MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM PADDY FIELDS IN MIZORAM

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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IN PARTIAL FULFILMENT OF THE REQUIREMENS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANY OF MIZORAM UNIVERSITY, AIZAWL

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CERTIFICATE

This is to certify that the thesis work entitled**, "Molecular and Biochemical Characterization of Phosphate Solubilizing Bacteria from Paddy Fields in Mizoram"**, submitted by Lalrampani Chawngthu (**MZU/Ph.D./619 of 23.05.2014**) in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Botany is a record of bonafide work carried out by her under my supervision and guidance.

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DECLARATION BY THE CANDIDATE

I, **Lalrampani Chawngthu**, hereby declare that the subject matter of this thesis entitled "**Molecular and Biochemical Characterization of Phosphate Solubilizing Bacteria From Paddy Fields in Mizoram**" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of ***Doctor of Philosophy*** in Botany.

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Preface

Due to the uncontrolled over-application of chemical fertilizers by farmers during intensive agricultural practices has led to excess nutrients accumulation in soils particularly phosphorous, which, as a result, makes the soils more lifeless. That is why, at the present time, major research interest lies in the production of competent and sustainable biofertilizers for crop plants, wherein inorganic fertilizer application can be reduced significantly to avoid further pollution problems. Development of microbes as biofertilizers is considered to some extent an alternative to chemical fertilizers because of their eco-friendly, non-hazardous, nontoxic nature and due to their extensive potentiality in enhancing crop production and food safety in agricultural sector.

Phosphorus is a vital macro- nutrient element which is essential for plant nutrition. Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular applications of chemical fertilizers. However, a huge proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to the plants. Out of added phosphorus fertilizer only 10-20% is available for the plants. The rest remains in the soil as insoluble phosphate in the form of rock phosphate and tri-calcium phosphate. In such cases, it is crucial to implement the use of an eco-friendly fertilizer which can help in P solubilization. Phosphate solubilizing Bacteria (PSB) significantly helps in the release of this insoluble inorganic phosphate and makes it available to the plants. PSB have been introduced to agricultural community as phosphate 'Biofertilizer'.

This study is primarily designed by focussing on the emergence and development of biofertilizers and taking notes on the lack of studies in the relevant subject in Mizoram state, India, assessment of location specific biofertilizer with special context to phosphorous has been undertaken. The thesis is broadly categorized into nine chapters. Chapter 1 and 2 includes the general introduction and literature review respectively. Chapter 3 deals with the study of the physico-chemical

and biochemical properties of the soil collected from the study site which is a paddy field where rice is grown during the khariff cropping season. Chapter 4 deals with the morphological study by biochemical characterization and the evaluation of the plant growth promoting ability by the isolates. Chapter 5 includes the molecular characterization of the isolated phosphate solubilzing bacteria using the 16srRNA gene sequence. The next chapter, chapter 6 contains the study of the mechanism of the phosphate solubilizers which is the ability to produce organic acids. This is done by quantitative analysis of organic acids by using UHPLC. This chapter also includes evaluation of the ability of the isolates to solubilize inorganic phosphorous quantitatively.

Chapter 7 covers the assessment of the selected PSB strains against two xenobiotics; an insecticide and a herbicide. The level of tolerance and alteration in terms of growth, enzyme acid phosphatase activity, the phytohormone IAA production and how the xenobiotics affect the phosphate solubilizing capacity were estimated. Chapter 8 deals with the study of the plant growth promoting ability of selected and identified PSB on the local rice variety. The parameters of this study includes, seed germination, the growth level of the seedlings and chlorophyll content of the rice plant which were monitored at 15 and 30 days. This experiment is undertaken to proof the evidence that the identified PSB strains were able to promote the growth of rice plant and can be used as a location specific biofertilizer thus, beneficially substituting the use of chemical fertilizer. Lastly, the thesis is concluded in chapter 9, summarizing all the findings and novel of work undertaken.

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ABBREVIATIONS

μM	micromolar
μl	microlitre
Al	Aluminium
AN	Available Nitrogen
ANOVA	Analysis of variance
AK	Available potassium
AP	Available phosphorus
APase	Acid phosphatase
BD	Bulk density
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Celsius
CFU	Colony Forming Unit
cm	centimeter
CTAB	Cetyl Trimethyl Ammonium Bromide
DAS	Days after sowing
DAI	Days after inoculation
DHA	Dehydrogenase
dNTPs	Deoxyribonucleotide triphosphate

EC	Electrical conductivity
EDTA	Ethylene diamine tetraacetic acid
Fig.	Figure
g	gram
ha	hectare
hrs	hours
HCN	Hydrogen cyanide
HPLC	High performance liquefied chromatography
IAA	Indole acetic acid
in	inch
K	Potassium
kg	kilogram
L	Litre
M	Molar
mg	miligram
ml	mililiter
mm	milimeter
mM	Milimolar
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
MSL	Mean sea level

MT	Metric Ton
MUB	Modified universal buffer
N	Nitrogen
N_2	Dinitrogen
NH_3	Ammonia
NRCSS	Natural resources conservation services soil
OD	Optical density
OA	Organic acid
ONPG	O-nitrophenyl-beta-D-galactopyranoside
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
p-NPP	p-nitrophenyl phosphate
pmol	picomole
ppm	parts per million
rDNA	ribosomal Deoxyribo nucleic acid
RNA	Ribo nucleic acid
rRNA	ribosomal Ribo nucleic acid
rpm	revolutions per minute
RT	retention time
Secs	seconds
SOC	Soil organic carbon

SOM	Soil organic matter
SMC	Soil moisture content
sp.	species
sq cm	square centimetre
TN	Total nitrogen
U	unit
UHPLC	High performance liquefied chromatography
URES	urease
USDA	United states department of Agriculture
UV-vis	Ultraviolet ray visible
v/v	volume by volume
w/v	weight by volume
WRC	Wet rice cultivation
yr	year

Chapter 1
Introduction

Phosphorus (P) is a primary essential nutrient which plays both metabolic and structural functions in plants. It is an essential plant nutrient second subsequent to nitrogen which is also involved in energy transfer mechanism in the biological system. Phosphorus is a major growth-limiting nutrient, which has fewer atmospheric sources as compared to nitrogen that can be made biologically available (Ezawa *et al*., 2002). It is thus required in optimum amount for proper plant growth. It is involved in important cellular functions, metabolic pathways like photosynthesis, biological oxidation, nutrient uptake and cell division for the growth and development of plants (Illmer and Schinner, 1992). It is also considered an important role in balanced nutrition of plants and have a direct relationship with straw strength, root proliferation, formation of grain, maturation and quality of a crop. The major physiological role of P involves in certain essential steps in accumulation and release of energy during cellular metabolism (Alexander, 1977) and also plays an important role in nitrogen fixation in leguminous plants (Saber *et al*., 2005). Phosphorus is the 11th most naturally occurring abundant element in the Earth's crust, water, and all living organisms and is one of 16 elements that are essential for plant growth in modern agriculture.

Phosphorus in soil is immobilized or becomes less soluble either by chemical precipitation, adsorption or both. The status of Indian soils is characterized by poor and medium with respect to the available phosphorous (Baby, 2002; Li *et al.*, 2003; Ramanathan *et al.,* 2004). In most agricultural soils P is contained in large reserves in which a substantial part has accumulated as a consequence of regular applications of chemical fertilizers. However, a large proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to plants (Rodriguez and Fraga, 1999; Arpana *et al.,* 2002). Although the average P content of soil is about 0.05% (W/W), only 0.1 % of the total phosphorus exists in plant accessible form (Illmer and Schinner, 1995). Inorganic P supplements the Worldwide soil as chemical fertilizers to support crop production but repeated use of fertilizers deteriorates soil quality (Tewari *et*

al., 2004). Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total. 'Over phosphatization' of soil has occurred thus causing global ecological problem of agriculture and forestry.

Microorganisms as phosphorus biofertilizers can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Ryu *et al*., 2006). Phosphate solubilizing micro-organisms are those microorganisms capable of converting insoluble form of mineral phosphates into primary and secondary orthophosphate and are present in almost all types of soil. A large number of bacteria, cynobacteria and actinomycetes are known to solubilize phosphate (Yadav and Dadarwal, 1997). Several soil microorganisms, notably species of *Bacillus, Pseudomonas, Penicillium, Aspergillus* etc. lowers the pH by secreting organic acids to bring about dissolution of bound phosphates in soil (Gerretsen, 1948; Sundara Rao and Sinha, 1963; Gaur and Ostwal, 1972). The phosphate-solubilizing bacteria have the ability to convert insoluble Phosphorous to soluble form by releasing organic acids, chelation and ion exchange and thus increases the soil fertility (Omar, 1998; Narula *et al*., 2000; Whitelaw, 2000). Organic acids produced by PSB solubilize insoluble phosphates by lowering the pH and competing with phosphate for adsorption sites in the soil (Nahas, 1996). Besides organic acid production, the enzyme phosphatase has a role in P-solubilization (Al-Ghazali *et al.,* 1986). Bacteria also produces indole acetic acid which is known to promote extensive root architecture that are capable of absorbing nutrient elements efficiently from the surroundings which ultimately improves rice growth (Naher, 2009, 2011).

Some powerful phosphate solubilizers come from the genera *Pseudomonas, Bacillus* and *Rhizobium* (Rodriguez and Fraga, 1999). Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and

mineralization (Hilda and Fraga, 1999). Plant growth-promoting rhizobacteria (PGPR); the bacteria that colonizes the rhizosphere and plant roots, and enhance plant growth by any mechanism have been applied to various crops to enhance growth, seed emergence and crop yield, and some have also been commercialized (Dey *et al*., 2004; Herman *et al*., 2008; Minorsky, 2008). The use of PGPR as biofertilizer has become a promising tool for improving primary production lowering the inputs of chemical fertilizers through any possible mechanisms such as nitrogen fixation, nutrient mobilization, biocontrol or phytohormone production (Glick, 1995). Therefore, P-solubilization ability of micro-organisms is considered to be one of the most important traits associated with plant P nutrition. The characteristic study of phosphate solubilization is rather complex because they belong to a diverse group not closely related under a phylogenetic point of view. Therefore, good molecular techniques based on nucleic acid composition are needed to perform the analysis and identification of phosphorus solubilizing microorganisms.

One of the advances realized in the past decade or so has been the analysis of the nucleotide sequences of the 16S ribosomal RNA gene (16SrDNA), which has emerged as the single best method to identify bacteria (Kolbert and Persing, 1999; Drancourt *et al*., 2000). As more 16SrDNAs were sequenced and studied, it was realized that (1) the nucleotide sequences among various bacteria are highly conserved; (2) the conservation and divergence reflect bacterial evolution; and (3) each bacterial species has its unique 16SrDNA sequences (Fox *et al*., 1980). So, the use of 16SrRNA gene sequences to study bacterial phylogeny and taxonomy has become the most common housekeeping genetic marker used. In reference to Patel (2001) the reason of 16srRNA being used as a genetic marker are (i) its presence in almost all bacteria, often existing as a multigene family, or operons (ii) the 16SrRNA gene (1,500 bp) is large enough for informatics purposes; and (iii) The function of the 16SrRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution). The invention of polymerase chain reaction (PCR) technology in the mid-1980s has become popular for its amplification power, speed and simplicity and its application for bacterial 16SrDNA has flourished. The entire 16SrDNA or a portion of it may be amplified by

PCR using a machine called Thermal cycler. Conserved regions of 16SrDNA allow design of highly conserved primers for nearly universal amplification of most bacterial species (Greisen *et al*., 1994; Han *et al*., 2002). The steps that are required to reach bacterial identification through 16SrDNA sequencing include DNA extraction, PCR amplification, nucleotide sequencing, and database homology search and reporting.

The test of the relative efficiency of isolated strains is carried out by selecting the microorganisms that are capable of producing a halo/clear zone on a specialized culture medium for Phosphate solubilizers on a plate owing to the production of organic acids into the surrounding medium (Katznelson *et al.*, 1962). In recent years**,** biofertilizers has emerged as promising components of integrating nutrient supply system in agriculture. Our whole system of agriculture depends in many important ways, on microbial activities and there appears to be a tremendous potential for making use of microorganisms in increasing crop production. Biofertilizers are composting the area with the objective of increasing the number of such microorganisms and accelerate microbial process to augment to extent of the availability of the nutrient in a form which can easily be assimilated by plants (Subba-Rao, 1986). Thus, isolation and identification of plant growth promoting microorganisms and to use it as fertilizers are an important part of environment friendly sustainable agricultures practices (Bloemberg *et al*., 2000).

Soil is a complex matter which comprises of biotic and abiotic components of which there is a strict relationship between them. Soil microbial occurrence depends mainly on the soil physical and chemical parameters, which sequentially brings out the structural stability and fertility (Ketterings *et al*., 1997). Several agro ecosystems are inclined by the strength and distribution of a variety of living organisms (Paoletti, 1999; Narayan and Gupta, 2018). The microbial community is influenced directly by every parameter of soil and thereby resulting into sustainability in agriculture (Ivask *et al*., 2008). The atmospheric boundary layer plays a crucial role in regulating weather and climate by exchanging huge quantity of surface water and energy with the overlying atmosphere (Zhang *et al*., 2004). In this context, the seasonal rainfall variability affects the variability in soil moisture, which influences the wetness/dryness of monsoon season (Douville, 2002). Monitoring the soil quality is

very essential in assessing land sustainability practices in ecosystems (Carter, 2002). Doran and Zeiss (2002) defined soil quality as “the capability of soil to function as an essential living system, within ecosystems and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health.” So, since soil functions as the key source for the production of food, and interrelates with the surrounding environment, the enhancement and preservation of the soil quality is crucial for sustaining land productivity under different land uses and management (Unger *et al*., 1991). Soil organic carbon (SOC) is a key attribute in maintaining soil tilt and quality and is an energy source for microorganisms in soils. It also influences other soil functions, such as the charge characteristics, aggregate stability, water holding capacity, and many more (Lal *et al*., 2004) (Gregorich *et al*., 1994). A variety of factors can affect SOC dynamics, while soil enzyme activities and soil physico-chemical properties are more important among these factors and more easily determined (Chandra *et al*., 2016; García-Ruiz *et al*., 2009; Robertson *et al*., 2016). Soil enzymes play important role in nutrient cycling in soil ecosystems (Burns *et al*., 2013; Hill *et al*., 2009). The types and amount of enzymes depend on the soil quality and environmental conditions; hence, enzyme activities can be used as good indicators for soil fertility in different ecosystems.

The nutrient level of the soil can also be decreased by the addition of xenobiotics which can thus lead to decrease in microbial community. Xenobiotics are by definition unnatural compounds (e.g. pesticides, industrial wastes, chemical fertilizers etc) which include naturally occurring compounds (e.g. heavy metals) that are synthesized or are present in unnaturally high concentrations in the environment (Skladany and Metting, 1993). Such compounds are of crucial concern in the soil environment as they could affect many biological and biochemical reactions in soils (Dick, 1997).

The present study will focus on diversity of phosphate solubilizing bacteria based on their molecular and biochemical characteristics by using 16srRNA gene profiling from the wetland paddy field of Serchhip District, Mizoram. Further, the investigation will find out the effect of xenobiotic action on the biomass and phosphatase activity of selected isolated strains. Organic acids production by the

isolates will also be determined. Overall, plant growth promotion activity will be monitored by inoculating the local rice seeds with the selected bacterial isolates.

Noting and taking all these background facts into account, the major goal of the present investigation was designed with five objectives. These objectives include the following points:

1. To isolate phosphate solubilizing bacteria from paddy field soil.
2. To perform biochemical characterization and 16SrRNA gene profiling of phosphate solubilizing bacteria.
3. To quantify organic acid produced by the selected isolates by HPLC.
4. To study the influence of two xenobiotics on phosphate solubilizing activity of the selected isolates
5. To evaluate the effect of the PSB strains on the growth of rice plant

Chapter 2

Review of Literature

2.1. Phosphorous and its availability to plants

In the earth lithosphere, the content of phosphorous (P) is around 0.12% and in surface soils, the total content of P varies from 0.02 to 0.15% with an average of 0.06% (Lindsay, 1979). About 20–80% of total soil P in organic form is present in mineral soils, whereas the rest is in inorganic form. Inorganic soil phosphorous in the soil solution may exist both as non occluded and occluded form; where; non occluded are those which are present as exchangeable and adsorbed form in soil solution and present in minerals. Occluded P is those held by Fe and Al oxides and oxyhydroxides. The primary and secondary P minerals released enters the soil and can be absorbed by plants or precipitated as secondary P minerals or can be used by soil microbes or even lost due to leaching or runoff. Phosphorus in soil solution may range from 0.02 to 5.46 mg PL^{-1} (Morel and Plenchette, 1994) with an average value around 0.05 mg P L^{-1}. Most soil possesses considerable amounts of P, but a large proportion is bound to soil constituents. Soil having low total P can be enhanced with the help of P fertilizer but are not able to hold the added P. About 75–90% of the added chemical P fertilizer is precipitated by metal cation complexes and rapidly becomes fixed in soils and has long-term impacts on the environment in terms of eutrophication, soil fertility depletion, and carbon footprint (Sharma *et al.*, 2013). P cycle in the biosphere can be described as "sedimentary," as unlike in the case for nitrogen, there is no interchange with the atmosphere, and no large atmospheric source can be made biologically available (Walpola and Yoon, 2012; Rodriguez and Fraga, 1999).

Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants (Sagervanshi *et al.*, 2012). Plant cell can take up several P forms, but the greatest part is absorbed in the forms of

phosphate anions mainly $HPO4^{2-}$ or $H_2PO_4^{\ -}$ depending upon the soil pH (Mahidi *et al.*, 2011; Kumar *et al.*, 2018; Satyaprakash *et al.*, 2017). P is important for the functioning of key enzymes that regulate the metabolic pathways of a plant. P availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium. Since deficiency of P severely restricts the growth and yield of crops (Walpola and Yoon, 2012). Chemical phosphatic fertilizers are therefore widely used to achieve optimum yields. P fertilizers of soluble forms are effortlessly precipitated as insoluble forms, this directs to extreme and frequent application of P fertilizer to plantation area (Alam *et al*., 2002). Phosphorus nutrition has become a global issue and 30-40% crop yield of the world‘s cultivation land is hampered by the P availability (Rudresh *et al.*, 2005)

The first systematic soil fertility map of India was given in 1967 by Ramamurthy and Bajaj in 1969 which indicates that 4% samples were high in available P. However, the soil fertility map published in 2002 by Motsara indicates about 20% of the soil samples are high in available P which indicates the accumulation of P in soil. In recent 2011, GIS based district-wise soil fertility map of India showed that soil of around 51% districts were low, 40% were medium and 9% were high in available P in India (Muralidharudu *et al.,* 2011). This may be due to the non-judicious use of phosphatic fertilizers by the farmers and its subsequent fixation and accumulation in agricultural soil (Richardson, 2004).

The role of P in crop production systems is exemplified by the amount of fertilizer-P used during the last 35 years, which has doubled since 1960, stabilizing at slightly under two million tons/year over the last 10 years (Tirado and Allsopp, 2012). When P is added as fertilizer to the soil, it gets fixed. The soil microorganisms solubilize this fixed phosphates and converts into a form available to the plants. Such process is referred to as mineral phosphate solubilization (MPS) and microbes which solubilize it is called P-solublizing microorganism (PSM).

Plants utilize only a small portion of phosphatic fertilizer when added to the soil (Tallapragada and Seshachala, 2010). The requirement of P varies considerably in plants. Tree require lowest among all plants with critical values ranging from 0.12 to 0.15% while grasses have higher requirement from 0.20 to 0.25 %, legumes and some vegetable crops require higher values from 0.25 to 0.30% or even higher (Saha and Biswas, 2009). The accumulated phosphates in agricultural soils are adequate to maintain maximum crop yields worldwide for about 100 years (Walpola and Yoon, 2012) if it could be mobilized and are converted into soluble P forms using of phosphate solubilizing microorganisms. The microorganisms in the rhizosphere region play significant role in improvement of soil productivity by solubilizing inorganic P due to production of organic acids, causing changes in soil pH, producing chelating substances, inorganic acids H_2S and CO_2, humic substances (Halvorson *et al.,* 1990; Illmer and Schinner, 1992). A major concern has, therefore, been made to get an economically low-priced technology and eco-friendly system that could supply adequate P to plants for a long run.

2.2. Phosphate solubilizing bacteria – Its diversity

Soil is a natural basal media for growth of microorganisms. Mostly, one gram of fertile soil contains 10^1 to 10^{10} bacteria, and their live weight may exceed 2,000 kg ha^{-1} (Khan *et al.,* 2009). Among the whole microbial population in soil, P solubilizing bacteria comprise 1–50% and P solubilizing fungi 0.1 to 0.5% of the total respective population (Khan *et al.,* 2009; Walpola and Yoon, 2012; Chen *et al*., 2006). PSMs are known to be metabolically active in the rhizosphere region of the soil (Selvi *et al*., 2017). Phosphate solubilizing microorganisms (PSMs) constitutes a group of beneficial microorganisms that are capable of hydrolyzing organic and inorganic phosphorus compounds from insoluble compounds. The existence of soil microorganisms comprising of bacteria, actinomycetes and some fungi that can solubilize soil precipitated or soil attached phosphate has been reported previously (Reyes *et al.*, 2001). Among these PSMs, many researchers have reported strains from bacterial genera (*Bacillus, Pseudomonas, Enterobacter* and *Rhizobium*) (Whitelaw, 2000), fungal genera (*Penicillium* and *Aspergillus*), actinomycetes, and arbuscular mycorrhizal (AM) are notable (Sharma *et al.*, 2013; Satyaprakash, 2017;

Chen *et al*., 2006; Thakur *et al*., 2014; Hajjam, 2017). According to Khan *et al.* (2007), Phosphorus solubilization by the rhizospheric microbes dates back to 1903. Most of the reported PSB belong *to Pseudomonas, Bacillus, Enterobacter, Rhizobium, Mesorhizobium, Burkholderia, Azotobacter*, *Azospirillum* and *Erwinia* genera and the following are the most widely studied species *Pseudomonas* sp., *Bacillus firmus* (Banik and Dey, 1982) . The bacteria like *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata*, and *Enterobacter* are often most referred PSM (Subbarao, 1988; Kucey *et al*., 1989). Studies revealed that inoculation of PSM's enhanced the crop yields by solubilizing the soil fixed and applied phosphates. Species of the genus *Bacillus*, *Pseudomonas, Rhizobium, Aspergillus* and *Penicillium* are the potential P-solubilzers commonly present in the soil (Rodriguez and Fraga, 1999). The variability in the *in situ* performance of phosphate solubilizing microorganisms (PSM) has greatly hampered the large-scale application of PSM in sustainable agriculture. Numerous reasons have been suggested for this, but none of them have been conclusively investigated. Despite the variations in their performance, PSM are widely applied in agronomic practices in order to increase the productivity of crops while maintaining the health of soils (Khan *et al*., 2007).

Kumar *et al.* (2010) isolated six phosphate solubilizing bacteria (PSB) from paddy fields of Eastern Uttar Pradesh, India harboring low available phosphorus. Taxonomic delineation employing morphological, biochemical, 16SrRNA gene sequences and phylogenetic affiliations suggests that they are members of *Enterobacter* and *Exiguobacterium* genera. Igual *et al.* (2001) reported that among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli* and endosymbiotic rhizobia have been described as effective phosphate solubilizers. Sharma *et al.* (2007) isolated two strains of phosphate solubilizing bacteria from soil sample and were identified as *Pseudomonas fluorescens* and *Bacillus megaterium* on the basis of their morphological, cultural and biochemical reactions. Twelve efficient PSB isolates were selected from the colonies based on their ability to form clear zone on Pikovskaya's agar medium from maize, onion, jasmine, and tomato rhizosphere soils from four different localities of Salem, Tamil Nadu India (Ranjan *et al*., 2013). In addition to *Pseudomonas* and *Bacillus*, other

bacteria reported as P-solubilizers include *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium, Gordonia*, *Phyllobacterium*, *Delftia* sp. (Wani *et al*., 2007; Chen *et al*., 2006)

2.3. Mechanism of Phosphate solubilization:

P-solubilization is the biological process of conversion of fixed form of inorganic phosphorous into primary ($H_2PO_4^-$) and secondary orthophosphate (HPO_4^{-2}) (Goldstein, 1986). There are two components of P in soil, organic and inorganic phosphates. The insoluble form comprises of the larger proportion and therefore, is unavailable for plant nutrition. Inorganic P in the soil occurs mostly in insoluble mineral complexes, some appears after application of chemical fertilizers and the precipitated form cannot be taken up by plants. On the other hand, organic matter accounts for 20–80% of soil P which is an important reservoir of immobilized P (Richardson, 1994a).

The release of soil solid P into the soil solution involves these major processes: dissolution-precipitation, sorption-desorption, and mineralization-immobilization (Sims and Pierzynski, 2005). Microorganisms in the soil effect solubilization of P through the release of complexing or mineral dissolving compounds e.g. organic acid anions, siderophores, H^+, OH^-, and CO_2, release of extracellular enzymes like phosphatases, and release of P during organic matter decomposition (McGill and Cole, 1981). The production of organic acids by microorganisms is the one of the mechanism for the solubilization process which is operational by several points: (1) lowering the pH in rhizosphere (2) precipitation of P for chelating of the cations, (3) competing with P for sorption sites on the soil and (4) forming of soluble complexes with the metal ions associated with insoluble P compounds like phosphates of Ca, Al, Fe. The reason for the lowering of pH in rhizosphere is due to the release of these organic acids (Whitelaw, 2000; Maliha *et al*., 2004) due to the direct oxidation respiratory pathway that is operative on the outer surface of the cytoplasmic membrane (Zaidi *et al*., 2009).

Soil organic P mineralization accounts to about 4–90% of the total soil P (Khan *et al*., 2009b) which takes part in the role of cycling of P in an agricultural system. Organic phosphorous compound mineralization depends mainly on the

physicochemical and biochemical properties of the organic P compounds. Soil organic P compounds that are mainly related to clay particles are simply mineralized as a fraction of them is easily accessible for fast microbial usage (Ruback *et al.*, 1999). There are also some enzymes which causes the mineralization of organic phosphorus in soil. Acid phosphatase is an enzyme that causes dephosphorylation of soil organic P compounds by breaking phosphoester or phosphoanhydride bonds. Acidic and alkaline phosphatases are the most abundant in nature among different phosphatase enzyme classes produced by PSM (Nannipieri *et al.*, 2011). Acid phosphatases are dominant in acid soils, while alkaline phosphatases are more abundant in neutral and alkaline soils (Eivazi and Tabatabai, 1977). In distinction to phosphatases made by plants, the enzyme phosphatase of microbial origin possesses a larger affinity for organic P compounds (Tarafdar *et al.*, 2001).

As reported by Altomare *et al.* (1999) a fungus *T. Harzianum* (T-22) solubilized insoluble rock phosphate P in *in vitro* cultures but there was no organic acids detected in the culture filtrates. The P solubilizing activity of *T. Harzianum* (T-22) was characterised to both reduction and chelation processes. This shows that acidification alone may not be the universal mechanism of solubilization. Inorganic acids such as sulphuric, nitric and carbonic acid are considered as other responsible acids for phosphate solubilization produced by some strains (Fankem *et al.*, 2006). As reported by many researchers some bacteria like *Nitrosomonas* and *Thiobacillus* species produce nitric and sulfuric acids which can also effect solubilization of insoluble phosphate compounds (Azam and Memon, 1996; Masood and Bano, 2016; Meena *et al.*, 2015e, 2016e; Teotia *et al.*, 2016; Bahadur *et al.*, 2016b). The assimilation of NH_4^+ resulting in the excretion of H^+ also results in indirect process (Parks *et al.*, 1990) or activation of plant metabolism promoting proton efflux and production of organic acids by plant roots (Carrillo *et al.*, 2002) have also been involved in the release of P.

2.4. Molecular approach for identification of Phosphate Solubilizing Bacteria:

During the 20th and 21st century, microbial identification techniques have undergone rapid development. Initially, conventional or phenotypic methods were the most used. Conventional techniques require acknowledgment of contrasts in

morphology, development, enzymatic action and metabolism to characterize genera and species (Petti *et al*., 2005). The conventional technique for characterization involve isolation and characterization of microorganisms using commercial growth media such as Nutrient medium, Luria-Broth medium, Triptic Soy Agar etc. (Kirk *et al*., 2004). This is usually followed by enzyme activities, morphological, physiological and chemotaxonomical properties to characterize upto species level (Ana *et al*., 2017). However, approaches to characterize and classify microbial communities by traditional or conventional cultivation strategy have changed to the molecular and genetic level. Cultivation-based method have permitted merely a glimpse of microbial diversity as only an estimated 1 % of the naturally occurring bacteria has been isolated and characterized up until this point (Muyzer, 1999). Understanding the patterns of bacterial diversity is of specific importance since bacteria may well comprise the most of earth's biodiversity and intervene critical environmental or ecosystem processes (Cavigelli and Robertson, 2000; Torsvik *et al*., 2002). Conventional cultivation methods for the improvement and isolation of microorganisms yield only a limited fraction of all microorganisms present. Polymerase chain reaction (PCR) based molecular techniques give a quick and sensitive alternative to conventional culture techniques. Molecular methods are based on the analysis of single cells, opening an opportunity to analyze the microbial community in its full diversity. Eventually, molecular based techniques for ecological studies on cloning/amplification of target genes isolated from environmental samples are more reliable (DeSantis *et al*., 2007). Due to tedious process of morphological and biochemical identification of bacteria, molecular tools has been adopted for this purpose (Widjojoatmodjo *et al*., 1995).

PCR-based 16SrDNA profile provides information about prokaryotic diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships (Pace, 1996, 1997, 1999). The analysis of the nucleotide sequences of the 16S ribosomal RNA gene (16SrDNA) has been one of the technological advances realized in the past decade, which has emerged as the single best method to identify bacteria (Kolbert and Persing, 1999; Drancourt *et al*., 2000). Ehresmann *et al*. (1972) sequenced the first bacterial 16SrDNA for Escherichia coli. This prototypic 16SrDNA (GenBank accession No. J01859) contains 1542

nucleotides. From the numerous sequencing and studies of more 16SrDNAs, it was realized that the nucleotide sequences among various bacteria are highly conserved; the conservation and divergence reflect bacterial evolution; and each bacterial species has its unique 16SrDNA sequences (Fox *et al.*, 1980). Therefore, 16SrDNA sequencing became a tool for studies of bacterial phylogeny. However, such 16SrDNA sequencing was quite difficult and sophisticated and could be performed uniquely only in a set number of research laboratories. In the mid-1980s, the invention of polymerase chain reaction (PCR) technology has changed it. As PCR became popular for its amplification power, speed, simplicity, and economy, its application for bacterial 16SrDNA has prospered. In this technique, the entire 16SrDNA or a portion of it may be amplified by PCR. Conserved regions of 16SrDNA allow design of highly conserved primers for nearly universal amplification of most bacterial species (Greisen *et al.,* 1994; Han *et al.*, 2002). When the nucleotide sequences of the amplicon are determined, it is compared to a database which matches with the homology and consequent identification of a specific bacterium is achieved. It is the variable regions of 16SrDNA that give the discriminatory power. There are numerous public and private databases available, such as GenBank, Ribosomal Database Project (RDP), Ribosomal Differentiation of Medical Microorganisms (RIDOM), and others. Bacterial identification through 16SrDNA sequencing requires four steps: DNA extraction, PCR amplification, nucleotide sequencing, and database homology search and reporting (Han, 2006). There is various numbers of advantages to the 16SrDNA sequencing method as compared with traditional phenotypic tests. First, it shortens turn-around time. Second, the results are definitive for those organisms that are difficult to be identified. At least upto the genus-level identification can be done (Drancourt *et al.*, 2000). Third, new taxa can be discovered. Fourth, for those uncultivated bacteria or subculture failures, this method remains the only way to find out the identity of the organism (Relman, 1999). Lastly, this culture-independent strategy remains the only way to study the diversity of non cultivable bacteria in different settings (Hugenholtz *et al.*, 1998).

Studies of 16srRNA identification have been made by many researchers. The isolation, screening and characterization of 36 strains of phosphate solubilizing

bacteria (PSB) from Central Taiwan were carried out using 16SrDNA sequencing (Chen *et al.,* 2006). Panhwar *et al.* (2014) identified three PSBs *viz., PSB7 Burkholderia thailandensis and PSB21 Burkholderia seminalis, whereas PSB17 Sphingomonas pituitosa with* accession numbers NR 074312.1, NR 042635.1, and NR 25363.1 from the acid sulfate soils in Semerak, Kelantan, Malaysia. Authors (Molla *et al*., 1984; Kim *et al*., 1998; Va´zquez *et al*., 2000; Whitelaw, 2000) have also identified and reported *Enterobacter* sp. and *Pseudomonas* sp. as a potential phosphate-solubilizing bacteria. Based on 16SrRNA gene sequence comparisons and phylogenetic positions, Zhang *et al.* (2017) identified the following strains*: Acinetobacter*, *Klebsiella sp., Enterobacter sp., Bacillus sp.*, *B. Megaterium*, *Paenibacillus taichungensis*, *Ochrobactrum.*

2.5. Biochemical approach of Phosphate solubilizing bacteria:

Bacteria may be conveniently grouped into a number of natural assemblages based characteristics such as cell shape, spore forming capabilities and whether they are aerobic / anaerobic or gram positive / gram negative (Sigee, 2005). The morphological and biochemical method of identification of bacteria is the classical method of characterization of bacteria. Classical identification of individual bacterial species in environmental samples typically involves isolation, laboratory culture and then taxonomic characterization. The classification of bacteria into families, genera and species is based on a wide range of phenotypic characteristics (Holt *et al*., 1994). These include culture conditions, colony morphology, biochemical characteristics and detailed morphology.

2.5.1 Acid phosphatase: Many soil microorganisms like *Aspergillus*, *Penicillium*, *Mucor, Rhizopus, Bacillus, Pseudomonas*, and mycorrhizal hyphae produce phosphatases which cause the dephosphorylation of soil organic P compounds by breaking phosphoester or phosphoanhydride bonds. The mineral phosphorous is solubilized by bacteria known as the phosphate solubilizing bacterial or PSBs. Assimilation of phosphorous takes place with the help of the enzyme called the phosphatase. Phosphatase enzyme hydrolyzes the phosphomonoesters from number of organic molecules like deoxy ribonucleotides, ribonucleotides, proteins and

phosphate esters. These are present in variable amount in the soil, and present in wide variety of soil microorganisms (Zaidi *et al*., 2003). Phosphatase enzyme occurs in all groups of organisms from bacteria's Bacillus, *E. Coli*, *Pseudomonas*, *Aerobactor* and *Bacillus* species (Mahesh *et al*., 2010), fungi *Aspergillus caespitosus*, *Mucor rouxii* (Luis *et al*., 2006), and algae *Cladophora* (El-Shahed *et al*., 2006) and *Chlamydomonas* (Loppes and Matagne, 1973).

2.5.2 IAA: Indole acetic acid (IAA) is one of the most physiologically active auxins, which can be produced by several microorganisms including PGPR through the L-tryptophan metabolism pathway. IAA is known to control organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, gene regulation and responses to light and gravity (Teale *et al*., 2006; Lambrecht *et al*., 2000). IAA, produced by PGPR are known to promote an extensive root architecture, which gives larger root surface, which are capable of absorbing nutrient components expeditiously from the environment that ultimately improves rice growth (Naher, 2009, 2011; Boiero *et al*., 2007). Microbial isolates from the rhizosphere of different crops seem to have more prominent potential to synthesize and release IAA as secondary metabolites. Production of IAA by microbial isolates varies enormously among different species and strains and relies upon the accessibility of substrates. Many bacteria isolated from the rhizosphere have the ability to synthesize IAA in the presence or absence of physiological precursors such as tryptophan (Davies, 1998).

2.5.3 Organic acid: PSM's produced the low molecular weight organic acids (gluconic, 2-ketogluconic, glyoxylic, citric, malic, lactic acids etc.) to solubilize the insoluble phosphates and lowering of pH in the cell surroundings (Maliha *et al*., 2004). There is experimental evidence to support the role of organic acids in mineral phosphate solubilization (Chen *et al*., 2006; Rashid *et al*., 2004). The major mechanism of mineral phosphate solubilization as observed in various microorganisms is through the release of organic acids which results in acidification of the microbial cell and its surroundings by lowering the level of pH. The quantity

and type of organic acid produced vary with the different strains of microorganisms. The amount of soluble phosphate released depends on the strength and type of acid produced. These organic acids chelate the cation that is bound to phosphate and being converted into soluble forms through their carboxyl and hydroxyl groups (Sagoe *et al*., 1998). The most important mechanism associated with the solubilization of insoluble phosphate is organic acid production, accompanied by acidification of the medium (Puente *et al*., 2004). Reductions in releasing rate of soluble phosphorous during the later stages of the incubation might be due to the depletion of nutrients in the culture medium, in particular, carbon source needed for the production of organic acids (Kang *et al*., 2002; Kim *et al*., 2005; Chaiharn and Lumyong, 2009). A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Khan *et al.,* 2009). Some common organic acids released by PSM are gluconic acid (Di-Simine *et al*., 1998; Bar-Yosef *et al*., 1999), oxalic acid, citric acid (Kim *et al*., 1997), lactic acid, tartaric acid, and aspartic acid (Venkateswarlu *et al*., 1984). Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. Strains from the genera *Bacillus, Pseudomonas* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez *et al*., 1999). Numerous range of phosphobacteria have been found to produce some organic acids such as monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketoglucenic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids in order to solubilize inorganic phosphate compounds.

2.6. PSB – Its role as a biofertilizer and as PGPR:

PSB has the ability to dissolve tricalcium phosphate from an insoluble form into a soluble form, as has been reported by many researchers (Chen, 2006; Gull, 2004; Illmer and Schinner, 1992; Rodriguez *et al.,* 2004). As P is major essential

macronutrients for plant growth and development, it is commonly applied to crops as fertilizer for optimizing the yield. PSB have been used to convert insoluble rock P material into soluble forms available for plant growth (Nahas, 1990; Bojinova *et al*., 1997). This conversion is through acidification, chelation and exchange reactions by PSB present in the soil. It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, P-solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al*., 1999). Application of phosphate solubilizing bacteria (PSB) as bioinoculants can solubilize the fixed soil P and applied phosphates resulting in higher crop yields (Gull *et al*., 2004). The interest in PSB has increased due to the prospective use of efficient strains as bio-inoculant (biofertilizer) components in organic agriculture, which is emerging as an alternative to chemical inputs in intensive agriculture (Ryder *et al.,* 1994). Phosphate solubilizing bacteria (PSBs) are important classes of plant growth promoting rhizaobacterias (PGPR) which are utilized as biofertilizers in different agricultural fields, as significant areas of cultivated soils are insufficient in nutrients like phosphorus (P) (Xie, 1996). Nahas *et al*. (1990) and Sheng *et al*. (2002) reported that PSBs have been used to convert insoluble phosphate into soluble forms, thus increasing P availability in soils and increase mineral content for plant growth. The use of PSB as inoculants concurrently increases P uptake by the plant and crop yield. The *Pseudomonas* spp. has been used for plant growth promotion and disease control in rice crop (Saikia *et al*., 2005). There are numerous plant growth-promoting rhizobacteria (PGPR) inoculants presently commercialized that seem to promote growth through at least one mechanism of the following: inhibition of plant disease (termed Bioprotectants), improved nutrient acquirement (Biofertilizers), or phytohormone production (Biostimulants). Commercial applications of PGPR are being tested and are frequently doing well; nonetheless, a better understanding of the microbial interactions that consequences in plant growth production will significantly increase the success

rate of field applications (Burr *et al*., 1984). Currently various bacterial strain such as *Azospirillum, Azotobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas* and *Serratia* have been identified as plant growth promoters (Khin *et al*., 2012).

Microorganisms that exert beneficial effect on plant growth and development through different means is termed as Plant growth promoting rhizobacteria (PGPR) (Vessey, 2003); rhizosphere is a narrow zone where the interaction occurs between soil, plants and microorganisms. The term PGPR was first coined by Klopper and Schroth in 1978. The use of plant growth promoting rhizobacteria, including phosphate solubilizing bacteria (PSB), as biofertilizers has become a great interest in developing countries as significant areas of cultivated soils are deficient in soil available P (Xie *et al*., 1998). Phosphate solublizing microbes are considered as important members of PGPR and their application in the form of biofertilizer has been shown to improve growth of cereals and other crops (Bhattacharyya and Jha, 2011; Gyaneshwar *et al*., 2002; Hu *et al*., 2006; Shahab *et al*., 2009; Vessey, 2003). PGPR that have the potential of phosphate solubilization improved the growth hormone production, availability of phosphorus and rate of nitrogen fixation (Ponmurugan and Gopi, 2006). Seed or soil inoculation with PSB has been known to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Abd-Alla, 1994; Jones and Darrah, 1994; Yadav and Dadarwal, 1997).

2.7. Xenobiotic – An overview

Xenobiotics are those chemical compounds which are foreign to the biosphere. The word, xenobiotic, is a combination of two different words; ‘xenos’ from the Greek word meaning strange/unnatural or foreign or foreigner and ‘biotic’ meaning life. They are thus, chemically synthesized (Fetzner, 1999). Xenobiotic pollutants may become available to microorganisms in different environmental compartments whether in air, soil, water or sediments. Some important classes of pollutants with xenobiotic structural features includes polycyclic aromatic hydrocarbons (PAHs), halogenated aliphatic as well as aromatic hydrocarbons,

nitroaromatic compounds, azo compounds, s-triazines, organic sulfonic acids, and synthetic polymers (Sinha *et al*., 2009). Xenobiotics also include many compounds that are involved in both industrial and agricultural activities. Fungi and aerobic as well as anaerobic bacteria are implicated in the degradation of xenobiotics. Occasionally these microbial alteration processes are fortuitous, an occurrence that is not uncommon in microbiology. Alternatively, microorganisms may also use xenobiotic compounds as a source of energy, carbon, nitrogen, or sulfur. Degradation of many xenobiotic chemicals requires microbial communities. Some xenobiotics, however, appear to resist microbial attack as well. The fate of industrial solvents and other industrial chemicals in the soil environment is an important domain of soil biochemistry.

The use of synthesized agro chemicals and fertilizers has been a point of discuss in the public field in the past. Though their advantage tend to be immediate, they still render a lasing environmental and public health threat to man by (1) possible entrance of heavy metals to the food chain, (2) death of soil biotic life (3) environmental deterioration and degradation and (4) alteration or damage of soil structure (Alalaoui, 2007). Most of the xenobiotic compounds for example, pesticides/insecticides however, are recalcitrant (the compounds that resist biodegradation and thereby persist in environment) and some of them are biomagnified to dangerous/toxic level. Xenobiotic waste/residues may be biodegradable, poorly biodegradable or recalcitrant and non biodegradable (Jha *et al*., 2015). According to Tropel and Meer (2004) most organisms, particularly bacteria are known for detoxifying abilities. They mineralize, transform or immobilize the pollutants. Bacteria play a crucial role in biogeochemical cycles for sustainable development of the biosphere. According to Chowdhury *et al*. (2008) and Varsha *et al*. (2011) example of aerobic degradative bacteria of xenobiotics are *Pseudomonas, Gordonia, Bacillus, Moraxella, Micrococcus, Escherichia, Sphingobium, Pandoraea, Rhodococcus* and anaerobic xenobiotics degradative bacteria are *Pelatomaculum, Desulphovibrio, Methanospirillum, Methanosaeta, Desulfotomaculum, Syntrophobacter, Syntrophus*. However, the community of soil microbes can be affected by many conditions of xenobiotic applications, one of which are insecticides; their effects are variable according to types, doses and field

conditions. Malathion, diazinon and dimethoate are anon-systemic, wide-spectrum organophosphate insecticides which affects on soil diversity microbes, dimethoate (0.2%), phorate at 300 fg/g and malathion at 100-300 fg/g had specifically toxic effect on one type of microorganisms but stimulated the growth of another type (Martinez *et al*., 1993; Gonzalez *et al*., 1993; Mandic *et al*., 1997; Digrak and Kazanici, 2001).

Chapter 3

Soil Physico-chemical and Bio-chemical Properties of the Study Site

3.1. Introduction

Soil is a significant component of agricultural system. It is important not only for agriculture but is also useful for living organisms. Soil as a component of the terrestrial ecosystem fulfils many functions including those that are essential for sustaining plant growth (Nwachokor *et al*., 2009). It is composed of particles of broken rock that have been caused by chemical and mechanical processes like weathering and erosion and has a complex function which is beneficial to all living organisms (Sumithra *et al*., 2013). In fact, the most important factors that govern the physico-chemical properties of soil are the chemistry of parent rock, climate and vegetation. Besides being a group of mineral particles, it also has a biological system of living organism as well as some other components. Agriculture, whichis an art of raising plants from the soil is one of the most economical factors for human beings (Wagh *et al*., 2013). A famous position in worldwide cultivation of wheat, rice, jawar, pulses, sugarcane, vegetables and fruits etc. is occupied by Indian agriculture and reason of physical, chemical condition of any land is indispensable for appropriate implementation of the other management practices (Kekane *et al*., 2015). Use of agrochemicals intensively may lead to soil degradation, residues of agrochemicals in crop or ground soil and water includes adverse effects on human health, causes accumulation of inorganic minerals in the soil, especially in intensive commercial horticulture, especially in vegetable and fruit production (Fotio *et al*., 2004). Thus, all agricultural productions and development of forest depends upon the soil physico-chemical parameters. It is a dynamic system which is an ecological niche where constant biological activity influence the chemical nature of its parent material and the plant growth it supports. So, it is very important to know the basic knowledge about the physico-chemical properties of soil as it provides the conditions of available nutrients which indicates the fertility and productivity of the soils (Sahrawat and Wani, 2013).

The importance of soil quality data is to provide appropriate anti-degradation measures and designing sustainable agricultural management practices that promote both maximum crop performance and minimum environmental degradation (Reynolds *et al.*, 2007). Soil fertility is an important factor, which determines the development of plant. It depends on the concentration of Carbon, Nitrogen, Phosphorus, Potassium, organic and inorganic materials, micronutrients and water. In general soil chemical fertility and in particular lack of nutrient inputs is a major factor in soil degradation (Hartemink *et al.*, 2010).The study and understanding of soil properties and their distribution over an area has proved to be useful for the development of soil management plan for efficient utilization of limited land resources. Moreover, it is very important for agrotechnology transfer (Buol *et al.*, 2003). Seasonal variations in physico-chemical properties of the soil can occur due to significantly alteration of seasonal changes in climate conditions.

pH (potential of Hydrogen) is the most significant property of the soil. It is the negative logarithm of the active hydrogen ion (H^+) concentration in the soil solution. It is the measure of soil sodicity, acidity or neutrality. It is an important consideration for agriculture workers due to many reasons, including the fact that many plants and soil life forms prefer either alkaline or acidic condition (Pandeeswari *et al.*, 2012). It also affects microbial population in soils. Most nutrient elements are available in the pH range of 5.5–6.5.pH is therefore, considered while analysing any kind of soil.

Moisture is also an important physical property of soil as the absorption of nutrients depends on it. The texture and structure of soil is also much related to its water content. The soil moisture commonly depends on void ratio, particle size, clay minerals, organic matter and ground water conditions (Yennawar *et al.*, 2013)

Soil temperature can influence the chemical, physical and biological processes associated with plant growth. Soil temperature fluctuates with climatic conditions. Sun being the major source of heat is generated by the chemical and biological activity of the soil (Jain *et al.*, 2014). It also plays an important role in

seed germination. The alterations of temperature will have an impact on the growth of biomass and the activity of the microorganisms (Naranjo *et al*., 2004)

Electrical conductivity is used to measure ions present in solution. The electrical conductivity of a soil solution increases with the increased concentration of ions (Tale *et al*., 2015). It is a measurement that correlates with soil properties that affect soil texture, cation exchange capacity, drainage condition, organic matter level, salinity and subsoil characteristics (Solanki *et al*., 2012).If the electrical conductivity is less than 1 (dS/cm) it is a normal soil, 1-2(dS/cm) then critical for germination, 2-3(dS/cm) critical for growth of salt sensitive crops and greater than 3(dS/cm) it is severely injurious to crops (Deshmukh *et al*., 2012).

Bulk density is an indicator of soil compaction. It is calculated as the dry weight of soil divided by its volume. This volume includes the volume of soil particles and the volume of pores among soil particles. Bulk density is typically expressed in g/cm^3. It reflects the soil's ability to function for structural support, water and solute movement, and soil aeration.

Nitrogen is the most crucial fertilizer element obtained by plants from the soil and is a bottleneck in plant growth (Gorde, 2013). About 80% of the atmosphere is nitrogen gas and plant roots obtain nitrogen in the form of NO_3 and NH_4 (Sumithra *et al*., 2013). Nitrogen cycle plays a significant role in soil system and is influenced by biological processes. It is required for the development of plants and is a constituent of chlorophyll, plant protein and nucleic acid (Jain *et al*., 2014). Although it has many beneficial effect, it could also have negative effect on the soil due to the excessive use of inorganic nitrogen fertilizers by farmers and cause soil acidification. Study has been made by Jain and Singh from Madhya Pradesh, India, on the available nitrogen which ranges from 172 ±2.1 to 193.3 $Kgha^{-1}$ for red, and brown soil and197±4.9 to 215±21$Kgha^{-1}$for black soil, 183±19 $Kgha^{-1}$nitrogen investigate in yellow soil (Jain and Singh, 2014).

Phosphorus being the most important micronutrient present in every living cell in plants and is considered the major essential element for plant growth (Tale *et al*., 2015). It is the most often limiting nutrient remains present in plant nuclei and

act as energy storage. It also helps in energy transfer (Jain *et al*., 2014). High organic matter content of soil has better supplies of organic phosphate for plant uptake (Miller and Donahuer, 2001). Sufficient phosphorus availability for plants triggers early plant growth and hasten maturity (Solanki *et al*., 2012). Wagh and Sayyed reported that phosphorus is necessary for seed germination and essential for flowering and fruits formation, its deficiency symptoms are purple stem and leaves, poor yield of fruit, poor crop yield (Wagh and Sayyed, 2013). A survey of Indian soils revealed that 98% of soils are deficient in P because the concentration of phosphorus available to plants in fertile soil is generally not higher than 10µM even at pH level of 6.5 where it is mostly soluble (Gyaneshwar *et al.,* 2002).

Unlike N and P, Potassium (K) is not an integral part of any major plant component. It functions as a key role in an enormous array of physiological process essential to plant development from protein synthesis to protection of plant water balance (Sumithra *et al*., 2013). It is involved in many plant metabolic reactions. High available potassium content on soil surface may be accredited to the application of potassium fertilizers (Miles and Hammer, 1989). It decreases with an increase in depth of the soil (Campbell and Rouss, 1961).Water loss from plants is controlled by potassium and it plays an important role in improving the plant growth. Muhar *et al.* have reported 25% samples were categorized as low (<125 kg ha^{-1}), 67.5% medium (125 to 300 kg ha^{-1}) and 2.5% high (>300 kg ha^{-1}) (Muhar *et al*., 1963).

Soil organic carbon (SOC) plays a vital role in carbon cycle and soil quality of agro-ecosystems. For better soil management and sustainable agriculture, understanding the factors that affect SOC and the main indicators for soil quality can be helpful (Zhao *et al*., 2018). Soil organic carbon acts as a store house for plant nutrients maintaining physical condition of soil and supports soil biota communities (Lungmuana *et al*., 2016). It is generally perceived that organic matter/SOC per se increases the activity of soil microbes. SOC influences other soil functions, such as the charge characteristics, cumulative stability, water holding capacity, and so on (Lal *et al*., 2004; Gregorich *et al*., 1994). Various factors could affect SOC dynamics, while soil enzyme activities and soil physico-chemical properties are more important

among these factors and more easily determined (Chandra *et al*., 2016; Robertson *et al*., 2016).

The enzymatic activity in the soil is mainly of microbial origin, being derived from intracellular, cell-associated or free enzymes. Healthy soils are necessary for the integrity of terrestrial ecosystems to remain intact, such as drought, climate change, pest infestation, pollution, and human exploitation including agriculture (Ellert *et al*., 1997). Soil enzymes are a group of enzymes whose usual inhabitants are the soil and are continuously playing an important role in maintaining soil ecology, physical and chemical properties, fertility, and soil health. The overall process of organic matter decomposition in the soil system is managed by these enzymes (Sinsabaugh *et al*., 1991). They play a crucial role in agriculture by catalyzing several important reactions necessary for the life process of different microbes in the soil and stabilization of soil structure, the decomposition of organic wastes, organic matter formation, and nutrient cycling (Dick *et al*., 1994). Activities of soil enzyme have been used as indicators of rates of soil nutrient cycling due to the fact that the relative assays are easily used and these measurements are susceptible to changes in soil management (Bending *et al*., 2004; Kandeler *et al*., 2006). Putting into points; the activities of these enzymes: (1) are often closely related to soil organic matter, microbial activity and soil physical properties or biomass, (2) changes much quicker than other parameters, which provides early indications of change in soil health, and (3) involve simple procedures (Dick *et al*., 1996). These enzymes may include amylase, arylsulphatases, b-glucosidase, cellulose, chitinase, dehydrogenase, phosphatase, protease, and urease released from plants (Miwa *et al*., 1937), microorganisms (James *et al*., 1991) and soil (Gupta *et al*., 1993).

Studies on the activities of dehydrogenase enzyme in soil is very important as it may signify potential of the soil to support biochemical processes which are fundamental for maintaining soil fertility. Dehydrogenase enzyme is known to oxidize soil organic matter by transferring protons and electrons from substrates to acceptors. These processes are part of respiration pathways of soil micro-organisms and are closely related to the type of soil and soil air-water conditions (Doelman and

Haanstra, 1979; Kandeler *et al*., 1996; Glinski and Stepniewski, 1985). It is frequently used as an indicator of biological activity in soils (Burns, 1978).

Phosphatases are believed to play vital roles in P cycles (Speir and Ross, 1978). They are a wide group of enzymes that are capable of catalyzing hydrolysis of esters and anhydrides of phosphoric acid (Schmidt and Lawoski, 1961) and are excellent indicators of soil fertility and play key role in the soil system (Dick and Tabatai, 1992; Eivazi and Tabatabai, 1977; Dick *et al*., 2000). If there is P deficiency in the soil system, plant roots secrete acid phosphatase and enhance the solubilization and remobilization of phosphate, thus influencing the ability of the plant to cope with P-stressed conditions (Karthikeyan *et al*., 2002; Mudge *et al*., 2002)

Soil enzyme urease originate mainly from plants (Polacco, 1977) and microorganisms found as both intra and extracellular enzymes (Burns, 1986; Mobley and Hausinger, 1989). It is responsible for the hydrolysis on application of urea fertilizers to the soil into NH_3 and CO_2 with the concomitant rise in soil pH (Andrews *et al*., 1989; Byrnes and Amberger, 1989). So, it results in a rapid N loss to the atmosphere through NH_3 volatilization (Simpson *et al.,* 1984; Simpson and Freney, 1988). There are several factors which influence urease activity in soil. These include cropping history, organic matter content of the soil, soil depth, soil amendments, heavy metals, and environmental factors such as temperatures (Tabatabai, 1977; Yang *et al*., 2006).

3.2. Study site and soil sampling

Serchhip district, Mizoram is divided into three Sub-division namely Serchhip, North Vanlaiphai and Thenzawl, and two blocks namely Serchhip and East Lungdar. A wetland paddy field of North Vanlaiphai was selected for this study which is from Serchhip district, Mizoram, India. The region is located at latitude 23° 7'47" N and longitude 93° 4'11" E (Fig. 3.1). North Vanlaiphai has an average elevation of 1284 MSL. The air temperature of the site varies between 18-30°C and the soil temperature ranges from 25-27°C.Soil samples were collected from the rhizosphere region of the rice plant in pre harvest season during the month of July-August and post harvest season during the month of February-March for two

consecutive years and the average was taken into account (Fig. 3.2 a & b). Within the plots of the paddy field, soil samples from the surface 0-30cm were collected from six random plots and mixed as one composite sample. Soil samples were kept in plastic bags contained with ice pack and transported to the laboratory and stored at 4°C for further investigation.

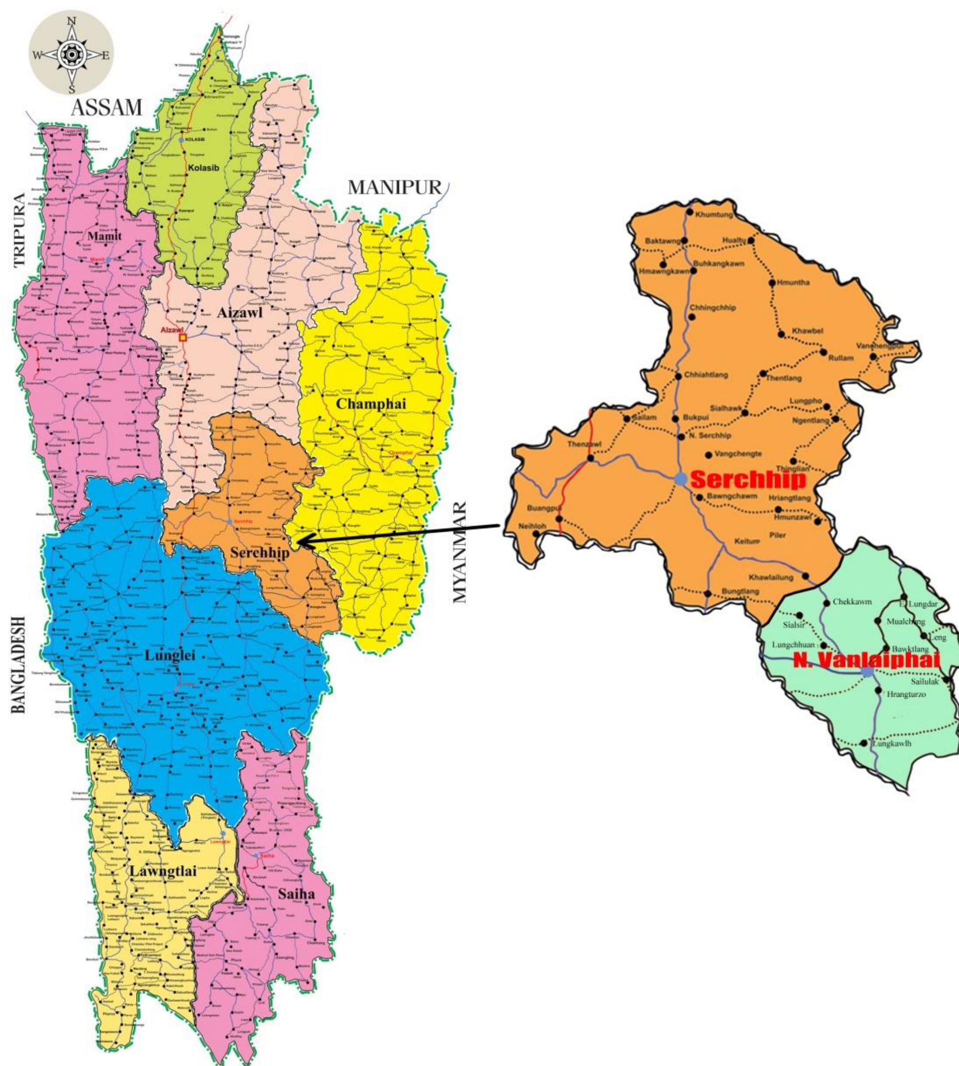


Fig 3.1. Map of Mizoram highlighting Serchhip district and North Vanlaiphai

Fig. 3.2(a) North Vanlaiphai paddy field during pre harvesting season

Fig. 3.2(b) North Vanlaiphai paddy field during post harvesting season

3.3. Methodology

3.3.1. *Analysis of soil physical properties*

3.3.1.1. Bulk density (BD) (Bashour and Sayegh, 2007)

Bulk density of the soil was measured by using cylindrical soil core sampler. Soil was collected from the surface at around 6 inches deep from random plots of the study site. Soil collected was oven dried at 105°C for 12 hours and the weight was taken. The exact volume of the soil was determined by measuring the cylinder volume.

$$\text{Bulk Density (BD gm}^{-3}\text{)} = \frac{Weight\ of\ Oven\ Dried\ soil\ (g)}{Volume\ of\ Soil\ Core\ (\text{cm}^2)}$$

Where,

Volume of soil core = $3.14r^2h$

r = inside radius of cylinder (cm)

h = height of cylinder (cm)

3.3.1.2. Soil moisture content (SMC) (Hot air oven method)

Weight of 10g of collected soil was taken and was kept under hot air oven at 105°C for 24 hrs. After the soil was completely dried, it was weighed again and recorded. The percentage of moisture content was calculated by the formula:

$$\text{Moisture content (\%)} = \frac{W1 - W2}{W1}$$

Where,

W_1 = initial weight

W_2 = final weight

3.3.2. *Analysis of Soil chemical properties*

3.3.2.1. Soil pH

Freshly collected soil sample was weighed into 10g and kept in a glass beaker containing 50ml of distilled water. The suspension was then stirred with a glass rod and then stirred continuously with magnetic stirrer for 30 minutes. After stirring, the solution was kept at room temperature for 12 hrs and the pH readings were taken using electronic digital pH meter.

The most common classes of soil pH according to the USDA, NRCS are:

Extremely acid	3.5 – 4.4	Neutral	6.6 – 7.3
Very strongly acid	4.5 – 5.0	Slightly alkaline	7.4 – 7.8
Strongly acid	5.1 – 5.5	Moderately alkaline	7.9 – 8.4
Moderately acid	5.6 – 6.0	Strongly alkaline	8.5 – 9.0
Slightly acid	6.1 – 6.5		

3.3.2.2. Available nitrogen in soil (AN) (Subbiah and Asija, 1956)

Reagent preparation

1. 0.32% $KMnO_4$solution : 3.2g of pure KMnO4 was dissolved in distilled water and diluted to 1L
2. 2.5% NaOH solution : 25g of NaOH was dissolved in distilled water and diluted to 1Lwith distilled water
3. 2% Boric acid solution was made containing 20-25ml of mixed indicator / liter
4. Mixed indicator :0.066g methyl red was mixed with 0.099g bromocresol green and dissolved in 100ml 95% alcohol
5. 0.02N Sulphuric acid (H_2SO_4)was also prepared

Procedure

5g of dried and sieved soil was transferred in the digestion tube. The tube was loaded into the distillation unit, and then to the other side of the hose, 20ml of 2% boric acid was kept and mixed with indicator in 250ml conical flask. 25ml of each potassium permanganate (0.32%) and sodium hydroxide (2.5%) solution was automatically added by distillation unit programme. The block containing the sample was heated at 410°C by passing steam at a steady rate and the liberated ammonia absorbed in 20ml of 2% boric acid containing mixed indicator solution kept in a 250ml conical flask. The pink colour turned green due to the absorption of ammonia.

Nearly 150ml of distillate was collected in about 10 minutes. Then, the green colour distillate was titrated with 0.02N $H_2SO_4$and the colour changed to the original shade again (pink colour). Simultaneously, blank sample without soil was run alongside. The blank and titre reading was noted and available nitrogen in the soil was calculated.

The available nitrogen was expressed in kg ha^{-1} and calculated according to the formula:

$$\text{Available Nitrogen (kg ha}^{-1}\text{)} = \frac{R(Titerreading - Blankreading) \times Normalityofacid \times AtomicweightofN \times Weightofonehectareofsoil}{Sampleweight(g) \times 1000}$$

$$= \frac{R \times 0.02 \times 14 \times 2.24 \times 10^{6}}{5 \times 1000}$$

Interpretation of results:

Available nitrogen (kg ha^{-1})	Soil rating
<280	Low
280-560	Medium
>560	High

3.3.2.3. Available Phosphorus (Olsen *et al.,* 1954)

100 ml conical flask was taken; to it, 2.5g of dried soil and 50ml of sodium bicarbonate solution was mixed and shaken with the help of Tarson's horizontal orbital plate shaker at 6000 rpm for 30 minutes. The suspension was filtered through Whatman filter paper No.42 and activated carbon was added to obtain a clear filtrate. 5ml of the extract was taken in a 25ml volumetric flask to which 5ml of Dickman and Bray's reagent was added drop by drop with constant shaking till the effervescence due to CO_2 evolution ceases and 1ml of diluted $SnCl_2$ was added. The volume was then made up to the mark (of 25ml volumetric flask). The colour is stable for 24 hours and maximum intensity was obtained in 10 minutes. The absorbance was read with a UV-VIS spectrophotometer (Dynamica HALO DB-20 UV-VIS double beam spectrophotometer) at 660nm.

Preparation of standard curves:

Different concentration of phosphorous (1ml, 2ml, 3ml, 4ml, 5ml and 10ml of 2 ppm phosphorous solution) were taken in 25 ml volumetric flask. The standard concentration was prepared in the range of 0.08 µg/ml to 0.80 µg/ml. the curve was plotted taking calorimetric reading on the vertical axis and the amount of phosphorous (in µg/ml) in horizontal axis.

It was calculated by the following formula:

$$\text{Available Phosphorus}\left(\frac{\text{Kg}}{\text{ha}}\right) = R \times \frac{V}{v} \times \frac{1}{S} \times \frac{(2.24 \times 10^{6})}{10^{6}}$$

Where,

V = total volume of extractant (ml)

v= volume of aliquot taken for analysis (ml)

S = weight of soil (g)

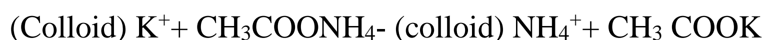
R = weight of phosphorus in the aliquot in µg (from standard graph)

Interpretation of results:

Available Phosphorous (P_2O_5) (kg ha^{-1})	Soil rating
<20	Low
Between 20 and 50	Medium
>50	High

3.3.2.4. Available Potassium (Metson, 1980)

Available K is determined by extracting the soil by shaking with N neutral ammonium acetate solution. The ammonium ions replace potassium ions absorbed on the soil colloids.


(Colloid) K^++ CH_3COONH_4- (colloid) NH_4^++ CH_3 COOK

The estimation of potassium in the extract is carried out with the help of flame photometer.

Reagents

1. N neutral ammonium acetate solution: 700ml of distilled water was taken in a 1L volumetric flask. To it, 57ml of glacial acetic acid (99.5%) was added and 69ml of concentrated ammonium hydroxide was added. It was diluted to about 900ml and the pH adjusted to 7.0 by adding 3N NH_4OH or 3N CH_3COOH and made up to 1L.

2. Standard solution: 1.908g of KCl was dissolved in distilled water and the volume was made upto 1L. This solution contains 1000 mgK/L i.e. 1000ppm K.

3. Working solution of K: 10ml of 1000ppm K solution was taken in a 100ml volumetric flask and the volume was made up to the mark. This solution contains 100ppm K.

Procedure

5g of dried and sieved soil was taken in a 150ml conical flask and 25ml N neutral ammonium acetate solution was added and shaken for 5 minutes on a shaker.

The contents were filtered through Whatman No. 1 filter paper. The first few drops of the filtrate were rejected. The filtrates were fed to flame photometer after necessary setting and calibration of the instrument. A blank without soil was also run using the same procedure.

Preparation of standard curve

From the 100ppm solution, 10ml, 20ml, 30ml and 40ml of K was taken in each 100ml volumetric flask and the volume was made up to the mark. This gives 10ppm, 20ppm, 30ppm and 40ppm of K. The concentration of K may be reduced according to the working range of flame photometer. The curve was obtained by plotting the readings of flame photometer against the various concentration of K by setting the reading at zero for the blank and 100 for the standard K solution having maximum concentration to be used.

Observation and calculation,

Available K kg/ha = R x 5 (dilution factor) X 2.24

Available K_2O kg/ha = R x 5 x 2.24 x 1.23

Where,

Weight of soil taken = 5 g

Volume of extractant = 25 ml

Reading of flame photometer = X

ppm K as obtained from standard curve corresponding to X = (R) ---ppm

Here 1.23 is conversion factor for converting K into K_2O

Rating of soil based on available potassium (K_2O) in soil:

Available potassium (K_2O) (kg ha^{-1})	Soil rating
<125	Low
Between 125 and 250	Medium
>250	High

3.3.3. *Analysis of soil biochemical properties*

3.3.3.1. Soil Organic Carbon (SOC) (Walkey and Black, 1934)

A handful of the collected soil was sun dried and ground completely. It was then sieved through 0.2mm aperture Lab Standard Sieve mesh 20cm in diameter. 0.5g of the fine soil which was sieved was taken in a dry conical flask and to it 10ml of 1N potassium dichromate solution was added. Then, 20ml of concentrated sulphuric acid was added to the flask in slant position carefully and swirled several times and was allowed to stand for about 30 minutes. 200ml of distilled water with 10ml ortho-phosphoric acid was added to get a sharper end point of titration. After the addition of 1ml diphenylamine indicator, the content was titrated with 0.5N ferrous ammonium sulphate solutions till the colour flashes from blue-violet to green. The final reading after the development of green colour was recorded for each replicate. A blank without soil was run simultaneously. The SOC is then calculated by the following formula and expressed in percentage.

Organic carbon (%) $= \frac{10\ (B-T)}{B} \times 0.003 \times \frac{100}{S}$

Where,

B = Volume of ferrous ammonium sulphate required for blank titration in ml

T = Volume of ferrous ammonium sulphate needed for soil sample in ml

S =Weight of soil in gram

3.3.3.2. Soil Organic Matter (SOM)

This is calculated in percentage by using Van Bemmelen factor

SOM (%) = SOC (%) $\times$ 1.72

3.3.3.3. Dehydrogenase (DHA) (Casida *et al*., 1964)

1 g of fresh soil with three replicates were placed in a test tube (15 x 2cm) and carefully mixed with 0.1g of $CaCO_3$. Then, 1ml of 1 % 2, 3,5triphenyl tetrazolium chloride (TTC) solution was added and the tubes were incubated at 30°C for 24 hrs (incubator should be pre-set at 30°C prior to the experiment). The resulting slurry was transferred on Whatman filter paper No.1 and triphenyl formazan (TPF) was extracted with successive aliquots of concentrated methanol in a 50 ml volumetric flask. The extinction of the pink colour was read with the help of Spectrophotometer (Dynamica Halo DB-20) at 485nm using methanol as blank (without soil). The enzyme activity was expressed in terms of µg TPFmg^{-1} 24hrs^{-1}.

3.3.3.4. Acid Phosphatase (APase) (Tabatabai and Bremner, 1969)

0.1 g of air-dried soil with three replicates was taken in a 50ml conical flask and mixed with 4ml of modified universal buffer (MUB pH-6.5), 0.25ml toluene and 1ml of 0.115M p-nitrophenyl phosphate (p-NPP) solution (p-NPP should be prepared fresh and kept in dark bottle). The flask was swirled manually for a few seconds and plugged tightly with cotton plug and incubated for 1 hr at 37°C at an incubator. To this, 1ml of 0.5M $CaCl_2$ and 4ml of 0.5M NaOH solutions were added simultaneously into the mixture before transferring into Whatman No.1 filter paper. The yellow coloured filtrate of p-nitrophenol phosphate (phosphoric acid) was read with the help of spectrophotometer (Dynamica Halo DB-20) at 410nm. For blank, 1ml p-NP was added after $CaCl_2$ and NaOH were added into the mixture without soil just before filtration. The enzyme activity was expressed in terms of µg p-NPP mg^{-1} hr^{-1}.

3.3.3.5. Urease (McGarity and Myers, 1967)

100 ml volumetric flask were taken, to this 1g of fresh soil with three replicates each was placed and treated with 1ml of toluene which was allowed to stand for 15 minutes for complete penetration of toluene into the soil. Then, 10ml buffer (pH-7) and 5ml of 10% urea solution (freshly prepared) were added. After a thorough mixing the flask was incubated for 3 hrs at 37°C in an incubator. For blank,

5ml of 10% urea solution was replaced by 5ml of sterile distilled water. After incubation, the volume of the flask was made up to the mark (100ml) with distilled water and shaken thoroughly and filtered through Whatman No.5 filter paper. The ammonia released as a result of urease activity was measured by Indophenol Blue method in which 0.5ml of the filtrate was taken into a 25ml volumetric flask and 5ml of distilled water was added. Then, 2ml of phenolate solution was added and thereafter, 1.5ml of sodium hypochlorite solution was added. The final volume of the flask was increased up to 25ml with distilled water and the blue colour was read with the spectrophotometer (Dynamica Halo DB-20) at 630nm. The enzyme activity was expressed in terms of mg NH_4^+-Nmg^{-1} 3hrs.

3.4. Statistical analysis

All data were presented as means of three replicates with standard error. Differences between variables were tested with standard one-way analysis of variance (ANOVA). Correlation coefficient (r) was also performed between the soil biochemical and physical analysis. Differences were considered as significant at $P \leq 0.05$ levels. The statistical analyses were performed using SPSS 16.0 software (Standard release version 16 for windows, SPSS Inc., IL, and USA).

3.5. Results

3.5.1. Soil physico-chemical properties

All of the experiments were done in triplicates and the average was considered as result. Physical properties of soil are represented graphically in Figure 3.3. Soil temperature was 25.2°C in pre harvest and 26.5°C in post harvest season. Soil moisture content was found to be much higher during pre harvest season which is 67.97% and lower during post harvest with 35.8%. Bulk density is 1.66 gm cm^{-3} which is slightly higher during pre harvesting season and 1.31 gm cm^{-3} in post harvest season.

Chemical properties of the soil are shown in Figure 3.4. The average soil pH level ranges between 4.13 during pre harvest and 5.29 during post harvest season which is under the very strongly acidic range according to the USDA, NRCSS.

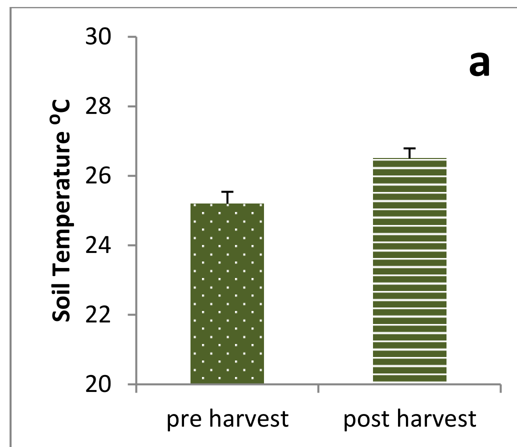
Available nitrogen was 287kgha^{-1} during pre harvest season and 274kgha^{-1} during post harvest season. Available phosphorus was recorded as 12.26kgha^{-1} during pre harvest season and 12.18kgha^{-1}during post harvest season. Available potassium was observed as 125.67kgha^{-1}and 117.00kgha^{-1} during pre and post harvest season respectively.

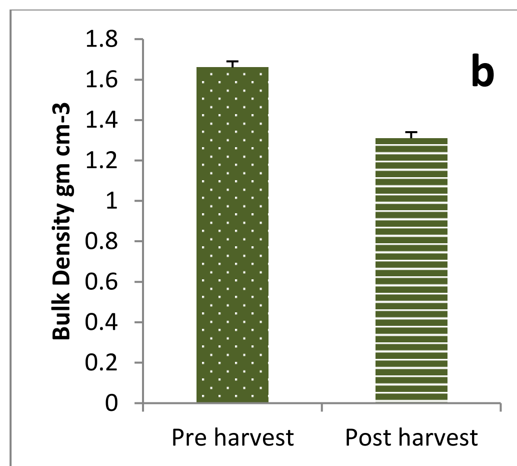
3.5.2. Soil biochemical activity

Soil biochemical activities are shown in Figure 3.5. Soil enzyme activity during pre harvest season was recorded as: dehydrogenase 0.864µg TPFmg^{-1}24hrs^{-1}, acid phosphatase 93.458µg p-NPmg^{-1}hr^{-1}and urease 0.931NH4^{+}-Nmg^{-1}3hrs^{-1}. During post harvest season, dehydrogenase activity was 0.182µgTPFmg^{-1}24hrs^{-1}; activity of acid phosphatase was 59.542µg p-NPmg^{-1}hr^{-1}and urease activity was 0.708NH_4^+-Nmg^{-1} 3hrs^{-1}. Soil organic carbon (SOC) was 2.1% and soil organic matter (SOM) was 3.61% during pre harvest season and 0.57% SOC and 0.97% SOM during post harvesting season.

3.5.3. Statistical analysis

Differences between variables were tested with standard one-way analysis of variance (ANOVA); significant differences existed in all the parameters except parameter 9 that is Available Phosphorous (Table 3.1). Bivariate correlations were performed between each parameter separately during pre-harvest and post-harvest season by Pearson's correlation coefficient using one tailed test of significance (Table 3.2 & 3.3).





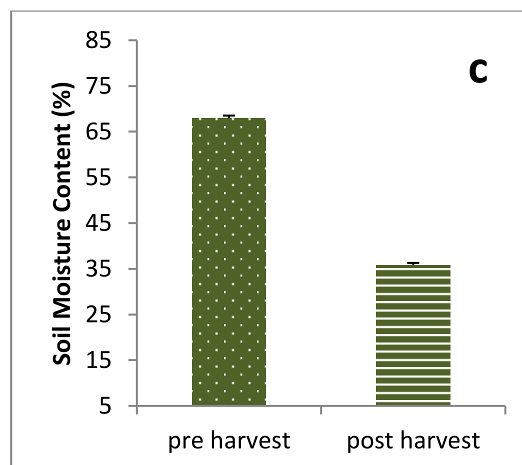


Fig 3.3: Physical properties of the soil sample during pre and post harvest season

(a) Soil temperature

(b) Bulk density

(c) Soil moisture content

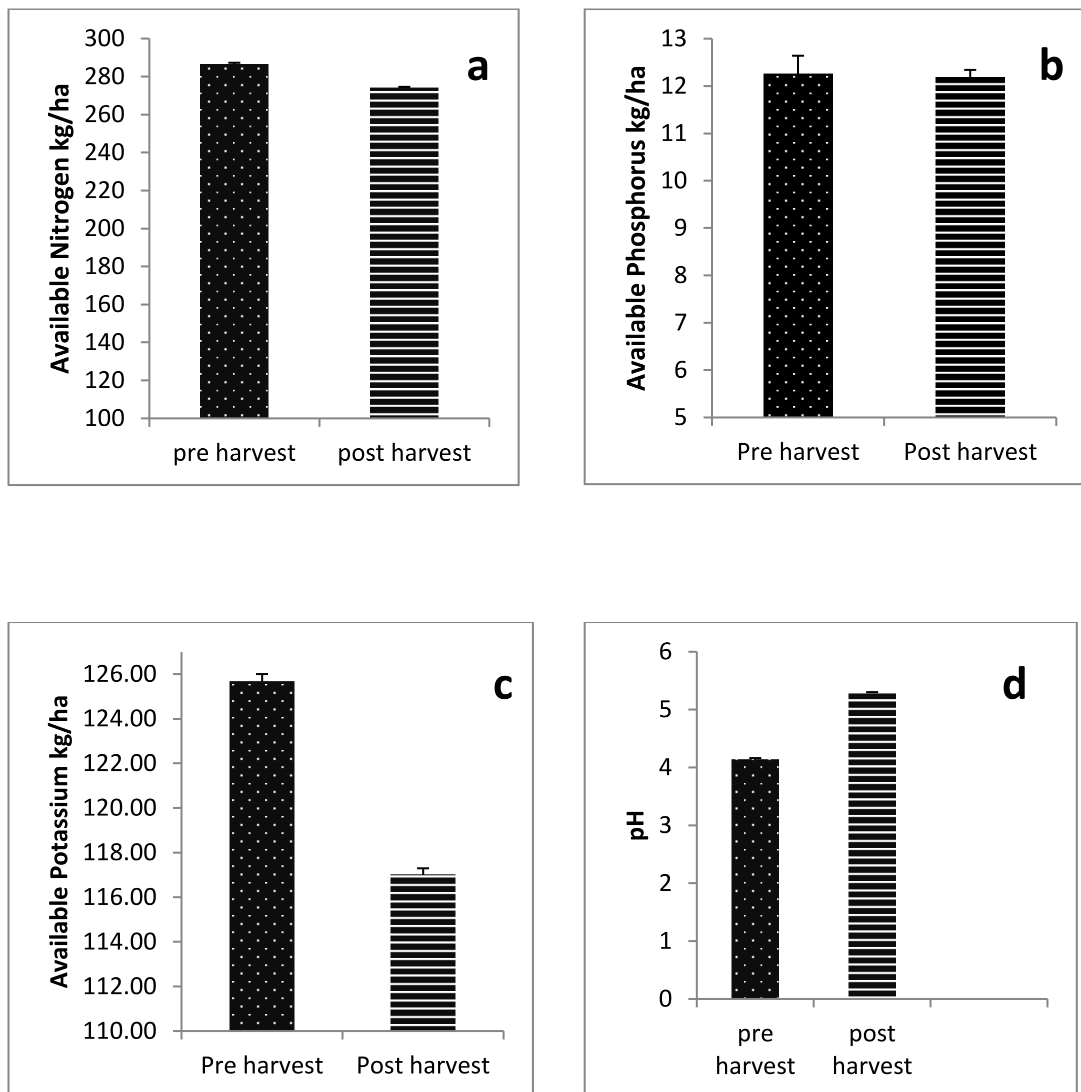


Fig 3.4: Chemical properties of the soil sample during pre and post harvest season

(a) Available Nitrogen

(b) Available Phosphorous

(c) Available Potassium

(d) Soil pH

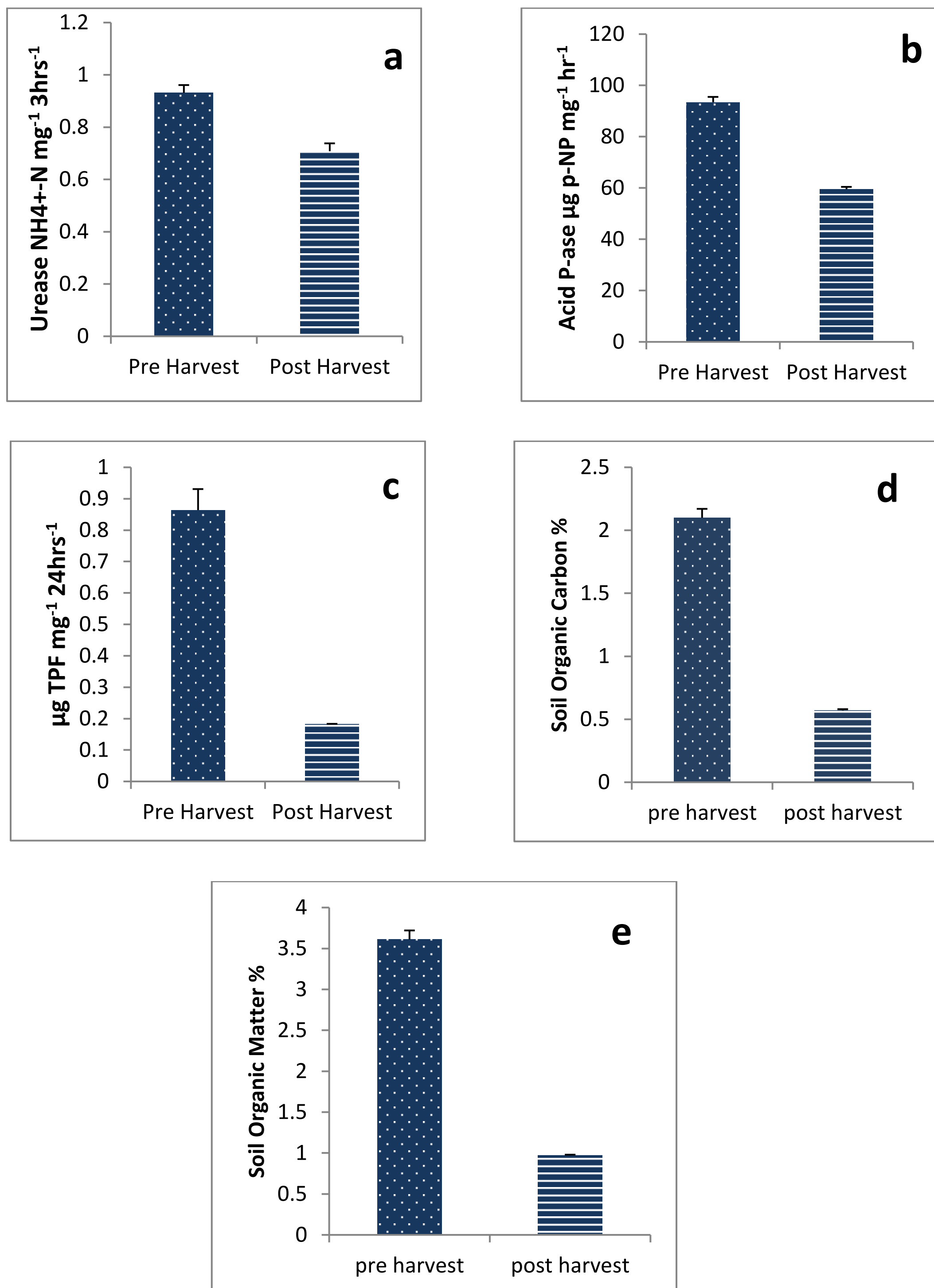


Fig 3.5: Biochemical properties of the soil sample during pre and post harvest season

(a) Urease activity

(b) Acid phosphatase activity

(c) Dehydrogenase activity

(d) Soil Organic Carbon

(e) Soil Organic matter

Table 3.1: One way analysis of variance (ANOVA) among biochemical and physico-chemical properties of soil between pre and post harvest season. * Marked effects are significant at $p \leq 0.05$.

S. N	Parameters	Source of Variation	F-value	p-value
1	Soil pH	Pre harvest x Post harvest	1.132E3	0.000*
2	Soil Moisture Content	-do-	1.900E3	0.000*
3	Bulk Density	-do-	83.200	0.001*
4	Soil temperature	-do-	8.112	0.046*
5	Soil Organic Carbon	-do-	175.748	0.000*
6	Soil Organic matter	-do-	174.869	0.000*
7	Available Potassium	-do-	386.286	0.000*
8	Available Nitrogen	-do-	170.455	0.000*
9	Available Phosphorus	-do-	0.035	0.860
10	Dehydrogenase activity	-do-	104.910	0.001*
11	Urease activity	-do-	36.386	0.004*
12	Phosphatase activity	-do-	225.680	0.000*

Table 3.2: Correlation coefficient (r) values among soil biochemical and physicochemical properties during pre harvesting period ** Marked correlation is significant at the 0.01 level (1-tailed) and * marked correlation is significant at the 0.05 level (1-tailed)

Parameters	ST	SMC	BD	SOC	AN	AP	AK	SOM	pH	URES	APase
DHA	-0.353 0.383	0.846 0.179	0.942 0.109	-0.556 0.312	0.661 0.270	-0.354 0.385	-1.000** 0.003	-0.554 0.331	-0.507 0.331	0.912 0.134	-0.958 0.093
ST		0.201 0.436	-0.019 0.494	-0.581 0.303	-0.935 0.115	1.000** 0.000	0.345 0.388	-0.583 0.302	0.985 0.055	0.061 0.480	0.607 0.293
SMC			0.976 0.070	-0.914 0.133	0.199 0.436	0.199 0.436	-0.850 0.176	-0.913 0.134	0.030 0.490	0.990* 0.045	-0.657 0.272
BD				-0.803 0.203	0.371 0.379	-0.020 0.494	-0.945 0.106	-0.801 0.204	-0.189 0.439	0.997* 0.025	-0.806 0.202
SOC					0.255 0.418	-0.580 0.303	0.564 0.309	1.000** 0.001	-0.434 0.357	-0.848 0.178	0.295 0.405
AN						-0.936 0.115	-0.655 0.273	0.258 0.417	-0.982 0.061	0.296 0.404	-0.849 0.177
AP							0.346 0.387	-0.582 0.302	0.986 0.054	0.060 0.481	0.608 0.292
AK								0.562 0.310	0.500 0.333	-0.961 0.132	0.955 0.095
SOM									-0.436 0.356	-0.847 0.179	0.292 0.406
pH										-0.110 0.465	0.733 0.238
URES											-0.756 0.227

DHA=Dehydrogenase; ST=Soil Temparature; SMC=Soil Moisture Content; BD=Bulk Density; SOC=Soil Organic Carbon; AN=Available Nitrogen; AP=Available Phosphorus; AK=Available Potassium; SOM=Soil Organic Matter; pH=Soil pH; URES= Urease; APase= Acid Phosphatase

Table 3.3: Correlation coefficient (r) values among soil biochemical and physicochemical properties during post harvesting period. ** Marked correlation is significant at the 0.01 level (1-tailed) and * marked correlation is significant at the 0.05 level (1-tailed).

Parameters	ST	SMC	BD	SOC	AN	AP	AK	SOM	pH	URES	APase
DHA	0.000 0.500	0.000 0.500	0.977 0.068	-0.756 0.227	0.000 0.500	0.419 0.362	0.866 0.167	-0.803 0.203	0.189 0.439	0.753 0.229	-0.466 0.346
ST		-0.995* 0.032	0.212 0.432	0.655 0.273	-1.000** 0.000	-0.908 0.138	-0.500 0.333	0.596 0.297	0.982 0.061	0.658 0.271	-0.885 0.154
SMC			-1.111 0.464	-0.728 0.240	0.995* 0.032	0.946 0.105	0.585 0.301	-0.674 0.264	-0.958 0.093	-0.578 0.304	0.833 0.187
BD				-0.600 0.295	-0.212 0.432	0.218 0.430	0.741 0.235	-0.659 0.271	0.392 0.372	0.875 0.161	-0.643 0.278
SOC					-0.655 0.273	-0.911 0.135	-0.982 0.061	0.997 0.024	0.500 0.333	-0.139 0.456	-0.227 0.427
AN						0.908 0.138	0.500 0.333	-0.596 0.297	-0.982 0.061	-0.658 0.271	0.885 0.154
AP							0.817 0.196	-0.878 0.159	-0.812 0.198	-0.282 0.409	0.608 0.292
AK								-0.993 0.037	-0.327 0.394	0.323 0.395	0.039 0.488
SOM									0.434 0.357	-0.213 0.432	-0.153 0.451
pH										0.788 0.211	-0.957 0.094
URES											-0.933 0.117

DHA=Dehydrogenase; ST=Soil Temparature; SMC=Soil Moisture Content; BD=Bulk Density; SOC=Soil Organic Carbon; AN=Available Nitrogen; AP=Available Phosphorus; AK=Available Potassium; SOM=Soil Organic Matter; pH=Soil pH; URES= Urease; APase= Acid Phosphatase

3.6. Discussion

The soil quality check includes an analysis of parameters and procedures which effects on soil to work efficiently as a component of a sound ecosystem (Tale and Ingole, 2015). Soil analysis for different parameters was taken within 30cm depth of the soil due to the greatest abundance of plant roots, biological activity and highest nutrient levels (Mahler and Tindall, 1994). Change in the soil chemical properties in the form of P mineralization-immobilization of organic P, are strongly influenced by seasonal variations in temperature, moisture, plant growth and root activity, and by organic matter accumulation from litter fall (Perrot *et al.*, 1990; Mc Grath *et al.*, 2000). All parameters were studied on two seasonal variations which were termed as pre harvest season during the month of July-August and post harvest season during the month of February-March for two consecutive years and the average or mean was taken into account. Our result shows that all the soil parameters varied under the influence of the seasonal variations whereas the soil temperature fluctuates within the season during time of day, night and local climatic situation and ranges from 24.8°C to 27°C throughout the year.

Soil pH level was lower during pre harvest season (4.13) and slightly higher during post harvest season (5.29). According to the USDA, NRCS, the soil rating for pH falls between 4 to 5 which is very strongly acidic. Also, Kekane *et al.* (2015) stated that if the pH is less than 6 then it is said to be an acidic soil, the pH ranges from 6-8.5 is normal soil and greater than 8.5 then is said to be alkaline soil. So, the soil sample is found to be acidic throughout the year. Soil sample was collected during the month of July which is rainy season and the planting period termed for the pre harvesting season, and post harvest during the month of February. According to Natural Resources Conservation Service (NRCS), United States Dept. of Agriculture, soil pH decreases over time in a process called soil acidification, due to leaching from high amounts of rainfall. Conyers *et al.* (1995) and Yan *et al.* (1996) also stated that the release of organic acids from decomposition of organic matter can lead to leaching of bases under existing high rainfall which can cause lowering of the soil acidity or low pH of the soil. As a matter of fact, study reveals that the soil of Mizoram are the product of slow digenesis changes of acidic parent material causing intrinsic soil acidity and high precipitation further aggravates this problem due to

leaching of basic cations (Mishra and Saithantluanga, 2000). According to the soil quality information sheet, USDA, soil pH affects many micro-organisms. The type and population densities change with pH. A pH of 6.6 to 7.3 is favourable for microbial activities that contribute to the availability of nitrogen, sulfur and phosphorus in soils.

Soil bulk density is one of the major physical factors affecting root growth (Pabin *et al*., 1998). The BD is 1.66gm cm^{-3} and 1.31gm cm^{-3} pre and post harvest respectively.

SOC or organic carbon is a key feature in maintaining soil tilt and quality and energy source for microorganisms in soils. It also influences other soil functions, such as the charge characteristics, aggregate stability, water holding capacity, and so on (Lal *et al*., 2004; Gregorich *et al*., 1994).The organic carbon content and organic matter was exceptionally higher during pre harvest season or the rainy season. SOC was 2.1% and 0.57% during pre and post harvest respectively. The organic matter was 3.61% and 0.97% during pre and post harvest season respectively. Soil carbon content is positively correlated with soil organic matter which is in accordance with the study of Soon and Arshad (1996). Miller and Donahuer (2001) reported that the soil with high organic matter content have better supplies of organic phosphate for plant uptake than have the soils with low organic content. Our result is close to those reported by Colney and Nautiyal (2013) who also studied on Mizoram soil and found the result of SOC ranges from 0.38 to 1.94 % and most of the soils were in medium category and in almost all the soils the organic carbon content decreased with depth which could be due to the reduction inorganic matter content with increasing depth. Study by Sevgi and Tecimen (2008) on natural forest reported that higher organic carbon was due to production and return of higher amount of litter in natural forest and that of decomposition rates (microbial respiration) doubles with every 10°C increase in the temperature (Schlesinger, 1997; Hartel, 2005).Soil moisture content was exceptionally higher during the planting period or the pre harvesting season with 67.97% and 35.80% during post harvest season, which could be due variations in soil organic carbon content (Sathyavathi and Reddy, 2004). As from our

study we also found that there are huge variations between pre and post harvest season in SOC and SOM.

There was a slight increase in the content of available nitrogen during pre harvest season with 287kg/ha and 274kg/ha during post harvest season. The amount of available N falls in between low to medium level according to the soil rating chart. This could possibly be owing to mineralization of high temperature during the month. The result is in support with the finding of Xiao-gang *et al.* (2007). Higher values of available nitrogen in the soil profile during rainy season reflects blue green algae fixation, rain water input and higher rate of release of mineral nitrogen through microbial decomposition (Birch, 1958;Choudhri and Sharma, 1975). Singh and Singh (2006) reported that during dry periods, plant uptake of nutrients is greatly reduced and the N-mineralization and nitrification are either immobilized in microbial biomass or accumulate in the soil as inorganic nitrogen. Bergeron *et al.* (2002) studies showed that increased biological nitrogen fixation along with increased mineralization rates occur during rainy season, which resulted in increased nitrogen content at this time.

According to the USDA, NRCS guidelines phosphorus availability is controlled by three primary factors: soil pH, amount of organic matter, and proper placement of fertilizer phosphorus. The result revealed in Fig. 3.4 shows that available phosphorus in pre harvest or the rainy season was 12.26 kg/ha and 12.18 kg/ha during post harvest season. According to the soil rating chart AP is low. Since soil pH falls in a very strongly acidic range (between 5 to 5 pH), it typically results in reduction of availability of P in soil solution by 30% or more. Acidic soil also results in reduction of root growth, which is critical to P uptake. Soil pH values below 5.5 and between 7.5 and 8.5 limit phosphate availability to plants. The result however is more or less the same between pre and post harvest season with only 0.08 differences between the mean. Ashraf *et al.* (2014) reported that soil with maximum leaching are known to contain low amount of phosphorus as compared to the soil with minimum leaching. Low AP could also be attributed to the growth of plants and accumulation of biomass during growing season (Styles and Coxon, 2007).

The available potassium of the soil accounts to be 125.67kg/ha in pre harvest and 117.33kg/ha during post harvest season. According to the soil rating chart, the

level of AK falls under medium fertility level. The decline in the potassium content in the agricultural fields might have been due to large uptake of this major nutrient by the cultivated rice crops (Mishra *et al.*, 1979; Cleveland *et al.*, 2003).

Enzymes are known to play a substantial role in maintaining soil health and its environment. An equilibrium of balance between chemical, physical, and biological (including microbial especially enzyme activities) components contribute to maintaining soil health. Enzymes play key roles in the cycling of nutrients in nature and their activity is sensitive to agricultural practices and considered as an index of soil fertility (Nannipieri *et al.*, 2002; Yao *et al.*, 2006). Activities of soil enzymes are greatly affected by organic matter content of soil (Dalal, 1975) and often are used as indices of microbial activity and soil fertility (Kumar *et al.*, 1992). In our study site, three soil enzyme activities which are urease, acid phosphatase and dehydrogenase were conducted on two seasonal variations *viz.* pre harvest season or the rainy season and post harvest season or the dry season. Nannipieri *et al.* (1990) have pointed out that activity of enzyme is substrate specific and are related to specific reactions. Because of this, it is not easy to obtain a general picture of soil fertility status from one enzymatic activity assessment. Thus, three enzymatic activities have been assessed from the study site. The concurrent measurement of different enzymes, on the other hand, happens to be useful to evaluate soil biochemical activity and the process related to soil fertility dynamics (Pascual *et al.*, 1998). One way analysis of variance showed significant seasonal variation between pre and post harvest season in all of the enzyme activities.

Urease was found to have higher activity during pre harvest season with 0.931 and 0.708NH_4^+-Nmg^{-1} 3hrs^{-1}during post harvest season.Veeraragavan *et al.*, (2018) also studied soil enzyme activity on acid phosphatase and urease and found the levels are higher in the rainy season or the monsoon season and less in dry weather. Result of higher activity of urease in growth stages of rice were also supported by Apoorva *et al.* (2018) and Senthil Kumar *et al.* (2000). According to Speir and Ross (1978) higher urease activity may possibly be due to the presence of higher metabolic activity and larger biomass of microorganisms which contributes to most of the soil enzyme activity during a short period of time under favourable conditions.

The activity of enzyme dehydrogenase was exceptionally higher in pre harvest as compared to the post harvest season with 0.864 and 0.182µgTPFmg^{-1} 24hrs^{-1}. This may possibly be due to higher organic matter content, which coincides with the previous study made by Wlodarczyk *et al*. (2002). The characteristic of being associated with viable microbial populations has made dehydrogenase activity the most widely studied enzyme activity in soils (Burns and Dick, 2002). Dehydrogenase enzyme is known to oxidize soil organic matter by transferring protons and electrons from substrates to acceptors. The higher activity of SOM in pre harvest season may perhaps lead to the increase in the activity of DHA during the pre harvest season. Our finding was supported by many workers (Baruah and Mishra, 1984; Benckiser *et al*., 1984; Tiwari *et al*., 1989.) whose studies have shown revealed that dehydrogenase enzyme was higher in wet land or flooded soil compared to non-flooded soil.

Phosphatases are broad groups of enzymes that are capable of catalyzing hydrolysis of esters and anhydrides of phosphoric acid. In soil ecosystems, these enzymes are believed to play critical roles in P cycles (Speir and Ross, 1978) as evidence shows that they are correlated to P stress and plant growth. Enzyme acid phosphatase activity was assessed due to pH level of the soil falling in acidic region ranging from 4.13 to 5.29. The activity of phosphatase was very high in pre harvest season with 93.458 and 59.452µgp-NPmg^{-1}hr^{-1}**.** This could be due to phosphatases are directly related to plant growth.

The higher activity of the soil enzymes in our findings during the cropping period or the pre harvest period where the field is flooded throughout the growing phase which can also be the monsoon season or the rainy season was supported by many researchers like Yang *et al*. (2008) that determined the activities of soil urease, phosphatase and other enzymes at various growth stages of cucumber and found higher activity during early and late growth stages. Wen-Hui *et al*. (2007) in a longterm field experiment in rice crop found significantly higher enzymes (urease, acid phosphatase, dehydrogenase and invertase) in growing stages. Gu *et al*. (2019) also determined the activities of soil enzymes which include urease, phosphatase, invertase, and catalase, which were highly influenced by flooding the field.

Statistical analysis by one way ANOVA showed significant variations in all the parameters except parameter 9 which are available phosphorous between pre and post harvest season as the amount of AP content in both seasons showed no significant variation. The bivariate correlation gave the evidence that the parameters between available phosphorous and soil temperature, available potassium and enzyme dehydrogenase, soil organic matter and soil organic carbon were highly significant during pre harvest season. The enzyme urease was found to be significant with soil moisture content and bulk density. During post harvest season, soil moisture content was significantly correlated with the soil temperature. Available nitrogen was highly significant with soil temperature and significantly correlated with soil moisture content.

Chapter 4

Biochemical Characterization and Plant Growth Promoting Activity of Isolated Phosphate Solubilizing Bacteria

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4.1. Introduction

Microbial diversity is the variety that is present amongst microorganisms and their environments. Microorganisms exist in all ecosystems. The bacteria are omnipresent and are remarkable in the abilities to live in environments that are hospitable for life and the greatest among energy sources (Ganesan and Muthuchelian, 2009). Soil bacteria are one of the most important groups of microbes, which are found to be copious in rhizosphere region of the soil ranging between 10^{-6} to 10^{-8} colony-forming units (cfu) per gram, and some of them have shown great potential for plant growth promotion, development and as biocontrol agents (Siddiqui and Mahmood, 1999).

Bacteria may be suitably grouped into a number of natural assemblages (Holt and Krieg, 1994) based on their characteristics such as cell shape, spore forming capabilities and whether they are aerobic/anaerobic or Gram positive/Gram negative. (Sigee, 2005). The classical method of characterization of bacteria is the morphological and biochemical method of identification. Classical identification of individual bacterial species in environmental samples typically involves isolation, laboratory culture and then taxonomic characterization. The classification of bacteria into families, genera and species is based on a wide range of phenotypic characteristics (Holt *et al.*, 1994). These include culture conditions, colony morphology, biochemical characteristics and detailed morphology. Study of bacterial communities by biochemical characterization has significant potential for environmental studies, which was used by Findlay *et al.* (2003) in their study of stream bacteria.

Microorganisms including bacteria must be identified for various reasons, one of which includes the comparison of biochemical activities for taxonomic

purposes. All microorganisms have their unique identifying biochemical characteristics which are the properties controlled by the cells' enzymatic activity, and they are responsible for bioenergetics, biodegradation and biosynthesis. The addition of all these chemical reactions is defined as cellular metabolism, and the biochemical transformations that take place both outside and inside the cell are governed by biological catalysts called enzymes which are extracellular and intracellular enzymes. Extracellular enzymes or exoenzymes act on substances outside of the cell. Some raw materials like foodstuffs which are high molecular weight substances cannot pass through cell membranes, and so, these substances such as polysaccharides, lipids, and proteins must be degraded to low molecular weight materials nutrients before they can be passed onto the cell. Due to this reaction, exoenzymes are generally hydrolytic enzymes so as to reduce high molecular weight materials into their building blocks by incorporating water into the molecule which results in liberation of smaller molecules, which may then be transported into the cell and assimilated. Extracellular enzymes include starch hydrolysis, lipid hydrolysis, casein hydrolysis and gelatine hydrolysis. Intracellular enzymes or endoenzymes are those enzymes that function inside the cell and are generally responsible for synthesis of new protoplasmic requirements and production of cellular energy from assimilated materials. The ability of cells to act on nutritional substrates permeating cell membranes indicates the presence of many endoenzymes capable of transforming the chemically specific substrates into essential materials. As a result of these metabolic processes, metabolic products are formed and excreted by the cell into the environment. Assay of these end products not only aids in identification of specific enzyme systems but also serves to identify, separate, and classify microorganisms. Intracellular enzymes include carbohydrate fermentation, nitrate reduction, catalase reactions, urease test, oxidase test, litmus milk reaction, H_2S production and IMViC test (Cappucino and Sherman, 2014). Based on these biochemical characteristics, bacteria can be detected at genus level morphologically.

Soil bacteria play an important role in biogeochemical cycles and have been utilized for crop production for decades. Plant–bacterial interactions in the rhizosphere region are the determinants of plant health and soil fertility. Free-living

soil bacteria beneficial to plant development, usually referred to as plant growth promoting rhizobacteria (PGPR), are capable of promoting plant growth by colonizing the plant root (Kloepper and Schroth, 1978; Kloepper *et al*., 1989; Cleyet-Marcel *et al*., 2001) PGPR have the capability to supply or contribute to sustainable plant growth development (Hayat *et al*., 2010). The main functions of these bacteria are: to provide nutrients to plants; to augment plant development, by the production of plant hormones; to control or restrain the activity of plant pathogens; to improve soil structure; and bioaccumulation or microbial leaching of inorganics (Brierley, 1985; Ehrlich, 1990; Davison, 1988). However, the proper mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood (Dey *et al*., 2004) although it has been proven that PGPR have the potential to contribute in the development of sustainable agricultural systems (Schippers *et al*., 1995). The possible explanation accounts to various reasons. First, the ability to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease the amount of ethylene in the root of developing plants thereby increasing the length and growth of the root (Li *et al*., 2000); Second, the capability to release hormones like indole acetic acid (IAA) (Patten and Glick, 2002) gibberellic acid (GA) and cytokinins (Dey *et al*., 2004) etc., the function of symbiotic nitrogen fixation (Kennedy *et.al*., 1997, 2004) and can also have antagonistic activity against phytophatogenic bacteria (Cattelan *et al*., 1999; Pal *et al*., 2001; Glick and Pasternak, 2003) the ability of solubilization and mineralization of nutrients, particularly mineral phosphates (de Freitas *et al*., 1997; Richardson 2001; Banerjee and Yasmin, 2002). Another mechanism by which PGPR can inhibit phytopathogens is the production of hydrogen cyanide (HCN) (Hayat *et al*., 2010).

The symbiotic and non-symbiotic bacteria associated with plants may promote the growth of plants directly through production of plant hormones (Dangar and Basu, 1987; Lynch, 1990) and other PGP activities (Dobbelaere *et al*., 2003). These PGPR synthesizes and produce phytohormones which are called plant growth regulators (PGRs). Some of the well-known PGRs are auxins, gibberellins, cytokinins, ethylene and abscisic acid (Zahir *et al.,* 2004). Attention has been given to phytohormone auxin to a great extent since indole-3-acetic acid (IAA) is the most

physiologically active hormone in plants which is known to increase in cell elongation and cell division and differentiation (Cleland, 1990; Hagen, 1990). IAA is the most common and best characterized phytohormone. It has been estimated that 80% of bacteria isolated from the rhizosphere can produce plant growth regulator IAA (Patten and Glick, 1996). The investigation for microorganisms to be utilised in agricultural field should also assess indirect plant growth mechanisms. The production of siderophores defined as Fe^{3+} binding agents, hydrogen cyanide (HCN), ammonia and chitinases can prevent the harmful effects caused by phytopathogenic organisms. Ammonia is also involved in the supply of nitrogen to plants. Other important enzymes which take part in the colonization of plants by microorganisms are pectinases and cellulases (Hayat *et al*., 2010).

PSB which is also a PGPR increases P uptake by the plant and crop yield. The enzymes and mechanism that affect the plant growth regulation in PSB involves the production of organic acids and acid phosphatases which plays major role in the mineralization of organic phosphorous in soil (Gupta *et al*., 2014; Greaves and Webley, 1965; Tarafdar and Junk, 1987; Garcia *et al*., 1992). Activity of various phosphatases in the rhizosphere of maize, barley, and wheat showed that phosphatase activity was considerable in the inner rhizosphere at acidic and neutral soil pH (Burns, 1983). Phosphate solubilizing microbes have a special quality to secrete enzymes like phosphatase, phytase, and C–P lyase that helps in mineralization of organic P (Othman and Panhwar, 2014). Thus, the enzyme phosphatase is the most commonly secreted enzymes that hydrolyze phosphoric acid into P ion and a free OH group molecule, consequently eliminating P from its substrate. Soil bacteria expressing a significant level of acid phosphatases include strains from the genus*Rhizobium* (Abd-Alla, 1994a, b), *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*and *Klebsiella* (Thaller *et al*., 1995a), as well as Pseudomonas (Gügi *et al*., 1991) and *Bacillu*s (Skrary and Cameron, 1998).

In order to study the beneficial bacteria with plant growth promoting activities associated within the rhizosphere region of the crop, morphological studies including biochemical activities of the isolates is a huge necessity with the different

plant growth promoting trait like the IAA, HCN, NH_3 and the enzyme that plays major role in P solubilization; the acid phosphatase activity were investigated.

4.2. Methodology

4.2.1. Gram's staining

Gram's staining of all of the PSB isolates was done using Himedia gram staining kit. A purified colony was maintained for each isolate, and with the help of a sterile metal loop which was sterilized under the flame in the laminar air flow chamber, one loop of the colony was taken and spread on a clean slide, smeared and fixed in the flame. The instructions on the Himedia grams staining kit was followed which was first stained using crystal violet, then washed in distilled water followed by grams iodine, washed and then decolorized using ethyl alcohol, washed and then blot dried carefully which was counterstained using saffranin. This is then washed and dried which was finally examined under oil immersion objective using compound microscope.

4.2.2. Biochemical characterization

Biochemical characterization of all of the PSB isolates was done under aseptic conditions for the morphological characterization on the basis of different biochemical tests following Cuppucino and Sherman (2007).

4.2.2.1. Carbohydrate fermentation (Glucose/sucrose) test: For this test, phenol red carbohydrate broth was used as media which include carbohydrate source (glucose or sucrose). The test media were autoclaved at 121°C for 15 minutes. After the medium was cooled completely the test bacteria were inoculated aseptically and were incubated at 35±2°C for 24 hours. Positive result showed yellow colour after incubation and negative showed pink colour.

4.2.2.2. Starch hydrolysis test: 24 hours of nutrient broth bacterial culture were streaked on starch agar plates and were incubated at 35±2°C for 48 hours. After incubation the plates were flooded with gram's iodine. Positive result indicates clear zone around the colonies.

4.2.2.3. Gelatin hydrolysis test: This method was done by the nutrient gelatin stab method. 24 hour old test bacteria were inoculated aseptically by stabbing four to five times (half inch) into the tube containing nutrient gelatin medium. The inoculated tubes along with uninoculated tube were incubated at 35±2°C for up to 14 days. The tubes were removed daily and kept at 4°C for 15 mins to check gelatine liquefaction. The tubes were tilted to observe whether the gelatin was hydrolyzed. Positive results showed partial or total liquefaction after exposure to cold temperature.

4.2.2.4. Catalase activity: A loop full of bacterial culture (24 hours old) was placed on a clean slide and 3 drops of 3% H_2O_2 (Hydrogen peroxide) was added to it. Bubbles are developed within 10 seconds indicating positive result which are rapid evolution of oxygen.

4.2.2.5. Oxidase activity: Himedia oxidase disc DD018 was used for the test. The disc was kept on a clean slide and the test bacteria were spread onto the disc and the reaction is observed within 5-10 seconds at room temperature. Purplish blue colour development indicates positive result.

4.2.2.6. Casein hydrolysis: The test bacteria were inoculated by streaking onto petri plates containing skim milk agar media (autoclaved and cooled) and incubated at 35±2°C. After 24 hours the plates were examined for the presence of clear zone around the colonies indicating zone of proteolysis.

4.2.2.7. ONPG test (Ortho-nitrophenyl beta-D-galactopyranoside): Himedia ONPG disc DD008 was used for the test. One ONPG disc was placed in a sterile tube, 0.1ml of sterile 0.85% w/v sodium chloride solution was added to it, then, test inoculums was inoculated into the tube and incubated at 35±2°C 24 hours. Development of yellow colour indicates positive result.

4.2.2.8. Nitrate reduction test: The pure culture of the isolates was sub cultured in a Nitrate broth medium which consists of nutrient broth supplemented with 0.1% KNO_3 (potassium nitrate) as the nitrate substrate and were incubated at 37°C for 48 hours. Then, Solution A (sulfanilic acid), Solution B (α-naphthylamine), and zinc powder were prepared separately. After 48 hrs of incubation, five drops of Solution

A and five drops of Solution B were added to all nitrate broth cultures. The development of red coloration was observed. Then, to the cultures where no red colour developed, minute quantity of zinc powder was added and then observed. No red colour development indicates negative nitrate reduction test.

4.2.2.9. Motility test: Semi solid agar media was used for motility test. 5ml of 1% TTC solution was added to the melted agar medium then autoclaved. When the medium is in a semi solid state, using sterile loop, the test bacteria were stabbed once down the canter of the tube to about half the depth of the medium. The tubes were incubated at 35±2°C for 18 hour or more until growth is developed. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque while non-motile bacteria generally give growths that are confined to the stab-line.

4.2.3. Plant growth promoting activities

4.2.3.1. Acid phosphatase activity (Tabatabai and Bremner, 1969)

Pure bacterial culture was maintained in nutrient broth medium and incubated at 37°C for 48hours. After 48 hours, 3ml of aliquot of the broth culture was taken and to it 1 ml of modified universal buffer (MUB) and 1ml of 0.115M p-NPP were pipetted into a 20ml sterilized test tube and closed with cotton plug and aluminium foil. The mixture was incubated at 37°C for 1 hour at a bacteriological incubator. The enzyme phosphatase reaction was stopped by the addition of 20 ml 0.5N NaOH. The mixture was transferred to a sterilized 50 ml volumetric flask and the volume was made up to the mark with sterile distilled water. Yellow colour was observed in the mixture. The absorbance (yellow colour intensity) was read with a spectrophotometer at 410 nm along with standards that were prepared by using 20mg/ml p-NP.

4.2.3.2. Indole Acetic Acid (Gutierrez *et al.*, 2009)

The bacterial isolates were grown in NBRIP (National Botanical Research Institute's Phosphate) growth medium containing 0.2% L-tryptophan incubated for 72 hour with continuous agitation at 30±7°C at an incubator shaker. After incubation, the culture broth was centrifuged at 8000rpm for 10min and the supernatant was

collected. One ml aliquot of the supernatant was mixed vigorously with 4ml of Salkowski's reagent, incubated at 37°C for 30min. Development of pink color indicates the IAA production and the absorbance were measured at 530 nm in a UV-Visible spectrophotometer. The uninoculated medium mixed with Salkowski reagent served as blank. The concentration of IAA in each culture medium was compared with standard IAA curve.

4.2.3.3. Hydrogen cyanide (HCN) production (Lorck, 1948)

HCN production was determined following the method of Lorck (1948). The isolates were grown in Bennett agar amended with 4.4 g/l glycine. Whatman filter paper was flooded with 0.5% picric acid in 2% sodium carbonate and kept for about one minute and then was placed underneath the lid of the petri dish. The plates were then incubated at 30±7°C for 7 days and were checked every 24 hours. Development of orange to red colour on the filter paper indicates positive HCN production.

4.2.3.4. Ammonia (NH_3) production (Cappucino and Sherman, 1992)

The bacterial isolates were tested for the production of ammonia using the method described by Cappucino and Sherman (1992). In this method, nutrient broth cultures of the isolates were incubated at 37°C in an incubator shaker with continuous agitation at 80% rpm for 7 days. After incubation period is over, 10ml of the broth culture was taken and 0.5ml of nessler's reagent was added and the development of brown to yellow colour indicated a positive test for ammonia production.

4.3. Results

Biochemical screening

Biochemical characterizations of all bacterial isolates were done on few biochemical tests following Cappucino and Sherman (2007). The extracellular enzyme (exoenzymes) test includes starch hydrolysis, gelatin hydrolysis and casein. The intracellular enzyme (endoenzymes) test includes carbohydrate fermentation using glucose and sucrose, nitrate reduction, catalase and oxidase test. The observed

results are shown in table 4.1. The bacterial colonies were circular and some were irregular in shape, raised elevation, most colonies with transparent opacity producing milky/whitish to yellow pigment on the agar plates. There were also colonies that were smooth, shiny and slimy in texture. Mostly all the isolates looked rather similar compared to one another through the naked eye. Out of 43 PSB isolates, 27 isolates were gram positive and 16 isolates were gram negative comprising of 62.79% gram positive. 93 % were positive for catalase test, 48.84% were positive for starch hydrolysis test, 48.84 % were positive for gelatin hydrolysis and in casein test 76.74% were positive, 60.47% were positive for oxidase test, 76.74%were positive for nitrate reduction test,74.41% positive for sucrose and 100% positive for glucose test, 30.23% positive for ONPG. Out of 43 isolates total of 41 isolates were motile accounting to 95.35% motility rate.

pH level: pH of all of the isolates dropped from neutral 7±0.5 pH to acidic ranging from 3.91 to 6.77 (Table 4.3). The lowest pH drops or the most acidic strain was MZLRPC2 with 3.91 drop of pH and highest pH level was MZLRPA4 measuring 6.77.

Acid phosphatase activity: Before inoculating the nutrient broth with bacterial isolates pH was maintained at 7. The significant drop of pH and production of enzyme acid phosphatase activity are the factors which indicate the production of organic acid contributing in the phosphate solubilization. Thus, all the isolates were able to produce acid phosphatase enzyme which mineralizes unavailable form of P compound into available P in NBRIP medium during 48 h of incubation period. The range of the acid phosphatase activity production measured from 0.741 to 41.074 p-NPPμ$g^{-1}ml^{-1}$hr. The highest phosphatase activity producing strain was MZLRPC4 (41.074 p-NPPμ$g^{-1}ml^{-1}$hr) and the lowest was MZLRPC17 (0.74 p-NPPμ$g^{-1}ml^{-1}$hr) (Table 4.3) (Fig 4.1).

IAA production: It was observed that 34 PSB isolates were capable of producing IAA (Table 4.4.) (Fig 4.2) which was able to produce pink colouration then; positive isolates were quantified using UV-Vis spectrophotometer. The PSB isolate with highest IAA production wasMZLRPB3 producing 18.767±2.261 μg ml^{-1} in 72 hrs.

The PSB isolate with lowest IAA production was MZLRPB1 producing 0.867±0.135 µg ml^{-1} in 72 hrs. Strain MZLRPA8.2 did not show any production during 24 and 48 hrs, but showed production on 72 hrs with 3.311±1.473 µg ml^{-1}. Also strain MZLRPB5 was negative on 24 hrs, but showed positive result during 48 and 72 hrs with 0.622±0.078 µg $ml^{-1}$and 1.989±0.206 µg ml^{-1} respectively.

HCN production: Among the 43 isolates, 31 isolates were positive for HCN production with 72.09% rate. (Table 4.2). Isolate MZLRPC4 exhibited the highest amount of HCN production as indicated by a very deep red color on the filter paper. However, among the isolates, some strains showed negative production like MZLRPA7, MZLRPB19, MZLRPB21, MZLRPC12, MZLRPC25 and MZLRPC13. Other negative strains were MZLRPA3, MZLRPB10, MLZRPB11, MZLRPC2, MZLRPC11.1 and MZLRPD2.

NH_3 production: All 43 rhizospheric PSB isolates were positive for the production of ammonia showing 100% positive production (Table 4.2). After the addition of nessler's reagent all of the isolates developed yellow colour which indicated a positive test for ammonia production. This test was done only qualitatively.

Table 4.1: Qualitative biochemical screening of 43 isolated PSB strains

S.N	Strain name	Grams stain	Catalase	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	ONPG	Motility test	Sucrose	Glucose	Oxidase	Nitrate reduction
1	MZLRPA1	+	+	+	-	-	-	+	+	+	-	+
2	MZLRPA2	+	+	+	+	+	-	+	+	+	-	+
3	MZLRPA3	-	+	-	-	-	+	+	-	+	+	-
4	MZLRPA4	-	+	-	-	-	+	+	+	+	+	+
5	MZLRPA7	+	+	+	+	+	-	+	+	+	+	+
6	MZLRPA8.2	+	+	-	+	+	-	+	+	+	-	+
7	MZLRPA10	-	+	-	+	+	-	+	+	+	+	-
8	MZLRPA11	-	-	-	-	+	-	+	+	+	-	+
9	MZLRPA12	+	+	+	+	+	-	+	+	+	+	-
10	MZLRPB1	+	+	-	+	+	-	+	+	+	-	+
11	MZLRPB2	+	+	-	+	+	-	+	+	+	-	-
12	MZLRPB3	+	+	-	+	+	-	+	+	+	-	-
13	MZLRPB4	+	+	-	-	+	-	+	+	+	+	+
14	MZLRPB5	+	+	-	-	+	-	+	+	+	-	+
15	MZLRPB6	+	+	-	-	+	-	+	+	+	-	+
16	MZLRPB8	+	+	-	-	-	+	+	+	+	+	+
17	MZLRPB10	-	+	-	+	-	+	+	+	+	+	+
18	MZLRPB11	-	+	+	+	+	+	+	+	+	+	+
19	MZLRPB12	+	+	-	-	+	+	+	+	+	+	+
20	MZLRPB13	+	+	+	-	-	+	+	+	+	-	+

S.N	Strain name	Grams stain	Catalase	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	ONPG	Motility test	Sucrose	Glucose	Oxidase	Nitrate reduction
21	MZLRPB14.2	+	+	-	-	+	-	+	+	+	-	+
22	MZLRPB17	+	+	-	-	+	-	+	+	+	+	+
23	MZLRPB19	+	+	+	+	+	+	+	+	+	+	+
24	MZLRPB21	+	+	-	-	-	+	+	+	+	+	+
25	MZLRPC2	-	+	-	-	-	-	+	+	+	+	+
26	MZLRPC3	-	+	+	-	+	-	+	-	+	+	+
27	MZLRPC4	-	+	+	+	+	-	+	-	+	+	+
28	MZLRPC5	-	+	+	-	+	-	+	-	+	+	+
29	MZLRPC6	+	+	-	-	+	-	+	+	+	+	+
30	MZLRPC11.1	-	-	-	+	-	+	+	-	+	+	+
31	MZLRPC12	+	+	+	+	+	+	-	+	+	+	+
32	MZLRPC13	+	+	+	+	+	-	+	+	+	-	-
33	MZLRPC16	-	+	+	-	+	+	+	-	+	+	+
34	MZLRPC17	-	+	+	-	-	+	-	-	+	+	-
35	MZLRPC19	+	+	-	-	+	-	+	+	+	+	+
36	MZLRPC20	-	+	+	-	+	-	+	-	+	+	+
37	MZLRPC21	-	+	+	-	+	-	+	-	+	+	+
38	MZLRPC22	+	+	+	+	+	-	+	+	+	-	+
39	MZLRPC23	+	+	+	+	+	-	+	+	+	-	+
40	MZLRPC24	+	+	+	+	+	-	+	+	+	-	-
41	MZLRPC25	+	+	+	+	+	-	+	+	+	-	-
42	MZLRPC26	-	+	+	+	+	-	+	-	+	+	+
43	MZLRPD2	+	-	-	+	+	-	+	-	+	-	-

Table 4.2: Qualitative screening for HCN and NH_3 production

S.N	Strain name	NH_3 production	HCN production
1	MZLRPA1	+	+
2	MZLRPA2	+	+
3	MZLRPA3	+	-
4	MZLRPA4	+	+
5	MZLRPA7	+	-
6	MZLRPA8.2	+	+
7	MZLRPA10	+	+
8	MZLRPA11	+	+
9	MZLRPA12	+	+
10	MZLRPB1	+	+
11	MZLRPB2	+	+
12	MZLRPB3	+	+
13	MZLRPB4	+	+
14	MZLRPB5	+	+
15	MZLRPB6	+	+
16	MZLRPB8	+	+
17	MZLRPB10	+	-
18	MZLRPB11	+	-
19	MZLRPB12	+	+
20	MZLRPB13	+	+
21	MZLRPB14.2	+	+
22	MZLRPB17	+	+

S.N	Strain name	NH_3 production	HCN production
23	MZLRPB19	+	-
24	MZLRPB21	+	-
25	MZLRPC2	+	-
26	MZLRPC3	+	+
27	MZLRPC4	+	+
28	MZLRPC5	+	+
29	MZLRPC6	+	+
30	MZLRPC11.1	+	-
31	MZLRPC12	+	-
32	MZLRPC13	+	-
33	MZLRPC16	+	+
34	MZLRPC17	+	+
35	MZLRPC19	+	+
36	MZLRPC20	+	+
37	MZLRPC21	+	+
38	MZLRPC22	+	+
39	MZLRPC23	+	+
40	MZLRPC24	+	+
41	MZLRPC25	+	-
42	MZLRPC26	+	+
43	MZLRPD2	+	-

Table 4.3: Quantification of acid phosphatase activity and pH level of 43 isolated PSB strains

S.N	Isolated strains	AP-ase p-NPP$\mu g^{-1}ml^{-1}hr$	pH level
1	MZLRPA1	6.111± 0.231	5.50±0.111
2	MZLRPA2	6.074±0.037	4.23±0.210
3	MZLRPA3	6.926±0.161	5.55±0.033
4	MZLRPA4	9.667±0.898	6.77±0.221
5	MZLRPA7	6.481±0.074	5.61±0.031
6	MZLRPA8.2	7.741±0.643	4.97±0.024
7	MZLRPA10	11.37±0.074	5.11±0.051
8	MZLRPA11	6.185±0.206	4.99±0.042
9	MZLRPA12	34.889±0.723	4.00±0.022
10	MZLRPB1	11.000±0.064	4.22±0.021
11	MZLRPB2	6.852±0.225	6.70±0.023
12	MZLRPB3	13.852±0.037	4.91±0.033
13	MZLRPB4	32.593±1.397	4.77±0.022
14	MZLRPB5	11.704±0.89	4.87±0.034
15	MZLRPB6	22.704±3.121	4.69±0.054
16	MZLRPB8	6.000±0.064	4.04±0.055
17	MZLRPB10	12.963±0.185	4.68±0.022
18	MZLRPB11	6.667±0.577	4.76±0.032
19	MZLRPB12	22.185±0.481	4.75±0.043
20	MZLRPB13	22.111±0.78	5.41±0.012
21	MZLRPB14.2	7.519±0.098	4.78±0.112
22	MZLRPB17	16.667±0.064	4.76±0.221
23	MZLRPB19	8.481±0.76	4.50±0.301
24	MZLRPB21	18.481±0.353	4.76±0.210
25	MZLRPC2	9.852±0.134	3.91±0.231
26	MZLRPC3	6.556±0.064	4.23±0.033
27	MZLRPC4	41.074±0.643	3.98±0.043
28	MZLRPC5	6.963±0.134	5.62±0.024
29	MZLRPC6	5.852±0.303	5.77±0.038
30	MZLRPC11.1	12.593±0.134	5.00±0.059
31	MZLRPC12	1.407±0.243	4.23±0.099
32	MZLRPC13	10.111±0.525	5.44±0.029
33	MZLRPC16	4.037±0.225	4.99±0.114
34	MZLRPC17	0.741±0.316	6.00±0.231
35	MZLRPC19	8.37±0.392	5.70±0.451
36	MZLRP20	9.889±0.064	4.98±0.253
37	MZLRPC21	6.111±0.28	5.11±0.312
38	MZLRPC22	8.704±0.196	4.91±0..022
39	MZLRPC23	8.444±0.357	4.89±0.015
40	MZLRPC24	21.296±0.098	4.91±0.211
41	MZLRPC25	8.741±0.098	5.00±0.098
42	MZLRPC26	6.963±0.365	4.56±0.071
43	MZLRPD2	2.963±0.376	5.91±0.034

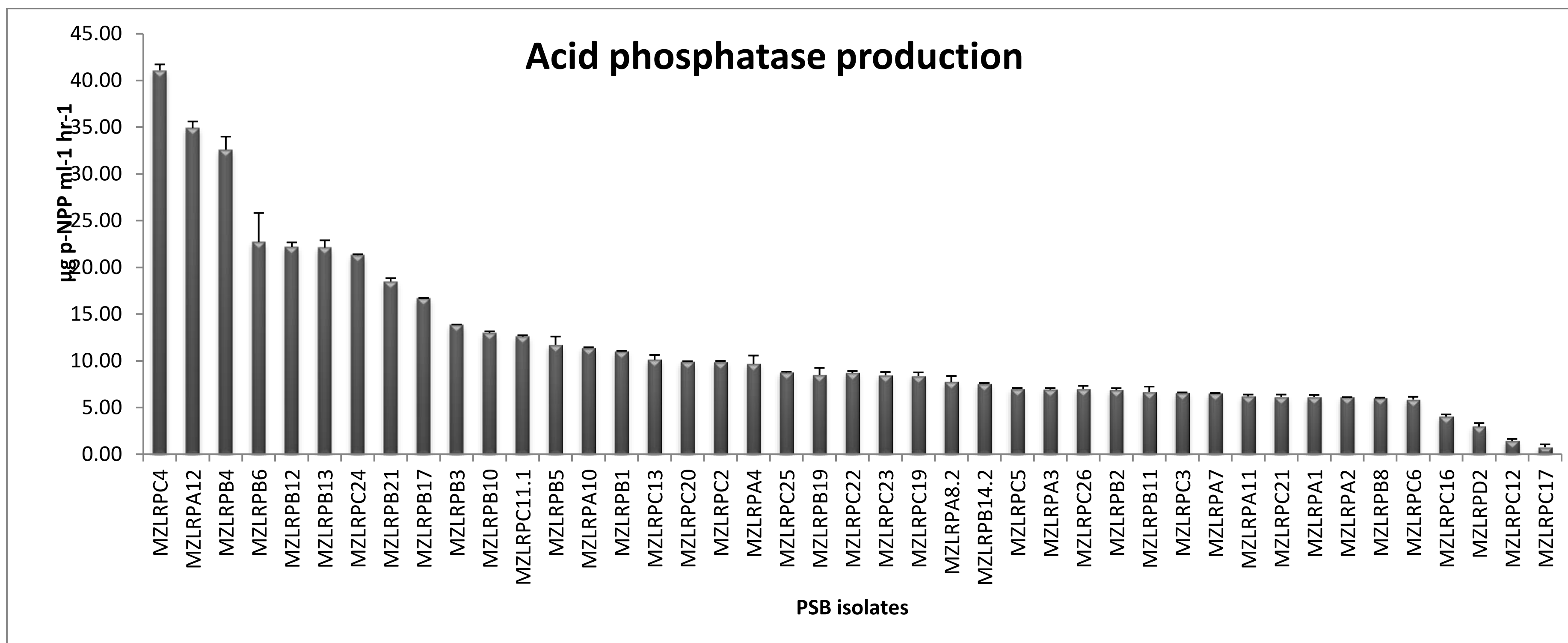

Figure 4.1: Acid phosphatase (APase) (µg p-NPP ml^{-1} hr^{-1}) activity of isolated PSB strains.

Table 4.4: Quantification of IAA production in µg ml^{-1} on 34 PSB isolated strains.

S.N	Strain Names	24hrs IAA µg ml^{-1}	48hrs IAA µg ml^{-1}	72hrs IAA µg ml^{-1}
1	MZLRPB3	4.566±0.051	7.978±0.345	18.767±2.261
2	MZLRPA3	11.988±0.262	19.444±1.161	18.656±1.002
3	MZLRPB17	12.485±0.010	16.067±0.117	16.511±0.029
4	MZLRPB19	15.666±0.019	15.856±0.011	16.311±0.128
5	MZLRPB21	16.322±0.029	16.711±0.266	16.456±0.263
6	MZLRPB6	8.355±0.149	18.256±1.897	16.311±0.649
7	MZLRPB14.2	13.566±0.561	15.233±0.468	15.967±0.192
8	MZLRPC4	14.344±0.011	16.601±0.351	15.811±0.785
9	MZLRPC20	12.000±0.117	15.801±0.192	14.302±0.203
10	MZLRPC21	14.211±0.106	15.822±0.212	12.978±0.517
11	MZLRPB8	0.066±0.096	3.278±0.941	12.389±3.344
12	MZLRPC13	12.311±0.097	12.844±0.213	12.089±0.414
13	MZLRPC23	4.377±0.022	5.044±0.022	11.889±0.222
14	MZLRPC3	11.700±0.051	11.933±0.301	11.456±0.495
15	MZLRPC2	8.300±0.153	9.744±0.678	11.322±1.113
16	MZLRPC12	7.333±0.019	9.402±0.867	11.400±0.353
17	MZLRPC19	10.477±0.106	10.489±0.095	11.044±0.029
18	MZLRPB11	13.411±0.247	13.578±0.475	10.333±0.656
19	MZLRPC5	7.900±0.117	8.378±0.212	8.411±0.225
20	MZLRPC6	7.788±0.106	8.522±0.228	8.478±0.495
21	MZLRPC16	8.733±0.019	9.378±0.595	8.244±0.124
22	MZLRPC25	5.455±0.011	5.322±0.239	6.667±0.426
23	MZLRPC26	5.055±0.011	5.089±0.011	6.656±0.422
24	MZLRPC24	4.366±0.033	4.733±0.019	5.667±0.773
25	MZLRPA12	1.222±0.545	8.002±1.367	4.978±0.235
26	MZLRPC22	5.066±0.019	5.211±0.438	4.489±0.319
27	MZLRPD2	4.366±0.019	5.122±0.563	4.444±0.095
28	MZLRPC17	4.4333±0.051	5.467±0.782	4.433±0.084
29	MZLRPB12	0.101±0.033	1.922±1.161	4.411±0.078
30	MZLRPA8.2	-	-	3.311±1.473
31	MZLRPB10	0.622±0.022	1.578±0.041	2.122±0.228
32	MZLRPB5	-	0.622±0.078	1.989±0.206
33	MZLRPB2	0.222±0.089	0.769±0.536	1.244±0.472
34	MZLRPB1	0.144±0.073	0.589±0.011	0.867±0.135

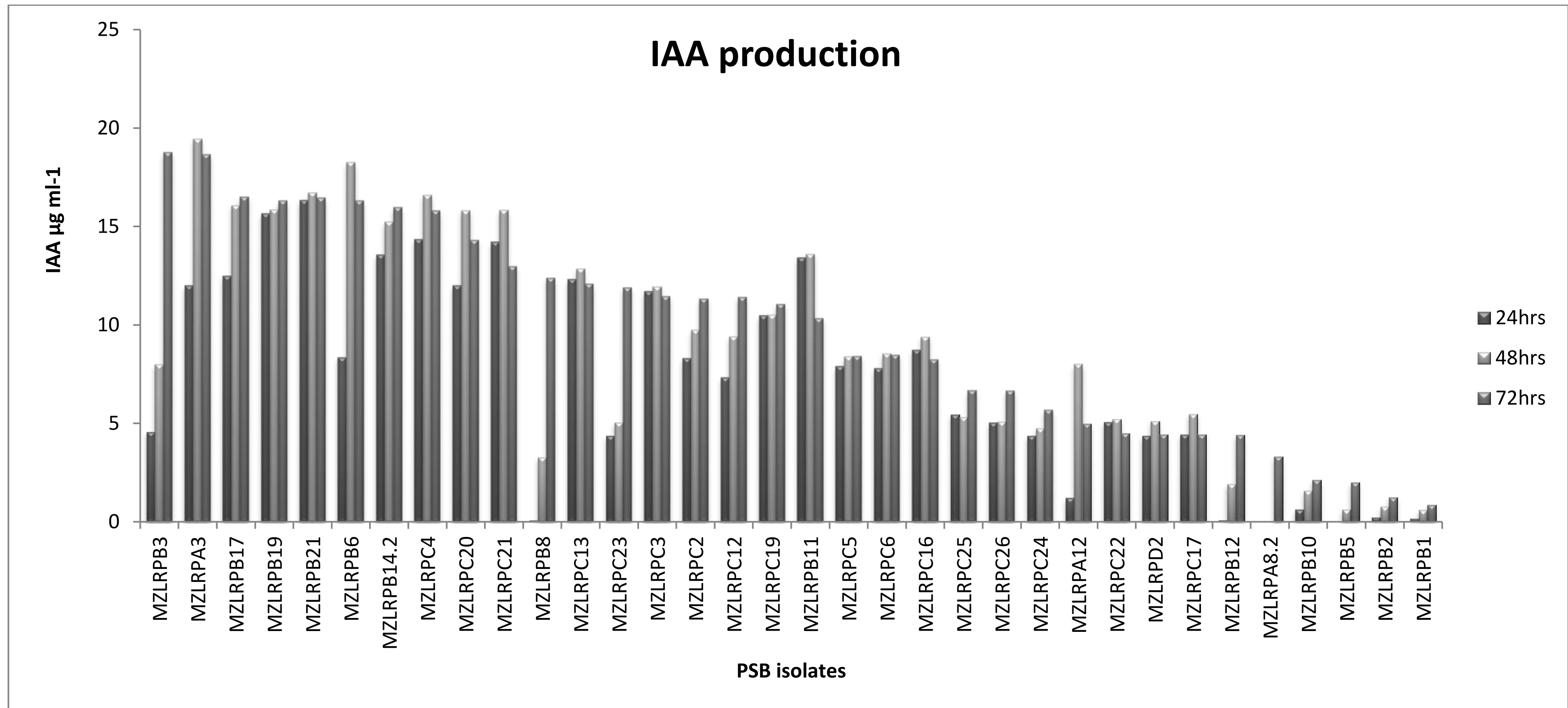

Figure 4.2: Indole acetic acid quantification (IAA µg ml^{-1}) of potential PSB strains.

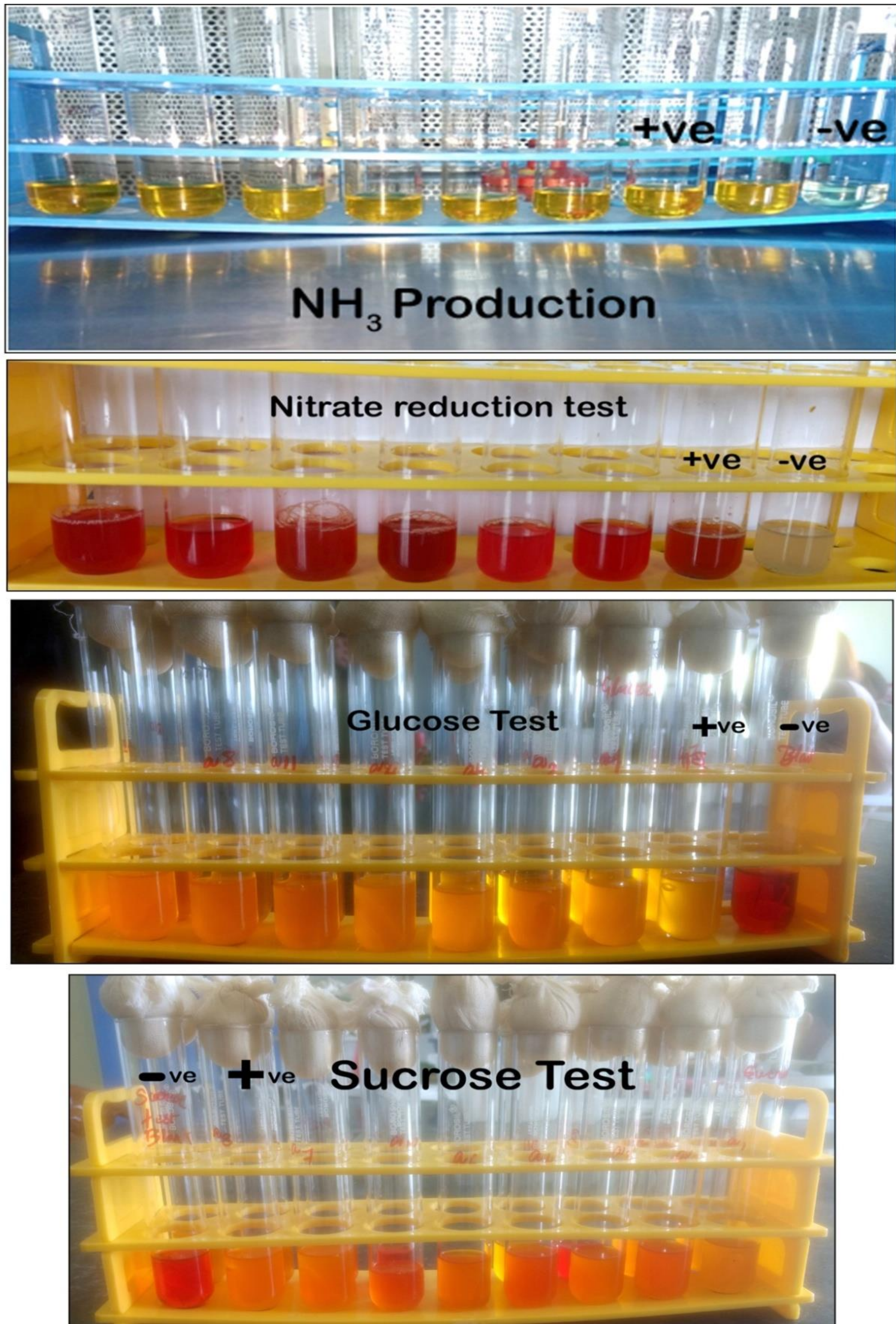

Figure 4.3a: Biochemical screening of potential PSB isolates.

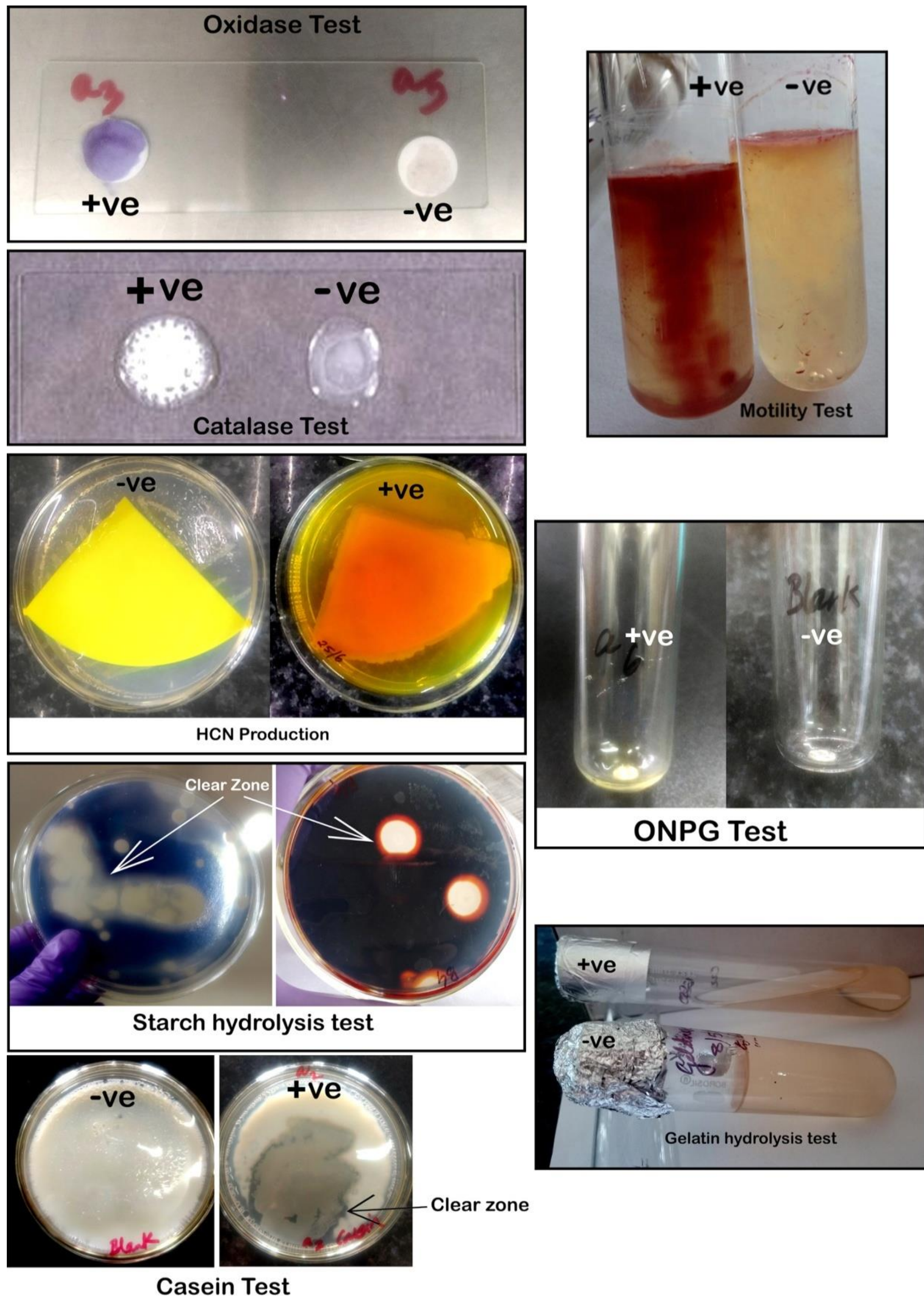

Figure 4.3b: Biochemical screening of potential PSB isolates.

4.4. Discussion

Phosphorus is a very important macronutrient required by plants second to nitrogen. It exists in soil as mineral salts or incorporated into organic compounds. Regardless of phosphorus compounds being abundant in soil, the majority of them occur in an insoluble form (Miller *et al.*, 2010). Due to this reason application of phosphate fertilizer in agricultural soil has become very popular which has constraints in that it is rapidly immobilized (fixed) to insoluble forms which leads lost as a result of run-off and leaching, leaving as little as 10–20% available for plant utilization (Sashidhar and Podile, 2009). So beneficial microorganisms in this aspect that have the ability to liberate organic phosphates or to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate and make it available to plants is of vital importance.

In this study, 43 phosphate solubilizing bacteria which were confirmed using pikovskaya medium for their P solubilizing ability which were morphologically characterized based on different biochemical test. The extracellular enzyme (exoenzymes) test includes starch hydrolysis, gelatin hydrolysis and casein. The intracellular enzyme (endoenzymes) test includes carbohydrate fermentation using glucose and sucrose, nitrate reduction, catalase and oxidase test.

Carbohydrate fermentation test is to determine the ability of an organism to ferment (degrade) a specific carbohydrate in a basal medium producing acid or acid with visible gas. The acid would change the colour of the medium in a positive test. Organisms use carbohydrates differently depending on their enzyme complement. In this study, monosaccharides sucrose and glucose were used as carbohydrate source and all the strains were able to ferment the glucose and 11 PSB isolates could not ferment sucrose. This finding is supported by Sagervanshi *et al.* (2012) who also found positive glucose fermentation test on all isolated PSB from the soil and negative sucrose test for two strains AB-01 and AB-02. The lack of carbohydrate fermentation by some organisms should not be construed as absence of growth. The organisms use other nutrients in the medium as energy sources. Among these nutrients are peptones present in nutrient broth (Cappucino and Sherman, 2014).

Starch hydrolysis was performed to test the utilization of starch by bacteria by producing the enzyme amylase which is an exoenzyme that hydrolyses starch. Only 21 of the PSB strains could hydrolyse starch *i.e*., they could produce exoenzyme amylase (Priest, 1977) and 22 PSB strains could not hydrolyse starch. It has been proposed that *Bacillus cereus* strains producing emetic toxin are unable to hydrolyze starch (Raevuori *et al.*, 1977) (Shinagawa *et al.*, 1985). So, the isolates that lack amylase activity could have emetic toxins. There were some strains that could hydrolyse starch among the same genus but were also negative in other strains. Draghi *et al.* (2014) reported two strains of *Burkholderia* that were well capable of starch hydrolysis. Khambalkar and Sridar (2015) also reported positive result of starch hydrolysis, gelatin hydrolysis and carbohydrate fermentation on *Burkholderia* sp.

Gelatin hydrolysis or the gelatin liquefaction test was performed to check the ability of the microorganism to produce the enzyme gelatinase. Gelatin is a protein derived from collagen, which is insoluble in cold but soluble in hot water and form gel on cooling it. The proteolytic organisms digest protein and may liquefy gelatin (Hemraj *et al*., 2013). Out of 43 psb strains tested, 21 strains could hydrolyse gelatin and 22 strains could not. So, 48.84% could hydrolyse gelatin. This finding is in support of Sabiha *et al*. (2010) where they tested *Bacillus* sp. from rhizosphere region of *Costus* sp. and found all strains were capable of hydrolysing gelatin. Pandey *et al*. (2005) also found *Burkholderia* sp. to hydrolyse gelatin which are also PSB.

40 isolates were capable of producing enzyme catalase which indicates that it detoxifies hydrogen peroxide by breaking it down into water and oxygen gas thus forming bubbles by addition of hydrogen peroxide. A positive result is detected by the formation of air bubbles (Hemraj *et al*., 2013).

Oxidase test is done to detect the presence of cytochrome C and hence the production of oxidase enzyme by given test organism. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of the test reagent p-aminodimethylaniline oxalate to colonies grown on a plate medium (Hemraj *et al*., 2013). Purple colour development indicates positive test. In this study 26 of total isolates test strains were positive for oxidase test and 17 were negative.

Nitrate reduction test determines the production of an enzyme called nitrate reductase, which results in the reduction of nitrate (NO_3). Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. In the tested strains, 33 PSB isolated strains were able to reduce nitrate to nitrite.

Casein hydrolysis test determines the ability to degrade the casein protein by producing proteolytic exoenzyme called proteinase (caseinase) by some microorganisms. 33 PSB isolated strains were able to hydrolyse casein.

O-nitrophenyl-beta-D-galactopyranoside (ONPG) is an artificial substrate structurally similar to lactose with the exception that glucose is substituted with an o-nitrophenyl group. Unlike lactose, the substrate O-nitrophenyl-beta-D-galactopyranoside (ONPG) is capable of penetrating the bacterial cell without the presence of permease. In the disk method, the organism to be tested is taken from a medium containing a high concentration of lactose. A dense suspension (turbidity equivalent to a McFarland 3) is prepared. An ONPG disk is added to 0.5ml of the suspension. If the organism possesses beta-galactosidase, the enzyme will split the beta-galactoside bond, creating a yellow color change in the suspension. Organisms with strong beta-galactosidase activity can produce a positive reaction a few minutes after inoculation of the ONPG medium; other organisms may take up to 24 hours. In this study, 13 PSB strains were positive for O-nitrophenyl-beta-D-galactopyranoside (ONPG) test.

Motility is the ability of an organism to move by itself by means of propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility. All of the tested isolated PSB strains were found to be motile. Mahantesh and Patil (2011) have also worked on biochemical test of phosphorous solubilizing microbes and have found similar results.

All of the 43 PSB isolates were able to produce the enzyme acid phosphatase using the substrate p-nitrophenyl phosphate of which Glick (2005) described the production of enzymes phosphatase and phytase have prominent effects on P solubilization as well as plant growth. Similar study was done by Behera *et al*. (2017) who determined acid phosphatase activity by performing p-nitrophenyl phosphate assay (p-NPP) of the P solubilizing bacterial broth culture. Ponmurugan

and Gopi (2006) also reported the production of phosphatase enzyme by PSB.Panhwar *et al*. (2011) also studied acid phosphatase activity on phosphate solubilizing bacteria from aerobic rice in Malaysia. As shown in Table 4.3, the highest phosphatase activity producing strain was MZLRPC4 (41.074 p-NPPμg^{-1}ml^{-1}hr) and the lowest producing strain was MZLRPC17 (0.74 p-NPPμg^{-1}ml^{-1}hr).

The PSB isolated strains had the potential for other characteristic like production of phytohormone Indole acetic acid (IAA) which is known to have important effects on plant growth and development (Glick, 2005). 79.1% of the total tested strain has the ability to produce IAA. This study is supported by other researchers like Naher *et al*. (2009) and Panhwar *et al*. (2012) who also found similar results that isolated PSB strains from rice field have the potential for the production of IAA. It has thus been reported that IAA production by microbes can vary among different species and strains, which is also influenced by culture condition, growth stage and substrate availability (Mirza *et al*., 2001). As studied by Sarwar and Kremer (1995) isolates from the rhizosphere soil are more efficient auxin producers than isolates from the bulk soil. From our study, it shows that incubation period have great impact on the IAA production as it keeps on increasing with increase in incubation time. The two strains MZLRPA8.2 and MZLRPB5 did not show any production during 24 and 48 hrs, but showed production on 72 hrs. The reason could be that in 24 hrs incubation there was minute production of IAA which was unable to detect it under UV light. However, as the incubation increases, increasing in the cell number, it was able to detect after 48 hrs of incubation.

72.09% of all the strains tested produced hydrogen cyanide. Karuppiah and Rajaram (2011) have also studied HCN production by *Bacillus* sp. strains isolated from rice rhizosphere and Kumar *et al*. (2012) have also found HCN production on several bacterial species like *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp., *Acinetobacter* sp., and *Micrococcus* sp. isolated from the beans rhizosphere. As described by Haas and Defago (2005) the hydrogen cyanide is part of powerful antifungal compounds produced by PGPR and involved in pathogens biological control.

Ammonia (NH_3) production is an important characteristic of plant growth promoting rhizobacteria which indirectly influences plants growth (Yadav *et al*., 2010). Out of the entire strains tested 100% were positive for ammonia production. Agbodjato *et al*. (2015) also tested ammonia production on several PGPR bacterial strains like *Serratia* strains *Bacillus* sp. and *Pseudomonas* sp. Joseph *et al.* (2007) have also observed NH_3 production on *Bacillus* sp. and *Pseudomonas* sp.

Chapter 5

Isolation and 16SrRNA Gene Sequencing of Phosphate Solubilizing Bacteria

5.1. Introduction

In the earth's ecosphere, the size of microbial diversity is much larger and unknown than previously thought (Rondon *et al.*, 1999). A sense of the biological dominance of microbes is given by estimates of the total number of living bacteria, roughly 4-6 x 10^{30} cells (Whitman *et al.*, 1998; Dunlap, 2001). However, the true extent of microbial diversity is assumed to be much higher, as many microorganisms are not recovered by using the employed culture techniques (Rondon *et al.*, 1999). So, the target of various researchers or workers has been determined towards further characterizing of different microorganisms from clinical, environmental or food etc. using molecular techniques and phylogenetic analysis based on the DNA sequence information in order to understand their diversity, habitat and relationship with the environment.

In the 1980s, a new standard for identifying bacteria began to be developed. In the laboratories of Woese and others, it was shown that phylogenetic relationships of bacteria and indeed all life-forms could be determined by comparing a stable part of the genetic code (Woese *et al.*, 1985, 1987). Hence, rRNA genes have been used as standard phylogenetic markers in molecular taxonomic studies since the pioneering studies on the tree of life by Woese and Fox (1977). In modern days, in the taxonomy of microorganisms, molecular biology methods like 16S ribosomal RNA (rRNA) gene sequencing (Duskova *et al.,* 2012; Pendharkar *et al.*, 2013), polymerase chain reaction (PCR) (Callaway *et al.*, 2013) and other related PCR-based methods (Deggim *et al.*, 2016; Adelfi *et al.*, 2013) are very popular. These techniques are characterized by high sensitivity and reproducibility. 16SrRNA gene sequencing is considered the most accurate method and claimed the gold standard for the identification of microorganisms' up to the species level (Cherkaoui *et al.*, 2010). This has also emerged as the single best method to identify bacteria (Kolbert and Persing, 1999; Drancourt *et al.*, 2000). Ehresmann *et al.* (1972) sequenced the first bacterial 16SrDNA for *Escherichia coli* (GenBank accession No. J01859) which

contains 1542 nucleotides. As more 16SrDNAs were sequenced and studied, it was realized that (1) the nucleotide sequences between different bacteria are highly conserved (2) the conservation and divergence reflect bacterial evolution and (3) each bacterial species has its unique 16SrDNA sequences (Fox *et al*., 1980). Therefore, 16SrDNA sequencing became a tool for studies of bacterial phylogeny. The utilization of 16SrRNA gene sequences for the characterization and distinguishing proof of prokaryotes is mostly subjected to comparisons against a database of known sequences. At present, the sequences of type strains of ~99% of prokaryotic species with validly published names are accessible in public databases (Chun and Rainey, 2014). Again, 16SrRNA sequence utilization has led to various bacterial genera and species reclassification and thus, renamed; classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species have been facilitated (Woo *et al*., 2008).

The application of the phylogenetic knowledge to microbial community has contributed to microbial diversity studies worldwide. The phylogenetic revolution and the progress of culture-independent molecular approaches have provided right estimates for the diversity of microbial communities (Amann *et al*., 1995). Microbial diversity constitutes an extraordinary reservoir of life in the biosphere that has only just begun to be explored and understood (Jain *et al*., 2005). Understanding patterns of bacterial diversity is of particular importance because bacteria may well comprise the majority of earth's biodiversity and mediate critical ecosystem processes (Cavigelli and Robertson, 2000; Torsvik *et al*., 2002).

Numerous soil bacteria and fungi notably species of *Bacillus, Pseudomonas, Penicillium*, *Aspergillus*, etc. secrete organic acids and lower the pH in their vicinity to bring about dissolution of bound phosphates in soil (Gerretsen, 1948; Sundara Rao and Sinha, 1963; Gaur and Ostwal, 1972).

The understanding of the molecular diversity of PSB can be helpful in the selection of the dominant types of the bacteria involved in P-Solubilization which find their use as biofertilizers.

5.2. Methodology

5.2.1. Collection of soil samples

Soil sample was collected from the rice rhizoshperic region of North Vanlaiphai paddy field situated in Serchhip district, Mizoram during cropping period in the month of July and September. The region is located at latitude 23° 7' 47" N and longitude 93°4' 11" E. Soil samples were collected randomly within the root rhizospheric region of 0-30cm deep from 10 different plots and within each plot; samples were collected from five different rice crops and mixed as one composite sample. Collected samples were kept in plastic bags contained with ice pack and transported to the laboratory and stored at 4°C for further investigation.

5.2.2. Isolation and purification of phosphate solubilizing bacteria

Isolation of Phosphate Solubilizing Bacteria from the collected soil sample was done by plating serial dilutions of the soil extracts in Pikovskaya's agar medium (PVK) also called the plate assay method (Pikovskaya, 1948). PVK medium was autoclaved at 121°C and pressure 15psi for 15 minutes. 1g of soil sample was dispersed in 9 ml of sterilized water and then serially diluted upto 10^{-8} dilution using autoclaved distilled water and maintaining replicates in each dilution. The mixture was shaken thoroughly. 100µl of each suspension were transferred on Pikovskaya's agar medium on petri plates and spread evenly using sterile L-shaped spreader and incubated at 30±37°C for 4 days or more until clear halo zones are formed. Colonies with clear halo zones start to appear from the second day of incubation (Fig 5.1).These colonies were selected for further sub-culturing, purified and maintained in agar plates at 4°C.The production of clearing zones around the colonies is an indication of the presence of phosphate-solubilizing bacteria.

5.2.3. Determination of Phosphorus Solubilization Index (SI)

Phosphorus solubilizing index (PSI) of each PSB isolates was assayed by spotting the cultures on Pikovskaya's agar media plates. The plates were incubated at 30±7°C for 7 days and observed for colony diameter and diameter of solubilization zone. The diameter of the colony was measured in cm.PSI was evaluated according to the ratio of the total diameter (colony+halo zone) and the colony diameter (Edi-Premono *et al.,* 1996).

$$\text{Solubilization Index (SI)} = \frac{Colonydiameter\ + Halozonediameter}{Colonydiameter}$$

5.2.4. Molecular identification of phosphate solubilizing bacterial strains

Isolation of genomic DNA was done following the protocol of Kumar *et al.* (2010). The purified culture was transferred onto Nutrient Broth medium (Difco manual, 1953) in an aseptic condition and incubated at 30±7°C overnight in a bacteriological incubator. Exact 2ml of the overnight grown culture was centrifuged at 8000 rpm for 5 mins. The pellet was washed twice with TE buffer and suspended in 567µl TE buffer containing 2 mg/ml lysozyme. The suspension was incubated in a temperature controlled water bath at 37°C for 30 mins. Then, to the suspension, 3µl of proteinase K and 30 µl of 10% SDS was added and incubated at 37°C for 1 hour in a water bath. To this, 100µl of 5 mol/L NaCl was added and mixed thoroughly. Then, pre warmed 80µl of 10% CTAB (cetyl trimethyl ammonium bromide) was added and incubated at 65°C for 10 mins. After this, the samples were cooled down at room temperature and equal volumes of (P:C:I) phenol/chloroform/isoamyl alcohol (25:24:1) were added and mixed well by carefully inverting it until the phases were completely mixed. The tubes were centrifuged at 12,000 rpm for 5 mins at 4°C and the upper aqueous phase was aspirated out carefully using sterile micropipette. To the clear aqueous phase, equal volume of chloroform/isoamyl alcohol (CI) (24:1) was added and mixed by gently vortexing for few seconds and centrifuged at 12,000 rpm for 5 mins at 4°C. The clear aqueous phase was precipitated out and to it, double volume of chilled ethanol was added followed by

addition of 1/10th volume of 3M sodium acetate. Samples were left overnight at -20°C to allow DNA precipitation. The precipitated DNA pellet was collected by centrifuging at 12,000 rpm for 10 mins at 4°C. The pellet was washed with 70% ethanol and air dried followed by suspension in 50 µl of TE buffer. The DNA samples so prepared were qualitatively checked on 0.8% agarose gel. 1X TBE buffer was used as the running buffer and for preparation of the agarose gel. 3µl of 10mg/ml of the intercalating agent ethidium bromide (EtBr) was added to the 50 ml of agarose gel for DNA binding purpose. The electrophoresis gel was run at 80 volts. The genomic DNA banding patterns produced in the agarose gel was visualized under UV light and documented using a Protein simple Gel Doc Alphamagermini system (Taiwan). The genomic DNA were kept at -20°C for further analysis.

5.2.5. PCR amplification of 16srRNA gene

16SrRNA gene amplification was carried out by using universal bacterial primers 27f as forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and 1525r as reverse primer (5'-AGAAAGGAGGTGTACCAGCC-3').The reaction was performed on Mastercycler nexus gradient (Eppendorf AG, Germany) according to the conditions given in Table5.1 (a) (b). The total PCR reaction mixture was 25µl. A negative control reaction mixture without DNA template was also included with each set of PCR reactions. The amplified PCR product was checked by using 1.5 % agarose gel electrophoresis using 1X TBE buffer and stained with ethidium bromide. The PCR bands were analyzed under UV light and documented using a Protein simple Gel Doc Alphamagermini system (Taiwan). The PCR products of 16S rRNA gene were sent for sequencing commercially at Eurofins Genomics India Pvt. Ltd. Bangalore. The sequence was compared for similarity level with the reference strains of PSB from genomic database banks, using the NCBI Blast available at the http://www.ncbi.nlm.nih.gov/blast web site. The structures were analysed using the CLUSTAL W v.1.4 software.

Table 5.1(a) PCR mixture conditions

Components	Quantity (µl)
DNA template	1
10X buffer	2.5
25mM $MgCl_2$	1.5
Dntp	2
27F (10pmole/µl)	0.8
1525R (10pmole/µl)	0.8
DNA Polymerase (2U/µl)	0.5
Nuclease free H_2O	15.9
Total	**25**

Table 5.1 (b) Cycling conditions of PCR

Initial denaturation	95°C for 5 min
Denaturation	94°C for 1 min (30 cycles)
Annealing	57.5°C for 40 sec
Extension	72°C for 1.30 min
Final extension	72°C for 10 min

5.2.6. Phylogenetic analysis

The evolutionary models were selected based on lowest BIC (Bayesian Information Criterion) value using MEGA 7. The evolutionary history was inferred by using the Neighbour joining method based on the Kimura 2-parameter model (Kimura, 1980).

5.3. Results

5.3.1. Isolation and purification of phosphate solubilizing bacteria

Collection of the soil sample was done in a completely randomized block design. Samples were taken on pre harvesting period or the cropping stage while the paddy field remained flooded in the particular growing period. The temperature of the soil was approximately 25.2°C during the study period. The collection of the samples was done four times during two consecutive years in the month of July and September. Samples were collected only from the root rhizospheric region of the rice crop. Serial dilution ranging from 10^{-2} to 10^{-8} was done where maximum colonies of PSB was found in 10^{-3} and 10^{-4} dilutions. PSB were selected based on the colonies having clear halo zone on pikovskaya's agar medium. Clear zones started to develop

from the 2nd day of incubation and also 3rd day for some strains (Fig 5.1 to 5.6). The solubilization zones increases with the increase in the incubation period. So, S.I was taken on the 7th day of incubation. Most of the colonies were creamy, off-white in colour, some were pale yellow and one colony was pink in colour, beige or pale brown colour. The shape of the colonies was mostly round and some were root like structure. Texture was slimy or sticky and even rough dry surface was found. Total of 43 phosphate solubilizing bacteria were isolated and identified. These isolates were designated as MZLRPA1, MZLRPA2 and so on (Table 5.3). Among the 43 isolated strains, seven different genera were identified *viz*., *Bacillus* (25), *Burkholderia* (6), *Paenibacillus* (1), *Paraburkholderia* (8), *Edaphobacter* (1), *Dyella* (1) and *Paraclostridium* (1). The maximum species identified showed closest similarity with *Bacillus cereus* with total of 9 isolates. This is followed by *Paraburkholderia fungorum* with total of 5 isolates.The solubilization index of all the isolates were measured and recorded. The S.I ranges from 2.21cm to 4.6cm (Table 5.2). The PSB strain with highest S.I was MZLRPC4 *Paraburkholderia fungorum* with 4.64cm and MZLRPC25 *Bacillus cereus* with 4.6cm. The lowest S.I was MZLRPB11 *Burkholderia dolosa* with 2.08cm and MZLRPB5 *Bacillus cereus* with 2.11cm.

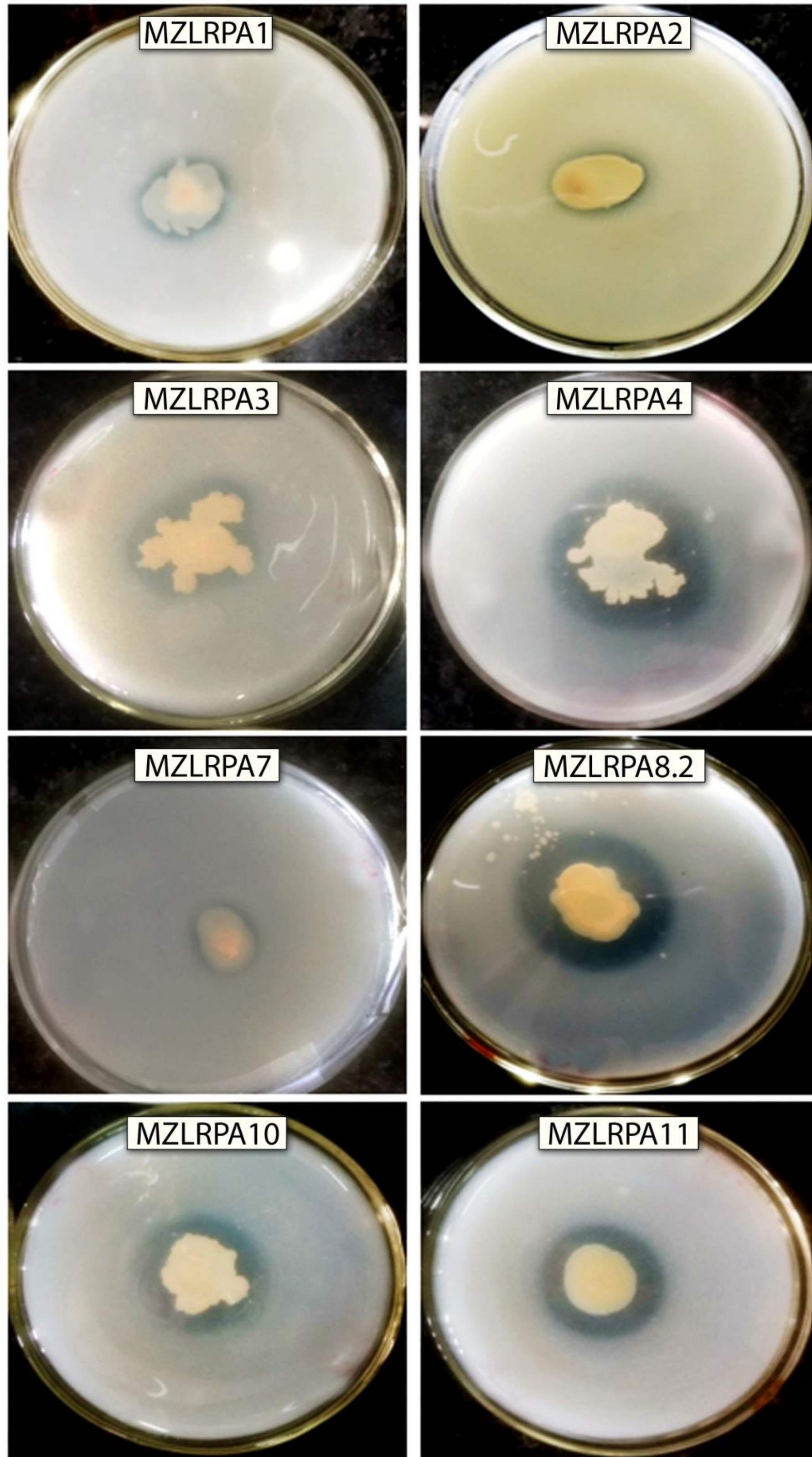

Figure 5.1: Potential PSB strains MZLRPA1-MZLRPA11 grown on PVK agar media

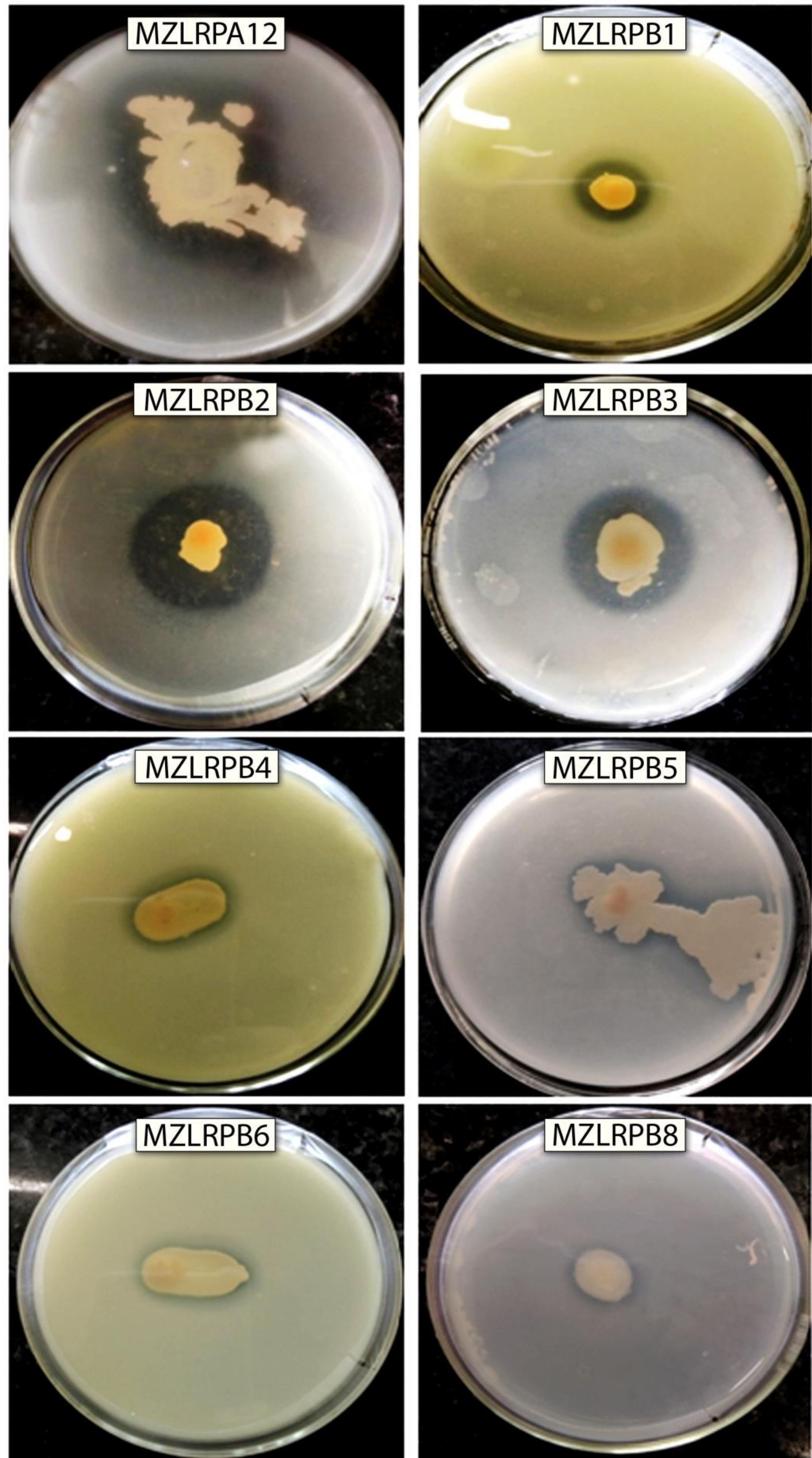

Figure 5.2: Potential PSB strains MZLRPA12-MZLRPB8 grown on PVK agar media

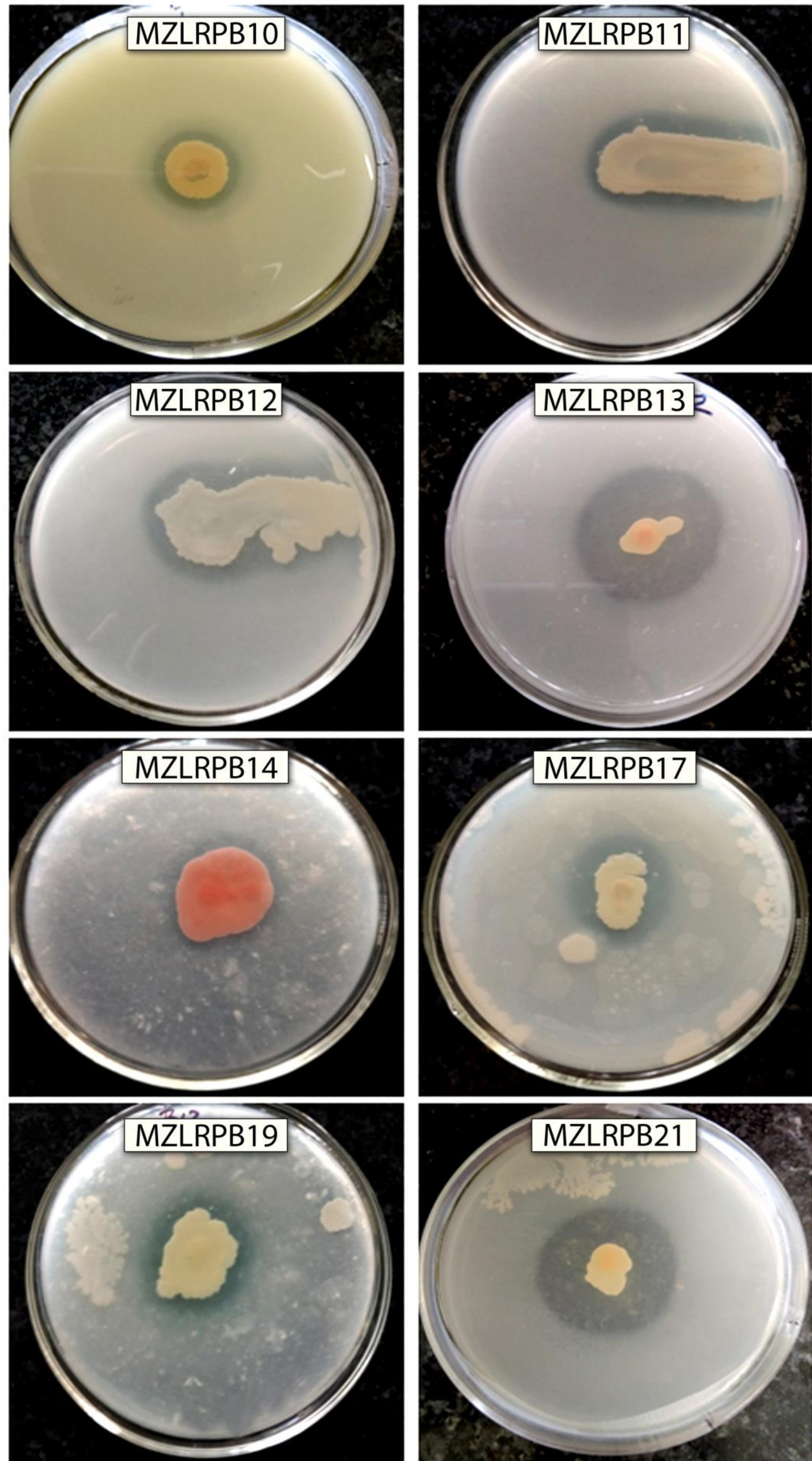

Figure 5.3: Potential PSB strains MZLRPB10-MZLRPB21 grown on PVK agar media

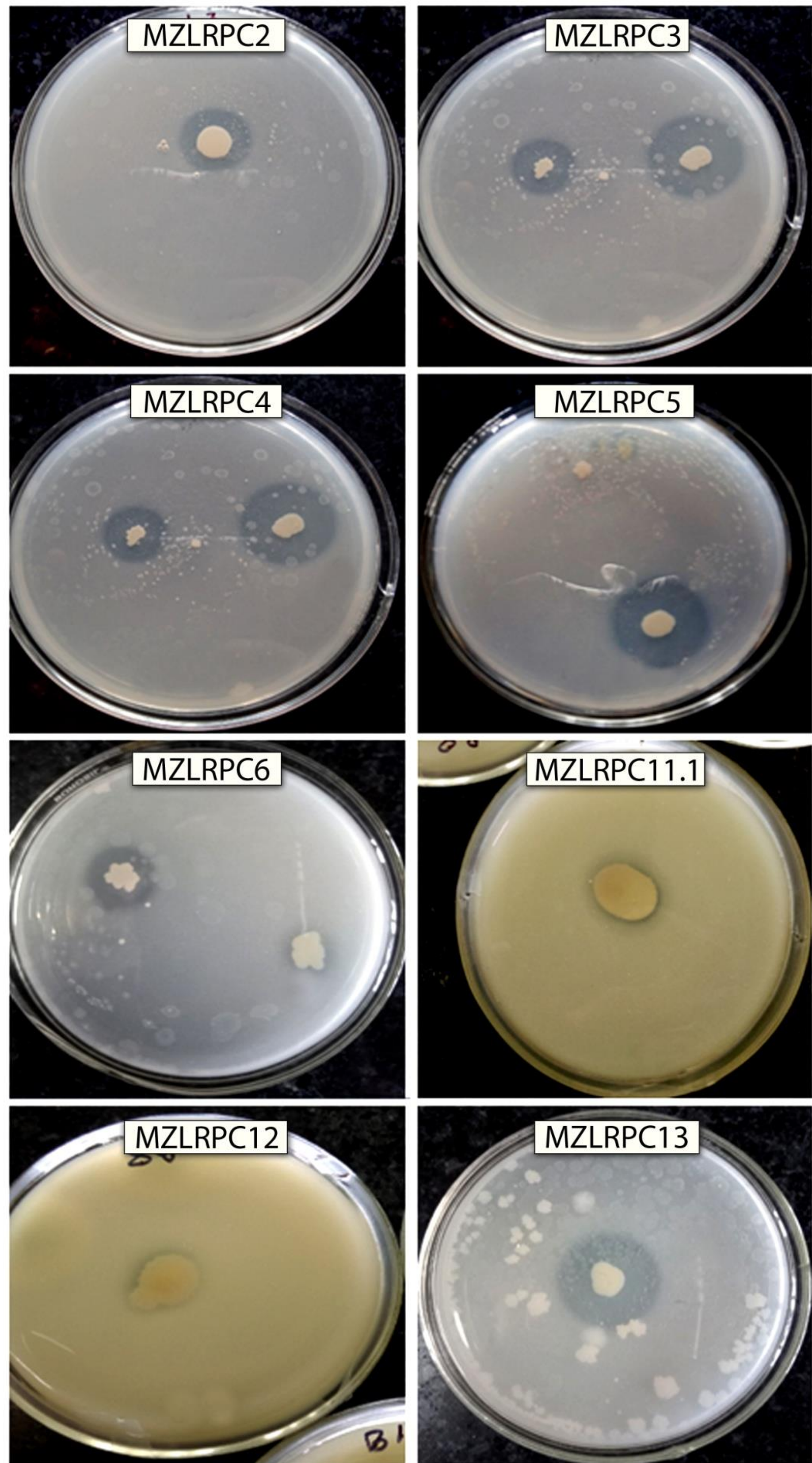

Figure 5.4: Potential PSB strains MZLRPC2-MZLRPC13 grown on PVK agar media

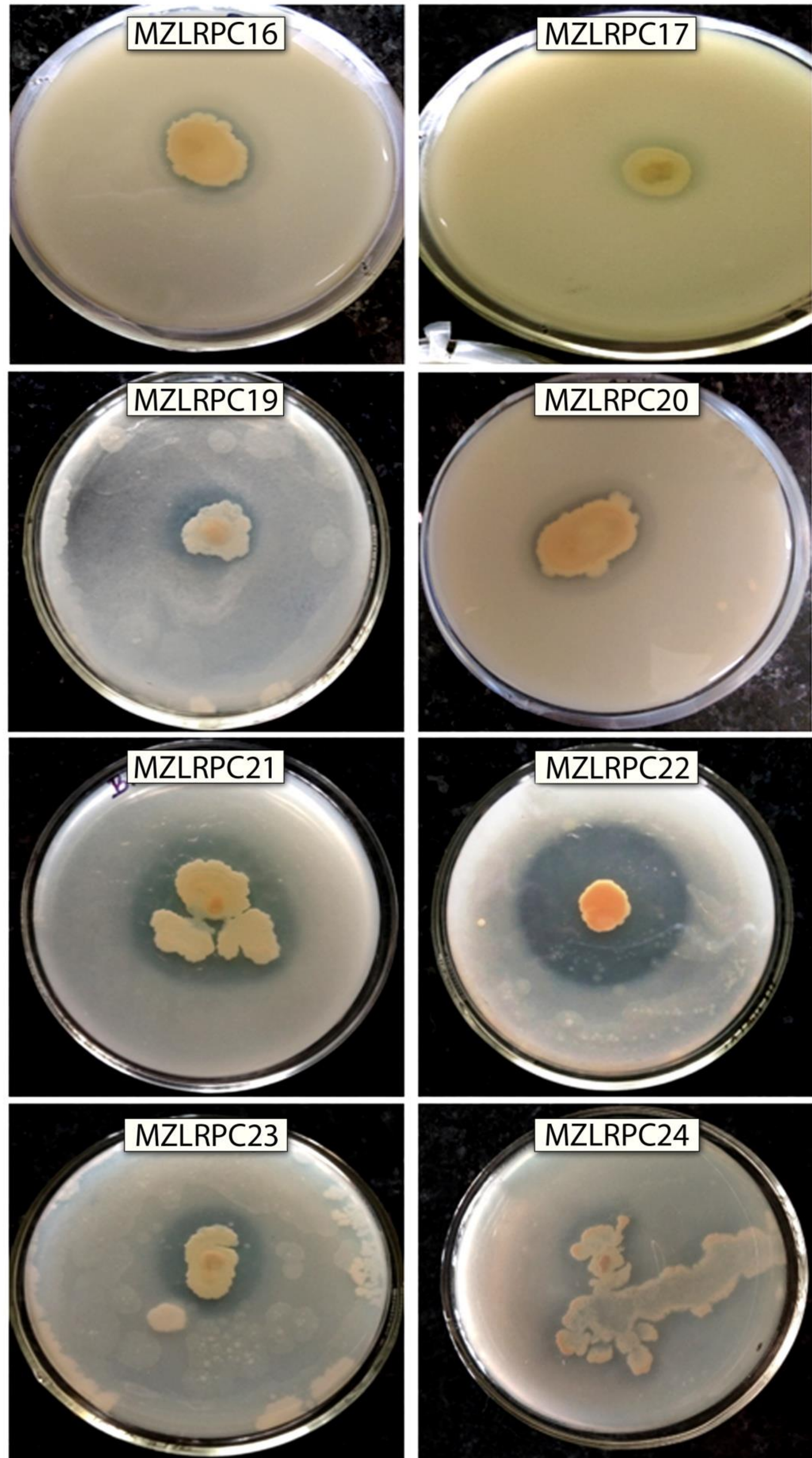

Figure 5.5: Potential PSB strains MZLRPC16-MZLRPC24 grown on PVK agar media

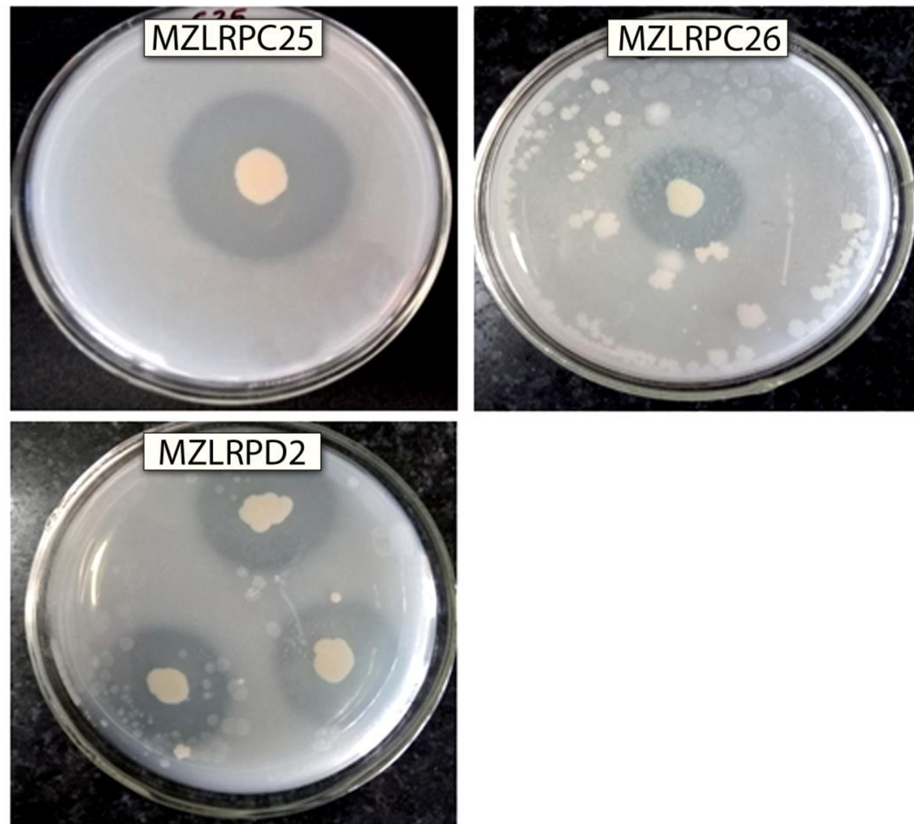

Figure 5.6: Potential PSB strains MZLRPC25, MZLRPC26 and MZLRPD2 grown on PVK agar media showing solubilization zones around the colony

Table 5.2: Table showing solubilizing index of all PSB isolates in centimetre

S.N	Strain name	S.I (cm)
1	MZLRPA1	2.24±0.01
2	MZLRPA2	2.2±0.02
3	MZLRPA3	2.4±0.01
4	MZLRPA4	2.64±0.05
5	MZLRPA7	2.21±0.05
6	MZLRPA8	2.70±0.05
7	MZLRPA10	2.65±0.02
8	MZLRPA11	2.6±0.01
9	MZLRPA12	3.13±0.01
10	MZLRPB1	3.10±0.02
11	MZLRPB2	3.60±0.12
12	MZLRPB3	2.60±0.11
13	MZLRPB4	2.21±0.05
14	MZLRPB5	2.11±±0.10
15	MZLRPB6	2.17±0.09
16	MZLRPB8	2.12±0.01
17	MZLRPB10	2.37±0.02
18	MZLRPB11	2.08±0.05
19	MZLRPB12	3.02±0.04
20	MZLRPB13	4.13±0.14
21	MZLRPB14	2.44±0.21
22	MZLRPB17	2.6±0.06
23	MZLRPB19	2.5±0.05
24	MZLRPB21	4.57±0.21
25	MZLRPC2	3.6±0.09
26	MZLRPC3	4.55±0.06
27	MZLRPC4	4.64±0.03
28	MZLRPC5	4.25±0.01
29	MZLRPC6	2.3±0.01
30	MZLRPC11.1	2.23±0.05
31	MZLRPC12	2.42±0.05
32	MZLRPC13	3.5±0.11
33	MZLRPC16	2.84±0.91
34	MZLRPC17	2.42±0.06
35	MZLRPC19	2.9±0.07
36	MZLRPC20	3.6±0.04
37	MZLRPC21	3.92±0.01
38	MZLRPC22	4.38±0.01
39	MZLRPC23	3.77±0.05
40	MZLRPC24	3.5±0.02
41	MZLRPC25	4.6±0.14
42	MZLRPC26	3.63±0.11
43	MZLRPD2	3.1±0.14

5.3.2. Molecular identification of phosphate solubilizing bacterial strains

5.3.2.1. Genomic DNA isolation

Genomic DNA was isolated using PCI method following Kumar *et al*. (2010). Total of 43 genomic could be obtained from the colonies having solubilization capacity. The isolated DNAs were kept in eppendorf tubes at -20°C and could last even upto six months for further analysis. The extracted DNA was checked for quality on 0.8% agarose gel using ethidium bromide as the intercalating dye. A single clear DNA band was obtained and visualized under UV light and documented using a Protein simple Gel Doc Alphamagermini system (Taiwan) (Figure 5.7 and 5.8).

5.3.2.2. PCR amplification

The PCR based amplification using 16srRNA gene was subjected to all of the genomic DNA isolates using Eppendorf Mastercycler Nexus Gradient (Germany). The universal primers used were 27f as forward primer and 1525r as reverse primer. The desired amplified size of the PCR product was 1500-bp. 1.5% of agarose gel was used to run the PCR product. Molecular marker of 3kb DNA ladder was used for reference. The single clear band was then visualized at Protein simple Gel Doc Alphamagermini system (Taiwan) and the image was captured for reference (Figure 5.9, 6.0 and 6.1). The products of the PCR obtained were then sent for Sanger sequencing commercially at Eurofins Genomics India Pvt. Ltd. Bangalore. The results obtained were then subjected to NCBI BLAST database to find the closest similarity of the sequence. All of 43 isolates were successfully sequenced and the closest similarity ranged from 98-100% (Table 5.3).

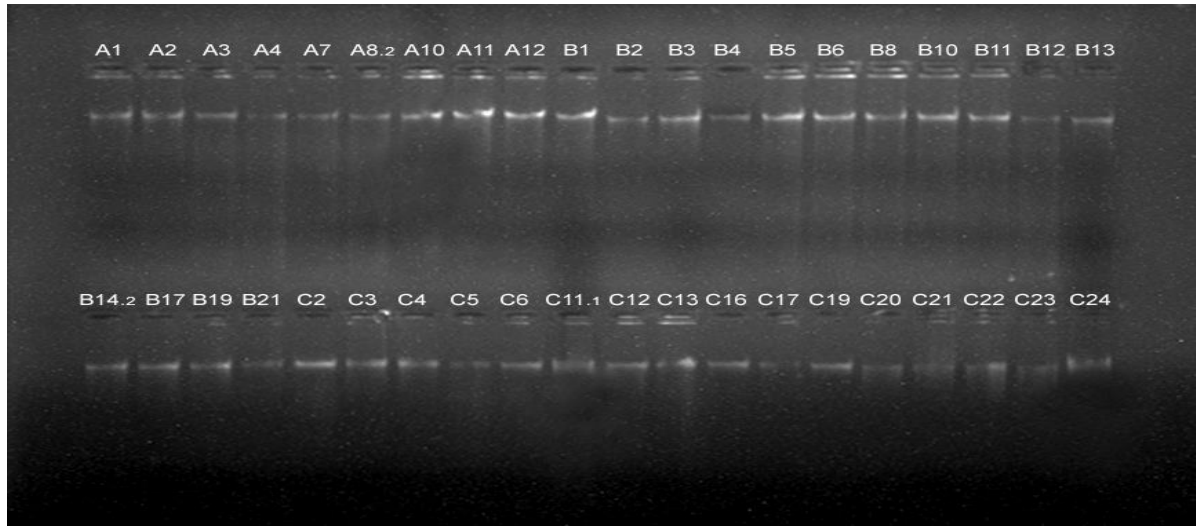

Figure 5.7: Quality of genomic DNA band of 40isolated PSB.

Figure 5.8: Quality of genomic DNA band of 3 isolated PSB.

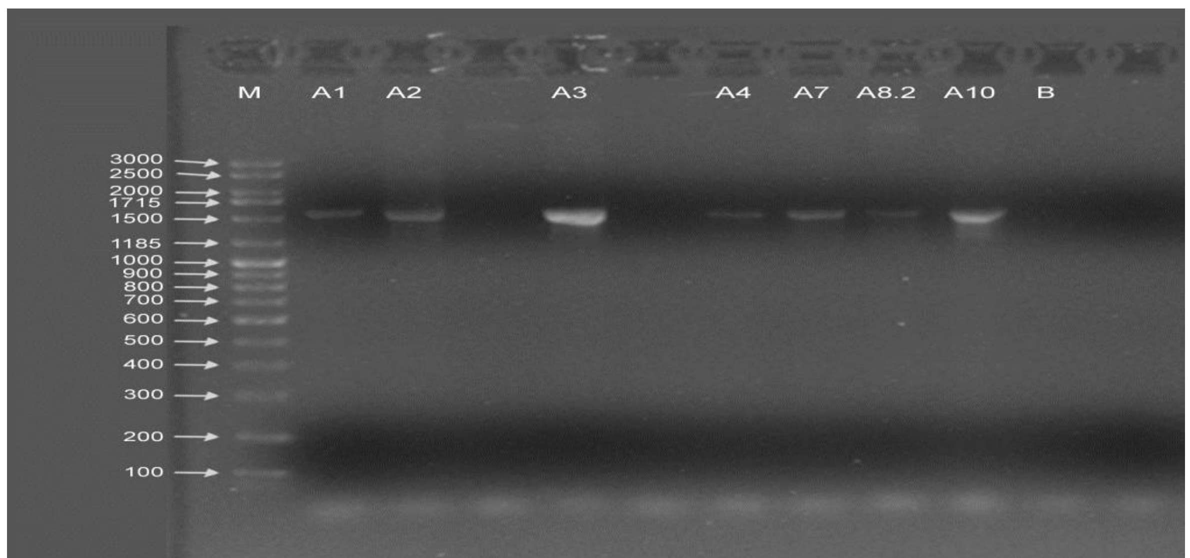

Figure 5.9: Band showing PCR amplification using 16srRNA gene of 7 PSB strains.

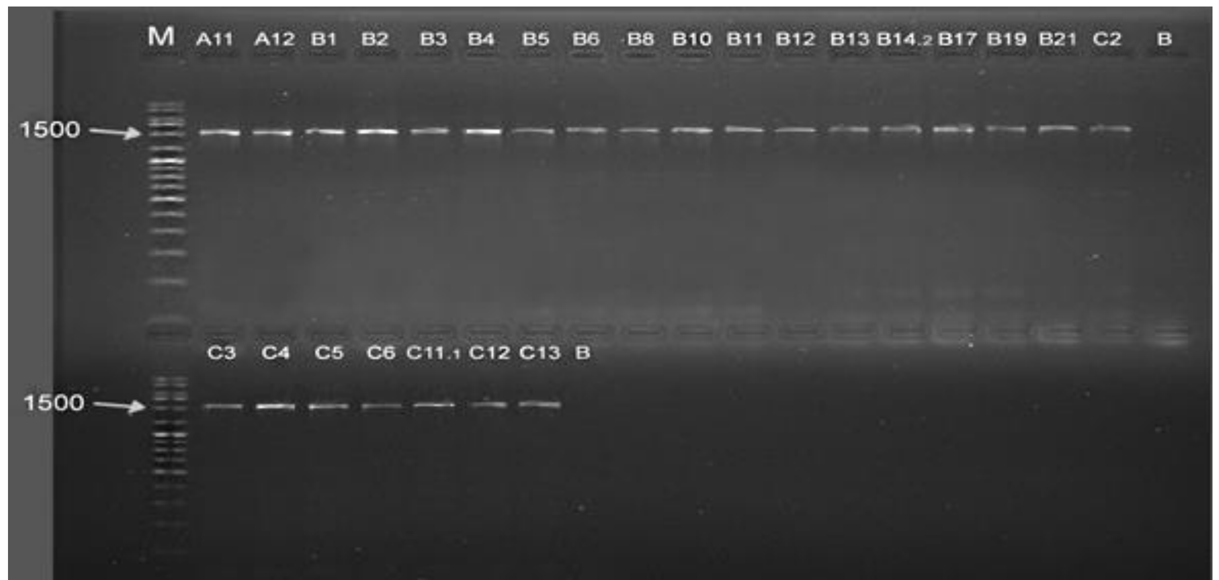

Figure 6.0: Band showing PCR amplification using 16srRNA gene of 25 PSB strains.
M; low range DNA ruler plus (100bp-3kb).
B; Blank sample

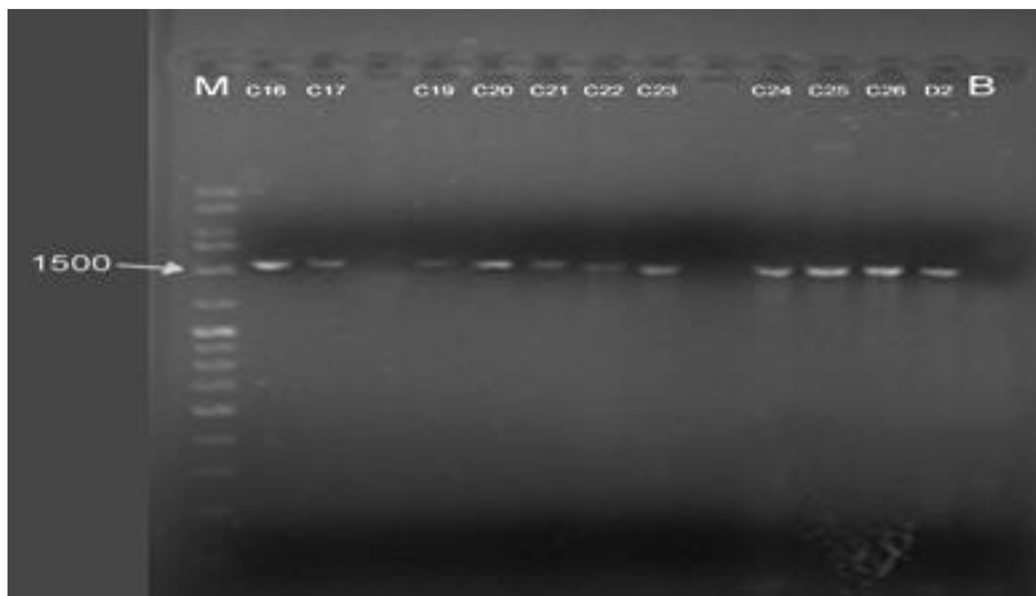

Figure 6.1: Band showing PCR amplification using 16srRNA gene of 11 PSB strains.
M; low range DNA ruler plus (100bp-3kb).
B; Blank sample

5.3.2.3. Phylogenetic analysis

The results of DNA sequencing classified all the 43 isolates into 7 genera; *Bacillus*, *Burkholderia*, *Paraburkholderia*, *Paenibacillus*, *Edaphobacter*, *Paraclostridium* and *Dyella*. The nucleotide sequences were further compared by BLASTn analysis tool to look for 16S rRNA gene homology along with sequences from type strains retrieved from EzTaxon-database (http://www.ezbiocloud.net/eztaxon). The nucleotide sequences were deposited in NCBI Genbank database under the accession numbers MK932023 – MK932068 (Table5.3.). The evolutionary history was inferred by using Neighbour joining method based on Kimura 2- parameter model (K2+G) (Saitou and Nei, 1987).The estimated transition/transversion bias (R) ratio is 1.15. The model was selected based on the lowest Bayesian information criterion (BIC) scores with 2214.201 values. The optimal tree with the sum of branch length = 0.54684421 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 163 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

In the phylogenetic tree (Fig.6.2), all the gram positive bacteria; the genus *Bacillus*, *Paenibacillus* and *Paraclostridium* were clustered together in three sub-clades. Clade I was divided into three smaller clades (Clade IA, Clade IB and Clade IC) with a bootstrap support value of 93%. In Clade IA, *Bacillus* group, 25 strains were clustered with a bootstrap support value of 98% along with a type strain from EzBiocloud database with bootstrap support value of 98%. In Clade IB, the strain *Paenibacillus* sp. was clustered along with the type strain from EzBiocloud database with bootstrap support value of 98%. Clade IC consist of the strain *Paraclostridium bifermentans* strain clustered along with the type strain with a bootstrap support

value of 99%.In Clade II, all the gram negative bacterial strains were clustered together with a bootstrap support value of 97%. In Clade IIA, *Dyella* sp. was clustered with the type strain from EzBiocloud database with a bootstrap value of 98%.While in Clade IIB, there are four sub clades. In clade IIBa, all *Burkholderia* sp. were clustered together with bootstrap support value of 48% and *Burkholderia contaminans* as type strain from Ezbiocloud database with 67% bootstrap value. In Clade IIBc, the strain *Paraburkholderia multivorans* is also clustered separately from the rest of the *Paraburkholderia* strain with bootstrap value of 48%. The genera *Paraburkholderia* are clustered in Clade IIBe alongwith the type strain from EzBiocloud with bootstrap value of 94%. The last gram negative strain *Edaphobacter* is clustered separately from the rest of the other strains with bootstrap support value of 99%. Scale is 0.020.

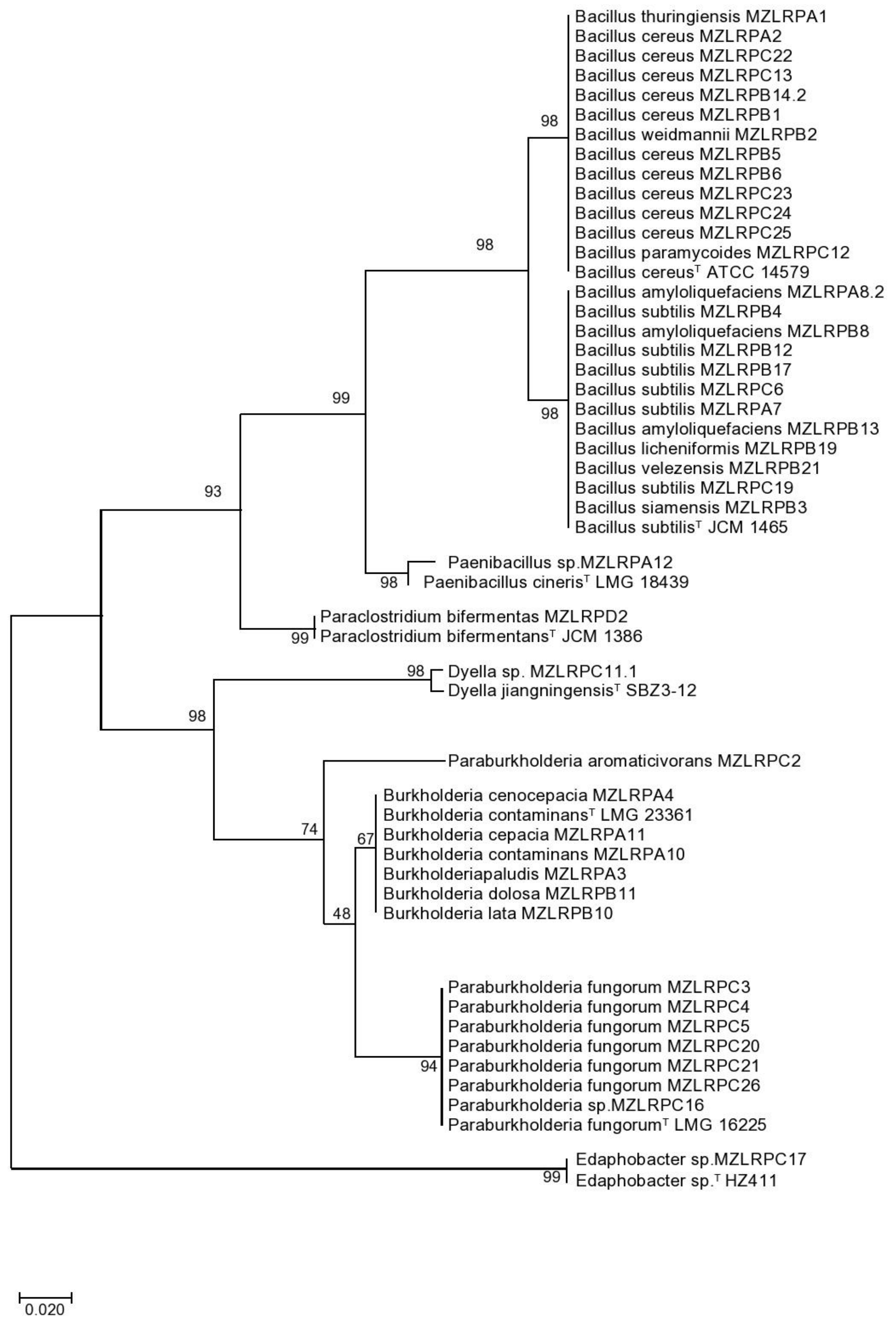

Figure 6.2: Phylogenetic tree based on 16S rRNA gene sequence of 43 PSB isolated strains using Kimura-2 parameter model with neighbour method under 1000 bootstrap replicates. Type strains are suffixed as superscript T.

Table 5.3: Table showing the result of NCBI Blast with the closest similarity strain

S.N	Strain name	Similarity Isolates name	% identity	NCBI Accession No
1	**MZLRPA1**	*Bacillus thuringiensis*	**99.84**	**MK932052**
2	**MZLRPA2**	*Bacillus cereus*	**100**	**MK932053**
3	**MZLRPA3**	*Burkholderia paludis*	**100**	**MK932026**
4	**MZLRPA4**	*Burkholderia cenocepacia*	**99.76**	**MK932036**
5	**MZLRPA7**	*Bacillus subtilis*	**100**	**MK932054**
6	**MZLRPA8.2**	*Bacillus amyloliquefaciens*	**99.74**	**MK932027**
7	**MZLRPA10**	*Burkholderia contaminans*	**100**	**MK932028**
8	**MZLRPA11**	*Burkholderia cepacia*	**100**	**MK932029**
9	**MZLRPA12**	*Paenibacillus sp.*	**98.43**	**MK932030**
10	**MZLRPB1**	*Bacillus cereus*	**100**	**MK932031**
11	**MZLRPB2**	*Bacillus wiedmannii*	**100**	**MK932025**
12	**MZLRPB3**	*Bacillus siamensis*	**100**	**MK932066**
13	**MZLRPB4**	*Bacillus subtilis*	**99.5**	**MK932032**
14	**MZLRPB5**	*Bacillus cereus*	**100**	**MK932055**
15	**MZLRPB6**	*Bacillus cereus*	**100**	**MK932056**
16	**MZLRPB8**	*Bacillus amyloliquefaciens*	**100**	**MK932037**
17	**MZLRPB10**	*Burkholderia lata*	**98.3**	**MK932023**
18	**MZLRPB11**	*Burkholderia dolosa*	**100**	**MK932024**
19	**MZLRPB12**	*Bacillus subtilis*	**100**	**MK932038**
20	**MZLRPB13**	*Bacillus amyloliquefaciens*	**99.15**	**MK932057**
21	**MZLRPB14.2**	*Bacillus cereus*	**100**	**MK932039**
22	**MZLRPB17**	*Bacillus subtilis*	**99.62**	**MK932040**
23	**MZLRPB19**	*Bacillus licheniformis*	**100**	**MK932058**
24	**MZLRPB21**	*Bacillus velezensis*	**100**	**MK932059**
25	**MZLRPC2**	*Paraburkholderia aromaticivorans*	**96.61**	**MK932033**
26	**MZLRPC3**	*Paraburkholderia fungorum*	**100**	**MK932034**
27	**MZLRPC4**	*Paraburkholderia fungorum*	**100**	**MK932042**
28	**MZLRPC5**	*Paraburkholderia fungorum*	**100**	**MK932043**
29	**MZLRPC6**	*Bacillus subtilis*	**99.61**	**MK932044**
30	**MZLRPC11.1**	*Dyella sp.*	**100**	**MK932045**
31	**MZLRPC12**	*Bacillus paramycoides*	**100**	**MK932067**
32	**MZLRPC13**	*Bacillus cereus*	**100**	**MK932046**
33	**MZLRPC16**	*Paraburkholderia sp.*	**100**	**MK932068**
34	**MZLRPC17**	*Edaphobacter sp.*	**98.66**	**MK932047**
35	**MZLRPC19**	*Bacillus subtilis*	**99.62**	**MK932060**
36	**MZLRPC20**	*Paraburkholderia fungorum*	**100**	**MK932048**
37	**MZLRPC21**	*Paraburkholderia fungorum*	**99.88**	**MK932049**
38	**MZLRPC22**	*Bacillus cereus*	**100**	**MK932050**
39	**MZLRPC23**	*Bacillus cereus*	**100**	**MK932061**
40	**MZLRPC24**	*Bacillus cereus*	**100**	**MK932062**
41	**MZLRPC25**	*Bacillus cereus*	**100**	**MK932063**
42	**MZLRPC26**	*Paraburkholderia fungorum*	**99.26**	**MK932064**
43	**MZLRPD2**	*Paraclostridium bifermentans*	**98.23**	**MK932065**

5.4. Discussion

It is well known that P is an important nutritional element for plant. However, it is one of the least soluble nutrient ions in the environment; so, less than 5% of total soil phosphate is available to plants (Epstein, 1972; Brown, 1974). Phosphate-solubilizing microorganisms play an important role in supplementing phosphorus to the plants, allowing a sustainable use of phosphate fertilizers.

The isolated bacterial colonies were confirmed as phosphate solubilizing bacteria by plate assay method following Gerretson (1948) using Pikovskaya agar (Pikovskaya, 1948) by producing clear halo zone around each colony which was formed due to solubilization of tricalcium phosphate (TCP) supplemented in PVK agar medium. The reason for using TCP as P source is based on the findings of Chakraborty *et al*. (2010), Chung *et al*. (2005), Kumar *et al*. (2010) and Parasanna *et al*. (2011) that PSB isolates solubilized tricalcium phosphate (TCP) to a greater extent than rock phosphate, aluminum phosphate and iron phosphate with $AlPO_4$ exhibiting poor solubilization. This could be due to the fact that rock phosphates are less amenable to microbial solubilization because of their structural complexity, while TCP being amorphous is more facile to solubilization (Kumari *et al*., 2008; Nahas, 1996). Pradhan and Sukla (2005) also proved PVK medium to be the most effective source for P solubilization and used only PVK medium for further studies. Numerous workers like Sagervanshi *et al*. (2012), Anbuselvi *et al.* (2015), Panhwar *et al.* (2014), Paul and Sinha (2016) and many more have also identified PSB using similar technique. According to Singal *et al*. (1991) microorganisms that are capable of producing a halo/clear zone is due to solubilization of organic acids in the surrounding medium and are thus, selected as potential phosphate solubilizers (Das, 1989). Several reports on bacteria and fungi isolated from soil have evaluated their mineral phosphate solubilizing (MPS) activity with various P sources such as calcium phosphate tribasic [$Ca_3(PO_4)_2$] (Illmer and Schinner, 1995), iron phosphate ($FePO_4$) (Jones *et al*., 1991) and aluminium phosphate ($AlPO_4$) (Illmer *et al*.,1995).

From our study, based on 16SrRNA gene sequence, we identified a total of 43 PSB constituting of the genera *Bacillus* (58.1%), *Burkholderia* (13.9%),

Paenibacillus (2.3%), *Paraburkholderia* (18.6%), *Dyella* (2.3%), *Edaphobacter* (2.3%) and *Paraclostridium* (2.3%) from the rhizospheric region of the flooded rice field of North Vanlaiphai, Mizoram. The reason for studying the rhizospheric region has been deliberated by Reyes *et al*. (2006) that higher amount of PSB population is found in the rhizosphere region as compared to the non rhizosphere region. Whipps and Lynch (1986) also observed higher percentage of PSB population in the rhizosphere of plants. Teng *et al*. (2018) on 16SrRNA gene sequencing identified PSB strains belonging to the genera *Pseudomonas, Aeromonas, Enterobacter, Bacillus* and *Providencia* from rhizospheric region of plants from Yeyahu Wetland. There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizosphere (Sperber, 1958). These consist of both aerobic and anaerobic strains, with an occurrence of aerobic strains in submerged soils. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non-rhizosphere soil (Raghu and MacRae, 1966).

Majority of the isolates belonged to the genus *Bacillus* with 25 strains. This genus *Bacilli* is known to occur at population levels of 10^6 to 10^7 per gram of soil (Alexander, 1977). The general overview of *Bacillus* species are Gram-positive, sporulating, chemoheterotrophic rod-shaped bacteria and are usually flagellated being motile; they are aerobic or facultative anaerobic and catalase positive (Waites *et al*., 2008). The members of genus *Bacillus* are usually found in soil and correspond to a wide range of physiological abilities and can also thrive well in every environment (Kuta *et al*., 2008). The large majority of *Bacillus* species are harmless saprophytes and usually non pathogenic to humans. In our findings, *Bacillus cereus* constitutes majority of the isolates with total of 10 isolated strains. Peter C.B Turnbull has quoted that *Bacillus cereus* could be occasional pathogens of humans and livestock. *Bacillus subtilis* strain is next to *Bacillus cereus* with total of 6 isolated strains. *B. subtilis* is also aerobic, but in the presence of glucose and nitrate, a few anaerobic growths can take place (Claus and Berkeley, 1986). It is non-pathogenic or neither toxigenic to humans, animals, or plants. Next is *Bacillus amyloliquefaciens* with 3 isolated strains. Based on previous studies it is considered to be a root-colonizing bio-control bacterium and can act as a bio-pesticide and provide benefits

to plants in both soil and hydroponic applications. It takes action against bacterial (Wu *et al*., 2014) and fungal pathogens. Each one isolated strain of *B. thuringiensis, B. weidmannii, B. siamensis, B. licheniformis* and *B. velezensis* were also identified. Among them, *B. thuringiensis* commonly abbreviated as *Bt* is a unique bacterium as it shares a common place with considerable amount of chemical compounds which are used commercially to control insects important to agriculture and public health (Ibrahim *et al*., 2010). All of the identified species of *Bacillus* genus were clustered together in the same clade with 98% bootstrap value meaning that they are very closely related to each other. Gordon *et al*. (1973) had earlier described the strains *B. subtilis*, *B. licheniformis*, and *B. Pumilus* with *B. subtilis* as the type strain. Later, other novel species which belongs to the *B. subtilis* species complex have been described by few researchers like *B. amyloliquefaciens* (Priest *et al*., 1987), *Bacillus velezensis* (Ruiz-García *et al*., 2005a), *Bacillus siamensis* (Sumpavapol *et al*., 2010) and recently *Bacillus weidmannii* by Miller (2016). One isolate of *Paenibacillus* species was also identified. There has also been a previous study on *Paenibacillus* sp. as a beneficial bacteria that can promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole‑3‑acetic acid (IAA) and release of siderophores that enable iron acquisition (Grady *et al*., 2016).

Next to the genus *Bacillus*, mostly identified genus belongs to *Burkholderia* with 6 isolates and 8 isolated strains of genus *Paraburkholderia.* The genus *Burkholderia* was first proposed by Yabuuchi *et al.* (1992) which later more than 100 new species of *Burkholderia* were described. *Burkholderia* sp. was transferred from rRNA group II of the former genus *Pseudomonas.* The genus *Burkholderia* currently consists of more than 60 species most of which have been assigned species names (Wisplinghoff, 2017). On the other hand, *Burkholeria* genus was not monophyletic; it eventually split based on 16SrRNA gene sequences and conserved sequence indels (CSIs), and the genus *Paraburkholderia* was established with *Paraburkholderia graminis* as the type species (Sawana *et al*., 2014). Up till 2014 the genus *Paraburkholderia* included 65 species with validly published names. There are no human pathogenic strains that have been reported in the genus *Paraburkholderia*

although there have been reports of animal and plant pathogen (Sawana *et al*., 2014; Dobritsa *et al*., 2016). Cells of the genus *Paraburkholderia* are Gram-stain-negative, straight, slightly curved or sometimes coccoid rods with one or more polar flagella. Among the isolates, *Burkholderia cepacia* could sometimes be pathogenic to humans causing pulmonary infections in people with cystic fibrosis (CF) (Woods and Sokol, 2006). Pande *et al*. (2019) in their study have reported that *Burkholderia cepacia* isolates could be used as a plant growth-promoting bacterium and can help in sustaining sweet corn productivity.

Two genus which have not been studied as a phosphate solubilizer in previous research has been identified. *Dyella* sp. which is a grams stain negative, aerobic, motile and non-spore forming rods. There are no previous records of *Dyella* sp. being a phosphate solubilizer. However, few researchers like Chen *et al*. (2017), Xie and Yokota (2005) and Weon *et al.* (2009) have isolated the genus from soil and have studied few characteristics about the genus. *Edaphobacter* sp. has also been isolated which is a grams stain negative. From massive reviews, regarding the plant growth promotion there are no studies yet for the particular genus. Koch *et al*. (2008) have isolated and studied the characteristic of *Edaphobacter* from forest soil.

The 16SrRNA gene sequence has been widely used as a molecular method to estimate phylogenetic relationships among bacteria. There has been copious amount of research in phylogenetic analysis of bacteria based on 16SrRNA gene sequencing. Some of these include the work done by Chung *et al*. (2005) who used the primers fD1 and rP2. They isolated and identified PSB which were under the genera *Enterobacter* sp., *Klebsiella* sp. and *Pantoea* sp. Sagervanshi *et al*. (2012) have also identified *Burkholderia cenocepacia* and *Bacillus* sp. based on 16SrRNA gene sequencing from Anand agricultural soil. Gupta *et al*. (2012) also identified PSB from the rhizospheric region of *Aloe barbadensis* using TCP as P-source and by 16SrRNA gene sequencing. They identified *Pseudomonas synxantha, Burkholderia gladioli, Enterobacter hormaechei* and *Serratia marcescens*. Liu *et al*. (2016) identified 10 strains of PSB using 16SrRNA *viz*. *Bacillus aryabhattai*, *Bacillus megaterium*, *Klebsiella variicola*, *Stenotrophomonas rhizophila*, and *Enterobacter aerogenes* from grapevine rhizospheres. According to Rodriguez and Fraga (1999)

strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers. From the work of Chen *et al*. (2006) identification and phylogenetic analysis of 36 isolates were carried out by 16SrDNA sequencing. Ten isolates belonged to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium*. Additionally, four strains *viz*., *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. are being reported for the first time as phosphate solubilizing bacteria (PSB) after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by organic acid secretion.

Based on our findings, the strain with the highest phosphate solubilization index is MZLRPC4 *Paraburkholderia fungorum* with S.I 4.64cm followed by MZLRPC25 *Bacillus cereus* with S.I 4.60cm and the lowest were MZLRPB11 *Burkholderia dolosa* with S.I 2.08cm, MZLRPB5 *Bacillus cereus* with S.I 2.11cm, MZLRPB8 *Bacillus amyloliquefaciens* S.I 2.12cm.

Chapter 6

Quantification of Organic Acid Production and Inorganic Phosphate Fixation by Phosphate Solubilizing Bacterial Isolates

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6.1. Introduction

Phosphorus is an essential element and one of the major macronutrients required in optimum amount for plant development and growth. Several important functions like cellular, metabolic and reproductive functions rely on sufficient phosphorus supply to the plants. Merely about 25% of the phosphorus applied to the soil is available for the crops and the rest become unavailable due to chemical fixation with aluminium and iron in acidic soils. Dadarwal *et al.* (1997) also noted that a huge portion of soluble inorganic phosphate when applied to agricultural soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants. Indian soils are characterized by poor and medium status with respect to available phosphorus (Baby, 2002; Li *et al.*, 2003; Ramanathan *et al.*, 2004).

Microorganisms play a very important role in agriculture by reducing the demand of chemical fertilizers by supplying nutrients to the plants (Cakmakci *et al.*, 2006). The production of organic acids by microorganisms is the one of the mechanism for the solubilization process which is effective by numerous points like the lowering of pH in rhizosphere which is due to the release of these organic acids (Whitelaw, 2000; Maliha *et al.*, 2004); due to the direct oxidation respiratory pathway that is operative on the outer surface of the cytoplasmic membrane (Zaidi *et al.*, 2009); precipitation of P for chelating of the cations, competing with P for sorption sites on the soil and forming of soluble complexes with the metal ions associated with insoluble P compounds like phosphates of Ca, Al, Fe. Among these microorganisms, phosphate solubilizing bacteria have the ability to solubilize P in soil and reduce inputs of chemical fertilizers (Arpana and Bagyaraj, 2007). There has been an investigation to support the role of organic acids in mineral phosphate solubilization (Chen *et al.*, 2006; Rashid *et al.*, 2004). In soil, P-solubilizing bacteria (PSB) have the ability for solubilizing the inorganic P and make it available to the

plants (Gothwal *et al.*, 2006). The ability to secrete microbial metabolite low molecular weight organic acids by phosphate solubilizing bacteria is the principal mechanism of the bacteria for the mineral phosphate solubilization (Cunningham and Kuiack,1992; Goldstein, 1995;Singh and Amberger, 1997; Gadd, 1999; Whitelaw, 2000)which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, hence converting it into soluble forms (Kpomblekou and Tabatabai, 1994; Chen *et al*., 2006, Rodriguez and Fraga, 1999).

Various researches have shown that phosphate solubilizing microbes release numerous organic acids like gluconic acid, oxalic acid, tartaric acid and lactic acid that help in lowering down the pH of soil making conditions practical for the occurrence of monovalent (available) form. Othman and Panhwar (2014) have studied and reported that phosphate solubilizing microbes have a special quality to secrete enzymes like phosphatase, phytase, and C–P lyase that helps in mineralization of organic P. Additional factors that affect phosphate solubilization includes soil pH, organic matter, physicochemical properties of the soil, vegetation type, environmental conditions and agronomic practices performed in that specific land use system and most importantly interaction of phosphate solubilizing microbes with other microbial community in soil (Seshachala and Tallapragada, 2012).

In this experiment, the phosphate solubilizing bacteria which were isolated from the paddy field of North Vanlaiphai were analysed for their mechanism of phosphate solubilizing capacity. Eight phosphate solubilizing strains such as- MZLPA12, MZLRPB1, MZLRPB4, MZLRPB10, MZLRPB11, MZLRPB13, MZLRPC4 and MZLRPC17 were selected based on their phosphate solubilizing capacity (highest and lowest) and genera. Eight organic acids such as- acetic, citric, formic, gluconic, malic, oxalic, succinic and tartaric acid were quantified. Inorganic phosphate fixations of all the isolates were evaluated in broth culture media for quantification of the released P in the medium.

6.2. Methodology

6.2.1. Quantification of organic acids

The isolates were cultured in NBRIP broth medium (Nautiyal, 1999), pH was maintained at 7 and incubated at 37°C with continuous agitation in an incubator shaker for 10 days. After incubation the pH of the broth cultures were observed and recorded. The cultures were then vortexed for 60secs with 5secs interval, and then were centrifuged at 10,000rpm for 10mins. Supernatant of each blended culture was filtered through 0.22µm millex-syringe driven filter unit. Organic acids in the supernatant were analyzed by Ultra High Performance Liquid Chromatography (UHPLC, Thermo scientific Ultimate 3000) equipped with Acclaim Organic Acid column, 3µm (3x150mm) and a Diode Array Detector. 2µl of the filtered supernatant were injected into the UHPLC using a glass syringe and eluted with the solvent 100mM Na_2SO_4 pH 2.65 adjusted with Methanesulfonic acid at a flow rate of 0.34mL min^{-1} for 15 min at 30°C. Peaks of organic acids were detected at a wave length of 210nm.The organic acid in the supernatant were identified by their retention time, corresponding with their standards. For the standard preparation, Organic acid kit, Sigma-Aldrich (Supelco) which were individually packaged and of analytical standard grade in neat form was used. Eight organic acids *viz*., acetic, citric, formic, gluconic, malic, oxalic, succinic and tartaric acid were selected and for each acid in different concentrations 30ppm, 50ppm, 70ppm and100ppm were prepared and diluted with the solvent. Standard for each organic acid were developed by injecting individually in different concentrations such as 30ppm, 50ppm, 70ppm and100ppm and thereby developing a line of best fit for standard curve. After developing best fit line for standard curve for a single acid, the retention time obtained by a single strain was processed and quantified. HPLC grade distilled water was used to perform the experiment.

6.2.2. Inorganic Phosphate fixation by PSB

Phosphate solubilizing activity in PVK broth medium was evaluated for each isolate. The culture was incubated in an incubator shaker at 30±7°C at 70%rpm for 7 days. The bacterial cultures were centrifuged at 10,000rpm for 10mins and supernatant was estimated for Phosphorous determination which was done by the Ascorbic acid method (Murphy and Riley, 1962). The procedure is as follows:

Reagent preparation:

(1) Sulphuric acid (H_2SO_4) 5N: 70ml of Sulphuric acid (H_2SO_4) was added with 500ml dH_2O

(2) Antimony potassium tartarate: 0.343g of Antimony potassium tartarate was dissolved in 100ml dH_2O then 25ml dH_2O was added

(3) Ammonium molybdate solution: 5g Ammonium molybdate was dissolved in 125ml dH_2O

(4) Ascorbic acid (was always prepared fresh) 0.1M: 0.88g Ascorbic acid was dissolved in 50ml dH_2O

(5) Combined reagent: 50ml of 5N H_2SO_4 was added with 5ml Antimony potassium tartarate, 15ml Ammonium molybdate solution and 30ml Ascorbic acid.

All the reagents must be at room temperature before they are mixed, and they should be mixed in the order given. If turbidity forms, shake and let it stand for a few minutes until it disappears. This combined reagent is stable for 4 hours.

After combined reagent was prepared, 50ml of the supernatant PSB sample was taken in a sterile dry tube. To it, 0.05ml or 1 drop of phenolphthalein indicator was added. If red colour was developed, 5N H_2SO_4 was added drop wise to discharge the colour. Then, 8ml of the combined reagent prepared was added and mixed thoroughly. After 20 to 30mins, the absorbance was measured at 880nm using a UV-Vis spectrophotometer using blank as a reference solution and compared with the standard curve of KH_2PO_4.

6.3. Results

6.3.1. Organic acid production

Organic acid productions were tested in eight isolates such as MZLPA12 *Paenibacillus sp.*, MZLRPB1 *Bacillus cereus*, MZLRPB4 *Bacillus subtilis*, MZLRPB10 *Burkholderia lata*, MZLRPB11 *Burkholderia dolosa*, MZLRPB13 *Bacillus amyloliquefaciens*, MZLRPC4 *Paraburkholderia fungorum* and MZLRPC17 *Edaphobacter sp*. There was significant decrease in pH level ranging

from 4 to 5 with initial pH 7 in liquid medium in all the isolates after 7 days of incubation with continuous agitation (Table 6.2). The quantity of all organic acids tested in all the PSB strains are given in Table 6.1 in parts per million for each 2µl injection. The UHPLC system analysis of culture filtrate showed the presence of multiple organic acids *viz*., gluconic, acetic, tartaric, citric, succinic, formic, malic and oxalic acid during the solubilization of tricalcium phosphate. All acids were able to be quantified in MZLRPA12 (Fig 6.5), but only formic acid could be displayed in the same chromatogram excluding gluconic acid since formic and gluconic acids have retention times which are very close to each other (2.823 and 2.953 RT respectively) In MZLRPB1 (Fig 6.5), MZLRPB10 and MZLRPB13 (Fig 6.6), all eight organic acids were able to be quantified and represented in the chromatogram. In MZLRPB4 (Fig 6.5), MZLRPC4 and MZLRPC17 (Fig 6.7) all eight OA were present and quantified but formic acid could not be represented in UHPLC chromatogram since formic and gluconic acids have retention times which are very close to each other. In MZLRPB11 (Fig 6.6), OA acetic acid and malic acid could not be quantified but trace of malic acid was shown in the chromatogram which indicates that the standard prepared could not detect the trace amount present in the sample. Formic acid was also present and quantified but could not be represented in the chromatogram. In all the PSB strains tested, the dominant OA were gluconic, citric, malic, oxalic, succinic, formic and tartaric acid.

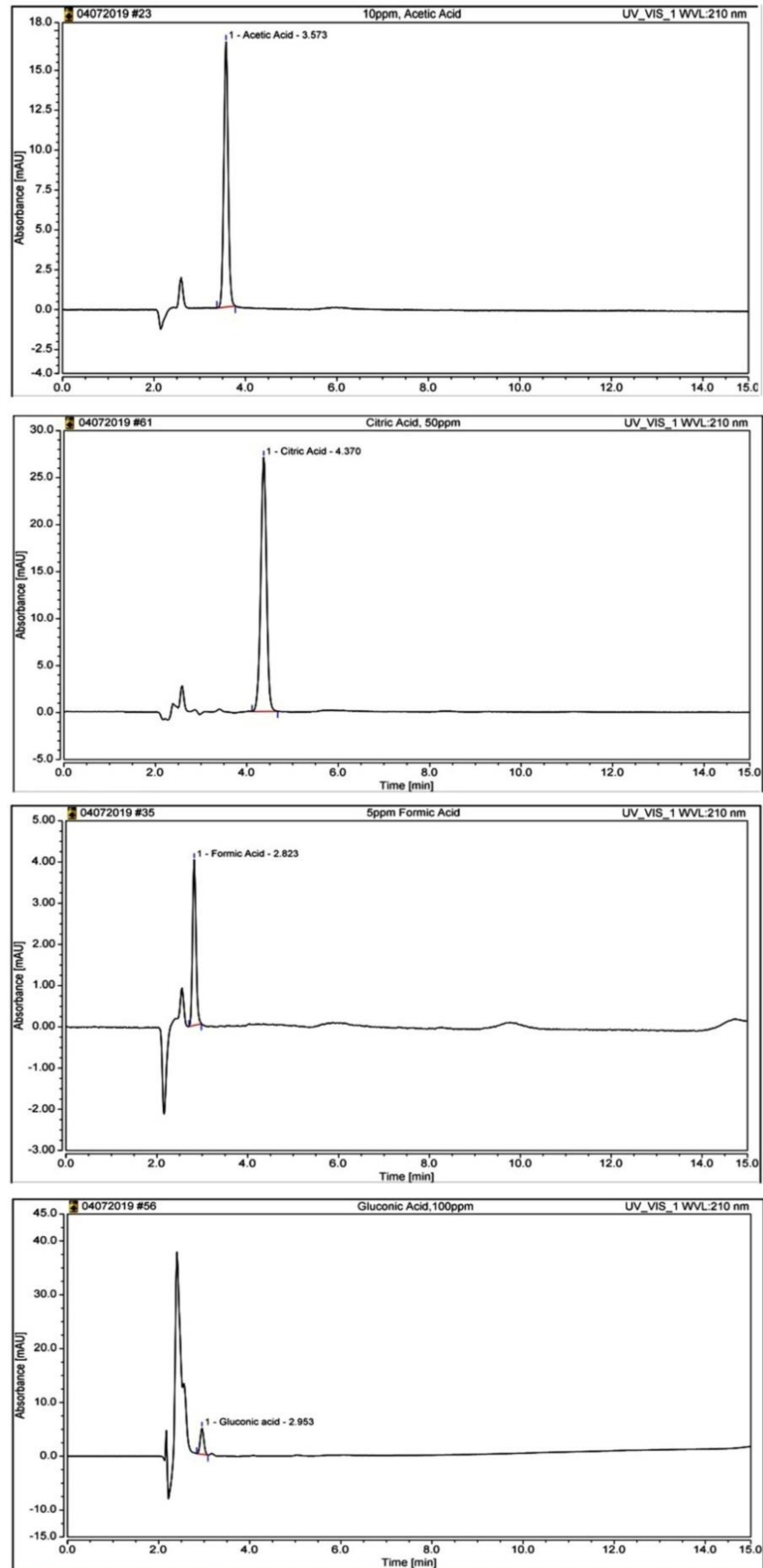

Figure 6.3: Chromatogram of single standard organic acids *viz*., acetic, citric, formic and gluconic

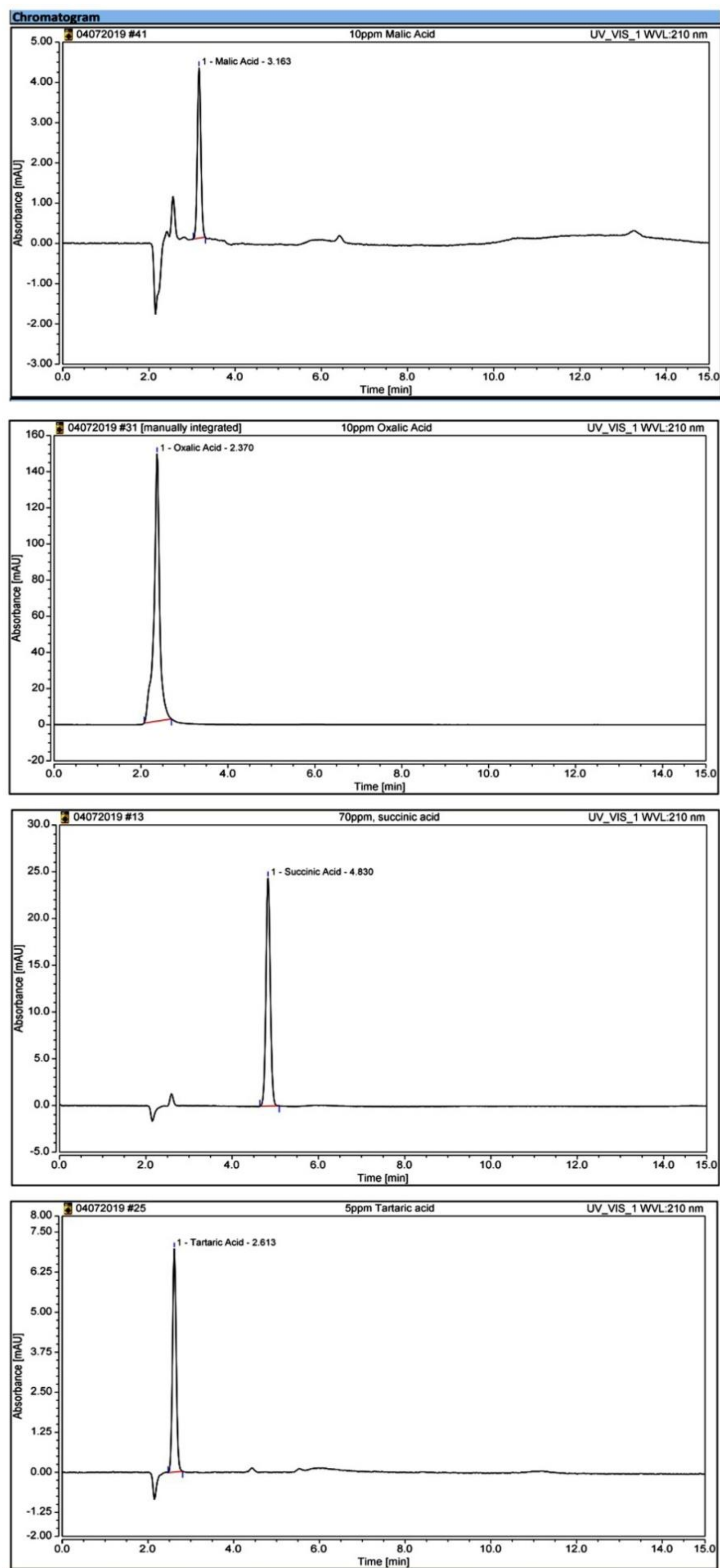

Figure 6.4: Chromatogram of single standard organic acids *viz.*, malic, oxalic, succinic and tartaric

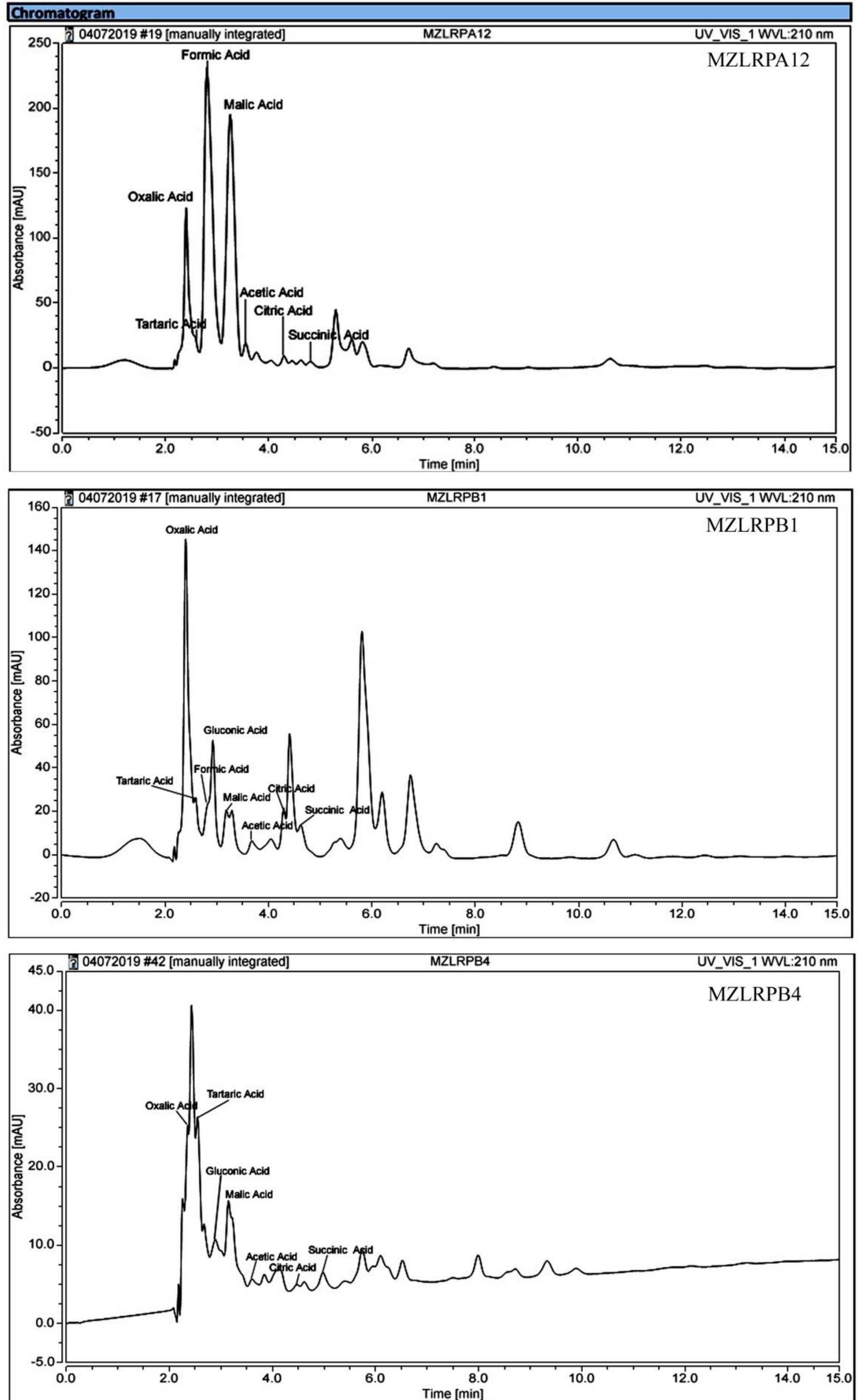

Figure 6.5: Chromatograms of MZLRPA12, MZLRPB1 and MZLRPB4 strains showing retention time of eight different organic acid using UHPL

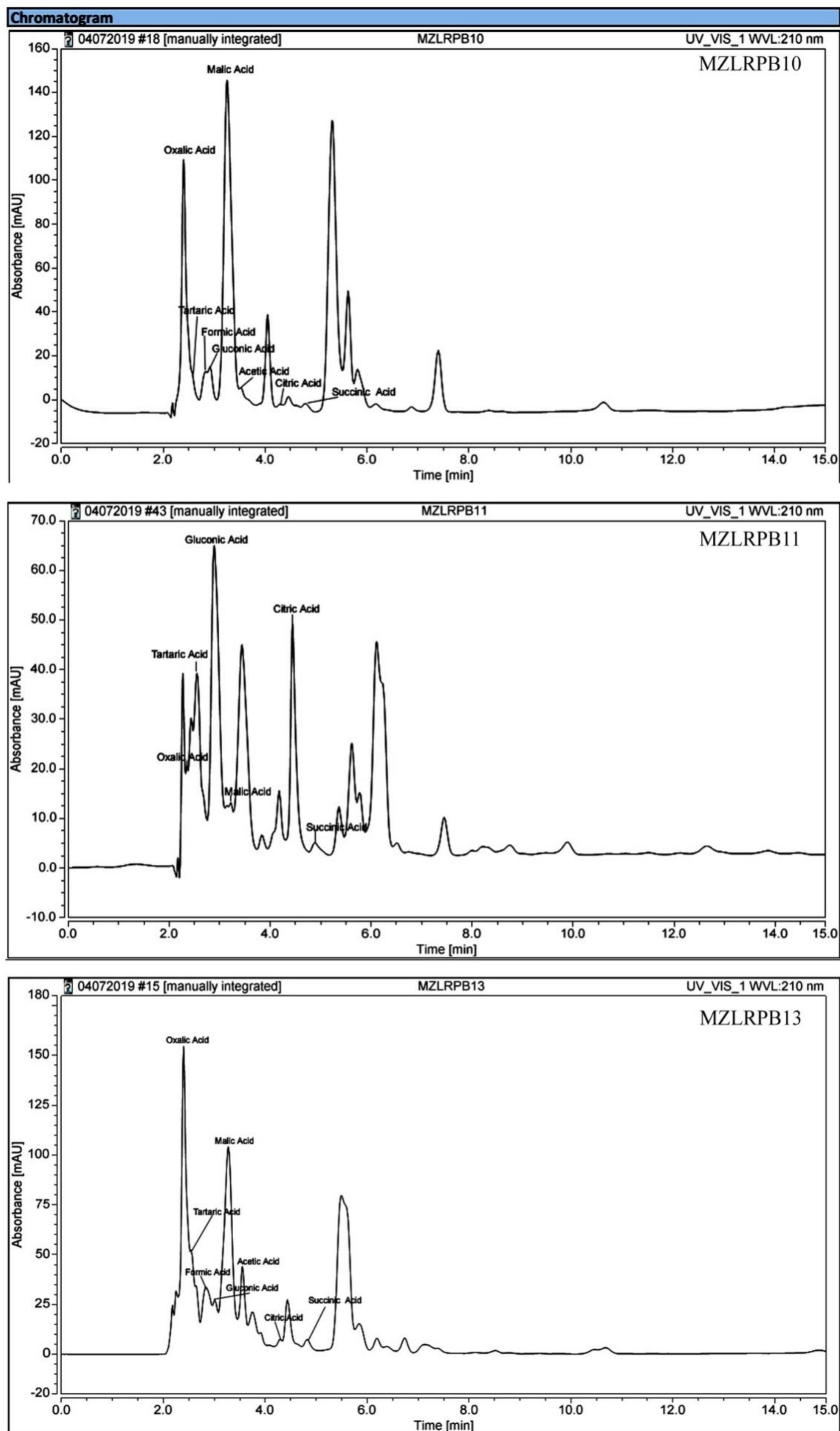

Figure 6.6: Chromatograms of MZLRPB10, MZLRPB11 and MZLRPB13 strains showing retention time of eight different organic acid using UHPL

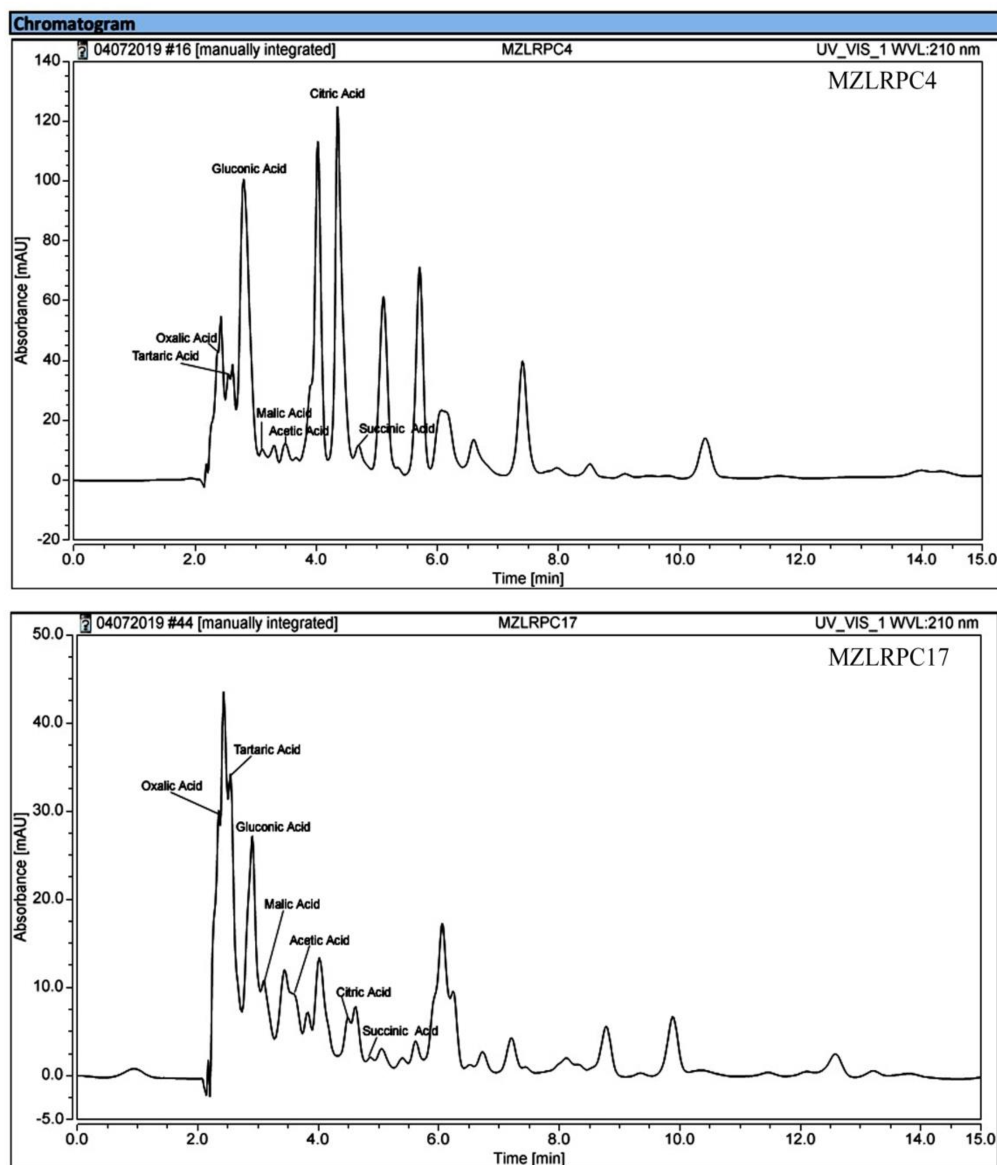

Figure 6.7: Chromatograms of MZLRPC4 and MZLRPC17 strains showing retention time of eight different organic acid using UHPL

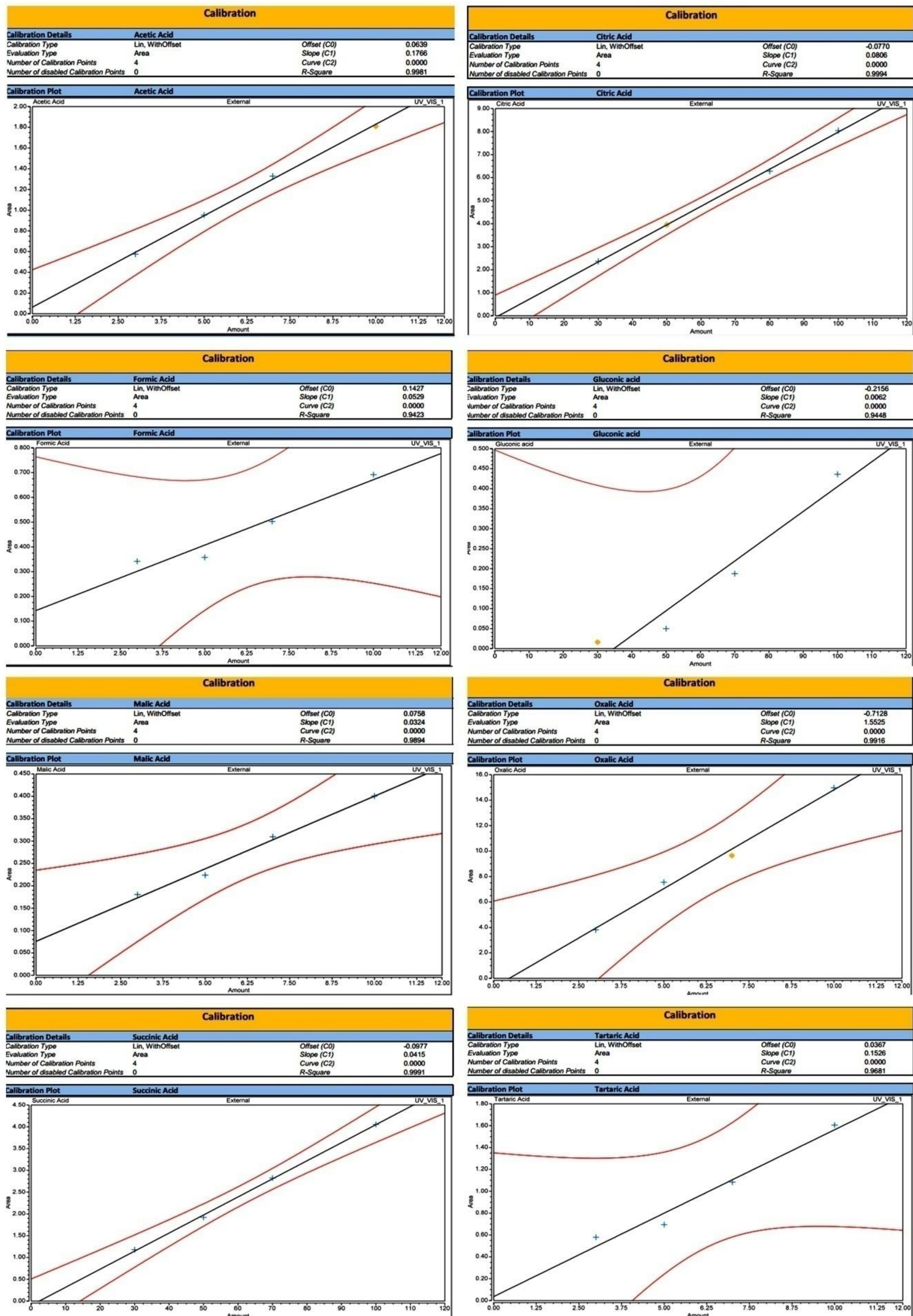

Figure 6.8: Standard curves of eight single organic acids showing R square value

Table 6.1: Quantity of organic acids present in parts per million in each 2μL injection of PSB samples

Organic acids	**Retention Time**	**MZLRPB4 (ppm)**	**MZLRPA12 (ppm)**	**MZLRPB1 (ppm)**	**MZLRPB10 (ppm)**	**MZLRPB11 (ppm)**	**MZLRPB13 (ppm)**	**MZLRPC4 (ppm)**	**MZLRPC17 (ppm)**
Acetic acid	3.573	0.1625	5.6854	2.9516	0.3526	n.a	13.3506	3.3997	0.5717
Succinic acid	4.830	8.12911	11.3383	4.0617	9.7254	9.8450	14.9080	13.0002	3.1411
Tartaric acid	2.613	2.7254	2.7254	2.4365	0.0455	10.2790	1.9851	4.3075	4.3075
Malic acid	3.163	28.3716	959.8454	18.3625	735.8752	n.a	427.0879	6.3351	6.3351
Oxalic acid	2.370	0.5692	6.1335	8.0239	6.5417	0.4760	6.9171	0.6061	0.5846
Formic acid	2.823	0.7324	690.9516	0.6510	4.6040	153.8101	32.5538	245.6142	40.8603
Citric acid	4.370	1.4552	7.9907	8.5101	1.7045	64.2066	2.5169	185.5943	2.7893
Gluconic acid	2.953	59.1459	508.0003	116.5555	74.5933	1375.9183	43.8003	242.3388	425.2257

6.3.2. Inorganic Phosphate fixation by PSB strains and pH level

Quantitative measurement of phosphate release by all the isolates in PVK broth culture are represented graphically in Figure 6.9. The quantities of inorganic phosphate fixed and pH level are revealed in Table 6.2. All the pH level of the PSB broth medium dropped from neutral pH (7) to acidic range (approx. 4) which is an initial indication of organic acid production by the isolates. Along with it the inorganic P supplemented in the medium was fixed by the PSB isolates which were then quantified using UV-Vis Spectrophotometer. The O.D readings were then subjected to the standard readings of P-determination for final data. It was observed that strain MZLRPC4 *Paraburkholderia fungorum* showed the highest P solubilization rate with 3.204 *p*-NPμg^{-1}ml^{-1}hr and lowest P-solubilizing strain is MZLRPC25 *Bacillus cereus* with 0.918 *p*-NPμg^{-1}ml^{-1}hr.

Table 6.2 Quantitative inorganic phosphate fixation by 43 PSB strains and their pH level

Strain names	pH	Phosphate $\mu g^{-1}ml^{-1}hr$
MZLRPA1	5.66±0.002	1.830±0.001
MZLRPA2	4.89±0.001	2.752±0.006
MZLRPA3	6.05±0.002	1.366±0.001
MZLRPA4	5.55±0.001	1.336±0.001
MZLRPA7	4.98±0.003	2.652±0.001
MZLRPA8	4.44±0.012	2.787±0.001
MZLRPA10	5.76±0.001	2.715±0.001
MZLRPA11	6.09±0.001	1.363±0.001
MZLRPA12	5.55±0.001	2.604±0.002
MZLRPB1	4.99±0.002	2.027±0.001
MZLRPB2	6.70±0.005	1.810±0.002
MZLRPB3	4.91±0.009	2.387±0.003
MZLRPB4	4.77±0.001	2.234±0.001
MZLRPB5	4.87±0.004	2.805±0.005
MZLRPB6	4.69±0.001	2.497±0.007
MZLRPB8	4.04±0.001	2.721±0.004
MZLRPB10	4.68±0.003	2.754±0.004
MZLRPB11	4.76±0.001	2.637±0.001
MZLRPB12	4.75±0.002	2.600±0.006
MZLRPB13	4.55±0.003	2.340±0.001
MZLRPB14	5.41±0.004	2.874±0.014
MZLRPB17	4.76±0.001	2.942±0.002

Strain names	pH	Phosphate $\mu g^{-1}ml^{-1}hr$
MZLRPB19	4.50±0.001	2.483±0.002
MZLRPB21	4.76±0.011	1.727±0.003
MZLRPC2	4.41±0.040	2.450±0.001
MZLRPC3	5.87±0.021	1.485±0.001
MZLRPC4	3.44±0.001	3.204±0.001
MZLRPC5	6.11±0.003	1.472±0.002
MZLRPC6	3.76±0.001	3.104±0.001
MZLRPC11.1	5.56±0.002	2.586±0.001
MZLRPC12	5.43±0.002	1.506±0.003
MZLRPC13	6.76±0.024	1.470±0.001
MZLRPC16	6.88±0.001	1.451±0.001
MZLRPC17	6.11±0.001	2.373±0.007
MZLRPC19	6.02±0.001	1.216±0.001
MZLRPC20	6.55±0.003	1.330±0.006
MZLRPC21	5.74±0.005	1.249±0.001
MZLRPC22	5.11±0.002	1.425±0.001
MZLRPC23	4.54±0.001	1.280±0.001
MZLRPC24	4.65±0.001	1.475±0.004
MZLRPC25	6.22±0.001	0.918±0.024
MZLRPC26	3.88±0.033	1.492±0.005
MZLRPD2	4.93±0.060	1.551±0.001

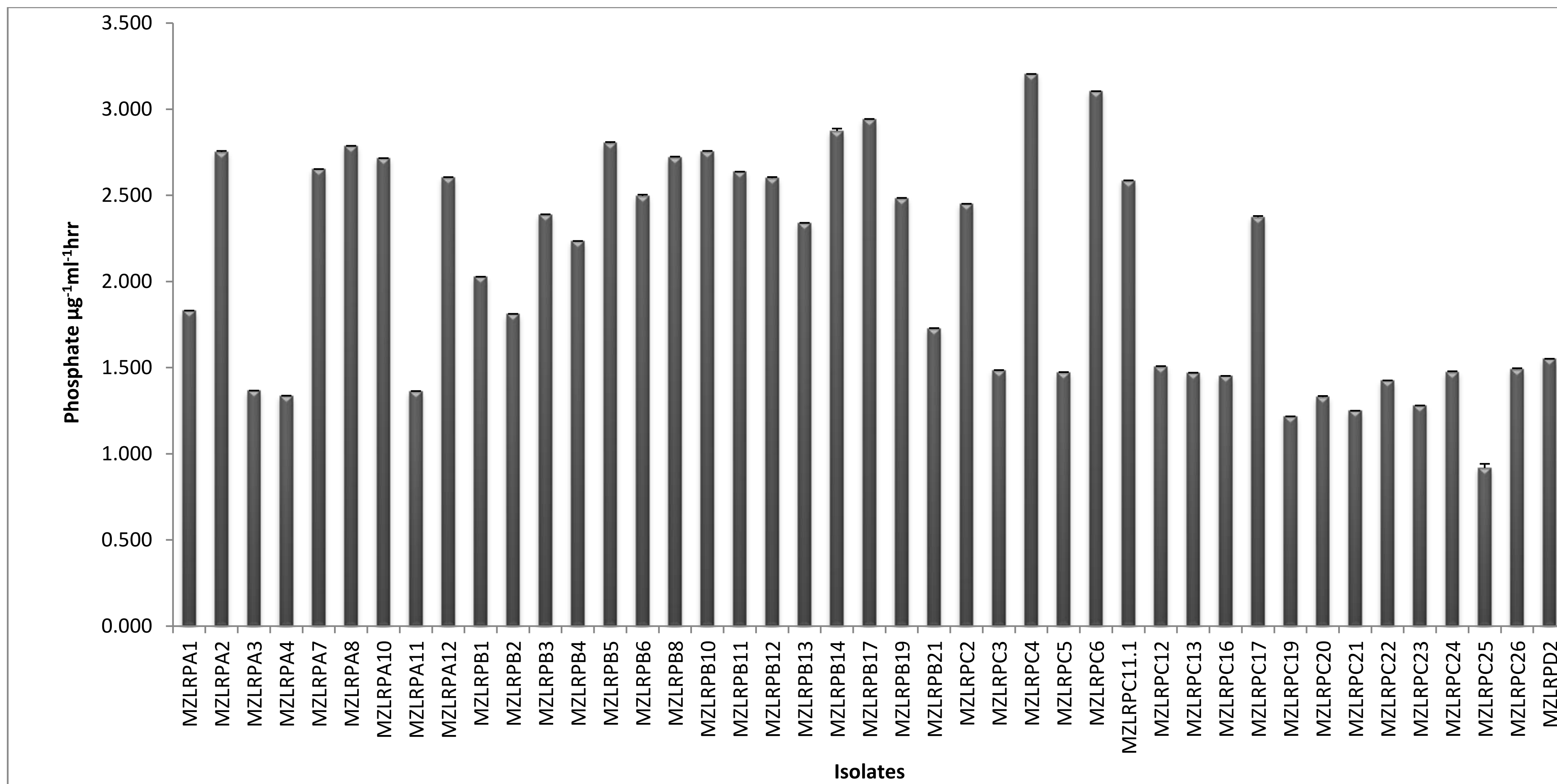

Figure 6.9: Phosphate quantification in broth medium of all PSB isolates

6.4. Discussion

In the present study, two types of phosphate solubilizing bacterial culture media which contain TCP as a P source (Pikovskaya, 1948) such as solid PVK medium (with agar) and PVK broth medium (without agar) were used to estimate phosphate solubilization quality and quantity respectively. The reason for using TCP as P source is based on the findings of Chakraborty *et al*. (2010), Chung *et al*. (2005), Kumar *et al*. (2010) and Parasanna *et al*. (2011) that PSB isolates solubilized tricalcium phosphate (TCP) to a greater extent than rock phosphate, aluminium phosphate and iron phosphate with $AlPO_4$ exhibiting poor solubilization. This could be due to the fact that rock phosphates are less amenable to microbial solubilization because of their structural complexity, while TCP being amorphous is more facile to solubilization (Kumari *et al*., 2008; Nahas, 1996). Pradhan and Sukla (2005) also proved that PVK medium to be the most effective source for P solubilization and used only PVK medium for studying P-solubilizing activities.

The most important mechanism utilized by microorganisms has been well documented that the solubilization of tricalcium phosphate (TCP) is due to the acidification of the medium through biosynthesis and release of a wide variety of organic acids (Rodríguez and Fraga, 1999). Singal *et al.* (1991) have also documented that the development of clear halo zone around the colonies is due to organic acids production in the media plates and are selected as potential phosphate solubilizers (Das, 1989). A significant decline in the pH of the medium during incubation period from 7pH up to 3pH was observed which is in support of the study of Mardad *et al.* (2013) which proved the secretion of organic acids. Similar study has also been reported by Chen *et al*. (2006) and Illmer and Schinner (1995). The production of organic acids by bacteria can reduce the pH and increase phosphorus solubilization and can also chelate cations of phosphorus compounds to release phosphate ion (Kpomblekou and Tabatabai, 1994).

The use of High-performance liquid chromatography (HPLC) has generally replaced the time-consuming enzymatic or chemical analyses of single compounds. HPLC on a strong cation-exchange resin in the H^+ form allows the separation of carbohydrates in the presence of organic acids (Bonn, 1984 and 1985).In our study, both NBRIP and PVK broth medium were used for organic

acid detection in Ultra HPLC. However, good peak detection was observed only at NBRIP medium and was taken as the final result. This is strongly supported by the study of Nautiyal (1999) that NBRIP broth was about 3-fold more efficient as compared to PVK broth. The eight PSB strains tested for organic acid production were able to produce succinic, oxalic, malic, gluconic, acetic, tartaric, formic and citric acid by comparing the retention time with those of authentic standards thus, confirming the mechanism for P-solubilization. This finding is in agreement with the findings of Panhwar *et al*. (2012) who determined the presence of succinic, oxalic, malic and propionic acids in PSB isolates with NBRIP broth medium in HPLC. Many other researchers like Vikram *et al*. (2007) and Leyval and Berthelin (1989) also found the production of organic acids *viz.,* oxalic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glyconic, fumaric, adipic and 2-ketogluconic acid by PSB in the broth culture. Mardad *et al.* (2013) have also studied inorganic-phosphate solubilizing bacteria (PSB) isolated from a phosphate rock deposit and determined the phosphate solubilization index of the isolates in National Botanical Research Institute's phosphate (NBRIP) medium supplemented with tribasic calcium phosphate. From their study gluconic acid was strongly produced by all strains and succinic, acetic, glutamic, oxaloacetic, pyruvic, malic, fumaric acid and alpha ketoglutaric acid were also detected and quantified.

Under UHPLC analysis each OA individual standard were prepared using the solvent as the diluent and different concentrations such as 30ppm, 50ppm, 70ppm and100ppm of each eight OA were primed. Only 2µl of the PSB supernatant filtered samples were injected and the quantities were estimated in parts per million. MZLRPB13 *Bacillus amyloliquefaciens* produced highest acetic acid with 13.35ppm and highest succinic acid with 14.90ppm while MZLRPB11 did not produce any acetic acid. MZLRPB11 *Burkholderia dolosa* produced the highest amount of tartaric acid 10.28ppm and gluconic acid with 1375.92ppm. MZLPA12 *Paenibacillus sp.* produced considerably higher amount of malic acid with 959.85ppm and highest amount of formic acid with 690.95ppm. MZLRPB1 *Bacillus cereus* produced highest oxalic acid with 8.02ppm. MZLRPC4 produced highest amount of citric acid with 185.59ppm. Our results indicate that the most abundantly produced organic acid among all the eight organic acids was, gluconic acid reaching concentration ranging

from 43.80 to 1375.92ppm per 2 µl of each sample. The least amount of OA was oxalic acid ranging from 0.47 to 8.02ppm. In case of MZLRPA12 the retention time for gluconic acid and formic acid were very close to each other with 2.82 min for formic and 2.95 min for gluconic acid. When individually injected the specific sample could detect both acids and could be quantified. However, in the mixture chromatogram, only formic acid was detectable hence, showing only formic acid in the chromatogram. The production of gluconic acid as an outcome of the activity of periplasmic or cell-membrane bound NADP-dependent glucose dehydrogenase (GDH) is one of the best studied mechanisms by which phosphate solubilizing microorganism (PSM) release P from inadequately soluble mineral phosphate (Goldstein, 1995).Furthermore, Liu *et al*. (1992) suggested that solubilization is as the result of acidification of the periplasmic space because of the immediate oxidation of glucose (not phospholytic oxidation) or other aldose through the activity of quinoprotein glucose dehydrogenase (PQQGDH). Glucose is changed over to gluconic acid which produces a transmembrane proton usable for bioenergetics and transport functions of the membrane, while the GA protons are available for solubilizing phosphates.

Chapter 7

Influence of Xenobiotics on Phosphate Solubilizing Capacity of Isolated PSB

7.1. Introduction

Biological features of soil health for maintaining the functions of natural and managed ecosystems are essential for sustainable agricultural fertility and productivity (Enriqueta-Arias *et al.*, 2005). As microbes form the life blood of soil system, it is therefore imperative that the impact on these organisms of any xenobiotic compound entering the soil be studied carefully.

Xenobiotics are those chemical compounds that are foreign to a living organism. Human activity creates a lot of recalcitrant xenobiotic compounds. According to Sinha *et al.* (2009) principal xenobiotics include alkanes, polycyclic aromatic hydrocarbons (PAHs), antibiotics, synthetic azo dyes, pesticides, fuels, solvents, pollutants (dioxins and polychlorinated biphenyls), polyaromatic, chlorinated and nitro-aromatic compounds. The word, xenobiotic, is a combination of two different roots, “xeno” and “biotic.” Xeno is a Greek word which means strange, unnatural, or different. Biotic is a word that implies life. Xenobiotic, therefore, refers to an organic compound that mimics natural biochemical that are essential for life, but which have characteristics that are strange and unnatural. They are often toxic to life. Xenobiotics comprise numerous compounds that are involved in both industrial and agricultural activities. These include synthetic organic pesticides that are commonly used in agricultural field which are added to the soil in large amounts every year. Chemicals with pesticide activity were designed primarily to control insect, weed, fungal or nematode pests (Alexander, 1965 and 1980; Ankumah *et al.,* 1995; Skladany and Metting, 1992). In the present study two commonly utilized agrochemicals were selected as xenobiotics *viz.* Dimethoate (insecticide) and Butachlor (herbicide).

Dimethoate is an anon-systemic, wide-spectrum organophosphate insecticide which effects on soil microbial diversity. These chemicals act by interfering with the activities of cholinesterase, an enzyme that is essential for the proper working of the

nervous systems of both humans and insects. It is an insecticide used to kill mites and insects systemically and on contact. It is used against a wide range of insects (Hayes, 1990; Meister, 1992).

Among the commonly used herbicides, the chloroacetanilide group *viz*., acetochlor, alachlor, butachlor and metachlor, propachlor, are the most consumed chemicals all over the world in agriculture (Eurostat, 2007). Butachlor (N-(butoxymethyl) -2-chloro-2′, 6′-diethyl acetanilide) is a widely recommended herbicide for use in rice cultivation. It is a systemic selective pre-emergent herbicide applied on rice, tea, wheat, beans and other crops *viz*. corn, soybean etc (Dwivedi *et al*., 2012).

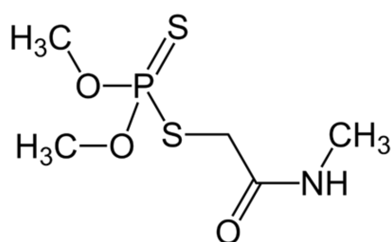

Figure 7.0: Structure of Dimethoate

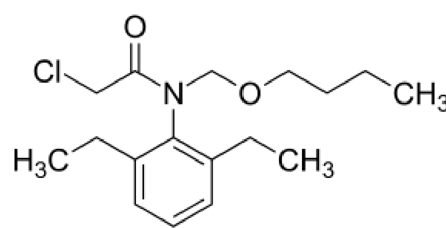

Figure 7.1: Structure of Butachlor

Synthetic organic compounds are one of the major sources of contamination in the natural environment (Kolwzan, 2009; Sekutowski and Sadowski, 2009; Kowalska, 2009). Herbicides are the property of various chemical groups, which can be highly effective in weed control and can provide good quality and quantity of crops (Brikhofer *et al*., 2008). Uncontrolled farming practices may lead to the increase of herbicides to various ecosystems, posing a threat for living organisms in those habitats, mainly microbes (Cederlund, 2007; Cycoń, 2007). A dependable source of indicator for monitoring soil conditions is the activity of soil enzymes which adds to the valuable source of information on soil changes induced by xenobiotics. The enzymes that are produced by the soil microbes and plants play a major role in the environment as they actively contribute in the route of circulation of organic matter (Li *et al*., 2004; Singh, 2009). The global utilization of pesticides assures production capabilities, although their significant use, persistence and

transfer cross-ecosystems and into trophic food webs can cause major environmental pollution (Pimentel, 1995; Ackerman, 2007).

Consequently, disturbances of microbial communities by foreign synthetic substances like xenobiotics ensuring various ecological processes in soil such as organic matter degradation and nutrient cycling, could lead to decrease of growth of microbes, declining of their enzyme activity and could negatively affect soil fertility and sustainable agricultural productivity.

7.2. Experimental design

A completely randomized design (CRD) was adopted for the study of influence of the xenobiotics on five different parameters which were biomass dry weight, bacterial growth, acid phosphatase enzyme, IAA production and phosphate determination which were all performed separately. For the treatment, the xenobiotics were prepared into three concentrations *viz*. 50ppm, 100ppm and 150ppm separately. All the treatments were then monitored for 72 hours taking observations on 24, 48 and 72 hrs consecutively which acted as the groups. All tests were done in triplicates. All results in each treatment were analysed in each group and the result were statistically analysed. The objective of this study was to study the effect of two Xenobiotic compounds *viz*. Butachlor 50% EC (Grass Kill-Herbicide) and Dimethoate 30% EC (Rogorus-Insecticide) on bacterial dry weight biomass, growth, acid phosphatase enzyme activity, IAA production and determination of phosphate released. The approximate dosage of Butachlor 50% EC (Grass Kill-Herbicide) as recommended for use in the field is 2.4-4.5 L in 250-500 L of water and for Dimethote 180-750 gm active ingredient per hectare.

7.3. Selection of test organisms

Two phosphate sollubilizing bactrerial isolates based on P solubilization efficiency and enzyme phosphatase activity were used such as MZLRPC4 *Paraburkholderia fungorum (*Accession number MK932042*)* and MZLRPA12 *Paenibacillus* sp. (Accession number MK932030).

7.4. Methodology

7.4.1. Xenobiotics used

Two agrochemicals which were commonly used in agricultural field were selected for the study. Butachlor 50% EC (herbicide) with the trade name as "Grass Kill" and Dimethoate (insecticide/pesticide) with trade name as "Rogorus" was selected. Recommended dosage of butachlor ($C100_{17}H_{26}NO_2Cl$ and molecular weight 311.89) per hectare is 2.5-4.0 L in dilution with 250-600 L water. The packaging recommended dosage dimethoate ($C_5H_{12}NO_3PS_2$. and molecular weight is 229.2) per hectare is 180 to 750 gm a.i. per hectare.

7.4.2. Preparation of solution

The different concentration of agrochemical solution (butachlor and dimethoate) for the experiment were prepared following Azhar *et al*. (2013) at 50ppm, 100ppm and 150ppm individually using Pikovskaya broth medium as diluent for measurement of bacterial biomass, growth, P-determination and acid phosphatase activity and NBRIP medium as diluent for IAA production test. They were then autoclaved at 121°C for 30 minutes by using saturated steam under 15psi of pressure.

7.4.3. Dry weight Biomass

Three different concentrations viz. 50ppm, 100ppm and 150ppm were prepared using Pikovskaya (PVK) broth medium (Pikovskaya, 1948) and autoclaved. After it was cooled down, 1 ml of pure PSB culture was transferred to 100 ml conical flask with Pikovskaya broth treated with Butachlor and Dimethoate in 50ppm, 100ppm and 150ppm each with replicates. Control bacterial culture (without treatment) was also maintained. Cultures were incubated at refrigerated incubator shaker with continuous shaking at 80% rpm at 30±7°C. After 24, 48 and 72 hrs each, 10ml of each treated culture were centrifuged at 10,000rpm for 10 minutes. The supernatant was discarded and the pellet was air dried. After completely dried, the centrifuge tubes were weighed along with the pellet. Weights of the empty tubes were all recorded as well. The dry biomass was calculated as-

Dry weight of bacteria = W_2-W_1

Where, W_1 = Initial weight of centrifuge tube

W_2 = Final weight of centrifuge tube

7.4.4. Bacterial growth test

To a clean and dry cuvette, 2ml of incubated culture media solution was added and optical density was read at 600nm absorbance in UV-VIS Spectrophotometer. Each reading was observed and recorded.

7.4.5. Determination of Indole-3-acetic acid production (Gutierrez *et al.*, 2009)

The bacterial isolates were grown in NBRIP (National Botanical Research Institute's Phosphate) growth medium containing 0.2% L-tryptophan incubated for 72 hours with continuous agitation at 30±7°C at an incubator shaker with each of the xenobiotic treatment. Reading was taken in 24, 48 and 72 hours. After incubation, the culture broth was centrifuged at 8000rpm for 10 min and the supernatant was collected. One ml aliquot of the supernatant was mixed vigorously with 4ml of Salkowski's reagent, incubated at 37°C for 30 min. Development of pink colour indicates the IAA production and the absorbance were measured at 530nm in a UV-Visible spectrophotometer. The untreated medium mixed with Salkowski reagent served as blank. The concentration of IAA in each culture medium was compared with standard IAA curve.

Chemical preparation:

Salkowski reagent: In a clean and dry 100 ml conical flask, 50 ml of 35% perchloric acid was added and mixed with 1ml of 0.5 M $FeCl_3$ solution

7.4.6. Estimation of Phosphatase Activity (Huang and Shindo, 2000)

10 ml of treated and incubated culture media solution was taken into the centrifuge tube and centrifuged at 10,000rpm for 10 minutes. To an empty clean dry test tube 1ml of 10mM p-NPP was added. To it 1ml of 0.1M acetate buffer pH 5.6 was added, and then 2ml of the enzyme stock (treated and untreated culture medium solution supernatant) was added immediately. It was incubated for 1 hour at 37°C in

temperature controlled water bath. Then the phosphatase reaction was stopped by the addition of 2ml 1M NaOH. The absorbance yellow colour intensity was read with UV-Vis spectrophotometer at 410nm.

Chemical preparation:

10mM p-NPP : In a clean and dry amber conical flask, 0.186 g of p-NPP was added and mixed with 50 ml dist.H_2O (to be prepared fresh).

1M NaOH : 8g of NaOH was mixed with 200ml dist. H_2O

7.4.7. Phosphate determination (Murphy and Riley, 1962)

Phosphate solubilizing activity in treated PVK broth medium was evaluated for each isolate. The treated culture medium was incubated in an incubator shaker at 30±7°C at 80 rpm for 7 days. The bacterial cultures were centrifuged at 10,000rpm for 10 mins and supernatant was estimated for Phosphate determination which was done by the Ascorbic acid method. Control was maintained without xenobiotic treatment. Each reading was taken on 24, 48 and 72 hours respectively. The procedure is as follows-

Chemical preparation:

Sulphuric acid (H_2SO_4) 5N: 70ml of H_2SO_4 was mixed with 500 ml dist. H_2O.

Antimony potassium tartarate $K_2Sb_2(C_4H_2O_6)_2$: 0.343g of $K_2Sb_2(C_4H_2O_6)_2$ was dissolved in 100ml dist. H_2O and 25ml dist. H_2O was again added.

Ammonium molybdate $(NH_4)_2MoO_4$ solution: 5g of $(NH_4)_2MoO_4$ was added with 125 ml dist. H_2O

Ascorbic acid ($C_6H_8O_6$) 0.1M: 0.88g of $C_6H_8O_6$ was taken and mixed with50 ml dist. H_2O

Combined reagent: 50ml of 5N H_2SO_4 was added with 5ml Antimony potassium tartrate, 15ml Ammonium molybdate solution and 30ml Ascorbic acid.

All the reagents must be at room temperature before they are mixed, and they should be mixed in the order given. If turbidity forms, shake and let it stand for a few minutes until it disappears. This combined reagent is stable for 4 hours.

After combined reagent was prepared, 50ml of the supernatant PSB sample was taken in a sterile dry tube. To it 0.05ml or 1 drop of phenolphthalein indicator was added. If red colour was developed, 5N H_2SO_4 was added drop wise to discharge the colour. Then, 8ml of the combined reagent prepared was added and mixed thoroughly. After 20 to 30mins, the absorbance was measured at 880nm using a UV-Vis spectrophotometer using blank as a reference solution and compared with the standard curve of KH_2PO_4.

7.5. Results

7.5.1. Effect of xenobiotic on the dry weight biomass

The dry weight biomass of two PSB test organisms MZLRPA12 and MZLRPC4 which were treated with different concentrations (50ppm, 100ppm and 150ppm) of butachlor and dimethoate were recorded on the 24 hours, 48 hours and 72 hours from the period of incubation. Both bacterial strains showed decreased in dry weight biomass with an increase in concentration *i.e.*, higher the concentration, lower the weight of biomass. On the contrary, there was an increase in biomass weight with increase in incubation period *i.e.* the longer the incubation period, the higher the weight of biomass. The unit for dry weight biomass is given in g/ml (Fig. 7.2 and 7.3).

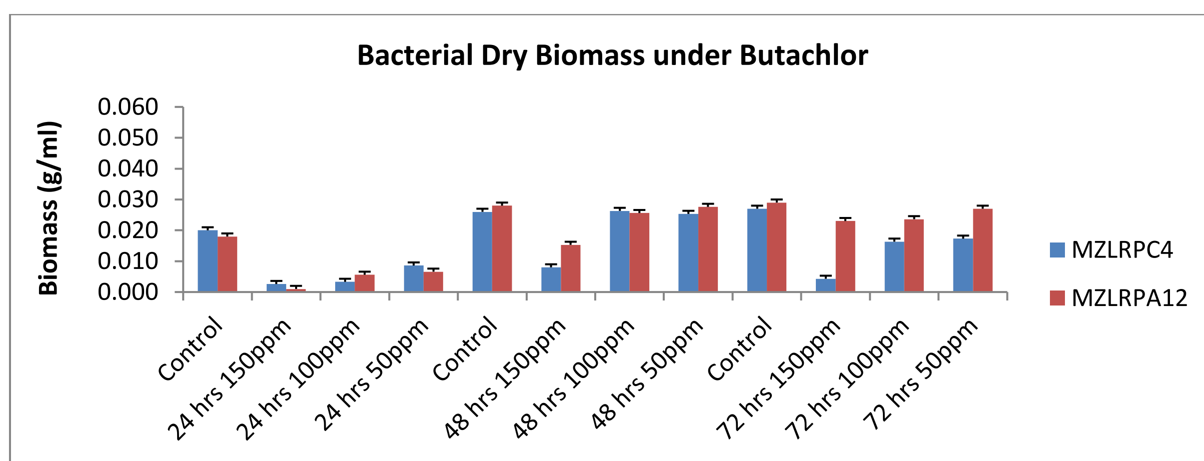

Figure 7.2: Biomass of MZLRPC4 and MZLRPA12 at different concentrations of butachlor

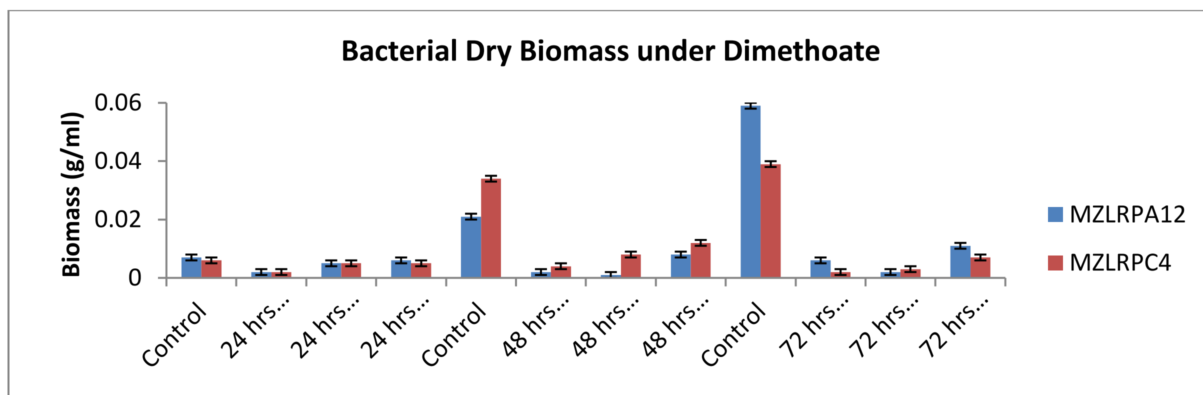

Figure 7.3: Biomass of MZLRPC4 and MZLRPA12 at different concentrations of dimethoate

7.5.2. Effect of xenobiotic on the bacterial growth

The bacterial growth of two PSB test organisms MZLRPA12 and MZLRPC4 which were treated with different concentrations (50ppm, 100ppm and 150ppm) of butachlor and dimethoate were harvested on the 24 hours, 48 hours and 72 hours and the intensity of the treated culture broth were read at 600nm using UV-Vis spectrophotometer. Both bacterial strains resulted with a decrease in growth with an increase in concentration while at the same time there was an increase in growth with increase in incubation period (Fig. 7.4 and 7.5).

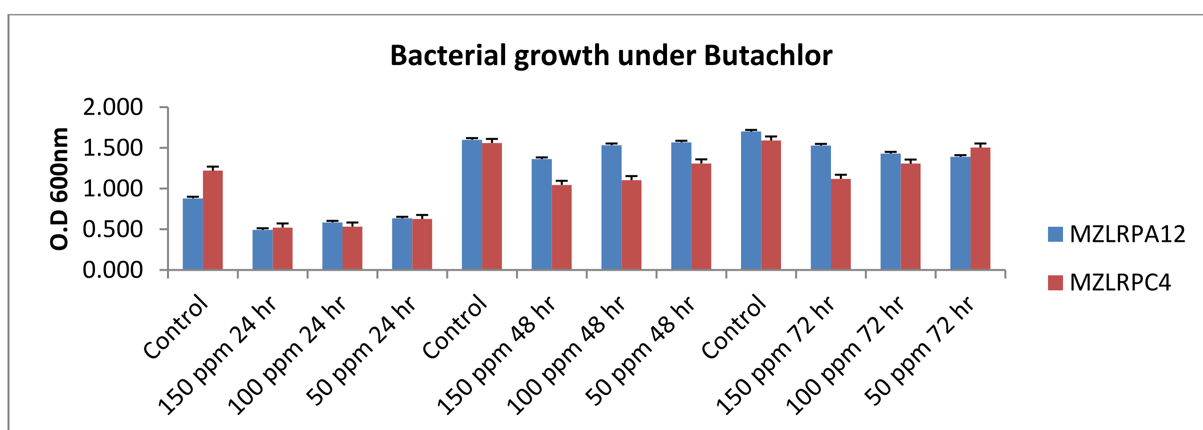

Figure 7.4: Bacterial growth of MZLRPC4 and MZLRPA12 at different concentrations of butachlor

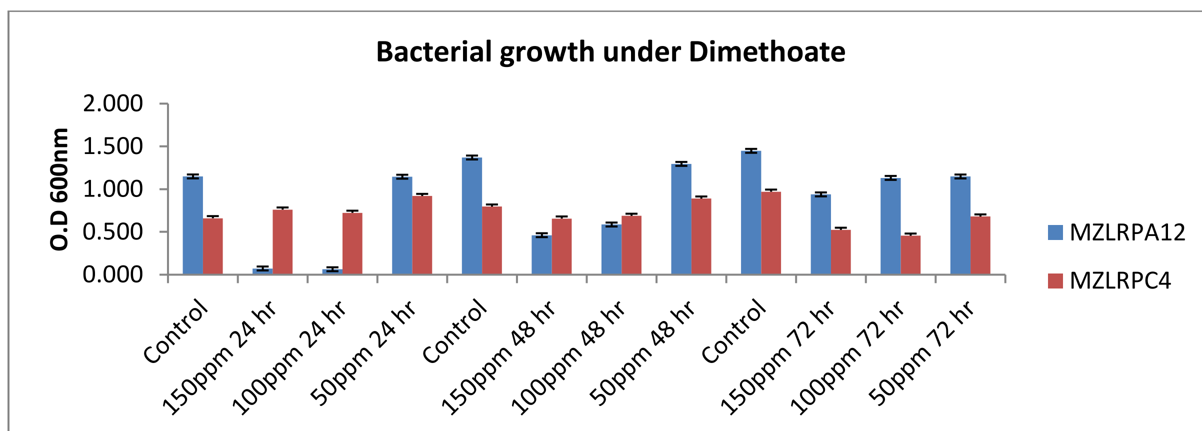

Figure 7.5: Bacterial growth of MZLRPC4 and MZLRPA12 at different concentrations of dimethoate

7.5.3. Effect of xenobiotic on IAA production

The IAA production of two PSB test strains MZLRPA12 and MZLRPC4 treated with different concentration of two xenobiotics (50ppm, 100ppm and 150ppm) were analysed on 24 hours, 48 hours and 72 hours from the incubation period. In 24 hours, IAA production was suppressed both in butachlor and dimethoate treatment. In 48 hours in butachlor treatment (Fig 7.6), MZLRPC4 decreased considerably with increase in concentration and showing higher IAA production than control and MZLRPA12 also showed slightly decrease with increase in concentration but lower than control. In 72 hours butachlor treatment (Fig 7.6), control was higher than both treatments hence showing same trend as 48 hrs but MZLRPC4 showed lesser IAA production than 48 hours. In control treatment, the IAA production increased with increase in incubation period. In dimethoate treatment (Fig. 7.7), MZLRPC4 showed decrease in IAA production with increase in concentration in 48 and 72 hrs hence increase during incubation period. MZLRPA12 showed production only on 50ppm concentration in 48 and 72 hrs. The unit for IAA production is µg/ml.

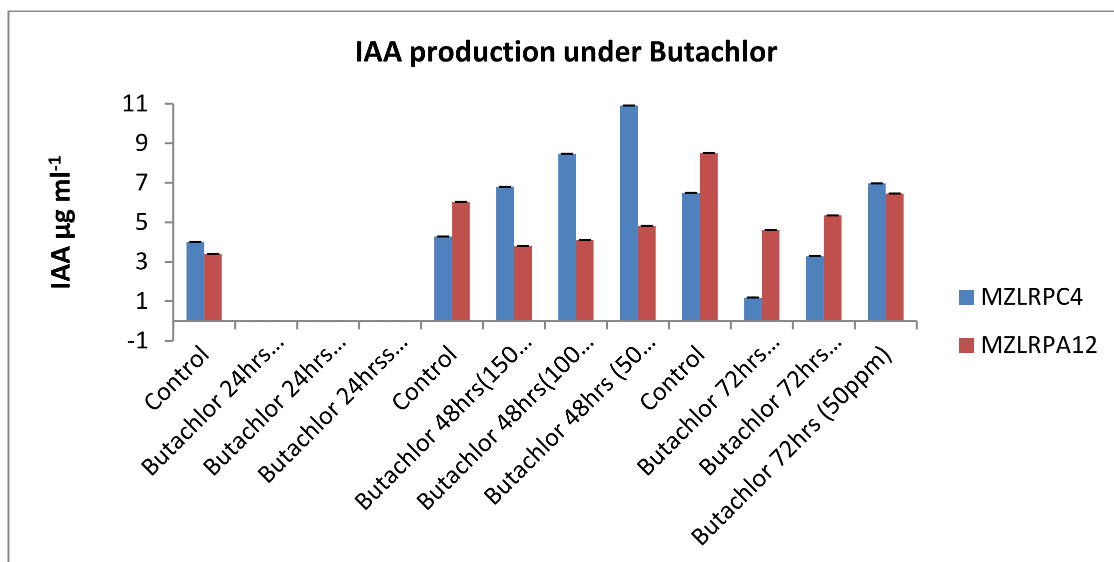

Figure 7.6: IAA production of MZLRPA12 and MZLRPC4 in butachlor treatment

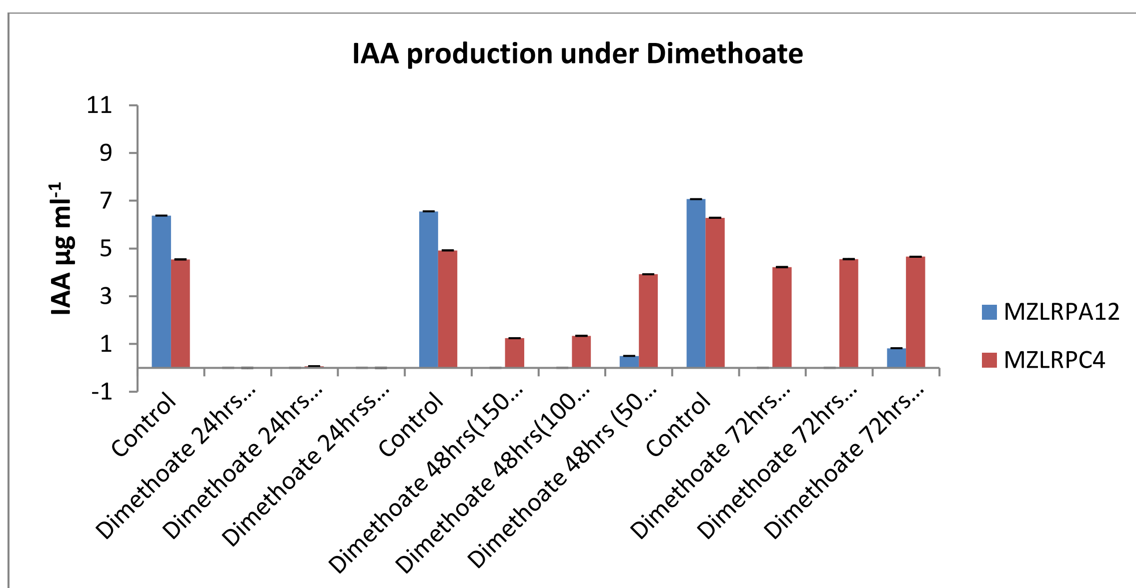

Figure 7.7: IAA production of MZLRPA12 and MZLRPC4 in dimethoate treatment

7.5.4. Effect of xenobiotic on acid phosphatase activity

The acid phosphatase activity of two PSB test strains MZLRPA12 and MZLRPC4 treated with different concentration of two xenobiotics (50ppm, 100ppm and 150ppm) were analysed on 24 hours, 48 hours and 72 hours from the incubation period. Control treatment showed increase in enzyme activity with increase in

incubation period. In butachlor treatment (Fig.7.8), decrease in enzyme activity with increase in the concentration was observed. In MZLRPA12 and MZLRPC4, the duration of the incubation period had very minute to no effect on the enzyme activity. However, in MZLRPA12 72hrs, the three concentrations show slightly lower enzyme activity as compared to 24 and 48 hrs. So in this case, the duration of incubation period played inconsequential role in increasing the enzyme activity. Out of the two isolates, tolerance capacity of MZLRPC4 was found to be higher against the herbicide butachlor. In dimethoate treatment (Fig. 7.9), decrease in enzyme activity with increase in the concentration was observed similar to butachlor treatment. In MZLRPA12, there was decrease in enzyme activity with increase in incubation period. In MZLRPC4, there was slight increase in 48 hrs compared to 24 hrs and then dropped to some extent in 72 hrs of incubation. So, the incubation period did not play much role in effecting or suppressing the enzyme activity. Both strains showed somewhat similar level of tolerance to dimethoate.

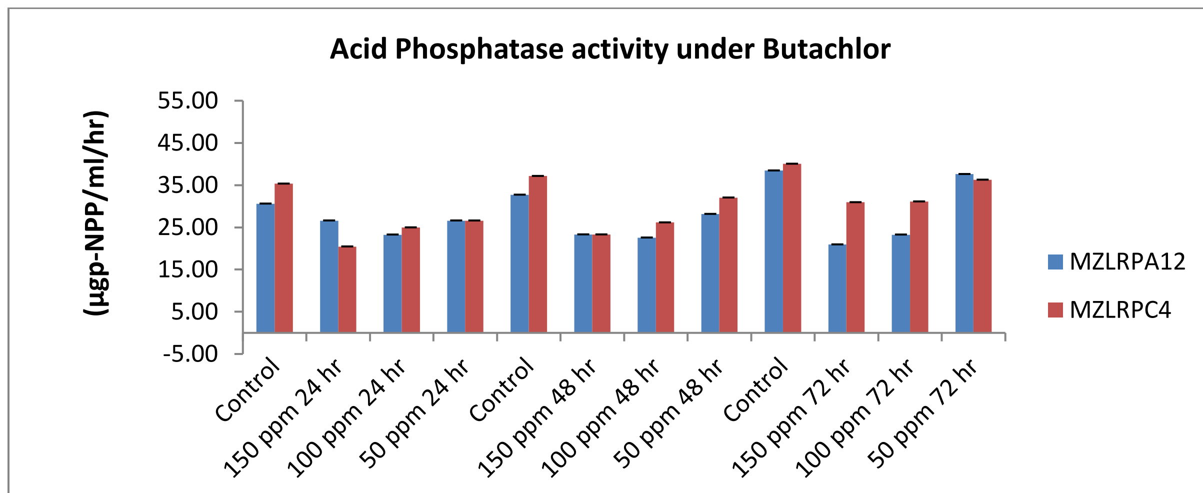

Figure 7.8: Acid phosphatase activity of MZLRPC4 and MZLRPA12 on butachlor treatment

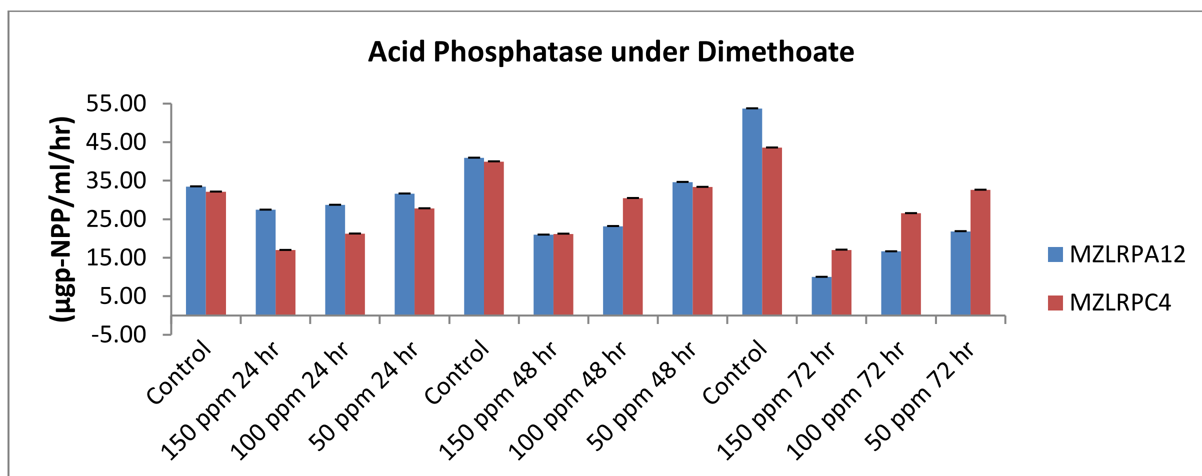

Figure 7.9: Acid phosphatase activity of MZLRPC4 and MZLRPA12 on dimethoate treatment

7.5.5. Effect of xenobiotic on phosphate determination

The determination of released phosphate by two PSB test strains MZLRPA12 and MZLRPC4 treated with different concentration of two xenobiotics (50ppm, 100ppm and 150ppm) were analyzed on 24 hours, 48 hours and 72 hours from the incubation period. Control treatment showed increase in enzyme activity with increase in incubation period. In butachlor treatment (Fig.8.0), decrease in P-release with increase in concentration was observed. In MZLRPA12, during 24 hrs incubation 100ppm showed higher amount compared to 50ppm and in 72 hrs, 50ppm showed least amount of P release as compared to 100 and 150ppm. In MZLRPC4, 24 hrs showed decrease in amount of P with increase in concentration. So is the same in 48 hrs, in 72 hrs, there is no amount produced in 150ppm and minute amount in both 100 and 50ppm. So, in this case the duration of incubation period have effect on the phosphate determination and is stable only up to 48 hrs due to the treatment. In dimethoate treatment (Fig.8.1), MZLRPA12 in 24 and 48 hrs did not show much change in the concentrations. However, in 48 hrs the overall P release amount was higher than 24 hrs in 72 hrs; it is slightly higher than 48 hrs and highest in 50ppm. Increase in the incubation period increased the amount of P release but did not affect much in the concentrations. MZLRPC4 in 24 hrs showed slightly decrease in P with increase in concentrations. In 48 and 72 hrs, 150ppm did not show any amount of P

released, whereas in 50ppm, the amount increased with increase in incubation period. In this case, the highest concentration 150ppm is most susceptible to the release of phosphate by the isolates.

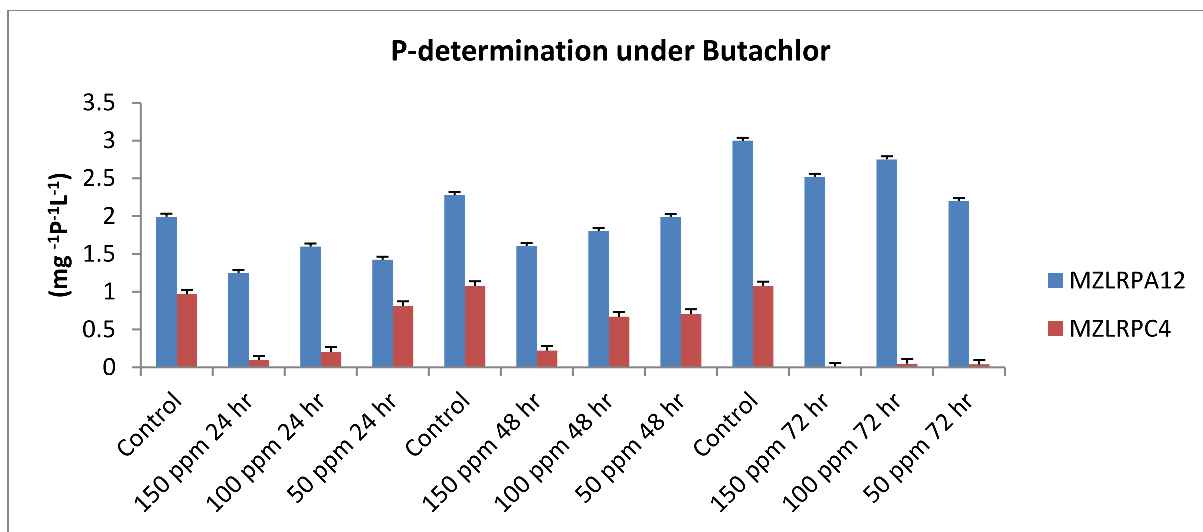

Figure 8.0: P-determination of MZLRPC4 and MZLRPA12 on butachlor treatment

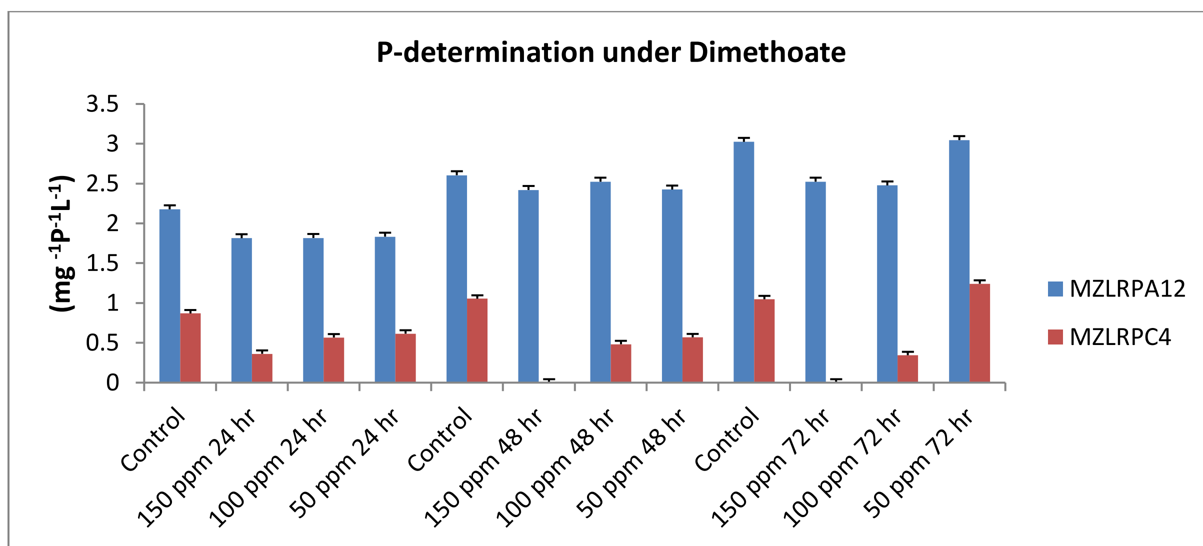

Figure 8.1: P-determination of MZLRPC4 and MZLRPA12 on dimethoate treatment

Table 7.1: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of butachlor at 24 hours of incubation. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters (24 hrs Butachlor)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	8.645	0.007*
2	Acid Phosphatase MZLRPC4	-do-	14.790	0.001*
3	IAA MZLRPA12	-do-	9.919	0.005*
4	IAA MZLRPC4	-do-	66.640	0.000*
5	P-determination MZLRPA12	-do-	17.370	0.001*
6	P-determination MZLRPC4	-do-	57.325	0.000*
7	Bacterial growth MZLRPA12	-do-	315.490	0.000*
8	Bacterial growth MZLRPC4	-do-	66.993	0.000*
9	Biomass MZLRPA12	-do-	422.250	0.000*
10	Biomass MZLRPC4	-do-	192.889	0.000*

Table 7.2: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of butachlor at 48 hours of incubation. Marked effects are significant at $p \leq 0.05$

S.N	Parameters (48 hrs Butachlor)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	6.041	0.019*
2	Acid Phosphatase MZLRPC4	-do-	19.976	0.000*
3	IAA MZLRPA12	-do-	2.543	0.130*
4	IAA MZLRPC4	-do-	4.092	0.049*
5	P-determination MZLRPA12	-do-	30.098	0.000*
6	P-determination MZLRPC4	-do-	64.155	0.000*
7	Bacterial growth MZLRPA12	-do-	21.097	0.000*
8	Bacterial growth MZLRPC4	-do-	3.263	0.080*
9	Biomass MZLRPA12	-do-	19.127	0.001*
10	Biomass MZLRPC4	-do-	10.163	0.004

Table 7.3: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of butachlor at 72 hours of incubation. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters (72 hrs Butachlor)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	298.116	0.000*
2	Acid Phosphatase MZLRPC4	-do-	4.123	0.048*
3	IAA MZLRPA12	-do-	19.126	0.001*
4	IAA MZLRPC4	-do-	30.358	0.000*
5	P-determination MZLRPA12	-do-	91.468	0.000*
6	P-determination MZLRPC4	-do-	7.174	0.000*
7	Bacterial growth MZLRPA12	-do-	64.938	0.000*
8	Bacterial growth MZLRPC4	-do-	22.327	0.000*
9	Biomass MZLRPA12	-do-	0.766	0.544
10	Biomass MZLRPC4	-do-	26.223	0.000*

Table 7.4: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of dimethoate at 24 hours of incubation. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters (24 hrs Dimethoate)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	14.663	0.001*
2	Acid Phosphatase MZLRPC4	-do-	239.481	0.000*
3	IAA MZLRPA12	-do-	4.720	0.000*
4	IAA MZLRPC4	-do-	1.292	0.000*
5	P-determination MZLRPA12	-do-	6.428	0.016*
6	P-determination MZLRPC4	-do-	25.514	0.000*
7	Bacterial growth MZLRPA12	-do-	5.949E4	0.000*
8	Bacterial growth MZLRPC4	-do-	30.055	0.000*
9	Biomass MZLRPA12	-do-	17.143	0.001*
10	Biomass MZLRPC4	-do-	25.667	0.000*

Table 7.5: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of dimethoate at 48 hours of incubation. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters (48 hrs Dimethoate)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	57.875	0.000*
2	Acid Phosphatase MZLRPC4	-do-	39.365	0.000*
3	IAA MZLRPA12	-do-	5.427	0.000*
4	IAA MZLRPC4	-do-	180.247	0.000*
5	P-determination MZLRPA12	-do-	6.308	0.017*
6	P-determination MZLRPC4	-do-	1.030	0.000*
7	Bacterial growth MZLRPA12	-do-	22.456	0.000*
8	Bacterial growth MZLRPC4	-do-	11.968	0.003*
9	Biomass MZLRPA12	-do-	50.870	0.000*
10	Biomass MZLRPC4	-do-	11.027	0.003*

Table 7.6: One way analysis of variance (ANOVA) of MZLRPC4 and MZLRPA12 on APase, IAA, P-determination, growth and biomass under the influence of different concentration of dimethoate at 72 hours of incubation. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters (72 hrs Dimethoate)	Source Of Variation	F-value	p-value
1	Acid Phosphatase MZLRPA12	CTRLx150ppmx100ppmx50ppm	1.381	0.000*
2	Acid Phosphatase MZLRPC4	-do-	267.528	0.000*
3	IAA MZLRPA12	-do-	388.758	0.000*
4	IAA MZLRPC4	-do-	8.270	0.008*
5	P-determination MZLRPA12	-do-	24.771	0.000*
6	P-determination MZLRPC4	-do-	56.403	0.000*
7	Bacterial growth MZLRPA12	-do-	85.451	0.000*
8	Bacterial growth MZLRPC4	-do-	147.127	0.000*
9	Biomass MZLRPA12	-do-	28.810	0.000*
10	Biomass MZLRPC4	-do-	567.259	0.000*

7.6. Discussion

A number of studies on widely-used pesticides have already shown that pesticide application leads to changes in soil nutrient levels and alterations to soil microbial activity, diversity and/or genetic structure (Girvan *et al*., 2004; Ros *et al*., 2006).

In our study, the effect of the two agrochemicals serving as the xenobiotics Dimethoate (insecticide) and Butachlor (herbicide) at different concentration *i.e.,* 50ppm, 100ppm and 150ppm on MZLRPC4 *Paraburkholderia fungorum* (Accession number MK932042) and MZLRPA12 *Paenibacillus sp*. (Accession number MK932030) gave the evidence that their growth decreased with increase in the concentration of the two xenobiotics while at the same time following the same trend, the overall growth increased with increase in the incubation period (Fig.7.4 & 7.5). The tested PSB strains were affected by the xenobiotic used and they have a variable effect depending on its type, field condition and doses used. So, it is clear that the impact is associated with the concentration and incubation period. Our finding is in support with the study of Haleem *et al*. (2013) who also found that there was a reduction and decreased in the total bacterial count with increase in organophosphorous insecticide concentrations and incubation time as compared to control.

The dry weight biomass decreased with increase in the level of concentration (Fig. 7.2 & 7.3). However, total growth increased with the incubation period. Comparing the two xenobiotics, insecticide dimethoate had more effect towards the biomass. Bacterial growth showed decrease with increase in xenobiotic concentration in the broth medium by reading the level of turbidity through UV-Vis spectrophotometer. This finding is in support with the findings of Dubey *et al*. (2012) who gave the evidence that total count of PSB decreased to a high level with the application of different types of pesticides.

The measurement of phosphatase activity has been used as a synthetic index to evaluate the effect of xenobiotic compounds on the overall microbial catalytic activity in soil (Mathur and Sanderson, 1978; Doelman and Haanstra, 1989; Dumontet *et al*., 1993). The experimental result showed that the activity of acid

phosphatase of both *Paenibacillus sp.* and *Paraburkholderia fungorum* decreased with increase in the concentration of both xenobiotics treatments (Fig. 7.8 & 7.9). The strain MZLRPA12 *Paenibacillus* sp. showed decline in the enzyme activity with the increase in the incubation period although it did show decrease with increase of the xenobiotic concentration i.e., in every hour observation, 150ppm which was the highest concentration showed least amount of enzyme activity but in 72 hours the overall result was slightly lower as compared to 48 and 24 hrs. The strain MZLRPC4 *Paraburkholderia fungorum* showed highest activity in 48 hrs. From our study we can conclude that the enzyme acid phosphatase activity had inconsequential influence on the incubation period.

The analysis of variance (ANOVA) (Table 7.1 to 7.6) result showed that the differences in the activity of acid phosphatase under different concentrations. Butachlor as compared to dimethoate have less effect on PSB on regards of acid phosphatase production (enzyme). The increased in the enzyme acid phosphatase activity was due to decreased in intracellular phosphatase, which caused depression of the APase gene (Galabova *et al.,* 1993). The decreased in acid phosphatase activity could be due to self repression mechanism by Pi available from the organic phosphate sources in the culture medium as reported by Nahas (2015).

Both PSB strains were able to produce the phytohormone IAA. It has been reported that IAA production by bacteria can vary among different species and strains, and is also influenced by culture condition, growth stage and substrate availability (Mutluru and Konada, 2007). The effect of three concentrations (150ppm, 100ppm, 50ppm) of the two xenobiotics on IAA synthesized by the both PSB strains strains MZLRPA12 and MZLRPC4 varied considerably (Fig.7.6 & 7.7). In untreated medium or control both PSB strains produced significant amount of IAA but did not vary much with the increase in incubation period. In contrast, the quantity of IAA released by the PSB strains, however, decreased progressively with graded-increment of each xenobiotic in the medium. Of the herbicide butachlor, severe effect on IAA synthesis was evident during 24 hrs incubation where there was no IAA production and in 48 hrs MZLRPC4 showed slightly higher IAA production than MZLRPA12. In 72 hrs, there was drastic change in MZLRPC4 IAA production

with sudden drop of the amount of IAA production. Of insecticide dimethoate, severe effect was again evident in 24 hrs with no IAA production. In 48 hrs, only MZLRPA12 showed production only on 50ppm (the lowest concentration) while MZLRPC4 showed evidence of the production. In 72 hrs, MZLRPA12 again showed production only on 50ppm while MZLRPC4 could give evidence for all concentrations. Hence, from this study, it can be concluded that the PSB MZLRPC4 *Paraburkholderia fungorum* have higher tolerance capacity towards the two xenobiotics for IAA production. This study is in support of Ahemad and Khan (2011) who also studied different pesticides, herbicides and insecticides at different doses towards the plant growth promoting traits of *Mesorhizobium* strain and found significant variations on different doses and different agrochemicals used.

The two xenobiotics also had considerable variation on the phosphate solubilizing efficiency in the broth culture medium (Fig. 8.0 & 8.1). Butachlor treatment showed decline in the P efficiency with higher xenobiotic concentration. Out of the two strains, MZLRPA12 showed higher tolerance towards butachlor treatment. MZLRPC4 was not able to solubilize Phosphorous at 72 hrs of incubation. Dimethoate treatment showed decline in the P efficiency by the two strains with MZLRPA12 higher activity as the incubation period increased. MZLRPC4 showed comparatively lesser P solubilizing efficiency in comparison with MZLRPA12.

From the experiment conducted it can be considered that the two xenobiotics butachlor and dimethoate have negative impact on the two PSB strains by reducing their growth, biomass, enzyme activity, hormones production and their ability to solubilize phosphorous. Depending on the concentration of the xenobiotics, their effect may vary. However, higher concentration will have severe consequence on the microbial community.

Chapter 8

In vitro and *in vivo* Study on Efficacy of Selected Phosphate Solubilizing Bacteria on Rice Plant Growth

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8.1. Introduction

Phosphorus is an essential macronutrient that is required for maximizing the yield of crops (Griffith, 2009) as it is involved in essential metabolic pathways, which includes photosynthesis, biological oxidation, uptake of nutrients, and cell division (Illmer and Schinner, 1992; Gupta *et al*., 2012).P supplies energy needed for metabolic processes and it is considered obligatory for the synthesis of nucleic acid molecules (Lal, 2002).There are two components of P in soil *viz.,* organic and inorganic phosphates. Larger proportion is present in insoluble forms, and therefore, not accessible for plant nutrition. In soil, inorganic P occurs predominantly in insoluble mineral complexes, some of which appear after the application of chemical fertilizers and therefore, plants cannot take up the precipitated forms. Contrarily, organic matter is an essential reservoir of immobilized P that accounts for around 20–80% of soil P (Richardson, 1994). But plants cannot uptake P as organic form directly, so it must first be transformed into inorganic form after being mineralized and catalysed by different soil enzyme processes (Sarapatka, 2003). So, microorganisms are helpful in releasing P from organic complexes of total soil P by mineralization to help plants to take up P (Abd-Alla, 1994; Bishop *et al*., 1994). Subsequently, in order to convert both organic and inorganic insoluble phosphates to a form that is accessible to the plants, microorganisms play vital role in increasing plant yields. Also, some bacterial species can mineralize and solubilize soil organic and inorganic P (Hilda and Fraga, 2000; Khiari and Parent, 2005; Sarker *et al*., 2012).

Due to the severe concern on climate change which eventually leads to global rising population, there is a requirement to uphold food security by increasing crop production worldwide. Consequently, farmers use vast quantity of chemical fertilizers and pesticides to attain maximum crop yield. These agrochemicals are chemically synthesized, industrial substances made of N, P and K of which the

excess usage leads to pollution to soil, air, and water directly or indirectly (Galloway *et al*., 2008; Youssef and Eissa, 2014). The constant use of chemical fertilizers, biocides, and pesticides negatively influence the natural micro flora such as bacteria, fungi, cyanobacteria, and protozoan present in the rhizosphere or the applied field and causes imbalance in the natural ecosystem (McLaughlin and Mineau, 1995; Dash *et al*., 2017a,b; Dash *et al*., 2018) and also could eventually damage the environment. In this situation, sustainable agriculture is the need for the intricate crisis of chemical fertilizers, pesticides, or finally for improvement of climate changes (Kumar *et al*., 2017a). For an eco-friendly sustainable agriculture, use of biofertilizers in substitute to agro-chemicals can ensure food security and safety as well as sustain the microbial diversity in soil. Such type of agriculture is driven by the microflora of the soil, which accounts to the plant growth promoting microorganisms including bacteria, actinomycetes, arbuscular mycorrhiza fungi (AMF), cyanobacteria, which are directly or indirectly associated with plants or soil for production, disease control, salt and drought tolerance, and also for the mitigation of heavy metal stress (Gupta *et al*., 2012; Govers *et al*., 2012; Kaushal and Wani, 2016).

The plant rhizosphere is a thin layer of soil, cohering to the root surface. Microorganisms present in the rhizosphere region constitute of various taxa. Majority of these taxa comprises of bacteria, followed by actinomycetes, fungi and protozoa etc. (Hiltner, 1904; Kumar *et al*., 2015b). In the plant rhizosphere , root exudates are excreted which exert a pull on microbial population and colonize the plant roots, which helps in growth promotion and control of diseases or stress (Oku *et al*., 2012, Kumar *et al*., 2015a,b,c, 2016b, 2017a,b; Singh *et al*., 2017a,b,c). These microorganisms that are involved either directly or indirectly in production of crop yield are hence called plant growth promoting microorganism, and the bacterial species that are related with plant growth is termed as plant growth promoting bacteria (PGPB) (Kloepper *et al*., 2004; Glick *et al*., 2009; Babalola, 2010; Kumar *et al*., 2016b, 2017a) or that are able to colonize plant root systems and promote growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1989). Antoun and Prevost (2006) gave evidence in the literature indicating that "PGPRs can be a true success story in sustainable agriculture".

Bacteria that are able to solubilize the mineral phosphorus and make it available to the plants are called phosphate solubilizing bacteria (PSB) (Vessey, 2003). Assimilation of phosphorous takes place with the help of the enzyme phosphatase. PSB are present in variable amount in the soil (Zaidi *et al.*, 2003). Recently, phosphate-solubilizing bacteria (PSB) have attracted the interest of agriculturists for their use as biofertilizers to improve plant growth and yield. PSB have the ability to solubilize insoluble P and release soluble P by producing various organic acids, mineral acids, siderophores, protons, humic substances, CO_2 and H_2S (Illmerand Schinner, 1995). PSB from the soil transport the insoluble phosphorous to a soluble form of phosphate by the process which includes the consumption of the tricalcium phosphate. The plants take up the nutrient in the orthophosphate forms (HPO_4^{2-} or $H_2PO_4^-$). For that reason PSB acts as a natural bio-fertilizer which is essential for the plants for their growth (Banerjee *et al.*, 2017). PGPR are essential for farmers as best alternative to chemical fertilizers due to the fact that application of PGPR as biofertilizer reduces the cost of crop production. A large number of PGPR like *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter, Pseudomonas*, *Klebsiella* and *Paenibacillus* has been isolated from rhizosphere of diverse crops (Saharan and Nehra, 2011; Vessey, 2003). The use of PGPR, including phosphate solubilizing bacteria (PSB), as biofertilizers has developed into huge interest for developing countries as large areas of cultivated soils are deficient in soil available P (Xie *et al.*, 1998).

It has been assumed that, global crop yield up to 30-40% of arable land is limited by low P availability (Von Uexkull and Mutert, 1995). In soils, P may exist in many different forms, which can be thought of existing in 3 "pools": solution P, active P and fixed P (Busman *et al.*, 2009). Generally, a major portion of soil P remains as insoluble forms with cations (Al^{3+} and Fe^{3+} in acidic soils, and Ca^{2+}in calcarious soils), which are usually unavailable for uptake by crop plants (Abd-Alla, 1994; Yadav and Dadarwal, 1997).

Rice (*Oryza sativa*), the premier food crop not only in India but also the world is considered the prince among cereals (Chhabra, 2002). It is probably the most important cereal in the world and serves as food for about 50% of the world's population (Ladha *et al.*, 1997). India is the second largest

producer of rice first being China and its production in India has increased from 20 million tons during 1950–51 to 96.69 million tonnes during 2007–08. It contributes 48% to the cereals and 42% to the total food production. According to Statistical abstract, Department of Agriculture (crop husbandry) (Govt. of Mizoram, 2009-2010) Mizoram produced 66,132 metric tonnes production of paddy crop.

Introduction of biofertilizer into soil is a good preference (Saber *et al*., 2009) because not only does it give the positive effect on the physical, chemical and biological properties of soil, biofertilizers also save on expensive inputs which are currently consumed from non-renewable energy sources (Shariati *et al*., 2013).

8.2. Selection of bacterial strains

The PSB strains selected were MZLRPB13, MZLRPC17, MZLRPA12, MZLRPB4, MZLRPC4, and MZLRPB11. The bacterial strains were selected based on the two highest S.I (MZLRPC4 and MZLRPB13), two lowest S.I (MZLRPB11, MZLRPC17) and with single genus isolated such as MZLRPB4 and MZLRPA12.

Table 8.1: List of selected PSB isolates for the study showing phosphate solubilizing ability

S.N	Strain name	Similarity strain from NCBI	P.S.I	P.S.E
1	MZLRPB13	*Bacillus amyloliquefaciens*	4.13±0.14	2.340±0.001
2	MZLRPC17	*Edaphobacter sp.*	2.42±0.06	2.373±0.007
3	MZLRPB4	*Bacillus subtilis*	2.21±0.05	2.234±0.001
4	MZLRPC4	*Paraburkholderiafungorum*	4.64±0.03	2.234±0.001
5	MZLRPB11	*Burkholderiadolosa*	2.08±0.05	2.637±0.001
6	MZLRPA12	*Paenibacillus sp.*	3.13±0.01	2.604±0.002

8.3. Experimental design

Out of the 43 isolated strains, six best strains based on their solubilizing index, P-efficiency test and IAA production were selected for the seed bacterization test on rice seeds. The experimental set up was designed in a Completely Randomized Design (CRD). The PSB strains selected were MZLRPB13, MZLRPC17, MZLRPA12, MZLRPB4, MZLRPC4, and MZLRPB11. The rice variety used was a local variety called “Fartesen” in the local Mizo language. A number of 20 rice seeds were used for inoculation with each single bacterial strain i.e. a total of 120 rice seeds was used with rice seed inoculated with sterilized distilled water (SDW) as control. All the seeds were surfaced sterilized prior to the day of inoculation. One day old bacterial broth culture in Nutrient medium was used for the treatment. The cultures were washed twice with SDW and the culture residue was used for serial dilution. Dilution of 10^5 cfu/ml of the bacterial culture was used for inoculation of the seeds. Each single strain inoculation was done for all six PSBs. After 7 days of incubating, the germination rate was counted and then transplanted onto autoclaved soil in plastic pots (33cm diameter and 8cm length) and kept out under indirect sunlight on the balcony of the laboratory building where it could get enough sunlight and air. The seedlings were watered daily using only sterilized water and were monitored within 30 days. Two harvesting were done; one on the 15th day and second on the 31st day after incubation. Inoculation with the PSBs was also done within the growing period. The shoot length, root length, dry biomass and chlorophyll content were the parameters used for this analysis. All of the experiments were performed in triplicate.

8.4. Description of rice

The rice plant is a member of Poaceae (old Gramineae) family. The common cultivated rice plant is an annual which usually grows to about 1-2 meters depending on the variety and soil fertility. It has long, slender leaves 50–100 cm (20–39 in) long and 2–2.5 cm (0.79–0.98 in) broad. Rice is a kharif crop and is sown in the month of June and is solely grown in the wetland paddy field of North Vanlaiphai (study area). Seed germination starts after two days of sowing (DAS) and flowering starts during

the month of September. The rice grains ripen and are harvested by the month of November.

Table 8.2: Details of rice plant

Plant character	**Description**
Seed variety	*Oryza sativa*
Local name	Fartesen
Crop	Kharif
Date of sowing	June
Seed germination	2 - 3 DAS
Flowering	September
Harvest	November

8.5. Methodology

8.5.1. Preparation of Bacterial inoculants

The selected six bacterial strains were cultured in 250ml conical flask containing 100ml of nutrient broth medium at a refrigerated incubator shaker maintaining the temperature at 30±7°C with continuous agitation at 70% rpm for 72 hours. The bacterial cells were harvested via centrifugation at 10000rpm for 5 mins at room temperature and the supernatant were discarded. The pellet was washed twice with sterile distilled water and then centrifuged at 8000rpm for 5 mins. The bacterial pellet were suspended in 10ml SDW vortexed for few seconds and used for seed treatment.

8.5.2. Seed surface sterilization

25 rice seeds locally known as "Fartesen" were used for each bacterial strain each maintained in triplicates. The seeds were surface-sterilized with 4% w/v NaClO (sodium hypochlorite) solution for 1-2 minutes, then washed three times with SDW and dried under a sterile air stream.

8.5.3. *In vitro* seed treatment and inoculation

The pre suspended bacterial inocula were serially diluted upto 10^{-5}cfu/ml and kept in separate test tubes. The sterilized seeds were immersed in each serially diluted bacterial suspension and the preparation was stirred frequently for five minutes and then kept overnight. So, the number of bacterial cells per seed determined via serial dilution was 10^{-5}cfu/seed. Sterilized seeds without bacterial treatment were used as control. Sterilized petri plates were used for germinating the treated seeds.

8.5.4. Seed germination test

The effect of the isolates on seed germination was also determined. 25 seeds inoculated with each six different bacterial strains were incubated in sterile petri plates on two layers of moistened filter paper and kept at room temperature with proper light source. Sterilized seeds without bacterial treatment were used as control. For proper germination, sufficient moisture is required in which 5 ml of SDW was added to each petri plates every other day. Germination started to occur after 72 hrs of incubation. The number of seeds germinated was recorded from the 3rd day after sowing till the 7th day. This experiment was planned in a completely randomized design with three replicates for each isolate.

$$\text{Germination rate (\%)} = \frac{Number\ of\ seeds\ germinated \times 100}{total\ number\ of\ seeds}$$

8.5.5. Pot experiment

Pot experiment was carried out to investigate the effect of the PSB strains on single inoculations. The seedlings after 7 days of germination were transplanted to clean plastic pots using sterilized sandy loam soil and kept under indirect sunlight *in vivo*. Root and shoot length with number of leaves were recorded on 15 days after sowing (DAS) and 31DAS. The rice plants were watered everyday with SDW and

inoculated with each bacterial inoculum after every 5 days. Each experiment was conducted in triplicates.

8.5.6. Plant growth and Biomass measurement

Determination of the growth of the plant by recording the shoot, root length and number of leaves and dry weight biomass was done. Data were recorded after 15 and 31 DAS. The rice plant were carefully uprooted and washed to remove soil. The, root length, shoot length and number of leaves were recorded. Fresh weight of the whole plant was recorded and then was oven dried at 60°C until constant weight was obtained. Then, the dry weight was recorded. Biomass of the plant was expressed in g $plant^{-1}$.

8.5.7. Chlorophyll content (Arnon, 1949)

100mg of fresh leaves were taken, crushed in mortar and pestle and kept at 50ml borosil glass beaker and suspended in 10ml of 80% acetone and covered with aluminium foil. This was mixed well and kept at 4°C overnight in dark. The mixture was then centrifuged at 10,000rpm for 10 mins and the supernatant was withdrawn carefully. The absorbance was read at 645nm and 663nm using UV-Vis spectrophotometer (Dynamica Halo DB-20) taking 80% acetone solution as blank. The reading was taken in a triplicate sample and average was considered for calculation of chlorophyll content. The chlorophyll a,b and a+b (total chlorophyll) contents were calculated by using the formulae (Arnon, 1949).

mg chlorophyll a/g tissue = $\frac{12.7(A663)-2.69(A645)\times V}{1000\times W}$

mg chlorophyll b/g tissue = $\frac{22.9(A645)-4.68(A663)\times V}{1000\times W}$

mg total chlorophyll /g tissue = $\frac{20.2(A645)+8.02(A663)\times V}{1000\times W}$

Where, A = absorbance at specific wavelength

V = final volume of chlorophyll extract in 80% acetone

W = fresh weight of tissue extracted

8.5.8. Statistical analysis

All the experiments were performed in triplicate and mean values with ± SE were calculated. One way ANOVA was done for each parameter and correlation coefficient (r) values among plant growth performance, germination rate, biomass and chlorophyll content was calculated by using SPSS 16.0. Statistical significance at $p \leq 0.05$ was considered.

8.6. Results

Study on the effect of seed bacterization of rice seeds with PSBs and monitoring on the plant growth yield and chlorophyll content was conducted, observed and recorded within one month which is harvested at 15 DAS and 31 DAS (Table 8.3 and 8.4). Treated rice plants along with untreated sample were harvested and processed for further observations (Fig. 8.2 to 8.5).

Seeds inoculated with each single PSB strain showed increase in root length, shoot length and dry weight biomass in all the replicates in both the harvests *i.e.*, 15 DAS and 31 DAS as compared to control plant. During the first harvest (15 DAS) it was observed that rice plant inoculated with PSB strain MZLRPC4 showed maximum shoot length (22.87cm), maximum root length (6.67cm)and maximum dry biomass (0.16g) and maximum chlorophyll a (1.387mg g^{-1}) and second highest chlorophyll b (1.954mg g^{-1}) and total chlorophyll content (3.419mg g^{-1}) while strain MZLRPC17 showed highest chlorophyll b (2.164mg g^{-1})and highest total chlorophyll content (3.489mg g^{-1}). Strain MZLRPB4 showed minimum shoot (20.37cm) and root length (4.97cm) and three strains (MZLRPA12, MZLRPB4, and MZLRPB11) showed minimum dry biomass (0.09g). MZLRPB11 showed minimum chlorophyll a content (0.142mg g^{-1}) and minimum total chlorophyll content (0.309mg g^{-1}) and MZLRPB13 showed minimum chlorophyll b content (0.143mg g^{-1}). Control plant showed 19.97cm of shoot length, 4.57cm of root length and 0.07g of dried biomass, 0.284mg g^{-1} of chlorophyll a, 0.140mg $g^{-1}$of chlorophyll b and 0.432mg g^{-1} of total chlorophyll content.

During the second harvest (31 DAS) MZLRPC4 showed the maximum shoot length (47.33cm) root length (21.13cm) and highest dry weight biomass (0.48g) and maximum chlorophyll b content (3.405mg g^{-1}) and total chlorophyll content

(6.795mg g^{-1}) while MZLRPC17 showed maximum chlorophyll a content (3.319mg g^{-1}). MZLRPB11 showed minimum shoot length (38.90g) and MZLRP B13 showed minimum root length (13.43g) and two strains (MZLRPB4 and MZLRPB13) showed minimum dry biomass as in the same range with control (0.22g). Control plant showed 36.20cm shoot length, 9.50cm root length, 0.22g dried biomass, 3.215mg g^{-1} chl a, 2.846mg g^{-1} chl b, 6.190mg g^{-1} total chlorophyll content.

One way ANOVA showed a significant variation ($p \leq 0.05$) of root, shoot length, dried biomass, chlorophyll a, chlorophyll b and total chlorophyll content on 15 DAS and 31 DAS (Table 8.5-9.8). However, there are few cases where the p level is > 0.05 which indicates there is no significant variation in such cases. In 15 DAS, Shoot length of CTRLxMZLRPA12 (p-value 0.178), CTRLxMZLRPB13 Chlorophyll a (p-value 0.108), chlorophyll b (p-value 0.870) and total chlorophyll (p-value 0.260), CTRLxMZLRPB11 Dried Biomass (p-value 0.092), Shoot length (p-value 0.079), Chlorophyll b (p-value 0.274), CTRLxMZLRPC17 Shoot length (p-value 0.115), CTRLxMZLRPB4 Root length (p-value 0.311) Shoot length (p-value 0.228), Dried Biomass (p-value 0.063). In 31 DAS, All crossed with CTRL Chlorophyll a (p-value 0.108), CTRLxMZLRPC4 Chlorophyll a (p-value 0.445) Chlorophyll b (p-value 0.070) Total chlorophyll (p-value 0.043), CTRLxMZLRPB13 Dried Biomass (p-value 0.087), CTRLxMZLRPB11 Dried Biomass (p-value 0.057), CTRLxMZLRPB4 Dried Biomass (p-value 0.097), Chlorophyll a (p-value 0.106).

Figure 8.2: Rice plant before harvesting

Figure 8.3: *In vivo* rice plant culture on soil

Figure 8.4: Harvested rice plants

Figure 8.5: Harvested rice plants focussing root region

Table 8.3: Growth performance and chlorophyll content of rice plant inoculated with selected PSB isolates and control at 15 DAS

Potential PSB isolates	Shoot length (cm)	Root length (cm)	Dried biomass (g)	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Total chlorophyll (mg g^{-1})
CTRL	19.97± 0.09	4.57±0.07	0.07±0.00	0.284	0.140	0.432
MZLRPB11	22.20±0.95	6.10±0.21	0.09±0.01	0.142	0.160	0.309
MZLRPB13	22.17±0.50	5.83±0.45	0.11±0.00	0.304	0.143	0.455
MZLRPC4	22.87±0.13	6.67±0.19	0.16±0.02	1.387	1.954	3.419
MZLRPA12	21.67±0.69	6.00±0.17	0.09±0.01	0.388	0.278	0.679
MZLRPC17	21.10±0.56	5.83±0.13	0.11±0.01	1.242	2.164	3.489
MZLRPB4	20.37±0.27	4.97±0.34	0.09±0.01	1.013	0.824	1.875

Table 8.4: Growth performance and chlorophyll content of rice plant inoculated with selected PSB isolates and control at 31 DAS

Potential PSB isolates	Shoot length (cm)	Root length (cm)	Dried biomass (g)	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Total chlorophyll (mg g^{-1})
CTRL	36.20±0.61	9.50±0.87	0.22±0.00	3.215	2.846	6.190
MZLRPB11	38.90±0.21	16.83±0.93	0.31±0.04	3.253	3.358	6.732
MZLRPB13	40.10±0.06	13.43±1.11	0.22±0.00	3.248	3.344	6.739
MZLRPC4	47.33±1.45	21.13±0.64	0.48±0.05	3.112	3.405	6.795
MZLRPA12	44.33±1.45	17.20±0.61	0.28±0.02	3.312	2.692	6.125
MZLRPC17	43.10±0.61	15.57±0.47	0.37±0.01	3.319	2.711	6.149
MZLRPB4	41.87±0.63	16.33±0.88	0.22±0.00	3.253	3.189	6.735

Table 8.5: ANOVA of plant growth performance among rice seeds inoculated with 6 isolated strains at 15 DAS. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB13xMZLRPB11xMZLRPC17x MZLRPB4xMZLRPA12xMZLRPC4	7.964	0.001*
2	Shoot length	-do-	4.393	0.011*
3	Dried Biomass	-do-	6.356	0.002*
4	Chlorophyll a	-do-	440.615	0.000*
5	Chlorophyll b	-do-	224.718	0.000*
6	Total chlorophyll	-do-	1.021	0.000*

Table 8.6: ANOVA of plant growth performance among rice seeds inoculated with MZLRPC4 strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPC4	113.400	0.000*
2	Shoot length	-do-	71.343	0.001*
3	Dried Biomass	-do-	18.544	0.013*
4	Chlorophyll a	-do-	516.704	0.000*
5	Chlorophyll b	-do-	135.500	0.000*
6	Total chlorophyll	-do-	705.052	0.000*

Table 8.7: ANOVA of plant growth performance among rice seeds inoculated with MZLRPA12 strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPA12	59.645	0.002*
2	Shoot length	-do-	2.661	0.178
3	Dried Biomass	-do-	4.157	0.111*
4	Chlorophyll a	-do-	726.448	0.000*
5	Chlorophyll b	-do-	81.617	0.001*
6	Total chlorophyll	-do-	206.408	0.000*

Table 8.8: ANOVA of plant growth performance among rice seeds inoculated with MZLRPB13 strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB13	7.805	0.049*
2	Shoot length	-do-	18.938	0.012*
3	Dried Biomass	-do-	76.421	0.001*
4	Chlorophyll a	-do-	4.245	0.108
5	Chlorophyll b	-do-	0.030	0.870
6	Total chlorophyll	-do-	1.722	0.260

Table 8.9: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPB11 strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB11	49.209	0.002*
2	Shoot length	-do-	5.474	0.079
3	Dried Biomass	-do-	4.864	0.092
4	Chlorophyll a	-do-	206.692	0.000*
5	Chlorophyll b	-do-	1.604	0.274
6	Total chlorophyll	-do-	50.041	0.002*

Table 9.0: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPC17 strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPC17	72.200	0.001*
2	Shoot length	-do-	4.042	0.115
3	Dried Biomass	-do-	12.318	0.025*
4	Chlorophyll a	-do-	8.781	0.000*
5	Chlorophyll b	-do-	1.097	0.000*
6	Total chlorophyll	-do-	2.077	0.000*

Table 9.1: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPB4strain at 15 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 15 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB4	1.346	0.311
2	Shoot length	-do-	2.028	0.228
3	Dried Biomass	-do-	6.547	0.063
4	Chlorophyll a	-do-	310.057	0.000*
5	Chlorophyll b	-do-	1.446	0.000*
6	Total chlorophyll	-do-	1.661	0.000*

Table 9.2: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with 6 PSB strains at 31 DAS. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB13xMZLRPB11xMZLRPC17x MZLRPB4xMZLRPA12xMZLRPC4	19.490	0.000*
2	Shoot length	-do-	21.412	0.000*
3	Dried Biomass	-do-	15.201	0.000*
4	Chlorophyll a	-do-	2.176	0.108
5	Chlorophyll b	-do-	13.609	0.000*
6	Total chlorophyll	-do-	11.399	0.000*

Table 9.3: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPC4 strain at 31 DAS. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPC4	116.892	0.000*
2	Shoot length	-do-	49.891	0.002*
3	Dried Biomass	-do-	31.440	0.005*
4	Chlorophyll a	-do-	0.715	0.445
5	Chlorophyll b	-do-	1.060	0.070
6	Total chlorophyll	-do-	8.510	0.043

Table 9.4: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPA12 strain at 31 DAS. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPA12	52.780	0.002*
2	Shoot length	-do-	43.343	0.003*
3	Dried Biomass	-do-	16.471	0.015*
4	Chlorophyll a	-do-	497.633	0.000*
5	Chlorophyll b	-do-	1.737	0.000*
6	Total chlorophyll	-do-	137.200	0.000*

Table 9.5: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPB13 strain at 31 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB13	7.849	0.049*
2	Shoot length	-do-	40.381	0.003*
3	Dried Biomass	-do-	5.088	0.087
4	Chlorophyll a	-do-	46.173	0.002*
5	Chlorophyll b	-do-	1.112	0.000*
6	Total chlorophyll	-do-	1.041	0.000*

Table 9.6: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPB11 strain at 31 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB11	33.379	0.004*
2	Shoot length	-do-	17.496	0.014*
3	Dried Biomass	-do-	7.049	0.057
4	Chlorophyll a	-do-	191.118	0.000*
5	Chlorophyll b	-do-	867.832	0.000*
6	Total chlorophyll	-do-	849.670	0.000*

Table 9.7: One way analysis of variance (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPC17 strain at 31 DAS. Marked effects are significant at p≤0.05.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPC17	37.899	0.004*
2	Shoot length	-do-	64.049	0.001*
3	Dried Biomass	-do-	80.480	0.001*
4	Chlorophyll a	-do-	139.367	0.000*
5	Chlorophyll b	-do-	97.826	0.001*
6	Total chlorophyll	-do-	10.995	0.029*

Table 9.8: One way analysis (ANOVA) of plant growth performance among rice seeds inoculated with MZLRPB4 strain at 31 DAS. Marked effects are significant at $p \leq 0.05$.

S.N	Parameters 31 DAS	Source of variation	F-value	p-value
1	Root length	CTRLxMZLRPB4	30.564	0.005*
2	Shoot length	-do-	41.463	0.003*
3	Dried Biomass	-do-	4.646	0.097
4	Chlorophyll a	-do-	4.343	0.106
5	Chlorophyll b	-do-	1.241	0.000*
6	Total chlorophyll	-do-	15.459	0.017*

Table 9.9: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPB4 treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters MZLRPB4	Chl a	Chl b	TC	SL	RL	BM
GR	-0.486 0.338	-0.500 0.333	0.484 0.339	-0.974 0.073	0.189 0.439	-0.918 0.130
Chl a		-0.514 0.328	-1.000** 0.001	0.274 0.412	0.766 0.222	0.793 0.208
Chl b			0.516 0.327	0.684 0.260	-0.945 0.106	0.115 0.463
TC				-0.271 0.412	-0.768 0.221	-0.792 0.209
SL					-0.408 0.366	0.803 0.203
RL						0.217 0.430

Table 10.1: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPB11 treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$.

Parameters MZLRPB11	Chl b	TC	SL	RL	BM
Chl a	-0.382 0.375	-0.997 0.023	-0.110 0.465	0.959 0.092	-0.810 0.200
Chl b		0.314 0.398	0.961 0.89	-0.629 0.284	0.851 0.176
TC			0.039 0.488	-0.936 0.115	0.766 0.222
SL				-0.388 0.373	0.672 0.265
RL					-0.943 0.108

Table 10.2: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPB13 treated pot. Marked correlation coefficient (r) values are significant at p ≤ 0.05.

Parameters MZLRPB13	Chl b	TC	SL	RL	BM
Chl a	-0.554 0.313	-0.178 0.443	-0.064 0.480	-0.194 0.438	-0.051 0.484
Chl b		0.918 0.130	-0.866 0.167	-0.709 0.249	-0.803 0.203
TC			-0.993 0.037	-0.931 0.119	-0.974 0.073
SL				0.967 0.083	0.993 0.037
RL					0.990 0.046

Table 10.3: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPC4 treated pot. Marked correlation coefficient (r) values are significant at p ≤ 0.05

Parameters MZLRPC4	Chl a	Chl b	TC	SL	RL	BM
GR	-0.327 0.394	-1.000** 0.000	-1.000** 0.000	0.115 0.463	0.809 0.200	-0.253 0.419
Chl a		0.327 0.394	0.329 0.393	-0.976 0.070	0.290 0.406	-0.997* 0.025
Chl b			-1.000** 0.000	-0.115 0.463	-0.809 0.200	-0.253 0.419
TC				-0.116 0.463	-0.808 0.200	-0.254 0.418
SL					-0.491 0.337	0.990* 0.045
RL						-0.364 0.381

Table 10.4: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPA12 treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$

Parameters MZLRA12	Chl a	Chl b	TC	SL	RL	BM
GR	1.000** 0.000	-0.500 0.333	0.817 0.196	-0.941 0.110	0.982 0.061	-0.667 0.268
Chl a		0.500 0.333	0.817 0.196	0.941 0.110	0.982 0.061	-0.667 0.268
Chl b			0.091 0.471	0.763 0.224	-0.655 0.273	-0.312 0.399
TC				-0.575 0.305	0.693 0.256	-0.975 0.072
SL					-0.988 0.049	0.376 0.377
RL						-0.514 0.328

Table 10.5: Correlation coefficient (r) values among plant growth performance and chlorophyll content at MZLRPC17 treated pot. Marked correlation coefficient (r) values are significant at $p \leq 0.05$

Parameters MZLRC17	Chl b	TC	SL	RL	BM
Chl a	-0.638 0.280	0.348 0.387	0.820 0.194	-0.999 0.014	0.257 0.417
Chl b		0.500 0.333	-0.082 0.474	0.603 0.294	-0.908 0.137
TC			0.822 0.193	-0.390 0.373	-0.816 0.196
SL				-0.845 0.180	-0.342 0.389
RL					-0.214 0.431

**. Correlation is significant at the 0.01 level (1-tailed).

*. Correlation is significant at the 0.05 level (1-tailed).

Chl a: Chlorophyll a; Chl b: Chlorophyll b; TC: Total chlorophyll; SL: Shoot Length; RL: Root Length; BM: Biomass; GR: Germination Rate

8.7. Discussion

Application of PGPR as biofertilizer has resulted in improved growth and grain yield of various crops such as wheat, rice, maize and sugarcane (Bhattacharyya and Jha, 2011; Moutia *et al*., 2010; Saharan and Nehra, 2011). Promotion of plant growth by bacteria has been well documented by Reed and Glick (2004), Babalola *et al*. (2007) and Babalola (2010). Phosphate Solublizing microbes are considered as important members of PGPR and their application in the form of biofertilizer has been shown to improve growth of cereals and other crops (Bhattacharyya and Jha, 2011; Gyaneshwar *et al*., 2002; Hu *et al*., 2006; Shahab *et al*., 2009; Vessey, 2003). An increase in P availability to plants through the inoculation of PSBs has also been reported previously in pot experiments and under field conditions (Banik and Dey, 1981; Chabot *et al*., 1996; deFreitas *et al*., 1997; Zaidi *et al*., 2003). Plant growth promotion has been reported by the solubilization of insoluble P in the soluble form by the activities of PGPR (Rodríguez and Fraga, 1999; Richardson, 2001).

Several workers reported that PGPR and PSB strains were used as efficient bio-inoculants for enhancing growth attribute, yield and nutrient content of rice crops (Khalid *et al*., 2009; Singh *et al*., 2011; Manivannan, 2011).

Seed inoculation with PSB has been known to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Abd-Alla, 1994; Jones and Darrah, 1994; Yadav and Dadarwal, 1997). Several lines of support imply that application of PSB improves plant P nutrition and increases the yield of cereals including wheat (Afzal and Asghari, 2008; Ashrafuzzaman *et al*., 2009; Islam and Hossain, 2012).

There is slight increase in the chlorophyll content as compared to the control sample (without PSB inoculation). Steffan *et al*. (2013) have also found that chlorophyll content with PGPR inoculation with PSB strains increased significantly the chlorophyll content at 42 and 59 DAI. Similar results were also reported by Han and Lee (2005) that inoculation increased the chlorophyll content in lettuce. One way analysis of variance (ANOVA) showed significant difference between all isolates together with control in different plant growth performance. Also, the single PSB

strains inoculated with rice seeds which showed significant variation were MZLRPC4, MZLRPA12, MZLRPC17 and MZLRPB4 in 15 DAS and in 31 DAS all isolates showed significant variation except MZLRPC4. There is significant correlation between total chlorophyll and chlorophyll ‘a’ at MZLRPB4. In MZLRPC4, correlation coefficient was significant at chl ‘b’ and germination rate, total chl and germination rate, total chl and chl ‘b’, biomass and chl ‘a’, biomass and shoot length. In MZLRPA12, correlation coefficient was significant at chl ‘a’ and germination rate.

Treatments with PGPR enhance germination percentage, seedling vigour, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields etc (Ramamoorthy *et al*., 2001). The enhancement of plant growth by PGPR indicates their potential as biofertilizers in the field of agriculture. It was found that inoculation of rice seedlings with *Bacillus* sp. significantly increased the number and length of root & shoots and dry weight (Biswas *et al*., 2000)

PGPB as biofertilizer has been proven as a safe and efficient methods of increasing crop yields (Premachandra *et al*., 2016; Vejan *et al*., 2016). Recently from last few decades numerous bacterial genera such as *Azotobacter, Bacillus, Klebsiella, Enterobacter, Arthrobacter, Burkholderia, Bacillus, Pseudomonas, Azotobacter Serratia*, etc. had been used as biofertilizers as reported by various authors and called these isolates as PGPB (Kloepper *et al*., 2004; Saharan and Nehra, 2011; Kumar *et al*., 2014, 2015a, 2016a,b, 2017a,b; Singh *et al*., 2017a).

It is stated that organic fertilizer applications increased the chlorophyll content (Belal, 2006). This is related to increased generation of nitrogen, magnesium and iron intake and thus total chlorophyll amount increases (Harhash and Abdel-Nasser, 2000).

The strain MZLRPC4 which has closest similarity to *Paraburkholderia fungorum* have the best performance for root and shoot development and biomass production as compared to the other tested strains in both the harvesting period. So, this strain can be concluded as the best potential PGPR for better growth performance of the local rice seeds. Similar bacteria was studied by Rahman *et al*. (2018) on *Paraburkholderia fungorum* strain named asBRRh-4 along with *Bacillus*

amyloliquefaciens strain named as BChi1 as a probiotic bacteria that have significant improvement of fruit yield and antioxidant contents in strawberry fruits by the application in a field condition and has thus, included in the PGPR group.

From the experiment conducted, we have the evidence that the isolated PSB strains could be used as a phosphate solubilizer in replacement of inorganic chemical fertilizers in agricultural field. This could also serve as a prior experiment to field trial.

Chapter 9
Summary and Conclusion

India's population as well as the world's is rapidly expanding and with that expansion it puts considering pressure on the agricultural lands which leads to the need for more resources. Agriculture plays a significant role in India's economy to provide means of livelihood to rural masses. Therefore, to increase the agricultural development is a challenging issue and a crucial step has to be initiated not only in India but in a worldwide context. Generally, in conventional agriculture there are two major inputs necessary for crop production, which are fertilizer and pesticide. In other words, it can be said that fertilizer is food and pesticide is medicine for plants. But, most of the traditional and conventional farm practices are not ecologically sustainable. The immense use of chemicals, pesticides, and fertilizers can have adverse affect to the local ecology as well as the population. Indiscriminate use of pesticides, improper storage etc. may lead to health problems. Another consequence can also lead to the downward yield of crops due to high utilization of chemical fertilizers leading to poor soil health due to lack of organic matter, loss of inherent fertility; and by affecting the soil micro flora and fauna. Sustainable agriculture reduces the use of hazardous chemical and control pests. Furthermore, plants cannot uptake all the nutrients applied through chemical fertilizers; so, some amount of nutrients are either fixed in the soil or leached out and ultimately mixed with water bodies. Taking all this into account, and to make agriculture more sustainable it is essential to implement a balanced and reasonable use of nutrients which are cost effective and eco-friendly of which biofertilizer could be a suitable option. According to various literature reviews, study of factors leading to sustainable agriculture like diversity of biofertilizers have been made in few states of India out of which the state Mizoram is highly neglected. So, looking into this situation, assessment or development of beneficial microbial flora and fauna especially biofertilizers is of vital importance.

Taking the inevitability for development of sustainable agriculture and also taking steps to acquire the main aim of the ever green revolution *i.e.*, is to produce more using less land, less water and less inorganic chemical fertilizer, development of location specific biofertilizers in the form of phosphate solubilizing bacteria have been chosen and further studies.

In this work, phosphate solubilizing bacteria from the paddy field of North Vanlaiphai, Serchhip district, Mizoram, India has been studied. The soil samples of the study site were collected and analysed for the physico-chemical properties. Then, the rhizospheric soil samples of the rice crop were collected. Phosphate solubilizing bacteria were isolated and biochemically and molecularly characterized. Then, the mechanism of the phosphate solubilizing bacteria which are the ability to produce organic acids were further studied and quantified. Selecting six different PSB strains based on different genus and species and the ability to solubilize phosphorous, experiment on their efficacy on the rice plant growth *in vitro* and *in vivo* were conducted to monitor whether the strains would be able to be used as an effective biofertilizer. Lastly, the study of the influence of two selected xenobiotics *viz*., an insecticide (Dimethoate) and a herbicide (Butachlor) on two selected PSB strains were conducted by taking different parameters like bacterial growth, dry weight biomass and to check their tolerance capacity on whether the phosphate solubilizing efficiency and IAA production of the PSBs were affected by these xenobiotic treatments.

The first step of the experiment was the collection of soil samples from the paddy field of North Vanlaiphai and analysis were done on two seasonal variations which were termed as pre harvest season during the month of July-August and post harvest season during the month of February-March for two consecutive years and the average was taken into account. For analysis of soil samples, soil pH, soil moisture content, bulk density, soil organic carbon, soil organic matter, available nitrogen, available phosphorous and available potassium were done. Soil enzyme test such as dehydrogenase, urease and phosphatase were also performed. The texture of the soil was found to be sandy loam soil. Soil pH ranged from 4.13 to 5.29 which are acidic. In fact, from numerous studies by other researchers Mizoram soil is mostly acidic in nature. Soil temperature was somewhat the same during pre and post

harvest season with 25.6°C and 26.5°C respectively. Soil moisture content (SMC) was 67.97% in pre harvest comparatively higher than post harvest 35.8%. Bulk density (BD) was 1.66gm cm^{-3} during pre harvest and 1.31gm cm^{-3} during post harvest.

Soil enzyme properties during pre harvest were all very high compared to the post harvest which is the dry season. During the pre harvest season, soil dehydrogenase activity (DHA) was 0.864µg TPFmg^{1} $24hrs^{-1}$, acid phosphatase activity (APase) was 93.458µg p-NP $mg^{-1}hr^{-1}$ and urease activity (URES) was 0.931 NH_4^{+}-N mg^{-1} $3hrs^{-1}$. During post harvest season, DHA was 0.182µg TPF mg^{1} $24hrs^{-1}$, APase was 59.548µg p-NP $mg^{-1}hr^{-1}$ and URES was 0.708 $NH4^{+}$-N mg^{-1} $3hrs^{-1}$.

Physico-chemical properties of the soil were also analysed on both seasons. During pre harvest season, available nitrogen (AN) was 287 kg/ha and 274 kg/ha during post harvest. Available potassium (AK) during pre harvest was 125.65kg/ha and 117.33 kg/ha during post harvest season. Available phosphorous (AP) was 11.78 kg/ha and 12.05 kg/ha during pre and post harvest season respectively. In all cases, during pre harvest season, the chemical properties were found to be relatively higher.

From the rhizospheric soil sample of the rice crop, phosphate solubilizing bacteria were isolated. First, serial dilutions of the soil were done with sterile distilled water. Then, using specialized medium called Pikovskaya (PVK) agar, PSBs were detected and isolated. PSBs were isolated based on the production of clear halo zone around the colonies on the agar plates. Then, these isolates were further purified by repeated sub-culturing. Then, the phosphate solubilizing efficiency in broth PVK medium was tested for final confirmation of the PSBs using the ascorbic acid method. Most of the colonies appeared creamy or white opaque colour, some appeared translucent off-whitish, pale yellow and even pink colour. Majority were smooth surface and wet, some were rough, and some were sticky and dry. There was also a root like structure colony. Biochemical characterization of all isolates was performed qualitatively for morphological identification which was followed by DNA isolation using the P:C:I method. Then, molecular characterization using 16srRNA gene profiling was performed on all of the isolated PSBs. The gene amplification was performed by using the primer 27f and 1525r. The PCR products

of 16S rRNA gene were sent for sequencing commercially at Eurofins Genomics India Pvt. Ltd. Bangalore.

After sequencing of the PCR purified product, the sequence data were compared for similarity level with the reference strains of PSB from genomic database banks using the NCBI Blast available at the http://www.ncbi.nlm.nih.gov/blast website. The gene sequences were submitted to GenBank database bearing accession numbers MK932023 to MK932068. So, based on the biochemical and partial 16S rRNA gene sequence analysis a total of 43 PSB isolates were identified using the NCBI Blast with percentage of similarity identity level ranging from 98.23 to 100%. These isolated strains were designated as MZLRPA, MZLRPB, MZLRPC and MZLRPD followed by numerals. Total of 7 genera were identified *viz*., *Bacillus* (58.1%), *Burkholderia* (13.9%), *Paenibacillus* (2.3%), *Paraburkholderia* (18.6%), *Dyella* (2.3%), *Edaphobacter* (2.3%) and *Paraclostridium* (2.3%). Maximum species identified was *Bacillus cereus* with total 9 isolates followed by *Paraburkholderia fungorum* with total of 5 isolates. From the critical scrutiny of literature, it has been observed that the genera *Dyella, Edaphobacter* and *Paraclostridium* are the first ever report of phosphate solubilizing bacteria.

The phosphate solubilizing capacity were scrutinized both on PVK agar medium qualitatively and on PVK broth medium quantitatively. Qualitative screening gave the evidence that the highest PSB strain was MZLRPC4 *Paraburkholderia fungorum* with S.I 4.64cm and MZLRPC25 *Bacillus cereus* with 4.60cm. The lowest S.I was MZLRPB11 *Burkholderia dolosa* with 2.08cm and MZLRPB5 *Bacillus cereus* with 2.11cm. Quantitative estimation confirmed the evidence that the strain MZLRPC4 *Paraburkholderia fungorum* is the highest P solubilizing efficiency with 3.204 p-NPμg^{-1}ml^{-1}hr and lowest P-solubilizing efficiency strain is MZLRPC25 *Bacillus cereus* with 0.918 p-NPμg^{-1}ml^{-1}hr.

All of the isolated 43 PSB nucleotide sequences which were obtained after NCBI BLASTn search analysis were then compared to the type strains retrieved from EzBiocloud-database. However, based on the 16srRNA gene sequence the variety of species is less diverse and majority of the species belongs to the genus *Bacillus* and *Burkholderia*. Each of one isolate of *Dyella*, *Paraclostridium* and

Edaphobacter were identified as well. The phylogenetic tree analysis of the evolutionary history was inferred using Kimura-2 parameter model with neighbouring method under 1000 bootstrap replicates. It was evident from the phylogenetic analysis that of all the gram positive bacteria was clustered together with a bootstrap support value of 93%. First, *Bacillus* group, 25 strains were clustered with a bootstrap support value of 98%, then, the strain *Paenibacillus* sp. was clustered along with the type strain from EzBiocloud database with bootstrap support value of 98%. The strain *Paraclostridium bifermentans* strain clustered along with the type strain with a bootstrap support value of 99%. In another clade, all the gram negative bacterial strains were clustered together with a bootstrap support value of 97%. *Dyella* sp. was clustered with the type strain from EzBiocloud database with a bootstrap value of 98%. All 6 strains of *Burkholderia* sp. were clustered together with bootstrap support value of 48% and *Burkholderia contaminans* as type strain from EzBiocloud database with 67% bootstrap value. The strain *Paraburkholderia multivorans* is also clustered separately from the rest of the *Paraburkholderia* strain with bootstrap value of 48%. The genera *Paraburkholderia* 7 strains are all clustered in together along with the type strain with bootstrap value of 94%. The last gram negative strain *Edaphobacter* is clustered separately from the rest of the other strains with bootstrap support value of 99%. The type strains were all superscripted with suffix "T" after the species name.

Since the main mechanism of phosphate solubilization is the production of organic acids, eight organic acids *viz*., acetic, citric, formic, gluconic, malic, oxalic, succinic and tartaric acids were selected and for each acid in different concentrations 30ppm, 50ppm, 70ppm and100ppm. Eight PSB strains such as MZLPA12 *Paenibacillus sp.*, MZLRPB1 *Bacillus cereus*, MZLRPB4 *Bacillus subtilis*, MZLRPB10 *Burkholderia lata*, MZLRPB11 *Burkholderia dolosa*, MZLRPB13 *Bacillus amyloliquefaciens*, MZLRPC4 *Paraburkholderia fungorum* and MZLRPC17 *Edaphobacter sp*. The strains were selected based on the highest P-solubilizer MZLRPC4 *Paraburkholderia fungorum,* MZLRPC17 *Edaphobacter sp*. being the first reported strain as phosphate solubilizer, MZLRPB11 *Burkholderia dolosa* as the lowest solubilization index, MZLPA12 *Paenibacillus sp.* being the only genera isolated and highest acid phosphatase activity, MZLRPB1 *Bacillus*

cereus being the maximum number of isolated species identified, MZLRPB4 *Bacillus subtilis* being the most common *Bacillus* species and second highest acid phosphatase sctivity, MZLRPB10 *Burkholderia lata* and MZLRPB13 *Bacillus amyloliquefaciens* randomly chosen. The analysis was carried out using UHPLC. All eight organic acids were able to be identified and quantified. All of the isolates reduced the level of pH in the NBRIP broth culture from 7pH to approximately 4pH which is a clear indication of organic acid production. Among all the OA tested, all of the PSB strains tested were able to produce all the organic acids excluding MZLRPB11 which did not produce acetic acid. This shows the evidence of the main mechanism of phosphate solubilizing ability by the bacterial isolates which proofs that the PSB isolates are true phosphate solubilizers.

Experiment was carried out to evaluate the plant growth promoting activity of the PSB strains on local rice seeds *in vitro* by seed bacterization or in another term by inoculating the rice seeds in the isolated bacterial broth culture and checking the seed germination. Then, the seedlings were transplanted into sterilized soil pots and observed daily within 31 days and harvested on 15th and 31st day after sowing. The PSB strains selected were MZLRPB13, MZLRPC17, MZLRPA12, MZLRPB4, MZLRPC4, and MZLRPB11. All the strains were able to enhance the growth of rice as compared to control (without bacterial inoculation). The strain MZLRPC4 *Paraburkholderia fungorum* showed maximum shoot length, maximum root length and maximum dry biomass and maximum chlorophyll content during 15 DAS and 31 DAS. Thus, among all the tested strains MZLRPC4 was the best plant growth promoter.

From the experiments conducted, based on the phosphate solubilization and the ability to promote the activity of the plant growth, the strain MZLRPC4 *Paraburkholderia fungorum* is found to be the best PSB strain and all the strains tested for plant growth promotion could be further developed as a location specific fertilizers for efficient phosphate solubilizers.

Xenobiotic test which was conducted to test two PSB strains for their tolerance capacity against an insecticide and a herbicide revealed that the two strains tested MZLRPA12 and MZLRPC4 were both weekly tolerant to the activity of butachlor and dimethoate on the three concentrations 150, 100 and 50ppm.

Dimethoate which is an insecticide was more toxic in the phosphate solubilization, growth, IAA production and enzyme activity towards the strains tested. So, it is clear from the evidence that xenobiotics like the agrochemicals used in agricultural field can degrade the activity of soil beneficial bacteria.

This research concludes that the isolation and identification of the 43 phosphate solubilizing bacteria from North Vanlaiphai paddy field is the first ever report from the study site. The strains *Dyella, Edaphobacter* and *Paraclostridium* are the first report as phosphate solubilizers. Biochemical characterization was done for preliminary identification morphologically followed by molecular identification. The ability to produce organic acids gave the evidence that the isolated PSBs are inorganic phosphate solubilizers. Soil analysis of the study site gave significant variations among all the parameters between the two seasons studied *i.e*., pre harvest and post harvest season. The plant growth promoting activity tested *in vitro* and *in vivo* showed that the PSB isolates were able to promote the growth of the rice plant by root and shoot development, high dry weight biomass content and in chlorophyll content. Xenobiotic stress revealed the tolerance capacity of two PSB strains against a common herbicide butachlor and an insecticide dimethoate which are commonly used in Mizoram agricultural field. The placid tolerance capacity showed that use of agrochemicals can thus lead to degradation of phosphate solubilizers if used in higher concentrations. Thus, the novel PSB strains isolated from this vicinity can be an imperative for further development of location specific biofertilizer in Mizoram, India.

Appendix – I

Media composition for PSB

1. Pikovskaya's medium (Pikovskaya, 1948)

Component		Amount
Glucose	–	10.00 g
$MgSO_4.7H_2O$	–	10.00 ml
$CaCl_2$	–	10.00 g
Tricalcium phosphate ($Ca_3(PO_4)_2$)	–	5.00 g
Distilled water	–	1000 ml
Agar	–	18.00 g
pH	–	7.00

2. Nutrient medium (Difco Manual, 1953)

Component		Amount
Peptone	–	5.00 g
Beef extract	–	3.00 g
Sodium chloride	–	8.00 g
Distilled water	–	1000 ml
pH	–	7.30

3. NBRIP medium (Nautiyal, 1999)

Component		Amount
Glucose	–	10.00 g
$Ca_3(PO_4)_2$	–	5.00 g
$MgCl_2.6H_2O$	–	5.00 g
$MgSO_4.7H_2O$	–	0.25 g
KCl	–	0.20 g
$(NH_4)_2SO_4$	–	0.10 g
Distilled water	–	1000 ml
pH	–	7.00

Appendix-II

Reagents prepared for soil physico-chemical and biochemical properties

1% 2,3,5 triphenyl tetrazolium chloride (TTC)

TTC	–	1.0 g
Distilled water	–	100 ml

0.115 M p-nitrophenyl phosphate (p-NP)

p- NP	–	4.26 g
Distilled water	–	100 ml

Modified universal Buffer (MUB pH 6.5)

Tris (hydroxymethylaminomethane)	–	2.42 g
Maleic acid	–	2.3 g
Citric acid	–	2.8 g
Boric acid	–	1.26 g
MilliQ water	–	800 ml

Then adjusted to pH 6.5 with 10 M sodium hydroxide (NaOH)

10M NaOH

NaOH	–	40 g
Distilled water	–	100 ml

0.5 N NaOH

NaOH	–	2 g
Distilled water	–	100 ml

0.5 M $CaCl_2$

$CaCl_2$	–	7.351 g
Distilled water	–	100 ml

10 % urea solution

Urea	–	10.0 g
Distilled water	–	100 ml

Phenolate solution preparation:

Solution A: Dissolve 62.5 g of phenol in 20 ml of methanol.

Then, add 18.5 ml of acetone to it and the mixture is then made up to 100 ml with ethyl alcohol.

Solution B: Dissolve 27 g of NaOH in 100 ml of distilled water.

For phenolate solution, mix together 20 ml of solution A and B and make up the whole volume to 100 ml with distilled water. This has to be prepared fresh before use.

1 N potassium dichromate solution

Potassium dichromate	–	29.418 g
Distilled water	–	100 ml

0.5 N ferrous ammonium sulphate solution

Ferrous ammonium sulphate	–	19.606 g
Distilled water	–	100 ml

0.32% $KMnO_4$ solution

Potassium permanganate ($KMnO_4$)	–	3.2 g
Distilled water	–	1000 ml

2 % Boric acid

Boric acid	–	20 g
Distilled water	–	1000 ml

Mixed indicator

Methyl red	–	0.066 g
Bromocresol green	–	0.099 g
95% alcohol	–	100 ml

2.5 % NaOH

Sodium Hydroxide (NaOH)	–	25 g
Distilled water	–	1000 ml

0.05 M Sodium bicarbonate ($NaHCO_3$)

$NaHCO_3$	–	21 g
Distilled water	–	100 ml
pH	–	8.5

0.02 N Sulphuric acid (H_2SO_4)

Conc.H_2SO_4	–	1 ml
Distilled water	–	1.8 L

Dickman's and Bray's reagent

Ammonium molybdate	–	15 g
Distilled water	–	600 ml
10 N HCl	–	400 ml

40 % stannous chloride ($SnCl_2.2H_2O$)

$SnCl_2.2H_2O$	–	10 g
Conc. HCl	–	25 ml

Diluted Stannous chloride solution (Make a fresh solution every 2 hrs as needed)

40 % $SnCl_2.2H_2O$	–	0.5 ml
Distilled water	–	66 ml

1 M Ammonium acetate (NH_4OAc)

NH_4OAc	–	77.09 g
Distilled water	–	1000 ml
pH	–	7.0

Neutral Ammonium acetate solution

99.5% Glacial acetic acid (CH_3COOH)	–	57 ml
Distilled water	–	700 ml
Concentrated (NH_4OH)	–	69 ml
(Diluted upto 900 ml with dist.water)		
pH (adjusted by 3 N NH_4OH or 3 N CH_3COOH)	–	7.0

Appendix-III

Media and Reagents Used For Biochemical Screening of Potential Phosphate Solubilizing Bacteria

Phenol red carbohydrate broth medium

Peptone	–	5.00 g
Beef extract	–	3.00 g
Sodium chloride	–	8.00 g
Carbohydrate source	–	10 g
Distilled water	–	1000 ml
pH	–	7.3±0.1
Phenol red indicator	–	few drops till medium turn pink

Starch agar medium

Beef extract	–	3g
Peptone	–	5g
Starch, soluble	–	2g
Agar	–	15 g
Distilled water	–	1000 ml
pH	–	7.2±0.1

Nutrient gelatin medium

Gelatin	–	120g
Peptone	–	5g
Beef extract	–	3g
Distilled water	–	1000 ml
pH	–	7.2±0.1

Skim milk agar medium

Skim milk powder	–	5g mixed with 50 ml distilled water (stir until dissolved)
Agar	–	1 g mixed with 50 ml distilled water, (stir until dissolved). Add the mixture and autoclave.

Semi solid agar medium (Motlity test)

Beef extract	–	3g
Peptone	–	10g
Sodium chloride	–	5g
Agar	–	4g
Distilled water	–	1000ml
		(Melt agar mixture; add 1% TTC solution, then autoclave)

1% Triphenyltetrazolium Chloride (TTC) solution – 1g of TTC in 100ml distilled water

Bennett's agar medium for HCN

Yeast extract	–	1g
Beef extract	–	1g
Casein enzymatic hydrolysate	–	2g
Dextrose	–	10g
Agar	–	15g
Glycine	–	4.4g
Distilled water	–	1000 ml
pH	–	7.3±2

Nitrate broth medium

Peptone	–	5 g
Beef extract	–	3 g
Potassium nitrate (KNO_3)	–	1 g
Distilled water	–	1000 ml
pH	–	7.0±2.0

Appendix-IV

Reagents Used For Isolation and Phylogenetic Affiliation of Phosphate Solubilizing Bacteria

TE Buffer (pH 8.0)

10mM Tris-HCl	-	0.157 g
Distilled water	-	100 ml
1mM EDTA	-	3.722 g
Distilled water	-	10 ml

5M Sodium chloride

Sodium chloride	-	29.22 g
Distilled water	-	100 ml

3M Sodium acetate (pH 5.2)

Sodium acetate	-	24.69 g
Distilled water	-	100 ml
pH	-	5.2

5x Tris- borate- EDTA (TBE) buffer

Tris	-	54 g
0.5M EDTA	-	3.722 g
Boric acid	-	27.50 g
Distilled water	-	1000 ml
pH	-	8.2

1x TBE buffer (500 ml)

5x TBE	-	100 ml
Distilled water	-	400 ml

Composition of the PCR reaction mixture (25 µl tube^{-1})

10 x buffers	-	2.5 µl
$MgCl_2$ (25 mM)	-	1.5 µl
dNTPs (2.5 mM)	-	2.0 µl
Primers	-	0.8 µl
Template DNA (100 ng)	-	1.0 µl
Taq DNA polymerase (2U/µl)	-	0.5 µl
Nuclease free water	-	15.9 µl

0.8% agarose gel (50 ml)

Agarose	-	0.4 g
1x TBE	-	50 ml
Ethedium Bromide (EtBr) (10 mg/ml)	-	2 µl

1.5% agarose gel (50 ml)

Agarose	-	0.75 g
1x TBE	-	50 ml
Ethedium Bromide (EtBr) (10 mg/ml)	-	2 µl

1.5% agarose gel (100 ml)

Agarose	-	1.5 g
1x TBE	-	100 ml
Ethidium Bromide (EtBr) (10 mg/ml)	-	4 µl

70% ethanol (100 ml)

Ethanol (99.9%)	-	70 ml
Distilled water	-	30 ml

10% Cetyl trimethyl ammonium bromide (CTAB)

CTAB	-	10.0 g
Distilled water	-	100 ml

10% Sodium dodecyl sulphate (SDS)

SDS	-	10.0 g
Distilled water	-	100 ml

Lysozyme (2 mg/ml): Dissolve 2 mg of lysozyme in 1 ml of 1x TBE buffer (pH 8.0) and store at -20°C for further use.

Proteinase K (20 mg/ml): Dissolve 20 mg of proteinase K in 1 ml of sterile distilled water and store at -20°C for further use.

Ethidium Bromide (EtBr) (10 mg/ml): Dissolve 10 mg of EtBr in 1 ml of sterile distilled water and store at 4°C for further use.

Appendix-V

Reagents used for organic acid quantification using UHPLC

25% Sodium Sulphate (Na_2SO_4)	10 g Na_2SO_4 in 30 g HPLC water (w/w)
100 mM Sodium Sulphate (Na_2SO_4)	10.67 ml of 25% Na_2SO_4 is added to 500 ml HPLC water. Then, pH is adjusted to 2.65 with Methanesulfonic acid
1000 ppm organic acid stock solution	10mg of organic acid in neat form in 10 ml HPLC water
30 ppm organic acid (100 µl)	3µl of 1000ppm stock solution added with 97µl 100mM Na_2SO_4
50 ppm organic acid (100 µl)	5µl of 1000ppm stock solution added with 95µl 1000mM Na_2SO_4
70 ppm organic acid (100 µl)	7µl of 1000ppm stock solution added with 93µl 100mM Na_2SO_4
100 ppm organic acid (100 µl)	10µl of 1000ppm stock solution added with 90µl 100mM Na_2SO_4

Appendix-VI

Reagents used for xenobiotic stress

0.2% L-tryptophan

L-tryptophan	–	0.200 g
Distilled water	–	100 ml

Salkowski's reagent

35% perchloric acid	–	50 ml
0.5 M $FeCl_3$	–	1 ml

0.5 M Ferric chloride ($FeCl_3.6H_2O$)

$FeCl_3$	–	6.757 g
Distilled water	–	50 ml

10mM *p*-Nitrophenyl Phosphate (p-NPP)

10mM p-NPP	–	0.186 g
Distilled water	–	50 ml

1M Sodium Hydroxide (NaOH)

NaOH	–	8 g
Distilled water	–	200 ml

5N Sulphuric acid (H_2SO_4)

Concentrated H_2SO_4	–	70ml
Distilled water	–	500 ml

Antimony potassium tartarate $K_2Sb_2(C_4H_2O_6)_2$

$K_2Sb_2 (C_4H_2O_6)_2$	–	0.343 g
Distilled water	–	125 ml

Ammonium molybdate $(NH_4)_2MoO_4$ solution

$(NH_4)_2MoO_4$	–	5 g
Distilled water	–	125 ml

0.1M Ascorbic acid ($C_6H_8O_6$)

$C_6H_8O_6$	–	0.88 g
Distilled water	–	50 ml

Combined reagent : 50 ml of 5N H_2SO_4 is added with 5 ml Antimony potassium tartarate, 15 ml Ammonium molybdate solution and 30 ml Ascorbic acid.

Preparation of stock solution for xenobiotic:

Dimethoate 30% EC

1000 ppm stock solution	0.33 ml + 99.67 distilled water

Butachlor 50% EC

1000 ppm stock solution	0.02 ml + 99.98 distilled water
50 ppm	5ml stock solution + 95ml Nutrient broth medium
100 ppm	10ml stock solution + 90ml Nutrient broth medium
150 ppm	15ml stock solution + 85ml Nutrient broth medium

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Title of Research Molecular and Biochemical characterization of Phosphate Solubilizing bacteria from Paddy fields in Mizoram

Supervisor Dr. R. Lalfakzuala

List of Publications

Research Journal

1. Lalrampani chawngthu, Remruattluanga Hnamte, R. Lalfakzuala. 2020. Isolation and characterization of Rhizospheric Phosphate Solubilizing Bacteria from Wetland Paddy Field of Mizoram, India. *Geomicrobiology journal*.Taylor and Francis ISSN: 0149-0451 (Print) 1521-0529.
2. Aayushi Biswas, Lalrampani Chawngthu, C. Vanlalveni, Remruattluanga Hnamte, R. Lalfakzuala, Lalthazuala Rokhum. 2018. Biosynthesis of silver nanoparticles using *Selaginella bryopteris* plant extracts and their antimicrobial and photocatalytic activities. Journal of Bionanoscience.Vol.12:227-232.
3. R. Lalfakzuala, Lalrampani, C. Vanlalveni, Lalmuankimi Khiangte, Remruattluanga hnamte. 2014. Antibacterial activity of methanolic extracts of selected weeds against two phosphorous solubilizing bacteria. International Journal of Current Microbiology and Applied Science. ISSN: 2319-7706 Vol.3 (4)1014-1019.
4. R. Lalfakzuala, C. Vanlalveni, Lalmuankimi Khiangte, Lalrampani, Remruattluanga Hnamte and Lianthangpuii. 2015. Effects of insecticides on growth of soil fungi. Asian journal of microbiology, biotechnology and environmental science ISSN: 0972-3005 Vol.17 (1): 199-203.

Conference Proceeding

5. Lalrampani and R. Lalfakzuala. 2016. Influence of heavy metals, pH and salt on biomass and phosphatase activity of an isolated PSB strain. Proceedings of the Mizoram Science Congress 2016. ISBN: 978-93-85926-49-5.
6. Lalrampani and R. Lalfakzuala. 2017. Diversity of phosphate solubilizing bacteria isolated from paddy field of Mizoram. Proceedings of the Bioderversity, Conservation and utilization of natural resources with reference to Northeast India. ISBN: 978-818653578-0.
7. Lalrampani Chawngthu and R. Lalfakzuala. 16srRNA gene profiling of potential bacterial biofertilizer with phosphate solubilizing ability and its influence on the growth promotion of rice. Proceedings of the National conference on natural resources management and sustainable agriculture with reference to NE India. Arunachal University of Studies, Namsai. ISBN 81-944507-0-5 ISBN 978-81-944507-0-2.

Paper Presented in Seminar/Workshop

1. Lalrampani and R. Lalfakzuala. “Influence of heavy metals, pH and salt on biomass and phosphatase activity of an isolated PSB strain”. Mizoram Science Congress held at Mizoram University during 13th -14th October 2016. ISBN: 978-93-85926-49-5.
2. Lalrampani and R. Lalfakzuala. “Diversity of phosphate solubilizing bacteria isolated from paddy field of Mizoram”. Bioderversity, Conservation and utilization of natural resources with reference to Northeast India (BCUNRNEI) organized by the Department of Botany, Mizoram University during 30th-31st March, 2017. ISBN: 978-818653578-0.
3. Lalrampani and R. Lalfakzuala. “Isolation and screening of Phosphate Solubilizing bacteria from paddy field of North Vanlaiphai, Mizoram” National conference on PGPR for sustainability of agriculture and environment organized by the Department of Biotechnology, Mizoram University during 11th-12th May, 2018.
4. Lalrampani Chawngthu and R. Lalfakzuala. “Influence of plant growth promoting rhizobacteria on growth and chlorophyll content of rice”. National conference on emerging trends in environmental research (NACETER) organized by the Department of Environmental Science, Pachhunga University College, Mizoram during 31st October-2nd November, 2019.
5. Lalrampani Chawngthu and R. Lalfakzuala. “Biodiversity of phosphate solubilizing bacteria in wetland paddy field of Mizoram, India”. International conference on recent advances in animal sciences (ICRAAS-2019) organized by the Department of Zoology, PUC; Mizo Academy of Sciences (MAS); Mizoram University; Directorate of Fisheries and Climate Change; Directorate of Agriculture (Research and Education) Govt.of Mizoram, India during 6th -8th November, 2019.
6. Lalrampani Chawngthu and R. Lalfakzuala. “16srRNA gene profiling of potential bacterial biofertilizer with phosphate solubilizing ability and its influence on the growth promotion of rice”. National conf. on natural resources management and sustainable agriculture with reference to NE India, Arunachal University of Studies, Namsai organized by the Faculty of Agriculture Sciences. Arunachal University of Studies, Namsai, Arunachal Pradesh during 28th-29th January, 2020.

Seminar and Workshop Attended

1. Seminar on Oil and Natural Gas in Mizoram: Present Scenario and Prospects Organized by Mizo Post Graduate Science Society in Collaboration with the Directorate of Geology and Mineral Resources, Govt.of Mizoram held on 28th August, 2013 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.
2. One Day Seminar on Genetically Modified Crops and Food Security Organized by Mizo Academy of Sciences and Govt.Zirtiri Residential Science College held on 23rd January, 2014 at Govt. Zirtiri Residential Science College, Aizawl, Mizoram.
3. One day Advocacy workshop on Oil and Natural Gas Exploration in Mizoram organized by Mizo Academy of Sciences held on 18th July, 2014 supported by Directorate of Geology and Mineral Resources, Govt.of Mizoram.
4. Seminar on Make in India: Science and Technology Driven Innovations Organized by Mizo Academy of Sciences In Collaboration with the Mizoram Science, Technology and Innovation Council (MISTIC), Govt. of Mizoram held on 4th November, 2016 at Pi Zaii Hall, Synod Conference Centre, Aizawl, Mizoram.
5. Workshop on 'Statistical and Computing Methods For Life-Science Data Analysis' held During 5th-10th March, 2018 Organized By Biological Anthropology Unit, Indian Statistical Institute, Kolkata and Department of Botany, MZU, Aizawl.
6. Seminar nn Science and Technology For a Sustainable Future Organized by Mizo Academy of Sciences with the Mizoram Science, Technology and Innovation Council (MISTIC), Govt. Of Mizoram and National Council for Science And Technology Communication, Dept.Of Science And Technology, New Delhi On 30th April, 2018.
7. One Day Awareness Programme Cum Workshop on Invasive Alien Plants in Himalayas: Status, Ecological Impact and Management (Mizoram and Tripura chapter), Organized by Botanical Survey of India in Collaboration with the Dept. of Botany, MZU, Aizawl on 26th April, 2019 under National Mission for Himalayan Studies.
8. One day workshop on IPR and Plant Protection with special reference to Northeast India jointly organized by Dept. of Botany, MZU and Dept. of Horticulture, Govt.of Mizoram on 18th December, 2019.

PARTICULARS OF THE CANDIDATE

NAME OF THE CANDIDATE	: Lalrampani Chawngthu
DEGREE	: Ph.D.
DEPARTMENT	: Botany
TITLE OF THESIS	: Molecular and Biochemical Characterization of Phosphate Solubilizing Bacteria from Paddy Fields in Mizoram
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APPROVAL OF RESEARCH PROPOSAL	
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ABSTRACT

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM PADDY FIELDS IN MIZORAM

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DEPARTMENT OF BOTANY

SCHOOL OF LIFE SCIENCE

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Abstract

One of the most important macronutrient elements which are a necessity for plant development is Phosphorous. It is involved in vital cellular functions, metabolic pathways like photosynthesis, respiration, nitrogen fixation andthe whole plantgrowth.Due to the valuable role it plays it cannot be substituted by any other nutrient. Soilphosphorous deficiency can occur due to various reasons and many agronomists have tried to fulfill the requirement of soil phosphorous by application of chemical fertilizers as well as other organic sources like manure. If utilization of chemical fertilizers is practiced for a longer period,it is inevitable that serious soil degradation, nitrogen leaching, soil compaction, reduction in soil organic matter, and loss of soil carbon, etc will occur. The reaction of plant under Pstress or even when it is available in sufficientquantity is quiteplacid. The fundamentalconstriction in theavailability of P is the solubilization as it gets fixed both in acidic and alkaline soil. Soil fixed P can only be solubilized by some microbes in the soil which are called“phosphate solubilizing microorganisms” (PSMs) or particularly “phosphate solubilizing bacteria” (PSB) for bacterial solubilizers. These bacteria released a specific enzyme protein called acid phosphatase and different types of organic acids in the soil which makesphosphorous soluble and available to plants. The type of beneficial bacteria which are able to promote plant growth and development are termed as plant growth promoting bacteria (PGPB) and those that are present within the rhizospheric region are called plant growth promoting rhizobacteria (PGPR).The utilization of PGPR has shown massive potentials to be a promising technique in the practice of sustainable agriculture.

The study of beneficial microorganisms in the contribution to a sustainable agriculture is an imperative call to look forward to. In the context of Mizoram, the study of beneficial microorganisms is still infancy. So, study of phosphate solubilizing bacteria (PSB) which can also be termed as PGPR or PGPB have been done in this research. A paddy field with khariff crop plantation with rice cultivation in North Vanlaiphai (23̊ 7'47" N latitude and 93̊ 4'11" Elongitude) located at Serchhip district in Mizoram was selected.

The rhizospheric soil samples of the rice crop were collected and analysed for the physico-chemical properties. Analysis were done on two seasonal variations which were termed as pre harvest season during the month of July-August and post harvest season during

the month of February-March for two consecutive years and the average was taken into account. Physical properties such as soil moisture content, bulk density, soil temperature and chemical properties such as soil pH,available nitrogen, available phosphorous and available potassiumwere performed. Biochemical properties of soil *viz.* dehydrogenase, urease, phosphatase, soil organic carbon and soil organic matter were also analysed.Soil pH ranged from 4.13 to 5.29 which are acidic.Soil temperature was 25.6°C and 26.5°C during pre and post harvest season respectively. Soil moisture content (SMC) was 67.97% in pre harvest comparatively higher than post harvest 35.8%. Bulk density (BD) was 1.66 gm cm^{-3} during pre harvest and 1.31 gm cm^{-3} during post harvest.During the pre harvest season, soil dehydrogenase activity (DHA) was 0.864 µg TPF $mg^{1}24hrs^{-1}$, acid Phosphatase activity (APase) was 93.458 µg p-NPP $mg^{-1}hr^{-1}$ and urease activity (URES) was 0.931 NH_4^+-N $mg^{-1}3hrs^{-1}$. During post harvest season, DHA was 0.182 µg TPF mg^{1} $24hrs^{-1}$, APase was 59.548 µgp-NPP $mg^{-1}hr^{-1}$ and URES was 0.708 $NH4^+$-N mg^{-1} $3hrs^{-1}$. Available nitrogen (AN) was 287 kg/ha and 274 kg/ha, available potassium (AK) was 125.65 kg/ha and 117.33 kg/ha and available phosphorous (AP) was 11.78 kg/ha and 12.05 kg/ha during pre and post harvest season respectively.

From the rhizospheric soil sample of the rice crop, phosphate solubilizing bacteria (PSB) were isolated and screened using specialized medium called Pikovskaya (PVK) medium. The PSB were selected based on the solubilization zone around each colonies produced in PVK agar media. Biochemical characterizations of all isolates were performed qualitatively for morphological identification which was followed by DNA isolation using the P:C:I method. Then, molecular characterization using 16srRNA gene profiling was performed on all of the isolated PSBs. The gene amplification was performed by using 27f as forward and 1525r asreverse primer.

The PCR product obtained were then sent to Eurofins Genomics India Pvt. Ltd. for commercial sequencing.The gene sequences obtained were then submitted to GenBank database bearing accession numbers MK932023 to MK932068 with total of 43 PSB isolateswith percentage of similarity identity level ranging from 98.23 to 100%.These isolated strains were designated as MZLRPA, MZLRPB, MZLRPC and MZLRPD followed by numerals.Total of 7 genera were identified *viz.*, *Bacillus* (58.1%), *Burkholderia* (13.9%), *Paenibacillus* (2.3%), *Paraburkholderia* (18.6%), *Dyella* (2.3%), *Edaphobacter* (2.3%) and *Paraclostridium* (2.3%).The phylogenetic tree analysis of the evolutionary history was inferred using Kimura-2 parameter model with neighbouring method under 1000 bootstrap

replicates. Maximum species identified was *Bacillus cereus* with total 9 isolates followed by *Paraburkholderia fungorum* with total of 5 isolates. From significant enquiry of literature, it has been observed that the genera *Dyella, Edaphobacter* and *Paraclostridium* are the first report of phosphate solubilizing bacteria.

The phosphate solubilizing capacity were scrutinized both on PVK agar medium qualitatively and on PVK broth medium quantitatively.Quantitative estimation confirmed the evidence that the strain MZLRPC4 *Paraburkholderia fungorum* is the highest P solubilizing efficiency with 3.204 p-NP $\mu g^{-1} ml^{-1}$hr and lowest P-solubilizing efficiency strain is MZLRPC25 *Bacillus cereus* with 0.918 p-NP $\mu g^{-1} ml^{-1}$hr.

Since the main mechanism of phosphate solubilization is the production of organic acids, eight organic acids *viz*., acetic, citric, formic, gluconic, malic, oxalic, succinic and tartaric acids were quantified. After incubation of the PSB isolates in broth culture media, the pH level dropped from 7.0 to 4.0 pH approximately which indicates the organic acid production by the isolates. Eight PSB strains such as MZLPA12 *Paenibacillus sp.*, MZLRPB1 *Bacillus cereus*, MZLRPB4 *Bacillus subtilis*, MZLRPB10 *Burkholderia lata*, MZLRPB11 *Burkholderia dolosa*, MZLRPB13 *Bacillus amyloliquefaciens*, MZLRPC4 *Paraburkholderia fungorum* and MZLRPC17 *Edaphobacter sp*. were selected for organic acid quantification. All eight organic acids were able to be identified and quantified.

In vitro and *in vivo* experiment was conducted to evaluate the plant growth promoting activity of the PSB strains on local rice seeds by seed bacterization and checking the seed germination. Then, the seedlings were transplanted into sterilized soil pots and observed daily within 31 days. Harvesting was done twice during 15 and 31 DAS.Six PSB strains MZLRPB13, MZLRPC17, MZLRPA12, MZLRPB4, MZLRPC4, and MZLRPB11 were selected. All the strains were able to improve the growth of rice as compared to the control sample. The strain MZLRPC4 *Paraburkholderia fungorum* showed maximum shoot length, maximum root length and maximum dry biomass and maximum chlorophyll. Thus, among all the tested strains MZLRPC4 was the best plant growth promoter.

Xenobiotic treatement was also done to check the tolerance capacity of selected two PSB strains *viz*., MZLRPA12 and MZLRPC4. Different parameters were tested which are IAA production, acid phosphatase activity, bacterial growth, dry weight biomass and phosphate determination. The stress test revealed that the two strains tested were both not highly tolerant to the activity of butachlor and dimethoate on the three concentrations used *i.e*. 150, 100 and 50ppm. Dimethoate which is an insecticide was more toxic towards the

strains tested. So, it is clear from the evidence that xenobiotics like the agrochemicals used in agricultural field can degrade the activity of soil beneficial bacteria.

This study reveals identification and characterization of potential phosphate solubilizing bacteria from a remote area in Mizoram. The study also uncovers the beneficial influence of phosphate solubilizing bacteriaon rice growth promotion. Therefore, the novel bacterial strain isolated from this vicinity can be used to develop location specific biofertilizer for sustainable agriculture.

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