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Isolation and Identification of Soil-Derived Pseudomonas Species with Potential Plant Growth Promoting Traits

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Abstract:- Microbial biofertilizers have proven to be a better substitute to the chemical fertilizers. Pseudomonas is a beneficial bacterium in enhancing growth of the plants. In this study, attempt was made to collect soil sample and isolate the pseudomonas strains, study their morphological, cultural characteristics. These isolates were subjected to various biochemical tests such as Indole Acetic Acid (IAA) Test, Triple-Sugar-Iron Test, etc., and out of nine isolates namely PF1,PF2,PF9 , PF2, PF7 and PF8 have yield maximum results and have shown a potential for their use as biofertilizers.

Keywords:- *Pseudomonas, Plant growth promoters, Phosphate Solubilization.*

I. INTRODUCTION

The use of chemical-based pesticides and fertilizers has created destruction in the environment. These agents are both dangerous to animals and humans, and they have the potential to persist and accumulate in natural ecosystems. A potential remedy to this problem is substituting these chemicals with biological approaches, considered more environmentally friendly in the long run (Suman et al., 2016). Microorganisms have the capacity to replace these chemical-based pesticides and fertilizers they have been proven to be a great source of biofertilizers and plant growth promoters. Few such plant growth promoting bacteria are *Pseudomonas, Enterobacter, Rhizobium, Erwina* etc., (Harsha Sharma, Kalpana Sharma).

Pseudomonas sp. is a common bacterium in agricultural soils with many characteristics that make it a good candidate for PGPR. The most effective Pseudomonas strains have been fluorescent Pseudomonas sp. (K Manasa, R Subhash Reddy, S Triveni). Recently different strains of Pseudomonas fluorescens are used as seed inoculants to enhance growth and promote greater yields. They can be considered as important PGPR and make excellent biofertilizers to enhance the crop yield by direct or indirect methods. Some strains even produce chelating agents with higher affinity to absorb iron thus enhance the growth of the plant by increasing the solubility of inorganic iron in plant, these agents are named siderophores (Intl. J. Microbial. Res., 4 (3): 227-233, 2013).

II. MATERIALS AND METHODS

A. Soil Sampling

Soil sample was collected from swamp near Sonegaon HB Estate Lake [21º06'15.5"N 79º05'37.5" E]. Soil sample collected from randomly selected plant and intact root system was dug out from the subsurface of soil 10-15 cm deep. Soil intimately adhering to the root was collected and bagged.

Fig. 1: Geolocation of Sampling Site- Swamp near Sonegaon Lake.

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B. Isolation of bacteria from the soil sample

➢ *Preparation of Soil Serial dilution*

1g of soil sample was weighed using weighing balance and dissolved in 10ml of saline water. It was label Sample Solution (SS). 1ml of sample was pipette out from the SS and add to the 9ml dilutant in test tube, and labeled 10^{-1}. Dilutants till 10^{-7} are made. 10^{-6} dilution is used for spreading (Jackie Reynolds).

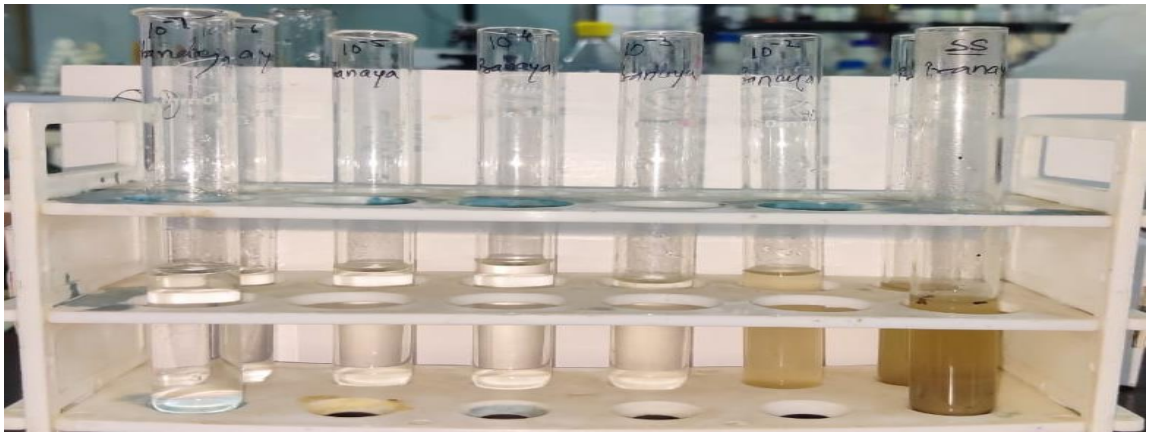

Fig. 2: Serial Dilution of Soil Sample

➢ *Preparation of Spread plate for bacterial culture*

For the bacterial growth take pre-prepared plate of King's B Media [protease peptone 20g, Glycerol 10ml, K_2HPO_4 1.5g, $MgSO_4$ 1.5g, Agar 15g in 1000ml Distilled water], label them accordingly. Pipette out 0.1ml of dilutant from 10^{-6} serially diluted tube onto the petri plate. Heat the L spreader to sterilize it. Open the plate and using the L spreader spread the inoculum around the agar surface until the traces of free liquid disappears (Deshwal VK, Vig K, Amisha DM). Incubate the petri plate for 24h at 27-30ºC.Make sure the plate is kept inverted to prevent condensed droplets to fall on agar surface.

C. Enumeration

The plate incubated for 24h at 27-30ºC were observed for bacterial growth on King's B Media. Different visible colonies were enumerated manually and recorded (Jackie Reynolds). (Table 1).

D. Identification

➢ *Morphological Characterization*

The colonies on the King's B media were observed and checked for purity and studied for Colony morphology, Odor and pigmentation. Were also recorded for Cell morphology (gram reaction) and reaction to UV light (Holt et al., 1994). (Table 2)

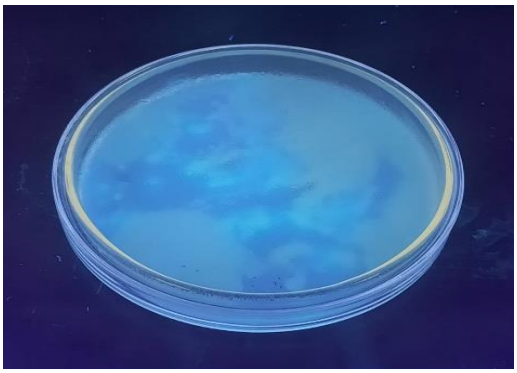

Fig. 3: Plate under UV light flouresence Emission

➢ *Culture Characterization*

Selected Isolate colonies of bacteria were studied for their colour, shape, margin, surface, elevation, growth, sporulation and tabularly recorded. (Table 2).

E. Pure Culturing

Nutrient agar slants were prepared by pouring 6ml of Nutrient Agar Media [Nutrient Broth 13g, Agar 17g in 1000ml Distilled water] into test tubes. Cap them with cotton plug and autoclave at 121ºC 15lbs for 20 mins. Once Autoclaved allow them to cool and solidify in a slant position. Make sure the media does not touch cotton plugs. Isolates were picked using a sterile inoculation loop and streaked on to the surface of Nutrient Agar Slants and labeled accordingly. The tubes were incubated for 24h at 27-30ºC for pure colony growth.

F. Biochemical and Physiological Characterization

For identification of *Pseudomonas species* certain biochemical tests were performed on the isolates and recorded as follow:

➢ *Indole Acetic Acid (IAA) Production Test*

To check for the production of Indole Acetic Acid. SIM Agar [Peptone 30g, Beef Extract 3g, Ferrous Ammonium Sulfate 0.2g, Sodium thiosulfate 0.3g, Agar 13.5g in 1000ml Distilled water] butts are prepared. The isolates are stabbed and capped with cotton plug. Incubate for 48h at 27-30ºC. After incubation, add drops of Kovac's Reagent. Formation of Red oil like ring confirms the production Indole Acetic Acid (Aneja, 2001).

➢ *Phosphate Solubilization*

To study the capacity of the bacteria to solubilize inorganic phosphate. Pre-prepared plates of Pikovskaya Agar Media [Pikovskaya Agar 31.3g in 1000ml Distilled water] were made. Isolates were picked using a sterile inoculation loop and placed at the center of the agar surface. Label the plates accordingly and sealed with parafilm to prevent contamination from foreign sources. Incubate at 27-30ºC for 24h (Shilpi Damor, Dr. Praveen Goswami). Record the

colony diameter and halozone diameter on a regular interval. Calculate and record the Phosphate Solubilization Index and Efficiency in tabularly. (Table 3) (Table 5).

$$\text{Phosphate Solubilization Index, (PSI)} = \frac{Bacterial\ Colony\ Diameter + Halozone\ Diameter}{Bacterial\ Colony\ Diameter}$$

- *Oxidase Test*

It is performed to check for the presence of cytochrome oxidase. Trypticase Soy Agar or Tryptone Soy Agar (TSA) media [Trypticase Soy Broth 30.3g, Agar 15g in 1000ml Distilled water] plates were prepared. Isolates were streaked using a sterile inoculation loop on to the surface of Agar media. Labeled and incubated for 48h at 27-30ºC.Wurster's Blue Reagent (N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride) was prepared by dissolving 0.1g of reagent in 10ml Distilled water. After incubation ,2-3 drops of Wurster Blue reagent were placed on the bacterial colony, turns blue in few mins marks a positive result. Change in colour after 15 mins must be taken as false positive (K Manasa, R Subhash Reddy and S Triveni). (Table 3).

- *Catalase Test*

It is tested to study for the presence of catalase enzyme. On a clean and sterile glass slide, the isolate is placed. 1-2 drops of 30% H_2O_2 is added. Appearance of gas bubbles confirms the presence of catalase enzyme thus marks a positive result (Cheesbrough M. (2006)). (Table 3).

- *Starch Hydrolysis*

Tested for determining the capability of bacteria to hydrolyze starch. Starch Agar Media [Soluble Starch 10g, Beef Extract 3g, Agar 15g in 1000ml Distilled water] plates are prepared and inoculated by streaking on the agar surface by isolates. Incubated for 24h at 27-30ºC for growth. After incubation the surface is flooded with Iodine solution. Starch turns deep purple in colour in the presence of iodine, the hydrolyzed region around the colony turns transparent conforming a positive result (Cappucino, 1983). (Table 3).

- *Citrate Utilization*

Is performed to determine whether the bacteria can use sole citrate as source of carbon. Simmon Citrate Agar [Simmon Citrate Agar 24.25g in 1000ml Distilled water] slants are prepared and inoculated by stabbing the butt and streaking on the surface of slant with the isolates. Incubated for 48h at 27-30ºC.Change in colour from green to blue indicates utilization of citrate thus indicating positive result (Begum et al.).

- *Hydrogen Sulfide Production Test*

Tested to check for the production of Hydrogen Sulfide Gas (H_2S). SIM Agar [Peptone 30g, Beef Extract 3g, Ferrous Ammonium Sulfate 0.2g, Sodium thiosulfate 0.3g, Agar 13.5g in 1000ml Distilled water] butts are prepared. (Clarke, 1953).The isolates are stabbed and capped with cotton plug. Incubate for 48h at 27-30ºC.Presence of black precipitate confirms the production of Hydrogen Sulfide Gas.

- *Motility Test*

To determine whether the bacteria possess flagella/cilia. SIM Agar [Peptone 30g, Beef Extract 3g, Ferrous Ammonium Sulfate 0.2g, Sodium thiosulfate 0.3g, Agar 13.5g in 1000ml Distilled water] butts are prepared. The isolates are stabbed and capped with cotton plug. Incubate for 48h at 27-30ºC. Hazy appearance along the stab line confirms the ability of the bacteria to move, thus confirms the presence of flagella/cilia. (Table 3)

- *Methyl Red Test*

Tested to determine bacteria's capacity to utilize mixed acid fermentation pathway. If the pathway is utilized the pH falls under 4.4(red), if the pathway has not been utilized the pH is above 6.6(yellow). Pipette 6ml of Glucose-phosphate Broth [Protease peptone 7g, Glucose 5g, Dipotassium phosphate 5g in 1000ml Distilled water] in test tubes, Cap them with cotton plug and autoclave at 121ºC 15lbs for 20 mins. Inoculate using a sterile inoculation loop and label them accordingly. Incubate at 27-30ºC for 24h. Methyl Red Reagent was prepared by dissolving 0.1g methyl red in 300ml 95% Ethyl Alcohol. Add 2-3 drops of reagent to the inoculated test tube. (Olutiola PO, Famurewa O and Sonntag HG). Appearance of red colour indicates positive test and yellow indicates negative. (Table 3)

- *Triple-Sugar-Iron (TSI) Test*

It is to determine the capability of a bacteria to ferment sugar and gas production. Triple-Sugar-Iron Agar Slants are prepared by pouring 6ml of TSI Agar Media [Triple-Sugar-Iron Agar 64.4g in 1000 ml Distilled water] in test tubes. Cap them with cotton plug and autoclave at 121ºC 15lbs for 20 mins. Once Autoclaved allow them to cool and solidify in a slant position(Suman et al.). Make sure the media does not touch cotton plugs. Stab the butt and then streak the surface of the slant with isolates in each tube using a sterile inoculation loop and label them accordingly. Incubate the tubes for 24h at 27-30ºC. Change in colour in the butt and slant determines the fermentation capability of the bacteria and indicates Alkaline(K) and Acid(A) nature, also note for the production of gas by the isolates in the tube. (Table 3) (Table 4).

III. RESULT AND DISCUSSION

A. Isolation and Enumeration

The microbial population from swamp soil was collected. Nine gram-negative isolates were isolated from the soil. Deep yellow colony have the maximum growth and bacterial enumeration compared to the other six.

Table 1: Enumeration of bacteria (x10^7 cfu/gm)

S.no.	Morphological Character	Bacterial Enumeration (x 10^7 cfu/gm soil)
1	Cream White	63
2	Pale Yellow	52
3	Red Brown	11
4	Deep Yellow	110
5	Green	4

B. Morphological and Cultural Characterization

All the isolates developed small to medium smooth, shiny and mucous colonies. Out of 9 isolates, 3 showed green pigment ,3 brown pigment and remaining showed yellowish pigments. The isolates showed negative gram reaction, small, rod shaped with no sporulation property.

Table 2: Morphological and Cultural Characteristics on King's B Media

S. no.	Isolate Name	Colony Colour	Colony Shape	Margin	Surface	Elevation	Pigmentation	Type of Growth	Gram Reaction	Sporulation	Reaction to UV light fluorescence emission
1	PF1	Dull Yellow	Irregular	Irregular	Smooth	Convex	Pale Yellow	Fast	Negative	Negative	Bright
2	PF2	Green	Round	Regular	Smooth and Shiny	Convex	Green	Fast	Negative	Negative	Bright
3	PF3	Red Brown	Irregular	Irregular	Shiny	Convex	Red Brown	Fast	Negative	Negative	Bright
4	PF4	Yellow	Round	Regular	Smooth	Convex	Deep Yellow	Fast	Negative	Negative	Bright
5	PF5	Brown	Spots	Regular	Mucous	Convex	Brown	Fast	Negative	Negative	Dull
6	PF6	Dull Yellow	Irregular	Irregular	Smooth	Convex	Green	Fast	Negative	Negative	Bright
7	PF7	Brown	Round	Regular	Mucous	Convex	Brown	Fast	Negative	Negative	Bright
8	PF8	Brown	Round	Regular	Mucous	Convex	Green	Medium	Negative	Negative	Bright
9	PF9	Red Brown	Irregular	Irregular	Smooth	Convex	Pale Yellow	Slow	Negative	Negative	Bright

C. Biochemical and Physiological Characterization

After studying the morphological and cultural characteristics for the nine isolates they were subject to various test to study their biochemical activities viz., Oxidase Test, Catalase Test, Starch Hydrolysis, Indole Production, Triple-Sugar-Iron Test etc.

All the nine isolates gave positive test for oxidase test, catalase test, Starch Hydrolysis and Indole Acetic Acid (IAA) Production Test. For Methyl Red test, except for PF6 other isolates tested positive. Out of nine isolates eight showed positive for Citrate Utilization, seven for Motility, four for both Phosphate Solubilization and Triple-Sugar-Iron test and three for gas production. PF5, PF7, PF9 tested positive for H_2S Production Test.

Table 3: Biochemical and Physiological Characteristics of Isolates

S.no.	Isolate Name	IAA Test	PS Test	Citrate Utilization	MR Test	TSI Test	Starch Hydrolysis	Oxidase Test	Catalase Test	H2S Test	Gas Production Test	Motility Test
1	PF1	++	-	++	+	-	+	+	+	-	-	+
2	PF2	++	+++	+++	+	-	+++	+++	++	-	-	+++
3	PF3	++	-	++	++	-	+	++	+++	-	-	++
4	PF4	++	-	++	+	+	++	+++	+	+++	-	+
5	PF5	++	-	+	+	-	+++	+	+++	-	-	+
6	PF6	++	++	+++	-	-	++	+	+	-	-	-
7	PF7	+++	+	-	++	+++	+	+	++	++	++	++
8	PF8	+++	+++	+++	+++	+++	+++	+++	+	-	+++	-
9	PF9	++	-	+	+	+	+	+	++	+	+	+++

[IAA – Indole Acetic Acid, H_2S- Hydrogen Sulfide, MR – Methyl Red, TSI – Triple-Sugar-Iron, PS – Phosphate Solubilization, +++ highest activity, ++ average activity, + low activity, - negative result]

Fig. 6: Catalase Test Result

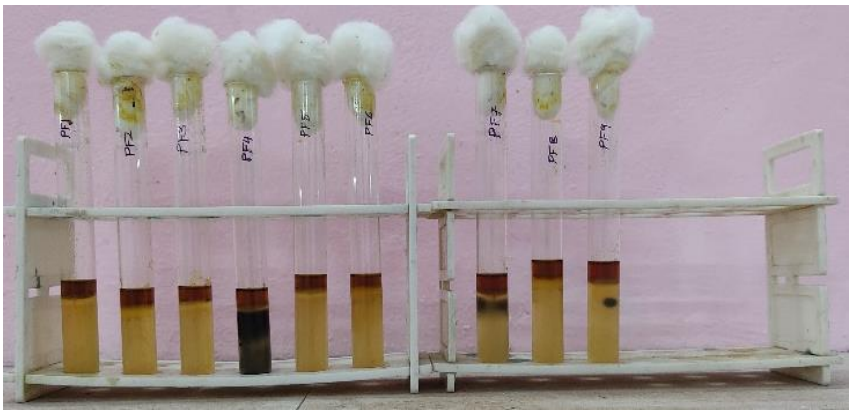

Fig. 8: IAA Test Result

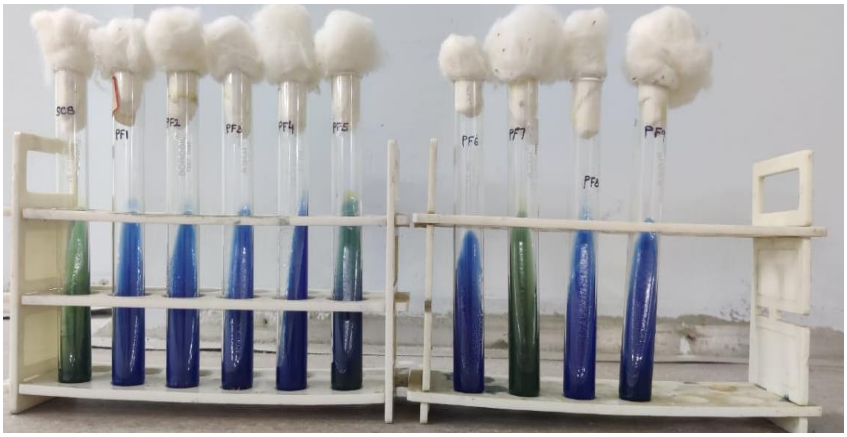

Fig. 9: Citrate Utilization Test Result

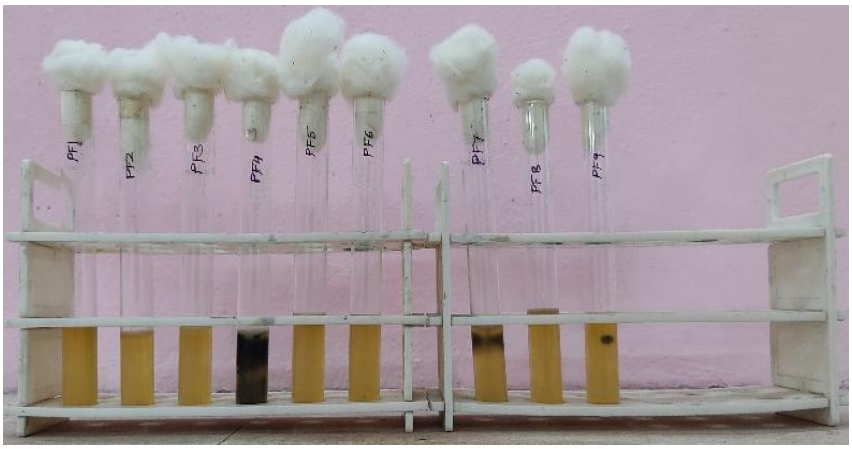

Fig. 10: H_2S Test Result

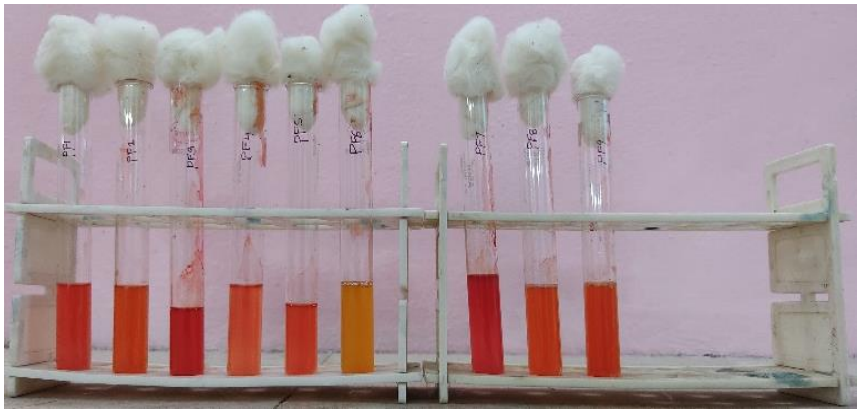

Fig. 11: Methyl Red Test Result

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Fig. 12: Triple-Sugar-Iron Test Result

Table 4. Triple-Sugar-Iron Test Result

S.no	Isolate Name	Slant/Butt Colour	Slant/Butt Nature	Fermentation
1	PF1	Red/Red	K/K	Glucose, lactose and sucrose non fermenter
2	PF2	Red/No Change	K/NC	Glucose, lactose and sucrose non fermenter
3	PF3	Red/No Change	K/NC	Glucose, lactose and sucrose non fermenter
4	PF4	Red/Yellow	K/A	Glucose Fermenter only
5	PF5	Red/No Change	K/NC	Glucose, lactose and sucrose non fermenter
6	PF6	Red/Red	K/K	Glucose, lactose and sucrose non fermenter
7	PF7	Yellow/Yellow	A/A	Glucose, Lactose and sucrose fermenter
8	PF8	Yellow/Yellow	A/A	Glucose, Lactose and sucrose fermenter
9	PF9	Red/Yellow	K/A	Glucose Fermenter only

[K – Alkaline, A – Acid, NC – No Change]

➢ *Phosphate Solubilization*

All isolates were subjected to the study of solubilization of inorganic phosphate. Out of nine isolates 4 should solubilization out of which PF2 and PF8 has shown best solubilization.

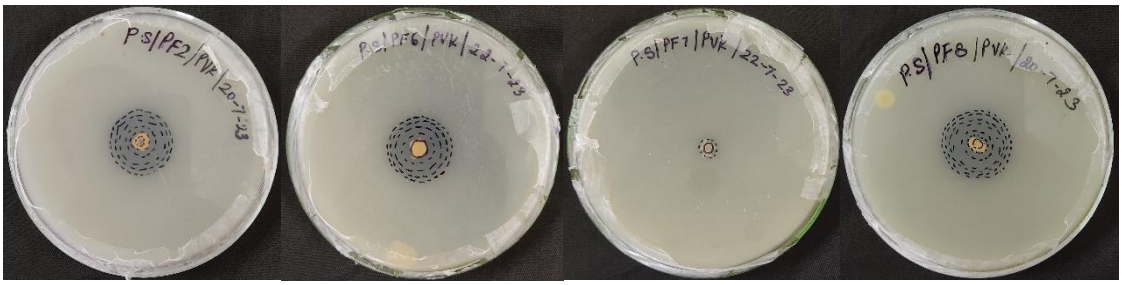

Fig. 13: Phosphate Solubilization Test Result of PF2, PF6, PF7, PF8.

Table 5: Phosphate Solubilization Index

INCUBATION PERIOD	SOLUBILIZATION INDEX								
	PF1	**PF2**	**PF3**	**PF4**	**PF5**	**PF6**	**PF7**	**PF8**	**PF9**
1	1	2.8	1	1	1			2.8	1
2	1	2.57	1	1	1	2.83	3.33	2.71	1
3	1	3	1	1	1	3.83	2	2.93	1
4	1	3.28	1	1	1	4	2	3.42	1
5	1	3.85	1	1	1	4	2	3.71	1
6	1	4.14	1	1	1	4.42	2	3.75	1
7	1	4.14	1	1	1	4.45	2	4.12	1
8	1	4.14	1	1	1	4.7	2	4.12	1
9	1	4.14	1	1	1	4.85	2	4.12	1
10	1	4.54	1	1	1	4.85	2	4.15	1

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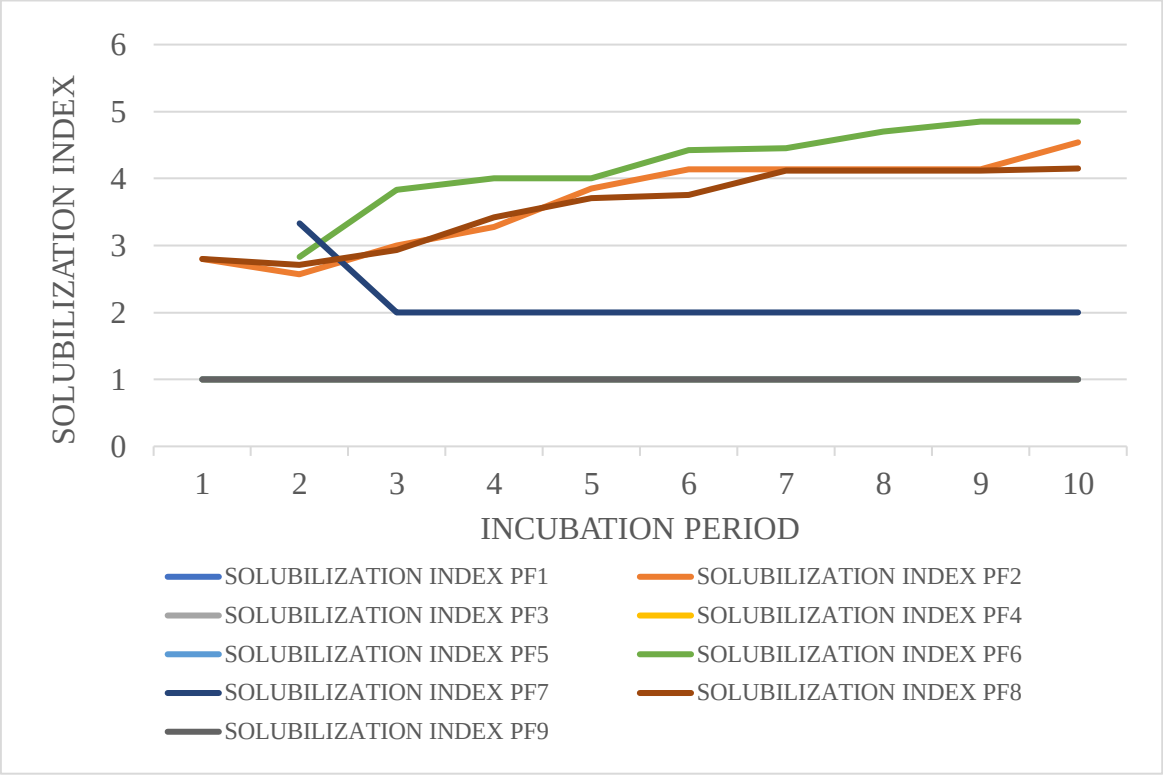

Graph 1: Phosphate Solubilization Index (PSI) of 9 Isolates on PVK Agar from 1st to 10th day

IV. CONCLUSION

Further studies on the nine isolates using them in pot analysis and field experiment would help us understand their potential as plant growth promoters and their contribution in agricultural lands. The data from the present study suggests that out ff the nine PF2, PF7 and PF8 have the maximum activities and would be ideal organism for further studies in pot analysis and field experiment with combination of two organisms to exploit their true potential for a good biofertilizer production.

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