ISOLATION AND SCREENING OF MULTITRAIT PHOSPHATE SOLUBILIZING BACTERIA AND THEIR GROWTH-PROMOTING EFFECT ON LETTUCE (*Latuca sativa*) VAR. GREEN WAVE

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This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation.

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To Whom It May Concern

Mr. **Suraj Chaudhary**, who enrolled in Master of Science in Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal, conducted successfully a part of his M.Sc. thesis work entitled "**ISOLATION AND SCREENING OF MULTITRAIT PHOSPHATE SOLUBILIZING BACTERIA AND THEIR GROWTH‑PROMOTING EFFECT ON LETTUCE (*Lactuca sativa*) VAR. GREEN WAVE**" in my laboratory for the partial fulfillment of his academic programme. The work conducted by Mr. Suraj Chaudhary was mutually supervised by me and his supervisor Dr. Bhushan Shrestha.

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Glossary Acronyms

ACC	1-aminocyclopropane-1-carboxylate
ADP	Adenosine diphosphate
AICL	Agriculture Inputs Company Limited
ATP	Adenosine triphosphate
BNF	Biological nitrogen fixation
CTAB	Hexadecyl trimethyl ammonium bromide
DAI	Days after incubation
DAP	Diammonium phosphate
DAPG	2,4-diacetylphloroglucinol
DCP	Dicalcium phosphate
DNA	Deoxyribonucleic acid
DTPA	Diethylene triamine penta acetic acid
EDTA	Ethylene diamine tetra acetate
EPS	Extracellular polymeric substances
GTP	Guanosine triphosphate
HAP	Hydroxyapatite
HC	Hydrolysis capacity
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
LHCP	Light harvesting chlorophyll a/b binding proteins
MLST	Multilocus sequence typing
MR-VP	Methyl red-Voges Proskauer
NARC	Nepal Agricultural Research Council
NPK	Nitrogen phosphorous potassium
NTA	Nitrilo-2,2',2"-tri acetic acid
OF	Oxidative fermentative
PCR	Polymerase chain reaction
PDA	Potato Dextrose agar
PGP	Plant growth promotion
PGPR	Plant growth promoting rhizobacteria
PPM	Parts per million
PSB	Phosphate solubilizing bacteria
PSE	Phosphate solubilization efficiency
PSF	Phosphorous solubilizing fungi
PSI	Phosphate Solubilization Index
PSI	Photosystem I
PSII	Photosystem II
PSM	Phosphate solubilizing microorganisms

PVK	Pikovskaya medium
RNA	Ribonucleic acid
SIM	Sulphur indole motility
STCL	Salt Trading Corporation Limited
TCP	Tricalcium phosphate
TE	Tris EDTA
Tg	Teragram (equal to 10^{12} g)
TSI	Triple sugar iron
TSP	Triple superphosphate
UTP	Uridine triphosphate

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ABSTRACT

Phosphorus (P) is a crucial growth-limiting plant nutrient that influences a variety of key metabolic processes in plants, including cell division and development, macromolecular biosynthesis, photosynthesis, and respiration. It is typically advised to apply P fertilizer to increase the soil's availability of P. Unfortunately, 75–90% of applied P fertilizers soon precipitate as a result of Fe, Al, and Ca complexes present in the soil, making them unavailable to plants soon after application. An effective strategy for enhancing the soil's P availability to plants is to employ PSB to release insoluble and fixed forms of P. This study was undertaken to isolate effective phosphate solubilizing bacteria along with various plant growth promoting traits. A total of 19 bacterial isolates were isolated from rhizosphere soil of which 5 were selected for further study with the highest solubilization index. Studies on quantitative P solubilization of TCP and bone meal revealed increased solubilized P concentration with days of incubation. At 15 days of incubation the highest solubilization of TCP was observed with isolate KP2 (73.59 µg/mL) followed by L2 (65.98 µg/mL).However in case of bone meal solubilization maximum solubilization was observed with isolate L2 (49 µg/mL) followed by KP2 (45 µg/mL) at 15 days of incubation. Reduction in pH of medium during both TCP and bonemeal solubilization was observed with increase in days of incubation. Upon 16s rRNA sequencing of most effective PSB isolate L2 was identified to be *Burkholderia* sp. Different PGP features were found with isolated PSBs. The most promising isolate was L2, which possessed PGP characteristics such as zinc solubilization, nitrogen fixation, auxin synthesis, ammonia generation, and antifungal activity. Isolate SM1 displayed PGP characteristics including nitrogen fixation, antifungal activity, ammonia generation, zinc solubilization, and cellulase synthesis. Different PGP characteristics were also present in the isolates KP2, KP3, and P1. The results of a greenhouse experiment using PSB isolate for lettuce growth promotion showed that PSB had a beneficial effect on growth promotion. The most promising isolate was L2, which resulted in increased leaf area, root volume, and dry biomass. Except for isolate SM1, other PSB isolates accelerated the growth of lettuce. Thus, we may recommend an efficient PSB isolate as a viable biofertilizer to be used to boost crop yields.

Keywords: bio-control, IAA, nitrogen fixation, phosphate, phosphate solubilization, plant growth promotion, rhizobacteria

Chapter-1

Introduction

1.1 Background

The demand for agricultural products, both food and non-food, worldwide is constantly rising, driven by dietary changes, population growth, increased income, and rising urbanization (FAO, 2018). We may satisfy this rising demand for agricultural products by applying chemical agro-inputs like fertilizers and insecticides. However, the extensive use of chemical fertilizers is linked to greenhouse gas emissions that will have a major effect on climate change and global warming (Aloo et al., 2022). Additionally, continued usage of these chemical inputs will harm the productivity and health of the soil (Garcia, 2020). As a result, a modern farming strategy known as sustainable agriculture necessitates the use of agricultural techniques that are more eco-friendly and uphold the long-term balance of the soil ecosystem.

Phosphorus is a crucial growth-limiting plant nutrient that influences a variety of key metabolic processes in plants, including cell division and development, macromolecular biosynthesis, photosynthesis, and respiration (Sanjotha & Manawadi, 2016). Phosphorus increases root development and helps flower formation, as well as the quality and quantity of fruits and seed formation. Furthermore, an adequate phosphorus content may improve plant's ability to fight illnesses and harsh environments (Maheshwari et al., 2013). Since phosphate resources are naturally non-renewable, proper management of these resources is necessary to extend the life of phosphate reserves (Dhillon et al., 2017). Due to its extremely low diffusion rate, P is not readily available in the soil. It is typically advised to apply P fertilizer to increase the soil's availability of P. Because of the constant application of chemical fertilizers, most agricultural soils have large stocks of P. Unfortunately, Fe, Al, and Ca complexes existing in the soil quickly precipitate 75-90% of P fertilizers, rendering it unavailable to plants shortly after application (Mihalache et al., 2015). In this context, research on alternatives to chemical fertilizers that can be locally available to farmers at low cost is of prime importance. Mineral phosphate solubilization can become the best alternative to these chemical phosphatic fertilizers.

Release of insoluble and fixed forms of P is an important issue to increase availability of the soil P to plants (Mihalache et al., 2015). On the basis of available data, soil microorganisms play an important role in transformation of insoluble P to soluble P in soil. P solubilization and mineralization capacities have been shown by a greater variety of soil microorganisms, including bacteria, fungus, actinomycetes, and algae (Suleman et al., 2019). Bacteria are predominant amongst them and are proven to be more effective in phosphorus solubilization than other microbes (Ingle & Padole, 2017). *Pseudomonas*, *Agrobacterium*,

Bacillus, *Rhizobium*, *Enterobacter*, *Alcaligenes*, *Aerobactor aerogenes*, *Achromobacter*, and *Burkholderia* are among the most prevalent P solubilizers found in plant roots (Aloo et al., 2020; Shrivastava et al., 2018).

In contrast to phosphorus-solubilizing fungi (PSF), which have a P solubilization capability of only 0.1 to 0.5%, PSB make up 1 to 50% of the total microbial population in soil. However, fungus in soils can travel farther than bacteria, which makes them potentially significant for the solubilization of P in soils (Ingle & Padole, 2017). PSF include *Penicillium*, *Aspergillus*, *Rhizoctonia*, *Trichoderma* and mycorrhizal fungi, among others (Kaur & Reddy, 2017). One yeast with the ability to solubilize phosphate is *Yarrowia lipolytica*. P solubilization activity has also been discovered in algae like cyanobacteria (Ingle & Padole, 2017).

Phosphate-solubilizing microorganisms not only make P available to plants, but also they speed up the accessibility of other trace elements, improve nitrogen fixation efficiency, and produce critical growth-promoting substances like siderophores and antibiotics (Mehta et al., 2013; Saida et al., 2015). They also protect plants from soil-borne pathogens (Aliyat et al., 2020). These microorganisms also support soil health by increasing soil porosity (Idrees, 2021). Utilizing plant growth-promoting rhizobacteria (PGPR) like PSB could be an effective strategy for boosting plant growth, nutrition, root development, plant competitiveness, and environmental stress tolerance (Mohamed et al., 2019).

As PSBs supply soluble phosphate to plants and plants supply root-borne carbon molecules (mostly sugars), the link between PSB and plants is synergistic in nature. Recent research has demonstrated that PSB inoculation is a promising strategy for enhancing growth and yield of maize (Viruel et al., 2014), rice (Cavite et al., 2021), wheat (Elhaissoufi et al., 2020), lettuce (Suryatmana et al., 2021), green gram (Abhishali et al., 2023) and soyabean (Diep et al., 2017). The demand for extra P fertilizers can be reduced by utilizing PSB to make P fixed in the soils available to the plants.

1.2 Rationale of the study

Nepal imports significant quantities of inorganic soluble P fertilizers, including triple superphosphate (TSP) and diammonium phosphate (DAP), to supply P that is in a form that plants can utilize. The majority of applied P, however, appears to become inaccessible to plants quickly after application, according to a number of studies (Mihalache et al., 2015). Due to the high cost of these fertilizers, which are usually beyond the capacity of small farmers, this method of increasing low soil P fertility with soluble P fertilizers has not been successful (Takeshima, 2019). The issue of farmers being unable to afford high fertilizer prices can be resolved by the use of microorganisms that can solubilize fixed P in the soil. Thus, this study's objective is to identify multi-trait phosphate solubilizing bacteria from rhizosphere soils of different location of Lalitpur, Nepal, that may dissolve insoluble phosphate. The isolates may potentially be utilized as bio-fertilizers to solubilize phosphate.

1.3 Objective

1.3.1General objective

- To isolate and characterize multi trait phosphate solubilizing bacteria and evaluate their effect on growth promotion of lettuce plants.

1.3.2 Specific objective

- To isolate and screen phosphate solubilizing bacteria from plant rhizosphere soil.
- To study phosphate solubilization efficiency of the isolated phosphate solubilizing bacteria.
- To characterize the selected phosphate solubilizing bacteria by physical and biochemical methods.
- To identify selected PSB isolates by 16S rRNA sequencing
- To estimate the amount of phosphate released by phosphate solubilizing bacteria during phosphate TCP solubilization and bone meal solubilization.
- To determine the changes in pH during phosphate solubilization
- To screen for plant growth promoting traits of PSB isolates (nitrogen fixation, IAA production, ammonia production, HCN production, cellulase production, antifungal activity)
- To evaluate effect of phosphate solubilizing bacteria on growth of lettuce plants under green house condition

1.4 Research hypothesis

Null hypothesis: Phosphate solubilizing bacteria with multiple plant growth promoting traits will not be isolated from plant rhizosphere soil

Alternate hypothesis: Phosphate solubilizing bacteria with multiple plant growth promoting traits will be isolated from plant rhizosphere soil

Chapter -2

Literature Review

The world's population is expected to reach 9.7 billion by 2050, 10.8 billion by 2080, and 11.2 billion by 2100, according to the United Nations (FAO, 2018). The demand for food is anticipated to rise dramatically as a result of the population growth. Agricultural intensification can increase crop production, but doing so will increase dependency on chemical agro-inputs like fertilizers and pesticides, which have a number of negative environmental consequences. A greater focus on the environmental implications of agricultural intensification, including impacts on land use change, air and water quality, soil fertility, biodiversity, and disease transmission, has, however, sparked worries in more recent times (Garcia, 2020). Due to the aforementioned issues, research into alternate crop fertilization techniques is expanding globally in an effort to create sustainable food production systems.

Food insecurity and self-insufficiency are issues being faced by Nepal as a result of an expanding population, shrinking agricultural land, and static production of the principal grains. To achieve the goal of the nation's food security, it is necessary to boost the yield of grain crops. Use of chemical fertilizers and pesticides, while ignoring their harmful effects on the environment, is one of the current method to boost agricultural yield (Devkota et al., 2018). Agrochemical demand has been steadily rising as agriculture has become more commercialized. Chemical fertilizer is the most crucial agricultural input, and its lack in the necessary quantity or timing has a significant negative impact on crop yield. In Nepal, the supply of fertilizer is still much behind the demand. The public corporation Salt Trading Corporation Limited (STCL) and the state-owned Agriculture Inputs Company Limited (AICL) have not been successful in distributing fertilizer in a timely, adequate, and lucrative manner (Panta, 2018).

There is currently no chemical fertilizer production facility in Nepal. Fertilizer is typically imported into Nepal through Indian ports, namely Kolkata port, from countries like India, China, Turkey, Egypt, and other Gulf nations (Kaini, 2020). Since the beginning of 2022, there has been a global shortage of fertilizers and a price increase due to rising ingredient costs and supply disruptions brought on by sanctions (against Belarus and Russia). Due to increased natural gas prices, notably in Europe, production of ammonia, an essential component of nitrogen-based fertilizers, was significantly curtailed (Baffes & Koh, 2022). Starting in late July 2021, China, the largest producer of phosphate in the world, halted or severely restricted exports of phosphate fertilizers (Prasain, 2021). It will be very challenging for nations like Nepal to maintain a steady supply of chemical fertilizer.

2.1 Phosphorous

The chemical element phosphorus has an atomic number of 15 and the letter P as its chemical symbol. Phosphorus is an element that exists in two main forms, red and white phosphorus, but is never found on Earth as a free element due to its strong reactivity. One of the primary raw materials needed to make phosphatic fertilizers like single superphosphate, diammonium phosphate, nitrophosphates, etc. is rock phosphate (Samreen & Kausar, 2019). However, rock phosphate is a finite resource, and the majority of supply are mined in areas with unstable political systems creating concerns to the several nations with small or no reserves (Carrington, 2019).

Mineral phosphorus makes approximately 0.12% of the crust of the earth. However, not all of the phosphate reserves found in the world can be mined because it would not be profitable to do so (Samreen & Kausar, 2019). Sedimentary marine phosphorites are the main form of phosphate rock resources. Northern Africa, the Middle East, China, and the United States are the regions with the largest sedimentary deposits. Brazil, Canada, Finland, Russia, and South Africa all have notable igneous occurrences. There are more than 300 billion tons of phosphate rock resources in the world (U.S. Geological Survey, 2022). At present rate of production, it is estimated that there will be 259 years' worth of rock phosphate available, which means there are no immediate concerns about phosphate supplies. However, there will be problems with the worldwide supply of phosphate fertilizer because of a number of 'bottlenecks' or restrictions in the physical, ecological, technical, geopolitical, social, and legal arenas (Cordell & White, 2011). Despite being the largest producer of rock phosphate and possessing the second-largest reserves in the world, China only has 24 years of supply left at present production rates, compared to 29 and 37 years for India and the USA, respectively (Blackwell et al., 2019). Another risk to the supply of phosphate fertilizer is that some other countries with the greatest resources, such Algeria, Jordan, and Syria, are situated in recently active war zones (Blackwell et al., 2019; Cordell & White, 2011). It is urgent to reconsider how phosphate fertilizer is utilized, where it can be obtained, how to utilize it most effectively, and possible substitute sources for the production of phosphate fertilizer in this situation of potential crisis in rock phosphate availability.

2.2 Phosphorous in soil

A vital mineral for all life on Earth, phosphate is extensively applied to agricultural areas. Phosphorus, despite being essential to plant development and metabolism, is the least available macronutrient and, as a result, the nutrient that is most frequently deficient in agricultural soils. This is a result of both its limited availability and its weak recovery from applied fertilizer (Balemi & Negisho, 2012). Phosphorus cycling in soils has drawn a lot of interest in terms of ecosystem development and fertilization. On the continents, soil holds nearly 98% of the roughly 122,600 Tg P that is present in the soil/biota system. The average residence duration of P in soils is 600 years, whereas the exchange of P between biota and soils occurs very quickly, on the average of 13 years (Filippelli, 2002). The P nutrition cycle is

a complicated system that is dependent on a number of physical, chemical, microbiological, vegetation dynamics, and climate variables which is shown in Figure 1. Three types of phosphorus can be found in soil: soil soluble P, insoluble inorganic P, and insoluble organic P.

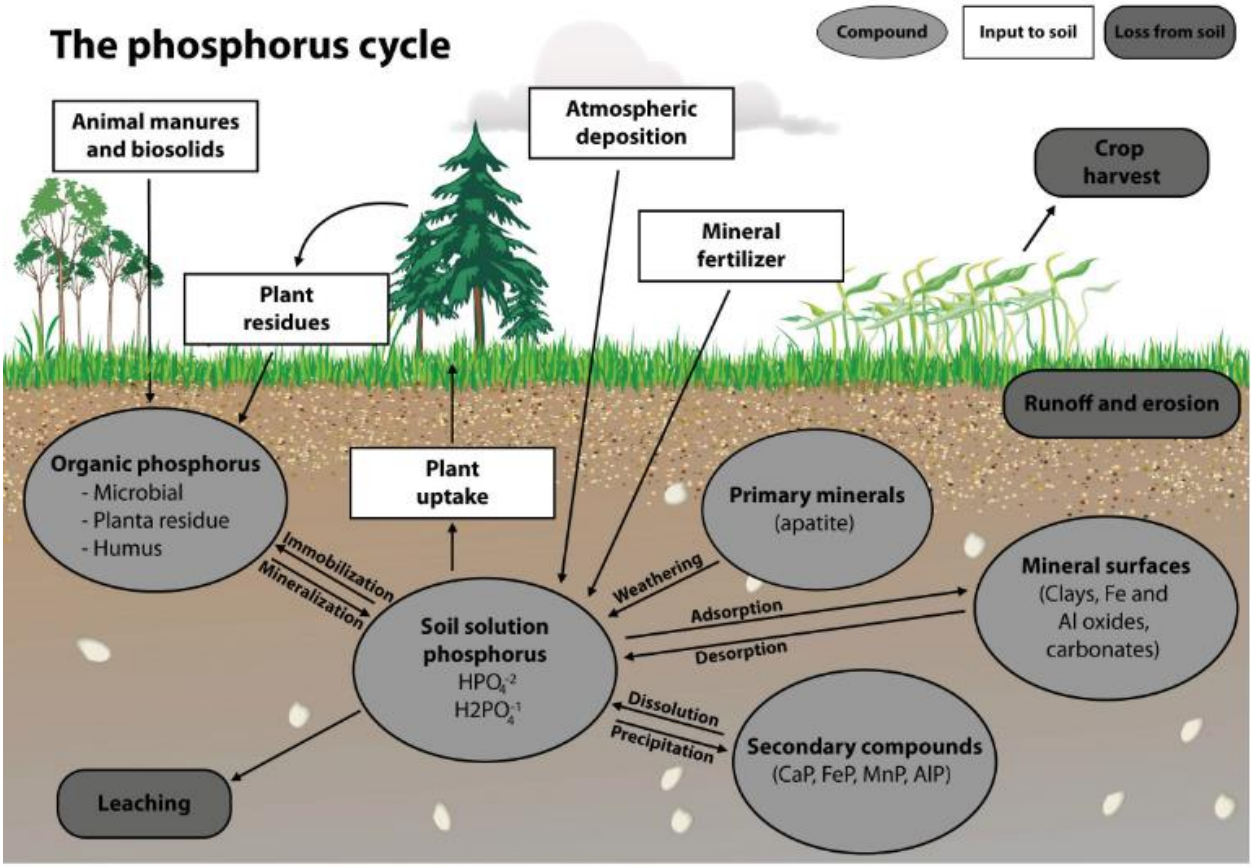


Figure 1: Illustration of Phosphorous cycle (Mangini & Aeschbach, 2017)

2.2.1 Phosphorus in soil solution

The term "soil solution P" represents the phosphorus fractions that are dissolved in the soil solution that can be easily absorbed by plant roots (Mwende Muindi, 2019). Only a little portion of soil P (1 ppm or 0.1% of total soil P) is present in soil solution because of its poor solubility and fixation in soil. Orthophosphate (HPO_4^{2-} and $H_2PO_4^-$ ions), which is readily absorbed by plant and microbial cells, is the predominant inorganic P in soil solution (Ziadi et al., 2013).

2.2.2 Insoluble inorganic P

35% to 70% of the total P in soil generally occurs in inorganic forms (Shen et al., 2011). There are about 200 phosphorus minerals in nature. Apatite, which includes hydroxyapatite, fluorapatite, and chlorapatite, is the most prevalent class of phosphate minerals among all (Liu & Chen, 2014). The weathering process causes primary phosphates to release soluble P. These released phosphorus compounds could be leached, used by microbes and plants, added to the labile pool, or changed into secondary phosphorus minerals. Soil acidity

accelerates apatite weathering (Smeck, 1985). A significant fraction of applied P fertilizers precipitates or get fixed, making them unavailable to plants. Through chemical precipitation or physical adsorption, phosphorus is frequently bonded to iron and aluminum oxides and hydroxides which leads to the formation of secondary phosphorous minerals (Mwende Muindi, 2019). Phosphorus can be primarily absorbed by Al/Fe oxides and hydroxides in acidic soils, but in alkaline soils, P gets precipitated to produce dicalcium phosphate (DCP), octocalcium phosphate, and hydroxyapatite (HAP) (Shen et al., 2011). Only 10–30% of phosphate fertilizer supplied may be retrieved by the crop produced after the fertilization due to adsorption, precipitation, and conversion to organic forms (Balemi & Negisho, 2012).

2.2.3 Insoluble organic P

The biota, which includes bacteria, plants, and animals, serve as an additional source of phosphorus in the environment by assimilating it into their cellular biomass. Biota can greatly affect the amount of phosphorus in the environment; for instance, microbial communities contribute 0.5-7.5% of the total phosphorous in topsoil from grassland and pastures and up to 26% in native forests (Liu & Chen, 2014). Inositol phosphates and phosphonates, which are stable forms of organic P, and orthophosphate monoesters and organic polyphosphates, which are active forms, make up the majority of soil organic P (Mwende Muindi, 2019). Nucleic acids make up the majority of microbial P, with polyphosphates, phospholipids, and inositols appearing in decreasing order of abundance (Hedley & Stewart, 1982). In contrast to phospholipids and nucleic acids, which can easily be hydrolyzed and returned to soluble and labile pools, inositols are more difficult to break down and have a tendency to build up in soil (Ölinger et al., 1996). Organic P transformations are basically cyclical in nature. P is absorbed by plants for use in the synthesis of biomass, part of which is reintroduced periodically to the soil. Microorganisms break down biomass, accumulating organic matter and organic phosphorus as a result. The soluble P pool receives periodic additions of mineralized organic phosphorus. This soluble P is necessary for both organic and inorganic transformation processes. If given sufficient time, the inorganic transformation pathway will result in the continual transfer of a part of the soluble phosphorus into an inaccessible P sink. In this manner, soil accumulates stable organic P and occluded inorganic P (Smeck, 1985).

2.3 Phosphate fixation in soil

The conversion of soluble phosphate to a less soluble form is referred to as phosphate fixation or retention by soils (Dean, 1949). Many studies have described phosphate fixation as the process by which phosphate ions are kept on active portion of soil particle surfaces. Chemically unstable phosphate ions quickly react with clay surfaces' oxides and hydroxides of Al and Fe, in acidic soils, and with $Ca(OH)_2$ in calcareous soils to generate less or more stable compounds (Kenneth et al., 2017; Parfitt et al., 1975). P adsorption in soils is frequently reported to increase when pH decreases (Arai & Sparks, 2007).

2.3.1 P-fixation in acidic soil

The majority of P-fixation occurs in acidic soils where $H_2PO_4^-$ interacts with the surfaces of insoluble oxides of Fe, Al, and Mn. This reaction starts a chain of chemical fixation reactions that interlock the P (Dean, 1949).

A. Precipitation Reaction

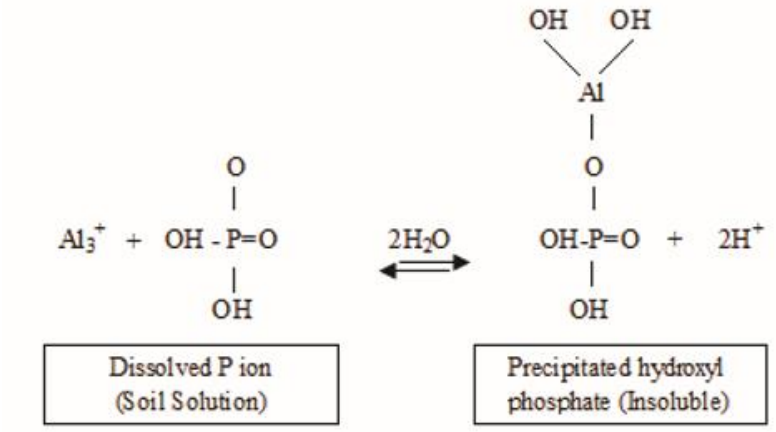


Figure 2: Precipitation reaction of phosphate in soil (Mahdi et al., 2012)

B. Anion exchange reaction

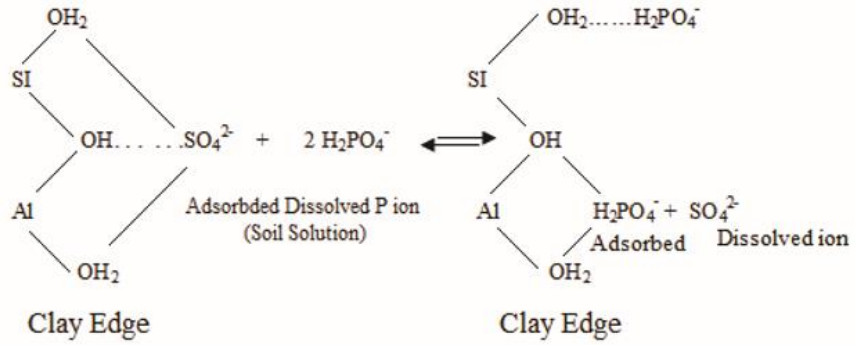


Figure 3: Anion exchange reaction of phosphate in soil (Mahdi et al., 2012)

C. Reaction with Al or Fe oxide surfaces

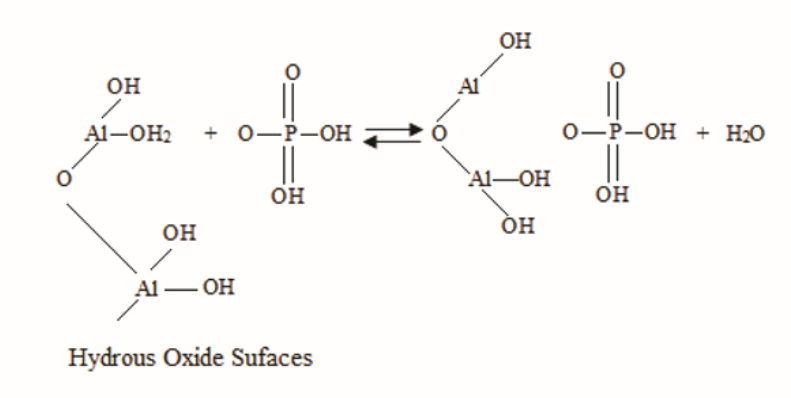


Figure 4: Reaction of Al oxide surfaces with phosphate in soil (Mahdi et al., 2012)

D. Formation of stable binuclear bridge

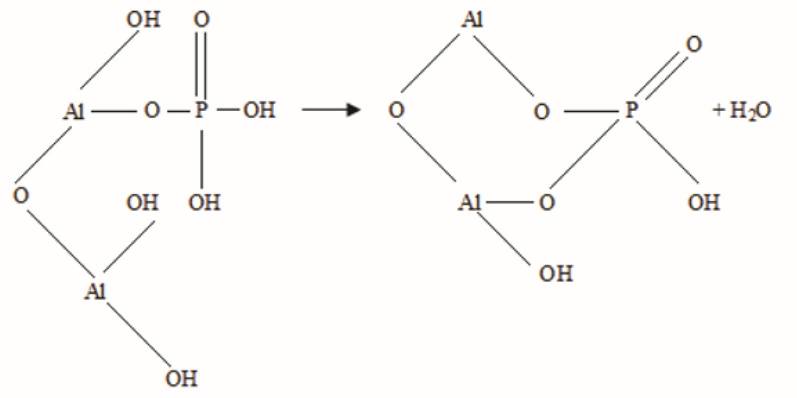


Figure 5: Formation of stable binuclear bridge by phosphate in soil (Mahdi et al., 2012)

There are significant amounts of "free" and "combined" Si, Al, and Fe oxides in the soils' inorganic colloidal fraction. Allophane, imogolite, Al and Fe oxides, as well as organo-mineral complexes, enable soils to adsorb significant amounts of phosphate, which has a large surface area and reactivity (Chatterjee et al., 2014). Figures 2, 3, 4, and 5 shows how previously mentioned soil elements hold P through ligand exchange, precipitation reactions, and adsorption.

2.3.2 P-fixation in alkaline soil

Typically, calcium phosphate complexes are responsible for phosphorus fixation in alkaline and calcareous soils (Hemwall, 1957). The only stable substance that can exist in an alkaline soil is hydroxy apatite. When soluble phosphate is added to calcareous soils, it eventually transforms into a fluorphosphate with properties resembling those of raw rock (Dean, 1949). Dicalcium phosphate (DCP), which is accessible to plants, is created when phosphate precipitates with calcium. Both octocalcium phosphate and hydroxyapatite (HAP), which can be generated from dicalcium phosphate and are less accessible to plants at alkaline pH levels between 7 and 14, are stable forms of the compound (Gustafsson et al., 2012;

Midgley, 1941). More than half of the total inorganic P observed in calcareous soils from extensive fertilization trials is attributed to HAP. HAP dissolving rises as soil pH drops (Gustafsson et al., 2012; Hanyabui et al., 2020).

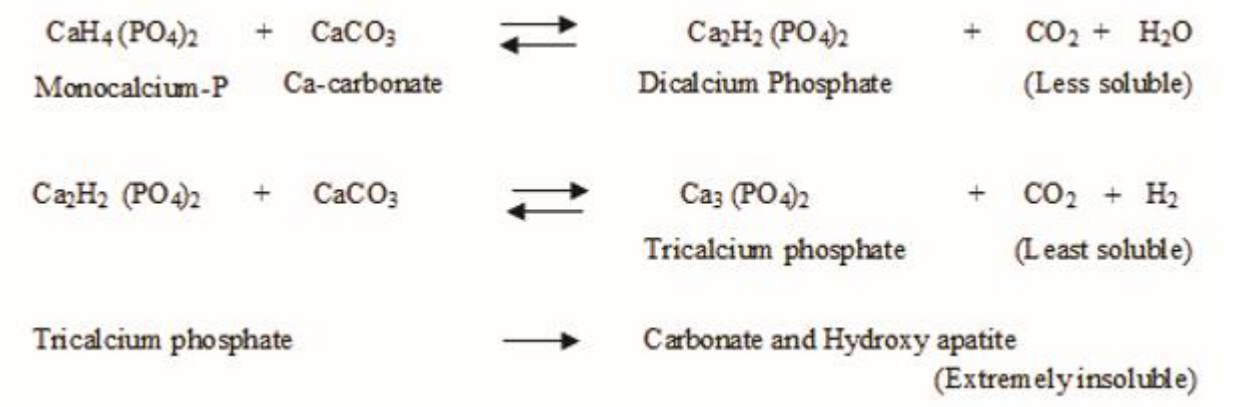


Figure 6: Fixation of Phosphate in calcareous soil (Mahdi et al., 2012)

2.4 Phosphorous as a major plant nutrient

After nitrogen, phosphorus is the second-most crucial macronutrient to plants. It is a crucial part of genetic material, metabolic substances, structural elements, and regulatory chemicals. The majority of P is found in plants as membrane phospholipids, phosphorylated intermediates of energy metabolism, and nucleic acids and nucleotides (Tiessen, 2008). In properly fertilized plants, tissue P concentrations range between 0.4% and 1.5% of the dry matter (Broadley et al., 2004). Additionally, P aids in glucose metabolism, enzyme activation and inactivation, and cell division. P also promotes the growth of roots, stalks, and stems as well as flower and seed formation, crop yield, and crop quality. The capacity of leguminous plants to fix nitrogen has been found to increase when P is available (Razaq et al., 2017). P is therefore necessary at every stage of development, from germination to maturity.

2.4.1 Structural role of P

P can be found in the tissues of plants either as organic phosphate esters or free inorganic orthophosphate (Pi). The cytoplasm contains the metabolically active Pi form, whereas the vacuole stores extra P. The esterified P can be found in proteins, phospholipids, phosphorylated metabolites, and nucleic acids (Hasanuzzaman et al., 2018). Nucleic acids, such as DNA and RNA, which transmit genetic information from one generation to the next, require phosphorus as an essential element. They make up the greatest pool of total organic P in a plant, and depending on the crop, they can range from 0.3 to 2.0 mg P g^{-1} dry weight (Suzuki et al., 2010). Monosaccharide sugars undergo phosphorylation after reacting with ATP to produce Pi esters. These phosphorylated chemicals are the key intermediaries in starch synthesis and breakdown as well as photosynthesis. Phospholipids, which create lipophilic and hydrophilic zones, are a crucial part of cell membranes (White & Hammond, 2008).

2.4.2 Growth and developmental role of P

The growth parameters of plants, such as shoot dry biomass, plant height, leaf number, and leaf area, are all influenced by phosphorus, from the cellular to the whole plant level. It also plays a critical role in cell division and cell enlargement (Assuero et al., 2004). Plants' reproductive growth, including the development of flowers and seeds, is greatly aided by phosphorus. According to studies, the majority of the phosphorus (P) absorbed by plants is retained in seeds and fruits. P availability issues can affect seed size, viability, and quantity. The number of seeds, seed dry matter, seed yield, and harvest index all increases when the soil has an appropriate amount of P (Rufino et al., 2017). For better seed germination and increased seedling vigor, seed P concentration is a crucial component. P aids in the quicker establishment of new seedlings (Zhu & Smith, 2001).

2.4.3 Energy transfer reactions by P

Phosphorus plays a significant part in cellular metabolism and is a necessary component of high-energy bonds such as acyl phosphate, phosphoanhydride, and enol phosphate. These phosphate-containing compounds with high energy serve as sources for vital biological functions by transferring energy to acceptor molecules. Adenosine triphosphate (ATP) or other highly energetic phosphorylated molecules transfer nutrients across the membranes of the root, leaf, and other plant organs. Numerous cellular functions need ATP, such as the generation of macromolecules, the phospholipids in membranes, and the transfer of nutrients across a concentration gradient. In gluconeogenesis and saccharide metabolism, phosphoanhydride bonds GTP and UTP serve as crucial electron suppliers (Hasanuzzaman et al., 2018).

2.4.4 Physiological role of P

The presence of P is crucial for the photosynthetic process. Pi, CO_2, and H_2O serve as the main building blocks for photosynthesis, which involves the production of ATP and sugars. The maintenance of the photosynthetic systems, which consists of the PSI, PSII, LHCP, cyt-f, cyt-b, and antenna mobility, also depends on phosphorus (Rychter & Mikulska, 1990). Pi is crucial for the chloroplasts' starch production. The distribution of freshly fixed C between the synthesis of starch in chloroplasts and the synthesis of sucrose in the cytoplasm is controlled by the level of Pi (Nielsen et al., 1998). The plant's respiratory systems depend heavily on phosphorus. Roots typically respire using a different, non-phosphorylating route when P is scarce. Reduced ATP and ADP generation from this cyanide-resistant respiratory route has an impact on the plant's energy-dependent functions (Hasanuzzaman et al., 2018; Rychter et al., 1992).

2.5 Sustainability of phosphorous use

P resources must be used sustainably because P limits agricultural production and Pi fertilizers are a finite supply. The establishment of crop genotypes with improved soil P acquisition, better agronomic practices, increasing uses of alternative P-fertilizers such manures, animal waste, biofertilizers, and recovered phosphates, may be some of the

approaches for P sustainability (White & Hammond, 2008). Improved knowledge of P dynamics in the soil, rhizosphere, and plant serves as a crucial foundation for P management. Based on the balance of P's inputs and outputs, P input into cropland can be optimized. The relative stability of P inside soils demands a long-term management plan for soil-based P management to keep the soils-available for optimum crop yield.

2.5.1 Agronomic practice

Good agronomic practices boost nutrient accumulation in the soil and subsequently enhance their bioavailability, which may enhance nutrient uptake and nutrient utilization. Agronomic practices can improve soil phosphorus availability and decrease phosphorus fixation (Horst et al., 2001). The most effective agronomic practice is to band phosphorus fertilizers to maintain the amount of phosphorus that is available to plants. P fertilizer placements inside bands have a better chance of lowering P-fixation and increasing fertilizer effectiveness in P-fixing soils (Dev et al., 2022). Another agronomic tactic can be the selection and development of crop varieties with high P-uptake efficiency. On soils with poor P-sorption capability, this may be the only practical alternative when used in conjunction with periodic P treatment (Lynch, 1998). The incorporation of P mobilizing plant species into the agricultural system as intercrops or in rotation appears to be another effective agronomic strategy. The ability of some plant species, including *Lupinus albus*, to mobilize sparingly soluble soil P has been demonstrated by research (Hocking et al., 2000).

2.5.2 Biotechnological approach

Breeding crop genotypes or cultivars that are more effective at acquiring and using P can be one approach in successful P management. Plants that have been genetically altered may be able to use applied fertilizers more effectively and function better in nutrient-limited environments. Genetic selection, breeding for nutrient-efficient plants and biotechnological methods can all be used to achieve this (Raghothama, 2005). High total plant P content, broad root systems, enhanced root architecture, and phosphatase and organic acid exudation into the rhizosphere are multiple factors responsible for P usage efficiency (Noushahi et al., 2019). It will be helpful to analyze the genes responsible for these features and integrate those genes into crop plants by traditional or marker-assisted breeding programs, directed gene identification and genetic engineering, or a combination of these methods (Cordell & White, 2011; Rose et al., 2011). Engineering crops to release more phytase, which is produced by over expressing a fungal phytase gene, has improved the ability to utilize insoluble P molecules in soils (George et al., 2005).

2.5.3 Organic amendments

Maintenance fertilization is promising method for preserving the concentration of phosphorus that is readily available in the soil. Only the P that is lost from the soil with the harvested crops is supplemented in this process. Mineral P fertilizer treatment is required to replace the P lost along with the removal of cereal grains, other edible vegetable

components, and livestock products like cow manure, milk, and meat utilized for human use (Hanyabui et al., 2020). When crop residues are fed to animals, some of the P can be recycled by adding the animal dung and bone meal to the soil. On the other side, bacteria can break down crop residues that have been left in the field (Balemi & Negisho, 2012).

P limitation in the soil can be solved by adding organic manures to the soil. Large amounts of organic phosphorus are also present in organic matter, including phospholipids and nucleic acids, which can be liberated and increase the quantity of inorganic P in the soil via mineralization. Applying organic materials may drastically reduce the amount of phosphorus that is absorbed by soil. The humic acids compete with inorganic phosphorus for the adsorption sites due to their abundance of negative charges, carboxyl, and hydroxyl groups (Abou Seeda et al., 2020). Earthworm casting, specifically in tropical soils, increases nutrient availability by mobilizing P. Because of the increased microbial population and subsequent increase in enzyme activity in the castings, earthworms have a positive impact on the availability of N and P to plants (Yadav et al., 2021).

2.5.4 Microbial activity

Microorganisms, which can efficiently mineralize soil organic phosphorus and convert insoluble inorganic phosphorus into soluble forms, play a critical role in regulating phosphorus availability. In soils with little available P, studies have shown that mycorrhizae like *Claroideoglomus etunicatum* and *Acaulospora longula* typically provide the growth and nutritional advantages (Teixeira-Rios et al., 2016). The solubilizing of phosphate involves a number of bacterial species as well as a few species of fungus. The phosphate-solubilizing bacteria (PSB) use a variety of methods, including the formation of organic acids, chelation, and ion exchange reactions, to transform the insoluble phosphate, such as HPO_4 and H_2PO_4, into the soluble form (Nosheen et al., 2021).

2.6 Phosphate solubilizing microbes and their role in P availability

Insoluble phosphorous compounds are solubilized by a variety of soil microflora, which enables plants to take phosphorus with ease. Insoluble forms of phosphate are mobilized by plants with the help of certain fungal and bacterial species (Karpagam & Nagalakshmi, 2014). A number of rhizobacteria strains have been described and extensively examined for their P solubilizing ability. According to estimates, 0.1–0.5% of fungi and 50% of all soil bacteria can solubilize P (Alori et al., 2017; Ingle and Padole, 2017). Rhizospheres, rhizoplanes, and even non-rhizosphere soils can be used to isolate P-solubilizing microorganisms. The density and P-solubilizing capacity of microbes varies from soil to soil. In plant rhizospheres, they are known to be better P solubilizers and more metabolically active (Sharma et al., 2013).

Numerous microbial species, such as bacteria, fungi, actinomycetes, and even algae, play an important part in the solubilization of phosphorus. Several bacteria from the genera *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Enterobacter*, as well as some fungi like *Penicillium* and *Aspergillus*, are capable of solubilizing P in soil microorganisms (Nosheen et al., 2021).

Others species of bacteria like *Rhodococcus*, *Micrococcus*, *Serratia*, *Arthrobacter*, *Acinetobacter*, *Chryseobacterium*, *Burkholderia*, *Gordonia*, *Phyllobacterium*, *Delftia* sp. , *Enterobacter*, *Erwinia*, *Pantoea*, *Flavobacterium*, *Vibrio*, *Agrobacterium*, *Kushneria*, *Azotobacter*, *Chryseobacterium*, *Xanthobacter*, *Pantoea*, *Klebsiella*, and *Achromobacter* are also reported with P solubilizing ability (Li et al., 2019; Nosheen et al., 2021; Sharma et al., 2013).

Fungi in soils may be more important for P solubilization than bacteria since they can spread more widely (Alori et al., 2017). The fungal strains of *Penicillium*, *Alternaria*, *Aspergillus*, *Trichoderma*, *Populospora*, *Myrothecium*, *Saccharomyces*, *Schizosaccharomyces*, *Paecilomyces*, *Cephalosporium*, *Cladosporium*, *Rhizopus*, *Curvularia*, *Arthrobotrys*, *Chaetomium*, *Fusarium*, *Mortierella*, *Phoma*, *Pythium*, *Rhizoctonia*, *Torula*, etc have been observed to have P solubilization capacity (Javeria et al., 2020; Mauch-Mani et al., 2017; Sharma et al., 2013). Actionmycetes have the ability to survive in harsh conditions (such as drought and fire) and have the capability to produce phytohormones and antibiotics in addition to solubilizing P. Actinomycetes from the genera *Actinomyces*, *Micromonospora*, and *Streptomyces* were among the 20% of actinomycetes that could solubilize P (Mauch-Mani et al., 2017). Due to their ability to withstand high temperatures and increase P availability, actinomycetes have been used in the composting of animal and municipal waste (Jones & Oburger, 2011). In addition to bacteria, fungi, and actinomycetes, P solubilization activity has also been documented in algae and mycorrhiza. P solubilizing capacity has been reported in cyanobacteria such as *Anabena* sp., *Calothrix braunii*, *Nostoc* sp., *Scytonema* sp., etc (Sharma et al., 2013; Shrivastava et al., 2018).

2.7 Mechanism for P availability

The soil's nutrient density, the organism's physiological state, and its growth status are factors that affect phosphate solubilizing microorganisms (PSM)'s capacity to solubilize phosphorus. By absorbing and excreting organic and inorganic ions and molecules, microorganisms have the ability to change the chemical composition of their immediate surroundings (Alori et al., 2017). The three main ways that soil microorganisms solubilize P are: (1) the release of compounds that complex with or dissolve minerals (such as protons, hydroxyl ions, siderophores, and organic acid anions), (2) the liberation of extracellular enzymes (biochemical organic phosphate (Po) mineralization), and (3) the release of Po during substrate degradation (biological Po mineralization) (Shown in Figure 7). Although P may be briefly immobilized in the microbial biomass, it still exists in a bioavailable state that can be released through microbial turnover (re-mineralization) (Jones & Oburger, 2011).

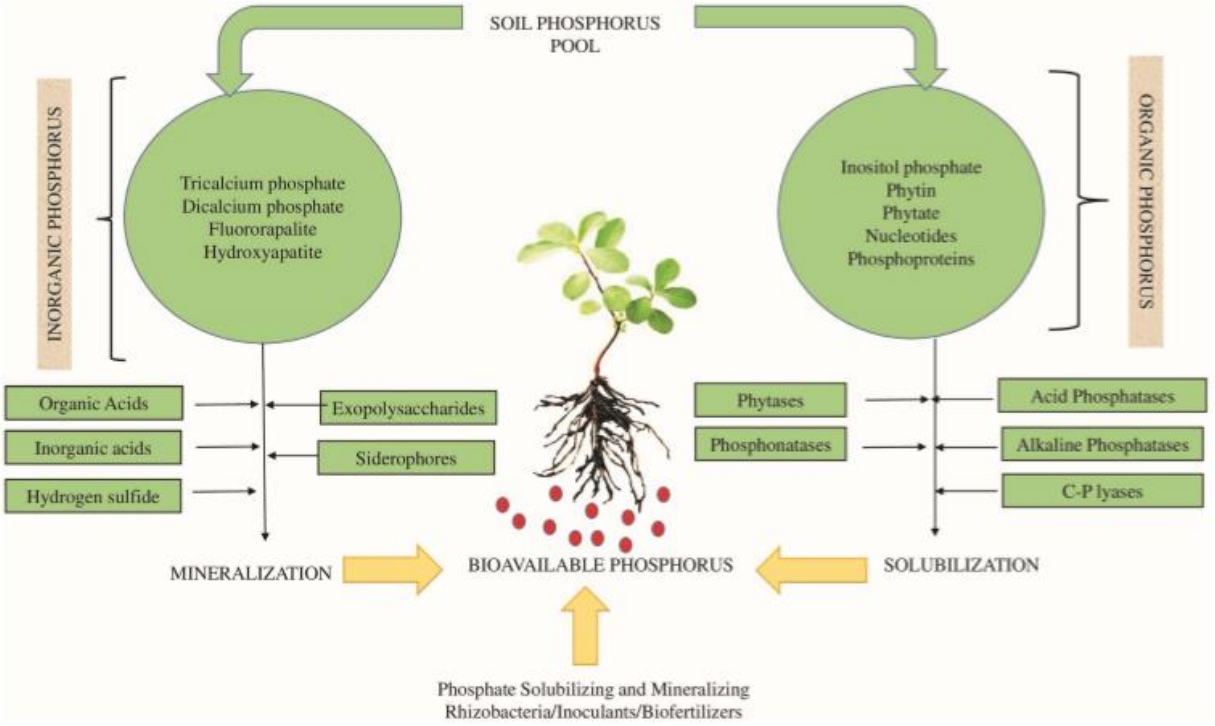


Figure 7: Phosphate solubilizing mechanism for organic and inorganic sources (Aloo et al., 2022)

2.7.1 Inorganic P solubilization

There are several ideas that explain the process of inorganic phosphate solubilization. The primary process, as seen in numerous investigations, is the production of substances that dissolve minerals, such as siderophores, organic acids, hydroxyl ions, protons, and CO_2 (Hameeda et al., 2008; Ingle & Padole, 2017). The dissolution of insoluble P by inorganic acid (such as HCl, H_2SO_4) has also been documented, despite the fact that organic acid has been proposed as the primary mechanism of P solubilization. Their effectiveness, though, has received less support (Kim et al., 1997). Production of H_2S has also been reported to dissolve Pi. H_2S interacts with ferrous phosphate to produce ferrous sulphate while simultaneously releasing phosphate (Sharma et al., 2013).

2.7.1.1 Organic acid production

The medium's pH dropping shows that the P-solubilizing bacteria are releasing organic acids through the direct oxidation pathway that takes place on the cytoplasmic membrane's outer face (Sharma et al., 2013). In research on the solubilization of Pi, low molecular weight organic acid anions (carboxylates) produced by microorganisms have frequently been discovered. Gluconic, citric, malonic, 2-ketogluconic, malic, lactic, oxalic, succinic, glycolic acids, and tartaric are among the reported organic acid anions released by PSM (Karpagam & Nagalakshmi, 2014). Organic acids can either chelate the Fe, Al, and Ca ions associated with P or they can directly dissolve the mineral P as a result of the anion exchange of phosphate by acid anion (Gyaneshwar et al., 2002). By competing with P for soil adsorption sites and acidifying microbial cells and their surroundings, organic acids displace metal ions like Ca^{2+}, Al^{3+}, or Fe^{3+} and liberate P-ions from insoluble metal salts. Functional groups,

especially carboxylic acids, in chelating agents like EDTA (ethylene diamine tetra acetate), DTPA (diethylene triamine penta acetic acid), and NTA (nitrilo-2,2',2"-tri acetic acid) form a stable complex with metal ions of insoluble phosphate salts like Ca^{2+}, Al^{3+}, or Fe^{3+} and influence P-solubilization (Yadav, 2021). Tri-carboxylic anions, like citrate, typically have a higher potential for solubilizing Pi than di-carboxylic acids (gluconate, oxalate, etc.). The high affinity of oxalate to generate Ca precipitates has also been found to make it particularly effective at mobilizing P in calcareous soils (Jones & Oburger, 2011).

2.7.1.2 Exopolysaccharides production

Extracellular polymeric substances (EPS) of large molecular weight are produced by many bacteria around their cell walls (Yadav, 2021). Microorganisms primarily create exopolysaccharides in reaction to stress and biofilm formation. Research on microbial exopolysaccharides has shown that they are capable of binding to metals in soil (Prabhu et al. 2019). Studies on microbially produced EPS have demonstrated their capacity to complex metals in soil (Al3+ > Cu2+ > Zn2+ > Fe3+ > Mg2+ > K+), from which it can be inferred that they influence P solubility in soil in some manner (Jones & Oburger, 2011).

2.7.1.3 Siderophore production

Microorganisms respond to iron deprivation by producing siderophores, which are complexing substances that have a strong affinity for iron (Jones & Oburger, 2011). In iron-limited environments, some microbes survive by secreting siderophores, which may have an impact on the solubilization of iron from minerals or organic substances. P availability is indirectly increased by siderophore-producing PSMs since molecules, like ferric phosphate, are mineralized to extract Fe from them (Prabhu et al., 2019; Sharma et al., 2013; Yadav, 2021).

2.7.2 Organic P solubilization

Nucleic acids, polyphosphonates, phosphonates, phospholipids, phytic acid, and sugar phosphates are a few examples of the different organic phosphate compounds found in soil (Prabhu et al., 2019). Three classes of enzymes—(i) nonspecific phosphatases, (ii) phytases, (iii) phosphonatases, and C-P lyases—are capable of releasing phosphorus from organic substances in the soil (Ingle & Padole, 2017). Phosphatase enzymes break down ester phosphate bonds to release phosphate ions, converting high-molecular-weight organic phosphate into low-molecular-weight molecules. These enzymes are classified as acid or alkaline phosphomonoesterases based on their pH optimum. Typically, alkaline phosphatases are more prevalent in neutral and alkaline soils, while acid phosphatases are more prevalent in acidic soils (Ingle & Padole, 2017; Sharma et al., 2013). P is released from phytate, the main organic P component in soil, by phytases. The C-P bond of organophosphonates is broken by phosphonatases and C-P lyases. This transforms phosphonates into phosphate ions and hydrocarbons (Jones & Oburger, 2011; Prabhu et al., 2019; Yadav et al., 2021).

2.8 Plant growth promoting traits of PSB

In addition to solubilizing P, certain rhizobacterial PSB also produce PGP hormones and siderophores, fix nitrogen, and solubilize other crop essential elements including zinc and potassium. Compared to plants that simply have the P solubilization function, such PSB can be more beneficial to plants (Aloo et al., 2020). Both direct and indirect methods can be utilized by PGPR to promote plant growth. Direct PGP methods include processes that improve the availability of mineral nutrients, such as phosphate solubilization, nitrogen fixation, and iron sequestration by producing siderophores, as well as the production of organic plant growth regulators like gibberellins, cytokinin, auxins and ethylene. On the contrary, indirect approaches include actions which restrict the growth of phytopathogenic microbes, such as the generation of HCN, enzymes that break down fungal cell walls, and other antibiotic components (Arif et al., 2017; Kaur & Reddy, 2017). Table 1 lists the several growth-promoting traits of PSMs.

The following are some methods that phosphate-solubilizing bacteria use to promote plant growth.

2.8.1 Phytohormones production

PSM produces phytohormones such as Indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, Cytokinins, and Gibberellins. These phytohormones have an impact on the physiological, morphological, and biochemical processes in plants. IAA (indole-3-acetic acid) is essential for the control of several physiological processes, including cell division and elongation, vascular differentiation, gravitropism, and phototropism (Luziatelli et al., 2021; Sarwar et al., 1992; Wagi & Ahmed, 2019). Plants will produce less ethylene when ACC deaminase cleaves the ACC, the direct precursor of ethylene. Plant growth is accelerated when ethylene levels are reduced. Gibberellin is a growth hormone that influences seed germination, promotes plant development, and slows the aging process. Plant senescence, blooming, fruiting, and seed dormancy are influenced by the cytokinins produced by PSM. They also affect both cell division and cell enlargement (Kaur & Reddy, 2017; Richardson & Simpson, 2011; Yadav, 2021).

Table 1: Growth promoting substances released by PSB

Phosphate solubilizing bacteria	Plant growth promoting traits	References
Pseudomonas spp., *Burkholderia cepacia*	ammonia, IAA, HCN, Siderophore, N fixation	(Rahman et al., 2017)
Bacillus amyloliquefaciens, *Bacillus cereus*, *Burkholderia cepacia*	antifungal activity, IAA, siderophore	(Pradhan et al., 2022)
Bacillus spp.	IAA, Antagonistic activity, N fixation	(Panhwar et al., 2012)
Bacillus subtilis, *Bacillus cereus*	IAA, siderophore, ACC deaminase	(Wagi & Ahmed, 2019)
Enterobacter cloacae	IAA	(Thakur & Parikh, 2015)
Rhizobium leguminosarum, *Pseudomonas* sp.	IAA, Gibberellin	(Afzal & Bano, 2008)
Bacillus aryabhattai, *Klebsiella variicola*, *Enterobacter aerogenes*	IAA, ACC deaminase, protease, siderophore, chitinase, N fixation	(Liu et al., 2016)
Enterobacter aerogenes, *Enterobacter ludwigii*, *Enterobacter amnigenus*, *Enterobacter cancerogenus*	IAA	(Aarab et al., 2013)
Pseudomonas brassicacearum, *Serratia rubidaea*, *Enterobacter bugandensis*, *Pantoea agglomerans*, *Pseudomonas lactis*, *Pantoea stewartii*	siderophore	(Aliyat et al., 2020)
Bacillus cereus, *Acinetobacter calcoaceticus*, *Chryseobacterium indologenes*, *Bacillus licheniformis*, *Bacillus subtilis*, *Pseudomonas mendocina*, *Pseudomonas fluorescens, Azotobacter vinelandii*, *Achromobacter xylosoxidans, Micrococcus luteus*	IAA, Phosphatase activity	(Dahlia et al., 2021)
Pseudomonas azotoformans, *Pantoea agglomerans*	HCN, siderophore, IAA	(Rfaki et al., 2020)
Bacillus thuringiensis, *Pseudomonas panipatensis*, *Sinomonas atrocyanea*, *Pseudomonas monteilii*	IAA	(Raj et al., 2014)
Bacillus megaterium, *Staphylococcus haemolyticus*, *Bacillus licheniformis*	IAA, Cu and Zn metal resistance	(Biswas et al., 2018)
Streptomyces roseocinereus	Antimicrobial activity, ACC deaminase, IAA,	(Chouyia et al., 2020)
Pseudomonas brassicacearum, *Pseudomonas corrugata*, *Aeromonas media*, *Enterobacter ludwigii*	IAA, Siderophore	(Saida et al., 2015)
Pseudomonas spp., *Pseudomonas poae*, *Bradyrhizobium japonicum*	IAA, ACC deaminase, Zinc solubilization	(Poonguzhali et al., 2008)

2.8.2 Release of biocontrol agents

In comparison to pesticides and other antimicrobial treatments, biological control agents created by PSM are generally regarded as being more environmentally friendly. PSM produces numerous antifungal substances, antibiotics, HCN, lytic enzymes, and other substances to manage phytopathogens (Ganeshan & Kumar, 2005; Jinal & Amaresan, 2020).

PSM generates antibiotics such cyclic lipopepdes, oligomycin A, kanosamine, zwittermicin A, xanthobaccin, and viscosinamideare as well as antifungal agents like DAPG (2,4-diacetylphloroglucinol), and pyrrolnitrin (Sattiraju et al., 2019). Plant diseases can also be controlled and reduced by microbial hydrolytic enzymes produced by PSM, including chitinase, 1,3-glucanase, peroxidase, protease, and lipase (Saeed et al., 2021).

2.8.3 Nitrogen fixation

In addition to P solubilization, N_2 fxation by several PGPRs (*Rhizobium*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, *Azotobacter*, and *Bacillus*) has also been documented (Joshi et al., 2023). Although 78% of the atmospheric gases are composed of nitrogen (N_2), plants cannot use this form. Biological nitrogen fixation (BNF) is the process by which specific bacteria and archaea convert nitrogen to ammonia (NH_3) via a nitrogenase protein complex. The *nif* genes encode enzymes that convert atmospheric N_2 into a plant-available form of nitrogen. N_2 fixation regulatory proteins are also encoded by *nif* genes in addition to enzymes. *Nif* gene expression is induced by the oxygen (O_2) concentration and low nitrogen level in the host plant's root environment (Aloo et al., 2022; Saeed et al., 2021).

2.8.4 Release of trace elements

Release of trace elements like Zn, Cu, K, Fe etc has been reported by PSM. By increasing the soil's availability of iron or by competing with pathogens for iron uptake, siderophores produced by microorganisms either directly or indirectly stimulate plant development (Kaur & Reddy, 2017). According to research, the most effective K solubilizers are *Bacillus mucilaginosus*, *B*. *edaphicus*, and *B. circulans* (Saeed et al., 2021).

2.9 Effect of PSB on crop production

Several phosphate solubilizing bacteria are found in soil, but generally their populations are too low to effectively compete with other rhizobacteria. Because of this, the P they release typically isn't enough to significantly stimulate *in situ* plant growth. Therefore, in order to increase plant yield, a higher concentration of highly effective PSB must be inoculated in the soil (Rodríguez & Fraga, 1999). According to several studies, the application of PSM improved growth, yield, and quality in a variety of crops, including potatoes, walnuts, apples, maize, rice, mustard, oil palm, aubergine, and chilies. Other crops that profited from the use of PSM included wheat, soybeans, sugarbeet, sugarcane, chickpeas, and other legumes (Ahmad et al., 2013; Kalayu, 2019; Singh et al., 2013).

Chen and Liu (2019) observed that the inoculation of P solubilizing *Pantoea* sp. increased the height and dry biomass of rice plants. In comparison to the rice plant used as a positive control, they also noticed longer, larger, and more volumetric roots. Application of *Enterobacter lugwigii* and inorganic P considerably boosted all rice growth-promoting characteristics like fresh root weight, fresh shoot weight, root length and shoot length, in comparison to control, with the exception of chlorophyll content (Lee et al., 2019). In laboratory settings, PSB strains increased *Triticum aestivum* root and shoot length by 10–

95% and seed germination by 80–90% as compared to control. While under natural conditions, seed germination rose by 80–96% and shoot length by 5–34.8 percent (Batool & Iqbal, 2019). According to reports, wheat that received PSB inoculation had higher average levels of chlorophyll a, b, total chlorophyll, and carotenes (Dasila et al., 2022). With *Paenibacillus polymyxa* inoculation, a sizable increase in chlorophyll content and P acquisition was seen in the durum wheat plant (Cherchali et al., 2019). An experiment conducted in a greenhouse found that inoculating barley with nitrogen fixers and PSB either independently or jointly boosted grain yield and straw production (Belimov et al., 1995).

Application of PSB inoculants to *Falcataria moluccana* seed considerably increased root length, shoot length, fresh and dry weight, seedling P content, and P accessible in soil during Suliasih and Widawati (2017)'s greenhouse trials. The increase in root and shoot length ranged from 19 to 112% and 12 to 27%, respectively. Silva et al. (2021) discovered that *Ochrobactrum pseudogrignonense* enhanced all metrics related to millet growth promotion, including foliar area, plant height, root dry weight, shoot dry weight, etc. In maize seedlings, Hameeda et al. (2008) found that PSB inoculation enhanced plumule length, root length, plant weight, germination, and seed vigor index. In addition, they noted that seed treatment with *Serratia marcescens* and *Pseudomonas* sp. boosted field grain maize yield by 85% and 64%, respectively, in comparison to the uninoculated reference.

According to Suryatmana et al. (2021), the yield of lettuce can be increased by between 17.52 and 49.24% fresh weight by using consortium inoculants (*Azotobacter*, P solubilizing *Pseudomonas* sp.) along with N, P, and K fertilizers. Maldonado et al. (2020) observed inoculation with *Erwinia* sp. and 50% fertilization of lettuce were shown to produce the same amount of yield as 100% fertilization of the control plants. Valetti et al. (2018) reported that inoculating a field with phosphate-solubilizing bacteria dramatically boosted both the growth and crop output of *Brassica napus* (from 21 to 40%); reaching levels that were comparable to or even exceeded the fertilized control. The treatments that received both insoluble $Ca_3(PO_4)_2$ and *Pantoea* sp. had considerably larger dry weights for the tomato plant shoots and roots that were examined in the greenhouse trials. In comparison to $Ca_3(PO_4)_2$ alone, growth was observed to increase by 3 times, and root dry weight by 6 times, in the combined treatment with *Pantoea* sp (Sharon et al., 2016).

Chapter-3

Materials and Methods

3.1 Materials required

The list of reagents, instruments and media utilized (their compositions) in this study are listed in Appendix I.

3.2 Isolation of Phosphate Solubilizing Bacteria

3.2.1 Collection of soil samples

The soil samples were collected from three different localities (Khumaltar, Lele and Saibubhanjyang) of Lalitpur District of Nepal. The soil samples were collected from Potato, Spinach, and Mustard rhizosphere at a depth of 0-15 cm. Soil samples were collected and maintained in aseptic polythene bags at the laboratory for further examination. Ten soil samples were used for the PSB isolation.

3.2.2 Isolation and purification of Phosphate Solubilizers

PSBs were isolated from soil samples using the serial dilution spreadplate technique on Pikovskaya agar medium (PVK), a selective medium used for the isolation of phosphate solubilizers. One gram of soil from each sample was suspended in 10 mL of sterilized water in test tubes. These tubes were agitated to break the clogs. Then, a series of dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} were made. From dilutions of 10^{-3}, 10^{-6} and 10^{-9}, 0.1 mL of the suspension was transferred to petridishes containing PVK. The suspensions were spread uniformly on petridishes using a glass rod spreader and incubated at 30±1 °C for 7 days. After incubation morphologically different colonies showing clear zones were selected and, purified by streak plate method (Walpola & Yoon, 2013).

3.2.3 Preparation of Glycerol Stock

Glycerol Stocks for PSB isolates were prepared by adding 500 µL of 50% glycerol to 500 µL of 24 hour bacterial culture in sterile cryotubes. The tubes were then stored by gradual cooling from 4 ˚C to -20 ˚C temperature.

3.3 Qualitative Screening for P solubilization

On PVK agar medium, the phosphate solubilization of all suspected colonies was examined. Spot inoculations of the isolates were made in the center of the Pikovskaya's plate and incubated at 30±1 °C. Diameter of clearance zone was measured after 7 days of incubation. The Phosphate Solubilization Index (PSI) and Phosphate solubilization efficiency (PSE) was calculated using following formula:

$$PSI = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}$$

$$\mathrm{PSE} = \frac{\mathrm{Z}}{\mathrm{C}} \times 100\%$$

where, Z- Clearance zone including bacterial growth, C- Colony diameter

(Cherchali et al., 2019)

3.4 Identification and Characterization of the bacterial Isolates

Selected PSB isolated were characterized by a series of physical and biochemical reactions as described by Green and Goldman (2021) in Practical Handbook of Microbiology. One most efficient PSB isolate was identified, by using a molecularly based, 16S rRNA sequencing approach.

3.4.1 Morphological and Biochemical characterization of the isolates

Selected PSB isolates were studied for the colony morphology, cell shape, colony type and biochemical features.

3.4.1.1 Gram staining

For gram staining, clear grease-free slides were used. They were properly cleaned with alcohol, a drop of sterile distilled water was added, and then colonies were picked up using a sterile inoculation loop. A thin bacterial smear was made, air dried, and heat fixed. For one min, crystal violet was flooded over the smear. The smear was then washed using distilled water and flooded in gram's iodine for a min. The stain was decolored with decolourizer (95% alcohol) for 20-30 second. After one minute of counterstaining with saffranin, it was properly washed, let to air dry, and examined under a microscope. Gram-negative cells were observed pink in color, while gram-positive cells were purple.

3.4.1.2 Sulfur, Indole, motility test

The SIM medium was inoculated with pure isolate by stabbing in the middle, within the 2\3 of the tube, with an inoculating needle. The tubes were incubated for 24 h at 37 ˚C. H_2S production and motility were evaluated. 3–4 drops of the Kovac's reagent were added for the indole production test. Blackening along the line of inoculation indicated the formation of H_2S, whereas red color ring development in the upper portion of the medium indicated the production of indole. Regarding motility, growth just along the line of inoculation indicated negative, but dispersed growth outward from the stabline or turbidity throughout the medium indicated positive.

3.4.1.3 Citrate utilization test

Simmon's citrate agar slants were inoculated with the 24 h old culture of microbes. At 37 °C, the slants were incubated for 24 h. The medium's color shift from green to blue indicated a positive reaction.

3.4.1.4 Triple sugar iron test

The TSI medium was inoculated with inoculating needle by stabbing into the medium in the butt of the tube and then streaked along the surface of the slant. After 18 to 24 hours of 37

°C incubation, the tubes were evaluated for the production of hydrogen sulfide, gas, and carbohydrate fermentation.

3.4.1.5 Oxidative fermentative test

Using an inoculating needle, pure isolated colonies were introduced to the OF medium in duplicate by stabbing with the needle halfway to the bottom of the tube. A 1 cm thick layer of liquid paraffin was poured on one tube of each pair. Both tubes were incubated for 48 h at 37 °C. A yellow color that appeared in the medium indicated that acid was being produced. Acid production in both tubes (open and paraffin-covered) suggested a fermentative organism, but acid production in an open tube solely indicated an oxidative organism.

3.4.1.6 MR-VP test

For the MR test, the isolated organisms were inoculated to MR-VP broth and cultured for 24 h at 37 °C. Methyl red indicator was added after incubation. The appearance of a red color indicated a positive test. For VP test, MR - VP broth was inoculated with the isolated bacteria and incubated for 24 h at 37 °C. After incubation, a few drops of 5% alpha-naphthol solution and 40% KOH were added. Development of pink colour in about 10-15 min indicated positive reaction.

3.4.1.7 Urease test

To verify the production of urease enzyme, a urease test was performed. The isolated culture was inoculated to urea agar slants, and the slants were then incubated for 24 h at 37 °C. By observing the development of pink color, urea hydrolysis was confirmed.

3.4.1.8 Catalase test

Using the slide test method, the catalase activity of test isolates was evaluated. On a clean glass slide, a small amount of bacteria was transferred. It was then mixed with a drop of 3% H_2O_2. Catalase activity was considered to be present when bubbles evolved quickly within 5–10 s.

3.4.1.9 Oxidase test

Oxidase paper was taken and placed on the clean glass slide. Bacterial culture was smeared over the paper with the help of glass rod. A deep purple blue color that formed within 5–10 s was an indication of a positive reaction.

3.4.1.10 Starch hydrolysis test

The sterile starch agar medium was streaked with a loopful of the culture, and the plates were incubated at 37 °C for 24 h. The plates were flooded with iodine solution for 15-20 min after incubation. The bacterial colony was observed for halo zone formation.

3.4.1.11 Pectinase production test

A loopful of an isolated, pure bacterial colony was spread across the sterile pectinase production medium, and the plates were then incubated at 37 °C for 24 h. After incubation,

the plates were submerged in iodine solution for 15 to 20 min. Halo zone development was examined along the bacterial colony.

3.4.2 Molecular Identification of selected Isolates

3.4.2.1 DNA extraction

DNA was isolated using the CTAB technique from a chosen, highly effective PSB isolate (William et al. 2012). 3 mL of bacterial culture was centrifuged for 1 min at 12000 rpm. The supernatant was discarded and the cells were re-suspended in 567 µL of TE buffer. After that, 3 µL of proteinase k (20 mg/mL) and 30 µL of 10% SDS were added to the tube and the mixture was incubated at 37 ˚C for an hour. Following incubation, 80 µL of CTAB-NaCl solution and 100 µL of 5 M NaCl were added. For 10 min, the mixture was incubated at 65 ˚C. Equal volume of chloroform: Isoamyl alcohol mixture (24:1) were added to it and mixed well. Then it was centrifuged for 8 minutes at 13000 rpm. Without disrupting the interface, the top aqueous layer was transferred into a new epi-tube. After that, an equivalent volume of PCI (Phenol: Chloroform: Isoamyl – 25:24:1) was added, and it was mixed by vertexing. The tubes were centrifuged for 8 min at 13000 rpm, and the supernatant was collected in new epi-tubes. An equal quantity of Chloroform:Isoamyl alcohol (24:1) was added and mixed. Once more, the tubes were centrifuged for 8 min at 13000 rpm, and the supernatant was collected in new epi-tubes. The tube was filled with 0.6 mL of chilled isopropanol, and it was left to stand for 5 min. For 8 min, the tubes were centrifuged at 13000 rpm. The pellets were washed with 70% ethanol (13000 rpm for 5 min) and the supernatant was discarded. The pellets were air dried and re-suspended in 40 µL of TE buffer following an ethanol wash. DNA was quantified using Nano-drop and visualized by performing gel electrophoresis.

3.4.2.2 Amplification of DNA sequence

At Intrepid Nepal, Thapathali, Kathmandu, Nepal, the 16s rRNA region was amplified from extracted DNA. Using two universal bacterial primers, 16sBakt341F: 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3' and 16sBakt805R:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3', the 16S rRNA genes were amplified by polymerase chain reaction (PCR). The amplification was carried out in 25 µL using 12.5 µL of 2X Kapa, 2.5 µL of template DNA, and 5 µL of each primer (forward and reverse). The following conditions were used for the reactions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 98 °C for 30 s, 65 °C for 25 s, 72 °C for 20 s, and the final extension at 72 °C for 5 min. The products were kept at hold at 4 ˚C. Amplified PCR products were visualized by 1% Agarose gel with Gel red for DNA visualization and bromophenol as loading dye. Solis biodyne of 100bp was used as ladder during gel electrophoresis.

3.4.2.3 Sequencing and sequence analysis

Sequencing was carried out at Intrepid Nepal, Thapathali. The sequencing was carried out using Native barcoding kit 24 (SQK-NBD112.24) in Flongle (FLO-FLG001). The raw sequences were quality trimmed. The 16S rRNA gene sequences were retrieved, and the NCBI Basic

Local Alignment Search Tool (BLAST) was used to compare them to the GenBank database. Using MEGA v.11, the phylogenetic tree was constructed using the neighbor-joining method with the Jukes-Cantor evolutionary distance measurement (Saitou and Nei, 1987; Tamura et al., 2021). Phylogenetic tree was generated using the bootstrap value of 1000 (Felsenstein, 1985).

3.5 Evaluation of Isolates for Phosphate Solubilization

The selected PSB isolates were used for the quantitative P solubilization. 250 mL Erlenmeyer flasks were added with 50 milliliters of Pikovskaya's broth (without phosphorus source), to which 250 mg of tricalcium phosphate and 200 mg of old bone meal were added separately. At 121 °C and 15 psi pressure, the flasks were sterilized for 15 minutes. A 0.1 mL aliquot of each isolate's 24 hour active culture was added to this autoclaved and cooled medium. For each PSB isolate, three replicate flasks were used, and the amount of P released in the broth was estimated at 5, 10, and 15 days following inoculation.

After incubation, culture broth was centrifuged at 10,000 rpm for 30 min. The available P was quantified by a modified single solution reagent method' as described by Murphy and Riley (1962). Method for P estimation by a modified single solution reagent is elaborated in Appendix III. KH_2PO_4 solution was used as standard for estimation of available P. Standard calibration curve of KH_2PO_4 for P estimation is listed in Appendix II.

3.6 Estimation of pH During Phosphate Solubilization in PVK medium inoculated with PSB isolates

Using a pH meter, the pH of the culture broth during P solubilzation was measured at different time of incubation (5th, 10th, and 15th days of incubation) to determine the pH change caused by the growth of PSB (Panda et al., 2015).

3.7 Screening of Isolates for Nitrogen Fixation

To assess bacteria's capacity to fix atmospheric nitrogen, nitrogen-free media (Jensen medium) were used to culture isolated bacteria (Rahim et al., 2021; Sherpa et al., 2021). The Jensen media was streaked with a single colony of the isolated bacteria, which was then cultured for 7-8 days at 30 °C. Bacterial colonies on plates were seen as qualitative proof of atmospheric nitrogen fixation.

3.8 Evaluation of Isolates for Ammonia Production

Selected PSB isolates were qualitatively tested for ammonia production by the method of Cappuccino and Sherman (1991). For quantitative estimation of ammonia isolates were cultured in peptone water at 30 ˚C for 5 days. To obtain cell-free supernatants, the broth was centrifuged at 3000 rpm for 20 min. After that, it was added with 5% Nessler's reagent. Uninoculated nutrient broth with Nessler’s reagent was used as a control. Positive isolates showed a change in the supernatant's color from pale to deep yellow. By measuring the absorbance at 425 nm, the amount of ammonia produced was determined. For the standard

curve, ammonium sulfate was used at concentrations ranging from 0-10 µg/mL (Chrouqi et al., 2017). Standard curve of ammonium sulfate for ammonia estimation is listed in Appendix II.

3.9 Evaluation of Isolates for Auxin Production

The Zakry et al. (2010) method was used to evaluate PSBs potential for producing auxin hormone. The test isolates were grown in nutrient broth for 3 days at 28 ± 2 ℃. Cell suspensions from bacterial cultures were separated by centrifuging at 6000 rpm for 30 min. 2 mL of the centrifuged supernatant was added with 2 drops of orthophosphoric acid, 4 mL of Solawaski's reagent (50 mL of 35% perchloric acid and 1 mL of 0.5 (M) $FeCl_3$), and left at room temperature for 25 min in the dark. The observation of a color change from yellow to pink or red indicated the production of auxin.

For quantitative estimation of auxin production, 3 mL of broth from a bacterial culture that had been cultured for 7 days was taken. It was then centrifuged for 30 minutes at 6000 rpm. To 2 mL of supernatant, 4 mL of Salkowski's reagent and 1-2 drops of orthophosphoric acid were added (Kshetri et al. 2018). After that, the mixture was let to stand at room temperature in the dark for 25 minutes. With a UV-VIS spectrophotometer set at 530 nm, the absorbance of color change was determined. Indole acetic acid (IAA) was used as the standard in the preparation of a standard graph to determine the concentration of auxin produced by isolates. Standard graph of IAA is listed in Appendix II.

3.10 Dual culture Assay for Screening of Biocontol Activities of Isolates against selected phytopathogen

Dual-culture assay, as described by Sakthivel and Gnanamanickam (1987), was used to assess bacterial isolates for *in vitro* antagonistic activity against the three fungal phytopathogens *Fusarium solani*, *Rhizoctonia solani*, and *Alternaria solani*. The National Plant Pathology Research Centre, Khumaltar, Lalitpur, provided the fungal phytopathogens. In a potato dextrose agar (PDA) plate, a fungal disc (approx 5 mm^2) was placed at the center. On both sides, bacterial isolates were streaked 3 cm from the agar plug. As a control, plates inoculated with sterile water and fungal disc was used. For 5-7 days, the plates were incubated at 28±2 °C. The following equation was used to express the results as the percentage inhibition of the fungal mat's growth in the presence and absence of bacterial isolates:

$$Inhibition(\%) = [1 - \frac{Fungal\ growth}{Control\ growth}] \times 100\%$$

(Ali et al., 2020)

3.11 Screening of Isolates for HCN production

Using the approach developed by Bakker and Schippers (1987), the ability of the effective PSB to create hydrogen cyanide (HCN) was evaluated. The lids of the Petri plates were placed with pads of Whatman No. 1 filter paper, and the plates were sterilized. For bacterial

culture, tryptic soy agar medium containing glycine (4.4 g/L) was used. The medium was streaked with each isolate. Two milliliters of sterile picric alkaline solution (2% sodium carbonate in 0.5% picric acid) were used to soak the filter paper padding in each plate. Parafilm was used to seal the inoculated plates in order to keep the gaseous metabolite produced by the antagonistic bacteria inside and permit a chemical reaction with picric acid to occur on top. After one week of incubation at 28 °C, a change in color from yellow to orange or brown showed the formation of HCN.

3.12 Screening of Isolates for Zinc solubilization

A modified Pikovaskaya's media containing 0.1 % zinc oxide (ZnO) as Zinc source was inoculated with isolated PSB. The plates were then incubated at 28 ± 2 ˚C for 96 h. After 48 and 96 h of incubation, halo zones were measured around colonies, and solubilization efficiency was calculated using the formula below:

$$Solubilization\ Efficiency\ (S.E) = \frac{Halo\ zone\ diameter}{Colony\ diameter} \times 100\%$$

(Sunithakumari et al., 2016)

3.13 Screening of Isolates for Cellulose degradation

By streaking on the cellulose CongoRed agar media, the ability of bacterial isolates to breakdown cellulose was evaluated (Hendricks et al., 1995). The presence of Congo-Red discoloration around colonies was a sign of cellulose-degrading activity. The ability of positive isolates to breakdown cellulose was qualitatively assessed using the hydrolysis capacity (HC), also known as the ratio of clearing zone to colony diameter.

3.14 Evaluation of isolates for Organic acid production

PSB were grown and multiplied in liquid PVK medium to determine organic acid levels. Cultures were blended on the seventh day, and they were centrifuged for 20 min at 2000 rpm. Each culture's supernatant was filtered using a 0.45 µm, non-sterile, 4 mm micro filter syringe. Each culture's 20 µL purified solution was injected into an Aminex 87-H (25*4.6mm) bio-rad ion exchange column. The column was operated at 50 ˚C with 5 mM H_2SO_4 as the mobile phase and a constant (isochratic) flow rate of 0.6 mL min^{-1}. The RI detector was used to calculate the levels of organic acid in the samples. Control panel for Open lab software was used for the HPLC. The retention periods and peak regions of the chromatogram were compared with the standards of tartaric acid, citric acid, lactic acid, and succinic acid in order to identify the unknown organic acids.

3.15 Biocompatibility assay of Bacterial isolates

The cross streak method was used to quickly test bacteria for biocompatibility (Prasad and Babu, 2017). By placing a single streak in the center of the agar plate, the targeted microbial strain was inoculated. After 48 hours of incubation, the test isolates were inoculated on the agar plate by a single streak that ran perpendicular to the central streak. The microbial

interactions were investigated by observing the inhibitory zone size after an additional 48 hours of incubation. The microbial interactions with no inhibitory zone were considered biocompatible with each other.

3.16 Green House Evaluation of Efficient Phosphate Solubilizing Bacteria (PSB) On Growth of Lettuce Plants

The effects of inoculation with particular strains of P solubilizing bacteria on the growth and yield of lettuce were examined using soil gathered from agricultural fields on the NARC premises in Khumaltar. The top 15 cm of surface soil was collected, dried, and ground. The soil was autoclaved at 121 ˚C for three subsequent days. Autoclaved soil was filled in alcohol sterilized plastic pots of 4 kg capacity. Tricalcium phosphate at a rate of 200 g/hectare (17.2 mg P_2O_5/kg of soil) was weighed separately and added in to the soil as basal dose before sowing (Fenta & Assefa, 2017). Five effective phosphate-solubilizing bacteria were used in a soil pot culture experiment to examine how well they supported the growth and development of lettuce plants. The treatments used for soil pot culture experiment are shown below. Three replications for each of the nine treatments were designed.

1. Absolute Control (No inoculation, no phosphate (p) source)
2. Control (No inoculation, TCP)
3. L2 +TCP
4. P1+TCP
5. KP2+TCP
6. KP3+TCP
7. SM1+TCP
8. Consortium (L2, P1, KP2, KP3) +TCP
9. NPK full dose (118kg urea/hectare, 78.65 kg DAP/hectare, 40 kg/hectare)

Lettuce (*Lactuca sativa*) seeds of green wave variety were surface sterilized with sodium hypochlorite (1%) for 15 min and 70 % ethanol for 1 min. After that, sterile water was used to properly rinse it. The seeds were then overnight air dried in a dry flask. The seeds were sown in pots at 8 seeds per pot in three replications. One milliliter of each undiluted 4 days old PVK broth culture (about 10^9 cells) of each isolate was inoculated by pipetting on to the base of the seedlings of lettuce plant when they emerged. Following growth, thinning was carried out to keep just three plants in each pot. The pots were routinely irrigated with distilled water to maintain the ideal moisture level, and other usual precautions were taken to safeguard the plants against pests and diseases.

The observations were carried out at 60 days of sowing seeds. The height of the plant was measured in centimeters (cm) from the plant's base to the tip of a fully opened leaf. By uprooting the plants, the root length was measured from the tip of the longest root to the neck area and represented in centimeters (cm). Plants were uprooted and dried in hot air oven at 70 ˚C. Dry biomass of both shoot and roots were measured.

Chapter-4

Results

4.1 Isolation and qualitative screening of PSB

The phosphate solubilizing bacteria in this study were isolated on PVK agar plates, and they were recognized as P solubilizers by observing the clear zone around the colonies. From 10 different soil samples, a total of 19 distinct PSB were isolated. In PVK agar media enriched with tricalcium phosphate, all the isolates were evaluated for their ability to solubilize phosphate source. The phosphate solubilizing efficiency and solubilization index of the isolates was determined after seven-day incubation. With an efficiency of 128.58% and an index of 2.29, SM1 had the highest P solubilizing ability among the isolates, whereas SCS1 had the lowest with an efficiency of 50% and an index of 1.5. The five isolates SM1, KP2, KP3, L2, and P1 with the highest capacity to dissolve P were selected for further research. These isolates (SM1, KP2, KP3, L2 and P1) showed P solubilization efficiencies of 128.57, 112.5, 118.18, 100, and 90%, respectively.

Table 2: Tricalcium phosphate solubilization index and efficiency of soil-isolated phosphate solubilizing bacterial strains

Isolates	Colony diameter (mm)	Total diameter (colony + halozone) (mm)	Solubilization Index (S.I)	Solubilization efficiency (S.E) (%)
P1	5	9.5	1.9	90
P2	7	12	1.71	71.42
P3	7.5	11.5	1.53	53.33
P4	6.5	11	1.69	69.23
L1	6.5	11.5	1.78	76.92
L2	5.5	11	2	100
N1	6	10.5	1.75	75
N2	7	11	1.57	57.14
SA1	6.5	10.5	1.62	61.54
SB1	4.5	8	1.78	77.78
SC1	5	8.5	1.7	70
NS1	4	6.5	1.63	62.5
KP2	4	8.5	2.13	112.5
KP3	5.5	12	2.18	118.18
SM1	3.5	8	2.29	128.57
SM2	3.5	6.5	1.86	85.71
SCS1	5	7.5	1.5	50
SCS2	4.5	8.5	1.89	88.89
SCS3	8	13.5	1.69	68.75

4.2 Identification and Characterization of the Bacterial Isolates

The selected SM1, KP2, KP3, L2, and P1 phosphate solubilizing strains were subjected to physical and biochemical tests for identification. Highly efficient L2 isolate was molecularly characterized.

4.2.1 Morphological and Biochemical Characterization of the Isolates

In Table 3, the phosphate-solubilizing strains SM1, KP2, KP3, L2, and P1 are characterized physically and biochemically. Figure 8 displays the Gram staining of a PSB isolate.

According to the findings, the isolates L2 and P1 exhibited G-ve, immobile rods with positive biochemical reaction tests for urease, citrate utilization, oxidase, catalase, and oxidative, as well as growth on citrimide agar. All other tests were negative. SM1 displayed G+ve, motile rods with positive oxidase, catalase, and fermentative biochemical reaction tests, but all other tests were negative. KP2 was detected with G+ve, cocci showed a positive biochemical response for MR, and all other tests came out negative. KP3 showed G-ve, motile rods with positive biochemical reaction tests for catalase, oxidase, gas, MR, fermentative and citrate utilization. All other tests were negative.

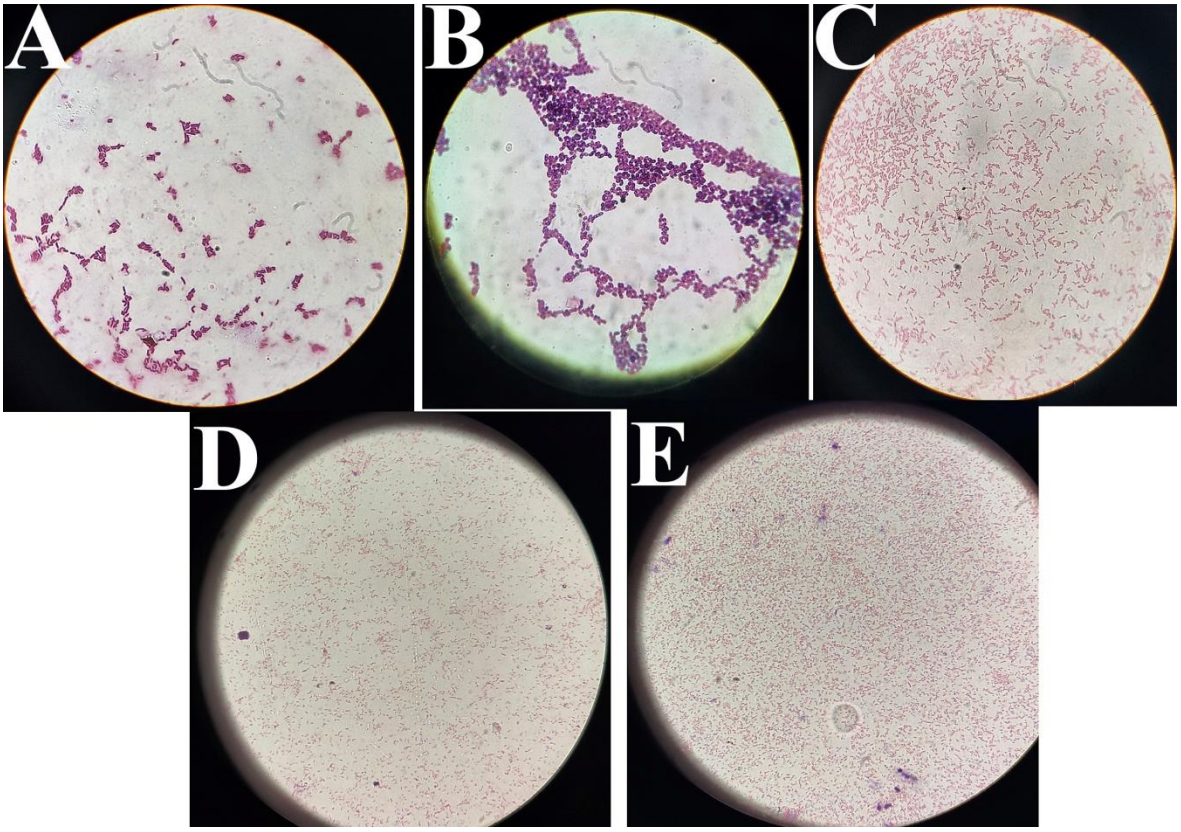


Figure 8: Gram staining of PSB isolates; A-Isolate SM1, B-isolate KP2, C-isolate KP3, D-isolate L2 and E-isolate P1

Table 3: Physical and biochemical attributes of selected phosphate solubilizing bacterial strains

Test	Media	Inference				
		L2	**P1**	**SM1**	**KP2**	**KP3**
Gram Stain		-ve	-ve	+ve	+ve	-ve
Shape		rod	rod	rod	cocci	rod
Oxidative	Hugh Leifson	+ve	+ve	+ve	-ve	+ve
Fermentative		-ve	-ve	+ve	-ve	+ve
Urease	Urea Agar	+ve	+ve	-ve	-ve	-ve
Citrate	Simmon's Citrate Agar	+ve	+ve	-ve	-ve	+ve
H_2S	TSI	-ve	-ve	-ve	-ve	-ve
Gas		-ve	-ve	-ve	-ve	+ve
Slant/Butt		R/NC	R/NC	R/NC	NC/NC	Y/Y
H_2S	SIM	-ve	-ve	-ve	-ve	-ve
Indole		-ve	-ve	-ve	-ve	-ve
Motility		-ve	-ve	+ve	-ve	+ve
MR	MR-VP medium	-ve	-ve	-ve	+ve	+ve
VP		-ve	-ve	-ve	-ve	-ve
Oxidase	Oxidase test disc	+ve	+ve	+ve	-ve	+ve
Catalase	3% H_2O_2	+ve	+ve	+ve	-ve	+ve
Growth on Cetrimide agar	Cetrimide Agar	+ve	+ve	-ve	-ve	-ve
Starch hydrolysis	Starch agar	-ve	-ve	-ve	-ve	-ve
Pectinase activity	Pectinase production medium	-ve	-ve	-ve	-ve	-ve
Most probable Organism		*Burkholderia* sp.	*Burkholderia* sp.	*Bacillus* sp.	*Streptococcus* sp.	*Klebsiella* sp.

Note: R=red; NC=No change; Y=yellow, +ve=positive; -ve=negative

4.2.2 Molecular characterization of the Isolate

One highly efficient PSB isolate was subjected to 16S rRNA sequencing. Genomic DNA was initially isolated using the CTAB technique. Approximately 550 kb of the 16S rRNA gene were amplified by PCR and sequenced. Obtained DNA sequence is listed in Appendix IV.

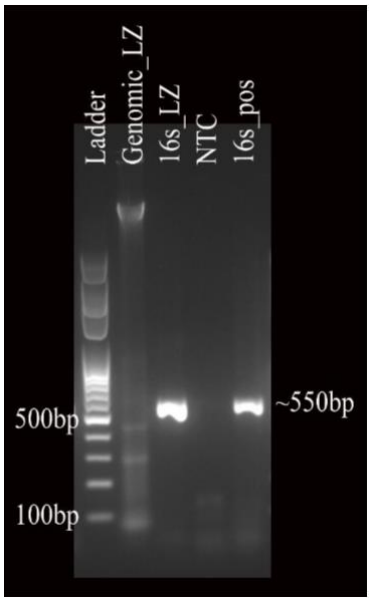


Figure 9: Agarose gel electrophoresis of genomic DNA and 16S PCR products of sample L2. 16s_pos- 16s positive PCR product, NTC- negative control, 16s_LZ- 16S PCR product of isolate L2, Genomic_LZ- extracted genomic DNA of isolate L2.

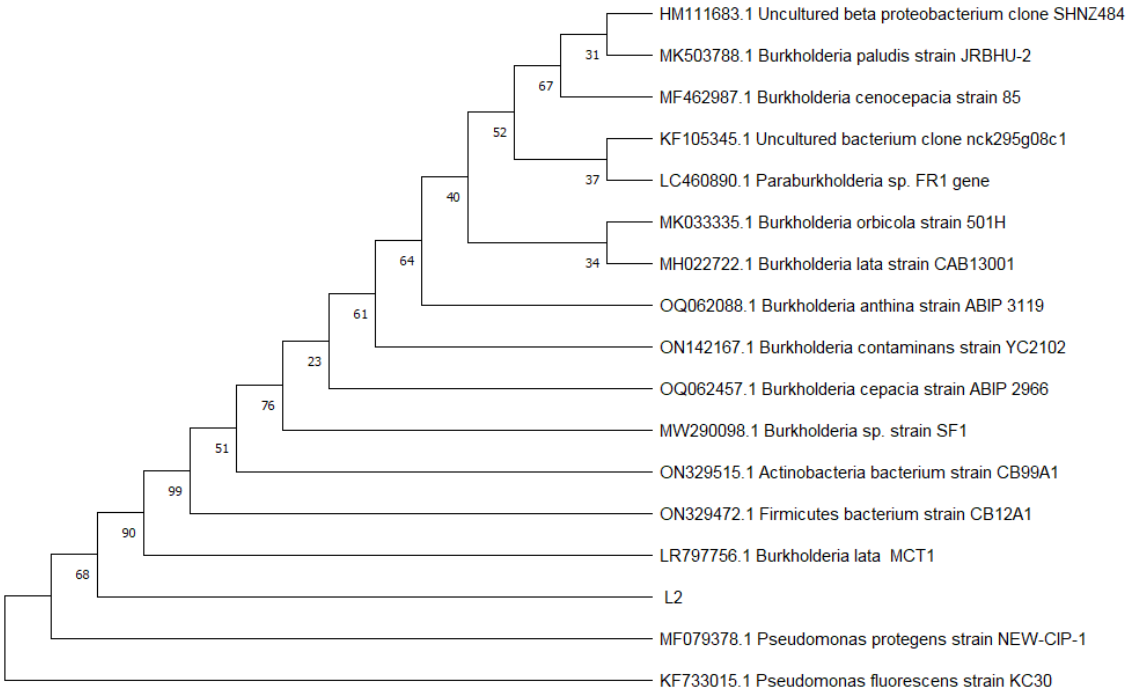
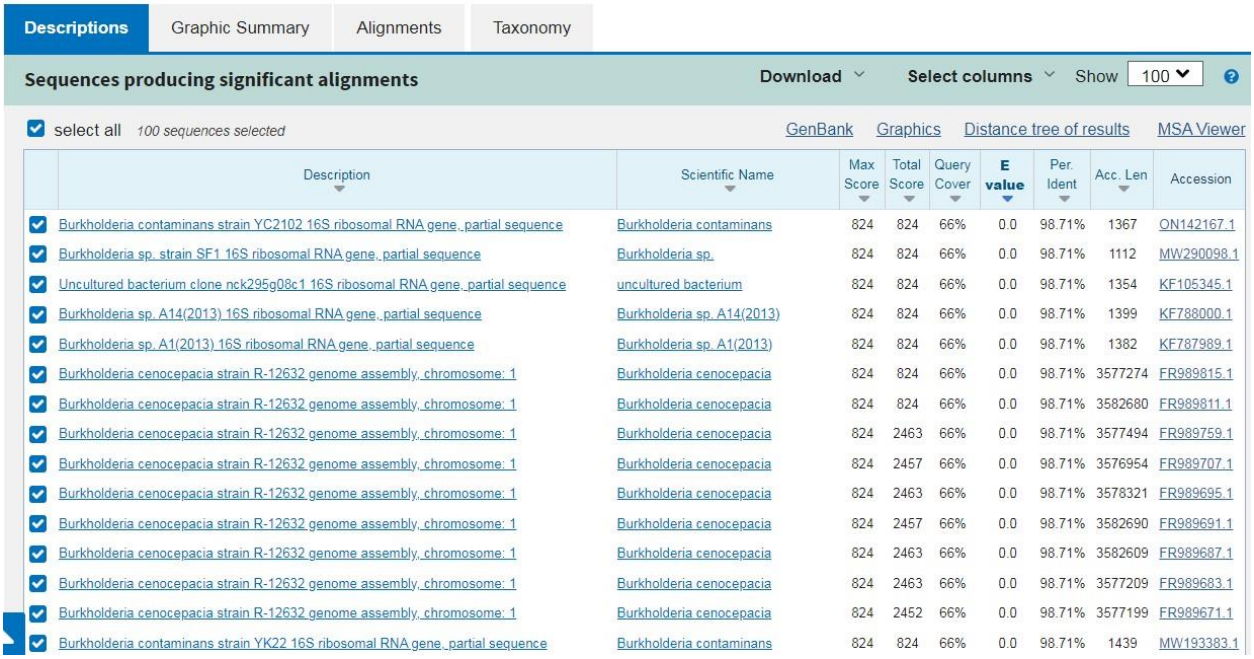


Figure 10: Cladogram of PSB isolate L2 shows Phylogenetic neighbourjoining tree based on 16S rRNA sequences showing the phylogenetic relationship between related species of the genus *Burkholderia*. *Pseudomonas protegens* strain NEW-CIP-1 and *Pseudomonas fluorescens* strain KC30 was used as an out-group.

Figure 9 illustrates the gel electrophoresis of the amplified PCR product and extracted genomic DNA. The resulting rRNA sequence was subjected to nucleotide BLAST's sequence homology analysis. The PSB isolate L2's sequence displayed a high degree of similarity

(98.71%) to *Burkholderia* sp. (shown in Figure 11). The 16S rRNA sequences that were acquired were added into the MUSCLE algorithm for multiple sequence alignment in MEGA11 software. The data from the batch received were put through a phylogenetic analysis. Figure 10 displays the cladograms obtained by phylogenetic analysis. PSB isolate L2 was determined to be *Burkholderia* sp. based on the results.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Burkholderia contaminans strain YC2102 16S ribosomal RNA gene, partial sequence	Burkholderia contaminans	824	824	66%	0.0	98.71%	1367	ON142167.1
Burkholderia sp. strain SF1 16S ribosomal RNA gene, partial sequence	Burkholderia sp.	824	824	66%	0.0	98.71%	1112	MW290098.1
Uncultured bacterium clone nck295g08c1 16S ribosomal RNA gene, partial sequence	uncultured bacterium	824	824	66%	0.0	98.71%	1354	KF105345.1
Burkholderia sp. A14(2013) 16S ribosomal RNA gene, partial sequence	Burkholderia sp. A14(2013)	824	824	66%	0.0	98.71%	1399	KF788000.1
Burkholderia sp. A1(2013) 16S ribosomal RNA gene, partial sequence	Burkholderia sp. A1(2013)	824	824	66%	0.0	98.71%	1382	KF787989.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	824	66%	0.0	98.71%	3577274	FR989815.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	824	66%	0.0	98.71%	3582680	FR989811.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2463	66%	0.0	98.71%	3577494	FR989759.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2457	66%	0.0	98.71%	3576954	FR989707.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2463	66%	0.0	98.71%	3578321	FR989695.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2457	66%	0.0	98.71%	3582690	FR989691.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2463	66%	0.0	98.71%	3582609	FR989687.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2463	66%	0.0	98.71%	3577209	FR989683.1
Burkholderia cenocepacia strain R-12632 genome assembly, chromosome: 1	Burkholderia cenocepacia	824	2452	66%	0.0	98.71%	3577199	FR989671.1
Burkholderia contaminans strain YK22 16S ribosomal RNA gene, partial sequence	Burkholderia contaminans	824	824	66%	0.0	98.71%	1439	MW193383.1

Figure 11: BLAST sequence homology analyis of obtained rRNA sequence of isolate L2

4.3 Evaluation of Isolates for Phosphate Solubilization

4.3.1 Tricalcium phosphate solubilization by selected isolates

The findings showed that all strains released more P from TCP as incubation time increased, with the quantity released at 15 days after incubation (DAI) being the highest. The isolates released P levels that ranged from 56.08 µg/mL to 73.59 µg/mL at 15 DAI (Table 4). At 15 DAI, isolate KP2 released the most P (73.59 µg/mL), isolate L2 released the second-most P (65.98 µg/mL), and isolate SM1 solubilized the least P (56.08 µg/mL). At 5 DAI, isolate KP2 (28.66 µg/mL) released the most P, followed by SM1 (26.56 µg/mL). With 22.82µg/mL, isolate L2 released the least quantity of P.

Table 4: Amount of phosphate released by selected PSB isolates during Tricalcium phosphate solubilization

Isolates	Phosphate concentration (µg/mL)		
	5th Day	10th day	15th day
SM1	26.56±0.57	41.40±0.74	56.08±7.79
KP2	28.66±0.58	52.65±2.56	73.59±4.93
KP3	25.26±0.93	47.67±1.52	64.91±5.98
L2	22.82±0.28	38.38±2.17	65.98±2.24
P1	23.89±0.74	42.27±2.65	60.15±1.74

4.3.2 Bone phosphate solubilization by selected isolates

Table 5 displays the findings of bone phosphate solubilization by the selected isolates. There was a clear correlation between the amount of soluble P and the number of days following incubation.

At 15 days of incubation, the bacterial isolate L2 had the maximum amount of solubilization (49.02 µg/mL), followed by KP2 (45.81 µg/mL). At various incubation days, the isolate KP3 had the least P solubilization. KP2 demonstrated maximum P solubilization on days 5 and 10 of incubation, with concentrations of 29.06 µg/mL and 37.11 µg/mL, respectively.

Table 5: Amount of phosphate released by selected PSB isolates during bone phosphate solubilization

Isolates	Phosphate concentration (µg/mL)		
	5th Day	10th day	15th day
SM1	23.33±2.52	26.21±1.15	34.85±3.89
KP2	29.06±0.11	37.11±2.56	45.81±0.51
KP3	21.85±0.84	24.91±0.72	32.13±1.71
L2	23.35±0.91	35.86±1.93	49.02±0.28
P1	24.39±1.39	29.12±1.55	34.54±1.54

4.4 Estimation of pH During Phosphate Solubilization in PVK medium inoculated with PSB isolates

4.4.1 pH change during TCP solubilization

At the fifth, tenth, and fifteenth days of incubation, the pH variations of the PVK medium inoculated with PSB isolates during TCP solubilization were examined (shown in Figure 12). A maximum pH drop from the starting pH of 7.0 to 3.93 in 15 days was observed in isolate L2 inoculated on PVK medium. The isolate SM1 showed least pH drop with final pH of 4.7 at 15 day of incubation.

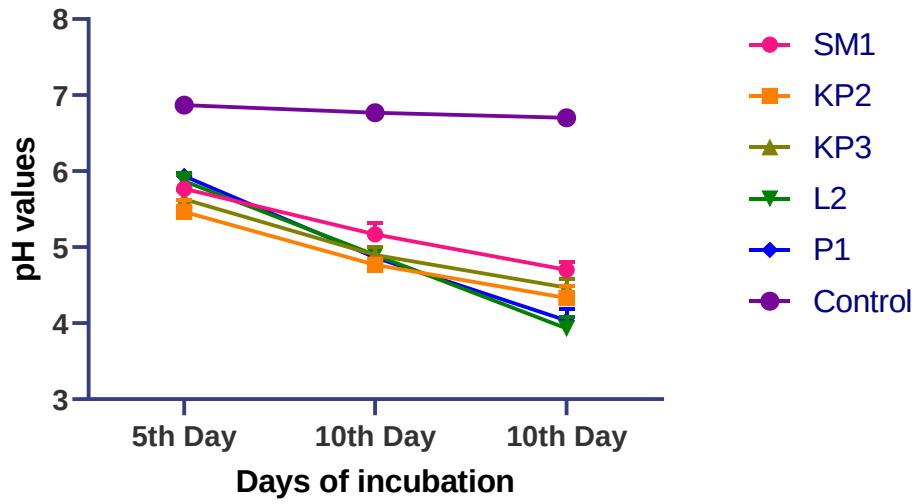


Figure 12: pH change in PVK medium during TCP solubilization by PSB isolates

4.4.2 pH change during bone phosphate solubilization

The pH of PVK medium being reduced and the number of days after incubation were found to be positively correlated. Figure 13 demonstrates the ability of PSB isolate to reduce pH along with days of incubation during bone phosphate solubilization. Maximum reduction in pH was observed with isolate KP2 from initial pH of 7.0 to 4.73. The isolate P1 had the second-highest pH decrease, resulting in a final pH of 5.1. Isolate SM1 showed least drop in pH.

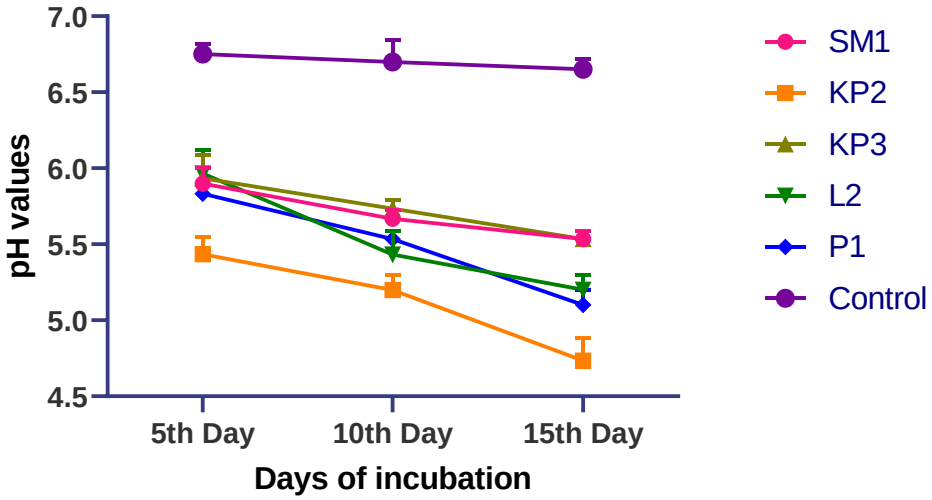


Figure 13: pH change in PVK medium during bone phosphate solubilization by PSB isolates

4.5 Screening of Isolates for Nitrogen Fixation

By growing the selected PSB strains on nitrogen-free Jensen medium, the ability of the PSB strains to fix nitrogen was evaluated in this study (shown in Figure 14).

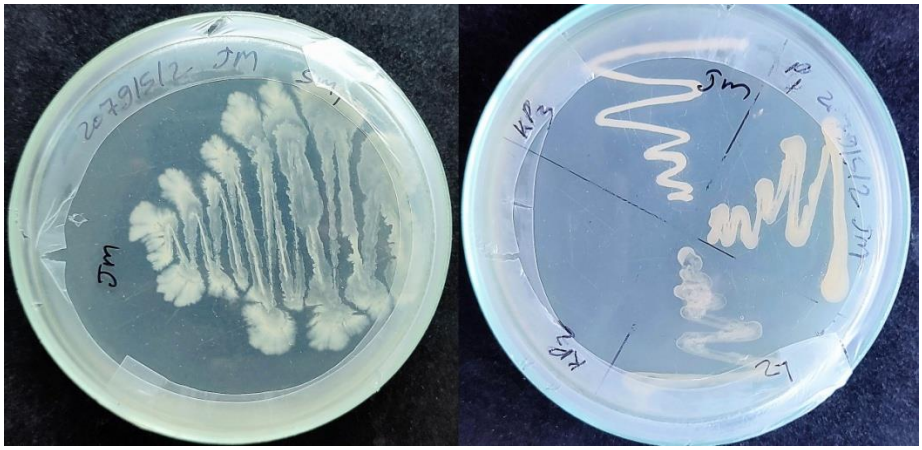


Figure 14: Growth of PSB isolates in Jensen Media (growth indicated positive for N-fixation)

After 7-8 days of incubation, the qualitative growth of the bacterial isolates on Jensen media was examined, and the findings are shown in Table 6. Isolate SM1 showed rapid swarming growth. Isolate KP3 had medium growth, compared to isolates L2 and P1, which had luxuriant growth. KP2 exhibited no growth. This finding suggests that isolates SM1, L2, KP2 and P1 have nitrogen fixation abilities. In KP2, the capacity to fix nitrogen was lacking.

Table 6: Growth of selected P solubilizers on nitrogen free Jensen media

Isolates	Nitrogen fixing activity
SM1	+
KP2	-
KP3	+
L2	+
P1	+

(- no growth, + positive growth)

4.6 Evaluation of Isolates for Ammonia Production

The amount of $NH4^+$ produced in the culture medium was used to assess the ammonia producing ability of PSB strains. Depending on the quantity of $NH4^+$, ammonium reacts with Nessler's reagent to generate a yellow complex or a yellow-brown complex. As shown in Figure 15, the $NH4^+$ produced by isolates ranged from 1.98 µg/mL to 4.25 µg/mL. With a concentration of 4.25 µg/mL, isolate KP3 had the highest capacity for ammonia synthesis. It was followed by isolate P1 with the concentration of 3.88 µg/mL. The least amount of ammonia was produced by KP2.

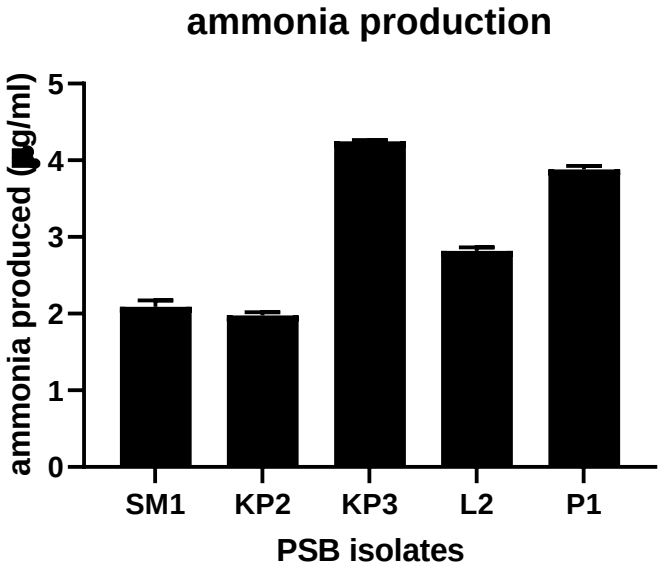


Figure 15: Production of ammonia by PSB isolates in peptone water

4.7 Evaluation of Isolates for Auxin Production

Qualitative screening of auxin production was determined to be positive for three PSB isolates, KP2, KP3, and L2, based on their appearance of color change to red during auxin production assay.

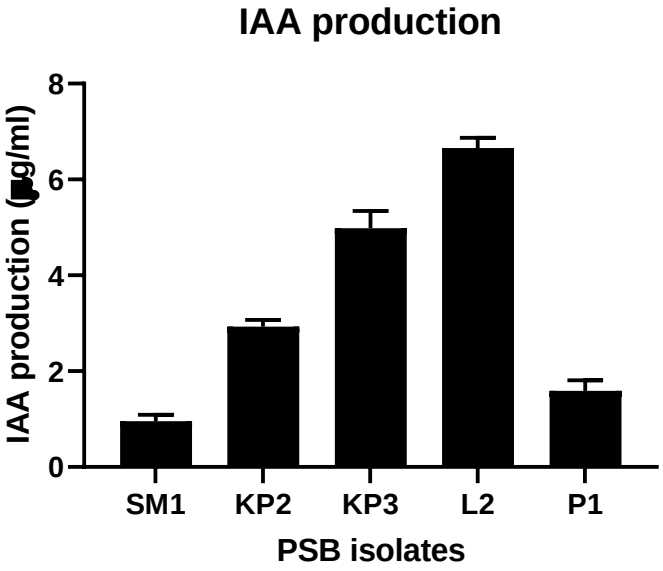


Figure 16: IAA production in LB medium by PSB isolates

After 7 days of incubation, quantitative estimation of auxin production was evaluated. Figure 16 illustrates the range of auxin production by PSB isolates (0.95 to 6.66 µg/mL). Isolate L2 produced the highest quantity of IAA with a concentration of 6.66 µg/mL,

followed by KP3 with a concentration of 4.98 µg/mL. The isolate SM1 inoculated medium contained the least amount of IAA with concentration of 0.95 µg/mL.

4.8 Dual culture Assay for Screening of Biocontol Activities of Isolates against selected phytopathogens

In PDA media, the antagonistic activity of the PSB isolates against *Fusarium* sp., *Rhizoctonia* sp., and *Alternaria* sp. were assessed. Dual Culture assay of PSB isolates against *Rhizoctonia sp.* is depicted in Figure 17.

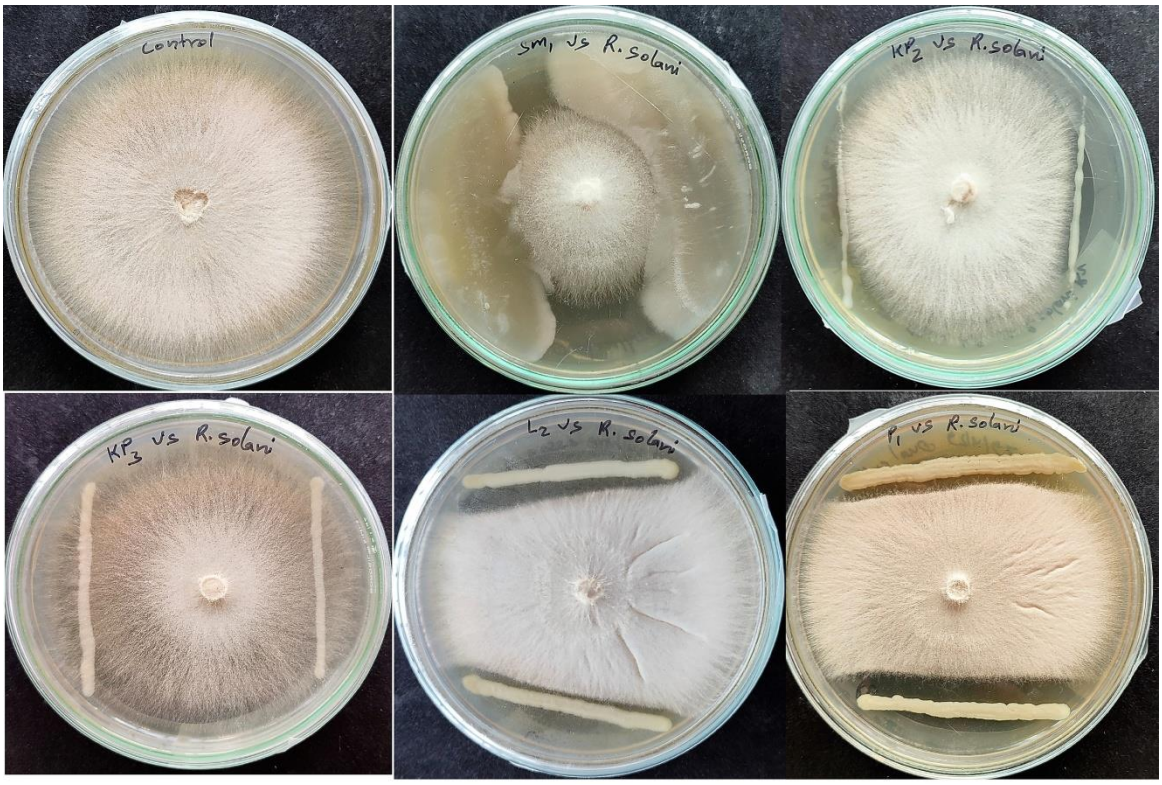


Figure 17: Dual culture assay of PSB isolates against *Rhizoctonia* sp.

Four isolates exhibited some degree of antagonistic activity toward the all investigated phytopathogenic fungi which is shown in Table 7. Only antagonism toward *Fusarium* sp. was demonstrated by isolate KP3. When the fungus was not exposed to the isolated rhizobacteria, the fungal pathogens continued to grow unchecked on the control plates. The SM1 isolate showed the greatest mycelial growth inhibition, with inhibitions of 56.28%, 52.93%, and 49.87% against *Alternaria* sp., *Rhizoctonia* sp., and *Fusarium* sp. respectively. The KP3 isolate showed the lowest amount of inhibition against *Fusarium* sp., with an inhibition of 16.4%.

Table 7: Inhibition percentage of the selected P solubilizer against *Fusarium* sp., *Rhizoctonia* sp., and *Alternaria* sp.

Isolates	*Fusarium* sp.	*Rhizoctonia* sp.	*Alternaria* sp.
SM1	49.87±7.96	52.93±5.23	56.28±5.97
KP2	20.66±3.42	28.17±1.99	35.12±3.26
KP3	16.4±2.56	-	-
L2	36.84±5.13	42.87±0.75	40.35±3.28
P1	42.18±5.85	43.28±3.15	37±10.41

4.9 Screening of Isolates for HCN production

All five PSB isolates showed no evidence of HCN production. Following incubation, no color change from yellow to orange or brown was seen.

4.10 Screening of Isolates for Zinc solubilization

In this research, plate assay was used to evaluate the isolates for zinc solubilization (shown in Figure 18). The modified Pikovskaya agar medium, which contained 0.1% ZnO as an insoluble source of Zn, was used to inoculate the PSB isolates. By measuring the diameter of the colony growth and the solubilization zone, the solubilization efficiency (S.E) was calculated. Three PSB isolates were able to solubilize zinc after two days of incubation (Table 8). Isolate SM1 had highest S.E with 240.71%. P1, with S.E of 240.47%, came in second place. Isolates KP2 and KP3 isolates did not exhibit solublization zone.

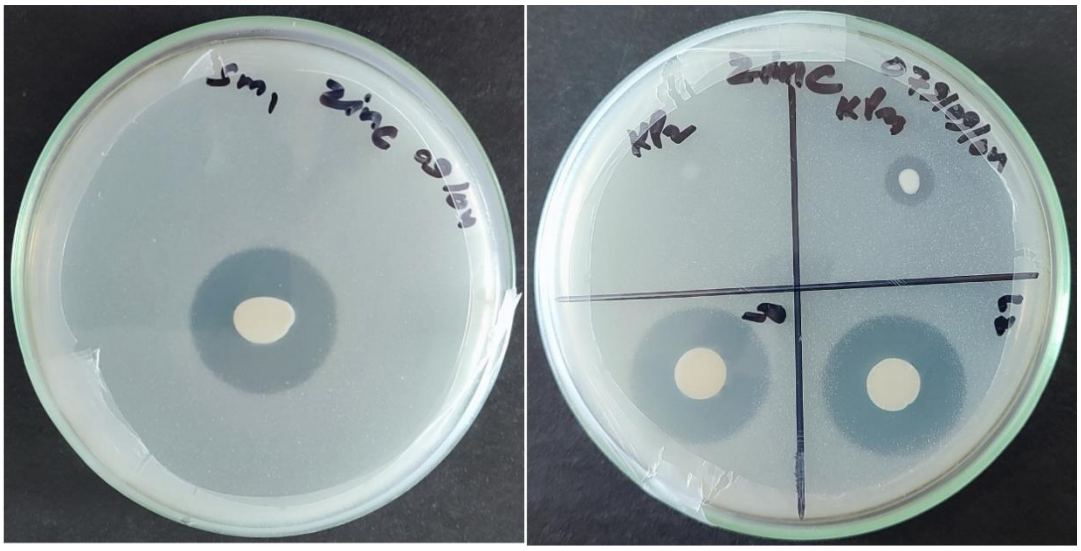


Figure 18: Halozone around PSB isolates on modified PVK media supplemented with insoluble zinc

After 96 hour of incubation four isolates were observed to solubilize ZnO. Isolate P1 showed highest ability for zinc solubilization with S.E of 280%. It was followed by isolate SM1 with S.E of 276.39%. Although isolate KP3 did not exhibit solubilization activity on day 2 of incubation, it did so on day 4 with S.E of 215%.

Table 8: Qualitative evaluation of PSB isolates for zinc solubilization

Isolates	Day 2			Day 4		
	C.D	H.D	S.E (%)	C.D	H.D	S.E (%)
SM1	0.85±0.21	2±0.14	240.71±43.44	1.05±0.21	2.85±0.07	276.39±49.1
KP2	-	-	-	-	-	-
KP3	-	-	-	0.45±0.07	0.95±0.07	215±49.5
L2	0.65±0.07	1.35±0.07	208.33±11.79	1.05±0.07	2.65±0.07	253.18±23.78
P1	0.65±0.07	1.55±0.07	240.47±37.04	0.95±0.07	2.65±0.07	280±28.28

C.D = Colony diameter; H.D = Halo zone diameter; S.E = Solubilization efficiency

4.11 Screening of Isolates for Cellulose degradation

Only SM1 was discovered to be positive for cellulose degrading activity among the five PSB isolates which exhibited a clear zone on screening media (cellulose Congo-Red agar). The hydrolytic capability (clear zone to colony ratio) of isolate SM1 was found to be 1.96.

4.12 Evaluation of isolates for Organic acid production

High Performance Liquid Chromatography (HPLC) was used to examine the organic acids produced during phosphate solubilization by the isolated PSB strains SM1, KP2, KP3, L2, and P1. The retention time of citric acid, succinic acid, tartaric acid and lactic acid were found to be 7.65, 11.42, 8.34 and 12.01 min respectively by running standards of those organic acids. By comparing the retention time of standards, it was discovered that all PSB isolates were able to produce lactic and citric acids (Figure 19, 20, 21, 22, 23 and 24). Tartaric and succinic acid production by isolates was not detected.

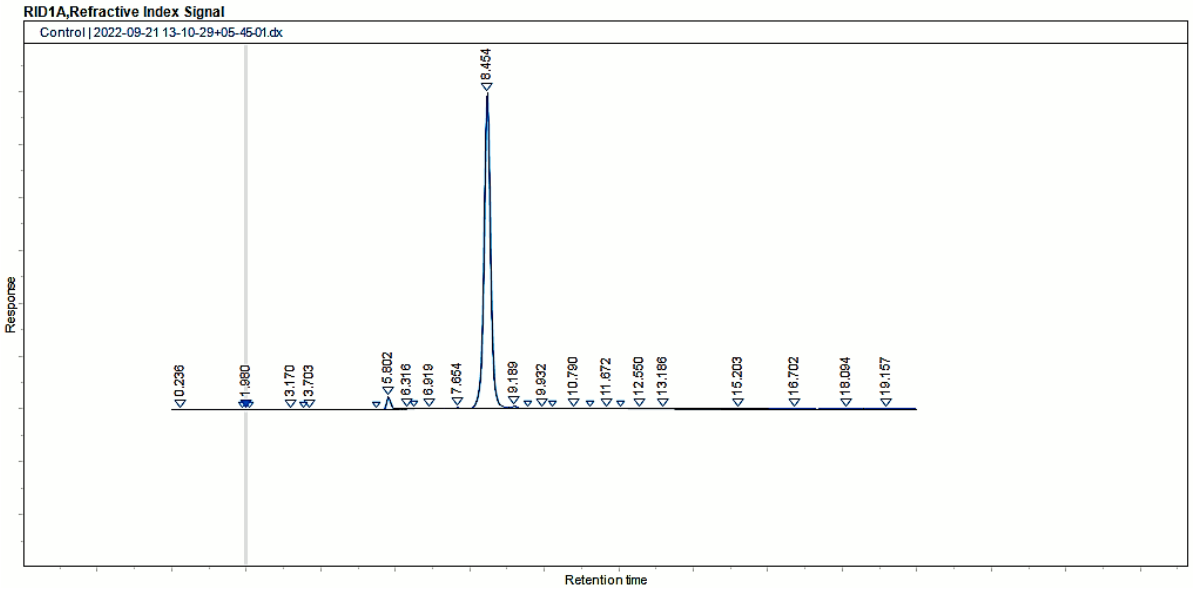


Figure 19: Chromatogram of culture filtrate of uninoculated broth for presence of organic acid

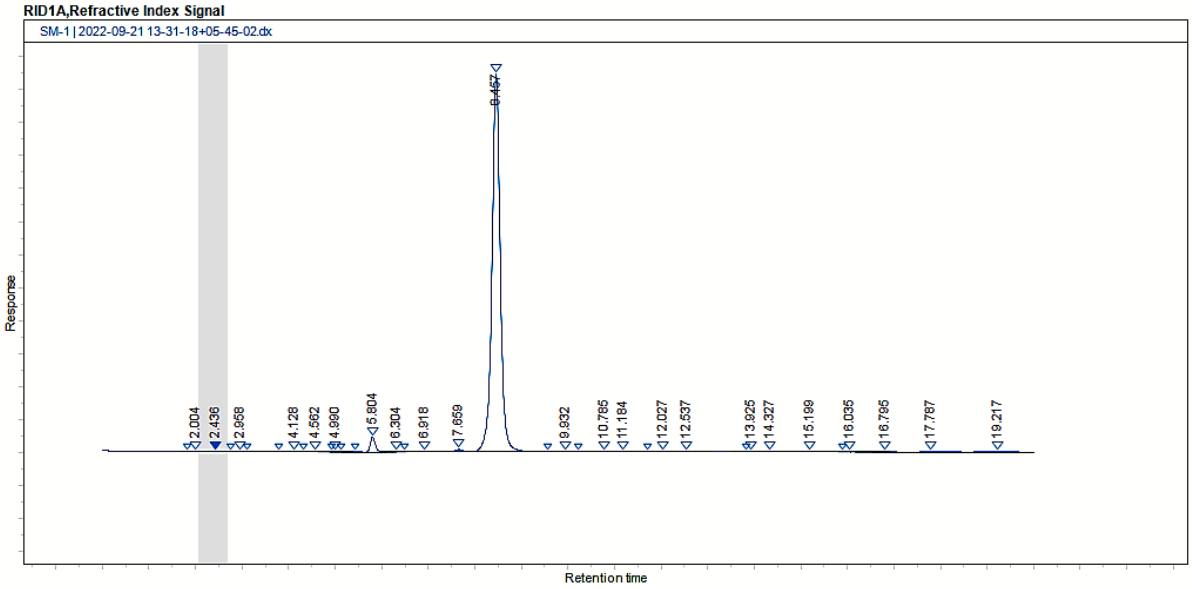


Figure 20: Chromatogram of culture filtrate of isolate SM1 for presence of organic acid

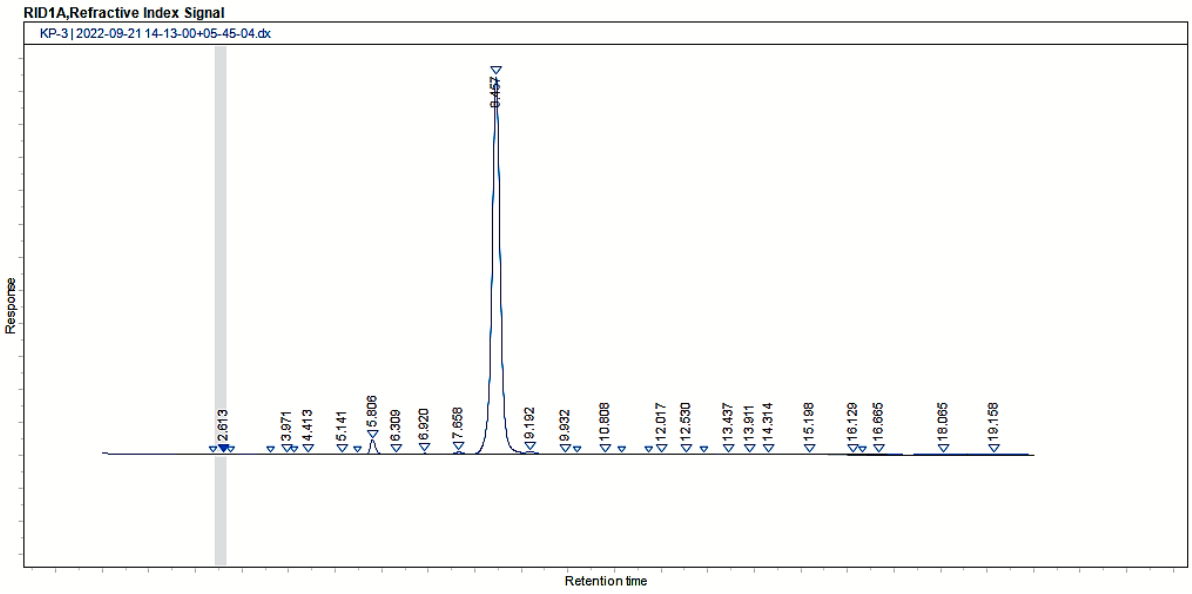


Figure 21: Chromatogram of culture filtrate of isolate KP2 for presence of organic acid

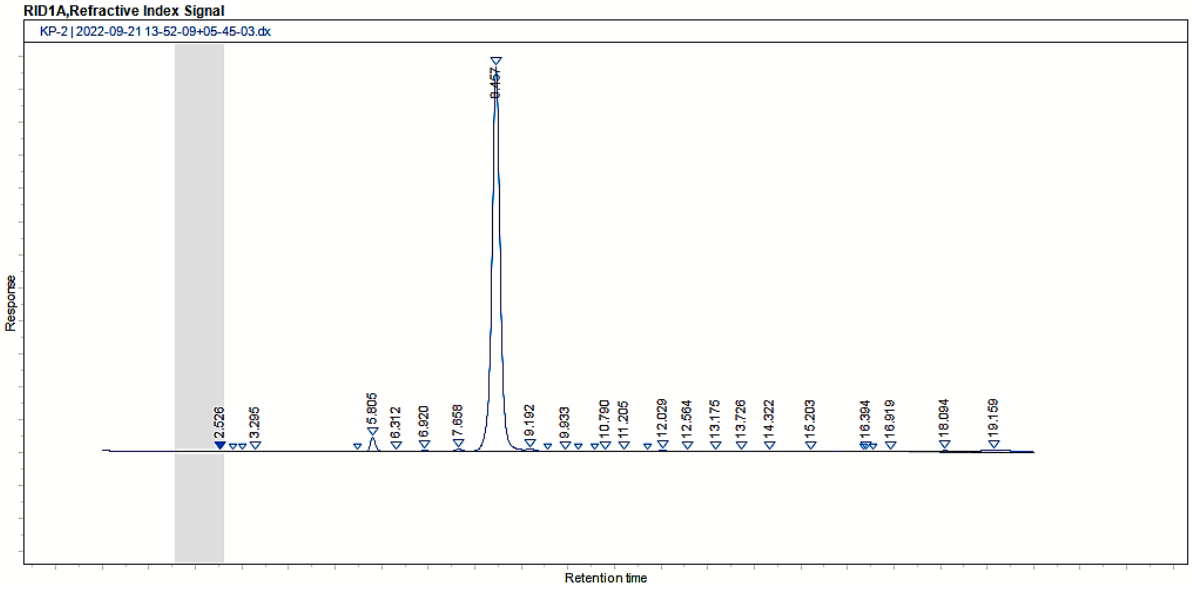


Figure 22: Chromatogram of culture filtrate of isolate KP2 for presence of organic acid

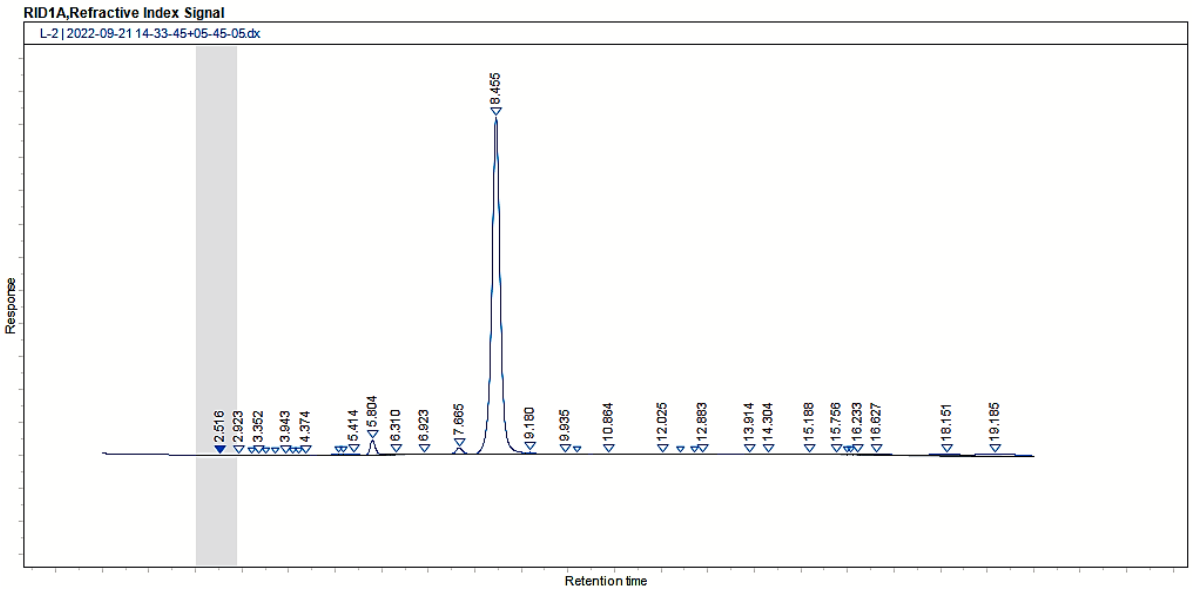


Figure 23: Chromatogram of culture filtrate of isolate L2 for presence of organic acid

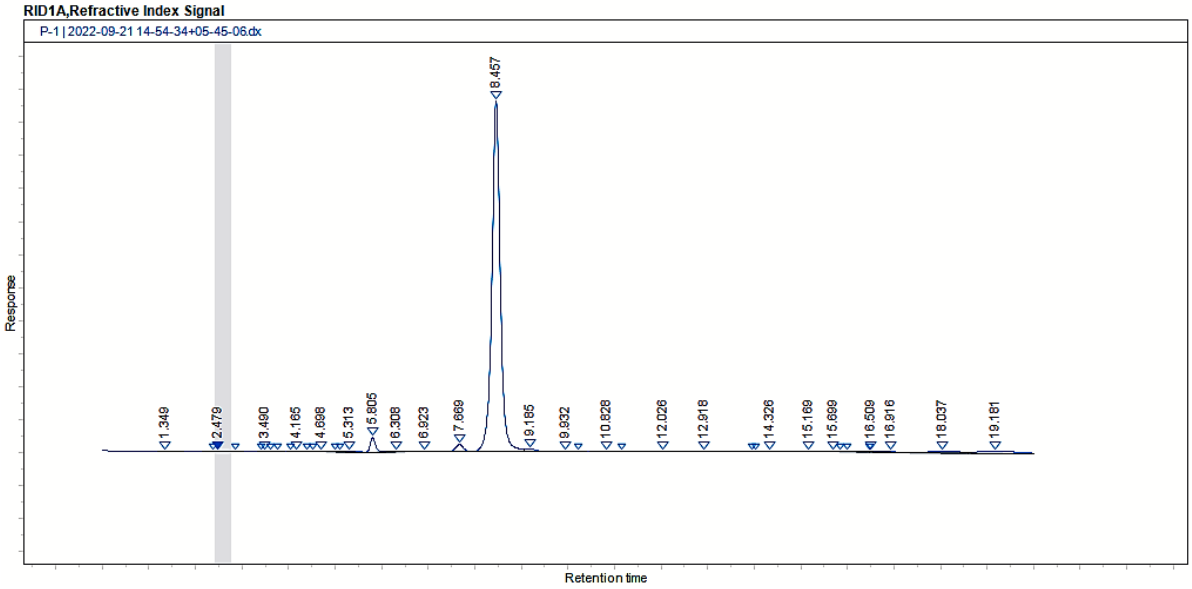


Figure 24: Chromatogram of culture filtrate of isolate P1 for presence of organic acid

By comparing the peak region with the organic acid standard curve (listed on Appendix II), quantitative estimates of the organic acid generated by PSB were determined. The highest concentration of citric acid was produced by SM1 (413 µg/mL), followed by L2 (407.65 µg/mL), as indicated in Table 9. With a concentration of 151.3 µg/mL, SM1 produced the most lactic acid, followed by P1 (117.98 µg/mL). KP3 produced the least amount of lactic acid and citric acid, with concentrations of 9.48 µg/mL and 91.12 µg/mL, respectively.

Table 9: Production of organic acid by PSB isolates during TCP solubilization

Isolates	Citric acid(µg/mL)	Lactic acid(µg/mL)
SM1	413.48	151.3
KP2	140.19	74.02
KP3	91.12	9.48
L2	407.65	107.37
P1	195.68	117.98

4.13 Biocompatibility assay of Bacterial isolates

The cross streaking method was used to assess the biocompatibility of PSB isolates. The isolates that showed inhibition were thought to be bio incompatible. According to our observations, isolate SM1 shared an inhibitory zone with all other isolates. Thus SM1 was regarded as bio incompatible with all other isolates. The remaining four isolates were considered to be biocompatible since they lacked inhibition zone with each other.

4.14 Green House Evaluation of Efficient Phosphate Solubilizing Bacteria (PSB) On Growth of Lettuce Plants

This study evaluates the efficiency of selected PSB strains with varying P solubilizing capacity for enhancing the growth and development of lettuce grown in greenhouse conditions.

Figure 25: Effect of PSB on growth and development of Lettuce roots. A- uninoculated soil with no TCP, B- uninoculated soil with TCP, C-SM1 with TCP, D-KP2 with TCP, E-KP3 with TCP, F-L2 with TCP, G-P1 with TCP, H-Consortium with TCP and I-NPK

According to the findings, isolates KP2, KP3, and L2 increased lettuce shoot length compared to the uninoculated control. KP2, KP3, and L2 were discovered to have shoot lengths of 20.97, 22.53, and 21.1 cm, respectively (shown in Table 10). However, when compared to lettuce with no inoculums +TCP, shoot length of plant inoculated with SM1, P1, and PSB consortium showed lower metrics. The treatment without TCP and without inoculums produced the shortest shoots, measuring 18.76 cm. Comparing PSB inoculations to control treatments, visual inspection of lettuce revealed increased leaf area. With the recommended amount of NPK treatments, the largest leaf area was seen. Appendix V lists a photograph of a green house experiment for evaluating the effect of PSB on lettuce growth promotion.

The data from the current investigation showed that the application of PSB isolates resulted in a reduction of primary root length, as shown in Table 10. The treatment without TCP and without inoculums produced the longest root length, measuring 18.5 cm. The shortest root length, 8.6 cm, was found in the treatment with SM1 and PSB consortium. Plants treated with PSB and NPK showed an increase in the quantity and/or length of lateral roots and root hair. In our pot experiment, visual inspection of the roots revealed that isolate L2 had significantly more developed root hairs than the others (shown in Figure 25). Except for isolate SM1, all other PSB isolates enhanced lettuce's secondary roots. As compared to the control treatments, isolate SM1 resulted with inferior roots qualities.

Table 10: Effect of PSB on shoot and root length of lettuce

Treatment	Shoot length (cm)	Root length (cm)
1. No inoculation, No TCP	18.76± 3.17	18.5±4.70
2. No inoculation +TCP	20.77±2.89	11.7±2.45
3. SM1+TCP	19.8±1.54	8.6±1.5
4. KP2+TCP	20.97±2.25	11.13±1.0
5. KP3+TCP	22.53±0.75	8.77±2.85
6. L2+TCP	21.1±2.53	11.07±0.15
7. P1+TCP	18.03±1.14	12.77±3.74
8. Consortium +TCP	19.27±3.25	8.6±0.17
9. NPK Full Dose	20.33±2.95	10.4±1.93

The results in Table 11 made it clear that PSB strains had an impact on the dry weight of lettuce roots and shoots. Plants inoculated with L2 showed the highest dry weight of root per pot (1.48g). It was followed by treatment with TCP and without inoculums with root dry weight per pot of 1.09g. NPK-treated plants had the highest average dry weight of shoots per pot (8.75g), followed by L2-inoculated plants (6.24g). When compared to the control plant that received TCP supplements, the plant inoculated with SM1 had a lower dry shoot weight per pot (1.94g).

Table 11: Effect of PSB on shoot and root dry biomass of lettuce

Treatment	Root dry weight (g per pot)	Shoot dry weight (g per pot)	Total dry weight (g per pot)
1. No inoculation, No TCP	0.9± 0.14	1.02±0.07	1.92±0.07
2. No inoculation +TCP	1.09±0.16	2.04±0.09	3.13±0.25
3. SM1+TCP	0.68±0.1	1.94±0.07	2.62±0.02
4. KP2+TCP	0.61±0.042	2.29±0.09	2.91±0.05
5. KP3+TCP	0.74±0.014	3.25±0.1	3.99±0.09
6. L2+TCP	1.48±0.06	6.24±0.11	7.72±0.16
7. P1+TCP	0.63±0.05	4.26±0.13	4.89±0.18
8. Co. innoculum +TCP	0.28±0.06	3.51±0.18	3.79±0.23
9. NPK Full Dose	0.36±0.09	8.75±0.21	9.14±0.30

With the exception of SM1, the results showed that all of the PSB strains examined increased the dry weight of lettuce above the uninoculated control. It was noted that the plant given the full dose of NPK had the maximum 9.14 g of dry weight per pot. Plant inoculated with L2 with a dry weight of 7.72 g per pot came next. The treatment without TCP and without inoculums had the lowest dry weight (1.92 g per pot).

CHAPTER 5

Discussion, Summary, Conclusions, and Recommendations

5.1 Discussion

It is necessary to establish a productive, affordable, and ecologically sustainable method for fertilizing soil with P. The utilization of specific microbe species with distinct properties is frequently employed as an alternative to chemical fertilizers (Damo et al., 2022). The objective of this study was to find effective phosphate-solubilizing bacteria and numerous features that support plant growth. Efficient P-solubilizing bacteria have been isolated in the current study using PVK medium. By observing the solubilization zone around the colony in PVK medium, many researchers have successfully isolated PSB (Baliah & Begum, 2011; Sharon et al., 2016). Under in vitro conditions, the efficacy of PSB from plant rhizospheres was examined using tricalcium phosphate as the only source of insoluble P. Nineteen bacterial isolates tested positive for PSB based on the clear zone formation. Only the isolation and initial characterisation of phosphate-solubilizing bacteria have been described as being reliable using the plate method, according to researchers. Mihalache et al. (2015) noticed that the strain that was most effective at solubilizing the identical phosphate source from liquid media was not the one with the highest solubilization zone. Similar results were reported by our observation as well.

The PSB isolates SM1, KP2, KP3, L2, and P1 were examined morphologically and biochemically for this study. Isolate L2 displayed a high degree of sequence similarity (98.71%) with *Burkhoderia* sp. using partial 16s rRNA sequence analysis. As these bacteria share a significant degree of genetic similarity across species, 16s rRNA sequence analysis is insufficient for species-level identification (Devanga et al., 2019). Due to these challenges, multilocus sequence typing (MLST) of house-keeping genes, including *atpD*, *gltB*, *gyrB*, *recA*, *lepA*, *phaC*, and *trpB*, must be investigated for more precise identification (Suárez-Moreno et al., 2010). Numerous studies have identified *Burkholderia*, *Bacillus*, *Klebsiella*, and *Pseudomonas* as frequent P solubilizers (Sanjotha & Manawadi, 2016; Sharon et al., 2016; Suleman et al., 2019).

During this study, the concentration of accessible P during TCP solubilization varied from 56.08 µg/mL to 73.59 µg/mL. Similar results were obtained in a study of tricalcium phosphate solubilization by Kumar et al. (2020). They observed P concentration between 20.80 µg/mL-159.55 µg/mL. According to Cao et al. (2018), who isolated PSB from a natural wetland, showed solubilized P up to 125.88 mg/L. In the study of Aliyat et al. (2020), PSB isolates showed high solubilization efficiency, with soluble-P concentrations in the NBRIP medium varying from 101.91 µg/mL to 174.33 µg/mL. Different parameters, such as nitrogen supplies, phosphate sources, TCP concentration, temperature, potassium sources,

etc., may have an impact on the PSB's ability to solubilize phosphate (Yadav et al., 2013). In our study, TCP and bone meal both were solubilized by all of the isolates. However, a lesser amount of bone meal was solubilized compared to TCP solubilization. This was congruent with the findings of the Mulissa et al. (2015), which had similar results. Our findings demonstrated that as the incubation period is increased, phosphate solubilization efficiency rises. A rise in P solubilization with increasing incubation period has also been noted by several researchers (Goswami et al., 2021; Tagele et al., 2018). For isolating highly effective PSB strains from plant rhizosphere, we have also taken PGP properties into consideration in addition to P solubilizing capacity. A number of factors, including IAA, nitrogen fixation, biocontrol activity, siderophore synthesis and HCN generation have been utilized to isolate effective PGPR (Chouyia et al., 2020; Mei et al., 2021).

All isolates displayed the highest soluble P content at the lowest pH. The finding of Wang et al. (2020) is in favor of this. It is well acknowledged that bacteria solubilize P through processes like acidity, chelation, and exchange reactions (Mahdi et al., 2012). By supplying protons and complexing anions, or by facilitating ligand exchange events or the release of metal ions into solution, organic acids play a crucial part in the process of solubilizing inorganic P (Rfaki et al., 2020).

Since they were able to grow on nitrogen-free Jensen medium, four isolates from our study were considered positive for nitrogen fixation. The ability of bacteria to grow on Jensen media revealed that they could use the atmospheric nitrogen because Jensen media lack nitrogen (Rahim et al., 2021). Numerous studies have suggested that the bacteria *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp and *Azotobacter* sp. are capable of fixing nitrogen (Chrouqi et al., 2017; Rahman et al., 2017; Sherpa et al., 2021). Ammonia, which is synthesized by bacteria, can be used by plants as nitrogen for growth (Jimtha et al., 2014). All of the isolates used in our study were capable of producing some ammonia. Ha and Chu (2020) noticed that certain strains of *Azospirillum* and *Azotobacter* could produce ammonia. Sherpa et al. (2021) noted the ammonia-producing capacities of *Bacillus megaterium*, *Paenibacillus polymyxa*, *Pseudomonas rhodesiae*, *Pseudomonas kribbensis*, *Kosakonia oryzendophytica*, *Burkholderia cenocepacia*, and *Bacillus aryabhattai*. Particularly in nutrient-poor and degraded soils, adding nitrogen-fixing bacteria to soil coupled with PGP characteristics can be an effective method to reduce the usage of costly chemical fertilizers (Rahman et al., 2017).

As IAA have a significant impact on plant growth, the ability of rhizobacterial isolates to produce IAA is particularly useful (Chandra et al., 2018). IAA promotes root development and growth, which could lead to improved nutrient uptake (Jimtha et al., 2014). In the present study, the PSB isolate was able to generate some amount of IAA. Similar outcomes were discovered by other researchers that isolated PSB strains with the capacity to produce IAA and fix N_2 (Kshetri et al., 2018; Thakur & Parikh, 2015). Primary root growth can be significantly impacted by IAA in even tiny doses (Chrouqi et al., 2017). The synthesis of IAA

by bacteria has been shown to increase root surface area (Ali et al., 2020). IAA has been discovered to be produced by several P solubilizing bacterial strains, including *Pseudomonas*, *Bacillus*, *Aeromonas*, *Azotobacter*, *Burkhoderia*, and *Enterobacter* (Jain et al., 2021; Rahman et al., 2017; Thakur & Parikh, 2015).

The bacterial isolates from this investigation showed a range of fungal pathogen inhibition *in vitro* that varied among species. One isolate was able to control only one tested fungal pathogen, while the other controlled all three pathogens (*Fusarium* sp., *Rhizoctonia* sp., and *Alternaria* sp.). Pradhan et al. (2022) noticed that *Pseudomonas fluorescens* and *Paenibacillus polymyxa* had antifungal effects against a number of soil-borne fungi, including *Thanatephorus cucumeris*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Pythium debaryanum*, and *Aspergillus niger*. Numerous bacteria, including *Pseudomonas*, *Bacillus*, and *Agrobacterium*, have been applied to soil or plants as biocontrol agents (Abo-Elyousr et al., 2019). According to reports, *Burkholderia contaminans* is antagonistic to a number of phytopathogens, including *Rhizoctonia solani*, *Stemphylium botryosum*, *Colletotrichum dematium*, *Stemphylium lycopersici*, *Alternaria alternate*, *Pythium graminicola*, *Fusarium moniliforme*, *Fusarium graminearum*, *Alternaria solani*, and *Fusarium oxysporum* (Tagele et al., 2018). This supports our finding. Bacterial isolates uses a variety of biocontrol strategies, including the generation of siderophores, diffusible and volatile antibiotics, lytic enzymes, etc (Tariq et al., 2010). It is known that *Bacillus* sp. uses the processes of lipopeptide production and competitive inhibition to prevent the growth of phytopathogens (Chrouqi et al., 2017). Studies have shown that the bacterial metabolite HCN has antifungal properties (Ali et al., 2020). In our study, no PSB isolates had the ability to produce HCN.

The most prevalent micronutrient deficiency in crops, zinc deficiency causes significant production losses. Utilizing zinc fertilizers to reduce zinc deficiency and boost crop yield may not be cost-effective (Kamran et al., 2017). In our investigation, qualitative screening of ZnO solubilization revealed that four out of five PSB isolate were able to do so. The ability of *Bacillus*, *Pseudomonas*, *Azotobacter*, and *Rhizobium* to solubilize zinc has been identified by a number of researchers (Hussain et al., 2015; Jagana et al., 2019; Joshi et al., 2013). According to a number of research studies, adding zinc-solubilizing bacteria (ZSB) to the soil can increase Zn uptake by plants, which would therefore improve growth and yields (Dasila et al., 2022; Manasa et al., 2019; Sharma, 2012). According to a study by Joshi et al. (2013), using zinc-solubilizing bacteria increased wheat root and shoot growth as well as wheat seed zinc content. ZSB application in the soil may aid in the effective utilization of Zn fertilizers (Perumal et al., 2019).

Only one PSB isolate was able to produce cellulase in this study. The ability of *Bacillus*, *Pseudomonas*, *Salmonella*, and *Acinetobacter* to produce lytic enzyme like cellulase, chitinase and β-1, 3 glucanase has been reported (Gupta et al., 2012; Mahmood et al., 2014). The lytic enzymes breakdown the glycosidic linkages in phytopathogens' cell walls, thus preventing their spread in the area (Ali et al., 2020; Chrouqi et al., 2017).

It is generally known that the primary method by which microorganisms solubilize TCP involves acidifying the medium through the production and release of a wide range of organic acids (Rodríguez & Fraga, 1999). The generation of organic acid by PSB isolates was assessed in the current investigation. HPLC examination of the culture filtrate revealed the production of citric acid and lactic acid. Similar observation by Vyas and Gulati (2009) found evidence of the production of lactic and citric acids by various *Pseudomonas* sp. strains. Major organic acids like succinic acid, oxalic acid, citric acid, and 2-ketogluconic acid have been found in significant quantities among phosphate solubilizers (Marra et al., 2015; Serrano & Mardad, 2013; Yadav et al., 2013). However, Mihalache et al. (2015) found that isolated strains from the rhizosphere of runner beans mostly produce lactic, isocitric, tartaric, and pyruvic acids. Researchers have found a link between the release of aluminum-bound phosphate and organic acids like citric and tartaric acids (Prijambada et al., 2009). Numerous roles for organic acids in the soil have been hypothesized, such as microbial chemotaxis, root nutrient uptake, mineral weathering, and metal detoxification (Lee et al., 2019).

Numerous studies have been conducted to assess PSB's potential to promote plant development. They've seen improvements in a number of plant growth indices, including increased shoot length, leaf area, root area, dry biomass, and P content in plants (Batool & Iqbal, 2019; Kumari et al., 2020). An in vivo investigation by Jinal and Amaresan (2020) revealed that tomato plants that had been treated with *Bacillus subtilis* had improved root, shoot growth and biomass. These concur with our research on the impact of PSB on lettuce growth. When PSB was applied, we noticed a shorter root length. Numerous in vitro studies have demonstrated that a number of PGPR enhance the number and/or length of lateral roots, restrict the growth of the main root, and promote the elongation of root hairs (Grover et al., 2021). Cavite et al. (2021) also noticed that the application of PSB to rice seedlings improved the root surface area.

In a lettuce greenhouse experiment, Maldonado et al. (2020) found that significant improvements were made to the plant's root and leaf area, fresh and dry weight and leaf count by PSB. Our results agree with them, with the exception of isolate SM1. These findings are consistent with research conducted by other authors on the impacts of PSB on a variety of crops, including *Emblica officinalis* (Kumari et al., 2020), tomato (Jinal & Amaresan, 2020), rice (Stephen et al., 2015), barley (Chouyia et al., 2020), Chinese cabbage (Poonguzhali et al., 2008) and Tea (Panda et al., 2015). SM1 did not exhibit any increase in the measured parameters, despite the fact that the isolates had a range of PGPR characteristics. This can be the result of inadequate population establishment in lettuce plant rhizosphere regions. The PSB isolate consortium did not perform well in lettuce growth promotion. This may be the result of isolates competing with one another and inhibiting one another.

In our investigation, the addition of PSB isolates resulted in a significant increase in the dry weight of the lettuce roots and shoots. Numerous studies looking into how PSB affects

lettuce growth promotion have shown comparable results, although to varied degrees (Chabot et al., 1996; Maldonado et al., 2020; Martínez-Cano et al., 2021; Suryatmana et al., 2021). Maldonado et al. (2020) noted that PSB isolate inoculation and 50% fertilization of lettuce both matched the output of the control plants at 100% fertilization. Researchers have noted that PSB has a good effect on the dry weight of many plants, including rice (Rawat et al., 2021), barley (Chouyia et al., 2020), maize (Viruel et al., 2014), green gram (Singh et al., 2020), and wheat (Mohamed et al., 2019).

5.2 Summary

In many agroecosystems, phosphorus is a key nutrient that restricts crop growth and is essential for a number of physiological and biochemical processes (Zhu et al., 2018). For the best crop yields, its exogenous application as chemical fertilizer has been determined to be essential, but its rising cost and lack of availability are difficulties faced by farmers. Intriguingly, between 75% to 90% of phosphatic fertilizer applied precipitates to form insoluble complexes with Ca, Fe, and Al ions (Eramma et al., 2020). Therefore, in this situation, strategies are required to enable plants to access this insoluble/precipitated phosphorus. One potential approach for the efficient and sustainable use of phosphate resources is the use of microorganisms that can dissolve insoluble P. The current work intends to identify and characterize effective multi trait bacteria that can solubilize P for increased agricultural output.

In the current investigation, phosphate-solubilizing bacteria from 10 different soil samples were isolated and screened on PVK agar plates with a clear zone around the colonies. Five effective isolates out of a total of 19 unique PSBs were chosen for further research. Morphological and biochemical characterization of selected PSB isolates were carried out. The 16s rRNA sequencing and phylogenetic analysis of isolate L2 confirmed that it is *Burkholderia* sp.

Study on TCP and bone meal solubilization by PSB revealed that the number of incubation days enhanced the amount of P that was available in the medium. At 15 days of incubation during TCP solubilization, the isolates showed P release in the following order: KP2 (73.59 µg/mL), L2 (65.98 µg/mL), KP3 (64.91 µg/mL), P1 (60.15 µg/mL), and SM1 (56.08 µg/mL). The isolates, however, released P in the following order during bone meal solubilization: L2 (49.54 µg/mL), KP2 (45.81 µg/mL), SM1 (34.85 µg/mL), P1 (34.54 µg/mL), and KP3 (32.13 µg/mL) during 15 days of incubation.

The pH of the broth was found to decrease with selected PSB strains during TCP solubilization. The medium inoculated with L2 witnessed a maximum pH drop from 7.0 to 3.93 after 15 days of incubation. The pH also decreased similarly in the bone meal-supplemented medium that had been inoculated with PSB isolates. After 15 days of incubation, isolate KP2 demonstrated a maximum pH lowering of 4.73 from initial pH of 7.0.

Testing PSB isolates for nitrogen fixation capacity revealed isolates SM1, L2, KP3 and P1 to have N fixing capacities. Nessler's reagent was used to assess the PSB isolates' ability to produce ammonia. The findings showed that KP3 (4.25 µg/mL) and P1 (3.88 µg/mL) produced the most ammonia. The potential of PSB isolates SM1, KP2, KP3, L2, and P1 to produce IAA was assessed. According to the findings, IAA was produced in the following amounts: L2 (6.66 µg/mL), KP3 (4.98 µg/mL), and P1 (2.93 µg/mL).

The capacity of PSB isolates to combat the phytopathogens (*Fusarium* sp., *Rhizoctonia* sp., and *Alternaria* sp.) was investigated. Inhibition percentages of PSB against *Fusarium* sp., *Rhizoctonia* sp., and *Alternaria* sp. were from 16.4 to 49.87%, 52.93 to 28.17%, and 56.28 to 35.12%, respectively. It was also investigated whether PSB isolate might dissolve ZnO in modified PVK. The results showed that PSB's with a ZnO solubilizing efficiency of P1 (280%), SM1 (276.39%), L2 (253.18%), and KP3 (215%).

In the PVK medium inoculated with the isolates, our analysis showed that the presence of organic acids was obvious. Citric acid and lactic acid were discovered in the medium that had been inoculated with SM1, KP2, KP3, L2, and P1. PSB generated citric acid in the range of 91.12 – 413.48 µg/mL. Lactic acid was found in the range of 9.48 to 151.3 µg/mL.

The outcomes of our pot experiment showed that PSB had a favorable effect on lettuce plant growth. Visual inspection showed that PSB inoculation enhanced the root volume and leaf area. Longer shoot length were observed with isolate KP2, KP3 and L2 compared to control. In case of root length control treatment exhibited longest root length. The inoculation of isolates KP2, KP3, L2, P1, and consortium of PSB with tri calcium phosphate boosted the lettuce's dry weight. Highest lettuce dry weight per pot was observed with NPK treatment followed by isolate L2 inoculation. However, dry weight of lettuce with SM1 isolate was lower than the control treatment.

5.3 Conclusions

In this study five phosphate-solubilizing bacteria, denoted as SM1, KP2, KP3, L2, and P1, were isolated. The isolate L2 was identified as *Burkholderia* sp. after 16s rRNA sequencing and phylogenetic analysis. The plant growth-promoting properties of PSB isolates were investigated, including their capacity to fix nitrogen, generate IAA, solubilize zinc, and exhibit antagonistic action. L2 was the most productive strain in this study due to its capacity to solubilize P from TCP and bone meal, produce IAA, fix nitrogen, solubilize zinc, and exhibit antagonistic activity toward phytopathogen. Additionally, it considerably enhanced the growth of lettuce leaf area, plant height, root volume, and dry biomass.

Numerous PGPR features, including the synthesis of auxin, nitrogen fixation, ammonia production, antifungal activity, and zinc solubilization, were also present in the other PSB isolates SM1, KP2, KP3, and P1. With the exception of isolate SM1, they have encouraged growth of the lettuce leaf, root, and dry biomass. The results of this study indicate that isolated PSB may be employed as biofertilizers. The P usage efficiency of applied chemical

fertilizers can be improved using these PSB isolates. Additionally, they can help reduce the need of chemical P fertilizers.

5.4 Recommendations

The following suggestions can be made in light of our study's findings:

- It is necessary to conduct extensive field experiments so that the significance of these PSB in plant growth promotion may be well understood.
- It is necessary to do additional research on PSB isolates for plant growth enhancement, including potassium solubilization, siderophore synthesis, and organic phosphate solubilization.
- Investigating the many environmental elements that either directly or indirectly influence the functions of phosphate-solubilizing bacteria is important.
- To obtain more effective PSB isolates, more PSB isolates from various ecological zones should be isolated and characterized.
- Efficient PSB isolates need to be produced in sufficient quantities, commercialized, and made available to farmers.

References

Aarab, S., Ollero, F. J., Megías, M., Laglaoui, A., Bakkali, M., & Arakrak, B. (2013). Isolation and Identification of Potential Phosphate Solubilizing Bacteria from the Rhizosphere of Lupinus hirsutus L. in the north of Morocco. *Moroccan Journal of Biology*, *10*, 7–13.

Abhishali, Debbarma, V., & Chauhan, A. (2023). Effect of Biofertilizers and Foliar application of Boron on Growth and Yield of Greengram (Phaseolus radiatus L .). *Biological Forum-An International Journal*, *15*(3), 277–281.

Abo-Elyousr, K. A. M., Khalil Bagy, H. M. M., Hashem, M., Alamri, S. A. M., & Mostafa, Y. S. (2019). Biological control of the tomato wilt caused by Clavibacter michiganensis subsp. michiganensis using formulated plant growth-promoting bacteria. *Egyptian Journal of Biological Pest Control*, *29*(1). https://doi.org/10.1186/s41938-019-0152-6

Abou Seeda, M., Yassen, A., Abou Ei-Nour, E. A., & Sahar, M. Z. (2020). Improving of phosphorus use efficiency in Plant-Soil-System. A review. *Middle East Journal of Agriculture Research*, *August*. https://doi.org/10.36632/mejar/2020.9.3.39

Afzal, A., & Bano, A. (2008). Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (Triticum aestivum). *International Journal of Agriculture and Biology*, *10*(1), 85–88.

Ahmad, M., Zahir, Z. A., Khalid, M., Nazli, F., & Arshad, M. (2013). Efficacy of Rhizobium and Pseudomonas strains to improve physiology, ionic balance and quality of mung bean under salt-affected conditions on farmer's fields. *Plant Physiology and Biochemistry*, *63*, 170–176. https://doi.org/10.1016/j.plaphy.2012.11.024

Ali, S., Hameed, S., Shahid, M., Iqbal, M., Lazarovits, G., & Imran, A. (2020). Functional characterization of potential PGPR exhibiting broad-spectrum antifungal activity. *Microbiological Research*, *232*(December), 126389. https://doi.org/10.1016/j.micres.2019.126389

Aliyat, F. Z., Maldani, M., Guilli, M. El, Nassiri, L., & Ibijbijen, J. (2020). Isolation and characterization of phosphate solubilizing bacteria from phosphate solid sludge of the moroccan phosphate mines. *Open Agriculture Journal*, *14*(1), 16–24. https://doi.org/10.2174/1874331502014010016

Aloo, Becky N., Tripathi, V., Makumba, B. A., & Mbega, E. R. (2022). Plant growth-promoting rhizobacterial biofertilizers for crop production: The past, present, and future. *Frontiers in Plant Science*, *13*(September), 1–15. https://doi.org/10.3389/fpls.2022.1002448

Aloo, Becky Nancy, Makumba, B. A., & Mbega, E. R. (2020). Phosphate-Solubilizing Rhizobacteria: Diversity, Mechanisms, and Prospects in Sustainable Agriculture. *Preprints*, *September*, 1–32. https://doi.org/10.20944/preprints202009.0681.v1

Alori, E. T., Glick, B. R., & Babalola, O. O. (2017). Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Frontiers in Microbiology*, *8*(JUN), 1–8. https://doi.org/10.3389/fmicb.2017.00971

Arai, Y., & Sparks, D. L. (2007). Phosphate Reaction Dynamics in Soils and Soil Components: A Multiscale Approach. *Advances in Agronomy*, *94*(06), 135–179. https://doi.org/10.1016/S0065-2113(06)94003-6

Arif, M. S., Shahzad, S. M., Yasmeen, T., Riaz, M., Ashraf, M., Ashraf, M. A., Mubarik, M. S., & Kausar, R. (2017). Improving Plant Phosphorus (P) Acquisition by Phosphate-Solubilizing Bacteria. In M. Naeem, A. A. Ansari, & S. S. Gill (Eds.), *Essential Plant Nutrients* (pp. 513–556). Springer International Publishing. https://doi.org/10.1007/978-3-319-58841-4_21

Assuero, S. G., Mollier, A., & Pellerin, S. (2004). The decrease in growth of phosphorus-deficient maize leaves is related to a lower cell production. *Plant, Cell and Environment*, *27*(7), 887–895. https://doi.org/10.1111/j.1365-3040.2004.01194.x

Baffes, J., & Koh, W. C. (2022). Fertilizer prices expected to remain higher for longer. In *World Bank Blogs*. https://blogs.worldbank.org/opendata/fertilizer-prices-expected-remain-higher-longer

Bakker, A. W., & Schippers, B. (1987). Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas SPP-mediated plant growth-stimulation. *Soil Biology and Biochemistry*, *19*(4), 451–457. https://doi.org/10.1016/0038-0717(87)90037-X

Balemi, T., & Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: A review. *Journal of Soil Science and Plant Nutrition*, *12*(3), 547–561. https://doi.org/10.4067/s0718-95162012005000015

Baliah, N. T., & Begum, P. J. (2011). *Original Research Article Isolation , identification and characterization of phosphate solubilizing bacteria (PSB) isolated from economically important crop plants*. *4*(2015), 915–924.

Batool, S., & Iqbal, A. (2019). Phosphate solubilizing rhizobacteria as alternative of chemical fertilizer for growth and yield of Triticum aestivum (Var. Galaxy 2013). *Saudi Journal of Biological Sciences*, *26*(7), 1400–1410. https://doi.org/10.1016/j.sjbs.2018.05.024

Belimov, A. A., Kojemiakov, A. P., & Chuvarliyeva, C. V. (1995). Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. *Plant and Soil*, *173*(1), 29–37. https://doi.org/10.1007/BF00155515

Biswas, J. K., Banerjee, A., Rai, M., Naidu, R., Biswas, B., Vithanage, M., Dash, M. C., Sarkar, S. K., & Meers, E. (2018). Potential application of selected metal resistant phosphate solubilizing bacteria isolated from the gut of earthworm (Metaphire posthuma) in plant growth promotion. *Geoderma*, *330*(May), 117–124. https://doi.org/10.1016/j.geoderma.2018.05.034

Blackwell, M., Darch, T., & Haslam, R. (2019). *Phosphorus use ef fi ciency and fertilizers : future opportunities for improvements*. *6*(4), 332–340.

Broadley, M. R., Bowen, H. C., Cotterill, H. L., Hammond, J. P., Meacham, M. C., Mead, A., & White, P. J. (2004). Phylogenetic variation in the shoot mineral concentration of

angiosperms. *Journal of Experimental Botany*, *55*(396), 321–336. https://doi.org/10.1093/jxb/erh002

Cao, Y., Fu, D., Liu, T., Guo, G., & Hu, Z. (2018). Phosphorus Solubilizing and Releasing Bacteria Screening from the Rhizosphere in a Natural Wetland. *Water*, *10*(2), 195. https://doi.org/10.3390/w10020195

Cappuccino, J. Sherman, N. (1992). *Microbiology Laboratory Manual*. Benjamin/Cummins Science Publishing, California.

Carrington, D. (2019, September 6). Phosphate fertiliser "crisis" threatens world food supply | Farming | The Guardian. *Guardian*. https://www.theguardian.com/environment/2019/sep/06/phosphate-fertiliser-crisis-threatens-world-food-supply

Cavite, H. J. M., Mactal, A. G., Evangelista, E. V., & Cruz, J. A. (2021). Biochemical characteristics and inoculation effects of multi-trait plant growth-promoting rhizobacteria on upland rice (Oryza sativa L. cv PSB Rc23) seedling growth. *Archives of Microbiology*, *203*(6), 3533–3540. https://doi.org/10.1007/s00203-021-02337-z

Chabot, R., Antoun, H., & Cescas, M. P. (1996). Growth promotion of maize and lettuce by phosphate-solubilizing Rhizobium leguminosarum biovar . phaseoli. *Plant and Soil*, *184*(2), 311–321.

Chandra, S., Askari, K., & Kumari, M. (2018). Optimization of indole acetic acid production by isolated bacteria from Stevia rebaudiana rhizosphere and its effects on plant growth. *Journal of Genetic Engineering and Biotechnology*, *16*(2), 581–586. https://doi.org/10.1016/j.jgeb.2018.09.001

Chatterjee, D., Datta, S. C., & Manjaiah, K. M. (2014). Fractions, uptake and fixation capacity of phosphorus and potassium in three contrasting soil orders. *Journal of Soil Science and Plant Nutrition*, *14*(3), 640–656. https://doi.org/10.4067/s0718-95162014005000051

Chen, Q., & Liu, S. (2019). Identification and Characterization of the Phosphate-Solubilizing Bacterium Pantoea sp. S32 in Reclamation Soil in Shanxi, China. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/FMICB.2019.02171/FMICB_10_02171_PDF.PDF

Cherchali, A., Boukhelata, N., Kaci, Y., Abrous-Belbachir, O., & Djebbar, R. (2019). Isolation and identification of a phosphate-solubilizing Paenibacillus polymyxa strain GOL 0202 from durum wheat (Triticum durum Desf.) rhizosphere and its effect on some seedlings morphophysiological parameters. *Biocatalysis and Agricultural Biotechnology*, *19*(March), 101087. https://doi.org/10.1016/j.bcab.2019.101087

Chouyia, F. E., Romano, I., Fechtali, T., Fagnano, M., Fiorentino, N., Visconti, D., Idbella, M., Ventorino, V., & Pepe, O. (2020). P-solubilizing streptomyces roseocinereus ms1b15 with multiple plant growth-promoting traits enhance barley development and regulate rhizosphere microbial population. *Frontiers in Plant Science*, *11*(August), 1–10. https://doi.org/10.3389/fpls.2020.01137

Chrouqi, L., Ouahmane, L., Jadrane, I., Koussa, T., & Al Feddy, M. N. (2017). Screening of soil rhizobacteria isolated from wheat plants grown in the Marrakech region (Morocco, North Africa) for plant growth promoting activities. *Journal of Materials and Environmental Science*, *8*(9), 3382–3390.

Cordell, D., & White, S. (2011). Peak Phosphorus: Clarifying the Key Issues of a Vigorous Debate about Long-Term Phosphorus Security. *Sustainability*, *3*(10), 2027–2049. https://doi.org/10.3390/su3102027

Dahlia, A., Jabbar, F. B. A., & Indrayani. (2021). The abundance and diversity of phosphate solubilizing bacteria as a function of harvesting regime of duckweed (Lemna minor L.). *AACL Bioflux*, *14*(5), 3055–3067.

Damo, J. L. C., Ramirez, M. D. A., Agake, S. I., Pedro, M., Brown, M., Sekimoto, H., Yokoyama, T., Sugihara, S., Okazaki, S., & Ohkama-Ohtsu, N. (2022). Isolation and Characterization of Phosphate Solubilizing Bacteria from Paddy Field Soils in Japan. *Microbes and Environments*, *37*(2). https://doi.org/10.1264/jsme2.ME21085

Dasila, H., Sah, V. K., Jaggi, V., & Sahgal, M. (2022). Phosphate solubilizing bacteria (PSB) a potential tool to enhance soil health and wheat vigor parameters in pot trial experiment. *The Pharma Innovation Journal*, *11*(3), 1829–1835.

Dean, L. A. (1949). Fixation of Soil Phosphorus. In *Advances in Agronomy* (Vol. 1, Issue C, pp. 391–411). https://doi.org/10.1016/S0065-2113(08)60754-3

Dev, P., Paliyal, S. S., Mridula, Rana, N., & Upadhyay, R. G. (2022). Strategies and methods for improving phosphorus acquisition and its use efficiency: A review. *Environment Conservation Journal*, *23*(1&2), 22–30. https://doi.org/10.36953/ecj.021887-2153

Devanga, R., Naveen, K., & Veeraraghavan, B. (2019). Accurate identification and epidemiological characterization of Burkholderia cepacia complex: An update. *Annals of Clinical Microbiology and Antimicrobials*, *18*(1), 1–10. https://doi.org/10.1186/s12941-019-0306-0

Devkota, K. P., Devkota, M., Khadka, L., Khadka, A., Paudel, G., Acharya, S., & McDonald, A. J. (2018). Nutrient responses of wheat and rapeseed under different crop establishment and fertilization methods in contrasting agro-ecological conditions in Nepal. *Soil and Tillage Research*, *181*(March), 46–62. https://doi.org/10.1016/j.still.2018.04.001

Dhillon, J., Torres, G., Driver, E., Figueiredo, B., & Raun, W. R. (2017). World phosphorus use efficiency in cereal crops. *Agronomy Journal*, *109*(4), 1670–1677. https://doi.org/10.2134/agronj2016.08.0483

Diep, C. N., Trung, N. B., & Nhu, V. T. P. (2017). Effects of Bradyrhizobia and Phosphate-solubilizing bacteria on soybean (Glycine max L . Merrill) cultivated on Ferrasols of Cujut district , DakNong province , Vietnam. *International Journal of Environmental & Agriculture Research*, *4*, 70–79.

Elhaissoufi, W., Khourchi, S., Ibnyasser, A., Ghoulam, C., Rchiad, Z., Zeroual, Y., Lyamlouli, K., & Bargaz, A. (2020). Phosphate Solubilizing Rhizobacteria Could Have a Stronger

Influence on Wheat Root Traits and Aboveground Physiology Than Rhizosphere P Solubilization. *Frontiers in Plant Science*, *11*(July), 1–15. https://doi.org/10.3389/fpls.2020.00979

Eramma, Devaswamy, M., Rao, S., Ramesh, Y. M., & Naik, N. M. (2020). Isolation and Screening of Phosphate Solubilizing Bacteria from Paddy Rhizosphere Soil. *International Journal of Current Microbiology and Applied Sciences*, *9*(2), 477–485. https://doi.org/10.20546/ijcmas.2020.902.059

FAO. (2018). *The future of food and agriculture – Alternative pathways to 2050*. http://www.fao.org/3/I8429EN/i8429en.pdf

Felsenstein, J. (1985). CONFIDENCE LIMITS ON PHYLOGENIES: AN APPROACH USING THE BOOTSTRAP. *Evolution*, *39*(4), 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Fenta, L., & Assefa, F. (2017). Isolation and characterization of phosphate solubilizing bacteria from tomato (Solanum l.) rhizosphere and their effect on growth and phosphorus uptake of the host plant under green house experiment. *International Journal of Advanced Research*, 1–49.

Filippelli, G. M. (2002). The Global Phosphorus Cycle. *Reviews in Mineralogy and Geochemistry*, *48*(1), 391–425. https://doi.org/10.2138/rmg.2002.48.10

Ganeshan, G., & Kumar, A. M. (2005). Pseudomonas fluorescens, a potential bacterial antagonist to control plant diseases. *Journal of Plant Interactions*, *1*(3), 123–134. https://doi.org/10.1080/17429140600907043

Garcia, A. (2020). The environmental impacts of agricultural intensification. *SPIA Technical Note*, *9*.

George, T. S., Simpson, R. J., Hadobas, P. A., & Richardson, A. E. (2005). Expression of a fungal phytase gene in Nicotiana tabacum improves phosphorus nutrition of plants grown in amended soils. *Plant Biotechnology Journal*, *3*(1), 129–140. https://doi.org/10.1111/j.1467-7652.2004.00116.x

Goswami, A., Ghosh, S., Pramanik, P., & Goutam, K. G. (2021). Characterization of Potent Phytate Solubilizing Bacterial Strains of Tea Garden Soils as Futuristic Potent Bio-Inoculant. *International Journal of Current Microbiology and Applied Sciences*, *10*(4), 470–484. https://doi.org/10.20546/ijcmas.2021.1004.049

Green, L. H., & Goldman, E. (2021). Practical Handbook of Microbiology. In L. H. Green & E. Goldman (Eds.), *Practical Handbook of Microbiology*. CRC Press. https://doi.org/10.1201/9781003099277

Grover, M., Bodhankar, S., Sharma, A., Sharma, P., Singh, J., & Nain, L. (2021). PGPR Mediated Alterations in Root Traits: Way Toward Sustainable Crop Production. *Frontiers in Sustainable Food Systems*, *4*(January), 1–28. https://doi.org/10.3389/fsufs.2020.618230

Gupta, P., Samant, K., & Sahu, A. (2012). Isolation of cellulose-degrading bacteria and

determination of their cellulolytic potential. *International Journal of Microbiology*, *2012*. https://doi.org/10.1155/2012/578925

Gustafsson, J. P., Mwamila, L. B., & Kergoat, K. (2012). The pH dependence of phosphate sorption and desorption in Swedish agricultural soils. *Geoderma*, *189–190*, 304–311. https://doi.org/10.1016/j.geoderma.2012.05.014

Gyaneshwar, P., Naresh Kumar, G., Parekh, L. J., & Poole, P. S. (2002). Role of soil microorganisms in improving P nutrition of plants. *Plant and Soil*, *245*(1), 83–93. https://doi.org/10.1023/A:1020663916259

Ha, T. Q., & Chu, T. T. H. (2020). Selection of nitrogen fixation and phosphate solubilizing bacteria from cultivating soil samples of Hung Yen province in Vietnam. *Journal of Vietnamese Environment*, *12*(2), 162–168. https://doi.org/10.13141/jve.vol12.no2.pp162-168

Hameeda, B., Harini, G., Rupela, O. P., Wani, S. P., & Reddy, G. (2008). Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. *Microbiological Research*, *163*(2), 234–242. https://doi.org/10.1016/j.micres.2006.05.009

Hanyabui, E., Apori, S. O., Frimpong, K. A., Atiah, K., Abindaw, T., Ali, M., Asiamah, J. Y., & Byalebeka, J. (2020). Phosphorus sorption in tropical soils. *AIMS Agriculture and Food*, *5*(4), 599–616. https://doi.org/10.3934/AGRFOOD.2020.4.599

Hasanuzzaman, M., Fujita, M., Oku, H., Nahar, K., & Hawrylak-Nowak, B. (2018). Plant Nutrients and Abiotic Stress Tolerance. In M. Hasanuzzaman, M. Fujita, H. Oku, K. Nahar, & B. Hawrylak-Nowak (Eds.), *Plant Nutrients and Abiotic Stress Tolerance*. Springer Singapore. https://doi.org/10.1007/978-981-10-9044-8

Hedley, M. J., & Stewart, J. W. B. (1982). Method to measure microbial phosphate in soils. *Soil Biology and Biochemistry*, *14*(4), 377–385. https://doi.org/10.1016/0038-0717(82)90009-8

Hemwall, J. B. (1957). The Fixation of Phosphorus by Soils. *Advances in Agronomy*, *9*(C), 95–112. https://doi.org/10.1016/S0065-2113(08)60110-8

Hocking, P. J., Randall, P., Delhaize, E., & Keerthisinghe, G. (2000). The role of organic acids exuded from roots in phosphorus nutrition and aluminum tolerance in acidic soils. *Management and Conservation of Tropical Acid Soils for Sustainable Crop Production*, 61–73.

Horst, W. J., Kamh, M., Jibrin, J. M., & Chude, V. O. (2001). Agronomic measures for increasing P availability to crops. *Plant and Soil*, *237*(2), 211–223. https://doi.org/10.1023/A:1013353610570

Hussain, A., Zahir, Z. A., & Asghar, M. (2015). Prospects of zinc solubilizing bacteria for enhancing growth of maize PROSPECTS OF ZINC SOLUBILIZING BACTERIA FOR ENHANCING GROWTH OF MAIZE. *Pakistan Journal of Agricultural Sciences*, *54*(4), 915–922.

Idrees, F. (2021). Effect of phosphorus levels and phosphate solubilizing bacteria on growth and seed production in coriander. *Pure and Applied Biology*, *10*(3), 617–627. https://doi.org/10.19045/bspab.2021.100063

Ingle, K. P., & Padole, D. A. (2017). Phosphate Solubilizing Microbes: An Overview. *International Journal of Current Microbiology and Applied Sciences*, *6*(1), 844–852. https://doi.org/10.20546/ijcmas.2017.601.099

Jagana, C. S., Ahmad Baba, Z., Krishnanand, S. I., Zargar, M. Y., Badri, Z., & Khan, I. J. (2019). Isolation and Characterization of Zinc Solubilizing Bacteria from Kashmir Himalayas. *International Journal of Current Microbiology and Applied Sciences*, *8*(06), 1248–1258. https://doi.org/10.20546/ijcmas.2019.806.152

Jain, D., Kaur, G., Bhojiya, A. A., Chauhan, S., Khandelwal, S. K., Meena, R. H., Rajpurohit, D., & Mohanty, S. R. (2021). Phenetic characterization of nitrogen fixing azotobacter from rhizospheric soil of Southern Rajasthan. *Journal of Pure and Applied Microbiology*, *15*(1), 428–436. https://doi.org/10.22207/JPAM.15.1.40

Javeria, S., Kumar, A., Kharkwal, A. C., Varma, A., Srinivasa, N., & Sharma, P. (2020). Evaluation of rhizospheric Trichoderma species strains for producing cell wall-degrading and defense related enzymes in response to Fusarium oxysporum f. sp. lentis. *Indian Phytopathology*, *73*(3), 461–467. https://doi.org/10.1007/s42360-020-00262-7

Jimtha, J. C., Smitha, P. V., Anisha, C., Deepthi, T., Meekha, G., Radhakrishnan, E. K., Gayatri, G. P., & Remakanthan, A. (2014). Isolation of endophytic bacteria from embryogenic suspension culture of banana and assessment of their plant growth promoting properties. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *118*(1), 57–66. https://doi.org/10.1007/s11240-014-0461-0

Jinal, N. H., & Amaresan, N. (2020). Evaluation of biocontrol Bacillus species on plant growth promotion and systemic-induced resistant potential against bacterial and fungal wilt-causing pathogens. *Archives of Microbiology*, *202*(7), 1785–1794. https://doi.org/10.1007/s00203-020-01891-2

Jones, D. L., & Oburger, E. (2011). *Solubilization of Phosphorus by Soil Microorganisms* (E. Bünemann, A. Oberson, & E. Frossard (eds.); Vol. 26, pp. 169–198). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-15271-9_7

Joshi, D., Negi, G., Vaid, S., & Sharma, A. (2013). Enhancement of Wheat Growth and Zn Content in Grains by Zinc Solubilizing Bacteria. *International Journal of Agriculture, Environment and Biotechnology*, *6*(3), 363. https://doi.org/10.5958/j.2230-732X.6.3.004

Joshi, S., Gangola, S., Jaggi, V., & Sahgal, M. (2023). Functional characterization and molecular fingerprinting of potential phosphate solubilizing bacterial candidates from Shisham rhizosphere. *Scientific Reports*, 1–12. https://doi.org/10.1038/s41598-023-33217-9

Kaini, B. R. (2020). What's the cause behind fertilizer shortage? What can be done? -

myRepublica - The New York Times Partner, Latest news of Nepal in English, Latest News Articles. *My Republica*. https://myrepublica.nagariknetwork.com/news/whats-the-cause-behind-fertilizer-shortage-what-can-be-done/

Kalayu, G. (2019). Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers. *International Journal of Agronomy*, *2019*, 1–7. https://doi.org/10.1155/2019/4917256

Kamran, S., Shahid, I., Baig, D. N., Rizwan, M., Malik, K. A., & Mehnaz, S. (2017). Contribution of Zinc Solubilizing Bacteria in Growth Promotion and Zinc Content of Wheat. *Frontiers in Microbiology*, *8*(December). https://doi.org/10.3389/fmicb.2017.02593

Karpagam, T., & Nagalakshmi, P. K. (2014). *Original Research Article Isolation and characterization of Phosphate Solubilizing Microbes from Agricultural soil*. *3*(3), 601–614.

Kaur, G., & Reddy, S. M. (2017). Role of phosphate-solubilizing fungi in sustainable agriculture. *Developments in Fungal Biology and Applied Mycology*, 391–412. https://doi.org/10.1007/978-981-10-4768-8_20

Kenneth, M., Aaron, S., Cheo, E. S., Norbert, N. F., & Vivian, B. C. (2017). Phosphorus fixation and its relationship with physicochemical properties of soils on the Eastern flank of Mount Cameroon. *African Journal of Agricultural Research*, *12*(36), 2742–2753. https://doi.org/10.5897/ajar2017.12530

Kim, K. Y., McDonald, G. A., & Jordan, D. (1997). Solubilization of hydroxyapatite by Enterobacter agglomerans and cloned Escherichia coli in culture medium. *Biology and Fertility of Soils*, *24*(4), 347–352. https://doi.org/10.1007/s003740050256

Kshetri, L., Pandey, P., & Sharma, G. D. (2018). Rhizosphere mediated nutrient management in Allium hookeri Thwaites by using phosphate solubilizing rhizobacteria and tricalcium phosphate amended soil. *Journal of Plant Interactions*, *13*(1), 256–269. https://doi.org/10.1080/17429145.2018.1472307

Kumar, M., Kumar, V., & Prasad, R. (2020). *Phyto-Microbiome in Stress Regulation* (M. Kumar, V. Kumar, & R. Prasad (eds.)). Springer Singapore. https://doi.org/10.1007/978-981-15-2576-6

Kumari, V., Bahadur, V., Mishra, S., Topno, S. E., & Wilson, D. (2020). Effect of Phytohormones and Plant Growth Promoting Microorganisms on Germination and Plant Growth of Aonla (Emblica officinalis Gaertn.). *International Journal of Current Microbiology and Applied Sciences*, *9*(11), 76–83. https://doi.org/10.20546/ijcmas.2020.911.008

Lee, K. E., Adhikari, A., Kang, S. M., You, Y. H., Joo, G. J., Kim, J. H., Kim, S. J., & Lee, I. J. (2019). Isolation and characterization of the high silicate and phosphate solubilizing novel strain enterobacter ludwigii GAK2 that promotes growth in rice plants. *Agronomy*, *9*(3). https://doi.org/10.3390/agronomy9030144

Li, Y., Zhang, J., Zhang, J., Xu, W., & Mou, Z. (2019). Characteristics of inorganic phosphate-solubilizing bacteria from the sediments of a eutrophic lake. *International Journal of*

Environmental Research and Public Health, *16*(12). https://doi.org/10.3390/IJERPH16122141

Liu, M., Liu, X., Cheng, B. Sen, Ma, X. L., Lyu, X. T., Zhao, X. F., Ju, Y. L., Min, Z., & Fang, Y. L. (2016). Selection and evaluation of phosphate-solubilizing bacteria from grapevine rhizospheres for use as biofertilizers. *Spanish Journal of Agricultural Research*, *14*(4). https://doi.org/10.5424/sjar/2016144-9714

Liu, Y., & Chen, J. (2014). Phosphorus Cycle. In *Encyclopedia of Ecology* (pp. 181–191). Elsevier. https://doi.org/10.1016/B978-0-12-409548-9.09043-6

Luziatelli, F., Melini, F., Bonini, P., Melini, V., Cirino, V., & Ruzzi, M. (2021). Production of indole auxins by enterobacter sp. Strain p-36 under submerged conditions. *Fermentation*, *7*(3). https://doi.org/10.3390/fermentation7030138

Lynch, J. (1998). The Role of Nutrient-Efficient Crops in Modern Agriculture. *Journal of Crop Production*, *1*(2), 241–264. https://doi.org/10.1300/J144v01n02_10

Mahdi, S. S., Talat, M. A., Dar, M. H., Hamid, A., & Ahmad, L. (2012). Soil phosphorus fixation chemistry and role of phosphate solubilizing bacteria in enhancing its efficiency for sustainable cropping - A review. *Journal of Pure and Applied Microbiology*, *66*(4), 1905–1911.

Maheshwari, D. K., Aeron, A., & Saraf, M. (2013). Bacteria in agrobiology: Crop productivity. *Bacteria in Agrobiology: Crop Productivity*, 1–507. https://doi.org/10.1007/978-3-642-37241-4

Mahmood, R., Afrin, N., Jolly, S. N., & Shilpi, R. Y. (2014). AIsolation and Identification of Cellulose-Degrading Bacteria from Different Types of Samples Riad. *World Journal of Environmental Biosciences*, *9*(2), 8–13.

Maldonado, S., Rodríguez, A., Ávila, B., Morales, P., González, M. P., Araya Angel, J. P. A., Olalde, V., Bravo, J., Jana, C., Sierra, C., & Stoll, A. (2020). Enhanced Crop Productivity and Sustainability by Using Native Phosphate Solubilizing Rhizobacteria in the Agriculture of Arid Zones. *Frontiers in Sustainable Food Systems*, *4*(December), 1–14. https://doi.org/10.3389/fsufs.2020.607355

Manasa, S., Naik, N. M., Ramesh, Y., & Mahadevaswamy. (2019). In vitro screening of the isolates for zinc solubilization and growth promoting attributes. *Journal of Pharmacognosy and Phytochemistry*, *8*(5), 1205–1209.

Mangini, A., & Aeschbach, W. (2017). *Potentials and Limitations of multi-proxy records in speleothem research case studies in comples climate systems*. University of Heidelberg, Germany.

Marra, L. M., Oliveira-Longatti, S. M. de, Soares, C. R. F. S., Lima, J. M. De, Olivares, F. L., & Moreira, F. M. S. (2015). Initial pH of medium affects organic acids production but do not affect phosphate solubilization. *Brazilian Journal of Microbiology*, *46*(2), 367–375. https://doi.org/10.1590/S1517-838246246220131102

Martínez-Cano, B., García-Trejo, J. F., Sánchez-Gutiérrez, A. E., Toledano-Ayala, M., & Soto-

Zarazúa, G. M. (2021). Isolation and Characterization of Plant Growth-Promoting Compost Bacteria That Improved Physiological Characteristics in Tomato and Lettuce Seedlings. *Agriculture*, *12*(1), 3. https://doi.org/10.3390/agriculture12010003

Mauch-Mani, B., Valdes-Lopez, O., Torres, Y. T., Babalola, O. O., Alori, E. T., & Glick, B. R. (2017). *Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture*. https://doi.org/10.3389/fmicb.2017.00971

Mehta, P., Walia, A., Chauhan, A., & Shirkot, C. K. (2013). Plant growth promoting traits of phosphate-solubilizing rhizobacteria isolated from apple trees in trans Himalayan region of Himachal Pradesh. *Archives of Microbiology*, *195*(5), 357–369. https://doi.org/10.1007/s00203-013-0881-y

Mei, C., Chretien, R. L., Amaradasa, B. S., He, Y., Turner, A., & Lowman, S. (2021). Characterization of phosphate solubilizing bacterial endophytes and plant growth promotion in vitro and in greenhouse. *Microorganisms*, *9*(9). https://doi.org/10.3390/microorganisms9091935

Midgley, A. R. (1941). Phosphate Fixation in Soils-A Critical Review. *Soil Science Society of America Journal*, *5*(C), 24–30. https://doi.org/10.2136/sssaj1941.036159950005000c0004x

Mihalache, G., Zamfirache, M. M., Mihasan, M., Ivanov, I., Stefan, M., & Raus, L. (2015). Phosphate-solubilizing bacteria associated with runner bean rhizosphere. *Archives of Biological Sciences*, *67*(3), 793–800. https://doi.org/10.2298/ABS141003038M

Mohamed, A. E., Nessim, M. G., Abou-el-seoud, I. I., Darwish, K. M., & Shamseldin, A. (2019). Isolation and selection of highly effective phosphate solubilizing bacterial strains to promote wheat growth in Egyptian calcareous soils. *Bulletin of the National Research Centre*, *43*(1), 203. https://doi.org/10.1186/s42269-019-0212-9

Mulissa, J. M., Carolin, R. L., Ruth, A. S., & Fassil, A. (2015). Characterization of phosphate solubilizing rhizobacteria isolated from lentil growing areas of Ethiopia. *African Journal of Microbiology Research*, *9*(25), 1637–1648. https://doi.org/10.5897/AJMR2015.7473

Murphy, J., & Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

Mwende Muindi, E. (2019). Understanding Soil Phosphorus. *International Journal of Plant & Soil Science*, *December*, 1–18. https://doi.org/10.9734/ijpss/2019/v31i230208

Nielsen, T. H., Krapp, A., Röper-Schwarz, U., & Stitt, M. (1998). The sugar-mediated regulation of genes encoding the small subunit of Rubisco and the regulatory subunit of ADP glucose pyrophosphorylase is modified by phosphate and nitrogen. *Plant, Cell and Environment*, *21*(5), 443–454. https://doi.org/10.1046/j.1365-3040.1998.00295.x

Nosheen, S., Ajmal, I., & Song, Y. (2021). Microbes as biofertilizers, a potential approach for sustainable crop production. *Sustainability (Switzerland)*, *13*(4), 1–20. https://doi.org/10.3390/SU13041868

Noushahi, H., Hussain, M., Bilal, M., & Salim, M. A. (2019). Improving Phosphorus Use Efficiency by Agronomical and Genetic Means. *World Journal of Agricultural Sciences*, *15*(2), 47–53. https://doi.org/10.5829/idosi.wjas.2019.47.53

Ölinger, R., Margesin, R., & Kandeler, E. (1996). Enzymes Involved in Phosphorus Metabolism. In *Methods in Soil Biology* (pp. 208–227). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-60966-4_13

Panda, P., Chakraborty, S., Ray, D. P., Mahato, B., Pramanik, B., & Choudhury, A. (2015). Solubilization of Tricalcium Phosphate and Production of IAA by Phosphate Solubilizing Bacteria Isolated from Tea Rhizosphere Soil. *Economic Affairs*, *60*(4), 803. https://doi.org/10.5958/0976-4666.2015.00113.8

Panhwar, Q. A., Othman, R., Rahman, Z. A., Meon, S., & Ismail, M. R. (2012). Isolation and characterization of phosphate-solubilizing bacteria from aerobic rice. *African Journal of Biotechnology*, *11*(11), 2711–2719. https://doi.org/10.5897/ajb10.2218

Panta, H. K. (2018). Supply Chain of Subsidized Chemical Fertilizers in Nepal. *Journal of the Institute of Agriculture and Animal Science*, *35*(1), 9–20. https://doi.org/10.3126/jiaas.v35i1.22509

Parfitt, R. L., Atkinson, R. J., & Smart, R. S. C. (1975). The Mechanism of Phosphate Fixation by Iron Oxides. *Soil Science Society of America Journal*, *39*(5), 837–841. https://doi.org/10.2136/sssaj1975.03615995003900050017x

Perumal, M. D., Selvi, D., Chitdeshwari, T., & Balachandar, D. (2019). Enhanced Zinc Nutrient and Enzyme Activity of Rice Crop by Zinc Solubilizing Bacteria with Zn sources in Zn Deficient Rice Soil. *Madras Agricultural Journal*, *106*(Spl). https://doi.org/10.29321/MAJ.2019.000242

Poonguzhali, S., Madhaiyan, M., & Sa, T.-M. (2008). Isolation and Identification of Phosphate. *Journal of Microbiology and Biotechnology*, *18*(4), 773–777. https://koreascience.kr/article/JAKO200818259606273.pdf

Prabhu, N., Borkar, S., & Garg, S. (2019). Phosphate solubilization by microorganisms: Overview, mechanisms, applications and advances. In *Advances in Biological Science Research: A Practical Approach*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-817497-5.00011-2

Pradhan, M., Das, R., Dhali, S., Lakra, P. B., Pradhan, C., & Mohanty, S. (2022). Plant growth promoting activities of P solubilizing bacteria and their impact on disease resistance in groundnut, Arachis hypogaea L. against soil borne fungal pathogens. *Indian Journal of Experimental Biology*, *59*(September), 606–616. https://doi.org/10.56042/ijeb.v59i09.54938

Prasad, A. A., & Babu, S. (2017). Compatibility of Azospirillum brasilense and Pseudomonas fluorescens in growth promotion of groundnut (Arachis hypogea L.). *Anais Da Academia Brasileira de Ciências*, *89*(2), 1027–1040. https://doi.org/10.1590/0001-3765201720160617

Prasain, S. (2021, December 1). Fertiliser crisis could spell economic disaster, experts warn.

Kathmandu Post. https://kathmandupost.com/national/2021/12/03/fertiliser-crisis-could-spell-economic-disaster-experts-warn

Prijambada, I. D., Widada, J., Kabirun, S., & Widianto, D. (2009). Secretion of Organic Acids by Phosphate Solubilizing Bacteria. *Journal of Tropical Soils*, *14*(3), 245. https://doi.org/10.5400/jts.2009.v14i3.245-251

Raghothama, K. G. (2005). Phosphorus and Plants Nutrition: An Overwiev. *Agronomy Monograph*, *46*, 353–378.

Rahim, A. A., Ibrahim, N. A., Ishak, F. N., Mean, L. J., Ayub, N. A. M., & Fazilah, N. N. (2021). Investigation of Newly Isolated Methylobacterium sp. as Potential Biofertilizer. *IOP Conference Series: Earth and Environmental Science*, *765*(1), 012063. https://doi.org/10.1088/1755-1315/765/1/012063

Rahman, C. H., Ahcene, B., Miloud, B., & Rachid, D. (2017). Screening and characterization of plant growth promoting traits of phosphate solubilizing bacteria isolated from wheat rhizosphere of Algerian Saline soil. *Malaysian Journal of Microbiology*, *13*(2), 124–131.

Raj, D. P., Linda, R., & Babyson, R. S. (2014). Molecular characterization of Phosphate Solubilizing Bacteria (PSB) and Plant Growth Promoting Rhizobacteria (PGPR) from pristine soils. *International Journaal of Innovative Sience, Enginering and Technology*, *1*(7), 317–324.

Rawat, P., Das, S., Shankhdhar, D., & Shankhdhar, S. C. (2021). Phosphate-Solubilizing Microorganisms: Mechanism and Their Role in Phosphate Solubilization and Uptake. *Journal of Soil Science and Plant Nutrition*, *21*(1), 49–68. https://doi.org/10.1007/S42729-020-00342-7

Razaq, M., Zhang, P., Shen, H. L., & Salahuddin. (2017). Influence of nitrogen and phosphorous on the growth and root morphology of Acer mono. *PLoS ONE*, *12*(2), 1–13. https://doi.org/10.1371/journal.pone.0171321

Rfaki, A., Zennouhi, O., Aliyat, F. Z., Nassiri, L., & Ibijbijen, J. (2020). Isolation, Selection and Characterization of Root-Associated Rock Phosphate Solubilizing Bacteria in Moroccan Wheat (Triticum aestivum L.). *Geomicrobiology Journal*, *37*(3), 230–241. https://doi.org/10.1080/01490451.2019.1694106

Richardson, A. E., & Simpson, R. J. (2011). Soil microorganisms mediating phosphorus availability. *Plant Physiology*, *156*(3), 989–996. https://doi.org/10.1104/pp.111.175448

Rodríguez, H., & Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, *17*(4–5), 319–339. https://doi.org/10.1016/S0734-9750(99)00014-2

Rose, T. J., Rose, M. T., Pariasca-Tanaka, J., Heuer, S., & Wissuwa, M. (2011). The frustration with utilization: Why have improvements in internal phosphorus utilization efficiency in crops remained so elusive? *Frontiers in Plant Science*, *2*(NOV), 1–5. https://doi.org/10.3389/fpls.2011.00073

Rufino, C. de A., Tavares, L. C., Martín-Ramos, P., Fernandes-Vieira, J., Abreu Júnior, J. de S.,

Silva, F. J. A., Fernandes-Correa, M., & Martín-Gil, J. (2017). Performance of Soybean Seedlings Upon Nutrient Application by Seed Coating. *Brazilian Archives of Biology and Technology*, *60*(December), 1–11. https://doi.org/10.1590/1678-4324-2017160128

Rychter, A. M., Chauveau, M., Bomsel, J., & Lance, C. (1992). The effect of phosphate deficiency on mitochondrial activity and adenylate levels in bean roots. *Physiologia Plantarum*, *84*(1), 80–86. https://doi.org/10.1111/j.1399-3054.1992.tb08768.x

Rychter, A. M., & Mikulska, M. (1990). The relationship between phosphate status and cyanide-resistant respiration in bean roots. *Physiologia Plantarum*, *79*(4), 663–667. https://doi.org/10.1111/j.1399-3054.1990.tb00041.x

Saeed, Q., Xiukang, W., Haider, F. U., Kučerik, J., Mumtaz, M. Z., Holatko, J., Naseem, M., Kintl, A., Ejaz, M., Naveed, M., Brtnicky, M., & Mustafa, A. (2021). Rhizosphere bacteria in plant growth promotion, biocontrol, and bioremediation of contaminated sites: A comprehensive review of effects and mechanisms. *International Journal of Molecular Sciences*, *22*(19). https://doi.org/10.3390/ijms221910529

Saida, A., Ollero, F. J., Megias, M., Laglaoui, A., Bakkali, M., & Arakrak, A. (2015). Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. *International Journal of Current Microbiology and Applied Sciences*, *4*(1), 260–269.

Saitou, N., & Nei, M. (1987). ESCALA CIWA-AR Escala CIWA-Ar(Clinical Institute Withdrawal Assesment for Alcohol) Evaluación del Síndrome de Abstinencia Alcohólica. *Mol. Biol. Evol*, *4*(4), 406–425.

Sakthivel, N., & Gnanamanickam, S. S. (1987). Evaluation of Pseudomonas fluorescens for Suppression of Sheath Rot Disease and for Enhancement of Grain Yields in Rice (Oryza sativa L.) . *Applied and Environmental Microbiology*, *53*(9), 2056–2059. https://doi.org/10.1128/aem.53.9.2056-2059.1987

Samreen, S., & Kausar, S. (2019). Phosphorus Fertilizer: The Original and Commercial Sources. *Phosphorus - Recovery and Recycling*. https://doi.org/10.5772/intechopen.82240

Sanjotha, G., & Manawadi, S. (2016). Isolation, Screening and Characterization of Phosphate Solubilizing Bacteria from Karwar Costal Region. *International Journal of Research Studies in Microbiology and Biotechnology*, *2*(2), 1–6. https://doi.org/10.20431/2454-9428.0202001

Sarwar, M., Arshad, M., Martens, D. A., & Frankenberger, W. T. (1992). Tryptophan-dependent biosynthesis of auxins in soil. *Plant and Soil*, *147*(2), 207–215. https://doi.org/10.1007/BF00029072

Sattiraju, K. S., Kotiyal, S., Arora, A., & Maheshwari, M. (2019). Plant Growth-Promoting Microbes: Contribution to Stress Management in Plant Hosts. In *Environmental Biotechnology: For Sustainable Future*. https://doi.org/10.1007/978-981-10-7284-0_8

Serrano, A., & Mardad, I. (2013). Solubilization of inorganic phosphate and production of organic acids by bacteria isolated from a Moroccan mineral phosphate deposit. *African*

Journal of Biotechnology, *7*(8), 626–635. https://doi.org/10.5897/AJMR12.1431

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, *2*(1), 1–14. https://doi.org/10.1186/2193-1801-2-587

Sharma, S. K. (2012). Characterization of Zinc-Solubilizing Bacillus Isolates and their Potential to Influence Zinc Assimilation in Soybean Seeds. *Journal of Microbiology and Biotechnology*, *22*(3), 352–359. https://doi.org/10.4014/jmb.1106.05063

Sharon, J. A., Hathwaik, L. T., Glenn, G. M., Imam, S. H., & Lee, C. C. (2016). Isolation of efficient phosphate solubilizing bacteria capable of enhancing tomato plant growth. *Journal of Soil Science and Plant Nutrition*, *16*(2), 525–536. https://doi.org/10.4067/S0718-95162016005000043

Shen, J., Yuan, L., Zhang, J., Li, H., Bai, Z., Chen, X., Zhang, W., & Zhang, F. (2011). Phosphorus dynamics: From soil to plant. *Plant Physiology*, *156*(3), 997–1005. https://doi.org/10.1104/pp.111.175232

Sherpa, M. T., Sharma, L., Bag, N., & Das, S. (2021). Isolation, Characterization, and Evaluation of Native Rhizobacterial Consortia Developed From the Rhizosphere of Rice Grown in Organic State Sikkim, India, and Their Effect on Plant Growth. *Frontiers in Microbiology*, *12*(September), 1–15. https://doi.org/10.3389/fmicb.2021.713660

Shrivastava, M., Srivastava, P. C., & D'Souza, S. F. (2018). Phosphate-Solubilizing Microbes: Diversity and Phosphates Solubilization Mechanism. In V. S. Meena (Ed.), *Role of Rhizospheric Microbes in Soil* (Issue June, pp. 137–165). Springer Singapore. https://doi.org/10.1007/978-981-13-0044-8_5

Silva, U. C., Cuadros-Orellana, S., Silva, D. R. C., Freitas-Júnior, L. F., Fernandes, A. C., Leite, L. R., Oliveira, C. A., & Dos Santos, V. L. (2021). Genomic and Phenotypic Insights Into the Potential of Rock Phosphate Solubilizing Bacteria to Promote Millet Growth in vivo. *Frontiers in Microbiology*, *11*(January), 1–17. https://doi.org/10.3389/fmicb.2020.574550

Singh, P., Singh, P., & Kanwar, P. (2013). Effect of Rhizobium, PSB and Phosphorous on yield and Economics of MUNGBEAN. *Annals of Plant and Soil Research*, *15*(2), 164–166.

Singh, R., Singh, P., Singh, V., & Yadav, R. A. (2020). *Effect of phosphorus and PSB on growth parameters , yield , quality and economics of summer greengram (Vigna radiata L .)*. *6*(4), 2798–2803.

Smeck, N. E. (1985). Phosphorus dynamics in soils and landscapes. *Geoderma*, *36*(3–4), 185–199. https://doi.org/10.1016/0016-7061(85)90001-1

Stephen, J., Shabanamol, S., Rishad, K. S., & Jisha, M. S. (2015). Growth enhancement of rice (Oryza sativa) by phosphate solubilizing Gluconacetobacter sp. (MTCC 8368) and Burkholderia sp. (MTCC 8369) under greenhouse conditions. *3 Biotech*, *5*(5), 831–837. https://doi.org/10.1007/s13205-015-0286-5

Suárez-Moreno, Z. R., Devescovi, G., Myers, M., Hallack, L., Mendonça-Previato, L.,

Caballero-Mellado, J., & Venturi, V. (2010). Commonalities and Differences in Regulation of N -Acyl Homoserine Lactone Quorum Sensing in the Beneficial Plant-Associated Burkholderia Species Cluster. *Applied and Environmental Microbiology*, *76*(13), 4302–4317. https://doi.org/10.1128/AEM.03086-09

Suleman, D., Sani, A., Ambardini, S., Boer, D., & Arfa, N. (2019). Isolation and identification of phosphate solubilizing bacteria (PSB) from various plant rhizospheres and its ability to dissolve tricalcium phosphate under in vitro condition. *Bioscience Research*, *16(3)*, 2486–2495.

Suliasih, S., & Widawati, S. (2017). Laboratory and Greenhouse Assays of Phosphate Solubilizing Rhizobacteria to Improve Growth of Falcataria moluccana Seedling. *International Journal of Agricultural Technology*, *13*(5), 699–714.

Sunithakumari, K., Padma Devi, S. N., & Vasandha, S. (2016). Zinc solubilizing bacterial isolates from the agricultural fields of Coimbatore, Tamil Nadu, India. *Current Science*, *110*(2), 196–205. https://doi.org/10.18520/cs/v110/i2/196-205

Suryatmana, P., Setiawati, M. R., Hindersah, R., Satria, A., & Fitriatin, B. N. (2021). THE POTENTIAL OF THE CONSORTIUM (Azotobacter spp. and Phosphate Solubilizing Bacteria) IN INCREASING PLANT N UPTAKE, PLANT NITROGEN CONTENT, Azotobacter spp. POPULATION AND LETTUCE (Lactuca sativa L) CROP YEALD. *International Journal of Agriculture, Environment and Bioresearch*, *06*(01), 77–86. https://doi.org/10.35410/IJAEB.2021.5604

Suzuki, Y., Kihara-Doi, T., Kawazu, T., Miyake, C., & Makino, A. (2010). Differences in Rubisco content and its synthesis in leaves at different positions in Eucalyptus globulus seedlings. *Plant, Cell and Environment*, *33*(8), 1314–1323. https://doi.org/10.1111/j.1365-3040.2010.02149.x

Tagele, S. B., Kim, S. W., Lee, H. G., Kim, H. S., & Lee, Y. S. (2018). Effectiveness of multi-trait Burkholderia contaminans KNU17BI1 in growth promotion and management of banded leaf and sheath blight in maize seedling. *Microbiological Research*, *214*(March), 8–18. https://doi.org/10.1016/j.micres.2018.05.004

Takeshima, H. (2019). Agricultural Transformation in Nepal. In G. Thapa, A. Kumar, & P. K. Joshi (Eds.), *Agricultural Transformation in Nepal*. Springer Singapore. https://doi.org/10.1007/978-981-32-9648-0

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Tariq, M., Yasmin, S., & Hafeez, F. Y. (2010). Biological control of potato black scurf by rhizosphere associated bacteria. *Brazilian Journal of Microbiology*, *41*(2), 439–451. https://doi.org/10.1590/S1517-83822010000200026

Teixeira-Rios, T., De Oliveira, J. R. G., & Yano-Melo, A. M. (2016). Arbuscular mycorrhizal fungi and phosphorus in the initial development of Mimosa tenuiflora (Willd.) Poir. *Revista Brasileira de Botanica*, *39*(4), 997–1004. https://doi.org/10.1007/s40415-016-

0297-4

Thakur, A., & Parikh, S. C. (2015). Auxin Hormon Production and Plant Growth Promotion by Phosphate Solubilizing Bacteria of Groundnut Rhizosphere. *International Journal of Innovative Researchin Science, Engineering and Technology*, *4*(9), 8539–8548. https://doi.org/10.15680/IJIRSET.2015.0409078

Tiessen, H. (2008). Phosphorus in the global environment. In P.J White & J. . Hammond (Eds.), *The Ecophysiology of Plant-Phosphorous Interactions* (pp. 1–7). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/978-1-4020-8435-5_1

U.S. Geological Survey. (2022). Mineral Commodity Summaries 2022. In *Angewandte Chemie International Edition, 6(11), 951–952.* (Issue 703). https://pubs.usgs.gov/periodicals/mcs2022/mcs2022.pdf

Valetti, L., Iriarte, L., & Fabra, A. (2018). Growth promotion of rapeseed (Brassica napus) associated with the inoculation of phosphate solubilizing bacteria. *Applied Soil Ecology*, *132*(January), 1–10. https://doi.org/10.1016/j.apsoil.2018.08.017

Viruel, E., Erazzú, L. E., Calsina, L. M., Ferrero, M. A., Lucca, M. E., & Siñeriz, F. (2014). Inoculation of maize with phosphate solubilizing bacteria : effect on plant growth and yield. *Journal of Soil Science and Plant Nutrition*, *14*(4), 819–831.

Vyas, P., & Gulati, A. (2009). Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. *BMC Microbiology*, *9*(1), 174. https://doi.org/10.1186/1471-2180-9-174

Wagi, S., & Ahmed, A. (2019). Bacillus spp.: Potent microfactories of bacterial IAA. *PeerJ*, *2019*(7). https://doi.org/10.7717/peerj.7258

Walpola, B. C., & Yoon, M.-H. (2013). Isolation and characterization of phosphate solubilizing bacteria and their co-inoculation efficiency on tomato plant growth and phosphorous uptake. *African Journal of Microbiology Research*, *7*(3), 266–275. https://doi.org/10.5897/AJMR12.2282

Wang, Y. Y., Li, P. S., Zhang, B. X., Wang, Y. P., Meng, J., Gao, Y. F., He, X. M., & Hu, X. M. (2020). Identification of phosphate-solubilizing microorganisms and determination of their phosphate-solubilizing activity and growth-promoting capability. *BioResources*, *15*(2), 2560–2578. https://doi.org/10.15376/biores.15.2.2560-2578

White, Philip J., & Hammond, J. P. (2008). *Phosphorus nutrition of terrestrial plants* (pp. 51–81). https://doi.org/10.1007/978-1-4020-8435-5_4

William, S., Feil, H., & Copeland, A. (2012). Bacterial DNA Isolation CTAB Protocol Bacterial genomic DNA isolation using CTAB Materials & Reagents. In *Sigma*. https://jgi.doe.gov/wp-content/uploads/2014/02/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf

Yadav, A. N. (2021). *Soil Microbiomes for Sustainable Agriculture* (A. N. Yadav (ed.); Vol. 27). Springer International Publishing. https://doi.org/10.1007/978-3-030-73507-4

Yadav, B. D., Yadav, R. K., Nogiya, M., Yadav, M., Mahala, D., Meena, S., Yadav, B., Verma, A., Yadav, S., Devi, S., Kumawat, R., & Bijarniya, A. (2021). Phosphorus management strategies to enhance P-use efficiency and sustainable crop production. *International Journal of Chemical Studies*, *9*(1), 3562–3567. https://doi.org/10.22271/chemi.2021.v9.i1ax.11786

Yadav, H., Gothwal, R. K., Nigam, V. K., Sinha-Roy, S., & Ghosh, P. (2013). Optimization of culture conditions for phosphate solubilization by a thermo-tolerant phosphate-solubilizing bacterium Brevibacillus sp. BISR-HY65 isolated from phosphate mines. *Biocatalysis and Agricultural Biotechnology*, *2*(3), 217–225. https://doi.org/10.1016/j.bcab.2013.04.005

Zakry, F. A. A., Halimi, M. S., Rahim, K. A., Osumanu, H. A., Wong, S., Franklin, R. K., Stephen, L. T., & Make, J. (2010). Isolation and plant growth-promoting properties of rhizobacterial diazotrophs from pepper vine (Piper nigrum L.). *Malaysian Applied Biology*.

Zhu, J., Li, M., & Whelan, M. (2018). Phosphorus activators contribute to legacy phosphorus availability in agricultural soils: A review. *Science of the Total Environment*, *612*, 522–537. https://doi.org/10.1016/j.scitotenv.2017.08.095

Zhu, Y. G., & Smith, S. E. (2001). Seed phosphorus (P) content affects growth, and P uptake of wheat plants and their association with arbuscular mycorrhizal (AM) fungi. *Plant and Soil*, *231*(1), 105–112. https://doi.org/10.1023/A:1010320903592

Ziadi, N., Whalen, J. K., Messiga, A. J., & Morel, C. (2013). Assessment and modeling of soil available phosphorus in sustainable cropping systems. In *Advances in Agronomy* (1st ed., Vol. 122). Elsevier Inc. https://doi.org/10.1016/B978-0-12-417187-9.00002-4

Appendix I: List of Equipment and materials

1. Reagents and Chemicals

Ammonium molybdate
Ammonium sulfate
Ascorbic acid
Bone meal
Chloroform
CTAB
Distilled water
DNA ladder (100kb)
Ethanol
Ethidium bromide (EtBr)
Ferric chloride
Glycerol
Glycine
Gram staining reagents (Crystal violet, iodine, ethanol, safranin)
Hydrogen peroxide
Indole acetic acid
Isoamyl alcohol
Isopropanol
Loading dye
NaCl
Nessler's reagent
Nuclease free water (NFW)
Orthophosphoric acid
Oxidase test disc
Percholoric acid
Phenol
Picric acid
Potassium antimonyl tartarate
Potassium dihydrogen phosphate
Potassium hydroxide
Potassium iodide
Proteinase K
SDS
Sodium carbonate
Sulphuric acid
TE buffer

Tricalcium phosphate
Tryptophan
Urea
Zinc oxide
Methyl red indicator

2. Media

A. Cellulose CongoRed agar media	**g/L**
KH_2PO_4	0.5 g
$MgSO_4$	0.25 g
Cellulose	2 g
Agar	15 g
Congo-Red	0.2 g
Gelatin	2 g
Final pH	6.8–7.2

B. Jensen media g/L	
Sucrose	20 g
Calcium Carbonate	2 g
Magnesium sulphate	0.5 g
Dipotassium phosphate	1 g
Sodium chloride	0.5 g
Ferrous sulphate	0.1 g
Sodium Molybdate	0.005 g
Agar	15 g

C. Luria bertani (LB)	**g/L**
Peptone	10 g
Yeast extracts	5 g
Sodium chloride	5 g

D. Nutrient broth (NB)	**g/L**
Peptones	10 g
Beef extract	1 g
Yeast extract	2 g
Sodium chloride	5 g
Final pH	6.8-0.2

E. Pectinase production medium	**g/L**
Pectin	10 g
Di-ammonium orthophosphate	3 g
K_2HPO_4	2.0 g
$MgSO_4$	0.1 g
Agar	20 g
Final pH	5.5

F. Pikovskaya medium (PVK)	**g/L**
Yeast Extract	0.5 g

Dextrose	10.0 g
Calcium Phosphate	5.0 g
Ammonium Sulphate	0.5 g
Potassium Chloride	0.2 g
Magnesium Sulphate	0.1 g
Manganese Sulphate	0.0001 g
Ferrous Sulphate	0.0001 g
Agar	15.0 g

G. Potato dextrose agar (PDA) g/L

Potato infusion	200 g
Dextrose	20 g
Agar	20 g

H. Starch agar medium g/L

Beef extract	3 g
Soluble starch	10 g
Agar	12 g

I. Tryptic soy agar (TSA) g/L

Pancreatic digest of casein	15 g
Peptic digest of soybean meal	5 g
Sodium chloride	5 g
Agar	15 g
Final pH	7.3±0.2

J. Modified PVK for zinc solubilization g/L

Glucose	10 g
Ammonium Sulphate	1 g
Potassium chloride	0.2 g
Dipotassium hydrogen phosphate	0.2 g
Magnesium sulphate	0.1 g
Yeast extract	0.2 g
Manganese sulphate	0.1 g
Iron sulphate	0.0001 g
Agar	15 g
Insoluble ZnO	1 g

3. Equipments

Autoclave
Centrifuge
Electrophoresis apparatus
Gel documentation system
High performance liquid chromatography (HPLC)
Hot air oven
Incubator

Laminar airflow cabinet
Microscope
Nanodrop
pH meter
Refrigerator
UV spectrophotometer
Water bath

Appendix II: Standard Calibration Curves

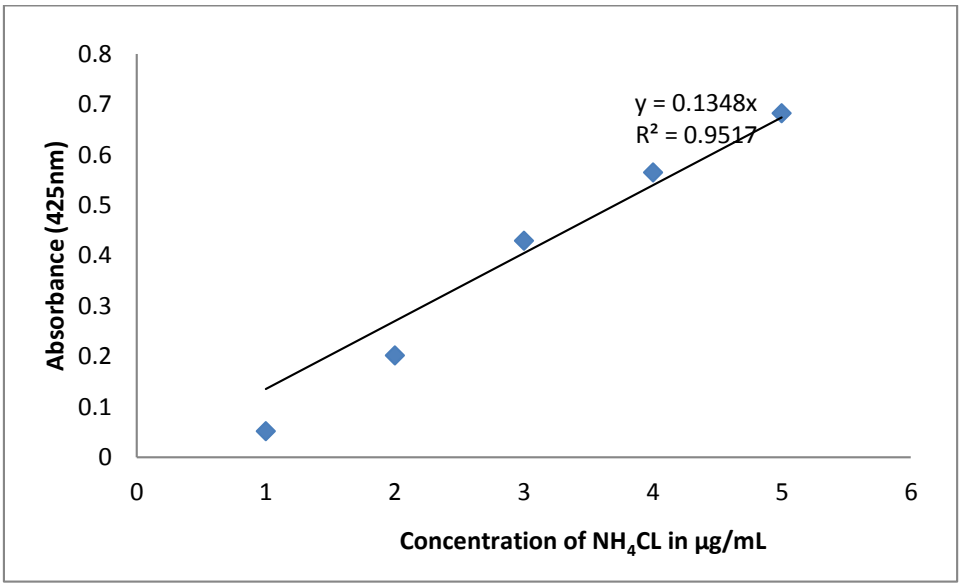


Fig 1: Standard curve prepared for Ammonia production assay

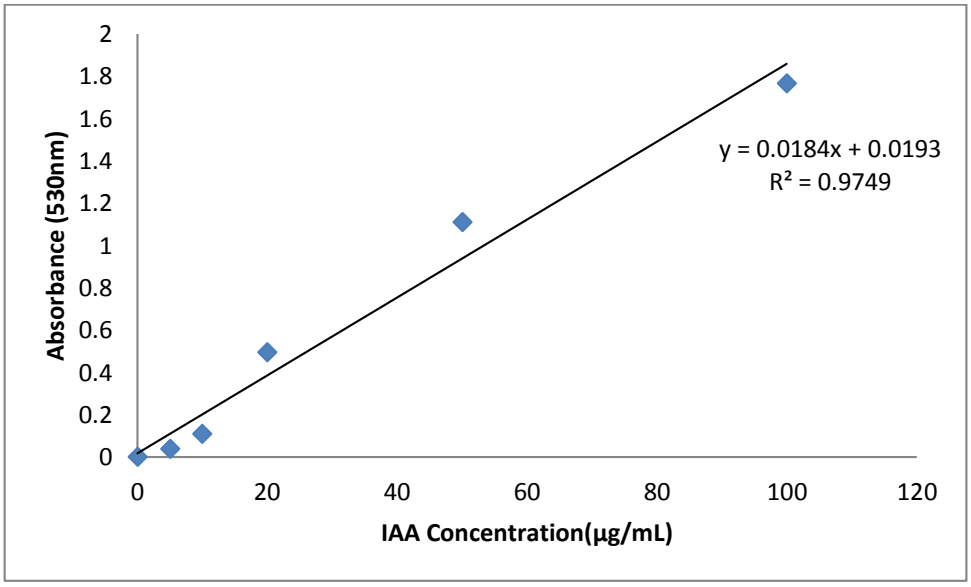


Fig 2: Standard curve for IAA production assay

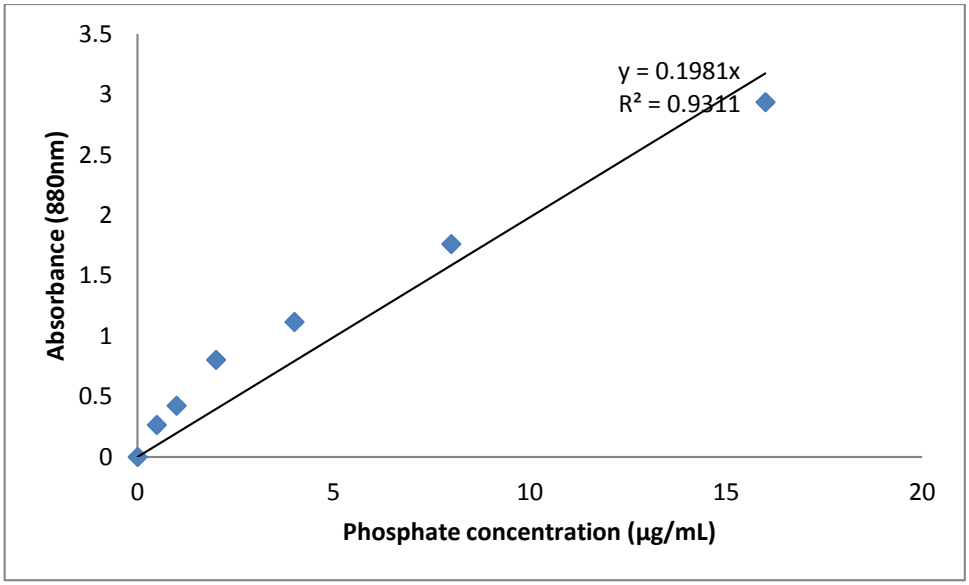


Fig 3: Standard curve for soluble P estimation assay

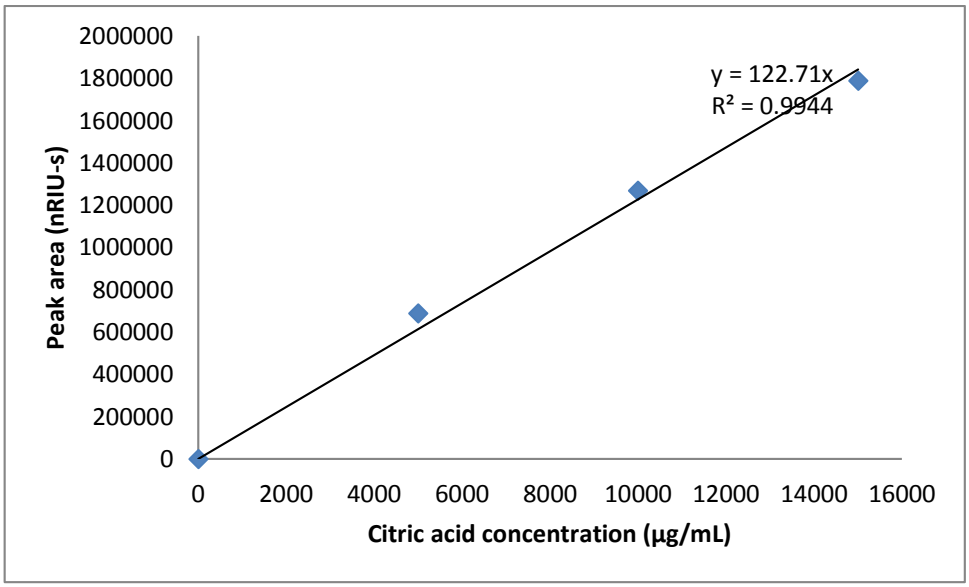


Fig 4: Standard curve for Citric acid production assay

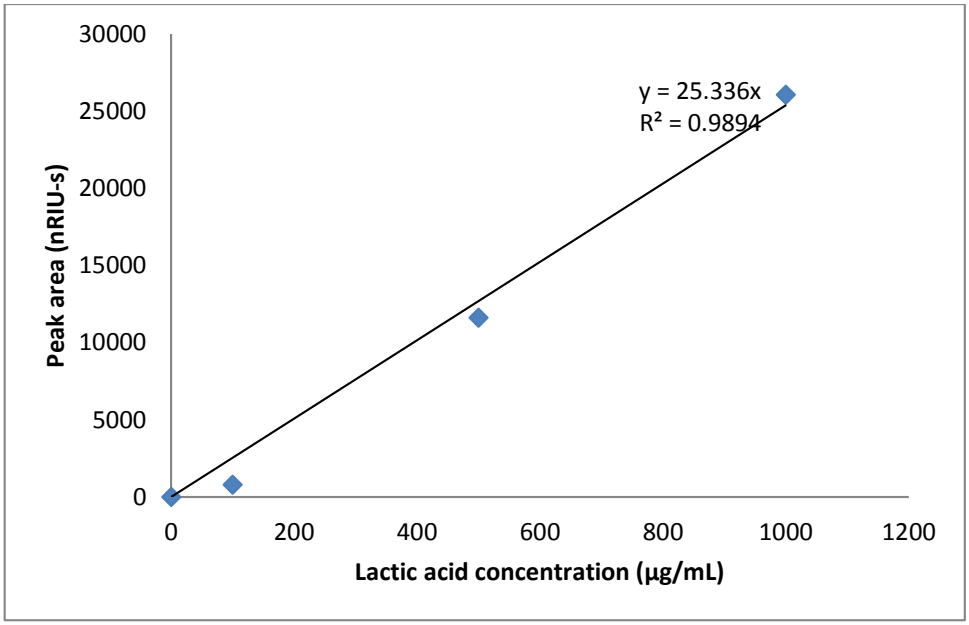


Fig 5: Standard curve for Lactic acid production assay

Appendix III: Reagent Preparation

A. Reagents for phosphate estimation (Murphy & Riley, 1962)

i. Sulphuric acid (5N): 70 mL of conc. sulphuric acid was diluted to 500 mL with distilled water.

ii. Ammonium molybdate: Ammonium molybdate (20g) was dissolved in distilled water to a final volume of 500 mL.

iii. Ascorbic acid (0.1M): In 75 mL of distilled water, 1.32g of ascorbic acid was dissolved. (Should be prepared freshly)

iv. Potassium antimonyl tartarate (1 mg Sb/mL): 0.2743 g of potassium antimonyl tartarate was dissolved in distilled water to a final volume of 100ml.

v. Mixed reagent: 125 mL of 5N sulphuric acid and 37.5 mL of ammonium molybdate was mixed throughly. To this 75 mL of ascorbic acid solution and 12.5 mL of potassium antimonyl tartarate solution was added.

For phosphate estimation, 8 mL of mixed reagent was added to 40 mL of sample and it was diluted to 50 mL with distilled water. After 10 min, optical density of solution was measured at 880 nM wavelength.

B. Nessler's reagent ($K_2[HgI_4]$)

It is made by mixing 2 g of potassium iodide with 5 mL of water. This solution is mixed with 3 g of mercury (II) iodide, and the resulting mixture is diluted to 20 mL. To produce the alkaline base, 40 g of potassium hydroxide (30%) is added.

Appendix IV: 16S rRNA sequence result of isolate L2

>bar14_16s_LZ_2_clus265_reads642

ACAAATCATGTACTTCGTTACTTCGTTCAGTTACGTATTGCTAAGGTTAAAACGAGTCTCTTGGGACCCAT
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CGTGGGGAGCAAACGGGATTAGATACCCTGGTAGTCAAGGCTGTCTCTTATACACATCTCCGAGCCCAC
GAGACGGGTGCTGTCTATGGGTCCCAAGAGACTCGTTTTAACCTTAGCAATAGCCAAGAGACTCGTTTT
AACCTTAGCAATAC

Appendix V: Photograph of lettuce plant during pot experiment

Figure 1: Effect of PSB on growth and development of Lettuce. A- uninoculated soil with no TCP, B-uninoculated soil with TCP, C-SM1 with TCP, D-KP2 with TCP, E-KP3 with TCP, F-L2 with TCP, G-P1 with TCP, H-Consortium with TCP and I-NPK

ISOLATION AND SCREENING OF MULTITRAIT PHOSPHATE SOLUBILIZING BACTERIA AND THEIR GROWTH-PROMOTING EFFECT ON LETTUCE (*Latuca sativa*) VAR. GREEN WAVE

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