JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 905-914

Statistical Optimization of Phosphate Solubilization by *Erwinia* sp.hdds3fr

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(Received: 11 January 2015; accepted: 03 March 2015)

Phosphate solubilizing bacteria (PSB) are known to convert phosphate from the insoluble to soluble form , and make it available for plant uptake. The use of microbial inoculants (biofertilizers) possessing P-solubilizing activities in agricultural soils is considered as an environmental-friendly alternative to further applications of chemical based P fertilizers. Therefore, the objective of this research was to mine for phosphate solubilizing microorganisms from our local environments. Upon screening, 45 isolates were obtained showing varying degrees of phosphate solubilization on agar plates using PVK and NBIRP media. One bacterial isolate exhibited the highest phosphate solubilization on both media. The phosphorus solubilizing potential of the isolate was evaluated using solid and liquid media under in vitro conditions. The bacterial isolate was identified based on 16S rRNA gene sequencing data as *Erwinia* sp.hdds3fr. It was also aimed in this study to investigate nutritional factors affecting P solubilization using the Plackett-Burman experimental design. On the basis of the calculated t-values, the main factor that had significant positive effect on phosphate solubilization by *Erwinia* sp. hdds3fr was $Ca_3(PO_4)_2$. In order to validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out. A significant increase near 2 fold in SP was achieved.

Keywords: Phosphate solubilization; *Erwinia* sp. hdds3fr; optimization; Plackett-Burman statistical design.

Phosphorus (P) is one of the major plant nutrients limiting plant growth, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration [1,2] and nitrogen fixation in legumes [3]. Although P is abundant in soils in both inorganic and organic forms, its availability is restricted as it occurs mostly in insoluble forms. These insoluble, precipitated forms cannot be absorbed by plants [4]. The P content in average soil is about 0.05% (w/w) but only 0.1% of the total P is available to plant because of poor solubility and its fixation in soil [5].Moreover, the efficiency of applied P fertilizers in chemical form rarely exceeds 30% due to its fixation, either in the form of iron/aluminium phosphate in acidic soils [6] or in the form of calcium phosphate in neutral to alkaline soils [7]. It has been suggested that the accumulated P in agricultural soils would be sufficient to sustain maximum crop yields worldwide for about 100 years if it were available[8, 9, 10].Furthermore P is a finite resource and based on its current rate of use, it has been estimated that the worlds known reserves of high quality rock P may be depleted within the current century [11]. Beyond this time, the production of P based fertilizers will require the processing of lower grade rock at significantly higher cost[12]. The realization

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of all these potential problems associated with chemical P fertilizers together with the enormous cost involved in their manufacture, has led to the search for environmental compatible and economically feasible alternative strategies for improving crop production in low or P-deficient soils [13].The use of microbial inoculants (biofertilizers) possessing P-solubilizing activities in agricultural soils is considered as an environmental-friendly alternative to further applications of chemical based P fertilizers.

The objective of the present investigation was to isolate PSB that could solubilize insoluble phosphate efficiently and optimizing its culture conditions to improve solubilization process using Plackett-Burman design.

MATERIALS AND METHODS

Isolation of phosphate solubilizing bacteria

Soil employed to isolate PSM was collected from garden at Faculty of Science, Alexandria University in Egypt. One gm of soil was suspended in 9 ml distilled water and serially diluted up to 10^{-5}. A sample (100µl) from each serial dilution was platted on agar plates of PVK medium[14] containing in gL^{-1}, glucose, 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4.7H_2O$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.002; $FeSO_4.7H_2O$, 0.002; and on NBRIP medium [15]containing in gL^{-1}glucose,10; tricalcium phosphate (TCP), 5; $MgCl_2.6H_2O$, 5; $MgSO_4.7H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1. Medium pH was adjusted to 7 with HCl. Plates were incubated for 2 to 3 days at 30 °C. Colonies with clear halos were considered as phosphate solubilizing microorganisms (PSM) [16]. A total of 45 isolates that exhibited clear zones on the agar plates were selected. Based on the size of the clear zone, isolate H3fr was the most efficient phosphate solubilizer, and was selected for further studies.

Identification of isolate H3fr

Partial sequencing of 16S rRNA for the bacterial isolate H3fr was performed with the help of a DNA sequencing service with universal primers. Polymerase chain reaction (PCR) was performed with initial denaturation at 95°C for 2 min followed by 30 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 45 s at 72 °C. The final extension was held for 5 min at 72 °C. The online program BLAST (NCBI, 2012) was used in identifying the related sequences with known taxonomic information available at the databank of National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA). A phylogenetic tree was constructed with the CLUSTAL X program [17], which involved sequence alignment by the neighbor-joining method [18] and maximum parsimony with the MEGA4 program [19]. The grouping of sequences was based on confidence values obtained by bootstrap analysis of 1000 replicates. Gaps were edited in the BioEdit program [20] and evolutionary distances were calculated by Kimura's two-parameter model [21].

Qualitative estimation of phosphate solubilization

Phosphate solubilizing efficiency was assessed on PVK and NBRIP solid media [22,23,24]. The isolate was spotted on solid media using sterile tooth picks ,and plates were incubated at 30 ±2°C for 2days and the diameter of each colony as well as the halo zone formed were measured.

Quantitative estimation of soluble phosphate

For quantitative estimation of soluble P [24], a pre-culture of the bacterial isolate was prepared in 20 ml LB medium dispensed in 100 ml Erlenmeyer flask and incubated under shacked condition (170 rpm) at 30 ±2°C till OD_{600} = 1.0 (~ 6 x 10^9 CFU/ml).For phosphate solubilization experiment, aliquots of 20 ml of either NBRIP or PVK were dispensed in 100 ml Erlenmeyer flasks and inoculated with 0.2 ml (1%v/v) of previously prepared seed cultures. Liquid cultures were incubated in a shaker incubator (170 rpm) for 5 days at 30 ±2°C, unless otherwise stated. Uninoculated flasks were kept as control. The content of each flask was then centrifuged at 10,000 rpm for 10 min to remove cells and the supernatants were analyzed for soluble P content using the molybdenum-blue method [25]. All experiments were carried in triplicates and the values are the average of data obtained.

Experimental Design and Optimization

Plackett-Burman design a rapid screening multifactor to find the most significant independent factors [26,27,28], was used in the present study to screen the important variables that significantly influenced phosphate solubilization. In this experiment, seven independent variables were screened in eight combination organized according

to the Plackett-Burman design matrix (Table2). Each variable was evaluated at two levels, a high (+) and a low (-) level (Tables 3 and 4.).All trials were performed in triplicates and the averages of phosphate solubilization (PS%) were treated as a response. Higher PS % is directly correlated with higher solubilization efficiency. This efficiency was evaluated based on PS%, all treatments were obtained at 12 h of inoculation.

The main effect of each variable can be calculated using the following standard equation:

Main effect = [Ó R(H)-Ó R(L)]/N

Where R(H) and R(L) are observations of trials where the independent variable was present in high and low concentrations, respectively, and N is the number of the trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low concentration of this variable is near to optimum.

Data Analysis of the Result of Plackett-Burman Design

Excel (Microsoft Office, 2010) was used for the experiment design and all statistical analyses. The variables with confidence levels above 95% were considered as influencing phosphate solubilization significantly. The application of statistical design was carried out in a 'two phases' of optimization approach. The first step was to evaluate the relative importance of various constituents within a complex culture medium and selecting of levels of the variables, which have significant influences on the solubilization process. The second was the verification experiments to validate the results under specific optimized experimental conditions.

RESULTS AND DISCUSSION

Identification of isolate H3fr

A total of 45 isolates that exhibited clear zones on the agar plates were selected as phosphate solubilizing organisms. One isolate (H3fr) was selected as an efficient phosphate solubilizing organism. The selected isolate had a marked insoluble phosphate solubilizing ability as visualized by the clear zone development around the colonies after 2 days of incubation. Comparison of the 16S rRNA sequence among available strains of *Erwinia* species showed the highest sequence homology to *Erwinia* sp. CMG3059 EU048331.1 with the similarity percentage of 98.5% and to *Erwinia* sp. v427 EF088378.1 and *Enterobacter* sp.EU567059.1 with the similarity percentage of 98%. This strain was designated as *Erwinia* sp. hdds3fr. GenBank accession number of 16S rDNA gene partial sequence of the selected isolate and the highest sequence similarity as well as the closest neighbour(s)are shown(Table1).Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees. The obtained sequence was deposited in the NCBI Genebank under accession numberGU182309.

Compared with all sequences, the results are presented as a phylogenetic tree (Fig.1), showing the evolutionary position of H3fr amongst related organisms

The genus *Erwinia* belongs to family *Enterobacteriacea*, *Gamma Proteobacteria*. Indeed, several Gram-negative, non-spore-forming, facultatively anaerobic, rod-shaped bacteria have been classified traditionally into the genus *Erwinia*, mainly on the basis of their association with plants as pathogens, epiphytes or saprophytes [29,30].

In accordance with our data, it was reported that the majority of PSB isolates was affiliated to the *Beta* or *Gamma* sub-divisions of the *Proteobacteria*; three were similar to *Pantoea* species and two were closely related to *Serratia* species [31]. *Erwinia* was listed as one of the commonly reported inorganic phosphate mineralization genera[32, 33, 34, 35, 36]. From a total of 395 rhizobacterial isolates, two *Erwinia* sp. (AUEY28 and AUEY29) were described as the top mineral P mobilizers isolated [36].

Qualitative and quantitative estimation of P solubilization efficiency by *Erwinia* sp. hdds3fr

Selection of suitable medium is an important issue. Data in Fig. 2 depict that medium NBRIP appeared to support the solubilization process for *Erwinia* sp. hdds3fr as observed by the clear zone formed. For quantitative measurement, standard inoculum (1%) of *Erwinia* sp. hdds3fr was used to inoculate 20 ml portion of NBRIP and PVK liquid media. Flasks were then incubated at 30°C for 5 days. As shown in Fig.3 the value of soluble phosphorus (84.5%, 839µg/ml) obtained with NBRIP was almost double the amount produced on PVK medium (42.2%, 418 µg/

ml). These data are in good agreement with those previously reported who found a maximum production of 450ìg/ml on NBRIP broth by isolate NBRIP12601[37,38]. Similarly, the isolate EnHy-401 had the maximum value of soluble phosphorus (632.6 ± 23.0ìg/ml)[39] after 5 days incubation [40]. Also, isolate BIHB752 recorded a maximum solubilization which liberated 805.0ìg/ml phosphorus [40]. The values recorded in this study were higher than those isolates from Korean soils that reported TCP solubilisation (96 to 139 (ìg/ml) [41]. *Pantoea aglomerans* isolated from the acidic soils of Korea recorded 900 ìg/ml soluble phosphorus [42].

Under our experimental condition, *Erwinia* sp. hdds3fr was able to grow on the two tested media and solubilize tricalcium phosphate. As clearly observed the NBRIP medium supported better solubilization compared to PVK [41]. Therefore, further experimentation was done using NBRIP medium

Table 1. Accession number of isolate H3fr and its similarity percentage to the nearest neighbours

Isolate	Genbank accession number	Similarity (%)	Nearest neighbour(s)
H3fr	GU182309	98.5	*Erwinia* sp. *CMG3059 EU048331.1*
		98	*Erwinia* sp. *v427 EF088378.1*
		98	*Enterobacter* sp. *EU567059.1*

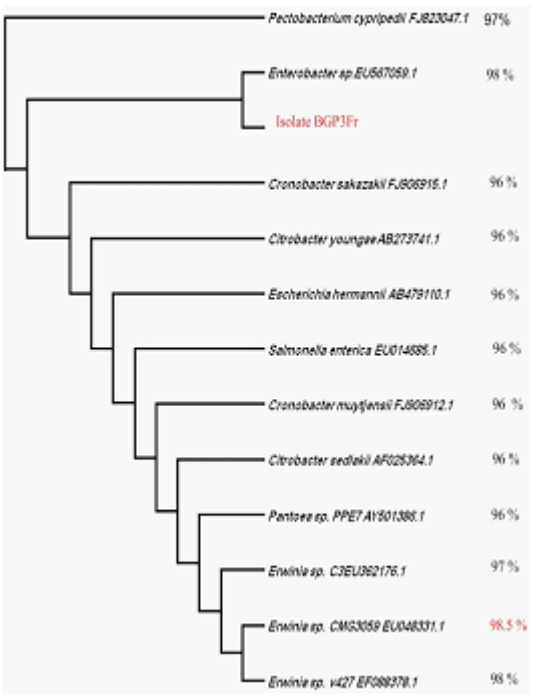

Fig. 1. Phylogenetic tree of isolate H3fr. 16S rDNA-based dendogram showing the phylogenetic position of isolate H3fr among representatives of related bacterial species. The tree was constructed by Bioedit method [20]. Percentages (right) indicate phylogenetic similarities

Optimization of P solubilization by *Erwinia* sp.hdds3fr

For screening of the most important factors affecting PS, the Plackett-Burman design was applied with nine different fermentation conditions as shown in Table 3. All experiments were performed in triplicate and the average of results of phosphate solubilization (%) was

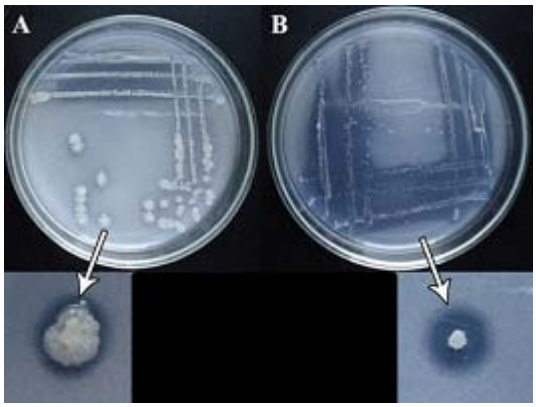

Fig. 2. Clear zone formation by *Erwinia* sp.hdds3fr grown for 48 h on PVK (A), and NBRIP (B) media

presented as response as shown in Table 3. The main effect of each variable on phosphate solubilization as well as t-values were estimated for each independent variable as shown in Table 4 and graphically presented in Figure 4. The values of PS% for different treatments showed a variation from 23to 72% (Table 3), suggesting that the studied variables had a significant effect on the PS% and therefore on phosphate solubilization.

Every microorganism has its own peculiar nutritional and physico-chemical requirements for the enzyme production[43]; where the main effect with a positive sign indicates that the high concentration of this variable is nearly optimum and a negative sign indicates that the low concentration of this variable is nearly optimum. In this study with respect to *Erwinia* sp. hdds3fr, the analysis of the main effect, t-values and P-values of 7 factors presented graphically (Fig. 4) show that the four variables G, MS, N and V positively affected phosphate solubilisation. Meanwhile, C, Mc and K had negative effects.Positive effect explains that if a higher concentration was used, a better response was

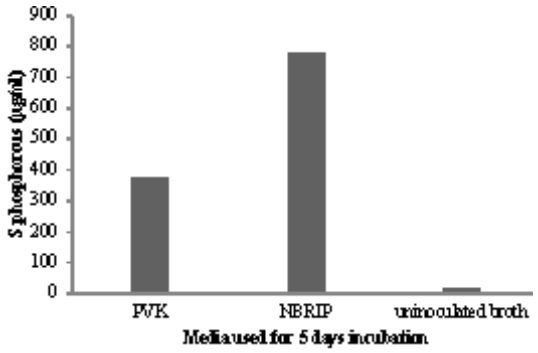

Fig. 3. Soluble P (µg ml^{-1}) in culture filtrate of *Erwinia* sp. hdds3fr as affected by different cultivation medium. Cultures were incubated at 30°C,170 rpm for 5 days

Table 2. Factors examined as independent variables affecting phosphate solubilization by *Erwinia* sp. hdds3fr and their levels in the Placket-Burman experimental design

Variable	Symbol	Level (g/l)		
		Lower	0 level	Higher
Glucose	G	5	10	15
$Ca_3(PO_4)_2$	C	2.5	5	7.5
$MgCl_2$	Mc	2.5	5	7.5
$MgSO_4.7H_2O$	Ms	0.125	0.25	0.375
KCl	K	0.1	0.2	0.3
$(NH_4)_2SO_4$	N	0.05	0.1	0.15
culture volume	V	10 ml	20 ml	30 ml

Table 3. The Placket-Burman experimental design (in coded levels) with PS % as response

Trials	Factor under study	Component							Response [Soluble P$^-$ (%)]
		G	C	Mc	Ms	K	N	V	
1	Ms K N	- 1	- 1	- 1	+1	+1	+1	- 1	55
2	G N V	+1	- 1	- 1	- 1	- 1	+1	+1	72
3	C V	- 1	+1	- 1	- 1	- 1	- 1	+1	24
4	G C K	+1	+1	- 1	- 1	+1	- 1	- 1	23
5	McMs V	- 1	- 1	+1	+1	- 1	- 1	+1	50
6	G McMs K	+1	- 1	+1	+1	+1	- 1	- 1	52
7	C Mc N	- 1	+1	+1	- 1	- 1	+1	- 1	23
8	G C McMs K N V	+1	+1	+1	+1	+1	+1	+1	25
9	Basal medium	0	0	0	0	0	0	0	46

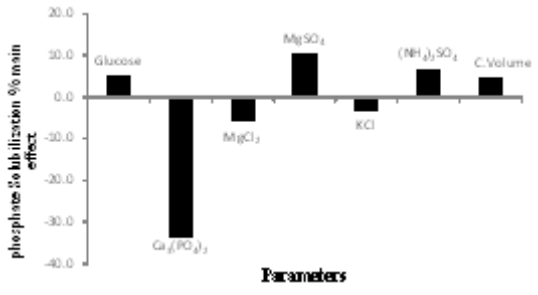

Fig. 4. Elucidation of culture conditions affecting phosphate solubilization (PS%) by *Erwinia* sp. hdds3fr

Table 4. Statistical analysis of the Plackett-Burman experimental result for phosphate solubilization by *Erwinia* sp. hdds3fr

Factor	Phosphate solubilization			
	Main effect	t-value	p-value	Significance level
Glucose	5.106	0.351461	0.74	26%
$Ca_3(PO4)_2$	-33.779	-6.726512	0.01	99%
$MgCl_2$	-5.779	-0.398964	0.71	29%
$MgSO_4$	10.442	0.743560	0.49	51%
KCl	-3.271	-0.223790	0.84	16%
$(NH_4)_2SO_4$	6.716	0.465845	0.66	34%
Culture Volume	4.532	0.311251	0.77	23%

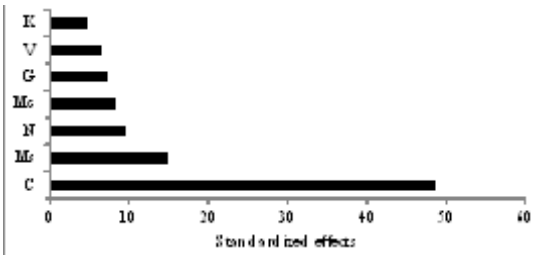

Fig. 5. Pareto plot of standardized effects

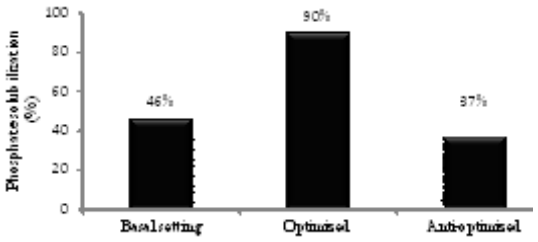

Fig. 6. Verification experiment of the applied Plackett-Burman statistical design by comparing the percentage of soluble phosphorus in *Erwinia* sp.hdds3fr culture filtrate, growing on the optimized, basal and anti-optimized media

achieved, while a negative effect means lower concentrations are favored for better results.

Phosphate solubilisation was increased with the increase of glucose in the culture of *Erwinia*sp.hdds3fr as it was reported with *Burkholderia cepacia* DA23[44], *Acinetobacter calcoaceticus* YC-5a [45], *Serratia marcescens* CTM 50650[45], *Pantoea agglomerans* R-42 [42]and *Pseudomonas* sp.[38]. The positive effect of magnesium sulphate in enhancing P solubilization observed in this study was also reported with *Acinetobacter calcoaceticus* YC-5a[44]. It was reported that, the rate of phosphate solubilization by *Pseudomonas* sp. was increased with increasing concentrations of $MgSO_4.7H_2O$ and $MgCl_2$; suggesting that, KCl is the less required ingredient and the phosphate solubilization ability improved when $(NH_4)_2SO_4$ was used at a lower concentration[38]. On the contrary, in the present study, P solubilisation required high concentration of $(NH_4)_2SO_4$. The presence of the insoluble tricalcium in low level enhanced the solubilization process. On the other hand and in contrast with *Erwinia* sp.hdds3fr; it was found that, the soluble P production was enhanced with increasing amount of insoluble phosphatesby *Burkholderia cepacia* DA23 and *Acinetobacter calcoaceticus* YC-5a,respevtively[44,45]. From the data obtained, it was concluded that increasing the volume of culture medium (low aeration) might increase the amount of soluble phosphorus. In another study, it was found that phosphate solubilization by *Pantoea agglomerans* (KCTC 2564) increased independently of aeration[47], while phosphate removal by *Burkholderia* spp. from sediments containing mineral-phosphates was facilitated in the absence of aeration [48]. On the contrary, in a study on phosphate mobilization by *Burkholderia*

caribensis FeGL03, it was found that restricted air supply in cultures would then adversely affect gluconic acid production[49].

The significant variables were identified by statistical analysis of the Plackett-Burman experiment using the t-test supported by Excel (Microsoft Office, 2010) to determine the statistical significance of the measured response (Table 4). Some researchers employed confidence levels greater than 70 % as significant effect levels [50, 51]. Other investigators find that, confidence levels greater than 90 % are acceptable [52, 53, 54] or even levels greater than 95 % to be a significant effect on the response [55, 45]. In the present study, any component showing a statistical confidence equal and/or higher than 95 % ($P<0.05$) was considered significant parameter. It was clear that only one variable [$Ca_3(PO_4)_2$] had a significant level ($P = 0.01$) with main effects of 33.779 %, while the other six variables with $P>0.05$, were considered insignificant.

Fig.5. shows the Pareto's chart that represents the estimated effects, in decreasing sequence of magnitude, of C, MS, N, Mc, G, V, and K. .The measure of each bar is proportional to the estimate effect. Pareto's chart is a graphical representation of Student's t-test. This test presents each of the estimated effects. To plot the estimates in decreasing order of importance Pareto's chart is selected [56, 52].

Verification experiment

In order to validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. Consequently, based on the results obtained from the Plackett-Burman experiment, a medium of the following formula was predicted to be near optimum for phosphate solubilization (g/l): glucose, 15; $Ca_3(PO_4)_2$, 2.5; $MgCl_2$, 2.5; $MgSO_4 \cdot 7H_2O$, 0.375; KCl, 0.1; $(NH_4)_2SO_4$, 0.15 and culture volume, 30 ml. The data were examined and compared to the basal and anti-optimized medium. The anti- optimized medium was the opposite concentrations of the optimized one and its formula was in (g/l): glucose, 5; $Ca_3(PO_4)_2$, 7.5 ; $MgCl_2$, 7.5; $MgSO_4 \cdot 7H_2O$, 0.125; KCl, 0.3; $(NH_4)_2SO_4$, 0.05 and culture volume, 10 ml.

The verification experiment applied indicated that the solubilized P before and after optimization were 46% and 90%, respectively. Thus, a significant increase near 2 fold in SP was achieved by Plackett-Burman optimization (Fig. 6). On the other hand, *Acinetobacter calcoaceticus* YC-5ashowed 1.8 fold phosphate solubilisation increase compared with that using the original medium [45].

CONCLUSION

The results presented here emphasize the importance of exploring the natural biodiversity of local environments and studying P-solubilizing microorganisms. Data also demonstrate optimum metabolic capacity at different environmental parameters by the application of statistical experimental design that would probably enhance the solubilization process.It is suggested that researchers must continue to examine microbial interactions for use as biofertilizers, and growth promoters in order to improve communities in field soils.

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