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Isolation and Identification of Insoluble Inorganic Phosphate Solubilizer Bacteria and Fungi Species from the Soil

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Abstract:- Many plants benefit from the use of biofertilizers containing phosphate-solubilizing microorganisms found in soil. In order to achieve optimal results, it is necessary to identify and analyze the various phosphate-solubilizing microorganisms present in the soil. After isolating multiple strains, they were subjected to both biochemical and morphological analysis. A number of microorganisms were then tested for their ability to solubilize phosphate using Pikovskayas media. Three fungi (Rwii, Rwiii, and Rwiv) and one bacterial strain (RW3) were found to be more effective. The soil's productivity improved due to the higher phosphate solubilizing efficiency of the chosen strain.

Keywords:- *Phosphate Solubilising Microorganisms (PSMs), Phosphate Solubilization Index, Pikovskayas Media.*

I. INTRODUCTION

Phosphorus is an essential macronutrient for plant growth and development as it plays a critical role in key metabolic pathways, including photosynthesis, nutrient absorption, biological oxidation, and cell division (P. Illmer, F. Schinner). To improve agricultural production, inorganic P is added to soils worldwide as chemical fertilizers, but excessive usage degrades soil quality (Tewari et al. 2004). The current situation is gradually moving towards a more sustainable agriculture. This is due to the ability of phosphate-solubilizing bacteria to improve soil fertility by converting insoluble phosphorus to soluble phosphorus through various processes like the release of organic acids, chelation, and ion exchange (S.A. Omar). To avoid phosphorus deficit, phosphate-solubilizing microorganisms (PSM) might play an essential role in a more ecologically friendly and sustainable manner (G. Narasimha et al.). The main aim of the present work is to isolate phosphate-solubilising microorganisms from soil test their solubilization index and identify the microorganism with the highest efficiency that could be used as a biofertilizer.

II. MATERIALS AND METHODS

➢ *Collection of Soil Sample*

In a sterile polythene bag, a soil sample weighing about 20 grams was taken from the rhizosphere. The soil was taken from Krishna Mandir in Sonegaon, Nagpur, Maharashtra, 440025, India with Lat [21.09906] and Long [79.057126] respectively. The sample was taken to the lab for analysis and kept there at a temperature of 4 ^{0}C.

Fig 1 Geolocation of Sampling Site- Undisturbed Area Near Krishna Mandir.

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➢ *Isolation of Microorganisms from Soil*

- *Soil Sample Preparation*

1gm soil sample collected from the site was weighed using a weighing balance and dissolved in 10 ml of saline water. It was labelled Sample Soil. 6 test tube were prepared with 9ml of dilutant. 1ml of the soil sample is pipetted out from the Sample Soil test tube and added to the 1st dilutant tube labelled 10^{-1}. Dilutants till the factor 10^{-6} were prepared. 10^{-6} is used for spread plating and incubated at 37ºC for 24 hrs. (Jackie Reynolds).

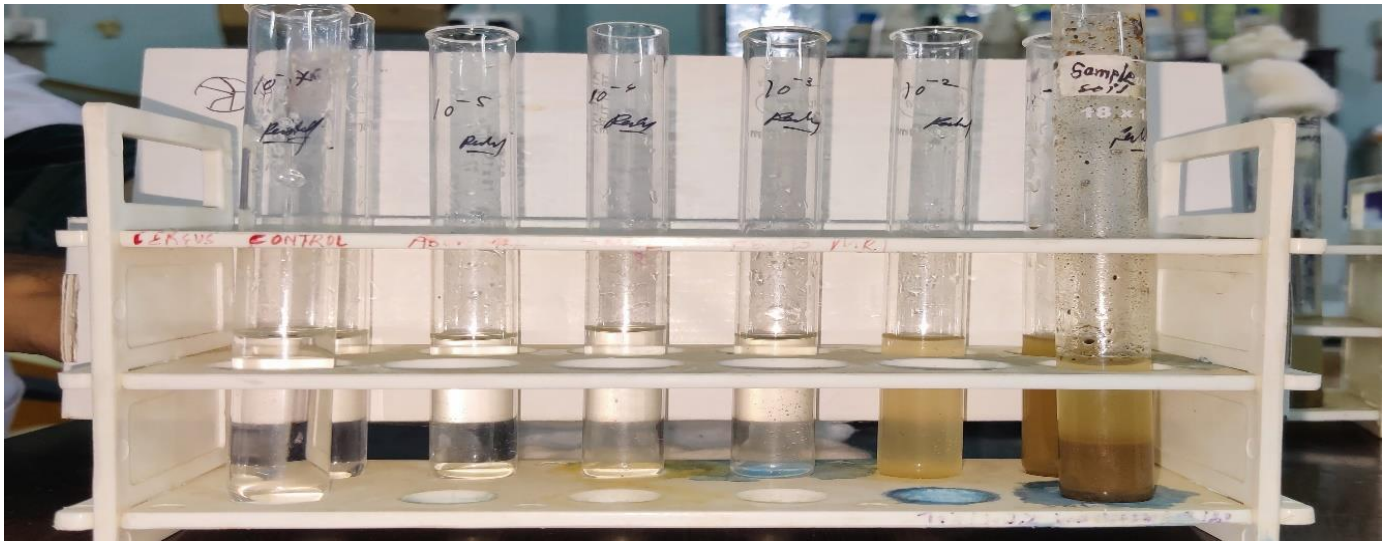

Fig 2 Serial Dilution of Soil Sample.

➢ *Isolation of Phosphate Solubilizing Microorganisms (PSMs)*

A scientific approach was employed in order to isolate and identify microorganisms that possess the ability to solubilize phosphate. Following the incubation period, PSM colonies were identified by visual means. The presence of PSM colonies was indicated by clear zones around both the bacterial and fungal colonies. The isolated microorganisms were then purified and maintained on Sabouraud dextrose agar (SDA) for fungi and Nutrient agar (NA) for bacteria (Ms. Shilpi Damor).

➢ *Microscopic Study of Fungi and Bacteria*

Observing the morphology of a strain is crucial for identification, and different methods are used for fungi and bacteria. Fungi were stained using the Lactophenol cotton blue method, while bacteria were stained using the Gram method. Both methods are important for differential staining.

The Lactophenol Cotton Blue (LPCB) staining solution is a clear blue combination of three reagents. Phenol disinfects, lactic acid preserves fungal structures, and cotton blue stains the chitin on the fungal cell wall and other structures. To apply LPCB staining, a drop of 70% ethyl alcohol was added to a glass slide, then a fungal specimen was added using a sterile inoculating loop. The sample was then teased with a sterile needle and a dropper adds one or two drops of the LPCB solution, which was covered with a sterile coverslip to avoid air bubbles (Astrid Leck).

The process of Gram staining involves the use of several reagents, including Crystal Violet as the primary stain, Iodine as the mordant, a decolourizer made of acetone and 95% alcohol, and Safranin as the counterstain. To perform the staining, a clean glass slide was taken and a loopful of samples was added to create a smear of suspension. Crystal Violet was then poured onto the slide and left to sit for approximately 30 seconds to 1 minute before being rinsed with water. Next, Gram's Iodine was flooded onto the slide for 1 minute and washed with water. After that, a solution of 95% alcohol or acetone was applied for 10-20 seconds, and the slide is rinsed with water. Finally, Safranin was added for approximately 1 minute and washed with water. Stains were observed at 40x under a microscope (Pranaya Pradeep Nampalliwar).

➢ *Identification of PSM Through Biochemical Tests.*

PSMs were characterized based on biochemical analysis, using various tests such as Citrate utilization test, Starch hydrolysis test, Catalase test, Hydrogen sulphite test, Indole Acetic Acid (IAA) test, Methyl Red test, and Oxidase test.

- *Citrate Utilization Test*

The purpose of the test is to determine if the microbes can use sodium citrate as their only carbon source and inorganic ammonium hydrogen phosphate ($NH_4H_2PO_4$) as their nitrogen source. To conduct the test, the media included 3 grams of sodium citrate, 1.5 grams of sodium hydrogen phosphate, 1 gram of potassium dihydrogen phosphate, and 0.2 grams of magnesium sulphate for every 1 liter of distilled water. The microorganisms were introduced by stabbing in the butt and streaking the slant surface. The test tubes were then incubated at 37°C for 48 hours. A positive test was indicated by a shift in colour from green to blue (Begum et al.).

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- *Starch hydrolysis Test*

A test is conducted to determine if a microbe has the ability to break down starch and use it as nourishment. The test involved combining 10 grams of soluble starch, 3 grams of beef extract, and 12 grams of agar in 1 liter of distilled water to create a specific media composition. Plates are then prepared, streaked with the isolates, and incubated at 27-30ºC for 24 hours. After incubation, an iodine solution is added to the plates. If there is a clear region around the isolates, it indicates a positive test (Cappucino, 1983).

- *Catalase Test*

In order to evaluate the capacity of the strains to decompose H_2O_2 by generating the catalase enzyme, an experiment was carried out. A minute quantity of culture was mixed to form an emulsion and a solitary droplet of 20% hydrogen peroxide was deposited onto a pristine glass slide. The manifestation of air bubbles indicated the activity of catalase (Cheesbrough M. (2006)).

- *Hydrogen Sulphite Test*

In order to determine if a microbe can produce H_2S gas using thiosulphate as a source of sulphur, a test is conducted. The media composition for this test includes 3gm of beef extract, 30gm of peptone, 0.2gm of ferrous ammonium sulphate, 0.025gm of sodium thiosulphate, and 3gm of agar to 1 litre of distilled water. Butts are prepared (Clarke, 1953). The butts are introduced with the isolates by stabbing and incubated for 24-48 h at 37ºC. The appearance of a Black mass confirms the test.

- *Indole Acetic Acid (IAA) Test*

Microbial ability to produce IAA, which aids in plant growth, is tested using a media composition of 15 grams of Tryptophan in 1 liter of distilled water. In each test tube, 6ml of the broth was taken with the isolates and incubated for 24-48 hours at 37ºC. The addition of Kovacs Reagent resulted in the formation of a red ring, indicating a positive test (Jiaqi Chen).

- *Methyl Red Test*

A test is conducted to determine an organism's ability to produce and maintain a stable level of acid from glucose fermentation while overcoming the buffering capacity of the system. To prepare the media for 1 liter of distilled water, combine 7 grams of protease peptone, 5 grams of glucose, and 5 grams of dipotassium phosphate. Distribute 6ml in each test tube and autoclave. The isolate was inoculated and incubated at 27-30ºC for 24 hours. The Methyl Red reagent is used to determine the result, where red color indicates a positive test, and yellow indicates a negative one (Olutiola PO, Famurewa O, and Sonntag HG).

- *Oxidase Test*

The oxidase test is a method used to detect microorganisms that produce cytochrome c oxidase, an enzyme present in the bacterial electron transport chain. To prepare the media, mix 15.5 grams of tryptone soya broth and 7.5 grams of agar into 1 liter of distilled water. Plates are then made and streaked with isolates and labelled accordingly. The plates are incubated at 37°C overnight, and visible signs of growth are observed. Following this, 2-3 drops of Wurster's reagent are added to the plates, which is made by adding 0.1gm into 10 ml of distilled water. A positive test result is indicated by the appearance of a blue colour, while a negative test result is indicated by a colourless outcome (K Manasa, R Subhash Reddy, and S Triveni).

III. RESULTS AND DISCUSSION

➢ *Isolation of PSMs and Phosphate solubilization index*

From the soil sample collected, a total of 9 PSM strains were isolated. Out of the 9 strains, 4 of them were found to be efficient, consisting of 3 fungal strains named Rwii, Rwiii, and Rwiv, and 1 bacterial strain named RW3. Certain microorganisms possess the remarkable ability to solubilize phosphate, creating a distinct and transparent zone around their colony. This zone serves as an indicator of their exceptional capacity for phosphate solubilization. The phosphate solubilization index for selected isolates is indicated in Table 1.

To measure the ability of specific Phosphate-solubilizing microbes to solubilize phosphate, we use the solubilization index (SI), which is the ratio of the total diameter (clearance zone) to the colony diameter. The formula to calculate the phosphate SI is as follows:

$$SI = \frac{Colony\ diameter + Halozone\ diameter}{Colony\ diameter}$$

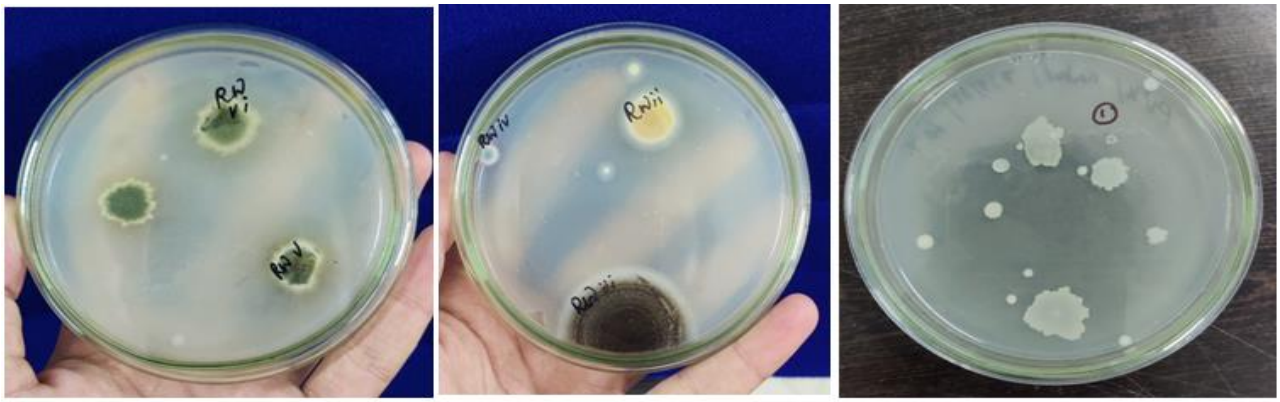

Fig 3 Isolated Colonies were Observed.

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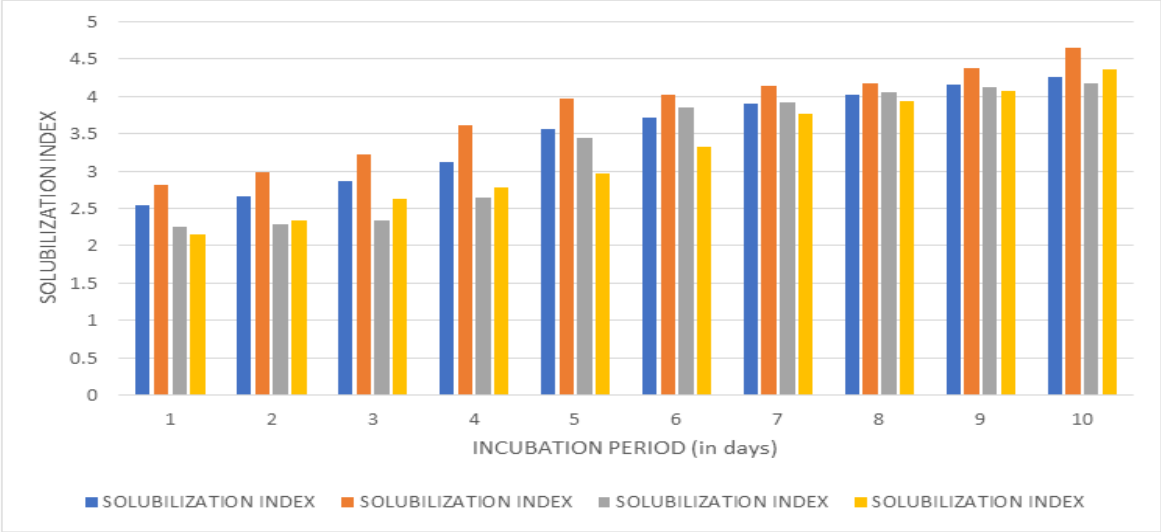
Table 1 Phosphate Solubilization Index of Selected Strains.

INCUBATION PERIOD (in days)	SOLUBILIZATION INDEX			
	Rwii	Rwiii	Rwiv	RW3
1	2.54	2.82	2.25	2.145
2	2.66	2.98	2.29	2.34
3	2.86	3.22	2.33	2.62
4	3.12	3.62	2.64	2.78
5	3.56	3.98	3.44	2.96
6	3.72	4.02	3.86	3.32
7	3.91	4.14	3.92	3.76
8	4.02	4.18	4.06	3.94
9	4.16	4.38	4.12	4.08
10	4.26	4.66	4.17	4.36



Graph 1 Phosphate Solubilization Index Shown by Isolates

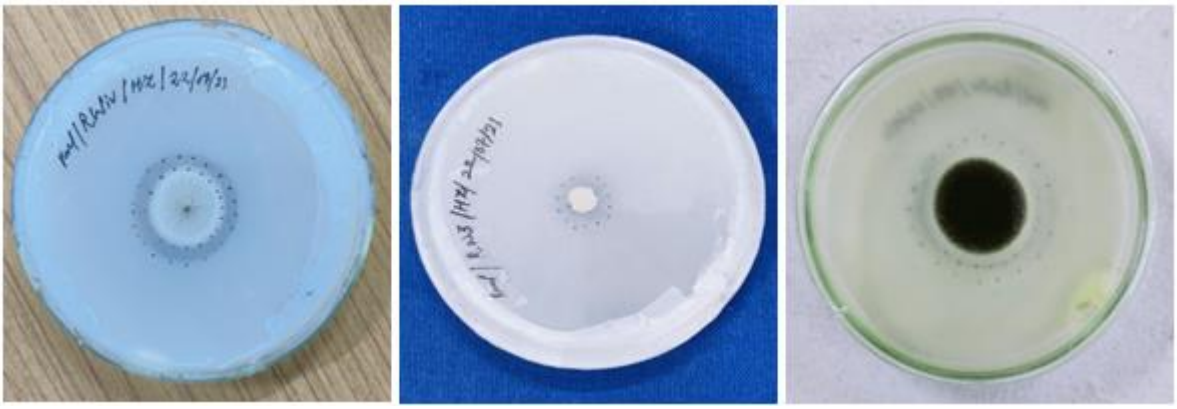


Fig 4 Phosphate Solubilization by Isolates.

➢ *Morphological and Biochemical Characterization*

After identifying the most efficient isolates, we conducted further studies on their morphology and performed biochemical analysis. The analysis revealed a significant variation in PSMs. We then chose the efficient isolates Rwii, Rwiii, Rwiv, and RW3 for additional biochemical tests, as they were found to be more closely related to Aspergillus sp. and Pseudomonas sp.

The results of various morphological tests on 4 strains are indicated in Table 2(a) and 2(b).

Table 2 (a) Morphological Characters of Fungi Isolates.

Strains	Staining (Lactophenol cotton blue)	Colony form	Colour	Type of growth	Sporulation
Rwii	Yes	Circular	Brown	Within 48hrs	Yes
Rwiii	Yes	Circular	Black	Within 48hrs	Yes
Rwiv	Yes	Circular	Greenish Red	After 3 days	Yes

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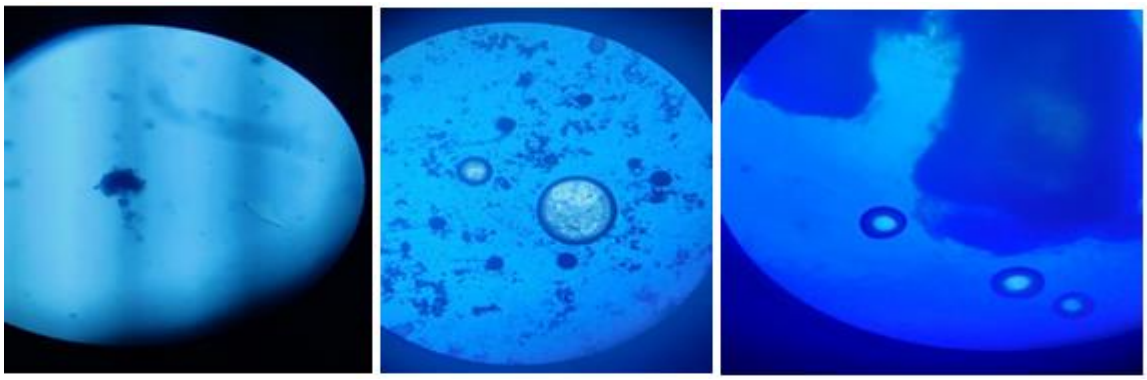

Fig 5 Fungal Colonies were Observed Under a 40x Microscope [Rwii, Rwiii, Rwiv(Left to Right)].

Table 2 (b) Morphological Characters of Bacterial Isolate.

Strains	Staining (Gram Staining)	Colony form	Shape	Colour	Type of growth	Pigmentation
RW3	Negative (Red colour stained)	Circular	Rod-shaped	Creamish white (before staining)	Within 24hrs	Yes

The results of various Biochemical tests on 4 strains are indicated in Table 3.

Table 3 Biochemical Analysis of PSMs

Strains	Citrate utilization	Starch hydrolysis	Catalase test	Hydrogen sulphite	Indole Acetic Acid (IAA)	Methyl Red	Oxidase test
Rwii	+	++	+	-	+	-	+
Rwiii	+++	+++	++	+++	+++	-	++
Rwiv	+	+	++	-	++	-	-
RW3	+++	+++	+++	+++	-	++	+++

[+++ highest activity, ++ average activity, + low activity, - negative result]

Fig 6 Citrate Utilization Test

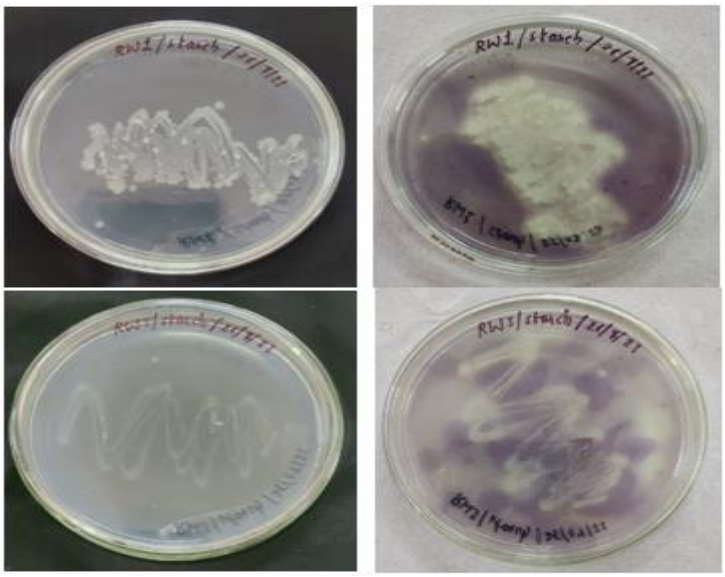

Fig 7 Results of Starch Hydrolysis Test

Fig 8 Results of Catalase Test

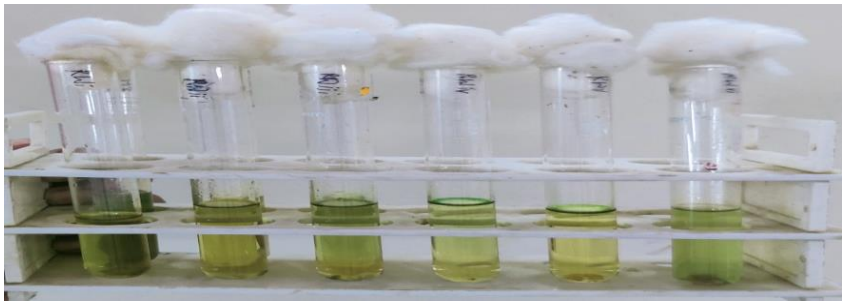

Fig 9 Results of Indole Acetic Acid (IAA) Test

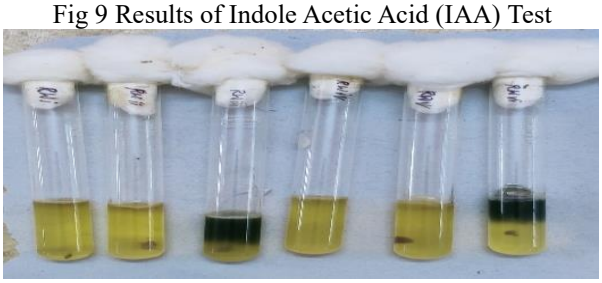

Fig 10 Results of Hydrogen Sulphite Test

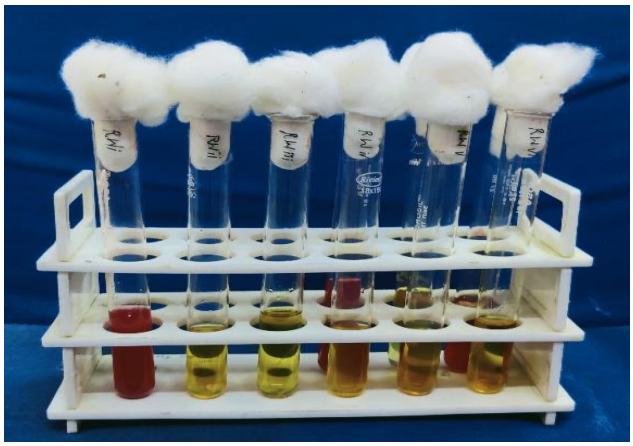

Fig 11 Results of Methyl Red Test

Fig 12 Result of Oxidase Test on Bacterial Isolate

IV. CONCLUSIONS

Microbes that can solubilize phosphate are essential for providing plants with this nutrient, making them valuable biofertilizers. Rwiii and RW3 strains were found to be the most efficient among fungal and bacterial strains, respectively showing maximum activity. By identifying potential candidates based on their morphological and biochemical characteristics, we isolated these microbes. This approach enables us to utilize them effectively, leading to higher crop yields in the soil.

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