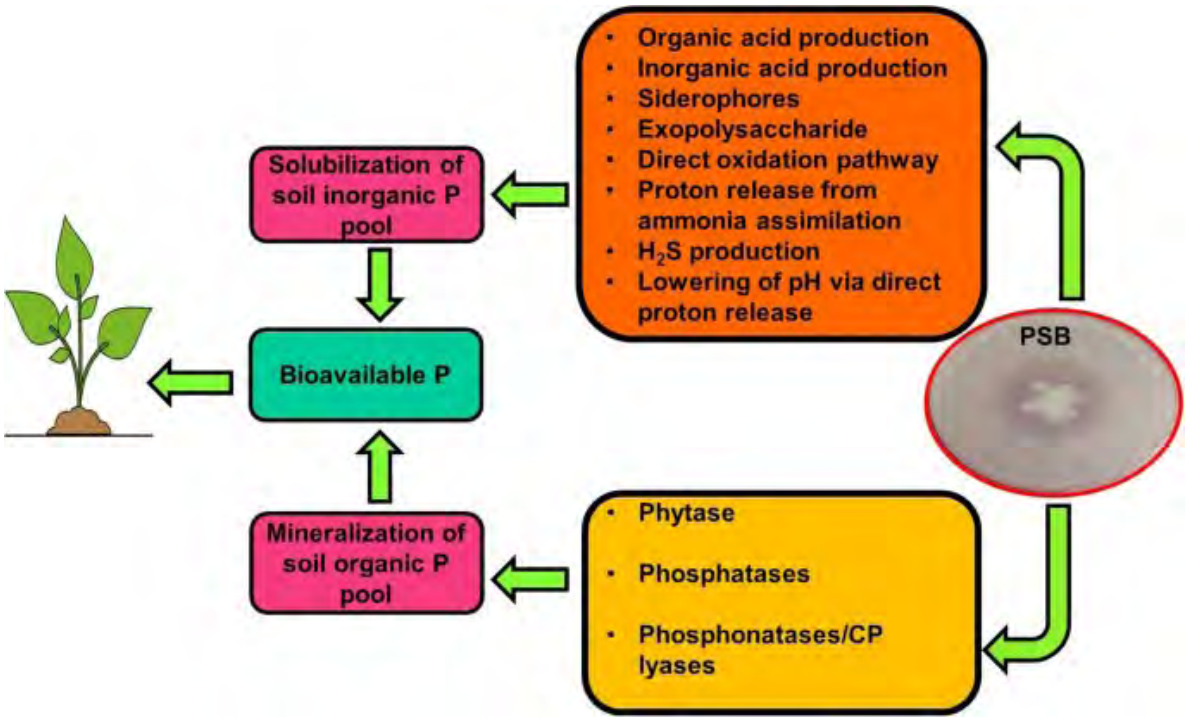

Fig 2.1. Schematic presentation of phosphate solubilization by PSB. The PSB plate shown in the figure represents the phosphate-solubilizing bacteria with halo zone formation that indicates the solubilization of tricalcium phosphate. P phosphorus, PSB phosphate-solubilizing bacteria, H_2S hydrogen sulfide, CP carbon-phosphorus

2.10.2. Mechanisms of organic phosphate solubilization

Organic phosphorus in the soil exists in the form of phytate, phosphomonoesters, phosphodiesters, phospholipids and nucleic acids, and xenobiotic phosphonates (pesticides, detergents, antibiotics, and flame retardants). By excreting microbial phosphatases, PSB can breakdown organic phosphate compounds into soluble forms, making them accessible for plant uptake and utilization. Before organic phosphate compounds can be absorbed by plants, they must undergo degradation through the action of enzymes like Phosphatase, phytase, or carbon-

phosphorus lyase (Kumar & Shastri, 2017). These enzymes play a vital role in breaking down organic phosphate esters or bonds, converting them into inorganic phosphate. This conversion allows plants to readily take up and utilize the inorganic phosphate for their growth and development. The majority of organic compounds containing phosphorus are often high molecular-weight substances that tend to be resistant to chemical hydrolysis. As a result, they require bioconversion processes to be transformed into soluble ionic phosphate forms (such as Pi, HPO_4^-, $H_2PO_4^-$) or low molecular-weight organic phosphates. Only in these converted states can they be assimilated by the cell for various biological processes. This bioconversion allows for the efficient utilization of organic phosphorus by living organisms. The sink theory, proposed by Halvorson *et al*. (1990), suggests the significant role of PSM in solubilizing and releasing organic P that can be easily absorbed by plants. According to this theory, a continuous removal of phosphorus (P) takes place, resulting in the dissolution of calcium-phosphate compounds. Different enzymes play important roles in this process. In this process, the first group of enzymes is known as non-specific acid phosphatases (NSAPs).

2.10.2.1. Non-specific acid phosphatases (NSAPs)

NSAPs possess non-specific substrate specificity, allowing them to hydrolyze various phosphorus-containing compounds. These enzymes are responsible for dephosphorylating the phosphor-ester or phosphoanhydride bonds that exist in organic compounds. Through the cleavage of these bonds, NSAPs facilitate the liberation of inorganic phosphate, thereby making it accessible for a range of biological processes. Phosphatase enzymes can either be acid phosphatases or alkaline phosphatases (Nannipieri *et al*., 2011). The classification of enzymes based on pH optima is a well-established scientific concept. Acid phosphatases are enzymes that function optimally in acidic environments, such as acidic soil. On the other hand, alkaline phosphatases work best in alkaline to neutral soil conditions. This categorization is based on the specific pH range in which these enzymes exhibit their highest activity. The presence of different types of phosphatases in soils helps to break down and release phosphate from organic matter, making it available for plant uptake and contributing to nutrient cycling in the ecosystem. Scientific studies have provided evidence that microorganisms in the soil secrete both acid and alkaline phosphatases, showing a higher preference for organic phosphatases. Notably, alkaline phosphatases play a significant role in hydrolyzing around 90% of the total organic phosphorus

present in the soil, thereby enhancing the availability of phosphorus to plants. In a specific research study, purified alkaline phosphatases derived from Bacillus licheniformis MTCC 2312 were introduced into the soil, resulting in significant improvements in the phosphorus content percentage in the root and stem of Zea mays L (Singh & Banik, 2019). The increase observed was approximately 2.35-fold for the root and 1.76-fold for the stem.

2.10.2.2. Phytases

Phytase enzymes are produced by PSM during the process of mineralization of organic phosphorus (P). In soil, organic material is stored in phytate form, and phosphorus is released from this due to phytase enzymes. Phytases break down the phytate molecule, releasing inorganic phosphate that can be utilized by plants. Plants have limited capacity to directly take up phosphorus from phytate due to the complex structure of the phytate molecule. The strong binding of phosphate groups within the phytate molecule makes it inaccessible to the plant's phosphorus uptake mechanisms. To make phosphorus in phytate available for plant uptake, the phytate molecule needs to be broken down into inorganic phosphate (Pi) through the action of enzymes like phytase. Phytase cleaves the phosphate groups from the inositol rings, releasing inorganic phosphate that can be taken up by plants' root systems. In many plant seeds and tissues, phosphorus is stored in phytate form and provides a reservoir of phosphorus for future plant growth (Sharma *et al*., 2013). However, plants themselves generally lack sufficient phytase activity to effectively degrade phytate. Instead, they rely on phosphate-solubilizing microorganisms in the soil, which produce phytase enzymes, to break down phytate and release inorganic phosphate that can be taken up by plant roots. The inoculation of cereal crops with phytase-producing bacteria led to an increase in phosphorus uptake, eliminating the need for traditional fertilizers (Martínez *et al*., 2015).

2.10.2.3. Phosphatases/Carbon–Phosphorus (C–P) Lyases

These enzymes play a crucial role in breaking down the C-P bond found in organophosphates, which ultimately enhances the availability of phosphorus for plants (Rodríguez *et al*., 2006).

However, the effectiveness of lyases in promoting significant organic phosphorus solubilization is limited due to the scarcity of their substrates in the soil. CP lyases have been observed to exhibit activity in diverse PSBs, including *Bacillus, Pseudomonas, Enterobacter,* and *Rhizobium* (Teng *et al*., 2019)as well as in endophytic fungi like *Aspergillus and Penicillium*(Mehta *et al*., 2019).

2.11. Factors influencing microbial phosphate solubilization

Various factors affect the activity of PSB to convert insoluble organic and inorganic phosphorus to a soluble form. Some are these:

Nutritional level in soil

1. Soil's pH 2. Soil type
3 Soil's organic matter4. Types of plants
5. Temperature 6. Physiology of microbes
7. Bacterial growth status 8. Nutritional level in soil
9. Interaction of PSB with other soil microorganisms in the soil
10. Agronomics practices
11. Metabolites produced and its released rate by PSB

In warm and humid climates, phosphorus solubilization is accelerated as compared to cool and dry climates. Moreover, rapid solubilization of phosphorus occurred in aerated soil as compared to saturated and wet soil. Rhizospheric bacteria are stimulated by adding inorganic phosphorus in small amounts. Then, these bacteria can be able to mineralize phytic acid, leading to enhanced phosphorus availability for plants. By adding lime and compost as soil amendments, the activity of microorganisms can enhance and facilitate the solubilization of phosphates in the soil. In the presence of soil's organic matter, microbial phosphorus solubilization is increased. For

phosphate solubilization, the pH range exists between 6 and 7.5 because when pH becomes acidic and basic, phosphorus will combine with Al, Fe, and Ca and become not available to plants.

2.12. Enzymatic breakdown of complex organic P compounds:

PSB in soil releases PS enzymes which are phosphatases and phytases. Phosphatase enzymes are exo-enzymes. Phosphatases are categorized into acid and alkaline phosphatases. Phosphatases utilize organic phosphorus compounds as substrates and enzymatically cleave the ester bonds, releasing inorganic phosphate (Pi).

In organophosphates, the Carbon–Phosphorus bond is broken by Phosphonatases and Carbon–Phosphorus lyases.

In soil, mostly organic phosphorus is distributed in phytate form and bound with other minerals. Plants can't utilize phytate directly from soil. Plants, some animal tissues, bacteria, and fungi are the source of phytase. Microbial-derived phytases are highly preferred in the biotechnological production of enzymes due to their efficient catalytic properties and ease of manufacturing. These enzymes hydrolyzed phytic acid at multiple positions on the inositol ring, leading to the release of vital minerals such as phosphorus and zinc in an inorganic form. Consequently, this process enhances mineral absorption in plants. While plants cannot directly acquire phosphorus from phytate. In the rhizospheric portion, the presence of phosphate-solubilizing microorganisms increases phosphorus availability. As compared to plant-derived phytase, microbial-derived phytasehas shown superior efficiency. At present, research on microbial phytase is highly active, focusing on addressing concerns related to food safety and security.

Phosphatases and Carbon–Phosphorus lyases are enzymes that have an important role in breaking Carbon–Phosphorus bonds in organophosphates.

2.13. Introducing phosphate solubilizing microbes for crop inoculation:

Previous research investigated the effects of introducing PSBs into crops. Among these microorganisms, Pseudomonads and Bacillus species have great importance in phosphate solubilization. Researchers have examined the growth-promoting potential of Pseudomonas isolates on a variety of crops including wheat, rice, lentils, etc. Similarly, Bacillus strains have been utilized to enhance the growth of rice and millet (Kumar *et al*., 2013; Malviya *et al*., 2012). (Ekin, 2010) conducted a study revealing that the application of PS Bacillus sp. leads to increased P solubilization in sunflowers, resulting in enhanced seed quality and oil yield. Researchers have conducted many experiments by inoculating PSBs with crops and proved that by inoculating PSBs with crops, the quality and yield of crops are increased. In their study,(Hussin *et al*., 2017) investigated the effects of two P solubilizers, B. polymyxa and Pseudomonas striata, on mung bean plants. They observed significant enhancements in various growth parameters upon inoculation with these microorganisms. Additionally, the application of the P solubilizers led to an increase the levels of P in the soil, resulting in enhanced uptake of vital macronutrients like Nitrogen, Potassium, phosphorus, Calcium, and Magnesium in mung bean plants' roots and shoots, as compared to the non-inoculated group. (Chauhan *et al*., 2017) investigated that PSBs have ability to produce IAA, anti-fungal activity, siderophores and HCN.

2.14. Impact of phosphate solubilizing bacteria on root development

Plant hormones, including well-known phytohormones like auxins, ethylene, abscisic acid, cytokinins, and gibberellins, play vital roles in regulating diverse plant processes. PSBscan impact the roots of plant systems by modulating signaling pathways and the transport of auxins. Research has demonstrated that *Phyllobacterium brassicacearum* (STM196) can cause modifications in auxin distribution within the roots of Arabidopsis (Contesto *et al*., 2008). PSBs can induce alterations in plant roots by providing a substantial amount of auxins that synergize with the plant's endogenous auxins (Haidar *et al*., 2018). As a result, the architecture of the root system becomes modified due to this interaction. This includes the increased lateral roots and root hair formation, ultimately enhancing the uptake of nutrients and water. Phosphorus mobilization is improved by enhanced symbiotic interaction of PSBs with root area. In the case

of wheat, it was observed that inoculation with bacteria led to elevated auxins content and higher plant biomass. This suggests that root growth and nutrient uptake are enhanced due to auxins. Phosphate solubilizing bacteria are known to produce various enzymes, among them ACC deaminase enzyme is present that cleaves substrate and modifies root system.

3. Materials and Methods

3.1. Sample Collection

Agriculture soil samples were collected from Charsada, KPK, Pakistan, for the isolation of phosphate solubilizing bacteria. From the rhizospheric portion, soil samples were collected. To avoid environmental contaminants, an upper soil layer was removed. By using a sterile spatula, soil samples were collected from the rhizospheric portion and transported to the laboratory in sterile sampling bags.

3.2. Sample enrichment

Enrichment was done in a Minimal Salt Medium (MSM). One gram of soil was added into sterilized Erlenmeyer flasks containing MSM and incubated in a shaker incubator at 150 rpm, at 37℃ for approximately one week. After one week, enrichment was done for further processing.

Table 3.1: Composition of Minimal salt medium

Components	Concentrations (g/L)
Na_2HPO_4	0.24g
K_2HPO_4	0.2g
NH_4NO_3	0.01g
$MgSO_4$	0.001g
$CoCl_2$	0.001g
Peptone	0.5g
$ZnSO_4$	0.14g
Glucose	10

3.3. Serial dilution:

After enrichment of the soil, 9 test tubes were taken, and 9ml sterile saline into 9 test tubes. Labeled all test tubes with proper numbering 1 to 9. One ml broth from an enriched sample was taken into 1 test tube and mixed with a pipette. From 1 test tube, one ml was poured into 2T and mixed. One ml from 2T was added into 3T and the same procedure was performed until the last tube 9T. Finally, 9 different serial dilutions were achieved. From 3T, 5T, 7T, and 9T-test tubes, about 100ul liquid was poured and spread on serial-wise labeled plates containing Hi Chrome Bacillus Agar. The plates were incubated at 37℃ for one day.

3.4. Isolation of *Bacillus* Strains on Hi Chrome agar

Bacteria were cultured on Hi Chrome agar. Based on color, *Bacillus* species were identified. On morphology based on shape, margin, and elevation, 34 Bacillus strains were isolated.

3.5. Identification of the *Bacillus* strain

3.5.1. Gram's staining

Gram staining serves the purpose of distinguishing between gram-positive and gram-negative bacteria by examining their distinctive cell wall characteristics.

3.5.1.1. Procedure for Gram's staining

In Gram staining, a 24-hour fresh bacterial culture was used. Aseptic techniques were employed to create a bacterial smear by placing a small drop of autoclaved water onto a clean glass slide using a sterile loop. The smear was allowed to air-dry and then carefully heat-fixed. Subsequently, the smear was covered with crystal violet solution and left for 1 minute before being rinsed with distilled water. The iodine solution was then applied for 45 seconds, followed by a thorough washing with distilled water. To remove excess stain, the smear was treated with 95% alcohol. Afterward, the smear was counterstained with safranin for 60 seconds, air-dried, and finally, observed under a microscope using a 100X lens.

3.6. Biochemical characterization of *Bacillus*

3.6.1. Catalase test

This test was used to identify the presence of the Catalase enzyme within a specific bacterial strain. A colony that was one day old was placed on a slide and combined with a 3% hydrogen peroxide solution. The Catalase enzyme, if present, would break down the H_2O_2 into water and oxygen, resulting in the production of bubbles. The formation of bubbles was then observed to confirm the presence of Catalase.

3.6.2. Oxidase Test:

This test was used to detect the presence of the cytochrome c oxidase enzyme in a specific bacterial strain. A small strip of filter paper was used, onto which one to two drops of oxidase reagent (tetramethyl para-phenylenediamine) were applied. Using an inoculating loop, a bacterial

culture was streaked onto the filter paper, and color change was noted. The presence of a dark purple color indicates the existence of cytochrome c oxidase, as it undergoes oxidation to produce an indole phenol product.

3.6.3. Citrate Utilization

This test is utilized to determine a bacterial strain's ability to utilize citrate as the sole carbon source in the absence of lactose, glucose, and ammonia as nitrogen sources. In Simmons citrate agar medium, ammonium ions serve as the nitrogen source, while sodium citrate acts as the carbon source. Bacteria with the capability to metabolize citrate salt can convert it into organic acids and carbon dioxide. The reaction of sodium with sodium carbonate and carbon dioxide results in the formation of an alkaline salt. The pH indicator in this medium detects the pH increase, leading to a color change from green (neutral) to Prussian blue (alkaline). The procedure involves streaking the bacterial strain onto the slant of Simmons citrate agar and incubating it for 24 hours at 37℃. A positive result is indicated by growth on the slant and a color change from green to blue.

3.6.4. Motility testing of *Bacillus* strains.

The motility of microorganisms was assessed using SIM agar as the testing medium. The agar was sterilized, and test tubes were prepared. Fresh bacterial cultures were inoculated by introducing a bacterial sample into the agar via stabbing. Subsequently, the inoculated tubes were placed in an incubator at 28°C for 24 hours. Following the incubation period, the growth patterns of the bacteria were examined. Microorganisms displaying limited growth along the inoculation line were categorized as non-motile, whereas those exhibiting a diffuse growth pattern were regarded as motile.

3.7. Isolation of Phosphate Solubilizing Bacteria (PSB)

Thirty-four bacterial strains were isolated on Hi Chrome ager. Then NBRIP media was prepared for the isolation of PSBs. For the preparation of NBRIP media, tricalcium phosphate was used as

a phosphate source and glucose was used as a carbon source. After autoclave, NBRIP media was poured into plates and all bacterial strains were point inoculated. For seven days, plates were incubated at 28^0C. After the required time, all bacterial strains formed clear zones around their colonies that indicated their phosphate solubilizing activity. Colony diameter and halozones were measured.

Then calculated phosphate solubilizing index by using a formula.

Phosphate solubilizing index (PSI) = (Colony diameter+ Halozone diameter) /Colony diameter

The phosphate solubilizing efficiency of all 34 strains was calculated by using the formula:

Table 3.2: Composition of NBRIP Media

Components	Concentration(g/L)
$Ca_3(PO_4)_2$	5
$MgCl_2$.6H2O	5
$MgSO_4$.7H2O	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
Technical agar	2
Glucose	10

3.8. Secondary screening of phosphate solubilizing bacteria on different media & pH

3.8.1. Solubilization of inorganic phosphate having tricalcium phosphate as phosphate source in solid medium at different pH

NBRIP media was prepared for screening of four bacteria strains (S5, S8, S14 and S32). For the preparation of NBRIP media, $Ca_3 (PO_4)_2$ was used as a phosphate source and glucose was used as a carbon source. pH was adjusted at different Ph values (5,7,9 and 11) before sterilization. After autoclave, NBRIP media was poured into plates and bacterial strains were point inoculated at 4 pH values (5, 7, 9, and 11). For seven days, plates were incubated at 28^0C. After the required time, all four bacterial strains formed clear zones around their colonies that indicated their phosphate solubilizing activity. Colony diameter and halo zones were measured. Then calculated phosphate solubilizing index and phosphate solubilizing efficiency.

3.8.2. Quantitative estimation of phosphate solubilizing bacteria having tricalcium phosphate as a phosphate source at different pH

To check the quantification of phosphate solubilizing bacterial isolates (5S, 8S,14S, and 32S), NBRIP broth media having $Ca_3(PO_4)_2$was prepared and their pH was adjusted at 5,7 and 9 before autoclaved. After sterilization, inoculated fresh bacterial culture 5S, 8S, 14S, and 32S in NBRIP broth. After inoculation, the cultures were kept on an orbital shaker at 150 rpm and incubated for 7 days at 28°C.After 7 days, bacterial cultures of 5S, 8S, 14S, and 32S are then centrifuged at 6000rpm for 20 minutes.

3.8.3. Quantitative estimation of phosphate solubilizing bacteria having Aluminium phosphate as a phosphate source at 5 pH values:

To check the quantification of PSB isolates (5S, 8S,14S, and 32S), NBRIP broth media having Al_3PO_4 as a phosphate source was prepared and their pH was adjusted at 5,7 and 9 before autoclaved. After sterilization, inoculated Fresh bacterial culture 5S, 8S, 14S, and 32S in NBRIP broth. After inoculation, the cultures were kept on an orbital shaker at 150 rpm and incubated for

7 days at 28°C. After 7 days, bacterial cultures of 5S, 8S, 14S, and 32S are then centrifuged at 6000rpm for 20 minutes.

To check the bacterial ability for PS, a method described by King (1932) was followed. In NBRIP broth, bacterial cultures were grown and centrifuged at 50000 rpm for 30 minutes. Reagents such as ammonium molybdate, stannous chloride, and standard phosphate solution were used. 10ml culture supernatant of PS strains (5S, 8S, 14S and 32S) was taken in conical flask. Added ammonium molybdate- sulfuric acid solution and mix well. Add 1 drop of stannous chloride and shake properly. A blue color was developed that indicates phosphorus solubilization. Then by using a spectrophotometer, OD was measured at 600nm.

3.9. Antifungal activity of *Bacillus* strains

Isolated strains were checked for their antagonistic activity against fungal phytopathogen *Aspergillus niger*. Fungus was spread on SDA and bacterial strains were point inoculated. The plates were kept at 28^0C for 5-7 days and zones of inhibition were observed.

3.10. Plant growth -promoting traits of PSBs

3.10.1. Indole acetic acid production

Bacterial strains were selected and introduced into 30 mL of LB broth that was supplemented with tryptophan. The culture was then incubated at 28°C for a period of three to five days with gentle shaking. After the specified incubation timeframe, the culture broth was centrifuged at conditions of 10,000 rpm and 4°C, lasting for 10 minutes. Following centrifugation, a 2 mL portion of the resulting cell-free supernatant was combined with an equal volume (2 mL) of Salkowski reagent. The Salkowski reagent consists of 35% HClO4 (Perchloric acid) and 0.5 M FeCl3 (iron chloride). The resultant mixture, formed by the combination of the cell-free supernatant and the Salkowski reagent, was shielded from light and allowed to react at room temperature for a duration of 20 to 30 minutes. The presence of a pink coloration following this incubation period was indicative of indole acetic acid (IAA) production(Ullah & Bano, 2019).

3.10.2. Hydrogen cyanide biosynthesis

We prepared nutrient agar plates supplemented with glycine and spread fresh bacterial cultures using a spread inoculation technique. Sterilized filter papers were dipped in a solution containing 0.5% picric acid and 2% sodium carbonate. These treated filter papers were then cautiously placed on the agar surface. Subsequently, the plates were incubated at 28°C for 4 days. A positive result was indicated by the appearance of an orange color on the filter paper."

3.11. Phosphatases production test

To check the phosphatases production, prepare a phenolphthalein diphosphate solution. Tryptic soya agar was prepared and phenolphthalein. Then media was poured into plates. Inoculated bacterial cultures and incubated at 28^{o}C for 48 hours. After incubation, add ammonium hydroxide solution drops and leave for 15 minutes. After 15 minutes by adding ammonium hydroxide, the pink color indicated the positive results of bacterial strains S5, S8, S14, and S32.

3.12. Effect of phosphate solubilizing bacteria on *Zea mays* growth

3.12.1. Pot experiment by using phosphate solubilizing bacteria and inorganic phosphate source

The experimental setup was conducted in a greenhouse during May-June 2023, with three replications. The pot experiment included three controls (non-inoculated with PSB), two PSB species, and two inorganic phosphate sources. Two different soil conditions were prepared, autoclaved soil and un-autoclaved soils. Each pot was filled with 42.9 mg of soil. Before putting the seeds into pots, pot soil was completely mixed with the respective phosphate sources. In half treatments, tricalcium phosphate (TCP) was evenly mixed into the soil while aluminum phosphate was added to the other half, based on maize nutrient requirements.

3.12.1.1. Sterilization of seeds:

Maize seeds were obtained from NARC Islamabad. In the lab, *Zea mays* seeds were surface sterilized with 3% bleach for approximately 1 minute and then rinsed thoroughly with autoclaved water three times. Following the sterilization process, the seeds were left to air-dry.

3.12.1.2. Inoculation of seeds into bacterial broth:

These prepared seeds were then inoculated into 4 separate flasks, each serving a distinct purpose. The 1stflask contained a bacterial broth culture of the S5 strain, 2nd flask contained the bacterial broth culture of the S32 strain, 3rd flask contained bacterial consortia and the fourth flask served as a control.

3.12.1.3. Seeds sowing:

Equal numbers of seeds (3 seeds per pot) were planted in autoclaved and unautoclaved soil. A 2 mL broth culture was applied to the seeds, followed by a uniform 3 cm layer of soil. 3 control pots were maintained without phosphate-solubilizing bacteria but with inorganic phosphate sources. Pots were watered daily to maintain soil moisture.

3.12.1.4. Agronomic variables

After 15 days, agronomic variables including shoot and root length, dry weight, wet weight, and chlorophyll content were measured to evaluate plant growth. Seed germination percentage was calculated. Before harvesting, chlorophyll content was measured. The roots were rinsed with tap water to remove soil particles.

3.12.1.5. Treatments used in *Zea mays* growth under controlled conditions

Autoclaved soil	Un-autoclaved soil
Soil + Seed + $Ca_3 (PO_4)_2$	Soil + Seed + $Ca_3 (PO_4)_2$
Soil+ Seed + Al_3PO_4	Soil+ Seed + Al_3PO_4
Soil+ Seed + $Ca_3(PO4)_2$+ Al_3PO_4	Soil+ Seed + $Ca_3(PO4)_2$+ Al_3PO_4
Soil+ Seed + S5	Soil+ Seed + S5
Soil+ Seed + S32	Soil+ Seed + S32
Soil+ Seed + S5+S32	Soil+ Seed + S5+S32
Soil+ Seed + S5+ $Ca_3 (PO_4)_2$	Soil+ Seed + S5+ $Ca_3 (PO_4)_2$
Soil+ Seed + S5+ Al_3PO_4	Soil+ Seed + S5+ Al_3PO_4
Soil+ Seed +S32+ $Ca_3(PO_4)_2$	Soil+ Seed +S32+ $Ca_3(PO_4)_2$
Soil+ Seed + S32+ Al_3PO_4	Soil+ Seed + S32+ Al_3PO_4
Soil+ Seed + S5+ S32+ $Ca_3(PO_4)_2$	Soil+ Seed + S5+ S32+ $Ca_3(PO_4)_2$
Soil+ Seed + S5+ S32+ Al_3PO_4	Soil+ Seed + S5+ S32+ Al_3PO_4
Soil+ Seed + S5+ S32+ $Ca_3(PO_4)_2$+Al3PO_4	Soil+ Seed + S5+ S32+ $Ca_3(PO_4)_2$+Al3PO_4

4. Results

4.1. Isolation of *Bacillus*

Based on colony characteristics like color, shape, size, appearance, texture, elevation, and margin, thirty-four bacterial strains were isolated on Hi chrome agar for screening purposes.

Figure 4.1: Isolation of the *Bacillus* strains by spread plate method on Hi chrome *Bacillus* agar

Table 4.1: Colony morphological characteristics of *Bacillus* species on Hi Chrome agar

Strains	Shape	Margins	Elevations	Size	Appearance	Color	Texture
S5	Circular	Undulate	Flat	Small	Rough	White	Mucoid
S8	Circular	Entire	Raised	Small	Glistering	Green	Mucoid
S14	Circular	Undulate	Raised	Small	Rough	Pink	Mucoid
S32	Punctiform	Entire	Flat	Small	Rough	Green	Mucoid

4.2. Identification of *Bacillus*

4.2.1. Gram staining:

All the *Bacillus* species were gram-positive and rod-shaped bacteria.

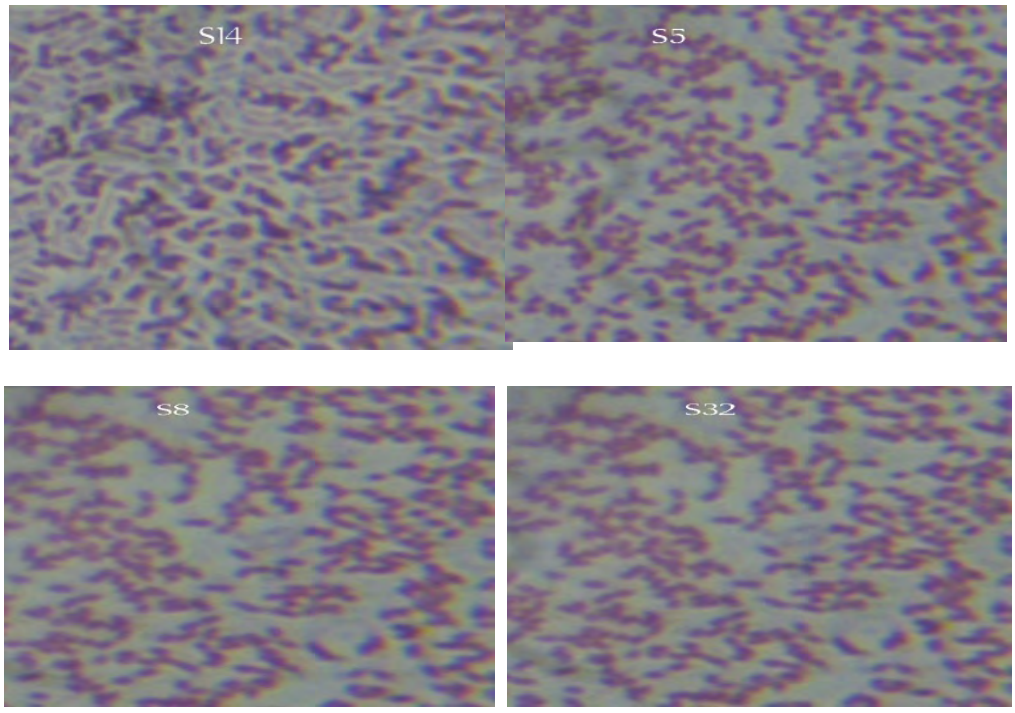

Figure 4.2: Gram staining of bacterial strains S5, S8, S14 and S32

4.3. Identification of *Bacillus* based on Biochemical Tests:

Table: 4.2: Biochemical identification of bacterial strains

Biochemical Tests	S5	S8	S14	S32
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	+	-	+	+
HCN	+	+	+	+
Citrate utilization	+	+	+	+

4.4. Isolation of phosphate solubilizing bacteria on NBRIP media

Out of thirty-four bacterial strains, thirty bacterial isolates showed clear holozones around their colonies. While four bacterial strains did not form holozones around colonies. By measuring colony diameter and holozones, the phosphate solubilizing index and phosphate solubilizing efficiency were calculated. Based on higher PSI values, four bacterial strains (S5,S8,S14, and S32) were selected for further screening. Bacterial strain S14 showed higher PSI value of 5.75 .

Figure 4.3.Zones indicating phosphate solubilization by bacterial strains S5, S8, S14 and S32

Table 4.3: Phosphate solubilizing index values of phosphate solubilizing bacterial strains ((S5, S8, S14 and S32)

Bacterial Strain code	Colony Diameter (cm)	Halo Zone (cm)	Phosphate solubilizing index (PSI)
S5	0.6	1.6	3.66
S8	0.8	2	3.50
S14	0.4	1.9	5.75
S32	0.6	1.8	4.00

4.5. Secondary screening of phosphate solubilizing bacteria on different media & pH

4.5.1. Phosphate solubilizing index of phosphate solubilizing bacterial strains having Ca_3PO_4 as a phosphate source in solid media at different pH values (5, 7, 9, and 11)

After incubation, colony diameter, and holozones were measured, and calculated PSI values. Zones indicated solubilization of phosphorus due to the presence of phosphatases and the production of organic acids. The colony diameter of bacterial strains (S5, S8, S14, and S32) ranged between **0.6cm** to **2cm** and the halo zone diameter of bacterial strains (S5, S8, S14, and S32) ranged between **1cm** to **2.3cm** at different pH values. PSI values ranged between **2.095** to **2.785**. Strain S5 shows maximum PSI value i.e., **2.714** at pH 7. Strain S8 shows maximum PSI value i.e., **2.731** at pH 7. Strain S14 shows a higher PSI value (**2.66**) at pH 5. Strain S32 shows a higher PSI value (**2.785**) at pH 5.

Figure 4.4: Phosphate solubilizing zones of bacterial isolates (S5, S8, S14, and S32) at different pH

Table 4.4: Phosphate solubilizing index values of bacterial strains (S5, S8, S14 and S32) at pH 5

Bacterial strains	Colony diameter (cm)	Holozones (cm)	PSI=(CD+HZ)/CD
S5	1±0.00	1.65±0.49	2.65
S8	0.70±0.14	1.2±0.14	2.714
S14	0.60±0.14	1±0.42	2.666
S32	0.70±0.14	1.25±0.07	2.785

Each value is Mean, ± indicates S.D

Table 4.5: Phosphate solubilizing index values of bacterial strains (S5, S8, S14 and S32) at pH 7

Bacterial strains	Colony diameter (cm)	Holozones (cm)	PSI=(CD+HZ)/CD
S5	1.05±0.64	1.8±0.57	2.714
S8	1±0.71	1.7±0.71	2.731
S14	2.1±0.14	2.3±0.14	2.095
S32	1.05±0.49	1.65±0.21	2.713

Each value is Mean, ± indicates S.D

Table 4.6: Phosphate solubilizing index values of bacterial strains (S5, S8, S14 and S32) at pH 9

Bacterial strains	Colony diameter (cm)	Holozones (cm)	PSI=(CD+HZ)/CD
S5	0.75±0.07	1.05±0.07	2.401
S8	1±0.28	1.7±0.71	2.665
S14	1.10±0.28	1.6±0.57	2.45
S32	1.25±0.35	2.15±1.20	2.72

Each value is Mean, ± indicates S.D

Table 4.7: Phosphate solubilizing index values of bacterial strains (S5, S8, S14 and S32) at pH 11

Bacterial strains	Colony diameter (cm)	Holozones (cm)	PSI=(CD+HZ)/CD
S5	1.25±0.07	1.6±0.14	2.28
S8	1.3±0.14	1.6±0.28	2.23
S14	1.2±0.42	1.45±0.35	2.20
S32	1.1±0.28	1.55±0.07	2.40

Each value is Mean, ± indicates S.D

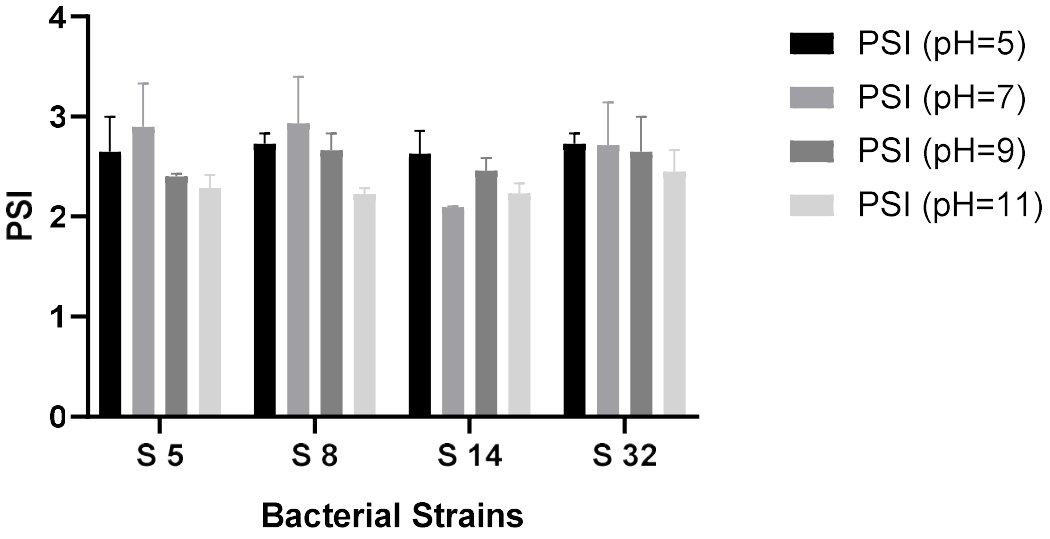

Fig 4.5. Phosphate solubilizing index of bacterial isolates (S5, S8, S14, and S32) on NBRIP agar at different pH values. Error bars Mean ± standard error (n=3)

4.5.2. Quantitative estimation of phosphate solubilizing bacteria having tricalcium phosphate at different pH

Bacterial isolates were inoculated in NBRIP broth having $Ca_3 (PO_4)_2$ at pH (5, 7 and 9). These bacterial cultures were placed on an orbital shaker at 150 rpm and incubated for 7 days at 28°C. After 7 days, bacterial cultures of 5S, 8S, 14S, and 32S are then centrifuged at 6000rpm for 20 minutes. Cultural supernatant was used for the assessment of solubilized $Ca_3 (PO_4)_2$ by phosphate solubilizing bacterial strains (S5, S8, S14 and S32). Using ammonium molybdate and stannous chloride with bacterial supernatant, OD values were observed at a spectrophotometer at 600nm. It was observed that all strains (S5, S8, S14, and S32) solubilized maximum $Ca_3 (PO_4)_2$ at pH 7 and less solubilized at alkaline pH as compared to acidic pH. Strain S32 showed maximum OD values of **2.816** at pH 7while S8 showed a minimum of **2.4618.**

Table: 4.8: Quantitative estimation of phosphate solubilizing bacteria by using $Ca_3 (PO_4)_2$at different pH (5, 7, and 9):

Bacterial strains	pH 5	pH 7	pH 9
S5	2.623±0.08	2.7065±0.01	2.3425±0.06
S8	1.9035±0.07	2.4618±0.07	1.6813±0.1
S14	2.533±0.04	2.782±0.05	2.117±0.09
S32	2.689±0.1	2.816±0.1	2.488±0.1

Each value is Mean, ± indicates S.D

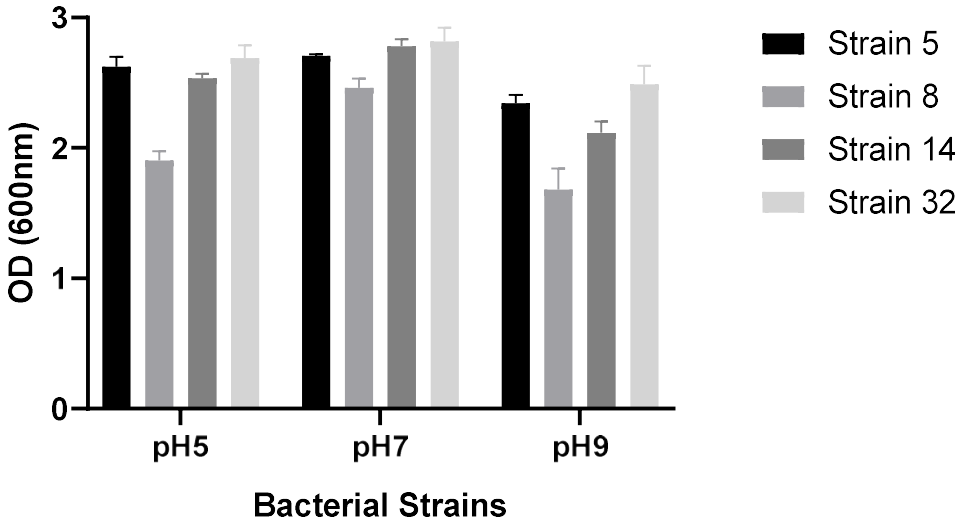

Fig 4.6: Solubilization of tricalcium phosphate at different pH values and their OD values of culture supernatant after 7 days of incubation at 28 C. Error bars Mean ± standard error (n=3)

4.5.3. Quantitative estimation of phosphate solubilizing bacteria having aluminum phosphate as a phosphate source at different pH

Bacterial isolates were inoculated in NBRIP broth having Al_3PO_4 at pH (5, 7, and 9). These cultures were placed on an orbital shaker at 150 rpm and incubated for 7 days at 28°C. After 7 days, bacterial cultures of 5S, 8S, 14S, and 32S are then centrifuged at 6000rpm for 20 minutes. Cultural supernatant was used for the assessment of solubilized Al3PO4 by phosphate solubilizing bacterial strains (S5, S8, S14, and S32). Using ammonium molybdate and stannous chloride with bacterial supernatant, OD values were observed at a spectrophotometer at 600nm. It was observed that all strains (S5, S8, S14, and S32) solubilized maximum Al_3PO_4 at pH 7. As compared to alkaline pH, more solubilization of phosphate sources by PSB occurred at acidic pH. Strain S5 showed a maximum OD value of **2.405** at pH 7while S8 showed a minimum of **1.847.**

Table: 4.9: Quantitative estimation of phosphate solubilizing bacteria by using $AlPO_4$at different pH (5, 7, and 9)

Bacterial strains	pH 5	pH 7	pH 9
S5	2.394±0.04	2.405±0.08	1.6105±0.12
S8	1.673±0.07	1.847±0.07	1.146±0.05
S14	1.413±0.03	2.0025±0.01	1.157±0.12
S32	2.0915±0.12	2.298±0.07	2.0955±0.05

Each value is Mean, ± indicates S.D

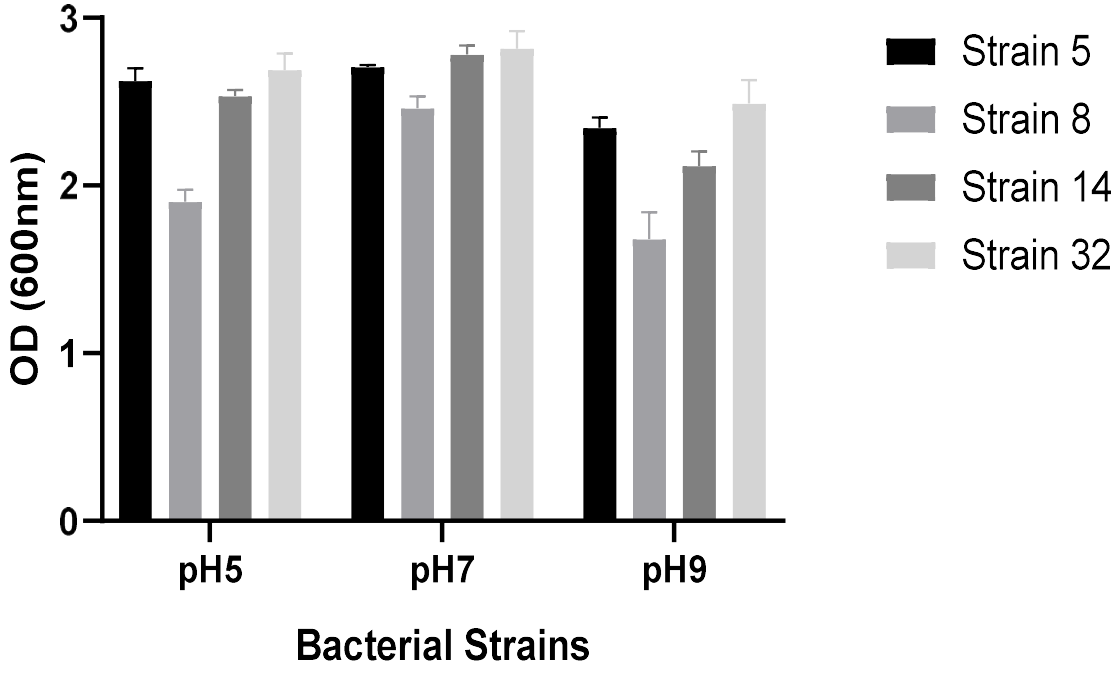

Fig 4.7: Solubilization of aluminum phosphate at different pH values and their OD values of culture supernatant after 7 days of incubation at 28 oC. Error bars Mean ± standard error (n=3)

4.6. Zone of inhibition against fungal phytopathogens by *Bacillus* strains S5 and S32

Fig 4.8: Antifungal activity of *Bacillus* strains S5 and S32

Table 4.10: Antifungal activity of *Bacillus* strains S5 and S32

Bacterial Strains	Antifungal activity against *Aspergillus niger*(mm)
S5	62nm±0.05
S32	65mm±0.01

Each value is Mean, ± indicates S.D

4.7. Detection of plant growth -promoting traits by *Bacillus*:

4.7.1. Indole acetic acid production test:

Bacterial strains S5 and S32 were introduced into Luria Bertani broth supplemented with tryptophan. The development of a pink color upon the addition of the Salkowski reagent into the cell-free supernatant indicated positive results. Bacterial strain S5 and S32 showed positive results.

4.7.2. HCN production

Plant growth-promoting rhizobacteria are known for their ability to produce a diverse range of chemical compounds that confer countless benefits to plants. One such compound is hydrogen cyanide (HCN), which has gained recognition as a biocontrol agent due to its demonstrated toxicity against plant pathogens. Bacterial strains S5 and S32 showed positive results for HCN production.

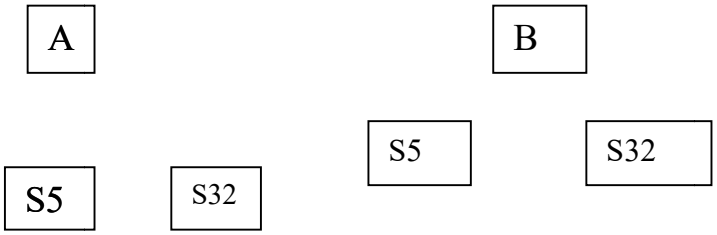

Fig 4.9: A=**IAA production, B=HCN production test by bacterial strains S5, and S32**

4.8. Detection of phosphatases

Figure4.10. Phosphatases detection by bacterial strains (S5, S8, S14 &S32)

4.9. Effect of different treatments on *Zea mays* growth under controlled condition

After 15 days of *Zea mays* seed sowing, agronomic variables including shoot and root length, dry weight, wet weight, and chlorophyll content were measured to evaluate plant growth. Seed germination percentage was calculated. Before harvesting, chlorophyll content was measured. Results have shown that due to the inoculation of PS bacterial strains S5 and S32 along with tricalcium phosphate, the significant increase in root length, shoot length, dry weight, wet weight, and chlorophyll content of maize plants in un-autoclaved soil as compared to autoclaved soil was observed. (Table 4.15 and Table 4.16).As compared to autoclaved soil, Maize growth is increased more in unautoclaved soil.

Fig 4.11: Effect of phosphate solubilizing bacteria and inorganic phosphates on *Zea mays* growth in unautoclaved soil

Fig 4.12: Effect of phosphate solubilizing bacteria and inorganic phosphates on *Zea mays* growth in autoclaved soil

Fig 4.13: Effect of different Treatments on *Zea mays* growth under controlled conditions, T1.Seed+ Ca_3 $(PO_4)_2$, T2.Seed+ Ca_3 $(PO_4)_2$+$AlPO_4$ T3. Seed + S5 +S32. T4. Seed + S5. T5. Seed+ S5+Ca_3 $(PO_4)_2$. T6.Seed+S32+ S5+Ca_3 $(PO_4)_2$ +$AlPO_4$

Fig 4.14: Effect of different Treatments on *Zea mays* growth, T1.Seed+ Ca_3 $(PO_4)_2$, T2.Seed+ Ca_3 $(PO_4)_2$+$AlPO_4$ .T3. Seed + S5+S32. T4. Seed + S32. T5. Seed+ S32+ Ca_3 $(PO_4)_2$. T6.Seed+S32+ S5+Ca_3 $(PO_4)_2$ +$AlPO_4$

Table 4.11: Effect of phosphate solubilizing bacteria and inorganic phosphates on *Zea mays* growth in autoclaved soil: Means of triplicate values ± S.D. Significance value ≤ 0.05

Treatments (Autoclaved soil)	Germination Rate(%)	Chlorophyll Content	Width of blade (cm)	Wet weight (g)	Dry weight (g)	Total plant length (cm)
Soil+ Seed + $Ca_3(PO_4)_2$	100%	19±0.10I	1.2±0.10C	2.8±0.15 E	0.26±0.05 E	27.67±1.53 GH
Soil+ Seed+ Al_3PO_4	100%	18.2±0.20J	1.1±0.10C	2.5±0.35 E	0.22±0.02 E	25.67±0.58 H
Soil+ Seed+ $Ca_3(PO_4)_2$+ Al_3PO_4	100%	19.2±0.25I	1.2±0.10C	3±0.15E	0.3±0.01E	28.33±1.15 G
Soil+ Seed+ S5	100%	24.6±0.55G	1.53±0.06 B	3.9±0.21 CD	0.72±0.05 D	35.33±0.58 F
Soil+ Seed+ S32	100%	23.6±0.55H	1.5±0.10B	3.7±0.32 D	0.65±0.07 D	34.67±1.53 F
Soil+ Seed+ S5+S32	100%	29.7±0.61D	1.6±0.20B	5.1±0.15 B	0.91±0.03 C	40.67± 2.08CD
Soil+ Seed+ S5+ $Ca_3(PO_4)_2$	100%	25.9±0.6E	1.56±0.06 B	4.3±0.57 C	0.77±0.04 CD	38.67±0.58 DE
Soil+ Seed+S5+ Al_3PO_4	100%	25.7±0.32F G	1.53±0.06 B	3.7±0.32 D	0.73±0.04 D	36.33±0.58 EF
Soil+ Seed+ S32+ $Ca_3(PO4)_2$	100%	25.1±0.61EF	1.56±0.06 B	4±0.15C D	0.72±0.04 D	38.67±1.53 DE
Soil+ Seed+ S32+ Al_3PO_4	100%	23.6±0.36H	1.5±0.10B	3.9±0.06 CD	0.68±0.04 D	35.67±1.53 F
Soil+ Seed+ S5+ S32+ $Ca_3(PO_4)_2$	100%	32.9±0.36B	1.86±0.06 A	5.5±0.45 A	1.24±0.27 AB	44.00±1.73 B
Soil+ Seed+ S5+ S32+ Al_3PO_4	100%	31.5±0.50C	1.63±0.15 B	5.3±0.25 AB	1.14±0.19 B	41.33±1.53 C
Soil+ Seed+S5+ S32+ $Ca_3(PO_4)_2$+Al_3PO_4	100%	34.5±0.50A	1.9±0.10A	5.7±0.21 A	1.39±0.02 A	47.00±2.00 A

Table 4.12: Effect of phosphate solubilizing bacteria and inorganic phosphates on *Zea mays* growth in unautoclaved soil: Means of triplicate values ± S.D. Significance value ≤ 0.05

Treatments (Unautoclaved soil)	Germination Rate (%)	Chlorophyll Content	Width of blade (cm)	Wet weight (g)	Dry weight (g)	Total plant length (cm)
Soil+ Seed + $Ca_3(PO_4)_2$	100%	19.2±0.26I	1.26±0.06F	3.1±0.36C	0.35±0.01 H	28.33±1.15 EF
Soil+ Seed+ Al_3PO_4	100%	18.8±0.26I	1.2±0.10F	2.9±0.25C	0.31±0.03 H	27.33±0.58 F
Soil+ Seed+ $Ca_3(PO_4)_2$+ Al_3PO_4	100%	20.1±0.32H	1.3±0.10F	3.3±0.47C	0.40±0.02 G	30.33±1.53 E
Soil+ Seed+ S5	100%	25.5±0.50F G	1.56±0.06 DE	4.3±0.50B	0.89±0.03 EF	37±2.00D
Soil+ Seed+ S32	100%	25.06±0.40 G	1.56±0.06 DE	4.1±0.95B	0.87±0.02 F	36.33±1.15 D
Soil+ Seed+ S5+S32	100%	35.46±0.50C	1.73±0.21C D	5.6±0.40A	1.31±0.02 C	43.33±1.53 B
Soil+ Seed+ S5+ $Ca_3(PO_4)_2$	100%	28.5±0.50D	1.59±0.01 DE	4.7±0.25B	0.95±0.03 D	40.33±1.53 C
Soil+ Seed+S5+ Al_3PO_4	100%	26.1±0.57EF	1.56±0.06 DE	4.5±0.40B	0.93±0.03 DE	38.67±1.53 CD
Soil+ Seed+ S32+ $Ca_3(PO_4)_2$	100%	27.7±0.38D	1.56±0.06E	4.6±0.38B	0.92±0.03 DE	40.67±2.08 C
Soil+ Seed+ S32+ Al_3PO_4	100%	26.6±0.55E	1.53±0.12 DE	4.4±0.45B	0.89±0.03 EF	37.33±0.58 D
Soil+ Seed+ S5+ S32+ $Ca_3(PO_4)_2$	100%	38.1±1.00B	1.96±0.06B	5.9±0.25A	1.43±0.03 B	48.67±1.53 A
Soil+ Seed+ S5+ S32+ Al_3PO_4	100%	36±0.15C	1.76±0.15C	5.7±0.30A	1.35±0.03 C	45.67±0.58 B
Soil+ Seed+S5+ S32+ $Ca_3(PO_4)_2$+Al_3PO_4	100%	39±0.15A	2.2±0.10A	6±0.15A	1.55±0.03 A	50.33±1.53 A

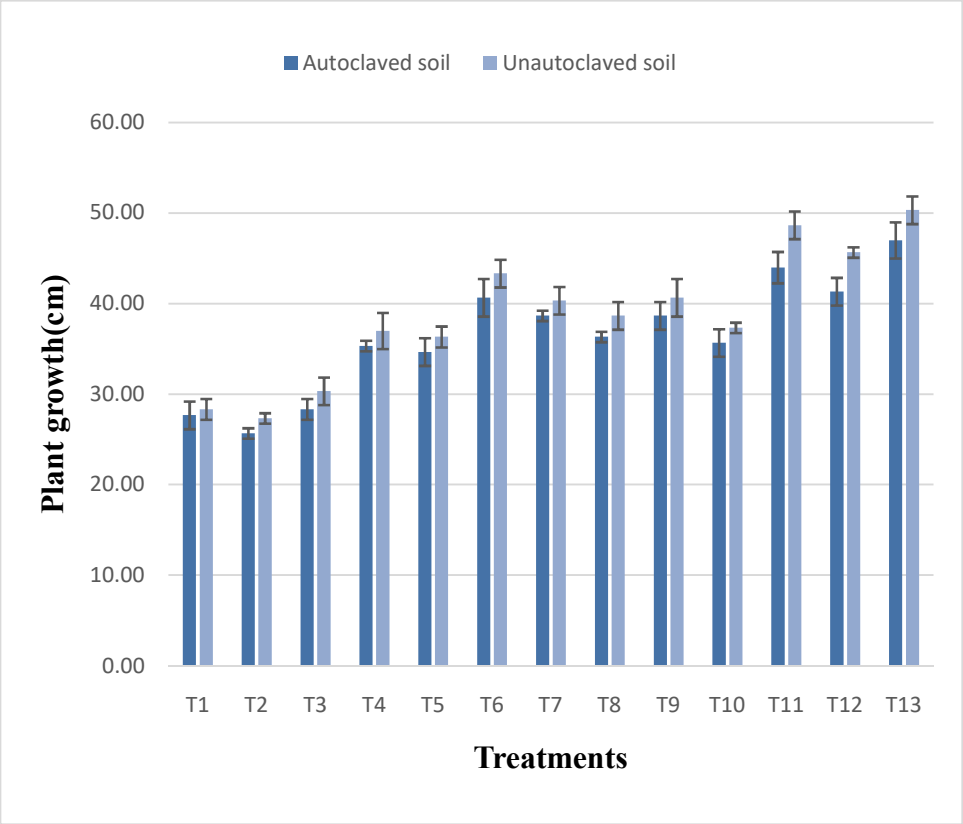

Fig 4.15: Effect of phosphate solubilizing bacteria and inorganic phosphates on *Zea mays* growth (cm) in autoclaved and unautoclaved soil. Means of triplicate values ± S.D. T1=Seed+Ca_3PO_4, T2=Seed+Al_3PO_4, T3= Seed+Ca_3PO_4+ Al_3PO_4, T4=Seed+S5, T5=Seed+S32, T6= Seed+S5+325, T7= Seed+S5+Ca_3PO_4,T8=Seed+S5+Al_3PO_4,T9=Seed+S32+Ca_3PO_4,T10=Seed+S32+Al_3PO_4,T11=Seed+S5+S32+Ca_3PO_4,T12=Seed+S5+S32 +Al_3PO_4, T13=Seed+S5+S32 + Ca_3PO_4+ Al_3PO_4

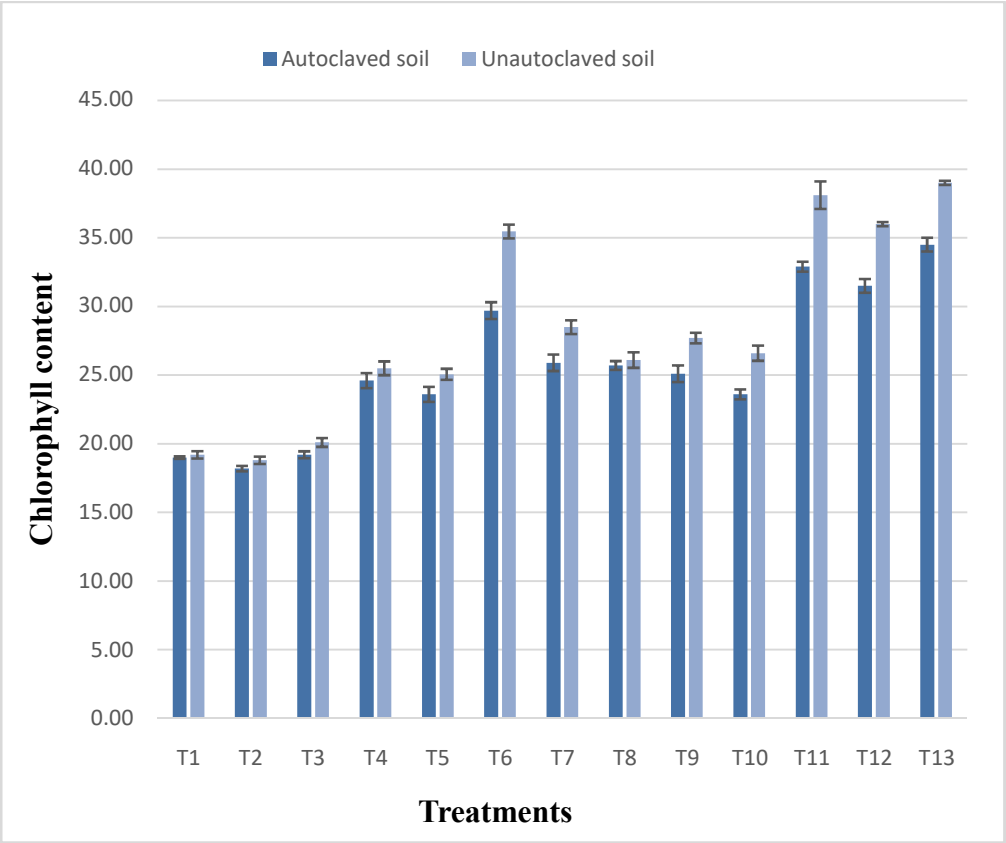

Fig 4.16: Effect of phosphate solubilizing bacteria and inorganic phosphates on chlorophyll content of *Zea mays* in autoclaved and unautoclaved soil. Means of triplicate values ± S.D. T1=Seed+Ca_3PO_4, T2=Seed+Al_3PO_4, T3= Seed+Ca_3PO_4+ Al_3PO_4, T4=Seed+S5, T5=Seed+S32, T6= Seed+S5+325,T7=Seed+S5+Ca_3PO_4,T8=Seed+S5+Al_3PO_4,T9=Seed+S32+Ca_3PO_4,T10=Seed+S32+ Al_3PO_4,T11=Seed+S5+S32+Ca_3PO_4,T12=Seed+S5+S32+Al_3PO_4,T13=Seed+S5+S32+Ca_3PO_4+Al_3PO_4

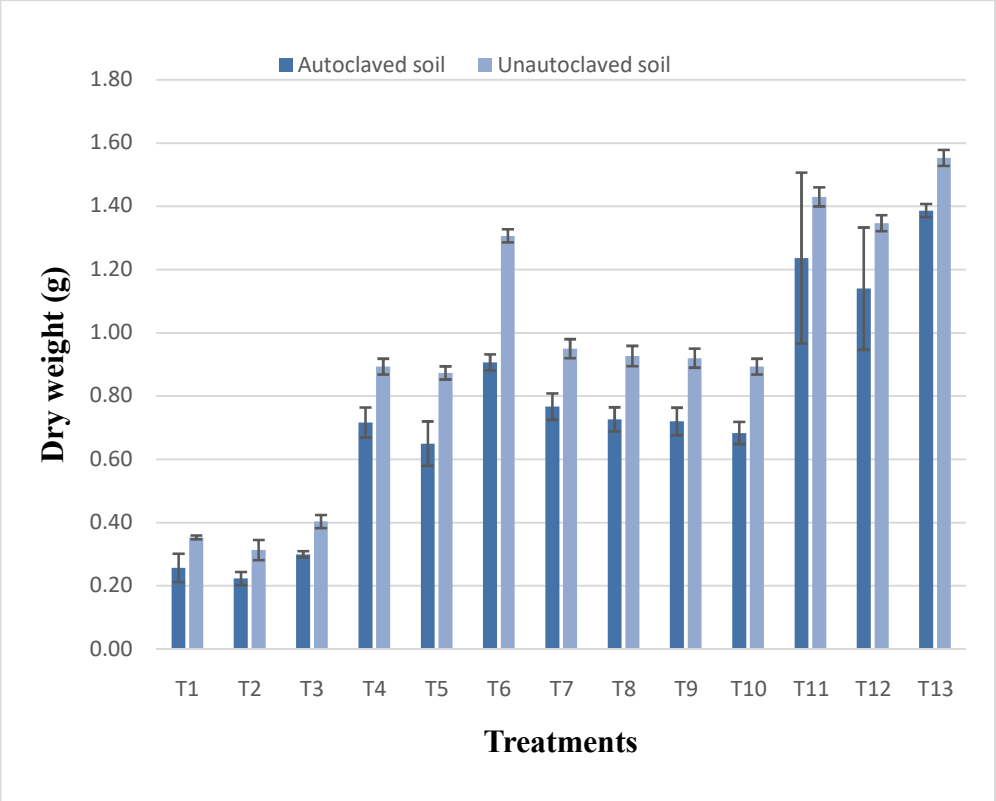

Fig 4.17: Effect of phosphate solubilizing bacteria and inorganic phosphates on dry weight (g) of *Zea mays* in autoclaved and unautoclaved soil. Means of triplicate values ± S.D. T1=Seed+Ca_3PO_4, T2=Seed+Al_3PO_4, T3= Seed+Ca_3PO_4+ Al_3PO_4, T4=Seed+S5, T5=Seed+S32, T6= Seed+S5+325, T7= Seed+S5+Ca_3PO_4,T8=Seed+S5+Al_3PO_4,T9=Seed+S32+Ca_3PO_4,T10=Seed+S32+Al_3PO_4,T11=Seed+S5+S32+Ca_3PO_4,T12=Seed+S5+S32 +Al_3PO_4, T13=Seed+S5+S32 + Ca_3PO_4+ Al_3PO_4

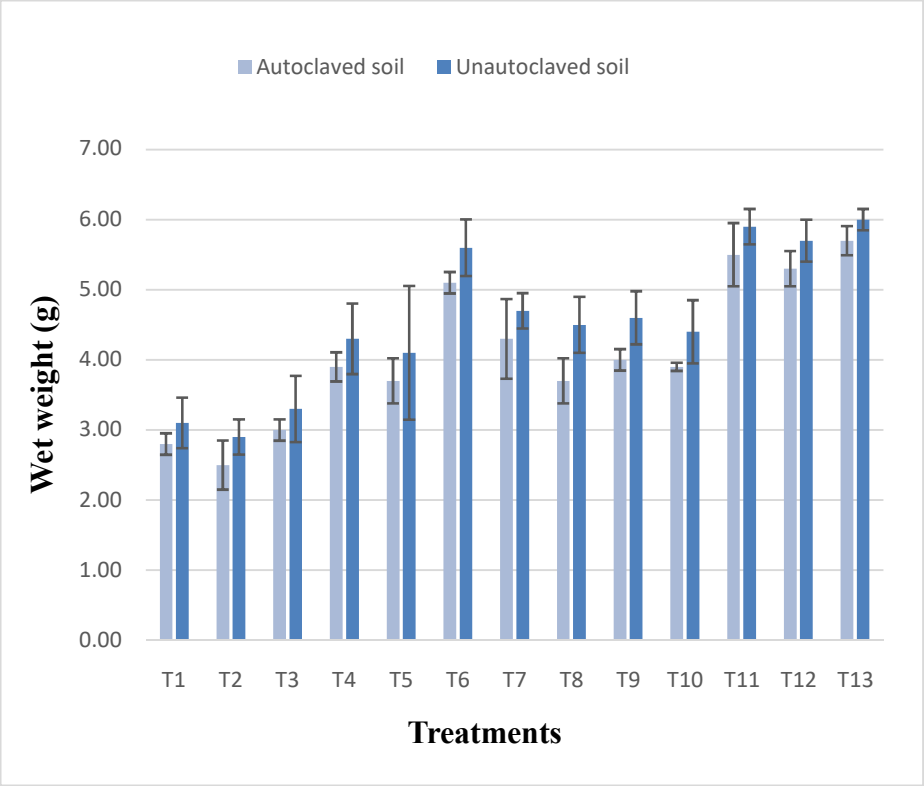

Fig 4.18: Effect of phosphate solubilizing bacteria and inorganic phosphates on wet weight (g) of *Zea mays* in autoclaved and unautoclaved soil. Means of triplicate values ± S.D. T1=Seed+Ca_3PO_4, T2=Seed+Al_3PO_4, T3= Seed+Ca_3PO_4+ Al_3PO_4, T4=Seed+S5, T5=Seed+S32, T6= Seed+S5+325, T7= Seed+S5+Ca_3PO_4,T8=Seed+S5+Al_3PO_4,T9=Seed+S32+Ca_3PO_4,T10=Seed+S32+Al_3PO_4,T11=Seed+S5+S32+Ca_3PO_4,T12=Seed+S5+S32 +Al_3PO_4, T13=Seed+S5+S32 + Ca_3PO_4+ Al_3PO_4

4. Discussion

In soil, phosphorus is a significant element for plant growth; however, it often becomes immobilized by various soil components, leading to its limited availability for plants (Zhang *et al*., 2017). This immobilization is primarily attributed to the adsorption of phosphorus by elements like aluminum, iron, calcium, and magnesium, as well as their respective oxides. Over time, these interactions can lead to the transformation of phosphorus into more complex forms. In alkaline soil, phosphorus is bound with calcium while in acidic soil, aluminum and iron-bound are formed (Maitra *et al*., 2015). To enhance plant productivity, it is commonly applied as fertilizer (Wei *et al*., 2017). In plant nutrition, phosphorus uptake is reliant on the availability of specific forms, namely H_2PO_4 and HPO_4^- (orthophosphates). Unfortunately, these accessible phosphorus forms are depleting rapidly from the plant rhizospheric region (Kumar *et al*., 2015). To address this deficiency, chemical phosphate fertilizers have been utilized. Although these fertilizers can increase plant growth. Nevertheless, a significant portion of the phosphorus introduced through these fertilizers becomes immobilized in the soil due to interactions with certain metals. Consequently, the effectiveness of applied phosphorus is compromised. This immobilized phosphorus can be liberated through the actions of phosphate solubilizing bacteria, which can dissolve these bound forms (Ahmad and Khan, 2012b).

In soil ecosystems, microorganisms play an important role in the conversion of precipitated P into soluble P form (Gronemeyer *et al*., 2011; Maitra *et al*., 2015). The region of soil surrounding plant roots accommodates a significant population of active bacterial species commonly known as plant growth promoting rhizobacteria (Reetha *et al*., 2014). An estimated majority, exceeding 95% of bacterial life exists within the rhizosphere of plants, playing a crucial role in facilitating plants' access to nutrients from the soil. A diverse range of microorganisms are present in the plant rhizosphere, many of which can enhance plant growth through their interaction with plant roots (Kumar *et al*., 2015). Various bacterial genera play essential roles in maintaining soil health and soil fertility through distinct biological activities and facilitating nutrient conversions. Beyond their role in making phosphorus more available to plants, these bacteria also exhibit other capabilities that promote plant growth.

In this study, thirty-four bacterial strains were isolated from soil samples by using Hi Chrome agar and identified as phosphate solubilizers. The selection of these strains was based on their

ability to solubilize inorganic phosphate on NBRIP media. Previous research indicates that bacteria possessing phosphate solubilizing capabilities belong to various families, including Enterococcaceae, Bacillaceae, etc(Azi*et al*., 2012; Acevedo *et al*., 2014; Yadav and Pandey, 2018). Several investigations have documented the isolation of phosphate solubilizing strains from the rhizosphere of diverse plant species, including wheat, within regular soil conditions (Linu*et al*., 2009; Iqbal *et al*., 2010; Ogut*et al*., 2010; Rajapaksha *et al*., 2011; Baig *et al*., 2012; Khan *et al*., 2017; Liu *et al*., 2018). Soil pH is a major factor that affects the adsorption of phosphorus.

Selected bacterial strains (S5, S8, S14, and S32) were identified as gram-positive and rod-shaped bacteria through Gram's staining as indicated in Fig 4.2. All bacterial strains showed positive results for various biochemical tests including Catalase, oxidase, Urease, citrate utilization, motility, and HCN production. Only bacterial strain S8 showed a negative motility test. These biochemical tests showed the identification of *Bacillus* strains. The results of different biochemical tests performed are listed in Table 4.2.Out of 34 bacterial strains, 30 bacterial strains showed clear colony zones and Holozones on NBRIP media that indicated phosphorus solubilization. Based on the highest PSI values, the four best bacterial strains (S5, S8, S14 and S32) were selected. Bacterial strain S14 showed the highest PSI value i.e., 5.75 as shown in Table 4.3. Higher phosphate solubilizing index values indicated that these selected strains have phosphate solubilization ability. Cleared zones indicated the presence of phosphatases enzymes that solubilized inorganic phosphates. Further analysis involved the quantitative and qualitative assessment of these selected strains (S5, S8, S14, and S32) by using two phosphate sources: $Ca_3(PO_4)_2$ and $AlPO_4$, at different pH values. Glucose was used as a carbon source. In qualitative assessment, after 7 days of incubation on NBRIP media containing tricalcium phosphate, clear zones were observed indicating phosphate solubilization. Colony diameter and holozones were measured and calculated phosphate solubilizing index. The phosphate solubilization index (PSI) exhibited variation, ranging from 2.095 to 2.785 at different pH values. Strain S32 and S14 showed the highest phosphate solubilizing index 2.785 and 2.66, respectively, at pH 5 due to the presence of acidic phosphatases indicated in Table 4.4. While bacterial strain S5 showed highest PSI value 2.714 and 2.731, respectively, at pH 7 as shown in Table 4.5. All bacterial strains showed maximum PSI values at acidic pH as compared to alkaline pH due to the production of acidic phosphatases as indicated in Tables 4.4 and 4.6. Results proved that at acidic pH,

tricalcium phosphates are more solubilized as compared to alkaline pH. PSBs have phosphatases enzymes that solubilize inorganic phosphate and form clear holozones around colony diameter as referred in Sharma *et al*., 2017; Behera *et al*., 2017.Enhanced phosphate solubilization is commonly observed when organisms have access to sufficient energy for the synthesis of various organic acids (Reza *et al*., 2017). Carbon sources serve as the primary energy reservoir, and it greatly affects the phosphate solubilization efficiency of the strains. Glucose was used as a carbon source. The concentration of carbon source also has a great effect on phosphate solubilization. Pallavi *et al*.(2020) also reported the same results. In quantitative assessment, the OD_{600} of PSB having $Ca_3 (PO_4)_2$ and $AlPO_4$ was measured by using a spectrophotometer. All selected bacterial strains (S5, S8, S14, and S32) showed higher OD values at pH 7 as indicated in Fig 4.6 and Fig 4.7. As compared to alkaline pH, bacterial strains showed higher OD values at acidic pH. PSB produces organic acids that create an acidic environment for inorganic phosphate solubilization. So, more solubilization of phosphate source occurs at acidic pH. Bacterial strains solubilized more tricalcium phosphate as compared to aluminum phosphate as a phosphate source because, at acidic pH, tricalcium phosphate is more soluble as compared to aluminum phosphate.

Bacillus strains S5 and S32 also showed antifungal activity against *Aspergillus niger* as shown in Table 4.10.Strains S5 and S32 showed positive IAA production test and HCN production test. Production of IAA indicated the plant growth-promoting traits. Selected bacterial strains S5, S8, S14, and S32 showed a pink color that indicated phosphatases production test as indicated in Figure 4.10.

In the pot experiment, inoculation of *Bacillus* strains S5 and S32 along with $Ca_3 (PO_4)_2$ and $AlPO_4$have a positive impact on the growth and development of *Zea mays* as indicated by an increase in germination rate, plant height, wet weight, chlorophyll content, the width of the blade, dry weight as compared to control as shown in Table: 4.11 and 4.12. Two types of soil were used, autoclaved and unautoclaved soil. *Zea Mays* growth is increased in unautoclaved soil as compared to autoclaved soil because, in unautoclaved soil, diverse types of microbes present that increase the plant growth by combining with selected strains. It means rhizospheric bacteria can increase the efficiency of PSB. When compared to the effect of solubilization of two phosphate sources on plant growth, it was shown that in the presence of tricalcium phosphate

with PSB (S5 and S32), a significant increase in the growth as compared to inoculation of aluminum phosphate along with PSB S5 and S32 due to the presence of acidic phosphatases. When compared to the soil from control pots, it was observed that due to inoculation of PSB, phosphatases activity is increased and solubilized more inorganic phosphates. Phosphatases enzymes help in the solubilization of inorganic phosphate and increase plant growth.

Conclusion

In the present study, *Bacillus* strains S5 and S32 were isolated and screened for their phosphate solubilizing activity by using different pH and inorganic phosphate sources. In both quantitative and qualitative assessments, it was shown that both bacterial strains S5 and S32 solubilized maximum tricalcium phosphate at acidic and neutral pH as compared to alkaline pH due to the presence of acidic phosphatases enzymes. At acidic pH, tricalcium phosphates are more soluble as compared to alkaline pH. Both bacterial strains S5 and S32 have antifungal activity against *Aspergillus niger* and have I.A.A production. In a greenhouse experiment, increased *Zea mays* growth was shown in the presence of *Bacillus* strains S5 &S32 along with Ca_3 $(PO_4)_2$in unautoclaved soil as compared to bacterial inoculation along with $AlPO_4$. Based upon the current study, it was shown that the simultaneous utilization of PSB *Bacillus* along with inorganic sources can be used as a biofertilizers in agricultural fields to enhance phosphorus utilization efficiency and boost crop yields.

Future Prospects

- To enhance the phosphate solubilizing bacterial use as efficient biofertilizers.
- In the coming years, further progress is anticipated in the field of Plant Growth-Promoting Soil Microorganisms (PSMs), specifically aimed at enhancing their efficiency in phosphate solubilization and enhancing plant growth. This advancement could involve the creation of fresh PSM strains using synthetic biology and other innovative methods.
- Moreover, the fine-tuning of existing PSMs for diverse environmental circumstances and applications is expected.
- Furthermore, the agricultural and environmental engineering sectors are expected to increase the utilization of PSMs. This trend is driven by the growing interest among farmers and environmental engineers in adopting sustainable and ecologically friendly approaches to address the challenges arising from depleting soil fertility and limited resources.
- Researchers should work to find out more data about the molecular mechanism used by these microbes to solubilize phosphate and make it available to plants.

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