Isolation, screening and identification of Zinc solubilizing bacteria from paddy fields

Syed jawad ul haq

Msc. Agriculture Microbiology
Sher-e-Kashmir
University of Agricultural Sciences and Technology of Kashmir India

***Abstract*- In the present investigation, There were 60 composite Zinc Solubilizing isolates obtained from paddy rhizosphere soil samples using the zinc growth medium supplemented with insoluble source of zinc such as zinc oxide (ZnO) and zinc carbonate ($ZncO_3$) individually. Among the ZSB isolates, There was maximum zinc Solubilizing halo zone observed with the isolate Znsb (Bara)S58 followed by Znsb(Bara)S41 with diameter of 24mm,5mm and 23mm,7mmrespectively.The isolate Znsb (Bara)S58 showed maximum solubilsing efficiency of 4.8% and solubilised 12.631ppm zinc after 10 days of incubation. Among all the 20 outstanding isolates, sixteen (16) were Gram positive rods belonging to *Bacillus* genera and four (4) were gram negative short rods belonging to *Pseudomonas.* Physico-chemical properties of paddy rhizosphere soil samples from Baramulla district like, the p^H ranged from 6.17 to 6.79. Electrical conductivity ranged from 0.101 to 0.164 dsm^{-1}, Organic carbon ranged from 0.44 to 14.3%. Available nitrogen content of the soils ranged from \296.42 to 627.07$kgha^{-1}$. Available zinc content of the soil samples ranged from 1.98 to 2.972 $ppmha^{-1}$. Status of biological properties of the rhizosphere soils from Baramulla district was as., Population of total viable bacteria ranged from 50-89 $cfug^{-1}$soil. Population of total viable potassium solubilizing bacteria ranged from 15 to 29 $cfug^{-1}$ soil. Population of total viable phosphate solubilizing bacteria ranged from 10 to 24$cfug^{-1}$soil. Population of total viable zinc solubilizing bacteria ranged from 10 to 22$cfug^{-1}$ soil respectively. The biochemical characteristic of Znsb (Bara) S53 and Znsb (Bara)S58 was carried out through utilization of catalase, starch, gelatin and hydrogen sulphide sources. The results of utilization of these sources confirmed the isolates as *Bacillus* sp and *Pseudomonas* sp. The strain Znsb(Bara)S53was identified as *Bacillus cereus by* 16SrRNA gene sequencing.**

***Keywords*: paddy, zinc Solubilizing bacteria, bacillus, ZnO, $ZncO_3$.**

1.Introduction

Microorganisms represent the major source of genetic diversity on earth. The prominence of microorganisms is due to their high metabolic rate which allows the conclusion about their potential for biotechnological applications including enzyme production for industrial and environmental uses by improving nutrient availability and use efficiency. In the present techno economic era, the multiplying population is exerting immense pressure on agricultural lands for higher crop yields which results in ever increasing use of chemical fertilizers. Consequently, there has recently arisen renewed interest in environmental friendly agricultural practices (Karlidag *et al.*, 2007). Long term studies show that intensive application of chemical fertilizer lead to reduction in crop production. This reduction is mainly due to increasing soil acidity, decreasing biological activities, change in soil characteristics and diminished micro elements.

Micronutrients are important for the optimum growth and productivity of the plants. Though these elements are required in critical amounts, they are very important to plant work behind the scene as activators of many plant functions. Zinc is an essential micronutrient that plays a vital role in various metabolic processes in plants and its deficiency adversely affects the growth and development of crop plants (Cakmak, 2008). More than 50% of Indian soils are deficient in zinc and warrents remedial measures to increase the zinc availability in these soils and sustain the growth in agriculture production. The available zinc content in Indian soils is low; however, the total zinc content is substantially high and exists in fixed forms such as smithsonite

($ZnCO_3$), sphalerite (ZnS), Zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite ($ZnSiO_4$) and hopeite ($Zn_3(PO_4)_2.4H_2O$). Zinc is a trace element found in varing concentrations in all soils, plants and animals and it is essential for the normal healthy growth of higher plants, animals and humans. Zinc is needed in small but critical concentrations and if the amount available is not adequate, plants and/or animals will suffer from physiological stress brought about by the dysfunction of several enzyme systems and other metabolic functions in which zinc plays a part.
The essentiality of zinc for plants was only scientifically established about 70 years ago and in some parts of the world the existence of deficiencies has only been recognized during the last 20 or 30 years. The relatively recent discovery of widespread zinc deficiency problems in rice and wheat is linked to the intensification of farming in many developing countries. This has involved a change from traditional agriculture with locally adopted crop genotypes and low inputs of nutrients, to growing modern high yielding plant varieties with relatively large amounts of micronutrient fertilizers and agrochemicals. Many of the new crop varieties are much more susceptible to zinc deficiency than the traditional crops and the increased use of macronutrient fertilizers, especially phosphorous can render a deficiency of zinc more likely. To increase food production by increasing the productivity of the land is essential in developing and developed countries.

Many bacterial enzymes contain zinc in the active center or in a structurally important site (Clarke and Berg, 1998). Zinc solubilizing potential of few bacterial genera has been studied. Hutchins *et al.*, (1986) reported that *Thiobacillus thioxidans*,

Thiobacillus ferroxidans and facultative thermophilic iron oxidizers solubilize zinc from sulphide ore (sphalerite). A few fungal genera possess immense potential of solubilizing zinc tolerating a high zinc level. *Aspergillus Niger* was found to grow fewer than 1000 mg zinc and this fungus is used to quantify zinc in soils containing low zinc (Bullen and Kemila, 1997). Exogenous application of soluble zinc sources, similar to fertilizer application, has been advocated to various crops. This causes transformation of about 96-99 percent of applied available zinc to various unavailable forms. This zinc thus made unavailable can be reverted back to available form by inoculating a bacterial strain capable of solubilizing it (Saravanan *et al.,* 2003).Since zinc is a limiting factor in crop production in alkaline and calcareous soils of the world. This study on zinc solubilization by bacteria has an immense importance in zinc nutrition of world wide. According to an estimate made under All India Coordinated Research Project (AICRP) on Micro and Secondary Nutrients and Polluted Elements in Soils and Plants, 49% of tested soil samples were found deficient in zinc (Singh, 2001). According to Cakmak (2009) 50% of the Indian soils under intensive cultivation of wheat and rice are deficient in plant available zinc. The deficiency of zinc in soils is usually attributed to low solubility of zinc rather than low total content of zinc in most agricultural soils. According to a current statistics, overall under nourished people in the world are estimated to be 1.02 billion (FAO, 2009). Hunger and malnutrition are the underlying causes of more than half of all the children's death, killing nearly 6 million children each year (FAO, 2005). To over come Zn deficiency constraint in plants is by the application of microbial inoculants as a biofertilizer. Recently bacterial based approach has been devised to solve these micronutrient deficiency problems (Anthoniadis, 2002).These bacterial isolates play a predominant role in the solubilization, transport and deposition of metals and minerals in the environment. Microorganisims play a major role in the transformation of unavailable form of metal to available form depending upon the reactions involved and the products formed (Lovely, 1991). The secretion of organic acids appear to be the functional metal resistance mechanism that chelates the metal ions extracellularly (Sunitha *et al*., 2014). Zinc (Zn) is an essential micronutrient and has particular physiological functions in all living systems, such as the maintenance of structural and functional integrity of biological membranes and facilitation of protein synthesis and gene expression. Among all metals, Zn is needed by the largest number of proteins. Zinc-binding proteins make up nearly 10 % of the proteomes in eukaryotic cells and 36% of the eukaryotic Zn-proteins are involved in gene expression (Andreini *et al.,* 2006). Zinc is an important component of enzymes that drive and increase the rate of many important metabolic reactions involved in crop growth and development. It exerts a great influence on basic plant life processes such as N_2 metabolism and uptake of N_2 and protein quality; photosynthesis and chlorophyll synthesis, resistance to abiotic and biotic stresses and protection against oxidative damage. When the supply of zinc to the plant is inadequate, crop yields and the quality of production will be adversely affected. Thus for proper function of crop plants a certain minimum level of zinc supply is essential. Certain groups of micro-organisms including bacteria, fungi and actinomycetes are known to solubilize zinc compounds into soluble form which can be utilized by the plants. Since the literature pertaining to solubilization of zinc compounds by bacteria is very scanty. The available literature on zinc solubilization by bacteria and their mechanisms of solubilization, other beneficial traits and their agronomic importance is reviewed in this chapter.

Zinc (Zn) deficiency is a serious constraint to rice production in many parts of the world and this could only be compensated by the application of costly chemical fertilizers either as foliar or soil application (Reyes and Brinkman 1989). Alternatively, numerous microorganisms especially those associated with roots have the ability to increase plant growth and productivity (Rodriguiz *et al*., 2004) by increasing the supply of mineral nutrients of low mobility in the soil like P, Zn and Cu (Thompson, 1996). Among these microorganisms a group of bacteria referred as Plant Growth Promoting Rhizobacteria (PGPR) are involved in nutrient cycling (Glick, 1995). The special focus on Zn solubilizer is due to the fact that zinc is one of the essential micronutrients required by all crops. It is a key element in many physiological and biochemical processes. Mineral zinc solubilisation by microbes directly enhances crop growth and yield. Zinc solubilising bacteria are capable of solubilizing various complex compounds of zinc like ZnO, $ZnCO_3$ and zinc phosphate through production and excretion of organic acids. Zinc solubilizing microorganisms solubilize zinc through various mechanisms one of which is acidification. These microbes produce organic acids in soil which sequester the zinc cations and decrease the pH of the nearby soil. Other mechanisms possibly involved in zinc solubilization include production of siderophores (Saravanan *et al*., 2003) and proton, oxido-reductive systems on cell membranes and chelated ligands (Chang *et al.,* 2007).

Zinc (Zn) one of the domineering micronutrients is required in small amount for the proper growth and development of living organisms (Hafeez *et al.,* 2009). In plants, specifically it is involved in auxin metabolism and acts as a significant anti-oxidant. Zn-finger transcription factors play an important role in the normal development of floral tissues, flowering, fertilization and fruiting.Plants can uptake zinc as divalent cation but only a very minor portion of total zinc is present in soil solution as soluble form. Rest of the zinc is in the form of insoluble complexes and minerals (Alloway, 2008). Due to unavailability of zinc its deficiency occurs widely in soils. To alleviate zinc deficiency, various methods have been applied since long. Zinc fertilizers in the form of zinc sulphate have been used but their usage puts an economical and environmental pressure further these are transformed into insoluble complex forms within 7 days of their application. As such better alternative to all these approaches is the use of zinc solubilizing rhizobacteria. Zinc solubilizing bacteria are potential alternatives for zinc supplementation and convert applied inorganic zinc to available forms**.** The plants need several macro and micro nutrients for their growth and reproduction.These nutrients supplemented through inorganic or organic forms are taken up by the plant roots along with water.

Zinc (Zn) is one of the essential micronutrient required for optimum plant growth and plays a vital role in metabolism (Hughes and Poole, 1989). It is essential element for crop production and for optimal size of fruit. Moreover, it is also required in the carbonic enzyme which is present in all photosynthetic tissues and required for chlorophyll biosynthesis (Ali *et al*., 1997).

Zinc plays a key role as a structural constituent or regulatory co-factor of a wide range of enzymes and proteins in many important biochemical pathways which are mainly concerned with carbohydrate metabolism both in photosynthesis and in the conversion of sugars to starch, protein metabolism, pollen formation, the maintenance of the integrity of biological membranes, the

resistance to infection by certain pathogens. Crop growth, vigor, maturity and yield are very much reliant upon essential micronutrient such as Zn. It is responsible for synthesis of auxin and catalyzes the photochemical reaction of chlorophyll. Zinc is also required for the stability of biological membranes and is important for the activity of various enzymes, e.g. Cu and Zn superoxide dismutase (SOD) and carbonic anhydrase which contain structurally bound Zn and plant growth regulator, i.e. Indole Acetic Acid (IAA). It influences the synthesis of nucleic acid, lipids and proteins by which the grain quality becomes superior.

2. Materials and methods

2.1 Rhizosphere soil characterization and sampling

Samples (rhizosphere soil) were collected from the rhizosphere of the natural population of paddy (*Oyzae sativa*) plant growing in district Baamulla of J&K. This is at an average elevation of 4270 m at above mean sea level. A total of 60 soil samples were used for isolation of total bacterial population total viable phosphate, potassium and zinc solubilizing bacterial population. Sampling was done in the month of july and August of the year 2018.The general laboratory techniques followed in the present study "Isolation, screening characterization of zinc solubilizing bacteria from paddy rhizosphere soil" were those described as by Cappuccino and Sherman (1992), Nene and Thapliyal (1993) and Aneja (2001) for preparation of media, sterilization, isolation and maintenance of bacterial cultures, with slight modifications wherever necessary. An account of the material used and methodology adopted is discussed in this chapter.

2.2 Collection of rhizosphere soil

Soil samples were collected from rice rhizosphere of twenty different physiographical locations in Baramulla district, J&K. All the collected samples were packed in sterile polythene bags and were transported immediately to the laboratory and preserved in refrigerator at 4^{o}c for further studies.

District name	No of locations	No of samples from each location	Total samples
Baramulla	20	3	60
		Total	60

2.3 Glassware

Petriplates, test tubes, microscopic slides, conical flasks of different capacities *i.e*., 1000, 500, 250 ml, pipettes of 1.0, 2.0, 5.0, 10.0 ml beakers and measuring cylinders of 50, 100, 500 and 1000 ml, micropipettes of 0.5-10, 10-100, 100-1000 µl were used. All the glassware used was Borosil made.

2.4 Cleaning of glassware

Glassware was first washed with a detergent, then cleaned with tap water and finally placed in the chromic acid solution prepared with following composition:

Potassium dichromate: 60 g Conc. H_2SO_4: 60 ml Distilled water: 1000 ml. The glassware was placed in the cleaning solution for 24 hr and then thoroughly washed with running tap water before its final cleaning with distilled water and dried.

2.5 Sterilization of glassware

Glassware was wrapped in butter paper and sterilized in hot air oven at 160 °C for 1 h before use. Media, distilled water, etc., were sterilized in an autoclave at 15 lbs psi and 121 °C for 20 min.

2.6 Precaution to avoid contamination

The inoculation work of microbial cultures was carried out under laminar air flow chamber. The laminar bench and air flow was disinfected by U.V lamp prior to commencement of work.

2.7 Equipment and Apparatus used

Hot air oven and autoclaves were used for sterilization of heat stable glassware and media respectively. BOD incubators were used for incubating cultures at different temperatures. Cultures were stored and maintained in a refrigerator. The pH was measured by using digital pH meter. Cyclomixer was used for homogenization of samples during serial dilution preparation. Master spreader was used for spread plate technique. Centrifuge was used for making cell-free filterates. Hi-media zonal scale was used to measure the zone around the colonies during phosphorus, zinc, potassium solubilization and biocontrol activity. Flame photometer and

Atomic Absorption Spectrophotometer (AAS) were used for detection of solubilization and release of potassium and zinc in liquid culture broths, Spectrophotometer for nitrogen analyser for nitrogen analysis. Samples were weighed using a single pan electronic balance. Compound electron microscope was used to observe the morphology of bacterial cultures.

2.8 Chemicals used

The chemicals used in the present investigation were of analytical and laboratory grades. The pH of the media was adjusted to the required level using 10 N NaOH and 10 N HCl. Formaldehyde 10 % solutions were used to fumigate the laminar air flow chamber and biological oxygen demand (BOD) incubators for disinfection.

2.9. Methodology

2.9.1 Preparation of inoculums

A bacterial cell suspension (O.D. 1.0 at 540 nm) of 24h old culture grown on nutrient agar slants at the rate of 10 per cent was used as inoculum in all experiments, unless mentioned otherwise.

2.9.2 Turbidity (Measurement of growth)

Growth was monitored by measuring the change in absorbance of cells in the medium at 540 nm using uninoculated medium as blank.

2.9.3 Viable count

Appropriate dilutions of bacterial population were used to seed the medium. The number of viable cells in the initial population was obtained by counting the number of colonies that developed after incubating the plates and multiplying this figure by dilution factor.

2.10 Isolation of rhizosphere soil bacterial population

Bacterial isolates were isolated from the rhizosphere soil of paddy plants during the month of July and August 2018. Soil samples collected from different sites under natural conditions. To estimate the number of soil bacteria, counts were calculated on the basis of serial 10 fold dilutions in duplicate, using the pour plate method and triplicate samples of 1 gm soil, and an appropriate dilutions (Johnson and Curl, 1972). Serial dilution method was used for isolating the total viable bacteria, total viable phosphorous solubilising bacteria, total viable potassium solubilising bacteria and total viable zinc solubilising bacteria (ZSB) from the rhizosphere soil samples. 1.0g of paddy rhizosphere samples was suspended in 9.0 ml of sterilized distilled water in test tubes. From the first dilution 1ml was transferred to the test tubes containing 9 ml of sterile distilled water to get 10^{-2} dilution. The same method was followed for preparing upto10^{-6} dilution. The Nutrient agar medium was prepared containing Beef extract -3g**,** Peptone -5g, Nacl -8 g and Agar -15g in Distilled water (1000ml and pH adjusted 7.3). The source of insoluble nutrient compound such as Nacl was supplemented at 8% and agar was added into the medium individually autoclaved at 121^{o}c for 30 minutes. The agar medium was poured in sterile petriplates under aseptic condition. The petri plates were made in replicates. After solidification, 0.1 ml from 10^{-4}, $10^{-5}$and 10^{-6} dilutions of rice rhizosphere soil samples were taken by sterile pipette, transferred and spread on to Petri plates. The inoculated plates were incubated at 27-30^{o}c for 48 h.

2.10.1 Similarly same method was followed for isolating the total viable phosphorous solubilising bacteria. The Pikovaskya's medium was prepared containing Glucose -10 g, Ca_3 $(PO_4)_2$ -5 g, $(NH4)_2SO_4$ - 0.5 g, KCl -0.2 g, $MgSO_4$ -0.1g, $MnSO_4$-Trace $FeSO_4$ –Trace and Yeast extract -0.5 g in Distilled water (1000ml). The medium was individually autoclaved at 121^{o}c for 30 minutes. The petri plates were made in replicates. After solidification, 0.1 ml from 10^{-4}, $10^{-5}$and 10^{-6} dilutions of rice rhizosphere soil samples were taken by sterile pipette, transferred and spread on to Petri plates. The inoculated plates were incubated at 27-30^{o}c for 48 h.

2.10.2. The total viable potassium solubilising bacteria was also isolated by serial dilution method. The Aleksandrov medium was prepared containing Glucose -5.0 g, Magnesium sulphate ($MgSO_4.7H_2O$) -0.005g, $FeCl_3$ -0.1 g, Calcium carbonate - 2.0 g, Potassium mineral -2.0 g and Calcium phosphate - 2.0 g in distilled water (1000 ml). The medium was poured in sterile petriplates under aseptic condition. The petri plates were made in replicates. After solidification, 0.1 ml from 10^{-4}, $10^{-5}$and 10^{-6} dilutions of rice rhizosphere soil samples were taken by sterile pipette, transferred and spread on to Petri plates. The inoculated plates were incubated at 27-30^{o}c for 48 h.

2.10.3 Serial dilution method was used for isolating the total viable zinc solubilising bacteria (ZSB) from the rhizosphere soil samples. 1.0g of paddy rhizosphere samples was suspended in 9.0 ml of sterilized distilled water in test tubes. From the first dilution 1ml was transferred to the test tubes containing 9 ml of sterile distilled water to get 10^{-2} dilution. The same method was followed for preparing upto10^{-6} dilution. The media containing dextrose – 1 0g, $(NH_4)_2$ SO_4 -1.0g, KCL -0.2g, K_2HPO_4 - 0.1g, $MGSO_4$ -0.2g in distilled water (1000 ml and pH was adjusted 7.0). The source of insoluble zinc compound such as $Znco_3$ was supplemented at 1% and agar was added into the medium individually and autoclaved at 121^{o}c for 30 minutes. The agar medium was poured in sterile petriplates under aseptic condition. The petri plates were made in replicates. After solidification, 0.1 ml from 10^{-4}, $10^{-5}$and 10^{-6} dilutions of rice rhizosphere soil samples were taken by sterile pipette, transferred and spread on to Petri plates. The inoculated

plates were incubated at 27-30ºc for 48 h. Colony forming units (CFU) were recorded on nutrient agar plates after 48 hour, the average number per gram oven dry weight of soil was calculated as:

$$\text{Colony forming unit} = \frac{\text{Bacterial plate count} \times \text{Di.factor}}{\text{Amount of sample plated}}$$

2.10.4 Physico chemical properties

The soil pH (1:2.5 Soil : water suspension) of rhizosphere soil samples from paddy fields were determined by Digital glass electrode pH meter, method as given by Jackson M.L., in 1973. Similarly the electrical conductivity (dsm^{-1}) of paddy rhizosphere soil samples was determined by solu-bridge conductivity meter, method as given by Jackson M.L., in 1973.

2.10.5 Organic carbon The organic carbon (%) content of paddy rhizosphere soil samples were determined by rapid titration, method as given by Walkley and Black in 1934**.**

The organic carbon (%) content in soil samples were determined by subjecting the samples to the oxidation by an oxidation agent ($K_2Cr_2O_7$) and then digestion by a standard acid followed by titration with a standard ferrous ammonium sulphate solution in presence of an indicator. Following formula was used for the calculation of total organic carbn content in the soil samples.

$$\text{Organic carbon (\%)} = \frac{\textbf{(B-S)} \times \textbf{N (FAS)} \times \textbf{0.003}}{\text{Weight of sample}} \times 1.732$$

2.10.6 Available Nitrogen The Available Nitrogen ($kgha^{-1}$) of paddy rhizosphere soil samples were determined by alkaline potassium permanganate method as given by Subbiah and Asijah, in 1956.

2.10.7 Available zinc The Available zinc (ppm) content of paddy rhizosphere soil samples were determined by Atomic Absorption Spectrophotometer method as given by Lindsay and Norvell, in 1978. Zinc solubilizing bacteria was isolated on modified Pikovaskyas medium (containing 0.1 % insoluble zinc sources as zinc carbonate) after 48 hou rs of incubation at 28 ± c^0 (Bunt and Rovira, 1955).

Serial dilution method was used for isolating the zinc solubilising bacteria (ZSB) from the rhizosphere soil samples. 1.0g of paddy rhizosphere samples was suspended in 9.0 ml of sterilized distilled water in test tubes. From the first dilution 1ml was transferred to the test tubes containing 9 ml of sterile distilled water to get 10^{-2} dilution. The same method was followed for preparing upto10^{-6} dilution. The tris-mineral salts medium was prepared containing dextrose – 1 0g, $(NH_4)_2 SO_4$ -1.0g, KCL -0.2g, K_2HPO_4 - 0.1g, $MGSO_4$ -0.2g in distilled water (1000 ml and pH was adjusted 7.0). The source of insoluble zinc compounds such as $Znco_3$ was supplemented at 0.1% and agar was added into the medium individually and autoclaved at 121ºc for 30 minutes. The agar medium was poured in sterile petriplates under aseptic condition. The petriplates were made in replicates.After solidification,1 ml from 10^{-4}, $10^{-5}$and 10^{-6} dilutions of rice rhizosphere soil samples were taken by sterile pipette, transferred and spread on to Petri plates. The inoculated plates were incubated at 27-30ºc for 48 h.

2.10.8 Screening of Zinc solubilizing bacteria (ZnSB) for mineral zinc solubilization:-

2.10.8.1 Plate assay (Qualitative):

Qualitative estimation of zinc solubilizing bacterial isolates were carried out on the modified Pikovaskayas medium by formation of hollow Zones (Bunt and Rovira, 1955). Zinc Solubilization Efficiency (SE) which is the ratio of total diameter of clearance zone including bacterial growth and the colony diameter was described by following formula (Poonam Sharma *et al.*, 2014).

$$\text{SE} = \frac{\text{Diameter of solubilisation halo zone}}{\text{Colony diameter}} \times 100$$

2.10.8.2 Atomic absorption spectrophoto metermethod (Quantitative):

Twenty most promising isolates that show highest zones of solubilization on the modified Pikovaskaya medium were tested for the quantum of zinc solubilized. Here the cultures were inoculated independently to 250 ml conical flasks containing modified Pikovaskayas broth amended with 0.1% of Zinc carbonate. There were three replicates maintained for each bacterial isolates. The selected isolates were examined for their ability to release Zn from broth media supplemented with 0.1 per cent zinc carbonate ($ZnCO_3$). The amount of Zn released in the broth medium by the isolates was studied at 5, 7 & 10days after incubation (DAI). These were incubated at 28±2°c for 10 days in incubating shakers.The broth was centrifuged at 1000 rpm for 5 minutes. The cultured broth was centrifuged and the supernatant was filtered through Whatman No. 42 filter paper individually. The supernatant was fed to AAS for estimation of the quantity of insoluble source of zinc solubilised (Bunt and Rovira, 1955).

2.10.9 Characterization of 20 outstanding isolates.

2.10.9.1Morphological

The isolates were characterized on following morphological features; Cell shape, size, colony features and pigmentation (Aneja 2003). Morphological characteristics of the colony of each isolate was examined on nutrient agar and specialized medium. Cultural characterization of isolates observed by different characteristics of colonies such as shape, colony features, pigmentation, etc were recorded.

2.10.9.2 Bio Chemical

The Biochemical characterization of selected ZSB isolates was carried out by following Bergey's Manual of determinative, Bacteriology.

Following bio chemical tests were conducted;

2.10.9.2.1 Gram's reaction

A drop of sterile distilled water was placed in the center of glass slide. A loopful of inoculum from young culture was taken, mixed with water, and placed in the center of the slide. The suspension was spread out on slide using the tip of inoculation needle to make a thin suspension. The smear was dried in air and fixed through mild heating by passing the slide 3 to 4 times over the flame. The smear was then flooded with crystal violet solution for 30 second and washed gently with flow of tap water. Then the slide was flooded with iodine solution. After 30 seconds, iodine solution was drained out followed by washing with 95 % ethanol. After that, it was washed with water within 15 to 30 sec and blotted carefully. The smear was flooded with safranin solution for 30 second. The slide was washed gently in flow of tap water and dried in air, after that smear was covered with coverslip by using immersion oil. The slide was examined under microscope at 100 X power with oil immersion and data were recorded.

2.10.9.2.2 Catalase test

This test was performed to study the presence of catalase enzyme activity of the isolates. Fresh cultures of pure isolates were taken on glass slides and one drop of H_2O_2 (30%) was added. Release of gas bubble indicated the presence of catalase enzyme activity which is positive reaction for the test.

2.10.9.2.3 Starch hydrolysis

Sterile starch agar plates were spotted with overnight grown cultures of the isolates and incubated at 28 ± 2 ^{0}C for 24 - 48 h. After incubation, the plates were flooded with iodine solution. The formation of a transparent zone around the colony was taken as positive reaction for the test.

2.10.9.2.4 Gelatin liquefaction

The overnight grown cultures of the test isolates were inoculated into sterilized nutrient gelatin deep tubes and incubated for 24 h at 28 ± 2^{o}C. Then the tubes were kept in the refrigerator for 30 min at 4^{o}C. The isolates which showed liquefied gelatin were taken as positive and which showed solidification of gelatin on refrigeration were recorded as negative for the test. Among all the isolates most were showing positive reaction for the test, some were recorded as negative for the test and few isolates showed weakly positive reaction for the test.

2.10.9.2.5 Hydrogen peroxide (H_2O_2) test

This test was performed to study the presence of peroxidase enzyme activity of the isolates. Fresh cultures of pure isolates were taken on glass slides and one drop of H_2O_2 (30%) was added. Release of gas bubble indicated the presence of peroxidase enzyme activity.

2.10.9.2.6 Hydrogen sulphide production test (Aneja 2003) Sterilized Hydrogen Sulfur Indole Motility (SIM) agar stabs were inoculated along the wall of the tubes with overnight cultures of the isolates and incubated for 48 h at 28 ± 2 ^{0}C. Visualization of black colour precipitation is the positive reaction for the test.

2.10.9.2.7 Molecular: The isolate that shows maximum zinc solubilisation was characterized on molecular basis (Krieg *et al*., 1984). The cultural sample was processed for identification in the following manner –

1. Genomic DNA was isolated from the culture by using Sigma's,"GenElute Bacterial Genomic DNA" Kit.
2. PCR was carried out using the following combination of primers – FDD2 – RPP2 (universal primers for 1.5 kb fragment amplification for eubacteria)

The PCR was set using the following Mix –

ddH_2O -	12.0ul
10 X PCR buffer –	2.0ul
dNTPs –	2.0ul
Forward primer -	0.4ul
Reverse primer -	0.4ul
Taq DNA Pol. -	0.2ul
Template DNA -	3.0ul
Total volume -	20.0ul

Template DNA – Genomic DNA
Sequences of the primer pair used for amplification -
RPP2 – CCAAGCTTCTAGACGGITACCTTGTTACGACTT
FDD2 – CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG

3. The PCR products obtained from above reactions were then processed for Cycle sequencing reaction (PCR performed using only one primer – SRV3-1).
4. Following the above reaction, the samples were cleaned up and loaded on the sequencer (Avant 3100 Gene Analyzer)

Protocol:

Total genomic DNA was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM $MgCl_2$; 1x PCR buffer (Bangalore Genei, Bangalore, India); 200 µM each d CTP, d GTP, d ATP, and d TTP; 2pmol of each, forward and reserve primer; and 1U of Taq DNA polymerase(Bangalore Genei, Bangalore, India) in a final volume of 20 µl.FDD2 and RPP2 pimers were used to amplify almost entire 16S r RNA gene as described previously (Rawlings 1995).The PCR was performed using the Eppendorf Gradient Master Cycler system with a cycle of 94^0C for 5 min. 30 cycles of 94^o, 60^o, and 72^oC for 1 min each; final extension at 10 min at 72^oC and the mixture was held at 4^oC. The PCR product was precipitated using polyethylene glycol (PEG, 60008.5%) washed thrice- using 70% ethanol and dissolved in Tris-HCL (10mM pH8.0).
The ABI prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems, foster city, Calif) was used for the sequencing of the PCR product.A combination of universial primers was chosen to sequence the nearly complete gene (Rawlings 1995, Muyzer et al. 1993).The sequence reaction and template preparation were performed and purified in accordance with the directions of manufacturer (Applied Biosystems). Samles were run on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems).The sequencing output was analyzed using the accompanyning DNA Sequence Analyzer computer software (Applied Biosystems).

Results

Zinc being an essential micro nutrient for plants is very limiting because of its very low use efficiency. Both the native and applied fractions of zinc are subjected to chemical fixation in soils. The rice growing soils of Kashmir valley have been exhibiting widely the zinc deficiency. A diverse range of micro organisms have been found to enhance zinc availability through solubilising the chemically complexed zinc. During the present investigation following results were obtained. Soil samples from rice rhizosphere at flowering stage of the crop were collected from twenty (20) physiographically different locations from district Baramulla of Kashmir valley (Table1). These samples were subjected to analysis of physico-chemical and biological properties as:-

3.1 Biological parameters

The various microorganisms such as total viable bacteria, phosphorous, potassium solubilising bacteria and zinc solubilising bacteria were determined from the collected soil samples. The total viable bacterial count ranged from 50-89×10^{-7}cfug^{-1} soil. The lowest count (50 × 10^{-7}cfug^{-1}soil) was recorded from Model town (S_{29}) and Jalalabad locations, while as the significantly highest population (89×10^{-7} cfug^{-1}soil) was found in Arampora (S_{19}). The population of total viable phosphorous solubilising bacteria ranged from 10 to 24×10^{-7}cfug^{-1} soil with minimum count of 10cfug^{-1} soil obtained from Singhpora (S_7) and significantly highest count of 24×10^{-7}cfug^{-1} soil recorded from many places like Singhpora (S_9), Zangam (S_{38}), Buran (S_{38}), Bandi Bala (S_{48}) and Bandi Payeen (S_{51}).
The total viable potassium solubilizing bacterial population varied from 14 to 29×10^{-7}cfug^{-1} soil, Nowpora (S_{16}) showed a minimum population of 14×10^{-7}cfug^{-1} soil while as the maximum KSB count (29 ×10^{-7} cfug^{-1} soil) was found from Arampora (S_{19}), Bumai (S_{23}) and Hanjiwera (S_{45}). Similarly population of total viable zinc solubilizing bacteria showed a wide diversity with respect to the locations and it ranged from 10 to 22×10^{-7}cfug^{-1} soil. Significantly the highest count (22×10^{-7}cfug^{-1} soil) was obtained from Zangam (S_{38})

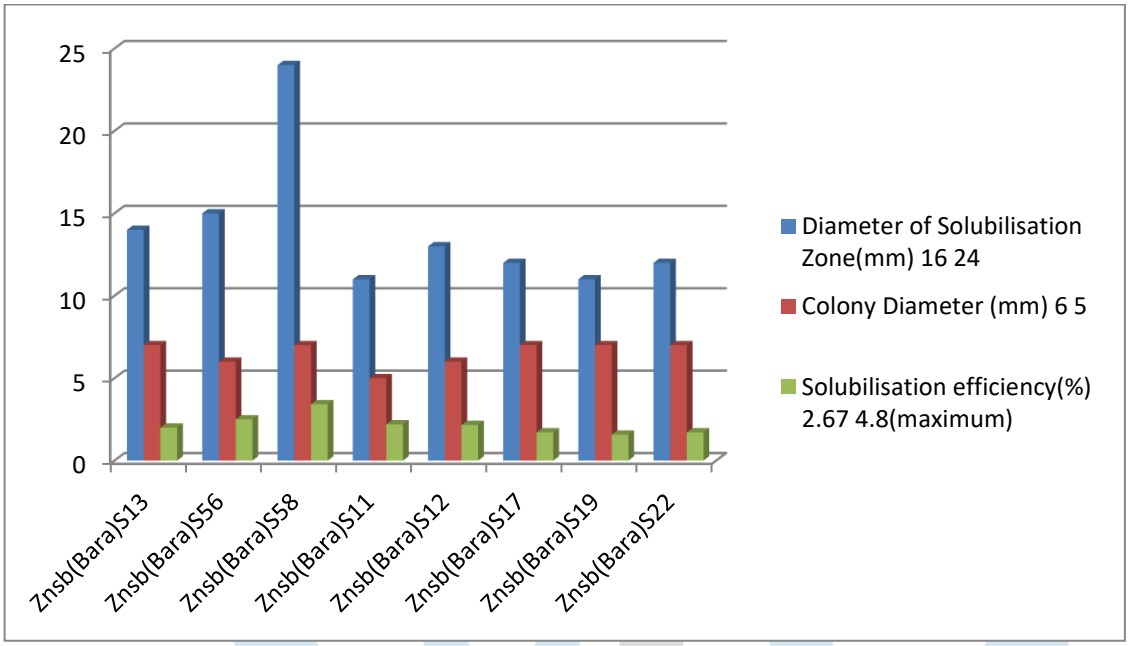
3.2 Physico chemical properties

The soil reaction pH (1:2.5) at all locations in the district was slightly acidic to neutral showing a range from 6.17 to 6.79. The significantly maximum pH value of 6.79 was recorded from Singhpora (S_8). Similarly the minimum pH value (6.17) was recorded from Buran (S_{40}).
The electrical conductivity was normal and ranged from 0.101 to 0.164 (ds^{-1}m). The maximum EC of 0.164 (dsm^{-1}) was obtained from Kanispora (S_1) and minimum value (0.101) was obtained from Delina (S_6).
Similarly the organic carbon content was medium to high ranged from 0.44 to 14.3%. The significantly maximum organic carbon content of (1.46%) was recorded from Bandi Payeen (S_{49}) and the minimum (0.44%) was recorded from Singhpora (S_9).
Available nitrogen content was medium to high and ranged from 296.42 to 627.07kgha^{-1}. Significantly highest available nitrogen content (627.07kgha^{-1}) was reported from Nehalpora (S_{31}) while as the lowest (296.42 kgha^{-1}) was found in Model town (S_{29}). Similarly the available zinc content was also medium to high showing a range from 1.98 to 2.972ppmha^{-1}. Significantly highest (2.972ppmha^{-1}) and lowest (1.98ppmha^{-1}) available zinc content was observed from Dawlatpora (S_{57}) and Kanispora (S_3) respectively.

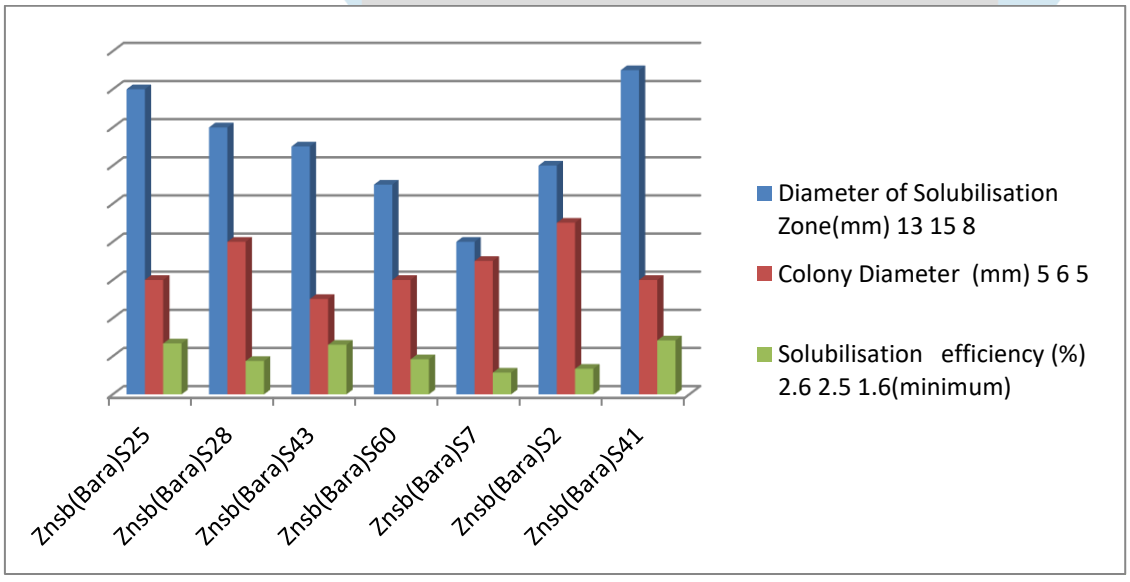
3.3. Screening of ZSB isolates (qualitative)

All the bacterial strains were screened for their ability to solubilise zinc in modified pikovaskays agar medium amended with 0.1% zinc carbonate ($ZnCO_3$). The diameter of zone of solubilization ranged from 8mm to 24mm. The isolate Znsb(Bara)S53 produced highest solubilization zone of 24mm while as Znsb(Bara)S37 isolate formed 8mm mm zone of clearance on modified Pikovaskaya medium. Colony diameter also varied widely from isolate to isolate with minimum size of 5mm formed by Znsb(Bara)S37, Znsb(Bara)S53 Znsb(Bara)S11,Znsb(Bara)S35,Znsb(Bara)S37 and Znsb(Bara)S43 isolates and the largest colony size of 9mm was formed by Znsb(Bara)S2 isolate.The zinc solubilization efficiency calculated on the basis of colony diameter and solubilization zone also showed a very wide variation among the isolates. It ranged from 1.6% to 4.8%.The isolate Znsb(Bara)S58 showed the maximum solubilization efficiency of (4.8%) followed by 3.42% shown by Znsb(Bara)S41

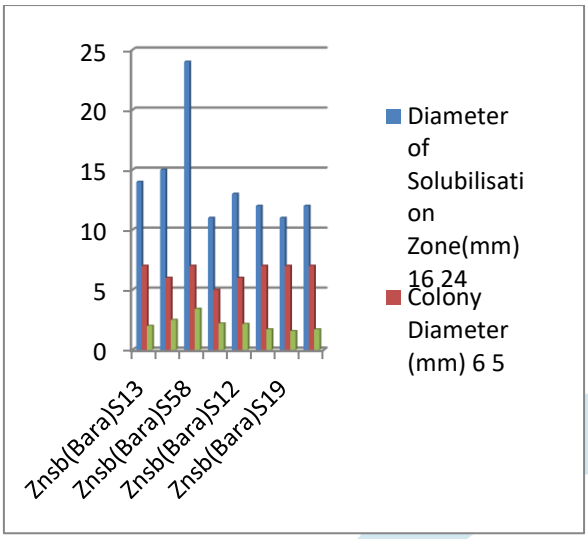
3.4 Zinc solubilization zone and efficiency by zinc Solubilizing isolates

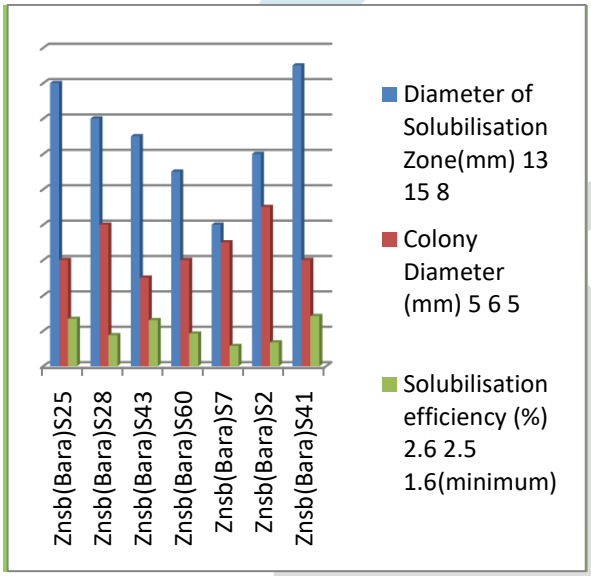


Zinc solubilization zone and efficiency by zinc Solubilizing isolates



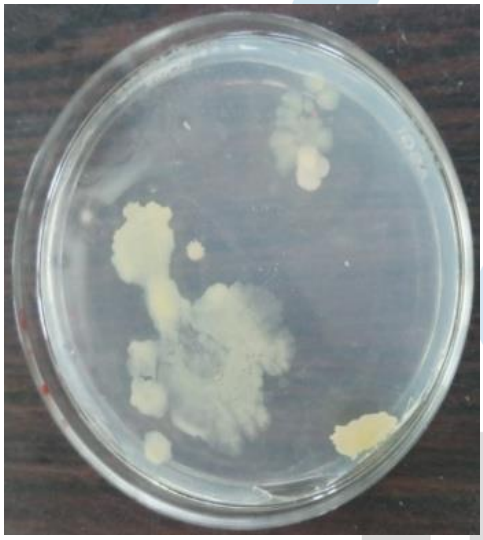
3.4 Zinc solubilization zone and efficiency by zinc Solubilizing isolate





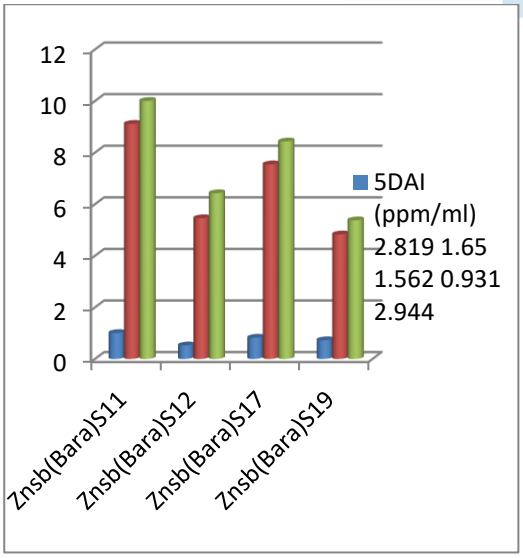
a) Znsb(Bara)S15

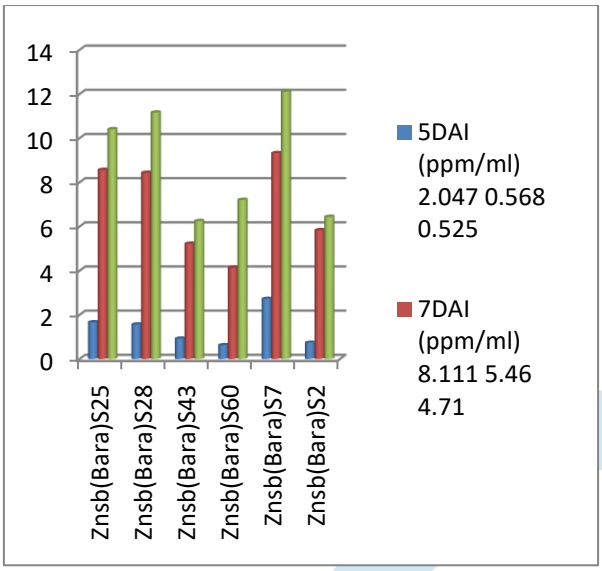
b) Znsb(Bara)S53



c) Znsb(Bara)S58

Figure 1: Quantification of Zinc Solubilizing isolates at 10DAI





3.5 Characterisation of out standing isolates (Morphological and bio-chemical characteristics)

Zinc solubilizing isolates were tested for morphological and biochemical characteristics.

The morphological colony characters such as cell shape and pigmentation were examined. Out of all twenty promising zinc solubiling isolates, sixteen isolates have showed rod shaped cell shape and yellow white pigmentation. The remaining four isolates showed short rod shaped cell shape and smooth, creamy pigmentation. Similarly the bio-chemical characters like catalase activity, starch hydrolysis, gelatin liquification, hydrogen sulphide production and hydrogen peroxide activity were observed. Out of 20 most promosing isolates. Four isolates (4) were Gram negative and rest of isolates (16) were Gram positive, similarly in case of Catalase activity all the twenty isolates were catalases producing. Catalase producing rhizobacteria must be highly resistant to abiotic stress viz., environmental, mechanical and chemical. In case of Starch hydrolysis nine (9) isolates were inable to hydrolyse starch while as remaining eleven (11) isolates hydrolyses the starch. The five isolates were able to liquefy gelatin, two isolates were weakly able to liquefy the same and thirteen isolates were positive for this test. The five isolates were negative, three isolates were weakly

positive and twelve isolates were able t to produce hydrogen sulphide. In case of hydrogen peroxide test, all the twenty isolates were able to produce peroxidases. These results were agreed with findings of Lahiri and Chakravarthi (1989), Najar *et al*. (2005) and Marzi (1988).

3.6 Morphological characterization

The twenty outstanding zinc solubilizing bacterial isolates were studied for various features like the colony characteristics, cell shape, pigmentation etc. The colonies of the studied isolates were by and large creamy, white, yellow, wheat white and light in colour. Most of the isolates showed flat, rough, small, white raised colonies and belonged to the probable genus *Bacillus* sp. Some of the isolates showed raised, smooth and circular colonies, and cells were short rod shaped. On the basis of these colony and morphological characteristics all the twenty zinc solubilizing isolates belonged to the probable genera of *Bacillus* sp and *Pseudomonas* sp. The cells of the isolate Znsb (Bara)S53 which showed maximum zinc solubilisation were rod shaped with Yellow, rough, large, raised colonies and it belonged to the probable genus *Bacillus*. Similarly The cells of the isolate Znsb(Bara)S37 which showed minimum zinc solubilization were Short rod shaped with Smooth, creamy white, raised colonies and it belonged to the probable genus *Pseudomonas* are presented in table

Morphological characters of screened zinc solubilising bacterial isolates

S.No	Isolates	Colony characters (pigmentation)	Cell shape	Probable genus
1	Znsb(Bara) S1	Yellow, rough, large, circular, raised	Rod	*Bacillus* sp.

2	Znsb(Bara)S58	Smooth, creamy white, raise	Short rods	*Pseudomonas* sp.
3	Znsb(Bara)S13	White, rough, Circular	Rod	*Bacillus* sp.
4	Znsb(Bara)S56	Sky blue, smooth, raised	Short Rods	*Pseudomonas* sp.
5	Znsb(Bara) S53	Wheat white color, rough, flat	Rod	*Bacillus* sp.
6	Znsb(Bara) S11	Light, rough, flat	Rod	*Bacillus* sp.
7	Znsb(Bara) S12	White rough flat	Rod	*Bacillus* sp.
8	Znsb(Bara) S17	White, rough flat	Rod	*Pseudomonas* sp.
9	Znsb(Bara) S19	White, rough, Small flat surface	Rod	*Bacillus* sp.
10	Znsb(Bara) S22	White, circular smooth	Rod	*Bacillus* sp.

S.No	Isolates	Colony characters (pigmentation)	Cell shape	Probable genus
11	Znsb(Bara) S35	White, rough circular	Rod	*Bacillus sp*
12	Znsb(Bara) S46	White creamy, flat, rough	Rod	*Bacillus sp*
13	Znsb(Bara) S37	Smooth, creamy white, raise	Short rods	*Pseudomonas sp*
14	Znsb(Bara) S25	White rough circular	Rod	*Bacillus sp*
15	Znsb(Bara) S28	White, rough, small, flat surface	Rod	*Bacillus sp*
16	Znsb(Bara) S43	White, circular smooth	Rod	*Bacillus sp*
17	Znsb(Bara) S60	Sky blue, smooth, raised	Short Rods	*Bacillus sp*
18	Znsb(Bara) S7	White, circular smooth	Rod	*Bacillus sp*
19	Znsb(Bara) S2	White, rough, circular	Rod	*Bacillus sp*
20	Znsb(Bara)S41	Yellow, rough, large, circular, Raised	Rod	*Bacillus sp*

Conclusion

The studied soil samples from paddy rhizosphere showed that soil pH was slightly acidic to neutral with normal electric conductivity low to medium available nitrogen; the available zinc content was low to medium in range. These nutrient complications are further aggravated by low nutrient use efficiency ranging from 30-50% (N) and 1-2% micronutrients (Zn). Optimum population of various microbes was reported from all the samples. Zinc solubilizing bacteria were found in each rhizosphere soil sample of the paddy crop. Out of a large number of isolates twenty most efficient (Znsb**)** bacteria were isolated on media suppplemented with zinc carbonate as a zinc source. The availability of zinc in soils is also very limited as major source of zinc. Soil minerals containing the zinc in tightly bound and complexed form (96-99%).

Further application of phosphate fertilizers also reduces zinc availability and concentrations of copper content in soils also reduce available zinc content. Solubilization of soil minerals by bacteria and fungi are well established. However less information was available about zinc solubilizing bacteria and their impact on plant growth and development. In this context, attempts were made for isolation of zinc solubilizing bacteria from soil samples of Paddy rhizosphere. The efficiency of the isolates to solubilize zinc minerals was tested. The rhizosphere soil samples of paddy crop were used in the study for isolation of zinc solubilizing bacteria. Morphologically all the isolates belonged tothe genera *Bacillus, Pseudomonas.* All the twenty isolates were identified. The most efficient isolate was characterised upto genus level and found as a *Bacillus* sp and *Pseudomonas* sp. All the isolates were able to solubilize respective mineral sources like zinc carbonate for zinc. Among all the isolates Znsb(Bara)S53(*Bacillus* sp.) showed maximum solubilization with 24µgml^{-1} and

maximum solubilizing efficiency of 4.8% and solubilized 12.631 ppm Zn after 10 days of incubation. The amount of zinc released by the zinc isolates ranged from 1.98 to 2.972 ppm^{-1}ml. All zinc solubilizing bacteria were tested for other beneficial activities like phosphate solubilization, only few isolates showed these beneficial properties.Among all the zinc solubilizing isolates Znsb (Bara) S53 (*Bacillus* sp.) showed all characterstics like solubilization of zinc, the studied soils are biologically active as reflected by the zinc solubilizing bacteria in every sample. All strains of zinc solubilizing bacteria were tested for other bio-chemical activities like Gram staining, catalase activity, starch hydrolysis, gelatin liquification, hydrogen sulphide production and hydrogen peroxide. Most isolates showed these beneficial properties.

Among all the zinc solubilizing isolates Znsb (Bara) S53 (*Bacillus cereus*) showed all characterstics like solubilization of zinc, Gram staining, catalase activity, starch hydrolysis, gelatin liquification, hydrogen sulphide production and hydrogen peroxide activity.

Zinc solubilizing bacteria can be used in paddy fields for enhancing the total factor productivity with reference to the zinc use efficiency which is generally > 1%. The screened zinc solubilizing bacterial isolates can be used with greater promise as biological inputs for enhancing nutrient availability, promoting plant growth and improving yield levels especially in Agricultural crops. The most potential Zn solubilizing isolate can be further screened for other plant growth promoting properties in future research programmes

References

1. Alloway, B.J. 2008. Zinc in soils and crop nutrition. Second edition, published by *IZA and IFA, Brussels, Belgium and Paris, France*.
2. Ali, S., Malik, K. A., Bilal, R., Rasul, M., Mirza, G. 2008. Association of nitrogen-fixing plant-growth promoting rhizobacteria (PGPR) with kallar grass and rice. *Journal* of *Plant and soil* **194**: 37-44.
3. Antoniadis, V. and Alloway, B. J. 2002. The role of dissolved organic carbon in the mobility of Cd, Ni and Zn in sewage sludge amended soils. *Environmental Pollution* **117**: 515-521.
4. Andreini, C., Banci, L. and Rosato, A. 2006. Zinc through the three domains of life. *Journal of Proteome Research* **5**: 3173–3178.
5. Aneja, K. R. 2001. Experiments in Microbiology. *Journal of Plant Pathology and Tissue culture*. Viswaprakasham, New Delhi. Bullen, Kemila, P. 1997. Influence of pH on the toxic effect of zinc, cadmium and pentachlorophenol on pure cultures of microorganisms. *Journal for Environmentally Toxic Chemicals* **16**: 146-153.
6. Bullen, Kemila, P. 1997. Influence of pH on the toxic effect of zinc, cadmium and pentachlorophenol on pure cultures of microorganisms. *Journal for Environmentally Toxic Chemicals* **16**: 146-153.
7. Cakmak, I. 2009. Enrichment of fertilizers with zinc: An excellent investment for humanity and crop production in *Indian \Journal of Trace Elements in Medicine and Biology* **23**: 281-289.471.
8. FAO, 2005. The state of food insecurity in the world. *Eradicating world hunger-Key to achieving the millennium development goals*. FAO, Rome.
9. FAO, 2009. The state of food insecurity in the world. *Journal of Economic crises-Impacts and lessons learned*. FAO, Rome.
10. Hafeez, F. Y., Naureen, Z., Price, A. H. and Roberts, M. R. 2013. Identification of rice blast disease-suppressing bacterial strains from the rhizosphere of rice grown in Pakistan. *Crop Protection* **2**: 1052-1060.
11. Karlidag, H., Esitken, A. and Turan, M. 2007. Effects of root inoculation of plant growth promoting rhizobacteria on yield growth and nutrient element content of leaves of apple. *International Journal of Current Microbiology and Applied Sciences* **3**: 139-155.

12. Karlidag, H., Esitken, A. and Turan, M. 2007. Effects of root inoculation of plant growth promoting rhizobacteria on yield growth and nutrient element content of leaves of apple. *International Journal of Current Microbiology and Applied Sciences* **3**: 139-155.
13. Karlidag, H., Esitken, A. and Turan, M. 2007. Effects of root inoculation of plant growth promoting rhizobacteria on yield growth and nutrient element content of leaves of apple. *International Journal of Current Microbiology and Applied Sciences* **3**: 139-155.
14. Lahiri, T. and Chakravarti, S. K. 1989. Characteristics of some soils of Sikkim at various altitudes. *Journal of the Indian Society of Soil Science* **37**: 451-454.
15. Marazi, A. R. 1988. Effect of different nitrogen levels and water regimes on yield and growth of rice. M.Sc thesis submitted to Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, Shalimar pp -120.
16. Poonam, S., Kumawat, K. C., Sabhjeet, K. and Navprabhjot, K. 2014. Assessment of zinc solubilization by endophytic bacteria in legume rhizosphere. *Indian Journal of Applied Research* **4.**
17. Reyes, R. Y. and Brinkman, R. 1989. The effect of zinc application and other land crop management measure on wetland rice productivity in a zinc-deficient soil. *Philippine. Journal of Crop Science* **61**: 7-13.
18. Rodriguiz, H., Gonzalez, T., Goire, I. and Bashan, Y., 2004. Gluconic acid production and phosphate solubilization by the plant growth promoting bacterium *Azospirillum spp. Naturwissenschaften* **91**: 552-555.
19. Saravanan, V. S., Kumar, R. M. and SA, M. T. 2011. Microbial zinc solubilization and their role on plants. *Bacteria in Agrobiology: Plant nutrient management.* pp: 47-63.
20. Saravanan, V.S. and Raj, S. A. 2004. Isolation and characterization of zinc solubalising bacteria from paddy soil. *Journal of Biofertiliser technology* **12**: 379-385.
21. Thompson, J. P. 1996. Correction of dual phosphorous and zinc deficiencies of linseed with cultivars of vesicular-arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **28**: 941- 951.