"Phosphate Solubilizing Alkaliphilic Bacteria from Coastal Ecosystems of Goa"

A thesis submitted to

Goa University

For the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

by

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July, 2017

CERTIFICATE

This is to certify that the thesis entitled **"Phosphate solubilizing alkaliphilic bacteria from coastal ecosystems of Goa"** submitted by **Ms. Neha Prabhu** for the award of the degree of **Doctor of Philosophy** in **Microbiology** is based on her original studies carried out by her under our supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institution.

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STATEMENT

As required under the University Ordinance O.D.09, I hereby state that the present thesis for Ph.D. degree entitled "Phosphate solubilizing alkaliphilic bacteria from coastal ecosystems of Goa" is my original contribution and that the thesis or any part of it has not been previously submitted for the award of any degree/diploma of any University/Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the problem has been duly cited. Due acknowledgement has been provided to the funding agency and the suggestions have been duly incorporated.

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DEDICATION

To my beloved Parents,

For their patience and faith

ACKNOWLEDGEMENT

This journey of Ph.D. has been quite adventurous, with plenty of ups and downs, but most importantly it has been an experience of relentless learning. I would like to take this opportunity to express my warmest gratitude to all those people whose support, encouragement, suggestions and criticism have left a mark on this work.

First and foremost, I would like to acknowledge my parents and brother for their unwavering and patient support throughout this time. If it were not for them, a lot in my life wouldn't even be conceivable. I thank them from the bottom of my heart!

I have been blessed to work under the supervision of two extremely proficient and intelligent guides, Dr. Sunita Borkar and Dr. Sandeep Garg, both of whom contributed a lot to this process. Dr. Borkar taught me both deliberately and inadvertently and I appreciate all her contribution of academic supervision, personal support and constant motivation in making my Ph.D. experience productive and stimulating. Dr. Garg, who has profoundly influenced and enhanced my knowledge of research and has been a guide in the literal sense. Both, Ma'am and Sir have provided guidance and wisdom at key moments in my research work and life and have been so kind as to overlook all my mistakes and helped me learn and evolve.

This work was a part of a major research project funded by Ministry of Earth Sciences, Government of India, under Dr. Sunita Borkar at P.E.S.'s R.S.N. College of Arts and Science, New Delhi. I'm greatly indebted to the Ministry and P.E.S College of Arts and Science.

I sincerely thank members of my faculty research committee, Dr. C Mohandass, Dr. M. Janathanam, Dr. Sarita Nazareth, Dr. S. K. Dubey for their insightful discussions

and suggestions and Dr. Saroj Bhosle for her invaluable suggestions and constant inspiration at every stage of this research.

I would like to express my appreciation to Dr. Gopakumar, Librarian and the library staff for being very supportive in the course of formulation of this thesis.

I am grateful to the Management and staff of P.E.S.R.S.N College of Arts and Science, Farmagudi for their constant encouragement. My beloved teachers and colleagues of the Department of Microbiology, Dr. Caroline Fernandes, Dr. Flory Pereira, Dr. Rasika Gaonkar, Mr. Mahesh Nadkarni, Mr. Dattaram Gaude and Mr. Vinayak Gaude.

A special note of thanks goes to Kiran Joshi, Abhay Mishra, Alok Dixit and Dr. Varada Damare for their expertise in statistics. I thank Dr. Nrupa Borkar, for making the endless list of research articles available to me and Dr. Neha Nancy, Shrestha Mathur and Kunica Asija for their support, personal and academic, over many years.

I also wish to thank the staff of those institutions which have supported me during the work; Dr. Anoop Tiwari and Ms. Shahina Gazi of National Centre for Antarctic and Ocean Research, Vasco; Dr. Samir Damare of National Institute of Oceanography, Dona Paula; Mr. Gaonkar of Krishi Vigyan Kendra; Indian Council for Agricultural Research, Goa Complex, Dr. Ashwini Kumar, National Institute of Malaria Research, Panaji.

My sincere gratitude to Saraswati, Deepa, Tanu, Budhaji, Domnic, Narayan, Rajesh, Gajanan, Sonia, and Prarthana for assistance rendered from time to time.

I have crossed paths with many researchers who have influenced and enhanced my knowledge of research. Thank you Dr. Trelita, Dr. Teja, Dr. Pramoda, Dr. Milind,

Dr. Lakshangy, Dr. Priya, Dr. Shyamolina, Dr. Bhakti, Dr. Gauri, Dr. Meghnath, Cristabell, Dr. Jaya, Dr. Kashif, Jyothi, Dr. Brenda, Dr. Dnyanada, Dr. Subhajit, Dr. Sanika, Dr. Sushma, Dr. Akshaya, Dr. Amrita, and Jyothi. I would specially like to thank and wish good luck to my budding juniors, Sulochana, Sanket, Alisha, Sajiya, Divya, Neekhat and Anchit.

I'm also deeply indebted to Mrs. Priya Naik who always welcomed and fed me whenever I was at their place during Ph.D. years.

I'd also like to thank a special set of teachers who taught me so much by technically playing the role of my students. They have been a source of valuable learning and I wish them luck in all their endeavours.

My thanks to all those who I might have missed out mentioning here, but that is not a reflection of your importance, contribution and value in my life, it's only a reflection of my weak memory. Please accept my gratitude!

I'm more grateful to everyone than I have been able to mention here, but I don't want a desperate verbal attempt to spoil my emotions, thus I end with,

Thank you very much for everything!

Neha Prabhu

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Chapter I

Introduction & Literature Survey

1.1 INTRODUCTION

Phosphorus is one of the major essential macronutrients required for plant growth (Ehrlich, 1990). It is taken up by plants as an orthophosphate ion (Illmer & Schinner, 1995). Phosphate containing compounds present in soil can be classified into 3 groups, viz. soluble orthophosphate, insoluble inorganic phosphate and insoluble organic phosphate (Johnston & Steen, 2000; Behera *et al.,* 2014). Orthophosphate ions react with numerous organic and inorganic constituents of soil and therefore become least mobile and unavailable for uptake by plants. Hence, a large quantity of phosphatic fertilizer is applied to achieve maximum plant productivity. However, it is observed that the orthophosphate ions of the applied fertilizer react with cations of the soil and precipitate into inorganic tricalcium phosphate, iron phosphate and aluminium phosphate. Organic phosphate enters in soil from decomposition of plant, animal and microbial matter. Different forms of organic phosphate compounds include nucleic acids, phospholipids, phosphoproteins, inositol phosphates, etc. (Zhu *et al.,* 2016). These forms are not taken up by the plants due to their high molecular weight. Therefore, phosphorus is a limiting element in soil for plants (Nopparat *et al.,* 2007; Fankem *et al.,* 2008).

The global cycling of insoluble inorganic and organic soil phosphates is attributed to microorganisms (Spohn *et al.,* 2015). Phosphate solubilizing bacteria can improve plant phosphorus nutrition by solubilizing insoluble inorganic and organic phosphates. Several mechanisms responsible for solubilization of inorganic and organic phosphates have been reported (Aspiras *et al.,* 1971; Roos & Luckner, 1984; Illmer & Schinner, 1992; Ionescu & Belkin, 2009; Park *et al.,* 2009; Nannipieri *et al.,* 2011; Ehrlich *et al.,* 2016).

Production of organic acids is the most reported mechanism for solubilization of inorganic phosphate (Illmer & Schinner, 1992; Paul & Sinha, 2015). Apart from this, inorganic acids such as sulphuric, nitric and carbonic acids produced by microorganisms have been reported to solubilize inorganic phosphate (Khan *et al.,* 2009a). Acidification of the medium as a result of H^+ excretion originating from NH_4^+ has been suggested as an alternate mechanism of inorganic phosphate solubilization (Park *et al.,* 2009). The high molecular weight microbial exopolysaccharides have been shown to play an indirect role in phosphate solubilization (Ionescu & Belkin, 2009). The rate of dissolution due to exopolysaccharide appears to be dependent on the microbial source and concentration of polymer. Since siderophores chelate iron, it has been attempted to correlate the amount of siderophore released to the amount of phosphate solubilized from insoluble iron phosphates (Vassilev *et al.,* 2006; Hamdali *et al.,* 2008). However, siderophore production has not been widely implicated as a phosphate solubilization mechanism. Other postulated mechanisms include production of hydrogen sulphide by sulphur reducing bacteria. H_2S reacts with phosphate containing minerals, thus releasing phosphate (Ehrlich *et al.,* 2016).

Organic phosphate solubilization is called as mineralization of organic phosphorus (Nannipieri *et al.,* 2011). It occurs due to production of extracellular phosphatase enzymes by microorganisms present in the soil. These enzymes act upon the organic phosphate by the hydrolysis of ester-phosphate bonds, leading to the release of phosphate ions. These low molecular weight molecules then can easily be assimilated by the living forms. Based on their pH optima, these enzymes are classified as acid, neutral and alkaline phosphatases (Eivazi & Tabatabai, 1977; Jorquera *et al.,* 2008).

High salinity and alkalinity of soil increase the precipitation of available phosphorus and interfere with activity of phosphate solubilizing microbes (Son *et al.,* 2006). Soil alkalinity and salinity are one of the most severe environmental factors limiting the productivity of agricultural crops through osmotic effects, toxicity of salt ions and the changes in the physical and chemical properties of soil (Sahay *et al.,* 2012). Therefore, the yield and profit of agricultural production in saline and alkaline soil are drastically lowered than neutral counterpart soil (Chookietwattana & Maneewan, 2012).

It is known that the ability of tolerance to stress such as high pH and salt concentration plays an important role in the survival of plant growth promoting bacterial strains in such environments. Recent studies indicate that stress tolerant microbes can help crops to cope with stress e.g. high pH and salt concentration (Shanker & Venkateswarlu, 2011). Therefore, exploring bacteria which are able to efficiently thrive and grow in such stressful environments is the need of the hour.

Alkaliphilic bacteria are organisms which require high pH for their growth. Most studies on alkaliphilic bacteria have so far focused on phylogenetic analysis and secondary metabolites. Only limited information is available on their agricultural and enzymatic potential. If these bacteria are also able to solubilize phosphate in alkaline and saline environments, they then can be considered as potential plant growth promoting bacteria to increase the crop productivity of alkaline and saline soils.

Coastal ecosystems provide a unique ecological niche to diverse microbes. These microbes play various roles in environmental activities and nutrient cycling such as nitrogen fixation, phosphate solubilization, sulfate reduction, etc. (Barea *et al.,* 2005; Appanna & Hamzehzarghani, 2008; Oyeyiola, 2010; Woyessa & Assefa, 2011;

Audipudi *et al.,* 2012). Goa, a tiny state on the west coast of India, with an area of 3,702 sq. km harbours diverse coastal ecosystems like mangroves, khazan lands, sand dunes and salt pans.

Mangroves are an ecological group of halophilic wetland shrubs and trees; mainly found at the intertidal zones of estuaries and mudflats (Augustinus, 1995). Khazan lands are low lying mangrove fringed areas which are situated close to coastal saline lands drained by the tidal estuaries (Sonak *et al.,* 2005). Sand is the by-product of weathered rocks from inland regions. Coastal dunes are made up of sand that is piled high by wind. Saltpans are man-made hyper saline ecosystem in which crude salt is extracted during summer. The diverse microbial community of the coastal ecosystems continuously transforms nutrients from decomposition of organic matter into sources of nitrogen, phosphorus and other nutrients (Borkar, 2015b).

Considering the immense potential of such indigenous bacteria from coastal eco-niches, the present investigation has been carried out to study phosphate solubilization by alkaliphilic bacteria from coastal ecosystems of Goa with an objective to understand,

a) **The mechanisms involved in solubilization of inorganic and organic phosphate compounds by alkaliphilic bacteria from coastal ecosystems of Goa.**
b) **The optimal physico-chemical and nutritional requirements for phosphate solubilization.**
c) **Effect of stress conditions such as pH, salt concentration and metal ions on phosphate solubilization.**

1.2 LITERATURE SURVEY

Phosphorus is the 11th most abundant element in earth's crust. It is vital to all known life forms because it is a key element in many physiological and biochemical processes (Ehrlich, 1990). A component of every cell in all living organisms, phosphorus is indispensible and cannot be replaced by any other element.

1.2.1 Phosphorus in plants

Phosphorus, one of the 17 chemical elements required for plant growth is classified as a major nutrient. It is frequently deficient for crop production and is required by crops in relatively large amounts. Its functions cannot be performed by any other nutrient. Adequate supply of phosphorus is required by plants for optimum growth and yield.

Phosphorus is taken up mostly as the primary orthophosphate ion (HPO_4^{-2} and $H_2PO_4^-$). It enters the plant from the water in soil through root hairs, root tips and outermost layers of root cells. Uptake of phosphorus is also facilitated by mycorrhizal fungi that grow in association with the roots of many crops. Once inside the plant root, phosphorus may be stored in the root or transported to the upper portions of the plant.

In plants, phosphorus is essential for a number of physiological functions that are involved with energy transformations. Phosphorus is a component of many cell constituents and plays a major role in several key processes, including photosynthesis, respiration, energy storage and transfer, cell division, and cell enlargement (Armstrong, 1988; Theodorou & Panxton, 1993). Adequate phosphorus is needed for the promotion of early root formation. Phosphorus also improves crop

quality and is necessary for seed formation (Mullins, 2009). It is also one of the primary structural components of membranes that surround plant cells.

The deficiency of phosphorus affects not only growth and development in plants, but also crop yield and the quality of fruit and formation of seeds. Deficiency of phosphorus can delay the ripening of crops which can set back the harvest, risking the quality of the produce (Johnston & Steen, 2000).

1.2.2 The occurrence of phosphorus in nature

The element phosphorus does not occur by itself in nature. It is always combined with other elements to form phosphates (McGrath *et al.,* 1995). Element phosphorus combines spontaneously and vigorously with oxygen to form phosphorus pentoxide. This pentoxide form combines with water to form an anhydride orthophosphoric acid. Further, orthophosphates combine with other elements to form minerals. The type of phosphorus bearing minerals that form in soil is highly dependent on soil pH. If we look at the global phosphorus fluxes, most of the phosphorus is insoluble or poorly soluble inorganic compounds. About 10^{15} metric tons in earth's crust is present as apatites. In aquatic environments, the total phosphate concentration is extremely small as compared to sediment. Phosphate rock deposits are found throughout the world and over 30 countries are currently producing phosphatic fertilizer for use in domestic markets and/or international trade. Phosphorous in soils originates from the weathering of residual minerals and from phosphorus additions in the form of fertilizers, plant residues, agricultural wastes and/or bio-solids (Behera *et al.,* 2014). The mineral family called apatite covers most of the phosphorus. Phosphorus is normally present in the range from 400 to 1200 mg/kg of soil (Fernandez & Novo, 1988). Phosphates can be very complex and more than one form

of phosphate is found in nature (Johnston & Steen, 2000; Mullins, 2009). The diverse soil phosphate forms can be categorized as soil solution phosphates, insoluble inorganic and insoluble organic phosphates **(Figure 1.1)**.

1.2.3 Types of phosphates

Phosphorus compounds in soil are classified into 3 groups based on their solubility and nature (Behera *et al.,* 2014).

1.2.3.1 Orthophosphate

In soil, orthophosphate occurs in three forms viz; monovalent ($H_2PO_4^-$), divalent (HPO_4^{2-}) and trivalent (PO_4^{3-}) (Beever & Burns, 1980). The type of orthophosphate ion present in the soil depends on soil reaction. Monovalent form is mostly found in acidic and neutral soils at a relatively low pH of 4 to 5, while divalent and trivalent phosphates are found in neutral to alkaline soils (Yadav & Tarafdar, 2001).

1.2.3.2 Inorganic phosphate

Most inorganic phosphorus compounds in soil fall into one of the two groups:

In neutral to alkaline soils, calcium is the most dominant controlling cation. Phosphorus reacts with calcium to form insoluble calcium phosphates. In acid soil, iron and aluminium are the controlling cations. Iron and aluminium react with phosphorus to form insoluble iron phosphates and aluminium phosphates (Rodriguez & Fraga, 1999; Khan *et al.,* 2009a; Yadav & Verma, 2012; Sharma *et al.,* 2013; Krishnaraj & Dahale, 2014).

1.2.3.2.1 Calcium phosphates

The original source of phosphorus is the mineral apatite, a calcium phosphate that is nearly insoluble. Apatite minerals may be found in even the more weathered soils,

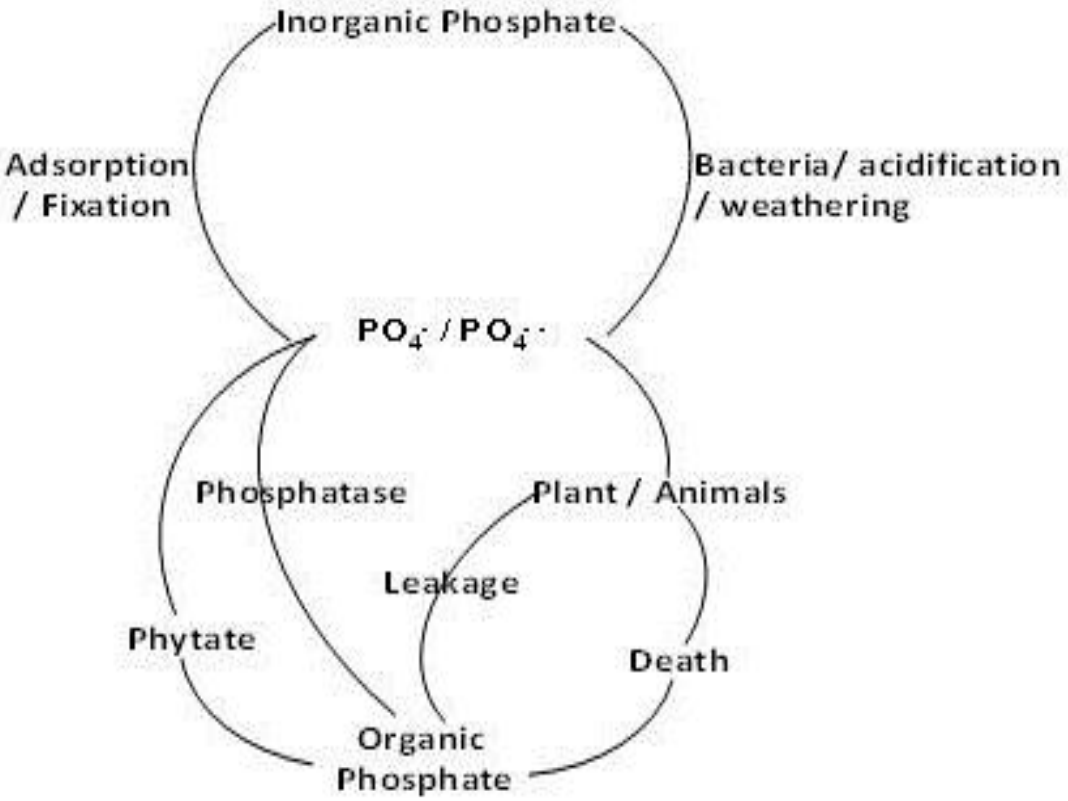


Figure 1. 1 - Movement of phosphorus in soil (Khan *et al.*, 2009a)

especially in their lower horizons. This fact is an indication of the extreme insolubility and consequent unavailability of the phosphorus. The simpler compounds of calcium such as mono and di-calcium phosphates are readily available for plant growth. These compounds are present in extremely small quantities only because they easily revert to the more insoluble forms (Rodriguez & Fraga, 1999; Khan *et al.,* 2009a; Yadav & Verma, 2012; Sharma *et al.,* 2013; Krishnaraj & Dahale, 2014).

1.2.3.2.2 Iron and aluminium phosphate

In this group, the compounds involved are probably hydroxyl phosphates such as strengite (iron phosphate) and variscite (aluminium phosphate). Strengite and variscite are too insoluble to contribute much to plant nutrition.

1.2.3.3 Organic phosphate

Organic phosphorus generally represents about 30-50% of the total phosphorus in soils and varies between 4 and 90% in most soils (Dalal, 1977). Most of the organic phosphorus compounds are esters of orthophosphoric acid and have been identified primarily as inositol phosphates, phospholipids and nucleic acids (Paul & Clark, 1988; Goldstein, 1994).

1.2.3.3.1 Inositol phosphate

The abundance of inositol phosphates is highly variable; however, they frequently represent the dominant form of organic phosphate in soil (Khan *et al.,* 2009a). Inositol phosphate esters in soil range from monophosphates to hexaphosphates. They are characterized by high acidity and are often found as components of polymers or insoluble complexes with proteins and lipids. The stability of inositol phosphates is closely linked to the number of phosphate groups, rendering esters of

higher order more recalcitrant to biodegradation and consequently more abundant. The most common isomer in soil is inositol hexaphosphate also known as phytic acid (Anderson, 1980; Harley & Smith, 1983).

1.2.3.3.2 Phytin

Phytin, a Ca-Mg salt of phytic acid and its derivatives are the most abundant approximately 50% of the known organophosphorus compounds in soil (Pederson, 1953; Dalal, 1977). Phytin has six orthophosphate groups attached to each carbon atom in the benzene ring.

1.2.3.3.3 Nucleic acids

Nucleic acids occur in all living cells and are produced during the decomposition of residues by soil microorganisms. Two distinct forms of nucleic acids, ribonucleic acid and deoxyribonucleic acid are released into the soil in greater quantities than inositol phosphates, and they are broken down more quickly. The nucleic acid content in soil is not known, although it is suggested that it comprises of not more than 5% of organic phosphates (Adams *et al.*, 1954; Yadav & Verma 2012).

1.2.3.3.4 Phospholipids

Phosphorus containing fatty compounds is known as phospholipids. They are insoluble in water. They are readily utilized and synthesized by soil microorganisms. Some of the most common phospholipids are derivatives of glycerol. The rate of release of phospholipids from organic sources in soil is rapid. Phospholipids constitute 1-5% of total organic phosphate in soils (Dalal, 1977).

Among the three forms of phosphate, only soil solution phosphate or orthophosphate can be taken up by plants. Orthophosphate ions readily react with numerous

inorganic and organic constituents of soil and therefore become least mobile and unavailable for uptake by plants (Hinsiger, 2001). The global cycling of insoluble inorganic and organic soil phosphate forms is attributed to microorganisms (Rodriguez & Fraga, 1999; Khan *et al.,* 2009a; Yadav & Verma, 2012; Sharma *et al.,* 2013; Krishnaraj & Dahale, 2014; Spohn *et al.,* 2015).

1.2.4 Phosphate solubilizing microorganisms

Microorganisms which solubilize phosphorus bearing insoluble inorganic and organic phosphate compounds are termed as phosphate solubilizing microorganisms (Rodriguez & Fraga, 1999). Pikovskaya (1948) was one of the first to report solubilization of insoluble inorganic phosphate by microorganisms. This report led the start of research on phosphate solubilization.

A significant increase in knowledge on phosphate solubilization has been observed ever since (Chung *et al.,* 2005; Sharma *et al.,* 2013). Numerous strains of bacteria, actinobacteria and fungi have been reported and investigated for their phosphate solubilizing abilities (Whitelaw, 2000; Igual *et al.,* 2001). Phosphate solubilizing microorganisms are ubiquitous in nature. However, they differ in mineral phosphate solubilizing ability. Phosphate solubilizing microorganisms have been reported from different layers of soil, such as rhizosphere, rhizoplane, phyllosphere. The density of phosphate solubilizing bacteria in each of the layers is different. They have also been isolated from non-rhizosphere soil, phosphate rock deposit soil and even stressed soil (Zaidi *et al.,* 2009).

Since the report of Pikovskaya (1948), several media, such as bromophenol blue dye method (Gupta *et al.,* 1994; Mehta & Nautiyal, 2001) and National Botanical Research Institute Phosphate medium (Nautiyal, 1999) for inorganic phosphate

solubilization have been proposed. Different sources of insoluble inorganic phosphate are used like aluminium phosphate, iron phosphate, tricalcium phosphate and zinc phosphate. Microorganisms exhibiting inorganic phosphate solubilization produce a clear zone or halo around their colonies. Mineral Salts Medium amended with organic phosphate compounds such as p-nitrophenyl phosphate and glycerophosphate have been used for organic phosphate solubilization (Dean, 2002). The selection of inorganic and organic phosphate compound for testing phosphate solubilizing microorganism depends upon the type of soil where the phosphate solubilizing microorganism will be used.

However, screening on agar medium is not a suitable test for phosphate solubilization. Phosphate solubilization ability of microorganisms must be performed in liquid media. The isolates which show solubilization in liquid medium can be further tested in field for plant growth promotion (Gupta *et al.,* 1994; Nautiyal, 1999; Mehta & Nautiyal, 2001). A substantial number of diverse microbial species like bacteria, fungi, actinobacteria and algae exhibit phosphate solubilization capacity.

1.2.4.1 Phosphate solubilizing bacteria

Scientists have studied phosphate solubilizing bacteria for more than 5 decades. Phosphate solubilizing bacteria have been isolated from numerous terrestrial and aquatic sources (Rodriguez & Fraga, 1999; Sharma *et al.,* 2013). Considerable amount of reports suggest that phosphate solubilizing bacteria are mostly concentrated in the rhizospheric region of plants (Vazquez *et al.,* 2000; Tripathi *et al.,* 2005; Rajkumar *et al.,* 2006; Khan & Zaidi, 2007; Selvakumar *et al.,* 2008; Rashid *et al.,* 2012; Khan & Zaidi, 2014a). Rhizospheric bacteria are metabolically more active than non-rhizospheric bacteria. Among the whole microbial population

in soil, phosphate solubilizing bacteria constitute 1 to 50% (Chen *et al.,* 2006). Phosphate solubilizing bacteria have also been reported from soil stressed with alkalinity, salinity and contamination of metals (Mittal *et al.,* 2003; Srinivasan *et al.,* 2012). These bacteria solubilize phosphate under the influence of abiotic stress such as, drought, low or high pH, salinity, and temperature (Son *et al.,* 2006; Li *et al.,* 2010; Vassilev *et al.,* 2012). Cold tolerant *Pseudomonas* sp., *Pantoea* sp., *Mycobacterium* sp., *Mycoplasma* sp. and *Acinetobacter* sp. have exhibited phosphate solubilizing ability at low temperature from 4 - 16°C (Das *et al.,* 2003; Egamberdiyeva & Hoflich, 2003; Pandey *et al.,* 2006; Trivedi & Sa, 2008; Gulati *et al.,* 2009). *Pseudomonas* sp. and *Azospirillum* sp. solubilized phosphate under the drought conditions (Sandhya *et al.,* 2010; Arzanesh *et al.,* 2011). *Aerococcus* sp., *Arthrobacter* sp., *Bacillus* sp., *Pantoea* sp., and *Pseudomonas* sp. solubilized phosphate under saline conditions (1 to 10% NaCl) (Johri *et al.,* 1999; Son *et al.,* 2006; Banerjee *et al.,* 2010; Srinivasan *et al.,* 2012). *Bacillus* sp., *Pantoea* sp. and *Pseudomonas* sp. solubilized phosphate at alkaline pH (11.0) (Mittal *et al.,* 2003).

Vassilev *et al.* (2012) suggest that ecological location plays an important role in isolating stress tolerating bacteria. It is evident from literature that stress tolerant bacteria help plant cope with abiotic stress. These bacteria induce plant responses to stress by mechanisms such as induced systemic tolerance (Kloepper *et al.,* 2004; Mayak *et al.,* 2004; Zhang *et al.,* 2008; Yang *et al.,* 2009; Grover *et al.,* 2011).

Most reported genera of inorganic and organic phosphate solubilizing bacteria include, *Acinetobacter, Arthrobacter, Bacillus, Burkholderia, Citrobacter, Enterobacter, Gluconacetobacter, Klebsiella, Micrococcus, Proteus, Pseudomonas, Rhizobium* and *Serratia* (Gugi *et al.,* 1991; Abd-Alla, 1994; Thaller *et al.,* 1995;

Freitas *et al.,* 1997; Skrary & Cameron, 1998; Rodriguez & Fraga 1999; Seshadri *et al.,* 2002; Chen *et al.,* 2006; Khan *et al.,* 2009a; Collavino *et al.,* 2010, Gulati *et al.,* 2010). **Table 1.1** presents the list of different genera and species of phosphate solubilizing bacteria. Bacteria belonging to genera, *Bacillus, Pseudomonas* and *Rhizobium* are said to be highly efficient inorganic and organic phosphate solubilizers among the phosphate solubilizing bacteria (Illmer & Schinner, 1995; Rodriguez & Fraga, 1999; Wani *et al.,* 2007b; Cherif-Silini *et al.,* 2013; Krishnaraj & Dahale, 2014). Most number of species reported as phosphate solubilizers belong to the genus *Bacillus* followed by *Pseudomonas*.

Phosphate solubilizing bacteria are known to solubilize different forms of inorganic phosphate (aluminium phosphate, iron phosphate, tricalcium phosphate and zinc phosphate) and organic phosphate. Most number of bacteria have exhibited solubilization of calcium phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, rock phosphate, etc. (Gyaneshwar *et al.,* 1998). *Acetobacter liquefaciens* isolated from rhizosphere soil from Kerala solubilized 72.9 mg/l of tricalcium phosphate (Joseph & Jisha, 2009). *Bacillus* sp. solubilized tricalcium phosphate from 236 to 395 mg/l (Kumar & Narula, 1999; Matsuoka *et al.,* 2013). Only a few bacteria have exhibited the ability to solubilize aluminium, iron and zinc phosphate. *Gluconacetobacter diazotrophicus, Pseudomonas fluorescens* and *Pseudomonas aeruginosa* strains exhibited the ability to solubilize zinc phosphate (Di Simine *et al.,* 1998; Fasim *et al.,* 2002; Saravanan *et al.,* 2007). Solubilization of aluminium phosphate has been by bacteria belonging to the genera *Bacillus* and *Rhizobium* (Halder *et al.,* 1990; Halder & Chakrabarty, 1993; Gyaneshwar *et al.,* 2002).

Table 1. 1 - Genera and species of bacteria reported as phosphate solubilizers

Achromobacter
Acinetobacter
Acinetobacter baumannii
Acinetobacter haemolyticus
Acinetobacter lwoffii
Acinetobacter rhizosphaerae
Actinomadura oligospora
Advenella incenata
Aerobacter
Aerobacter aerogenes
Aeromonas
Agrobacterium
Alcaligenes
Aquaspirillum autotrophicum
Arthrobacter ilicis
Azospirillum
Azospirillum brasilense
Azotobacter
Bacillus
Bacillus amyloliquefaciens
Bacillus atrophaeus
Bacillus cereus
Bacillus chitinolyticus
Bacillus circulans
Bacillus coagulans
Bacillus firmus
Bacillus fusiformis
Bacillus licheniformis
Bacillus megaterium
Bacillus mycoides
Bacillus polymyxa
Bacillus pumilus
Bacillus smithii
Bacillus subtilis
Bacillus thuringiensis
Bradyrhizobium
Brevibacterium
Burkholderia
Burkholderia ambifaria
Burkholderia caledonica
Burkholderia cepacia
Burkholderia gladioli
Chryseomonas luteola
Citrobacter freundii
Enterobacter aerogenes
Enterobacter asburiae
Enterobacter cloacae
Enterobacter hormaechei
Enterobacter taylorae
Erwinia
Erwinia herbicola
Escherichia coli
Escherichia intermedium
Exiguobacterium mexicanum
Flavobacterium
Gluconacetobacter diazotrophicus
Klebsiella
Kluyvera cryocrescens
Mesorhizobium ciceri
Mesorhizobium mediterraneum
Microbacterium laevaniformans
Micrococcus
Micrococcus luteus
Micrococcus roseus
Mycobacterium phlei
Mycoplana bullata
Nitrobacter
Nitrosomonas
Paenibacillus macerans
Pantoea
Pantoea agglomerans
Pantoea stewartii
Pseudomonas
Pseudomonas aeruginosa
Pseudomonas calcis
Pseudomonas corrugata
Pseudomonas fluorescens
Pseudomonas oryzihabitans

Cont.

Table 1.1 - Genera and species of bacteria reported as phosphate solubilizers

Pseudomonas poae
Pseudomonas putida
Pseudomonas striata
Pseudomonas savastanoi
Pseudomonas chlororaphis
Pseudomonas pickettii
Rhizobium
Rhizobium leguminosarum bv phaseoli
Rhizobium leguminosarum bv viciae
Rhizobium meliloti
Serratia
Serratia marcescens
Serratia phosphoticum
Sinorhizobium meliloti
Stenotrophomonas maltophilia
Thiobacillus ferrooxidans
Thiobacillus thiooxidans
Variovorax boronicumulans
Vibrio
Vibrio proteolyticus
Xanthobacter agilis
Xanthomonas
Xanthomonas maltophilia

1.2.4.2 Phosphate solubilizing actinobacteria and fungi

Actinobacteria are known to survive under extreme conditions and possess ability to produce metabolites such as antibiotics, pigments, and phytohormones. Phosphate solubilizing actinobacteria have been widely studied in recent years (Fabre *et al.,* 1988; Hamdali *et al.,* 2008). **Table 1.2** presents the list of actinobacteria reported as phosphate solubilizers.

Phosphate solubilizing fungi constitute about 0.1–0.5% of total fungal populations in soil (Kucey, 1983). Among phosphate solubilizing fungi, the genera *Aspergillus* and *Penicillium* are most common followed by *Trichoderma* and *Rhizoctonia* (Sperber, 1958; Kucey, 1983; Venkateswaram & Natarajan *et al.,* 1984; Altomare *et al.,* 1999; Fenice *et al.,* 2000; Jacobs *et al.,* 2002a; Khan & Khan, 2002; Reyes *et al.,* 2002). **Table 1.3** presents reported phosphate solubilizing fungi.

Phosphate solubilizing microorganisms can improve plant phosphorus nutrition by mobilizing inorganic and organic phosphates. Several mechanisms of phosphate solubilization have been reported.

1.2.5 Mechanisms of phosphate solubilization

Microorganisms exhibit numerous mechanisms to solubilize phosphate (Banik & Dey, 1982, Krishnaraj & Dahale, 2014). The different mechanisms leading to solubilization of inorganic and organic phosphates are presented below.

1.2.5.1 Mechanisms of inorganic phosphate solubilization

1.2.5.1.1 Organic acid production

This is said to be the principle mechanism of inorganic phosphate solubilization. The ability of organic acids to solubilize phosphate rock is due to acidification of the

Table 1.2- Genera of actinobacteria reported as phosphate solubilizers

Actinomadura sp.
Actinomyces sp.
Actinoplanes sp.
Agromyces soli
Angustibacter luteus
Candidatus Streptomyces philanthi
Isoptericola hypogeum
Isoptericola variabilis
Kitasatospora sp.
Kocuria flava
Kocuria palustris
Microbacterium aurantiacum
Microbacterium kitamiense
Microbacterium yannicii
Microbispora sp.
Micromonospora aurantiaca
Micromonospora echinaurantiaca
Micromonospora endolithica
Micromonospora flavogrisea
Micromonospora olivasterospora
Nocardia sp.
Rhodococcus sp.
Saccharopolyspora sp.
Streptomyces sp.
Streptomyces alboniger
Streptomyces ambofaciens
Streptomyces anulatus
Streptomyces cavourensis
Streptomyces cinereorectus
Streptomyces cinnabarinus
Streptomyces coelicolor
Streptomyces filipinensis
Streptomyces fimicarius
Streptomyces galbus
Streptomyces griseus
Streptomyces lienomycini
Streptomyces noboritoensis
Streptomyces venezuelae
Streptomyces violascens
Streptosporangium sp.
Thermobifida sp.

Table 1.3 - Genera of fungi reported as phosphate solubilizers

Achrothcium sp.
Alternaria tenuis
Aspergillus aculeatus
Aspergillus awamori
Aspergillus foetidus
Aspergillus japonicus
Aspergillus nidulans
Aspergillus niger
Aspergillus terreus
Aspergillus wentii
Aspergillus flavus
Cephalosporium sp.
Chaetomium globosum
Cladosporium sp.
Cunninghamella candida
Curvularia lunata
Eupenicillium parvum
Fusarium oxysporum
Helminthosporium sp.
Humicola insolens
Humicola lanuginosa
Micromonospora sp.
Mortierella sp.
Mucor sp.
Myrothecium roridum
Oidiodendron sp.
Paecilomyces fusisporus
Paecilomyces hepiali
Penicillium aurantiogriseum
Penicillium bilaii
Penicillium citrinum
Penicillium cyclopium
Penicillium digitatum
Penicillium funiculosum
Penicillium frequentans
Penicillium lilacinum
Penicillium puberulum
Penicillium purpurogenum
Penicillium radicum
Penicillium simplicissimum
Penicillium variabile
Phoma sp.
Populospora mytilina
Pullularia pullulans
Pythium sp.
Rhizoctonia solani
Rhizopus sp.
Schwanniomyces occidentalis
Sclerotium rolfsii
Torula thermophila
Trichoderma harzianum
Trichoderma viride
Yarrowia lipolytica

medium. A fall in pH due to production of organic acids during the growth of phosphate solubilizing microorganisms in liquid medium has been reported by various authors (Cunningham & Kujack, 1992; Illmer & Schinner, 1992; Nahas, 1996; Freitas *et al.,* 1997; Alam *et al.,* 2002; Seshadri *et al.,* 2002; Rashid *et al.,* 2004; Alikhani *et al.,* 2006; Chen *et al.,* 2006; Fankem *et al.,* 2008; Henri *et al.,* 2008; Chang & Shang, 2009; Collavino *et al.,* 2010; Gulati *et al.,* 2010; Scervino *et al.,* 2011; Song *et al.,* 2012; Sharma *et al.,* 2013; Kelel *et al.,* 2014; Paul & Sinha, 2015). Phosphate solubilizing microorganisms are found to produce acids like acetic, formic (monocarboxylic acids); lactic, gluconic, glycolic (monocarboxylic hydroxy acids); 2-keto gluconic (monocarboxylic keto acid); oxalic, succinic (dicarboxylic acids); malic (dicarboxylic hydroxy acids) and citric acid (tricarboxylic hydroxy acids) in liquid media. **Table 1.4** shows various organic acids produced by phosphate solubilizing bacteria to solubilize different inorganic phosphate sources.

However, studies indicating no correlation between drop in pH and phosphate solubilization have also been reported (Mehta & Bhide, 1970; Wani *et al.,* 1979; Goldstein, 1986; Asea & Kucey, 1988).

Bajpai and Rao (1971) reported that nonvolatile acids such as citric and lactic acids were produced by *Bacillus megaterium*, *Bacillus circulans* and *Escherichia freundii* from glucose in Pikovskaya's medium. Mishustin *et al.* (1972) demonstrated solubilization of tricalcium phosphate in liquid medium by critic, glutamic, succinic and oxalic acids depending on pH and formation of soluble calcium complexes. In contrast, Asea and Kucey (1988) and Parks *et al.* (1990) observed that there is no relationship between quantity of organic acids produced and the amount of phosphate solubilized, but the nature of organic acids produced had a relationship with amount

Table 1.4 - Organic acids produced by phosphate solubilizing bacteria to solubilize different inorganic phosphate sources

Name of bacteria	Phosphate source	Organic acids produced
Enterobacter agglomerans (Kim *et al.*,1997)	Hydroxyapatite	OA
Azospirillum lipoferum (Rodriguez *et al.*, 2004)	TCP	GA
Azospirillum brasilense (Rodriguez *et al.*,, 2004)	TCP	GA
Pseudomonas fluorescens (Vyas & Gulati, 2009)	TCP	GA, 2-KA, MA, SA, FA, CA
	URP	OA, GA, 2KA, LA, SA
	MRP	OA, GA, 2-KA, MA
	NCRP	GA, OA, 2-KA, SA, FA
Pseudomonas poae (Vyas & Gulati, 2009)	TCP	GA, 2-KA, MA, SA, FA, CA
	URP	OA, GA,2-KA, MA.
	MRP	OA, GA,2-KA, LA, MA.
	NCRP	OA, GA,2-KA, SA, CA.
Pseudomonas trivialis (Vyas & Gulati, 2009)	TCP	GA, 2-KA, MA, SA, FA, LA, CA.
	URP	GA, OA, 2-KA, MA, SA, FA, CA, LA
	MRP	GA, OA, MA, , FA,
	NCRP	GA, OA, 2-KA, , SA, FA, CA
Pseudomonas fluorescens (Fankem *et al*., 2008)	TCP	OA, TAA, CA, TA, GA, MA, SA, FMA, LA, OAA,
Pseudomonas aeruginosa	TCP	GA
Pseudomonas striata (Appanna *et al.*, (2007)		TA, CA, MA, SA and GXA
Serratia marcescens (Chen *et al.*, 2006)	Hydroxyapatite	GA, CA
	TCP	CA, GA, SA, LA.
Bacillus sp. (Vazquez *et al.*, 2000)	TCP	OA, 2-KA, SA, AA, LA, IVA
Bacillus licheniformis (Vazquez *et al.*,, 2000)	TCP	LA, VA, IBA, AA.
Burkholderia cepacia (Lin *et al.*, 2006)	TCP	GA, 2-KA
	RP	OA, CA, GA, SA, FMA, AA,
Rhizobium leguminosarum (Ok-Ryul *et al.*, 2008)	TCP	2-KA, OA, CA, GA, SA, FMA, AA.
Acinetobacter haemolyticus	TCP	CA, OA, SA, FA

Key: TCP: Tricalcium phosphate, URP: Udaipur Rock Phosphate, MRP: Mussorie Rock Phospahte, NCRP: North Carolina Rock Phosphate; 2-KA: 2-kelogluconic acid, AA: Acetic acid, CA: Citric acid, FA: Formic acid, FMA: Fumaric acid, GA: Gluconic acid, GXA: Glyoxalic acid, IVA: Isovaleric acid, LA: Lactic acid, MA: Malic acid, OA: Oxalic acid, SA: Succinic acid, TA: Tartaric acid, TAA: Transaconitic acid & VA: Valeric acid.

of orthophosphate released. Kim *et al.* (1997) suggested that production of organic acids like citric, oxalic and lactic acid may play an important role in hydroxyapatite solubilization but is not the sole reason for increase in concentration of soluble orthophosphate in the culture medium. *Pseudomonas fluorescens* produced higher concentrations of gluconic acid when cultured in the presence of zinc phosphate (Di Simine *et al.*, 1998). Among the different acids produced by bacteria, gluconic acid has been regarded as the key organic acid for phosphate solubilization. The biochemical basis of gluconic acid and 2-ketogluconic acid is well studied in Gram negative bacteria by Goldstein (1995). These bacteria oxidize glucose in periplasmic space by glucose dehydrogenase to gluconic acid. Latter causes solubilization of mineral phosphate. Some bacteria further convert gluconic acid to 2-ketogluconic acid by gluconate dehydrogenase, where, 2-keto gluconic acid is a stronger acid than gluconic acid. However, its role in phosphate solubilization is yet to be ascertained. Interestingly, in some bacteria gluconic acid secretion is dependent on orthophosphate deficiency (Bagyaraj *et al.,* 2000; Crespo *et al.,* 2011). Stephen and Jisha (2011) reported that gluconic acid production is the principle mechanism of phosphate solubilization by *Burkholderia* sp. (MTCC 8369) bacterium. Jog *et al.* (2014) have reported overproduction of malic acid as the mechanism of phosphate solubilization in *Streptomyces* spp.

1.2.5.1.2 Chelation

Chelating substances also play a role in solubilization of phosphate. 2-ketogluconic acid is known as a powerful chelator of cations such as calcium. It is effective in solubilization of inorganic phosphate compounds of calcium (Sperber, 1958; Katznelson & Bose, 1959). Luo *et al.* (1993) observed no significant relationship between pH and phosphate solubilization but there was significant relationship

between the total amount of succinic, oxalic, acetic and malic acid produced by bacteria. These authors opined that the amount of phosphate solubilized from aluminium, iron and calcium phosphates was due to chelating effects of organic acids. Humic and fulvic acids released during microbial degradation of plant debris are also good chelators of calcium, iron and aluminium present with insoluble phosphates (Stevenson, 2005).

1.2.5.1.3 Inorganic acid production

Sulphuric acid, nitric acid and carbonic acids are also reported to solubilize phosphate (Hopkins & Whiting, 1916; Rudolf, 1922; Sperber, 1958; Rodriguez & Fraga, 1999). However, the effectiveness of inorganic acid's contribution in orthophosphate release appears to be less effective or negligible as compared to organic acids (Rudolf, 1922; Vazquez, 1996). Inorganic acids are produced by nitrifying and sulphur oxidizing bacteria during oxidation of nitrogenous or inorganic sulphur compounds. Inorganic acids produced react with insoluble phosphate compounds and convert them into soluble forms (Khan & Zaidi, 2007).

1.2.5.1.4 Proton extrusion

Solubilization of phosphates has also been reported to occur even in the absence of acid production. Acidification of the medium due to H^+ excretion is reported as an alternate mechanism of inorganic phosphate solubilization. H^+ excretion originates from NH_4^+ assimilation and respiratory H_2CO_3 production (Juriank *et al.,* 1986). Illmer and Schinner (1995) reported that a species of *Pseudomonas* did not produce any organic acid even though the bacterium solubilized bound forms of phosphorus. A model highlighting the pumping out of the protons from the cell has been proposed by Krishnaraj (1996).

Typically, the release of protons is also linked to the extrusion of organic acid anions into the external media (Arvieu *et al.,* 2003; Casarin *et al.,* 2003). The amount of protons released into the external medium is often significantly influenced by nitrogen supply. In general, a greater reduction in pH together with more solubilized phosphate can be observed with NH_4 as the sole nitrogen source compared to NO_3, due to the extrusions of protons to compensate for NH_4 uptake (Roos & Luckner, 1984; Illmer & Schinner, 1995; Sharan *et al.,* 2008). In contrast, Reyes *et al.* (1999) found a decrease in phosphate solubilization by *Penicillium rugulosum* from various phosphorus bearing minerals like hydroxyapatite, iron phosphate and aluminium phosphate when higher concentrations of NH_4 were supplied. The authors attributed these findings to the repressive effect of easily metabolized nitrogen sources on secondary metabolic biosynthesis in fungi. The same study also showed that the assimilation of amino acid arginine as the sole nitrogen source can also lead to a decrease in pH and enhance mobilization of phosphate. For some microorganisms, release of protons due to assimilation of NH_4 is the only mechanism present to solubilize phosphate. Asea and Kucey (1988) screened *Penicillium bilaii* and *Penicillium fuscum*, for phosphate rock solubilization ability in the presence of NH_4 and absence of nitrogen source. Results of the study revealed that only *P. bilaii* exhibited decrease in pH and solubilized phosphate in the absence of nitrogen source.

In a study using *Pseudomonas fluorescens*, Park *et al.* (2009) showed that the form of carbon source (e.g. glucose versus fructose) has greater effect on release of protons than nitrogen source (e.g. NH_4^+ versus NO_3). This indicates that different species exhibit different mechanisms for release of protons. Proton release may only partly depend on the presence of NH_4^+ ions.

1.2.5.1.5 Exopolysaccharide production

The role of high molecular weight exopolysaccharides in phosphate solubilization from soil constituents has not yet been directly investigated *in situ*. Nevertheless, microbial exopolysaccharides can have an indirect effect on phosphate availability through their important role in soil aggregation and in increasing pore connectivity in soil, thereby facilitating soil water retention and movement (Aspiras *et al.,* 1971; Ionescu & Belkin, 2009). Exopolysaccharides and biosurfactants are produced by microorganisms largely in response to biofilm formation and stress. Studies on microbially produced exopolysaccharides have shown their ability to complex metals in soil (Ochoa-Loza *et al.,* 2001), from which it can be deduced that they must in some way influence phosphate solubility in soil. In pure culture, microbial exopolysaccharide has been shown to stimulate the dissolution of tricalcium phosphate synergistically with organic acid anions (Yi *et al.,* 2008). Furthermore, the rate of dissolution appears dependent on the microbial source and concentration of exopolysaccharide. Although there is some evidence to suggest that exopolysaccharide production is stimulated under phosphate deficiency, this does not appear to be a universal phenomenon in bacteria (Dephilippis *et al.,* 1991, 1993). Furthermore, exopolysaccharide production seems to be more dependent on the rate of nitrogen supply than on available phosphate (Danhorn & Fuqua, 2003; Wielbo & Skorupska, 2008).

1.2.5.1.6 Siderophore production

Siderophores are substances secreted by microorganisms that chelate iron. They show strong affinity for iron. Siderophores are produced by microbes during deficiency of iron. Many phosphate solubilizing microorganisms have exhibited production of siderophores (Vassilev *et al.*, 2006; Hamdali *et al.*, 2008). However,

direct relationship between siderophore production and phosphate solubilization is not known (Caballero-Mellado *et al.,* 2007). There is an impressive body of literature concerning iron mobilization by microbial siderophores, but only one study exists that has investigated the effect of microbial siderophores on phosphate availability. Reid *et al.* (1985) investigated the ability to increase iron and phosphate diffusion of two siderophores (desferrioxamine-B and desferriferrichrome) as compared to water using a root simulation technique. They found that desferriferrichrome increased phosphate diffusion 13-fold compared to water, whereas different concentrations of desferrioxamine-B exhibited only a small effect. Considering the occurrence of iron phosphates in soil, the large phosphate sorption capacity of iron hydroxides and the needs of microorganisms for iron, the lack of knowledge about siderophore enhanced phosphate solubilization is quite surprising.

1.2.5.2 Mechanisms of organic phosphate solubilization

1.2.5.2.1 Enzyme production

Organic phosphate solubilization is also called as organic phosphate mineralization. It occurs in soil by decomposition of plant, animal and microbial remains which contains complex organic phosphate compounds. The decomposition of complex organic matter is carried out by saprophytic bacteria, which release enzymes, which in turn release orthophosphates from organic compounds. The mineralization of organic phosphates like nucleic acids, phospholipids, phosphonates, phytic acid, polyphosphonates and sugar phosphates is carried out by the action of enzymes called as phosphatases or phosphohydrolases. They hydrolyse phosphoric acid monoester substrates into products which are orthophosphate ions and a free hydroxyl group molecule (Banerjee & John, 2005; Mahdi *et al.,* 2011).

Phosphatases are broad substrate range enzymes that catalyse the hydrolysis of both esters and anhydrides of H_3PO_4. These enzymes are responsible for soil organic phosphorus mineralization and the release of inorganic phosphorus needed by microorganisms and plants. They are classified as acid and alkaline phosphatases because their maximum activities occur at low and high pH ranges, respectively.

The biochemical mineralization of organic phosphate is mediated by either cell wall bound or free phosphatase enzymes, whose release is driven by phosphate demand. The role of these enzymes in phosphate cycling is reviewed extensively by Nannipieri *et al.* (2011). Typically, extracellular phosphatases than intracellular or membrane bound phosphatases are thought to be responsible for inducing large changes in soil solution phosphate concentration (Tabatabai, 1994). Its activities have been shown to be inhibited by increasing concentrations of orthophosphate, polyvalent anions and high concentrations of several metal ions like copper, iron, mercury and manganese. Lower concentrations of divalent cations like calcium, cobalt, magnesium and zinc have been found to act as enzyme activators (Quiquampoix & Mousain, 2005). These reports clearly show that the activity of enzymes released from phosphate solubilizing microorganisms are not simply related to their release rate but are also strongly influenced by soil properties such as mineral composition and pH. Both, acid and alkaline phosphatases are produced by phosphate solubilizing microorganisms based on the external environment (Priest, 1977). Acid phosphatase enzymes are found in acidic soil, whereas, neutral and alkaline phosphatase enzymes are found in soil with alkaline pH (Renella *et al.*, 2006). It is known that plant roots can also produce acid phosphatases. However, they are unable to produce alkaline phosphatases. Alkaline phosphatases are mainly produced by phosphate mineralizing microorganisms (Juma & Tabatabai, 1988;

Criquet *et al.,* 2004). Phosphatases produced by phosphate solubilizing microorganisms have higher affinity for organic phosphate compounds as compared to phosphatase enzymes from plants. Based on this, phosphatase enzymes from microorganisms and plants can be differentiated from each other (Tarafdar *et al.,* 2001; Richardson *et al.,* 2009). The relationship between phosphate solubilizing microorganisms and mineralization of organic phosphate due to phosphatase activity is poorly understood (Chen *et al.,* 2003). Ambiguous results have been reported about the correlation between increased phosphatase activity and orthophosphate concentrations in soil solution; with several authors reporting no relationship between the two (Olander & Vitousek, 2000; Criquet *et al.,* 2002, 2004). Some groups of researchers have found a positive correlation (Tate & Salcedo, 1988; Rojo *et al.,* 1990; George *et al.,* 2002) and some observed a negative relation between orthophosphate concentrations and phosphatase activity (Ali *et al.,* 2009). Extensive research on phosphatases is required to benefit sustainable organic farming especially in coastal ecosystems. Formulation of phosphate solubilizing bacterial biofertilizers will reduce the use of chemical fertilizers in agricultural fields.

1.2.6 Phosphate solubilizers as biofertilizers

Biofertilizers are the formulations containing viable microbial cells that are applied to soil for growth promotion of plants through direct or indirect mechanisms (Arora *et al.,* 2010). They are usually prepared as carrier based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy handling, long term storage and high effectiveness of biofertilizers. In a form of fine powder, the most common way of inoculation is seed inoculation in which the inoculant i.e. bacteria and carrier mixture is mixed with water to make slurry further mixed with seeds. Another way of inoculation is soil inoculation,

whereby a large population of a bacterial strain can be introduced into the soil (Mahdi *et al.,* 2010; Majid & Khan, 2014).

Various types of materials are used as carriers in powder form for seed or soil inoculation. They can be divided into four basic categories, soils (peat coal, clay and soil), plant waste materials (compost, farmyard manure, soybean meal, by product of sugar industry, agricultural waste, spent mushroom compost), inert materials (vermiculite, perlite, rock phosphate, calcium sulfate) and plain lyophilized bacterial cultures (Bashan, 1998). The properties of a good carrier material for seed inoculation are that, it should be: non-toxic to inoculant bacteria, plants and animals, easy to process and free of lump forming material, easy to sterilize by autoclaving or gamma radiation, available in adequate amounts, inexpensive and should have good moisture absorption capacity, good adhesion to seeds and good pH buffering capacity. Peat is the most frequently used carrier material for seed inoculation. For soil inoculation, carrier material with granular form such as peat, perlite, charcoal or soil aggregates are generally used. Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered like survival of the inoculant bacteria on seed, during storage period and in soil where it is applied (Thompson, 1984; Boraste *et al.,* 2009; Malusa *et al.,* 2012).

In recent years, inoculation of carrier based phosphate solubilizing bacteria have promoted growth and yield of *Cicer arietinum, Glycine max, Gossypium, Helianthus, Oryza sativa, Solanum lycopersicum, Vigna unguiculata.* and *Zea mays* plants (Kumar *et al.,* 2001; Sundara *et al.,* 2002; Sahin *et al.,* 2004; Wu *et al.,* 2005; Cakmakci *et al.,* 2007; Jilani *et al.,* 2007). Talc based formulation of *Bacillus subtilis* and *Pseudomonas fluorescens* has significantly promoted growth of *Cajanus cajan,*

Cicer arietinum, Gossypium, Oryza sativa, Saccharum officinarum, Solanum tuberosum and *Triticum* (Nakkeeran *et al.*, 2005).

Recent advancement in the field of biofertilizers is the formulation of liquid inoculants. Carrier based inoculants are progressively replaced by liquid inoculants as they have longer shelf life (Pindi & Satyanarayana, 2012).

With rise in world population, the demand for food by the end of 2030 will be much higher. Therefore, scientists are looking at alternatives to increase production through improvement in agricultural productivity in infertile and barren soil in sustainable manner. Infertile and barren land is associated with stress due to salinity, alkalinity, toxic metals and organic compounds. These limit the yields and profits of agricultural crops due to osmotic effects, toxicity of salt ions and the changes in the physical and chemical properties of soil (Yamaguchi & Blumwald, 2005; Shahbaz & Ashraf, 2013). Recent studies indicate that microbes can help crops to cope with salinity and alkalinity stress (Dimkpa *et al.,* 2009). They can be a relatively inexpensive way to develop barren and infertile land into agriculturally sound land.

Alkaline soils with pH greater than 8.0 due to presence of carbonate, bicarbonate and borate are often associated with high salinity. Such conditions increase the precipitation of available phosphorous and interfere with activity of phosphate solubilizing microbes. Therefore, microorganisms which thrive under alkaline and saline conditions and yet capable of phosphate solubilization are of importance in increasing soil fertility.

Most of the biofertilizers available in market have been formulated for use in neutral soil. Therefore, there is a pressing need to isolate efficient phosphate solubilizing

bacteria, capable of proliferation in alkaline and saline soil and yet capable of phosphate solubilization.

1.2.2. Alkaliphilic bacteria

Alkaliphiles are defined as a diverse group of microorganisms that thrive in highly alkaline environments with optimum pH for growth being 9 or above (Horikoshi, 1999). The term alkaliphiles denotes *alca*- Arabic soda ash, *phile* – loving. They consist of two main physiological groups, alkaliphiles and haloalkaliphiles.

Based on the pH preference, alkaliphilic microorganisms can be classified into two broad categories, facultative alkaliphilic and obligate alkaliphilic microorganisms (Horikoshi, 1991). Facultatively alkaliphilic microorganisms show optimal growth between pH 10.0 or above but can grow well in neutral pH range. Obligate alkaliphiles grow optimally above pH 10.0 but do not grow below pH 9.0 (Krulwich & Guffanti, 1989; Horikoshi, 2006). Another class of microorganisms, show optimal growth in the pH range of 7.0 – 9.0 but grow above pH 9.5. These are termed as alkalitolerant microorganisms (Horikoshi, 2006).

The key difference between these two groups of alkaliphiles is in the membrane lipids. The fatty acid composition of the phospholipids in membranes of facultative alkaliphiles appears to have greater membrane integrity at near neutral pH values than the more unsaturated, highly branched fatty acids in membranes of obligate alkaliphiles (Clejan *et al.*, 1986). In order to more clearly distinguish alkaliphiles and alkalitolerant, the characteristic feature of alkaliphiles is the growth at high pH and an inability to grow at near-neutral pH values such as 6.5 (Horikoshi, 1999).

The optimal pH value of 9.5 is chosen for growth of alkaliphiles as it is approximately the upper limit of cytoplasmic pH range that is compatible with the growth of alkaliphilic bacteria studied till date (Sturr *et al.,* 1994; Krulwich *et al.,* 1998). The term facultative alkaliphile has been applied to species and strains that are able to grow between pH 6.5 – 7.5 and also at alkaline pH range. Alkaliphiles that cannot grow at neutral pH range (pH 6.5 – 7.5) are termed as obligate alkaliphiles.

Haloalkaliphiles, are an interesting class of extremophiles. A group that grows optimally at pH values at or above 9 along with high salinity up to 33% (w/v) NaCl (Horikoshi, 2011). The organisms living in such dual extreme environments possess special adaptation strategies that make them interesting towards exploration of their applications (Grant & Sorokin, 2011).

1.2.2.1. Distribution and habitat of alkaliphilic bacteria

Alkaliphilic bacteria are widely distributed in diverse natural and man-made alkaline niches throughout the globe. Studies by Horikoshi *et al.* (2006) indicate that alkaliphilic bacteria thrive not only in alkaline regions but also in neutral environment; although, count of alkaliphiles is higher in alkaline environment than in neutral and acidic environment (Horikoshi, 1999; Desai *et al.,* 2004).

Naturally occurring soda lakes and soda desserts are hot spots for isolation of promising alkaliphilic bacteria (Grant *et al.,* 1979; Khmelenina *et al.,* 1997; Bryantseva *et al.,* 1999; Zhilina *et al.,* 2004; Banciu *et al.,* 2008; Mamo & Mattiasson, 2016). Alkaliphilic bacteria have been reported from the deep sea of the Mariana Trench and Iheya Ridge (Takami *et al.,* 1997; Lu *et al.,* 2001). Interestingly,

alkaliphilic bacteria have even been isolated from guts of termites and larvae (Taksawan *et al.*, 2005; Aizawa *et al.*, 2010).

Anthropogenic activities such as mining, paper and pulp processing, dye making, chemical fertilizer preparation in agro-chemical industries, food and textile processing, electroplating, etc. make the surrounding area alkaline (Grant & Tinball, 1986; Borkar, 2015a). This enables enrichment of alkaliphilic bacteria in such man-made alkaline environments (Ntougias & Russell, 2000; Horikoshi, 2006).

Few researchers have studied alkaliphilic bacteria for bio-prospecting from various non-alkaline coastal ecosystems viz. mangrove, salt pan and sand dunes (Desai *et al.*, 2004; Godinho *et al.*, 2010; Kamat & Kerkar, 2011; Surve *et al.*, 2012; Borkar, 2015a).

1.2.2.2. Mechanisms of adaptations to high pH

Alkaliphilic bacteria are faced with problems of pH homeostasis (Borkar, 2015a). In the course of evolution, they have developed active and passive adaptive mechanisms that allow them to flourish in extremely high pH environment. The mechanisms have been thoroughly studied and investigated during the past decades (Mamo & Mattiasson, 2016).

Alkaliphilic bacteria change the external pH to a value suitable for growth (Sturr *et al.*, 1994; Krulwich *et al.*, 1998). Cell wall of alkaliphiles plays a key role in protecting the cell (Aono *et al.*, 1999). Alkaliphilic bacterial cell walls contain basic proteins or polyamines which confer the property of high buffering capacity. Cell wall of alkaliphilic bacteria is made up of acidic polymers like galacturonic acid, glucuronic acid, glutamic acid, aspartic acid, phosphoric acid and teichuronopeptides. These acidic polymers help in absorbing sodium and hydronium

ions and repulsing hydroxide ions. High membrane lipid:protein ratio is present in the cell membrane of alkaliphiles. The cytoplasmic membrane of alkaliphiles lowers the cytoplasmic pH by means of solute transport, mainly by Na^+/H^+ antiporter (Kitada *et al.,* 1994; Krulwich *et al.,* 1997).

Growth at alkaline pH is associated with metabolic shift that promote increased acid production, enhanced expression of transporters and enzymes. This facilitates the capture and retention of protons and change in cell surface composition that helps to retain the cytoplasmic protons. This indicates that alkaliphiles use combination of different strategies to thrive in their pH environment. The ability of producing enzymes that remains active and stable at alkaline conditions is another important strategy that has often been less pronounced when it comes to adaptive mechanism of alkaliphiles.

Alkaliphilic bacteria have proved promising in the field of biotechnology and environment (Singh, *et al.,* 2012). They have been widely investigated for the production of enzymes, acids, antibiotics, degradation of pollutant compounds, etc. (Borchert *et al.,* 2007; Singh *et al.,* 2012; Kanekar *et al.,* 2014; Mamo & Mattiasson, 2016). Applications of alkaliphiles in the field of agriculture are hardly known. Godinho (2015) has reported occurrence of agriculturally important alkaliphilic bacteria from coastal sand dune ecosystems of Goa. The author isolated phosphate solubilizing and siderophore producing alkaliphilic bacteria which were identified as *Brevibacterium* sp., *Brochothrix* sp., *Cellulomonas* sp. and *Microbacterium* sp..

India has a long coast of about 7500 km including its island territories (Kumar *et al.,* 2006). Its coastal zones, the interface of terrestrial and marine, are highly fragile and dynamic entities subject to waves, tides, currents, winds, in addition to anthropogenic

activities. Coastal zones support highly productive diverse habitats and provide a unique ecological niche to diverse organisms (Venkataraman & Wafar, 2005; Senapati & Gupta, 2014).

1.2.3 Coastal ecosystems of Goa

Goa is a tiny state on the west coast of India with an area of 3702 km^2. It harbours natural and man-made ecosystems along its 120 km long coast. The diverse coastal ecosystems found in Goa are estuaries, mangroves, khazan lands, salt pans and sand dunes (Fernandes & Achutankutty, 2010; Prasanna *et al.,* 2015). Mandovi and Zuari are two major rivers which are lifeline of the state (Shetye *et al.,* 1995). The coast of Goa has linear and wide beaches with sand dunes in the north and rocky cliffs along the south. Goa has seven estuaries and on the intertidal zones of all these estuaries are flat lands formed with silty sand and silty clay along with abundant organic matter. The estuaries of rivers are Terekhol, Chapora, Mapusa, Mandovi, Zuari (North bank) in North Goa and Sal, Talpona, Galgibag and Zuari (South bank) in South Goa. Mangroves, mudflats, khazan lands and man-made saltpans occur along the estuaries (Govindrajan *et al.,* 1974; Gokul *et al.,* 1985).

Each of the above mentioned eco-niches have special structure, functions, productivity and biodiversity (Untawale, 2006). These ecosystems are conspicuously associated with the microbial flora responsible for several important biological processes (Uroz *et al.,* 2009). Microorganisms in these eco-niches survive with exposure to varying levels of salinity, pH, temperature and moisture content due to the continuous influence of tidal cycle, freshwater influx recurring daily and seasonal changes (Vassilev *et al.,* 2012).

1.2.3.1 Mangrove ecosystem

Mangroves are an ecological group of halophilic shrubs and trees. They are wetland forests, mainly found at the intertidal zones of estuaries and backwaters, playing a crucial role along the coastline (Augustinus, 1995). They are up to 30 m high with interlacing roots (pneumatophores) above the ground that inhabit the coasts of tropical or subtropical sea (Sahoo & Dhal, 2009). Mangrove area in Goa is approximately 2000 ha (Untawale *et al.,* 1982; Selvam, 2003).

The estuaries of rivers housing mangroves in Goa are Terekhol, Chapora, Mapusa, Mandovi, Zuari, Sal, Talpona and Galgibag. Besides these, mangrove cover is also present in Kumbharjua canal, which joins Mandovi and Zuari rivers. Diverse species of mangroves are found in Chorao Island. The mangrove flora of Goa consists of 15 species belonging to 10 genera and 7 families. The dominant mangroves are *Acanthus ilicifolius, Acrostichum aureum, Avicennia officinalis, Rhizophora mucronata, Sonneratia alba* and *Sonneratia caseolaris* (Wafar *et al.,* 1997). These coastal forests are important in maintaining the health of estuaries.

Mangrove ecosystem has a peculiar physical and nutritional environment. Its physical environment is characterized by high humidity, temperature and alternate fresh and saline water runoffs. It is nutritionally rich due to the continuous shedding of foliage. This ecosystem supports abundant and diverse microorganisms which tirelessly attack organic matter to form detrital matter, build up their own body blocks and become endless food sources for other animals in the mangrove ecosystem (Jagtap, 1987; Jagtap *et al.,* 1993). These microorganisms though have continuous availability of nutrients, are influenced by tidal variations, salinity and anthropogenic activities which add substances through run offs from the terrestrial

ecosystems. Anthropogenic activities include agriculture practices like excessive use of fertilizers, pesticides and activities of industries such as mining, barge building yards, dwellings, ground water pumping, railway embankment, sewage disposal, etc. The interaction of microbial flora with anthropogenic substances has resulted in the proliferation of physiologically diverse microflora in mangrove ecosystems.

Scientists have isolated bacteria for bio prospecting from mangrove ecosystems of Goa. Several studies have reported the occurrence of phosphate solubilizing bacteria in the coastal areas of Goa (Ayyakkannu & Chadramohan, 1971; Naik *et al.,* 1982; De Souza *et al.,* 2000; Dastager & Damare, 2013). Siderophore producing bacteria have been reported from mangrove ecosystem in Goa and Konkan (Panchanadikar, 1993; Gaonkar *et al.,* 2012; Kharangate-Lad & Bhosle, 2016). *Pseudomonas nitroreducens* TSB.MJ10, isolated from the mangroves at Merces, Goa, in the vicinity of a petroleum pump, showed interesting characteristics like polyhydroxyalkanoate production, feruloyl esterase activity and siderophore formation, besides exhibiting strong and stable hydrocarbon-emulsifying activity (Rawte *et al.,* 2002; De Sousa & Bhosle, 2012). Protease producing alkaliphilic bacteria have been reported from mangrove ecosystems of Goa (D Costa, 2013). Alkaliphilic bacteria degrading sodium benzoate, tyrosine and aniline have been studied from mangrove ecosystem of Goa (Desai *et al.,* 2004; Krishnamurthy, 2015).

1.2.3.2 Khazan land ecosystem

Khazans of Goa, are salty, low lying, flat agricultural lands which are subject to inundation by neighbouring river. Khazan lands consists of four main parts, the dyke (*bundh*), the sluice gate (*manas*), the internal water body (*poiim*) and the rice fields. The first three parts play a role in the cultivation and maintenance of the rice fields.

The dyke is a 2 - 2.5 m high dam. It is made up of clayey soil from the surrounding land. Its function is to protect the khazan land during high tide by preventing the entry of brackish water from the estuary. It also maintains the water level in the khazan during monsoon season. The sluice gate acts as a one way valve by allowing water from the internal water body to flow into the neighbouring estuary during low tide. It automatically closes during high tide preventing the estuarine water from entering the khazan land. However, during the monsoon, the functions of the sluice gate are stopped in order to maintain water level in the rice fields. The internal water bodies in rice fields are interlinked and connected to the estuary through the sluice gate. Rice fields are elevated portion of the khazan land for paddy cultivation during the monsoon season. Fishing in the khazan is a secondary activity (Rubinoff, 2001; Sonak *et al.,* 2005; De Souza, 2007).

The tidal estuaries of Goa stretch from some 30 km in length and on either side lie khazans (Shetye *et al.,* 1995). Cultivation of khazan lands dates back at least 3,000 years. Eight of the eleven talukas in Goa have a total of 17,500 ha under khazans. Prominent khazan lands in Goa are found in Divar, Markaim, Dongrim, Paliyem, Parcem, Mayem, Calapur, Rachol, Curtorim, Loutulim. Other locations of khazan lands include the estuarine basins of Tiracol, Chapora, Ribandar, Cavellosim, Sancoale, Siolim, Carambolim, Loutolim, Quellosim, Baga, Mandovi-Zuari, along Cumbarjua canal, Sal, Talpona and Galjibaga river (Kamat, 2004; Sonak *et al.,* 2005).

Salinity tolerant traditionally cultivated rice varieties are available which are grown in Khazan lands of Goa. The high salinity tolerant rice varieties namely, Korgut, Muno and Assgo are regularly cultivated in khazan lands of Goa. Rice varieties like

Kalo Novan, Kalo Damgo and Bello are cultivated rarely due to the introduction of high yielding rice varieties. Other salinity tolerant varieties of rice known are Damgo, Kalo Korgut, Khochro and Shiedi (Bhosle & Krishnan, 2011).

Occurrence of arbuscular mycorrhizal fungi belonging to *Acaulospora, Glomus* and *Scutellospora* have been reported from khazan land ecosystems in Goa (Rodriguez & Anuradha, 2009). There is however, no knowledge of microbial diversity in khazan lands of Goa. Therefore, biodiversity of khazan land needs attention (Kamat, 2013).

1.2.3.3 Sand dune ecosystem

Sand is the by-product of weathered rocks from inland regions. These inland rock formations are eroded by rain and wind and washed into rivers that eventually flow into the ocean. Coastal dunes are made up of sand that is piled high by wind. In sea, the sand is shifted up the coast by currents and wave action. Wave action deposits the sand on the beach. It is then blown into the dunes by prevailing onshore winds. Shells, corals, and other skeletal fragments provide sediments to some beaches, especially to those in the tropics.

According to Desai (1995), the sand dunes of Goa can be classified into embroynic dune, mid shore dune and hind shore dune. Embroynic dune is nearest from the sea and is commonly not vegetated. This zone is formed due to the delivery of sand by the action of waves. The predominant plants found growing in this zone are *Ipomea pes-caprae, Spinifex littoreus* and a few other herbaceous species. In the mid shore dune, mostly the shrubs make up the vegetation. The common species found in mid shore dune are *Clerodendrum inerme, Leucas aspera, Spermacoce stricta* and *Vitex negundo*. Hind shore dune is characterized by the presence of trees with long root system. The dominant plants growing in this zone are *Anacardium occidentale,*

Casuarina equisetifolia, Clerodendrum inerme, Cocos nucifera, Pandanus tectorius, Vitex negundo, etc.

Normal soil forming processes do not affect sand dunes. Vegetation plays a dominant role in determining the size, shape, and stability of the fore dunes. Until dunes are vegetated, they are constantly growing and shifting. Aerial parts of the vegetation obstruct the wind and absorb wind energy. Wind velocity near vegetation is thus reduced and hence, sand deposits around the vegetation. A characteristic of dune vegetation, particularly the grasses growing under these conditions, is its ability to produce upright stems and new roots in response to sand covering. If plants do not continue to grow more rapidly than the rate of sand deposition, the arresting action of the plant ceases. Successive stages of plant growth and sand deposition result in an increase in width and height of the dunes.

In Goa, five regions which have prominent sand dune belts are Querim – Morjim, Chapora – Sinquerim; Caranzalem – Miramar, Velsao – Mobor, and Talpona – Galgibag. In addition to these, the coastal stretch of Goa also consists of several sandy areas, secluded coves with cliffs, rocky shores, headlands, promontories and weeded or bare hill slopes. Some islands with forest cove are found (Mascarenhas, 1998).

Few reports on bacteria from sand dune ecosystems of Goa are available. Godinho, (2007) has investigated plant growth promoting neutrophilic and alkaliphilic rhizospheric bacteria from *Ipomoea pes-caprae* and *Spinifex littoreus* from Miramar and Morjim sand dunes of Goa. The isolates obtained in this study exhibited growth promotion of *Solanum melongena*. Sodium benzoate degrading *Pseudomonas aeruginosa* TMR2.13 was isolated from Miramar sand dune on western coast of Goa,

India (De Sousa & Bhosle, 2012). Nayak *et al.* (2013) identified polyhydroxyalkanoate accumulating bacteria belonging to genera *Bacillus* and *Paracoccus*. Protease producing alkaliphilic bacteria have been reported from salt pan ecosystems of Goa (D Costa, 2013).

1.2.3.4 Salt pan ecosystem

Salt making is a traditional occupation in coastal Goa. Saltpans are man-made hyper saline ecosystems in which crude salt is extracted during summer. They consist of series of rectangular beds; each bed is walled on all four sides and joined to the next one through an opening in the common wall. A sluice gate in a wall of the first bed allows influx of saline water from a creek during high tides. Here, the water is allowed to evaporate. As the water evaporates, it is allowed to enter into the next tank where it crystallizes. Salt crystals are sprinkled in the last tank to initiate and catalyse the crystallization process. A sequential precipitation of calcium carbonate, calcium sulphate and sodium chloride (crude salt) occurs, while concentrated magnesium chloride brine remains in the course of evaporation of sea water (Oren, 1990).

The fresh salt is then harvested and is allowed to dry naturally forming heaps on the bunds. Salt curing in saltpans takes place during February-May and crude salt is extracted during summer. During the remaining part of the year, salt pans are used as fields to raise fish and shrimps. The biological process that develops as salinity increases in the evaporating saltpan forms a unique saline ecosystem which is yet to be understood better. It is a sensitive process and depends on factors like temperature, depth, turbidity of brine, physicochemical process occurring during salt production and the overall design of the saltpan. Organisms developing in the saltpan

constitute a biological system, which interacts with the physico-chemical process and is vital to the production of salt.

The biological system affects production process of the salt pan in three ways. First, it produces the appropriate quantity of organic matter, which is a source of energy for the various organisms, and reduces the permeability of the bottom of the ponds, thus minimizing brine losses, particularly at low concentrations. Second, it renders red hue to the brine in the salt pan, facilitating enhanced absorption of solar radiation and shielding solar radiation reflection from the white salt bed. The red colour of the brines in the pans is attributed to *Aphanothece*, *Dunaliella salina* and *Halobacterium.* These bacteria act as catalysts in the crystallization of better quality salt, rich in beta-carotenoids adding nutritive value to the salt (Korovessis & Lekkas, 1994).

Microbial communities in salt pans have an immense potential as an untapped resource for the discovery of novel microbes. Cyanobacteria (Shaikh, 1996), fungi (Nazareth, 2005; Nayak *et al.,* 2012) archaea (Braganca & Furtado, 1999; Raghavan & Furtado, 2005; Chaudhary *et al.,* 2014; Srivastava *et al.,* 2015), actinobacteria (Ballav *et al.,* 2015) and bacteria (Kerkar, 2004; Raghavan & Furtado, 2005; Mani *et al.,* 2015), have been reported from salt pans of Goa. Diversity studies in the salt pans of Goa indicate that haloarchaea belonging to genera *Crenarchaeota, Euryarchaeota, Halococcus, Haloarcula, Haloferax* and *Halorubrum* are dominant (Ahmed *et al.,* 2011; Mani *et al.,* 2012). Kerkar *et al.* (2012) isolated bacteria from biofilms of salt pans, which produced indole acetic acid, a growth promoter. Goan salt pans produce bioactive compounds showing potential activity for anticancer, antifungal, antioxidant, anti-dementia, antidepressant, anti-Parkinson and antipsychotic (Kamat & Kerkar, 2011; Ballav *et al.,* 2012).

As reviewed, numerous bacteria with bioprospecting potential occur in coastal ecosystems of Goa. Considering the immense potential of such indigenous bacteria from these econiches, it is envisaged to study phosphate solubilizing alkaliphilic bacteria from coastal ecosystems of Goa.

Chapter II

The mechanisms involved in solubilization of inorganic and organic phosphate compounds by alkaliphilic bacteria from coastal ecosystems of Goa

2.A.1 INTRODUCTION

Phosphorus is known as the master key element among all the elements required for plant growth. It plays an important role in plant's physiological and biochemical processes (Rodriguez & Fraga, 1999; Igual *et al.,* 2001; Cordell & White, 2013). Phosphorus occurs naturally in soil (Rodriguez & Fraga, 1999). The diverse soil phosphate forms can be categorized as soluble phosphates (orthophosphate), insoluble inorganic and insoluble organic phosphates (Rodriguez *et al.,* 2006; Khan *et al.,* 2009a; Khan *et al.,* 2014b). Phosphorus is taken up by plants mostly as orthophosphate ion ($H_2PO_4^-$) (Jones & Oburger, 2011). Due to the large reactivity of phosphate ions with numerous soil constituents, it is the least mobile element in most soil conditions (Hinsinger, 2001; Mahdi *et al.,* 2011). Therefore, it is not readily available for plant's uptake. However, numerous bacteria can improve plant phosphorus nutrition by solubilizing inorganic and organic phosphates (Banik & Dey, 1982; Richardson, 2001). Environmental factors like high alkalinity and salinity of soil interfere with activity of phosphate solubilizing bacteria and also increase the precipitation of available phosphorus (Nautiyal *et al.,* 2000). Therefore, bacteria which are able to thrive in saline-alkaline conditions and also capable of phosphate solubilization can be useful in increasing the fertility of alkaline and saline soils.

In India, 6.73 million ha land is salt affected, out of which 3.77 and 2.96 million ha are covered with alkaline and saline soils, respectively. The total area under coastal saline in the country is estimated about 1 milion ha and these areas are situated in Andhra Pradesh, Goa, Kerala, Orissa, Tamil Nadu, and West Bengal.

Natural and man-made ecosystems observed along the coast of Goa are mangrove plantation, khazan lands, sand dunes and salt pans. Each of these eco-niches has

special structure, functions, biodiversity and productivity (Untawale. 2006). These ecosystems are conspicuously associated with the microbial flora responsible for several important biological processes (Uroz *et al.,* 2009). Bacteria in these eco-niches survive with exposure to varying levels of salinity, pH, temperature and moisture content due to the influence of tidal cycle, freshwater influx and recurring seasonal changes (Vassilev *et al.,* 2012). Studies indicate that stress tolerant bacteria may be found in such ecosystems which are in contact with different levels of salinity and alkalinity. Therefore, coastal ecosystems of Goa are hotspots for isolation of phosphate solubilizing bacteria promoting growth of plants in alkaline and saline environments. These bacteria could be beneficial for crops grown in saline-alkaline soils, e.g. khazan lands in Goa (Sonak, 2006). This chapter describes the isolation of alkaliphilic bacteria from coastal ecosystems of Goa and their ability to solubilize phosphate.

2.A.2 MATERIALS AND METHODS

2.A.2.1 Collection of samples

Samples were collected in the pre-monsoon period during the months from January to May 2010. Sediment and water samples were collected from 13 mangrove habitats and 4 khazan lands **(Figure 2.1, Table 2.1)**. Only sand and sediment samples were collected from 4 sand dunes and 2 man-made solar salterns. Samples were collected from a depth of 10 to 15 cm using sterile spatula. They were stored in sterile plastic bags and bottles, and brought to the laboratory under cold conditions. The samples were immediately processed for physico-chemical analysis and isolation of alkaliphiles as described below.

2.A.2.2 Physico-chemical analysis of samples

Ten gram of sediment sample was suspended in 50 ml of distilled water in a 100 ml Erlenmeyer flask. The flask was kept under shaking conditions for 15 min at 150 rpm. After 15 min, the sediment was allowed to settle down. This sediment suspension was used. Water samples were used directly. The pH and salinity of both sediment and water samples were determined using pH meter and argentometric titration method, respectively (Clesceri *et al.,* 1999). Phosphate content of the sediment samples was determined using Bray's method (Bray, 1948).

2.A.2.3 Isolation of alkaliphiles

Alkaliphilic bacterial population was enumerated by plate count method (Gee *et al.,* 1980). One gram of the sediment sample or 1 ml of water sample was suspended in sterile physiological saline making the total volume up to 10 ml. Sediment suspension was prepared in a 50 ml Erlenmeyer flask and incubated under shaking conditions for 30 min at 150 rpm. The sediment suspension was allowed to settle down before use. Serial dilutions of the sediment and water samples were prepared and 0.1 ml of appropriate dilutions were plated out. The medium used for isolation of alkaliphilic bacteria was Polypeptone Yeast Extract Glucose Agar (PPYG) **(Appendix A)**. Final pH of the sterile medium was adjusted aseptically to pH 10.5 using 10% Na_2CO_3 solution. Plates were incubated at 30°C and examined after 48 hours for bacterial colonies. Viable count of alkaliphilic bacteria was determined. Morphologically distinct colonies were selected, purified, and maintained on PPYG medium slants at 4°C.

2.A.2.4 Characterization of alkaliphiles

Gram reaction of the alkaliphilic bacterial isolates was determined by Gram's staining and Gram character was confirmed by string test by KOH method (Buck, 1982). Alkaliphilic nature of the isolates was studied by spot inoculating the isolates in triplicates on PPYG agar plates with pH 7, 8, 9, 10 and 11. Plates were incubated at 30°C and examined after 48 hours for bacterial colonies.

2.A.2.5 Screening of isolates for inorganic and organic phosphate solubilization:

2.A.2.5.1 Inorganic phosphate solubilization

Phosphate solubilizing activity of 141 isolates was evaluated by plate method on modified Pikovskaya's (PVK) medium **(Appendix A)** using tricalcium phosphate as a sole source of phosphorus (Pikovskaya, 1948). PVK medium was modified by adjusting the pH to 10.0 for the growth of alkaliphiles. Inoculum was prepared by inoculating isolates in modified PVK medium **(Appendix A)** and incubating at 30°C on 150 rpm shaker conditions till O.D. reached 0.700 at 600 nm. Inoculum was prepared in this manner for all the experiments of this chapter. Inoculation of 10 µl of inoculum was carried out in triplicates on PVK plates with freshly grown cultures of the isolates. Inoculated plates were incubated at 30°C and observed at 24 hours interval up to 5 days for phosphate solubilization. Bacterial isolates solubilizing inorganic phosphate show visible dissolution zone or halo around the colonies. Halozone diameter and colony diameter were recorded to determine the solubilization Index (SI) (Afzal & Bano, 2008). SI was determined by dividing halozone diameter with colony diameter.

2.A.2.5.1.1 Effect of different pH and salt concentration on inorganic phosphate solubilizing isolates

Isolates showing positive phosphate solubilization were spot inoculated on Pikovskaya's agar in triplicates at different combinations of pH and salinity. The four combinations were pH 8 with 10% NaCl, pH 8 with 25% NaCl, pH 10 with 10% NaCl and pH 10 with 25% NaCl. Growth and phosphate solubilization ability of the isolates were noted after 48 hours of incubation at 30°C.

2.A.2.5.2 Organic phosphate solubilization

Organic phosphate solubilizing activity of the 141 alkaliphilic isolates was determined using Mineral Salts Medium (MSM) **(Appendix A)** containing disodium-p-nitrophenyl phosphate (p-NPP; 1 mM) as a sole source of organic phosphate (Sitdhipol *et al.,* 2012). The tubes were inoculated with 0.1 ml of freshly grown cultures of the isolates and incubated at 150 rpm and 30°C for 24 hours. Bacterial isolates producing phosphatase showed yellow colouration due to the liberation of para-nitrophenol from p-NPP in the culture broth. The culture broths were centrifuged at 10,000 rpm for 10 min. Absorbance of the supernatant was recorded spectrophotometrically at 405 nm.

2.A.2.5.2.1 Effect of glucose and phosphate ions on organic phosphate solubilizing isolates

Freshly grown 18 hour old phosphatase producing isolates were spot inoculated on MSM agar with different combinations of glucose, K_2HPO_4 and p-NPP **(Appendix A)**. In the first medium combination, p-NPP and K_2HPO_4 were present and glucose was absent. Second medium combination had p-NPP and glucose present but the medium was devoid of K_2HPO_4 ions. In the third medium combination, p-NPP,

glucose and K_2HPO_4 ions were present while, the fourth combination was devoid of both K_2HPO_4 and glucose, containing only p-NPP as the only source of carbon and phosphate. The experiment was carried out in triplicates. Growth and zone of yellow colouration were recorded after 24 hours of incubation at 30°C.

2.A.2.6 Identification of selected phosphate solubilizing isolates:

Selected phosphate solubilizing isolates were identified using morphological, cultural, biochemical and molecular methods.

2.A.2.6.1 Morphological characterization

2.A.2.6.1.a Gram staining and spore staining of the selected phosphate solubilizing isolates

In addition to Gram staining, spore staining was performed after 48 hours of culture growth by Schaffer and Fulton's Method (Schaeffer & Fulton, 1933).

2.A.2.6.1.b Scanning electron micrographs of the selected phosphate solubilizing isolates

Scanning electron microscopy (SEM) was performed for morphological characterization of the selected isolates. Isolates were grown on Pikovskaya's agar. Eighteen hours grown cells of the isolates were washed twice with phosphate buffered saline (PBS) **(Appendix B)**, centrifuged and resuspended in phosphate buffered saline. Smear was prepared on coverslips (Prior & Perkins, 1974). After air drying, the smears were fixed with 2.5% (v/v) gluteraldehyde overnight at room temperature. The coverslips with the smears were transferred in PBS for washing. Coverslips were then placed in 30% (v/v) acetone for 10 minutes and subsequently transferred in 50% (v/v), 70% (v/v) and 90% (v/v) for 10 minutes each and finally in 100% acetone for 30 minutes. After dehydration step, the coverslips containing

samples were placed on stubs. Stubs were then placed in sputter coater (JOEL JFC 1600). After sputtering, each stub was placed into the electron microscope sample chamber and observed with JOEL JSM-6360LV electron microscope.

2.A.2.6.2 Biochemical characterization

Biochemical tests were carried out to characterize the isolates (Starr *et al.,* 1981). The tests were sugar fermentation (glucose, xylose, maltose, lactose, fructose, sucrose, trehalose, galactose, sorbitol and mannitol), indole production, Methyl-red test, Voges-Proskauer test, citrate utilization, enzyme production (urease, gelatinase, phosphatase, amylase, catalase, oxidase, ornithine decarboxylase and lysine decarboxylase), nitrate reduction and motility. Based on the results obtained, the isolates were tentatively identified using Bergey's Manual of Determinative Bacteriology, Volume III **(Appendix A, B)** (Garrity *et al.,* 2009).

2.A.2.6.3 Buffering capacity of the isolates

Buffering capacity was determined using the method proposed by Krulwich *et al.* (1985). Selected alkaliphilic bacterial isolates and neutrophilic *Bacillus subtilis* and *Escherichia coli* were used. Alkaliphilic isolates were grown in PPYG broth at pH 10.0 and neutrophilic isolates in PPYG broth at pH 7.3 **(Appendix A)**. After 18 hours of incubation at 30°C, cultures were harvested by centrifugation at 10,000 rpm for 15 min. Supernatant was discarded and pellet was washed twice with and finally resuspended in KCl solution (200 mM). One ml of cell suspension was mixed with 1 ml of NaOH (0.1 N) and centrifuged at 10,000 rpm for 10 min. A volume of 0.5 ml of the supernatant was diluted with equal volume of distilled water and used for estimation of protein by Folin-Lowry method. Bovine serum albumin was used as standard (Lowry *et al.*, 1951). Volume of cell suspension corresponding to 5 mg of cell protein was taken for titration against 0.05 M KOH. Cell suspension was taken in

a beaker and titrated using 10 µl aliquots of KOH (0.05 M). The pH change was noted using pH meter after every addition of 10 µl KOH. The whole cell buffering capacity (Bo) was measured as nanomoles of hydroxyl ions consumed to change one pH unit per mg of protein. Buffering capacity is expressed in terms of OH^- ions consumed per mg of cell protein per unit change in pH.

$$Bi = Bo - Bt$$

where, Bo – Whole Cell buffering capacity

Bt – Permeabilised cell buffering capacity

Bi – Cytoplasmic cell buffering capacity

Bo and Bt are determined experimentally for every small addition of base (10 µl/50 nanomoles of OH^- ions) in which pH change is approximately 0.1 pH unit or more.

2.A.2.6.3.1 Permeabilisation of cells using Triton X-100

Volume of cell suspension corresponding to 5 mg protein was treated with 10 ml of Triton X-100 (10%) prepared in KCl solution (200 mM). The contents were mixed and allowed to stand for 5 minutes and centrifuged. The pellet obtained was washed and resuspended in KCl (200 mM) and titrated against KOH (0.05 M) till the pH changed by 1 unit. The treated cell buffering capacity (Bt) was measured and the internal or cytoplasmic buffering capacity (Bi) was determined using the above formula.

2.A.2.6.4 Molecular characterization

2.A.2.6.4.1 Genomic DNA extraction

Selected isolates were grown in PPYG broth **(Appendix A)** at 30°C for 24 hours. The culture broth was centrifuged and pellet was used for extraction of DNA. Genomic DNA was extracted by method described by Sambrook *et al.* (1989) **(Appendix C)**. The cell pellet was washed in de-ionised water and resuspended in

465 µl of 1X Tris EDTA buffer (pH 8.0). Then, 5 µl of lysozyme (10 mg/100 µl) was added in to the suspension and incubated at 37°C for 45 min. Further, 30 µl of sodium dodecyl sulfate (10%) was added and incubated at 60°C for 15 min. An equal volume of phenol-chloroform solution (1:1) was added, mixed gently and centrifuged at 12,000 rpm for 10 min. This step was repeated twice. The aqueous layer was collected and to it an equal volume of chloroform-isoamyl alcohol (24:1) was added, mixed gently and centrifuged at 12,000 rpm for 10 min. The aqueous layer was collected and to it sodium acetate solution ($1/10^{th}$ of the aqueous layer, 3 M) and 0.6 ml volume of chilled isopropanol were added and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded. One ml of chilled ethanol was added to the pellet and centrifuged at 12,000 rpm for 10 min. The ethanol was discarded and the tube was allowed to dry completely. The isolated DNA was stored at -20°C for further use.

2.A.2.6.4.2 PCR amplification of 16S rRNA gene

Amplification of 16S rRNA gene was performed using universal primers (8F and 1429R) with a final reaction volume of 100 µl reaction (Devatkal *et al.*, 2013). The 16S rRNA gene was amplified by dissolving dried DNA in 50 µl Tris EDTA buffer **(Appendix C)**. Two µl of this sample was added to 98 µl master mix (PCR buffer 10 µl + F Primer 1 µl + R Primer 1 µl + dNTPs 2 µl + Taq DNA polymerase 0.75 µl + $MgCl_2$ 6 µl and deionized water 77.25 µl). PCR was run as follows:

Step 1- 94°C---------- 3’

Step 2- 94°C---------- 1’

Step 3- 52°C---------- 1’

Step 4- 72°C---------- 2’

Step 5- 72°C---------- 5’

Steps 2, 3 and 4 were repeated for 35 cycles. To check for amplification, the PCR product was electrophoresed on 0.8% agarose gel at 80 Volts for 20 minutes (2 µl sample + 8 µl loading dye). PCR product was sent to Xcelris Pvt Ltd., Ahemedabad, for sequencing 16S rRNA gene.

2.A.2.6.4.3 Phylogenetic analysis of 16S rRNA gene

The 16S rRNA gene sequence obtained was compared with sequences in the GenBank using BLAST search program (Altschul *et al.,* 1990) and aligned using the multiple sequence alignment program Clustal X (Thompson *et al.,* 1997). Phylogenetic tree was constructed using the Neighbour Joining method of Clustal X version 2.0. Final tree was drawn with MEGA 6.0 software. 16S rRNA gene sequence of selected isolates was deposited in GenBank and accession numbers were obtained.

2.A.3. RESULTS

2.A.3.1 Physico-chemical analysis of samples

Twenty-three sediment and 17 water samples were collected from different sites of Goa **(Figure 2.1)** and analyzed for pH, salinity and orthophosphate **(Table 2.1)**. The pH of the sediment samples ranged from 6.1 (Batim saltpan sediment) to 10.0. (Lotulim khazan sediment) and pH of water samples ranged from 5.0 (Madkai mangrove water) to 8.0 (Ribander mangrove water). Salinity of sediment samples was recorded and found to be from 0.0 (Cortalim and Madkai ferry point sediment, Old Goa mangrove sediment) to 228.4 g/kg (Batim saltpan sediment) whereas, salinity of water samples ranged from 0.082 (Rasai mangrove water) to 75.87 g/l (Cavelossim Khazan water). Least phosphate content of 47.3 g/kg was found in

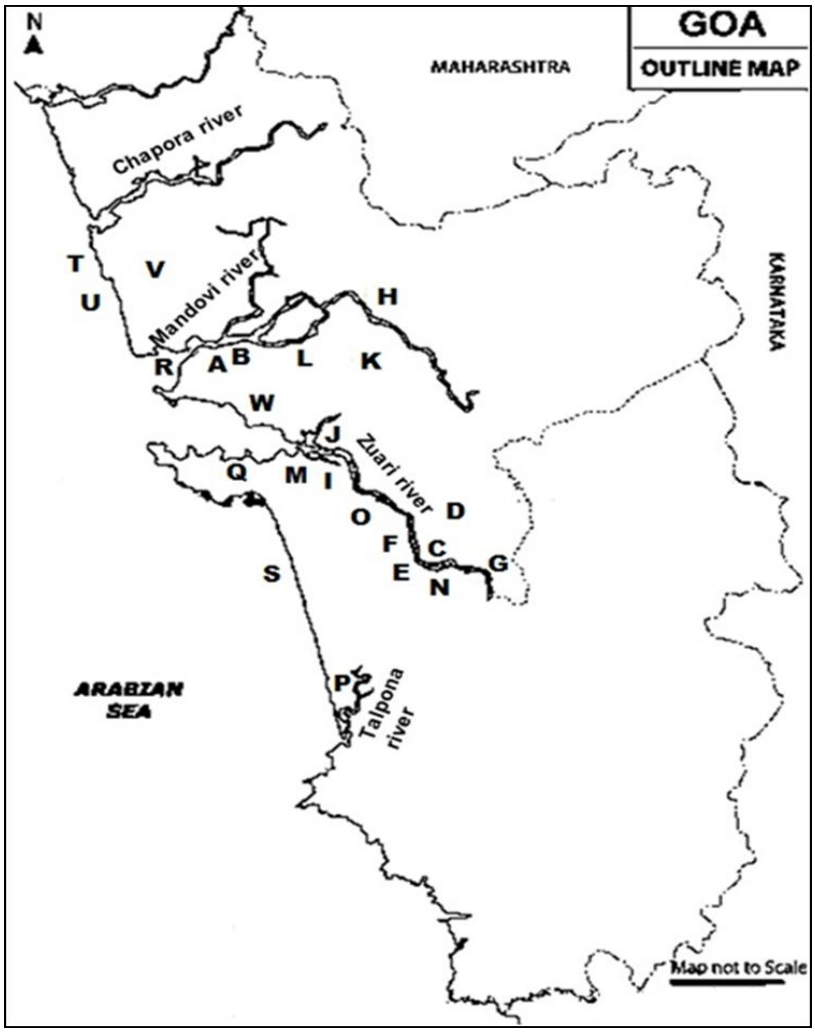


Figure 2.1 - Map of Goa showing location of sampling sites. Samples were collected from 23 sites representing four types of coastal ecosystems such as mangroves (A – M), khazan lands (N – Q), sand dunes (R – U) and salt pans (V – W).

Where, A - Merces; B - Ribander; C - Durbhat; D - Bandora; E - Rasai; F - Quellossim; G - Borim; H - Amona; I: - Cortalim Ferry Point; J - Madkai Ferry Point; K - Banastari; L - Old Goa; M - Cortalim. N - Lotulim; O - Quellossim; P - Cavellossim; Q - Sancoale; R - Miramar; S - Colva; T - Vagator; U - Anjuna; V - Arpora; W - Batim.

Table 2.1 - Physico-chemical analysis and viable count of alkaliphilic bacteria on PPYG agar of sediment and water samples collected from various coastal ecosystems of Goa

	Sampling Site	Type	Physico-chemical analysis			Viable count sediment (cfu/g) water (cfu/ml)
			pH	Salinity (NaCl) (g/kg) (g/l)	Phosphate (g/kg)	
	Mangrove					
A.	Merces	Sediment	6.4	19.90	59.1	1.5×10^4
		Water	7.8	0.800	N.D.	1.8×10^4
B.	Ribander	Sediment	6.7	33.81	59.1	2.7×10^4
		Water	8.0	2.800	N.D.	5.5×10^4
C.	Durbhat	Sediment	7.3	18.14	89.6	3.2×10^5
		Water	7.1	0.400	N.D.	3.2×10^4
D.	Bandora	Water	7.4	0.500	N.D.	2.9×10^3
E.	Rasai	Sediment	6.5	3.290	47.3	9.4×10^5
		Water	7.4	0.082	N.D.	3.2×10^5
F.	Quellossim	Sediment	6.3	0.820	47.3	4.0×10^5
		Water	6.0	0.164	N.D.	2.0×10^5
G.	Borim	Sediment	7.3	19.79	48.7	6.1×10^5
		Water	7.1	0.500	N.D.	1.3×10^7
H.	Amona	Sediment	7.8	0.000	56.1	7.8×10^4
		Water	7.1	25.97	N.D.	5.1×10^4
I.	Cortalim Ferry Point	Sediment	7.8	0.000	53.22	1.0×10^5
		Water	6.9	23.91	N.D.	3.2×10^4
J.	Madkai Ferry Point	Sediment	7.5	0.000	48.7	9.0×10^3
		Water	5.0	28.20	N.D.	4.1×10^4
K.	Banastari	Sediment	6.9	1.650	60.6	5.5×10^4
		Water	7.0	28.45	N.D.	2.5×10^4
L.	Old Goa	Sediment	7.4	0.000	60.6	3.2×10^4
		Water	7.1	33.48	N.D.	2.2×10^4
M.	Cortalim	Sediment	7.6	23.09	51.84	6.3×10^5
		Water	6.9	32.74	N.D.	9.4×10^5
	Khazan Land					
N.	Loutulim	Sediment	10.0	4.948	79.2	2.3×10^6
		Water	6.8	0.330	N.D.	4.1×10^4
O.	Quellossim	Sediment	6.2	4.120	60.6	7.0×10^3
		Water	6.4	0.412	N.D.	4.6×10^5
P.	Cavellossim	Sediment	6.2	82.47	60.6	2.6×10^7
		Water	7.3	75.87	N.D.	2.8×10^6
Q.	Sancoale	Sediment	7.1	64.33	45.83	4.2×10^6
		Water	6.9	59.13	N.D.	1.6×10^5
	Sand Dune					
R.	Miramar	Sediment	7.4	32.90	67.2	1.4×10^4
S.	Colva	Sediment	7.5	1.640	60.6	5.1×10^4
T.	Vagator	Sediment	7.8	6.590	69.3	8.7×10^5
U.	Anjuna	Sediment	8.2	4.940	65.2	3.1×10^6
	Salt Pan					
V.	Arpora	Sediment	7.0	202.0	47.3	4.2×10^5
W.	Batim	Sediment	6.1	228.4	59.1	1.0×10^7

Key: N.D – Not Done

sediment of Rasai mangrove and maximum value of 89.6 g/kg was observed in Durbhat mangrove sediment.

2.A.3.2. Isolation and characterization of alkaliphilic bacteria

Viable count of heterotrophic alkaliphiles from mangrove sediment samples ranged from $9.0 \times 10^3 - 9.4 \times 10^5$ cfu/g and count recorded in water samples ranged from 2.9×10^3 to 1.3×10^7 cfu/ml **(Table 2.1)**. In khazan samples, highest count of 2.6×10^7 cfu/g was obtained from sediment sample of Cavelossim khazan and lowest of 7.0×10^3 cfu/g in Quellossim khazan sediment sample. Sand dune samples gave the counts in the range of 1.4×10^4 to 3.1×10^6 cfu/g. Salt pan sediment sample from Batim gave the alkaliphilic count of the order of 10^7 cfu/g which was second highest amongst all the analysed samples. Higher counts of alkaliphiles were observed among sediment samples as compared to water samples.

A total of 141 alkaliphilic isolates were obtained from all the samples on the basis of different colony characteristics **(Figure 2.2)**. Eighty-eight isolates were obtained from mangrove ecosystem, twenty from khazan lands, twelve from sand dunes and twenty one from salt pans. Twenty-seven isolates were pigmented with colour ranging from yellow, orange, pink to brown. Sixteen isolates with different colony characters were obtained from sediment of Batim salt pans. Among the isolates 75.56% were Gram negative and 24.44% were Gram positive **(Figure 2.3)**. Gram staining of alkaliphiles also demonstrated wide variation in their morphology varying from rods, cocci, filamentous and pleomorphic forms.

Among these 141 isolates, 80 were alkalitolerant (56.7%), 43 were facultative alkaliphiles (30.5%) and 18 were obligate alkaliphiles (12.8%) **(Figure 2.4)**. In this study, obligate and facultative alkaliphiles were detected in mangrove, khazan and

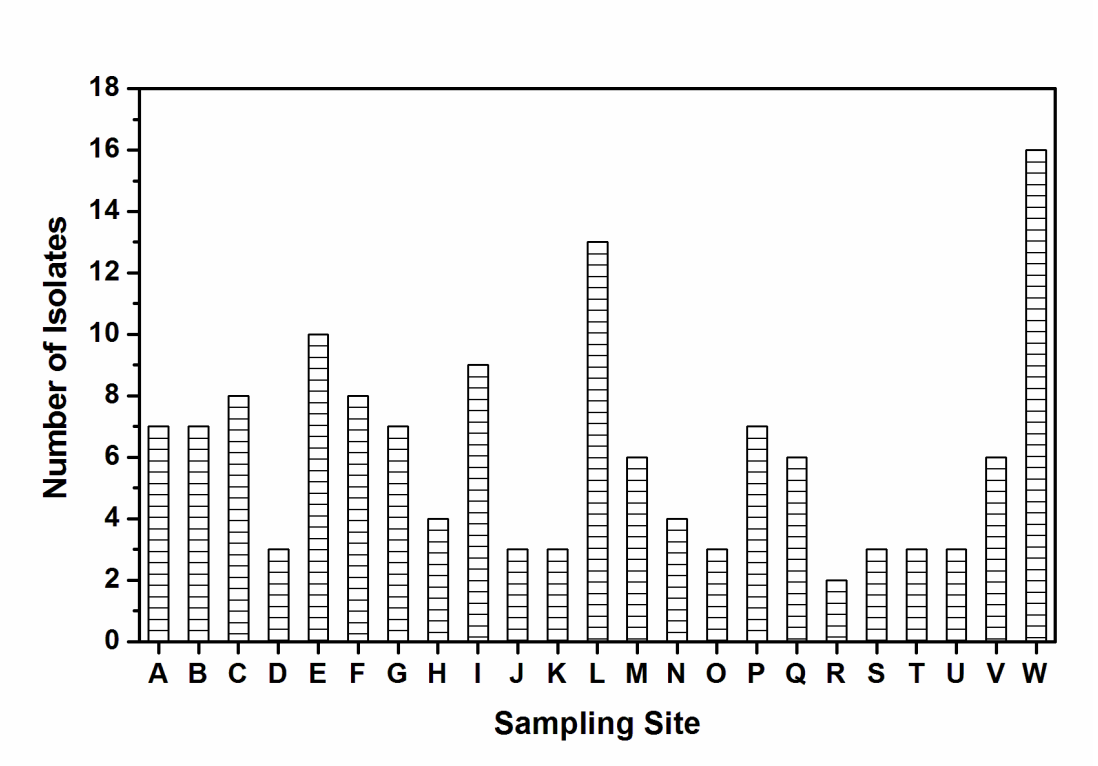


Figure 2.2 - Number of alkaliphilic bacterial isolates obtained from 23 sampling sites representing four types of coastal ecosystems being mangroves (A – M), khazan lands (N – Q), sand dunes (R – U) and salt pans (V – W).

Where, A - Merces; B - Ribander; C - Durbhat; D - Bandora; E - Rasai; F - Quellossim; G - Borim; H - Amona; I: - Cortalim Ferry Point; J - Madkai Ferry Point; K - Banastari; L - Old Goa; M - Cortalim. N - Lotulim; O - Quellossim; P - Cavellossim; Q - Sancoale; R - Miramar; S - Colva; T - Vagator; U - Anjuna; V - Arpora; W - Batim.

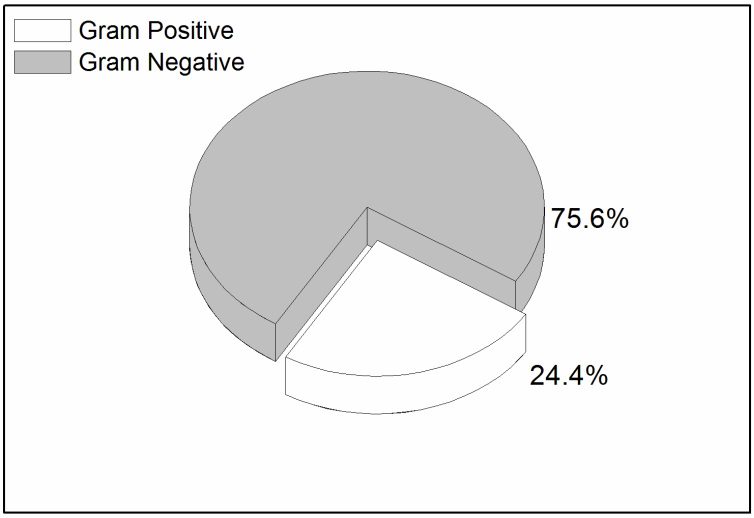


Figure 2.3 - Characterization of alkaliphilic bacterial isolates based on Gram character

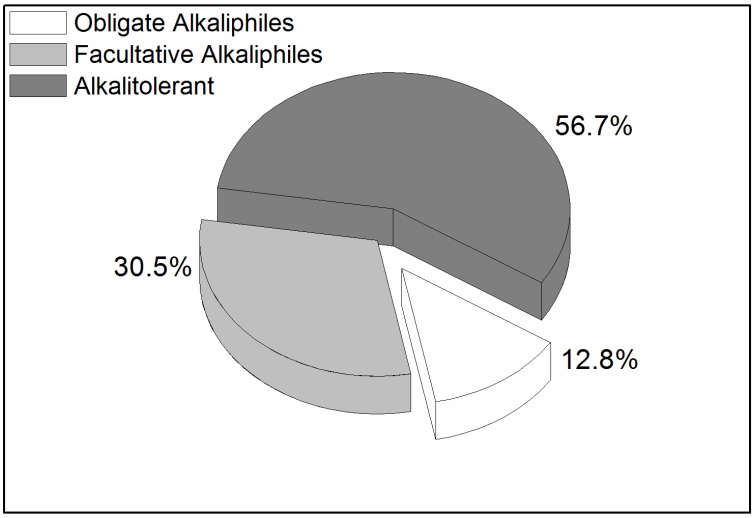


Figure 2.4 - Characterization of alkaliphilic bacterial isolates based on their alkaliphilic nature as obligate alkaliphiles, facultative alkaliphiles and alkalitolerant

sand dune ecosystems. However, all the isolates obtained from salt pan ecosystems were alkalitolerant.

2.A.3.3. Screening of alkaliphilic isolates for inorganic phosphate solubilization

Out of 141 isolates, 43 demonstrated tricalcium phosphate solubilization at pH 10.0 **(Figure 2.5)**. Forty three phosphate solubilizing isolates were obtained from sediment and water of fourteen sampling sites. Sediment and water of 9 sampling sites did not show the presence of any phosphate solubilizing bacteria.

Among the sampling sites, maximum numbers of phosphate solubilizers were obtained from Merces mangrove sample followed by Ribander mangrove, Madkai Ferry Point mangrove and Cavellossim khazan land sample. Varying degree of phosphate solubilization was observed among the isolates as indicated by SI **(Figure 2.6)**. Among the 43 isolates showing positive result, 3 isolates gave the SI above 2.5. These isolates were FA7, WA3 and DA3. Only 6 isolates demonstrated the index below 1.5. Remaining isolates showed index between 1.5 and 2.5. Highest zone of solubilization was given by isolate FA7 which was obtained from Quellossim mangrove sediment followed by WA3 from Batim salt pan sample.

Effect of pH and salinity on phosphate solubilizing isolates revealed that among forty-three phosphate solubilizing isolates, twenty-eight isolates showed growth at pH 8 with 10% NaCl out of which only three showed solubilization of tricalcium phosphate. Five isolates showed growth and among them only three showed solubilization at pH eight and 25% NaCl. On the combination of pH 10 and 10% NaCl five isolates exhibited growth and only three showed solubilization. Three isolates namely AA5, CA3 and WA3 showed growth and phosphate solubilization at pH 10 with 25% NaCl **(Table 2.2)**.

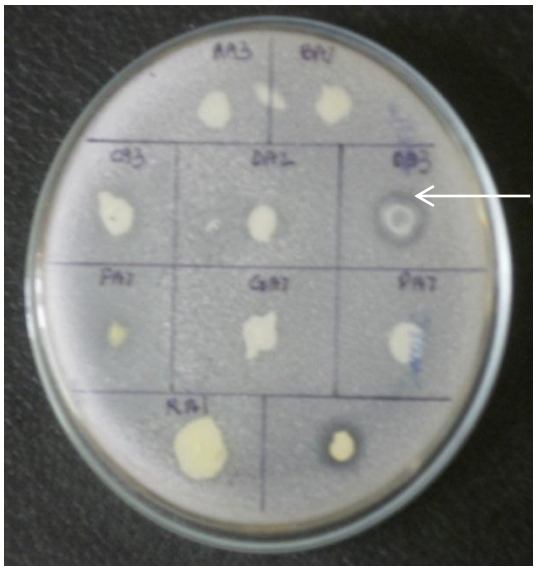


Figure 2.5 - Screening of alkaliphilic bacterial isolates for tricalcium phosphate solubilization on Pikovskaya's agar at pH 10.0. Bacterial isolates solubilizing inorganic phosphate show visible dissolution zones or halos around the colonies.

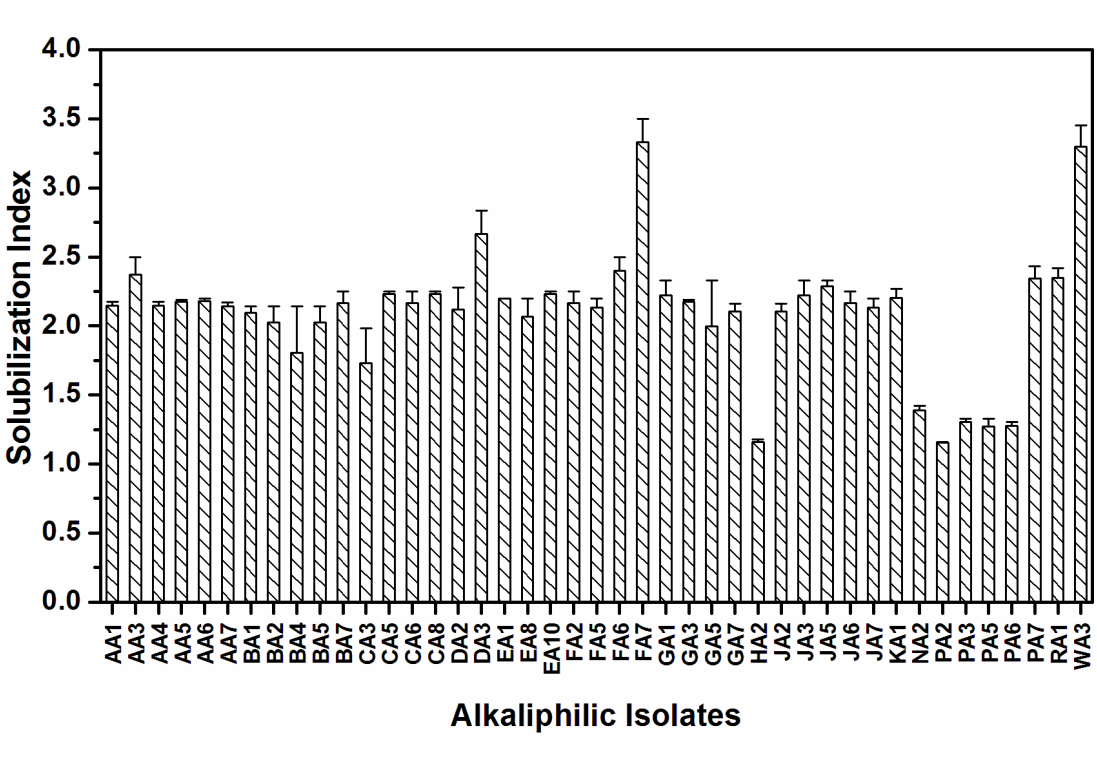


Figure 2.6 - Solubilization index of alkaliphilic isolates for solubilization of tricalcium phosphate on Pikovskaya's agar at pH 10.0. Error bars are standard error.

Table 2.2 - Effect of different pH and salt concentration on growth (G) and inorganic phosphate solubilization (PS) ability of alkaliphilic phosphate solubilizing isolates

Isolate	pH 8.0 + 10% NaCl		pH 8.0 + 25% NaCl		pH 10.0 + 10% NaCl		pH 10.0 + 25% NaCl	
	G	PS	G	PS	G	PS	G	PS
AA1	-	-	-	-	-	-	-	-
AA3	+	-	-	-	+	-	-	-
AA4	-	-	-	-	-	-	-	-
AA5	+	+	+	+	+	+	+	+
AA6	+	-	-	-	-	-	-	-
AA7	+	-	-	-	-	-	-	-
BA1	-	-	-	-	-	-	-	-
BA2	-	-	-	-	-	-	-	-
BA4	-	-	-	-	-	-	-	-
BA5	+	+	-	-	-	-	+	+
BA7	+	-	-	-	-	-	-	-
CA3	+	+	-	-	-	-	+	+
CA5	+	-	-	-	-	-	-	-
CA6	+	-	-	-	-	-	-	-
CA8	-	-	-	-	-	-	-	-
DA2	+	-	-	-	-	-	-	-
DA3	-	-	-	-	-	-	-	-
EA1	+	-	-	-	-	-	-	-
EA8	+	-	-	-	-	-	-	-
EA10	+	-	-	-	-	-	-	-
FA2	+	-	-	-	-	-	-	-
FA5	+	-	-	-	-	-	-	-
FA6	-	-	-	-	-	-	-	-
FA7	-	-	-	-	-	-	-	-
GA1	+	-	-	-	-	-	-	-
GA3	+	-	-	-	-	-	-	-
GA5	+	-	-	-	-	-	-	-
GA7	+	-	-	-	-	-	-	-
HA2	-	-	-	-	-	-	-	-
JA2	+	-	-	-	-	-	-	-
JA3	+	-	-	-	-	-	-	-
JA5	-	-	-	-	-	-	-	-
JA6	+	-	-	-	-	-	-	-
JA7	-	-	-	-	-	-	-	-
KA1	-	-	-	-	-	-	-	-
NA2	-	-	-	-	-	-	-	-
PA2	+	-	-	-	-	-	-	-
PA3	+	-	-	-	-	-	-	-
PA5	+	-	-	-	-	-	-	-
PA6	+	-	-	-	-	-	-	-
PA7	+	-	-	-	-	-	-	-
RA1	+	-	-	-	-	-	-	-
WA3	+	+	+	+	+	+	+	+

Key: + growth, phosphate solubilization; - no growth, no phosphate solubilization

2.A.3.4. Screening of alkaliphilic isolates for organic phosphate solubilization:

Twenty-six isolates out of 141 alkaliphilic isolates showed organic phosphate solubilizing ability by production of phosphatase at pH 10 **(Figure 2.7)**. These alkaliphilic bacteria were isolated from sediment and water of twelve sampling sites. Sediment and water from 11 sampling sites showed no phosphatase producing isolates. Highest number of phosphatase producing isolates was obtained from mangrove samples followed by sand dune samples, khazan land and salt pan samples. Among the 26 isolates, isolate FA7 obtained from Quellossim mangrove sample gave maximum absorbance at 405nm followed by isolate PA7 which was obtained from Cavellosim khazan land sample **(Figure 2.8)**.

Effect of phosphate ions, glucose, and p-NPP was studied on phosphatase enzyme production. It was observed that 16 isolates secreted low amount of phosphatase enzyme in the presence of phosphate ions. Phosphatase enzyme secretion decreased for two isolates in the presence of glucose. No change in phosphatase enzyme production was observed in six isolates due to presence of either phosphate or glucose. Among the isolates, one showed increased production of phosphatase in the presence of glucose. Maximum production of phosphatase enzyme was observed in media devoid of phosphate ions. Colour zone produced due to release of para-nitrophenol was found to be more in the fourth condition **(Table 2.3)**.

Figure 2.9 shows inorganic and organic phosphate solubilizing isolates obtained from each sample. Among the 141 isolates, 30 isolates showed only inorganic phosphate solubilizing activity, 13 isolates only organic phosphate solubilizing activity and 13 isolates demonstrated both inorganic as well as organic phosphate

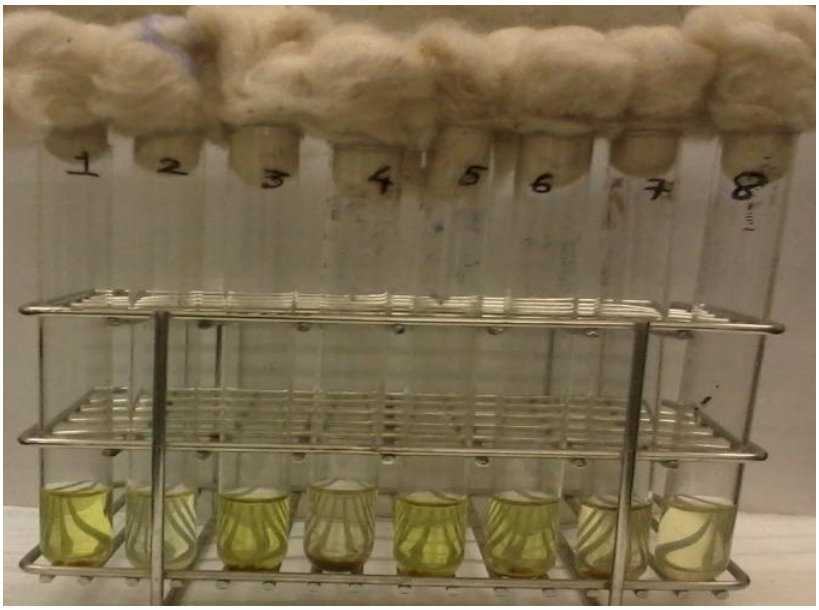


Figure 2.7 – Screening of alkaliphilic bacterial isolates for organic phosphate solubilization in MSM at pH 10.0

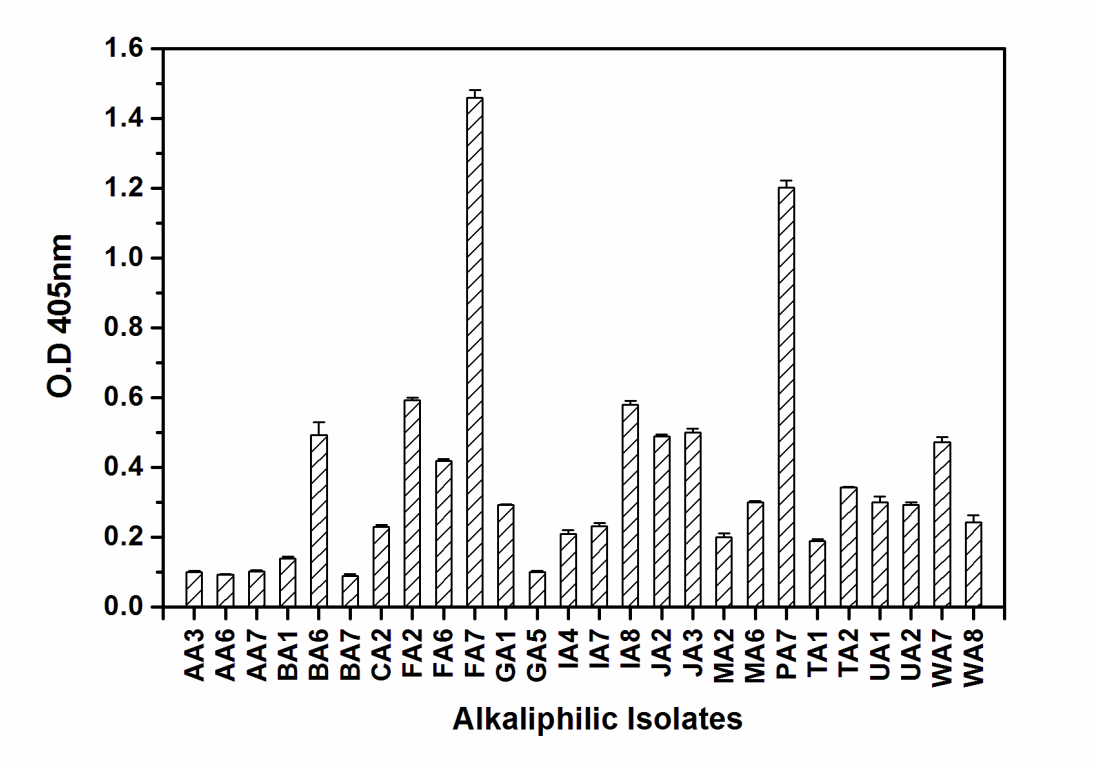


Figure 2.8 - Absorbance of p-nitrophenol released by organic phosphate solubilizing isolates in MSM at pH 10.0. Error bars are standard error.

Table 2.3 - Effect of inorganic phosphate ions, glucose and p-nitrophenyl phosphate (p-NPP) on phosphatase enzyme production by alkaliphilic isolates

Isolates	Colour zone diameter (mm)			
	KH_2PO_4 + pNPP	Glucose + pNPP	KH_2PO_4 + Glucose +pNPP	pNPP
AA3	5	6	5	8
AA6	5	10	5	10
AA7	5	6	4	11
BA1	6	7	5	10
BA6	7	8	7	9
BA7	7	10	7	11
CA2	7	10	8	9
FA2	7	10	8	10
FA6	6	8	7	10
FA7	15	17	16	16
GA1	5	6	4	11
GA5	8	10	8	16
IA4	-	9	9	10
IA7	7	10	8	10
IA8	8	13	9	14
JA2	7	8	7	8
JA3	7	11	11	13
MA2	8	10	9	10
MA6	9	10	9	10
PA7	10	12	10	13
TA1	7	10	9	10
TA2	7	8	7	9
UA1	8	10	7	10
UA2	7	9	8	9
WA7	7	9	8	9
WA8	6	10	7	10

Key: - no colour zone

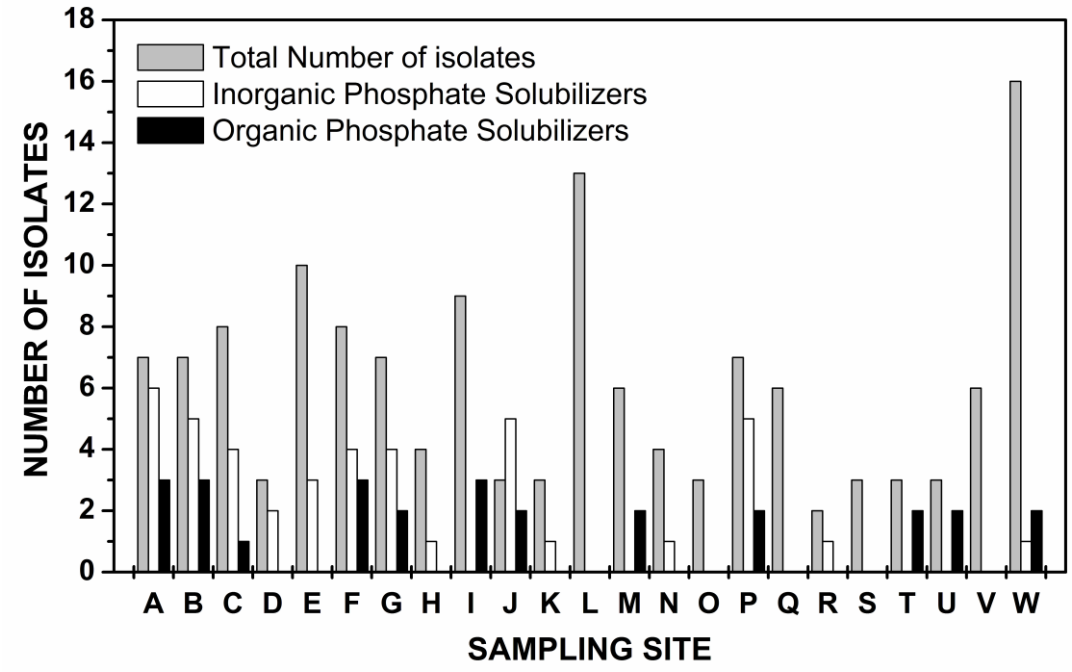


Figure 2.9 - Inorganic and organic phosphate solubilizing isolates obtained from 23 sampling sites representing four types of coastal ecosystems being mangroves (A – M), khazan lands (N – Q), sand dunes (R – U) and salt pans (V – W).

Where, A - Merces; B - Ribander; C - Durbhat; D - Bandora; E - Rasai; F - Quellossim; G - Borim; H - Amona; I: - Cortalim Ferry Point; J - Madkai Ferry Point; K - Banastari; L - Old Goa; M - Cortalim. N - Lotulim; O - Quellossim; P - Cavellossim; Q - Sancoale; R - Miramar; S - Colva; T - Vagator; U - Anjuna; V - Arpora; W - Batim.

solubilizing activity. Highest number of phosphate solubilizing isolates was recorded in mangrove sample from Merces.

2.A.3.5. Characterization and identification of selected isolates

Based on the results of tricalcium phosphate and organic phosphate solubilization, isolates FA7 and WA3 were selected for further characterization and identification.

Isolate FA7 was isolated from Quellossim mangrove sediment sample. It demonstrated both tricalcium phosphate solubilization **(Figure 2.10.a)** as well as organic phosphate solubilizing ability. Morphological characterization and scanning electron micrographs revealed FA7 to be rod shaped, Gram positive spore forming bacterium **(Figure 2.11.a)**. Length and breadth of FA7 were found to be 1.44 µm and 0.49 µm, respectively. The cytoplasmic buffering capacity of the isolate was found to be 1300 nmoles of OH^- ions **(Table 2.4)**. Isolate FA7 was found to belong to family Bacillaceae **(Table 2.5)**; it was further identified by 16S rRNA gene sequencing as *Bacillus marisflavi* **(Figure 2.12)**. 16S rRNA gene sequence of isolate FA7 has been deposited in Genbank with an accession number KX098478.

Isolate WA3 obtained from Batim salt pan sediment sample showed only tricalcium phosphate solubilizing activity **(Figure 2.10.b)**; however, it demonstrated the activity from 1 to 25% (w/v) salt concentration and pH 7.0 to 10.0. It was found to be a Gram negative rod shaped bacterium **(Figure 2.11.b)**. Length and breadth of WA3 were found to be 0.96 µm and 0.42 µm, respectively. The cytoplasmic buffering capacity of isolate WA3 was found to be 2300 nmoles of OH^- ions which was higher than 1300 nmoles of OH^- ions of isolate FA7 **(Table 2.4)**. Buffering capacity of whole cells of both the isolates was higher than triton X treated cells. Further, cells permiabilised with triton X-100 were found to lose their buffering capacity. Isolate

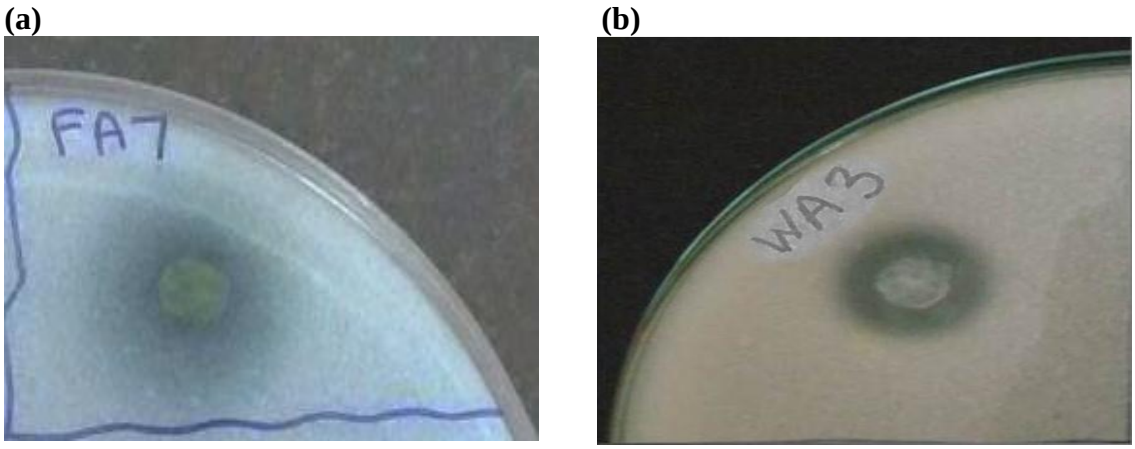


Figure 2.10 - Tricalcium phosphate solubilization on Pikovskaya's agar by selected alkaliphilic phosphate solubilizing isolates FA7 (a) and WA3 (b)

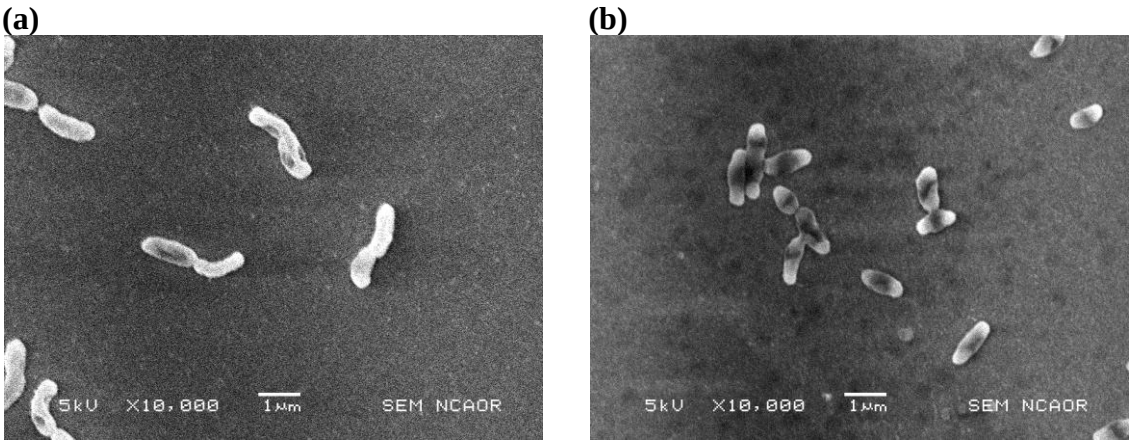


Figure 2.11 - Scanning electron micrographs of selected phosphate solubilizing bacterial isolates FA7 (a) and WA3 (b)

Table 2.4 - Buffering capacity of alkaliphilic isolates (FA7 and WA3) and neutrophilic bacteria (*E. coli* and *B. subtilis*)

Isolate	Whole Cell Buffering Capacity		Triton X treated Cells		Decrease in OH^- ion consumption after treatment	Buffering capacity		Cytoplasmic buffering capacity
	µl of 0.05M KOH consumed	nmoles of OH^- ions	µl of 0.05M KOH consumed	nmoles of OH^- ions		Whole cells (Bo)	Treated cells (Bt)	Bi=Bo-Bt
FA7	450	22500	320	16000	6500	4500	3200	1300
WA3	440	22000	210	10500	11500	4400	2100	2300
E. coli	15	750	11	550	200	150	110	40
B. subtilis	50	2500	20	1000	1500	500	200	300

Table 2.5 - Morphological and biochemical characteristics of selected potential alkaliphilic phosphate solubilizing isolates FA7 and WA3

Test	FA7	WA3
Gram character	Gram positive	Gram negative
Motility	Motile	Non-motile
Spore staining	Positive, Subterminal	Negative
Colony colour	Yellow	Cream
pH	8-11	7-10
Salt concentration (%)	0-5	1-25
Positive for production of	Catalase, Amylase, Phosphatase	Catalase, Lysine decarboxylase, Ornithine decarboxylase, MR
Negative for production of	Urease, Oxidase, Gelatinase, Indole.	Phospatase, Urease, Amylase, Oxidase, Gelatinase, Indole.
Acid produced from	Glucose, Maltose, Fructose, Mannitol, Sucrose, Xylose, Trehalose.	Glucose, Maltose, Fructose, Mannitol, Sucrose, Lactose, Galactose
Acid not produced from	Lactose, Sorbitol, Galactose.	Sorbitol, Trehalose.
Nitrate reduction	Negative	Positive
Citrate utilization	Negative	Positive
Tricalcium phosphate solubilization	Positive	Positive

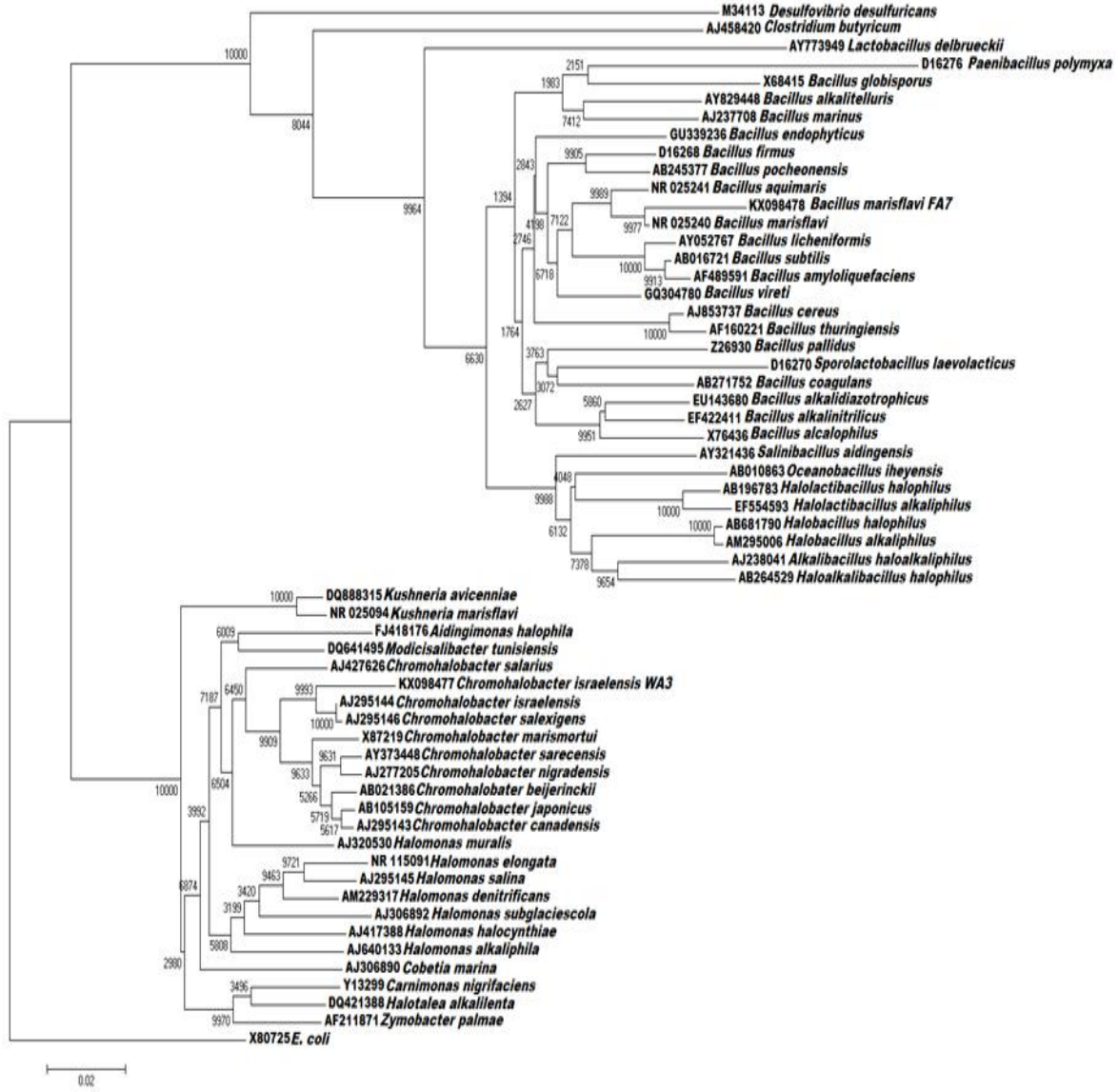


Figure 2.12 - Neighbour joining phylogenetic tree based on 16S rRNA gene sequence of FA7 *Bacillus marisflavi* (KX098478) and WA3 *Chromohalobacter israelensis* (KX098477) with closely related species of bacteria

FA7 and WA3 demonstrated high buffering capacity as compared with neutrophilic controls *E. coli* and *Bacillus subtilis*. The details of biochemical characteristics are summarized in **Table 2.5**. Based on the results of biochemical tests, isolate WA3 was tentatively identified using Bergey's Manual of Determinative Bacteriology, Volume III (Garrity *et al.,* 2009). It was found to belong to the family Halomonadaceae. Further, 16S rRNA gene sequencing revealed it to be *Chromohalobacter israelensis* **(Figure 2.12)**. 16S rRNA gene sequence of isolate WA3 was deposited in Genbank with an accession number KX098477.

2.A.4 DISCUSSION

Coastal ecosystems provide a unique ecological niche to diverse microbes which play various roles in environmental activities and nutrient cycling (Desai *et al.,* 2004; Barea *et al.,* 2005; Appanna *et al.,* 2007). In this study, alkaliphilic bacterial isolates were obtained from the various coastal ecosystems of Goa. Researchers have isolated facultative and obligate alkaliphilic bacteria for bio-prospecting from various coastal ecosystems of Goa, viz. mangrove, salt pan and sand dunes (Desai *et al.,* 2004; Godinho *et al.,* 2010; Kamat & Kerkar, 2011; Surve *et al.,* 2012; Gaonkar & Bhosle, 2013; Borkar, 2015b). Desai *et al.* (2004) have isolated alkaliphiles from mangrove ecosystems of Goa and among their isolates 18.30% were obligate alkaliphiles and 81.69% were facultative alkaliphiles.

The diverse microbial community of coastal ecosystems plays an essential role in the environment by contributing to the release of key nutrients from primary minerals that are required not only for their own nutrition but also for that of plants. Phosphorus is one such most essential macronutrient for the growth of plants (Illmer & Schinner, 1995). Its fixation is a serious problem in alkaline and saline soils

(Sharif *et al.,* 2000; Mujeeb *et al.,* 2010). Several studies have reported the occurrence of phosphate solubilizing bacteria in the coastal areas (Ayyakkannu & Chadramohan, 1971; Craven & Hayasaka, 1982; Naik *et al.,* 1982; Venkateswaram & Natarajan, 1984; De Souza *et al.,* 2000; Vazquez *et al.,* 2000; Gupta *et al.,* 2007; Ravikumar *et al.,* 2009; Dastager & Damare, 2013). Among the 141 predominant alkaliphilic bacteria obtained in this study, 30 isolates showed only inorganic phosphate solubilizing activity, 13 isolates only organic phosphate solubilizing activity and 13 isolates demonstrated both inorganic as well as organic phosphate solubilizing activity at pH 10.0. Significantly, alkaliphilic isolates also solubilized phosphate at a combination of high pH (10.0) and salt concentration (25% (w/v)). Nautiyal *et al.* (2000) have isolated phosphate solubilizing bacteria from alkaline soils which solubilized phosphate at pH 12 and 10% NaCl. Isolates screened by Mishra *et al.* (2009) in their study tolerated 10% NaCl while, isolates of Nakbanpote *et al.* (2014) demonstrated phosphate solubilization at 8% (w/v) salt concentration. Tolerance to high pH and salt concentration is important in the survival, multiplication and spread of bacterial strains in alkaline and saline soils (Nautiyal *et al.,* 2000).

Two potential isolates FA7 and WA3 were selected for identification. Isolate FA7 was identified as *Bacillus marisflavi* and isolate WA3 was identified as *Chromohalobacter israelensis*. To the best of our knowledge, this is the first report of phosphate solubilizing bacterium belonging to the genus *Chromohalobacter* able to solubilize phosphate at high pH and high salt concentration. *Bacillus* species are among the most successful bacterial communities in solubilization of phosphate (Illmer & Schinner, 1995). Organisms belonging to genera like *Achromobacter, Acinetobacter, Advenella, Aerobacter, Agrobacterium, Alcaligenes, Arthrobacter,*

Bacillus, Bradyrhizobium, Brevibacterium, Burkholderia, Chryseobacterium, Chryseomonas, Citrobacter, Corynebacterium, Delftia, Enterobacter, Erwinia, Flavobacterium, Gluconacetobacter, Gordonia, Klebsiella, Kluyvera, Micrococcus, Paenibacillus, Pantoea, Phyllobacterium, Pseudomonas, Rhizobium, Rhodococcus, Serratia, Thiobacillus, Vibrio, Xanthobacter and *Xanthomonas*, have been reported to solubilize inorganic and organic phosphate (Freitas *et al.*, 1997; Rodriguez & Fraga, 1999; Seshadri *et al.*, 2002; Khan *et al.*, 2009a; Collavino *et al.*, 2010; Gulati *et al.*, 2010).

Most phosphate solubilizing bacteria reported previously performed relatively low in alkalinity and salinity tolerance, being less appropriate for alkaline-saline soil based agriculture. Hence, it is urgently needed to develop alkaliphilic and haloalkaliphilic phosphate solubilizing bacteria for the agriculture in alkaline-saline soil.

2.B.1 INTRODUCTION

Phosphorus is an essential macronutrient required for growth of plants (Illmer & Schinner, 1995). However, it is a limiting element in soil as most of the soluble orthophosphate ions react with numerous organic and inorganic constituents of soil and thus become insoluble and unavailable for uptake of plants (Zhu *et al.,* 2016). Phosphate solubilizing bacteria present in soil convert this insoluble phosphate into soluble orthophosphate (Spohn *et al.,* 2015).

Several mechanisms responsible for solubilization of inorganic and organic phosphates are present in phosphate solubilizing bacteria. Acidification of the surrounding due to production of organic acids and H^+ ion excretion are widely reported mechanisms of inorganic phosphate solubilization (Roos & Luckner, 1984; Illmer & Schinner, 1992; Park *et al.,* 2009; Paul & Sinha, 2015). Production of inorganic acids such as sulphuric, nitric and carbonic acids has also been reported as a mechanism to solubilize inorganic phosphate (Khan & Zaidi, 2007). Microbial exopolysaccharides have been shown to play an indirect role in inorganic phosphate solubilization (Aspiras *et al.,* 1971; Ionescu & Belkin, 2009). Solubilization of iron phosphates has been attributed to production of siderophores (Vassilev *et al.,* 2006; Caballero-Mellado *et al.,* 2007; Hamdali *et al.,* 2008). Other postulated mechanism includes production of hydrogen sulphide by sulphur reducing bacteria. H_2S reacts with phosphate containing minerals, thus releasing phosphate (Ehrlich *et al.,* 2016).

Organic phosphate mineralization occurs due to production of extracellular phosphatase enzymes by microorganisms in soil (Nannipieri *et al.,* 2011). These enzymes render high molecular weight organic phosphate into low molecular weight

compounds by the hydrolysis of ester-phosphate bonds, leading to the release of phosphate ions (Eivazi & Tabatabai, 1977; Jorquera *et al.,* 2008).

Alkaliphilic bacteria were isolated from diverse coastal ecosystems of Goa and screened for phosphate solubilization at pH 10.0. Among the phosphate solubilizing alkaliphilic bacterial isolates, two isolates were selected for this study. Isolate FA7, identified as *Bacillus marisflavi* demonstrated both inorganic and organic phosphate solubilization at pH 10.0. Isolate WA3, identified as *Chromohalobacter israelensis,* had the ability to solubilize inorganic phosphate at pH 10.0 and 25% crude salt concentration. An attempt was made to study the mechanisms of phosphate solubilization employed by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3.

2.B.2 MATERIALS AND METHODS

2.B.2.1 Time course of tricalcium phosphate solubilization in shake flask

Bacillus marisflavi FA7 and *Chromohalobacter israelensis* WA3 were inoculated into six 50 ml Pikovskaya's broth **(Appendix A)** flasks (pH 10.0); one each for 12, 24, 48, 72, 96 and 120 hours. Sterile water inoculated medium was treated as control. Culture flasks were incubated at 30°C in a rotary shaker at 150 rpm. After every 24 hours for five days, the pH of the medium was recorded using pH meter equipped with a glass electrode; amount of residual phosphate and biomass were determined gravimetrically.

2.B.2.2 Estimation of biomass and residual phosphate

Culture broth was centrifuged at 10,000 rpm for 10 min to obtain pellet containing cells and residual tricalcium phosphate (Mirhadi *et al.,* 2011). The pellet was suspended in chilled HCl (1 N) to dissolve residual tricalcium phosphate. The

mixture was centrifuged at 10,000 rpm for 10 min to separate cell pellet from dissolved residual phosphate. Supernatant was collected in a separate tube. To the clear supernatant, NaOH (1 N) was added to re-precipitate tricalcium phosphate. Pellets of cells and tricalcium phosphate were washed in distilled water and centrifuged at 10,000 rpm. Pellets were dried in oven to estimate biomass and residual phosphate as described earlier.

2.B.2.3 Mechanisms of inorganic phosphate solubilization

2.B.2.3.a Production of organic acids

Bacillus marisflavi FA7 and *Chromohalobacter israelensis* WA3 were inoculated into 50 ml Pikovskaya's broth **(Appendix A)** flasks (pH 10.0); one each for day 1 (24 h) and day 5 (120 h). Culture flasks were incubated at 30°C in a rotary shaker at 150 rpm. After the incubation period, culture broths were centrifuged at 10,000 rpm for 15 min. Supernatants of day 1 and day 5 were analysed for the presence of organic acids by HPLC (Waters) using XBridge C8 column (4.6 x 250 mm). The supernatant samples were passed through 0.2 µm filter and 10 µl of the filtered sample was injected into HPLC for analysis. Elution was performed with an isocratic solvent consisting of 10.8% acetonitrile (HPLC grade) in 0.01% phosphoric acid with a flow rate of 1 ml/min and organic acids were detected at 210 nm (Lin *et al.*, 2006). Retention time of peaks obtained was recorded and compared with retention time of standard organic acids (0.1 mg/ml) which included acetic acid, citric acid, formic acid, gluconic acid, lactic acid, malic acid, oxalic acid and succinic acid.

2.B.2.3.b Production of H^+ ions due to inorganic nitrogen sources

Effect of different inorganic nitrogen sources on growth and phosphate solubilization was studied. *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 were

inoculated in 50 ml Pikovskaya's broth amended with different inorganic nitrogen salts (0.5 g/l) viz. NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3 and NH_4NO_3. Initial pH of the medium was adjusted to 10.0. Inoculated flasks were incubated under 150 rpm shaker condition at 30°C for 48 hours. Final pH, solubilized phosphate and biomass were estimated as described earlier.

2.B.2.3.c Production of exopolysacchride (EPS)

EPS production by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 was evaluated by spot inoculating on Congo red nutrient agar **(Appendix A)** containing 5% sucrose (Ferreira *et al.*, 2014). The plate was incubated at 30°C for 48 hours.

2.B.2.3.d Production of siderophore

Siderophore production by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 was detected by spot inoculating on Chrome Azurol Sulfonate agar (CAS) **(Appendix A)** (Schwyn & Neilands, 1987) at pH 10.0. The plate was incubated at 30°C for 48 hours.

2.B.2.4 Mechanisms of organic phosphate solubilization

Phosphatase enzyme production was carried out in MSM containing disodium-p-nitrophenyl phosphate (p-NPP) (1 mM) as the sole source of organic phosphate **(Appendix A)** (Helianti *et al.*, 2007). *Bacillus marisflavi* FA7 was inoculated in a 500 ml flask containing 200 ml of the medium. The flask was incubated at 150 rpm, 30°C for 24 hours. After the incubation period, broth was centrifuged at 10,000 rpm for 10 min at 4°C. Phosphatase enzyme activity and protein content of cell free culture broth were determined.

Chilled acetone was added to the cell free culture broth in the ratio of 1:3 (supernatant:acetone) and the precipitate obtained was collected by centrifugation at 10,000 rpm for 15 min at 4°C. Precipitate was dissolved in 1.5 ml of carbonate-bicarbonate buffer (0.1 M, pH 10.1). This crude enzyme extract was used for characterization of phosphatase enzyme.

2.B.2.4.1 Phosphatase enzyme assay

Phosphatase enzyme assay was determined by measuring the amount of liberated para-nitrophenol (p-NP) from p-NPP (Dean, 2002). Reaction was carried out in a total volume of 3 ml at pH 10.1 using carbonate-bicarbonate buffer (0.1 M). The reaction mixture consisted of 2 ml p-NPP substrate (5 mM), 0.95 ml buffer and 50 µl enzyme sample. The contents were incubated at 30°C; time course for 510 sec was followed at wavelength 405 nm using spectrophotometer. For cell free culture broth, enzyme reaction was carried out using 200 µl broth with 0.8 ml buffer. One enzyme unit is defined as liberation of 1 µM of p-NP in 1 sec. All solutions of substrate, additives, etc. were prepared in carbonate-bicarbonate buffer.

2.B.2.4.2 Protein estimation

The protein content of the supernatant and crude enzyme extract was determined by Folin Lowry's method (Lowry *et al.,* 1951). Bovine serum albumin was used as standard.

2.B.2.4.3 Characterization of phosphatase enzyme

2.B.2.4.3.a Effect of substrate concentration

Different concentrations of substrate p-NPP (33.33 - 3333.33 µM) were used to determine kinetic parameters K_m and V_{max}. The substrate p-NPP was dissolved in carbonate bicarbonate buffer (0.1 M, pH 10.1). All reaction mixtures were carried

out in a total volume of 3 ml. The controls were set up by incubating the substrate without enzyme to correct the products released due to chemical hydrolysis. The amount of p-NP released was determined at 405 nm using spectrophotometer. A graph of [S] v/s [V] was plotted. The Michaelis Menten kinetics of enzymatic reactions was analysed using Lineweaver-Burk reciprocal plot (1/S versus 1/V). Michaelis Menten constant (K_m) is indicative of enzyme substrate affinity and V_{max} is the maximum velocity at enzyme saturation.

2.B.2.4.3.b Effect of pH

Crude phosphatase enzyme preparation was assayed for enzyme activity at different alkaline pH (9.1, 9.6, 10.1 and 10.6) using carbonate–bicarbonate buffer (0.1 M). Substrate p-NPP (5 mM) solution was prepared in carbonate-bicarbonate buffer (0.1 M) of respective pH. All reaction mixtures were carried out in a total volume of 3 ml. The amount of p-NP released was determined at 405 nm using spectrophotometer.

2.B.2.4.3.c Effect of molarity

To study the effect of sodium ions on enzyme activity, carbonate-bicarbonate buffer (pH 10.1) of different molarity (0.01–2 M) was used. Substrate p-NPP (5 mM) solution was prepared in carbonate-bicarbonate buffer (pH 10.1) of respective molarity. All reaction mixtures were carried out in a total volume of 3 ml. The amount of p-NP released was determined by recording absorbance at 405 nm using spectrophotometer.

2.B.2.4.3.d Effect of additives

Effect of various additives like detergents [Sodium dodecyl sulfate (SDS) (1 mg/ml), Triton (0.10%)], chelating agent **[**Ethylenediaminetetraacetic acid (EDTA) (1 mM)],

and denaturing agents [Urea (0.5 mM), β-mercaptoethanol (1 mM)] was studied on activity of crude enzyme extract. Additive solutions were prepared in carbonate-bicarbonate buffer (pH 10.1, 0.1 M). Substrate p-NPP (5 mM) solution was prepared in carbonate-bicarbonate buffer (pH 10.1). All reaction mixtures were carried out in a total volume of 3 ml. The amount of p-NP released was determined at 405 nm using spectrophotometer.

2.B.2.4.4 Native polyacrylamide gel electrophoresis and Zymography

Native polyacrylamide gel electrophoresis (PAGE) of crude enzyme extract was carried out by method developed by Laemmli (1970). Crude enzyme concentrate was mixed with sample loading Tris buffer **(Appendix B)** and resolved on a 12% separating gel topped with a 1% stacking gel. Crude enzyme sample was loaded in two wells separated by a well in which native protein marker was loaded. Following the electrophoresis, the gel was cut vertically in two parts. First part containing native protein marker and crude enzyme sample was stained by silver staining method to visualize proteins bands (Wray *et al.,* 1981). Second part containing only a crude enzyme sample was used for zymography. The vertical gel strip was cut into 2 mm horizontal bands for preparation of zymography. Each cut portion was added in an eppendorf tube containing 1 ml p-NPP (5 mM) substrate, 0.4 ml carbonate-bicarbonate buffer (0.1 M, pH 10.1). The eppendorf tubes were incubated in dark and monitored for colour change. After 4 hours of incubation, absorbance of the samples was recorded at 405 nm.

2.B.2.4.5 Statistical analysis

Linear regression was carried out for time courses of enzyme activity. One way ANOVA and Student Newman Keuls method was performed to detect significant

difference among different treatments. All statistical analysis was carried out using Sigma Stat Ver 4.0.

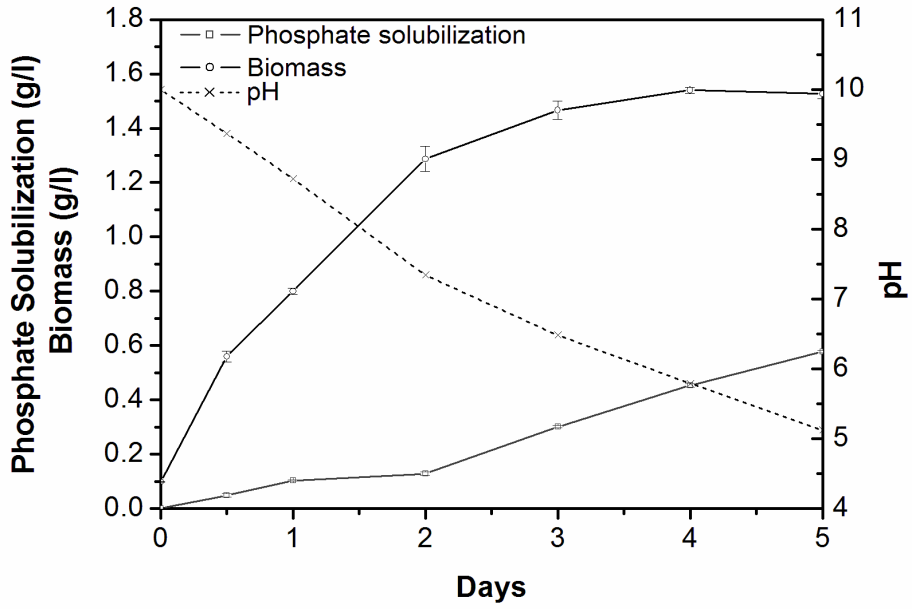
2.B.3 RESULTS

2.B.3.1 Time course of tricalcium phosphate solubilization

Bacillus marisflavi FA7 began to grow in the exponential phase within 12 hours of incubation in modified Pikovskaya's medium **(Figure 2.13.a)**. Stationary phase was observed within 72 hours of incubation. *Bacillus marisflavi* FA7 showed no growth but was found to survive and produce organic acids after broth attained neutral pH. Decrease in pH of the medium was observed from initial 10 to 5.12 over a period of 120 hours of incubation. Tricalcium phosphate solubilization started within 12 hours of incubation and continued through all the days up to 5 days, with final solubilized amount of 0.57 g/L of tricalcium phosphate. The pH of the medium had a negative correlation with phosphate solubilization by *Bacillus marisflavi* FA7 (r = - 0.954). Biomass and phosphate solubilization had a positive correlation (r = 0.8378).

Chromohalobacter israelensis WA3 was found to be in the exponential phase for 48 hours **(Figure 2.13.b)**. Stationary phase was observed till 120 hours. As the growth progressed, decrease in pH and phosphate solubilization was observed. *Chromohalobacter israelensis* WA3 was found to grow when the pH of the medium was neutral. Decrease in pH from 10.0 to pH 4.2 was recorded. At the end of 5 days, 1.8 g/l of tricalcium phosphate was solubilized by isolate *Chromohalobacter israelensis* WA3. The pH of the medium had negative correlation with phosphate solubilization (r = -0.985). Biomass and phosphate solubilization had a positive correlation (r = 0.961).

(a)



(b)

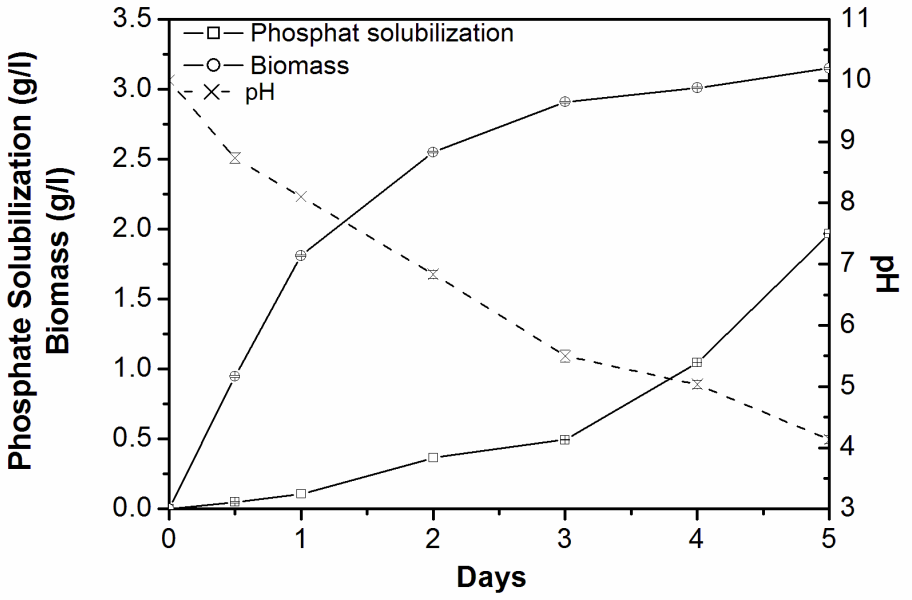


Figure 2.13 - Time course, growth and phosphate solubilization of *Bacillus marisflavi* FA7 (a) and *Chromohalobacter israelensis* WA3 (b) in Pikovskaya's medium in shaker flask condition with tricalcium phosphate as sole source of phosphate

2.B.3.2 Organic acid profile

Retention time of the peaks was compared with standard organic acid peaks **(Figure 2.14 and Table 2.6)**. HPLC analysis of day 1 and day 5 culture supernatants of *Bacillus marisflavi* FA7 showed four peaks **(Figure 2.15.a and 2.15.b)**. Two peaks were identified as oxalic acid and formic acid and remaining two were unidentified. Absorbance of acids produced on day 5 was five times higher as compared to day 1. HPLC analysis of day 1 and day 5 culture supernatants of *Chromohalobacter israelensis* WA3 showed four peaks in day 1 supernatant and five peaks in day 5 supernatant. Retention time of the peaks was recorded. **Figures 2.16.a** and **2.16.b** show peaks obtained after HPLC analysis of day 1 supernatant and day 5 supernatant, respectively.

2.B.3.3 Proton extrusion by ammonia assimilation

Bacillus marisflavi FA7 was found to grow in the presence of all nitrogen sources **(Figure 2.17.a)**. The biomass obtained with addition of inorganic nitrogen sources was higher than the control ($p<0.001$). Maximum growth was observed with NH_4NO_3 and $NaNO_3$ as compared to other treatments ($p<0.001$). Fall in the pH of culture broth was noted with all the substrates. Least decrease in pH was observed with $NaNO_3$ and KNO_3 ($p<0.001$). Significant decrease in pH was observed with NH_4Cl ($p<0.001$) when compared with other treatments. It was noted that the final pH of the control, $(NH_4)_2SO_4$ and NH_4NO_3 were similar ($p>0.01$). *Bacillus marisflavi* FA7 could solubilize tricalcium phosphate in all the treatments. Interestingly, highest phosphate solubilization was obtained with NH_4Cl as compared to other combinations ($p<0.05$).

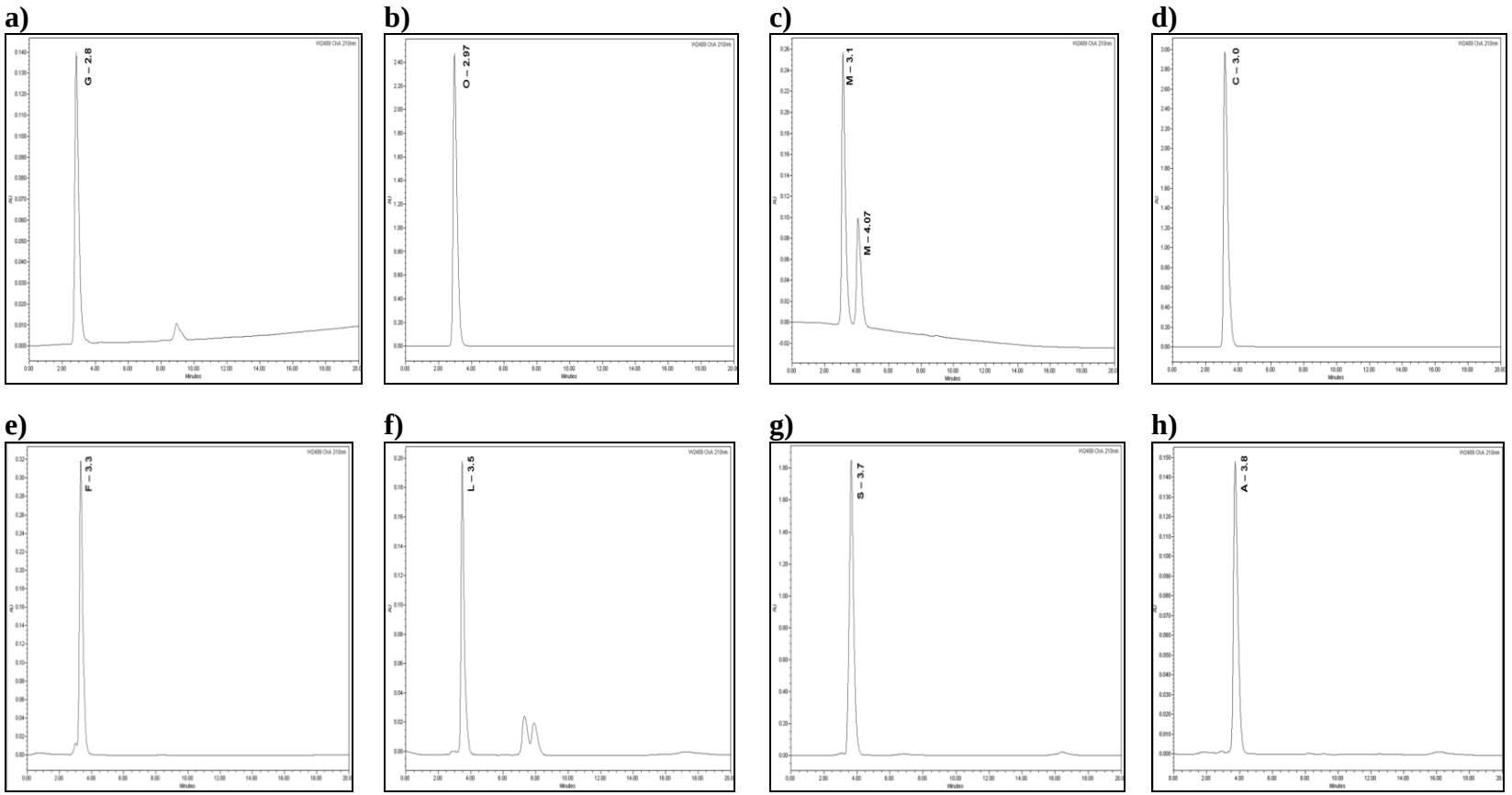
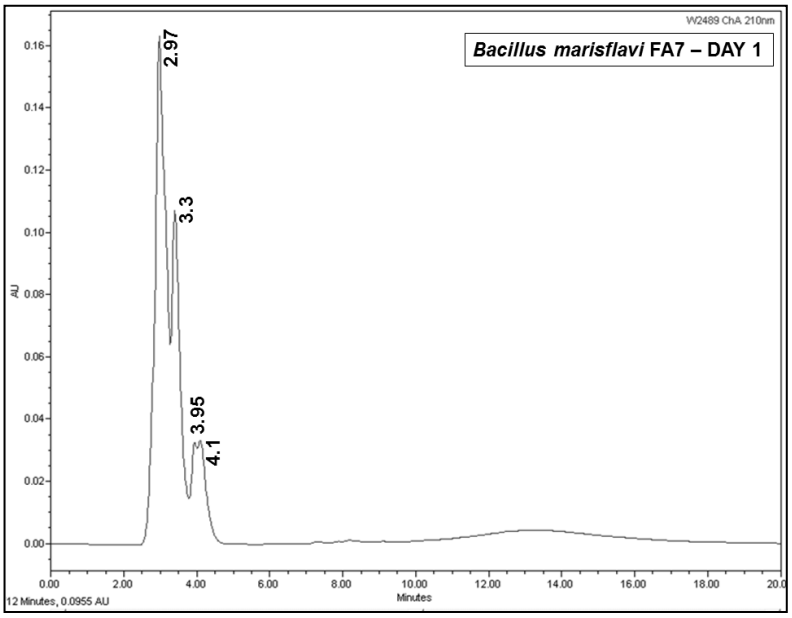


Figure 2.14 - Identification of standard organic acids by HPLC analysis; where, Gluconic acid (a), Oxalic acid (b), Malic acid (c), Citric acid (d), Formic acid (e), Lactic acid (f), Succinic acid (g) and Acetic acid (h)

Table 2.6 - Organic acids produced by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 in Pikovskaya's broth under shaker conditions on day 1 and day 5 (Refer Figure 2.15 and 2.16)

Organic acid	Retention time	*Bacillus marisflavi* FA7		*Chromohalobacter israelensis* WA3	
		Day 1	Day 5	Day 1	Day 5
Gluconic acid	2.8	-	-	-	-
Oxalic acid	2.97	+	+	+	+
Malic acid	3.1	-	-	-	+
Citric acid	3.2	-	-	-	-
Formic acid	3.3	+	+	+	+
Lactic acid	3.5	-	-	-	-
Succinic acid	3.7	-	-	-	-
Acetic acid	3.8	-	-	-	-
Unidentified	3.95	+	+	+	+
Unidentified	4.1	+	+	+	+

(a)



(b)

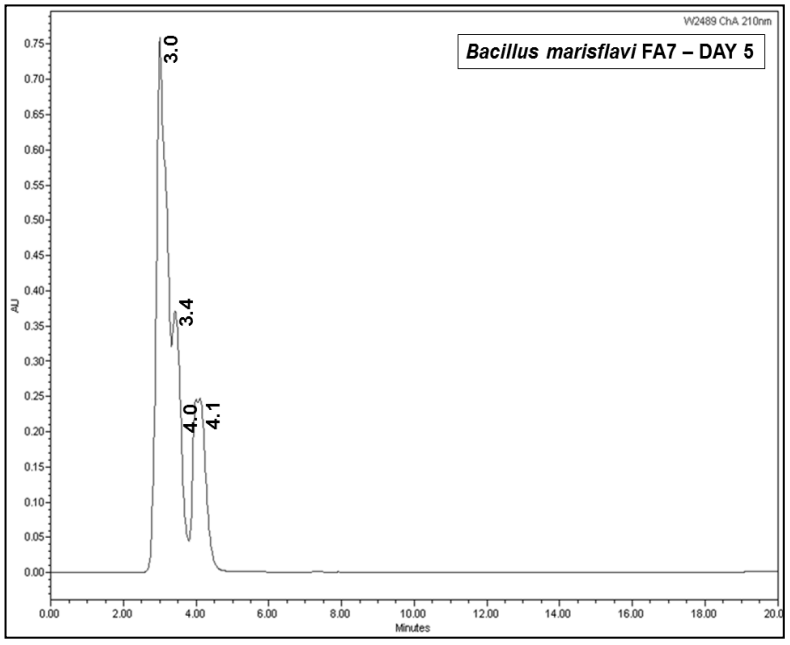
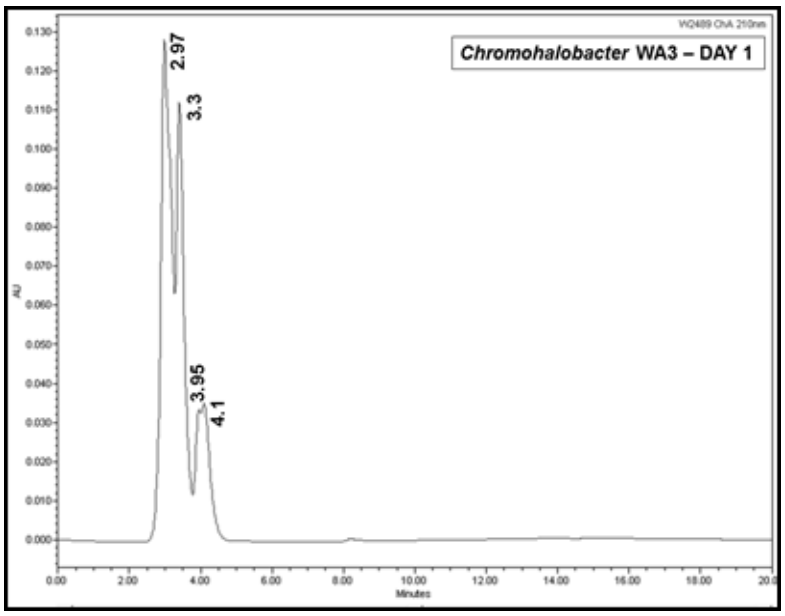


Figure 2.15 - HPLC analysis of culture supernatant of *Bacillus marisflavi* FA7 on Day 1 (a) and Day 5 (b) for determination of organic acids (Refer Table 2.6)

(a)



(b)

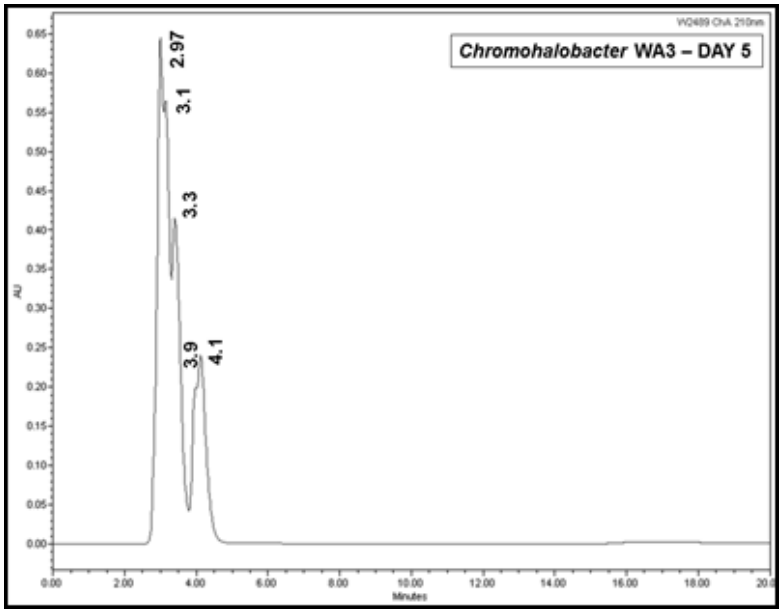


Figure 2.16 - HPLC analysis of culture supernatant of *Chromohalobacter israelensis* WA3 on Day 1 (a) and Day 5 (b) for determination of organic acids (Refer Table 2.6)

Chromohalobacter israelensis WA3 showed growth in all the treatments **(Figure 2.17.b)**. Maximum biomass was obtained with NH_4NO_3 compared to all treatments ($p<0.050$). Growth in NH_4Cl, $NaNO_3$ and KNO_3 was similar ($p>0.050$). There was no significant difference between the treatments NH_4SO_4 and control ($p>0.050$). Least decrease in pH was observed with control, $NaNO_3$ and KNO_3 ($p<0.001$). Among all treatments, significant decrease in pH was recorded with NH_4NO_3 ($p<0.050$). The final pH of NH_4Cl and $(NH_4)_2SO_4$ was similar ($0>0.050$). *Chromohalobacter israelensis* WA3 solubilized tricalcium phosphate in all the treatments. Highest phosphate solubilization was observed with NH_4NO_3 and NH_4Cl compared to other treatments ($p<0.001$).

2.B.3.4 EPS and siderophore production

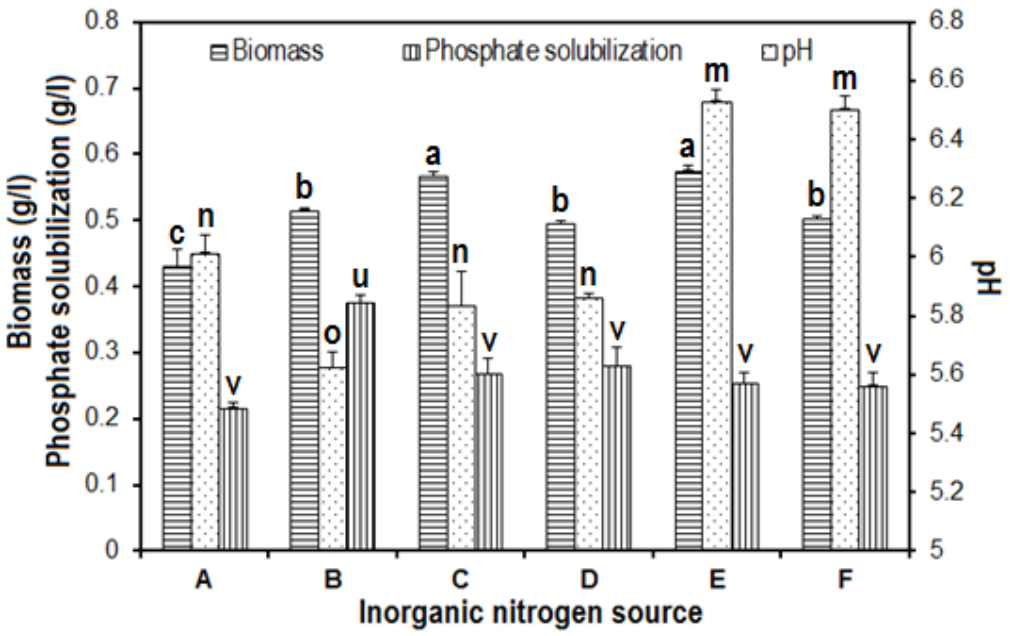
Bacillus marisflavi FA7 demonstrated change in colour from red to brown indicating production of EPS when inoculated on Congo red nutrient agar **(Figure 2.18)**. *Chromohalobacter israelensis* WA3 showed no brown colouration indicating that EPS was not produced.

Both *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 failed to produce siderophores.

2.B.3.5 Phosphatase activity

Bacillus marisflavi FA7 produced phosphatase in MSM containing p-NPP. Phosphatase enzyme activity and protein content of the cell free culture broth was found to be 5.14 U/ml and 368.5 µg/ml, respectively. After acetone precipitation, phosphatase enzyme activity of crude extract was 22.428 U/ml and protein concentration was 1000.5 µg/ml. Recovery during concentration step was 4.34 and 4% for enzyme and protein, respectively.

(a)



(b)

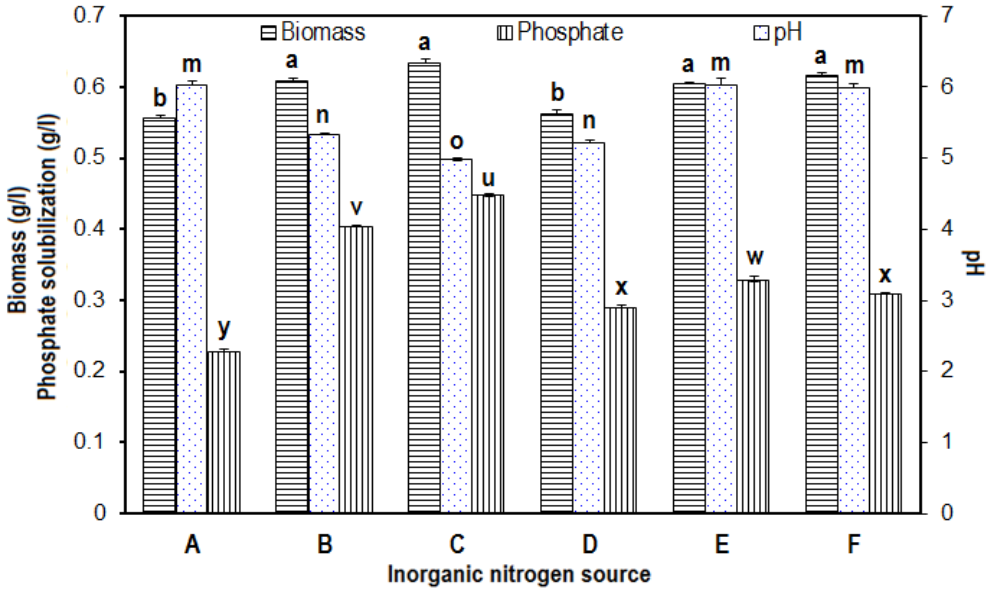


Figure 2.17 - Effect of different inorganic nitrogen sources on solubilization of tricalcium phosphate by *Bacillus marisflavi* FA7 (a) and *Chromohalobacter israelensis* WA3 (b); Where, A- Control (no inorganic nitrogen source), B- NH_4Cl, C- NH_4NO_3, D- $(NH_4)_2SO_4$, E- $NaNO_3$ and F- KNO_3. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.050.

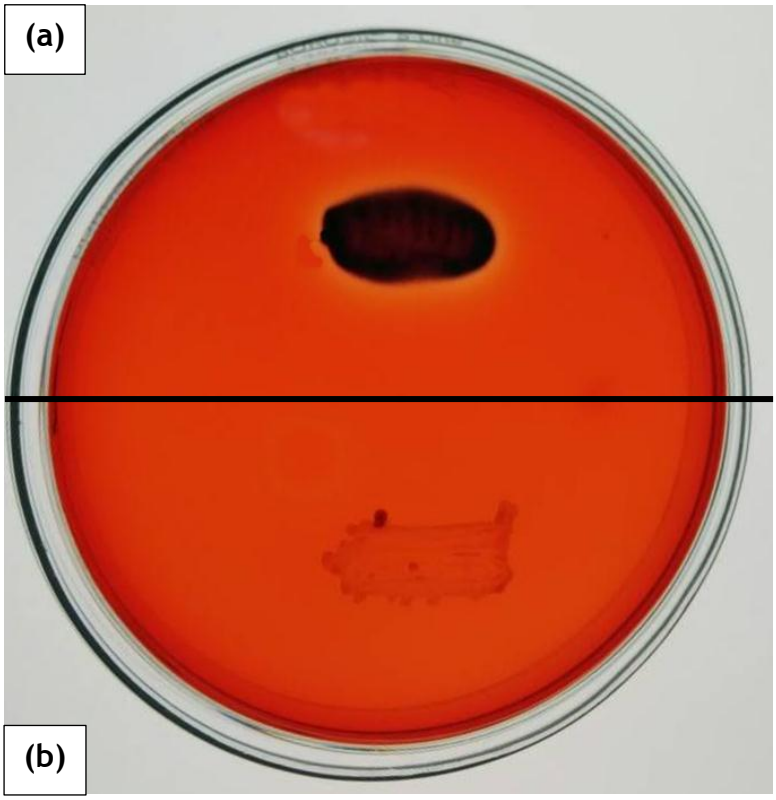


Figure 2.18 – Detection of exopolysaccharide on Congo red agar by *Bacillus marisflavi* FA7 (a) and *Chromohalobacter israelensis* WA3 (b)

2.B.3.6 Characterization of phosphatase enzyme

Activity of crude phosphatase enzyme extract was observed to increase with increase in concentration of p-NPP substrate **(Figure 2.19.a)**. However, after 1666 µM, no effect on rate of reaction was observed. The K_m and V_{max} values for the enzyme were 164.51 µM and 1.13 µM/sec, respectively **(Figure 2.19.b)**.

Phosphatase enzyme showed activity over a pH range from 9.1 to 10.6 **(Figure 2.20)**. Maximum activity was observed at pH 10.1. However, at pH 10.6 enzyme activity decreased drastically ($p<0.001$).

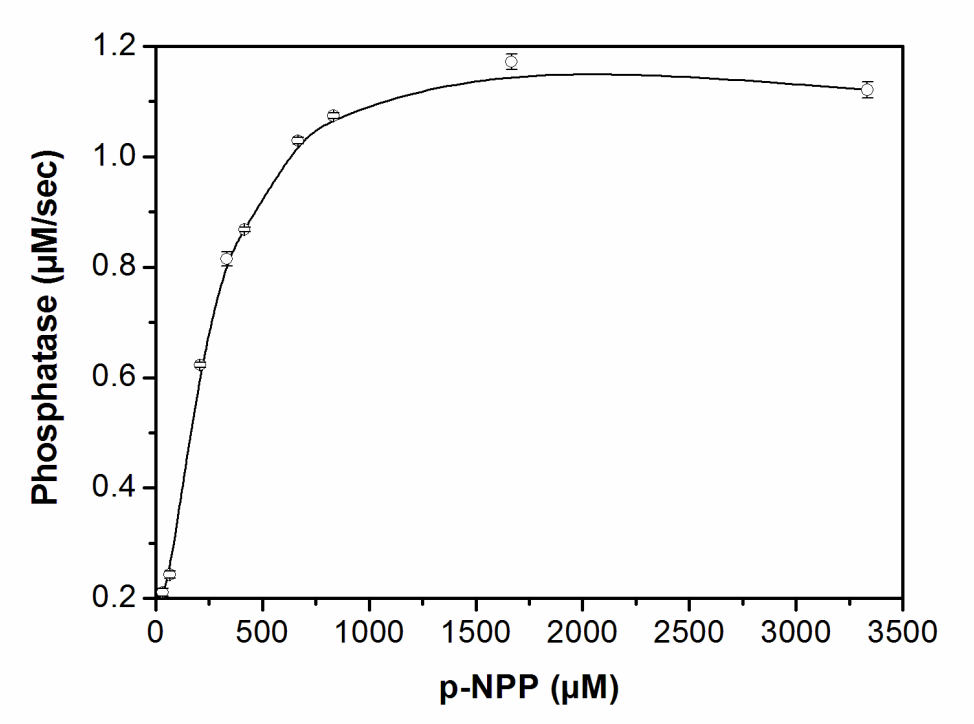
Activity of phosphatase enzyme increased with increase in molarity **(Figure 2.21)**. Further, from 0.1 M to 2 M of Na^+, the activity increase was gradual ($p<0.001$). No significant difference was observed in enzyme activity at molarities 0.1, 0.5 and 1.0. Phosphatase enzyme activity was significantly inhibited with different additives ($p<0.001$) **(Figure 2.22)**. β-mercaptoethanol caused maximum inhibition (69.58%) in activity compared to other additives. SDS, EDTA, Urea and Triton X100 showed reduction in activity but only to an extent of 10% to 26%.

Zymography of the protein gel demonstrated enzyme activity in the 3rd and 4th fractions of the gel **(Figure 2.23)**. Silver staining of the gel replicate showed 4 dark bands and 3 faint bands. Activity fraction showed that the protein is present in molecular weight range 175 – 200 kDa.

2.B.4 DISCUSSION

The scope of this study was to evaluate different mechanisms employed by alkaliphilic bacterial isolates *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 to solubilize inorganic and organic phosphate. Phosphate

(a)



(b)

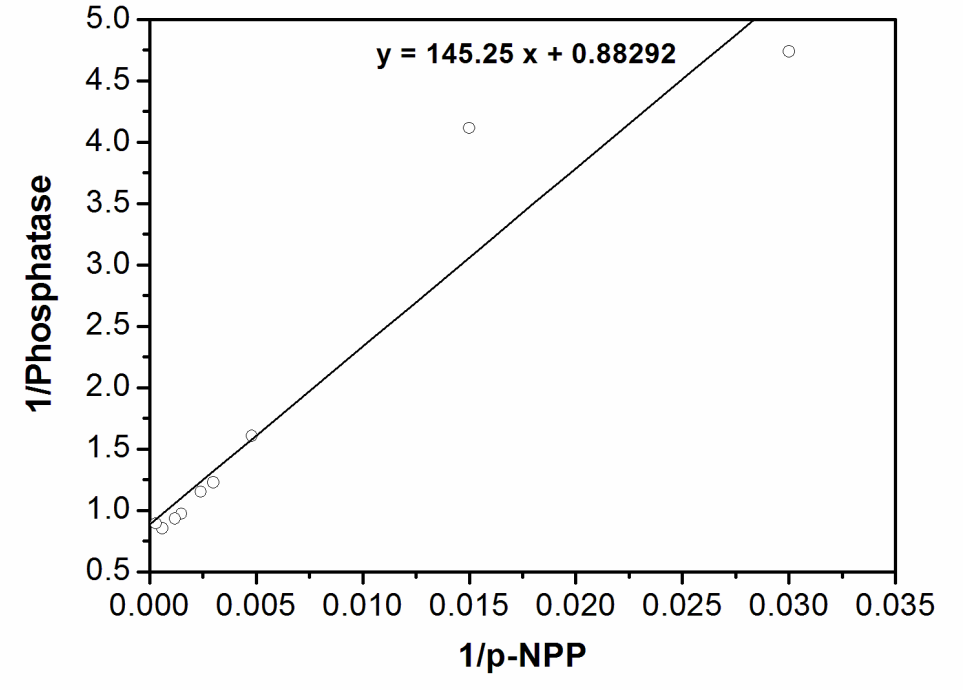


Figure 2.19 - Effect of different concentrations of p-NPP on phosphatase enzyme activity (a) along with Lineweaver-Burk plot for determination of K_m and V_{max} (b)

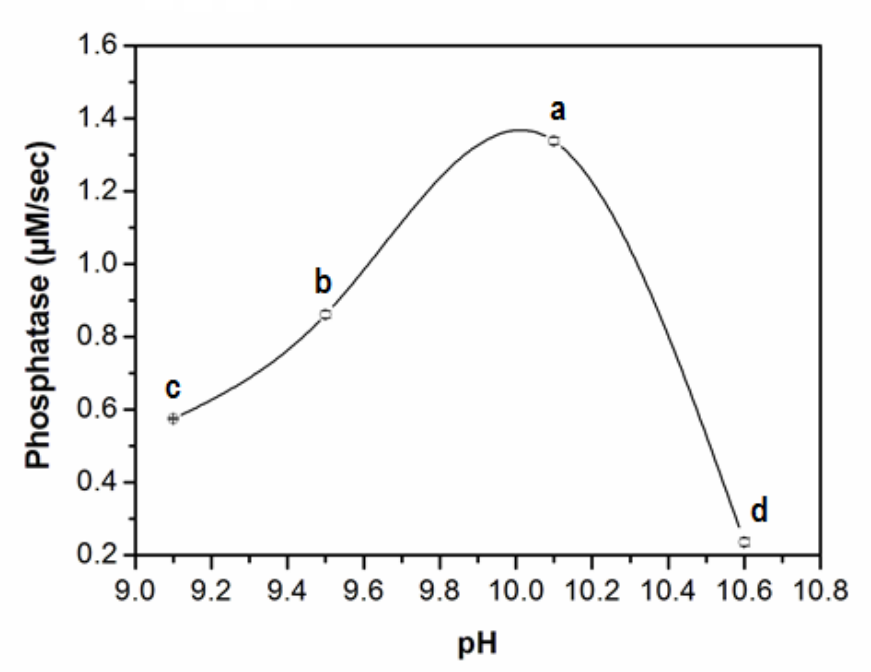


Figure 2.20 - Effect of pH on phosphatase enzyme activity. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.001.

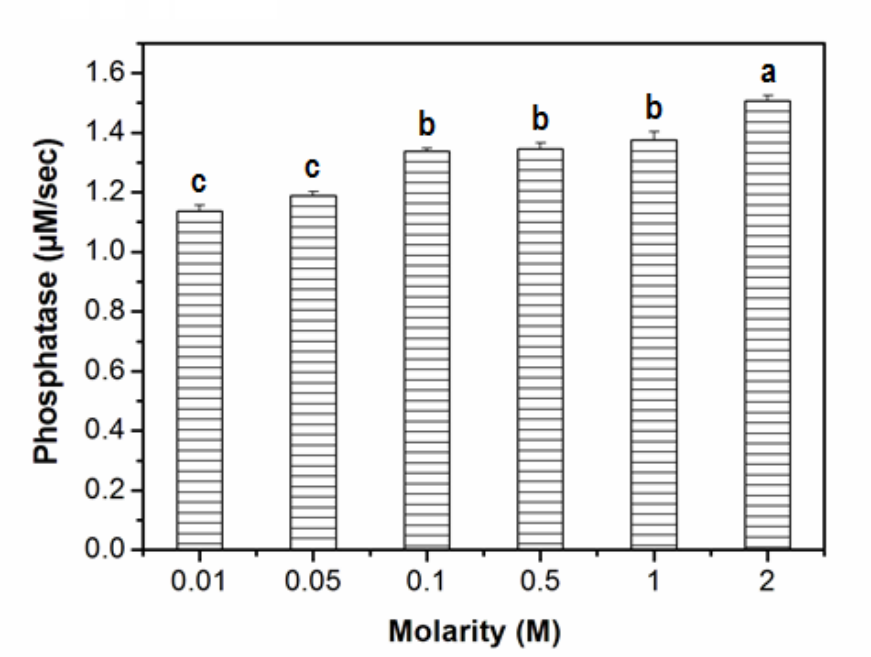


Figure 2.21 – Effect of molarity of sodium ions on phosphatase enzyme activity. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.001.

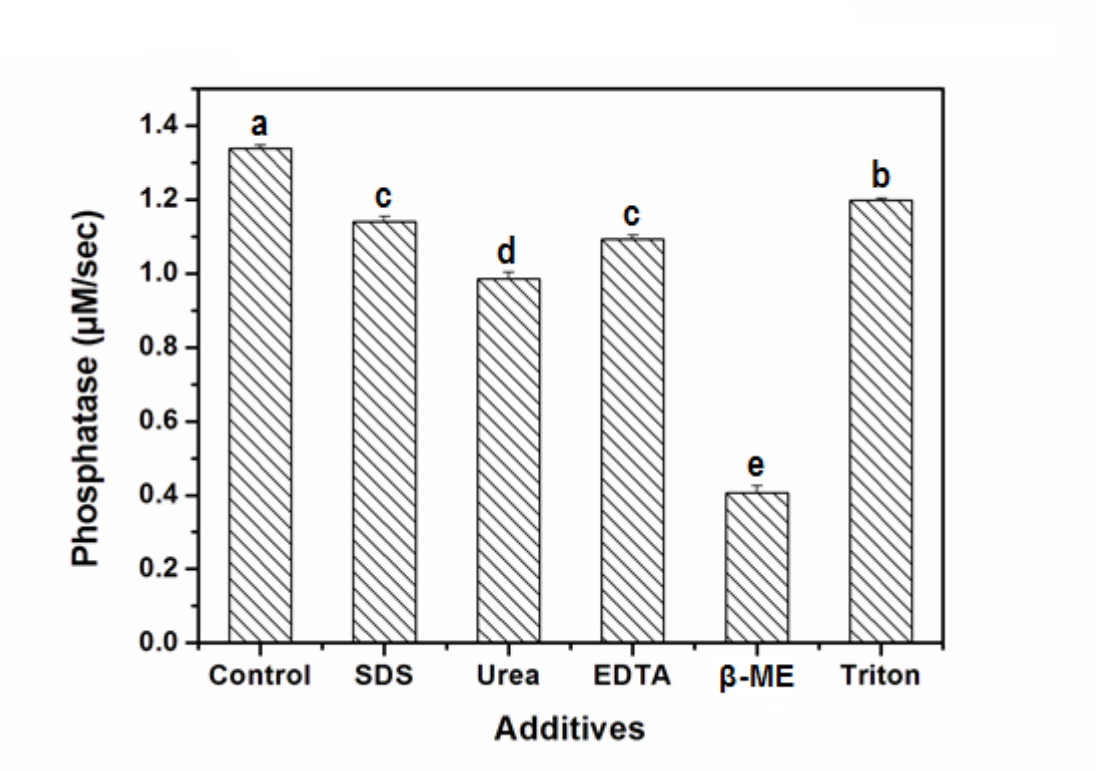


Figure 2.22 - Effect of additives on phosphatase enzyme activity (SDS -Sodium dodecyl sulfate, EDTA- Ethylenediaminetetraacetic acid, β-ME - β-mercaptoethanol). Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.001.

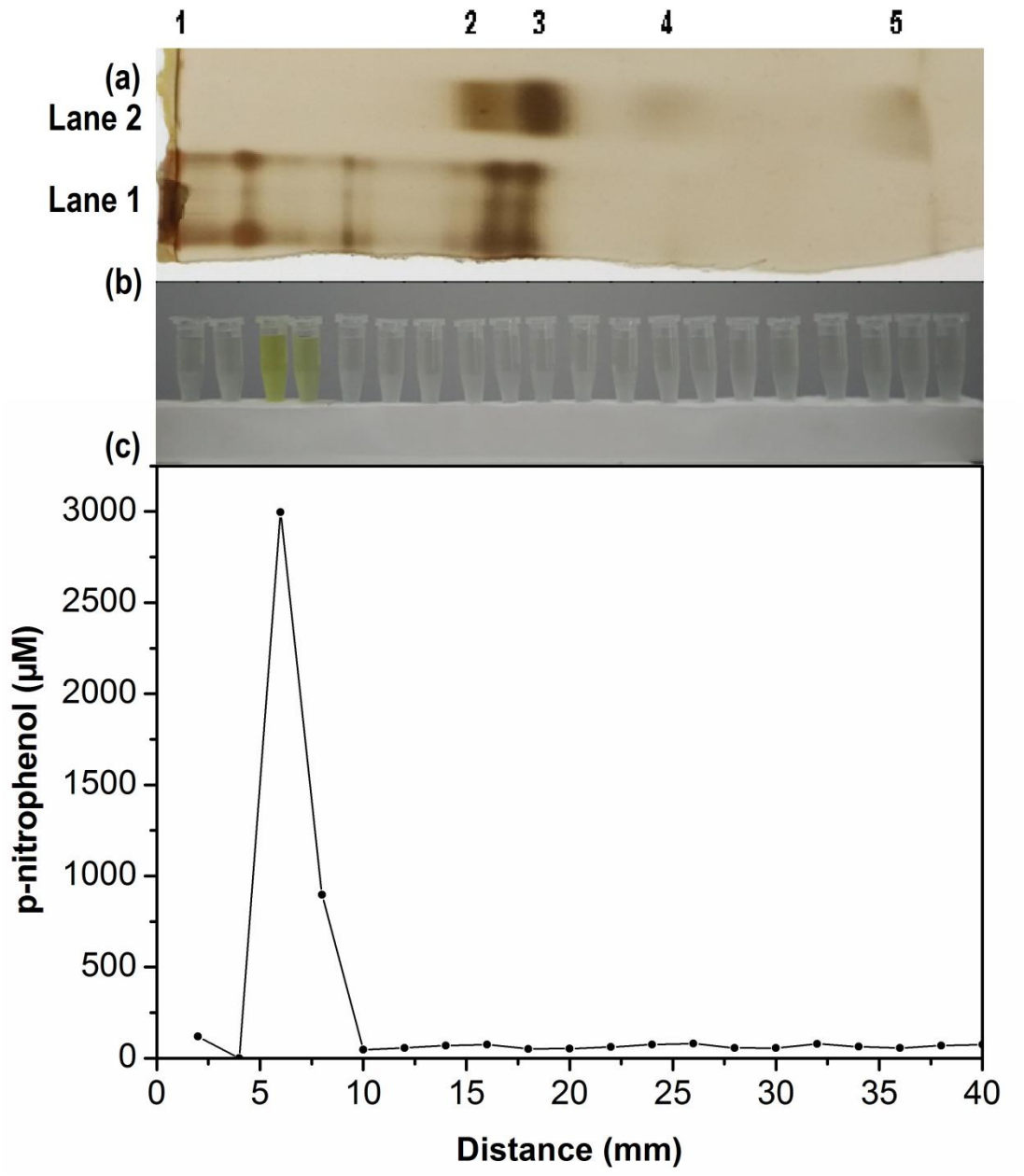


Figure 2.23 - Native PAGE and Zymography of crude alkaline phosphatase enzyme. Silver stained replicate of native gel (a); Lane 1 shows crude enzyme extract, Lane 2 shows native PAGE molecular weight marker; where, 1- 240kDa, 2- 67kDa, 3- 43kDa, 4- 20kDa, 5- 18kDa. Phosphatase enzyme activity of 2mm gel pieces as release of p-nitrophenol; where, b- pictorial and c- p-nitrophenol released.

solubilizing bacteria use diverse mechanisms to convert the insoluble phosphate into soluble forms.

Bacillus marisflavi FA7 isolated from Quellossim mangrove ecosystem solubilized 0.57 g/l tricalcium phosphate in five days of incubation. *Chromohalobacter israelensis* WA3 isolated from Batim salt pan ecosystem solubilized 1.8 g/l of tricalcium phosphate at the end of 5 days. Bacterial strains belonging to *Azotobacter, Bacillus* and *Pseudomonas* isolated from Chollangi, Godavari mangrove solubilized phosphate in the range of 0.080 – 0.1 g/l (Audipudi *et al.,* 2012). Pramod and Dhevendaran (1987) isolated phosphate solubilizing *Pseudomonas* and *Vibrio* from mangrove ecosystem of Cochin, India. They could solubilize phosphate in the range of 0.5 – 0.55 mg/l. So far, highest phosphate solubilization activity of 0.4 g/l was reported by the *Vibrio proteolyticus* in mangrove ecosystem in Mexico (Vazquez *et al.*, 2000).

Bacillus marisflavi FA7 and *Chromohalobacter israelensis* WA3 showed correlation between decrease in pH and phosphate solubilization. Similar findings have been reported in *Enterobacter intermedium* (Hwangbo *et al.*, 2003), *Serratia marcescens* GPS-5 (Tripura *et al.,* 2007), *Pseudomonas fluorescens* (Fankem *et al.,* 2008), *Burkholderia cepacia* DA23 (Ok-Ryul *et al.,* 2008) and *Pseudomonas trivialis* BIHB 747 (Vyas & Gulati, 2009). However, studies indicating no correlation between drop in pH and phosphate solubilization have also been reported (Mehta & Bhide, 1970; Wani *et al.,* 1979; Goldstein, 1986; Asea & Kucey, 1988).

Bacillus marisflavi FA7 showed 4 units decrease in the pH of the medium which is similar to the findings in phosphate solubilizing *Serratia* sp. (Behera *et al.,* 2017). Higher decrease in pH was recorded in *Chromohalobacter israelensis* WA3 as

compared to *Bacillus marisflavi* FA7. *Chromohalobacter israelensis* WA3 showed 6 units decrease in the pH of the medium.

Decrease in pH has been attributed to production of organic acids. The biochemical basis of gluconic acid and 2-ketogluconic acid is well studied in Gram negative bacteria by Goldstein (1995). *Bacillus marisflavi* FA7 released organic acids in the medium that were identified as oxalic acid, formic acid and two unidentified acids. Organic acids released by *Chromohalobacter israelensis* WA3 in the day 1 medium were identified as oxalic acid, formic acid and two unidentified acids; whereas, in day 5 supernatant, organic acids released were oxalic acid, formic acid, malic acid and two unidentified acids. Since, this is the first report of phosphate solubilization by Gram negative bacterium belonging to *Chromohalobacter* genus; no reports on the relationship of organic acid production and phosphate solubilization are available. However, Vargas *et al.* (2004) have studied genetics of osmoregulation in *Chromohalobacter* and proposed its potential in agriculture under osmotic stress conditions.

Pseudomonas strains produced 2-keto gluconic acid, citric acid, formic acid, gluconic acid, malic acid and succinic acid during solubilization of tricalcium phosphate (Vyas & Gulati, 2009). Citric and lactic acids were produced by *Bacillus megaterium* and *Bacillus circulans* from glucose in Pikovskaya's medium (Bajpai & Rao, 1971). Vazquez *et al.* (2000) reported production of acetic acid, iso-butyric acid, lactic acid and valeric acid during solubilization of tricalcium phosphate by *Bacillus licheniformis*. Another *Bacillus* strain produced 2-ketogluconic acid, acetic acid, isovaleric acid, lactic acid, oxalic acid and succinic acid (Vazquez *et al.*, 2000). Significant amount of reports from scientists suggest that 2-ketogluconic acid and

gluconic acid are major organic acids responsible for phosphate solubilization (Hwangbo *et al.,* 2003; Vyas & Gulati, 2009; Hsu *et al.,* 2015). However, HPLC analysis revealed that gluconic acid and 2-ketogluconic acids were not produced by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3.

Other reported mechanisms which decrease the pH are production of inorganic acids and H^+ ion excretion due to ammonia assimilation. For *Bacillus marisflavi* FA7, ammonium salts showed maximum decrease in pH compared to nitrate. When only nitrate salts were used decrease in pH was least. In line of this, solubilization was seen maximum with NH_4Cl for *Bacillus marisflavi* FA7. *Chromohalobacter israelensis* WA3 showed maximum solubilization with NH_4NO_3. This suggests that proton extrusion during ammonium assimilation is responsible to some extent of phosphate solubilization by *Bacillus marisflav*i FA7 and *Chromohalobacter israelensis* WA3.

Illmer and Schinner (1995) reported that a species of *Pseudomonas* did not produce any organic acid even though the bacterium solubilized inorganic phosphate. The amount of protons released into the external medium is often significantly influenced by nitrogen supply. In general, a greater reduction in pH together with more solubilized phosphate can be observed with NH_4^+ as the sole nitrogen source compared to NO_3, due to the extrusions of protons to compensate for NH_4 uptake (Roos & Luckner, 1984; Illmer *et al.,* 1995; Sharan *et al.,* 2008). In *Pseudomonas fluorescens,* the type of carbon source (e.g. glucose versus fructose) rather than nitrogen source (e.g. NH_4^+ versus NO_3) had the greatest effect on proton release (Park *et al.,* 2009). This indicates that mechanisms for proton release are different for different species.

Exopolysaccharides and biosurfactants are produced by microorganisms largely in response to biofilm formation and stress. Microbial exopolysaccharides form complex with metals in soil (Ochoa-Loza *et al.,* 2001). This suggests that exopolysaccharides play a possible role in release of phosphates from metal compounds (Zhao *et al.,* 2002; Chen *et al.,* 2006). *Bacillus marisflavi* FA7 showed the production of exopolysaccharide. However, its role in phosphate solubilization needs further investigation. In *Enterobacter* sp., exopolysaccharide contributed to the dissolution of tricalcium phosphate synergistically with organic acids (Yi *et al.,* 2008). *Chromohalobacter israelensis* WA3 showed no production of exopolysachharide.

Siderophores are chelating agents that have a high affinity for iron and are produced by microorganisms in response to iron deficiency. *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 did not produce any siderophores. Many studies have reported the production of siderophores from phosphate solubilizing microorganisms (Vassilev *et al.,* 2006; Caballero-Mellado *et al.,* 2007; Hamdali *et al.,* 2008). Reid *et al.* (1985) investigated the ability to increase iron and phosphate diffusion of two siderophores (desferrioxamine-B, desferriferrichrome) as compared to water using a root simulation technique. They found that desferriferrichrome increased phosphate diffusion 13-fold compared to water whereas different concentrations of desferrioxamine-B exhibited only a small effect. Siderophore production could be useful in solubilizing iron containing phosphate compounds.

Bacillus marisflavi FA7 produced alkaline phosphatase enzyme. Major source of phosphatase activity in soil is considered to be of microbial origin. Bacteria belonging to the genera *Bacillus, Citrobacter, Enterobacter, Klebsiella, Proteus,*

Pseudomonas, Rhizobium and *Serratia* have been reported to produce phosphatase enzyme (Luo *et al.*, 2017).

Cell membrane bound phosphatase enzyme has been reported to be responsible for phosphate solubilization in *Bacillus subtilis* and *Bacillus megaterium* (Wood & Tristram, 1970). However, phosphatase enzymes of these cultures are not detected in the culture broth. Extracellular alkaline phosphatase has been reported in *Bacillus amyloliquefaciens* and *Bacillus cereus* (Priest, 1977). Extracellular phosphatases hold promise for toxic metal bioremediation and bio-recovery through metal phosphate precipitation. Liang *et al.* (2016) observed that phosphatase from *Aspergillus niger* and *Paecilomyces javanicus* was able to precipitate lead when grown in medium containing glycerol-2-phosphate as the sole source of phosphorus. Similar results have been reported with copper metal (Tsekova *et al.*, 2000). Isolate *Bacillus marisflavi* FA7 demonstrated production of extracellular phosphatase enzyme with molecular weight of approximately 170 – 200 kDa in its native protein form. The molecular weight of extracellular alkaline phosphatase from *Bacillus licheniformis* was reported to be 121 kDa and was composed of four subunits (Priest, 1977). Alkaline phosphatase from *Vibrio* was recorded to be 60 kDa (Hauksson *et al.*, 2000).

The K_m and V_{max} values for the phosphatase enzyme of *Bacillus marisflavi* FA7 were 164.51 µM and 1.13 µM/sec, respectively. The alkaline phosphatase of *E. coli* has been reported to be an intracellular zinc metallo-enzyme with the molecular weight of 80,000 daltons, composed of two identical subunits. Its K_m was found to be 1 mM and the optimum pH of 10.0 (Halford, 1971). Aziz *et al.* (2017) determined

K_m and V_{max} for phosphatase enzyme of *Pyrobaculum calidifontis* as 60 µM and 4.0 µmol/min/mg, respectively.

Considerable variations have been observed in alkaline phosphatase activity in the presence of environmental factors such as pH, salt concentration, moisture, temperature, heavy metals, precipitation and nutrients in various ecosystems (Hou *et al.,* 2015). Phosphatase enzymes are pH sensitive. Phosphatase enzyme of *Bacillus marisflavi* FA7 was highly active at alkaline pH 10.1. It showed activity at higher molar concentration of sodium ions. Sodium ions are essential for growth of marine, alkaliphilic bacteria that live in sodium rich habitats (Dimroth, 1987). Phosphatase enzyme of *Bacillus marisflavi* FA7 showed decrease in activity in the presence of additives like beta-mercaptoethanol, SDS, EDTA, Urea and Triton X 100. Aziz *et al.* (2017) observed that in the presence of EDTA, phosphatase enzyme activity is inhibited as it requires metal ions for activity.

Bacillus marisflavi FA7 isolated from Quellosim mangrove ecosystem and *Chromohalobacter israelensis* WA3 isolated from Batim salt pan ecosystem of Goa exhibited more than one mechanism of phosphate solubilization **(Figure 2.24)**. Therefore, they could be used as potential phosphate solubilizer in alkaline-saline soils because of their innate phosphate solubilizing mechanisms under alkaline-saline conditions.

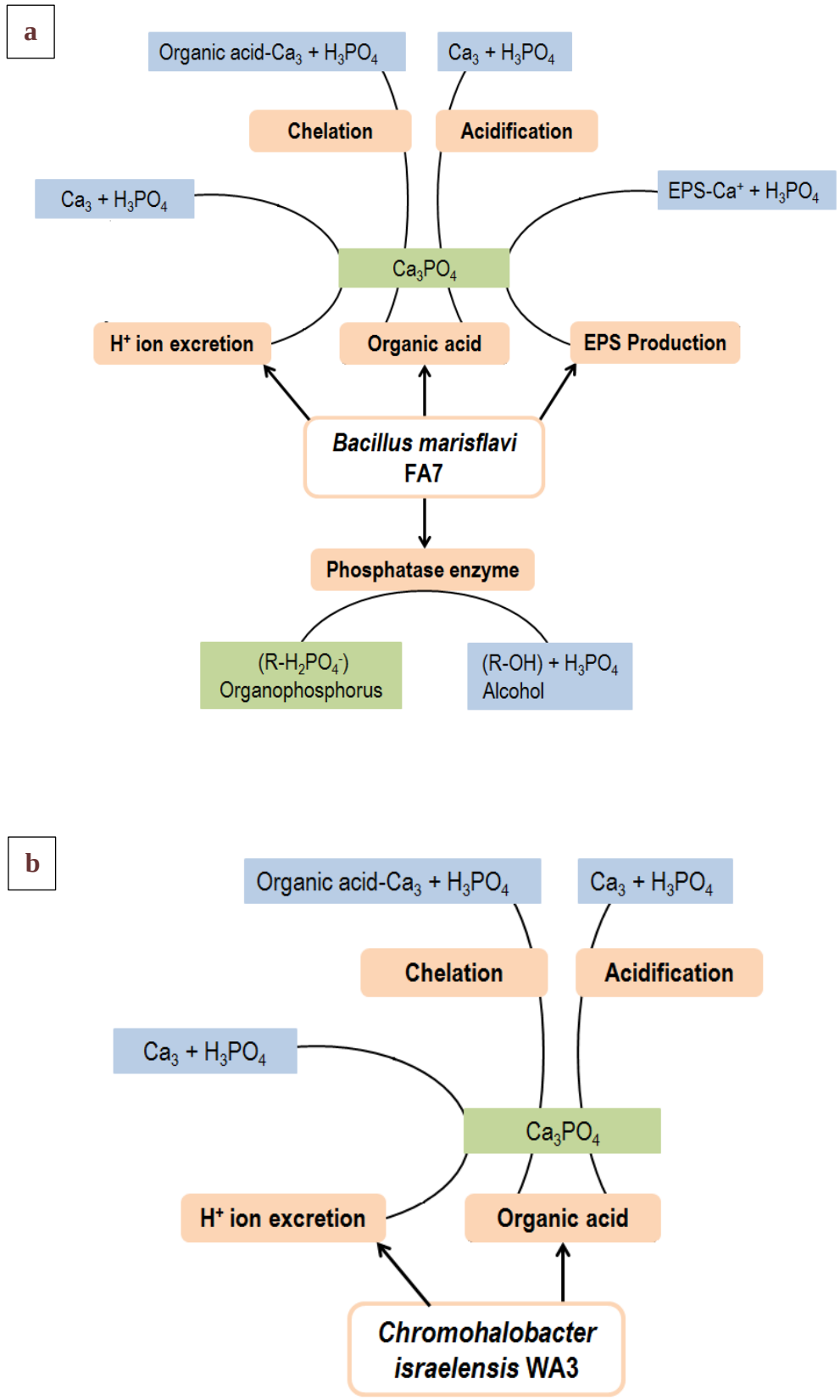


Figure 2.24 – Phosphate solubilization mechanisms in *Bacillus marisflavi* FA7 (a) and *Chromohalobacter israelensis* WA3 (b)

Chapter III

The optimal physico-chemical and nutritional requirements for phosphate solubilization

3.1 INTRODUCTION

Growth of phosphate solubilizing bacteria and the accumulation of metabolic products such as enzymes and organic acids is strongly influenced by medium components such as organic and inorganic salts (Sadaf & Nuzhat, 2008; Srividya *et al.,* 2009; Srinivasan *et al.,* 2012). The medium chosen should be inexpensive and should support good growth of microorganisms. It is a tedious job to search for multivariable major factors in a medium and to optimize them for biotechnological processes (Abdelwahed *et al.,* 2017). To formulate such an appropriate medium, one has to screen different substrates to find out the best suitable for maximum production. The conventional one factor at a time technique used for optimization of multivariable system is laborious, time consuming and often results in wrong inferences. Main effects and interactions of the factors that play fundamental roles in the production can be understood by applying statistical methods (Tabora & Domagalski, 2017).

Several methods can be found in literature (Rustagi, 1994; Montgomery, 2017). Only a few have got their way in biotechnological processes, especially fermentation studies. Some of these methods are Plackett-Burman Design, Response Surface Methodology and Simplex Search Technique (Myers *et al.,* 2016; Ekpenyong *et al.,* 2017). All the three methods have found different applications in the development of microbial processes. The objectives of statistically designed optimization study are to determine effects, interactions and optimal settings for the critical factors. Optimization through factorial design and response surface analysis is now a common practice in biotechnology (Ahuja *et al.,* 2003; Polak-Berecka *et al.,* 2011; Kaur & Kaur, 2013). Various research workers have applied this technique for

optimization of culture conditions. Once the critical factors have been identified via screening, the next step is optimization of experimental design.

Response Surface Methodology (RSM) was described five decades ago (Box & Wilson, 1951). It is a collection of statistical techniques for designing experiments, building models, evaluating the effect of single factor, interaction of many factors and searching optimum conditions of factors for desirable responses. Optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Using the mathematical model, the levels of the variables giving maximum response can be calculated.

The design can consist of two to five variables. In a three variable, composite design, variable can be presented as the three axis of a cube (X_1, X_2, X_3) **(Figure 3.1)**.
One end of the axis represents the high level and other represents low level of variable. Further, the centre of the axis is presented as central level of variable. According to the central composite design, complete randomization can be achieved by assuming all the levels as equally important and the central value assumed as the optimum for maximum or minimum of response. Boundary limits of the design are considered as two levels. All the five levels of three variables are presented in **Table 3.1**.

This requires a minimum of 15 experiments described as 8 corner points (high-low level combination), 6 face/star points (boundary limits) and one centre point (optimum level) of the cube **(Figure 3.1)**. To achieve the goal of regression and estimation of error within experiments, the centre point has to be repeated. These 20 experiments are presented in **Table 3.2**.

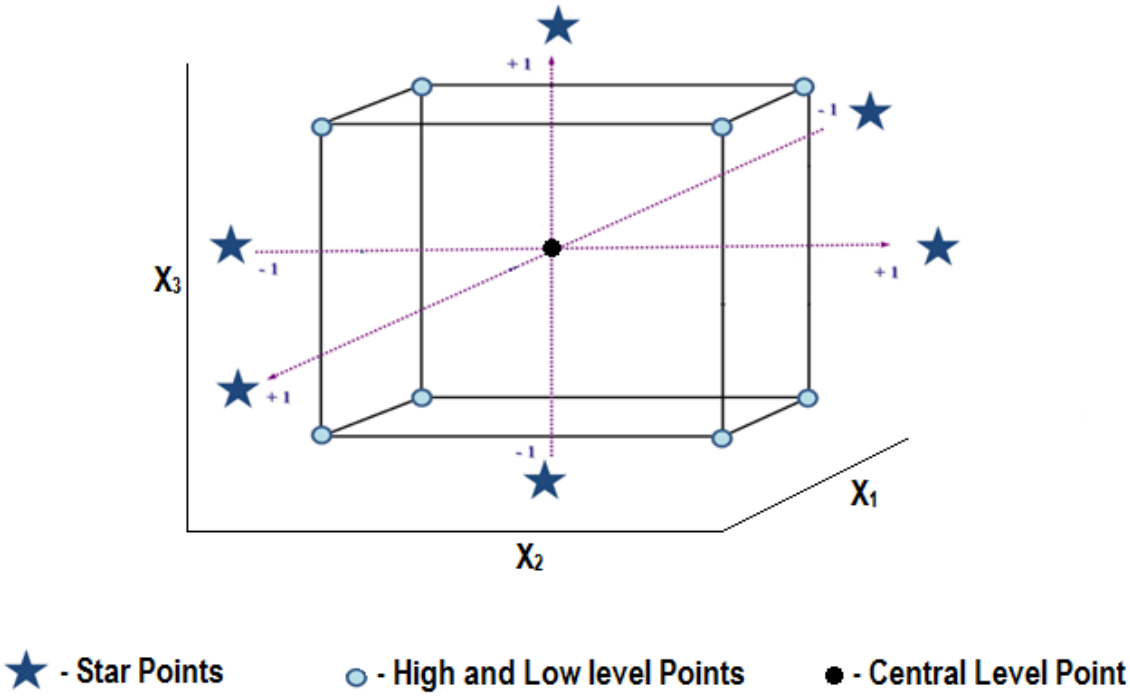


Figure 3.1 – Central Composite Design with three variables

Table 3.1 – Different levels of three variables used for Full Factorial Composite Design

Variable X	Levels				
	Star	Low	Centre	High	Star
X_1	-α	-1	0	+1	+α
X_2	-α	-1	0	+1	+α
X_3	-α	-1	0	+1	+α

Table 3.2 – Full Factorial Design of 15 experiments plus five experiments of repetition of central points

Experiment Number	Variable		
	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	-1.682	0	0
10	1.682	0	0
11	0	-1.682	0
12	0	1.682	0
13	0	0	-1.682
14	0	0	1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

The experimental data can be processed to fit second or quadratic level model as described below,

$$Y = (X_1 + X_2 + X_3)^2 = \beta_0 + \beta_1*X_1 + \beta_2*X_2 + \beta_3*X_3 + \beta_{11}*X_1^2 + \beta_{22}*X_2^2 + \beta_{33}*X_3^2 + \beta_{12}*X_1*X_2 + \beta_{13}*X_1*X_3 + \beta_{23}*X_2*X_3$$

Where, β_0 = constant coefficient

β_1, β_2, β_3 = linear coefficients

β_{11}, β_{22}, β_{33} = quadratic coefficients

β_{12}, β_{13}, β_{23} = second order interaction coefficients

Y = Response

Validation of the model developed is carried out by comparison between the experimental and calculated values of response observed of experiments. If the calculated value falls close to the experimental value, the model is said to be fitted well. To validate a model, the lack of fit should be less than 50% of the experiments.

With the development of computing software, RSM has been successfully applied in many areas of biotechnology. RSM has been applied to optimize variables for maximum phosphate solubilization in *Aspergillus japonicas* SA22P3406 (Nopparat *et al.,* 2009), *Pseudomonas putida* Rs-198 (Peng *et al.,* 2014), *Acinetobacter calcoaceticus* TM8 (Mishra *et al.,* 2015) and *Penicillium purpurogenum* (Scervino *et al.,* 2011). Culture conditions of *Bacillus subtilis* CM5 (Swain & Ray, 2008) and *Bacillus pumillus* (Iyyappan *et al.,* 2015) were optimized using RSM for production of phytohormone indole-3-acetic acid.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strain, growth medium and conditions

Physico-chemical and nutritional parameters for biomass and phosphate solubilization were optimized for *Bacillus marisflavi* FA7. Basal medium used for all optimization experiments was Pikovskaya's broth **(Appendix A)**. Initial pH of the medium was adjusted to 10.0. The flasks were inoculated with 1% inoculum of *Bacillus marisflavi* FA7 grown in modified Pikovskaya's broth and incubated at 30°C on 150 rpm shaker for 48 hours.

3.2.2 Effect of pH

Effect of different initial pH on growth, decrease in pH and phosphate solubilization was studied. Initial pH values selected were 7.0, 8.0, 9.0, 10.0 and 11.0. *Bacillus marisflavi* FA7 was inoculated in 50 ml Pikovskaya's broth at different initial pH values **(Appendix A)**. Biomass and solubilized phosphate were estimated gravimetrically.

3.2.3 Effect of crude salt concentration

Effect of different concentrations of crude salt on growth, decrease in pH and phosphate solubilization was studied. Different crude salt concentrations selected were 1.0, 2.0, 3.0, 4.0 and 5.0%. *Bacillus marisflavi* FA7 was inoculated in 50 ml Pikovskaya's broth with different concentrations of crude salt **(Appendix A)**. Biomass and solubilized phosphate were estimated gravimetrically.

3.2.4 Screening of carbon sources

Effect of different carbon sources on growth, decrease in pH and phosphate solubilization was studied. Different carbon sources selected for screening were fructose, glucose, lactose, maltose, mannitol, sucrose and xylose. *Bacillus marisflavi*

FA7 was inoculated in 50 ml Pikovskaya's broth with different carbon sources **(Appendix A)**. Biomass and solubilized phosphate were estimated gravimetrically.

3.2.5 Screening of inorganic nitrogen sources

Effect of different inorganic nitrogen sources on growth, decrease in pH and phosphate solubilization was studied. Different nitrogen salts selected for screening were ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrate and ammonium nitrate. *Bacillus marisflavi* FA7 was inoculated in 50 ml Pikovskaya's broth with different inorganic nitrogen sources **(Appendix A)**. Biomass and solubilized phosphate were estimated gravimetrically.

3.2.6 Optimization by Response Surface Methodology

A three factor five factorial complete rotatable Central Composite Design (CCD) (Haaland, 1989) was employed to find out the interactive effect of three variables tricalcium phosphate (TCP) (X_1), glucose (X_2) and ammonium chloride (X_3) on biomass and phosphate solubilization. Each factor in the design was studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) with 20 experimental flasks. The statistical software package Sigma Stat Version 4.0 was used to analyse the experimental design. The full experimental plan with respect to their values in actual form is listed in **Table 3.3**. A minimum number of experiments for three factors were 14 + 1= 15. A total of 20 experiments were placed with the repeat of experiments with central levels in order to estimate the residuals **(Table 3.4)**.

Table 3.3 – Experimental levels of distribution of variables (g/l) used for evaluation of biomass and phosphate solubilization response

Variable		-α	-1	0	+1	+α
X_1	Tricalcium phosphate	3.3	5	7.5	10	11.7
X_2	Glucose	2.45	5	10	15	17.55
X_3	Ammonium chloride	0.081	0.165	0.33	0.495	0.579

Table 3.4 – A set of 20 experiments with the actual values of three variables in each experiment

Flask no.	Actual		
	TCP (g/l)	Glucose (g/l)	Ammonium chloride (g/l)
1	5	5	0.165
2	10	5	0.165
3	5	15	0.165
4	10	15	0.165
5	5	5	0.495
6	10	5	0.495
7	5	15	0.495
8	10	15	0.495
9	3.3	10	0.33
10	11.7	10	0.33
11	7.5	2.45	0.33
12	7.5	17.55	0.33
13	7.5	10	0.081
14	7.5	10	0.579
15	7.5	10	0.33
16	7.5	10	0.33
17	7.5	10	0.33
18	7.5	10	0.33
19	7.5	10	0.33
20	7.5	10	0.33

A second order quadratic model, assumed to describe, the relationship between the response (Y) and the experimental factors (X_1, X_2 and X_3), was

$$Y = (X_1 + X_2 + X_3)^2 = \beta_0 + \beta_1*X_1 + \beta_2*X_2 + \beta_3*X_3 + \beta_{11}*X_1^2 + \beta_{22}*X_2^2 + \beta_{33}*X_3^2 + \beta_{12}*X_1*X_2 + \beta_{13}*X_1*X_3 + \beta_{23}*X_2*X_3$$

Where, β_0 = constant coefficient

β_1, β_2, β_3 = linear coefficients

β_{11}, β_{22}, β_{33} = quadratic coefficients

β_{12}, β_{13}, β_{23} = second order interaction coefficients

Y = Response

The flasks were incubated for a period of 48 hours at 30°C and then analyzed for amount of phosphate solubilized and biomass accumulation. Upon completion of experiments, the average phosphate solubilization and biomass production was taken as the dependent variable or response (Y). Experimental observations obtained from the central composite design were subjected to non-linear regression analysis using Sigma Stat for the above quadratic equation. Results obtained from non-linear regression analysis were used to describe the mathematical model for the biomass accumulation and phosphate solubilization. The optimum values and the models obtained were then used to generate combinations of variables for 2-D isotherm graphs using MS Excel 2010.

3.2.7 Estimation of biomass and residual phosphate

Culture broth was centrifuged at 10,000 rpm for 10 min to obtain pellet containing cells and residual tricalcium phosphate. The pellet was suspended in chilled HCl (1 N) to dissolve residual tricalcium phosphate. The mixture was centrifuged at 10,000 rpm for 10 min to separate cell pellet from dissolved residual phosphate. Supernatant

was collected in a separate tube. To the supernatant, NaOH (1 N) was added to re-precipitate tricalcium phosphate. Pellets of cells and tricalcium phosphate were washed in distilled water and centrifuged at 10,000 rpm. Pellets were dried in oven to estimate biomass and residual phosphate.

3.3 RESULTS

3.3.1 Effect of initial pH

Bacillus marisflavi FA7 showed growth from pH 8.0 to 11.0 **(Figure 3.2)**. Maximum growth was observed at pH 10.0 as compared to other pH values ($p<0.050$), followed by pH 11.0. It showed negligible growth at pH 7.0 ($p<0.001$). Growth was found to increase with increase in pH values from 8.0 to 10.0. *Bacillus marisflavi* FA7 showed highest decrease in pH when inoculated in a medium with pH 9.0 ($p<0.050$). No decrease in pH was observed in pH 7.0 flask ($p<0.001$). *Bacillus marisflavi* FA7 showed phosphate solubilization from pH values 8.0 to 11.0. Maximum phosphate solubilization was observed at pH 9.0 compared to other pH values ($p<0.050$). Negligible phosphate solubilization was observed at pH 7.0 ($p<0.001$). From pH 9.0, phosphate solubilization was found to decrease with increase in initial pH values. Phosphate solubilization at pH 8.0 and 10.0 was found to be similar ($p>0.050$).

3.3.2 Effect of crude salt concentration

Bacillus marisflavi FA7 exhibited growth from crude salt concentration of 0 to 5% **(Figure 3.3)**. However, growth was found to decrease with increase in salt concentration. Maximum growth was observed at 1.0% crude salt ($p<0.050$). Growth at crude salt concentrations 0% and 2% was found to be similar ($p>0.050$). At crude salt concentration of 5.0%, growth was the least ($p<0.001$). *Bacillus marisflavi* FA7 showed reduction in pH in all the treatments. Highest decrease in pH was observed at

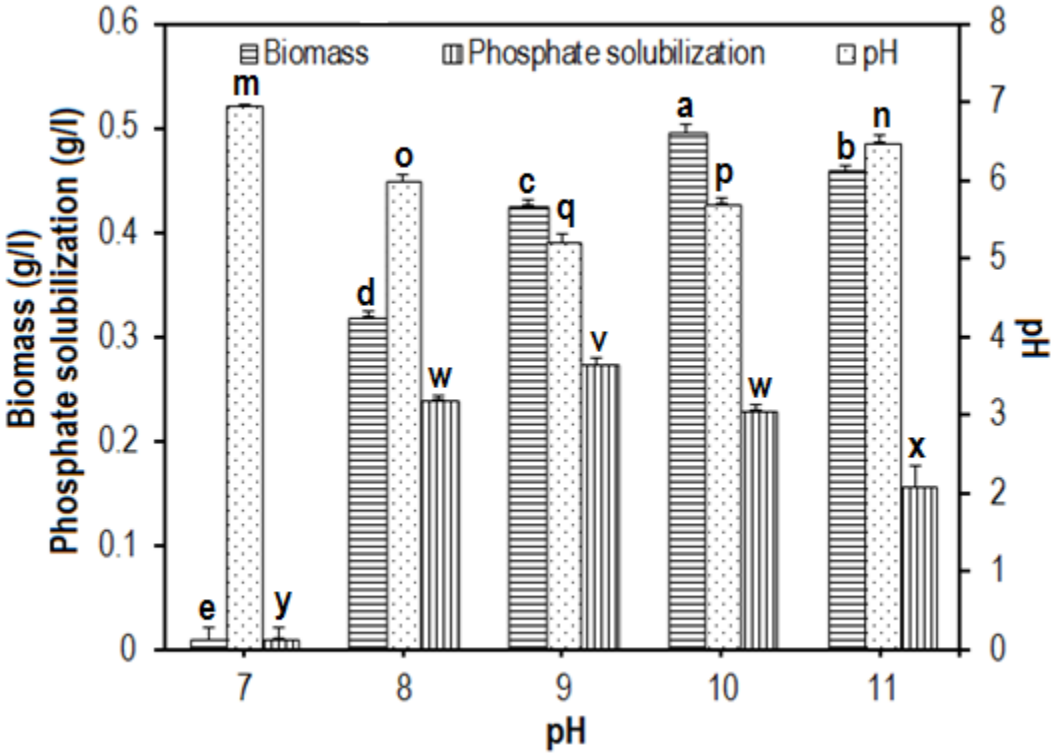


Figure 3.2 - Effect of different initial pH values on growth and phosphate solubilization by *Bacillus marisflavi* FA7 in Pikovskaya's broth. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.050.

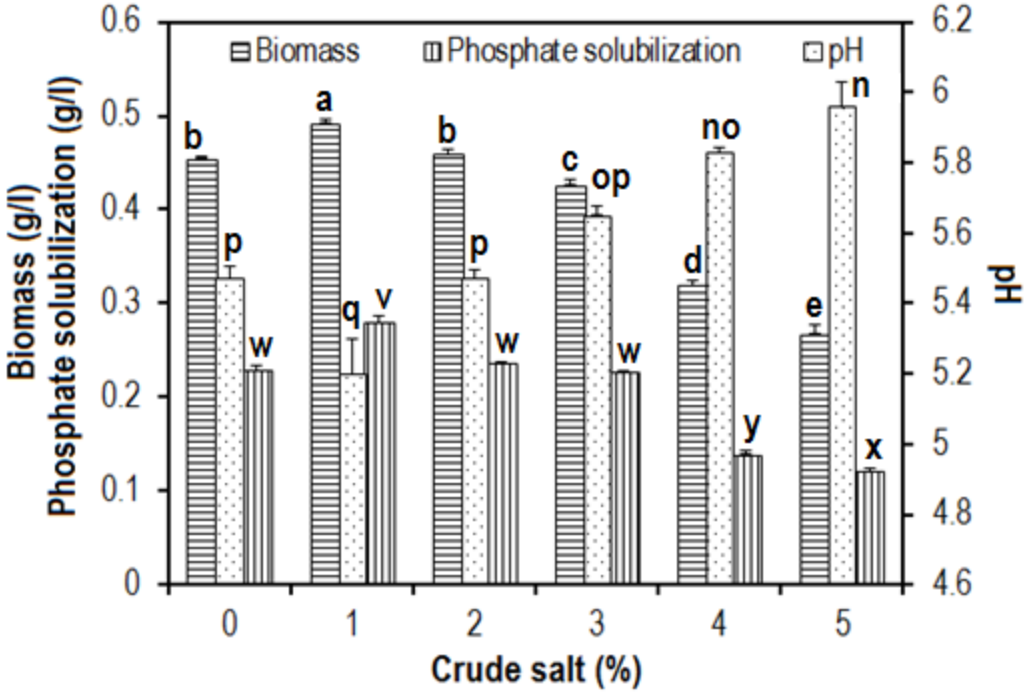


Figure 3.3 - Effect of different crude salt concentrations on growth and phosphate solubilization by *Bacillus marisflavi* FA7 in Pikovskaya's broth. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.050.

1.0% crude salt ($p<0.050$). Decrease in pH at 0%, 2% and 3% was found to be similar ($p>0.050$). Decrease in pH at pH 4.0 was found to be similar with decrease in pH at 3% and 5% ($p>0.050$). *Bacillus marisflavi* FA7 showed phosphate solubilization from 0 to 5% crude salt concentration. Maximum phosphate solubilization was observed at 1.0% crude salt ($p<0.001$). Decrease in phosphate solubilization was observed with increase in crude salt concentration. Phosphate solubilization at 0, 2.0 and 3.0%, was found to be similar ($p>0.050$).

3.3.3 Effect of carbon sources

Bacillus marisflavi FA7 exhibited growth in presence of all the tested carbon sources **(Figure 3.4)**. The biomass obtained with addition of carbon sources in medium was higher than the control ($p<0.001$). Maximum growth was recorded in sucrose. However, biomass obtained with sucrose, glucose, xylose, mannitol and lactose was of similar extent ($p>0.050$). Fall in the pH of culture broth was noted with all the substrates. Least decrease in pH was observed in control ($p<0.001$). Highest decrease in pH was observed with glucose and xylose when compared with other treatments ($p<0.001$). It was noted that the final pH of lactose and sucrose and maltose and fructose were similar ($p>0.050$). *Bacillus marisflavi* FA7 could solubilize tricalcium phosphate in all the treatments. Interestingly, highest phosphate solubilization was obtained with glucose and xylose as compared with other combinations ($p<0.01$). Phosphate solubilization in media containing sucrose, mannitol and lactose was similar ($p>0.050$).

3.3.4 Effect of inorganic nitrogen sources

Bacillus marisflavi FA7 grew in presence of all the tested inorganic nitrogen sources **(Figure 3.5)**. The biomass obtained with addition of inorganic nitrogen sources in

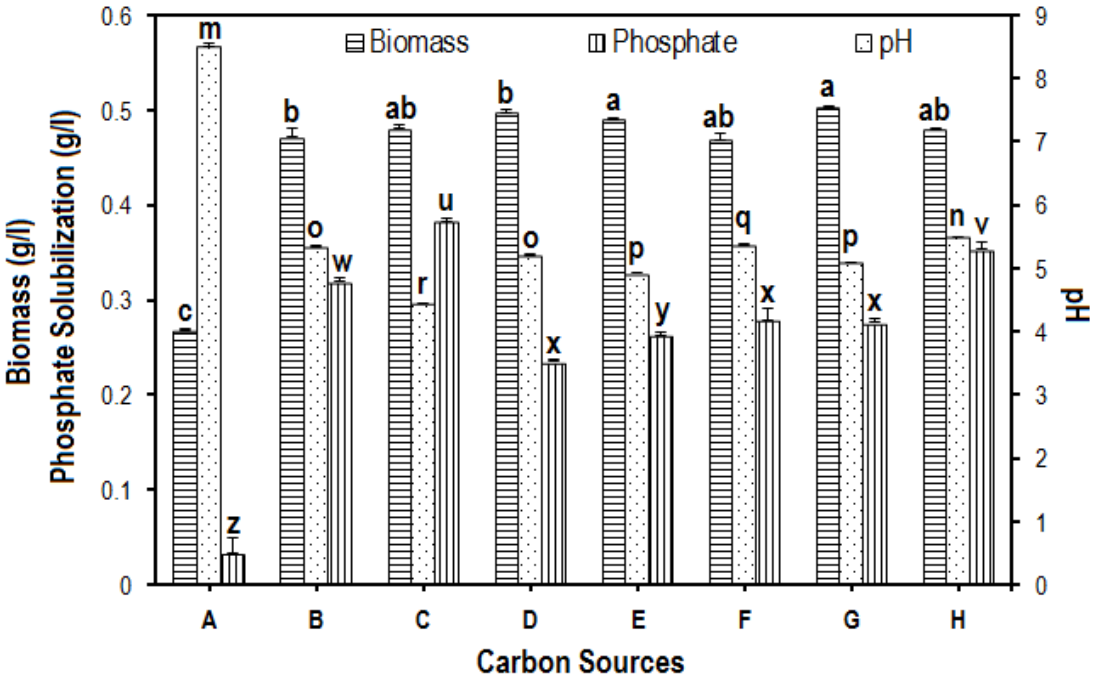


Figure 3.4 - Effect of different carbon sources on growth and phosphate solubilization by *Bacillus marisflavi* FA7 in Pikovskaya's broth. Where, A – Control (no carbon source), B - Fructose, C - Glucose, D - Lactose, E - Maltose, F - Mannitol, G - Sucrose, H - Xylose. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.050.

medium was higher than the control ($p<0.001$). Maximum growth was observed with NH_4NO_3 and $NaNO_3$ as compared to other treatments ($p<0.001$). Fall in the pH of culture broth was noted with all the substrates. Least decrease in pH was observed with $NaNO_3$ and KNO_3 ($p<0.001$). Significant decrease in pH was observed with NH_4Cl ($p<0.001$) when compared with other treatments. It was noted that the final pH of the control, $(NH_4)_2SO_4$ and NH_4NO_3 were similar ($p>0.01$). *Bacillus marisflavi* FA7 could solubilize tricalcium phosphate in all the treatments. Interestingly, highest phosphate solubilization was obtained with NH_4Cl as compared with other combinations ($p<0.01$).

3.3.5 Response Surface Methodology

On the basis of results obtained from preliminary screening, glucose and ammonium chloride were selected as carbon source and inorganic nitrogen source respectively as variables for optimization.

3.3.5.1 Models of optimization

3.3.5.1.A Biomass

Model 3.1 shows the various interactions of tricalcium phosphate, glucose and ammonium chloride and their impact on biomass accumulation of *Bacillus marisflavi* FA7. This model predicted high degree of similarity between the experimental observed values and calculated values with the r^2 observed as 0.653; indicative of good fitness. Maximum extent of coefficient was observed with linear and squared coefficients of ammonium chloride. All linear coefficients were negative for biomass accumulation. Squared coefficients of tricalcium phosphate and ammonium chloride were positive. Squared coefficient of glucose was found to be negative. All the interaction coefficients were positive. All the coefficients in association with the

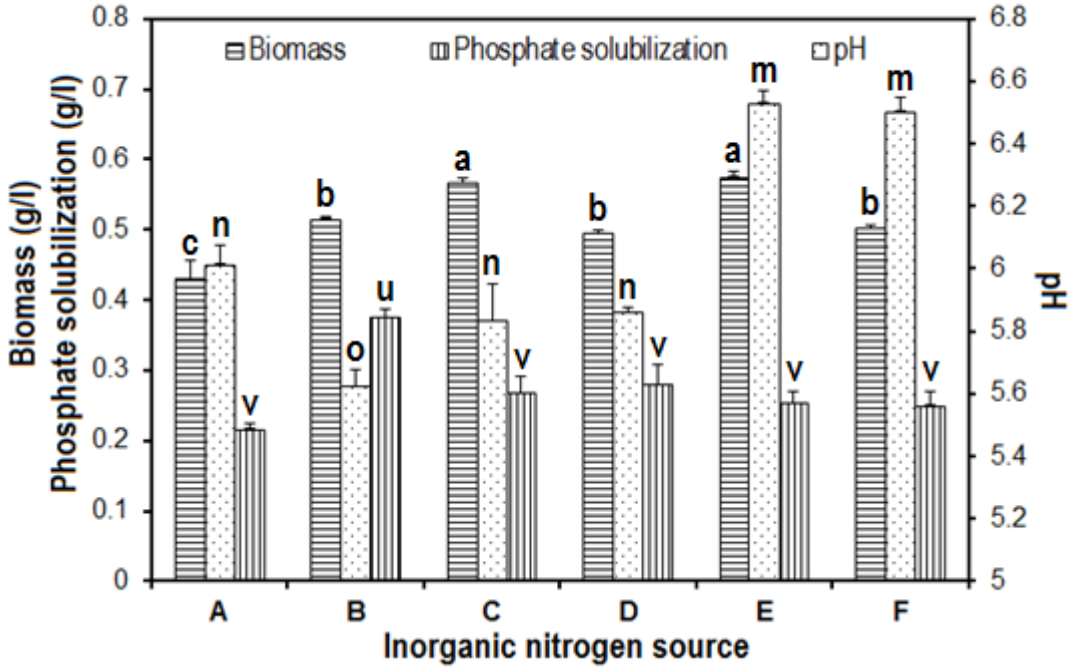


Figure 3.5 - Effect of different inorganic nitrogen sources on growth and phosphate solubilization by *Bacillus marisflavi* FA7 in Pikovskaya's broth. Where, A - Control (no inorganic nitrogen source), B - NH_4Cl, C - NH_4NO_3, D - $(NH_4)_2SO_4$, E - $NaNO_3$ and F - KNO_3. Error bars are standard errors and different alphabet labels indicate significant difference at p value of 0.050.

Model 3.1- Model for biomass production by *Bacillus marisflavi* FA7

$$\begin{aligned}\text{Biomass} = {} & 0.840 \\ & - 0.0621*\text{Tricalcium phosphate} \\ & - 0.0111*\text{Glucose} \\ & - 0.602*\text{Ammonium chloride} \\ & + 0.0032*(\text{Tricalcium phosphate})^2 \\ & - 0.0000326*(\text{Glucose})^2 \\ & + 0.212*(\text{Ammonium chloride})^2 \\ & + 0.00138*(\text{Tricalcium phosphate}*\text{Glucose}) \\ & + 0.0491*(\text{Tricalcium phosphate}*\text{Ammonium chloride}) \\ & + 0.00515*(\text{Glucose}*\text{Ammonium chloride})\end{aligned}$$

r^2 = 0.653

Normality test = Passed

Standard error of estimate = 0.043

Constant variance test = Passed

respective variables are presented in the **Model 3.1**. The results of CCD experiments for studying the effects of three independent variables, viz. tricalcium phosphate, glucose and ammonium chloride on biomass with the mean predicted and observed response are presented in **Table 3.5**.

Using **Model 3.1**, the optimum concentration of tricalcium phosphate, glucose and ammonium chloride for biomass accumulation was found to be 11.0 g/l, 15.0 g/l and 0.55 g/l, respectively.

3.3.5.B Phosphate solubilization

Model 3.2 shows the interaction of tricalcium phosphate, glucose and ammonium chloride and their impact on tricalcium phosphate solubilization by *Bacillus marisflavi* FA7.

This model predicted high degree of similarity between the experimental observed values and calculated values with the r^2 observed as 0.872, indicative of good fitness. Maximum extent was observed with squared coefficient and linear coefficient of ammonium chloride followed by interaction coefficient of tricalcium phosphate and ammonium chloride. Linear coefficients of glucose and ammonium chloride were positive for phosphate solubilization. Linear coefficient of tricalcium phosphate was negative. All squared coefficients were found to be negative. Coefficients of interaction of glucose with tricalcium phosphate and ammonium chloride were found to be negative. Interaction coefficient of tricalcium phosphate and ammonium chloride was found to be positive. All the coefficients in association with the respective variables are presented in the **Model 3.2**. The results of CCD experiments for studying the effects of three independent variables, viz. tricalcium phosphate,

Table 3. 5 - Amount of biomass produced in three factor five factorial complete rotatable central composite design experiment with actual values of TCP, Glucose and ammonium chloride

Flask no.	X1	X2	X3	Biomass (g/l)	
	TCP (g/l)	Glucose (g/l)	Ammonium Chloride (g/l)	Observed Values	Calculated Values
1	5	5	0.165	0.544	0.538*
2	10	5	0.165	0.570	0.543*
3	5	15	0.165	0.546	0.498
4	10	15	0.165	0.562	0.571*
5	5	5	0.495	0.530	0.475
6	10	5	0.495	0.558	0.561*
7	5	15	0.495	0.470	0.452*
8	10	15	0.495	0.646	0.606*
9	3.3	10	0.33	0.442	0.495*
10	11.7	10	0.33	0.618	0.629*
11	7.5	2.45	0.33	0.466	0.502*
12	7.5	17.55	0.33	0.462	0.506*
13	7.5	10	0.081	0.502	0.529*
14	7.5	10	0.579	0.456	0.508*
15	7.5	10	0.33	0.506	0.506*
16	7.5	10	0.33	0.514	0.506*
17	7.5	10	0.33	0.516	0.506*
18	7.5	10	0.33	0.516	0.506*
19	7.5	10	0.33	0.512	0.506*
20	7.5	10	0.33	0.512	0.506*

***predicted value is as observed value**

Model 3.2 - Model for phosphate solubilization by *Bacillus marisflavi* FA7

Phosphate Solubilization = 0.0421

- 0.0191*Tricalcium phosphate
+ 0.0872*Glucose
+ 0.480*Ammonium chloride
- 0.00280*(Tricalcium phosphate)^2
- 0.00345*(Glucose)^2
- 2.276*(Ammonium chloride)^2
- 0.00181*(Tricalcium phosphate*Glucose)
+ 0.178*(Tricalcium phosphate*Ammonium chloride)
- 0.0323*(Glucose*Ammonium chloride)

r^2 = 0.872

Normality test = Passed

Standard error of estimate = 0.057

Constant variance test = Passed

glucose and ammonium chloride on phosphate solubilization with the mean predicted and observed response are presented in **Table 3.6**.

Using **Model 3.2**, the optimum concentration of tricalcium phosphate, glucose and ammonium chloride for phosphate solubilization were found to be 4.0 g/l, 11.0 g/l and 0.2 g/l respectively.

3.3.5.2 Isoresponse analysis of models

3.3.5.2.1 Isoresponse analysis of biomass and phosphate solubilization with respect to parameters optimized for biomass production

3.3.5.2.1.1 Analysis of biomass

Figure 3.6 shows the interaction between glucose and ammonium chloride on biomass accumulation when tricalcium phosphate level was kept at +α level (11.7 g/l). It was observed that at low concentration of glucose and ammonium chloride biomass yield was low. Biomass yield increased with increase in concentration of both glucose and ammonium chloride. It increased further with increase in concentration of glucose when ammonium chloride was at its highest level (+α).

Figure 3.7 shows the interaction between tricalcium phosphate and ammonium chloride when glucose was kept at +α level (17.55 g/l). Below 7.5 g/l of tricalcium phosphate, biomass yield was decreasing at lower concentration of tricalcium phosphate and higher concentration of ammonium chloride. As tricalcium phosphate concentration increased, biomass yield also increased. At high concentration of ammonium chloride and tricalcium phosphate, biomass yield was higher.

Figure 3.8 shows the interaction between tricalcium phosphate and glucose when ammonium chloride was at + α level (0.579 g/l). Increase in the yield of biomass was

Table 3.6 - Tricalcium phosphate solubilized in three factor five factorial complete rotatable central composite design experiment with actual values of TCP, Glucose and ammonium chloride

Flask no.	X1	X2	X3	Phosphate solubilization (g/l)	
	TCP (g/l)	Glucose (g/l)	Ammonium Chloride (g/l)	Observed Values	Calculated Values
1	5	5	0.165	0.267	0.318*
2	10	5	0.165	0.164	0.115*
3	5	15	0.165	0.411	0.357*
4	10	15	0.165	0.082	0.062*
5	5	5	0.495	0.198	0.221*
6	10	5	0.495	0.253	0.311*
7	5	15	0.495	0.100	0.153
8	10	15	0.495	0.200	0.152*
9	3.3	10	0.33	0.456	0.413*
10	11.7	10	0.33	0.204	0.242*
11	7.5	2.45	0.33	0.280	0.226*
12	7.5	17.55	0.33	0.087	0.134
13	7.5	10	0.081	0.189	0.238*
14	7.5	10	0.579	0.289	0.330*
15	7.5	10	0.33	0.365	0.377*
16	7.5	10	0.33	0.372	0.377*
17	7.5	10	0.33	0.402	0.377*
18	7.5	10	0.33	0.380	0.377*
19	7.5	10	0.33	0.377	0.377*
20	7.5	10	0.33	0.362	0.377*

*** predicted value is as observed value**

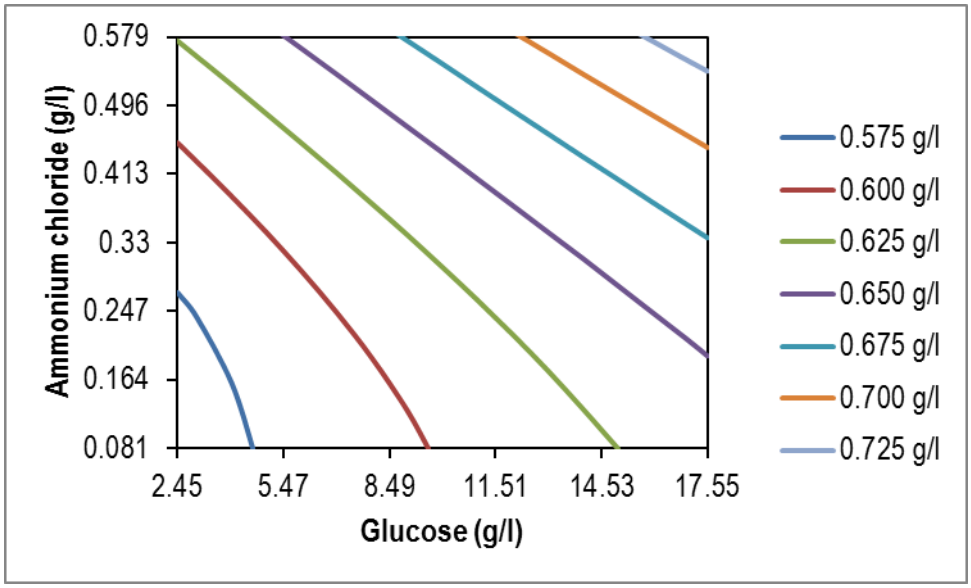


Figure 3.6 – Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of glucose and ammonium chloride at tricalcium phosphate concentration of 11.7 g/l

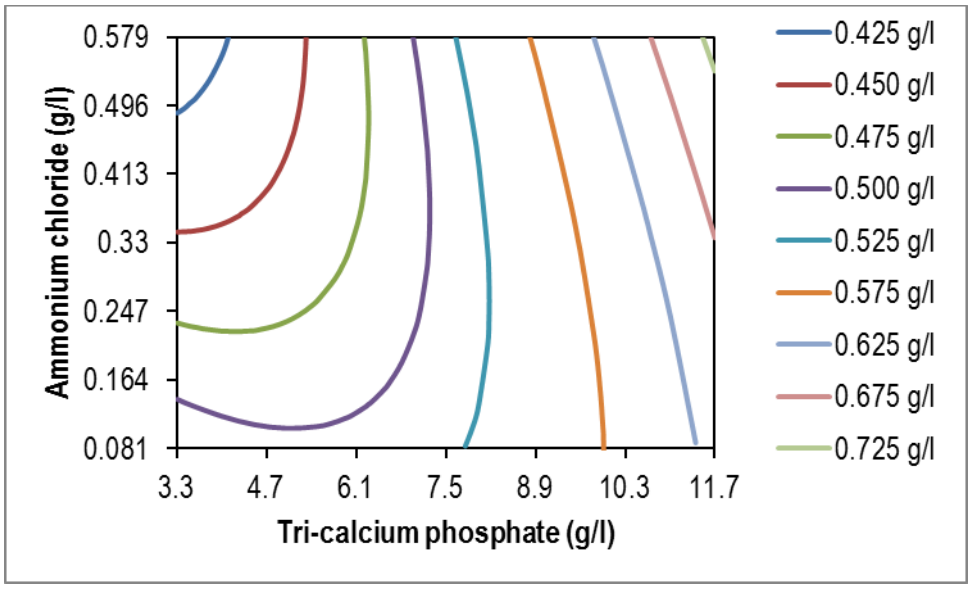


Figure 3.7 - Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium and ammonium chloride at glucose concentration of 17.55 g/l

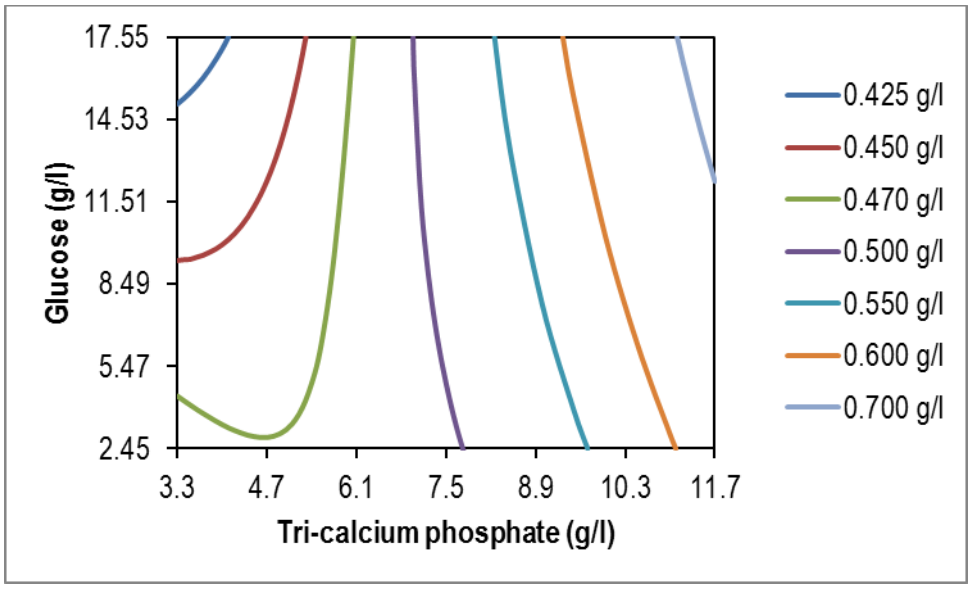


Figure 3.8 – Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium phosphate and glucose at ammonium chloride concentration of 0.579 g/l

observed with increase in the concentration of tricalcium phosphate. Below concentration of 6.1 g/l of tricalcium phosphate, with the increase in concentration of glucose, decrease in biomass accumulation was observed. At concentrations above 6.1 g/l of tricalcium phosphate, with increase in concentration of glucose, increase in biomass was observed.

3.3.5.2.1.2 Analysis of phosphate solubilization

Figure 3.9 shows the interaction of glucose and ammonium chloride on phosphate solubilization when tricalcium phosphate level was kept at +α level (11.7 g/l). Maximum phosphate solubilization was observed at high concentration of ammonium chloride and glucose concentration between 6.25 – 8.15 g/l. Bull's eye is depicted in the contour plot, indicating point of maximum response.

Figure 3.10 shows the interaction between tricalcium phosphate and ammonium chloride on phosphate solubilization when glucose concentration was 17.55 g/l. Phosphate solubilization was highest at low concentration of tricalcium phosphate and ammonium chloride. As the concentration of tricalcium phosphate and ammonium chloride increased, phosphate solubilization decreased.

Figure 3.11 shows the interaction between tricalcium phosphate and glucose when ammonium chloride was at + α level (0.579 g/l). In this combination, maximum phosphate solubilization was observed at high concentration of tricalcium phosphate and 5.0 to 8.5 g/l of glucose concentration. As the concentration of glucose increased or decreased beyond this point, phosphate solubilization decreased. Increase in concentration of tricalcium phosphate showed increase in phosphate solubilization.

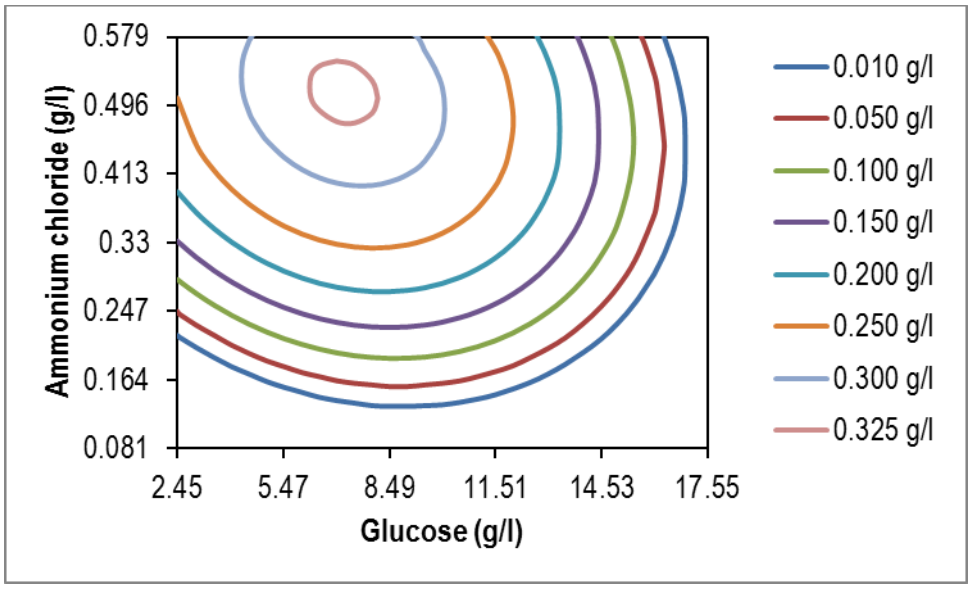


Figure 3.9 – Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of glucose and ammonium chloride at tricalcium phosphate concentration of 11.7 g/l

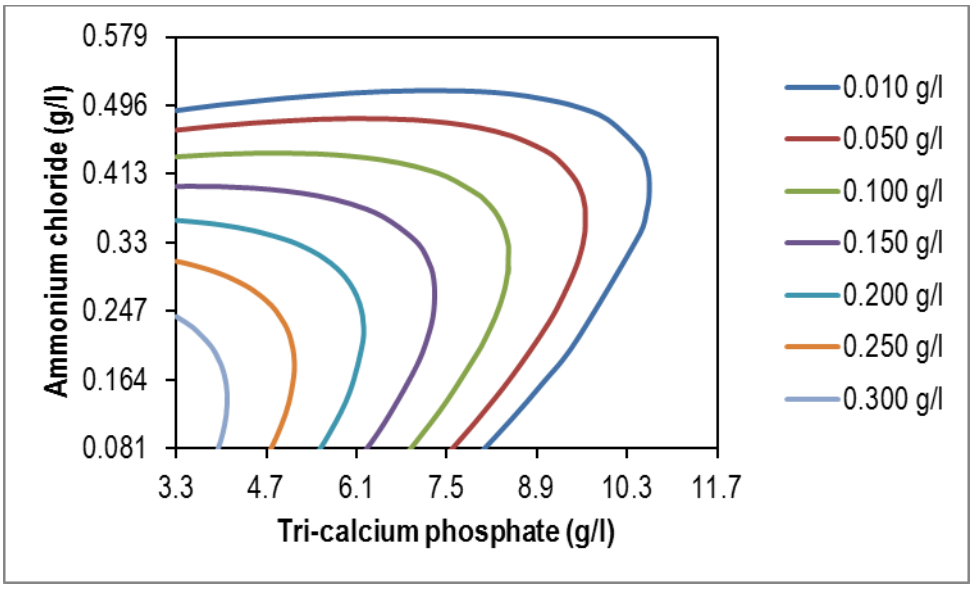


Figure 3.10 – Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium and ammonium chloride at glucose concentration of 17.55 g/l

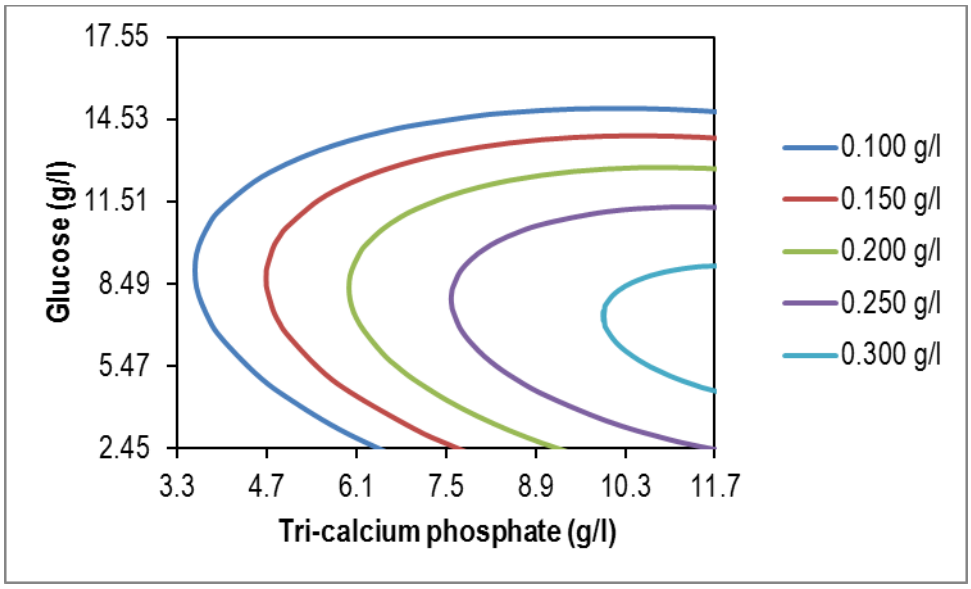


Figure 3.11 - Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium phosphate and glucose at ammonium chloride concentration of 0.579 g/l

3.3.5.2.2 Isoresponse analysis of biomass and phosphate solubilization with respect to parameters optimized for phosphate solubilization

3.3.5.2.2.1 Analysis of biomass

Figure 3.12 shows the interaction of between glucose and ammonium chloride on biomass accumulation when tricalcium phosphate level was kept at –α (3.3 g/l) level. It was observed that at low concentrations of glucose and ammonium chloride, biomass accumulation was high. As the concentration of glucose and ammonium chloride increased, biomass accumulation decreased.

Figure 3.13 presents the interaction between tricalcium phosphate and ammonium chloride when concentration of glucose was 13 g/l. It was observed that with increase in concentration of tricalcium phosphate, there was increase in biomass yield. When concentration of tricalcium phosphate was below 7.5 g/l, decrease in concentration of ammonium chloride led to increased biomass accumulation. When concentration of tricalcium phosphate was above 7.5 g/l of tricalcium phosphate, increase in concentration of ammonium chloride further increased accumulation of biomass.

Figure 3.14 shows the interaction between tricalcium phosphate and glucose when ammonium chloride was at - α level (0.081 g/l). A valley depicted in the contour plot indicated low biomass yield with increase in concentration of tricalcium phosphate. As the concentration of tricalcium phosphate and glucose decreased, biomass accumulation increased. As the concentration of tricalcium phosphate and glucose increased, biomass accumulation increased.

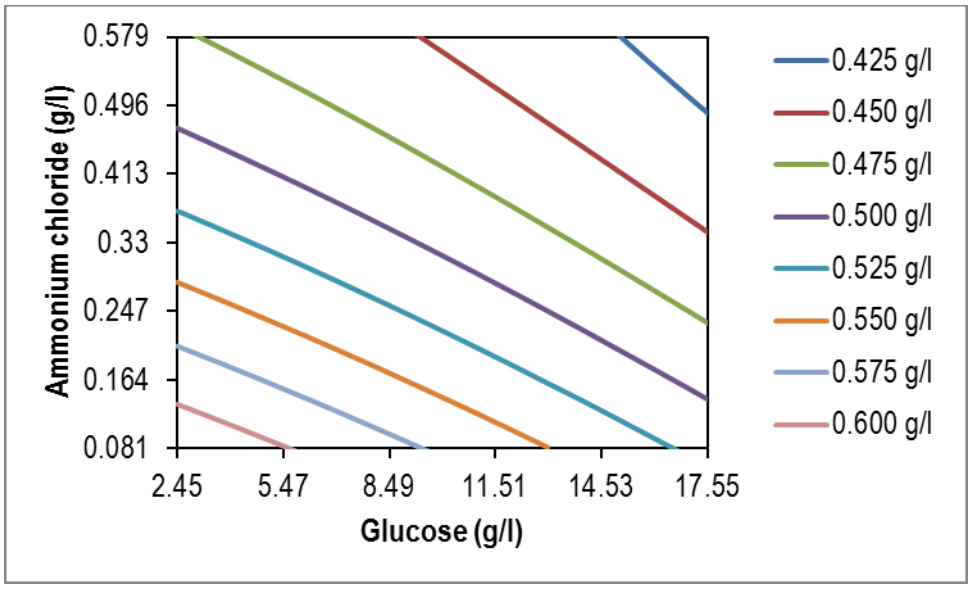


Figure 3.12 – Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of glucose and ammonium chloride at tricalcium phosphate concentration of 3.3 g/l

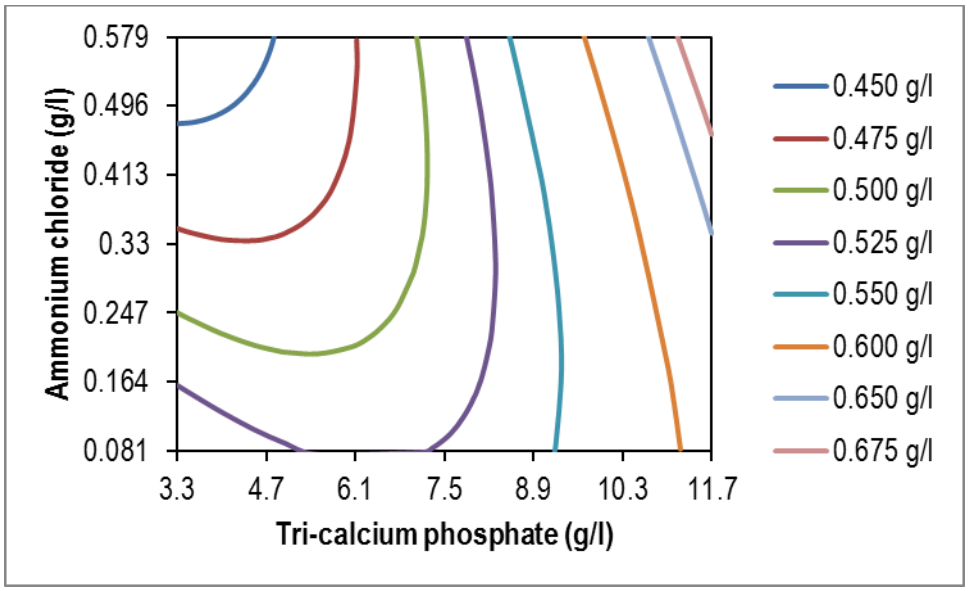


Figure 3.13 – Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium and ammonium chloride at glucose concentration of 13 g/l

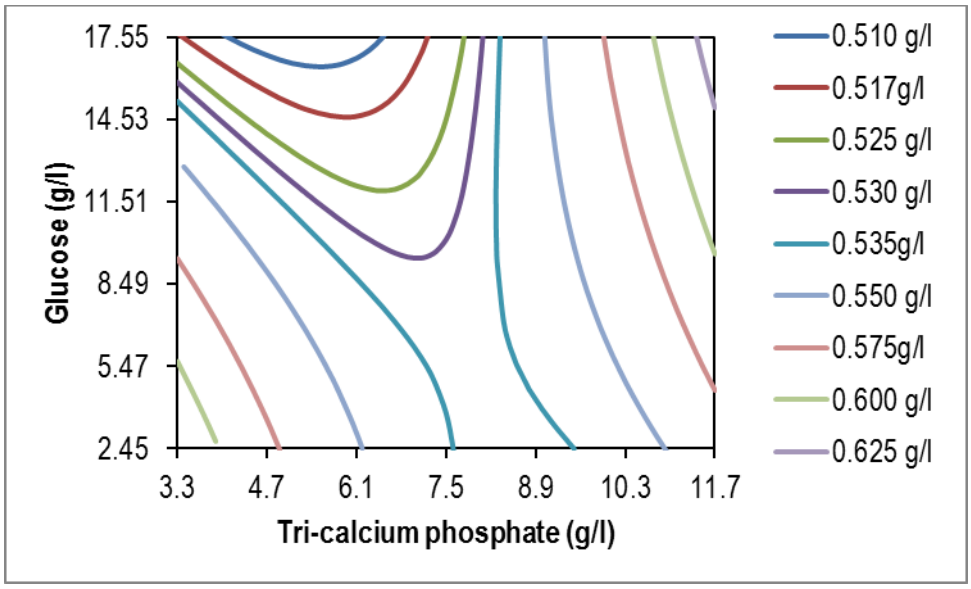


Figure 3.14 - Isoresponse contour plot showing biomass production of *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium phosphate and glucose at ammonium chloride concentration of 0.081 g/l

3.3.5.2.2.2 Analysis of phosphate solubilization

Figure 3.15 shows the interaction between glucose and ammonium chloride on phosphate solubilization when tricalcium phosphate level was kept at –α level (3.3 g/l). Maximum phosphate solubilization was observed at lower concentrations of ammonium chloride and 9.0 to 12.0 g/l of glucose. As indicated by Bull's eye in the contour plot. As the concentration of ammonium chloride increased, phosphate solubilization decreased.

Figure 3.16 shows the interaction between tricalcium phosphate and ammonium chloride when glucose concentration was 13 g/l. Maximum phosphate solubilization was observed at low concentration of tricalcium phosphate and ammonium chloride. As the concentration of tricalcium phosphate and ammonium chloride increased, phosphate solubilization decreased.

Figure 3.17 shows the interaction between tricalcium phosphate and glucose when ammonium chloride was at - α level (0.081 g/l). Maximum phosphate solubilization was observed at low concentration of tricalcium phosphate and 7.5 to 15.5 g/l of glucose concentration. As the concentration of tricalcium phosphate increased, phosphate solubilization decreased.

3.4 DISCUSSION

Soil phosphorous transformations are primarily mediated by microbial activity, which in turn is influenced by a combination of abiotic factors including soil type, environmental and nutritional factors. *Bacillus marisflavi* FA7 was selected for optimization of variables for biomass production and phosphate solubilization. Phosphate solubilization process is greatly influenced by carbon and nitrogen sources. In the presence of various carbon and nitrogen sources microorganisms

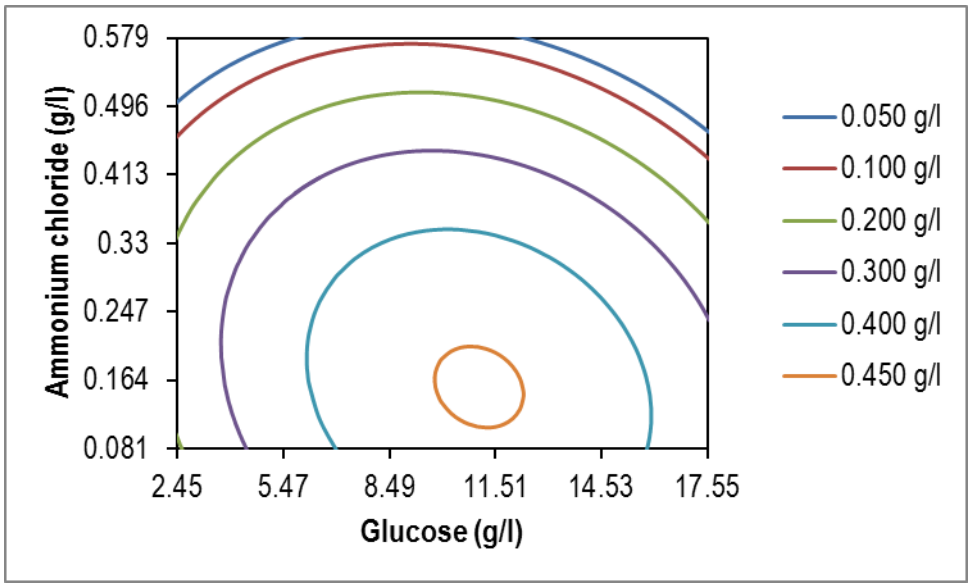


Figure 3.15 – Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of glucose and ammonium at tricalcium phosphate concentration of 3.3 g/l

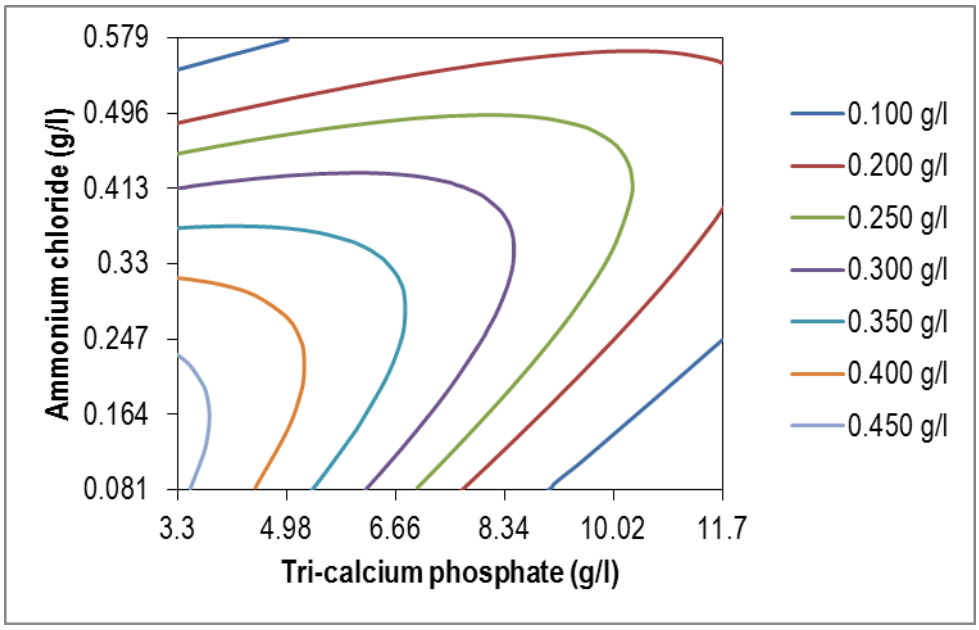


Figure 3.16 – Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium phosphate and ammonium chloride at glucose concentration of 13 g/l

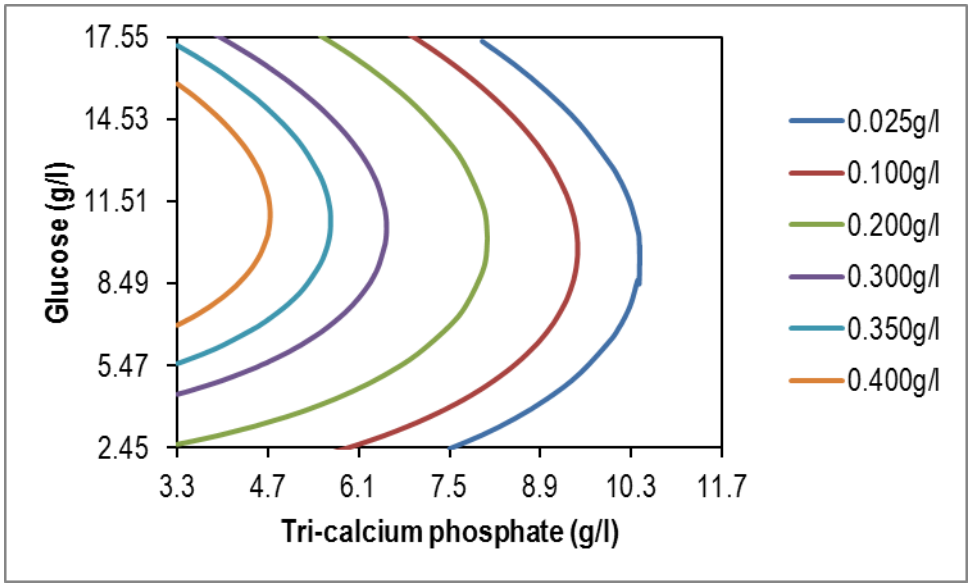


Figure 3.17 – Isoresponse contour plot showing phosphate solubilization by *Bacillus marisflavi* FA7 as a function of different concentrations of tricalcium and glucose at ammonium chloride concentration of 0.081 g/l

show diverse levels of phosphate solubilization activity. Carbon source is an important parameter for active proliferation of organisms and production of organic acids. Nitrogen source is important for the production of H^+ ions and inorganic acids.

Bacillus marisflavi FA7 yielded maximum biomass in the presence of sucrose. However, not much difference was observed in biomass yield with carbon sources such as glucose, xylose, mannitol and lactose ($p>0.050$). Highest decrease in pH was observed with glucose and xylose, which also reflected in highest phosphate solubilization in the two substrates.

Halder *et al.* (1991) report that carbon sources which support luxuriant growth of bacteria do not affect phosphate solubilization effectively. Several reports have shown the effect of different carbon sources on phosphate solubilization (Banik & Dey, 1983; Halder *et al.*, 1991; Nautiyal *et al.*, 2000; Hameeda *et al.*, 2006; Ahuja *et al.*, 2007; Sadaf & Nuzhat, 2008; Sharan *et al.*, 2008). Nautiyal *et al.* (2000) reported xylose, lactose and glucose to be best carbon sources for phosphate solubilization by four strains NBRI0603, NBRI2601, NBRI13246 and NBRI4003, respectively. Hameeda *et al*. (2006) have studied the effect of different carbon substrates on rock phosphate solubilization by *Enterobacter cloacae* EB 27, *Serratia* spp. (EB67 and EB 75) and *Pseudomonas* spp. (CDB 35 and BWB 21) and found glucose to be the best substrate. Wild type and mutant strain of *Xanthomonas campestris* RMLU-26 solubilized maximum phosphate with glucose as the carbon source (Sharan *et al.*, 2008). In a study conducted by Sadaf and Nuzhat (2008), strains of *Pseudomonas* (CMG852, CMG855, CMG856 and CMG860), *Bacillus thuringiensis* (CMG854 and CMG857) and *Acinetobacter lwoffli* (CMG851), showed higher zinc phosphate solubilization with glucose as the carbon source compared to fructose, lactose and

sucrose. *Pseudomonas* strain CMG853, showed sucrose as the best carbon source for zinc phosphate solubilization, whereas; *Pseudomonas* CMG858 and CMG 859 strains showed higher phosphate solubilization with lactose as the carbon source.

Screening of inorganic nitrogen sources revealed that maximum growth of *Bacillus marisflavi* FA7 was observed with NH_4NO_3 and $NaNO_3$. The bacterium showed maximum decrease in pH with ammonium salts compared to nitrate salts. When only nitrate salts were used decrease in pH was least. Maximum tricalcium phosphate solubilization was seen only with NH_4Cl.

In general, a greater reduction in pH together with more solubilized phosphate has been observed with NH_4^+ salts as the sole inorganic nitrogen source compared to NO_3, due to the extrusions of protons to compensate for NH_4 uptake (Roos & Luckner, 1984; Illmer & Schinner, 1995; Ahuja *et al.*, 2007; Sharan *et al.*, 2008). Nautiyal *et al.* (2000) have reported ammonium and nitrate salts to be equally effective for phosphate solubilization by strains NBRI0603, NBRI2601, NBRI13246 and NBRI4003. Ammonium sulfate was the preferred inorganic nitrogen source for wild type and mutant strain of *Xanthomonas campestris* RMLU-26 (Sharan *et al.*, 2008).

Glucose and ammonium chloride were selected as variables for optimization study by RSM as carbon and inorganic nitrogen sources respectively. Third variable selected was tricalcium phosphate. Most optimization studies are carried out by studying one variable at a time (Sadaf & Nuzhat 2008; Xiao *et al.*, 2008; Sagervanshi *et al.*, 2012). CCD increases the amount of data that can be obtained, while limiting the number of individual experiments (Kunamneni & Singh, 2005). In the present study, high similarity obtained between observed and calculated results,

reflected accuracy and applicability of RSM to optimize process for biomass and phosphate solubilization.

The models of biomass and phosphate solubilization facilitated to define optimal concentrations of variables for maximum response and interaction of variables **(Table 3.7)**. Model for biomass accumulation revealed that the negative value for ammonium chloride in the linear form illustrates decreased effect towards biomass production. Positive value for squared coefficient of tricalcium phosphate and ammonium chloride influences increased biomass production. All interaction coefficients indicated positive contribution towards increase in biomass.

In the phosphate solubilization model, the positive values of linear coefficients (glucose and ammonium chloride) suggest increase in phosphate solubilization with increase in concentration of the two variables. Negative values of linear and squared coefficients of tricalcium phosphate indicate decrease in solubilization with increase in concentration of tricalcium phosphate. Squared coefficients of both glucose and ammonium chloride indicate they influence negatively towards phosphate solubilization.

Contour plots obtained as a response to concentrations of tricalcium phosphate, glucose and ammonium chloride indicated maximum biomass production and phosphate solubilization. Highest biomass was observed at high concentration of tricalcium phosphate, glucose and ammonium chloride. Highest phosphate solubilization was observed at low concentrations of tricalcium phosphate and ammonium chloride and high concentration of glucose.

Table 3.7 – RSM optimized nutrient composition of the media to be used for maximum yield of biomass and solubilization of phosphate

Nutrient Components	Media composition (g/l) for	
	Biomass	Phosphate solubilization
$Ca_3(PO_4)_3$	11.0	4.0
Glucose	15.0	11.0
NH_4Cl	0.55	0.2
KCl	0.2	0.2
K_2HPO_4	0.1	0.1
$MgSO_4$	0.1	0.1
$MnSO_4$	0.0001	0.0001
$FeSO_4$	0.0001	0.0001
Yeast extract	0.5	0.5

The results of this study reveal that there is more than one factor responsible for biomass and phosphate solubilization. The factors interact with each other to produce maximum biomass and solubilize maximum phosphate.

Scervino *et al.* (2011) have optimized phosphate solubilization and organic acid production using RSM. They observed highest phosphate solubilization with highest glucose concentration and lowest ammonium sulfate concentration.

Mishra *et al.* (2015) optimized pH, temperature and incubation period to obtain maximum phosphate solubilization in *Acinetobacter calcoaceticus* TM8. They reported maximum phosphate solubilization of 273.50 g/ml at pH 7.3, temperature of 37.6 °C and incubation period of 7 days.

Bacillus marisflavi FA7 exhibited tricalcium phosphate solubilizing activity at alkaline pH. This study has presented optimized medium using statistical experiment design for biomass accumulation and phosphate solubilization for *Bacillus marisflavi* FA7. These results can be used to develop *Bacillus marisflavi* FA7 as a bio-fertilizer for inoculation in alkaline soil.

Chapter IV

Effect of stress conditions such as pH, salt concentration and metal ions on phosphate solubilization

4.1 INTRODUCTION

Agricultural research has long been focused on improving productivity after the green revolution. With rise in population, the demand for food will be much higher by the end of 2020. Therefore, scientists are looking at alternatives to increase production through improvement in agricultural productivity in fertile and barren soil in sustainable manner.

In a fast developing and densely populated country such as India, there is limited opportunity for opening up new land for agriculture (Shahbaz & Ashraf, 2013). It has been estimated that an approximate area of 7 million hectares of soil is affected by salinity and alkalinity in India. Most of the saline and alkaline soils occur in indo-gangentic plane that covers the states of Bihar, Haryana, Punjab, Uttar Pradesh and some parts of Rajasthan, arid tracts of Gujarat and Rajasthan and semi-arid tracts of Andhra Pradesh, Goa, Gujarat, Karnataka, Madhya Pradesh and Maharashtra (Patel *et al.,* 2011). Salinity and alkalinity limit the yields and profits of agricultural crops due to osmotic effects, toxicity of salt ions and the changes in the physical and chemical properties of soil (Yamaguchi & Blumwald, 2005; Shahbaz & Ashraf, 2013).

Salinity and alkalinity is further aggravated due to environmental pollution with the addition of toxic heavy metals and xenobiotic compounds to soil (Chookietwattana & Maneewan, 2012). Heavy metals are being added to the environment by a variety of sources, including mining activities, atmospheric deposition, municipal waste disposal, industrial, and agrochemicals. Along with salinity and alkalinity stress, heavy metals too are a major concern because of toxic effect of high metal concentration on plants.

Evolving efficient, low cost, easily adaptable methods for management of stress associated with salinity, alkalinity and metal is a major challenge. Soil associated with salinity and alkalinity suppresses plant growth (Paul, 2012). Under such circumstances, it requires suitable biotechnology not only to improve crop productivity but also to improve soil health through interactions of plant roots and soil microorganisms (Lugtenberg *et al.,* 2002). Recent studies indicate that microbes can help crops to cope with stress (Dimkpa *et al.*, 2009; Shanker & Venkateswarlu, 2011). Therefore, exploring bacteria which are able to efficiently survive or grow in such stressful environments is the need of the hour.

The objective of this study was to evaluate selected alkaliphilic bacterial strains to solubilize phosphate at different pH, salt concentration and in the presence of heavy metals. The isolates are expected to be promising bioresource for enhancing plant growth in saline-alkaline soil with heavy metal pollution.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and preparation of inoculum

Bacillus marisflavi FA7 and *Chromohalobacter israelensis* WA3 were selected for this study. The two strains were inoculated in 50 ml modified Pikovskaya's broth **(Appendix A)**. The cultures were incubated at 30°C on 150 rpm shaker conditions till O.D. reached 0.700 at 600 nm. This was used as inoculum (10 µl) for all experiments of this chapter.

4.2.2 Effect of salt concentration

Pikovskaya's agar **(Appendix A)** was used to study the effect of different concentrations of crude salt. The pH of the medium was adjusted to 10.0 using 10% (w/v) Na_2CO_3 solution. Crude salt concentrations tested for *Bacillus marisflavi* FA7

were 1%, 2%, 3%, 4% and 5%. For *Chromohalobacter israelensis* WA3, concentrations of crude salt tested were 2.5%, 5%, 10%, 15%, 20% and 25%. The plates were spot inoculated with 10 µl inoculum of *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 and incubated at 30°C for 48 hours. Colony diameter and halozone diameter were recorded to calculate the phosphate solubilization index of the isolates.

4.2.3 Effect of initial pH

The growth medium used to study the effect of different pH values was Pikovskaya's agar **(Appendix A)**. The different initial pH values selected for this experiment were 7, 8, 9, 10 and 11. Initial pH of the medium was adjusted using 10% (w/v) Na_2CO_3 solution. Pikovskaya's agar used for *Chromohalobacter israelensis* WA3 was amended with 15% (w/v) crude salt. Pikovskaya's agar plates of different pH were spot inoculated with 10 µl of inoculum of *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 and incubated at 30°C for 48 hours. Colony diameter and halozone diameter were recorded to calculate the phosphate solubilization index of the isolates.

4.2.4 Effect of metal ions

Effect of six metal ions was studied on growth and phosphate solubilization of *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3. Sulfate, chloride, and nitrate salts of six metals namely; aluminium [$Al_2(SO_4)_3$, $AlCl_3$, $Al(NO_3)_3.9H_2O$], cadmium [$3CdSO_4.8H_2O$, $CdCl_2$, $Cd(NO_3)_2$], copper [$CuSO_4$, $CuCl_2$, $Cu(NO_3)_2$], lead [$PbNO_3$], nickel [$NiSO_4.6H_2O$, $NiCl_2.6H_2O$, $Ni(NO_3)_3$] and zinc [$ZnSO_4.H_2O$, $Zn(NO_3)_2.6H_2O$] were selected. The concentrations of Al and Cd used for this experiment were 50, 100, 150 and 200 mg/l. Cu, Pb, Ni, and Zn were

used at concentrations of 200, 400, 600, 800 and 1000 mg/l. Stock solutions of the metal forms were prepared in deionized water and filter sterilized using 0.22 µm filters under aseptic conditions. The stock solutions were added to modify Pikovskaya's agar. The concentration of sulfate, chloride and nitrate was kept constant in all the flasks using K_2SO_4, KCl and KNO_3, respectively. The pH of the medium was adjusted to 10.0 with 10% (w/v) Na_2CO_3 solution. Pikovskaya's agar used for *Chromohalobacter israelensis* WA3 was amended with 15% (w/v) crude salt. The plates were spot inoculated with 10 µl inoculum of *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 and incubated at 30°C for 48 hours.

4.2.5 Scanning electron micrograph and EDX of *Chromohalobacter israelensis* WA3 in the presence of nitrate salts of six metal ions

Scanning electron microscopy (SEM) was performed to evaluate morphological changes in *Chromohalobacter israelensis* WA3 in the presence of nitrate salt of the six metals. Cells of *Chromohalobacter israelensis* WA3 exposed to nitrate salt of metals were suspended in 2% gluteraldehyde solution; a smear was prepared on glass coverslips and air dried ((Krishnaswamy & Namasivayam, 2010). The smears were fixed with 2.5% (v/v) gluteraldehyde solution overnight at room temperature. The smears were washed in PBS. Coverslips containing smears were then placed in 30% (v/v) acetone for 10 min and subsequently transferred in 50% (v/v), 70% (v/v) and 90% (v/v) for 10 min each and finally in 100% acetone for 30 min. After dehydration, the coverslips containing smears were placed on stubs. Stubs were placed in sputter coater (JOEL JFC 1600). After sputtering, the stubs were placed into the electron microscope sample chamber and observed with JOEL JSM-6360LV electron microscope. EDX spectrum was recorded to observe the presence of metals on the cell surface of *Chromohalobacter israelensis* WA3.

4.2.6 Statistical analysis

One way ANOVA and Student Newman Keuls method was performed to detect significant difference among different treatments. All statistical analysis was carried out using Sigma Stat Ver 4.0.

4.3 RESULTS

4.3.1 Effect of salt concentration

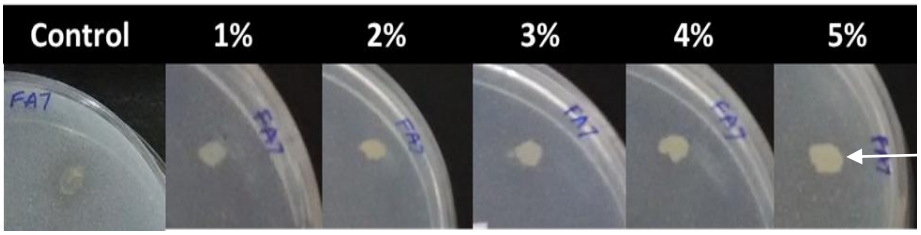
Bacillus marisflavi FA7 showed growth from 0 to 5% crude salt concentration **(Figure 4.1)**. Maximum growth was observed at 1% crude salt. Growth of *Bacillus marisflavi* FA7 was found to decrease with increase in crude salt concentration. *Bacillus marisflavi* FA7 solubilized tri-calcium phosphate from 0 to 5% crude salt. Solubilization index was higher at 1% crude salt concentration and it remained similar from 2 to 5% ($p>0.050$) **(Figure 4.2)**.

Chromohalobacter israelensis WA3 showed growth from 2.5 to 25% crude salt **(Figure 4.3)**. Maximum growth was observed at 15% crude salt. The strain showed solubilization of tri-calcium phosphate at all the concentrations of crude salt tested. Solubilization index of *Chromohalobacter israelensis* WA3 was found to be highest at 15% crude salt ($p<0.050$) **(Figure 4.4)**.

Among the two isolates, *Chromohalobacter israelensis* WA3 exhibited growth and phosphate solubilization at a wide range of crude salt concentration.

4.3.2 Effect of initial pH

Bacillus marisflavi FA7 showed growth as well as phosphate solubilization from pH 8.0 to pH 11.0 **(Figure 4.5)**. It showed no growth at pH 7.0. Maximum growth was observed at pH 9.0 and 10.0, followed by pH 8.0 and pH 11.0. Highest solubilization index of 1.57 was observed at pH 9.0 ($p<0.001$) **(Figure 4.6)**. At pH 10.0, solubilization index was 1.33 followed by 1.23 at pH 8.0 and 1.1 at pH 11.0.



Zone of clearance around the colony

Figure 4.1 - Effect of crude salt concentration on growth of and phosphate solubilization by *Bacillus marisflavi* FA7

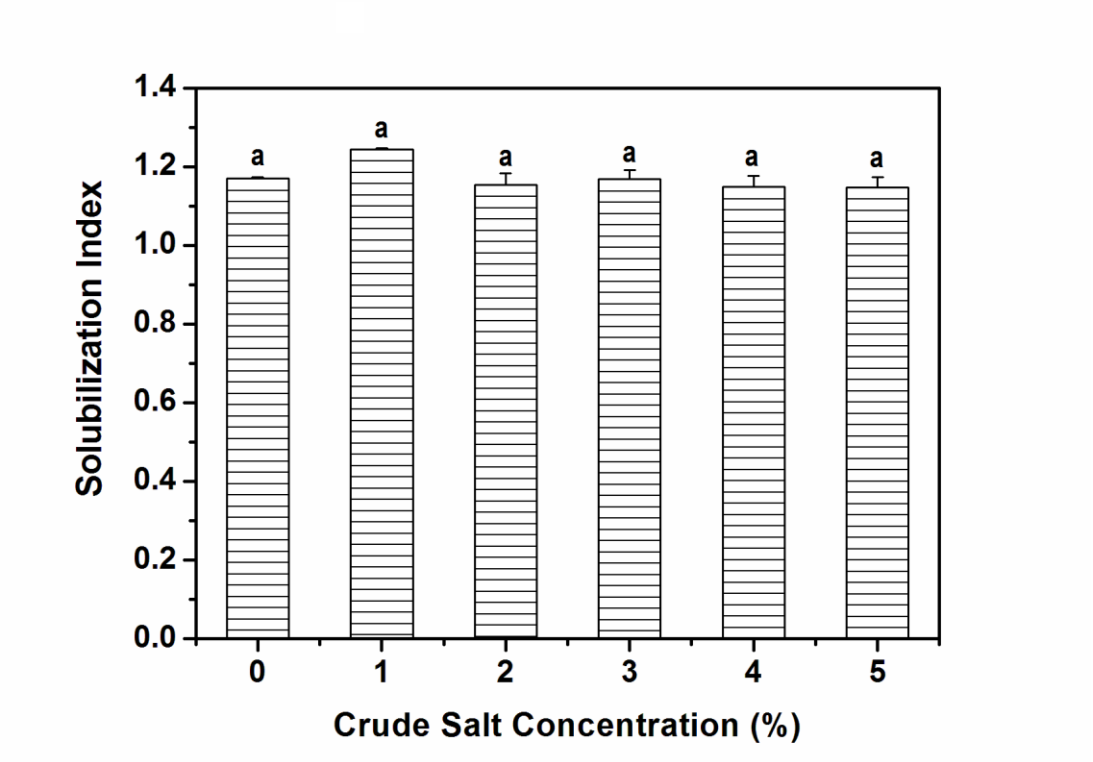


Figure 4.2 – Solubilization index of *Bacillus marisflavi* FA7 for tri-calcium phosphate solubilization at different concentrations of crude salt. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

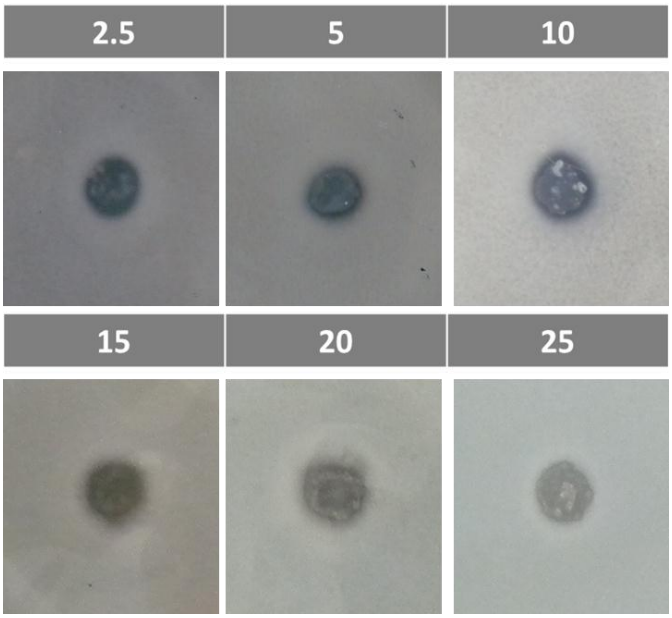


Figure 4.3 - Effect of crude salt concentration on growth of and phosphate solubilization by *Chromohalobacter israelensis* WA3

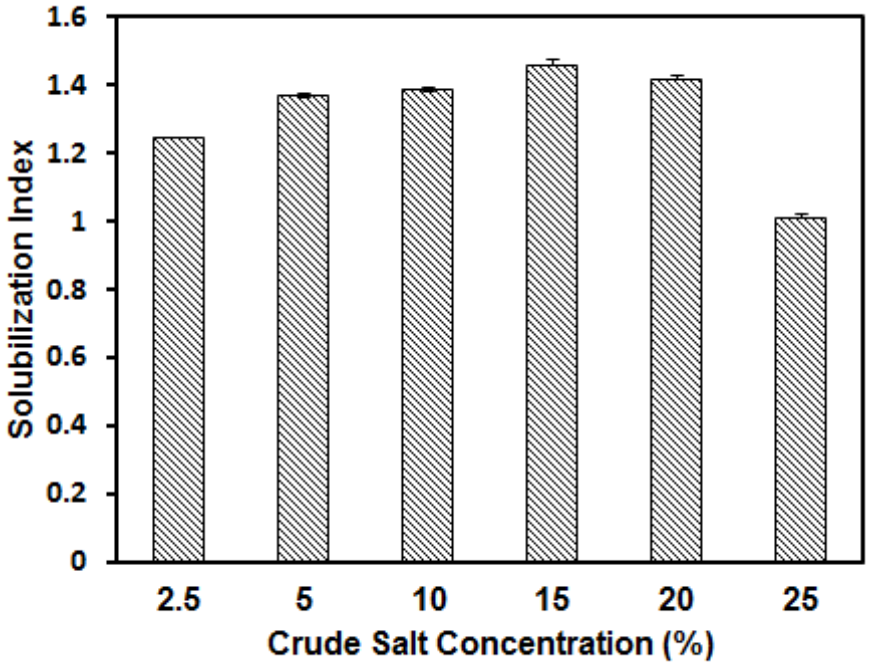


Figure 4.4 – Solubilization index of *Chromohalobacter israelensis* WA3 for tri-calcium phosphate solubilization at different concentrations of crude salt. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

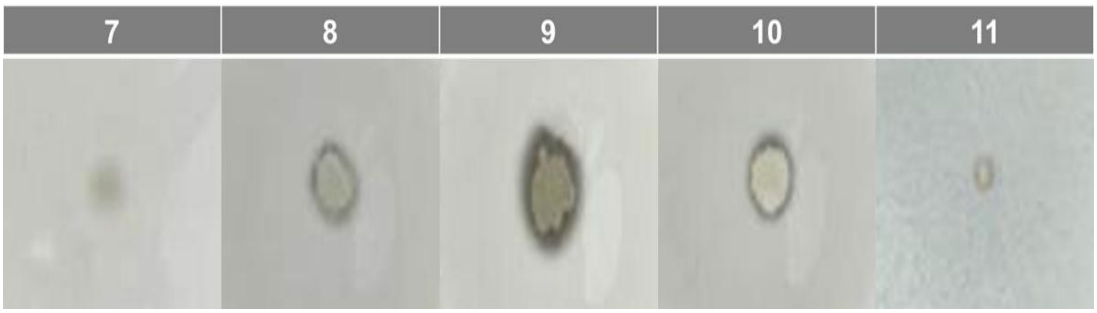


Figure 4.5 - Effect of initial pH of the medium on growth of and phosphate solubilization by *Bacillus marisflavi* FA7

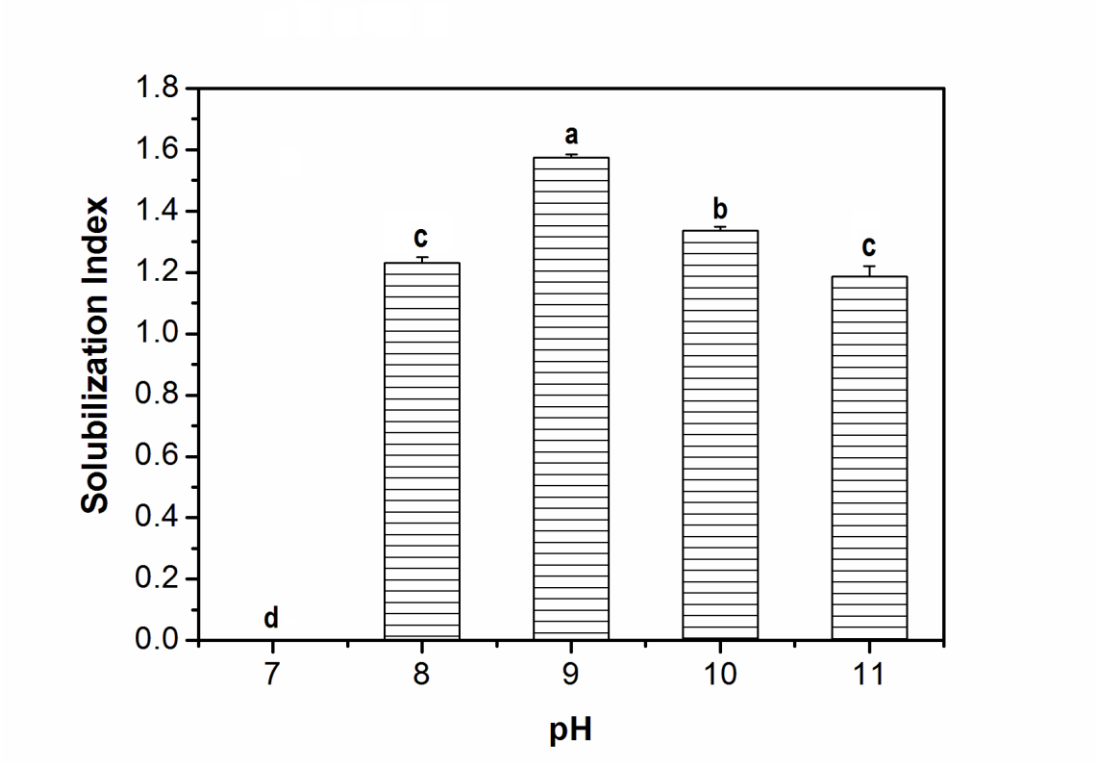


Figure 4.6 - Solubilization index of *Bacillus marisflavi* FA7 for tri-calcium phosphate solubilization at different initial pH values. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

Chromohalobacter israelensis WA3 showed similar growth at pH values from pH 7.0 to 11.0 **(Figure 4.7)**. Solubilization index was highest at pH 7 ($p<0.050$) **(Figure 4.8)**. For remaining pH values from 8 to 11.0, solubilization index remained constant ($p>0.050$).

Among the two isolates, *Chromohalobacter israelensis* WA3 exhibited growth and phosphate solubilization at neutral as well as alkaline pH. *Bacillus marisflavi* FA7 could grow and solubilize phosphate only in alkaline pH range.

4.3.3 Effect of metals on growth and phosphate solubilization

Bacillus marisflavi FA7 showed no growth in the presence of chloride, nitrate and sulfate salts of the six metal ions tested in the experiment. However, it showed growth and phosphate solubilization in absence of metals. *Chromohalobacter israelensis* WA3 showed growth as well as phosphate solubilization in the presence of chloride, nitrate and sulfate salts of the six metal ions tested.

4.3.3.a Effect of aluminium metal salts

Chromohalobacter israelensis WA3 showed growth in the presence of all three salts of Al metal **(Table 4.1 and Figure 4.9)**. However, growth and phosphate solubilization was higher in control. Growth in $AlCl_3$ was found to decrease with increase in concentration of the metal. The growth remained similar at all concentrations of $Al(NO_3)_3.9H_2O$ and $Al_2(SO_4)_3$. However, growth in $Al(NO_3)_3.9H_2O$ was higher as compared to $Al_2(SO_4)_3$.

Chromohalobacter israelensis WA3 exhibited exopolymer production at all the concentrations of Al metal salts. It showed phosphate solubilization at all the concentrations of the three Al metal salts. Higher phosphate solubilization was observed in $Al(NO_3)_3.9H_2O$ compared to $AlCl_3$ and $Al_2(SO_4)_3$.

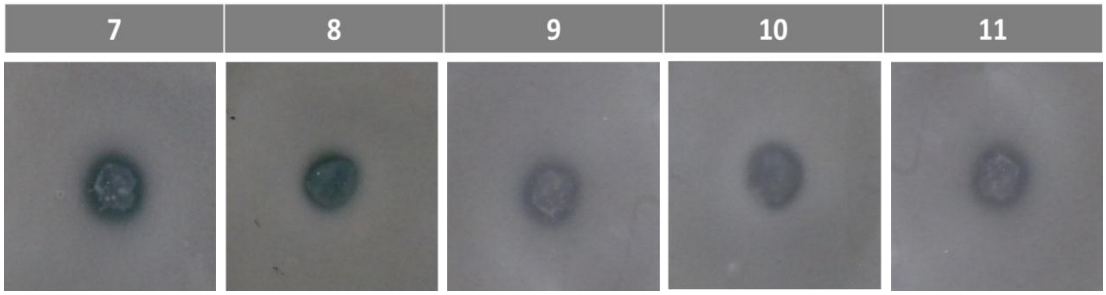


Figure 4.7 - Effect of initial pH of the medium on growth of and phosphate solubilization by *Chromohalobacter israelensis* WA3

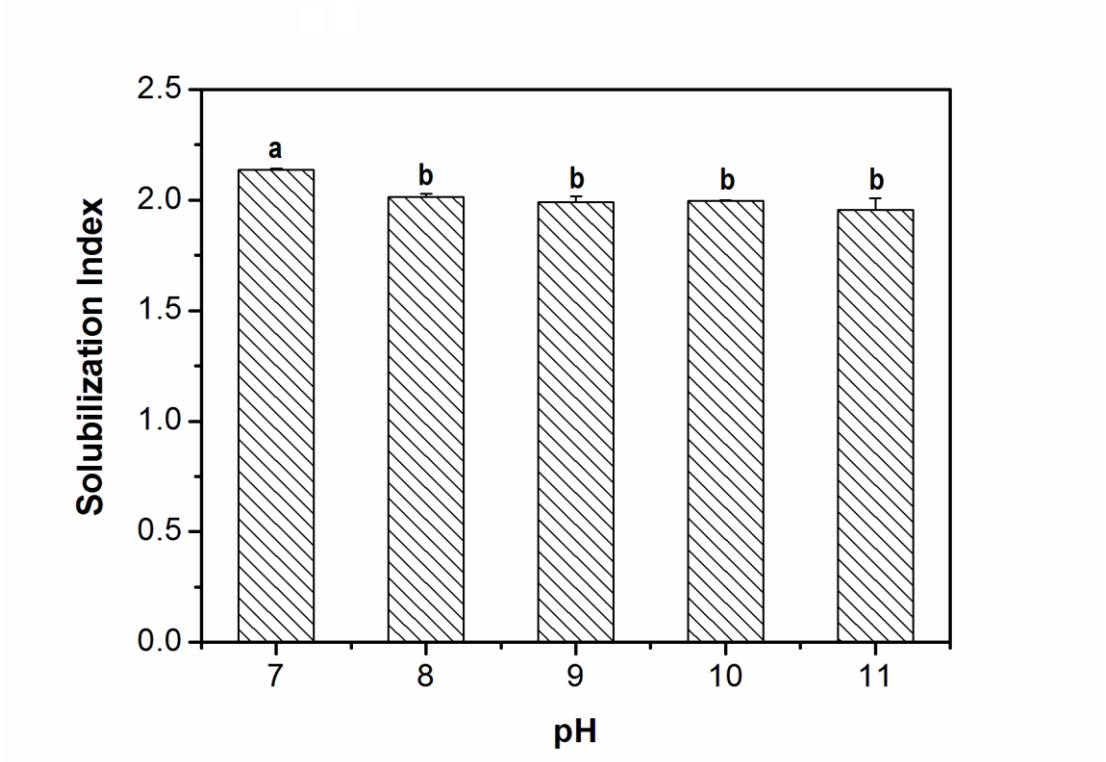


Figure 4.8 - Solubilization index of *Chromohalobacter israelensis* WA3 for tri-calcium phosphate solubilization at different initial pH values. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

4.3.3.b Effect of cadmium metal salts

Maximum growth of *Chromohalobacter israelensis* WA3 was observed at 50 mg/l of all the three salts of Cd as compared to control **(Table 4.1 and Figure 4.9)**. In the presence of $CdCl_2$, growth was observed from 50 to 150 mg/l which decreased with increase in concentration of $CdCl_2$. Growth was absent in 200 mg/l of $CdCl_2$. *Chromohalobacter israelensis* WA3 exhibited growth from 50 to 200 mg/l of $Cd(NO_3)_2$ and $3CdSO_4.8H_2O$. Decrease in growth was observed with increase in concentration of both $Cd(NO_3)_2$ and $3CdSO_4.8H_2O$. However, growth in $Cd(NO_3)_2$ was higher compared to $3CdSO_4.8H_2O$.

The isolate exhibited production of exopolymer in all salts of Cd. However, as in the case of growth, exopolymer production decreased with increase in concentration of the metal. *Chromohalobacter israelensis* WA3 exhibited highest tri-calcium phosphate solubilization at 50 mg/l of Cd in all the three salts. Phosphate solubilization decreased with increase in concentration of Cd. At 200 mg/l of $Cd(NO_3)_2$ and $3CdSO_4.8H_2O$, phosphate solubilization was absent.

4.3.3.c Effect of copper metal salts

Chromohalobacter israelensis WA3 showed maximum growth at 200 mg/l of all three salts of Cu metal **(Table 4.2 and Figure 4.10)**. Among the salts at 200 mg/l, maximum growth was observed in $CuSO_4$. Growth was found to decrease with increase in concentration of $CuCl_2$ and $CuSO_4$. The strain showed similar growth at all concentrations of $Cu(NO_3)_2$. At 200 mg/l of all three salts of Cu metal maximum exopolymer production was exhibited by the strain.

Production of exopolymer was found to decrease with increase in concentration of metal. Phosphate solubilization was observed at all the concentrations of $CuCl_2$ and $CuSO_4$. However, solubilization decreased with increase in concentration of metal. In

the presence of $Cu(NO_3)_2$, *Chromohalobacter israelensis* WA3 solubilized phosphate till 800 mg/l. Phosphate solubilization was absent at 1000 mg/l of $Cu(NO_3)_2$.

4.3.3.d Effect of lead metal salts

Chromohalobacter israelensis WA3 showed growth at all concentrations of $PbNO_3$ **(Table 4.2 and Figure 4.10)**. Highest growth was observed in control followed by 200 mg/l of $PbNO_3$. As the concentration of lead increased, growth was found to decrease. Interestingly, exopolymer was not produced by *Chromohalobacter israelensis* WA3 in the presence of $PbNO_3$. *Chromohalobacter israelensis* WA3 exhibited phosphate solubilization from 200 to 800 mg/l of lead. Phosphate solubilization was not observed at 1000 mg/l of lead. Phosphate solubilizing activity was found to decrease with increase in concentration of lead nitrate.

4.3.3.e Effect of nickel metal salts

Chromohalobacter israelensis WA3 exhibited growth at all concentrations of the three Ni salts ($NiCl_2.6H_2O$, $Ni(NO_3)_3$ and $NiSO_4.6H_2O$) **(Table 4.2 and Figure 4.10)**. Similar growth was observed at all concentrations of $NiCl_2.6H_2O$ and $Ni(NO_3)_3$ as compared to control. Growth in the presence of $NiSO_4.6H_2O$ salt was found to decrease with increase in concentration of metal **(Table 4.2)**. Exopolymer was produced at all the concentrations of the three salts of Ni. Tri-calcium phosphate was solubilized by *Chromohalobacter israelensis* WA3 in the presence of all three salts of Ni. However, phosphate solubilisation was higher in control. Similar extent of tri-calcium phosphate solubilization was observed at all concentrations of $NiCl_2.6H_2O$ and $Ni(NO_3)_3$. In the presence of $NiSO_4.6H_2O$, maximum tri-calcium phosphate solubilization was observed at 200 mg/l. However, as the concentration of $NiSO_4.6H_2O$ increased, phosphate solubilization was found to decrease.

Table 4.1 – Effect of salts (chloride, nitrate and sulphate salts) of metals (aluminium and cadmium) on growth of and phosphate solubilization by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3

Isolate	Metal	Concentration (mg/l)																											
		0						50						100						150						200			
		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate	
		G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS
Bacillus marisflavi FA7	Al	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cd	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chromohalobacter israelensis WA3	Al	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+	+ +	+ +	+	+	+ +	+	+ +	+ +	+	+	+ +	+	+ +	+ +	+	+	+ +	+ +	+	+	+	+
	Cd	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	++ + +	+	++ + +	+	++ +	+	+ +	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	-

Key – G : growth; PS : phosphate solubilization; + : low growth, phosphate solubilization; ++ : average growth, phosphate solubilization; +++ : high growth, phosphate solubilization; ++++ : Excellent growth, phosphate solubilization; - : no growth, phosphate solubilization.

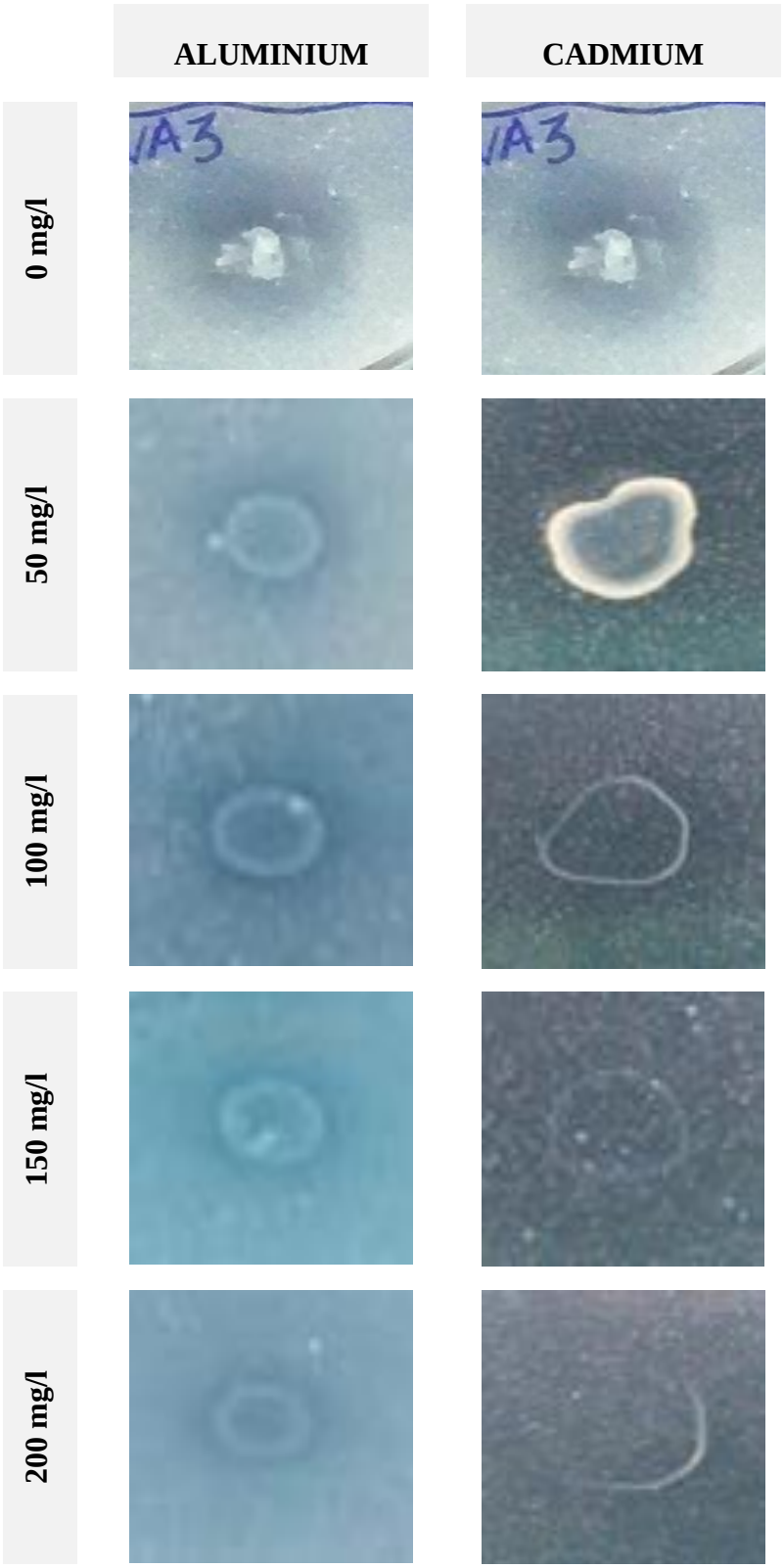


Figure 4.9 – Effect of nitrate salt of aluminium and cadmium on growth of and phosphate solubilization by *Chromohalobacter israelensis* WA3

4.3.3.f Effect of zinc metal salts

Chromohalobacter israelensis WA3 exhibited growth at all concentrations $ZnSO_4.H_2O$ and $Zn(NO_3)_2.6H_2O$ **(Table 4.2 and Figure 4.10)**. In the presence of $Zn(NO_3)_2.6H_2O$, maximum growth of *Chromohalobacter israelensis* WA3 was recorded at 200 mg/l and 400 mg/l. Growth was found to be similar from 600 to 1000 mg/l of $Zn(NO_3)_2.6H_2O$. In $ZnSO_4.H_2O$, growth of *Chromohalobacter israelensis* WA3 was lower as compared to $Zn(NO_3)_2.6H_2O$. Similar extent of growth was observed from 200 to 1000 mg/l of $ZnSO_4.H_2O$. Maximum amount of exopolymer was observed at 200 mg/l of $Zn(NO_3)_2.6H_2O$. Amount of exopolymer produced at 400 to 1000 mg/l of $Zn(NO_3)_2.6H_2O$ was similar. Similar extent of exopolymer production was observed at all the concentrations of $ZnSO_4.H_2O$. *Chromohalobacter israelensis* WA3 exhibited phosphate solubilization from 200 to 800 mg/l of $ZnSO_4.H_2O$. Phosphate solubilization was not observed at 1000 mg/l of $ZnSO_4.H_2O$. Phosphate solubilization was observed at all concentrations of $Zn(NO_3)_2.6H_2O$. Maximum phosphate solubilization was observed at 200 mg/l of $Zn(NO_3)_2.6H_2O$. As the concentration of $Zn(NO_3)_2.6H_2O$ increased, decrease in solubilization of tri-calcium phosphate was recorded.

4.2.5 Scanning electron micrograph and EDX spectrum of *Chromohalobacter israelensis* WA3.

Scanning electron micrographs of *Chromohalobacter israelensis* WA3 in the presence of 200 mg/l of the six nitrate salt metals revealed differences in the length and breadth of the cells as compared to control **(Figure 4.11)**. Length of *Chromohalobacter israelensis* WA3 cells was higher in the presence of $Al(NO_3)_3.9H_2O$, $Cd(NO_3)_2$, $Cu(NO_3)_2$, $PbNO_3$, and $Ni(NO_3)_3$ than the control ($p<0.050$) **(Figure 4.12.a)**. Cell length in the presence of $Zn(NO_3)_2.6H_2O$ was

Table 4.2 - Effect of salts (chloride, nitrate and sulphate salts) of metals (copper, lead, nickel and zinc) on growth of and phosphate solubilization by *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3

Isolate	Metal	Concentration (mg/l)																																	
		0						200						400						600						800						1000					
		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate		Chloride		Nitrate		Sulfate	
		G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS	G	PS
Bacillus marisflavi FA7	Cu	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pb	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ni	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Zn	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chromohalobacter israelensis WA3	Cu	++++	++++	++++	++++	++++	++++	+++	+	+++	+	+++	+	++	+	+	+	+++	+	+	+	+	+	++	+	+	+	+	+	-	++	+	+	+	+	-	+	+
	Pb	++++	++++	++++	++++	++++	++++	X	X	+++	+	X	X	X	X	+++	+	X	X	X	X	++	+	X	X	X	X	++	+	X	X	X	X	+	-	X	X
	Ni	++++	++++	++++	++++	++++	++++	+++	+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	+	+	++	+	++	+	+	+
	Zn	++++	++++	++++	++++	++++	++++	X	X	+++	+	++	+	X	X	++	+	++	+	X	X	+	+	++	+	X	X	+	+	++	+	X	X	+	+	++	+

Key – G : growth, PS : phosphate solubilization, + : low, phosphate solubilization; ++ : average growth, phosphate solubilization; +++ : high growth, phosphate solubilization; ++++ : Excellent growth, phosphate solubilization; - : no growth, phosphate solubilization; x : salt unavailable / salt not used because of precipitation

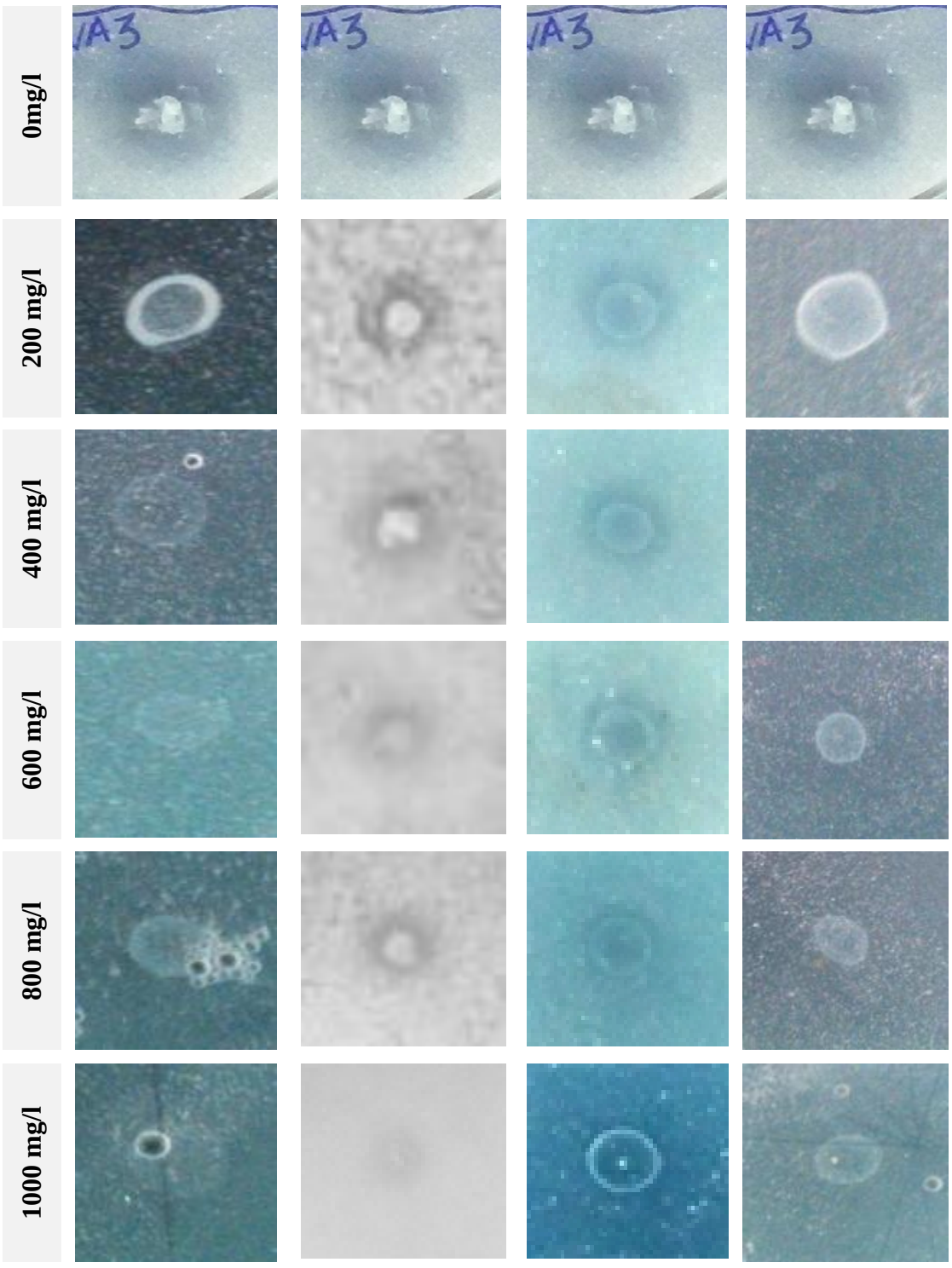


Figure 4.10 - Effect of nitrate salt of copper, lead, nickel and zinc on growth of and phosphate solubilization by *Chromohalobacter israelensis* WA3

similar to the control ($p>0.050$). Among the metals, maximum length was observed in the presence of $PbNO_3$ ($p\leq0.001$) followed by $Cd(NO_3)_2$ ($p<0.001$). Length of *Chromohalobacter israelensis* WA3 cells in the presence of $Al(NO_3)_3.9H_2O$, $Cu(NO_3)_2$, $Ni(NO_3)_3$ and $Zn(NO_3)_2.6H_2O$ was similar ($p>0.050$).

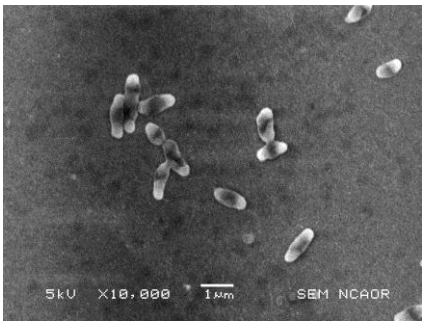
Breadth of *Chromohalobacter israelensis* WA3 cells was higher in the presence of the six metals as compared to control ($p<0.050$) **(Figure 4.12.b)**. Maximum breadth was observed in the presence of $Cd(NO_3)_2$ ($p<0.001$). Breadth of *Chromohalobacter israelensis* WA3 cells in the presence of $Al(NO_3)_3.9H_2O$, $Cu(NO_3)_2$, $Ni(NO_3)_3$ and $Zn(NO_3)_2.6H_2O$ was similar ($p>0.050$). In the presence of $PbNO_3$ cell breadth was lower as compared to other metals ($p\leq0.001$).

EDX spectrum showed peaks of the six nitrate metal salts which revealed that these metals were adsorbed by *Chromohalobacter israelensis* WA3 **(Figure 4.13)**. Weight percentage of metal adsorption is tabulated in **Table 4.3**.

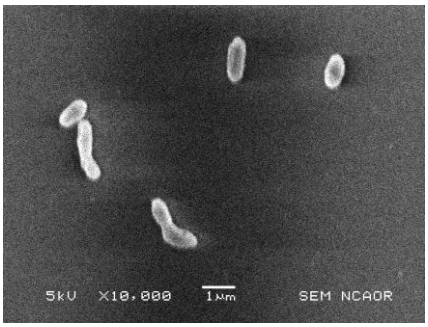
4.4 DISCUSSION

Survival, multiplication and spread of bacterial strains in soil depend on tolerance to environmental factors such as high salt, high pH and heavy metals. Stress tolerant bacteria are likely to be found in environments affected by osmotic, pH and temperature stresses (Nautiyal *et al.*, 2000). In this study, two phosphate solubilizing bacteria, *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 were isolated from coastal ecosystems of Goa. *Bacillus marisflavi* FA7 was isolated from sediment of Quellosim mangrove ecosystem and *Chromohalobacter israelensis* WA3 was isolated from sediment of Batim salt pan ecosystem. It is reported that bacteria in these ecosystems are exposed to varying levels of salinity,

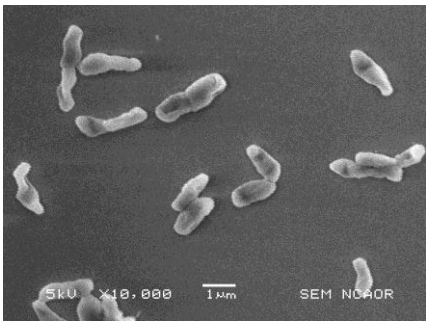
Control



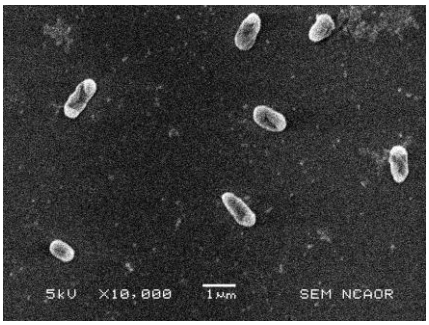
Aluminium



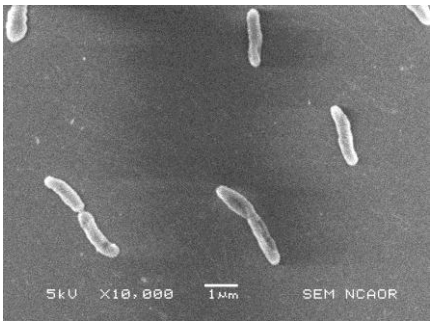
Cadmium



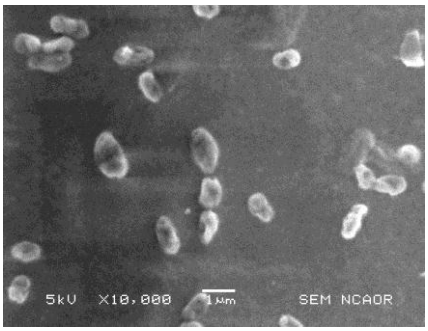
Copper



Lead



Nickel



Zinc

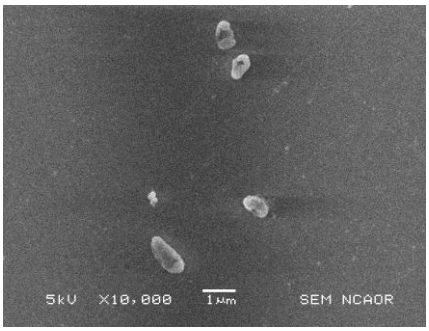
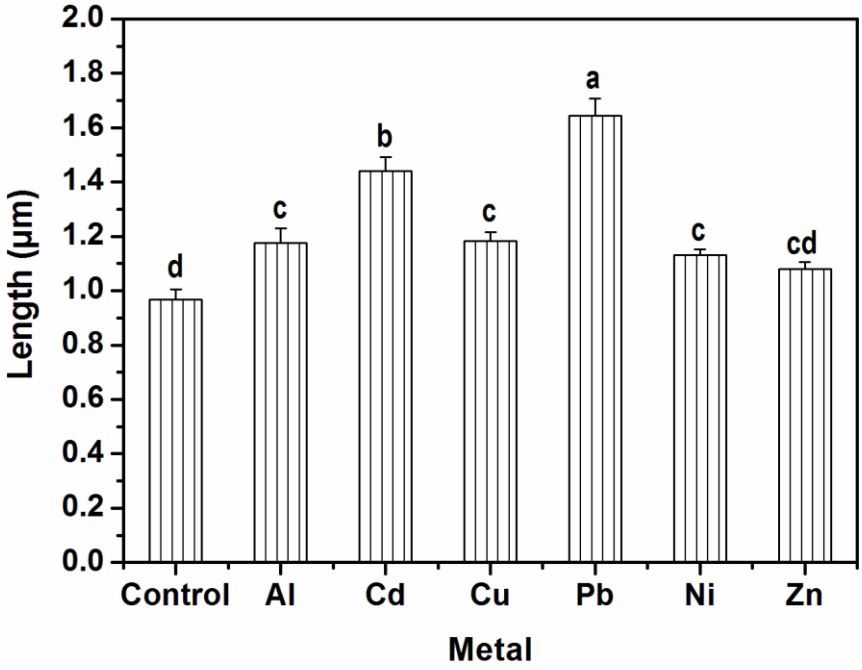


Figure 4.11 – SEM micrographs showing morphological changes in the cells of *Chromohalobacter israelensis* WA3 exposed to 200 mg/l of nitrate salts of aluminium, cadmium, copper, lead, nickel and zinc

(a)



(b)

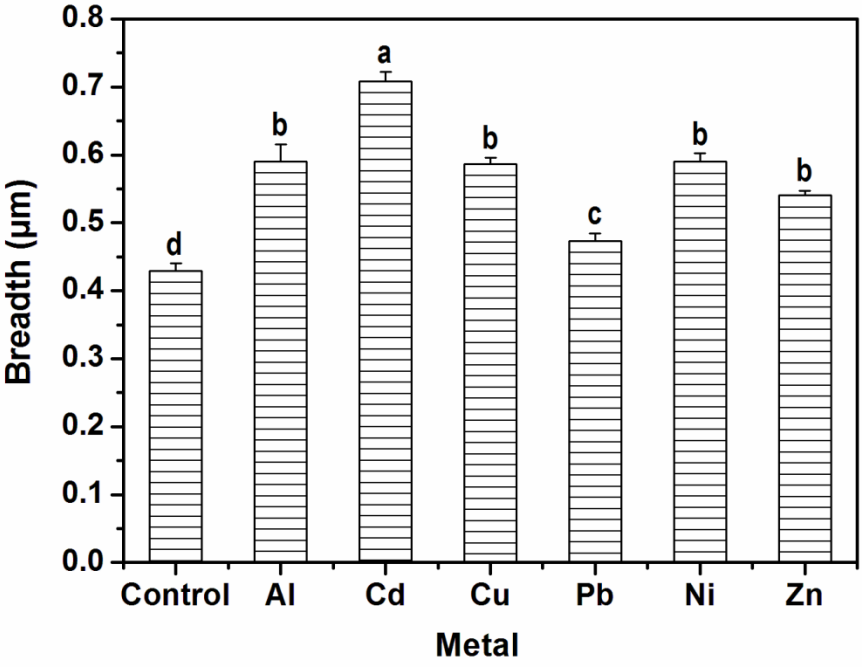


Figure 4.12 – Measurement of length (a) and breadth (b) of *Chromohalobacter israelensis* WA3 cells in the presence of nitrate salt of aluminium, cadmium, copper, lead, nickel and zinc. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.05.

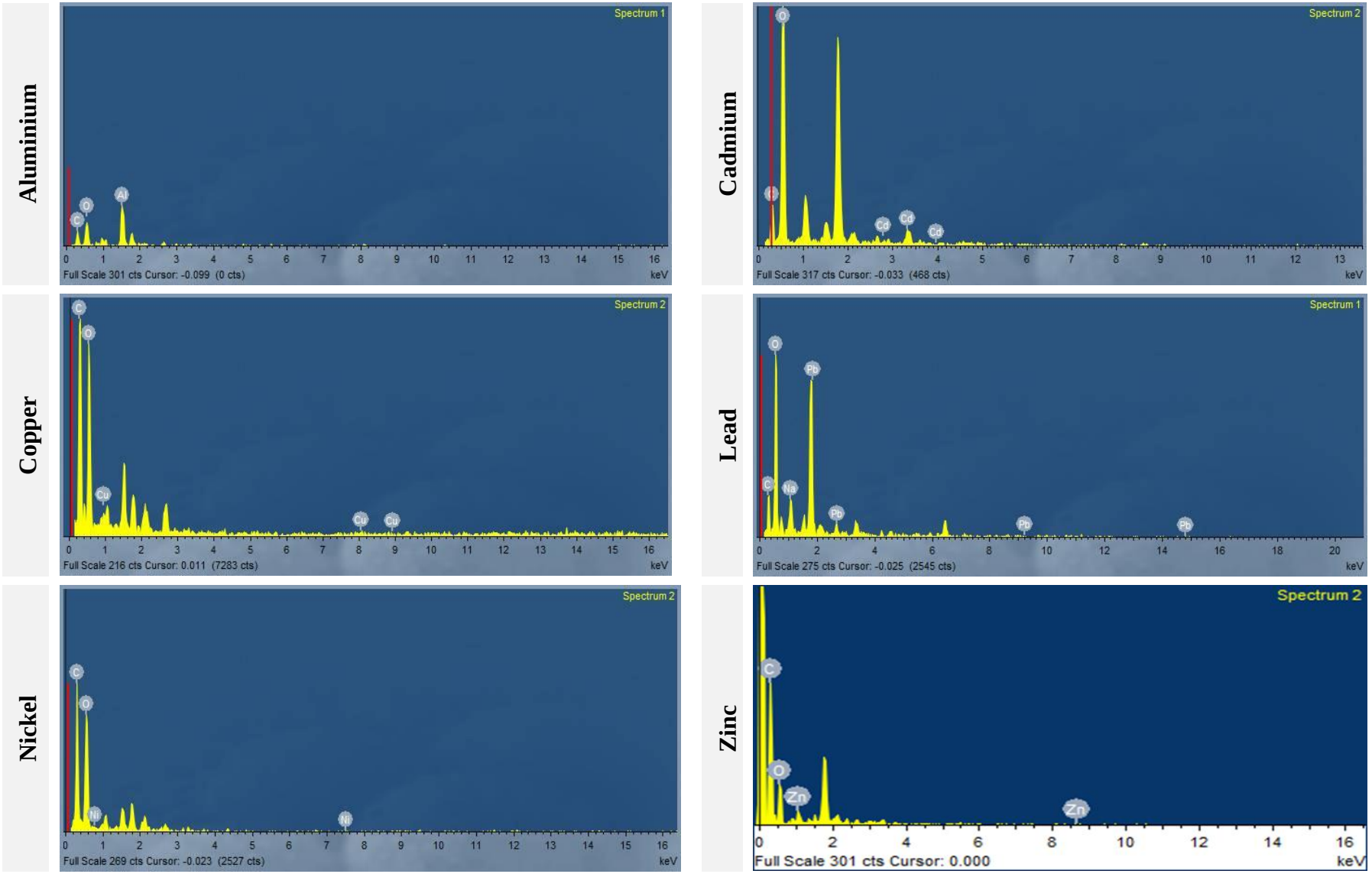


Figure 4.13 – EDX spectrum showing adsorption of nitrate salt of metal by *Chromohalobacter israelensis* WA3

Table 4.3 - Adsorption of nitrate salt of metals by *Chromohalobacter israelensis* WA3

Metal	Adsorption (weight %)
$Al(NO_3)_3.9H_2O$	8.10
$Cd(NO_3)_2$	0.75
$Cu(NO_3)_2$	0.10
$PbNO_3$	0.38
$Ni(NO_3)_3$	0.13
$Zn(NO_3)_2.6H_2O$	0.64

pH, temperature and moisture content due to the influence of tidal cycle, fresh water influx and recurring seasonal changes (Vassilev *et al.*, 2012).

Both the isolates demonstrated diverse level of growth and phosphate solubilization at different pH and crude salt concentrations. Effect of pH revealed that *Bacillus marisflavi* FA7 showed growth and phosphate solubilization only at alkaline pH range. It showed no grow at neutral pH. Highest growth and phosphate solubilization was observed at pH 9. *Chromohalobacter israelensis* WA3 exhibited better growth at neutral pH but tolerated pH as high as 11.0. Interestingly, phosphate solubilizing activity of *Chromohalobacter israelensis* WA3 remained constant over the pH range from 8.0 to 11.0. Effect of different salt concentration revealed that *Bacillus marisflavi* FA7 could tolerate crude salt up to 5%, whereas, *Chromohalobacter israelensis* WA3 could grow and solubilize phosphate till 25% crude salt concentration. Better growth and phosphate solubilization was observed at 15% crude salt concentration. The pH of medium for studying effect of different crude salt concentrations was maintained at 10.0. The results revealed that *Chromohalobacter israelensis* WA3 can grow as well as solubilize phosphate in extreme conditions of alkaline pH and high salt concentration.

In the literature, most phosphate solubilizing bacteria reported, performed relatively low in alkalinity and salinity tolerance, being less appropriate for alkaline-saline soil based agriculture. In a similar study, Johri *et al.* (1999) surveyed 4,800 bacterial strains isolated from alkaline soil. They found 857 phosphate solubilizing isolates from which 18 were able to solubilize phosphate at pH 9.0 and 5% salt concentration. Jacobs *et al.* (2002) reported the effect of different pH on growth and solubilization of insoluble calcium phosphate by fungus *Rhizoctonia solani*. The isolate efficiently

solubilized phosphate but solubilization activity decreased with increasing pH on medium containing tri-calcium phosphate. Nautiyal *et al.* (2000) have isolated phosphate solubilizing bacteria from alkaline soils which solubilized phosphate at pH 12 and 10% NaCl. Isolates screened by Mishra *et al.* (2009) in their study tolerated 10% NaCl while, isolates of Nakbanpote *et al.* (2014) demonstrated phosphate solubilization at 8% (w/v) salt concentration.

Effect of chloride, nitrate and sulfate salts of six heavy metals (Al, Cd, Cu, Pb, Ni and Zn) revealed that *Bacillus marisflavi* FA7 failed to grow in the presence of heavy metals. *Chromohalobacter israelensis* WA3 demonstrated growth as well as phosphate solubilization in the presence of chloride, nitrate and sulfate salts of all six heavy metals. *Chromohalobacter israelensis* WA3 presented different growth and phosphate solubilization capability in the presence of chloride, nitrate and sulfate metal salts. High concentrations of metals interfered with the phosphate solubilizing activity of *Chromohalobacter israelensis* WA3.

Phosphate solubilizing capability along with metal tolerance is a suitable combination for growth promotion of plants in metal contaminated soil. Khan *et al.* (2009b) have illustrated a number of mechanisms exhibited by microorganisms of agronomic importance used to tolerate the uptake of heavy metal ions and circumvent the metal stress. Such mechanisms include pumping of metal ions exterior to the cell, accumulation and sequestration of the metal to less toxic forms and adsorption or desorption of metals. *Chromohalobacter israelensis* WA3 is likely to have metal adsorption mechanism which was confirmed by EDX studies. It was interesting to note that as compared to control, morphology of *Chromohalobacter israelensis* WA3

cells in the presence of metals was different. Another interesting observation was elongation of cell to form short chains in the presence of lead nitrate salt.

Several authors have carried out similar studies and reported phosphate solubilizing activity of bacteria in the presence of heavy metals. Sen and Joshi (2015) have studied phosphate solubilization activity of lead tolerant *Pseudomonas aeruginosa* from Zawar mines of Udaipur. Rajkumar and Freitas (2008) reported the use of two plant growth promoting bacterial strains tolerant to heavy metals and able to promote the growth of *Ricinus communis* in a soil contaminated with nickel, copper and zinc. Gupta *et al.* (2002) developed heavy metal resistant phosphate solubilizing *Pseudomonas* sp. NBRI 4014. This bacterium was resistant to cadmium, nickel, and chromium. It also produced siderophore and indole acetic acid.

Microbial population is known to affect the mobility of trace metal due to the release chelators, acidification and redox changes; thereby making the metals available to plants (Abou-Shanab *et al.,* 2003). Studies have reported metal tolerant bacteria which are inoculated in soil, help reduce the toxicity of metals in soil and improve the overall growth and yield of chickpea, green gram and pea (Gupta *et al.,* 2004; Wani *et al.,* 2007a; Wani *et al.,* 2007b). A study conducted by Abou-Shanab *et al.* (2006) reported that inoculation of phosphate solubilizing bacteria enhance phytoremediation of nickel by *Alyssum murale.* Similar study for zinc was reported by Whiting *et al.* (2001); they inoculated *Microbacterium saperdae* and *Pseudomonas monteilii* to seeds of plant *Thlaspi caerulescens*.

The response of microorganisms to the surroundings is not always consistent, because soil environment and plant factors influence survival and the activity of phosphate solubilizing microorganisms. The results of this study suggest that

Bacillus marisflavi FA7 and *Chromohalobacter israelensis* WA3 isolated from coastal ecosystems have the ability of tolerance to stress such as high pH and salt concentration, with *Chromohalobacter israelensis* WA3 displaying additional tolerance to heavy metal ions. This ability plays an important role in the survival of plant growth promoting bacterial strains in environments. Therefore, such strains can be developed for further utilization as indigenous plant growth promoting bio-agents for alkaline and saline soils.

Chapter V

Plant growth promoting potential of alkaliphilic bacteria

5.1 INTRODUCTION

In recent years, plant growth promoting bacteria (PGPB) have gained increased attention (Lucy *et al.,* 2004). These occur naturally in soil and have various mechanisms for plant growth promotion (Hayat *et al.,* 2010). PGPB promote plant growth directly or indirectly by either facilitating resource acquisition or modulating plant hormone levels. Direct mechanisms of plant growth involve nitrogen fixation, inorganic and organic phosphate solubilization by production of organic acids and phosphatase enzyme, respectively and production of phytohormones such as indole acetic acid (Patten & Glick, 2002; Dobbelaere *et al.,* 2003; Chen *et al.,* 2006). Indirect mechanisms involve reducing the inhibitory effects of pathogenic agents on plant development by production of ammonia and organic compounds such as siderophores, thus acting as biocontrol agents (Dimkpa *et al.,* 2008).

PGPB have been studied extensively in agricultural research to improve productivity of crops in horticulture and forestry (Okon & Labandera-Gonzalez, 1994; Sturz *et al.,* 2000; Malik *et al.,* 2002; Matiru & Dakora, 2004). Commercialized PGPB strains include *Agrobacterium radiobacter, Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chroococcum, Bacillus firmus, Bacillus licheniformis, Bacillus megaterium, Bacillus mucilaginous, Bacillus pumilus, Burkholderia cepacia, Delftia acidovorans, Paenibacillus macerans, Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas solanacearum, Pseudomonas syringae, Serratia entomophila, Streptomyces griseoviridis, Streptomyces lydicus* and several species of *Rhizobium* (Antoun & Prevost, 2005; Figueiredo *et al.,* 2010; Glick, 2012; Bouizgame, 2013). These strains have been developed for application in agriculturally active soil.

With rise in world population, the demand for food by the end of 2020 will be much higher. Therefore, scientists are looking at alternatives to increase production through improvement in agricultural productivity in infertile and barren soil in sustainable manner. Infertile and barren land is associated with stress due to salinity, alkalinity, toxic metals and organic compounds. These limit the yields and profits of agricultural crops due to osmotic effects, toxicity of salt ions and the changes in the physical and chemical properties of soil (Yamaguchi & Blumwald, 2005; Shahbaz & Ashraf, 2013). Recent studies indicate that microbes can help crops to cope with salinity and alkalinity stress (Dimkpa *et al.,* 2009; Grover *et al.,* 2011). They can be a relatively inexpensive way to develop barren and infertile land into agriculturally sound land.

Application of bacteria exhibiting plant growth promoting characters from laboratory and greenhouse experiments to field trials is tedious. It requires a number of approaches for growth, storage, formulation and application of these bacteria. There is a limited understanding of effect of stress tolerant PGPB on plant interaction since these bacteria are often not studied in field experiments. Therefore, there is a need to isolate and develop bacteria for plant growth promotion under stress conditions.

5.2 MATERIALS AND METHODS

5.2.1 Evaluation of plant growth promoting traits

All the 141 alkaliphilic isolates were qualitatively screened for the production of plant growth promoting traits, like ammonia, indole acetic acid and siderophore.

5.2.1.1 Screening for ammonia production (Marques *et al*., 2010)

Ammonia production was checked by growing alkaliphilic isolates in 5 ml peptone water **(Appendix A)** at pH 10 in tubes and incubated at 30°C for 48 hours. After incubation, the accumulation of ammonia was detected by addition of 1 ml of

Nessler's reagent **(Appendix B)** in each tube. Appearance of yellow colour indicated production of ammonia.

5.2.1.2. Screening for indole acetic acid production (Chaiharn & Lumyong, 2011)

Indole acetic acid (IAA) in the medium was detected following the method of Chaiharn and Lumyong (2010). Isolates were inoculated in liquid Mineral Salt Medium (pH 10.0) **(Appendix A)** with tryptophan (1% (w/v)) and incubated for 48 hours at 30°C. After incubation, 1 ml of culture was centrifuged at 10,000 rpm for 5 min in a microfuge. A drop of ortho-phosphoric acid and 2 ml of Salkowski's reagent **(Appendix B)** was added to the clear supernatant. Development of a pink color indicated IAA production.

5.2.1.3 Screening for siderophore production (Schwyn & Neilands, 1987)

Siderophore production was detected by Chrome Azurol Sulfonate (CAS) plate assay at pH 10.0. Isolates were spot inoculated on CAS agar **(Appendix A)** and incubated at 30°C for 48 hours. Development of orange to yellow halo around the colony indicated production of siderophores.

5.2.2 Selection of carrier and formulation of carrier based phosphate solubilizing biofertilizer

Three different carriers viz. lignite, talc and vermiculite were used. The carriers were added to a conical flask, autoclaved at 121°C for 30 min and placed in an oven at 70°C for 3 h to dry. *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, Isolate AA5 and Isolate DA3 were cultured in Pikovskaya's broth **(Appendix A)** for 48 hours. Appropriate volume of culture broth corresponding to 10^{11} cells/g of carrier was added to the flask containing sterile carrier. Content of the flask was mixed thoroughly with the help of a sterile spatula. Each type of carrier was blended with

broths of each of the 4 isolates in separate flasks. All the flasks were incubated at 30°C. After an interval of 15 days for a period of 3 months, viable count of the four isolates was determined using serial dilution method. One gram of carrier was aseptically removed from each flask and suspended in 10 ml saline. Serial dilutions were prepared using saline and 0.1 ml of appropriate dilution was plated out on Pikovskaya's agar **(Appendix A)**. Plates were incubated at 30°C and examined after 48 hours for bacterial colonies. Viable count of the four isolates was determined.

5.2.3 Plant growth promoting potential of formulated biofertilizer on *Oryza sativa*

5.2.3.1 Preparation of soil pot

Soil was procured from Cavellosim khazan. Salinity and pH of the soil was determined by argentometric titration (Clesceri *et al.,* 1999) and pH meter respectively as described in chapter 2. Soil was air dried and sieved. It was filled in canisters and autoclaved three times at 121°C for 30 min. After cooling at room temperature, the soil was packed in pots. Plastic pots of 19 cm diameter and 15 cm height were used.

5.2.3.2 Treatment of inoculation

Effect of formulated biofertilizer was studied on *Oryza sativa* var Jyoti by seed inoculation method. The experiment comprised of total six treatments. The first four treatments were test cultures being *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, Isolate AA5 and Isolate DA3. Fifth and sixth treatments were positive control and negative control, respectively. Lignite based *Pseudomonas straita* biofertilizer, procured from University of Agricultural Sciences, Dharwad, was used as positive control. Uncoated seeds served as negative control.

5.2.3.3 Preparation of seeds

Seeds of *Oryza sativa* var Jyoti were obtained from Krishi Vigyan Kendra, Indian Council of Agricultural Research, Ela, Old-Goa. Seeds were surface sterilized with mercuric chloride solution **(Appendix B)** and rinsed three times with sterile distilled water. Carrier based slurry paste of the six treatments was prepared by mixing the formulated biofertilizer in sterile distilled water. The sterilized seeds were soaked with carrier based slurry and allowed to dry. Thirty seeds coated with each treatment were sown in plastic pots with sterile soil.

5.2.3.4 Analysis.

Pot experiments were conducted till 21 days. After the period, plantlets were carefully uprooted from the pots. Shoot length, root length, wet weight and dry weight of plantlets were recorded. Results of the experiment were statistically analyzed by ANOVA and Student Newman Keuls method using SigmaStat Version 4.0.

5.3 RESULTS

5.3.1 Plant growth promoting traits of alkaliphilic bacteria

Alkaliphilic isolates exhibited plant growth promoting traits at pH 10 **(Table 5.1)**. Twenty-one isolates showed production of ammonia out of 141 isolates **(Figure 5.1.a)**. Eight isolates showed high intensity of yellow colour whereas thirteen isolates showed low intensity of yellow colour. Seventy-three isolates out of 141 showed production of IAA **(Figure 5.1.b)**. Among seventy-three IAA producing isolates, 10 showed high intensity of pink colour formation. Ninety-two isolates out of 141 isolates showed siderophore production on CAS agar plates **(Figure 5.1.c)**. Five isolates showed maximum production of siderophores indicated by the zone of

Table 5.1 – Production of plant growth promoting traits by alkaliphilic isolates (Ammonia, IAA and Siderophore)

Isolate	Ammonia	IAA	Siderophore
AA1	-	-	-
AA2	-	-	-
AA3	-	+++	+
AA4	-	+++	-
AA5	-	-	-
AA6	-	+	+
AA7	-	+	+
BA1	+	-	+
BA2	++	+	-
BA3	-	-	+
BA4	-	-	-
BA5	-	+	-
BA6	-	+	+
BA7	-	-	+
CA1	-	-	-
CA2	-	-	-
CA3	-	-	-
CA4	-	++	+
CA5	-	-	-
CA6	-	+	-
CA7	-	++	-
CA8	-	-	-
DA1	-	-	-
DA2	-	-	-
DA3	-	-	-
EA1	-	-	+
EA2	+	-	-
EA3	-	+	+
EA4	-	-	+
EA5	+	-	+
EA6	++	-	+
EA7	+	-	+
EA8	-	-	+
EA9	-	-	+
EA10	-	+	+
FA1	-	+	+
FA2	-	+	+
FA3	-	-	-
FA4	-	-	-
FA5	-	+	-
FA6	+	+	+
FA7	-	-	-
FA8	-	+	+

Table 5.1 – Production of plant growth promoting traits by alkaliphilic isolates (Ammonia, IAA and Siderophore)

Isolate	Ammonia	IAA	Siderophore
GA1	-	++	-
GA2	-	+	+
GA3	-	+	-
GA4	-	+	+
GA5	-	++	-
GA6	+++	+	+
GA7	-	-	+
HA1	++	-	+
HA2	+	-	+
HA3	-	-	+
HA4	-	-	+
IA1	-	+	-
IA2	-	+	+
IA3	++	+	+
IA4	++	+	+
IA5	+++	+	+
IA6	-	+	+
IA7	-	+	+
IA8	-	+	+
IA9	+++	+	+
JA1	-	+	-
JA2	-	-	+
JA3	+	+	+
KA1	-	+	+
KA2	-	+	-
KA3	-	+	-
LA1	-	-	+
LA2	-	+	+
LA3	-	+	+
LA4	-	-	+
LA5	-	+	+
LA6	-	+	+
LA7	-	-	-
LA8	-	+	+
LA9	-	++	+
LA10	-	+	+
LA11	-	+	+
LA12	-	+	-
LA13	-	+	-
MA1	-	+	+
MA2	-	+	-
MA3	-	++	+
MA4	-	-	+

Table 5.1 – Production of plant growth promoting traits by alkaliphilic isolates (Ammonia, IAA and Siderophore)

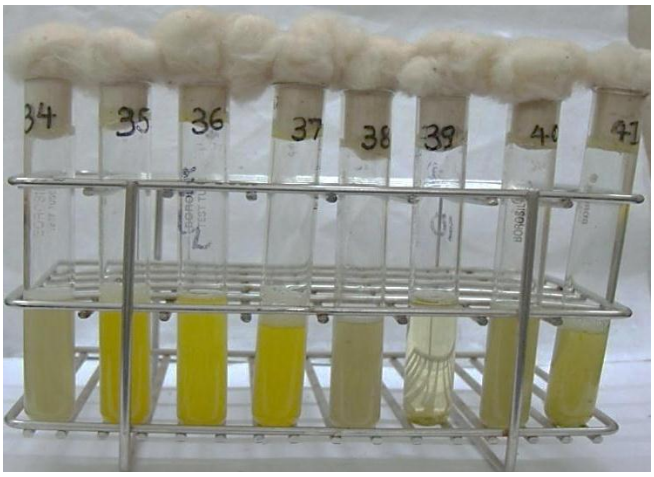
Isolate	Ammonia	IAA	Siderophore
MA5	-	-	-
MA6	-	-	+
NA1	-	+	+
NA2	-	+	+
NA3	-	+	+
NA4	-	-	+
OA1	-	+	+
OA2	-	-	+
OA3	-	+	+
PA1	-	+	+
PA2	-	-	+
PA3	-	+	+
PA4	-	+	-
PA5	+	+	+
PA6	-	+	+
PA7	-	-	-
QA1	+	-	+
QA2	-	-	-
QA3	-	++	+
QA4	-	+	+
QA5	-	+	-
QA6	-	-	+
RA1	-	+	+
RA2	-	+	+
SA1	-	-	+
SA2	-	-	+
SA3	-	++	+
TA1	-	-	+
TA2	-	-	+
TA3	-	-	-
UA1	-	+	+
UA2	-	+	+
UA3	-	-	+
VA1	-	-	+
VA2	-	+	+
VA3	-	-	+
VA4	-	+	+
VA5	-	-	-
VA6	-	-	-
WA1	-	+	-
WA2	-	-	+
WA3	-	-	-
WA4	-	-	+

Table 5.1 – Production of plant growth promoting traits by alkaliphilic isolates (Ammonia, IAA and Siderophore)

Isolate	Ammonia	IAA	Siderophore
WA5	-	+	-
WA6	+	-	+
WA7	-	-	+
WA8	-	-	-
WA9	-	-	-
WA10	-	-	-
WA11	+	-	+
WA12	+	+	+
WA13	+	-	+
WA14	-	-	+
WA15	-	-	-
WA16	-	-	-

Key: + positive test; - negative test

(a)



(b)

(c)

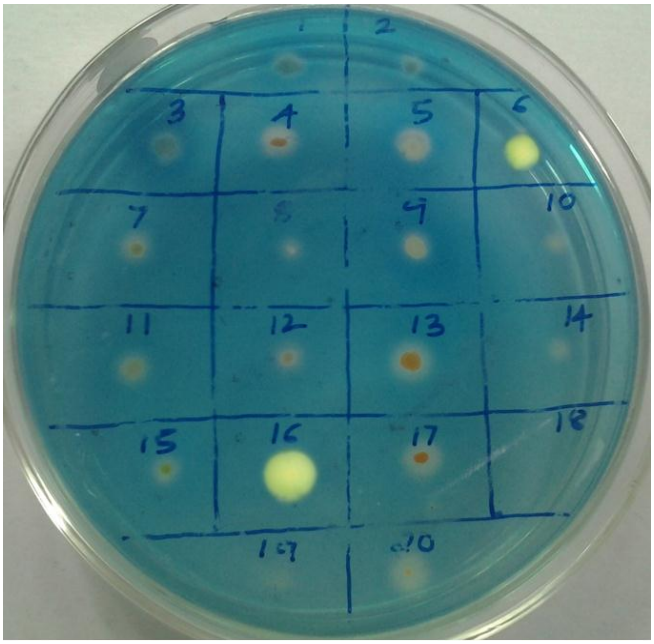


Figure 5.1 – Screening of alkaliphilic isolates for production of ammonia (a), indole acetic acid (b) and siderophore (c)

yellow colouration compared to other isolates. Nine isolates produced all three plant growth promoting traits. Twenty eight isolates did not exhibit any plant growth promoting trait.

5.3.2 Viability of test cultures in carriers

5.3.2.1 *Bacillus marisflavi* FA7

The viability of *Bacillus marisflavi* FA7 decreased with elapse of time **(Figure 5.2)**. At 15 days, the viability was similar in all 3 carriers, of the order of 10^{10} ($p>0.050$). After 30 and 45 days, count of *Bacillus marisflavi* FA7 in talc and vermiculite was of the order of 10^9. Viability of *Bacillus marisflavi* in lignite decreased to the order of 10^9 and 10^8 cells after 30 and 45 days, respectively. At 60 days, viability in vermiculite decreased from 10^9 to 10^8. Viability remained constant from 60 to 90 days in lignite (10^8) and talc (10^9). At the end of 90 days, viability in talc was significantly higher than lignite and vermiculite ($p<0.001$).

5.3.2.2 *Chromohalobacter israelensis* WA3

At 15 days of incubation, the viability of *Chromohalobacter israelensis* WA3 was of the order of 10^{11} in all the carriers ($p>0.050$) **(Figure 5.3)**. Viability in talc was of the order of 10^{11}, which was higher compared to lignite and vermiculite at the end of 30 days ($p<0.050$). At the end of 45 days, viability in lignite was least as compared to talc and vermiculite ($p<0.001$). Viability of *Chromohalobacter israelensis* WA3 in talc was close to 10^{11} at the end of 60 days and was higher than lignite and vermiculite ($p<0.001$). Viability in lignite and vermiculite was similar at the end of 60 days ($p>0.050$). Viability of *Chromohalobacter israelensis* WA3 remained 10 times higher in talc as compared with lignite and vermiculite at the end of 75 days ($p<0.001$) and 90 days ($p<0.050$).

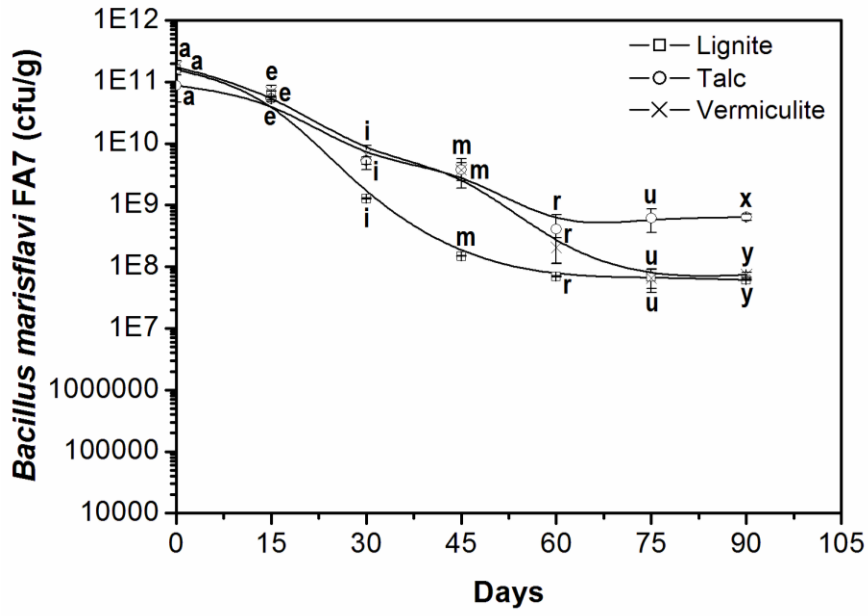


Figure 5.2 – Viability of phosphate solubilizing *Bacillus marisflavi* FA7 in lignite, talc and vermiculite. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

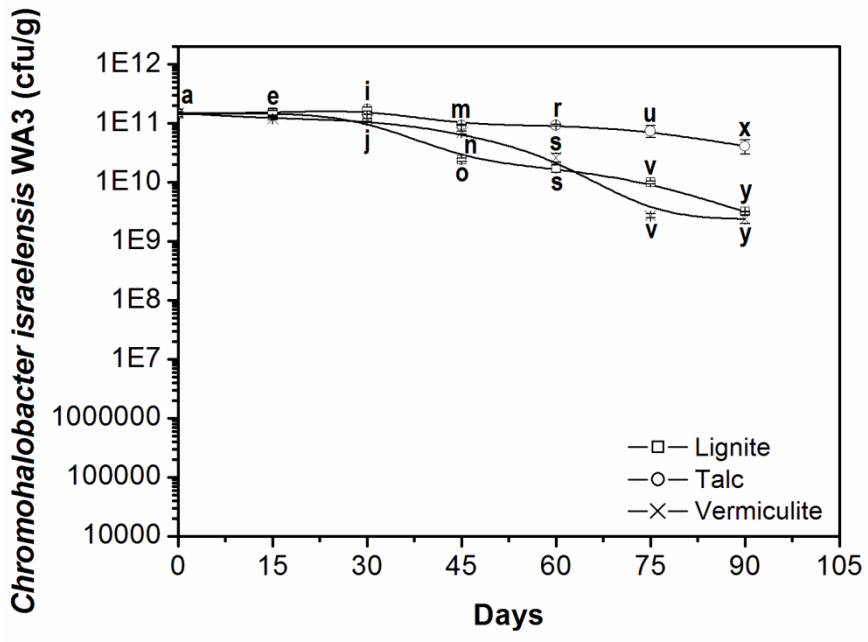


Figure 5.3 – Viability of phosphate solubilizing *Chromohalobacter israelensis* WA3 in lignite, talc and vermiculite. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

5.3.2.3 Isolate AA5

At 15 days, viability of isolate AA5 in lignite and vermiculite was similar ($p>0.05$) **(Figure 5.4)**. Count in talc carrier was higher as compared with lignite and vermiculite ($p<0.050$). From 30 to 90 days, the viability of AA5 decreased with elapse of time. However, viability in talc was significantly higher as compared to lignite and vermiculite ($p\leq0.001$). Count of isolate AA5 was least in lignite compared to vermiculite.

5.3.2.4 Isolate DA3

At the end of 15 days, count of isolate DA3 was of the order of 10^{11} in vermiculite **(Figure 5.5)**. It was higher as compared to lignite and talc ($p<0.001$). Count of DA3 was found to decrease with elapse of time in all the three carriers. At the end of 30 and 45 days, count in vermiculite was higher followed by talc and lignite ($p<0.001$). Count in lignite was 10 times lower as compared to talc and vermiculite. It was observed that at the end of 60 and 75 days, viability of isolate DA3 in talc was higher than lignite and vermiculite ($p<0.001$). At the end of 90 days it was observed that count in talc was higher followed by vermiculite and lignite ($p<0.001$).

During the three months of storage, the four isolates presented high variation in viable count in the three carriers. The viability of isolates in all carriers reduced through time. The number of viable cells detected in talc was higher than vermiculite and lignite with values of cfu/g after 90 days of storage for all the 4 isolates. Viability of *Chromohalobacter israelensis* WA3 was higher in talc followed by isolate AH5, *Bacillus marisflavi* FA7 and isolate DA3. Lignite carrier sustained less number of viable cells among the three carriers.

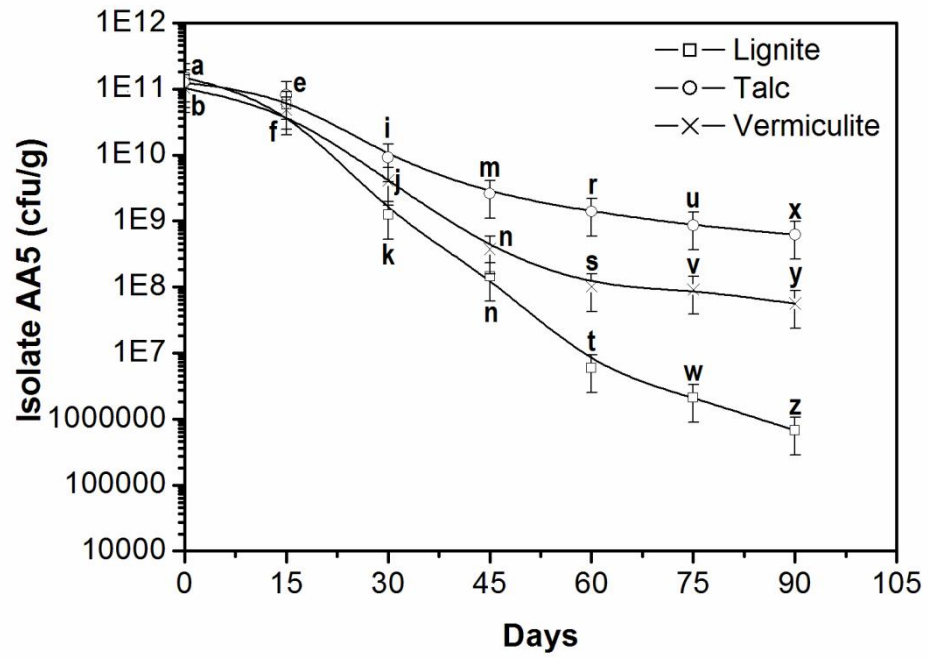


Figure 5.4 – Viability of phosphate solubilizing isolate AA5 in lignite, talc and vermiculite. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

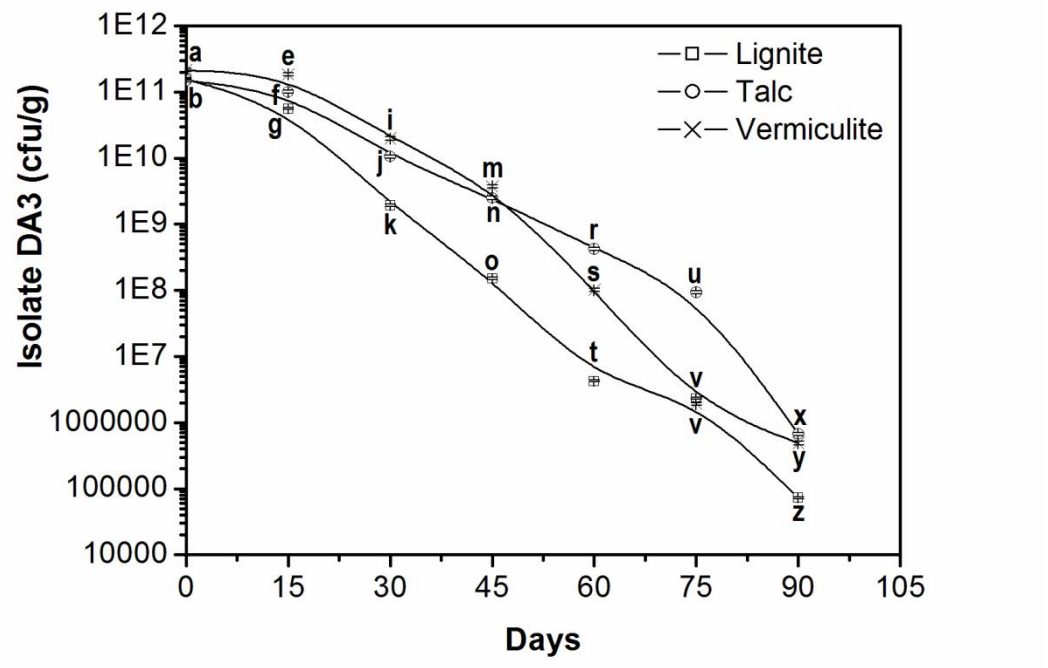


Figure 5.5 – Viability of phosphate solubilizing isolate DA3 in lignite, talc and vermiculite. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

5.3.3 Plant growth promoting potential of formulated biofertilizer on *Oryza sativa*

All the four selected isolates, *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, isolate AA5 and isolate DA3 promoted growth of *Oryza sativa* **(Figure 5.6)** as compared to negative control.

5.3.3.1 Shoot length

Shoot length of all treatments was significantly higher when compared with the negative control ($p<0.001$) **(Figure 5.7)**. Seeds inoculated with isolate DA3 had the highest shoot length among all the treatments ($p<0.001$). Shoot lengths of seeds inoculated with *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, isolate AA5 and positive control were found to be similar ($p>0.05$).

5.3.3.2 Root length

Root length of all treatments was significantly higher than root length of seeds inoculated with *Chromohalobacter israelensis* WA3 ($p<0.050$) **(Figure 5.8)**. Root length of positive control treatment was similar with root length of seeds inoculated with isolate AA5 and *Bacillus marisflavi* FA7 ($p>0.050$). Root length of seeds inoculated with isolate DA3 was significantly lower than positive control, isolate AA5 and *Bacillus marisflavi* FA7 treatments ($p<0.050$).

5.3.3.3 Wet weight

Wet weight of plantlets inoculated with isolate DA3 was significantly higher compared to all treatments ($p<0.001$) **(Figure 5.9)**. Positive control and negative control treatments were found to be similar ($p>0.050$). Wet weight of all treatments was significantly higher than both negative and positive control treatment ($p<0.001$).

Wet weight of seeds inoculated with *Chromohalobacter israelensis* WA3 was higher than treatments with *Bacillus marisflavi* FA7 ($p<0.001$) and isolate AA5 ($p<0.050$).

5.3.3.4 Dry weight

Dry weight of plantlets with *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, isolate DA3 and positive control treatments were higher compared to negative control treatment ($p<0.001$) **(Figure 5.10)**. There was no difference between treatments of isolate AA5 and negative control ($p>0.050$). Dry weight of plantlets inoculated with isolate DA3 was higher when compared to isolate AA5 ($p<0.001$) and *Bacillus marisflavi* FA7 ($p<0.050$) but similar when compared to positive control and *Chromohalobacter israelensis* WA3 ($p>0.050$). Dry weight of plantlets with treatments *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3 were not significantly different from positive control ($p>0.050$). Dry weight of *Chromohalobacter israelensis* WA3 treatment was higher than *Bacillus marisflavi* FA7 ($p<0.050$).

Among the treatments, isolate DA3 was the best growth promoter of *Oryza sativa* compared to other treatments. All the treatments performed better than the negative control. *Chromohalobacter israelensis* WA3 and positive control treatment showed similar results and performed better than *Bacillus marisflavi* FA7 and isolate AA5.

5.4 DISCUSSION

Chemical fertilizers cause drastic change in the chemical properties of soil. Hence, there is an urgent need for sustainable management of nutrients to reduce the adverse environmental impact of chemical fertilizers.

Alkaliphilic bacteria isolated in this study exhibited phosphate solubilization property at alkaline pH. Since phosphate solubilization is one of the plant growth promoting

Figure 5.6 – Effect of formulated phosphate solubilizing bio-fertilizer on growth promotion of *Oryza sativa*; where A – negative control, B – positive control, C – *Bacillus marisflavi* FA7, D – *Chromohalobacter israelensis* WA3, E – Isolate AA5 and F – Isolate DA3

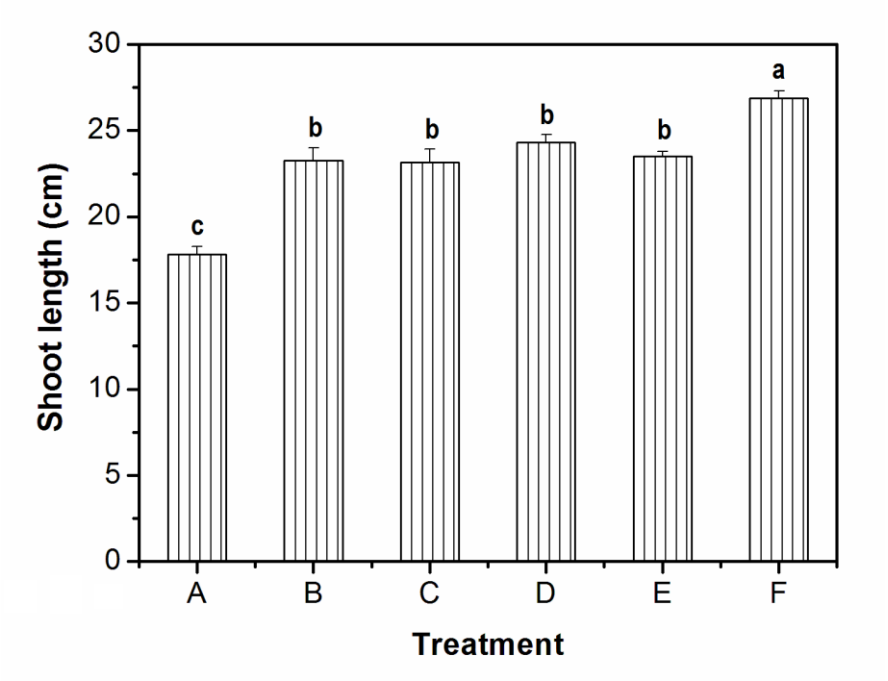


Figure 5.7 – Effect of formulated biofertilizer on shoot length of *Oryza sativa*; where A – negative control, B – positive control, C – *Bacillus marisflavi* FA7, D – *Chromohalobacter israelensis* WA3, E – Isolate AA5 and F – Isolate DA3. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

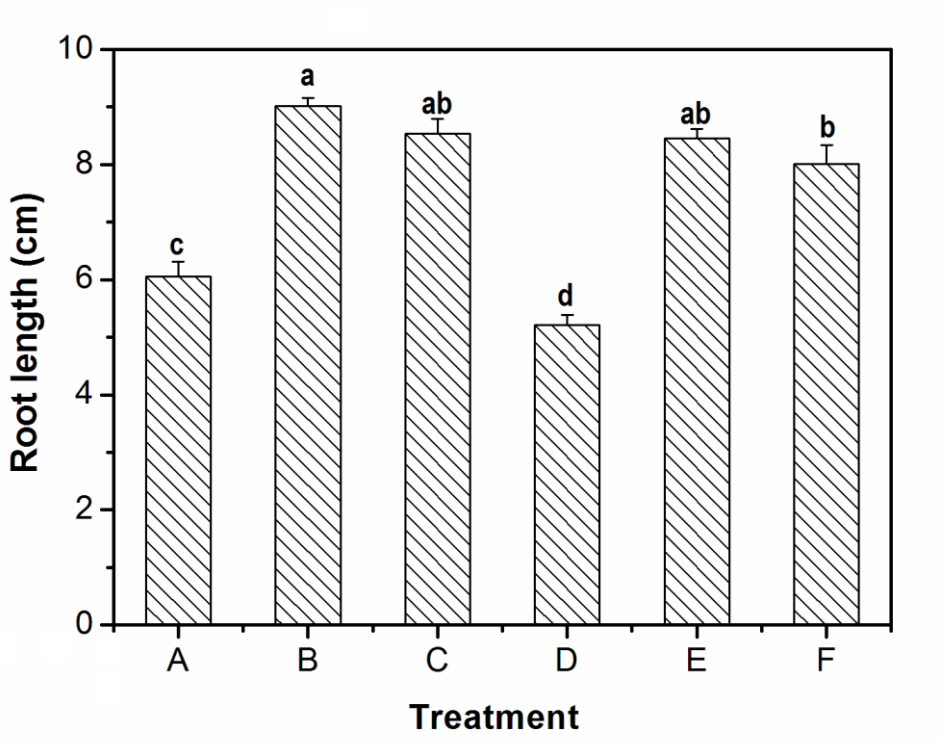


Figure 5.8 - Effect of formulated biofertilizer on root length of *Oryza sativa*; where A – negative control, B – positive control, C – *Bacillus marisflavi* FA7, D – *Chromohalobacter israelensis* WA3, E – Isolate AA5 and F – Isolate DA3. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

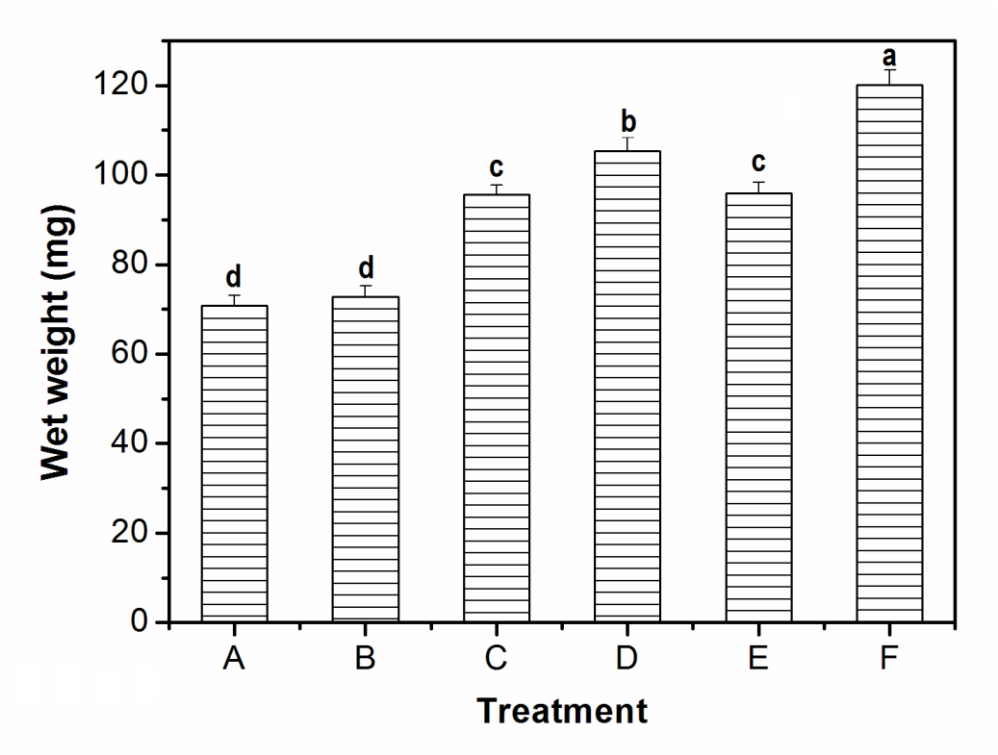


Figure 5.9 - Effect of formulated biofertilizer on wet weight of *Oryza sativa*; where A – negative control, B – positive control, C – *Bacillus marisflavi* FA7, D – *Chromohalobacter israelensis* WA3, E – Isolate AA5 and F – Isolate DA3. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

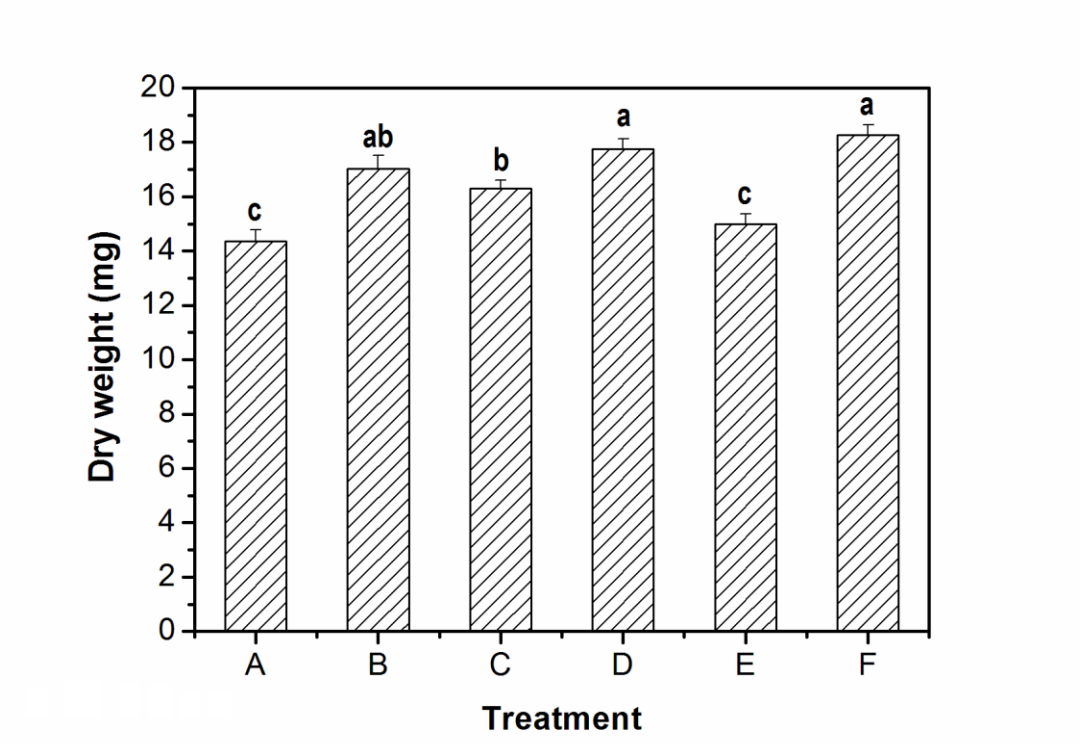


Figure 5.10 - Effect of formulated biofertilizer on dry weight of *Oryza sativa*; where A – negative control, B – positive control, C – *Bacillus marisflavi* FA7, D – *Chromohalobacter israelensis* WA3, E – AA5 and F – DA3. Error bars are standard errors and different alphabet labels on bars indicate significant difference at p value of 0.050.

activities, it was of interest to screen all alkaliphilic isolates for other plant growth promoting properties.

In the present study, 141 alkaliphilic isolates from coastal ecosystems of Goa were assessed for various plant growth promoting traits like production of ammonia, indole-3-acetic acid, and siderophores. Twenty-one alkaliphilic bacteria exhibited production of ammonia, Seventy-three isolates showed indole acetic acid production and siderophore was produced by ninety-two isolates.

Plant growth promoting bacteria bring about favourable changes in the soil. Phosphate solubilizers lead to solubilization of insoluble inorganic phosphate and make it available for plants uptake (Mehta *et al.,* 2010). Indole acetic acid (IAA) is a phytohormone and induces shoot and root elongation in plants (Fallik *et al.,* 1989; Kende & Zeevaart, 1997), cell division in tissues, cell differentiation, and the formation of adventitious roots (Bashan *et al.,* 2008).

Numerous reports of isolating plant growth promoting bacteria from coastal ecosystems are available. Isolation of eleven indole acetic acid producing bacteria has been reported from rhizosphere of *Ipomoea*, *Spinifex* and *Canavalia* in sand dune ecosystem of Uthandi, Chennai (Jayaprakashvel *et al.,* 2014). The isolates promoted growth of *Phaseolus vulgaris*, *Triticum*, *Vigna mungo* and *Vigna radiata*. In a similar study, 84 bacteria possessing plant growth promoting characters like phosphate solubilization, indole acetic acid production, siderophore and hydrogen cyanide production have been isolated from saline habitats (Ramadoss *et al.,* 2013). Out of the 84 isolates only 5 promoted growth of *Triticum*. Jha and Subramaniam (2014) observed growth enhancement of *Oryza sativa* by *Bacillus pumilus* and *Pseudomonas pseudoalcaligenes*. Viruel *et al.* (2014) observed positive effects on growth of *Zea*

mays inoculated with *Pantoea eucalypti* EV4, *Pseudomonas koreensis* SP28 and *Pseudomonas tolaasii* IEXb. They suggested that the growth promoting effect can be attributed to phosphate solubilization, siderophore and indole acetic acid production. Similar results have been observed in *Triticum* when inoculated with *Pseudomonas chlororaphis* sub sp. *aurantiaca* SR1 (Carlier *et al.,* 2008; Saxena *et al.,* 2013).

Production of carrier based biofertilizer has doubled over the past five years (Sruthilaxmi & Babu, 2017). Major biofertilizers developed are phosphate solubilizing *Azotobacter, Azospirillum, Rhizobium,* VAM (Vesicular arbuscular mycorrhiza), nitrogen fixing *Rhizobium* and potassium mobilizing bacteria. Formulated biofertilizers in India have proven to promote plant growth, induce resistance to pest and diseases, protect plants from abiotic stress and increase the yield in a variety of crops such as *Arachis hypogaea, Cajanus cajan, Cicer arietinum, Gossypium, Ipomoea batatas, Oryza sativa, Saccharum officinarum, Vigna radiata* and *Zea mays* (Singh *et al.,* 2016). These biofertilizers were formulated to mainly function in neutral soil.

An attempt was made to formulate phosphate solubilizing carrier based bio-fertilizer using four alkaliphilic bacteria isolated in this study. For all the four isolates, talc proved to be the best carrier supporting viability compared to lignite and vermiculite. To formulate biofertilizers, organic carriers are preferred over inorganic carriers. Among the carriers used in this study, lignite is an organic carrier with >35% carbon, whereas, talc and vermiculite are inorganic. High water holding capacity of talc may be the reason for high viability of all the isolates in talc compared to lignite and vermiculite (Chakraborty *et al.,* 2009; Gade *et al.,* 2014). Generally used carriers for biofertilizer formulation are coal, peat, clays, plant waste material (corn cobs, plant

compost) and inert materials (vermiculite, perlite and other minerals) (Saharana *et al.,* 2010; Sarma *et al.,* 2011; Maheshwari *et al.,* 2015; Sun *et al.,* 2016; Wei *et al.,* 2017).

Two carrier based formulations of *Bacillus cereus* and *Pseudomonas moraviensis* promoted growth in *Triticum*. Ground maize straw and sugarcane husk were used as carriers (Hassan & Bano, 2016). Biochar and flyash based formulations of *Bacillus* sp. strain A30 and *Burkholderia* sp. strain L2, promoted growth and yield of *Solanum lycopersicum* (Tripti *et al.,* 2017).

Results of our study indicate that the talc based formulations of phosphate solubilizing alkaliphilic bacteria promoted growth of *Oryza sativa*. The four alkaliphilic phosphate solubilizing strains used, showed potential for use as bioinoculants. In particular, isolate DA3 and *Chromohalobacter israelensis* WA3 were better growth promoters, followed by isolate AA5 and *Bacillus marisflavi* FA7. The application of these bacterial strains had beneficial effects on shoot length, root length, wet weight and dry weight of *Oryza sativa*.

Strains of *Pseudomonas tolaasii* exhibited biocontrol activity and plant growth promotion of *Oryza sativa* (Adhikari *et al*., 2001). *Pantoea dispersa* 1A promoted shoot and root length, shoot and root dry biomass and nitrogen, phosphorus and potassium uptake on *Triticum* when compared with uninoculated control (Selvakumar *et al*., 2011). Increased plant growth and nutrient uptake as a consequence of inoculation of phosphate solubilizing bacteria under field conditions were also reported in *Cicer arietinum* (Rudresh *et al*., 2005), *Oryza sativa* (Ashrafuzzaman *et al*., 2009; Sharma *et al*., 2014; Bakhshandeh *et al.,* 2017),

Triticum (Selvakumar *et al*., 2009; Selvakumar *et al*., 2011) and *Zea mays* (Kumar *et al*., 2007; Hameeda *et al*., 2008).

Based on the results of this study, it is inferred that talc based formulations of the four alkaliphilic bacterial isolates were promising in enhancing growth of *Oryza sativa*. Therefore, they can be developed as biofertilizers for saline-alkaline soil based agriculture.

Summary & Conclusion

SUMMARY & CONCLUSION

The present investigation was carried out considering the immense application of phosphate solubilizing alkaliphilic bacteria as biofertilizers for saline and alkaline soil. It elucidates the occurrence of phosphate solubilizing alkaliphilic bacteria in coastal ecosystems of Goa and the mechanisms of phosphate solubilization. It identified the optimum parameters for maximum growth of and phosphate solubilization by *Bacillus marisflavi* FA7. It evaluated the effect of pH, salt concentration and metal ions (Al, Cd, Cu, Pb, Ni, and Zn) on phosphate solubilization of selected strains. Finally, this study investigated the efficiency of selected isolates for formulation of biofertilizers for growth promotion of *Oryza sativa*.

The following points highlight the major outcome of this study,

Chapter II

- Highest count of alkaliphilic bacteria was obtained in Cavellossim khazan land sample.
- Out of a total of 141 alkaliphilic bacteria isolated, 12.8% of alkaliphiles were obligate, 30.5% were facultative alkaliphiles and 56.7% were alkalitolerant.
- Thirty alkaliphilic bacterial isolates solubilized inorganic phosphate, 13 solubilized organic phosphate and 13 isolates exhibited both inorganic and organic phosphate solubilizing ability.
- Selected two isolates FA7 and WA3 were identified as *Bacillus marisflavi* and *Chromohalobacter israelensis*, respectively. Both the isolates showed high buffering capacity when compared with neutrophiles *Bacillus subtilis* and *Escherichia coli*.

- This is the first study to report inorganic phosphate solubilization in *Bacillus marisflavi* species and genus *Chromohalobacter*.
- Both, *Bacillus marisflavi* FA7 and *Chromohalobacter israelensis* WA3, showed a negative correlation with pH and phosphate solubilization. *Bacillus marisflavi* FA7 solubilized 0.57 g/l of tricalcium phosphate at the end of 5 days. *Chromohalobacter israelensis* WA3 solubilized 1.8 g/l of tricalcium phosphate at the end of 5 days.
- *Bacillus marisflavi* FA7 produced organic acids and excreted H^+ ions to solubilize tricalcium phosphate. The strain solubilized organic phosphate by production of phosphatase enzyme.
- Phosphatase enzyme produced by *Bacillus marisflavi* FA7 was extracellular. Crude phosphatase enzyme extract of *Bacillus marisflavi* showed K_m and V_{max} of 164.51 µM and 1.13 µM/sec, respectively. Maximum enzyme activity was observed at pH 10.1. Its activity was found to increase with increase in molarity of carbonate – bicarbonate buffer. Reduction in the enzyme activity was observed in the presence of additives SDS, EDTA, Urea, β-mercapethanol and Triton X100.
- Presence of phosphatase enzyme was demonstrated using zymography. Activity fraction showed the protein to be present in molecular weight range of 175 – 200 kDa.
- *Chromohalobacter israelensis* WA3 produced organic acids and excreted H^+ ions to solubilize tricalcium phosphate.
- This is the first report on phosphate solubilization mechanisms by an alkaliphilic bacterium.

Chapter III

- For *Bacillus marisflavi* FA7, crude salt concentration and pH for maximum biomass was 1% and 10.0, respectively.
- Crude salt concentration and pH for maximum phosphate solubilization by *Bacillus marisflavi* FA7 was 1% crude salt concentration and pH 9.0.
- Best carbon and inorganic nitrogen sources for growth of *Bacillus marisflavi* FA7 were sucrose and NH_4NO_3, respectively.
- Best carbon and inorganic nitrogen sources for phosphate solubilization by *Bacillus marisflavi* were glucose and NH_4Cl, respectively.
- Using Response surface methodology model for biomass, the optimum concentration of tricalcium phosphate, glucose and ammonium chloride for highest biomass accumulation (0.67 g/l) were 11.0 g/l, 15.0 g/l and 0.55 g/l, respectively.
- Using the model for phosphate solubilization, the optimum concentration of tricalcium phosphate, glucose and ammonium chloride for phosphate solubilization (0.46 g/l) were 4.0 g/l, 11.0 g/l and 0.2 g/l, respectively.

Chapter IV

- *Bacillus marisflavi* FA7 exhibited phosphate solubilization from 0 to 5% crude salt concentration and from pH 8.0 to 11.0.
- *Chromohalobacter israelensis* WA3 showed phosphate solubilization from 2.5% to 25% crude salt concentration and from pH 7.0 to 11.0.
- *Bacillus marisflavi* FA7 showed no growth in the presence of chloride, nitrate and sulfate salts of the six metals namely, Al, Cd, Cu, Ni, Pb and Zn.

- *Chromohalobacter israelensis* WA3 showed growth as well as phosphate solubilization in the presence of chloride, nitrate and sulfate salts of the six metals namely, Al, Cd, Cu, Ni, Pb, and Zn.
- Scanning electron micrographs of *Chromohalobacter israelensis* WA3 in the presence of 200 ppm of the six nitrate salt metals (Al, Cd, Cu, Ni, Pb, and Zn) revealed differences in the length and breadth of the cells as compared to control.
- EDX spectrum showed peaks of the six nitrate metal salts which revealed that these metals were adsorbed by *Chromohalobacter israelensis* WA3.

Chapter V

- In addition to phosphate solubilization, alkaliphilic isolates exhibited other plant growth promoting traits like ammonia production, indole acetic acid production and siderophore production. Twenty-one isolates showed production of ammonia. Seventy-three isolates showed production of IAA. Ninety-two isolates showed siderophore production on CAS agar plates.
- Talc was the best carrier for viability of *Bacillus marisflavi* FA7, *Chromohalobacter israelensis* WA3, isolate AA5 and isolate DA3.
- It was observed that among the isolates, isolate DA3 was the best growth promoter of *Oryza sativa*. All the treatments performed better than the negative control. *Chromohalobacter israelensis* WA3 and positive control treatment showed similar results and performed better than *Bacillus marisflavi* FA7 and isolate AA5.

Alkaliphilic bacteria isolated from coastal ecosystems of Goa, India, exhibited phosphate solubilization using different mechanisms to solubilize inorganic and organic phosphate at high pH (10.0) and high salt concentration (25%). Selected alkaliphilic isolates also promoted the growth of *Oryza sativa* under saline conditions. The outcome of the present study has potential for formulation of biofertilizer for salt affected soil.

Appendices

APPENDIX – A

(Media)

(Composition in g/L)

A.1 Polypeptone Yeast Extract Glucose Agar

Peptone	5.0
Yeast extract	1.5
Glucose	5.0
Na_2HPO_4	1.5
NaCl	1.5
$MgCl_2$	0.1
Na_2CO_3	5.0
Agar:	15
pH	10.5

Solutions of 10% glucose and 10% Na_2CO_3 were sterilized separately by autoclaving.

A.2 Pikovskaya's Agar

$Ca_3(PO_4)_3$	5.0
Glucose	10.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
K_2HPO_4	0.1
$MgSO_4$	0.1
$MnSO_4$	0.0001
$FeSO_4$	0.0001
Yeast extract	0.5
Agar	2.0
pH	10.0

A.3 Pikovskaya's Agar for inoculum preparation

Glucose	10.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
K_2HPO_4	0.1
$MgSO_4$	0.1
$MnSO_4$	0.0001
$FeSO_4$	0.0001
Yeast extract	0.5
Agar	20.0
Distilled water	1000ml
pH	10.0

A.4. Mineral Salts Medium for phosphatase enzyme studies

$NaNO_3$	2.0
$MgSO_4$	0.2
$MnSO_4$	0.02
$CaCl_2$	0.02
$FeSO_4$	0.02
Glucose	5.0
Distilled water	1000ml
pH	10.0

A.5. Peptone water

Peptic digest of animal tissue	1.0
Sodium chloride	0.5
Distilled water (make the final volume to)	100ml

A.6. MSM for indole acetic acid production

$NaNO_3$	2.0
$MgSO_4$	0.2
$MnSO_4$	0.02
$CaCl_2$	0.02
$FeSO_4$	0.02
Glucose	5.0
Tryptophan	0.5
pH	10.0
Agar	2.0
Distilled water (make the final volume to)	100ml

A.7 Congo Red Agar

Peptone	0.5
Beef extract	0.3
Congo red	0.8
Agar	2.0
Distilled water (make the final volume to)	100ml

A.8. Media for biochemical characterization

i) Motility

Beef extract	3.0
Peptone	10.0
NaCl	5.0
Agar	4.0
pH	10.0

ii) Starch Agar

Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Soluble starch	20.0
Agar	20.0
pH	10.0

iii) Gelatin Hydrolysis Medium

Peptone	5.0
Beef extract	3.0
Gelatin	20.0
pH	10.0

iv) Christensen's Urea Medium

Peptone	1.0
KH_2PO_4	2.0
Sodium chloride	5.0
Urea	10.0
Glucose (10% w/v)	100ml
Phenol red (0.2% w/v)	6.0ml
Agar	20.0
pH	10.0

Sterile glucose and urea solutions were added to the medium prior to use.

v) Indole production Broth

Tryptone	10.0
Sodium chloride	5.0
pH	10.0

vi) Voges-Proskauer Broth

Peptone	5.0
K_2HPO_4	5.0
Glucose	5.0
pH	10.0

vii) Simmon's Citrate Agar

$NH_4H_2PO_4$	1.0
K_2HPO_4	1.0
Sodium chloride	5.0
Sodium citrate	2.0
$MgSO_4$	0.2
Bromothymol blue solution (0.04% w/v)	20.0ml
Agar	20.0
pH	10.0

viii) Medium for Acid production

Diammonium hydrogen phosphate	1.0
Potassium chloride	0.2
Magnesium sulfate	0.2
Yeast extract	0.2
Sugar solution (10% w/v)	100ml
Bromocresol purple (0.04% w/v)	15ml
Agar	20.0

ix) Nitrate Reduction Broth

Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
KNO_3	1.0
pH	10.0

x) Decarboxylase Broth (Ornithine, Lysine, Arginine)

L-ornithine monohydrochloride	5.0
Yeast extract	3.0
Glucose	1.0
Bromo cresol purple	0.015
pH	10.0

APPENDIX – B

(Stains and reagents)

B.1 Stains

B.1.1 Gram's staining

a. Crystal violet solution

Crystal violet (90% dye content)	1.0g
Ethyl alcohol	10.0ml
Distilled water (make final volume to)	100ml

b. Gram's iodine solution

Iodine	**1.0g**
Potassium iodide	2.0g
Distilled water (make final volume to)	100ml

c. Decolourizer

Ethanol	70ml
Distilled water (make final volume to)	100ml

d. Saffranin solution

Saffranin	2.5g
Ethyl alcohol	10ml
Distilled water (make final volume to)	100ml

B.1.2 Endospore staining

a. Malachite green solution

Malachite green	10ml
Distilled water	100ml

b. Saffranin solution

Saffranin	2.5g
Ethanol	10ml
Distilled water (make final volume to)	100ml

B.2 Reagents

B.2.1 Reagent for oxidase test

N,N,N,N-tetramethyl-p-phenylene diamine dihydrochloride	1.0g
Distilled water (make final volume to)	100ml

B.2.2 Reagent for catalase test

Hydrogen peroxide	10ml
Distilled water (make final volume to)	100ml

B.2.3 Reagent for nitrate reduction

Solution A

Sulphanilic acid	0.8g
Acetic acid (5N)	100ml

Solution B

A-naphthylamine	0.5g
Acetic acid (5N)	100ml

Acetic acid solution (5N)

Glacial acetic acid	57.27ml
Distilled water (make final volume to)	200ml

B.2.4 Reagent for methyl red test

Methyl red	0.1g
Ethanol	300ml
Distilled water (make final volume to)	500ml

B.2.5 Omeara's Reagent for Voges-Proskauer test

Iso-amyl alcohol	150ml
p-dimethyl-1-aminobenzaldehyde	10.0g
HCl (concentrated)	50ml

B.2.6 Kovac's reagent for Indole test

KOH	40.0g
Creatine	0.3g
Distilled water (make final volume to)	100ml

B.2.7 Nessler's reagent for ammonia production

Mercuric chloride	10.0
Potassium iodide	7.0
Sodium hydroxide	16.0
Distilled water (make final volume to)	100ml
pH	13.0

B.2.8 Salkowski's reagent for indole acetic acid production

0.5M $FeCl_3$	2ml
H_2SO_4	30ml
Distilled water	50ml

B.2.9. Mercuric chloride solution

Mercuric chloride	0.01g
Distilled water (make the final volume to)	100ml

APPENDIX C

1. Genomic DNA extraction

a) Saline-EDTA buffer

Sodium chloride	0.15 M
Sodium EDTA (pH 8.0)	0.1 M

b) TE Buffer

Tris (pH 7.3)	0.01 M
Sodium EDTA	0.001 M

c) Phenol-chloroform isoamyl alcohol

Phenol (pH 8.0)	25.0 ml
Chloroform	24.0 ml
Isoamyl alcohol	1.0 ml

2. Polymerase Chain Reaction

a) Primers for 16S rDNA gene sequence

8F – 5’ AGA GTT TGA TCC TGG CTC AG 3’

1429R – 5’ ACG GCT ACC TTG TTA CGA CTT 3’

b) PCR Master Mix (2X)

Taq DNA Polymerase	50 units/ml
dATP	400 µM
dGTP	400 µM
dCTP	400 µM
dTTP	400 µM
$MgCl_2$	3 mM

3. Native Polyacrylamide gel electrophoresis

Stock Solutions for native PAGE

a) Acrylamide-bis-acrylamide solution (monomer solution)

Acrylamide	29.0 g
N,N' methylene bis acrylamide	1.0 g
De-ionized water (make final volume to)	100 ml

Acrylamide and N,N' methylene bis acrylamide was dissolved in 80 ml of warm de ionized water. The pH of the solution was adjusted to 7.0. The final volume of the solution was made to 100 ml with de ionized water. The solution was stored in amber colour bottle at room temperature.

b) Resolving buffer (Tris 1.5 M, pH 8.8)

Tris	18.171 g
De-ionized water (make final volume to)	100 ml

Tris was dissolved in 60 ml of deionized water. The pH of the solution was adjusted to 8.8 using 6N HCl and the final volume was made up to 100 ml with de-ionized water. The solution was stored at 4°C

c) Stacking gel buffer (Tris 1.0 M, pH 6.8)

Tris	12.114 g
De-ionized water (make final volume to)	100 ml

Tris was dissolved in 60 ml of deionized water. The pH of the solution was adjusted to 6.8 using 6N HCl and the final volume was made up to 100 ml with de ionized water. The solution was stored at 4°C.

d) Ammonium per sulphate (APS) (10% w/v)

Ammonium oer sulphate	0.1 g
De ionized water	1 ml

e) Bromophenol Blue (1% w/v)

Bromophenol blue	0.1 g
De ionized water (make the final volume to)	10 ml

f) Tris-glycine electrophoresis buffer 5X (pH 8.3)

Tris base (25 mM)	3.02 g
Glycine (250 mM)	18.8 g
Deionized water (make final volume to)	200 ml

Preparation of 1X tank buffer

100 ml of 5X Tris – glycine electrophoresis buffer was made to 500 ml with de ionized water.

Sample buffer 2X (10ml)

Tris HCl (1 M, pH 6.8)	1 ml
Bromophenol blue (1% w/v)	2 ml
Glycerol	2 ml
De – ionized water	5 ml

Preparation of resolving and stacking gel

Solution	Resolving gel 12% (10 ml)	Stacking gel 5% (4 ml)
Monomer	4.0	0.67
Tris (1.5 M, pH 8.8)	2.5	-
Tris (1.0 M, pH 6.8)	-	0.5
APS (10% wv)	0.1	0.04
De ionized water	3.4	2.8
TEMED	0.005	0.005

4. Silver Staining of Native PAGE

a) Fixative solution (100 ml)

Methanol	50 ml
Glacial acetic acid	12 ml
Formaldehyde	0.05 ml
De ionized water	37.95 ml

b) $Na_2S_2O_3$ stock solution (2 ml)

$Na_2S_2O_3.5H_2O$	50 mg
De ionized water	2 ml

c) Pre-treatment solution (100 ml)

$Na_2S_2O_3$ stock solution	0.8 ml
De ionized water	99.2 ml

d) Silver solution (100 ml)

$AgNO_3$	0.2 g
Formaldehyde (37% w/v)	0.075 ml
De ionized water	99.925 ml

e) Developing solution (100 ml)

Na_2CO_3	6.0 g
$Na_2S_2O_3$ stock solution	0.016 ml
Formaldehyde (37% w/v)	0.05 ml
De ionized water	99.93 ml

f) Stop solution (100 ml)

Methanol	50 ml
Glacial acetic acid	12 ml
De ionized water	38 ml

Procedure:

The gel was placed on the gel rocker and all the specified solutions were added and replaced in the following steps

Sr. no	Step	Solution	Duration
1	Fixing	Fixative solution	4 hr
2	Washing	50% methanol	20 min
3	Washing	30% methanol	20
4	Pretreatment	Pretreatment solutiom	1 min
5	Rinsing	Deionized water	3 times
6	Impregnate	Silver solution	20 min in dark
7	Rinsing	Deionized water	3 times
8	Developing	Developing solution	Till protein bands appear
9	Stop	Stop solution	10 min
10	Washing	De ionized water	30 sec

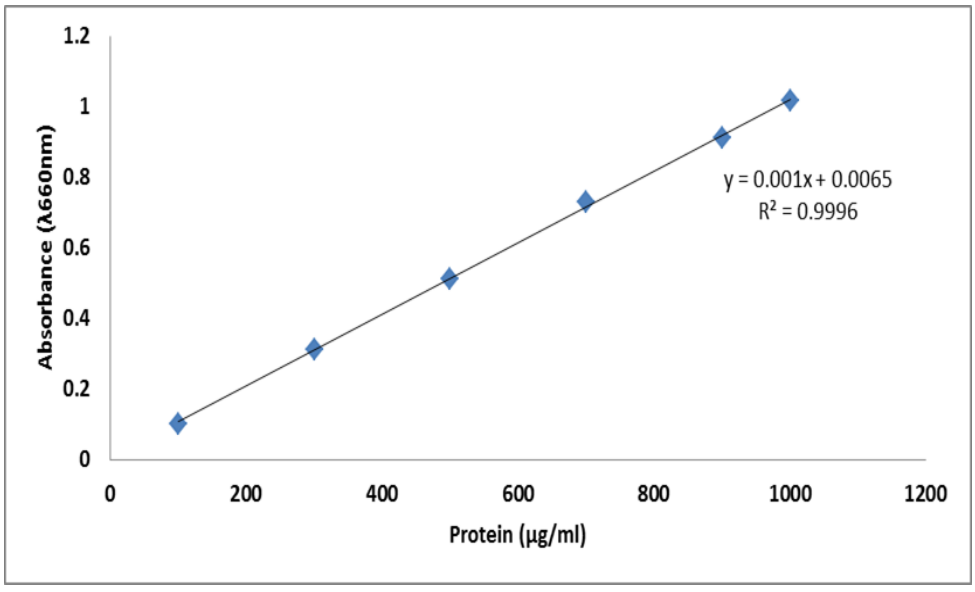
APPENDIX D

ESTIMATION AND STANDARD CURVE

1. Folin Lowry Method for protein estimation

Reagent A	**Sodium Carbonate solution**	
	Sodium carbonate	2.0g
	0.1 N NaOH	100ml
Reagent B	**Copper sulphate solution**	
	Sodium potassium tartrate	1.0
	Copper sulphate	0.5g
	Distilled water	100ml
Reagent C	**Alkaline copper sulphate solution**	
	Reagent A	50.0ml
	Reagent B	1.0ml
Reagent D	**Folin-Ciocalteau Reagent**	
	Folin-Ciocalteau Reagent (Commercial grade)	10ml
	Double distilled water	20ml

Procedure : To 1ml of the sample, add 5ml of Reagent C and keep at room temperature for 10min. 0.5ml of Reagent D is added and kept in dark for 20min. Absorbance is measured at 660nm against reagent blank.

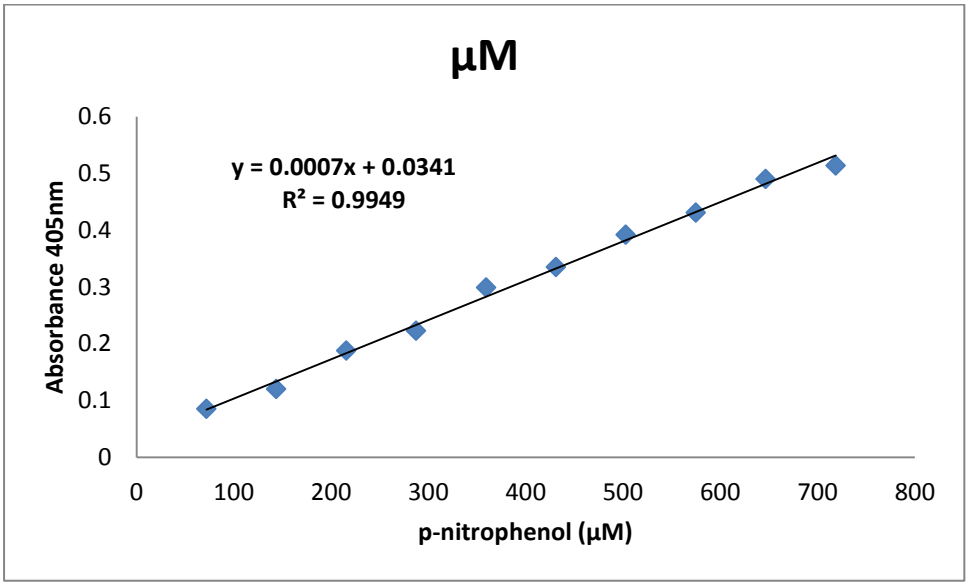


2. Standard curve of para-nitrophenol

Stock solution : 1mg/ml p-nitrophenol

Diluent : distilled water

Procedure : Prepare dilutions of stock solution of p-nitrophenol with distilled water as the diluent. Record the absorbance at 405nm.



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Publications & Presentations

LIST OF PUBLICATIONS

1) Prabhu, N., Borkar, S. & Garg, S., 2018. Phosphate solubilization mechanisms in alkaliphilic bacterium *Bacillus marisflavi* FA7. Current Science, 114(4), pp.845-853.

2) Prabhu, N., Borkar, S. & Garg, S., 2017. Alkaliphilic and haloalkaliphilic phosphate solubilizing bacteria from coastal ecosystems of Goa. Asian Journal of Microbiology Biotechnology and Environmental Science, 19(3), pp.703-714.

3) Prabhu, N., Garg, S. & Borkar, S., 2015. Formulation of biofertilizer using alkaliphilic and halophilic phosphate solubilizing bacteria and their effect on growth of *Oryza sativa*. Recent Developments in Biotechnology, pp.72-78.

PRESENTATIONS AT CONFERENCES

1) Presented poster entitled "Halo-alkalitolerant bacterium as Phosphate Solubilizer from Coastal Ecosystems of Goa" at 52nd International Conference on "Microbial biotechnology for sustainable development organized by Association of Microbiologists of India at Panjab University, from November 3rd – 6th, **2011**.

2) Presented a poster entitled "Biodiversity of phosphate solubilizing alkaliphiles and halophiles from coastal ecosystems of Goa" at 3rd International Conference on "Ecotoxicology & Environmental Sciences" (ICEES) from 28th – 30th November, **2011**.

3) Presented a poster entitled "Alkaliphiles from Coastal Ecosystems as Plant Growth Promoting Bacteria" at 53rd Annual Conference of Association of Microbiologists of India held at KIIT University, Bhubaneswar, Odisha from 22nd – 25th November, **2012**.

4) **Won the first prize for oral presentation** of paper entitled "Potential of Alkaliphiles from Coastal Ecosystems of Goa as Plant Growth Promoting Bacteria" at the National Seminar on Plant Sciences organized by Smt. Parvatibai Chowgule College of Arts and Science, Margao – Goa on 7th – 8th February, **2013**.

5) Presented a paper entitled "Response of phosphate solubilizing alkaliphile from coastal ecosystem of Goa to abiotic factors" as virtual presentation at the Vth International Conference on Environmental, Industrial, and Applied Microbiology – BioMicroWorld 2013, held at Madrid (Spain) from 2-4th October, **2013**.

6) Presented a paper "Land reclamation using plant growth promoting alkaliphiles obtained from coastal ecosystems of Goa" in the form of a oral presentation at the

4th National Conference on Pollution in Urban and Industrial Environment, held at Jadhavpur University, Kolkata on 8th – 9th December, **2014**.

7) Presented a poster entitled "Effect of Stress Conditions such as pH, Salt Concentration and Metal Ions on Haloalkaliphilic Phosphate Solubilizing Bacterium" at the 4th Bhartiya Vigyan Sammelan and EXPO, at Kala Academy, Panjim, Goa – India, from 5th – 8th February, **2015**.

8) Presented a paper "Efficacy of plant growth promotion by phosphate solubilizing bacteria from coastal ecosystems of Goa as potential biofertilizers" at National seminar on "Advances in Life Sciences, at St. Xavier College of Arts, Science and Commerce, Mapusa, from 7th and 8th December, **2015**.

9) Presented a paper entitled "Plant growth promoting potential of alkaliphilic and haloalkaliphilic phosphate solubilizing bacteria" at International Seminar on "New Frontiers in Microbiology and Applied Biology", at St. Xavier College of Arts, Science and Commerce, Mapusa, from 7th – 8th January, **2016**.

10) Presented a paper entitled "Potential stress tolerant bacterial bio-inoculant from a salt-pan of Goa" at National Conference on "Frontiers in Biofertilizers and Biopesticides" organized by R.J.S.P.M. Arts, Commerce & Science College, Pune from 9th – 10th January, **2018**.

11) Presented a poster entitled "Alkaliphilic phosphate solubilizing bacteria from coastal ecosystems of Goa, India" at "12th Edition of the International Congress on Extremophiles" (Extremophiles2018) at The Continental Hotel, Ischia, Italy from 16th – 20th September, **2018**.