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Isolation of total heterotrophic bacteria and phosphate solubilizing bacteria and *in vitro* study of phosphatase activity and production of phytohormones by PSB

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ABSTRACT

Water samples from the Karangadu coast, Palk Strait, Southeast coast of India were sampled bimonthly for enumeration of total heterotrophic bacteria as well as phosphate solubilizing bacteria between January and December 2008.THB was high in November month and PSB was also high in September and November months, population density of THB ranged from 4.25-8.25x10^6 cells ml^{-1} and phosphate solubilizing bacterial population from 2.02-2.6x10^3 cells ml^{-1}. A wide variation in the capacity to solubilize phosphorous by the isolates of PSB were observed. Further, all the isolates were able to secrete phytohormones like Indole acetic acid (IAA) and Gibberellic acid (GA_3) and also studied phosphatse activity by efficient PSB isolates under in vitro condition.

Key words: Heterotrophic bacteria, Phosphate solubilizing bacteria, Phosphate solubilization, Phosphatase activity, IAA and GA_3.

INTRODUCTION

Phosphorus exists in nature in a variety of organic and inorganic forms, primarily in either insoluble or very poorly soluble inorganic forms. Soluble forms of P fertilizers applied to the soil are easily precipitated as insoluble forms [1]. Phosphate solubilizing microorganisms solubilize insoluble P by producing various organic acids. Plants take up this available P [2]. However, P solubilization ability of microorganisms in soil may be different from that found under laboratory conditions [3]. Microorganisms distributed in the marine environments play an important role in the decomposition of organic matter and mineralization. Limited studies reported on the occurrence of phosphate solubilizing microbes in the Indian marine environment

[4, 5]. De Sousza *et al.,* [6] reported an extensive study on PSB around the Indian peninsula. If bacteria, with salinity tolerance and phosphate solibilization potential, can be used efficiently to help the crop plants growing in saline soils through amelioration. As phosphate solubilization is a complex biochemical phenomenon, an understanding of the bacterial populations capable of P-solubilization is a prerequisite in realizing the multiple roles the native bacteria perform. Phosphobacteria have been found to produce some organic acids such as monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketogluconic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids in order to solubilize inorganic phosphate compounds [7]. The present study was undertaken to study in detail about the distribution and population density of total heterotrophic bacteria (THB) and the constituent phosphate solubilizing bacteria (PSB) from the Karangadu coast, Palk Strait, Southeast coast of India were enumerated and PSB isolates were also screened for their performance under *in vitro* conditions.

MATERIALS AND METHODS

Enumeration and isolation of THB and PSB

Water samples were collected on alternate months (January-December 2008) from four different stations viz. Karangadu intertidal zone, Karangadu back waters, Karangadu river mouth and Karangadu open sea representing marine biotopes. Water samples collected in sterile McCartney bottles were transported to the laboratary in an icebox immediately for further studies. Serial dilutions were made and one ml of aliquots of 10^2-10^6 dilutions were transferred to petriplates containing Zobell's Marine Agar 2216 (HiMedia, India) for enumerating THB and Pikovskaya's agar media (HiMedia, India) for enumeration of phosphate solubilizing bateria plating was done in triplicate and incubated at room temperature 28±2^0C. After 48hrs the CFUs of THB were recored and after 72 hrs the CFUs of PSB were recorded.

Estimation of phosphorous solubilization

The phosphorous solubilization potential of selected strains of phosphate solubilizing bacteria was tested *in vitro* by estimating available phosphorous in the Pikovaskaya's medium amended with tricalcium phosphate as a substrate. The flasks were inoculated with culture broth of cultures OD at 2 (A_{600}). The flasks were incubated at 30 °C for seven days and centrifuged at 15000 rpm. Phosphorous was determined in supernatant as per the procedure outlined by Natarajan and Buvana [8]. Phosphorous solubilization on solid medium was measured in terms of solubilization efficiency (SE): (%) = (Z-C)/C x 100 where Z is solubilization zone, C is colony diameter.

Estimation of phytohormones produced by PSB

Five days old cultures of phosphate solubilizing bacteria were transferred to Pikovaskaya's broth containing L-tryptophan (biological precursor) as a substrate for the production of IAA and GA_3. Inoculated cultures were kept in a shaker for about five days under room temperature. Culture filtrates were centrifuged and subjected to IAA and GA_3 analysis following the procedure of Tien *et al.*,[9].

Determination of phosphatase activity

For phosphate solubilization, phosphobacteria produce phosphatase enzyme. In an attempt to study the phosphatase activity in response to phosphorous enrichment, experiments were set up using known bacterial broth cultures in flasks with and without adding phosphorous source (β-glycerophosphate used as a substrate). Culture filtrates were centrifuged and subjected to estimate phosphatase activity following the procedure of Tabatabai and Bremner [10].

RESULTS AND DISCUSSION

The physico-chemical parameters are presented in Table 1. Between different locations, the mean values of temperature fluctuated between 29.25 and 30.12 °C; the pH, 7.85 and 8.1; salinity, 29.51 and 32.25 ‰ and DO, 3.98 and 5.26 ml L^{-1}. However, there were little variations in these parameters between the months.

Population densities of THB and PSB with respect to different stations during various months are presented in Table 2. It is generally observed that there was a significant difference on the population density. THB population in all the stations remained almost between 4.25-8.25 x 10^6 cells ml^{-1} expecting a very few samples. The phosphate solubilizers recorded were less in number was found to fluctuate between 2.02-2.60x10^3 cells ml^{-1}. This variation in the population of PSB might be attributed to many factors such as nutrients, pH, organic matter, salinity level and some enzyme activities in water column. Seshadri et al., [11] carried out an investigation on microbial dynamics in the water column reported that there was a significant difference on the population level of THB and PSB in different stations of Chennai coast.

The phosphate solubilizing efficiency of isolated strains of PSB indicated that all the strains were solubilized inorganic phosphate contents effectively in the medium (Table 3). Among the 12 strains KPB6 (38.44±1.15 ppm ml^{-1}) was found as the best in solubilizing phosphates followed by KPB5 and KPB9. The phosphate solubilization efficiency in the solid media ranged between 40 and 83% the results showed a wide range of variations in P-solubilization efficiency. Similar findings have been reported by many researchers [12, 13, 14, 15]. There was no correlation between P-solubilization efficiency on solid and liquid medium. The phosphatase activity of the isolates showed that the strain KPB6 had higher activity (28.78±1.18 µmoles/g/h) followed by the strain KPB5 (26.13±1.10 µmoles/g/h) (Table 3). The phosphatase activity was low in KPB11, KPB2 and KPB12. However, there was a positive correlation between phosphate solubilizing capacity and phosphatase activity. This might be due to availability of higher amount of P in the medium and the ability of the strains [16]. Temperature and pH caused a delay the expression of phosphatase activity in all the isolates studied *in vitro* situation, although 32-37 ^{0}C was found to be much more suitable of all the isolates. There is increasing evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing available phosphorous.

The result on the production of PGPS (Plant growth promoting substances) indicated that all the 12 isolates of phosphate solubilizing bacteria were able to produce phytohermones such as IAA and GA_3 under *in vitro* condition. Table 4 showed that the strain KPB3 produced higher amount (31.55) of IAA followed by KPB9, KPB1 and KPB4. The production GA_3 by the strain KPB4 (16.55) had reached higher amount follwed by KPB9, KPB3 and KPB2. Vikram *et al.*, [17]

reported that PSB isolated from vertisols proeduced IAA, GA_3 and cytokinin-like substances which ultimately enhanced the plant metabolism. Phosphobacteria isolated from different crops soils are known to produce IAA and GA_3 [18], and some of them are capable of dissolving phosphates [19], the PSB culture release a maximum quantity of IAA in the presence of a physiological precursor, L-tryptophan in a liquid culture medium. Production of phytohormones varies greatly among different crops and is also influenced by culture conditions, growth stage and availability of substrates [20]. Hence the present findings may be concluded that among the 12 isolates of PSB strains like KPB6, KPB3 and KPB4 are efficient strains than others. These strains may be more effective and perform better under field condifions in the view of enhancing plant metabolism. Further studies would add new dimensions to their role in any particular area.

Table-1 Variations (Range and Annual mean*) of different physico-chemical parameters monitored during January-December, 2008

Station	Temperatute (°C)	pH	Salinity (‰)	DO (ml L^{-1})
Station 1	27 – 32 (30.12)	7.5 – 7.9 (7.85)	29 – 33 (30.2)	3.1 – 4.86 (3.98)
Station 2	27.5–32.5 (29.25)	7.6 – 7.8 (7.65)	27 – 32 (29.2)	3.52 – 5.48 (4.45)
Station 3	27 – 32 (28.6)	7.5 – 7.9 (7.7)	28 – 32 (29.5)	3.25 – 6.8 (5.06)
Station 4	26– 31.5 (29.4)	7.6 – 8.2 (8.1)	29 – 35.5 (32.25)	3.8 – 6.26 (5.26)

**Figures in parenthesis indicates annual mean*

Table-2 Total heterotrophic bacteria (THB) and phosphate solubilizing bacteria (PSB) at different sampling stations

Months	Station 1		Station 2		Station 3		Station 4	
	THB	PSB	THB	PSB	THB	PSB	THB	PSB
January	6.32	2.02	6.59	2.08	6.03	2.12	6.46	2.05
March	6.13	2.07	6.46	2.4	6.46	2.02	6.74	2.06
May	4.25	2.12	6.68	2.15	6.08	2.05	6.84	2.18
July	6.78	2.2	7.49	2.6	6.72	2.23	6.8	2.09
September	5.88	2.18	7.86	2.38	6.96	2.3	7.59	2.17
November	7.2	2.3	8.25	2.43	7.1	2.46	7.36	2.28

THB = No. x 10^6 cells $ml^{-1;}$ PSB = No. x 10^3 cells $ml^{-1;}$ Figures are average of three replicates

Table-3 Phosphorous solubilizing ability and phosphatase activity of PSB *in vitro* condition

Name of the strain	Available P (ppm ml^{-1})	Phosphatase activity (µmoles/g/h)	Phosphate solubilization efficiency (%)
KPB1	23.82±1.24	15.5±0.62	40
KPB2	26.42±1.3	10.82±0.23	55
KPB3	28.56±1.28	12.85±0.16	67
KPB4	24.65±1.4	18.9±0.58	70
KPB5	32.36±1.28	26.13±1.1	62
KPB6	38.44±1.15	28.76±1.18	83
KPB7	15.58±0.98	11.5±0.28	68
KPB8	30.35±1.25	13.82±0.85	62
KPB9	32.14±1.15	17.65±0.66	70
KPB10	29.18±1.08	14.71±0.8	50
KPB11	19.86±1.5	10.61±0.18	55
KPB12	20.1±1.65	10.84±0.23	57

Figures are average of three replicates

Table-4 Production of plant growth promoting substances by PSB strains under *in vitro* condition

Name of the strain	Plant growth promoting substances (ppm)	
	IAA	GA_3
KPB1	30.24	11.35
KPB2	25.31	12.0
KPB3	31.55	12.35
KPB4	30.52	16.55
KPB5	24.38	10.4
KPB6	15.86	11.26
KPB7	20.28	9.08
KPB8	23.4	13.08
KPB9	31.05	9.74
KPB10	21.42	11.12
KPB11	23.55	10.02
KPB12	24.8	10.84

Values indicates mean of three replicates

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