DEVELOPMENT OF INOCULANT CULTURES OF ZINC SOLUBILIZING MICROORGANISMS

by

AATHIRA S. KUMAR

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"DEVELOPMENT OF INOCULANT CULTURES OF ZINC SOLUBILIZING MICROORGANISMS"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani,
Date : 18-7-2016

Aathira S. Kumar
(2014-11-163)

CERTIFICATE

Certified that this thesis entitled **"DEVELOPMENT OF INOCULANT CULTURES OF ZINC SOLUBILIZING MICROORGANISMS"** is a record of bonafide research work done independently by Ms. Aathira S. Kumar (2014-11-163) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Dr. K. N. Anith
(Major Advisor, Advisory Committee)
Professor (Microbiology)
College of Agriculture, Vellayani,
Thiruvananthapuram- 695 522

Vellayani,
Date: 18-7-2016

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Aathira S. Kumar (2014-11-163), a candidate for the degree of **Master of Science in Agriculture** with major in Agricultural Microbiology, agree that this thesis entitled **"DEVELOPMENT OF INOCULANT CULTURES OF ZINC SOLUBILIZING MICROORGANISMS"** may be submitted by Ms. Aathira S. Kumar, in partial fulfilment of the requirement for the degree.

Dr. K. N. Anith
(Chairman , Advisory Committee)
Professor
Department of Agrl. Microbiology
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

Dr. Usha Mathew
(Member, Advisory Committee)
Professor
Department of Soil science and
Agrl. Chemistry
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

18/7/16

Dr. K.C. Manorama Thampatti
(Member, Advisory Committee)
Professor
Department of Soil Science and Agrl. Chemistry
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

Dr. Roy Stephen
(Member, Advisory Committee)
Professor and Head
Department of Agrl. Microbiology
College of Agriculture, Vellayani,
Thiruvananthapuram – 695 522

18/7/16

EXTERNAL EXAMINER

(Name and Address)

Dr. Jisha M. S.
Professor
School of Biosciences
Mahatma Gandhi University

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Per cent
nm	Nanometer
@	At the rate of
°C	Degree Celsius
CD	Critical difference
cm	Centimeter
et al.	And other co workers
Fig.	Figure
g	Gram
Hrs	Hours
i.e.	that is
L	Litre
ml	Milli litre
kg	Kilo gram
min	Minutes
mg	Milli gram
NS	Non Significant
sec	Seconds
sp or spp.	Species (Singular and plural)
viz.	Namely
pH	Negative logarithm of hydrogen ions
ppm	Parts per million
rpm	Revolution per minute
OD	Optical density
No.	Number
ZSB	Zinc Solubilising Bacteria

Introduction

1.INTRODUCTION

Micronutrients are essential for the optimum plant growth and productivity. They have an important role in development of plants and production of crops because they act as activators of many plant functions. Zinc plays an important role among several micronutrients that increase plant growth and productivity.

Zinc plays a vital role in metabolism, mitosis, seed development, mitochondrial activities etc. (Hughes and Poole, 1989). It is a constituent of about 59 enzymes and has an important role in auxin production from tryptophan and is present in relatively small amounts (5-100 mg kg^{-1}) in plant tissues.

Soil zinc deficiency is a worldwide problem in the production of crops thereby affecting the crop growth in over 50 percent of agricultural lands. According to Cakmak *et al.* (1998), zinc deficiency is closely related with 80 percent reduction in grain yield. The high dependency on foods with low zinc concentration leads to malnutrition and zinc deficiency among people (Welch and Graham, 2002). The All India Coordinated Research Project (AICRP) on Micro and Secondary Nutrients and Polluted Elements in Soils and Plants estimated that about 49 percent of soil samples tested was found to be deficient in zinc (Singh, 2001).

The available zinc content in soils of India are low whereas the total zinc content is high which exists in fixed forms such as smithsonite, sphalerite, zincite, franklinite, wellemite and hopeite.

Zinc deficiency in Kerala results from acidity of the soil assisted by high leaching due to rainfall. The available content of zinc in the surface samples of laterite soils in Kerala was found to be low (Mathew, 2006). Nair *et al.* (2007) reported acute zinc deficiency in low land rice soils of Palakkad.

The application of zinc fertilizers is a regular remedial practice which has use efficiency rarely exceeding one per cent whereas the remaining zinc is fixed

due to alkaline pH (Mandal and Mandal, 1986). The inappropriate addition of organic manures and imbalanced application of chemical fertilizers caused a widespread zinc deficiency in soil (Singh *et al.*, 2005).

Application of zinc has now become one of the important components of recommended package of practices of rice, maize and many other crops in most of the States of India. In Kerala, zinc deficiency is most prevalent in Wayanad and localised deficiencies are reported in the districts of Ernakulam, Idukki, Thiruvananthapuram, Kollam, Alappuzha, Thrissur and Kozhikode and need based application is recommended (Kerala State Planning Board, 2013). Forty per cent deficiency is reported in Agro ecological unit - 8- Southern laterites.

To correct the zinc deficiency, exogenous application of water soluble $ZnSO_4$ is advocated. In Kerala, zinc sulphate has been recommended @ 20 kg ha^{-1} for rice and sesamum, 30 kg ha^{-1} for ginger, turmeric and black pepper, 25 kg ha^{-1} for cardamom and 10 kg ha^{-1} for tomato and cassava as an adhoc recommendation in Package of Practices recommendation of KAU (KAU, 2011).

From the applied sources of soluble zinc about 96-98 percent of the zinc gets converted into unavailable forms. The water soluble zinc supplemented as zinc sulphate get converted into zinc hydroxide in soils with relatively high pH, zinc carbonate in alkali soils rich in calcium, zinc phosphate in near neutral to alkali soils with high phosphorus content and zinc sulphide under reduced conditions, leading to zinc deficiency. A microbial based approach could be an effective strategy to solve this problem. Zinc which is unavailable to plants can be reverted back to available form by inoculating microorganisms capable of solubilising the insoluble forms.

Once efficient zinc solubilising microorganisms are identified, they could be used as a biofertilizer for dissolving native zinc along with a cheaper zinc source like zinc oxide instead of costly zinc sulphate. The microbial mobilisation of unavailable forms of native and applied zinc is of much importance to avoid pollution by repeated application of zinc sulphate. Studies on zinc solubilisation

by microbes have immense potential in zinc nutrition of crop plants because zinc is a limiting factor in production of crops. Hence the present programme has been designed to develop inoculant cultures of zinc solubilising microorganisms with the following objectives:

1. Isolation and characterization of zinc solubilising microorganisms
2. Development of inoculants of efficient zinc solubilising microorganisms.

Review of literature

2. REVIEW OF LITERATURE

Plants require 17 essential elements for their growth and development. The elements like carbon, hydrogen, and oxygen are obtained from the atmosphere and soil water. The elements such as nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, iron, zinc, manganese, copper, boron, molybdenum, nickel and chlorine are supplied either from soil or by application of organic or inorganic fertilizers. If an agricultural system fails to supply enough products containing adequate quantities of all nutrients then it may lead to dysfunctional food systems that cannot support healthy lives (Graham *et al.,* 2001; Schneeman, 2001).

2.1 IMPORTANCE OF ZINC

Zinc plays a principal role in many biochemical pathways such as structural constituent or regulatory cofactor for a wide range of enzymes and proteins. They are associated in auxin metabolism, carbohydrate metabolism, protein metabolism, photosynthesis, conversion of sugars to starch, formation of pollen and also for imparting resistance to infection by pathogens.

Zinc is a component of several enzymes like superoxide dismutase and catalase that inhibit the oxidative stress in the cells of plants. It also influences early development of fruits and tubers. High levels of IAA or zinc in the plant will improve cell differentiation after flowering.

Zinc affects the regulation and sustainance of the gene expression which is associated in the tolerance of environment stresses in plants. This reveals the crucial role of zinc in oxidation- reduction reactions, metabolic processes and enzymatic reactions of plants (Cakmak, 2000).

According to the reports of Vallee (1991) zinc is an important component in more than 300 metalloenzymes. All the six enzyme classes such as oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases contain zinc. The DNA-binding proteins that perform principal role in the process of

transcription of DNA also contain zinc. Among the structurally and functionally diverse proteins which are assosiated with transcription, TFIIIA was found to be the first transcription factor as a zinc protein. The studies conducted by Laity *et al.* (2001), stated that zinc organises the domain in several proteins which make them capable of molecular recognition by proteins, nucleic acids and lipids. A proteome analysis done by Andrieni *et al.* (2006) concluded that about 4–10 percent of proteins in cell are basically zinc - binding proteins in all the three forms of life; bacteria, archaea and eukaryotes.

Sunitha *et al.* (2014) reported that application of NPK fertilizers has tremendously increased the food production. However it resulted in micronutrient deficiencies because many of the soils are devoid of micronutrients and among them zinc deficiency plays a vital role. To control these deficiencies, farmers started applying different zinc compounds as fertilizers and among them zinc sulphate was most regularly used zinc source. They estimated that the application of zinc sulphate either through straight fertilizer or by micronutrients mixture application in agriculture was about 85,000- 90,000 tones per annum.

2.2 DEFICIENCY OF ZINC IN PLANTS

Zinc is necessary in minimum levels for optimum functioning of crop plants. A zinc deficiency situation arises when the soil is impotent to supply a minimum amount of zinc for the plants. This leads to development of zinc deficiency symptoms such as rosetting, bronzing of chlorotic leaves, stunting, small and abnormally shaped leaves and interveinal chlorosis thereby impairing the physiological functions of plants.

According to Fageria (2004), the plant root system including root development was significantly influenced by zinc deficiency. For zinc deficient plants, there is a curtailment in protein synthesis which affect the nitrogen metabolism pathway.

Under serious condition of zinc deficiency, the flowering and fruiting in plants was greatly retarded (Epstein and Bloom, 2005). There was an increase in

declination of the quality of harvested produce, sensitivity to fluctuation in temperature intensity and to infection by several fungal pathogens in plants.

Tavallali *et al.* (2010) indicated that the quantity of uptake and transport of water in plants was influenced by zinc deficiency in plants. They also claimed that zinc decreases the impact of short periods of salt and heat stress. It was reported that there was a low level in auxin production especially IAA content in plants and this may be due to the fact that zinc is associated with tryptophan synthesis. The growth of internode and the size of leaves are much reduced due to low auxin production. The grain or seed yield are decreased due to direct effect of zinc deficiency.

2.3 SOIL ZINC CONTENT

The extent of zinc present in the soil is reliant on the type, intensity of weathering, climate and other dominating factors all along the process of soil formation (Saeed and Fox, 1977). Hafeez *et al.* (2013) stated that all types of soils may be affected by zinc including loam, sands, clays, alluvium and soils formed from basalt, sandstone, granite and volcanic ash.

The dearth of zinc occur in many parts of the world on a vast range of soil types such as semi- arid areas with calcareous soils, tropical regions with highly weathered soils and sandy textured soils in different climatic regions (Akay, 2011). The high pH, $CaCO_3$, organic matter, phosphate and copper can fix and minimise the availability of zinc in soils (Kapoor *et al.,* 2002).

The zinc deficient areas may link with weather conditions because it increases in cold and humid conditions (Alam *et al.*, 2010). The Indian soils are found to be lesser in zinc because half of the soils are classified to be deficient in zinc.

The available zinc content in Indian soils ranged from traces to 22 mg kg^{-1} (Nagarajan *et al.,* 1981). Katyal and Sharma (1991) reported that 47 per cent of soils in India are found to be insufficient in zinc. The zinc deficiency in soil is

expected to increase from 49 to 63 per cent by the year 2025 (Singh, 2009). The analysis of more than 2, 25,000 soil samples from 16 States and Union territories for micronutrient status indicated that zinc is the most limiting micronutrient for sustainable productivity of majority of States in India (Singh and Saha, 1995).

The zinc appears as sphalerite, olivine, hornblende, augite and biotite in soils. However, the availability of zinc from these insoluble sources is governed by several factors among which biochemical actions of rhizosphere microorganisms play a vital role in converting unavailable sources of zinc into available forms (Singh *et al.,* 2005). Within 7 days of application, about 96 to 99 per cent of the applied zinc is transformed into various insoluble forms which depend on the soil types and physicochemical reactions (Saravanan *et al.,* 2003).

2.4 ZINC IN KERALA SOILS

The critical level for DTPA extractable zinc for red and laterite soils of Kerala were fixed as 0.6 mg kg^{-1} and 1.2 mg kg^{-1} respectively. Praseedom (1970) reported that the available zinc status of Kerala soils ranged from 0.25 ppm to 8.0 ppm and it decreased with depth of soil.

Aiyer *et al.* (1975) observed the critical limit of available zinc in the acid rice soils of Kuttanad was 0.5 mg kg^{-1}. Sathyanarayanan (1997) reported that available zinc content was 0.3 mg kg^{-1} in major land resources of Southern districts of Kerala.

The mean status of total zinc in the wet land soils and garden land soils of Kerala were in the range of 24.1 mg kg^{-1} and 78.9 mg kg^{-1} and 11.5 mg kg^{-1} to 43.5 mg kg^{-1} 11.5 mg kg^{-1} and 43.5 mg kg^{-1} respectively (Mathew, 1999).

The available zinc ranged from 1.4 mg kg^{-1} to 2.9 mg kg^{-1} and 0.9 mg kg^{-1} to 3.2 mg kg^{-1} in the above soils. According to Mehara *et al.* (2005), the critical limit for DTPA zinc was 0.95 mg kg^{-1} in soil and 32.7 mg kg^{-1} in plants. John (2006) reported that the critical limit for zinc in Vellayani soils was 0.60 mg kg^{-1}.

2.5 UNAVAILABILITY OF ZINC TO PLANTS

There is enough zinc in soil to support growth of crops. However, the crop acquaint with zinc deficiency due to the presence of unavailable forms. These may be due to low total zinc content, high salt concentration, neutral or alkaline pH and high calcium carbonate in calcareous soil (Antoniadis and Alloway, 2002).

Even after application of zinc in soils with high contents of hydroxyl ions, it is hard to decrease zinc deficiency. The less availability of zinc under alkaline conditions is associated with precipitation of zinc as zinc hydroxide or zinc carbonate (Shukla and Mittal, 1979).

The high content of CO_3^{-2} in alkaline soils results in absorption of zinc and fix it in soils thereby making it unavailable to plants (Udo *et al.,* 1970). These are the factors that add to the low zinc availability at high pH. Stahl and James (1991) reported that the heavier textured soils with larger cation exchange capacity have higher capacities for adsorption of zinc when compared with light textured soils.

2.6 NUTRIENT ENHANCEMENT IN PLANT SYSTEM THROUGH ZINC SOLUBILISATION

The application of zinc in the form of chemical fertilizers like zinc sulphate remains futile since one to four percent is used by the crop and 75 percent of zinc applied is converted into other mineral fractions. Hence correction through fertilizer supplementation is not always successful due to agronomic and economic factors which include reduction in availability of zinc due to drying of top soil, subsoil constraints, incidence of diseases and price of fertilizers.

The supplementation of soluble zinc sources to mitigate zinc deficiency lead to conversion of about 96- 99 percent to unavailable forms whereas 1- 49 percent remains as available fraction in the soil. This requires a system that discharges the required fraction of available zinc that is transformed to

unavailable forms and remains in the soil to available form. The organic based zinc nutrition is best considering its zinc use capability is more in this system. So a bacterial based access was improvised to clear up the micronutrient deficiency problem (Lindsay and Norvel, 1978; Raj, 2002).

Numerous microorganisms have the capability to increase growth of plants and productivity, especially those associated with roots. This response was due to the involvement of these microbes in the solubilisation of unavailable forms of minerals (Cunningham and Kuiack, 1992).

The zinc solubilising capability of several micribial genera like *Bacillus* sp, *Pseudomonas* sp and *Aspergillus* sp were reported by researchers recently (Saravanan *et al.,* 2003). Hence microbial inoculants will be a substitute to overcome adverse effects of synthetic fertilizer thereby restoring soil fertility.

The bacterial strains are inoculated into soils with high content of total zinc and they work by releasing zinc from its unavailable complexed form. The term zinc solubilising bacteria refers to those bacteria that are capable of converting insoluble forms of zinc compounds into soluble forms.

The influence of zinc in the nutrition of microorganisms was first identified in *Aspergillus niger*, which was unable to grow without zinc (Raulin, 1869). Several genera of fungus exhibited the capacity for solubilisation, including *A. niger*, *Penicillium simplicissimum* (Franz *et al.*, 1993), *Abisidia cylindrospora*, *A. glauca* and *A. spinosa* (Coles *et al.,* 2001).

The coinoculation of plant growth-promoting rhizobacteria like *Pseudomonas putida* and arbuscular mycorhizae (AM) enhanced the level of iron, copper, aluminium, cobalt and nickel in shoot tissues (Meyer and Linderman, 1986). They further stated that *Pseudomonas putida* has the ability to produce 2-ketogluconic acid that can restore native micronutrients in the soil.

Certain *Penicillium* sp inoculated in soil increased iron, copper and zinc content inside plant system (Kucey, 1988). Franz *et al.* (1993) reported that the

addition of zinc oxide caused a rise in production of citric acid by *Penicillium simplicissimum*.

It was observed that arbuscular mycorhizae affect the transport of zinc in *Pinus radiata* and *Araucaria* sp (Bowen *et al.*, 1974). Altomare *et al.* (1999) reported that the biocontrol fungus, *Trichoderma harzianum* Rifai 1295–22 solubilised the metallic form of zinc.

Martino *et al.* (2003) isolated the ericoid mycorrhizae from unpolluted natural soils which proved to produce sufficient quantity of organic acids like fumarate, citrate and malate that enhanced solubilisation of micronutrients for plants and mycorrhizae.

Saravanan *et al.* (2004) reported that solubilisation of zinc oxide, zinc carbonate and sphalerite (zinc ore) under in vitro conditions by *Bacillus* sp and *P. fluorescens* from garden and paddy soils.

The success of *Bacillus* sp in turmeric proved the ability of the bacterial inoculants to change other macronutrient and micronutrient concentrations in soil (Kumar *et al.*, 2004). They also isolated a *Bacillus* sp from spheralite zinc ore that solubilised zinc and studied its effect on rice. This showed that the *Bacillus* sp can concurrently release magnesium, calcium, silicon and zinc from soil containing these ions.

A few ectomycorrhizal fungi appear as endophytic and entomopathogenic strains like *Beauveria caledonica*, easily solubilised zinc phosphate when compared to mineral forms such as pyromorphite. Oxalic acid production aided in solubilisation of these insoluble forms (Fomina *et al.*, 2004).

Wu *et al.* (2006) documented the ability of the soil bacteria *Azotobacter chroococcum*, *Bacillus megaterium* and *Bacillus edaphicus* to enhance the bioavailability of zinc and lead in the soil.

Fomina *et al.* (2006) stated that there was an increased solubilisation of zinc phosphate and accumulation of zinc by inoculated zinc-resistant mycorrhiza

under limited phosphorus conditions thereby revealing the effect of other nutrients on the solubilisation of insoluble zinc minerals.

The solubilisation of metallic zinc was observed in the Acetobacteraceae member, *Gluconacetobacter diaztorophicus*, which included the production of gluconic acid and its derivatives in the broth cultures (Saravanan *et al.*, 2007).

Several rhizosphere bacteria had influence on availability of zinc in the plant system. The metal-resistant bacterium *Burkholderia cepacia*, isolated from the rhizosphere of metal hyper-accumulating plants was able to improve the growth of plants and uptake of zinc by tartaric acid secretion in the zinc treated rhizosphere (Li *et al.,* 2007).

The ericoid mycorrhizae like *Paxillus involutus*, *Hymenoscyphus ericae, Oidiodendron maius*, *Suillus luteus* strains 21 and 22, *S. bovinus* LSt8 and *Beauveria caledonica* proved the solubilisation of insoluble zinc compounds (Fomina *et al.,* 2005; Gadd, 2007). Further studies strengthened the molecular mechanism of this solubilization phenomenon and have given evidence for production of gluconic acid by glucose dehydrogenase (Intorne *et al.,* 2009).

Arbuscular mycorrhizal (AM) fungi increases the absorption of immobile micronutrients viz., zinc and copper (Faber *et al.,* 1990; Kothari *et al.,* 1991; Li *et al.*, 1991; Liu *et al.*, 2000; Ryan and Angus, 2003). The enhancement of micronutrients in mycorrhizal plants is always related with acidification of rhizosphere (Dodd *et al.*, 1987), more external mycelium in the soil (Jakobsen *et al.,* 1992) and biochemical changes in the soil system (Subramanian and Charest, 1997).

2.7 MECHANISM OF ZINC SOLUBILIZATION

The microbes in rhizosphere play a central role in the improvement of crop production by the solubilisation of insoluble form of metallic zinc into soluble form. This metal solubilisation was mainly due to the discharge of organic acids followed by drop in pH by microorganisms.

A major mechanism of zinc solubilisation is the release of organic acids that impound cation and acidify the area near root system. A number of organic acids produced by microorganisms viz., acetic, citric, lactic, propionic, glycolic, oxalic, gluconic acid have been accounted to lower pH in the soil (Cunnigham and Kuiack, 1992). The organic acid released by microorganisms improve the soil zinc availability in two ways either through proton extrusion or as counter ions and therefore lower pH in the rhizosphere. Furthermore these anions can bind with zinc and intensify the solubility of zinc (Jones and Darrah, 1994) which brings about replenishing of soluble form (Zn^{2+}) to plants.

The organic acids dissociate in a pH dependent equilibrium into anions and protons contributing to the reduction of pH in the soil. They buffer pH and extend to dissociate as protons. Furthermore, microorganisms usually transport organic acids as anions in the soil system.

The gluconic acid biosynthesis is accomplished by the glucose dehydrogenase (GDH) enzyme and its co-factor, pyrroloquinoline quinone (PQQ). A gene from *Erwinia herbicola* was cloned by Goldstein and Liu (1987) that was involved in solubilisation of mineral phosphate. The expression of this gene permitted production of gluconic acid and solubilisation of mineral phosphate in *E.coli* HB101.

Fasim *et al.*(2002) observed solubilisation of insoluble zinc oxide and zinc phosphate by bacteria and stated that it was mediated by the production of gluconic and 2-ketogluconic acid. Other organic acids, such as lactic, isovaleric, isobutyric, acetic, glycolic, oxalic, malonic and succinic acids are also produced by various phosphate solubilising bacteria (Rodríguez and Fraga 1999).

The gluconic acid is the main organic acid produced by *Pseudomonas* sp (Illmer and Schinner, 1992), *Erwinia herbicola* (Liu *et al.,* 1992), *Pseudomonas cepacia* (Goldstein *et al.,* 1994), *Burkholderia cepacia* (Rodríguez and Fraga, 1999), *Rhizobium leguminosarum* (Halder *et al.,* 1990), *Rhizobium meliloti* (Halder and Chakrabartty, 1993) and *Bacillus firmus* (Banik and Dey, 1982).

Rodríguez *et al.* (2004) found that *Azospirillum brasilense,* when grown on fructose and glucose amended medium have shown in vitro solubilising capability by producing gluconic acid.

Gaur and Gaind (1999) stated that inorganic acids such as sulphuric acids and nitric are also generated by several nitrifying bacteria and *Thiobacillus* during the oxidation of inorganic compounds of sulphur or nitrogenous which react with calcium phosphate and convert them into soluble forms.

The plants inoculated with mycorrhiza were reported to produce organic acids that decrease pH of the rhizosphere thereby increasing the availability of nutrients (Koide and Kabir, 2000).

2.8 EFFECT OF ZINC SOLUBILIZING BIOFERTILIZERS ON CROP GROWTH

The studies conducted by Tariq *et al.* (2007) indicated that mixed inoculation of *Azospirillum*, zinc solubilizing bacteria *Pseudomonas* and *Agrobacterium* mitigated the zinc deficiency symptoms and enhanced the total biomass (23%), grain yield (65%) and harvest index as well as zinc content in the grains in rice. The inoculation improved the root length (54%), root weight (74%), root volume (62%), root area (75%), shoot weight (23%), panicle emergence index (96%) and offered the maximum solubilisation efficiency as compared with the uninoculated control.

Liu *et al.* (2000) reported that the external mycelium of mycorrhizal fungus supply 60 percent towards host plant nutrition of zinc under deficient conditions. It was claimed that mycorrhizal symbiosis enhances nutrition of zinc in maize.

Vaid *et al.* (2014) claimed that inoculation of *Burkholderia* and *Acinetobacter* in zinc deficient soils were found potent for wheat and significantly increased the dry matter yield per pot (12.9%), productive tillers per plant (15.1%), number of panicles plant^{-1} (13.3%), number of grains panicle^{-1} (12.8%),

grain yield (17.0%) and straw yield (12.4%) when compared with the control. The inoculation of bacteria significantly increased the total zinc uptake pot^{-1} (52.5%) as well as grain methionine concentration (38.8%).

The zinc solubilising organism, *Bacillus* sp has shown a favourable effect on the availability of N, P and K, thereby proving the essential role of these organisms in the conversion of these three nutrients in the soil (Kumar *et. al.*, 2004). The per cent rise in the uptake of nutrients in turmeric at harvest after supplementation of zinc solubilising bacteria for N (40.5 %); P (53.8 %); K (53.5 %), Zn (56 %) and Fe (70.0 %) was also reported.

Materials and methods

3. MATERIALS AND METHODS

A study was conducted to isolate, screen and evaluate zinc solubilizing microorganisms and to develop inoculant cultures of zinc solubilizing isolates from soils of Kerala. The experiments were carried out during 2014- 2016 in the Department of Agricultural Microbiology at College of Agriculture, Vellayani. The details of materials and the methods followed are given below:

3.1. SOIL SAMPLING

Soil samples were collected from zinc deficient locations such as Agroecological units 20, 21 (Wayanad) and 8 (Thiruvananthapuram). The locations of zinc deficiency were selected by analyzing the soil samples for the available zinc status by extraction method using 0.1N HCl followed by Atomic Absorption Spectrophotometer reading (Sims and Johnson, 1991).

Soil samples were collected from a non-cropped, undisturbed site that was covered by native vegetation and 50 g of soil samples were taken from the upper 30 cm of the soil profile from different sites and were pooled to get a representative sample.

Available Zinc Status was estimated by the method mentioned above. 2g soil was taken in 250ml conical flask and 20 ml 0.1 N HCl was added. This suspension was shaken for 5 minutes and filtered through Whatman No. 42 filter paper. The filtrate was collected and subjected to estimation of available zinc using Atomic Absorption Spectrophotometer.

3.2. ISOLATION OF ZINC SOLUBILIZING MICROORGANISMS

Microorganisms capable of solubilizing zinc were isolated from zinc deficient areas by serial dilution technique using zinc solubilizing medium (Bunt and Rovira, 1955) supplemented with 0.1% insoluble zinc oxide.

10g soil was transferred to 250ml flask containing 90ml sterile distilled water followed by vortexing and 1ml of the 10^{-1} dilution was transferred to 9ml sterile distilled water and vortexed (10^{-2} dilution). In similar way, dilutions were made upto 10^{-9}. 0.1ml of the serially diluted soil samples were spread uniformly by a sterilized glass spreader on petriplates containing solidified Bunt and Rovira agar medium supplemented with 0.1 percent of zinc oxide as sole zinc source for selectively screening the microorganisms. Two replications were maintained for each dilution. After 3-days of incubation at room temperature, zinc solubilizing microorganisms developed clear halo zones around colonies. The colonies with clearance zones were further purified by subculturing on Bunt and Rovira agar medium supplemented with 0.1 percent of zinc oxide. Ten zinc solubilizing bacterial isolates thus screened were selected for further studies.

3.3. ASSESSING THE SOLUBILIZATION POTENTIAL OF THE ISOLATES UNDER *IN VITRO* CONDITIONS

The zinc solubilization potential of the isolates was assessed by Plate assay and Broth assay.

3.3.1 Plate assay

Sterilized Bunt and Rovira agar medium supplemented with 0.1 per cent insoluble zinc compounds either zinc oxide or zinc phosphate was poured into sterilized pertiplates. After solidification of the media, spot inoculation of the bacterial strains were made with the help of flame sterilized loop on each plate aseptically and incubated at room temperature for 72 hours. The halo zones around the colonies were observed and measured in millimeters. Three replications for each isolate and a control without zinc solubilizing microorganisms were maintained.

3.3.2 Broth assay

Ten bacterial isolates showing maximum zinc solubilization in plate assay were selected for broth studies. The bacterial isolates were inoculated separately to basal medium with 0.1% insoluble zinc compounds either zinc oxide or zinc phosphate. The flask containing 100 ml of sterile broth were inoculated with 1 ml of 24 hours old actively growing test bacterial cultures and incubated at room temperatures with a shaking at 140 rpm. Three replications were maintained for each isolate along with an un-inoculated control.

The available zinc content in the broth culture was analyzed for 0, 7, 14, 20 and 30 days after inoculation. 10 ml each of the samples were transferred aseptically to centrifuge tubes and centrifuged at 10,000 rpm for 10 minutes to remove cell debris. The supernatant was collected and filtered through Whatman filter paper No. 42. The clear solution was collected in 30 ml vials and the quantity of available zinc released into the medium was assessed using Atomic Absorption Spectrophotometer (AAS).

The growth of the bacterial isolates was assessed based on optical density (OD) value at 660 nm in spectrophotometer at different intervals of 7, 14, 20 and 30 days after inoculation. 3ml of each sample was transferred aseptically to cuvette and read in spectrophotometer at 660nm.

3.4 SOIL INCUBATION STUDIES

3.4.1 Location

The experiment was conducted using soils with low available zinc collected from Agro ecological unit – 8 (Neyyatinkara taluk) in the Department of Agricultural Microbiology at College of Agriculture Vellayani.

Sterilized soil of 1 kg each was filled in plastic containers of dimension 24 cm x 13 cm x 10 cm and maintained at field capacity with sterile distilled water. Each box was covered with polythene sheet and incubated at room temperature. The isolate with maximum zinc solubilization potential that is ZSB - 4 was selected for the study.

A single colony of zinc solubilising bacteria (ZSB- 4) was picked aseptically and transferred into 250 ml Erlenmeyer flasks containing 100ml sterile nutrient broth. The flasks were incubated at 28°C on 120 rpm rotary shaker for 24 hours. After incubation, 100 ml of the culture was mixed aseptically with sterilized 1000g talc. The talc based zinc solubilising culture was added into the soil in the incubation trays at the rate of 2kg ha^{-1} under aseptic condition.

Wherever zinc oxide supplementation was required sterilized zinc oxide was added at the rate of 1 kg Zn ha^{-1} and 2 kg Zn ha^{-1} in respective treatments under aseptic condition.

The treatment details are as follows:

Design : CRD

Treatments: 6

Replication: 4

Treatments

T1 - ZnO alone @ 1 kg Zn ha^{-1}.

T2 - ZnO alone @ 2 kg Zn ha^{-1}.

T3 - Zn solubilising culture alone @ 2 kg ha^{-1}.

T4 - ZnO @ 1 kg Zn/ha + Zn solubilising culture @ 2 kg ha^{-1}.

T5 - ZnO + Zn solubilising culture each @ 2 kg ha^{-1}.

T6 – Control (without ZnO + without culture).

3.4.2. Soil analysis

The following parameters of soil were analyzed at monthly intervals for a period of 3 months.

3.4.2.1. Soil pH

pH of the air dried soil samples were determined with a soil water ratio of 1:2.5 (Jackson, 1958) using a pH meter.

10g soil sample were taken in 50ml beaker and 25ml distilled water was added and this suspension was stirred at regular intervals for 30 minutes. The pH meter was calibrated with standard buffer solutions of 4 and 7 and was measured.

3.4.2.2. Total zinc

The total zinc was extracted using concentrated hydrochloric acid and estimated using Atomic Absorption Spectrophotometer (Jackson, 1958).

1 g of soil (passed through a 0.2 mm sieve) was placed in a conical flask. 30 ml of concentrated hydrochloric acid was added followed by heating in low flame for one hour. Then the flame was gradually raised until the samples was completely charred and then clear. After cooling the digest was made upto 100ml. 1ml of this aliquot was taken in a volumetric flask and again made upto 100ml.

Total Zinc (ppm) = Concentration from instrument x 10,000

3.4.2.3. Available zinc

Available Zinc Status was estimated after extraction using 0.1N HCl and read in Atomic Absorption Spectrophotometer (Sims and Johnson, 1991).

2 g soil was taken in 250 ml conical flask and 20 ml 0.1 N HCl was added. This suspension was shaken for 5 minutes and filtered through Whatman No. 42 filter

paper. The filtrate was collected and estimation of available zinc using Atomic Absorption Spectrophotometer was done.

$$\text{Available Zinc (ppm)} = \frac{\text{Concentration from instrument x 20}}{10}$$

3.4.2.4. Available boron content

Available Boron content in the soil sample was estimated by using Hot water extraction method and estimated calorimetrically by Azomethine-H using spectrophotometer (Gupta, 1967).

20 g of sieved air dried soil sample was weighed in a 250 ml boron free conical flask, 40 ml distilled water and 0.5g of activated charcoal was added and boiled on hot plate for 5 minutes. The contents were filtered immediately through Whatman No. 42 filter paper and cooled to room temperature. 1ml aliquot was transferred to 10ml polypropylene tubes, 2ml of buffer and 2ml of azomethine – H was added and the absorbance was read at 420nm after 30 minutes on spectrophotometer.

Similarly standard Boron concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1ppm) were analyzed using the same procedure.

$$\text{Available B (mg kg}^{-1}\text{ soil)} = \frac{\text{Absorbance reading}}{\text{Slope from curve}} \times 40$$

3.4.2.5. Available phosphorus

Available Phosphorus in the soil was estimated as per Bray No. 1 extraction and ascorbic acid reduced molybdo-phosphoric blue colour method using spectrophotometer (Jackson, 1958).

5 g of soil was taken in 100 ml conical flask and Bray No. 1 reagent was added followed by shaking for 5 minutes. 7.5 ml of 0.8 M boric acid was added to 5 ml of the extract. Phosphorus was then estimated by ascorbic acid method.

5ml of the extract was pipetted into 25 ml volumetric flask and 4 ml of ascorbic acid was added followed by making up the volume to 25 ml with distilled water. After 10 minutes the intensity of colour was read at 660 nm.

$$\text{Available P (mg kg}^{-1}\text{ soil)} = \frac{\text{Absorbance for sample}}{\text{Slope of Std. curve}} \times 50$$

$$\text{Available P (kg ha}^{-1}\text{ soil)} = \text{Available P (mg kg}^{-1}\text{ soil)} \times 2.24$$

3.4.2.6. Oxidisable organic carbon content

Organic carbon content of the soil samples were determined by Walkly and Black chromic acid wet digestion method as defined by Walkley and Black (1934).

1 g of finely ground soil was taken in 500 ml wide mouth conical flask. 10 ml of 1 N $K_2Cr_2O_7$ was added and the flask was swirled gently to disperse the soil in the solution. Then 20 ml of concentrated H_2SO_4 was added rapidly and allowed the flask to stand for about 30 minutes followed by addition of 200 ml of distilled water. Three to four drops of o – phenanthroline indicator was added and titrated with 0.5 N ferrous ammonium sulphate.

$$\text{O C (\%)} = \frac{\{10 \times 1 - \text{Titre value (ml)} \times \text{Normality of Fe(NH4)2SO4}\} \times 0.39}{\text{Weight of soil}}$$

3.4.3. Analysis of population of zinc solubilising microorganisms

The soil samples were serially diluted and count of total zinc solubilizing bacteria was made by plating on zinc solubilizing medium containing 0.1 percent insoluble zinc oxide.

10g soil was transferred to 250 ml flask containing 90 ml sterile distilled water and 1ml of the 10^{-1} dilution was transferred to another test tube containing 9ml sterile distilled water followed by vortexing (10^{-2} dilution). In similar way, dilutions were made upto 10^{-9} dilution. 0.1ml of the serially diluted soil samples were spread uniformly by a sterilized glass spreader on petriplates containing solidified Bunt and

Rovira agar medium. Two replications for each dilution were maintained. The plates were then inverted and kept for incubation at room temperatures.

3.5 CHARACTERIZATION OF ISOLATES

Isolate with maximum zinc solubilization potential were selected and characterized based on morphological and molecular studies as detailed below.

3.5.1. Morphological characterization of isolates

The colony morphology, texture and appearance of the isolates on agar plates were studied. The Gram's reaction and biochemical characters were also analyzed.

For studying the colony characters, a single colony was picked with a flame sterilized loop and was added to 1ml sterile distilled water in eppendorf tube followed by vortexing. This sample was serially diluted upto 10^{-9} dilutions. 0.1ml of the serially diluted suspension was spread uniformly by a sterilized glass spreader on petriplates containing solidified Bunt and Rovira agar medium. The plates were then inverted and kept for incubation at room temperatures. The colony morphology, texture and appearance of the isolates were determined on appearance of full grown colonies on the agar medium.

3.5.1.1 *Gram's staining*

A thin smear of bacterial suspension was made on a glass slide. After heat fixing the smear it was stained with Crystal Violet for 1 minute followed by washing under tap water. Then it was stained with Gram' Iodine for 1 minute followed by washing with tap water. 95 percent alcohol was added drop by drop until the alcohol runs clear. It was washed then with tap water followed by addition of counter stain Saffranin for 45 seconds. This was again washed under tap water and blot dried and observed under microscope.

3.5.1.2. *Biochemical test*

The biochemical characters were identified using KB013 HiBacillus Identification Kit (Himedia Laboratories, Mumbai). The organism to be identified was first isolated and purified. A single isolated colony was picked up and inoculated in 5 ml Nutrient Broth followed by incubating at 28^0C for 6 to 8 hours until inoculum turbidity was ³1.0 OD at 620nm.

Method of Inoculation

The kit was opened aseptically and the sealing foil was peeled off. Each well was inoculated with 50µl of the above inoculum by surface inoculation method. It was then incubated at 28^0C for 24- 48 hours.

The results were interpreted as per the standards given in the Result Interpretation Chart. Addition of reagents in well no 2, 5 were done at the end of incubation period that is after 24 to 48 hours. Then it was analyzed for catalase, nitrate reduction, Voges-Proskauer's and carbohydarte fermentation test.

3.5.2. Molecular characterization of selected isolate by DNA sequencing using universal primers of 16SrRNA

The molecular characterisation of the selected isolate was done in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, by 16 SrRNA sequencing.

3.5.2.1. *Isolation of genomic DNA*

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

3.5.2.2. *Agarose gel electrophoresis for DNA quality and quantity check*

The isolated DNA was loaded in agarose gel and electrophoresis was performed. The gel was seen in a UV transilluminator and the image was captured using Gel documentation system under UV light.

3.5.2.3. *PCR analysis*

The PCR amplification was done in a PCR thermal cycler.

Primers used

Target	**Primer Name**	**Direction**	**Sequence (5' → 3')**
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

3.5.2.4. *Bioinformatics*

The rDNA sequences of the best isolate was searched in the NCBI (National Center for Biotechnology Information) database using BLAST(n) (Basic Local Alignment Search Tool (Nucleotide). Based on the genetic similarity the organisms were identified.

3.6. DEVELOPMENT OF FORMULATION AND ASSESSING SHELF LIFE

For developing the formulation and assessing the shelf life, talc, lignite, vermiculite, perlite and vermicompost were selected as carrier materials for the study.

1kg of each carrier materials was transferred into polythene bags and autoclaved for 3 consecutive days.

A single colony of zinc solubilising bacteria were picked aseptically and transferred into 250 ml Erlenmeyer flask containing 100 ml sterile nutrient broth. The flasks were incubated at 28°C on 120 rpm rotary shaker for 72 hours. After incubation, 100 ml of the culture was mixed aseptically with 1000 g of each sterile carrier, packed in polyethylene bags, sealed and incubated under room temperature.

The total count of zinc solubilising microorganism was monitored at monthly intervals for a period of three months by dilution plating technique on zinc solubilising medium supplemented with 0.1 percent insoluble zinc oxide.

For evaluation of survival of zinc solubilising bacteria after inoculation 1g of each sample was transferred to test tube containing 9 ml sterile distilled water and 1ml of the 10^{-1} dilution was transferred to another test tube containing 9 ml sterile distilled water and vortexed (10^{-2} dilution). In similar way, dilutions were made up to 10^{-8}. 0.1 ml of the aliquot from appropriate dilutions was transferred solidified Bunt and Rovira medium containing 0.1 percent of zinc oxide and spread uniformly with a sterilized glass spreader. The plates were inverted and incubated at room temperature.The number colonies after incubation was counted and expressed in cfu/g of carrier.

3.7 STATISTICAL ANALYSIS

The data generated from the experiments were statistically analyzed using analysis of variance techniques (ANOVA) as applied to Completely Randomized Design described by Panse and Sukhatme, 1985.

Results

4. RESULTS

The present study on "Development of inoculants cultures of zinc solubilising microorganisms" was conducted during the period from 2015-2016 in the Department of Agricultural Microbiology, Vellayani, Thiruvananthapuram, Kerala. Studies were carried out to isolate, screen and develop inoculants cultures of microorganisms capable of solubilising insoluble forms of zinc. Soil incubation studies were conducted to analyze the efficiency of the microorganisms. The results based on statistically analyzed data pertaining to the experiment conducted during the course of investigation are presented below:

4.1. ISOLATION OF ZINC SOLUBILISING MICROORGANISMS

Microorganisms capable of solubilising zinc oxide were isolated from zinc deficient areas such as Agroecological units 20, 21 (Wayanad) (Plate 1) and 8 (Thiruvananthapuram) (Plate 2) by serial dilution technique using zinc solubilizing medium.

Ten isolates of bacteria solubilising insoluble forms of zinc (zinc oxide) were obtained. These isolates were allotted code numbers from ZSB1- ZSB10 as shown in Table 1.

4.2. ASSESSING THE SOLUBILIZATION POTENTIAL OF THE ISOLATES UNDER *IN VITRO* CONDITIONS

All the ten isolates obtained were subjected to plate assay and broth assay in media supplemented with 0.1 per cent insoluble forms of zinc as zinc oxide or zinc phosphate.

All the selected isolates could effectively solubilize the insoluble Zn compounds used, namely, zinc oxide and zinc phosphate, under the assay conditions. The zone of solubilisation was comparatively high in zinc oxide amended medium when compared to zinc phosphate (Table 2 and 3).

Plate 1. Isolates from Wayanad soil in Bunt and Rovira medium supplemented with 0.1% zinc oxide

Plate 2. Isolates obtained from Thiruvananthapuram soil in Bunt and Rovira medium supplemented with 0.1% zinc oxide

Table 1 List of isolated microorganisms

Sl. No.	**Isolates**	**Type of Microorganism**
1.	ZSB 1	Bacteria
2.	ZSB 2	Bacteria
3.	ZSB 3	Bacteria
4.	ZSB 4	Bacteria
5.	ZSB 5	Bacteria
6.	ZSB 6	Bacteria
7.	ZSB 7	Bacteria
8.	ZSB 8	Bacteria
9.	ZSB 9	Bacteria
10.	ZSB 10	Bacteria

*ZSB – Zinc Solubilizing Microorganism

4.2.1. Plate assay

Zinc solubilizing potential varied with each isolate. The size of the solubilisation zone ranged from 8.67 mm to13.33 mm in zinc oxide and from 1.00 mm to 5.33 mm in zinc phosphate incorporated medium

After spot inoculation on zinc solubilizing medium, maximum zone of clearance of 13.33 mm was recorded with the isolate ZSB 4 which was significantly superior to all other isolates (Plate 3 and Plate 4). It was followed by ZSB 5 with a clearance zone of 10.67 mm. The mean value of ZSB 7,ZSB 2, ZSB 8, ZSB 6, ZSB 3, ZSB 1and ZSB 9 were found to be on par. The lowest clearance zone was recorded with the isolate ZSB 10 (8.33mm).

On spot inoculation of the ten isolates, the clearing zone produced by isolate ZSB 4 (Plate 5) was again significantly superior to all other isolates with a maximum clearance zone of 5.33 mm as shown in Table 3. The lowest mean value was observed in ZSB 5 and ZSB 8 with a clearance zone of 1 mm for each of them.

4.2.2. Broth assay

4.2.2.1. *Assessment of quantity of available zinc in mg/L released at different intervals in zinc oxide and zinc phosphate amended medium*

The efficacy of the isolates to solubilise the insoluble zinc was tested by analysing available zinc content by atomic absorption spectrophotometer and the results are presented in Tables 4 and 5.

The analysis of liquid medium supernatants by atomic absorption spectrophotometer showed a rise in the concentration of available zinc when compared with the uninoculated control.

On 1st day of inoculation, there was no significant difference in the available zinc content in all the treatments. From 7th day onwards the amount of zinc solubilized increased in all inoculated treatments ranging from 0.376 ppm to

Table 2. Zinc solubilizing potential of zinc solubilizing bacterial (ZSB) isolates with insoluble zinc oxide (Plate assay)

Sl. No.	Isolates	Clearing zone in mm *
1.	ZSB 1	9.00
2.	ZSB 2	10.00
3.	ZSB 3	9.33
4.	ZSB 4	13.33
5.	ZSB 5	10.67
6.	ZSB 6	9.67
7.	ZSB 7	10.33
8.	ZSB 8	10.00
9.	ZSB 9	8.67
10.	ZSB 10	8.33
	CD (0.05)	2.06

* Mean of three replications

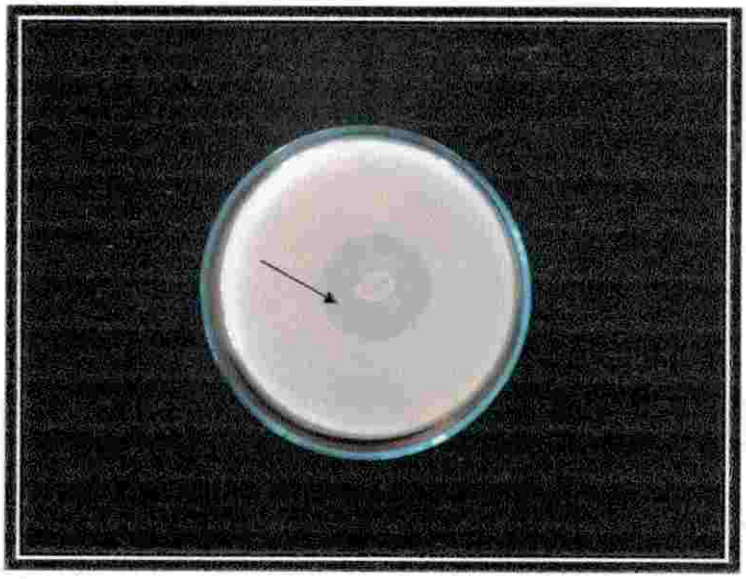


Plate 3. Spot inoculation of ZSB – 4 on Bunt and Rovira medium supplemented with 0.1 % zinc oxide

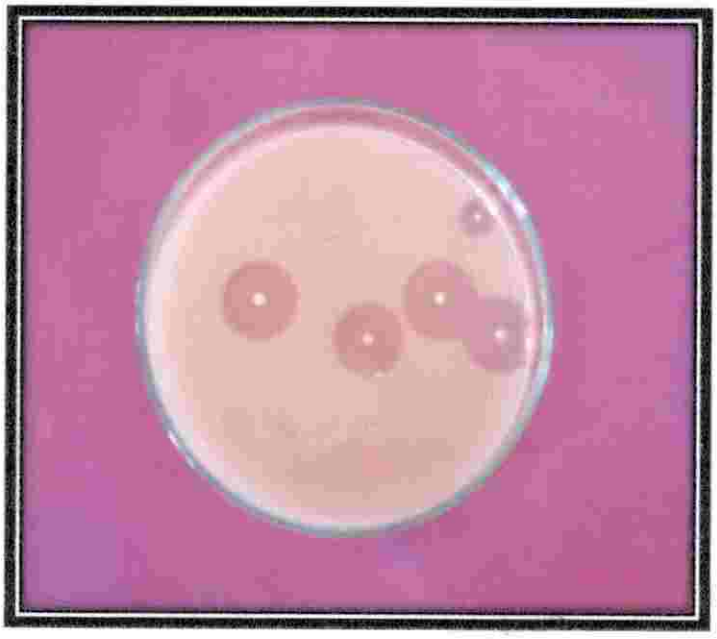


Plate 4. Spread plate of the isolate ZSB- 4 showing solubilisation zone

Table 3. Zinc solubilizing potential of zinc solubilizing bacterial (ZSB) isolates with insoluble zinc phosphate (Plate assay)

Sl. No.	Isolates	Clearing zone in mm *
1.	ZSB 1	1.33
2.	ZSB 2	2.00
3.	ZSB 3	1.66
4.	ZSB 4	5.33
5.	ZSB 5	1.00
6.	ZSB 6	2.33
7.	ZSB 7	1.33
8.	ZSB 8	1.00
9.	ZSB 9	2.33
10.	ZSB 10	2.00
	CD (0.05)	0.93

* Mean of three replications

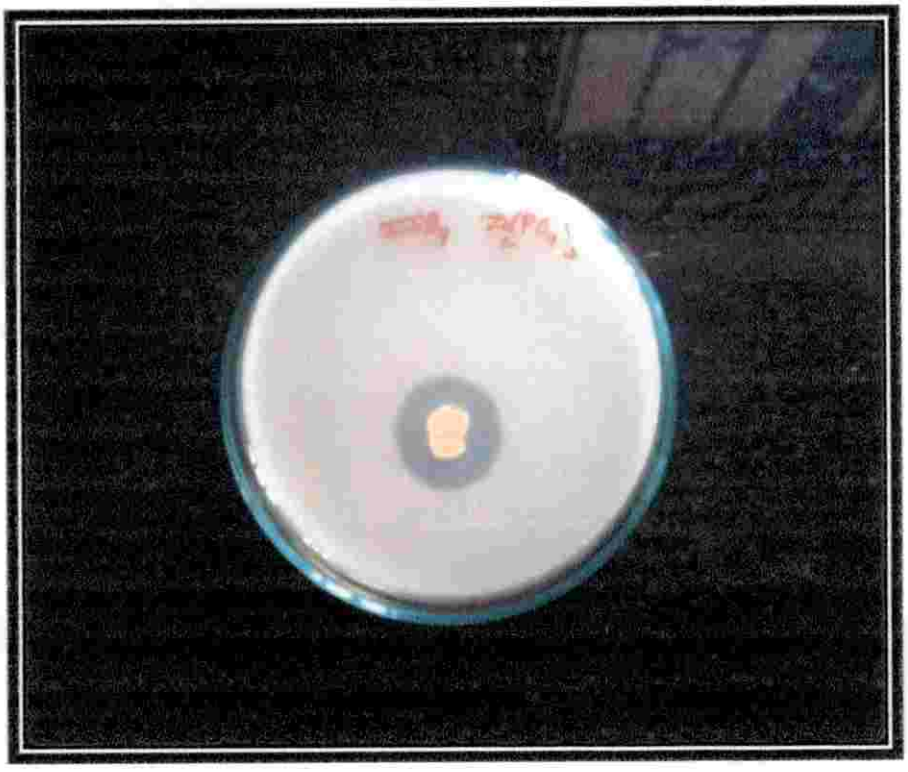


Plate 5. Spot inoculation of ZSB – 4 on Bunt and Rovira medium supplemented with zinc phosphate as zinc source

2.47 ppm in zinc oxide supplemented medium and 0.207 ppm to 0.91 ppm in zinc phosphate supplemented medium. On 14th day the concentration of available zinc ranged from 2.135 ppm to 8.159 ppm in zinc oxide amended medium and 0.477 ppm to 2.044 ppm in zinc phosphate amended medium. On 20th day the available zinc content increased upto a range of 14.03 ppm to 54.73 ppm in zinc oxide supplemented medium and 0.861 ppm to 3.654 ppm in zinc phosphate supplemented medium. The maximum solubilisation of zinc in both sources was observed on 30th day with a range of 35.917 ppm to 104.08 ppm in zinc oxide supplemented medium and 1.386 ppm to 4.15 ppm in zinc phosphate supplemented medium as shown in Table 4 and Table 5. The least amount of available zinc was found in uninoculated control.

Among the isolates, ZSB – 4 showed highest solubilisation of zinc in both sources that is upto 104.08 ppm in zinc oxide supplemented medium and 4.514 ppm in zinc phosphate supplemented medium and were significantly superior to all other isolates in all the days. This was followed by ZSB – 2 with available zinc concentration of 76.75 ppm in zinc oxide amended medium and ZSB – 7 with available zinc concentration of 3.348 ppm in zinc phosphate amended medium. ZSB – 9 showed lowest solubilisation (35.91 ppm available zinc) in zinc oxide amended medium. On the other hand in zinc phosphate amended medium, ZSB – 1 registered lowest solubilisation (1.318 ppm available zinc).

4.2.2.2. *Indirect assessment of growth of zinc solubilising bacteria in zinc oxide and zinc phosphate amended medium*

On first day of inoculation, the OD values of all the isolates in broth culture were on par in both the medium. On 7th day, maximum OD value was recorded with the isolates ZSB – 4 and ZSB – 2 (1.44). The least OD value was observed in ZSB – 3 with OD value of 1.03.

The data presented in Table 6 revealed that maximum OD value was obtained for all the isolates on 14th day after inoculation in zinc oxide supplemented medium and all the isolates were on par. On the other hand, in zinc

Table 4. Quantity of available zinc released at different intervals in zinc oxide medium

Isolates	Available zinc in mg/L in zinc oxide medium *				
	0^{th} day	7th day	14th day	20th day	30th day
ZSB 1	0.15	0.93	2.81	28.61	64.26
ZSB 2	0.15	0.85	3.66	28.53	76.75
ZSB 3	0.28	0.97	2.90	14.03	51.15
ZSB 4	0.34	2.47	8.14	54.73	104.08
ZSB 5	0.28	0.91	3.21	32.53	54.18
ZSB 6	0.22	0.94	3.89	32.30	59.56
ZSB 7	0.18	0.98	4.03	34.61	64.73
ZSB 8	0.24	0.83	2.98	30.04	69.81
ZSB 9	0.19	0.59	2.59	26.95	35.91
ZSB 10	0.17	0.37	2.13	25.71	49.08
CONTROL	0.08	0.22	0.23	0.19	0.13
CD (0.05)	NS	0.28	0.58	10.85	15.09

* Mean of three replications

Table 5. Quantity of available zinc released at different intervals in zinc phosphate medium

Isolates	Available zinc in mg/L in zinc phosphate medium*				
	0^{th} day	7th day	14th day	20th day	30th day
ZSB 1	0.009	0.349	0.892	0.976	1.318
ZSB 2	0.014	0.207	0.552	1.116	1.386
ZSB 3	0.011	0.369	0.921	1.248	2.051
ZSB 4	0.013	0.918	2.044	3.654	4.514
ZSB 5	0.036	0.582	0.661	1.922	2.698
ZSB 6	0.006	0.312	1.061	2.012	3.077
ZSB 7	0.010	0.313	1.035	1.845	3.348
ZSB 8	0.013	0.546	0.898	1.982	3.098
ZSB 9	0.013	0.268	0.835	1.084	2.475
ZSB 10	0.005	0.255	0.477	0.861	1.386
CONTROL	0.025	0.022	0.024	0.020	0.025
CD (0.05)	NS	0.230	0.291	0.551	0.574

* Mean of three replications

Table 6. OD value at 660nm at different intervals in zinc oxide medium

Isolates	OD value at 660nm in zinc oxide medium *				
	0^{th} day	7th day	14th day	20th day	30th day
ZSB 1	0.006	1.046	1.266	0.483	1.083
ZSB 2	0.003	1.441	2.089	1.053	0.953
ZSB 3	0.004	1.033	2.049	1.084	1.096
ZSB 4	0.006	1.446	2.116	1.033	0.986
ZSB 5	0.006	1.317	2.139	0.996	1.142
ZSB 6	0.002	1.213	2.143	1.023	1.136
ZSB 7	0.003	1.326	2.073	1.046	1.116
ZSB 8	0.003	1.324	2.176	1.106	1.158
ZSB 9	0.003	1.243	1.779	1.046	1.096
ZSB 10	0.006	1.046	1.553	0.483	1.06
CD (0.05)	0.002	0.225	0.162	NS	NS

* Mean of three replications

Table 7. OD value at 660nm at different intervals in zinc phosphate medium

Isolates	OD value at 660nm in zinc phosphate medium*				
	0^{th} day	7th day	14th day	20th day	30th day
ZSB 1	0.006	0.466	1.399	1.343	1.146
ZSB 2	0.006	0.314	1.697	1.496	1.224
ZSB 3	0.006	0.211	1.456	1.423	1.123
ZSB 4	0.002	0.543	1.559	1.585	1.116
ZSB 5	0.002	0.300	1.455	1.315	1.03
ZSB 6	0.006	0.226	1.251	1.183	0.706
ZSB 7	0.003	0.452	1.321	1.181	1.106
ZSB 8	0.003	0.326	1.135	1.193	1.045
ZSB 9	0.006	0.546	1.206	1.266	1.116
ZSB 10	0.006	0.466	1.399	1.343	1.146
CD (0.05)	NS	NS	0.259	0.240	0.255

* Mean of three replications

phosphate amended medium on 20th day maximum OD value of 1.58 was attained by the isolate ZSB – 4. A similar trend was also observed by the same isolates when grown in zinc phosphate amended medium as shown in Table 7.

On 20th and 30th day after inoculation there was a decrease in OD value for all the ten isolates and there was no significant difference among the isolates when grown in zinc oxide supplemented medium and zinc phosphate supplemented medium.

4.3. SOIL INCUBATION STUDIES

Soil incubation study was conducted to analyse the performance of the best isolate that is ZSB – 4. For this experiment was laid out using soils with low available zinc collected from Agro ecological unit – 8 (Neyyatinkara taluk) in the Department of Agricultural Microbiology at College of Agriculture Vellayani (Plate 6). Soil samples were analysed for the soil chemical parameters viz. soil pH, total zinc, available zinc, available boron, available phosphorus, oxidisable organic carbon content and for population of zinc solubilising microorganisms in soil for a period of three months. The results are presented in Tables 8 – 14.

4.3.1. Soil pH

The results with respect to soil pH are represented in Table 8. The pH of the soil ranged from 4.9 to 5.There was no significant difference observed in soil pH among treatments inoculated with zinc solubilising microorganisms (T3, T4, T5) and for uninoculated treatments (T1, T2, T6). On 3rd month, all the treatments were showing a pH of 4.9.

4.3.2. Total zinc

It is inferred from Table 9 that the mean values in case of total zinc had no significant difference among treatments. The total zinc content of the soil ranged from 0.05 % to 0.08 % during the entire three months. During the 3rd month, highest total zinc content was recorded by T2 with 0.08 % while lowest mean value was registered by T5 with 0.07 %.

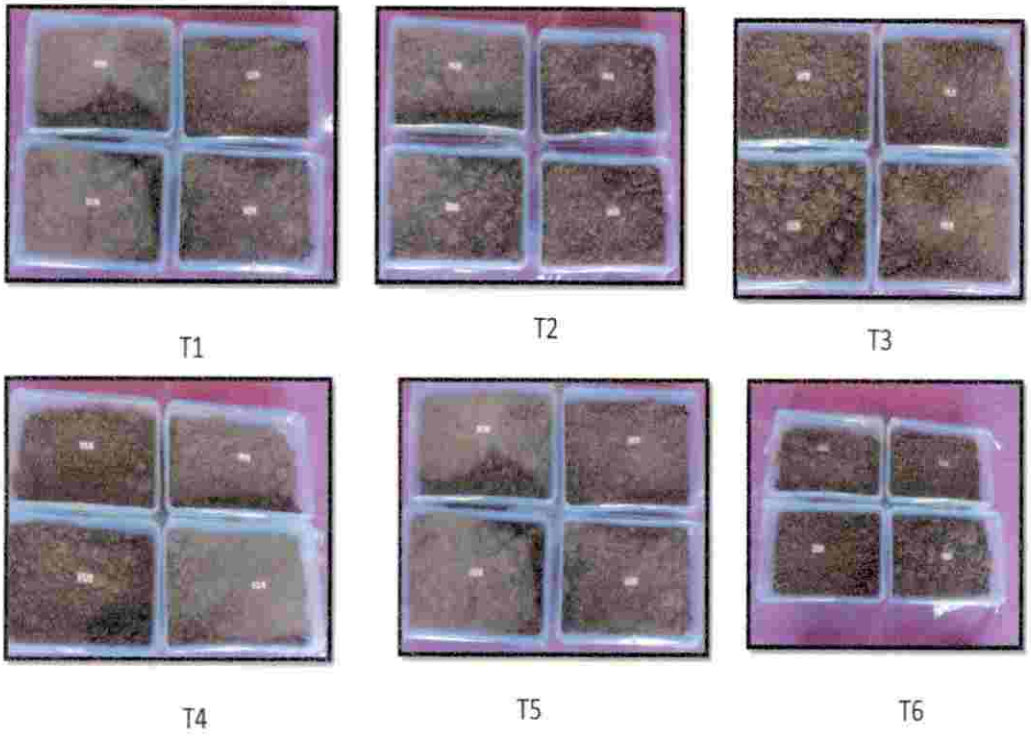


Plate 6. Design of soil incubation experiment

Table 8.Soil pH at monthly intervals for a period of three months

Treatments	Soil pH *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	4.9	4.9	4.9	4.9
T2	4.9	4.9	5.0	4.9
T3	5.0	4.9	4.8	4.9
T4	4.9	4.9	4.9	4.9
T5	4.9	4.9	4.9	4.9
T6	4.9	4.9	4.9	4.9
CD(0.05)	NS	NS	NS	NS

* Mean of four replications

Table 9. Total zinc in soil during incubation

Treatments	Total zinc (%) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	0.07	0.07	0.07	0.07
T2	0.05	0.06	0.07	0.08
T3	0.06	0.07	0.06	0.07
T4	0.07	0.07	0.06	0.07
T5	0.07	0.06	0.07	0.07
T6	0.07	0.07	0.07	0.07
CD(0.05)	NS	NS	NS	NS

* Mean of four replications

4.3.3. Available zinc

Various treatments had significantly influenced the available zinc content in soil as observed from Table 10.

On the first day, there was no significant difference in the available zinc content of the soil among the treatments. The mean values ranged from 0.37 ppm to 0.55 ppm. Treatment T4 registered the highest mean value of 0.55 ppm followed by treatment T3 with 0.52 ppm. The lowest value of available zinc was recorded by T2 (0.37 ppm).

After analysing the data on 1st month, it was revealed that the applied treatments had significant effect on the available zinc content in soil. The mean values ranged from 0.77 ppm to 0.97 ppm. The highest value was recorded by treatment T5 (0.97 ppm) followed by T4 (0.95) which were statistically on par. Treatment T3 (0.08 ppm) was found to be on par with T2 (0.07 ppm) and T1 (0.07 ppm). The lowest mean value was recorded by T6 the absolute control which was significantly lower than other treatments.

On analysing the data on 2nd month revealed that the treatments had influenced the available zinc content in soil. The mean values ranged from 0.52 ppm to 1.25 ppm. The highest value was registered by treatment T5 (1.25 ppm) which was significantly superior to other treatments. Treatment T4 (0.09 ppm) was found to be on par with T3 (0.87 ppm), T2 (0.57 ppm) and T1 (0.52 ppm). Treatment T6 (control) registered the lowest mean value 0.52 ppm.

There was an increase in the available zinc content in soil during the 3rd month. The mean values ranged from 0.62 ppm to 9.47 ppm. The highest value was registered by treatment T4 (9.47 ppm) which was significantly superior to other treatments. Treatment T6 (control) registered the lowest mean value 0.54 ppm.

Table10. Available zinc in soil during incubation

Treatments	Available zinc (ppm) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	0.42	0.77	0.52	0.67
T2	0.37	0.77	0.57	0.62
T3	0.52	0.87	0.87	2.37
T4	0.55	0.95	0.91	9.47
T5	0.47	0.97	1.25	6.95
T6	0.42	0.57	0.52	0.55
CD(0.05)	NS	0.20	0.35	0.64

* Mean of four replications

4.3.4. Available boron

Table 11 presents the available boron concentration in the soil. There was significant difference in the boron content of soil during the 3rd month.

On the first day, there was no significant difference in the available boron content of the soil among the treatments. The mean values ranged from 0.27 ppm to 0.39 ppm. Treatment T2 registered the highest mean value of 0.39 ppm followed by treatment T5 (0.36 ppm). The lowest value of available boron was recorded by T6 (0.27 ppm).

After analysing the data on 1st month, it revealed that the applied treatments had significant effect on the available boron content in soil. The mean values ranged from 0.10 ppm to 0.36 ppm. The highest value was recorded by treatment T3 (0.36 ppm) followed by T5 (0.33 ppm) and T4 (0.32 ppm) which were statistically on par. Treatment T2 (0.19 ppm), T1 (0.28 ppm) and T6 (0.10 ppm) were found to be on par.

Perusal of 2nd month data revealed that there was significant difference in the available boron content in soil. The mean values ranged from 0.39 ppm to 0.13 ppm. The highest value was recorded by treatments T4 (0.39 ppm) and T5 (0.39 ppm) followed by T3 (0.38 ppm) which were statistically on par. Treatment T2 (0.18 ppm), T1 (0.19 ppm) and T6 (0.13 ppm) were found to be on par.

On the 3rd month, there was a decrease in the available boron content in soil for the treatments T3 (0.30 ppm) and T5 (0.34 ppm) when compared with previous months and the treatment T4 (0.33) maintained the level of boron. The mean values ranged from 0.16 ppm to 0.34 ppm. T2 (0.17 ppm), T1 (0.16 ppm) and T6 (0.16 ppm) were found to be on par.

4.3.5. Available phosphorus

Various treatments had significantly influenced the available phosphorus content in soil as observed from Table 12.

Table11. Available boron in soil during incubation

Treatments	Available boron (ppm) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	0.35	0.28	0.19	0.16
T2	0.39	0.19	0.18	0.17
T3	0.34	0.36	0.38	0.30
T4	0.33	0.32	0.39	0.33
T5	0.36	0.33	0.39	0.34
T6	0.27	0.10	0.13	0.16
CD(0.05)	NS	0.16	0.10	0.09

* Mean of four replications

It is inferred from Table 12 that the mean values in case of available phosphorus ranged from 8.58 kg ha^{-1} to 9.37 kg ha^{-1} on the first day and on 1st month it ranged from 9.47 kg ha^{-1} to 10.56 kg ha^{-1}. There was no significant difference among the treatments.

During the 2nd and 3rd month the treatments had significant effect showing an increase in soil phosphorus content. Treatment T4 registered the highest mean value of 12.09 kg ha^{-1} and 12.26 kg ha^{-1} during the 2nd and 3rd month respectively and was on par with T3 and T5. Treatment T6 (absolute control) registered lowest mean value of 8.87 kg ha^{-1} and 8.88 kg ha^{-1} during the 2nd and 3rd month.

4.3.6. Oxidisable organic carbon content

The results revealed that the applied treatments had significant effect on the organic carbon content of the soil as shown in Table 13.The mean values of organic carbon content ranged from 1.04% to 1.19% on the first day. There was an increase in organic carbon content for 1st, 2nd and 3rd month for treatments T4 (1.29%, 1.23 %, 1.44%), T5 (1.21%, 1.23%, 1.43%) and T3 (1.21%, 1.18%, 1.38%) which were statistically on par. Treatment T6 (absolute control) registered lowest mean value of 1.02%, 1.00% and 0.95% during the 1st, 2nd and 3rd month respectively.

4.3.7. Population of zinc solubilising microorganisms

The population of zinc solubilising microorganisms significantly increased as shown in Table 14. At the time of inoculation, the mean values ranged from 1.37 x 10^2 cfu g^{-1} to 1.87 x 10^2 cfu g^{-1} of soil and the treatments T3, T4 and T5 were statistically on par. During the 2nd month the T3 registered a highest mean value of 3.80 x 10^2 cfu g^{-1} which was on par with T4 (7.2 x 10^3 cfu g^{-1}) and T5 (7.0 x 10^3 cfu g^{-1}). Maximum colony count of 9.3 x 10^3 cfu g^{-1} of soil was recorded in the treatment T4 during the 3rd month which was on par with T5 (9.1 x 10^3 cfu g^{-1}) and T3 (8.8 x 10^3 cfu g^{-1}). There was no population of zinc solubilising microorganisms in uninoculated treatments that is T1, T2 and T3 for the entire period.

Table12. Available phosphorus in soil during incubation

Treatments	Available phosphorus (kg ha^{-1})*			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	9.17	9.50	9.59	9.26
T2	9.10	9.47	8.66	9.40
T3	8.60	9.99	11.33	12.04
T4	9.37	10.56	12.09	12.21
T5	8.58	10.30	12.08	12.16
T6	9.22	9.74	8.87	8.88
CD(0.05)	NS	NS	**1.57**	**1.15**

* Mean of four replications

Table13. Oxidisable organic carbon content in soil during incubation

Treatments	Oxidisable organic carbon content (%) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	1.19	1.12	1.01	1.06
T2	1.08	1.11	1.03	1.01
T3	1.04	1.21	1.18	1.38
T4	1.08	1.29	1.23	1.44
T5	1.08	1.21	1.23	1.43
T6	1.12	1.02	1.00	0.95
CD(0.05)	NS	0.14	0.11	0.12

* Mean of four replications

Table14. Population of zinc solubilising microorganisms in soil during incubation

Treatments	Total viable count (log cfu g^{-1} soil) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
T1	0.00	0.00	0.00	0.00
T2	0.00	0.00	0.00	0.00
T3	2.23	3.60	3.80	3.91
T4	2.11	3.62	3.77	3.94
T5	2.16	3.60	3.75	3.91
T6	0.00	0.00	0.00	0.00
CD(0.05)	0.12	0.05	0.06	0.03

* Mean of four replications

4.4. CHARACTERIZATION OF ISOLATE

The isolate with maximum zinc solubilisation potential was selected for characterization studies.

4.4.1. Morphological characterisation of the isolate

Morphological characters comprising colony colour, texture and appearance of isolate was studied and data are presented in Table – 15 (Plate 7 and Plate 8).

4.4.2. Biochemical characterization of the isolate

The biochemical characters of the isolate were studied and data are presented in Table –16 (Plate 9).

4.4.3. Molecular characterisation of the isolate

The molecular characterisation of the selected isolate was done in collaboration with Rajiv Gandhi Centre for Biotechnology, Trivandrum. The genomic DNA was extracted from the isolate ZSB – 4. The agarose gel electrophoresis (0.8%) of the extracted DNA showed the presence of good quality, unshared DNA bands on the gel as shown in Plate 10.

4.4.3.1. *rDNA Amplification*

The 16S rRNA universal primers were used for amplification of the genomic DNA of the isolate ZSB – 4. The amplicons of size 1.5 KB were observed as shown in Plate 11.

4.4.3.2. *Sequencing of 16S rRNA fragments*

16S rRNA sequence of selected isolate obtained is presented in Table 17. The BLAST details of the most matching sequence are presented in Table 18 and Plate 12.

The isolate ZSB - 4 was identified as *Bacillus cereus.*

Table 15. Morphological characterisation of the Isolate ZSB – 4

Sl no.	Morphological characters	ZSB – 4
1.	Cell shape	Rod
2.	Colony shape and size	Irregular and large
2.	Margin	Undulated
3.	Elevation	Flat to raised
4.	Colour	Creamy white
5.	Texture	Smooth
6.	Gram's staining	G+

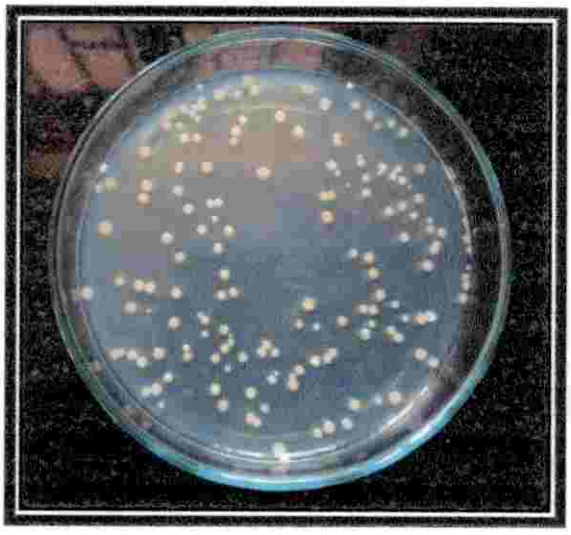


Plate 7. Colony morphology of the isolate ZSB - 4

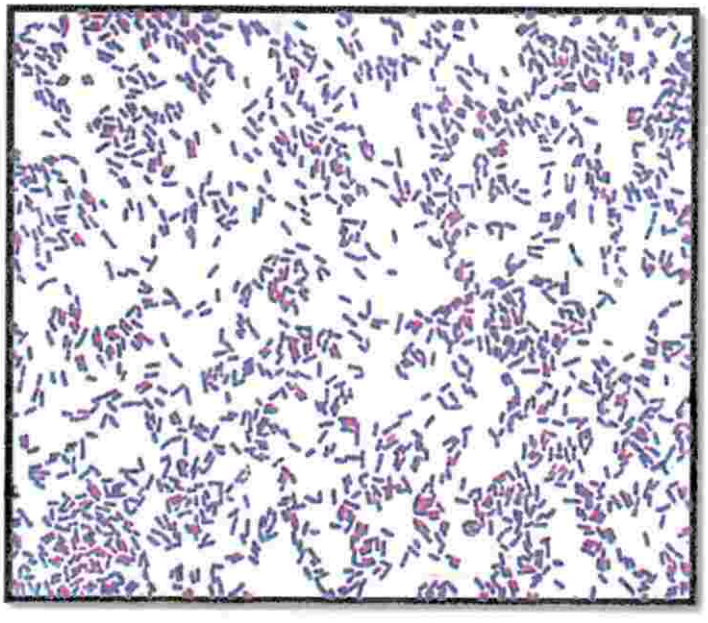


Plate 8. Gram positive rod shaped cells

Table 16. Biochemical characterisation of the Isolate ZSB – 4

Sl. No.	Test	Reaction
1	Malonate	-ve
2	Voges Proskauer's	-ve
3	Citrate	-ve
4	ONPG	-ve
5	Nitrate Reduction	+ve
6	Catalase	+ve
7	Arginine	-ve
8	Sucrose	+ve
9	Mannitol	-ve
10	Glucose	+ve
11	Arabinose	-ve
12	Trehalose	+ve

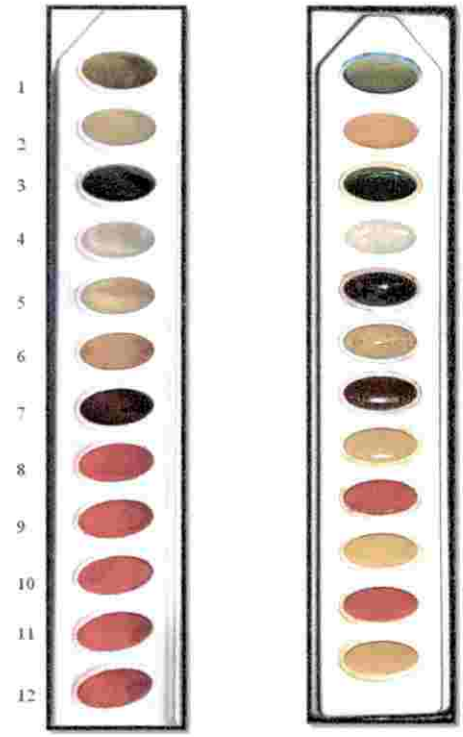


Plate 9. Biochemical reactions of the isolate ZSB - 4

Plate 10. Agarose gel (0.5%) electrophoresis showing band of genomic DNA

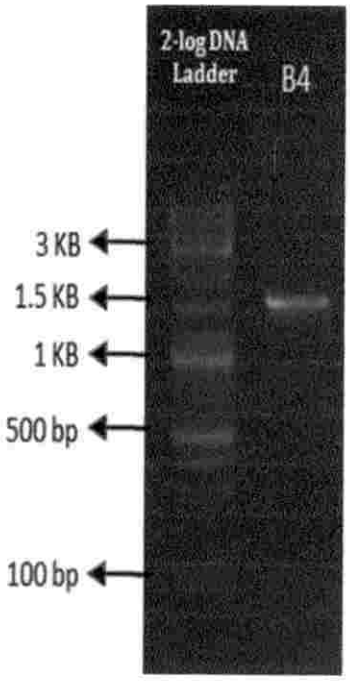
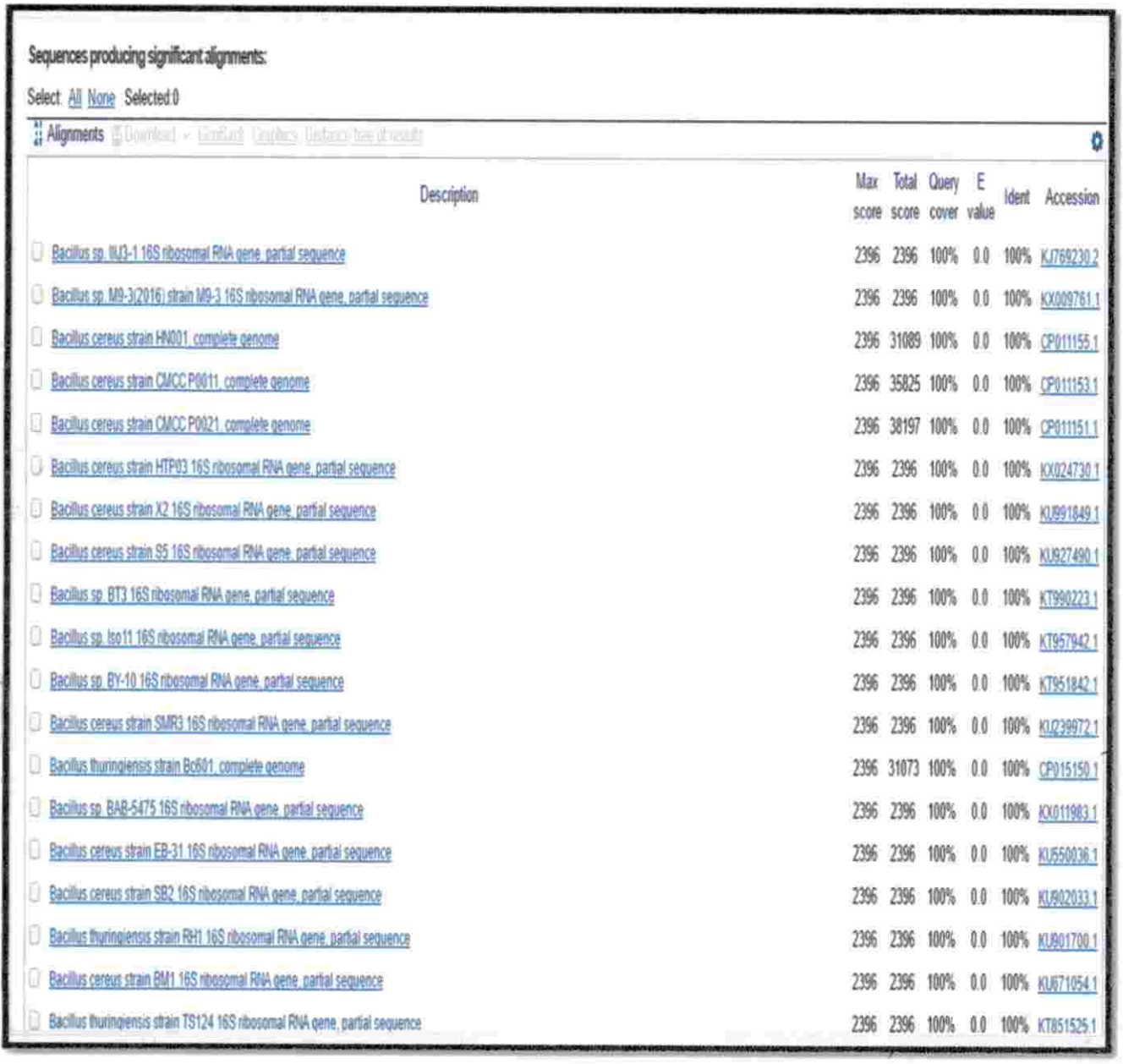


Plate 11. Agarose gel (0.8 %) showing PCR products

Table 17. 16S rRNA sequence of isolate ZSB – 4

Isolate	Sequence
ZSB - 4	>B4-16S TTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAA GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACAT TTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCAC TTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGG CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGG CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC GCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGT ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAA GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCT CAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAA GAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGAT ATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACT GACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGG GTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCC TGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG CGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGA TAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTG TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG CGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAA GGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA AATCATCATGC



Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download Compare Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. IIU3-1 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KJ769230.2
Bacillus sp. M9-3(2016) strain M9-3 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KX009761.1
Bacillus cereus strain HN001, complete genome	2396	31089	100%	0.0	100%	CP011155.1
Bacillus cereus strain CMCC P0011, complete genome	2396	35825	100%	0.0	100%	CP011153.1
Bacillus cereus strain CMCC P0021, complete genome	2396	38197	100%	0.0	100%	CP011151.1
Bacillus cereus strain HTP03 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KX024730.1
Bacillus cereus strain X2 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU991849.1
Bacillus cereus strain S5 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU927490.1
Bacillus sp. BT3 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KT990223.1
Bacillus sp. Iso11 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KT957942.1
Bacillus sp. BY-10 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KT951842.1
Bacillus cereus strain SMR3 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU239972.1
Bacillus thuringiensis strain Bc601, complete genome	2396	31073	100%	0.0	100%	CP015150.1
Bacillus sp. BAB-5475 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KX011983.1
Bacillus cereus strain EB-31 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU550036.1
Bacillus cereus strain SB2 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU902033.1
Bacillus thuringiensis strain RH1 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU901700.1
Bacillus cereus strain BM1 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KU871054.1
Bacillus thuringiensis strain TS124 16S ribosomal RNA gene, partial sequence	2396	2396	100%	0.0	100%	KT851525.1

Plate 12.BLAST search details of the sequences producing most significant alignment of the isolate ZSB- 4

TABLE 18. BLAST search details of the sequences producing most significant alignment of the isolate ZSB- 4

Isolate	Description	Max. score	Total score	Query cover (%)	E - value	Identity (%)	Accession no.
ZSB-4	*Bacillus cereus* strain HN 001, complete genome sequence	2396	31089	100	0.0	100	CP011155.1

4.5. ASSESSMENT OF SHELF LIFE OF ZINC SOLUBILISING MICROORGANISMS IN DIFFERENT CARRIER MATERIALS

The growth of zinc solubilising microorganisms was assessed in different carrier materials at monthly intervals for a period of three months (Plate 13).

On the first day of inoculation, highest mean value of 6.8 x 10^8 cfu g^{-1} was recorded in lignite followed by talc (6.6 x 10^8 cfu g^{-1}), vermicompost (6. x 10^8 cfu g^{-1}), perlite (3.9 x 10^8 cfu g^{-1}) and vermiculite (3.6 x 10^8 cfu g^{-1}). On 2^{nd} and 3^{rd} month talc was significantly superior to all other carriers with highest population count of 4.6 x 10^8 cfu g^{-1} and 3.9 x 10^8 cfu g^{-1} respectively which were followed by lignite (3.6 x 10^8 cfu g^{-1}, 2.9 x 10^8 cfu g^{-1}), vermicompost (3.2 x 10^8 cfu g^{-1}, 3.6 x 10^8 cfu g^{-1}), vermiculite (1.8 x 10^8 cfu g^{-1}, 1.8 x 10^8 cfu g^{-1}) and perlite (2.1 x 10^8 cfu g^{-1}, 1.1 x 10^8 cfu g^{-1}).

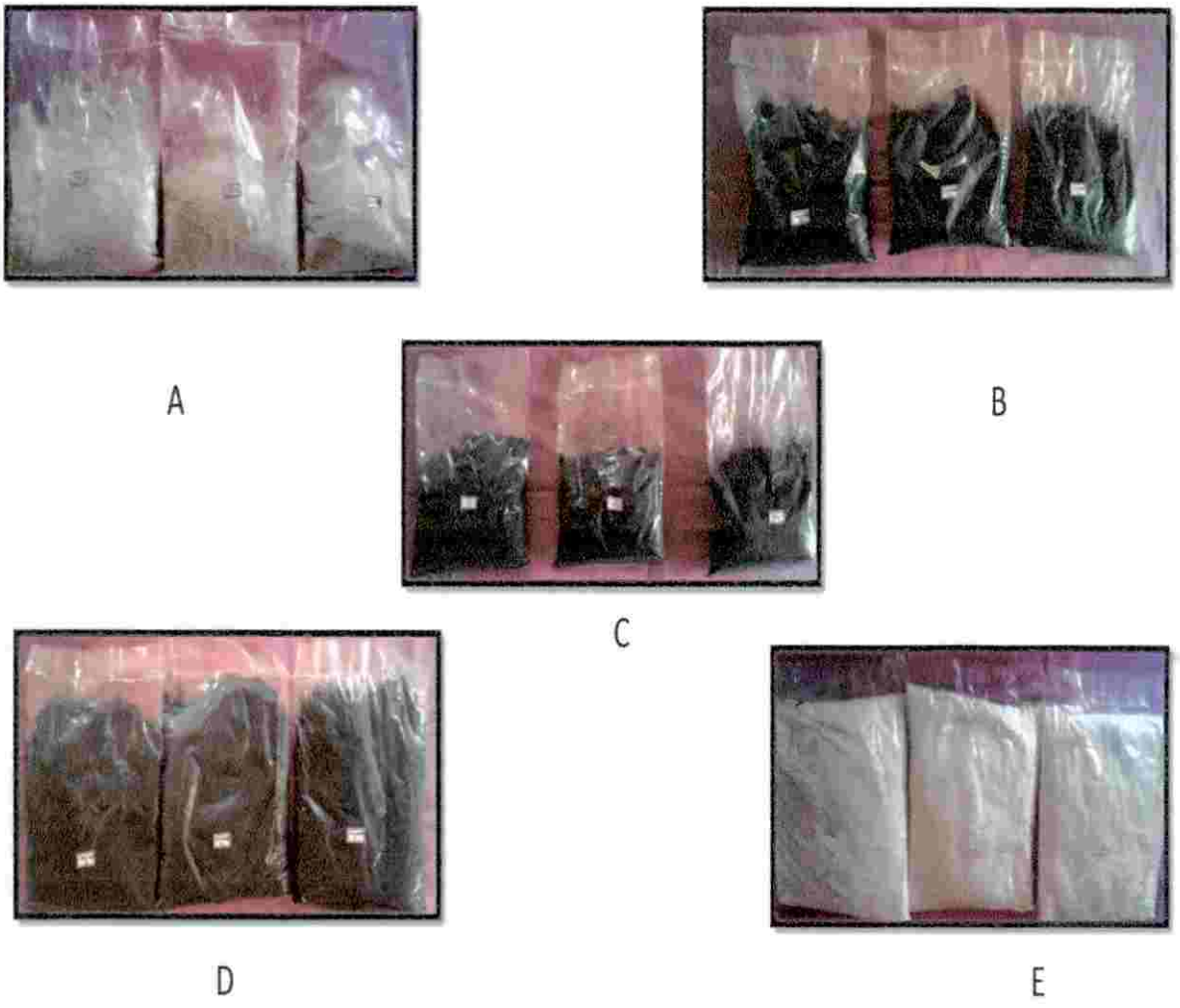


Plate 13. Different carriers used for assessment of shelf life of zinc solubilising microorganisms: (A) Talc (B) Vermicompost (C) Lignite (D) Vermiculite (E) Perlite

Table 19. Assessment of shelf life of zinc solubilising microorganisms in different carrier materials

Carriers	Total viable count (log cfu g^{-1}) *			
	0^{th} day	1^{st} month	2^{nd} month	3^{rd} month
Talc	8.64	8.64	8.63	8.56
Vermiculite	8.55	8.44	8.19	8.20
Lignite	8.83	8.60	8.50	8.30
Perlite	8.58	8.50	8.30	8.00
Vermicompost	8.73	8.59	8.46	8.20
CD (0.05)	0.16	NS	0.27	0.34

*Mean of three replications

Discussion

5. DISCUSSION

The present investigation has been carried out as an initial step to develop inoculants of zinc solubilising microorganisms for the use in zinc deficient soils that can increase the availability of zinc to crop plants.

Ten bacterial isolates capable of solubilising insoluble forms of zinc were obtained during the initial isolation from zinc deficient soils. Bunt and Rovira medium was used to isolate zinc solubilising microorganisms as it enabled detection of zinc solubilisation through formation of halo zone around the microbial colonies on agar plates. The solubilisation potential was found to be potentially varied among different isolates and this might be due to differences in the location from which they were isolated.

This study agrees with earlier findings (Di Simine *et al.*, 1998; Fasim *et al.,* 2002; Saravanan *et al.,* 2007) in which strains belonging to different genera like *Acinetobacter, Bacillus, Gluconacetobacter* and *Pseudomonas* were successfully isolated as zinc solubilizers. Zinc solubilising capability has already been reported in *Thiobacillus thioxidans, Thiobacillus ferroxidans*, facultative thermophilic iron oxidizers (Hutchins *et al.,* 1986), *Gluconacetobacter diaztorophicus* (Saravanan *et al*., 2007) and *Pseudomonas fluorescens* (Di Simine *et al.,* 1998; Saravanan *et al.,* 2003) and a few fungal genera such as *Trichoderma harzianum* Rifai 1295–22 (Altomare *et al*., 1999), *Aspergillus niger* (White *et al*., 1997), *Penicillium simplicissimum* (Franz *et al*., 1993), *Abisidia cylindrospora*, *A. glauca* and *A. spinosa* (Coles *et al.,* 2001). The ericoid mycorrhizae exhibited capability for solubilization of insoluble zinc compounds and these include *Paxillus involutus*, *Hymenoscyphus ericae, Oidiodendron maius*, *Suillus luteus* strains 21 and 22, *S. bovinus* LSt8 and *Beauveria caledonica* (Fomina *et al.,* 2005; Gadd, 2007).

All the ten isolates obtained in the isolation process were subjected to plate assay (Fig.1- 2) and broth assay (Fig.3- 4). The growth of the 10 isolates obtained was evaluated by measuring their optical density at 660nm (Fig.5- 6). The isolate

ZSB – 4 which recorded the maximum growth was selected for subsequent studies. The higher the clearance zone, the greater was the activity of the tested isolate.

It was apparent from the zinc solubilisation data that the solubilisation of different forms of insoluble zinc potentially varied with each isolate. Earlier studies conducted in plate assay with *Gluconacetobacter diazotrophicus* strains evaluated for zinc solubilisation, revealed variability at strain level (Madhaiyan *et al.,* 2004). It was inferred from Fig. 1 that the isolate ZSB – 4 has registered a highest solubilisation zone of 13.33 mm after spot inoculation on zinc solubilising medium supplemented with 0.1 per cent of zinc oxide. Similar results were reported by Desai *et al.* (2012) for organisms like *Pseudomonas, Azospirillum* and *Bacillus* with a solubilisation zone ranging from 5 mm to 7.6 mm. The results are also in accordance with the findings of Saravanan *et al.* (2003) who stated that *Bacillus* sp and *Pseudomonas* sp produced a clearance zone of 1.8 cm and 3.3 cm respectively when grown in Bunt and Rovira media containing 0.1% insoluble zinc oxide.

The data on Fig. 2 reveals that the isolate ZSB 4 was again significantly superior to all other isolates with a maximum clearance zone of 5.33 mm when grown on medium amended with zinc phosphate. Related results were also retrieved in studies conducted by Ramesh *et al.* (2014) with *Bacillus aryabhattai* strains recording a solubilisation zone upto 13 mm on Tris-minimal medium supplemented with zinc phosphate at a rate of 0.1 percent zinc concentration. Studies conducted by Goteti *et al.* (2013) was also in agreement with the above results and showed that the strains of *Pseudomonas* and *Bacillus* could effectively solubilize the insoluble zinc compounds, viz., $ZnCO_3$ and ZnO, under the assay conditions. The zone of solubilisation was comparatively high in ZnO amended medium as compared to $ZnCO_3$. However, with plate assay alone we could not be able to quantify the concentration of zinc released into the medium at the end of the incubation time. Hence liquid culture experiments were done to evaluate the amount of zinc released into the medium. The isolate ZSB - 4 solubilised both the

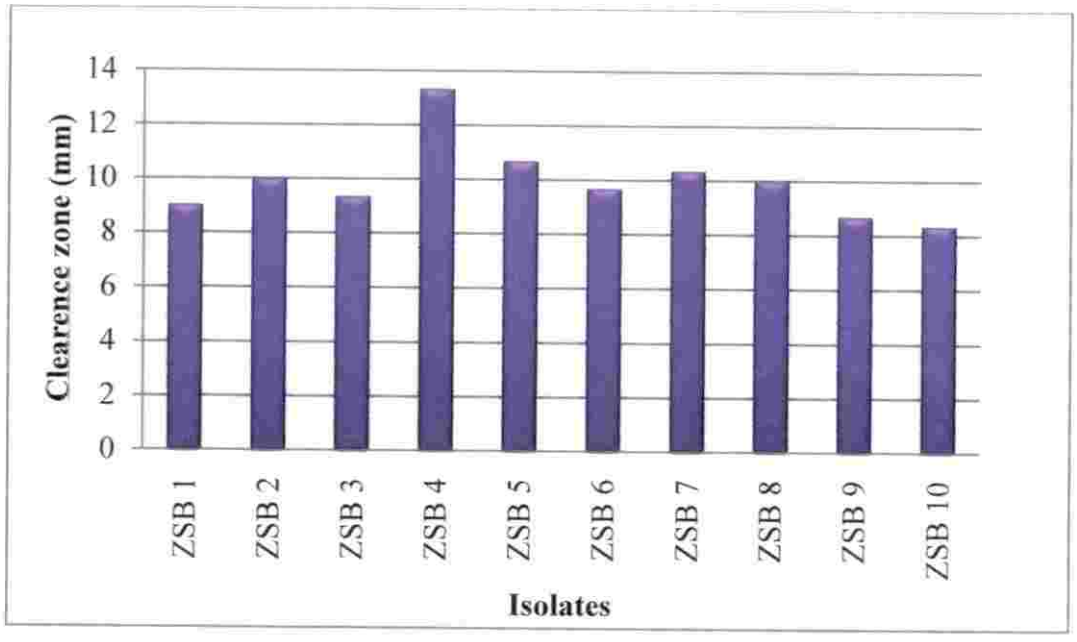


Fig.1. Zinc solubilizing potential of zinc solubilizing bacterial (ZSB) isolates with insoluble zinc oxide (Plate assay)

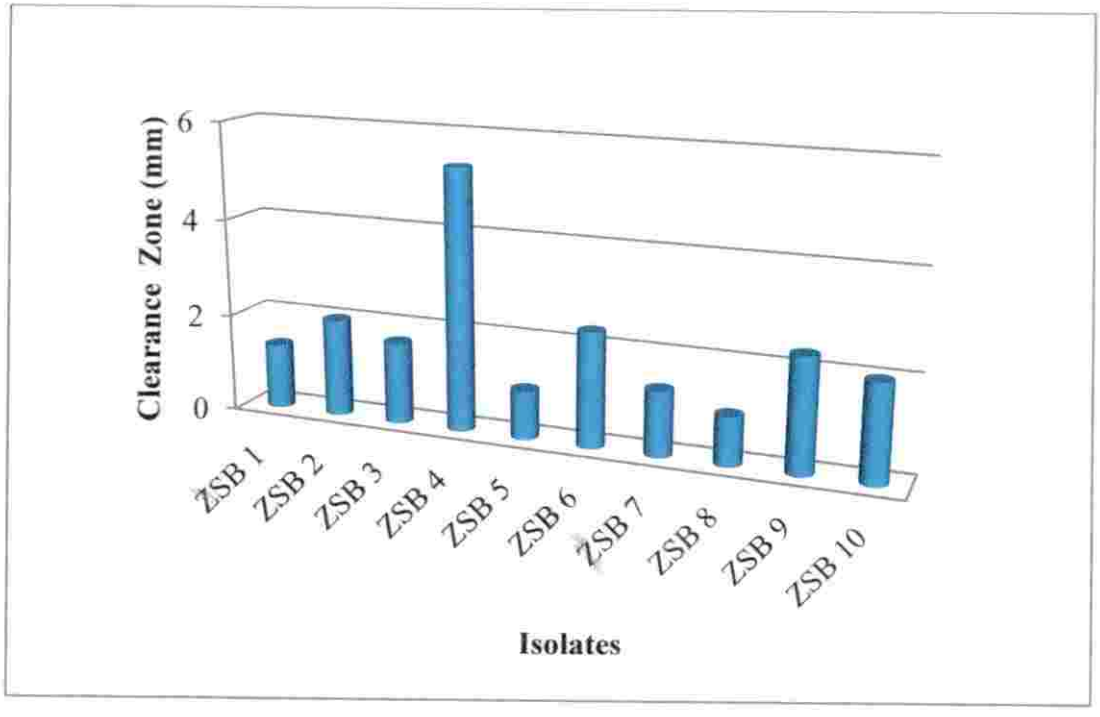


Fig. 2. Zinc solubilizing potential of zinc solubilizing bacterial (ZSB) isolates with insoluble zinc phosphate (Plate assay)

zinc compounds appreciably over other isolates in zinc oxide and zinc phosphate supplemented medium (Fig.3 and Fig.4). Similar results was reported by Desai *et al.* (2012) for organisms like *Pseudomonas, Azospirillum* and *Bacillus* with available zinc ranging from 9.46 ppm to 13.12 ppm on 9th day of incubation. This is in contrast to the finding of Ramesh *et al.* (2014), wherein these authors established that *Bacillus aryabhattai* strains efficiently solubilised the zinc compounds and zinc phosphate was effectively solubilised than zinc oxide. Their studies in broth assay recorded a release of available zinc up to 40.03 ppm and 153.47 ppm on Bunt and Rovira medium supplemented separately with two insoluble zinc compounds (zinc oxide and zinc phosphate) at 0.1% zinc concentration.

From the results, it appears that the solubilisation rate varied from one medium to another depending on the source of insoluble zinc. Among the zinc compounds, solubilisation of zinc was found to be higher in zinc oxide supplemented medium as compared to zinc phosphate. This was in agreement to the finding of Saravanan *et al.* (2007) wherein these authors reported that *Gluconacetobacter diazotrophicus* PAL5 readily solubilised the zinc compounds tested and zinc oxide was efficiently solubilised than zinc carbonate and zinc phosphate. This might be due to the more solubility of zinc oxide than zinc phosphate.

Organic acid production by microbial isolates has been reported to be a primary mechanism of solubilisation of insoluble minerals (Nguyen *et al.,* 1992; Fasim *et al.*, 2002). The studies conducted by Whiting *et al.* (1976) reported that conversion of glucose to gluconic acid by the glucose oxidative pathway in bacteria has been explained as a competitive approach by microorganisms. Similar findings were reported by Da Costa and Duta (2001) who stated that solubilisation of insoluble zinc compounds was dealt with production of organic acids and sequential release of zinc in external environment and bioaccumulation of zinc in the cells of bacterial species.

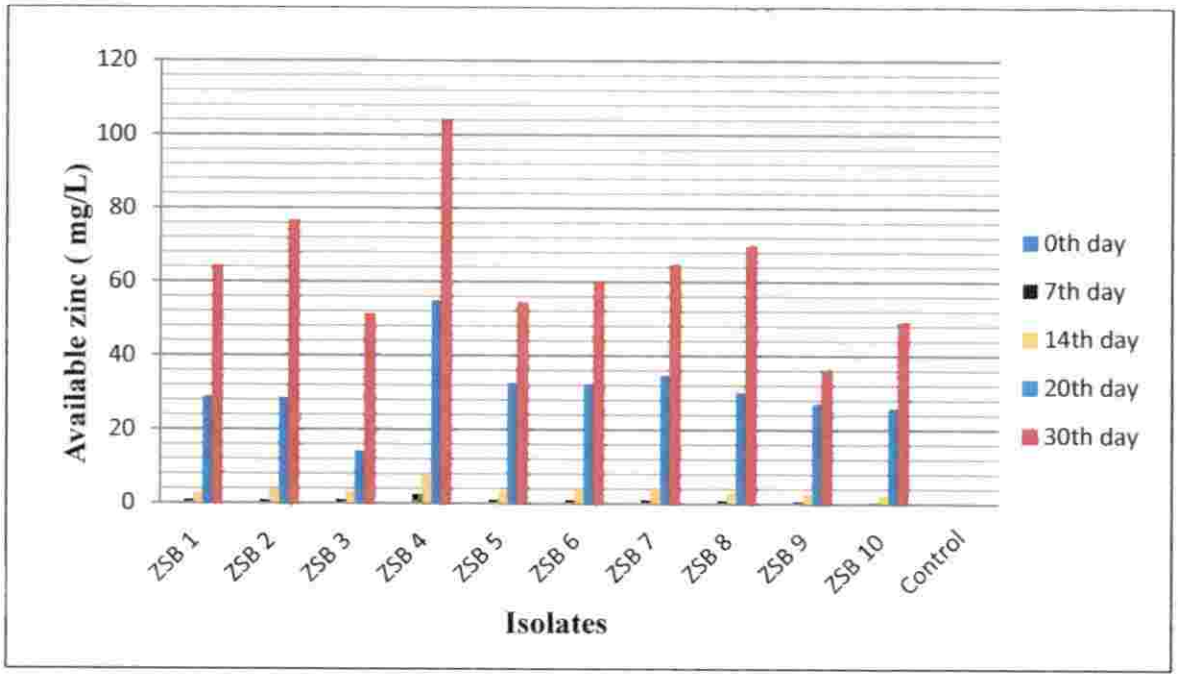


Fig. 3.Quantity of available zinc released at different intervals in zinc oxide medium

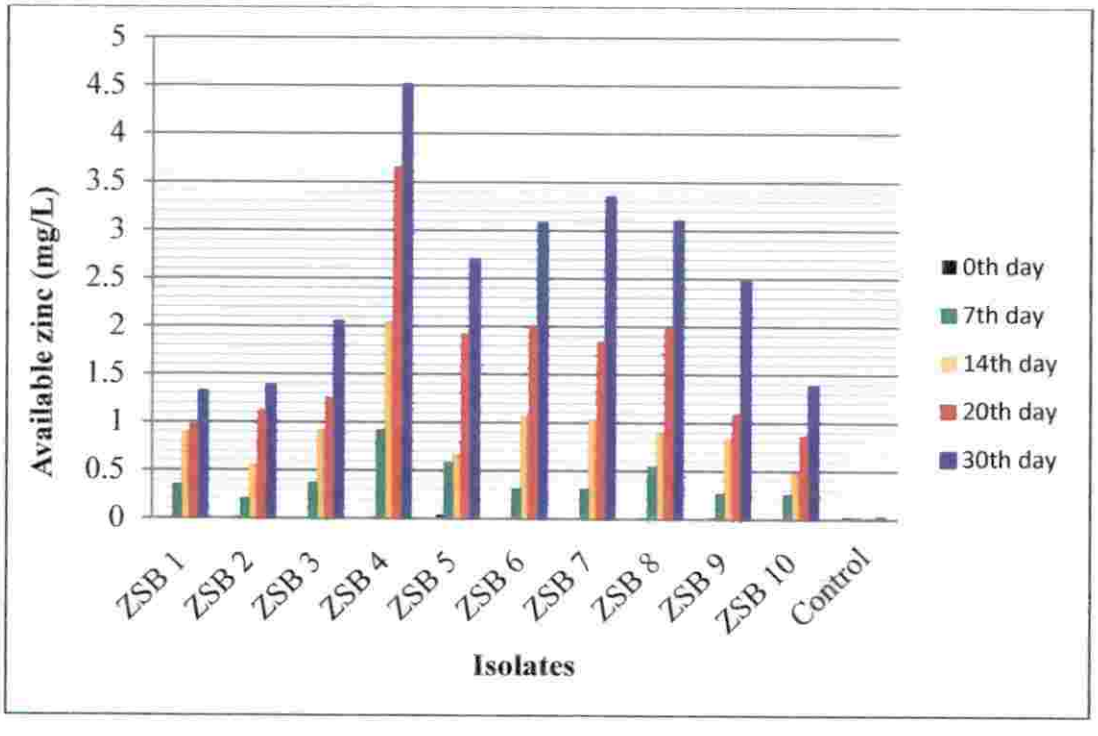


Fig.4. Quantity of available zinc released at different intervals in zinc phosphate medium

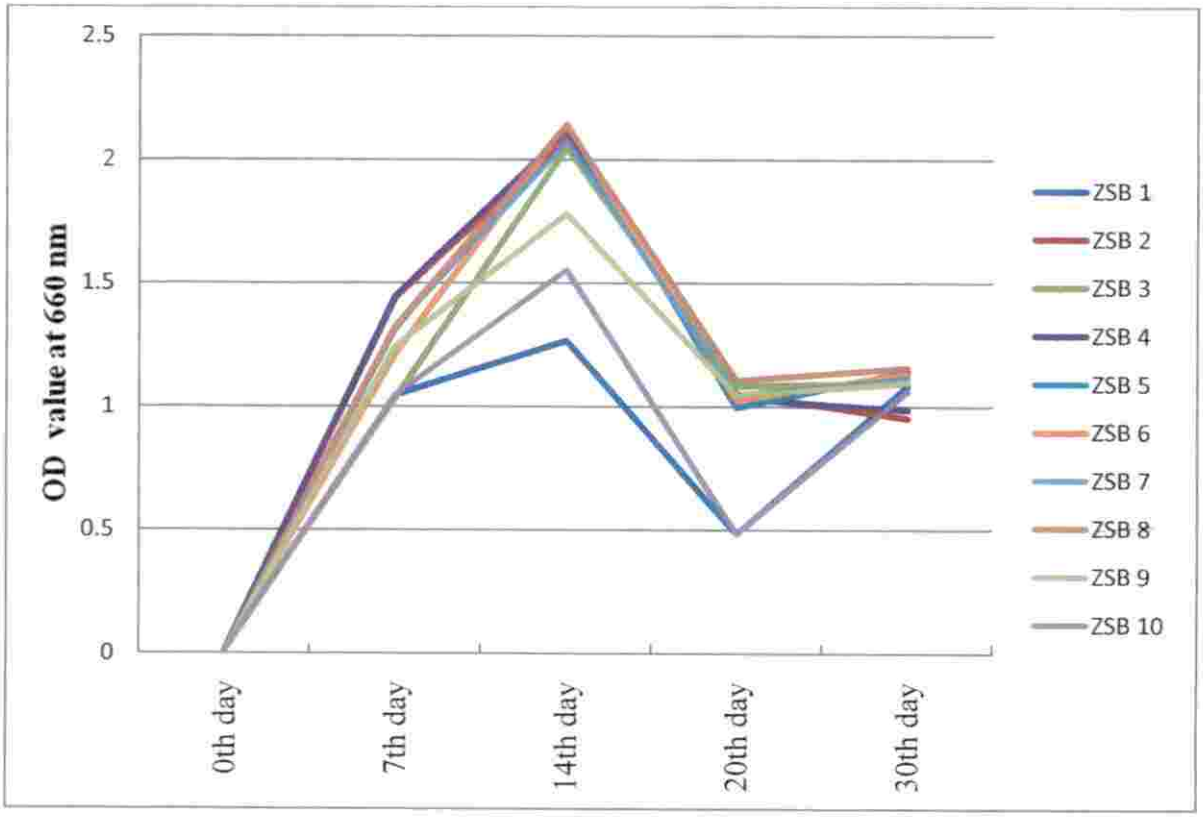


Fig. 5. OD value at 660nm at different intervals in zinc oxide medium

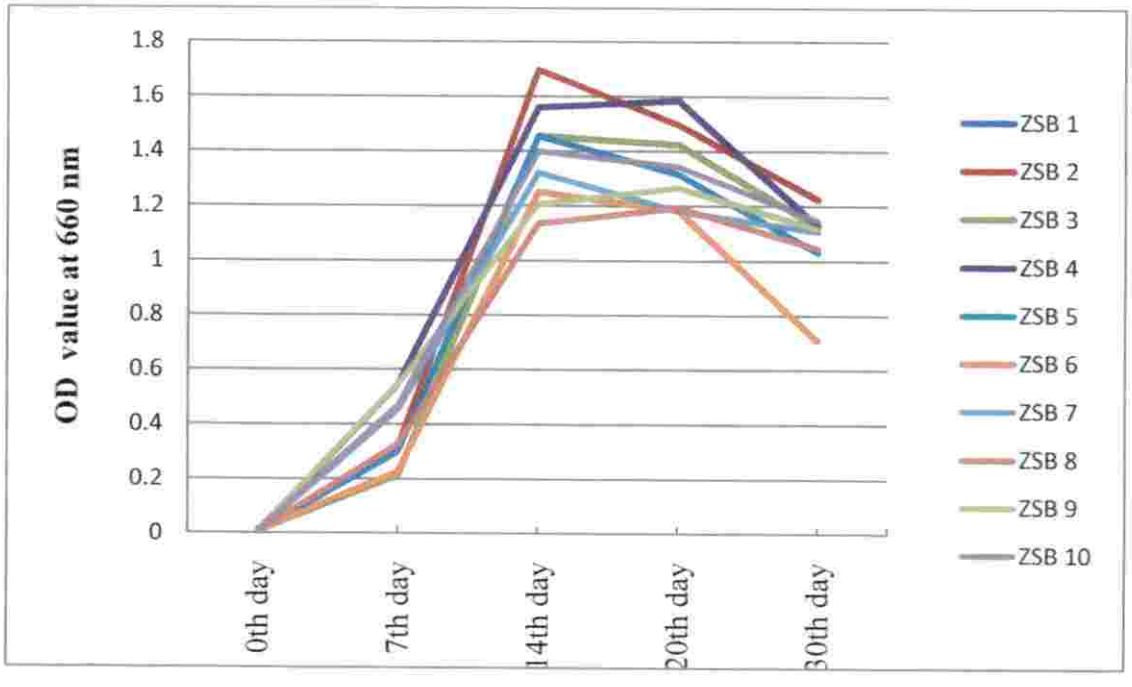


Fig. 6. OD value at 660nm at different intervals in zinc phosphate medium

The best isolate in the current study was identified as ZSB – 4 among the ten isolates and subjected to molecular characterization and the organism was identified as *Bacillus cereus*. The isolate was Gram positive, rod shaped and endospore forming.

It is well known that zinc solubilising capabilities tend to be widespread within bacterial taxa (Fasim *et al.*, 2002; Saravanan *et al.*, 2007; He *et al.*, 2010). The genus *Bacillus* is one of the well explored microorganism as they are found to be pervasive in nature and have multiple growth promoting characters (Kohler *et al.*, 2007; Ramirez and Kloepper, 2010; Zhao *et al.*, 2011).

In addition to zinc solubilisation, *Bacillus cerus* act as plant growth promoting rizhobacterium. *Bacillus cereus* was found active in changing phosphate solubility and solubilising several kinds of phosphate bonds so that phosphate could be made available easily and absorbed immediately by the plants (Zhao *et al.*, 2011). Karadeniz *et al.* (2006) observed that *Bacillus cereus* was able to produce several growth regulators like zeatin, kinetin, gibberellins and auxin. They observed that *Bacillus cereus* biofertilizers can be used as an alternative fertilizer in rice cultivation in dry land systems that are less fertile.

Sharma *et al.* (2013) stated that inoculation with *Bacillus firmus*, *Bacillus amyloliquefaciens*, *Bacillus* sp., and *Bacillus cereus* significantly increased zinc assimilation in soybean seeds as compared with the uninoculated control under microcosm conditions. Some *Bacillus* sp. posses a higher ability to bind heavy metals and a higher capacity to accumulate heavy metals to the cellular biomass.

It is inferred from Fig. 7 – 13, that the treatments showed varied effect on the soil chemical parameters for a period of three months when incubated under sterile conditions. The isolate ZSB – 4 significantly increased the available zinc content in soil from 0.55 ppm to 9.47 ppm in treatment T_4 (ZSB – 4 @ 2kg ha^{-1} + zinc oxide @ 1 kg ha^{-1}) during the incubation period. Similar results were also obtained by Vaid *et al.* (2014) and Sirohi *et al.* (2015) who reported an increase in DTPA extractable Zn in soils inoculated with bacterial strains. The inoculation with the phosphorus solubilizing bacteria *Bacillus* M-13 has shown significant

enhancement in manganese, zinc and copper contents in soil (Canbolat *et al.*, 2006).

From the data on Fig. 8, it is clear that available zinc levels in soil increased with the increase in incubation period. This might be due to the presence of more available zinc even after the absorption by the microbial cells. Da Costa and Duta (2001) studied the bioaccumulation of copper, zinc, cadmium and lead in the cells *of Bacillus* sp. *Bacillus cereus*, *Bacillus sphaericus* and *Bacillus subtilis*. This was in accordance with the findings of Coles *et al.* (2001) who reported that the complexing agents formed by microorganisms balanced the inorganic ions in the solution thereby preventing from precipitation or from being oxidized or reduced and converted to unavailable forms.

During the 3rd month, highest total zinc content was recorded by the treatment T_2 with 0.08%. Earlier studies have shown that the total zinc content was more in uninoculated control (Sarathambal *et al.*, 2010). The current observation may be due to more zinc available with solubilisation of insoluble zinc compounds by microorganisms in sterile soil.

It was observed that the soil pH showed no significant difference observed among treatments inoculated with zinc solubilising microorganisms (T_3, T_4, T_5) and for uninoculated treatments (T_1, T_2, T_6). This was in agreement with the findings of Iqbal *et al.*, (2010) wherein it was observed that there was no effect of bacterial inoculation in soil pH. Similar results were also reported by Canbolat *et al.* (2006) that the bacterial inoculation did not influence soil pH. This might be due to the buffering capacity of soil to withstand the change in soil pH.

It was found that, during the 2nd and 3rd month the treatments had significant increase in available phosphorus content especially for treatment T_4. Canbolat *et al.*, (2006) also reported that *Bacillus* M-13 and *Bacillus* RC01 inoculations enhanced the available phosphorus by 16.9% and 8.8% respectively. The studies conducted by Rodríguez and Fraga, (1999) have shown that *Pseudomonas striata* and *Bacillus polymyxa* solubilised 156 mg P L^{-1} and 116 mg

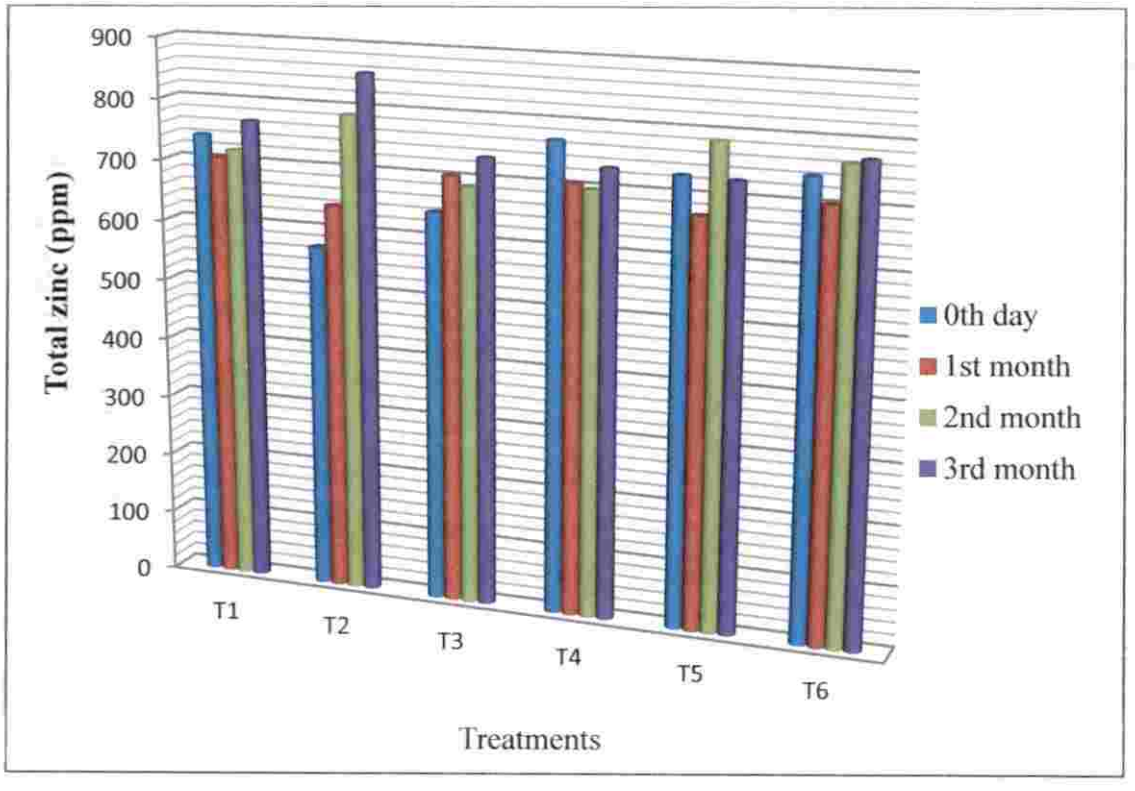


Fig.7. Total zinc in soil during the incubation

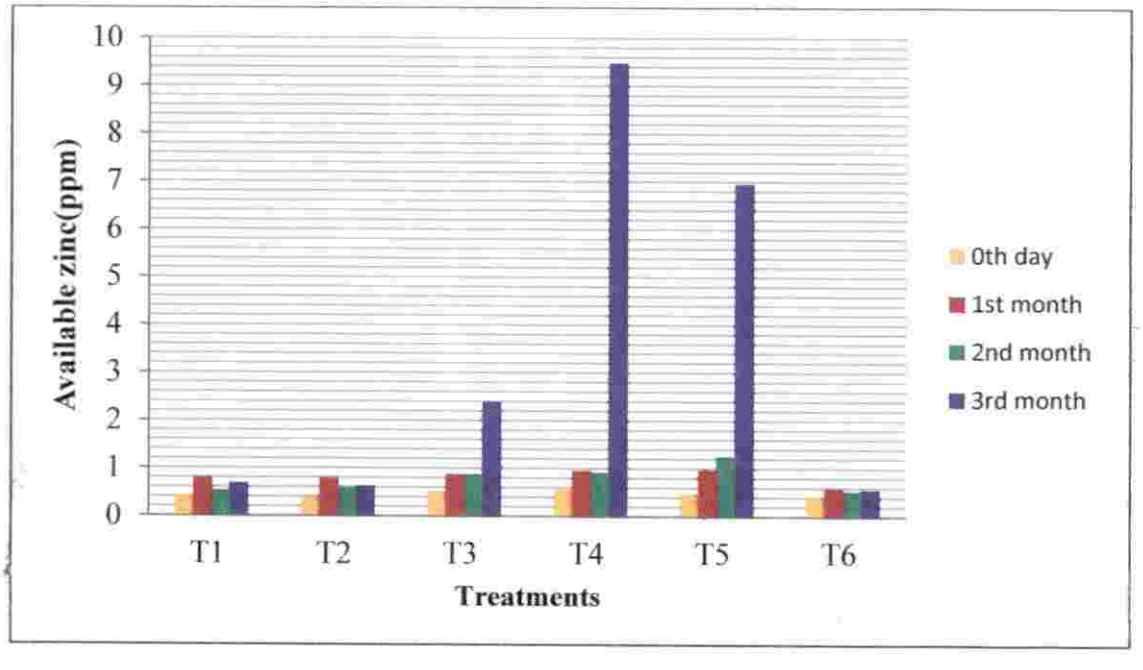


Fig. 8. Available zinc in soil during the incubation

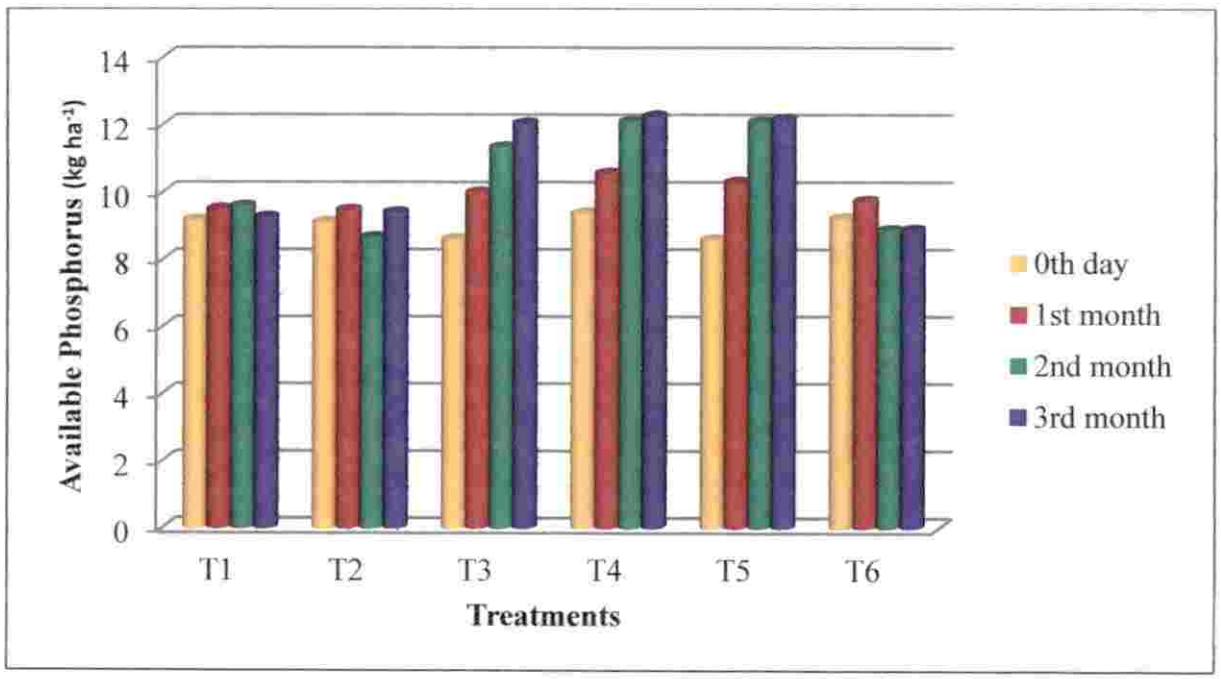


Fig. 9. Available phosphorus in soil during the incubation

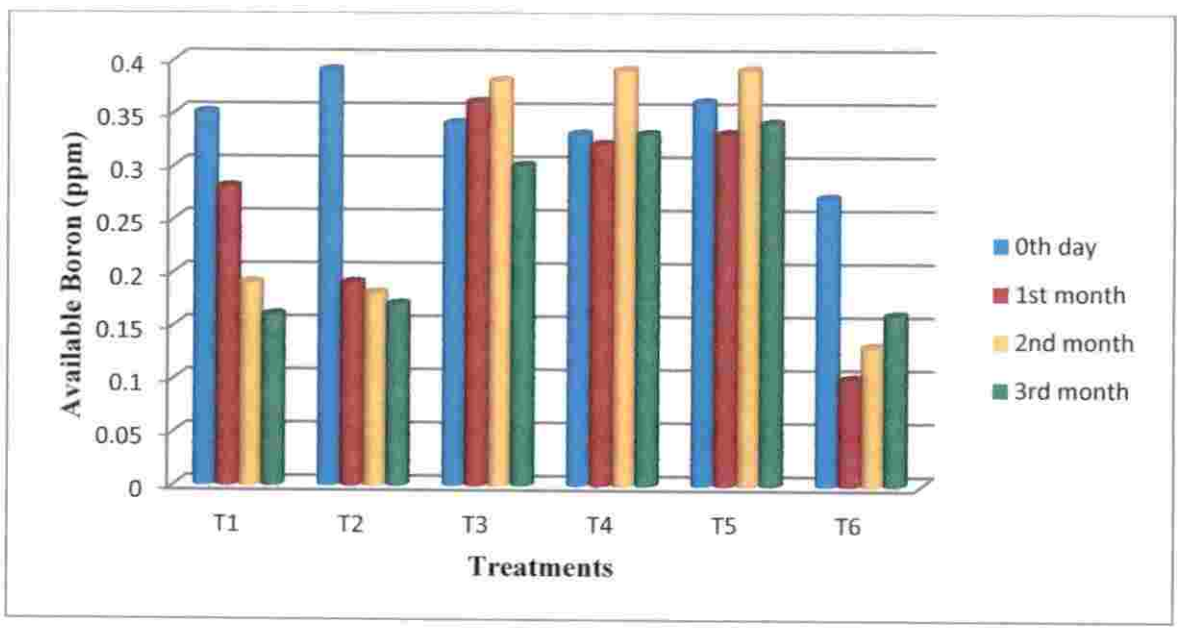


Fig. 10. Available boron in soil during the incubation

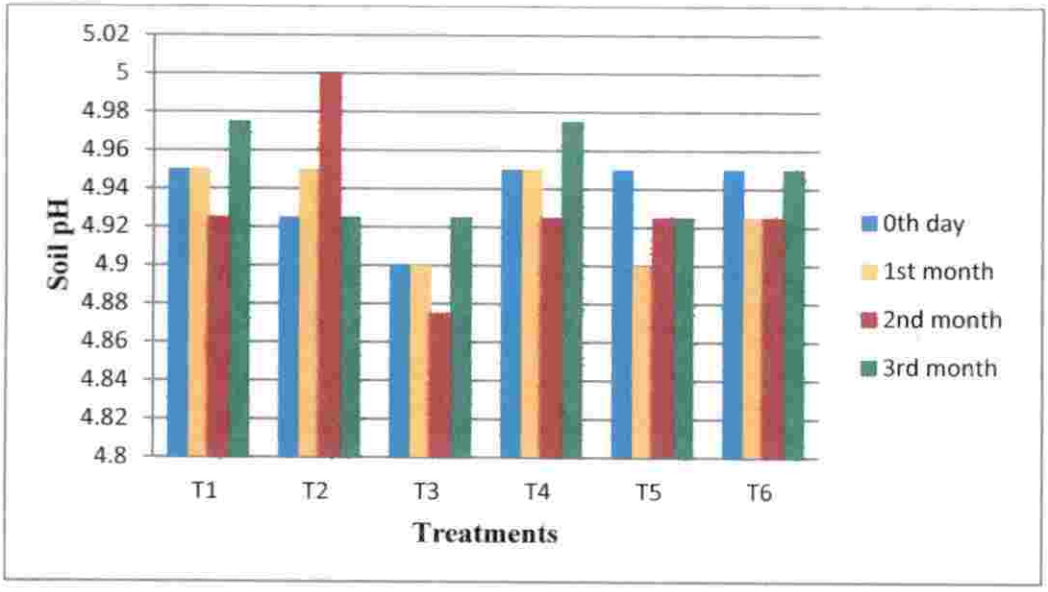


Fig. 11. Soil pH at monthly intervals during the incubation

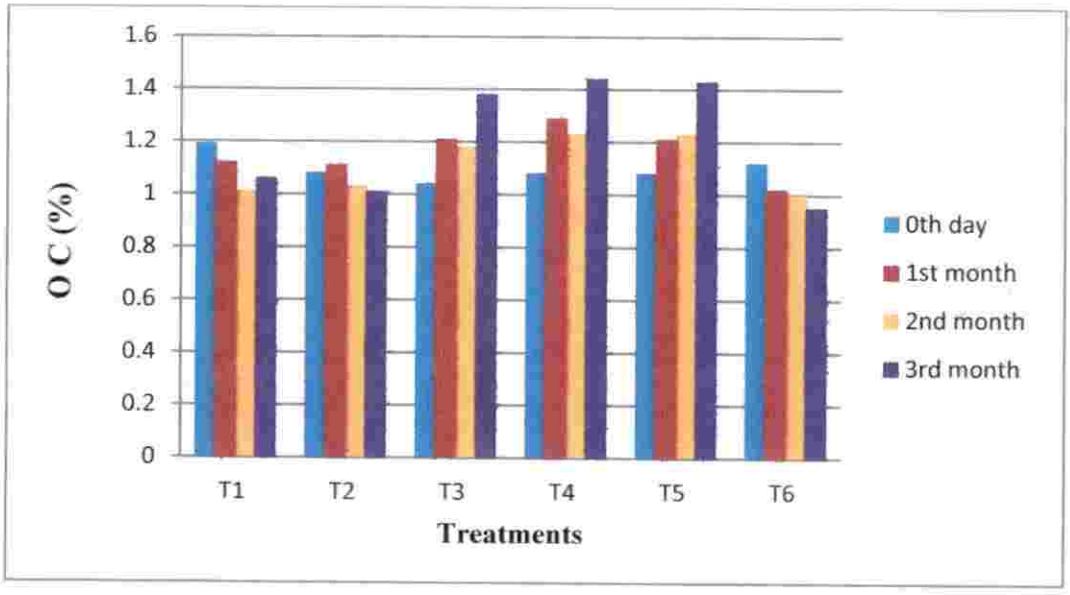


Fig. 12. Oxidisable organic carbon content in soil during the incubation

P L^{-1}, respectively. This might be due to the liberation of organic acids by the microorganisms which not only solubilised insoluble forms of zinc but also other insoluble sources of phosphorus and boron in the soil. There was a slight decrease in available boron content during the entire period of incubation and this might be due to the antagonistic effect of zinc and boron.

The practical implication of inoculation in soil is based on the maintenance of effective cell population in rhizosphere (Viveros *et al.,* 2010). The population of zinc solubilising microorganism was significantly increased during the incubation period. From Fig.13, it was implied that the maximum colony count in soil was recorded in the treatment T_4 during the 3rd month which was on par with treatments T_5 and T_3. Similar results were reported by Sirohi *et al.* (2015) that the strain of *P. fluorescens* was able to maintain the cell density of approximately 10^8 cfu g^{-1} for 4 weeks in soil. However, the soil generally acts as a "biological buffer" (Bashan, 1998) and hence, any difference in the composition and abundance of microbial population can be temporary.

Carrier materials viz., talc, lignite, vermiculite, vermicompost and perlite were used in the current study to assess shelf life of *Bacillus cereus* (Fig.14). The physico-chemical characters of carrier materials have got profound effect on the survival of microorganisms. The optimal characteristics of an inoculant carrier includes high water holding capacity, more surface area, richness in organic matter, neutral pH, easy availability and inexpensiveness (Gade *et al.,* 2014).

Among the different carriers tested, talc powder supported the maximum population of 3.9 x 10^8 cfu g^{-1} during the 3rd month. Studies conducted by Gade *et al.* (2014) were also in agreement with the above results and talc powder sustained the highest population of 18.00×10^8cfu g^{-1} for *P. fluorescens* at 180 days of storage. The results of this study are in accordance with the findings of Suryadi *et al.* (2013) who stated that the viability of *P. aeruginosa* lowered from 2.39 % to 18.30 % among various bioformulations and concluded that talc based formulation was stable during storage with no loss in viability.

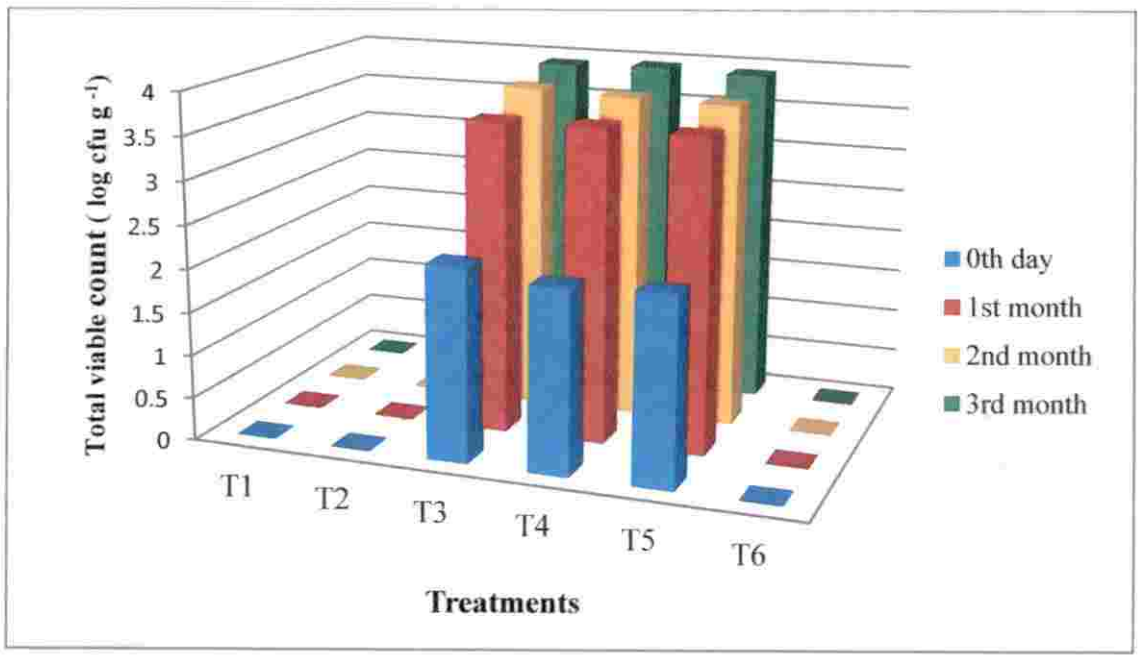


Fig. 13. Population of zinc solubilising microorganismsin soil during the incubation

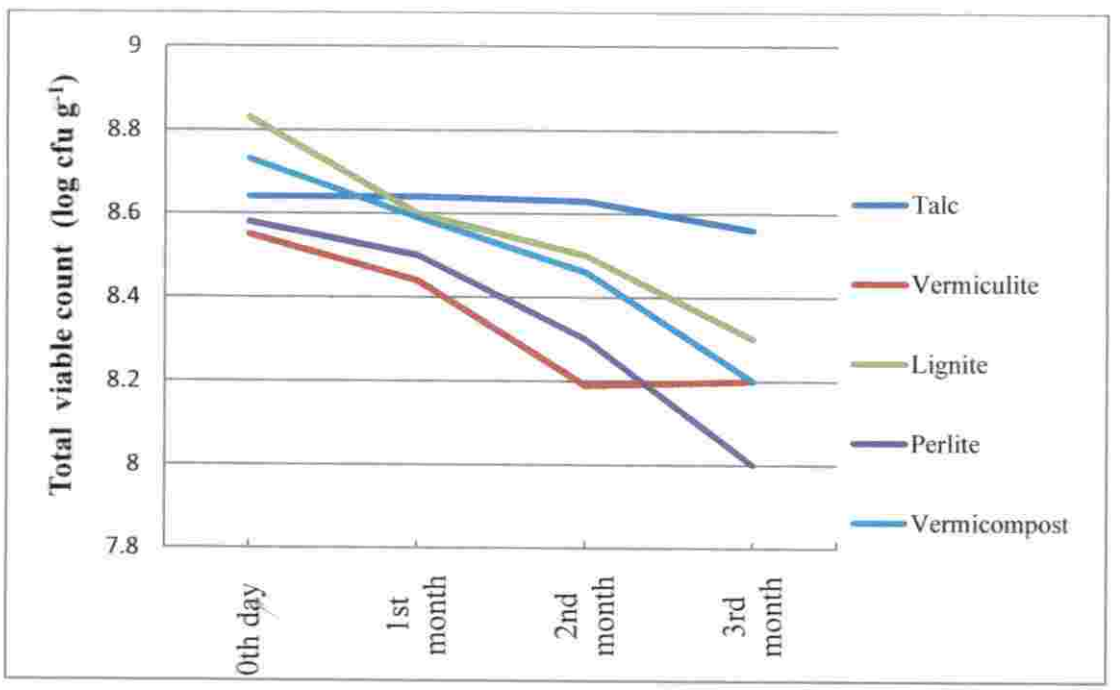


Fig. 14. Shelf life of zinc solubilising microorganisms in different carrier materials

Rajalaxmi *et al.* (2012) observed that talc based formulations of *P. fluorescens* retains a population of 11×10^{7}cfu g^{-1} at 300 days of storage. The studies conducted by Sivkumar *et al.* (2000) reported that talc supported the high population level of 18.3×10^{7} cfu g^{-1} after forty days of storage among the three carriers for the survival of *P. fluorescens*. Related findings were also reported by Mythukumar (2009), Prathuangwong *et al.* (2013) and Chenna *et al.* (2013) in respect of *Pseudomonas fluorescens* in talc based formulations. This might be due to the maximum water holding capacity and low bulk density of talc.

Talc has been traditionally used as carrier materials for effective bacterial formulations because constant bacterial populations have been observed for 240 days in talc based formulations (Vidhyasekaran and Muthamilan, 1995). The frequently used formulations involve strains of *Pseudomonas fluorescens, P. aeruginosa, P. putida, B. subtilis* and *B. amyloliquifaciens*. Dey *et al.* (2014) reported that *Pseudomonas putida* strains 30 and 180 have survived for 6 months in talc-based formulations.

Talc used in this present study is a natural product composed of several minerals in combination with chloride and carbonate (Nakkeeran *et al.*, 2005). Talc has very low moisture equilibrium, relative hydrophobicity and less moisture absorption. Furthermore, its properties to prevent the hydrate bridges formation has enabled for longer period of storage. The chemically inert nature and easy availability of talc make it a regularly used carrier for development of formulation (Kloepper and Schroth, 1981). The observations of this study are in agreement with earlier results in talc-based formulations for *B. subtilis* (78.13 %) (El-Hassan and Gowen, 2006) and *B. megaterium* (68 %) (Omer, 2010) after 180 days of storage at room temperature.

Summary

6. SUMMARY

Zinc is one of the indespensable micronutrients required for optimum growth of plants. After application of zinc fertilizers, a substantial quantity of inorganic zinc in soil is converted into unavailable forms. The external application of zinc as fertilizer in crop production increases cost of cultivation. The zinc in soil in different form may be unavailable to plants, but it can be made available by inoculating zinc solubilizing bacterial species as an inoculant. The selection and inoculation of zinc solubilizing bacteria along with low cost sources of insoluble zinc compounds, like zinc oxide, will aid in saving loss in crop cultivation.

In this context, isolation and identification of effective zinc solubilising bacteria for improving zinc availability as well as saving soil fertility is considered as an important aspect in sustainable crop production. The present programme was ensivaged to isolate, screen and evaluate zinc solubilizing microorganisms and to develop inoculant cultures of zinc solubilizing isolates from soils of Kerala. The salient results of the study are summarized below.

Ten isolates of bacteria solubilising insoluble forms of zinc (zinc oxide) was isolated from zinc deficient areas such as Agroecological units 20, 21 (Wayanad) and 8 (Thiruvananthapuram) by serial dilution technique on zinc solubilising medium containing 0.1% insoluble zinc oxide. These isolates were allotted code numbers from ZSB1- ZSB10. All the ten isolates when subjected to plate assay and broth assay effectively solubilized the insoluble Zn compounds used, namely, zinc oxide and zinc phosphate. The zone of solubilisation was comparatively high in zinc oxide amended medium when compared to zinc phosphate in plate assay and it ranged from 8.67 mm to13.33 mm in zinc oxide and from 1.00 mm to 5.33 mm in zinc phosphate incorporated medium. The maximum zone of clearance of 13.33 mm was registered with the isolate ZSB - 4 which was significantly superior to all other isolates. The assessment of quantity of available zinc in mg L^{-1} released at different intervals in zinc oxide and zinc phosphate amended medium revealed that the maximum solubilisation of zinc in

both sources was observed on 30th day with a range of 35.91 ppm to 104.08 ppm in zinc oxide supplemented medium and 1.38 ppm to 4.15 ppm in zinc phosphate supplemented medium, with highest release of zinc in broth assay by the isolate ZSB – 4. The indirect assessment of growth of zinc solubilising bacteria in zinc oxide revealed that maximum OD value was obtained for all the isolates on 14th day after inoculation in zinc oxide supplemented medium. On 20th and 30th day after inoculation there was a decrease in OD value for all the ten isolates and there was no significant difference among the isolates when grown in zinc oxide supplemented medium and zinc phosphate supplemented medium.

The selected isolate ZSB – 4 which was capable of solubilising insoluble forms of zinc were characterized based on morphological and molecular studies. The isolate ZSB – 4 was identified as *Bacillus cereus*.

With the objective of developing formulation, the selected isolate was inoculated in different carriers like talc, lignite, vermiculite, perlite and vermicompost. Talc was found to be significantly superior to all other carriers with highest population count of 4.6 x 10^8 cfu g^{-1} and 3.9 x 10^8 cfu g^{-1} during the 2nd and 3rd month respectively and was followed by lignite (3.6 x 10^8 cfu g^{-1}, 2.9 x 10^8 cfu g^{-1}), vermicompost (3.2 x 10^8 cfu g^{-1}, 3.6 x 10^8 cfu g^{-1}), vermiculite (1.8 x 10^8 cfu g^{-1}, 1.8 x 10^8 cfu g^{-1}) and perlite (2.1 x 10^8 cfu g^{-1}, 1.1 x 10^8 cfu g^{-1}).

Soil incubation study was conducted with the best isolate ZSB – 4. When formulated product of zinc solubilising culture @ 2 kg ha $^{-1}$, supplemented with 1 kg Zn as zinc oxide were applied, there was an increase in the available zinc content of the soil from 0.55 ppm to 9.47 ppm during the incubation period. The same treatment recorded the highest mean value for available phosphorus content, 12.09 kg ha^{-1} and 12.26 kg ha^{-1} respectively for the 2nd and 3rd month. There was no significant difference in soil pH among treatments inoculated with zinc solubilising microorganisms and uninoculated treatments. The mean values of total zinc did not differ significantly among treatments. The total zinc content of the soil ranged from 0.05 % to 0.08 % during the entire three months. The mean

values for the available boron content ranged from 0.16 ppm to 0.34 ppm. There was an increase in organic carbon content for 1st, 2nd and 3rd month for treatments inoculated with zinc solubilising bacteria and highest amount of oxidisable organic content was registered during the 3rd month for treatment T_4 (zinc solubilising culture @ 2 kg ha $^{-1}$ supplemented with 1 kg Zn as zinc oxide). The absolute control registered lowest mean value of 1.02%, 1.00% and 0.95% during the 1st, 2nd and 3rd month respectively. The population of zinc solubilising microorganisms significantly increased during the incubation period. On the day of inoculation, the viable colony count ranged from 1.37 x 10^2 cfu g^{-1} to 1.87 x 10^2 cfu g^{-1} of soil. The maximum colony count of 9.3 x 10^3 cfu g^{-1} of soil was recorded during the 3rd month when the zinc solubilising culture @ 2 kg ha $^{-1}$ supplemented with 1 kg Zn as zinc oxide was applied.

From the present investigation, based on zinc solubilizing potentiality, among the ten zinc solubilizing isolates, *Bacillus cereus* ZSB-4 was selected as elite zinc solubilizer which was capable of making zinc available from unavailable zinc sources such as zinc oxide and zinc phosphate. Even though the isolate ZSB - 4 showed better results in laboratory level, further it should be tested by conducting field experiments to confirm its performance.

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Appendices

8. APPENDIX - I

8.1.MEDIA COMPOSITION

8.1.1. Nutrient agar

Glucose	- 5.0 g
Peptone	- 5.0 g
Beef Extract	- 3.0 g
NaCl	- 5.0 g
Agar	- 20.0 g
Distilled water	-1000 ml
pH	-7.0

8.1.2. Bunt and Rovira medium

Glucose	- 20.0 g
Peptone	-1.0 g
Yeast Extract	-1.0 g
$(NH_4)_2 SO_4$	- 0.5 g
K_2HPO_4	- 0.4 g
$MgCl_2$	-0.1 g
$FeCl_3$	-0.01 g
Agar	-20.0 g
Distilled Water	-1000 ml
pH	- 6.6–7.0

Abstract

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DEVELOPMENT OF INOCULANT CULTURES OF ZINC SOLUBILIZING MICROORGANISMS

AATHIRA S. KUMAR

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ABSTRACT

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COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA

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ABSTRACT

The study entitled "Development of inoculant cultures of zinc solubilising microorganisms" was conducted at College of Agriculture, Vellayani during the period 2014 – 2016 with the objective of isolation, characterization and evaluation of zinc solubilising microorganisms from soils of Kerala and to develop inoculant culture of the best zinc solubilising isolate.

Microorganisms capable of solubilising zinc were isolated from Agroecological units 20, 21 (Wayanad) and 8 (Thiruvananthapuram) by serial dilution technique on Bunt and Rovira medium containing 0.1% insoluble zinc oxide. Ten isolates of bacteria capable of solubilising insoluble forms of zinc (zinc oxide) were obtained and allotted code numbers from ZSB-1 to ZSB-10. These were subjected to plate assay and broth assay in media supplemented with 0.1 per cent insoluble forms of zinc as zinc oxide or zinc phosphate. After three days of incubation of test plates, all the ten isolates solubilised zinc and produced clearing zone around the colonies on solid media. The size of the solubilisation zone ranged from 8.67 mm to13.33 mm in zinc oxide and from 1.00 mm to 5.33 mm in zinc phosphate incorporated medium. In broth culture, maximum solubilisation of zinc in both sources was observed on 30th day in the range of 35.91 ppm to 104.08 ppm in zinc oxide supplemented medium and 1.38 ppm to 4.15 ppm in zinc phosphate supplemented medium. The isolate ZSB – 4 showed maximum solubilisation of zinc in plate assay and broth assay.

For soil incubation study, the isolate with maximum zinc solubilisation (ZSB – 4) was inoculated in zinc deficient soils and analysed for the soil chemical parameters and population dynamics of the bacterial isolate for a period of three months. The treatments were designed as T_1 and T_2 with Zn at two levels as ZnO, T_3 with Zn solubilising culture alone @ 2 kg ha^{-1}, T_4 and T_5 with Zn solubilising culture @ 2 kg ha^{-1} supplemented with Zn at two levels as ZnO and T6, the absolute control had no insoluble zinc supplementation and inoculation with the bacterial isolate. The isolate ZSB – 4 significantly increased the available zinc content in soil from 0.55 ppm to 9.47 ppm in treatment T_4 (ZSB – 4 @ 2 kg ha^{-1} + zinc oxide @ 1 kg ha^{-1}) during the incubation period. The same treatment registered the highest mean value for available phosphorus content, 12.09 kg ha^{-1} and 12.26 kg ha^{-1} respectively for the 2nd and 3rd month. There was an increase in oxidisable organic carbon content in the 3rd month for treatments T_4 (1.44%),

T_5 (1.43%) and T_3 (1.38%) and it was maximum in T_4 which was statistically on par with T_3 and T_5. On the 3rd month, there was a decrease in the available boron content in soil for the treatments T_3 (0.30 ppm) and T_5 (0.34 ppm) when compared with previous months and the treatment T_4 (0.33) maintained the level of boron. The total zinc content of the soil ranged from 0.05 % to 0.08 % during the entire three months and the mean values had no significant difference among treatments. The maximum colony count of 9.3×10^3 cfu g^{-1} of soil was recorded in the treatment T_4 during the 3rd month which was on par with T_5 (9.1×10^3 cfu g^{-1}) and T_3 (8.8×10^3 cfu g^{-1}).

The best isolate ZSB – 4 was subjected to molecular characterization and it was revealed that the organism is *Bacillus cereus*. Different carrier materials like talc, lignite, vermiculite, vermicompost and perlite were used in the present study to assess survival of ZSB – 4. Among the different carriers tested, the talc powder supported the maximum population of 3.9×10^8 cfu g^{-1} during the 3rd month.

Based on the results of present study it can be concluded that application of talc based formulation of the zinc solubilising bacteria ZSB – 4 (*Bacillus cereus*) @ 2 kg ha^{-1} along with zinc oxide @ 1 kg ha^{-1} was found to increase zinc content in soil after incubation. Based on the survival of the isolates in different carriers for the development of inoculant cultures, talc was found to be most suitable carrier for the formulated product.