Study of Microbial Phosphate Solubilizers and their Potential Usage in Sustainable Agricultural Practices

A Dissertation Report submitted

for the partial fulfillment of the Degree of Master of Science

By

Nandaniya Jensi

Enrolment No.- 210622056

[M.Sc. Microbiology, IV Semester]

Under the supervision of

Dr. Chitra Bhattacharya

Assistant professor

Department of Microbiology

DEPARTMENT OF MICROBIOLOGY

ATMIYA UNIVERSITY

'YOGIDHAM GURUKUL' KALAWAD ROAD

RAJKOT (GUJARAT) – 360005

2022-2023

ATMIYA UNIVERSITY
(Established under the Gujarat Private University Act 11, 2018)
Yogidham Gurukul, Kalawad Road, Rajkot - 360005, Gujarat (INDIA)

AU/MB/O/230406/03

C E R T I F I C A T E

This is to certify that this dissertation work entitled "**Study of Microbial Phosphate Solubilizers and their Potential Usage in Sustainable Agricultural Practices**" was successfully carried out by **Nandaniya Jensi** towards the partial fulfillment of requirements for the degree of Master of Science in Microbiology of Atmiya University Rajkot. It is an authentic record of his own work, carried out by him under the guidance of **Dr. Chitra Bhattacharya** during the academic year of 2022-23 The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

Dr. Rohan Pandya
Head,
Department of Microbiology
Atmiya University, Rajkot,
Gujarat

Dr. Chitra Bhattacharya (Supervisor)
Assistant Professor
Department of Microbiology
Atmiya University, Rajkot, Gujarat

D E C L A R A T I O N

I hereby declare that the work incorporated in the present dissertation report entitled "**Study of Microbial Phosphate Solubilizers and their Potential Usage in Sustainable Agricultural Practices**'' is my own work and is original.

This work (in part or in full) has not been submitted to any University for the award of an any Degree or a Diploma.

Date: 8th April 2023
Nandaniya Jensi

Enrolment No.- 210622056

Chapter 6:- ACKNOWLEDGEMENT

Firstly, with deep regards and profound respect, I avail this opportunity to express my deep sense of gratitude and indebtedness to **Dr. Rohan Pandya**, the department of Microbiology for his inspiring gaudiness, constructive criticism, and valuable suggestions throughout the dissertation work. It would have not been possible for me to bring out this dissertation without his permission.

First and foremost, I am extremely grateful to my guide **Dr. Chitra Bhattacharya**, for her invaluable advice, continuous support, encouragement, thoughtful discussion, and untiring supervision throughout the dissertation.

I am very much thankful to **Ms. Dhruvi Vekariya** and **Ms. Janvi Hirani** for providing us with all the requirements needed for the work, gaudiness, and their support for my dissertation work.

I express my thanks to my dear friends **Amisha Parmar**, **Saloni Makadiya** and **Adroja Bansi** for their constant encouragement and ample support at all stages of the dissertation, who constantly inspired me to study and helped me in modeling my life.

I am thankful to the non-teaching staff of the Department of Microbiology, **Maheshbhai** for his help and care during my dissertation.

My appreciation also goes out to my family and friends for their encouragement and support throughout my dissertation work.

It's a great fortune that in a vast University, we worked on the omnipotent creator of Almighty **God**.

INDEX

List of Tables

List of Figures

Appendix

Short form	Full form
PVK agar	Pikovskaya's Agar
PDA	Potato Dextrose Agar
SCA	Simmon Citrate Agar
TSI	Triple iron sugar
MR	Methyl red
VP	Vogus-Prosker
GPB	Glucose Phosphate Broth
SE	Solubilizing Efficiency
cm	Centimeter
µg	Microgram
ml	Milliliter
pH	Potential of Hydrogen ion
OD	Optical Density
nm	Nanometer
SD	Standard Deviation
ºC	Celsius
µmol	Micromoles
min	Minute
U	Unit
mg	Milligram
PSMs	Phosphate solubilizing microorganisms
PS1	*Pseudomonas* sp.
PS2	*Penicillium* sp.
PS3	*Aspergillus* sp.

ABSTRACT:-

Phosphorus (P) is one of the essential macro elements which plays a vital role in the proper functioning of plants. So deficiency of P can diminish plant growth and development. Soil possesses total P in the form of organic and inorganic compounds, most of them remain inactive and thus unavailable to plants. Usage of synthetic Phosphate fertilizers show adverse effect and it is also expensive so many farmers cannot afford them. So there is a need to develop alternative techniques to provide P. Soil has the presence of several Phosphate solubilizing microbes (PSMs) which are beneficial microorganisms and have the capability of hydrolyzing organic and inorganic insoluble phosphorus compounds to soluble P form that can easily be assimilated by plants. . Under the present investigation identification, characterization, and optimization of the phosphate solubilizing activity of these microbes at different pH, temperature and salt concentrations was carried out. Isolation of PSMs have been carried out from the agricultural soil sample of Kankot, Rajkot. Collected soil sample undergone serial dilution followed by inoculation on Pikovskaya's Agar (PVK) medium. Out of 7 isolates only 3 isolates showed significant zone of hydrolysis on Pikovskaya's Agar (PVK) medium. Based on morphological and cultural characteristics, 3 isolates were tentatively identified *A.niger, Penicillium sp.* and *Bacillus sp.* Further extraction of phosphatase enzyme has been carried out and it's activity had been determined qualitatively by zone of clearance which is measured as 5 cm for *Penicillium* sp. and *Aspergillus* sp. and quantitatively by Spectro vanadomolybdate phosphoric yellow color method which was observed and measured as the highest value 83 μg/ml for *Pseudomonas* sp.. Partial purification of crude alkaline phosphatase enzyme has been carried out and followed by it enzyme assay was done by using Alkaline phosphatase assay. As result we found maximum enzyme activity is 3.410 of *Pseudomonas* sp., 1.829 of *Aspergillus* sp.), and 1.783 of *Penicillium* sp.. Enzyme optimization has been carried out at different pH, temperature, salt concentration ,and incubation periods. As result we found that alkaline phosphatase enzyme shows the maximum result at *Pseudomonas* sp. 9 pH, *Penicillium* sp. 10 pH and *Aspergillus* sp. 12 pH maximum effects for temperature was carried out for *Pseudomonas* sp as 5.147, *Penicillium* sp. as 4.341, and *Aspergillus* sp. as 5.519 ; the maximum effect of salt concentration for *Pseudomonas sp.* was calculated as 7.81,

Penicillium sp as 7.03, and *Aspergillus* sp. as 7.8. The maximum effects of incubation period was observerd as *Pseudomonas sp.*4.05, *Penicillium* sp. 4.12, and *Aspergillus* sp.4.36. Further these optimized enzyme was used in the *in-vitro* pot assay experiment to check it's efficiency in plant growth promotion. Purpose of this review is to understand the role of PSMs in crop production as biofertilizer.

Key Words: Phosphate Solubalizing Organisms, Phosphatase activity, *Pseudomonas* sp., *Penicillum* sp., *Aspergillus* sp., Bifertilizer

Chapter 1: INTRODUCTION

Phosphorus is one of the most important minerals for all forms of global life. The total phosphorus content present on the surface of the soil is low, averaging only 0.6% phosphorus, which is compared to the soil content of 0.14% nitrogen and 0.83% potassium. The phosphorus content is quite variable in soil, ranging from less than 0.04% P_2O_5 present in sandy soils. Soil phosphorus is classified into two comprehensive groups, organic and inorganic phosphorus. Organic phosphorus is mostly found in plant residues, microbial tissues, manures, and soil microorganisms. Soils contain approximately 30-65% of total phosphorus in organic forms, which are not available for plants, while the remaining 35-70% is in inorganic forms (Dadarwalet et al., 1997).

Soluble phosphorus formed by natural weathering or fertilizer reacts with clay, aluminum, and iron compounds in the soil. These forms of compounds are transformed into less available forms by the process of phosphorus fixation. These phosphorus fixation processes through phosphorus transfer very little in most soil and stay close to their original place. About more than 20% of phosphorus is used during the first season of cropping. So as a result, minute soil phosphorus is lost by leaching.

Phosphorus is one of the second most important micronutrients required for the maximum yield of crops productions (Schefferet al., 1998). Soil contains a very trace amount of phosphorus, so people used chemical phosphorus fertilizers for crop production (Richardson, 1994). Though, a large amount of soluble inorganic phosphorus applied to the soil as a chemical fertilizer is quickly immobilized soon application and becomes unattainable to plants (Dey, 1988; Yadav and Dadarwal, 1997). Farmers were applying chemical phosphorus fertilizers in numerous-time to overcome these problems. continuous application of chemical fertilizers may not be sustainable for soils which may decline soil fertility and soil pH, and this may lead to a decrease in the yield (Nyalemegbe et al., 2009). Recovery of phosphorus is very low (8- 20%) due to the high cost of mineral superphosphate fertilizers (Anonymous et al., 1997).

Next to nitrogen, phosphorus (P) is a non-renewable macronutrient, the greatest important nutrient required for plant growth (Li X et al., 2015). Phosphorus plays important role in biological growth and development such as photosynthesis, energy storage, respiration, and transfer in living plant cells (Solangi, M. K., Solangi, S. K., Solangi, N. K., 2016) but then again limited organic phosphorus is available for plant (Vessey K.J. 2003). Plants need 0.01- 3.0 mg per litter of phosphorus from the soil solution and remain as the main source of phosphorus for plants (Khan AA et al., 2009). This less amount of phosphorus taken by a plant which causes the deficiency in the plant resulting in leaves turning brown at the growing stage, weak stems, and slow development.

In the earliest time, use of the animal manure to provide phosphorus content for plant growth which is common agricultural practice. At that moment role of microflora on soil fertility was hardly understood (Kannaiyan S. et al., 2004). Accumulation of phosphate from organic compounds by plants and microorganisms takes place through the enzyme "phosphatase" which is present in a broad variety of soil microorganisms. A huge number

of soil microorganisms can dissolve the inorganic insoluble phosphate in soil and make them available for plants (Sonam Sharma et al., 2011).

Insoluble phosphate compound has been solubilized by phosphatase enzyme and organic acid produced by plants and microbes for example, phosphate-solubilizing microorganisms have been exposed to enhance the solubilization of insoluble P compounds from the release of phosphatase enzyme and organic acids (Sharma et al., 2005). Plants gain phosphorus from soil solution as a phosphate anion. Phosphorus is the minimum mobile element in plants and soil differing from other macronutrients. In plants, Phosphorous increases the strength of promotes, flower formation cereal straw, and fruit production, stimulates root development, and is also important for seed formation. Acceptable P fertilization may expand the quality of fruits, vegetables, and grain crops and rise their resistance to diseases and adverse conditions. It is vital for the stimulation of early root growth, the development of meristematic tissue, and in tending plant maturity. Because of the negative charge of phosphate ions, they are rapidly absorbed after weathering of clays or detritus particles, forming insoluble forms of aluminum, iron, or calcium phosphates, all unavailable to plants. Fungi and bacteria have the ability to solubilize these compounds (Bisen et al., 1996).

Phosphate-solubilizing microorganisms (PSMs) have been considered as one of the conceivable alternatives for inorganic phosphate fertilizers for promoting plant growth and yield (de-Freitas et al., 1997; Rodrı´guez and Fraga, 1999; Richardson, 2001; Vessey, 2003; Thakuria et al., 2004). Phosphate-solubilizing microorganisms as probable suppliers of soluble phosphorus confer and a great advantage for plants through solubilization and mineralization (Hong, Geun, Mi, & Moon, 2006). From solubilizing process mineral phosphorus solubilized by phosphate solubilizing microorganisms is generally associated with the release of low molecular weight of organic acids (Goldstein et al., 1995). Their hydroxyl and carboxyl groups can form complexes with the aluminum and iron of similar phosphate compounds in soil, so liberating bioavailable phosphate into the soil which can be used by plants (Gyaneshwar, Kumar, Pareka, & Podle, 2002). Solubilization of phosphate-rich compounds is also passed out by the action of a phosphatase enzyme known as alkaline phosphatase (AlPase). In all bacteria, this enzyme catalyzes the hydrolysis of a wide variety of catalyzes transphosphorylation and phosphomonoesters reactions by transferring the phosphoryl group to alcohol in the presence of certain phosphate acceptors (Coleman, 1992).

AIM:

Study of microbial phosphate solubilizers and their potential usage in sustainable agricultural practices.

OBJECTIVE:

- Isolation and identification of phosphate solubilizing Bacterial and Fungal Strains
- In-vitro optimization of growth of phosphate solubilizing microorganisms at different pH, temperature and salt concentration in growth medium
- Qualitative and Quantitative phosphatase assay
- To check the phosphatase enzyme activity
- Partial purification of phosphatase enzyme
- Enzyme optimization at different pH, temperature, substrate concentration and incubation period
- Application

Chapter 2: REVIEW OF LITERATURE

Plants require an appropriate proportion of Macro and Micro elements for their optimum growth. Among these macro elements are two types:

1. Primary macro elements
2. Secondary macro element

Primary macro elements include Nitrogen(N), Phosphorus(P), and potassium(K). Whereas secondary macronutrients include calcium(Ca), magnesium(Mg), and sulfur(S). Phosphorus is one of the essential elements for the growth and development of the plant. Approximately 0.2% of a plant's dry weight is composed of phosphorus (Azziz et al., 2012; Tak et al., 2012). In soil 0.05%(w/w) phosphorus is present and only 0.1% of this content is available for plant use (Zhu et al., 2011). With the increasing demand for agricultural production and as the peak in global production will occur in the next decades, phosphorus is receiving more attention as a non-renewable resource (Shen et al., 2011). Due to lower levels of phosphorus content agricultural production highly be influenced by the application of phosphorus fertilizers derived from phosphate rocks (David L. Jones et al., 2011). After the application of chemical fertilizer in the soil as inorganic phosphate form, it will quickly immobilize and becomes inaccessible to plant. Hence overall phosphate use efficiency becomes low (Rodriguez & Fraga et al., 1999). However, in recent year chemical fertilizer consumption increased exponentially throughout the world, causing serious environmental problems. Fertilization may affect the accumulation of heavy metals in soil and plant systems. Plants absorb the fertilizer through the soil, they can enter the food chain. Thus, fertilization leads to water, soil, and air pollution (Serpil Savci et al., 2012). The chemical fertilizer used per hectare in India is 1214 kg/ha (Serpil Savci et al., 2012).

The access amount of phosphorus in water leads to eutrophication which results in the reduction of living species in the aquatic environment, fish-killing, odor problem, proliferation of unwanted species and water will be non-potable. Chemical fertilizer leads to an elemental imbalance in soil due to soil degradation and decreases the fertility of the

soil. In addition, toxic substances accumulate within the vegetables and cause negative effects on humans and animals that are fed.

2.1 Effect of chemical fertilizers

When chemical fertilizers too much applied it emits some gases into the atmosphere such as carbon dioxide, methane, and hydrogen sulfide with chloro-fluoro hydrocarbons and halon gases also associate with these compounds leading to the greenhouse effect (Serpil Savci et al., 2012). In nature, insoluble inorganic phosphate is present in the soil, and several microorganisms have the capability to solubilize phosphate. Thus, the plant can directly absorb these soluble forms of phosphate. Plants procure phosphorus from soil solution as phosphate anion.

2.2 Phosphate solubilizing microorganisms

Phosphate Solubilizing microorganisms (PSM) help in the solubilization of insoluble phosphate compounds through the liberation of phosphatase enzymes and organic acids (Sonam Sharma et al., 2011). Phosphatase is the hydrolytic enzyme that cleaves the ester bond between the phosphate group and the organic residue of the organic phosphates (M. L. Dotaniya et al., 2019).

Enzymes are very essential for life, without enzymes life can not exist. Enzymes are produced by living cells and give new life to them. Unlimited variabilities of enzymes are existing from microorganisms found in diverse and extreme conditions (P. Nalini et al., 2015). Phosphatase has been classified as alkaline phosphatase and acid phosphatase as per the optimum pH required for their catalytic activity (Barret-Lanned et al., 1982; Shahbazkia et al., 2009).

2.3 phosphate solubilizing Bacteria

Microorganisms perform a vital role in the natural phosphorus cycle and among them are strained from genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Enterobacter, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium* (Fankem et al., 2006 and Zaheer et al., 2016). *Rhizobia, including R. leguminosarum, R. meliloti, M. mediterraneum, B. japonicum,* and *Bradyrhizobium* sp.

are probable P solubilizers (Afzal A et al., 2008). Among these microorganisms *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata*, and *Enterobacter* are actually effective for growing the plant available P in the soil, and the growth (Zaheer et al., 2016). A current report by Pindi et al.(Roy M et al.,2013) , was also indicated that *Pseudomonas, Bacillus, Micrococcus,* and *Flavobacterium* are lively in the process of solubilization.

2.3.1 Mechanism of Phosphate Solubilization

Phosphate-solubilizing bacteria solubilize inorganic soil phosphates, such as $FePO_4$, $Ca_3(PO_4)_2$, and $AlPO_4$, by the production of siderophores, organic acids, and hydroxyl (Sharma P et al., 2013). The major mechanism for phosphate solubilization happens in two ways. Proclamation of inorganic phosphate from the insoluble chemical form is typically improved by the action of acids produced by bacteria to produce organic compounds such as citrate, oxalate, gluconate, and succinate, and accordingly to increase free phosphate in the environment or medium (Illmer et al.,1995). Determine the quantity of acid in liquid culture, filtrates can be determined by thin-layer chromatography or high-performance liquid chromatography or paper chromatography (Gyaneshwar P et al., 1998).

2.4 Phosphate solubilizing Fungi

Strains of fungi that are part of the phosphate solubilization consist of *Achrothcium, Alternaria, Myrothecium, Oidiodendron, Paecilomyces, Penicillium, Phoma, Pichia fermentans, Populospora, Pythium, Rhizoctonia, Rhizopus, Saccharomyces, Arthrobotrys, Aspergillus, Cephalosporium, Cladosporium, Curvularia, Cunninghamella, Chaetomium, Fusarium, Glomus, Helminthosporium, Micromonospora, Mortierella, Schizosaccharomyces, Schwanniomyces, Sclerotium, Torula, Trichoderma,* and *Yarrowia* (Sharma P et al.,2013; Alori et al.,2017 and Khan et al., 2010) described that fungi are extra effective and have a better perspective to solubilize insoluble phosphate in the release of P from insoluble inorganic compounds than the bacteria. Additionally, their capability to withstand abiotic and biotic stress under soil conditions makes them to the probable applicant for rising bio-inoculant. *Penicillium* and *Aspergillus spp*. are the

presiding P-solubilizing filamentous fungi created in the rhizosphere (Chuang et al.,2007).

2.4.1 . Mechanisms of Phosphorus Solubilization

The phosphate solubilization mechanism in fungi is attended to and facilitated through acid production such as citric, gluconic, fumaric, malic, oxalic, lactic, 2-keto gluconic, malonic acids, succinic, propionic, and acetic acid by fungi. Which results in a diminution in the pH of the medium. Malic acid is said to be additional effectual than succinic acid and this may be due to the wide number of hydroxyl groups in malic acid related to succinic acid (Gaind et al., 2016). Phosphate solubilizing fungi are additional imperative to the solubilization of inorganic phosphate in soils than bacteria as they usually produce and secrete extra acids than bacteria (Sharma P et al., 2013). Hence, the quality and quantity of organic acid bring out are fully reliant on the type of P-solubilizing microorganisms. So, considerable differences may happen among various fungal isolates for the creation of available P from the same phosphate source (Toro et al., 1997).

2.5 Alkaline phosphatase enzyme

Microorganisms produced the alkaline phosphatases enzyme that is broad spread in nature. Alkaline phosphatase is a hydrolase enzyme working at alkaline pH. Phosphatase enzyme contains the two zinc ions that are involved in catalysis whereas the magnesium ion is important in the stabilization of structure (Simpson et al., 1968; Anderson et al., 1975). The enzymatically active alkaline phosphatase hydrolyzes phosphatase from various types of molecules like proteins, nucleotides, phosphate esters, alkaloids, and anhydrides of phosphoric acid. Alkaline phosphatase plays an essential role in phosphate metabolism and the making of alkaline phosphatase is regulated by the phosphoester compounds existing in the environment (Nalini et al., 2014). Alkaline phosphatases have high thermal stability and were described by several strains of bacterial and fungi such as *Thermus thermophilus* (Pantazali et al., 1998), *Thermus caldophilus* (Park et al., 1999), *Thermotoga neapolitana* (Dong and Zeikus et al., 1997), *Bacillus stearothermophilus* (Mori et al.,1999), *Humicola grisea var. thermoidea* (Buainain et al., 1998), *Scytalidium*

thermophilum (Guimaraes et al., 2001), and *Bacillus licheniformis* (Pandey and Banik et al., 2011).

2.6 Application of alkaline phosphatase:

Alkaline phosphatases play a significant role in various forms as a biofertilizer, in genetic engineering and molecular biology, clinical diagnosis, biosensor, dairy industries, pharmaceuticals, food industries, etc.

2.6.1 Dairy Industries

A maximum othe activity of the enzyme is inactivated in milk pasteurization. The evaluation of alkaline phosphatase activity is utilized as an indicator for acceptable pasteurization of milk (Harding, 1991; Fenoll et al., 2002; Rankin et al., 2010). Kay and Graham (1935) established the first enzymatic test for the detection of the efficiency of pasteurization based on the deactivation of alkaline phosphatase. Microbial alkaline phosphatases are noticeably more thermally resistant than milk alkaline phosphatases.

2.6.2 Agroecosystem

The mainstream of agricultural soils is huge capitals of phosphorus of which a considerable part has amassed as a result of systematic applications of phosphate fertilizers (Richardson, 1994). Alkaline phosphatase activity by soil alterations rises the availability of phosphorus to plants (Nalini et al., 2014). Alkaline phosphatase activity is afflicted by agricultural practices such as residue and tillage management (Deng and Tabatabai, 1997). Wang et al. (2011) reported a rise in alkaline phosphatase activity in non-till treatments with an increase in residue input amounts.

2.6.3 Aquatic ecosystem

Alkaline phosphatases are protruding enzymes in aquatic environments. Alkaline phosphatases in aquatic and sewage systems have been studied by (Reichardt et al., 1967; Berman, 1970; Jones, 1972; Flint and Hopton, 1977). Evaluation of the alkaline phosphatase activity of microorganisms which is considered a significant biochemical tool in limnological studies (Pandey and Parveen, 2011). The extracellular alkaline phosphatase liquified in natural waters is held to play a role in nutrient dynamics

(Berman, 1969; Kobayashi et al., 1984). López et al. (2006) determined. Alkaline phosphatases of lake water samples are contributive meaningfully to the phosphate pool. Alkaline phosphatase activity is one of the greatest existing approaches for the growth and survival of cyanobacteria under phosphate lack conditions (Bhaya et al., 2000).

Chapter 3:- MATERIALS & METHOD

3.1 Collection Soil Samples

The soil samples were collected from the different agricultural areas of Saurashtra regain. Each soil sample was mixed thoroughly and homogenized. Soil samples were collected in sterile polyethylene bags.

3.2 Isolation of phosphate solubilizing microorganisms

Phosphate solubilizing microorganisms were isolated using the serial dilution method and samples were diluted up to (10^{-8}). Pour plate and spread plates method were used for the isolation, screening, enrichment, and maintenance of phosphate solubilizing microorganisms (PSMs) by Pikovskaya's agar medium (Pikovskaya,1948) which contain tri-calcium phosphate (TCP). Phosphate solubilizing activity confirmed by the plates was incubated at 28ºC for 7 days till the clear halo zones were formed around the colonies of microorganisms. Phosphate solubilizing microorganisms produce a different tri-calcium phosphate solubilization zone around the colony with a distinct morphological appearance (Renu Gupta et al.,2021).

3.3 Characterization of isolated phosphate solubilizing microorganisms

3.3.1. Identification of phosphate solubilizing bacteria (PSB) strain

For the identification of phosphate solubilizing bacterial isolates were studied based on the colour, texture, and shape of a bacterial colony. The isolated bacteria were identified on the morphological and biochemical parameters by gram staining and different biochemical test. Then stained smear was observed under a 100× oil immersion lens by light microscopy (Aneja 2002).

3.3.2 Identification of phosphate solubilizing fungal (PSF) strain

Phosphate solubilizing fungal isolates were identified on basis of colony morphology and microscopic examination. For morphological identification presence or absence of aerial mycelium, width, and length of the colony, pigment production, and wrinkled furrows characteristics are to be evaluated (Gilman 2001). For the microscopic identification of

fungal strains Lactophenol Cotton Blue staining method has been performed by using of inoculating needle and Bunsen burner under 40× light microscopy (Aneja 2002).

3.4 Biochemical characterization of bacterial isolates

3.4.1 Indole test

First, prepare the peptone water tubes were incubated with the bacterial culture broth culture using sterile needle techniques. The uninoculated broth was used as a control and both tubes were incubated at 37ºC for 24 hours. After complete incubation, add 1 ml of Kovac's reagent in both tubes. After the addition of the reagent observed the cherry red colour on the top layer of the tube is a positive test and the absence of red coloration is indole negative (P.P.joy et al., 2014).

3.4.2 Methyl Red test

First, prepare the MR broth tubes were autoclaved and sterilized then add the experimental bacterial culture using sterile techniques. Uninoculated MR broth is used as a control. Both tubes were incubated at 37ºC for 24-48 hours. After appropriate incubation adds 2-3 drops of methyl red indicator to observe the colour changes. After the addition of the indicator remaining the red colour is a positive test and the colour changed to yellow is a negative test (P.P.joy et al., 2014).

3.4.3 Voges-Proskauer test

The experimental organism was inoculated into VP broth by loop inoculation using sterile techniques. The uninoculated tube was used as a control. Both tubes were incubated at 37ºC for 24-48 hours. After incubation adds alpha-naphthol and 40% KOH as an indicator. After adding the indicator the crimson-red colour formation indicates the positive result and colour change is the negative result of the test (P.P.joy et al., 2014).

3.4.4 Citrate utilization test

Simmons citrate agar slant was prepared and autoclaved for sterilization. Then slant was inoculated with the test organism by stab and streak inoculation. An uninoculated tube was kept as control. Both tubes were incubated at 37ºC for 24-48 hours. After proper

incubation observed the tubes for the growth and coloration of the medium. The colour of the medium if changed to blue indicates a positive result and green colour indicates a negative result (P.P.joy et al., 2014).

3.4.5 Triple sugar iron agar test

Triple sugar iron agar slants were prepared and sterilized by autoclaving. After sterilization add the test organisms by stab and streak inoculation method. An uninoculated slant was used as a control and both slants were incubated at 37ºC for 24-48 hours and seen the colour changes of both but and slant (P.P.joy et al., 2014).

3.4.6 Oxidase test

For the oxidase test, the test organisms were rubbed over the oxidase disc and saw the colour changes of the disc. If the colour changes to purple that gives a positive result on the test (P.P.joy et al., 2014).

3.4.7 Glucose fermentation test

First, prepare the peptone broth and add the 1% glucose into the fermentation tubes. Then inoculate the bacterial culture and uninoculated tube kept as a control. Both tubes were incubated at 37ºC for 24-48 hours and observed the colour changes in the fermentation broth. If the medium change the colour from red to yellow that is a positive result and if the colour change that is a negative result (P.P.joy et al., 2014).

3.5 Screening of phosphate solubilization activity

Solubilization efficiency is used as a parameter for assorting the possible isolates of PSMs. This phosphate solubilization activity helps to determine the quantitative measurement of the culture-mediated solubilizing degree of insoluble organic phosphate (Chen et al., 2006). The capability of phosphate solubilization of isolated strain was determined by both qualitative and quantitative methods using the methodology of Srivastav et al.,2004.

3.6 Qualitative analysis

For the qualitative analysis, first, pikovskaya's agar plates were prepared and inoculated with the culture. The plates were incubated at 37ºC for 3-7 days till a clear halo zone appeared around the colony. The phosphate solubilizing efficiency was found by the formula placed by Nguyen et al. (1992).

Solubilizing efficiency$= \frac{Halo\ zone\ diameter}{Colony\ diameter} \times 100$

3.7 Quantitative analysis

Soluble orthophosphate is available in the form of µg/ml in the supernatant and KH_2PO_4 is used as a standard at different days' intervals using the Spectro vanadomolybdate phosphoric yellow color method (Jackson, 1973).1 ml of 24 hours old culture was inoculated into 50 ml pikovskaya's broth which was incubated at $28^{º}\pm2$ for 1 week. The amount of released phosphorus in broth was estimated from supernatant tanking after centrifuging the broth at 5000 rpm for 10 min. Take 10 ml of supernatant and add 5 ml of ammonium molybdate reagent was added followed by rigorous shaking. Then 1 ml chlorostannous acid was added from the working solution making the total volume of the reaction mixture up to 50 ml with distilled water. The presence of phosphorus was determined by optical density at 600 nm. The presence of phosphorus present was obtained through the curve of potassium dihydrogen phosphate.

3.8 In-vitro optimization of growth at different pH, temperature, and salt concentration in growth medium

3.8.1. In-vitro optimization of growth at different pH

In-vitro categorization of the selected isolates was checked for growth at different pH where the broth culture of selected isolates was inoculated in four test tubes containing 10 ml of pikovskaya's medium maintained at 5.5, 6.5, 7.5, 8.5 pH with 1N HCL and 1N NAOH. Test tubes were incubated under shaking conditions for 48 h to check the growth with an optical density of 610nm (Renu Gupta et al.,2021).

3.8.2. In-vitro optimization of growth at different temperatures

Similar to pH, broth culture inoculated in four test tubes containing 10 ml pikovskaya's medium was incubated at different temperatures 28℃, 33℃, 37℃ for 48 h under shaking conditions. Measurement of growth by spectrophotometer at 610nm (Renu Gupta et al.,2021).

3.8.3. In-vitro optimization of growth at different salt concentrations

In-vitro optimization of growth at the different salt concentrations of pikovskaya's medium containing different TCP concentrations 1.0%, 1.5%, 2.0%, and 2.5% was incubated the broth culture for 48 h to check the growth at the different salt concentrations in growth medium (Renu Gupta et al.,2021).

3.9 Alkaline phosphatase assay

Enzyme extraction was carried out by inoculating the 100 µl of bacterial or fungal culture in 250 ml of a conical flask containing 100 ml of sterilized PVK broth. The inoculated flasks were incubated at 37ºC up to 192 h. The samples were taken every 24 h and centrifuged the sample at 10,000 rpm for 10 min at 4ºC (If the sample is fungi first filtered the sample and then centrifuged). The cell-free supernatants were analyzed for crude alkaline phosphatase activity following the method of Tabatabi and Bremner (1969). Alkaline phosphatases were examined using *p*-nitrophenol phosphate (pNPP), a colorless substrate that produces a colorimetric end-product as *p*-nitrophenol which produces a yellow colour. Take 1 ml cell-free supernatant was mixed with 4 ml modified universal buffer (pH 6.5). Then add 1 ml of 0.025 mM disodium *p*-nitrophenol phosphate (tetrahydrate) was mixed with the supernatant and incubated at 37ºC for 1 h. Add 1 drop of toluene that stop the microbial activity and incubate the mixture for 1 h. After incubation, the reaction was stopped by adding 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$. The mixtures were filtered through the Whatman filter paper no. 42. The concentration of *p*-nitrophenol was measured by taking the absorbance at 420 nm via UV-Vis spectrophotometer and values were examined using a standard curve by serially dilution solution of *p*-nitrophenol as a standard. One unit (U) of phosphatase activity was well-defined as the amount of enzyme required to liberate 1 µmol of *p*-

nitrophenol/ml/min from di-Na *p*-nitrophenyl phosphate (tetrahydrate) pNPP under examine conditions (B.C. Behera et al., 2017).

3.10 Partial purification of crude alkaline phosphatase enzyme

Partial purification of the crude alkaline phosphatase enzyme was the addition of 70% ammonium sulfate precipitation up to saturation followed by overnight dialysis at 4ºC (B.C. Behera et al., 2017). Quantification of protein content in both crude and partially purified alkaline phosphatase was carried through Lowry, Rosebrough, Farr, and Randall (1951), with bovine serum albumin as a standard (Sigma, Germany).

3.11 Enzyme assay

Enzyme assay has been carried out by following alkaline phosphatase assay as mentioned above (B.C. Behera et al., 2017).

3.12 Enzyme optimization at different pH, temperature, salt concentration and incubation periods

3.12.1 Enzyme optimization at different pH

Alkaline phosphatase activity was determined in a pH range of 3.0 to 12.0 by changing the ionic strength of the substrate. Acetate buffer was maintained at the pH. Blank was used without adding the enzyme. Enzyme activity was carried out at 37ºC for 30min (Surchandra et al., 2012).

3.12.2 Enzyme optimization at different temperature

Alkaline phosphatase activity was determined by different temperatures 25ºC to 85ºC, incubating the enzyme-substrate mixture at different temperatures for 30 min under assay conditions (Surchandra et al., 2012).

3.12.3 Enzyme optimization at different salt concentrations

For determination of the effect of substrate p-nitrophenyl phosphate concentration on enzyme activity. 1 ml of extracting enzyme containing 1 ml of different concentrations

ranging from 2 to 9 mg/ml. Enzyme substrate concentration was determined at 37ºC for 30 min under assay conditions (Surchandra et al., 2012).

3.12.4 Enzyme optimization at different incubation periods

The effect of incubation time on enzyme activity was monitored by incubating the enzyme-substrate mixture at various periods ranging from 10 to 80 min (Surchandra et al., 2012).

3.13 physicochemical analysis of soil

The soil sample was examine for chemical and physical parameter such as pH, electrical conductivity (EC), organic carbon (OC), nitrogen (N), phosphorus (P), potassium (K). This physicochemical analysis caried out from the Envitro Laboratories PVT. LTD, Rajkot.

3.14 Pot assay

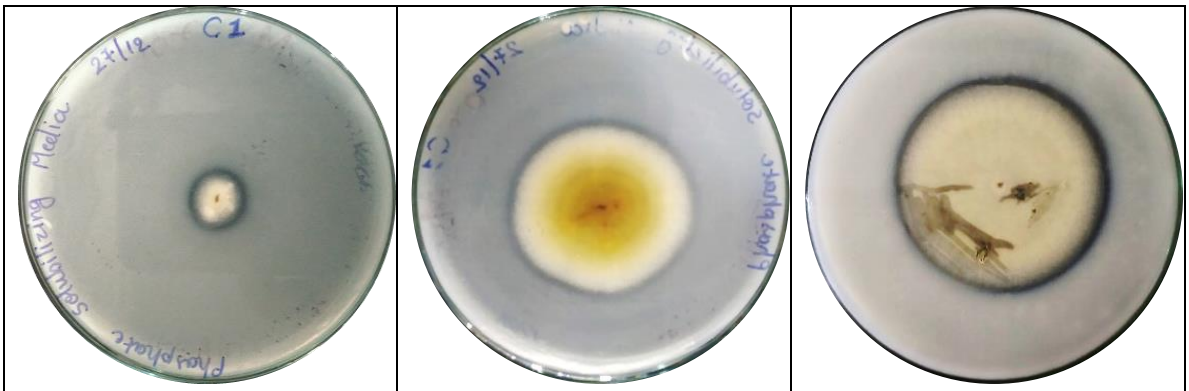
In-vitro pot assay has been carried out in the laboratory of the microbiology department, at Atmiya university Rajkot. Five different types of seeds i.e. Tomato seeds, Bean seeds, Chana seeds, Groundnut seeds, and Mug seeds have been taken to carry out a pot assay experiment. Following combinations were prepared for the pot assay experiment.

A. 150 gm autoclaved soil + 1ml of inoculum of Aspergillus spp., Penicillium spp., Pseudomonas spp. + seeds
B. 150 gm autoclaved soil + 1ml of crude enzyme of Aspergillus spp., Penicillium spp., Pseudomonas spp. + seeds
C. 150 gm autoclaved soil + 1ml of enzyme of Aspergillus spp., Penicillium spp., Pseudomonas spp. + seeds
D. 150 gm autoclaved soil + seeds as a positive control

Chapter 4:- RESULT & DISCUSSION

4.1 Isolation of phosphate solubilizing microorganisms

A total of 5 bacterial and 2 fungal strains were isolated from the soil samples of Saurashtra regain. Out of 7 isolates, 3 isolates were given maximum halo clear zone around the colonies that indicates better phosphate solubilizing activity. The isolates were recovered and purified on PVK agar. They displayed a significant variance in halo zone diameter which was developed up to 7 days incubation period.



Pseudomonas sp. (PS1)	*Penicillium* sp. (PS2)	*Aspergillus* sp. (PS3)

Fig. 1: Pure isolated culture from agricultural soil of Saurashtra region

The isolated phosphate solubilizing microorganisms were shown similar morphology and were characterized by the study of Khan et al. (2010) & Mujahid et al. (2014)

4.2. Characterization of isolated phosphate solubilizing microorganisms

4.2.1 identification of phosphate solubilizing bacteria (PSB) strain

For identification of isolate phosphate solubilizing bacterial strain were observed the colony morphology on nutrient agar plates. Characterization of isolated bacterial strains by size, shape, margin, elevation, consistency, opacity, and colour have been assessed as per Bergey's manual. In microscopic identification of the arrangement of isolated

bacterial strain and Gram's staining observed under 100× lens. The characterization details was given in Table-1.

Table 1: Morphological and Microscopic identification of Bacterial isolate form agricultural soil of Saurashtra region

Sample	Size	Shape	Colou-r	Texture	Elevation	Margin	Pigmentation	Gram's nature
***Pseudomonas* spp. (PS1)**	Small	Round	Light yellow	Slimy	Low convex	swell	Light yellow	Gram-negative

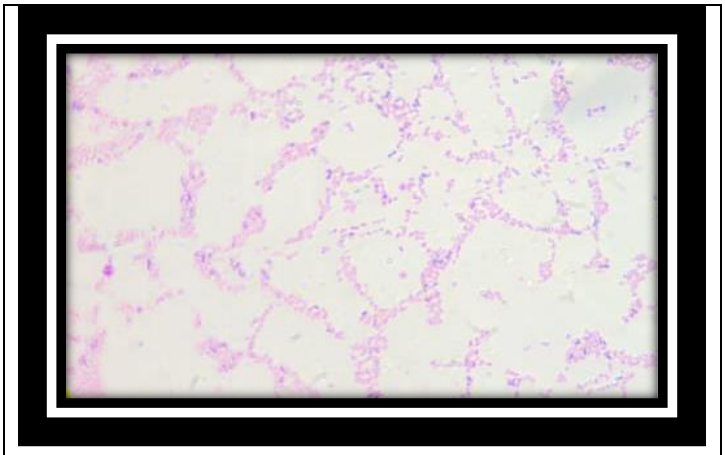


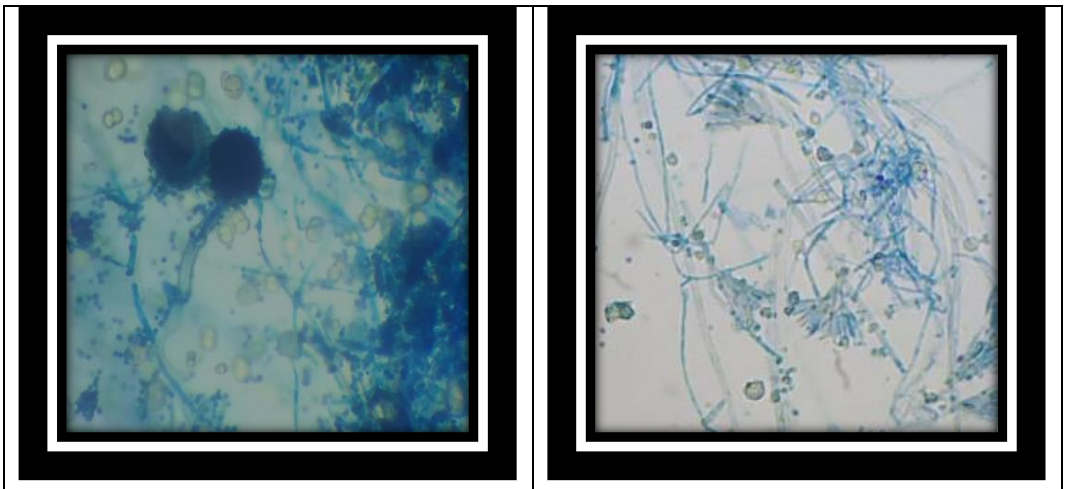
Fig.2 Gram staining of bacterial isolate

4.2.2 Identification of phosphate solubilizing fungal (PSF) strain

Characterization of isolated fungal species (PS2 and PS3) observed on PVK agar medium. In microscopic identification, fungal isolates by the arrangement of hyphae, conidia design, color, etc. were identified using Lacto-phenol Cotton blue and observed under 40× light microscopy. The details was given in Table-2.

Table 2: Microscopic and morphological identification of fungal strains from Saurashtra reagin

Sample	**Colony Colour**	**Front View Arial Mycelia Vegetative / Arise**	**Area (cm)**		**Pigmentation**	**Wrinkle-e Furrow**	**Microscopic Observation**	**Identified fungal isolats**
			Height	Width				
PS2	White to dark greenish	Vegetative	9	9	Yellowish to wine red	Yes	Septate hyphae, Hyaline, Brush-like penicillin, Conidia unicellular	*Penicilliu m* spp.
PS3	Brow to dark brown	Vegetative	9	9	No	No	Septate hyphae and hyaline, Biseriate conidial, smooth-walled conidiophore s	*Aspergillu s* spp.



Aspergillus sp.	*Penicillium* sp.

Fig. 3 Microscopic identification of fungal strains by Lacto-phenol Cotton Blue staining method

4.3 Biochemical characterization of Bacterial isolates

Biochemical characterization of bacterial isolates was carried out by different biochemical tests. Bacterial isolates were given the positive test of Oxidase test, Methyl red test, citrate test, triple sugar iron test, and Voges-Proskauer test. Table-3 shows all biochemical tests for bacterial isolates PS1.

Table 3: Biochemical Characterization of bacterial isolated strain

Sample	Oxidase test	Glucose fermentation	Indole test	Methyl red test	Voges-Proskeer test	Simmon citrate test	Triple sugar iron test
PS1 (*Pseudomonas* spp.)	+ve	-ve	-ve	+ve	+ve	+ve	+ve

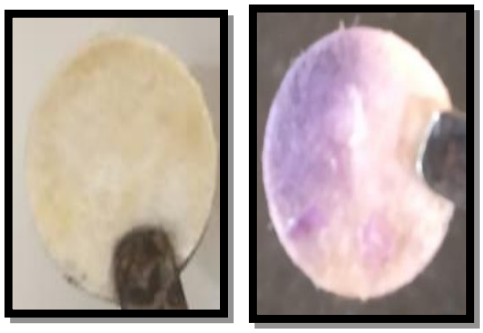
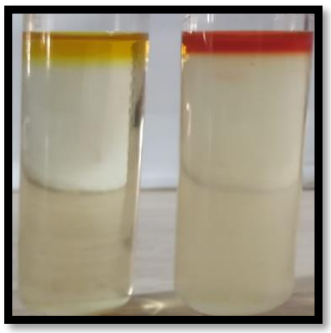
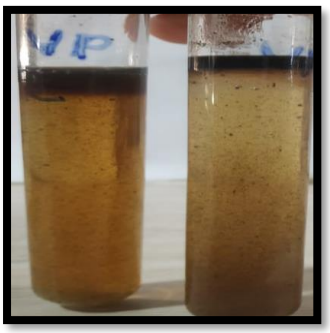
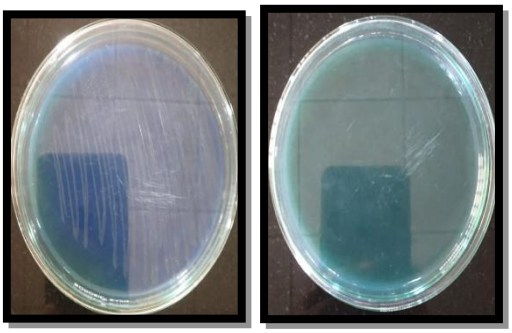
	
Oxidase test with Control	**Glouse fermentation test with Control**
	
Indol test with Control	**Methyle Red test with Control**
	
Voges-Proskauer test with Control	**Simmon Citrate test with Control**
	
Triple sugar iron test with Control	

Fig. 4 Biochemical analysis of bacterial isolated strain by Bergey's manual

4.4 Screening of phosphate solubilization activity

The isolated phosphate solubilizers were separated from the soil sample which was screened for the production of phosphate solubilization activity by the qualitative and quantitative methods in Pikovskaya's broth.

4.5 Qualitative analysis

Phosphate solubilization capacity was determined as a clear zone around the colonies incubated at 28ºC for 48 hours on PVK agar. All isolates give a clear zone on PVK agar and the selected three isolates give maximum clearance zone of different diameters. The phosphate solubilizing efficiency (SE) was determined for the selected isolates which range from 100-120 with a maximum value of 120 by PS1 followed by PS2 (166.27), and PS3 (104.16) respectively Table-4.

Table 4: Qualitative Analysis of PSMs

	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
Colony diameter(cm)	0.5	4.3	4.8
Halozone diameter(cm)	0.6	5	5
Solubilizing efficiency	120	166.27	104.16

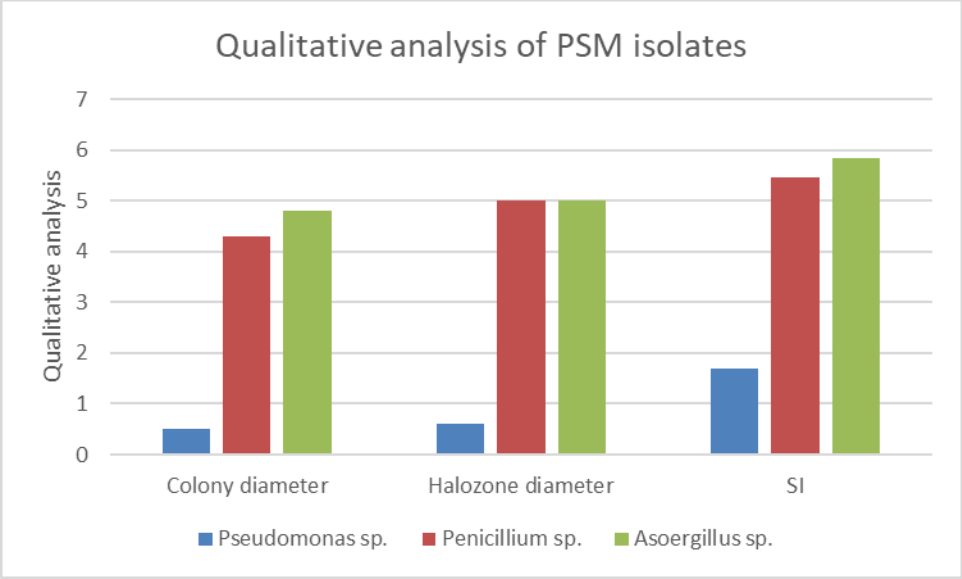


Fig. 5 Qualitative analysis of PSMs isolates.

The qualitative analysis of the phosphate solubilizing activity of selected isolates was given the solubilizing efficiency range from 100-120 cm. These studies compared with Muleta et al.,(2013) and Sasdiq et al., (2013) which found the solubilization efficiency varied between 2.56-4.50 and also finding the solubilizing efficiency according to R. Gupta et al., (2022) gives 2.1-3.2 mm. Hence selected isolates were good solubilizers of phosphate.

4.6 Quantitative analysis

The capacity of phosphate solubilization of isolated strains is diverse and showed an increase with the increase in the time interval but maximum P solubilization was noted by PS1 on the 7^{th} day at 83 µg/ml followed by PS2 77 µg/ml and PS3 78 µg/ml respectively Table-5.

Table 5: Quantitative Analysis of PSMs

	P solubilized (µg/ml)		
	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
3 DAYS	16	17	10
5 DAYS	72	62	53
7 DAYS	83	77	78

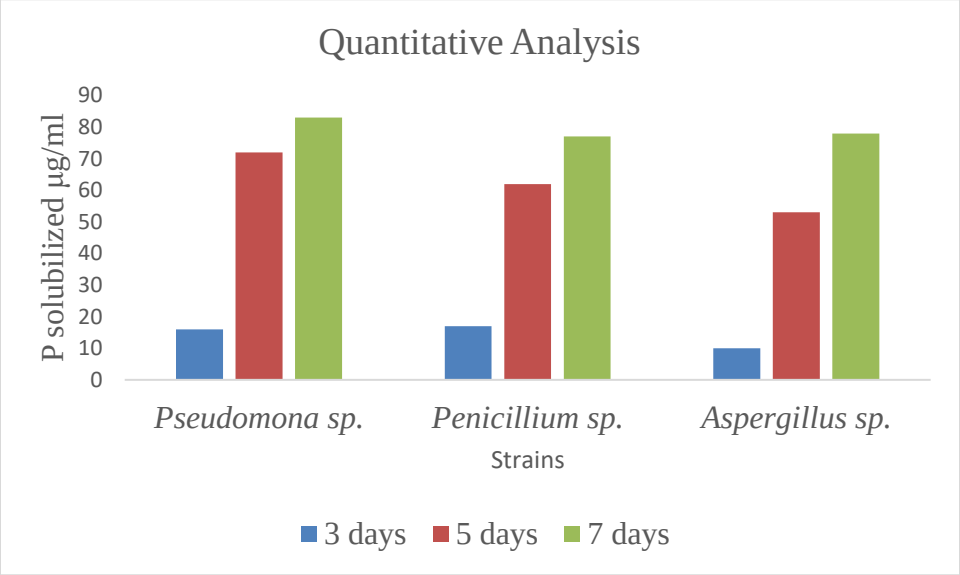


Fig. 6 Quantitative analysis of PSMs isolates.

The quantitative analysis of phosphate solubilizers has the capacity to solubilize P which increases the P solubilization at the time interval but produced the maximum P solubilization on the 7th day 19.12 µg/ml for *Bacillus pumilus* due to the production of organic acid was recorded by Mihalache et al., (2018). R. Gupta et al., (2022) reported 10.22 µg/ml of P solubilization on the 7th day from the *Bacillus subtilis* strain.

4.7 In-vitro optimization of growth at different pH, temperature, and salt concentration in growth medium

4.7.1 In-vitro optimization of growth at different pH

Different isolated strains were grown at different pH and the growth of three isolates at optimum culture conditions was found at maximum OD i.e. 610nm. Studies have shown that PS1 and PS2 showed their maximum growth at 5.5 pH and PS3 showed maximum growth at 8.5 pH.

Table 6: Optimization of growth of PSMs isolates at different pH

	Parameters	**Optical Density at 600nm**		
pH		***Pseudomonas* sp.**	***Penicillium sp.***	***Aspergillus* sp.**
		0.912	0.37	0.257
		0.935	0.48	0.285
5.5		0.928	0.29	0.222
	Mean	0.912	0.29	0.222
	SD	0.011789826	0.09539392	0.031564748
		0.49	0.91	0.181
		0.59	0.87	0.287
6.5		0.39	0.102	0.157
	Mean	0.39	0.102	0.157
	SD	0.1	0.455391407	0.069176104
		0.55	0.299	0.408
		0.69	0.384	0.423
7.5		0.49	0.289	0.309
	Mean	0.49	0.289	0.309
	SD	0.102632029	0.052201533	0.061943523
		0.506	0.332	0.89
		0.439	0.235	0.942
8.5		0.642	0.359	0.85
	Mean	0.439	0.235	0.85
	SD	0.103435971	0.065209917	0.04613025

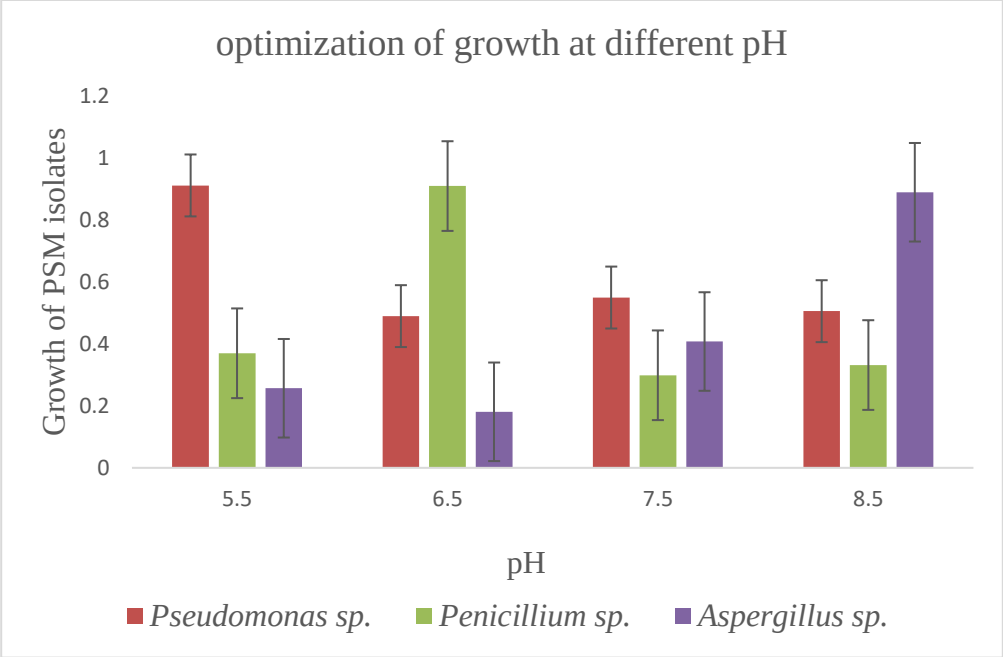


Fig. 7 Effect of different pH on growth of PSMs isolates.

4.7.2 In-vitro optimization of growth at different temperatures

Similarly, when growth at different temperatures (28-37 ºC) PS1 and PS3 showed their maximum growth at 28ºC and PS2 showed maximum growth at 33 ºC.

Table 7: Optimization of growth of PSMs isolates at different temperature

		Optical Density at 610nm		
Temperature (ºC)	**Parameters**	***Pseudomonas* sp.**	***Penicillum* sp.**	***Aspergillus* sp.**
28		0.361	0.927	0.533
		0.482	0.958	0.423
		0.327	0.852	0.684
	Mean	0.327	0.852	0.423
	SD	0.081467785	0.054500765	0.131035619
33		0.146	0.958	0.15
		0.245	0.982	0.236
		0.127	0.923	0.123
	Mean	0.127	0.923	0.123
	SD	0.063358767	0.029670412	0.059011298
37		0.299	0.51	0.251
		0.358	0.684	0.246
		0.256	0.485	0.384
	Mean	0.256	0.485	0.246
	SD	0.051208723	0.108398954	0.078270897

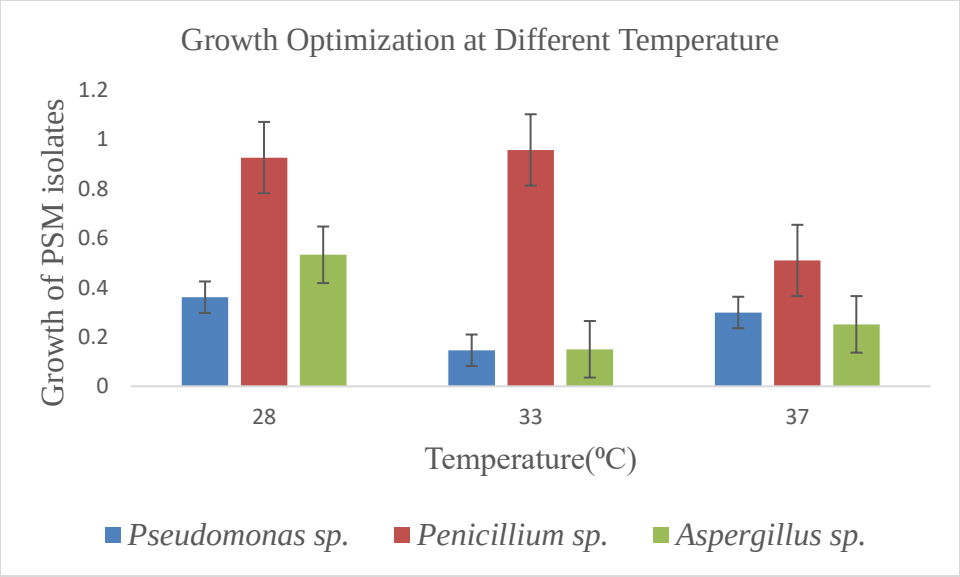


Fig. 8 Effect of different temperatures on the growth of PSMs isolates.

4.7.3 In-vitro optimization of growth at different salt concentrations

Different isolated strains were grown at different salt concentrations and the growth of three isolates at optimum culture conditions was found at maximum OD i.e. 610nm. Studies have shown that PS1 and PS3 at 1% salt concentration and PS2 show maximum OD at 2.5 % salt concentration.

Table 8: Optimization of growth of PSMs isolates at a different salt concentrations

		Optical Density at 610nm		
Salt concentration	**Parameters**	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
1		0.305	0.63	0.656
		0.325	0.623	0.648
		0.301	0.645	0.658
	Mean	0.301	0.623	0.648
	SD	0.012858201	0.01123981	0.005291503
1.5		0.176	0.809	0.47
		0.154	0.785	0.428
		0.189	0.828	0.654
	Mean	0.154	0.785	0.428
	SD	0.017691806	0.021548395	0.12020538
2		0.153	0.82	0.532
		0.149	0.816	0.523
		0.169	0.902	0.548
	Mean	0.149	0.816	0.523
	SD	0.010583005	0.048538644	0.01266228
2.5		0.293	1.04	0.556
		0.283	1.08	0.489
		0.292	1.03	0.589
	Mean	0.283	1.03	0.489
	SD	0.005507571	0.026457513	0.050954228

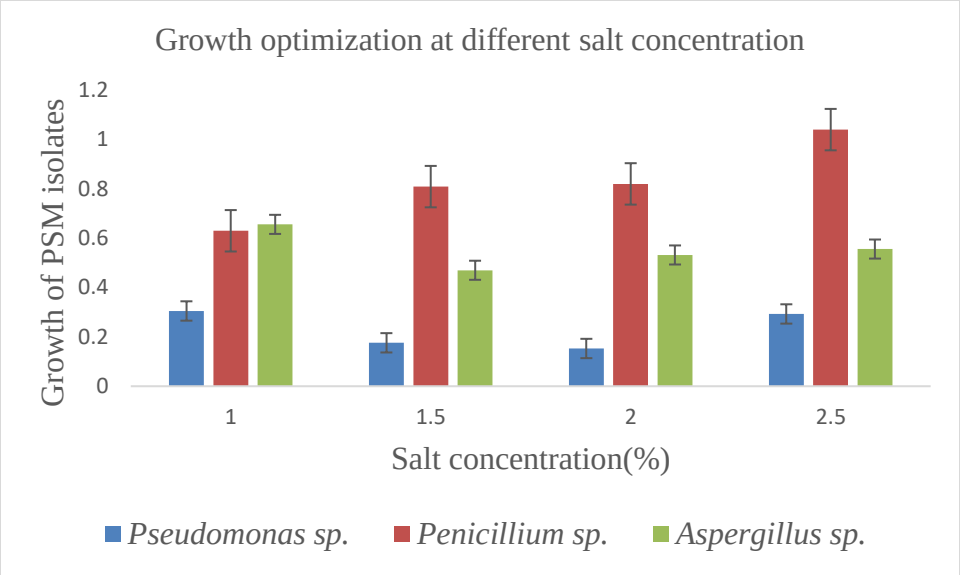


Fig. 9 Effect of salt concentration on growth of PSMs isolates.

Maheswar and Sathiyavani et al., (2012) and Patil et al., (2014) found comparable results from different microorganisms grown at slightly similar environmental conditions. *Bacillus subtilis* strain 2 has grown at maximum pH of 5.5, 1% salt concentration, and 37ºC temperature. *Bacillus subtilis* strain 4 grown at maximum pH 7.5, 28ºC temperature, and 1.5% of salt concentration. This outcome is also supported by Shruti et al., (2013) and Arindam et al., (2014).

4.8 Alkaline phosphatase assay

The maximum alkaline phosphatase production of the bacterial isolate was (PS1) 3.410 µmol/min recorded at 168 hours of incubation and decreased after incubation up to 192 hours. PS2 isolated strain shows phosphatase production of 11.783 µmol/min recorded at 168 hours and the PS3 isolated strain shows produce 2.821 µmol/min recorded at 48 hours. Further data was shown in Table-9.

Table 9: Enzyme activity for PSMs to check enzyme production

	Optical density at 420nm	Enzyme Activity (µmol/min)	Optical density at 420nm	Enzyme Activity (µmol/min)	Optical density at 420nm	Enzyme Activity (µmol/min)
	***Pseudomonas* sp.**		***Penicillium* sp.**		***Aspergillus* sp.**	
Day-1	0.035	1.085	0.036	1.116	0.041	1.271
Day-2	0.046	1.426	0.04	1.240	0.049	1.519
Day-3	0.056	1.736	0.053	1.643	0.091	2.821
Day-4	0.083	2.573	0.034	1.054	0.056	1.736
Day-5	0.049	1.519	0.055	1.705	0.064	1.984
Day-6	0.04	1.240	0.123	3.814	0.043	1.333
Day-7	0.11	3.410	0.38	1.783	0.059	1.829
Day-8	0.051	1.581	0.037	1.147	0.03	0.930

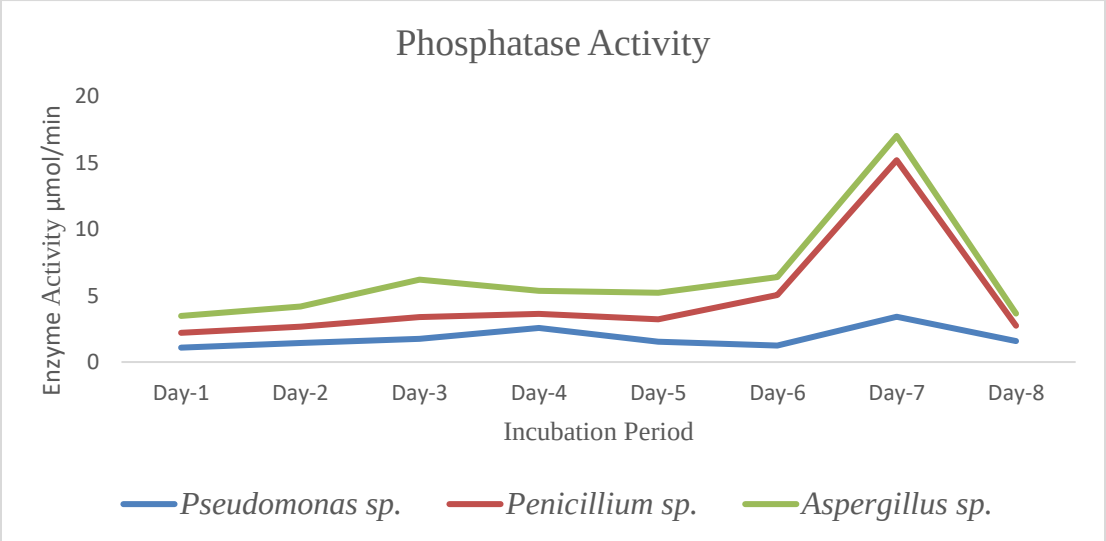


Fig. 10 Phosphatase production of PSMs isolates

B.C. Behera et al.,(2017) described the maximum alkaline phosphorus produced by the phosphate-solubilizing microorganisms at 71.531 U/ml at 48 h of incubation. Similar information also reported by Prasanna, Joshi, Rana, Shivay, & Nain et al.,(2011) and Walpola & Yoon et al (2013).

4.9 Partial purification of crude alkaline phosphatase enzyme

The crude enzyme of PS1 has a specific activity of 0.084 U/mg, PS2 0.144 U/mg, and PS3 0.133 U/mg using partial purification by ammonium sulfate precipitation method. The result of the purification of the enzyme is given in Table-10.

Table 10: Partial purification of crude phosphatase enzyme from PSMs isolates

Samples	**Purification step**	**Volume of fraction**	**Concentration of protein (µg/ml)**	**Concentration of enzyme (U/ml)**	**Specific activity (U/mg)**	**Total amount (Units)**	**Yield (%)**	**Purification factor (fold)**
***Pseudomonas* sp.**	Crude	450	83	7	0.084	3150	100%	1
	30-70% Ammonium sulphate precipitation	210	43	5	0.116	1050	33.33 %	1.38
***Penicilliu m* sp**.	Crude	370	69	10	0.114	3700	100%	1
	30-70% Ammonium sulphate precipitation	170	74	6	0.081	1020	27.56 %	0.71

***Aspergillus* sp.**	Crude	348	45	6	0.133	2088	100%	1
	30-70% Ammonium sulphate precipitation	148	155	4	0.025	592	28.35 %	0.18

Afterward purification, the enzyme was purified up to 1.82 fold with a yield and 16.33 U/mg and specific activity of 41.09% respectively. Our discovery is lesser than the verdicts of Poirier and Holt (1983), who recorded a 64% yield of alkaline phosphatase later partial purification from *Capno-cytophaga ochracea*. González et al. (1994) also spotted 108.3 U/mg specific activity and 48.8% yield from *Myxococcus coralloides D*.

4.10 Enzyme optimization at different pH, temperature, salt concentration and incubation periods

4.10.1 Enzyme optimization at different pH

The effect of different pH on alkaline phosphatase activity was verified at pH ranging from 3- 12. The enzyme activity of PS1 increased up to 3- 9 pH, PS2 increased its activity up to pH 3-10, and PS3 increased phosphatase activity up to 3-12 pH. Further data are shown in Table-11.

Table 11: Enzyme optimization at different pH

pH	Enzyme Activity (µmol/min)		
	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
3	1.023	0.899	2.201
4	1.209	0.930	2.449
5	1.023	1.209	2.697
6	1.085	1.147	2.604
7	0.992	1.023	2.635
8	1.116	1.116	2.790
9	1.395	1.364	3.038
10	1.333	4.212	2.914
11	1.147	1.488	2.728
12	1.395	1.488	3.162

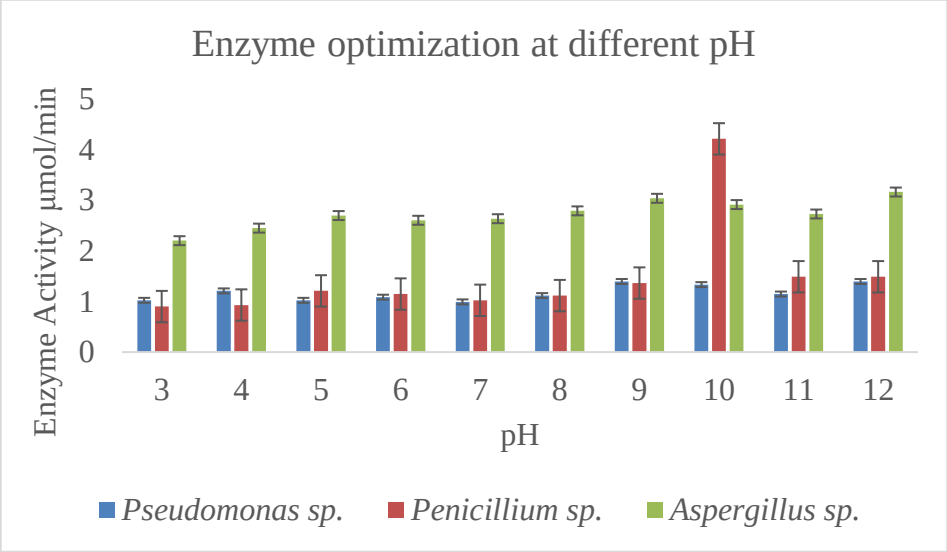


Fig. 11 Effect of different pH on alkaline phosphatase activity

Glew and Heath et al.,(1971) reported the maximum phosphatase activity at pH 9.0 from the *Micrococcus sodonensis*.

4.10.2 Enzyme optimization at different temperature

The alkaline phosphatase enzyme was active over a broad range of temperatures up to 25-85ºC. Maximum enzyme activity was observed at 85ºC. The isolated enzyme act as a thermostable which isolates from the soil. Further details was shown in Table-12.

Table 12: Enzyme Optimization at different Temperature

Temperature (ºC)	Enzyme Activity (µmol/min)		
	Pseudomonas sp.	***Penicillium* sp.**	***Aspergillus* sp.**
25	0.465	0.496	4.186
35	0.775	0.434	2.356
45	0.620	0.527	2.232
55	2.139	1.364	4.124
65	4.13	2.387	5.519
75	1.023	0.589	0.527
85	5.147	4.341	4.186

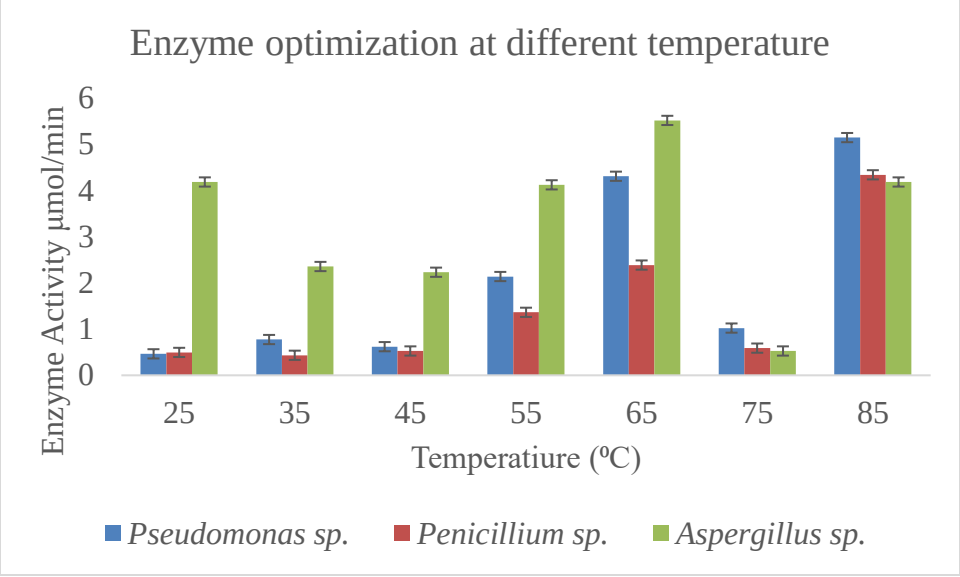


Fig. 12 Effect of different temperatures on alkaline phosphatase activity.

Gonzalez, Esther Farez-Vidal, Arias, and Montoya (1994) described that 37 ºC was the optimum temperature for the alkaline phosphatase activity which was purified from

Myxococcus coralloides D. while alkaline phosphatase purified from *Pseudomonas aeruginosa* shown its optimum activity at 41ºC temperature was reported by Day & Ingram et al., (1973).

4.10.3 Enzyme optimization at different salt concentrations

Alkaline phosphatase activity was calculated at various substrate (p-NPP) concentrations from 2-9 mg/ml. phosphatase activity was increased up to 2- 8 mg/ml concentration of p-NPP and details are given in Table-13.

Table 13: Enzyme optimization at different salt concentrations

Salt concentration(mg/ml)	**Enzyme Activity (µmol/min)**		
	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
2	2.13	2.32	2.57
3	2.63	2.97	3.03
4	1.51	2.17	1.58
5	2.91	3.00	3.59
6	2.79	2.97	2.72
7	7.81	5.82	6.97
8	6.94	7.03	7.81
9	5.17	5.45	6.17

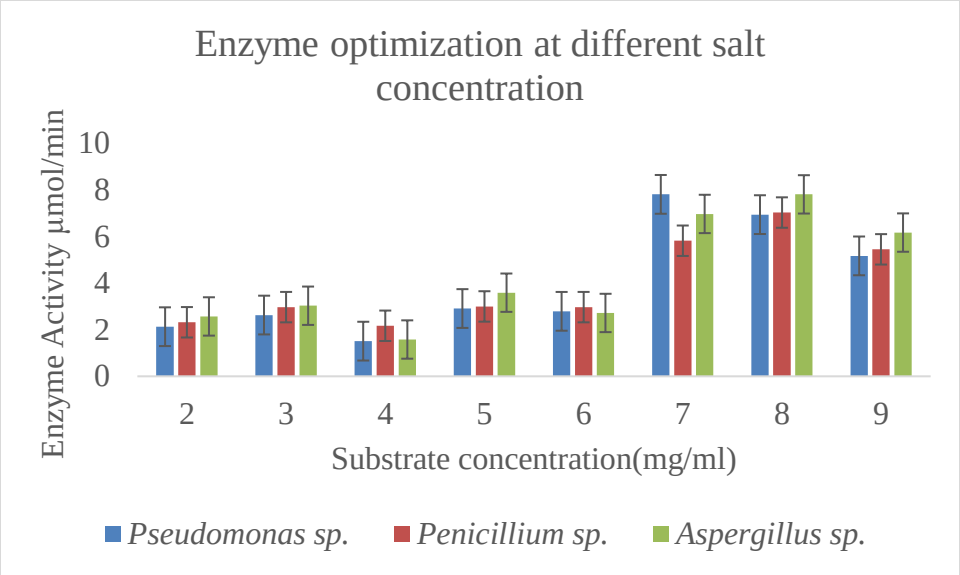


Fig. 13 Effect of different salt concentrations on alkaline phosphatase activity

The effect of various concentrations of pNPP (0.5-2.5 mg/ml) on alkaline phosphatase activity exposed that alkaline phosphatase activity was increased concentration up to 2 mg/ml. Further, the activity was found to be constant at higher concentrations reported by Ul qadar, Iqbal, and Niazi (2009).

4.10.4 Enzyme optimization at different incubation periods

Alkaline phosphatase activity was recorded at different periods of incubation at 10-80 min. the activity of phosphatase was increased slowly up to 10- 70 min and then decreased regularly. Further details was given in Table-14.

Table 14: Enzyme optimization at different incubation periods

Incubation period(min)	Enzyme Activity (µmol/min)		
	***Pseudomonas* sp.**	***Penicillium* sp.**	***Aspergillus* sp.**
10	4.05	3.09	3.49
20	3.25	3.07	3.51
30	3.28	3.31	3.64
40	3.25	3.32	3.71
50	3.27	4.01	3.74
60	3.66	3.76	4.36
70	3.36	4.12	3.83
80	3.32	3.73	3.81

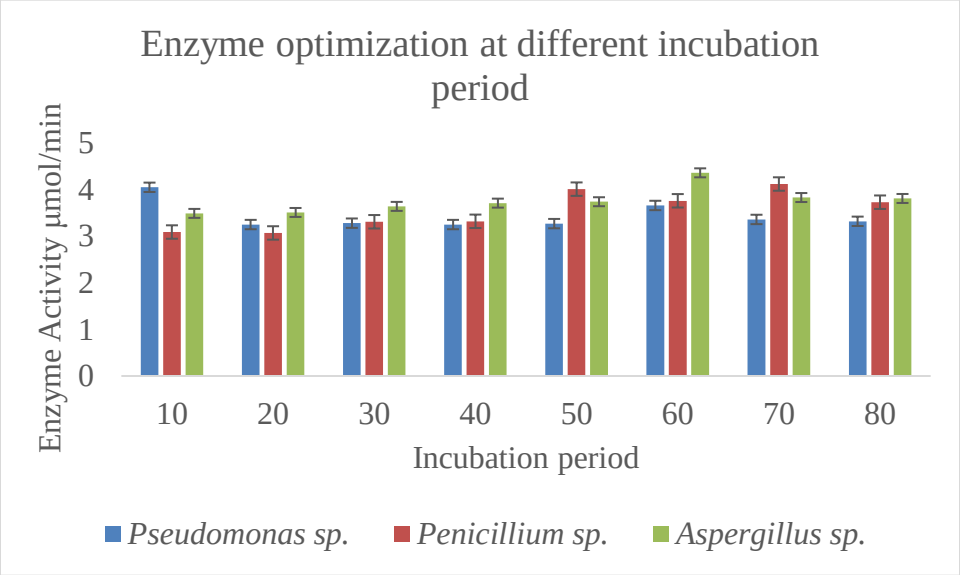


Fig. 14 Effect of different incubation periods on alkaline phosphatase activity.

The maximum alkaline phosphatase activity was found at 70 min of incubation which is similar to the above study while this result was obtained by Okuda et al., (1987) and Mohammed et al., (2010).

4.11 Physicochemical analysis of soil

The data was obtained by outsourcing at Envitro Laboratories PVT. LTD, Rajkot. The calculated values were found in the range of normal to higher values.

Table 15: Soil sample analysis

Test parameter	Quantity
pH	7.92
Electrical conductivity (EC)	0.465
Organic carbon (%)	3.60
Nitrogen (kg/ha)	420.0
Phosphorus (kg/ha)	364.47
Potassium (kg/ha)	155.78

4.12 Pot assay

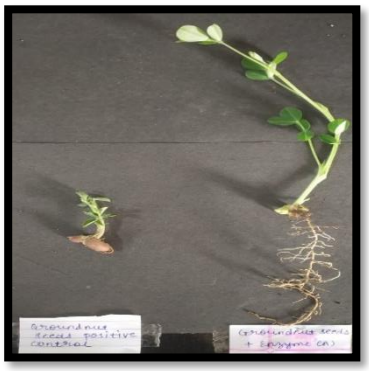
In-vitro pot culture experiment has measured the root length and shoot length, after the completion of 20 days and detail have been given in Tables.

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 15 . Effect of Phosphate Solubilization Activity of Crude Enzymes of Bacteria and Fungal Isolates on Growth of Groundnut Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 16 . Effect of Phosphate Solubilization Activity of Inoculum of Bacteria and Fungal Isolates on Growth of Groundnut Seedlings



Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 17 . Effect of Phosphate Solubilization Activity of partial purified enzyme of Bacteria and Fungal Isolates on Growth of Groundnut Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

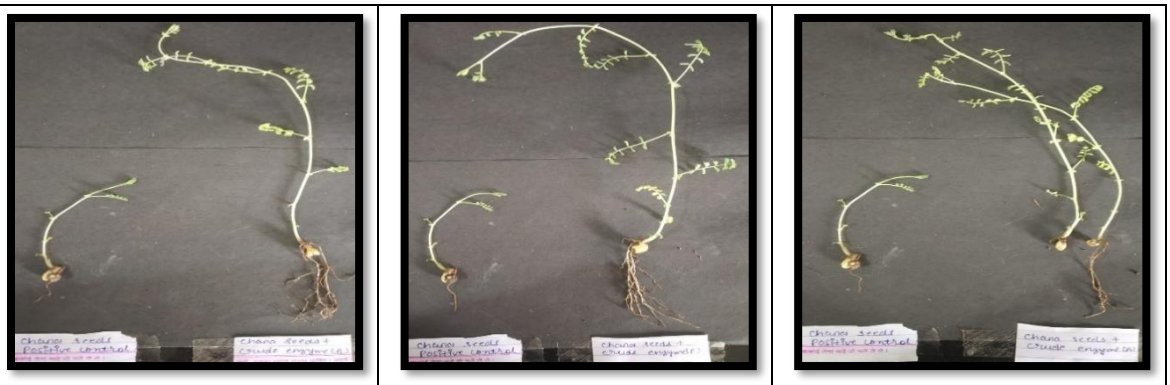
Fig 18 . Effect of Phosphate Solubilization Activity of Crude Enzyme of Bacteria and Fungal Isolates on Growth of Tomato Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 19 . Effect of Phosphate Solubilization Activity of Inoculum of Bacteria and Fungal Isolates on Growth of Tomato Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 20 . Effect of Phosphate Solubilization Activity of Partial Purified Enzyme of Bacteria and Fungal Isolates on Growth of Tomato Seedlings



Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 21 . Effect of Phosphate Solubilization Activity of Crude Enzyme of Bacteria and Fungal Isolates on Growth of Chana Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 22 . Effect of Phosphate Solubilization Activity of Inoculum of Bacteria and Fungal Isolates on Growth of Chana Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 23 . Effect of Phosphate Solubilization Activity of Partial Purified Enzyme of Bacteria and Fungal Isolates on Growth of Chana Seedlings

Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 24 . Effect of Phosphate Solubilization Activity of Crude Enzyme of Bacteria and Fungal Isolates on Growth of Mung Seedlings

		
Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 25 . Effect of Phosphate Solubilization Activity of Inoculum of Bacteria and Fungal Isolates on Growth of Mung Seedlings

		
Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 26 . Effect of Phosphate Solubilization Activity of Partial Purified Enzyme of Bacteria and Fungal Isolates on Growth of Mung Seedlings

		
Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 27. Effect of Phosphate Solubilization Activity of Crude Enzyme of Bacteria and Fungal Isolates on Growth of Bean Seedlings

		
Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 28. Effect of Phosphate Solubilization Activity of Inoculum of Bacteria and Fungal Isolates on Growth of Mung Seedlings

		
Pseudomonas sp.	*Penicillium* sp.	*Aspergillus* sp.

Fig 29. Effect of Phosphate Solubilization Activity of Partial Purified Enzyme of Bacteria and Fungal Isolates on Growth of Mung Seedlings

Table 16: Effect of Inoculum on Root of Plant Seedlings

Organisms	Parameters	Groundnut	Mung	Bean	Tomato	Chana
	Control	2.0	3.5	4.6	4.0	4.5
		5.0	2.9	3.9	5.0	3.6
		3.0	4.3	5.2	5.9	6.2
	Mean	2.0	2.9	3.9	4.0	3.6
	SD	1.527	0.70237692	0.65064	0.95044	1.32035
***Pseudomonas* sp.**	Inoculum	6.4	4.7	5.6	5.3	17.0
		5.2	3.8	5.2	4.9	16.0
		8.8	5.3	4.8	6.4	18.0
	Mean	5.2	3.8	4.8	4.9	16.0
	SD	1.833	0.75498344	0.4	0.77675	1.0
***Penicillium* sp.**	Inoculum	19.2	5.3	4.5	5.2	14.4
		18.5	4.9	3.6	4.9	13.9
		20.6	6.5	5.9	6.2	15
	Mean	18.5	4.9	3.6	4.9	13.9
	SD	1.069	0.8326664	1.15902	0.68069	0.55076
***Aspergillus* sp.**	Inoculum	15.4	5.4	4.7	5.2	9.5
		13.2	4.8	3.9	4.6	8.9
		17.9	6.8	5.2	6.9	10.2
	Mean	13.2	4.8	3.9	4.6	8.9
	SD	2.351	1.02632029	0.65574	1.19304	0.65064

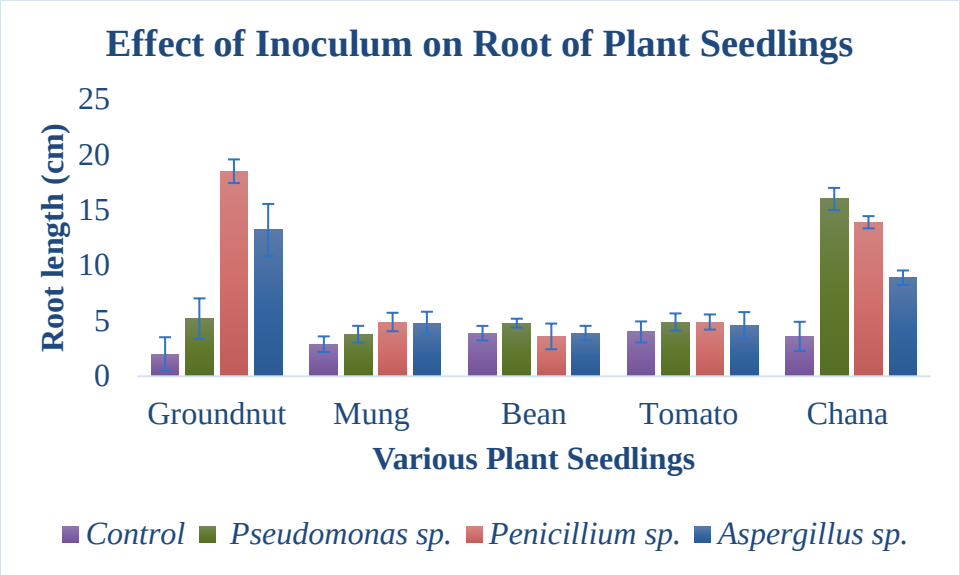


Fig. 30. Effect of Inoculum on Root of Plant Seedlings

Table 17: Effect of Inoculum on Shoot of Plant Seedlings

Organisms	Parameters	Groundnut	Mung	Bean	Tomato	Chana
	Control	4.8	16.0	13.2	3.2	10.9
		3.9	15.0	12.6	4.2	9.8
		5.2	18.0	14.5	3.1	11.2
	Mean	3.9	15.0	12.6	3.1	9.8
	SD	0.665	1.52752523	0.97125349	0.60827625	0.7371115
***Pseudomonas* sp.**	Inoculum	12.0	25.7	17.6	7.0	31.0
		13.0	24.7	16.8	6.9	30.0
		15.0	22.5	17.9	7.6	34.0
	Mean	12.0	22.5	16.8	6.9	30.0
	SD	1.527	1.63707055	0.56862407	0.37859389	2.081666
***Penicillium* sp.**	Inoculum	23.7	28.4	17.2	9.4	22.0
		21.2	27.9	16.8	10.2	20.0
		24.7	29.6	18.2	8.9	25.0
	Mean	21.2	27.9	16.8	8.9	20.0
	SD	1.802	0.87368949	0.72111026	0.65574385	2.5166115
***Aspergillus* sp.**	Inoculum	22.2	29.4	21.6	8.7	31.0
		21.9	30	20.7	9.1	35.0
		23.7	29.8	22.5	8.2	33.0
	Mean	21.9	29.4	20.7	8.2	31.0
	SD	0.964	0.30550505	0.9	0.45092498	2.0

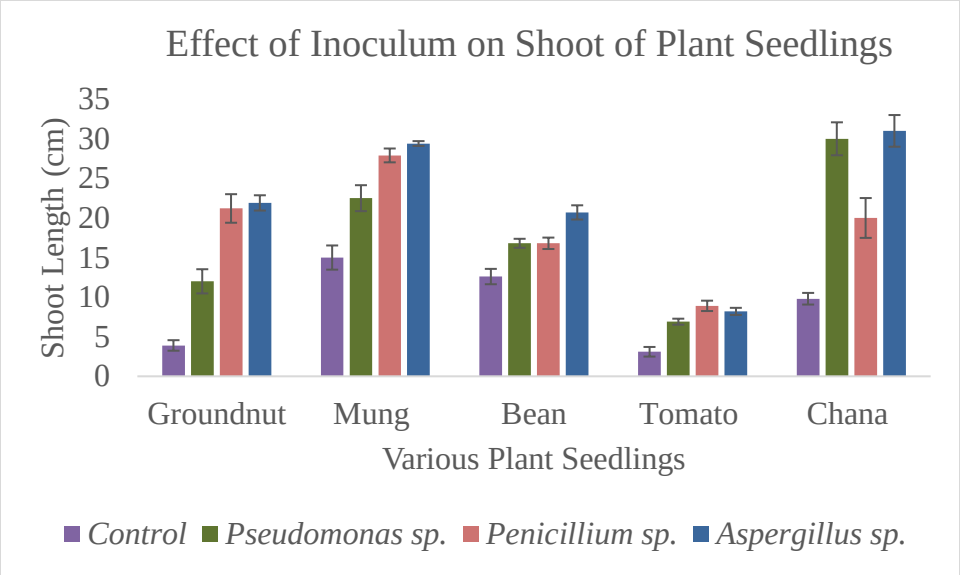


Fig. 31. Effect of Inoculum on Shoot of Plant Seedlings

Table 18: Effect of Crude Enzyme on Root of Plant Seedlings

	Parameters	Groundnut	Mung	Bean	Tomato	Chana
Organisms	Control	2.0	3.5	4.6	4.0	4.5
		5.0	2.9	3.9	2.0	3.9
		3.0	4.3	4.9	6.0	4.9
	Mean	2.0	2.9	3.9	2.0	3.9
	SD	1.527	0.70237692	0.51316	2.0	0.50332
***Pseudomonas* sp.**	Crude Enzyme	15.5	4.5	5.2	4.8	10.0
		13.5	3.6	6.3	4.5	9.0
		17.5	5.9	4.9	3.2	12.0
	Mean	13.5	3.6	4.9	3.2	9.0
	SD	2.0	1.15902258	0.73711	0.85049	1.52753
***Penicillium* sp.**	Crude Enzyme	17.0	5.4	4.7	3.8	10.0
		15.0	4.8	3.9	3.5	12.0
		12.0	6.5	5.2	4.2	10.6
	Mean	12.0	4.8	3.9	3.5	10.0
	SD	2.516	0.86216781	0.65574	0.35119	1.02632
***Aspergillus* sp.**	Crude Enzyme	19.0	3.8	4.1	4.2	14.3
		18.0	2.3	3.9	5.2	13.9
		21.0	4.8	4.6	4.1	14.2
	Mean	18.0	2.3	3.9	4.1	13.9
	SD	1.527	1.25830574	0.36056	0.60828	0.20817

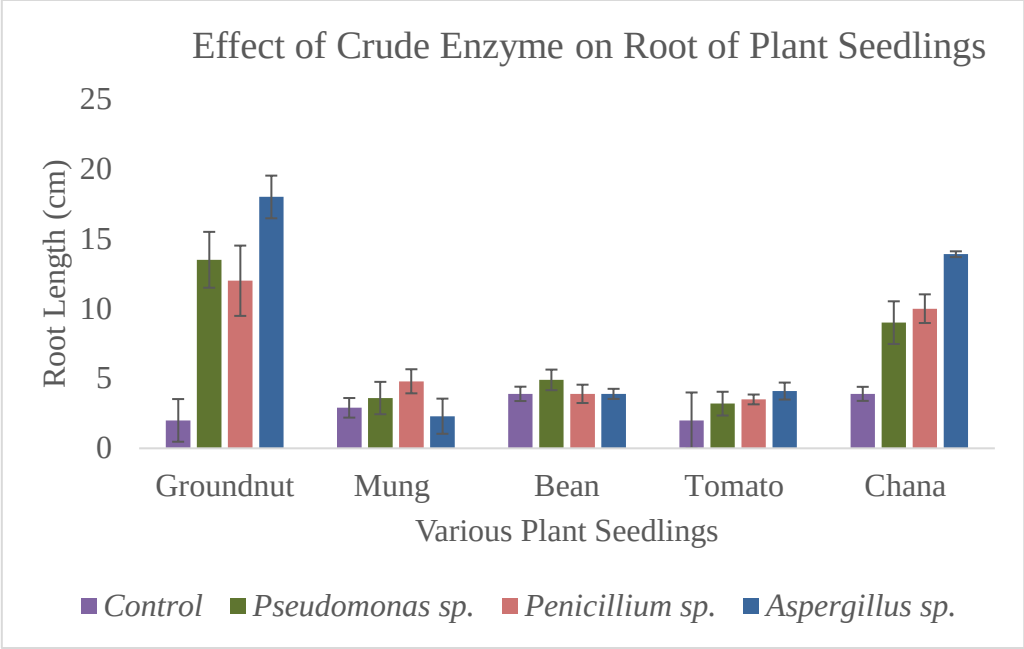


Fig. 32. Effect of Crude Enzyme on Root of Plant Seedlings

Table 19: Effect of Crude Enzyme on Shoot of Plant Seedlings

Organisms	**Parameters**	**Groundnut**	**Mung**	**Bean**	**Tomato**	**Chana**
	Control	4.8	16.0	13.2	3.2	10.9
		3.9	15.0	12.9	2.9	11.2
		5.2	18.0	14.9	4.2	9.8
	Mean	3.9	15.0	12.9	2.9	9.8
	SD	0.665	1.52752523	1.07858	0.68069	0.73711
***Pseudomonas* sp.**	Crude Enzyme	24.2	24.3	16.5	7.2	29.7
		23.7	23.9	15.8	6.7	28.9
		25.8	25.6	16.8	7.6	30.0
	Mean	23.7	23.9	15.8	6.7	28.9
	SD	1.096	0.88881944	0.51316	0.45092	0.56862
***Penicillium* sp.**	Crude Enzyme	18.5	20.0	15.4	6.5	35.6
		17.9	18.0	14.9	7.2	34.9
		19.6	22.0	15.9	5.9	35.8
	Mean	17.9	18.0	14.9	5.9	34.9
	SD	0.862	2.0	0.5	0.65064	0.47258
***Aspergillus* sp.**	Crude Enzyme	26.0	24.8	23.6	7.4	31.2
		24.0	23.9	22.8	6.5	30.8
		27.0	25.1	23.8	7.8	32.9
	Mean	24.0	23.9	22.8	6.5	30.8
	SD	1.527	0.6244998	0.52915	0.66583	1.11505

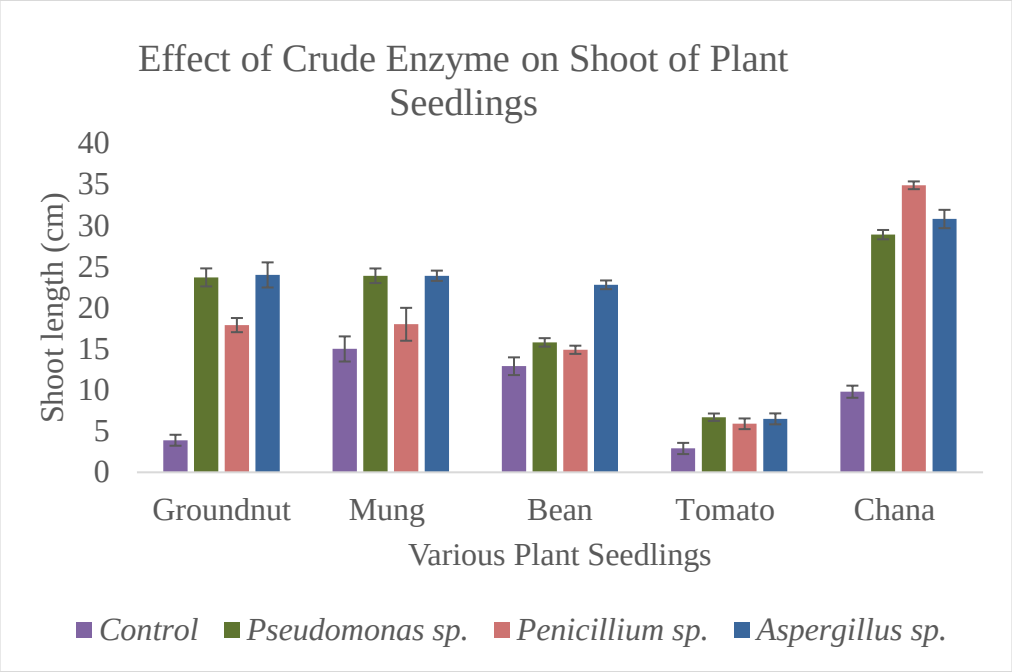


Fig. 33. Effect of Crude Enzyme on Shoot of Plant Seedlings

Table 20: Effect of Partial Purified Enzyme on Root of Plant Seedlings

	Parameters	Groundnut	Mung	Bean	Tomato	Chana
Organisms	Control	2.0	3.5	4.6	4.0	4.5
		5.0	2.9	4.2	3.9	3.7
		3.0	4.3	3.9	5.8	5.2
	Mean	2.0	2.9	3.9	3.9	3.7
	SD	1.527	0.70237692	0.35119	1.06927	0.75056
***Pseudomonas* sp.**	Partial Purified Enzyme	17.2	4.0	5.1	5.0	19.0
		16.8	3.0	5.9	4.9	18.0
		18.2	6.0	4.0	5.6	21.0
	Mean	16.8	3.0	4.0	4.9	18.0
	SD	0.721	1.52752523	0.95394	0.37859	1.52753
***Penicillium* sp.**	Partial Purified Enzyme	18.9	3.9	4.9	4.9	11.0
		17.5	2.6	3.6	3.7	10.0
		19.3	4.6	5.2	5.6	12.9
	Mean	17.5	2.6	3.6	3.7	10.0
	SD	0.945	1.01488916	0.85049	0.9609	1.47309
***Aspergillus* sp.**	Partial Purified Enzyme	18.7	3.6	4.4	4.5	13.0
		20.3	2.7	3.8	4.9	12.6
		19.3	4.6	4.2	3.5	13.8
	Mean	18.7	2.7	3.8	3.5	12.6
	SD	0.808	0.9504385	0.30551	0.72111	0.61101

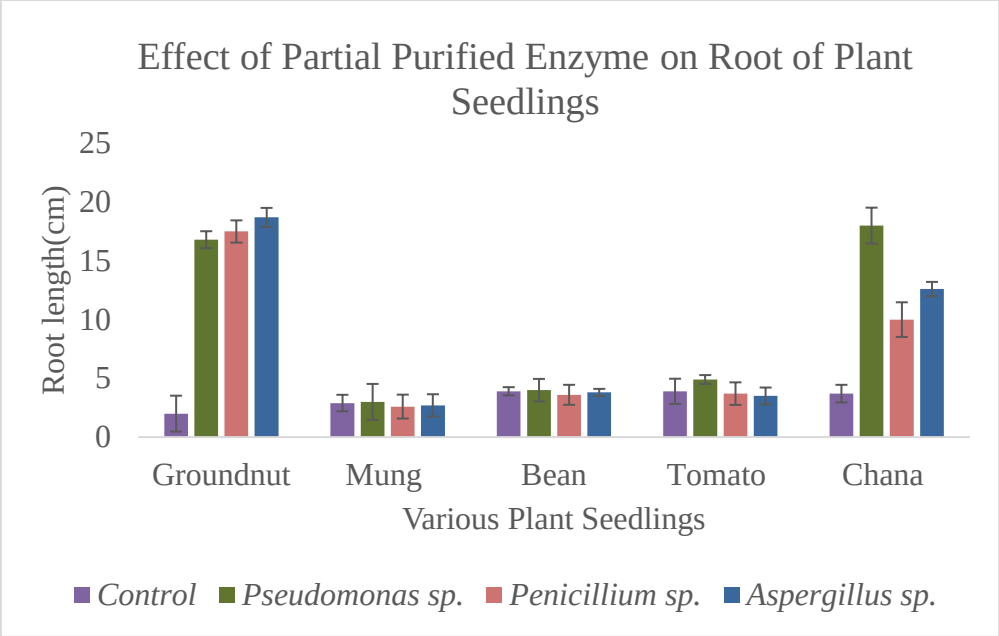


Fig. 34. Effect of Partial Purified Enzyme on Root of Plant Seedlings

Table 21: Effect of Partial Purified Enzyme on Shoot of Plant Seedlings

	Parameters	Groundnut	Mung	Bean	Tomato	Chana
Organisms	Control	4.8	16.0	13.2	3.2	10.9
		3.9	15.0	12.4	2.6	11.2
		5.2	18.0	13.8	3.7	10.6
	Mean	3.9	15.0	12.4	2.6	10.6
	SD	0.665	1.52752523	0.70238	0.55076	0.3
***Pseudomonas* sp.**	Partial Purified Enzyme	23.8	28.7	17.4	8.0	29.7
		22.9	27.9	16.8	10.0	28.9
		25.3	26.9	17.9	7.0	30.2
	Mean	22.9	26.9	16.8	7.0	28.9
	SD	1.212	0.90184995	0.55076	1.52753	0.65574
***Penicillium* sp.**	Partial Purified Enzyme	20.5	24.0	17.8	6.2	37.0
		19.8	23.0	16.5	5.9	35.0
		21.3	26.0	18.9	7.2	38.0
	Mean	19.8	23.0	16.5	5.9	35.0
	SD	0.75	1.52752523	1.20139	0.68069	1.52753
***Aspergillus* sp.**	Partial Purified Enzyme	22.5	25.0	22.2	6.5	24.7
		21.9	24.0	21.5	5.9	23.8
		23.9	27.0	23.8	7.2	25.2
	Mean	21.9	24.0	21.5	5.9	23.8
	SD	1.026	1.52752523	1.17898	0.65064	0.70946

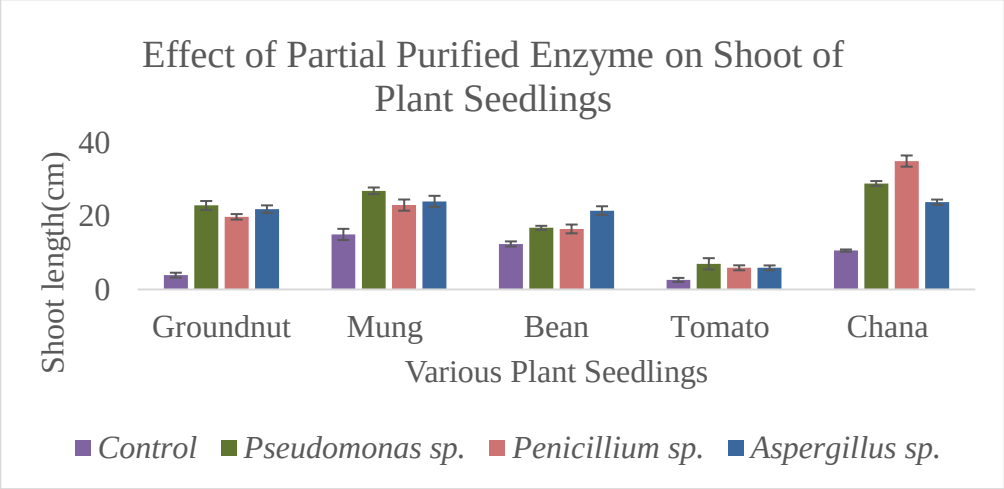


Fig. 35. Effect of Partial Purified Enzyme on Shoot of Plant Seedling

Chapter 5:- CONCLUSION

Recently, a bulk amount of phosphogypsum waste is being formed due to the high demand for phosphate fertilizers; The above study provides a sign of the existence of agricultural chief microorganisms. In-vitro pot assay gives information about plant growth promotion of plant. Alkaline phosphatase extract from the phosphate solubilizing microorganisms was thermostable up to 85ºC and active at pH 10 and high activity was found up to 70 min. Alkaline phosphatase acts as a thermostable enzyme that uses as a biofertilizer that decreases the inorganic phosphorus in soil. Phosphate solubilizing microorganisms solubilize the unobtainable phosphorus in the soil and also reestablish the nourishing status of the soil. From the above study *Pseudomonas spp.*, *Aspergillus spp.*, and *Penicillium spp.* microorganisms act as a biofertilizer for plant growth and also help for the P uptaken from the soil.

Phosphate solubilizing microorganisms (PSMs) stipulates an excellent opportunity to develop eco-friendly phosphorus biofertilizers to be used as an alternative or supplement to chemical fertilizers. Phosphate-solubilizing microorganisms can contribute to the effective use of the limited resource of phosphorus fertilizers under low-input farming systems and eco-friendly for environmental livelihood. Phosphate solubilizing microorganisms mobilized the soil's inorganic phosphate which can increase the bioavalibalitiy for plant use, improve soil fertility, increase crop productivity, and hence promote sustainable agriculture. Thus, phosphate solubilizing microorganisms (PSMs) give a wide range of possibilities for the development of conventional agriculture in different economics, localities, and cultural background.

Phosphate solubilizing microorganisms use as a biofertilizer that decreases the use of chemical fertilizer which minimizes the chemical application that is harmful to the environment. It also promotes sustainable agricultural application. Furthermore, the importance of organic matters in vermicompost technologies can underwrite to increase the inoculum's efficiency and the survival rate of bacteria and fungi advocate the seeds, which are other significant factors for fruitful inoculation. Indeed biofertilization techniques need a lesser amount of chemical effort on the soil and facilitate the combination of organic and inorganic phosphate with Phosphate solubilizers which

characterize pertinent reductions in the environmental effects associated with agricultural activities. Finally, the search for valuable bacteria and fungi is essential for the development of novel and effective inoculants for agriculture. Thus, the introduction of advantageous bacteria and fungi in the soil tends to be less violent and causes a smaller amount of impact on the environment than chemical fertilization, which makes it an inexpensive agronomical input and a mechanism to diminish the rate of production to the farming system.

ACHIEVEMENTS

Sr. No.	Name of Achievements
1.	Participated in National Conference on EMERGING PARADIGM IN AGRICULTURAL MICROBIOLOGY organised by Department of Microbiology, Atmiya University, Rajkot, Gujarat.
2.	Isolation and Identification of PGPR Traits from Soil Sample of the Saurastra Region published in INDIAN JOURNAL OF SCIENTIFIC RESEARCH.

• REFERENCES:

Afzal A, Bano A (2008) Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (Triticum aestivum L.). Int J Agric Biol 10: 85–88.

Alori, E. T., Glick, B. R. and Babalola, O. O. (2017). Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture, vol. 8, no. June, pp. 1–8.

Anderson, R.A., Bosron, W.F., Kennedy, F.S., Vallee, B.L. 1975. Role of magnesium in Escherichia coli alkaline phosphatase. Proc. Natl. Acad. Sci. U.S.A. 72, 2989– 2993.

Anonymous (1997), Fertilizer Recommendation Guide,1997. Bangladesh Agricultural Research Council (BARC), Farmgate, Dhaka-1215, Bangladesh, pp. 22-29.

Arindam, C., Mala, R.H., Roshni, R., Mohini, J., Rashmi, Y., Siddalingeshwara, K., Pramod, T., 2014. Isolation and characterization of potential plant growth promoting rhizobacteria from non-rhizospheric soil. Int. J. Curr. Microbiol. Appl. Sci. 3, 432–438.

Azziz G., Bajsa N., Haghjou T., Taulé C., Valverde A., Igual J., et al. (2012). Abundance, diversity and prospecting of culturable phosphate solubilizing bacteria on soils under crop–pasture rotations in a no-tillage regime in Uruguay. *Appl. Soil Ecol.* 61 320–326. 10.1016/j.apsoil.2011.10.004

Barret Lannerd ED, Roberson AD, Greenway BH (1982). Effect of phosphorous deficiency and water deification phosphatase activity from wheat leaves. J. Exp. Bot. 33:682-693.

Bikash Chandra Behera, Himendra Yadav , Santosh Kumar Singh, Bijay Kumar Sethi, Rashmi Ranjan Mishra , Sony Kumari , Hrudayanath Thatoi (2017). Alkaline phosphatase activity of a phosphate solubilizing Alcaligenes faecalis, isolated from Mangrove soil, Biotechnology Research and Innovation (2017) 1, 101---111, https://doi.org/10.1016/j.biori.2017.01.003.

Bisen, P.S. and Verma, K. In: “Handbook of Microbiology.” CBS publishers and distributors, New Delhi (1996).

Buainain, L.B., Kadowaki, M.K., Polizeli, M.L.T.M., Terenzi, H.F., Jorge, J.A. 1998. Characterization of a conidial alkaline phosphatase from the thermophilic fungus Humicola grisea var. thermoidea. J. Basic. Microbiol. 38, 85– 94.

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

Chuang, C.-C., Kuo, Y.-L., Chao, C.-C. and Chao, W.-L. (2007). Solubilization of inorganic phosphates and plant growth promotion by Aspergillus niger, Biology & Fertility of Soils, vol. 43, no. 5, pp. 575–584.

Coleman, J. E. (1992). Structure and mechanism of alkaline phosphatase. Annual Review of Biophysics & Biomolecular Structure, 21, 441-483.

Dadarwal ,Yadav K.S and, K.R.(1997). Phosphate solubilization and mobilization through soil microorganisms. In: biotechnological approaches in soil microorganisms for sustainable crop production (Dadarwal, K.R., Ed.)pp.293-308. Scientific Publishers, Jodhpur.

Day, D. F., & Ingram, J. M. (1973). Purification and characterization of Pseudomonas aeruginosa alkaline phosphatase. Canadian Journal of Microbiology, 19, 1225-1233

De Freitas J. R., Banerjee M. R., and Germida J. J. (1997), Phosphate solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of Canola (Brassica napus L.). Biol. Fertil. Soils 24, 358-364.

Dey K. B. (1988), Phosphate solubilizing organisms in improving fertility status. In: Biofertilizers: Potentialities and Problems (Sen S. P. and Palit P., eds.). Plant Physiology Forum, Naya Prokash, Calcutta, pp. 237-248.

Dong, G. and Zeikus, J.G. 1997. Purification and characterization of alkaline phosphatase from Thermotoga neapolitana. Enzyme Microb. Technol. 21, 335–340.

Fankem, H., Nwaga. D., Deubel, A., Dieng, L., Merbach, W. and Etoa, F. X., (2006). Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree

(Elaeisguineensis) rhizosphere in Cameroon. African Journal of Biotechnology, 5: 2450-2460.

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

Glew, R. H., & Heath, E. C. (1971). Studies on the extracellular alkaline phosphatase of Micrococcus sodonensis: Isolation and characterization. Journal of Biological Chemistry, 246, 1556-1565.

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

González, F., Esther Fárez-Vidal, M., Arias, J. M., & Montoya, E. (1994). Partial purification and biochemical properties of acid and alkaline phosphatases from Myxococcus coralloides D. Journal of Applied Bacteriology, 77, 567-573.

Guimarães, L.H.S., Terenzi, H.F., Jorge, J.A., Polizeli, M.L.T.M. 2001. Thermostable conidial and mycelial alkaline phosphatases from the thermophilic fungus Scytalidium thermophilum. J. Ind. Microbiol. Biotechnol. 27, 265–270.

Gyaneshwar P., Naresh K. G., Parekh L. J. (1998a). Effect of buffering on the phosphate solubilizing ability of microorganisms, World J. Microb. Biot. 14, 669–673.

Gyaneshwar, P., Kumar, N. J., Pareka, L. J., & Podle, P. S. (2002). Role of soil microorganisms in improving nutrition of plants. Plant & Soil, 245, 83-93.

Hong, S., Geun, P., Mi, C., & Moon, H. (2006). Solubilization of insoluble inorganic phosphate by a novel salt and pH tolerant Pantoea agglomerans R-42 isolated from soybean rhizosphere. Bioresource Technology, 97, 204-210.

Illmer, P., Barbato, A., Schinner, F., (1995). Solubilization of hardly soluble AlPO4 with P solubilizing microorganisms. Soil Biology and Biochemistry 27, 265–270.

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

Kang J., Amoozegar A., Hesterberg D., Osmond D. L. (2011). Phosphorus leaching in a sandy soil as affected by organic and incomposted cattle manure. *Geoderma* 161 194–201. 10.1016/j.geoderma.2010.1

Kannaiyan,S., Kumar,K. and Govindarajan, K. Scientific pub. (India), Jodhpur 2004.

Khan AA, Jilani G, Akhtar MS, Naqvi SMS, Rasheed M (2009a) Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J Agric Biol Sci 1 (1): 48–58.

Khan, M.S., Zaidi, A., Ahemad, M., Oves, M., Wani, P.A., 2010. Plant growth promotion by phosphate solubilizing fungi–current perspective. Arch. Agron. Soil Sci. 56 (1), 73–98.

Khan, S, Hesham, AE, Qiao, M, Rehman S, He, JZ (2010). Effects of Cd and Pb on soil microbial community structure and activities. Environ Sci Pollut Res 17: 288–296.

Li X, Luo L, Yang J, Li B, Yuan H. (2015a). Mechanisms for solubilization of various insoluble phosphates and activation of immobilized phosphates in different soils by an efficient and salinity-tolerant Aspergillus niger strain An2. Appl Biochem Biotech. 175 (5): 2755±2768.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

M. L. Dotaniya (2019) Role of Soil Enzymes in Sustainable Crop Production, Enzymes in Food Biotechnology , 2019 Elsevier Inc. All rights reserved. https://doi.org/10.1016/B978-0-12-813280-7.00033-5

Maheswar, N.U., Sathiyavani, G., 2012. Solubilization of phosphate by Bacillus Sps, from groundnut rhizosphere (Arachis hypogaea L). J. Chem. Pharma. Res. 4, 4007–4011.

Mihalache, G., Mihasan, M., Zamfirache, M.M., Stefan, M., Raus, L., 2018. Phosphate solubilizing bacteria from runner bean rhizosphere and their mechanism of action. Romanian Biotechnol. Lett. 23, 13853–13861.

Mohammed A Al-Omair (2010). Purification and biochemical characterization of acid phosphatase from Vigna aconitifolia. Am. J. Plant Physiol. 5:361-370.

Mori, S., Okamoto, M., Nishibori, M., Ichimura, M., Sakiyama, J., Endo, H. 1999. Purification and characterization of alkaline phosphatase from Bacillus stearothermophilus. Biotechnol. Appl. Biochem. 29, 235–239.

Mujahid, T.Y., Siddiqui, K., Ahmed, R., Kazmi, S.U., Ahmed, N., 2014. Isolation and partial characterization of phosphate solubilizing bacteria isolated from soil and marine samples. Pak. J. Pharm. Sci. 27, 1483–1490.

Nalini, P., Prabhakar, T., Ellaiah, P., Girijasankar, G. 2014. Isolation of alkaline phosphatase producing bacteria employing a novel screening medium for phosphatases. J. Pure Appl. Microbiol. 8, 3237–3244.

Naveen Varghese and P.P.joy, (2014) Microbiology Laboratory Manual, Kerala Agricultural University ,January 2014. Pineapple Research Station, Vazhakulam-686 670.

Nguyen, C., Yan, W., Le Tacon, F., Lapeyrie, F., 1992. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus Laccaria bicolor (Maire) PD Orton. Plant Soil 143 (2), 193–199.

Nyalemegbe, K., Oteng & Asuming-Brempong, J. (2009). Integrated organic-inorganic fertilizer management for rice production on the vertisols of the Accra Plains of Ghana, W. Afric. J. App. Ecol., 16, 23-33.

Okuda S, Kaneko J, Ogawa T, Yamaguchi T, Izaki K, Takahashi H (1987). Increase in enzyme activities in embryonic axes of soybean seeds during germination. Agric. Biol. Chem. 51:109-113.

P. Nalini, P. Ellaiah , T. Prabhakar and G. Girijasankar (2015), Microbial alkaline phosphatases in bioprocessing, International Journal of Current Microbiology and Applied Sciences ISSN: 2319-7706 Volume 4 Number 3 (2015) pp. 384-396 http://www.ijcmas.com.

Pandey, S.K. and Banik, R.M. 2011. Extractive fermentation for enhanced production of alkaline phosphatase from Bacillus licheniformis MTCC 1483 using aqueous two-phase systems. Bioresour. Technol. 102, 4226–4231.

Pantazaki, A.A., Karagiorgas, A.A., Liakopoulou-Kyriakides, M., Kyriakidis, D.A. 1998. Hyperalkaline and thermostable phosphatase in Thermus thermophilus. Appl. Biochem. Biotechnol. 75, 249–259.

Park, T., Lee, J-H., Kim, H-K., Hoe, H-S., Kwon, S-T. 1999. Nucleotide sequence of the gene for alkaline phosphatase of Thermus caldophilus GK24 and characteristics of the deduced primary structure of the enzyme. FEMS Microbiol. Lett. 180, 133–139.

Patil, V., 2014. Bacillus subtilis: A potential salt tolerant phosphate solubilizing bacterial agent. Int. J. Life Sci. Biotechnol. Pharma. Res. 3, 141.

Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Microbiology. 17, 362–370.

Poirier, T. P., & Holt, S. C. (1983). Acid and alkaline phosphatases of Capnocytophaga species. II. Isolation, purification, and characterization of the enzymes from Capnocytophaga ochracea. Canadian Journal of Microbiology, 9(10), 1361-1368.

Prasanna, R., Joshi, M., Rana, A., Shivay, Y. S., & Nain, L. (2011). Influence of co-inoculation of bacteria-cyanobacteria on crop yield and C-N sequestration in soil under rice crop. World Journal of Microbiology & Biotechnology, 28, 1223-12.

Renu Gupta.,2021. Identification, characterization and optimization of phosphate solubilizing rhizobacteria (PSRB) from rice rhizosphere, Saudi Journal of Biological Sciences 29 (2022) 35–42.

Richardson A. E. (1994), Soil microorganisms and phosphorus availability. In: Soil Biota, Management in Sustainable Farming Systems (Pankhurst C. E., Doube B. M., Grupta V. V. S. R., and Grace P. R., eds). CSIRO, Melbourne, Australia, pp. 50-62.

Richardson A. E. (2001), Prospects for using soil micro-organism to improve the acquisition of phosphorus by plants. Aust. J. Plant Physiol. 28, 897-906.

Rodrı´guez H. and Fraga R. (1999), Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 17, 319-339.

Rodriguez, H. and Fraga, R. (1999) Phosphate Solubilizing Bacteria and Their Role in Plant Growth Promotion. Journal of Biotechnology Advances, 17, 319-339. http://dx.doi.org/10.1016/S0734-9750(99)00014-2

Roy M, Srivastava RC (2013) Assembling BNF system in rice plant: frontier areas of research. Curr Sci 2013, 104: 3–10.

Scheffer F, Schachtschabel P. Lehrbuch der Bodenkunde. Stuttgart: Enke, (1998).The role of rock phosphate solubilizing fungi and vesicular arbuscularmycorrhiza (VAM).

Serpil Savci (2012) An Agricultural Pollutant: Chemical Fertilizer International Journal of Environmental Science and Development, Vol. 3, No. 1, February 2012

Shahbazkia HR, Sharifi S, Shareghi B (2009). Purification and kinetic study of bone and liver alkaline phosphatase isoenzymes in the dog. Comp. Clin. Pathol. 19:81-84.

Sharma P, Padh H, Shrivastava N (2013) Hairy root cultures: a suitable biological system for studying secondary metabolic pathways in plants. Eng Life Sci 13: 62–75.

Sharma, K. In: Manual of Microbiology. Isolation, Purification and Identification of Bacteria. Ane Books Pub. New Delhi, p. 41 2005

Shrivastava, U.P., 2013. Isolation and initial characterization of diazotrophic plant growth promoting rhizobacteria (PGPR) from rice rhizosphere of Parsa and Bara district of Nepal. Int. J. Pharm. Life Sci. 4.

Shruti, K., Arun, K., Yuvneet, R., 2013. Potential plant growth-promoting activity of rhizobacteria Pseudomonas sp in Oryza sativa. J. Nat. Prod. Plant Resour. 3, 38– 50.

Simpson, R.T., Vallee, B.L., Tait, G.H. 1968. Alkaline phosphatase of Escherichia coli. Composition. Biochemistry 7, 4336–4342.

Solangi, M. K., Solangi, S. K., Solangi, N. K., (2016). Grain yield, phosphorus content and uptake of wheat (Triticum aestivum L.) as affected by phosphorus fertigation. Pak. J. Biotechnol. 13, 205–209.

Sonam Sharma (2011) Isolation of Phosphate Solubilizing Microorganism (PSMs) From Soil, Journal of Microbiology and Biotechnology Research, 1 (2): 90-95

Sonam Sharma, Vijay Kumar and Ram Babu Tripathi (2011), Isolation of Phosphate Solubilizing Microorganism (PSMs) From Soil, Institute of Biomedical Education and Research, Mangalayatan University, Beswan- Aligarh, U.P. India, 2011, 1 (2): 90-95.

Tabatabi, M. A., & Bremner, J. M. (1969). Use of P-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology & Biochemistry, 1, 301-307.

Tabatabi, M. A., & Bremner, J. M. (1969). Use of P-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology & Biochemistry, 1, 301-307.

Th. Surchandra, S. S. Roy, N. Rakesh Singh, M. R. Sahoo and N. Prakash (2012). Partial purification and biochemical characterization of acid phosphatase from germinated mung bean (Vigna radiata) seeds, ICAR Research Complex for North Eastern Hill Region Manipur Centre, Lamphelpat, Imphal-795004, India.16 November, 2012, African Journal of Biotechnology Vol. 11(103), pp. 16777-16782, DOI: 10.5897/AJB12.2552.

Thakuria D., Talukdar N. C., Goswami C., Hazarika S., Boro R. C., and Khan M. R. (2004), Characterization and screening of bacteria from rhizosphere of rice grown in acidic soils of Assam. Curr. Sci. 86, 978-985.

Toro, M., Azcon, R., Barea, J. M., (1997). Improvement of Arbuscular Mycorrhizadevelopment by inoculation of soil with phosphate solubilizing rhizobacteriato improve rock phosphate bioavailability (p32) and nutrient cycling. Appl. Environ. Microbiol. 63 (11), 4408–4412.

Ul qader, S. A., Iqbal, S., & Niazi, Z. (2009). Partial purification and characterization of intracellular alkaline phosphatase from newly isolated strain of Bacillus subtilis KIBGE-HAS. The International Journal of Microbiology, 7, 1-9.

Vessey J. K. (2003), Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571-586.

Walpola, B. C., & Yoon, Min-Ho. (2013). In vitro solubilisation of inorganic phosphates by phosphate solubilizing microorganisms. African Journal of Microbiology Research, 7(27), 3534-4354.

Yadav K. S. and Dadarwal K. R. (1997), Phosphate solubilization and mobilization through soil microorganisms. In: Biotechnological Approaches in Soil Microorganisms for Sustainable Crop Production (Dadarwal R. K., ed.). Scientific Publishers, Jodhpur, India, pp. 293-308.

Zaheer, A., Mirza, B. S., Mclean, J. E., Yasmin, S., Shah, T. M., Malik, K. A., Mirza, M. S., (2016). Association of plant growth-promoting Serratia spp. with the root nodules of chickpea. Res. Microbiol. 167, 510–520.

Zhu F., Qu L., Hong X., Sun X. (2011). Isolation and characterization of a phosphate solubilizing halophilic bacterium *Kushneria* sp. YCWA18 from Daqiao Saltern on the coast of yellow sea of China. *Evid. Based Complement. Alternat. Med.* 2011:615032 10.1155/2011/615032