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A STUDY ON OPTIMIZATION OF PHOSPHATE SOLUBILIZING POTENTIAL OF BACTERIA AND THEIR EFFECT ON PLANT GROWTH WHEN APPLIED INDIVIDUALLY AND IN A CONSORTIUM

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ABSTRACT

The insoluble nature of a large portion of phosphorus present in soil restricts its uptake by plants. This often leads to plant abnormalities associated with phosphate deficiency, in spite of its ample concentration in soil. Nature amends this impediment with the help of phosphate solubilizing microorganisms that assist in making the phosphorus available to plants. In the current study, eighteen potential phosphate solubilizing bacteria (PSB) were isolated from rhizosphere soil of Maharashtra and Tamil Nadu (India). Among these isolates, P2 and P13 showed considerable solubilizing index of 2.35 and 3.06 corresponding to 16.47ppm and 17.88ppm phosphatesolubilization, respectively, in 48h. These isolates were identified as *Burkholderia cenocepacia* (P2) and *Enterobacter cloaceae* (P13) by cultural, morphological, biochemical and 16S rRNA sequence analysis. The optimum solubilization of phosphates was observed in NBRIP medium with 1% ammonium sulphate, 0.4 $O.D_{540nm}$, pH6-7 and temperature 28°C -37°C in 120h under shaker conditions by both isolates. Galactose and sucrose were effective carbon sources forthe activity of *E. cloaceae* and *B. cenocepacia* niraar respectively. Under optimized conditions, the consortium of PSB solubilized 59.52ppm phosphorus. The biofertilizer potential was also observed on wheat (*Triticum aestivum*) and mustard (*Brassica juncea*) seeds by individual cultures as well as its consortia. Significant enhancement of shoot length was observed on inoculation of test cultures in the soil. Further improvement was observed on addition of tricalcium phosphate (TCP) along with test cultures. However, the highest growth performance was observed in presence of the bacterial consortium and TCP.

Keywords: Phosphate solubilising bacteria, *Burkholderia cenocepacia*¸ *Enterobacter cloaceae*, consortium, biofertilizer, *Triticumaestivum*, *Brassica juncea*

1. INTRODUCTION

Phosphorus is a macronutrient required for plant growth and metabolism. Ideally, a 30-50ppm concentration of phosphates is sufficient for optimum plant growth and food production [1]. Although this concentration may seem relatively low, a constant availability or supply of phosphates in 394.6 million acres of agricultural land, supporting over 216 million acres of gross irrigated crop area in India is challenging [2]. Moreover, the growing population and the resulting increase in food requirement demandatleast doubling of the current food productivity by 2050, if not more [3]. This is impossible without a fertile land containing ample amounts of macro- and micro- nutrients especially phosphorus that stimulates the yield of fruits and grains in plants [4]. Statistically, it can be translated that the global demand of phosphate fertilizers is expected to increase from the current 21 million tons (in 2015) to 39 million tons by 2050 [5].

The biggest challenge faced by plants for phosphorus uptake, however, is due to its chemical nature. It can be roughly estimated that only 0.1% of the total 0.05% phosphorus (0.01-0.06ppm) present in soil, in the form of monobasic ($H2PO_4^-$) and dibasic (HPO_4^{2-}) ions, can be utilized by plants [6]. The remaining phosphates are present in the form of insoluble complex salts of inositol, calcium, aluminium, iron and sodium, orphospho mono-esters and phospho tri-esters that is unavailable to plants [6, 7]. Unfortunately, application of chemical phosphatic fertilizers cannot overcome this deficiency, since most of it gets converted into insoluble salts almost immediately after application [8]. Hence, often the plants show deficiency of phosphorus, resulting in low crop yield, despite its large reservoir in soil [9]. Furthermore, in order to maintain soil fertility, frequent application of chemical fertilizers is needed which ultimately leads to high production cost as well as soil toxicity. In addition,

the phosphate rocks used in the manufacture of chemical fertilizers are non-renewable, and keeping up with the current demand will result in its depletion by 2050 [5, 10].

The conventional agricultural practices cannot meet the rising food demands, and the use of chemical fertilizers has been exploited to its maximum potential. Beyond this, any further increase in crop yield cannot be expected. Thus a sustainable agricultural approach i.e., environment friendly, cost effective and capable of retaining ecological balance, is no longer a choice but a necessity. In this regard, the use of Phosphate Solubilizing Microorganisms (PSMs) appear to be a challenging approach which may lead to a green revolution without compromising environmental health [9, 11, 12]. In general, the rhizosphere microbes aid in nutrient decomposition, mobilization and mineralization of nutrients [13]. In addition to these characteristics, the PSMs convert the insoluble phosphatic compounds into soluble forms and make it available to plants [11]. This avoids the need for phosphate fertilizers.

The PSMs are ubiquitous and its existence is known since 1903 [14]. They solubilize phosphates mainly through substrate degradation and enzyme production. These enzymes include phosphatases (dephosphorylates the phospho-ester or phosphor-anhydride bonds in organic matter), phytases (release phosphorus by degrading phytates) and phosphonatases (cleave the C-P bond of organophosphonates) [15-17]. Other mechanisms include the production of organic acids as a byproduct of microbial metabolism that dissolves phosphatesin organic materials. They also chelate the cationic group (eg., Ca^{2+}, Fe^{3+}, Al^{3+}) of phosphorus containing compounds, to make stable complexes, and help in liberating phosphorus ions in the solution [18, 19]. In addition, the PSMs also stimulate nitrogen fixation in soil, mediate iron and zinc uptake by plants, and aid in synthesis of plant growth promoters like siderophores, indole acetic acid and gibberellic acid [13, 20, 21].

Among the PSM, the Phosphate Solubilizing Bacteria (PSB) is present abundantly (upto 50% microbial species) in soil and is considered as promising bio-fertilizers. The common genera include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Enterobacter Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium* and *Erwinia* [22]. Few published reports claim up to 70% increase in crop yield on use of PSB [23]. Recently, the development of microbial consortia has gained more importance given its further increased potential in phosphate solubilization [24-26]. Although significant research has been done in this area to classify sustainable agriculture using PSMs as commercial bio-inoculants, a promising approach; the current knowledge is still very basic for considering its practical implementation globally.

In the current study, potential bacteria were isolated and optimized for phosphate solubilization. Also, pot trial studies were carried out to observe the effect of direct inoculation of these isolates in to soil, on plant growth.

2. MATERIAL AND METHODS

2.1. Media and chemicals

All the reagents and culture media used in the current study were of analytical grade and purchased from Hi-media, India Ltd. or Difco laboratories.

2.2. Screening, enrichment and isolation of phosphate solubilizing bacteria

The rhizospheresoil samples were collected from different locations listed in Table 1 for screening and isolation of PSB.

Table 1: Sources of sample collection for screening and isolation of PSB

Sample source	No. of organisms
Satawali village, Ratnagiri, Maharashtra	3
Hanging garden, Mumbai, Maharashtra	7
Joseph BaptistaGarden, Mumbai, Maharashtra	4
Nagercoil, Tamil Nadu	4

A 1g soil sample was suspended in 10mL sterile saline, vortexed and allowed to stand for 30min. The enrichment of PSB was done by inoculating 1mL of above supernatant in 100mL of National Botanical Research Institute's Phosphate Solubilizing (NBRIP) broth [composition in (g/L): glucose (10), $Ca_3(PO_4)_2$ (5), $MgCl_2 \cdot 6H_2O$ (5), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (0.2), $(NH_4)_2SO_4$ (0.1)]. All constituents of the medium except $Ca_3(PO_4)_2$ were dissolved in distilled water (pH7) and autoclaved at 121°C, 15psi for 30min. The insoluble $Ca_3(PO_4)_2$ was autoclaved separately at 121°C, 10psi for 30min, cooled and added to the NBRIP medium. After inoculation, the enrichment broth was incubated on a rotary shaker for 7 days at Room Temperature (RT, 28°C). Successive enrichments were carried out twice by inoculating 2mL of the medium from previous batches

into 100mL of fresh medium and incubating it further for 7days [27].

The PSB was isolated from the enriched medium on NBRIP agar, incubated at RT and observed after 24h. The colonies showing a zone of clearance around them were further purified by re-streaking on NBRIP agar plate. The individual colonies thus obtained were dispensed in sterile saline to obtain the optical density of 0.2O.D_{540nm}. The phosphate solubilizing characteristic of test isolates were confirmed by spot inoculating the suspensions on NBRIP plate and observing for a zone of clearance after incubation of 24h at RT. The PSB were maintained on NBRIP slants at 4°C, after confirmation, until further studies.

2.3. Determination of phosphate solubilizing efficiency

The phosphate solubilizing efficiency of the potential isolates obtained after enrichment and isolation was determined qualitatively on the basis of Solubilization Index (SI) and quantitatively by spectrophotometric estimation of phosphate solubilization.

The SI is the ratio of diameter of the zone of clearance to the diameter of colony. It is a preliminary test to identify the potential of PSB to solubilize tricalcium phosphate (TCP) or suitable source of phosphorus in NBRIP or other medium [28]. The quantitative estimation of solubilised phosphates, by PSB, was adapted from the chlorostannous reduced molybdophosphoric acid blue method described by Gaur [29] with slight modifications.The cultures were grown in 50mL NBRIP broth by inoculating 1mL of the culture suspensions (0.2 O.D_{540nm}) of potential PSB in it, and incubating at RT for 48h on a rotary shaker (130rpm). To carry out the estimation, 5mL of sample was withdrawn from the above medium and centrifuged at 5000rpm for 40min.The supernatant obtained was decanted carefully and used for the analysis. The reaction mixture was prepared by adding 10mL of chloromolybdic acid and 2mL of chlorostannous acid to 0.5mL of the above supernantant and making up the volume to 40mL. The intensity of blue colour thus obtained is directly proportional to the amount of soluble phosphorus present in thesample. Hence, it was measured spectrophotometrically (BioEra's spectrophotometer A1603076) at 700nm. The concentration of phosphate solubilized by PSB was calculated using a standard graph of KH_2PO_4 (0.5-3.0ppm).

2.4. Identification of potential PSB

The potential isolates were identified primarily on the basis of morphological, cultural and biochemical tests using Bergey's manual [30] of bacteriology and confirmed by the 16S rRNA analysis carried out at SaiBiosystems Pvt. Ltd, Nagpur, India.

2.5. Qualitative assay for detection of phosphatase enzyme, organic acids and other plant growth promoters

The phosphatase enzyme production was determined by a simple agar plate method. In this method, the PSB is spot inoculated on the Mueller-Hinton agar medium (pH 5.6 - 5.8) containing p-nitro phenyl-phosphate (0.495mg/L), and incubated at RT for 24h.The presence of bright yellow color under and around the spot inoculum was indicative of phosphatase enzyme production [31]. Similarly, the organic acid production was determined by spot inoculating the PSB isolates on modified Pikovasky's agar plate containing 0.0024g/mL bromophenol blue as a pH indicator. The presence of yellow halo around the colonies indicated organic acid production [32].

The production of plant growth promoters like siderophores and IAA was detected using methods described by Schwyn and Neilands [33], and Holt *et al.* [30] respectively. Chrome Azurol S (CAS) blue agar was spot inoculated with potential PSB isolates and incubated at RT for 24h and observed for the appearance of orange halos around the colonies [33]. For detection of IAA, 1mL of PSB culture (0.2 O.D_{540nm}) was inoculated in 10mL nutrient broth containing tryptophan (0.1g/L) and incubated at RT for 96h. After incubation, the culture was centrifuged at 3000rpm for 30mins. A 2mL volume of the supernatant thus obtained was mixed with 2 drops of orthophosphoric acid and 4mL of Salkowski's reagent. The formation of pink colour indicated IAA production [33].

2.6. Optimization of nutritional and physicochemical parameters for phosphate solubilization

The optimization of different parameters was done by varying one factor while keeping the others constant. Two medium i.e., NBRIP and Pikovasky's agar medium were used to optimize phosphate solubilization by the potential isolates obtained in our study. The above media (50mL) was inoculated with 1mL of test cultures (0.2 OD_{540nm}) and incubated at RT for 24-120h. The efficiency of phosphate solubilization by test cultures was

estimated quantitatively using the method described above. After optimization of the medium, the next critical cultural parameter for bacterial isolates is aeration. Hence the growth and solubilizing potential of test isolates were studied under static and shaker conditions [34].

To study the nutritional parameters on the efficiency of phosphate solubilization, 1.5% concentration of different carbon (glucose xylose, sucrose, fructose, mannitol and lactose), and 0.01% organic nitrogen (tryptone, peptone, yeast extract and meat extract) and inorganic nitrogen (potassium nitrate, potassium nitrite, sodium nitrate, sodium nitrite, ammonium chloride) sources were used in our study. The various physicochemical parameters optimized in our study was pH (2, 4, 6, 8, 10, 12), temperature (RT, 37°C and 45°C) andoptical density (0.2, 0.4, 0.6, 0.8 and 1.0 OD_{540nm}) [35, 36].

2.7. Phosphorus solubilization by bacterial consortium

Two potential bacteria were co-inoculated in 50mL of optimized medium to study the effect of consortium on phosphorus solubilization and the efficiency was calculated spectrophotometrically as mentioned above.

2.8. Pot experiments to study the application of PSB as plant growth promoter

A culture suspension (0.2 $O.D_{540nm}$) was prepared for two potential strains showing phosphate solubilization isolated in our study. A 2mL volume of these suspensions was inoculated in 100mL NBRIP broth and incubated under optimum growth conditions. The bacterial suspension thus obtained was centrifuged at 5000rpm to obtain the cell pellet. The pellet was suspended in sterile Phosphate Buffered Saline (PBS) and centrifuged again to remove traces of insoluble TCP. The pellet was re-suspended in sterile PBS to obtain 0.2 $O.D_{540nm}$and used to treat mustard and wheat seeds for 30min before planting. For consortium studies, an equal volume (0.2 $O.D_{540nm}$) of two potential isolates were mixed and then employed to treat mustard and wheat seeds. The following 8 step treatment plan was followed to study the effect of PSBs as plant growth promoters in pot studies. At every step, phosphate fertilizer (160mg/Kg TCP), individual potential PSB and/or their consortium were added to the soil (30g) in plastic seedling traysand observed for plant growth. The pots used in our study included**:**

1. Control pot containing normal garden soil
2. Test pot containing soil and TCP
3. Test pot containing soil and PSB isolate 1 (PSB-1)
4. Test pot containing soil, PSB-1 and TCP
5. Test pot containing soil and PSB isolate 2 (PSB-2)
6. Test pot containing soil, PSB-2 and TCP
7. Test pot containing soil, PSB-1 and PSB-2
8. Test pot containing soil, PSB-1, PSB-2 and TCP

Six seeds were placed in each cup of the seedling tray. A previously prepared 1mL sample of each inoculant was uniformly applied on seeds as single and co-inoculum; seeds were then covered with 20g uniform layer of soil. Control plants received 1mL sterile PBS (no culture). Cups were watered twice daily (with an equal volume of water) during the period of study. After 4 weeks of germination, the effect of these promoters was assessed by measuring the shoot length [37].

3. RESULTS AND DISCUSSION

3.1. Screening and isolation of PSB

A total of 18 potential phosphate solubilizers were obtained from rhizosphere soil in our study. The SI of these isolates is presented in Table 2. Based on the observations of SI, two potential isolates i.e., P2 (SI = 2.35) and P13 (SI = 3.06) were selected for further studies. These isolates showed effective solubilization of 16.47ppm and 17.88ppm phosphates respectively in 48h.

Table 2: Solubilization index of potential PSB isolates

Culture	Solubilization Index
P1	1.31
P2	2.35
P3	1.56
P4	1.31
P5	2.05
P6	2.16
P7	2.22
P8	2.52
P9	2.11
P10	1.88
P11	1.76
P12	2.13
P13	3.06
P14	1.8
P15	1.82
P16	2.33
P17	1.9
P18	1.78

The SI index provides a qualitative measure of solubilization of inorganic phosphates by microbes. The published literature commonly reports SI values ranging from 1.2 to over 2.7 [38]. However, a study by Mardad *et al.*[39] reported a significantly higher SI of 4.40 by *Enterobacter hormaechei* subsp. *Steigerwaltii* strain NM23-1. This isolate successfully solubilized 505mg/L orthophosphate in NBRIP medium in 60-72h. Similarly, higher SI values were reported in a recent study where 5of the 23 potential PSB showed SI values between 4.3 and 4.9 [40]. These samples were obtained from less studied rhizosphere samples from different parts of Western India. Among these 5 PSB, three cultures identified as *Ralstonia pickettii*, *Burkholderia tropica* and *Burkholderia cepacia* showed solubilization of 574ppm, 400ppm and 375ppm phosphates under shaker conditions. Another study reported SI values in the range of 1.7 and 2.8 by 41 PSB including *Pseudomonas*, *Aeromonas*, *Bacillus*, *Burkholderia*, *Sphingomonas*, *Chryseomonas* and *Agrobacterium* [41].

Among the first published studies, Sackett*et al.* [42] demonstrated the solubilization of tri-calcium phosphate, di- calcium phosphate and calcium carbonate by soil microbes using agar plate method. The solubilization of calcium phosphate and iron phosphate by Bacillus sp., isolated from the glands of *Cassia occidentalis* was also reported in liquid medium [43]. In later studies, the abundance of phosphate solubilizing microorganisms in rhizosphere soil as compared to non- rhizosphere soil was noted [44]. Soon after this study, Katznelson and Bose [45] reported that over one third of rhizosphere bacteria were capable of solubilizing phosphates. Ahmed *et al.*[46] isolated PSMs including bacteria, fungi and actinomyces from soil samples of Bihar on carrot extract agar and noted the abundance of bacteria as compared to other microbes. Recent studies have also reported the biodiversity and abundance of PSMs in rhizosphere soil [47]. Hence collectively, these studies clearly suggest a high proportion and metabolic activity of PSMs in the rhizosphere.

3.2. Identification of potential PSB

The isolates P2 and P13 were observed to be rod-shaped, free-living, motile gram-negative bacteria. The colony characteristics of isolates P2 and P13 is given in Table 3. The cultural, morphological and biochemical tests identified these promising isolates as *Burkholderia cenocepacia niraar* and *Enterobacter cloaceae*. The 16s rRNA analysis further confirmed these findings. The DNA sequence of one of the isolates identified as *Enterobacter cloaceae* with 99% similarity was submitted to the NCBI gene bank (accession numberLC257681).

Table 3: Colony characteristics of potential PSB

Colony characteristics	P2	P1
Size	Pin-point	1mm
Shape	Circular	Circular
Color	Yellow	White
Margin	Irregular	Circular
Elevation	Low-convex	Convex
Opacity	Opaque	Opaque
Consistency	Butyrous	Mucoid
Grams nature & morphology	Gram negative rods	Gram negative rods

[P2]Burkholderia cenocepacia niraar, [P13] Enterobacter cloaceae

Several studies have reported the potential of Burkholderia sp. in solubilizing of phosphates [48-50]. Besides, they are also involved in nitrogen fixation, bioremediation and plant growth promotion [51-53]. Hence, there is much scope in its use as a biofertilizer. A study reported 4 of the 5 PSBs showing highest phosphate solubilization potential, isolated in their study, belonging to the genera Burkholderia [40]. Among other bacteria, a study reported isolation of 15 gramnegative bacteria and 5 diazotrophic free-living encapsulated Azotobacter species as potential PSB from Monte-Fresco rock phosphate mine in Táchira, Venezuela [47]. The potential of Aspergillus sp. (*A. niger*, *A. flavus*, *A. carbonum*, *A. fumigatous*, *A. wentii*) [54, 55], other fungi (*Fusariumoxysporum*, *Sclerotiumroltsii*, *Clindracladium* sp. *Penicillium* sp.) [47, 54] and *actinomycetes* (*Streptomyces*) [56] in solubilization of phosphates has also been reported.

3.3. Qualitative assay for detection of phosphatase enzyme, organic acid and other plant growth promoters

The test isolates in our study did not produce phosphatase enzyme, indicating alternative mechanism of solubilizing insoluble phosphates present in soil. The production of growth promoters i.e., siderophores and IAA were also not observed in our study. On further investigation, it was observed that *B.cenocepacia* niraar produced organic acid as determined by the formation of a yellow halo around its growth on modified Pikovasky's agar plate. Generally, the ability of solubilization depends

upon the production of organic acids and/or phosphatase enzyme in microbes [56]. However, *Enterobacter cloaceae* did not show production of acid or enzyme to help us understand the mechanism of phosphate solubilization.

In contrast to our study, Kailasan*et al.* [34] reported production of acid phosphatases by Burkholderia sp. and *Rhizobium radiobacter*. Gupta *et al.* [32] also used NBRIP medium for confirming acid production for phosphate solubilization by *Bacillus* and *Aspergillus* sp. In another study, the PSB isolated from Moroccan phosphate mine caused weathering of insoluble rock phosphate by production of siderophores, but the production of other organic acids or plant growth promoters was not detected [39].

A study reported the production of siderophores and IAA by three strains of PSB i.e., *B. cepacia, A. tumefaciens* and *R. pickettii* [40]. Another recently published study reported the isolation of 70 rhizobacteria sp. from various regions of south Punjab, Pakistan. Out of these samples, 10 isolates were identified as potential PSB with SI values in the range of 4-7 and all of them produced phytohormones (indole acetic acid), siderophore, ammonia and hydrogen cyanide [57]. *Kosakonia cowanii* MK834804 showed production of various organic acids viz., i.e. oxalic acid, malic acid, tartaric acid and gluconic acid, and solubilized phosphates under submerged fermentation conditions [58]. Another study reported the production of organic acids like gluconic, lactic, isovaleric, acetic, oxalic and citric acid by PSB [22].

3.4. Optimization of cultural, nutritional and physicochemical parameters for phosphate solubilization

The optimization of basic parameters was carried out in our study to optimize the phosphate solubilization potential of test isolates. Initially, the cultural growth parameters i.e., media, incubation time and aeration conditions were optimized. It has been reported that PSB can solubilize phosphates in the range of 30-900mg/L depending on the source of insoluble phosphates, media composition and initial pH [59]. Moreover, the type and characteristics of test microorganisms also affect the solubilization process significantly [24]. Furthermore, plant diversity affects the bacterial community, specifically the common *Bacillus* and *Pseudomonas* sp., thus indirectly influences the phosphate solubilizing ability of bacteria [18].

The Figures 1 and 2 represents the results for optimization of media by *B. cepacia*niraar and *E. cloaceae* respectively. Figure 3 represents the effect of aeration on test isolates. Based on a literature survey, two media i.e., NBRIP and PVK, were selected for optimization of phosphate solubilization by test isolates in our study. *B.cepacia* niraar and *E.cloaceae* showed better phosphate solubilization in NBRIP broth (27.41ppm, 29.90ppm) as compared to PVK broth (7.98ppm, 6.98ppm). These results were obtained in 120h under shaker conditions.

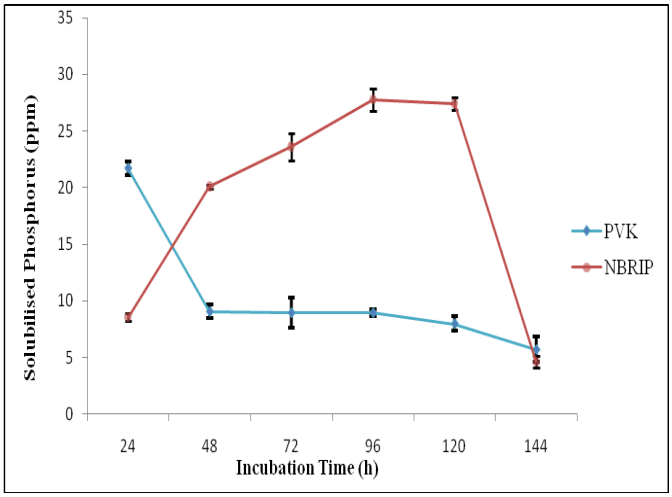

Fig. 1: Effect of media on phosphate solubilization by *Burkholderia cenocepacia niraar*

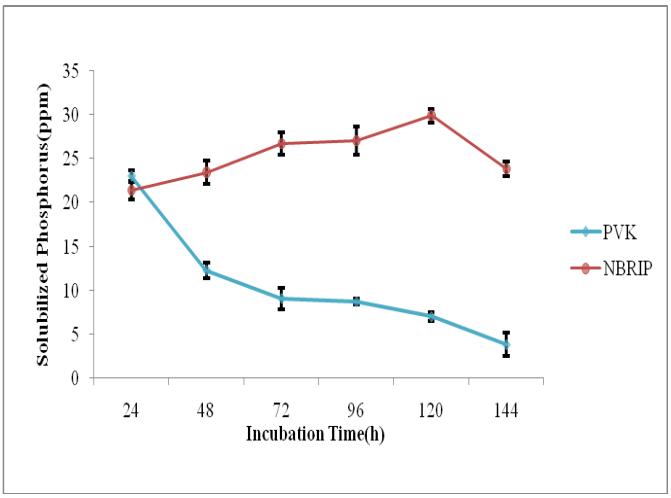

Fig. 2: Effect of media on phosphate solubilization by *Enterobacter cloaceae*

Similar to our findings, Nautiyal [36] reported significantly higher phosphate solubilization by the strain of Pseudomonas sp. in NBRIP (90ppm) compared to PVK (35ppm) medium in 120h. The solubilization of phosphorus by *P. fluorescens* (65.619µg/mL) and *Bacillus* sp. (560.667µg/mL) was also found to be optimum in NBRIP medium [60]. Earlier studies have reported

solubilization of phosphates within 24-48h of incubation [61, 62]. In other studies, a *Pseudomonas* sp. solubilized 61.78ppm phosphate in PVK broth in 48hwhereas *P. fluorescens* solubilized 36ppm phosphate in NBRIP medium in 72h [63, 64]. In a recent report, *Rhizobium tropici* solubilized 80ppm of phosphate ideally under shaker (130rpm) conditions [65]. Interestingly, *K. cowanii* showed a high SI (4.5) in PVK agar, however, under submerged fermentation conditions, it showed highest phosphate solubilization (70.2µg/mL) in NBRIP medium in 96h [58].

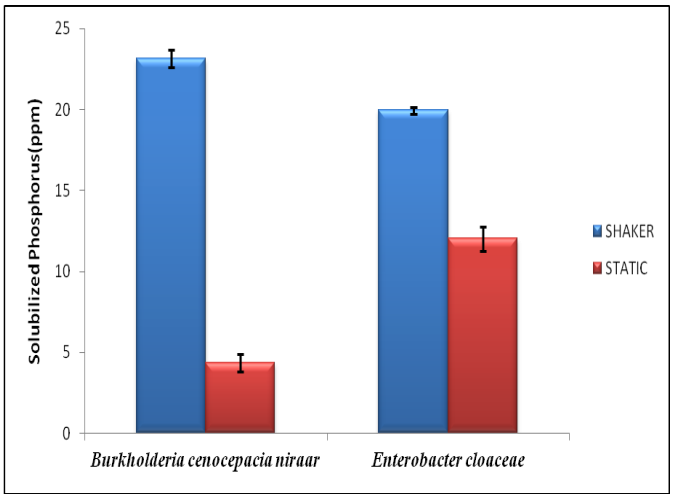

Fig.3: Effect of aeration on phosphate solubilization by test isolates

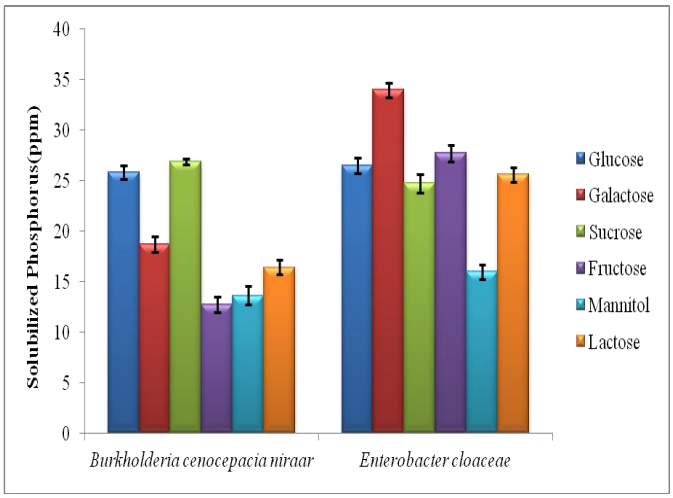

Fig. 4: Effect of 1.5% carbon source on phosphate solubilization by test isolates

After the optimization of cultural parameters, the nutritional parameters including the carbon and nitrogen sources were optimized. Figures 4 and 5 represents the results of ideal carbon and nitrogen source respectively for phosphate solubilization by test isolates. The results suggested that maximum phosphate solubilization was obtained when 1.5% galactose (33.98ppm) and 1.5% sucrose (26.89ppm) was used as a carbon source by *E.cloaceae* and *B. cenocepacia* niraar respectively. The growth of *E. cloaceae* in presence of fructose significantly supported phosphate solubilization (27.74ppm), however, *B. cenocepacia* showed least solubilization potential (12.73ppm) in presence of the same. The inorganic nitrogen source supporting maximum phosphate solubilization by *E. cloaceae* and *B. cenocepacia* niraar, respectively, was 0.01% ammonium sulphate (25.55ppm, 37.97ppm) followed by 0.01% potassium nitrate (23.64ppm, 36.092ppm) for both isolates.

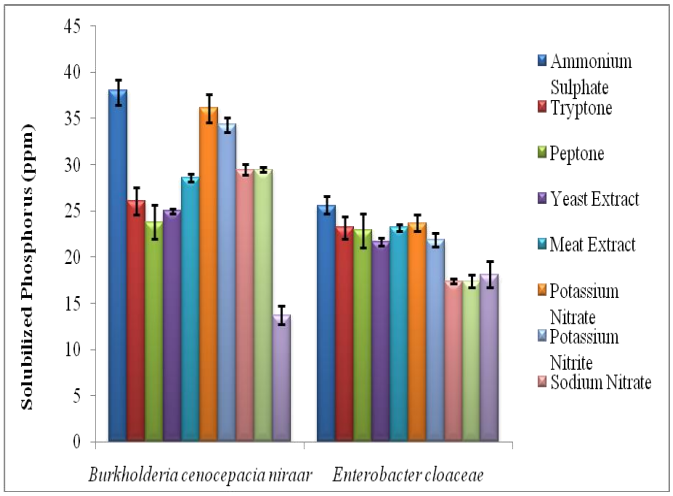

Fig. 5: Effect of 0.01% nitrogen source on phosphate solubilization by test isolates

The observations reported by Saini*et al.* [66] indicated optimum phosphate solubilization in presence of glucose and ammonium nitrate as carbon and nitrogen source respectively, by *Pseudomonas* sp. and *Bacillus* sp. Similar observations were reported for a phosphate solubilizing rhizobacteria by Batool and Iqbal [57]. Glucose and ammonium sulphate was found to be a better source of nitrogen for PSB species in studies carried out by Rahman*et al.* [60] and Vora *et al.* [67].A proteobacteria, *B. glathei,* isolated from rhizospheric soil,produced gluconate and acetate using glucose as a carbon source [68]. In another study, a considerably high concentration of glucose (10%) was reported to be optimum for phosphate solubilization activity of *Ralstonia pickettii* [34]. A study on ten PSB isolates reported best activity in presence of glucose and least in presence of lactose [69].Three PSB i.e., *Maricaulis virginensis*, *Kosakoniaoryzae*

and *Klebsiella pneumonia* showed optimum phosphate solubilization in presence of dextrose, ammonium sulphate and 1.2% sodium chloride [70]. Optimum phosphate solubilization by *K. cowanii*MK834804 was achieved in presence of 0.5% ammonium sulphate and 2% Lactose [58].

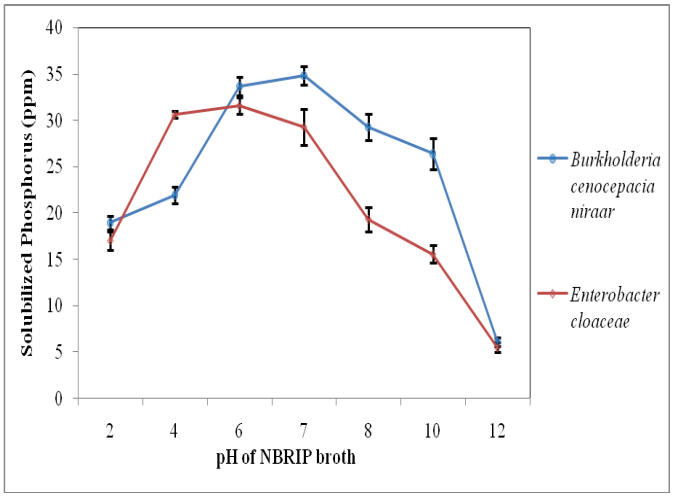

Fig. 6: Effect of pH on phosphate solubilization by test isolates

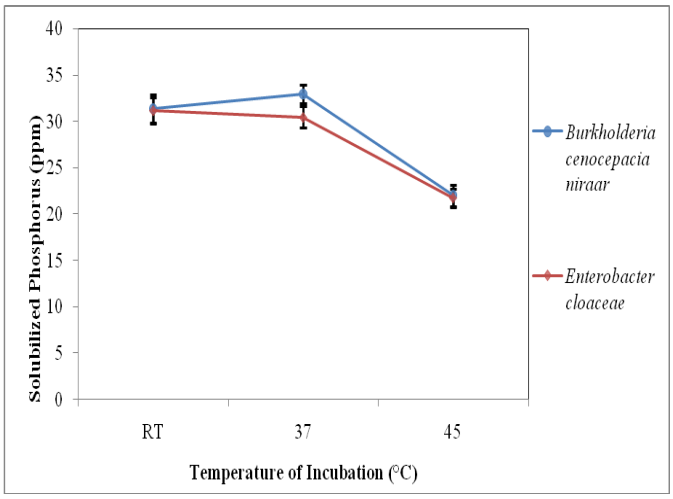

Fig. 7: Effect of Temperature on phosphate solubilization by test isolates

After optimization of cultural and nutritional parameters, the physicochemical parameters were optimized for solubilization of phosphates by the test isolates. Figures 6, 7 and 8 represent the optimum pH, temperature and optical density respectively for optimum solubilization of phosphates by *E. cloaceae* and *B. cenocepacia* niraar. The optimum optical density was found to be 0.4 $O.D_{540nm}$ for both isolates. The optimum pH and temperaturefor *B. cenocepacia* niraar was pH 7 and 37°C, whereas *E. cloaceae* showed optimum activity at pH 6 and RT in our study.

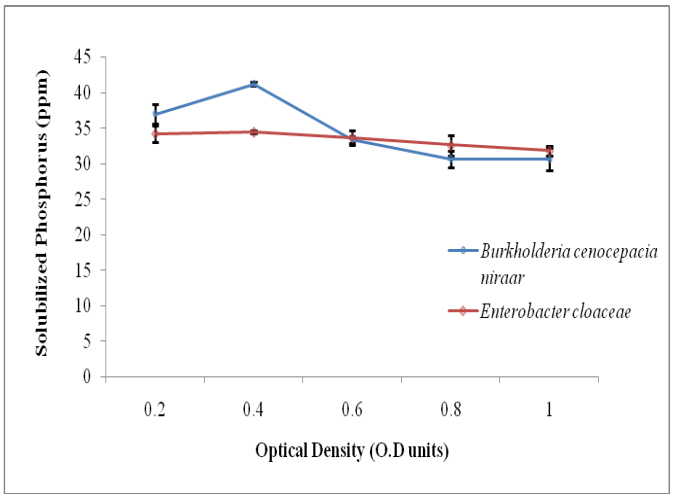

Fig. 8: Effect of optical density on phosphate solubilization by test isolates

In an earlier study, Islam *et al.* [71] reported optimum activity of PSB in the pH range of 5-7. A low pH of NBRIP medium was found to be ideal for phosphate solubilizing activity of *Alcaligenes faecalis* [72]. Several studies have reported a temperature range of 25-35°C to be optimum for activity of PSB [28,70]. A thermotolerant PSB was isolated from rock phosphate mines of Jhamarkotra that showed high ferric phosphate (Fe-P) and aluminum phosphate (Al-P) solubilizing abilities. The isolate was identified as *Brevibacillus* sp. and showed optimum solubilization at 50°C and pH7.5 [73]. In another study, 2 *Pseudomonas* sp. and 3 *Bacillus* sp. showed optimum phosphate solubilizing activity at 36°C and pH 7 in 3days [64]. Another PSB showed optimum conditions for phosphate solubilization at 35°C and pH7 [57], whereas *K. cowanii* MK834804 showed maximum activity at 36°C and pH8 [58].

3.5. Comparison of phosphate solubilization by test cultures individually and when used in consortium

The phosphate solubilization potential of test isolates were studied under optimized parameters. *B. cenocepacia* niraar showed 41.2ppm phosphate solubilization whereas *E. cloaceae* solubilized 34.39ppm phosphates under optimum cultural, nutritional and physicochemical parameters. Interestingly, the consortium of these two cultures showed 59.52ppm phosphate solubilization under optimum conditions (Figure 9).

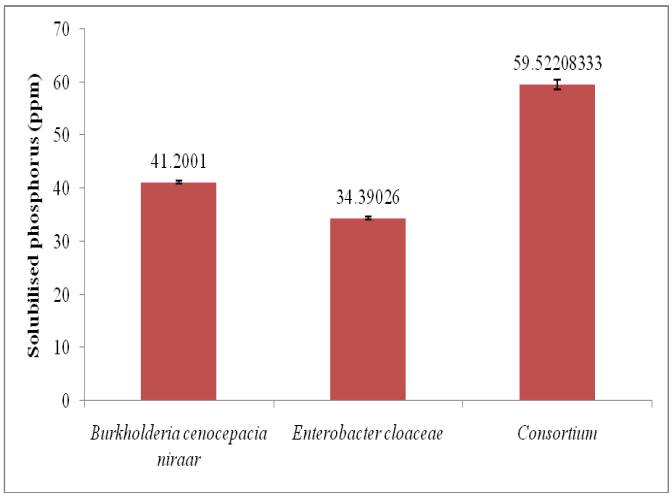

Fig. 9: Effect of consortium of test isolates on phosphate solubilization

A recent study reported that the PSB suspensions added with different phosphate ore samples had different microbial diversities. In this study, wheat rhizosphere soil samples were collected from the south campus of the University of Alberta in Edmonton, Canada. The rock phosphates (ore samples) were obtained from Yunnan phosphate mines in China. The microbial consortium from the same sample was allowed to grow in separate bioreactors containing ore sample 1 or 2. The bacterial suspension solubilizing ore sample 1 was mainly composed of alphaproteobacteria (49.71%) and TM7_class_incertae_sedis (15.68%). The other sample suspension showed presence of sphingobacteria (21.70%), TM7_class_incertae_sedis (16.92%), gammaproteobacteria (12.73%) and sphingobacteria (6.93%) [74].

The potential of microbial consortium as an effective biofertilizer has been reported in the current study and by several other researchers as discussed in the next section below.

3.6. Pot experiments to study the application of PSB as plant growth promoter

The Table 4 represents the effect of treatment of plant seed and soil with individual test isolates and its consortium. In the current study, an increase in shoot length of wheat and mustard plants were recorded from the seedlings raised with the PSB-inoculated seeds. Further improvement was observed on addition of TCP along with test cultures. However, the highest growth performance was observed in presence of the bacterial consortium and TCP.

Table 4: Pot experiments showing effect of test cultures and its consortium on plant growth

Treatment	Shoot length (cm)	
	Wheat	Mustard
Control	8.96	1.5
Soil +TCP	8.70	2.4
Soil +P2	10.42	1.5
Soil +P2+TCP	11.17	2.63
Soil+P13	9.75	2.55
Soil+P13+TCP	10.33	3
Soil +P2+P13	10.58	2.9
Soil+P2+P13+TCP	11.75	3.2

Key: P2 (B. cenocepacianiraar) and P13 (E.cloaceae)

The 8 parameters used for the assessment of the effect of test PSB isolated in our study, and its consortia, on plant growth were selected on the basis of literature survey [37, 75, 76]. Similar to our findings, the inoculation of soil with *Pantoea agglomerans* and *Burkholderia anthina* showed enhancement of shoot and root length as well as the shoot and root dry mass in moong bean plant. The growth was further improved on addition of TCP along with PSB inoculation. However, the highest growth performance of mung bean plants was observed on co-inoculation of both PSB strains and TCP [37]. In another study, an increase in grain yield of wheat by 9.3%, 14.8% and 13.1% was observed on inoculation of *Azospirillum*, *Bacillus* and *Enterobacter* strains to soil as compared to non-inoculated control [76]. The increased crop production is also reported under growth chamber and greenhouse conditions on co-inoculation of PSB [77, 78]. A 40% increase in shoot length was observed on inoculation of Burkholderia sp. PER2F soybean plants as compared to un-inoculated soil/seed [79]. Significant enhancement in the growth of rice seedlings and *Brassica napus* was observed on treatment of seed with *E. cloacae* [80, 81]. The PSB strains, in another study, showed an increase in shoot length from 14cm to 30cm and root length from 8cm to 10cm. The phosphorus content in plant was also found to increase from 15.1µg/mL to 70.9µg/mL [70]. In a recent study, over 80% increase in seed germination and 90% increase in root and shoot length was observed on inoculation of seed with PSB [57]. Another recent study reported improvement in safflower seed germination on inoculation of *Acinetobacter* sp. RC04 and *Sinorhizobium* sp. RC02. Further improvement was observed on co-inoculation of above isolates on the growth of seedling [82].

The PSB are emerging as a promising biofertilizer due to their positive effect on plant growth. The co-inoculation

of potential PSB, which may act synergistically, to improve growth performance in plants, has been reported by several researchers. Even commercial preparations of PSB bio-fertilizers containing powdered forms of *Burkholderia*, *Azotobacter*, *Rhizobium* and *Azospirillum* sp. are available in several countries [22].

4. CONCLUSION

The indispensable role of phosphorus in growth and metabolism of plants, and its unavailability due to formation of insoluble complexes in soil are compelling reasons to improvise a sustainable approach to improve plant growth. The rhizobacterial species play diverse roles in promoting plant growth and health. Among these, several species of PSMs have been identified and studied in previous years. The PSMs are involved in the biotransformation of phosphorus sources in soil and thus help in increasing the availability of phosphates to plants. The current study provides data on phosphate solubilizing activity of two potential strains, *B. cenocepacia* niraar and *E. cloaceae*. In addition, the promising biofertilizer characteristic of these isolates, as single cultures as well as in the form of consortia is demonstrated by pot studies. Further implementation of these cultures in field trials will enhance our knowledge, thus help in complete exploitation of these isolates to its full potential. The present study may also be useful in development of an efficient phosphate solubilizing consortium that can be used for improvement of crop yield.

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