

Original Research Article

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Potential Use of *Azotobacter* and Phosphate Solubilizing Bacteria as Biofertilizer

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ABSTRACT

In the present investigation rhizosphere soil was collected in and around Chennai. The rhizosphere soils were serially diluted and pour plated on selected medium of pikovskay's media and winogradsky's media for isolation of bacteria. The isolated bacteria were identified based on morphological and biochemical tests. The results were compared with Bergy's manual of determinative bacteriology. Screening is done by growth promoting bacteria by seed germination. The efficiency on different crop plants in terms of growth and yield both length and biomass. From the results, it was noted as *Azotobacter* and *Streptomyces*. Molecular characterization of microbial isolates studied by 16S RNA sequencing. *Azotobacter* and *Rastolnia sp.*, were mass cultured and used in the experiment of plants and in probiotic fishes. The length and weight was measured in plant and fish. The isolated bacteria were inoculated in nutrient broth and fungus was inoculated in potato dextrose broth and it is extracted by using ethanol. Artimia lethality test was done by using brain shrimp in 96 titre well plate by selected organisms in bacteria and in fungi. The antibacterial activity test was done by kirbby boyer method in *Azotobacter* and phosphate solubilizing bacteria.

Keywords

Rhizosphere, *Azotobacter*, *Rastolnia sp.*, PGPR, and Artimia lethality.

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Introduction

Biofertilizer contains living microorganisms and promotes growth by increasing the availability of primary nutrient (nitrogen and phosphorus) to the host plant. Biofertilizer also provides nutrients required by the plants and helps to increase the soil quality with natural microorganism (Vessey, 2003). Some of the beneficial microbes used in biofertilizers are N₂ –fixing bacteria, phosphate-solubilizing microbes and mycorrhizae which are able to fix atmospheric nitrogen or solubilize

phosphorus in the soil (Subba, 1999). Microorganisms are extensively used as biofertilizers in agricultural practices. The biofertilizers are the live or contain latent cell of beneficial microorganisms, which augment the availability of nutrients to the plants. The beneficial micro organisms are *rhizobium*, *Azotobacters*, *azospirillum*, *cyanobacteria*, *phosphobacteria* and *mycorrhiza*. Among these *Azotobacters* and phosphate bacteria play major role in the supply of nutrients and in the plant growth promoting activities. These bacteria are

present in low populations in the natural environment (Subba Rao, 1997).

The multiplied artificially and incorporated into the agricultural lands in the form of biofertilizers. A lot of research work is available on the biofertilizers in agriculture practices. Nitrogen and phosphorus are the major plant nutrients, which are referred to as the master key element in crop production. It is a greater part of soil phosphorus (approximately 95-99%) is present in the form of insoluble form; dominant in alkaline soil and unable to utilized by the plants (Lee *et al.*, 2005).

Plants and microbes species have developed symbiosis or mutually beneficial relationships. Rhizobium is the root of legumes host nitrogen fixing bacteria which can invade root and get sugars from the plant. They convert large amounts of dinitrogen (N₂) from the atmosphere into forms that the plants can use (Zahran, 1999). Some soil bacteria, isolated from the root region of plants, are known to enhance growth of the plants. These beneficial free-living soil bacteria are termed as plant growth promoting rhizobacteria (PGPR). The beneficial effect of PGPR is mediated through either direct or indirect mechanisms (Glick, 1995).

The PGPR also improve growth by indirect mechanisms such as, suppression of bacterial, fungal and nematode pathogens, and production of siderophores, HCN, ammonia, antibiotics, volatile metabolites *etc.* (Glick, 1995). Symbiotic relationship between phosphate solubilizing bacteria (PSB) and plants is synergistic in nature as bacteria provide soluble phosphate and plants supply root borne carbon compounds (mainly sugars), that can be metabolized for bacterial growth (Perez *et al.*, 2007).

Azotobacter naturally fixes atmospheric nitrogen in the rhizosphere. There are different strains of *Azotobacter* each has varied chemical, biological and other characters. However, some strains have higher nitrogen fixing ability than others. *Azotobacter* uses carbon for its metabolism from simple or compound substances of carbonaceous in nature.

Azotobacters are aerobic, free-living, heterotrophic bacteria with unique ability of fixing atmospheric nitrogen. The bacteria are gram-negative, non motile, but often motile by peritrichous flagella. *Azotobacters* are highly important for their ability to fix molecular nitrogen, contributing to the productivity of any environment. They were proved experimentally to fix 10 mg of atmospheric nitrogen per gram of carbohydrate consumed (Becking, 1971). A special group of bacteria (phosphate solubilising bacteria) occurring in sea water is capable of dissolving insoluble inorganic phosphorous into soluble inorganic phosphorous (Alexander, 1978; Chandrasekaran, 1966).

The present investigation is aimed towards the potential use of *Azotobacter* and phosphate solubilizing bacteria as biofertilizer.

Materials and Methods

Sample collection

The soil samples were collected from around Chennai. The samples were serially diluted and spread plate method was done. The pure cultures were streaked in pikovskay's medium and winogradsky medium. The pure cultures were stored in 4⁰ C for further study.

Pikovskay's medium – In this medium is used to identify the phosphate solubilizing

microorganisms. In that halo zone formed as positive result.

Winogradsky medium- In this medium is used to identify the *Azotobacter*.

Two strains have been selected for their efficiency of fixing the atmospheric nitrogen and or solubilizing the insoluble phosphate. These strains have scope for their possible utility as bio-fertilizers. It is necessary to identify the two strains at species levels prior to their economic utility. This chapter therefore, deals with the identification of most potent strains based on their cultural, morphological and biochemical characteristics.

Cultural Characteristics

The cultural characterization was studied based on morphological and biochemical characterization, such as abundance of growth, pigmentation, optical characteristics, form size margin and elevation were studied on nutrient agar plates.

Germinating seed methods

Seed germination test

The selected strains were bioassayed for their ability to promote/ inhibit seedling growth by using the method as described by Shende *et al.*, (1977) and Ellicot and Lynch (1934) with modifications. Rice seeds were immersed in 95% ethanol for 20 sec. subsequently the seeds were immediately washed with sterile distilled water (3X10min) and incubated in 0.1% mercuric chloride solution for 4 minutes. Following this treatment, the seeds were washed repeatedly in excess amounts of sterile distilled water for 5-6 hours on a shaker before seeding them in petridishes containing in their respective medium

containing at 10^8 cells ml^{-1} . The seeds were kept for 1 hour in the culture medium and decanted the medium. Five replicates of 100 seed of rice, green gram and brinjal were maintained for each bacterial species and incubated at 30 ° c for 3 days. After three days the percentage of seed germination was measured, shoot length and root length were measured in rice after 5 days of inoculation. In the case of green gram and rice, only root length was measured after 3 days of inoculation.

Antibacterial Assay

Antibacterial activity was carried out using disc diffusion method. The media was used Muller Hinton Agar (MHA). The tests were conducted at 3 different concentration of crude extract (5mg, 2.5mg and 1.5mg/disc). The plates were incubated for 24hours at 37°C.

Artemia Lethality Assay

After extraction with ethyl acetate the crude extract was tested for the *Artemia salina* toxicity assay, which is a simple, rapid and inexpensive bench top assay and considered as a useful tool for preliminary assessment of general toxicity (Meyer *et al.*, 1982).

Cultivation of Fungal Strains

The fungal strains were inoculated in a 1000 mL Erlenmeyer flask containing 250 mL of potato dextrose broth (PDB). Cultivation was performed at room temperature under static conditions and daylight. Depending on the fungal growth, cultures on liquid medium were incubated for 2 weeks.

Isolation of Genomic DNA

The Genomic DNA was performed in selected bacterial isolates using QIAGEN DNA isolation kit (Qiagen). The sequence

of 16S rRNA experiment was carried out.

Polymerase Chain Reaction

A pair of oligonucleotide primers is designed complementary to a target DNA molecule such that they can be extended by a DNA polymerase towards each other then the region of the template bounded the primers can be greatly amplified by carrying out cycles of denaturation, primer annealing and polymerization.

Result and Discussion

Isolation of bacteria from rhizosphere soil

The rhizosphere soils were collected from around Chennai. The rhizosphere soils were used for isolation of bacteria from its soil. Zone formation occurs in the phosphate solubilizing bacteria. The total cultivable bacterial count of the entire soil was found to be 2.48×10^{-3} CFU/ml and 3.19×10^{-3} CFU/ml in *Azotobacter* and phosphate solubilizing bacteria.

Different colony characteristics of the bacterial strains were observed on the surface of the Pikoskays medium and Winogradsky's medium which was shown in (Figure.1 and 1.1). The isolated colonies were pure cultured and stored at 4°C for further use.

Identification of the Isolated Bacteria

The organisms were identified based on morphological and biochemical tests (Table: 1) according to the Bergey's manual of determinative bacteriology. Belong to the genus *Azotobacter* and *Ralstonia* respectively. Determinations of biochemical characterization of bacteria isolated from rhizosphere soil were identified as *Azotobacter* and *Ralstonia*.

Evaluation based on Seed Germination in Green Gram and Rice

Seed germination in paddy was 40% and green gram was 80% in control. However, the seeds inoculated with bacteria exhibited an enhanced germination. The germination varied from 20 to 60% in rice and 80 to 100% in green gram depending on the strains *Azotobacter* used and 20 to 60% in rice and 80 to 90% in green gram depending on the strains of phosphate solubilizing bacteria (Table: 2 and Figure:2 and 2.1).

Root Length in Rice and Green Gram

The phosphobacterial strain increased the root nodules by 50%. However *Azotobacter* strains decrease the root nodules by 29% in rice. The *Azotobacter* strain increased the root nodules by 34.75% in green gram. However phosphobacterial decrease the root nodules by 29% in green gram. (Table: 2 and Figure: 3 upto 3.3).

Growth Measurement of Shoot in Green Gram and Rice

The phosphobacterial strain increased the shoot by 60%. However, *Azotobacter* strains decrease the shoot by 50% in green gram. The *Azotobacter* strain decreased the shoot by 30% in rice. However, phosphobacterial increase the shoot by 60% in rice. (Table:3).

Antibacterial Activity Test

In antibacterial activity test disc diffusion method is applied. Erythromycin, Chloramphenicol, Nalidixic acid are sensitive in phosphate solubilizing bacteria. Chloramphenicol, Nalidixic acid are sensitive and Erythromycin is resistant in *Azotobacter* (Table.4 and Figure.4).

Artimia Lethality Test (Phosphate Solubilizing Bacteria and *Azotobacter*)

Phosphate solubilizing bacteria and *Azotobacter* are used in the artimia lethality test. The two strains are inoculated in nutrient broth. Kept for 24 hours incubation. Equal amount of ethyl acetate is added and extracted. Compare to *Azotobacter* phosphate solubilizing bacteria is good (Table.5 upto 5.4).

Identification of Fungi

In order to select the most promising fungal strains, the isolated fungi were cultivated in a small scale level in potato dextrose broth in an Erlenmeyer flask to gain enough extract for the bioactivity screening. A total of 10 strains were isolated and identified on the basis of their morphological characteristics (Table: 6). Based on the results of enzyme production, all the fungal strains, *Mucor* sp., *Alternaria* sp., *Aspergillus terreus*, *Aspergillus flavipes*, and *Aspergillus niger* were identified and chosen for their biological activities using different bioassay systems, and extract of fungus (Figure.5).

Antibacterial Activity Test in Fungi

In nutrient agar plate by using well cutter well is cut around the plate. The pathogenic bacteria such as *Bacillus subtilis*, *Staphylococcus aerus*, *Salmonella paratyphi* were swapped in the nutrient agar plates. Then the extract of fungus such as *Aspergillus niger*, *Alternaria* sp., *Aspergillus flavipes*, *Mucor* sp., *Aspergillus terreus*, *Aspergillus flavus* were inoculated in the petriplates. The zones were formed around the well (Table: 6.1 and Figure: 5.1).

Artimia Lethality Assay in Fungi

Aspergillus Niger and *Aspergillus flavus* are good in artimia lethality assay in fungi

(Table: 6.2).

16 S RNA Sequencing

The bacterial species was identified as *ralstonia species*. The PCR amplification profile of selected strain. The condition is about 1% electrophoresis. (Figure: 6). The genomic DNA of two bacterial isolates in figure (6.1).

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5'TGCCCCACATGATAACGGTTCCCTT
GCAACTCTCACGCAGTGGGGGAG
CCTTGCTTCCCTGCCAGCGAGTGGCG
AACCGGTTATTAATCATCAGTAATGC
CCTGTAGTGGGGGATAACTAGTCGAA
AGATTAATAATACCGCAAACACCTG
AGGGTGAAAGTGGGCGACCGCAGGC
CTCATGCTATATGAGCGGCGATGTCT
GATTACCTAGTTGGTGGGGTAAAGGC
CTACCAAGGCGACCATCAGTAGCTGG
TCTGACAGGACGATCAGCCACACTGG
GACTGACACACGGCCCACACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTGG
ACATGGGCGAAACCCTGATCCAGCA
ATGACGCGTGTGTGAAGAAGGCCTTC
GGGTTGTAAAGCACTTTTGTCCGGAA
AGAAATGGCTCTGGTTAATACCCGGA
GTTGATGACGGTACCGGAAGAATAAC
GACCGGCTAACTCCGTGCCAGCAGCC
GCCGTAATACGTAGGGTCCAAGCGTT
AATCGGAATTACTGGGCATAAAGCGT
GCGCAGGCGGTTGTGC3'.
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Icl64817
Ralstonia sps. M1 16S ribosomal RNA partially sequenced. PCR amplification profile of selected strain and genomic DNA. It has 1500 base pair sequence. *Ralstonia maninitolytica* strain is found by sequencing and biochemical characterization.

In conclusion, Out of 10 strains, the best strains of *Azotobacter* and phosphate solubilizing bacteria were selected for their high beneficial activities. Further study was designed for characterization of the bacterial strains. Two selected strains were identified

at species level based on their morphological, culture and biochemical characteristics. Further experiment was

designed to test the utility of the identified strains as biofertilizer for rhizosphere soil.

Table.1 Cultural and biochemical characteristics of the selected bacterial strains

S.NO	Characteristics	AZ0	PSB
1	Gram staining	Gram –negative	Gram-negative
2	Bacterial shape	Ovoid	Rods
Culture characteristics on agar slants			
3	Abundance of growth	Moderate	Moderate
4	optical characteristics	Transparent	Opague
5	cultural characteristics on nutrient agar plates		
6	Size	Small	Small
7	Elevation	Convex	Flat
	Appearance on nutrient broth cultures	Sediment	Pellicle
8	Motility	Motile	Motile
Intra cellular enzyme			
9	Carbohydrate Fermentation		
10	Sucrose	-	-
11	Fructose	-	-
12	Maltose	-	-
13	Mannitol	-	-
14	Hydrogen sulphide production	Not tested	Not tested
15	Nitrate reduction	-	-
	IMViC test		
16	Indole production	-	-
17	Methyl red reaction	-	+
18	Voges proskauer Reaction	-	-
19	Citrate utilization	+	+
20	Urease activity	-	-
21	Catalase activity	+	+
22	Oxidase activity	+	+
Extra cellular enzyme			
23	Starch hydrolysis	+	+
24	Lipid hydrolysis	+	-
25	Gelatin hydrolysis	+	-

Table.2 Evaluation based on seed germination and root nodules in green gram and rice

S.NO	GREEN GRAM		RICE		
	Strains	Seed germination (%)	Root nodules (mm)	Seed germination (%)	Root nodules (mm)
Control		80	30.25	40	27.15
Azo 1		100	28.8	20	23
Azo2		90	20.6	30	14.3
Azo3		80	34.75	60	14
Azo4		100	16	20	29
.Psb1		80	25.75	20	13.5
Psb2		80	34.25	60	12
Psb3		90	22.6	20	10.16
Psb4		80	30	-	-
Psb5		90	21.3	20	50
Psb6		80	36.25	50	37.8

Table.3 Growth measurement of green gram and rice

Strains	Green gram		Rice	
	Millimeter	Percentage	Millimeter	Percentage
Control	99	40	62.4	50
Azo 1	-	-	62.6	30
Azo 2	90	20	47.5	20
Azo 3	103.66	60	94.6	30
Azo 4	73.88	50	63	30
Psb 1	75	10	58.5	20
Psb 2	107.8	50	84	60
Psb 3	104.6	30	117	40
Psb 4	94.4	50	50	10
Psb 5	91.6	30	41	10
Psb 6	-	-	89.8	60

Table.4 Antibacterial activity test

S.No	Antibiotic discs	Phosphate Solubilizing Bacteria	Mm	<i>Azotobacter</i>	Mm
1	Erythromycin	Sensitive	13	Resistant	-
2	Chloramphenicol	Sensitive	15	Sensitive	8
3	Nalidixic acid	Sensitive	14	Sensitive	16

Table.5 Artimia lethality test (Phosphate solubilizing bacteria and *Azotobacter*)

Content	Psb1		Azo1		Psb2		Azo2		Psb3		Azo3		Positive (Seawater)		Negative (Dms0)		
	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	
µg																	
1000 µg	-	10	-	10	-	10	-	10	-	10	-	10	10	-	-	10	
500 µg	-	10	-	10	-	10	-	10	-	10	-	10	10	-	-	10	
250 µg	-	10	-	10	-	10	-	10	-	10	-	10	10	-	-	10	
125 µg	1	9	1	9	1	9	1	9	1	9	1	9	10	-	-	10	
62.5 µg	3	7	4	6	3	7	4	6	3	7	4	6	10	-	-	10	
31.25µg	5	5	7	3	5	5	7	3	5	5	7	3	10	-	-	10	

Table.5.1 Initial weight and length of Probiotic in fish (1st week)

S.NO	Control		Phosphate solubilizing bacteria		<i>Azotobacter</i>	
	Weight	Cm	Weight	Cm	Weight	Cm
1	2.51	4.3	2.11	4.2	2.54	4.6
2	2.49	4.6	2.80	5	1.62	3.8
3	1.66	4.0	2.90	4.5	2.12	4.5
4	1.99	4.5	2.23	4.4	2.42	4.5
5	0.94	2.5	2.27	4.0	1.52	4.3
6	1.76	4.0	2.48	4.5	1.78	4.0
7	1.89	4.7	1.92	4.0	2.62	4.5
8	1.89	4.0	2.10	4.0	2.60	4.6
9	1.63	3.5	2.47	4.3	3.20	4.8
10	1.49	4.0	2.9	4.5	1.90	4.0
11	1.37	3.5	0.75	3.2	1.36	3.8
12	1.53	3.8	1.32	3.5	1.1	3.4
13	2.63	4.6	1.23	3.8	1.77	4.0

Table.5.2 Weight and length of Probiotic in fish (2nd week)

S.No	Control		Phosphate solubilizingbacteria		<i>Azotobacter</i>	
	Weight	Cm	Weight	Cm	Weight	Cm
1	1.65	3.6	3.58	4.5	2.06	4.0
2	1.84	4.1	3.76	4.9	5.24	4.9
3	2.44	4.2	3.20	4.5	3.36	4.7
4	3.16	4.6	2.68	5.0	5.75	4.4
5	2.90	4.7	1.81	3.6	3.03	4.7
6	2.56	5.0	4.11	5.2	2.62	4.3
7	1.91	3.9	3.54	4.3	4.70	4.4
8	2.25	4.9	3.34	4.2	1.60	3.7
9	1.88	4.8	2.71	4.2	2.89	4.5
10	3.06	4.2	1.48	4.0	2.68	4.0
11	2.91	4.6	.96	3.3	3.33	4.3

Table.5.3 Weight and length of probiotic in fish (3rd week)

S.No	Control		Phosphate solubilizing bacteria		<i>Azotobacter</i>	
	Weight	Cm	Weight	Cm	Weight	Cm
1	2.81	4.6	2.25	4.3	2.21	5.0
2	3.32	4.0	2.13	4.9	2.23	4.3
3	2.81	4.6	2.15	4.4	2.34	4.2
4	2.68	4.4	1.88	4.0	2.02	4.5
5	2.98	4.8	1.75	4.2	3.11	5.3

Table.5.4 Weight and length of probiotic in fish (4th week)

S.No	Control		Phosphate solubilizing bacteria		<i>Azotobacter</i>	
	Weight	Cm	Weight	Cm	Weight	Cm
1	2.57	4.6	2.05	4.1	2.77	4.8
2	2.26	4.5	1.80	3.6	3.68	4.9
3	2.0	4.0	2.11	4.7	2.19	4.0
4	1.59	3.5	2.11	4.4	2.32	4.5
5	2.30	4.3	1.98	4.1	2.40	4.2

Table.6 *Artimia* lethality assay in fungi

S.No.	Strain code	Name of the Fungi
1	R-f-leaf 1	<i>Aspergillus niger</i>
2	R-f-stem 3	<i>Aspergillus niger</i>
3	A- stem 1	<i>Alternaria sp.</i>
4	A-f-leaf 3	<i>Aspergillus flavipes</i>
5	R- root 1	<i>Mucor sp.</i>
6	A-F-leaf 1	<i>Aspergillus terreus</i>
7	R-f-stem 2	<i>Aspergillus niger</i>
8	A-f-leaf 2	<i>Aspergillus niger</i>
9	A-f-root 1	<i>Aspergillus flavus</i>
10	R-f-stem 1	<i>Aspergillus flavus</i>

Table.6.1 Antibacterial activity test in fungi

S.NO	FUNGUS	BS	SA	SP
1	R-f-leaf 1	-	-	3mm
2	A- stem 1	12mm	13mm	14mm
3	R- root 1	13mm	8mm	12mm
4	A-F-leaf 1	12mm	11mm	12mm
5	A-f-root 1	11mm	7mm	18mm

Table.6.2 Artimia lethality test (fungi)

	1		2		3		4		5		6		7		8		9		10	
Concentration	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A
1000µg	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-
500µg	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-
250µg	10	-	10	-	10	-	9	1	10	-	10	-	10	-	10	-	9	1	10	-
125µg	10	-	9	1	9	1	7	3	8	2	9	1	10	-	10	-	8	2	10	-
62.5µg	10	-	9	1	8	2	4	6	7	3	8	2	9	1	10	-	7	3	10	-
31.25µg	10	-	8	2	7	3	2	8	5	5	7	3	7	3	10	-	7	3	10	-
NEGATIVE CONTROL	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-
POSITIVE CONTROL	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10	-	10

Figure:1. Pure culture of microorganisms from root soil in selected media (Pikovskay's Media)

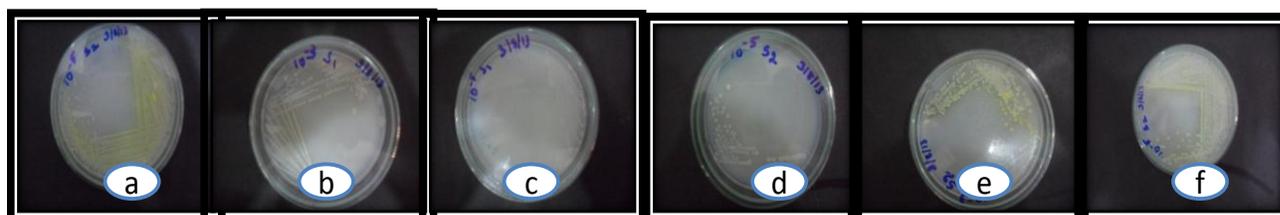


Figure: 1.1: Pure culture of microorganisms from root soil in selected media (winogradskay's)



Figure:2: Seed Germination (Green Gram) Figure: 2.1 Seed Germination (Rice)

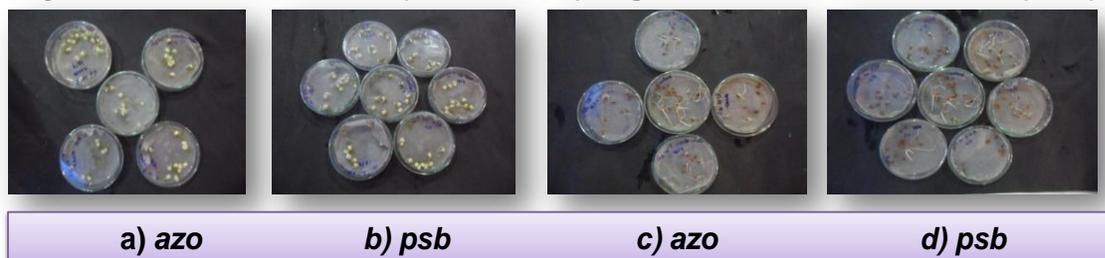


Figure: 3: Plant growth (Green Gram)



Figure: 3:1: Plant Growth (Rice)



Figure:3.2: Brinjal

Figure:3.3: Vermicompost

Figure:4:Antibacterial activity

Figure: 5 : Extract of fungus



Fig: 5.1: Activity test in fungi

Fig: 6.:PCR

Fig:6:1: G-DNA

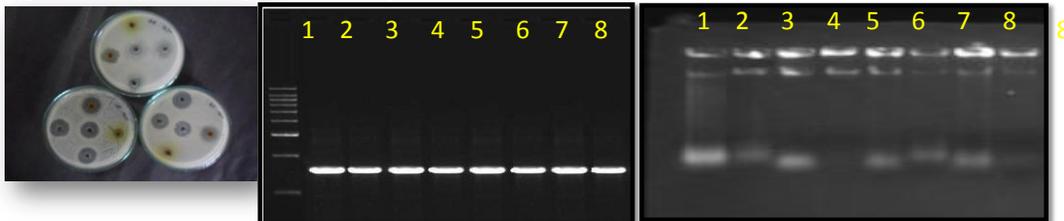


Fig: 6: PCR amplification profile of selected strain Conditions: 1% agarose gel electrophoresis Lane M: 1 KB DNA Ladder; 1: Sample 1 KB DNA Ladder (bp):1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000. Fig :6:1: Genomic DNA of selected two bacterial Isolates.

The bacterial strains especially were proved to be potent biofertilizers for raising green gram and rice. Further experiment was designed to study their probiotic potential in the fishes. The bacterial strains especially *ralstonia* beneficial effect on the fish culture tank, by enhancing the growth and production of fish. Mass culture was prepared. The mortality in % is determined after the formula $(A-N-B) Z^{-1}100$, with A = number of dead larva after 24 h; N = number of dead larva before the addition of test compound; B = number of dead larva in the negative control; Z = total number of larva. A mortality of *Artemia salina* at 10 μ g/mL is interpreted as high cytotoxic in bacteria and fungal extract diluted in DMSO. The selected strains were bioassayed in seedling growth. The percentage of seed germination was measured, shoot length and root length were measured in rice and green gram. Antibacterial activity was carried out using disc diffusion method by Muller Hinton Agar (MHA) to find sensitive and resistant.

Zone of inhibition was recorded in mm and the experiment was performed in duplicate. In bacteria after extraction with ethyl acetate the crude extract was tested for the *Artemia salina* toxicity assay, which is a simple, rapid and inexpensive bench top assay and considered as a useful tool for preliminary assessment of general toxicity. The best culture is selected from bacteria and fungi. The mass culture of *Azotobacter* and phosphate solubilizing bacteria are used as biofertilizer in green gram, rice and probiotics of fish. The weight and length of probiotic fish is measured from initial stage, 1st week, 2nd week and 3rd week. The fungal strains are isolated from root, stem, leaf. Then the extract of fungus such as *Aspergillus niger*, *Alternaria sp.*, *Aspergillus flavipes*, *Mucor sp.*, *Aspergillus terreus*, *Aspergillus flavus* were inoculated in the petriplates leaf. In fungi after extraction with ethyl acetate the crude extract was tested for the *Artemia salina* toxicity assay, which is a simple, rapid and

inexpensive bench top assay and considered as a useful tool for preliminary assessment of general toxicity.

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