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Investigation of Phosphate Solubilizing Bacteria (PSB) from Rhizospheric Niches of Healthy Plants

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Abstract:

Rhizospheric niches of healthy plants play an important role in the maintenance of health and growth of plant either by providing valuable nutrients or protecting plant from soil borne pathogens. The aim of present study was to use the rhizospheric niches of healthy plants to isolate efficient phosphate solubilizing bacteria. The soil samples from rhizospheric niches of Neem, Soya bean, Bavanchya and Tur were collected from different fields in the sterile polythene bags and brought to the laboratory. All the rhizospheric soil samples were tested for phosphate solubilizing bacteria on Pikovskaya agar by serial dilution method. Among the rhizospheric soil samples screened, rhizospheric niches from the Soya bean shown highest phosphate solubilizing bacteria, 114 than the other rhizospheric soil sample. The rhizospheric niches of Tur, Neem and Bavanchya have shown 47, 07, and 02 phosphate solubilizing bacteria. Over all 171 phosphate solubilizing bacterial isolates were isolated from different niches. Out of which 4 isolates, RRR18, SMD36, SMD38 and SMD40 were found to produce more than 5 mm zone of tri-calcium phosphate solubilization on Pikovskaya's agar plates after 9 days of incubation.Out of these 4 bacterial isolates, two bacterial isolates, namely SMD 38 and SMD 40when quantitatively tested, showed maximum P solubilization on 7th day in PKV broth supplemented with tri – calcium phosphate as 444 µg/ml and 421 µg/ml respectively.

Key Word: Rhizospheric niches, Pikovskaya's Agar, Phosphate Solubilizing bacteria.

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1.0 Introduction:

Crops neednumerous nutrients toreach their maximum potential yield. The nutrients, which are required by the plants, occurnaturally in the soil, but sometimes these are added as lime or fertilizer into the soil. Afternitrogen, phosphorous (P) is one of the major vitalmacronutrient for plant growth anddevelopment (Bagyaraj *et al.*, 2000). About 98 % soils have inadequate supply of availablePhosphorus (Hansan 1996) and likely to induce shortage of this mineral. Phosphorus hasseveral roles in the plants and is involved in functioning of nucleic acids, proteins,photosynthesis and in the formation of oils, sugars and starches etc. It is helpful in the rapidgrowth of the roots and shoots (Kaur, 2014).

Most of the soils contain the considerable reserves of total P;large part of it relatively remains inert and only less than 10 % of soil P enters the plant, animalcycle (Kucey *et al.*, 1989). When P is added as fertilizer to the soil, it gets fixed. Thesoil microorganisms solubilize this P and make it available to the plants (Hilda and Fraga1999).From several years, great attention has been devoted to study the role, that soilmicroorganisms play in the changing aspects of phosphate, particularly those able to solubilise insoluble P forms (Rao 1992). These microorganisms are bacteria and fungi that inhabitantthe rhizosphere (Barea and Azcon 1975, Bowen and Rovira 1999, Kaur, 2014).The present scenario is shifting towards a more sustainable agriculture by using Phosphate Solubilizing Bacteria.

Natural solubilization of mineral phosphates is an important mechanism exhibited by different microorganisms, knownas phosphate solubilizing microorganisms (PSM). Bacteria arethe predominant microorganisms that solubilize mineralphosphate in nature, as compared to other microorganisms (Yin, 1988, Paul and Sinha, 2017).Application of phosphate-solubilizing bacteria increases soil fertility due to their ability to convert insoluble P to soluble P by releasing organic acids, chelation and ion exchange [Omar (1998), Narula *et al.*,(2000), Whitelaw,(2000)].The important genera of P-solubilizing bacteria include *Achromobacter, Aerobacter, Alcaligenes, Azotobacter, Bacillus,Escherichia, Pseudomonas, Serratia* and *Xanthomonas* (Li, 1981; Datta *et al.*, 1982; Venkateswariu *et al.*, 1984; Gaur, 1990), *Azospirillum* (Seshadri et al., 2000).

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The present study mainly gives emphases to i) investigate for high Phosphate Solubilizing Bacteria from rhizospheric niches of different plants, ii) qualitative and quantitative estimation of phosphate solubilizing efficiency.

2.0 Materials and Methods:

2.1 Chemicals:

All the chemical compounds used during the study were obtained from M/S Hi-media, Mumbai, Glaxo Ltd., Mumbai, Sigma Aldrich, USA, unless and otherwise specified in the text. Analytical/Guaranteed (AR/GR) grade chemicals and double glass-distilled water was used.

2.2 Soil Sample Collection from Rhizospheric Niches:

Soil samples from the rhizospheric niches of plants *viz., Tur, Soya bean, Neem, and Bavanchya* etc grown were collected in the farmer field (Photo Plate 2.0), near the Purna City. For this purpose, the plants were uprooted carefully, shoots were cut off and roots along with rhizosphere soils were brought to the laboratory in polythene bags. The soil samples were processed immediately or stored at 4 – 8 ºC for the isolation of Phosphate solubilizing microorganisms.

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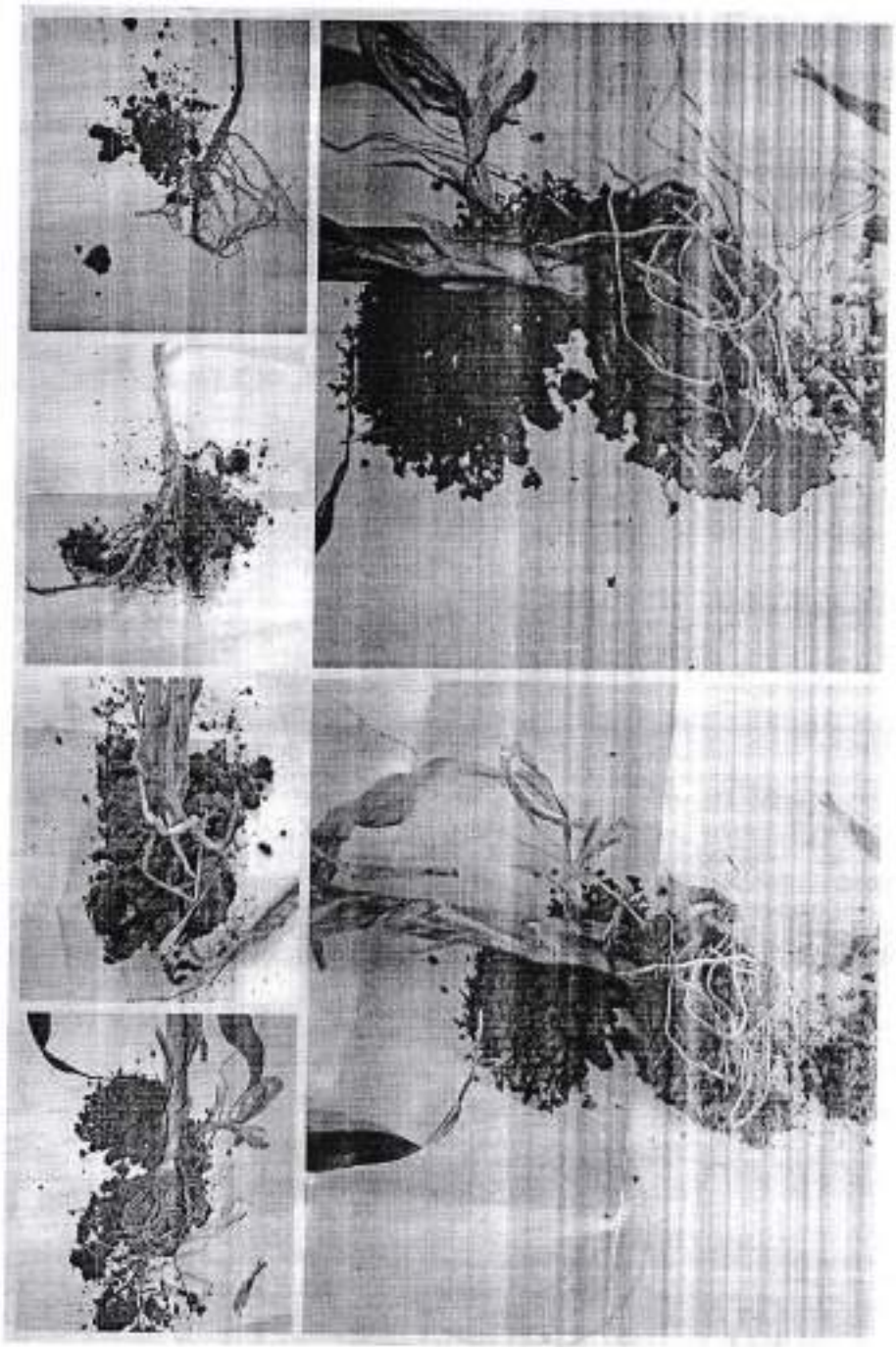

Photo plate 2.0: Soil sample collection from different Rhizospheric niches of plants

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2.3 Isolation of Phosphate Solubilizing Bacteria (PSB):

To isolate Phosphate Solubilizing Bacteria (PSB) from the rhizospheric soil samples dilution plate technique was used on Pikovskaya's medium (Pikovskaya 1948) containing tri-calcium phosphate (TCP) (Gupta *et al.*, 2012, Kaur, 2014). One gram each of the soil sample was transferred to 9 ml sterile dilution blank under aseptic conditions and serial dilutions were made. Appropriate soil dilutions (10^{-5}) were plated on Pikovskaya's agar medium by spread plate technique and incubated at 30 ± 1 ºC for 2-3 days. The colonies forming halo zone of clearance (Pikovskaya's medium) around them were counted as P - Solubilizers. All the bacterial colonies exhibiting halo zones were selected, purified and maintained on nutrient agar slants for further studies.

2.4 Assessment of Phosphate Solubilization Efficiency:

The assessment of phosphate solubilization efficiency of the bacterial colonies exhibiting strong halo zone was carried out qualitatively and quantitatively.

2.4.1. Qualitative Assessment of Phosphate Solubilization Efficiency:

Pure cultures of phosphate solubilizing bacteria were spot inoculated on the plates containing Pikovskaya's medium (Jackson 1973, Katoch, 2003 and Kaur 2012). The plates were incubated at 28±1°C and halo zone around colonies were recorded at regular interval upto 10 days. The capabilities of the isolated phosphate solubilizing bacterium to solubilize TCP on Pikovskaya's agar media were determined in terms of solubilization index (SI). Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter and the colony diameter, using the following formula of Edi-Premono *et al.*, (1996).

Phosphate Solubilization Index (SI) =

2.4.2. Quantitative Assessment of Phosphate Solubilizing Efficiency:

The quantitative assessment of solubilized Phosphate by bacterial isolates was done by Chloromolybdic Acid Method (Jackson, 1973, Kaur, 2014).

2.4.2.1. Chloromolybdic acid Method:

The phosphate solubilizing activity of the selected bacterial isolates as citrate soluble P was determined quantitatively in liquid medium following Chloromolybdic Acid Method (Kaur, 2014, Jackson, 1973).

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2.4.2.1.1. Chloromolybdic Acid:

15 gm Ammonium molybdate was dissolved in about 400 ml of distilled water, filtered and then 400 ml of 10 N HCl was added slowly with rapid stirring. Volume was made to 1000 ml with distilled water and stored in amber glass bottle.

2.4.2.1.2. Chlorostannous Acid:

Stock solution:

$SnCl_2.2H_2O$	10 gm
Con. HCl	25.0ml

$SnCl_2$ crystals were dissolved in Con. HCl and solution was kept in glass bottle under airtight stopper.

*Working Solution:*Fresh working solution was prepared by adding 1.0 ml of the above solution to 132.0 ml of distilled water.

2.4.2.1.3. Quantitative Assessment P Solubilization by Chloromolybdic Acid Method:

The phosphate solubilizing bacteria were grown in 50 ml nutrient broth for 24 hr. at 30 °C in incubator shaker. 0.1 ml of each phosphate solubilizing bacteria was aseptically transferred to 100 ml PKV broth contained in 250 ml conical flask. The flasks were incubated 30 °C in a rotary shaker at 130 rpm. Five ml of culture was taken out in sterile condition at regular interval of 2 days from third day onward and centrifuged at 10000 rpm for 10 min. Then 500 µl of each supernatant was transferred to 50 ml volumetric flask. This was followed by addition of 10.0 ml Chloromolybdic acid. The content of the flask was diluted to 40.0 ml with distilled water. Then 1 ml of Chlorostannous acid was added. After mixing, the volume was made up to 50.0 ml with distilled water. The blue colour intensity of the solution was measured at 600 nm. The soluble P was estimated from standard curve of KH_2PO_4 (100 ppm) drawn against O.D. 600 nm.

2.5 Identification of Phosphate solubilizing bacteria:

The efficient Phosphate solubilizing bacteria obtainedfrom screening was identified according to Bergey's Manual ofSystematic Bacteriology (1984) by using cultural and biochemicalcharacteristics as well as 16s rRNA sequencing. 16s rRNA sequencingof culture was carried out at Agharkar Research Institute (ARI) Pune,Maharashtra.

3.0 Result and Discussion:

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Natural solubilization of mineral phosphates is an important phenomenon displayed by different microorganisms, known as *phosphate solubilizing microorganisms (PSM)*. Bacteria are the principal microorganisms that solubilize mineral phosphate in nature, as compared to other microorganisms (Yin, 1988). *Phosphate solubilizing bacteria (PSB)* play an important role in biogeochemical phosphorus cycling in both terrestrial and aquatic environments (Das *et al.*, 2007).

3.1 Isolation of Phosphate Solubilizing Bacteria (PSB):

In present investigation 170 phosphate solubilizing bacteria were isolated from different rhizospheric niches of healthy plants by using serial dilution method on Pikovskaya's (PKV) agar plates (Photo Plate 3.0). Out of 171, 114 phosphate solubilizing bacteria (PSB) were isolated on Pikovskaya Agar from the Soya bean rhizospheric niches, by using dilution technique, which were fare greater than the other rhizospheric niches sample. Similarly, from the rhizospheric niches of Tur, Neem and Bavanchya 47, 07, and 02 phosphate solubilizing bacteria were isolated (Table 3.0). Use of Pikovskaya's agar medium for isolation of Phosphate Solubilizing Bacteria (PSB) was a simple way to detect PSB through formation of halo zone on agar plate containing tri – calcium phosphate as a sole Phosphorous source (Kaur, 2014). These rhizospheric isolates were tentatively named as SMD 1 to SMD 170 and RRR18.

Table 3.0: Isolated PSB from different rhizospheric niches with their Phosphate solubilization index

PSB Isolate	Phosphate Solubilization Index	*PSB Isolate*	Phosphate Solubilization Index	*PSB Isolate*	Phosphate Solubilization Index
SMD 1	1	SMD 58	1	SMD 115	1
SMD 2	2	SMD 59	1	SMD 116	2
SMD 3	2	SMD 60	2	SMD 117	2
SMD 4	1	SMD 61	2	SMD 118	0
SMD 5	1	SMD 62	1	SMD 119	1
SMD 6	**5**	SMD 63	2	SMD 120	2
SMD 7	1	SMD 64	2	SMD 121	2
SMD 8	1	SMD 65	2	SMD 122	2
SMD 9	1	SMD 66	2	SMD 123	2
SMD 10	1	SMD 67	2	SMD 124	0

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SMD 11	2	SMD 68	1	SMD 125	1
SMD 12	2	SMD 69	1	SMD 126	1
SMD 13	2	SMD 70	2	SMD 127	0
SMD 14	2	SMD 71	2	SMD 128	2
SMD 15	3	SMD 72	2	SMD 129	2
SMD 16	4	SMD 73	2	SMD 130	1
SMD 17	1	SMD 74	2	SMD 131	1
SMD 18	4	SMD 75	2	SMD 132	1
SMD 19	4	SMD 76	2	SMD 133	2
SMD 20	4	SMD 77	1	SMD 134	2
SMD 21	1	SMD 78	1	SMD 135	0
SMD 22	**5**	SMD 79	1	SMD 136	0
SMD 23	1	SMD 80	1	SMD 137	1
SMD 24	1	SMD 81	2	SMD 138	1
SMD 25	1	SMD 82	1	SMD 139	1
SMD 26	4	SMD 83	1	SMD 140	1
SMD 27	2	SMD 84	1	SMD 141	0
SMD 28	**5**	SMD 85	2	SMD 142	0
SMD 29	4	SMD 86	2	SMD 143	0
SMD 30	**5**	SMD 87	2	SMD 144	0
SMD 31	4	SMD 88	1	SMD 145	0
SMD 32	2	SMD 89	2	SMD 146	0
SMD 33	4	SMD 90	2	SMD 147	0
SMD 34	4	SMD 91	2	SMD 148	0
SMD 35	2	SMD 92	2	SMD 149	0
SMD 36	**5**	SMD 93	2	SMD 150	0
SMD 37	**5**	SMD 94	1	SMD 151	0
SMD 38	**5**	SMD 95	1	SMD 152	1
SMD 39	4	SMD 96	2	SMD 153	1
SMD 40	**5**	SMD 97	2	SMD 154	1
SMD 41	4	SMD 98	1	SMD 155	1
SMD 42	2	SMD 99	1	SMD 156	1
SMD 43	1	SMD 100	1	SMD 157	1
SMD 44	2	SMD 101	0	SMD 158	2
SMD 45	1	SMD 102	2	SMD 159	2
SMD 46	1	SMD 103	0	SMD 160	2
SMD 47	1	SMD 104	1	SMD 161	1
SMD 48	1	SMD 105	0	SMD 162	2
SMD 49	1	SMD 106	2	SMD 163	2
SMD 50	2	SMD 107	1	SMD 164	1
SMD 51	1	SMD 108	2	SMD 165	2

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SMD 52	1	SMD 109	1	SMD 166	2
SMD 53	4	SMD 110	0	SMD 167	2
SMD 54	1	SMD 111	2	SMD 168	1
SMD 55	1	SMD 112	2	SMD 169	2
SMD 56	1	SMD 113	0	SMD 170	0
SMD 57	4	SMD 114	1	RRR18	5

The role of microorganisms in solubilizing insoluble phosphates in soil and making it available to plant is well known (Kundu and Gaur, 1981). Phosphate solubilizing microorganisms include several Bacteria, Fungi, Actinomycetes, Yeast and Cyanobacteria (Gerretsen, 1948; Banik and Dey 1982 and Illmer and Schinner 1992). The phosphate solubilizing microorganisms can be isolated from different sources such as soil (Gupta *et al.*, 1986; Kapoor *et al.*, 1989), rhizosphere (Sardinaet *al.*, 1986; Singh and Kapoor, 1994), root nodules (Suranga and Kumar, 1993), compost (Gupta *et al.*, 1993), and rock phosphates (Gaur *et al.*, 1973). These reports support the fact that phosphate solubilizing bacteria can be isolated from rhizospheric niches.

In this study, from rhizospheric niches of soya bean greater amount of phosphate solubilizing bacteria were isolated on Pikovskaya's agar plate. Kaur (2014) isolated 1270 bacteria was isolated on Pikovskaya's agar plate by serial dilution method at 10^{-5} dilutions. Out of these 1270 bacterial isolates only 169 bacteria isolates were observed to be formed a halo zone around the colonies. Of 171 bacterial isolates, only 9 bacterial isolates designated as SMD6, SMD22, SMD28, SMD30, SMD36, SMD37, SMD38, SMD40 andRRR18 displayed greater efficacy of solubilizing the phosphate.

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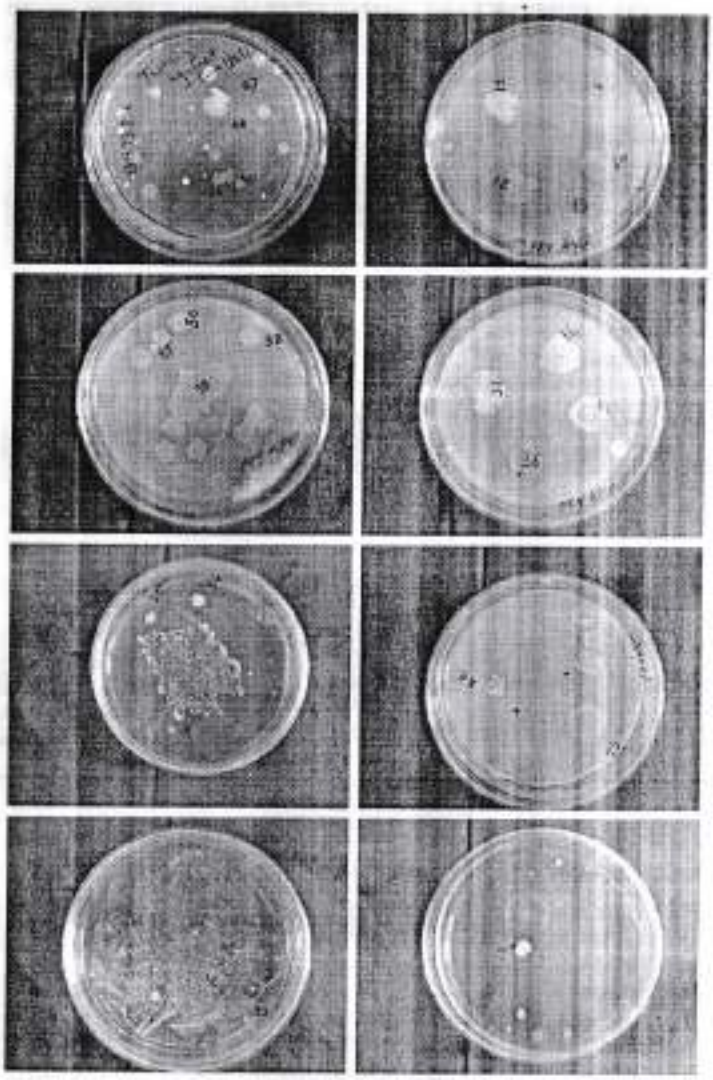

Photo plate 3.0: Isolation of Phosphate Solubilizing Bacteria (PSB) with clear halo zone

3.2 Estimation of Phosphate Solubilization Efficiency:

The phosphate solubilization efficiency of the bacterial colonies exhibiting strong halo zone was carried out qualitatively and quantitatively.

3.2.1. Qualitative Assessment of Phosphate Solubilization Efficiency:

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Qualitative screening of selected phosphate solubilizing bacterial isolates was completed by the method of Katoch, 2003 and Kaur 2012 revealed variations in phosphate solubilization efficiency. In total of 171 phosphate solubilizing bacterial isolates from different niches, 5 isolates, SMD36, SMD38, SMD40and RRR18 were found to be far more than 5 mm zone of solubilization on Pikovskaya's agar plates after 9 days incubation (Photo plate 3.1). The Phosphate solubilization activity of these isolates of PKV agar plates was ranged between 3.0 to 5.0 (Table 3.1). Kaur (2014) stated that 169 phosphate solubilizing bacteria isolated from different rhizospheric niches revealed phosphate solubilization index in range between 1.36 to 3.17. Our results were somehow like results of Kaur (2014).

Table 3.1: Phosphate Solubilization Index of Selected Rhizospheric Isolates after 9 days of incubation

Rhizospheric Isolates	Diameter of Colony + Halo zone (mm)	Diameter of Colony (mm)	Diameter Halo zone (mm)	Phosphate Solubilization Index
SMD 36	10	3	7	3.33
SMD38	10	2	8	5.0
SMD40	10	2	8	5.0
RRR18	9	2	7	4.5

Similar kind of results were also recorded by Katoch (2012) where maximum zone of P solubilization in isolates designates as FB1-PB1, SB1-PB1, CP1-PB1, DH2-PB1, KT14-PB1, AM8-PB1 and PT12-PB1 was observed on 8th day of incubation, whereas in isolates FB2-PB2, PEA-PB1, RM2-PB1, CP2-PB4, WC2-PB1 and WSP1-PB1 on 9th day of incubation. Nine isolates showed maximum zone of solubilization on 10th day, 2 isolates (WSP2-PB2 and PT13-PB1) on 7th day and one isolate (SP2-PB1) on 6th day of incubation period.

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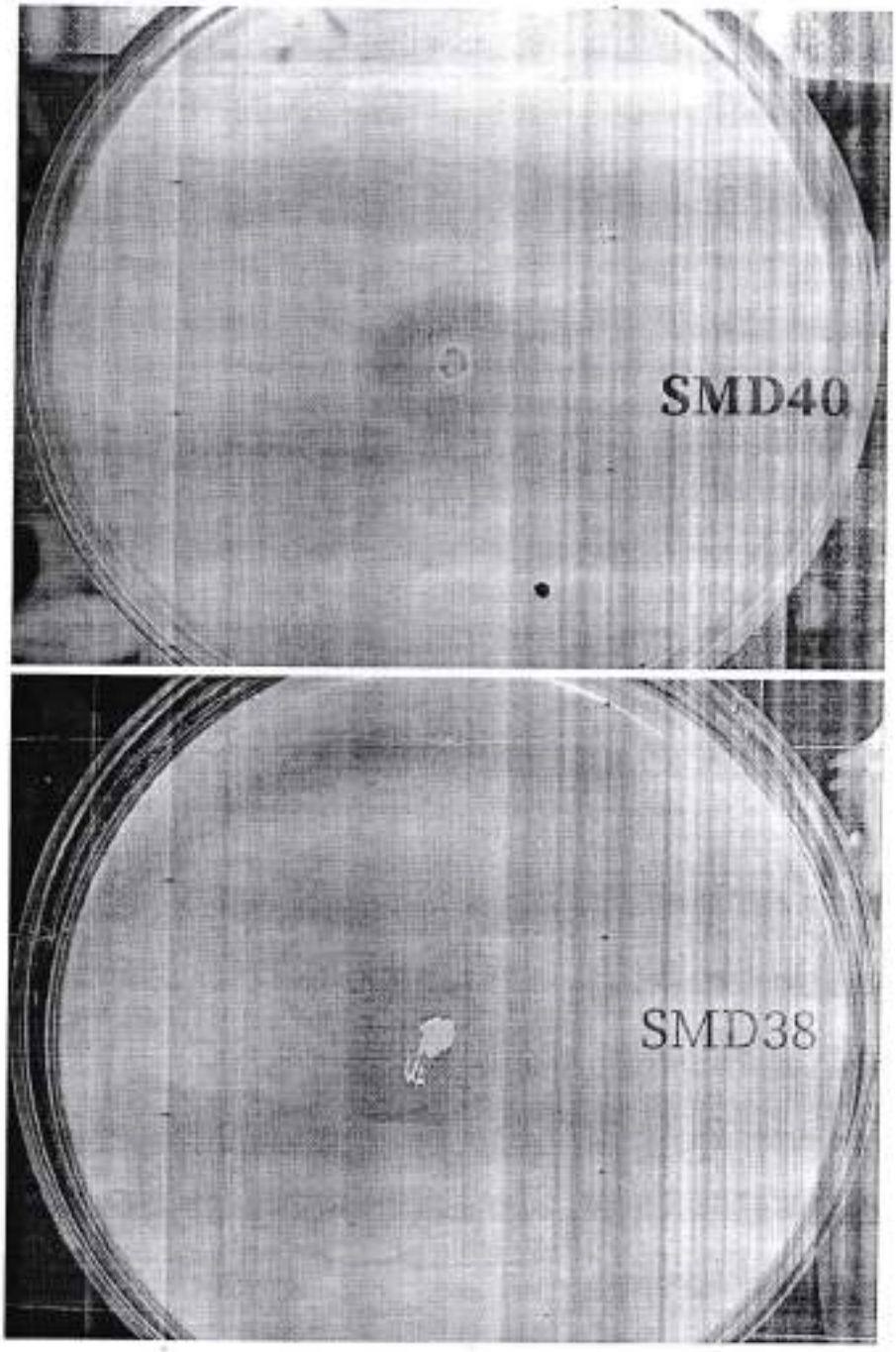

Photo Plate 3.1:Phosphate Solubilization Index of Selected Rhizospheric Isolates(Qualitative Estimation)

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3.2.2. QuantitativeAssessment of Phosphate Solubilizing Efficiency:

Quantitative estimation of selected five bacterial isolates was performed by the method of Jackson (1973) and Kaur (2012) in tri – calcium phosphate supplemented PKV broth. Results represented in Table 3.2 showed P solubilization was increased upto day seven of incubation and maximum solubilization was observed on day seven of incubation in most of the isolates. After seven day, it started to decrease. Out of these 4 selected bacterial isolates, two bacterial isolates, SMD 38 and SMD 40 showed maximum P solubilization in PKV broth supplemented with tri – calcium phosphate.

Table 3.2: Release of soluble P at different time intervals (Days) by selected bacterial isolates in PKV broth supplemented with tri – calcium phosphate

Bacterial isolates	3rd day Soluble P µg/ml	5th day Soluble P µg/ml	7th day Soluble P µg/ml	9th day Soluble P µg/ml	11thday Soluble P µg/ml
SMD 36	376	397	**400**	339	303
SMD 38	369	389	**444**	373	354
SMD 40	372	393	**421**	365	345
RRR18	365	386	**409**	399	355

An extensive range of microorganisms that can solubilize various form of soil bound phosphorous have been reported by Rodriguez and Fraga, (1999) and Whitelaw (2000) and among them, most prominent are *Bacillus spp.* and *Pseudomonas sp.*

When our results were compared with results of Kaur (2014), where the phosphate solubilizing bacteria PSB – B6, PSB – B8, PSB – P3, PSB – P7, PSB – P9, PSB – S1, PSB – S2, PSB – S8, PSB – S14, PSB – S15, PSB – MM10, PSB – MM11, PSB – MM16, PSB – SM4, PSB – SM5, PSB – SM9, PSB – SM10, PSB – SF12, PSB – SF15, PSB – SF16, PSB – 1, PSB – 3, PSB – , 4, PSB – 5, PSB – 6, PSB – 7, PSB – 8, PSB – 9, PSB – 10, PSB – 11, PSB – 12, and PSB – 13 showed maximum phosphate solubilization on day five of incubation in PKV broth supplemented with tri – calcium phosphate, as 163, 73, 221, 180, 144, 194, 290, 121, 166, 146, 182, 188, 171, 163, 291, 328, 70, 226, 285, 139, 365, **422**, 296, 429, 401, 410,**398**, 330,

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113, 199, **415 and 413** µg/ml respectively. While our phosphate solubilizing bacteria, SMD36, SMD38 SMD 40, and RRR18 shown maximum solubilization of insoluble phosphate provided in PKV broth on day seven as, **400, 444, 421,** and**409** respectively. Our results were far better than the results of Kaur (2014).

3.3 Identification of Phosphate solubilizing bacteria:

16S rRNA sequencing and Phylogenetic analysis identified SMD38 as *Sporolactobacillus laevolacticus* SMD38 and SMD 40 as *Sporolactobacillus laevolacticus* SMD40 (Fig. 3.0 and 3.1). The 16S rRNA sequence has been deposited in Genbank of National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine8600 Rockville Pike, Bethesda MD, 20894 USA with accession no. MN853532 for *Sporolactobacillus laevolacticus* SMD38 and MN853531for *Sporolactobacillus laevolacticus* SMD40.

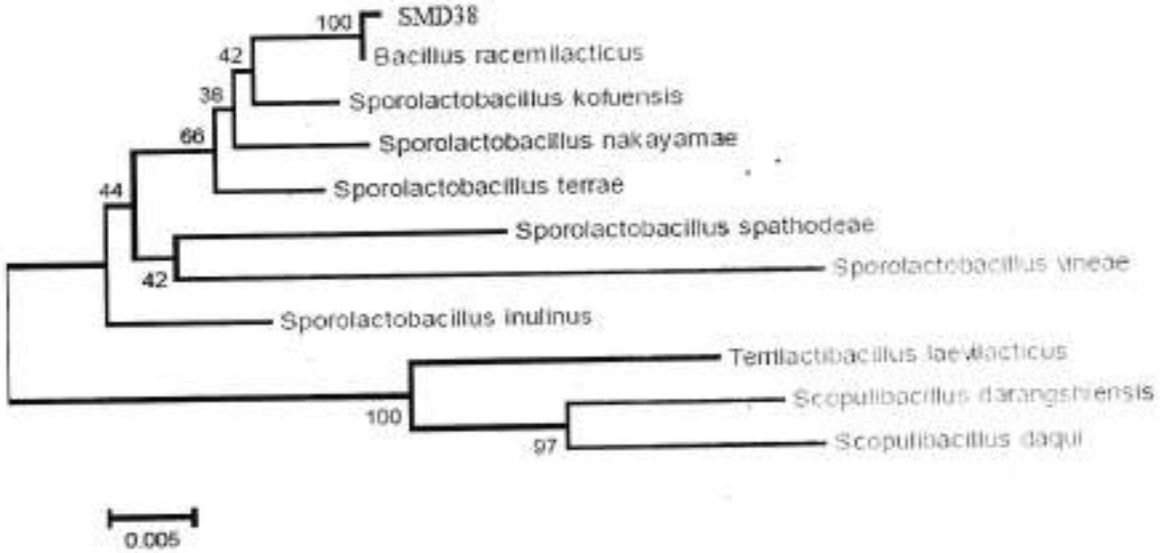

Figure 3.0 Phylogenetic tree of *Sporolactobacillus laevolacticus* SMD38 with closely related species after BLAST search of 16S rRNAsequences.The bootstrap values are indicated at the branch points.

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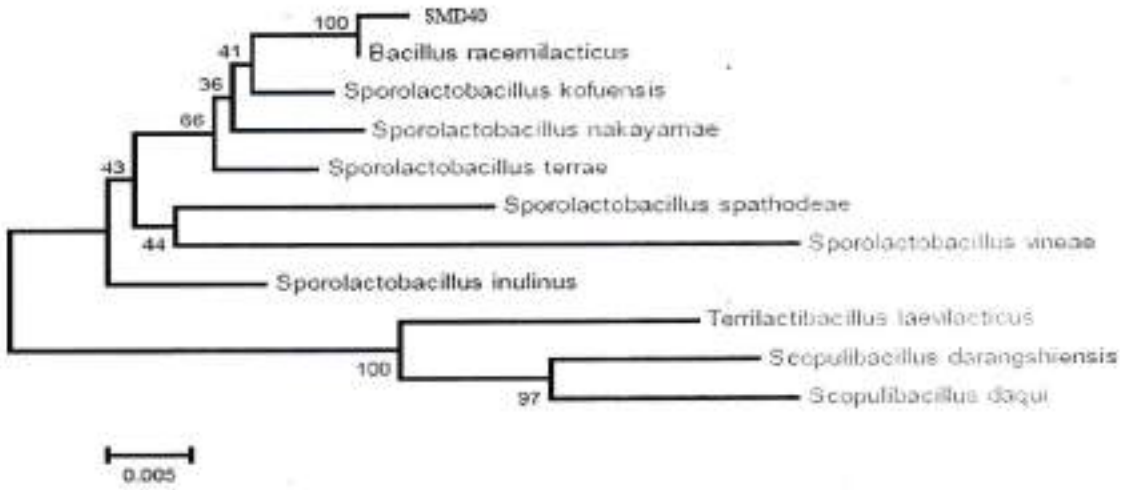

Figure 3.1 Phylogenetic tree of *Sporolactobacillus laevolacticus* SMD40 with closely related species after BLAST search of 16S rRNAsequences.The bootstrap values are indicated at the branch points.

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