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Unveiling the Potential of Zinc Solubilizing Microorganisms for Biofertilizer Application: Isolation and Identification

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Abstract:- Various micro- and macronutrients play a vital role in determining crop productivity. This study is aimed in isolating and identifying Zinc solubilizing microorganisms from rhizosphere soil. Zinc (Zn) is an essential trace element for life as it is required for the structural integrity and catalytic activity of all the classes of enzymes, and the zinc-binding domains are highly conserved among species. In plants, these enzymes are involved in carbohydrate metabolism, auxin metabolism, and antioxidant activity. The isolation of microorganisms from soil was implemented by inoculating a 1 mL dilution over the medium. Zinc oxide was used as an insoluble zinc source and modified the composition of Pikovskaya's agar. The plates were incubated at 37˚C for 48 hours, then the bacterial and fungal colonies were observed, and pure cultures were maintained on a Nutrient Agar slant and a Sabouraud Dextrose Agar (SDA) slant, respectively. Microscopic examination was done to at genus level to study morphology, and growth rate was observed. Isolates were maintained on Pikovskaya's agar for further screening of Phosphate solubilization. Some biochemical tests were performed to differentiate on the basis of their activities. The bacterial isolates were identified to be of Pseudomonas spp. while the fungal isolates were identified to be of Aspergillus spp., and Penicillium spp. The results showed that the isolates of Aspergillus spp. were the most abundant fungus. These results show the enormous potential of these microorganisms for biofertilizer production.

Keywords:- *Zinc Solubilizing Microorganisms, Pseudomonas spp., Aspergillus spp., Penicillium spp.*

I. INTRODUCTION

A. Biofertilizers for Sustainable Agriculture:

Biofertilizers are microbial inoculants that are applied to soil or seed to improve plant growth and productivity. They are a sustainable and environmentally friendly alternative to chemical fertilizers, and they can help improve soil health [1] and reduce the risk of environmental pollution. Biofertilizers provide nutrients needed for plant growth, improve soil health, and reduce the environmental impact of agriculture. Biofertilizers can provide plants with nitrogen, phosphorus, potassium, and other essential nutrients. This helps improve crop yield and quality. Biofertilizers help improve soil health by increasing the number of beneficial microorganisms in the soil. These microorganisms help break down organic matter, release nutrients from the soil, and suppress plant diseases. Healthy soil is more productive and better able to withstand drought, flooding, and other environmental stresses. Biofertilizers are a promising technology for sustainable agriculture. They offer a number of benefits over chemical fertilizers, including improved crop yields, improved soil health, reduced environmental impact, and cost-effectiveness, which can save farmers money. As research into biofertilizers is progressing, it is likely that they will be widely used in the future.

B. Plant Nutrients in Soil:

Plants produce their own food through photosynthesis and absorb the nutrients through their roots from soil. They require two types of nutrients: macro and micronutrients.

Macronutrients are essential nutrients that plants need in copious quantities. They include nitrogen, phosphorus, potassium, calcium, magnesium, and sulphur. Macronutrients are typically more abundant in soils. Biofertilizers can help to improve the availability of macronutrients to plants by fixing nitrogen from the air, solubilizing phosphorus from insoluble forms in the soil, or increasing the uptake of macronutrients by plants.

Micronutrients are essential nutrients that plants need in small quantities.[2]. They include boron, chlorine, copper, iron, manganese, molybdenum, nickel, and zinc. Micronutrients are often deficient in soils, and they can be a limiting factor for plant growth. Biofertilizers can help to improve the availability of micronutrients to plants by releasing them from insoluble forms in the soil or by increasing the uptake of micronutrients by plants.

C. Availability of Zinc in Soil[3]:

The zinc content within soil formation is determined by the geochemical composition and weathering of the parent rock. Environmental contamination and the use of zinc-rich products can modify this parent rock composition.

Zinc in soil exists as either divalent ions (Zn^{2+}) or complex forms like $ZnHCO_3^+$, $Zn(OH)_3^-$, and ZnO. These forms are categorized into five pools within the soil: water-soluble, exchangeable, organically bound, adsorbed, and chelated. The strength of these forms impacts their availability for plant uptake and the potential for leaching. Additionally, the distribution of these forms is influenced by factors such as zinc concentration, pH levels, and the presence of other metals, primarily iron and manganese.

Zinc is a crucial trace element essential for the growth of all crops. Serving as a key component in various biological enzymatic activities, it plays a vital role. Insufficient zinc can induce physiological stress, hampering the proper function of many enzymes and altering metabolic reaction rates. Plants absorb zinc primarily as divalent cations (Zn^{2+}), which are required for several vital processes, including metabolic functions, photosynthesis, auxin biosynthesis, fertilization, and resistance against specific pathogens.

However, zinc reservoirs are often not directly accessible to plants due to their insoluble nature. The bioavailability of zinc to plants is enhanced through the external application of zinc sulfate ($ZnSO_4$) as a chemical fertilizer. Yet only a small fraction (1-5%) of the applied zinc is utilized by crops, with the remaining 95% converting back into various insoluble forms. Furthermore, chemical fertilizers are costly and environmentally unfriendly, rendering crops more susceptible to diseases and gradually depleting soil fertility. The reckless use of fertilizers has led to detrimental water and soil pollution, posing risks to both plant and human health. Indeed, biofertilizers comprising living microbes are environmentally friendly and play a crucial role in maintaining long-term soil fertility and agro sustainability. Because microorganisms play a vital role in Zn solubilization, this ability of microorganisms can be used efficiently to solubilize the various insoluble Zn components to improve the bioavailability of zinc to crops.

D. Role of Zinc in Plants:

Plants require 20-100 mg/kg zinc, falling below of which, it is termed deficiency. Zinc serves diverse physiological and metabolic roles in plants. It is a component of essential enzymes like carbonic anhydrase and ribulose 1,5-biphosphate carboxylase (RuBPC), both crucial for photosynthesis. Additionally, zinc contributes to RNA and DNA synthesis, playing a regulatory function. Genes responsible for conferring environmental stress tolerance in plants are reliant on zinc. Visible signs of Zn deficiency include stunted growth, chlorosis, smaller leaves, short internodes (in cereals), and leaf rosetting[4]. Younger leaves are primarily affected and could visualize symptoms of interveinal chlorosis. Plants suffering from zinc deficiency fail to attain their maximum production potential and may become more susceptible to injury or infection. Notably, crops like maize, rice, and wheat, among various cereals, are particularly vulnerable to zinc deficiency.

E. Mechanism of Zinc Uptake by Plants:

Despite the soil containing an ample amount of zinc, its deficiency in plants is primarily attributed to the insoluble nature of zinc within the soil. In oxidizing, acidic soil conditions, zinc demonstrates enhanced mobility and is readily taken up by plants. Conversely, in poorly reducing, neutral, or alkaline soils, zinc remains immobile. Within the soil, zinc exists as a divalent cation or forms complexes with ligands. It is transported towards the roots through mechanisms involving mass flow, diffusion, and root extension[6]. The transportation of zinc from the soil solution into plants predominantly occurs through an active transport system, facilitating its movement from roots to shoots.[5] A small fraction of zinc is retained at the basal node, influencing the overall distribution of zinc within the plant. The transportation of zinc takes place through both xylem and phloem tissues, allowing for its movement to various parts of the plant.

F. Role of Zinc Fertilizers in Soil:

Nowadays, the most popular method used to resolve zinc deficiency is the use of many crop fertilizers, whose selectivity will rely on its cost, convenience, economic compatibility, mode of implementation, and acceptability in the environment. Zinc fertilizers are typically categorized into three main groups: inorganic, natural organic, and synthetic chelate complexes. Each category is chosen based on its specific merits and considerations.

Inorganic Zn source includes ZnO, $ZnCO_3$, $ZnSO_4$, $Zn_3(PO_4)_2$ and $ZnCl_2$. On the other hand, organic Zn fertilizers include different compounds, majorly Zn-phenolate, Zn-EDTA and Zn-lignosulfonate. Zn-EDTA enhances the phosphorus uptake and is more efficiently transported to plants as compared to $ZnSO_4$. The activity of biodegradable chelates IDHA [N-(1,2-carboxyethyl)-D, L-aspartic acid] and EDDS (ethylene diamine disuccinic acid) or complexes as gluconates and lignosulfonates which are the byproducts from the food and paper industries, has shown remarkable results[7]. Fertilizers such as zincate urea, zincate super and boronated super, when blended with micronutrients have shown long term effectiveness in increasing the soil fertility and micronutrient deficiency in plants. Due to inefficiency and economic factors, organic fertilizers are less accepted by farmer.

G. Microorganisms for Zinc Solubilization:

To overcome Zn deficiency, exploiting Zn soluble microorganisms may help in solubilizing the insoluble Zn into soluble form thereby making plant healthier with good yield[8]. Selection and inoculation of Zn solubilizing bacteria either in pure form or in conjunction with cheap insoluble Zn compounds would reduce the cost of production of the agricultural commodity. Several microorganisms are acknowledged for their pivotal role in solubilizing and mobilizing essential elements like potassium, phosphorus, iron, silicates, and zinc, facilitating their uptake by plant roots. Several research studies have highlighted the effectiveness of rhizospheric fungi to solubilize insoluble Zn compounds. Some fungi namely *Aspergillus spp.* and *Penicillium spp*. have highest activity

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in solubilizing insoluble Zn compounds ZnO, and $ZnCO_3$ through the secretion of acid. Like ahead bacteria like *Bacillus spp.* and *Pseudomonas spp.* have previously been reported to solubilize insoluble Zinc and thus can be employed as bio-fertilizers.

H. Mechanism of Zinc Solubilization by Zinc Solubilizing Biofertilizers (ZSBs):

Biofertilizers use diverse mechanisms to solubilize zinc in the soil. One prevalent mechanism entails the secretion of organic acids, such as citric acid or gluconic acid[10], by microorganisms like bacteria and fungi[9]. These acids play a crucial role in breaking down insoluble zinc compounds within the soil, rendering them accessible for plant absorption. This process involves the reduction of soil pH, which aids in dissolving zinc from its insoluble forms, thereby enhancing its availability to plants.

Furthermore, certain biofertilizers generate additional enzymes that directly participate in the solubilization of zinc. These enzymes contribute to the breakdown of complex zinc compounds, further facilitating its release into a form that can be taken up by plants. Through these multifaceted mechanisms, biofertilizers act as natural agents to enhance zinc availability and uptake, ultimately contributing to improved plant health and growth.

An additional significant mechanism employed by Zinc-Solubilizing Biofertilizers (ZSBs) involves the production of chelating agents for zinc solubilization. Chelating agents are molecules capable of binding to metal ions, forming soluble complexes[11]. Among the most common chelating agents produced by ZSBs are siderophores. Siderophores, which are originally iron-binding molecules, also possess the ability to bind to zinc ions. ZSBs also play a role in the mineralization of organic matter, a process through which nutrients are released from organic materials, rendering them more accessible to plants[11][12]. Zinc is one of the nutrients that can be liberated from organic matter by ZSBs. While the mineralization of organic matter is a gradual process, it can serve as a significant long-term source of zinc for plants.

In practice, ZSBs can be introduced to soil in liquid or powder form. They can also be combined with seeds or fertilizers to enhance their application. The most suitable method for applying ZSBs depends on the specific strain of ZSB and the prevailing soil conditions. This multi-faceted approach underscores the potential of ZSBs to enhance zinc availability for plants, contributing to improved agricultural productivity over time.

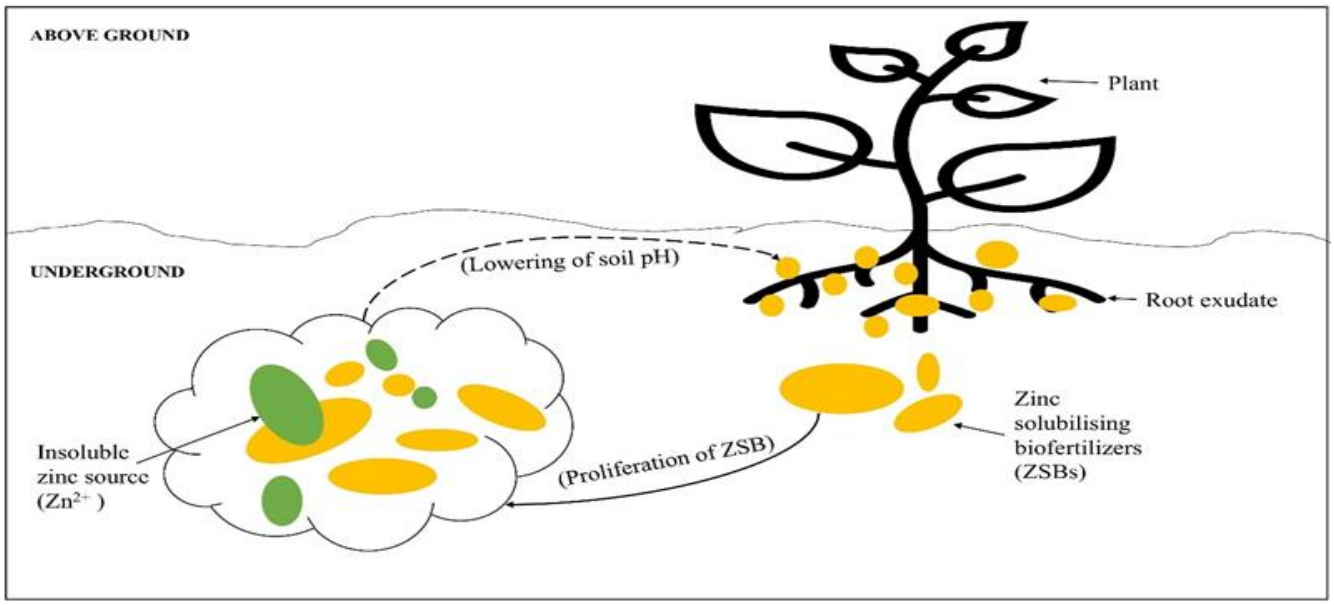

Fig 1 Overview of Zinc Solubilization by Zinc Solubilizing Biofertilizers (ZSBs).

II. MATERIALS AND METHODOLOGY

A. Collection of Soil Sample:

A rhizosphere soil sample was collected from the campus of the Rajiv Gandhi Biotechnology Centre, RTM Nagpur University, Nagpur, [21.144269^0 79.046737^0] by digging the earth surface to 10-15 cm. After collection, the sample was immediately transferred to the laboratory. The soil sample was sieved to separate stones, and rest of the sample was powdered and dried. It was stored at 4^0C for microbial analysis.

Fig 2 Soil Sample from the Campus.

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B. Isolation of Zinc Solubilizing Microorganisms from the Collected Soil Sample:

➢ *Serial dilution of Soil Sample[13]:*

For serial dilution, saline water (0.85%) was prepared by adding 0.85g NaCl in 100 ml distilled water. Prepared saline water, a conical flask, and 7 test tubes were autoclaved for 15 min at 15 psi at 121^0C. For serial dilution 1.0 g of rhizosphere soil was used. This 1.0 g soil sample was mixed with 10.0 g saline water in a conical flask. It was vigorously shaken to form a homogenous soil suspension, and the conical flask was marked 'S.' Then each of the seven test tube was filled with 9 ml saline water. From S, 1 ml soil suspension was added to first test tube making total volume of 10 ml with the help of micropipette. This test tube was marked 10^{-1}. From this test tube 1 ml of solution was then added to the second test tube which was then marked as 10^{-2}. Similar steps of serial dilution were followed for five more test tubes, marking them as 10^{-3} to 10^{-7} respectively. Thus, the soil sample was serially diluted upto the dilution factor of 10^{-7}.

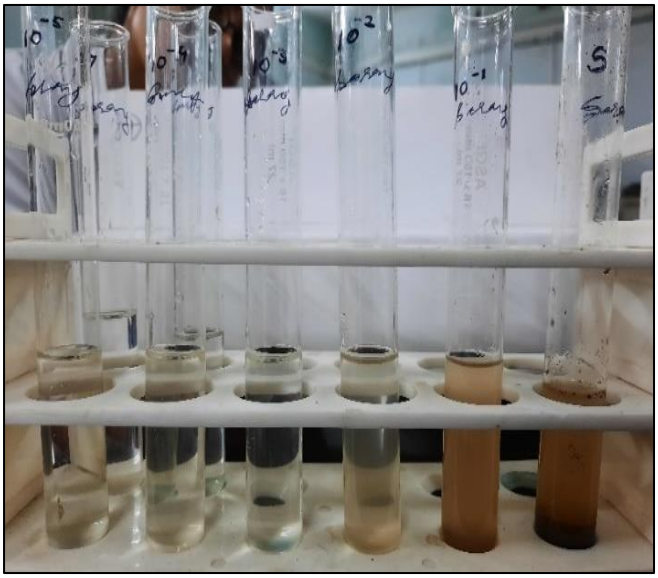

Fig 3 Serial Dilution of Soil Sample.

➢ *Media Preparation:*

The modified form of Pikovskaya's agar medium was used[14]. The composition of modified Pikovskaya's agar medium (m-PVK) is given below:

Table 1 The Composition of Modified Pikovskaya's Agar Medium (m-PVK)

Glucose	10.0 g
Ammonium Sulphate	1.0 g
Potassium chloride	0.2 g
Dipotassium hydrogen phosphate	0.2 g
Magnesium Sulphate	0.1 g
Yeast extract	0.2 g
Distilled Water	1000 mL
pH	7.0
Agar	15.0 g
Zinc oxide	0.1%

In this medium, Zinc oxide (ZnO) is used as the source of insoluble zinc for the isolation of zinc solubilizing microorganisms[14][15].

➢ *Spread Plate Technique[16]:*

The sterilized m-PVK medium was poured into each petri dish and allowed to solidify before inoculation. From the sixth dilution series (test tube marked as 10^{-6}), 0.1 mL solution was poured on the agar surface of first petri plate. The solution was uniformly spread all over the surface of agar medium with the help of L-shaped glass spreader and carefully rotated the plate at an angle of 45^0 at the same time. The experiment was performed by forming duplicates of this dilution series and then plates were kept for incubation at 37^0C inside the incubator.

➢ *Isolation of Zinc Solubilizing Microorganisms:*

After the incubation for 48 hours at 37^0C, the colonies of bacteria and fungi were observed growing on the surface of the medium. The zinc solubilizing bacteria (ZnSB) and zinc solubilizing fungi (ZnSF) were identified by the presence of a clear halo zone formed around the colonies. Zinc solubilizing bacteria and fungi were isolated from the plates and transferred to new plates for purification.[17].

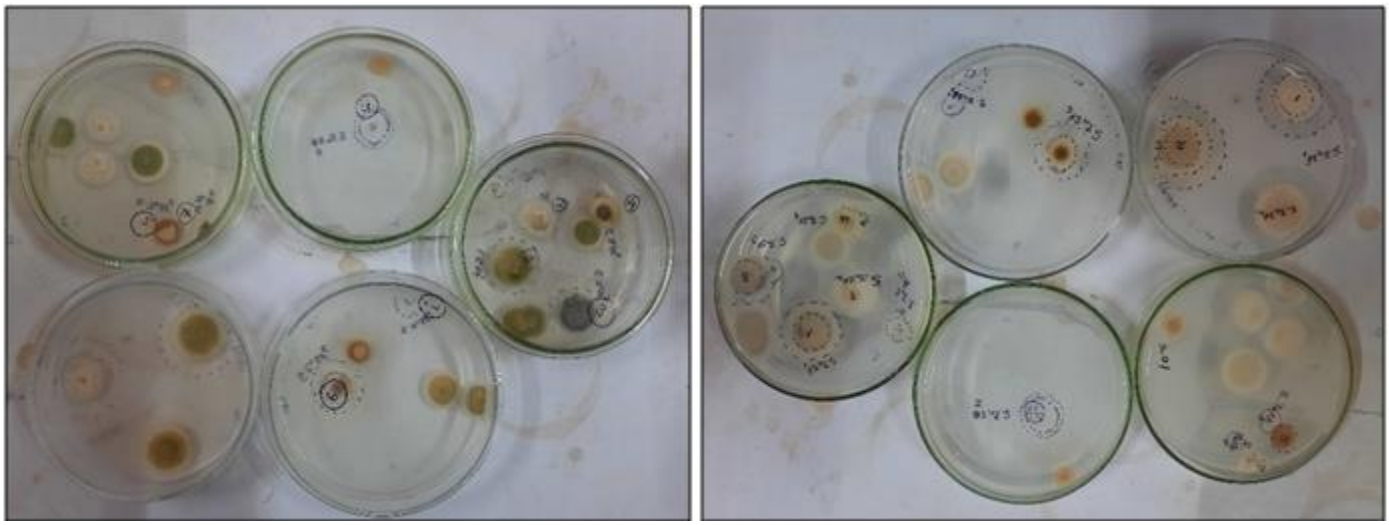

Fig 4 Bacterial and Fungal Colonies Grown on m-PVK.

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The pure cultures were preserved on the agar slants. The isolates of zinc solubilizing bacteria were cultured on the Nutrient Agar slants and isolates of zinc solubilizing fungi were cultured on Sabouraud Dextrose Agar (SDA) slants. Both media were autoclaved for 15 min at 15 psi at 121^0C. Under aseptic conditions, 10 mL each of Nutrient agar and SDA was poured in respective test tube. Test tubes were then placed on stage at an angle to form an inclined agar slant. As the agar solidified, streaking was performed over the surface. and test tubes were then left for incubation for 24 hours at 37^0C inside the incubator to obtain the pure culture.

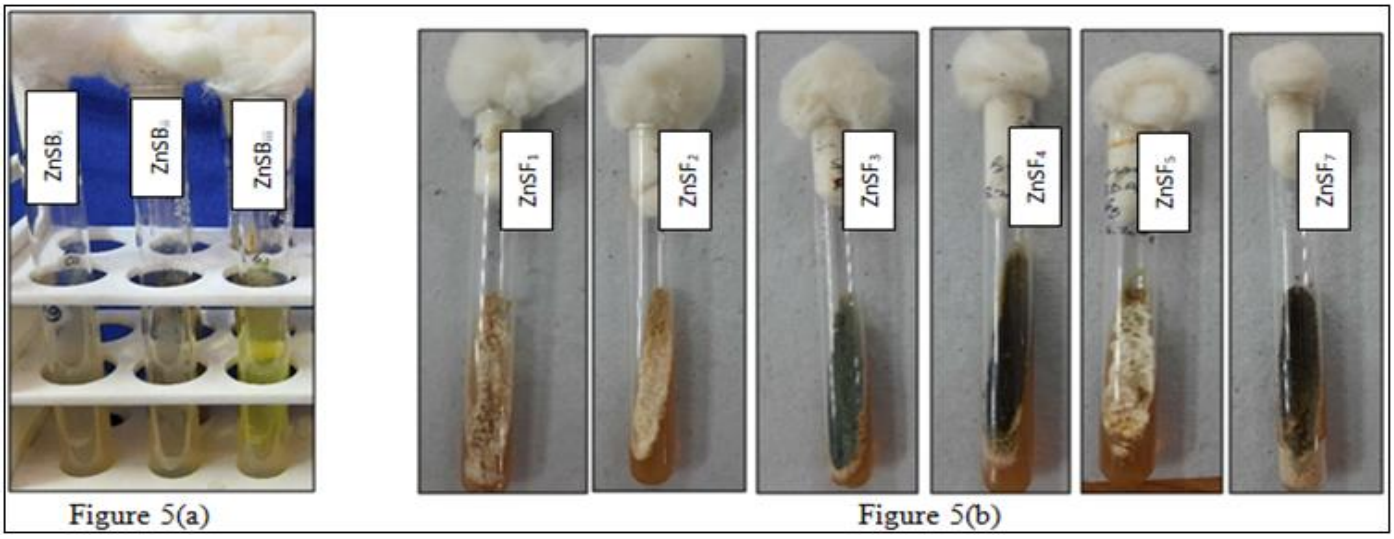

Fig 5 (a): Pure Culture of Bacteria Grown on Nutrient Agar Slants. (b): Pure Culture of Fungi Grown on SDA Slants.

C. Morphological Characterization:

➢ *Morphological Characterization of Zinc Solubilizing Bacteria.*

Gram's staining[18]- The 24-36 hour old culture suspension of the test bacterium was prepared. A loop full of bacterial suspension was smeared on a clean glass slide, air dried, and heat fixed on the flame of Bunsen burner. A drop of aqueous Crystal violet solution (0.5%) was spread over a fixed smear for 60 seconds and then washed with running tap water for another 60 seconds. The stained smear was then flooded with Gram's iodine solution for 60 seconds. Again, rinse off with tap water. Later, decolorize the smear with 95% Ethanol solution. As the color of crystal violet runoffs, wash it with running tap water. Finally counter stained with Safranin for next 10 seconds and then washed with flowing water. The slides were observed under the microscope.

➢ *Morphological Characterization of Zinc Solubilizing Fungi.*

Lactophenol Cotton Blue staining (Gillman, 1957.)[19]- A clean glass slide was taken, and a drop of Lactophenol Blue was placed. With the help of an inoculating needle, a fungal specimen was placed over the drop, and a clean coverslip was placed gently. The slide was then observed under the microscope, identifying the fungal structure.

D. Screening of Isolates for other Beneficial Effects:

Phosphate solubilizing test- The Pikovaskya's agar medium (PVK) was prepared by mixing commercially available Pikovaskya's Broth: 31.3 g, Agar: 15.0 g and Distilled water: 1000 ml. The composition was mixed in a flask; autoclaved and cooled media was then poured into the glass Petri dish and allowed to solidify for few minutes. After solidification, the pure culture of the test microorganism was streaked over surface and plates were incubated for 24 hours at 37^0C[20].

E. Biochemical Characterization of Zinc Solubilizing Microorganisms:

➢ *Starch Hydrolysis Test (MacFaddin, 2000)-*

The Starch Medium was Prepared using the Given Constituents:

Table 2 The Starch Medium

Beef extract	3.0 g
Soluble starch	10.0 g
Agar	12.0 g
Distilled water	1000 mL

The autoclaved and cooled media was poured on glass petri dishes and were left to solidify. On solidification, the pure culture of the test microorganisms was streaked on it and incubated for 24 hours at 37^0C. After incubation, these plates were flooded with Iodine solution and allowed to react for 30-45 seconds[20][22].

➢ *Indole Production Test (Isenberg and Sundheim, 1958)-*

Indole production test was done by preparing the Tryptone medium containing

Table 3 Indole Production Test

Tryptone	10.0 g
Sodium chloride	5.0 g
Distilled water	1000 mL
pH	7.5 ± 0.2

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Media was autoclaved at 15 psi for 15 min at 121^{0}C and after cooling, 5 ml of it was poured into test tubes. These test tubes were then inoculated with the pure culture of test microorganisms and left for incubation at 37^{0}C for 24 hours. After incubation for sufficient period, 0.5ml (5 drops) of spot indole reagent (1% p-Dimethylaminocinnamaldehyde) were added, and top layer was examined after 60 seconds[20][22].

➢ *Citrate Utilization Test (MacFaddin, 2000)-*

Simmon's citrate agar media was prepared for this test. The composition of this media was

Table 4 Citrate Utilization Test

Sodium chloride	5.0 g
Sodium citrate	2.0 g
Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate heptahydrate	0.2 g
Bromothymol Blue	0.08 g
Agar	15.0 g
Distilled water	1000 mL

The media was autoclaved, 5 ml of it was poured into each test tube and left to solidify in slant position. On solidification, the pure culture of the test microorganisms was stabbed and streaked over the slant and incubated for 24 hours at 37^{0}C.[22]. These slants were then left for observation.

➢ *Oxidase Test (Collins and Lyne, 1970)-*

The media was prepared by mixing commercially available Tryptone Soya Broth: 30.3 g and Agar: 15.0 g in 1000 mL distilled water. Media was then autoclaved at 15 psi for 15 min at 121^{0}C. media was then poured into petri plate and allowed to solidify. After solidification, the pure culture of test microorganisms was streaked over the media surface and incubated for 24 hours at 37^{0}C. After incubation, 2-3 drops of N,N,N,N tetramethyl-p-phenylenediamine-dihydrochloride (Wurster's Reagent) were added and then examined for color change over the surface[21].

➢ *H_2S Production Test (Beishir, 1991)-*

In this test Sulfide Indole Motility Medium (SIM) was used where Sodium thiosulfate acts as sulfur source. The composition of SIM medium was

Table 5 H_2S Production Test

Beef extract	3.0 g
Peptone	30.0 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.025 g
Agar	3.0 g
Distilled water	1000 mL
pH	7.3 ± 0.2

The media was then heated, autoclaved, and poured into glass test tube. The fresh and pure culture of test microorganisms was inoculated on media by stabbing to a depth of half an inch using a needle. Test tubes were then incubated at 37^{0}C and observed for the blackening of medium on the inoculation line for 2 days[21][22].

➢ *Methyl Red Test (Crown and Gen, 1998)-*

In this test, Glucose-Phosphate Broth was prepared using

Table 6 Methyl Red Test

Buffered peptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1000 mL
pH	6.9

The medium was equilibrated at room temperature on heating plate, later autoclaved, cooled, and poured in test tubes. The pure culture of test microorganisms was inoculated and incubated at 37^{0}C for 48 hours. Later methyl red indicator drops (2-3 drops) were added to the tubes and immediately observed for changes in color[20].

➢ *Catalase Test (Rangaswami and Bagyaraj, 1993)-*

The slide drop method was followed in this test. A loop full of 24-48 hours old cultures of test microorganisms was placed on the glass slide. A drop of commercially available Hydrogen peroxide (3% H_2O_2) was placed over the surface with the help of a dropper. It was then allowed to react for 60 seconds, and small bubble formations were examined on the glass slides[21][22].

III. RESULTS AND DISCUSSION

A. Isolation of Zinc Solubilizing Microorganisms from the Collected Soil Sample:

A total of 5 bacterial isolates and 7 fungal isolates were observed growing on the plates of modified Pikovaskya's Agar. The best isolates were selected based on the diameter of halo zones. The isolates showed halo zones, and out of them, the best 3 bacterial isolates and 6 fungal isolates were selected for further morphological and biochemical examination.

The best 3 bacterial isolates were named $ZnSB_i$, $ZnSB_{ii}$, $ZnSB_{iii}$ and the best 6 fungal isolates were named $ZnSF_1$, $ZnSF_2$, $ZnSF_3$, $ZnSF_4$, $ZnSF_5$, $ZnSF_7$.

The zinc solubilization index (S.I.) was calculated after 48 hours of incubation using the formula

$$\text{Solubilization Index (S.I.)} = \frac{\text{Colony Diameter (C.D)} + \text{Halo zone Diameter (H.D)}}{\text{Colony Diameter (C.D)}}$$

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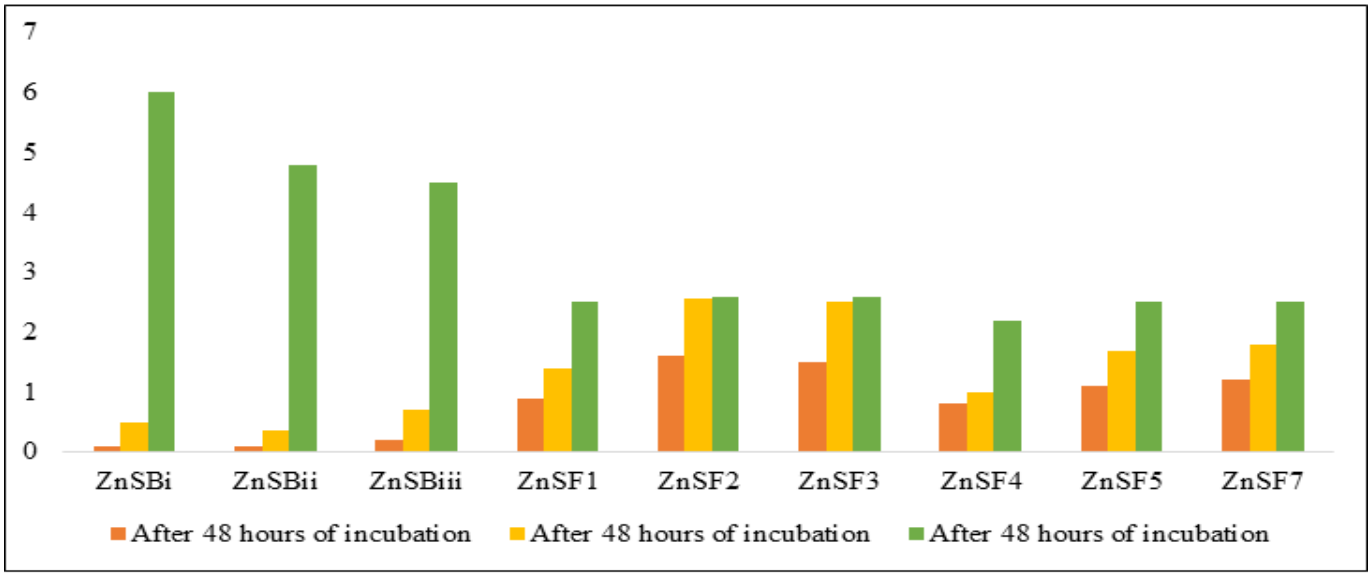
Table 7 Screening of Isolates for Zinc Solubilization on m-PVK Media (0.1% ZnO) after 48 Hours of Incubation.

Isolates	After 48 hours of incubation		
	C.D.* (cm)	H.D.* (cm)	Zinc Solubilization Index (S.I.)
$ZnSB_i$	0.10	0.50	6.0
$ZnSB_{ii}$	0.09	0.35	4.8
$ZnSB_{iii}$	0.20	0.70	4.5
$ZnSF_1$	0.90	1.40	2.5
$ZnSF_2$	1.60	2.55	2.6
$ZnSF_3$	1.50	2.50	2.6
$ZnSF_4$	0.80	1.00	2.2
$ZnSF_5$	1.10	1.70	2.5
$ZnSF_7$	1.20	1.80	2.5

C.D. = Colony Diameter H.D. = Halo zone Diameter

* All indices are measured using a scale and incorporate a margin of error of ±0.01cm.



Graph 1 Screening of Isolates for Zinc Solubilization on m-PVK Media.

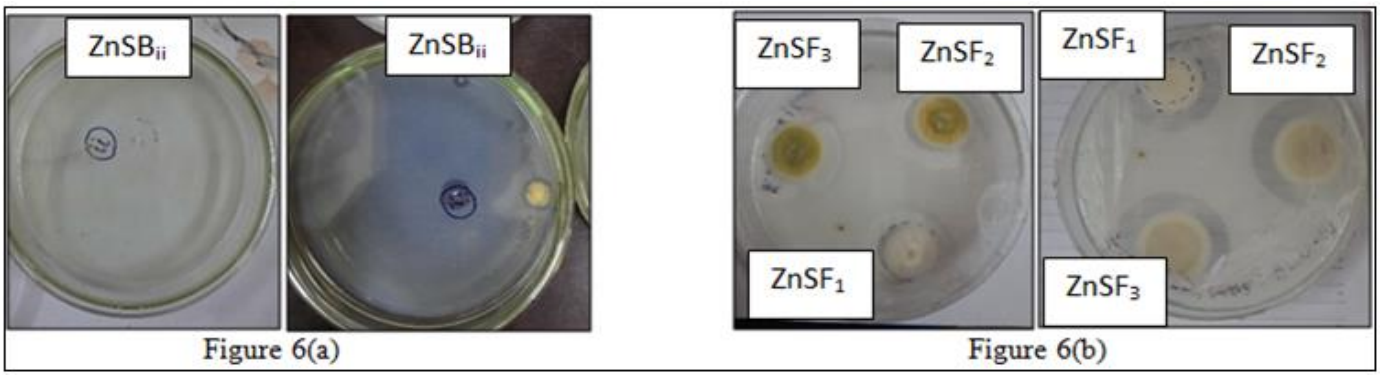


Fig 6(a): Zinc solubilization by bacterial isolate on m-PVK. (b): Zinc solubilization by fungal isolates on m-PVK.

B. Morphological Characterization:

➢ *Morphological Characterization of Zinc Solubilizing Bacteria.*

After gram staining, the bacterial isolates $ZnSB_i$, $ZnSB_{ii}$, $ZnSB_{iii}$ were identified to be of *Pseudomonas spp*.

Table 8 Morphological Characterization of Zinc Solubilizing Bacteria

Isolates	Colony characters	Cell shape	Gram reaction	Probable genera
$ZnSB_i$	Smooth, White	Rod shaped	Gram negative	*Pseudomonas sp.*
$ZnSB_{ii}$	Smooth, Creamy white	Rod shaped	Gram negative	*Pseudomonas sp.*
$ZnSB_{iii}$	Smooth, Light green	Rod shaped	Gram negative	*Pseudomonas sp.*

➢ *Morphological Characterization of Zinc Solubilizing Fungi.*

Lactophenol cotton blue staining was performed for identification of fungal components in the isolates. On microscopic visualization, the isolates were found to be of *Aspergillus spp*., and *Penicillium spp.*

Table 9 Morphological Characterization of Zinc Solubilizing Fungi

Isolates	Colony morphology	Colony surface	Growth rate	Probable genera
$ZnSF_1$	Yellow- Brown colony with thick white outer margin	Rough texture	Moderate	*Aspergillus sp.*
$ZnSF_2$	Yellow- Green colony	Velvety texture	Fast	*Aspergillus sp.*
$ZnSF_3$	Dark green colony	Velvety texture	Moderate	*Penicillium sp.*
$ZnSF_4$	Brown colony with black conidial head and thin yellow margin	Rough texture	Slow	*Aspergillus sp.*
$ZnSF_5$	Creamy to green colony with thin white outer margin	Smooth texture	Slow	*Aspergillus sp.*
$ZnSF_7$	Black colony, Excess conidia	Rough texture	Rapid	*Aspergillus sp.*

C. Screening of Isolates for other Beneficial Effects:

- *Phosphate Solubilizing Test-*

All the selected isolated were screened for phosphate solubilization, out of which 2 bacterial and 3 fungal isolates gave positive result by forming a halo zone area around the colony.

Table 10 Phosphate Solubilizing Test

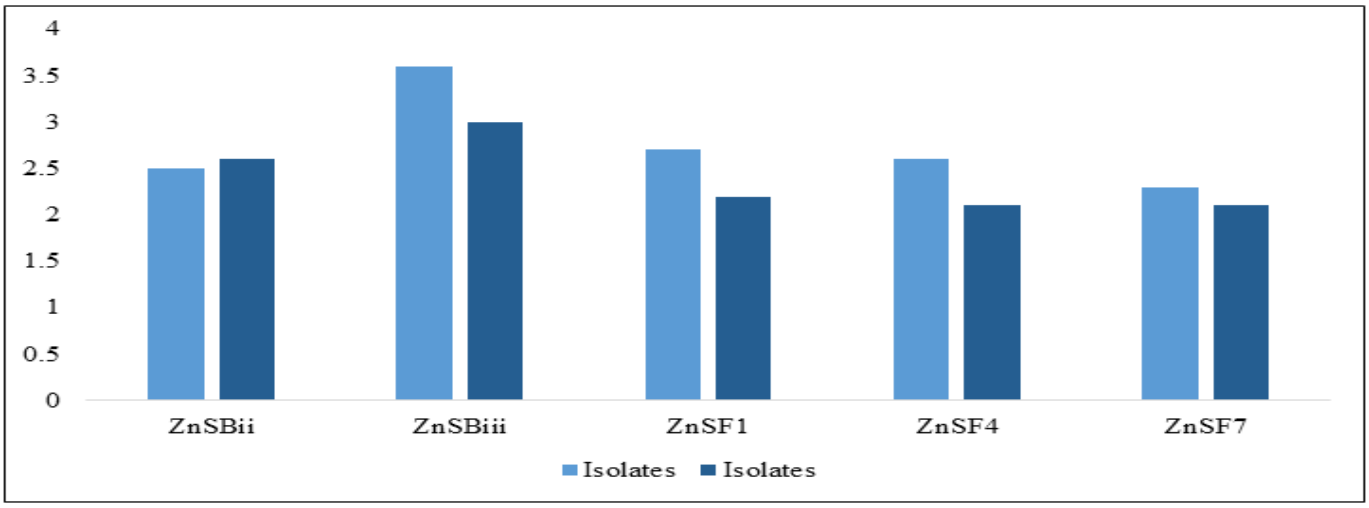
Isolates	Phosphate solubilization test
$ZnSB_i$	-
$ZnSB_{ii}$	+
$ZnSB_{iii}$	+
$ZnSF_1$	+
$ZnSF_2$	-
$ZnSF_3$	-
$ZnSF_4$	+
$ZnSF_5$	-
$ZnSF_7$	+

Table 11 Screening of Isolates for Phosphate Solubilization on PVK Media after Incubation.

Isolates	**24 hours incubation**			**After 10 days of incubation**		
	C.D. (cm)	H.D. (cm)	Phosphate solubilization Index	C.D. (cm)	H.D. (cm)	Phosphate solubilization Index
$ZnSB_{ii}$	0.40	0.60	2.5	0.80	1.30	2.6
$ZnSB_{iii}$	0.30	0.80	3.6	0.85	1.70	3.0
$ZnSF_1$	1.00	1.70	2.7	5.60	6.80	2.2
$ZnSF_4$	1.20	2.00	2.6	7.60	8.40	2.1
$ZnSF_7$	2.30	3.00	2.3	6.80	7.80	2.1

C.D. = Colony Diameter H.D. = Halo zone Diameter

* All indices are measured using a scale and incorporate a margin of error of ±0.01cm.



Graph 2 Comparison of Phosphate Solubilization Index on 24 Hours Incubation and after 10 Days of Incubation.

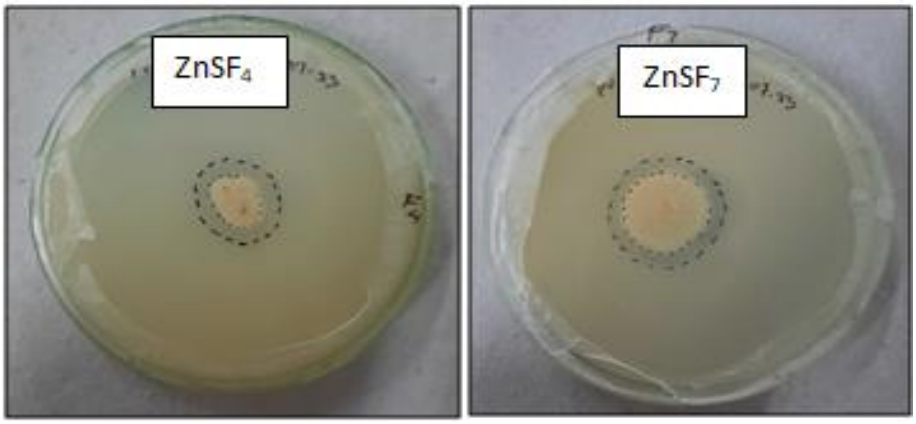


Fig 7(a) Phosphate Solubilization by Fungal Isolates on 24 Hours Incubation.

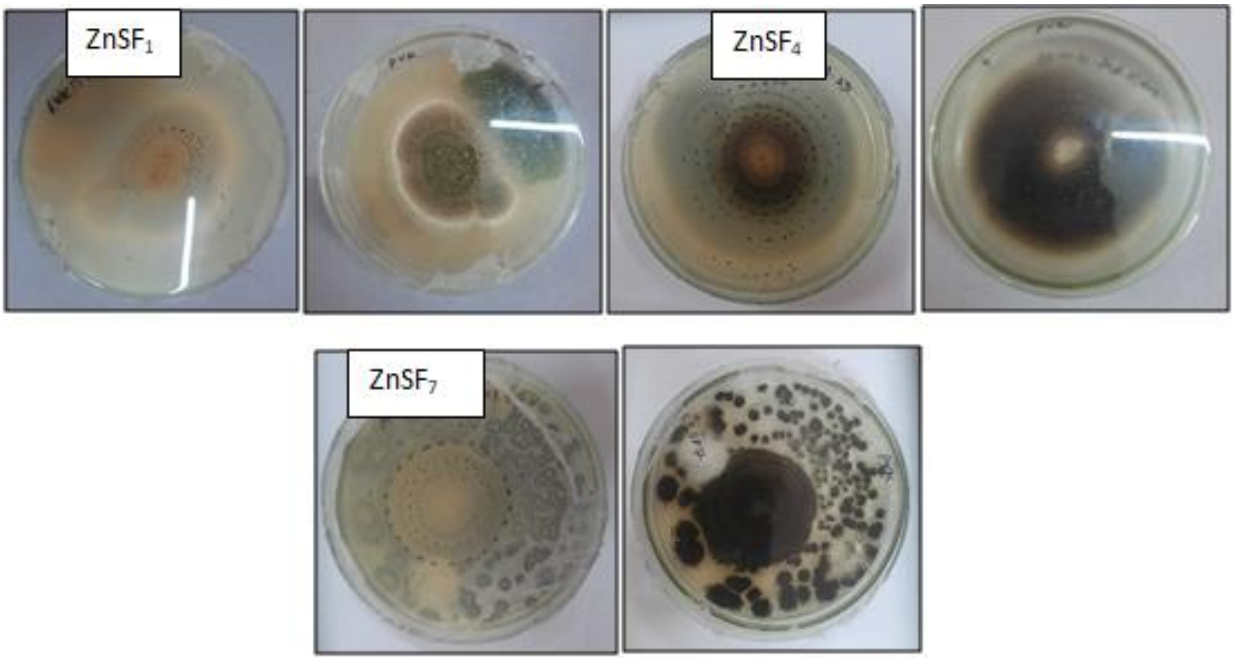


Fig 7(b) Phosphate Solubilization by Fungal Isolates after 10 Days Incubation.

D. Results and Discussion of Biochemical Test:

Table 12 Results of Biochemical Tests.

Isolates	Starch hydrolysis test	Indole production test	Citrate utilization test	Oxidase test	H_2S production test	Methyl red test	Catalase test
$ZnSB_i$	+	+	+	-	+	+	+
$ZnSB_{ii}$	+	-	+	-	-	+	+
$ZnSB_{iii}$	+	-	+	+	-	+	+
$ZnSF_1$	+	-	+	+	-	+	+
$ZnSF_2$	+	+	+	-	-	-	-
$ZnSF_3$	+	-	+	-	-	+	+
$ZnSF_4$	+	-	+	-	+	+	+
$ZnSF_5$	+	+	+	-	+	+	+
$ZnSF_7$	-	+	+	-	+	+	-

➢ *Starch Hydrolysis Test-*

The selected bacterial and fungal isolates were screened on starch agar to determine their ability to hydrolyse starch and check their amylase enzyme activity.

- *Positive Test:* On addition of an iodine solution, clear transparent zone is observed around the line of growth indicating organism has hydrolysed the starch.
- *Negative Test:* A blue area is formed around the growth.

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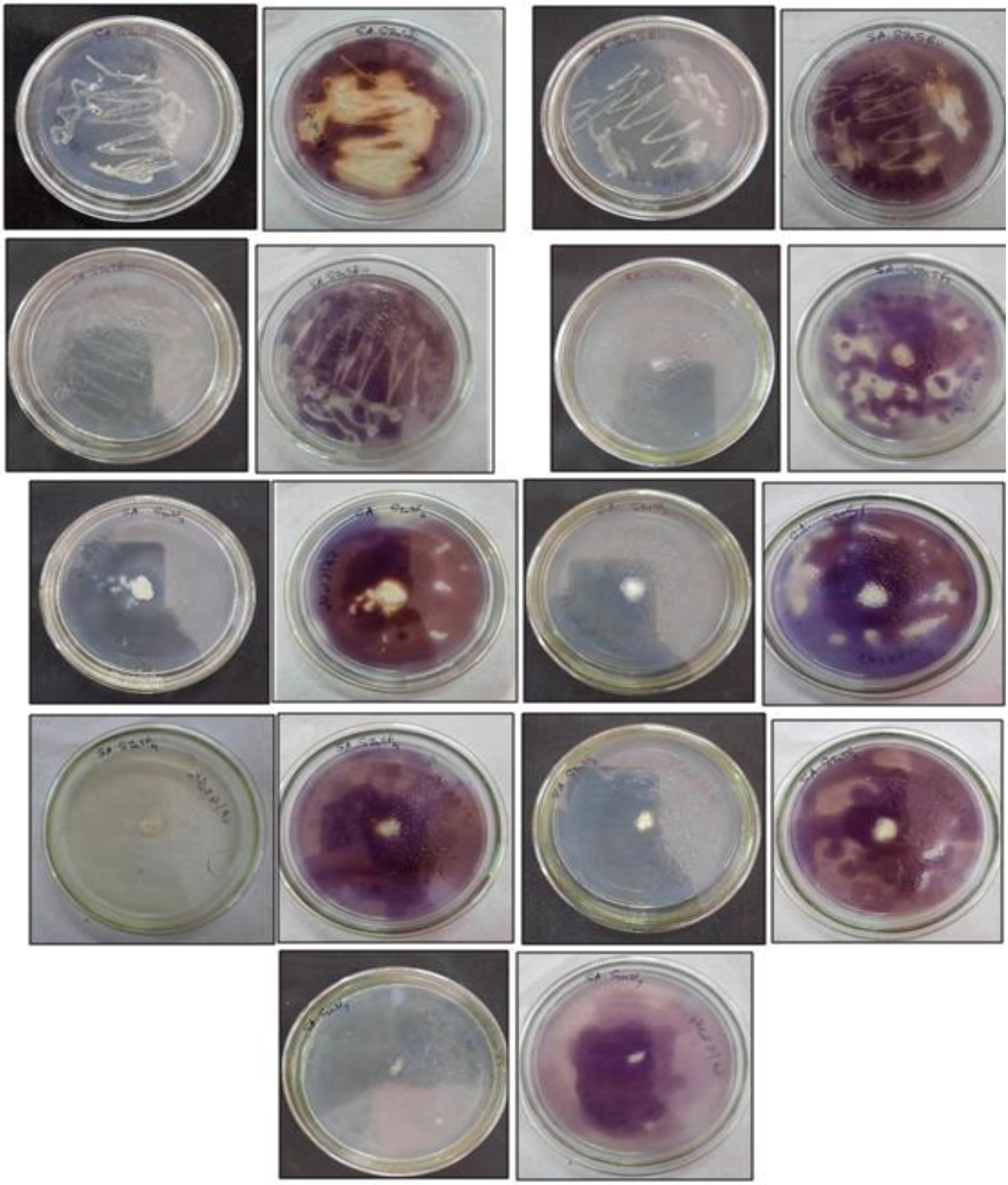

Fig 8 Results of Starch Hydrolysis Test.

➢ *Indole Production Test-*

The indole test determines the ability of microorganisms to degrade the tryptophan amino acid into indole. On addition of spot indole reagent (1% p- Dimethylaminocinnamaldehyde), the tryptone medium changed color.

- *Positive test:* Appearance of blue-green color which later turned to yellow-green color.
- *Negative test:* Colourless to slight yellow (same as color of tryptone media).

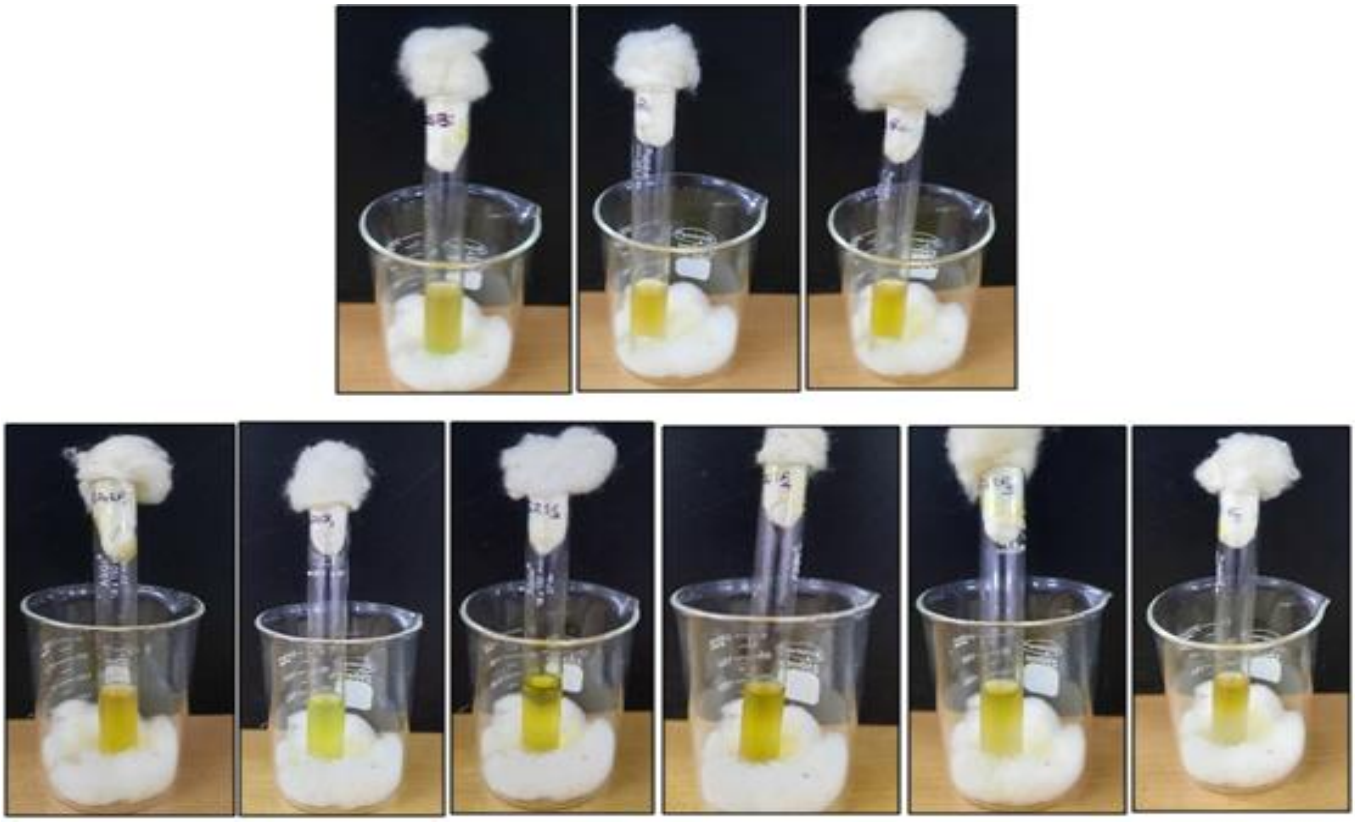

Fig 9 Results of Indole Production Test.

➢ *Citrate Utilization Test-*

A citrate test was performed to test the ability of isolates to utilize citrate as carbon source and the ammonium salt as a nitrogen source. When an organism metabolizes, the citrate is converted into pyruvate and the ammonium salt is broken down into ammonia, which increases the alkalinity. This shift in pH turns the green color of Simmon's citrate agar blue.

- *Positive test:* Color change from green to blue all over slant.
- *Negative test:* No color change.

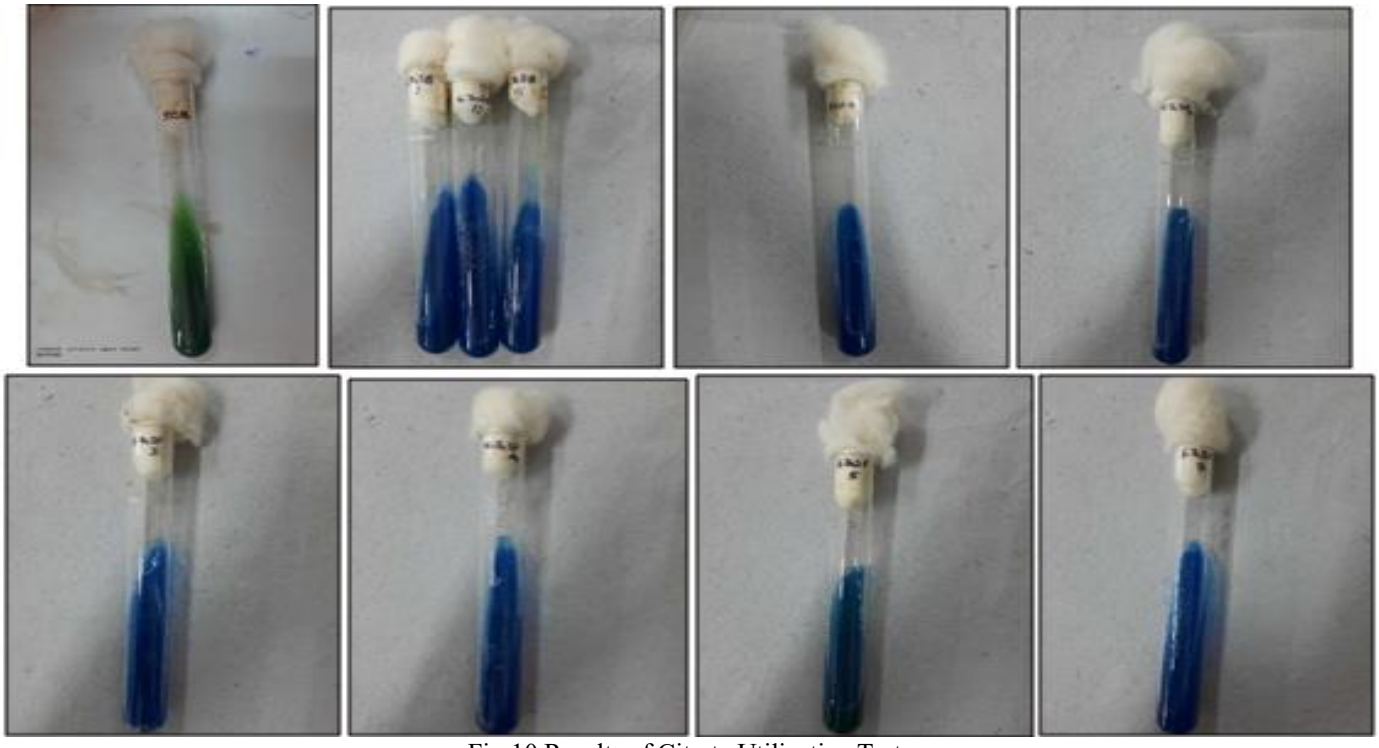

Fig 10 Results of Citrate Utilization Test.

➢ *Oxidase Test-*

The oxidase test was performed to detect the presence of cytochrome oxidase in the electron transport system in colony. The reagent acts as an electron acceptor in place of oxygen and the colony appears blue.

- *Positive test:* An extra electron takes the place of oxygen, and a blue color appears in 5-10 seconds.
- *Negative test:* Color does not change in the first 10 seconds.

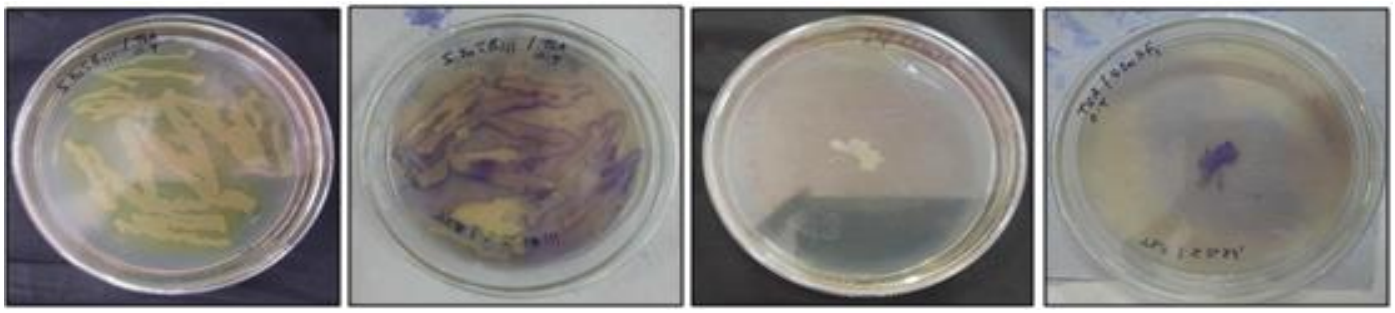

Fig 11 Results of Oxidase Test.

➢ *H_2S Production Test-*

This test was performed to determine whether the microorganisms have the ability to breakdown sulfur containing compounds into hydrogen sulfide gas during their metabolism. During protein degradation, inorganic thiosulfates are reduced. In the media, if H_2S is produced, it reacts with the iron component to form a black precipitate of ferric sulfide and thus the black color acts as an indicator.

- *Positive test:* Presence of black precipitate in/on medium.
- *Negative test:* No black compound in/on medium.

Fig 12 Results of H_2S Production Test.

➢ *Methyl Red Test-*

A methyl red test was performed to determine the capability of an isolate to produce stable acid as an end product during the acid fermentation of glucose.

- *Positive test:* Broth suspension turned dark red.
- *Negative test:* Broth suspension turned yellow.

Fig 13 Results of Methyl Red Test.

➢ *Catalase Test-*

This test was performed to demonstrate the presence of the catalase enzyme in colony. The catalase enzyme breaks down the hydrogen peroxide into oxygen and water.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2\ (gas\ bubble)$$

- *Positive test:* The appearance of the gas bubbles denotes the presence of catalase enzyme.
- *Negative test:* No bubbles were produced.

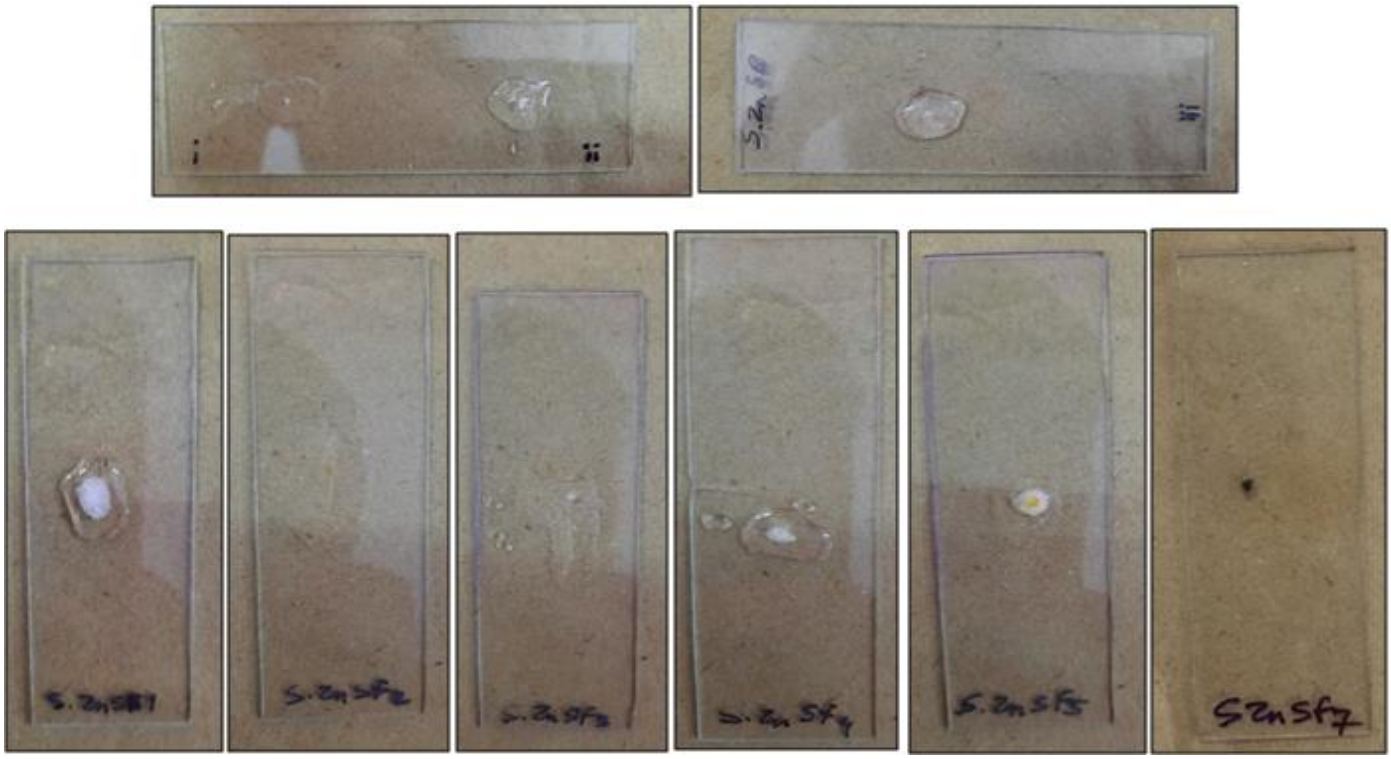

Fig 14 Results of Catalase Test.

IV. CONCLUSION

In this study, some natural bacterial and fungal strains were isolated from the soil of the campus of Rajiv Gandhi Biotechnology Centre, RTM Nagpur University, Nagpur. A total of 5 bacterial and 7 fungal isolates were identified, out of which 3 bacterial ($ZnSB_i$, $ZnSB_{ii}$, $ZnSB_{iii}$) and 6 fungal ($ZnSF_1$, $ZnSF_2$, $ZnSF_3$, $ZnSF_4$, $ZnSF_5$, $ZnSF_7$) isolates were characterized for their potential to solubilize zinc and phosphate by growing them on respective culture mediums. Some of the common soil bacterial isolates were identified as *Pseudomonas spp.,* and fungus were identified as *Aspergillus spp. and Penicillium spp.* Most of the fungal isolates were able to grow efficiently during the incubation period, while a few required more time for incubation. Certain biochemical tests were performed to study the activity of isolates. Hence, these microorganisms have the potential to become Zinc solubilizing biofertilizers (ZSBs).

Zinc holds a vital role as a micronutrient crucial for the growth and overall development of plants. However, its scarcity is a common issue in many soils. Zinc-solubilizing biofertilizers (ZSBs) offer a solution by effectively converting zinc from its insoluble forms within the soil, thereby enhancing its accessibility to plants. In light of the contemporary emphasis on sustainable agricultural practices, adopting zinc-solubilizing biofertilizers emerges as a cost-effective, environmentally friendly, and innovative approach. By leveraging zinc solubilizing biofertilizers, either independently or in conjunction with organic materials, the dual advantage of augmenting zinc's bioavailability and increasing nutrient content can be achieved. This approach extends its benefits beyond just zinc availability, as it can lead to enhanced uptake of other essential micro and macronutrients. This innovative strategy aligns harmoniously with the principles of sustainable agriculture, ensuring optimal resource utilization and contributing to the long-term vitality of both plants and ecosystems.

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