Acinetobacter sp. - An acid-tolerant phosphate solubilizing bacteria from lateritic soils

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Abstract

A study was conducted to screen the isolates of phosphate solubilizing bacteria (PSB) ls, for efficiency and acid tolerance. The rhizosphere and non-rhizosphere soil samples were collected from various lateritic soils in Thrissur district of Kerala and phosphate solubilizing bacteria were isolated on Pikovskaya's agar medium. Under *in vitro* conditions, the bacterial isolate PMD-7 (*Acinetobacter* sp.) solubilized 207.2 µg/ml of insoluble phosphorus. Its solubilization efficiency in Pikovskaya's agar medium was 247.62%. It grew in the pH range of 4.0 to 7.0 and tolerated upto pH 3.5. When it was grown in Pikovskaya's broth, it showed a reduction in pH from pH 7.0 to 4.35 indicating the production of organic acids, which is one of the mechanism involved in 'P' solubilization by microbes. The bacterial isolate also produced 33µg/ml of indole acetic acid (IAA) which is a phytohormone for the plant growth. The molecular characterization using 16s rDNA sequencing revealed the identity of bacteria as *Acinetobacter* sp. However, this new acid-tolerant PSB is the first report from Kerala, which could be a potential biofertilizer for acidic soils of Kerala.

Key words: *Acinetobacter* sp., phosphate solubilizing bacteria, acid-tolerant, lateritic soil

Introduction

Phosphorus is one of the essential macronutrients for plants and is applied to soil in the form of phosphatic fertilizers. The soluble form of phosphorus when applied to soil as phosphatic fertilizers are rendered insoluble due to chemical fixation (Dey, 1988). The poor availability of the phosphorus might influence plant quality and yield. Due to the negative impact of chemical fertilizers in the environment, the use of biofertilizers have advantages in the sustainable agricultural practices. Microorganisms are involved in a wide range of processes that helps in the transformation of soil phosphorus and are an integral part of the soil P cycle (Chen *et al*., 2006).The phosphate solubilizing bacteria secretes organic acids which act on insoluble phosphates and convert it into soluble form (Ponmurugan and Gopi, 2006). Microbial biomass assimilates soluble phosphorus, and prevents it from adsorption or fixation (Khan and Joergesen, 2009). Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in the growth environment have been reported to play a role in phosphate solubilization by phosphate solubilizing bacteria (He *et al*., 2002).

About 65% of Kerala soils are laterite with low activity clays, gravelly with low water holding capacity, low organic matter content and high phosphorus fixing capacity. The soil pH ranges from 4.5-6.2. The acidic nature of soil affects the field performance of biofertilizers in the region. Therefore, an acid tolerant phosphate solubilizing bacteria will be more suitable for Kerala soils as the currently available PSB such as *Bacillus megaterium* var. *phosphaticum* are suitable for only neutral soils. Hence, a study was undertaken to identify an acid-tolerant phosphate solubilizing bacteria from lateritic soils so that such bacteria can be exploited as phosphatic biofertilizers for Kerala.

Materials and Methods

Isolation of phosphate solubilizing bacteria

Rhizosphere and non-rhizosphere lateritic soil samples were collected at a depth of 5-10 cm from ten different locations of Thrissur district with pH ranging from 5.22 to 6.51. From each location, five samples were randomly collected and a composite sample was prepared.

Ten grams of soil sample from each location was suspended in sterile water (90 ml) and serial dilutions of the suspension were made in sterile water blanks. One milliliter of appropriate dilutions was plated in sterile Petri-plate to which Pikovskaya's Agar medium containing insoluble phosphate (tri-calcium phosphate) was added. The plates were incubated for 4-5 days at 28 ± 2^0C. Transparent and clear zone around the bacterial colonies indicated the extent of phosphate solubilization and such colonies were purified and maintained for further studies on Pikovskaya's agar slants.

Screening of PSB isolates for acid-tolerance in liquid and solid media

Assessment of acid-tolerance of various PSB strains in liquid medium were done in nutrient broth adjusted to pH 7.0, 6.5, 5.5, 4.5 and 3.5 (Pal, 1998). The pH was adjusted before and after sterilization using either 0.1N

NaOH or HCl. The 48 h old cultures were inoculated @ 0.1 ml into nutrient broth having pH 7.0, 6.5, 5.5, 4.5 and 3.5. Optical density values at 560 nm were taken after 72 h incubation.Growth at pH 6.5, 5.5, 4.5 and 3.5 were compared with pH 7.0 and per cent change in population over neutral pH was calculated using the following formula.

$$\text{Per cent change in population over neutral pH} = \left[\frac{(\text{O.D. at pH 7.0 - O.D. at pH x})}{\text{O.D. at pH 7.0}}\right] \times 100$$

where, pH x = pH 3.5, 4.5, 5.5 or 6.5. The cultures having less per cent change in acid-tolerance were rated as the most acid-tolerant strains.

Acid-tolerance of PSB isolates were also screened on solid medium adjusted to pH 7.0, 6.5, 5.5 and 4.5. Number of colonies in the media with pH 6.5, 5.5 and 4.5 were compared with that of pH 7.0 and per cent relative population was calculated as per the following formula.

$$\text{Per cent relative population} = \left[\frac{\text{population at pH x}}{\text{population at pH 7.0}}\right] \times 100$$

where, pH x = pH 4.5, 5.5 or 6.5. Isolates, which showed more than fifty per cent relative population, were ranked as most acid-tolerant strains.

Quantitative estimation of phosphate solubilization

The bacterial isolates positive for P-solubilization on Pikovskaya's agar medium were subjected to quantification of inorganic phosphorus released from Pikovskaya's broth medium containing tri-calcium phosphate as insoluble P-source. The available P content of the supernatant was estimated by using phosphomolybdic blue colour method (Olsen *et al.*, 1954).

Qualitative estimation of phosphate solubilization

Twenty microlitre of 24 h old PSB cultures grown in nutrient broth was spotted on Pikovskaya's agar plate and incubated for seven days at 28 ± 2^0C. The clear halo-zone and colony diameter were measured at 3, 5 and 7 days after incubation. The results were expressed as solubilization efficiency (SE) (Nguyen *et al.*, 1992.)

Screening of PSB isolates for Indole Acetic Acid (IAA) production

In vitro auxin production by selected isolates of PSB were determined (Khalid *et al.*,2004).

Molecular characterization of PSB using 16S rDNA

Most promising acid-tolerant and efficient phosphate solubilizing bacteria was identified based on their 16S rDNA gene sequencing (Sambrook *et al.*, 1989). Two microlitre of bacterial suspension was used as template for amplification of 16S rDNA gene. The primers used were 8F and 1522R. Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient, Germany) using PCR master mix 'Emerald Amp GT PCR'. The quality of isolated DNA was evaluated through agarose gel electrophoresis. The PCR product was purified and sequenced at Scigenome Pvt. Ltd., Cochin using the primers 8F and 1522r. The blastn programme (http://blast.ncbi.nlm.nih.gov/Blast.) was used to find out the homology of the nucleotide sequences.

Results and Discussion

Enumeration of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were isolated from ten different locations on Pikovskaya's agar media. A total of 35 isolates of PSB were obtained (Table 1). The maximum population ($100x10^5$ cfu g^{-1}) was recorded from soils of Mulayam followed by Madakkathara ($15x10^5$ cfu g^{-1}). Pal (1999) reported that one of the PSB isolates PAS-2 from pasture and waste land (pH 4.8) had highest P-solubilizing capacity which tolerated a wide range of soil acidity ranging from pH 4.5 to 6.1. However, in the present studies, higher population of PSB was observed in soil samples with a pH range from 6.3 to 6.5, indicating that PSB prefers near neutral pH. Population of the PSB was found to be less in soils below pH 6.0.

Acid-tolerant PSB isolates

All the 35 isolates of PSB were screened for acid-tolerance in both solid and liquid media. Isolates were able to tolerate acidic pH in the range of 4.5 to 6.5 (Table 2&3). However, only one isolate (POL-3) was able to tolerate pH upto 3.5. Pal (1999) reported that only 9 out of 23 bacterial isolates tolerated acidic pH under *in vitro*. He also reported that the strain PAS-2 isolated from pasture and wasteland of pH 4.8 had highest acid-tolerance. The present studies indicated that the PSB isolates obtained from acidic soils of pH 5.93 to 6.51 had high acid-tolerance.

Quantification of phosphate solubilization

The six most acid-tolerant phosphate-solubilizing bacteria (PKR-3, PKR-8, PMD-7, PMU-2, POL-1 and POL-3) were screened for quantity of P-solubilized. The amount of P-solubilized by the isolates ranged from 97 µg ml^{-1} to 207 µg ml^{-1} (Table 4., Fig. 1). The highest P-solubilized was recorded in the case of PMD-7 (207 µg ml^{-1}) followed by POL-1 (187.78 µg ml^{-1}) isolates. Pal (1998) reported the P-solubilizing capacity of PSB isolated from Garhwal, Himalaya region ranged between 11.4 and 45 µg $ml^{-1}day^{-1}$, which were capable of growing in low pH (5.4-5.6) but, showed variation in their growth rate. Jena and Rath (2013) reported that the production of acidity in the medium is directly correlated to the reduction in pH of the medium due to the production of variety of organic acids. In the present study, PMD-7 isolate recorded highest P-solubilization indicating its potential as an efficient P-solubilizer. However, pH reduction was not proportionate to the amount of P-solubilized. These results are in agreement with the studies conducted by Asea *et al.* (1988) who reported a lack of linear correlation between pH and the amount of P-solubilized in liquid media.

Qualitative estimation of phosphate solubilization

In the case of P-solubilization efficiency,the isolate POL-1 recorded maximum solubilization (450%) in the present studies (Table 4., Fig. 2). However, there was no correlation between P-solubilization in the liquid cultures and P-solubilization efficiency on the solid media. In a similar study, Ostwal and Bhide (1972) reported contradictory results between solubilization efficiency and P-solubilization in liquid cultures. However, POL-1 followed by PMD-7 was found to be the most acid-tolerant and efficient P-solubilizers in the present studies.

Efficiency for IAA production

The most acid-tolerant PSB isolates were also screened for IAA production. The PMD-7 isolate recorded highest IAA production (33.07 µg ml^{-1}) followed by PKR-3 (32.43 µg ml^{-1}) (Table 4., Fig. 3). In similar studies, Tien *et al*. (1979) reported that PSB isolated from rhizosphere soil of pearl millet produced indole acetic acid which enhanced the plant metabolism. However, production of IAA varied greatly among different species of bacteria and mainly influenced by culture conditions, growth stage and availability of substrate (Vijila, 2000) which is in agreement with the results of the present studies.

Morphological and biochemical characterization of PSB isolates

Only one isolate was gram-positive rod (POL-1), while others were gram-negative rods. All the isolates tested were motile except PKR-3. All the six isolates were positive for catalase and citrate utilization and negative for indole and methyl red tests. Oxidase was positive only for PMU-2 while urease was positive only for POL-1 isolate. The isolates PKR-3, PKR-8 and PMU-2 were positive for Voges- Proskauer test. The PSB isolates PMD-7, PMU-2, POL-1 and POL-3 were identified as *Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp. and *Acinetobacter* sp. respectively. Two isolates (PKR-3 and PKR-8) could not be identified due to their unusual characteristics (Table 5).

Identification of the most efficient PSB isolate

The isolate PMD-7 was found to be the most efficient and acid-tolerant PSB isolate based on acid-tolerance in both liquid and soild media, quantitative and qualitative estimation of phosphate solubilization, and indole acetic acid production. Based on the 16S rDNA sequencing, the nucleotide sequences obtained from the isolate showed more homology with *Acinetobacter* sp. Similar results on phosphate solubilizing bacteria of the genus *Acinetobacter* was reported from Bangladesh (Islam *et al*., 2007) and hence, it could be a potential biofertilizer.

Conclusion

Biofertilizer production from native microbial isolates has gained importance in recent years with respect to their field performance. Phosphorus solubilizing microorganisms have become a popular bioinoculant in Kerala for enhancement of the plant growth and yield due to its better performance under stress conditions. However, the present commercial PSB biofertilizer is suitable only for the neutral soils. As the soils in Kerala are acidic in nature, the new and native acid-tolerant *Acinetobacter* sp. obtained in the present studies will serve as a potential biofertilizer for acidic soils of Kerala.

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Table 1. Enumeration and morpho-types of phosphate solubilizing bacteria (PSB) from lateritic soils

Sl.No.	Location	pH	No. of isolates obtained from each location	Morpho-types	Population (x 10^5 cfu g^{-1} of soil)
1	Chelakkara	6.21	1	PCH – 2	10.0
2	Koratty	6.23	8	PKR – 2	1.0
				PKR – 3	4.0
				PKR – 4	1.0
				PKR – 1	2.0
				PKR – 5	1.0
				PKR – 6	3.0
				PKR – 7	2.0
				PKR – 8	5.0
3	Elanad	6.50	3	PEL – 6	4.0
				PEL – 4	2.0
				PEL – 3	6.0
4	Madakkathara	6.51	7	PMD – 6	1.0
				PMD – 5	3.0
				PMD – 4	15.0
				PMD – 3	6.0
				PMD – 1	1.0
				PMD – 2	9.0
				PMD – 7	1.0
5	Mulayam	6.29	4	PMU – 1	10.0
				PMU – 2	100.0
				PMU – 3	7.0
				PMU – 4	10.0
6	Nadavarambu	6.39	1	PND – 6	10.0
7	Ollur	5.93	4	POL – 1	10.0
				POL – 2	3.0
				POL – 3	4.0
				POL – 4	2.0
8	Perumpilavu	5.48	1	PPV-1	1.0
9	Vellanikkara	5.22	1	PVL – 3	14.0
10	Wadakkanchery	6.49	5	PWD – 1	1.0
				PWD – 2	1.0
				PWD – 3	7.0
				PWD – 4	0.10
				PWD – 9	4.0

Table 2. Screening of phosphate solubilizing bacteria isolates for acid-tolerance in nutrient broth

Isolates	Per cent change in population over neutral pH			Ranking of acid-tolerant PSB
	At pH 5.5	At pH 4.5	Sum	
PCH-2	63.08	81.54	144.62	32
PEL-3	19.35	35.48	54.84	12
PEL-4	30.95	36.90	67.86	17
PEL-6	25.93	65.43	91.36	23
PKR-1	41.77	86.08	127.85	31
PKR-2	17.65	23.53	41.18	8
PKR-3	10.27	21.23	31.51	4
PKR-4	42.86	62.50	105.36	25
PKR-5	13.70	75.34	89.04	22
PKR-6	74.68	74.68	149.37	33
PKR-7	3.30	52.75	56.04	14
PKR-8	9.83	24.28	34.10	5
PMD-1	27.00	52.00	79.00	21
PMD-2	13.54	25.00	38.54	6
PMD-3	59.02	91.80	150.82	34
PMD-4	12.73	85.45	98.18	24
PMD-5	25.42	88.14	113.56	28
PMD-6	6.12	44.90	51.02	11
PMD-7	8.87	21.77	30.65	3
PMU-1	21.05	26.32	47.37	10
PMU-2	12.24	28.57	40.82	7
PMU-3	8.93	60.71	69.64	19
PMU-4	26.60	81.91	108.51	26
PND-6	72.46	89.86	162.32	35
POL-1	8.09	19.65	27.75	2
POL-2	1.59	63.49	65.08	16
POL-3	5.74	21.31	27.05	1
POL-4	5.95	61.90	67.86	18
PPV-1	18.95	41.05	60.00	15
PVL-3	36.62	83.10	119.72	29
PWD-1	58.44	62.34	120.78	30
PWD-2	1.43	54.29	55.71	13
PWD-3	21.21	55.56	76.77	20
PWD-4	22.22	87.78	110.00	27
PWD-9	14.29	30.00	44.29	9

(Ranking was done statistically based on summation of per cent change in population at pH 5.5 and at pH 4.5 over pH 7.0. The cultures having less per cent change in acid-tolerance were rated as the most acid-tolerant strains)

Table 3. Screening of different isolates of phosphate solubilizing bacteria for acid-tolerance on Pikovskaya's agar media

Isolates	Per cent relative population			Ranking of acid-tolerant PSB
	At pH 5.5	At pH 4.5	Mean	
PCH-2	13.04	4.35	8.70	31.0
PEL-3	10.26	2.56	6.41	33.0
PEL-4	16.28	4.65	10.47	27.0
PEL-6	25.00	0.00	12.50	22.0
PKR-1	15.63	3.13	9.38	30.0
PKR-2	3.64	0.00	1.82	35.0
PKR-3	62.77	54.15	58.46	4.0
PKR-4	18.42	5.26	11.84	24.0
PKR-5	25.00	5.00	15.00	19.0
PKR-6	14.29	0.00	7.15	32.0
PKR-7	17.65	5.88	11.77	25.0
PKR-8	86.00	22.00	54.00	5.0
PMD-1	28.57	0.00	14.29	20.0
PMD-2	57.14	17.86	37.50	8.0
PMD-3	11.11	0.00	5.56	34.0
PMD-4	15.52	6.90	11.21	26.0
PMD-5	21.05	10.53	15.79	18.0
PMD-6	60.47	13.95	37.21	9.0
PMD-7	78.43	72.55	75.49	2.0
PMU-1	41.38	13.79	27.59	14.0
PMU-2	80.00	20.00	50.00	6.0
PMU-3	28.57	4.76	16.67	17.0
PMU-4	21.74	4.35	13.05	21.0
PND-6	35.29	8.82	22.06	16.0
POL-1	70.27	56.76	63.52	3.0
POL-2	43.48	17.39	30.44	13.0
POL-3	86.67	76.67	81.67	1.0
POL-4	35.14	13.51	24.33	15.0
PPV-1	54.55	9.09	31.82	11.0
PVL-3	17.24	3.45	10.35	28.0
PWD-1	20.00	0.00	10.00	29.0
PWD-2	65.71	25.71	45.71	7.0
PWD-3	25.00	0.00	12.50	23.0
PWD-4	50.00	12.50	31.25	12.0
PWD-9	56.25	12.50	34.38	10.0

(Ranking was done statistically based on the average of per cent relative population at pH 5.5 and at pH 4.5 over pH 7.0. Isolates, which showed more than fifty per cent relative population, were ranked as most acid-tolerant strains.)

Table 4. *In vitro* screening of different acid-tolerant PSB isolates for P-solubilization, solubilization efficiency and IAA production

Isolates	Quantity of P-solubilized (µg ml^{-1})	Reduction in pH*	Solubilization efficiency (%)	IAA production (µg ml^{-1})
PKR-3	97.44 (9.89^{e})**	5.68	183.33 (13.55^{c})**	32.43^{a}
PKR-8	86.78 (9.34^{f})	5.74	262.50 (16.21^{b})	19.57^{c}
PMD-7	207.22 (14.41^{a})	4.35	247.62 (15.75^{b})	33.07^{a}
PMU-2	145.67 (12.08^{c})	4.43	158.33 (12.60^{d})	20.09^{c}
POL-1	187.78 (13.72^{b})	3.41	450.0 (21.21^{a})	24.75^{b}
POL-3	100.67 (10.05^{d})	5.31	185.18 (13.60^{c})	12.92^{d}

Each value represents the average of three replications. Means followed by the same letter do not differ at $p<0.05$.

* Initial pH was adjusted to 7.0; ** Square root transformed values are given in bracket

Table 5. Characterization of phosphate solubilizing bacteria isolates

Characters	**Isolates**					
	PKR-3	**PKR-8**	**PMD-7**	**PMU-2**	**POL-1**	**POL-3**
Gram reaction	-	-	-	-	+	-
Morphology	Rods	Rods	Rods	Rods	Rods	Rods
Motility	-	+	+	+	+	+
Oxidase	-	-	-	+	-	-
Catalase	+	+	+	+	+	+
Indole	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-
Voges-Proskauer	+	+	-	+	-	-
Citrate utilization	+	+	+	+	+	+
Urease	-	-	-	-	+	-
Tentatively identified genus	UI	UI	*Acineto-bacter* sp.	*Pseudomonas* sp.	*Bacillus* sp.	*Acineto-bacter* sp.

UI : Unidentified; '+' : Positive for the test; '-' : Negative for the test

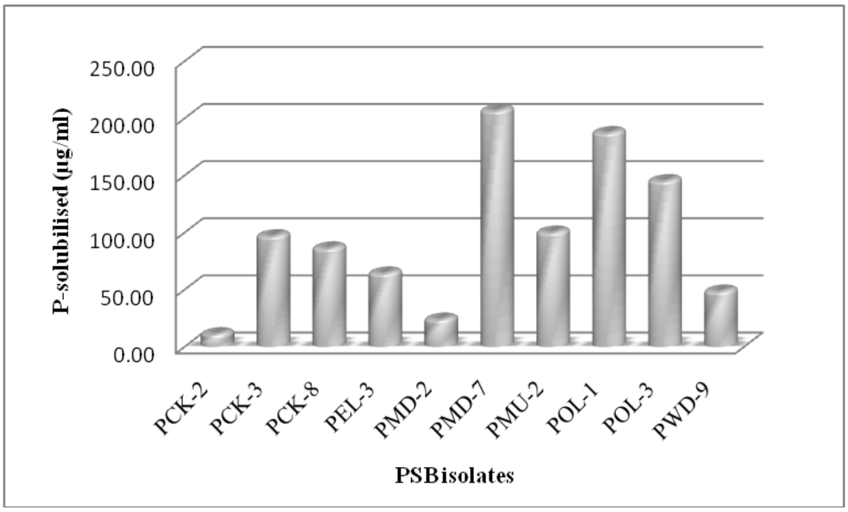

Fig.1. Phosphorus solubilization by PSB isolates

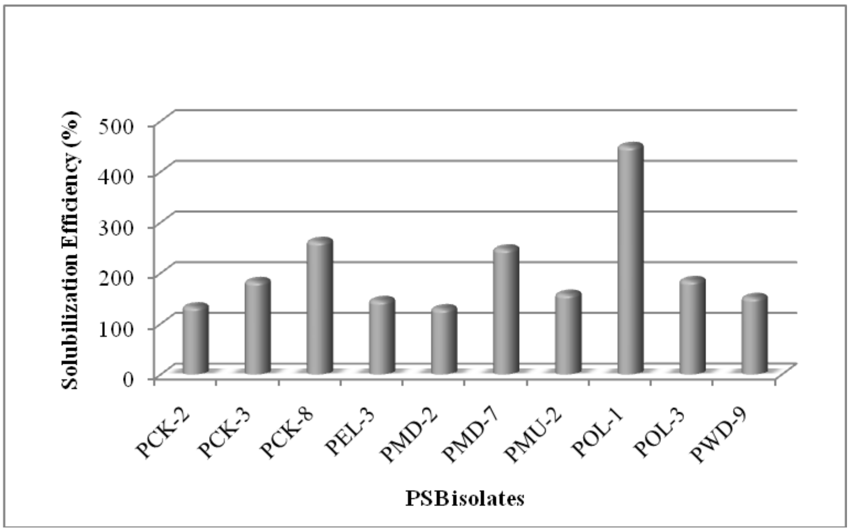

Fig.2. Solubilization efficiency of PSB isolates

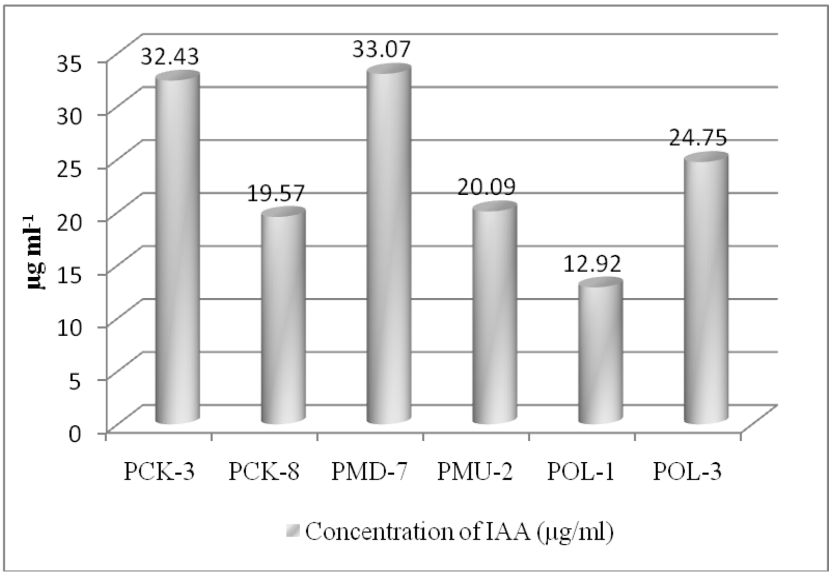

Fig.3. Indole acetic acid production by PSB isolates