Isolation, Purification And Liberation Of Free Phosphate By Indigenous Phosphate Solubilizing Bacteria And Effect On Plant Growth Promotion.

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Citation

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Abstract

Solubilization of insoluble organic phosphate has been the focus of many studies as it increases the availability of phosphorus to vegetation and improves plant growth. The aim of this study was to study those bacterial strains which were positive for phosphate solubilization in plate assay as well as in liquid media. The isolates which showed efficient phosphate solubilization activity both in plate and liquid media were examined for the release of free phosphate in liquid media. The amount of free phosphate liberated by the organisms was estimated. The efficient phosphate solubilizing bacteria which also released high amount of free phosphate in the media were scrutinized for auxin production via bioassay. Five bacterial isolates CMG851, CMG854, CMG857, CMG860 and CMG861 found positive for auxin production were checked for the production of indole acetic acid and indole butyric acid via high performance liquid chromatography (HPLC). The amounts of indole acetic acid and indole butyric acid were also estimated. These five efficient phosphate solubilizing and auxin producing bacterial isolates were identified via 16srRNA analysis. They were Acinetobacter lwofii (CMG851), Pseudomonas aeruginosa (CMG860) and Bacillus thuringiensis (CMG854, CMG857, CMG861) showed efficient phosphate solubilization and auxin production abilities. Mung beans were selected to study the effect of plant growth promotion abilities by the three phosphate solubilizing and auxin producing bacteria. The root and shoot length of Rye plants were measured and it was found that the selected bacteria had noticeable effect on them.

INTRODUCTION

The amelioration of phosphate deficiency by the application of costly and environmentally hazardous phosphate fertilizers is not an ideal solution and has generated serious issues about the continued viability of current agriculture practice. This has led to a search for more environmentally friendly and economically feasible strategies to improve crop production in low phosphorus soils. In an ideal manner, such strategies should enable the efficient use of phosphate solubilizing microorganisms. Several scientists have reported the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate and dicalcium phosphate (Gar and Gaind, 1999).

According to Statistics, the worldwide transaction amount of fertilizer is roughly US$40 billion. Out of this, 135 million metric tons of chemical fertilizer is applied each year, with sales volume of about US$30 billion. Although there are no clear application statistics for biofertilizer, however, its sales volume is estimated to be as much as US$3 billion.

MATERIALS AND METHOD

SAMPLE COLLECTION, MEDIA AND CHEMICALS

- Soil samples were collected from the following locations of Karachi.
- Nurseries (Glushan-e-Iqbal)
- Garden soil (Safari park)
- Staff town (Karachi University)
- Agriculture fields (Malir and Gaddap)

Nutrient broth and Nutrient agar were used for isolation and purification of the bacterial strains. Tris minimal salt medium (Fasim et al., 2002) was used for the initial screening of metal solubilization activity. NBRIP (Nautiyal, 1999), Pikovskya (1948) and MPVK medium (Son et al., 2006), were also used to check solubilization activity of selected bacterial strains.

Tris minimal medium (Appendix 7) was employed for solid and liquid culture, containing the carbon source, usually D-glucose10g l^{-1}. All chemicals were either of Oxoid, BDH or Merck.

ISOLATION OF BACTERIAL STRAINS FROM SOIL SAMPLES BY SERIAL DILUTION METHOD

Soil samples from nurseries, garden soils and agricultural fields (Malir and Gaddap) were brought to the laboratory in sterilized universals. Ten gram soil was aseptically weighed and transferred to Erlenmeyer flasks containing 100 ml nutrient broth and incubated at 37°C for 24 hrs on 100 rpm. After 24 hrs, incubated samples were diluted by serial dilution method. A series of tenfold dilution was made for each sample by pippeting 1 ml aliquots into 9 ml sterile water. Each dilution of (0.1 ml) series was then spread on the nutrient agar plates and incubated at 37°C. Morphologically distinct colonies were picked and purified by streaking on nutrient agar. The selected strains were stored at 4 ˚C as stabs and at -70 ˚C in 40% glycerol.

ISOLATION OF BACTERIA FROM PLANT ROOTS

Sadabahar plants of Karachi university staff town were picked along roots and soil. The plants were immediately brought to the laboratory in sterilized universals. Only the root portion of the plant was used for the isolation of bacteria. For this purpose, soil was removed gently by repeated washing with sterilized distilled water. The roots were then transferred to mortar and pestle and gently crushed. The paste obtained was transferred to Erlenmeyer flasks containing 100 ml nutrient broth and incubated at 37 °C for 24 hrs. A series of tenfold dilutions were made for each sample by pippeting 1ml aliquot into 9 ml sterile NaCl (0.89%). Each dilution of the series was spread (0.1 ml) on the nutrient agar plates and incubated at 37 °C for 24 hrs. Morphologically distinct colonies were picked and purified by streaking on nutrient agar plates.

SOLUBILIZATION OF INSOLUBLE METAL COMPOUNDS ON SOLID MEDIUM

All the bacterial strains were screened for solubilization activity towards the following insoluble compounds.

- Zinc phosphate
- Zinc oxide
- Calcium phosphate

All the above metal compounds were added to the tris minimal medium to give a final concentration of 5mM except for ZnO which was added to give a final concentration of 14mM. Overnight growth culture in nutrient broth was used as inoculum. A drop of the inoculum (10µl) placed on to the tris glucose agar plates amended with 5mM zinc phosphate in presence of 1% glucose and incubated at 30°C for 3 days. The plates were examined every day for metal solubilization activity by observing halos formation around colonies. The diameter of the zones of solubilization (halo formation) were observed and the efficiency of the solubilization was calculated according to the formula (Nguyen et al., 1992)

16SRIBOSOMAL RNA ANALYSIS

Four best Phosphate solubilizing bacterial strains were also identified by 16s ribosomal RNA gene sequence homology in addition to biochemical tests. PCR amplification was carried out using thermocycler (Eppendorf). Colony PCR was carried out and the PCR reaction mixture consisted of 2.5 µl (20 pico mole) of each forward (TAC CGC GGC TGC TGGCAC) and backward primer (TGG AGA GTT TGA TCC TGC CTC AC), MasterAmp Taq DNA polymerase; 0.5µl, MasterAmp Taq 10X PCR Buffer; 10µl, MasterAmp 10X PCR Enhancer; 15µl, 25 mM $MgCl_2$ Solution; 5µl, dNTP mix.; 8µl and dH2O; 56.5µl. Thermal cycling was done by initially denaturation at 96° C for10 minutes followed by 10 cycles at 94° C for 1minute. Then 52° C for 30 sec and 72° C for 1 min. Annealing temperature was 55° C for 30 sec for 30 cycles. Last elongation step was five min at 72°C. A 10 µl reaction mixture was used to visualize PCR product on 1% Agarose gel. The remaining PCR product was purified by using Qiagen purification kit and sequencing was done commercially by using universal 16sRNA primer on an automated ABI prism 377DNA sequencer. Sequence Data was analyzed by BLAST aligothirm (www.ncbi.nlm.nih.gov/blast/cgi)

SOLUBILIZATION OF INSOLUBLE METAL COMPOUNDS IN LIQUID MEDIUM

The bacterial strains were grown in liquid tris minimal media to study their solubilization activity of insoluble metal compounds in liquid tris minimal medium. Three replicates (each of 250 ml) samples of liquid tris minimal salt medium un supplemented (control), supplemented with 5mM zinc phosphate and 14mM zinc oxide were inoculated with an overnight grown pre culture of organisms (2.5% inoculum) and incubated in shakibutor (100 rpm) at 20 ˚C for 10 days.

EFFECT OF MEDIA AND ITS COMPONENTS

Effect of media on the efficiency of phosphate solubilization was studied by using following media

- Pikovskaya (Pikovskaya, 1948)
- Tris minimal salt medium (Fasim et al., 2002)
- NBRIP (National Botanical Research Institute's Phosphate growth medium) (Nautiyal, 1999)
- MPYK [(modified Pikovskya media) (Son2006)]

Effect of various/individual components of the Tris minimal media was further checked as described by Nautiyal (1999) on the efficiency of phosphate solubilization. Effect of each component was studied individually by deleting; each component one by one to note the minimum requirements of the strains for phosphate solubilization as described by Nautiyal (1999). Efficiency of solubilization was calculated as described in section 2.5.1. Experiment was done in 3 replicates and plates were incubated at 20 °C for 10 consecutive days.

QUANTIFICATION OF FREE PHOSPHATE IN LIQUID MEDIUM VIA CALORIMETRIC ASSAY

The bacterial strains which showed efficient phosphate solubilization activity in plate assay were selected to study phosphate release (PO_4) in liquid media by using colorimetric method (slight modification of EPA 424 F ascorbic acid method). Briefly the bacterial strains were grown in liquid tris minimal medium amended with 5 mM zinc phosphate and incubated at 20°C at 100rpm. An aliquot of 10 ml was aseptically removed from each flask at particular intervals of time (0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, 17, 20, 22 and 25 days) and centrifuged at 5,000g for 15 min. The supernatant was filtered through a sterilized 0.45µm millipore filter and assessed for free phosphate contents in the filtrate. Phosphate in the solution was visualized at 880 nm (UV visible thermo nicholet evolution-300). Working standards were prepared in the range of 0.01 to 6ppm from stock of 200ppm.

RESULTS

SELECTION OF PHOSPHATE SOLUBILIZING BACTERIA

The primary selection criteria for the phosphate solubilizing bacterial strains, which were picked for further studies, were as follows.

- Producing clear and sharp halos of more than 20mm
- Producing halos formation with in 48 hrs.
- Continually showing the solubilization trait up to 10 generations
- Continuously showing the gradual increase in the diameter of halos formation
- Showing the solubilization of both zinc phosphate and calcium phosphate

Only ten bacterial strains (7%) were fulfilled the above mentioned criteria and they were selected for future studies

SOLUBILIZATION OF INSOLUBLE PHOSPHATE COMPOUNDS ON SOLID MEDIUM

Qualitative screening of phosphate solubilization was carried out by using tris glucose agar plates amended with either zinc phosphate or calcium phosphate. All the ten bacterial strains showed solubilization of zinc phosphate up to 5mM except CMG860 which showed solubilization of zinc phosphate up to 14mM .

SOLUBILIZATION OF INSOLUBLE PHOSPHATE COMPOUNDS IN LIQUID MEDIUM

Ten efficient bacterial strains which showed solubilization of all tested compounds in plate assay were then grown on liquid tris minimal media to study their solubilization trait in liquid medium. Solubilization of zinc phosphate and calcium phosphate were inspected daily and clearance/transparency of the media against control was noted visually and consider as a positive sign of solubilization (Figure 64). Transparency of the media is a primary indicator of solubilization to be visualized as halos formation is considered a primary indicator of solubilization in plate assay. Although a plate represents a closer environment to the soil because of the contrary results in solid and liquid media, it is necessary to check the solubilization in both media. In plate bacteria come in contact with a limited patrticles at a time while in liquid media their tolerance as well as more thorough study was achieved.

IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIAL STRAINS

16SRRNA

Four best phosphate solubilizing bacterial strains CMG851,

CMG854, CMG857 and CMG860 were identified through 16s ribosomal RNA. CMG851 showed 98% homology with Acinetobacter lwoffi, (Table 1) CMG854, (Table 1) and CMG857 (Table 1) showed 99 % homology with Bacillus thuringiensis while CMG860 showed 99% homology with Pseudomonas aeruginosa (Table 1) Sequences were submitted to Genbank and the accersion numbers were obtained (Table 1)

ESTIMATION OF PHOSPHATE SOLUBILIZATION

QUANTIFICATION OF FREE PHOSPHATE VIA CALORIMERTIC ASSAY

Release of soluble phosphate in liquid media as a result of solubilization of zinc phosphate was determined at different time intervals. Results are shown in Figure 1 CMG860 released maximum amount of soluble phosphate in the culture broth with 4709 mg/L concentration after 6days of incubation (Figure 1). CMG851 released maximum amount of soluble phosphate in the culture broth with 2580mg/L after 4 days of incubation (Figure 1). CMG854 released maximum amount of soluble phosphate in the culture broth after five days of incubation with concentration of 1132mg/L (Figure 1). while CMG57 released maximum amount of soluble phosphate in the culture broth after 8th days of incubation with concentration of 1646mg/l (Figure 1).

Phosphate solubilization is a phenotype exhibited by many soil borne microorganisms known as PSM (phosphate solubilizing microorganisms). In the natural environment, rhizophere of different plant species interact with phosphate solubilizing bacteria (PSBs) which play an important ecophysiological role (Perez et al., 2007). In fact, they mobilize insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots, consecutively plants supply root borne carbon compounds, mainly sugars, which can be metabolized for bacterial growth (Deubel et al., 2000; Goldstein, 1995).

Figure 1

Figure1: Release of free phosphate in the media

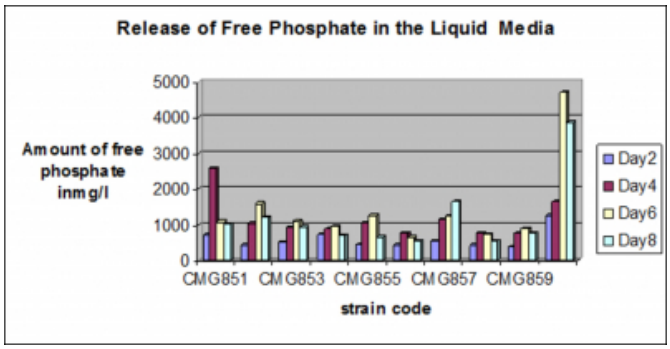
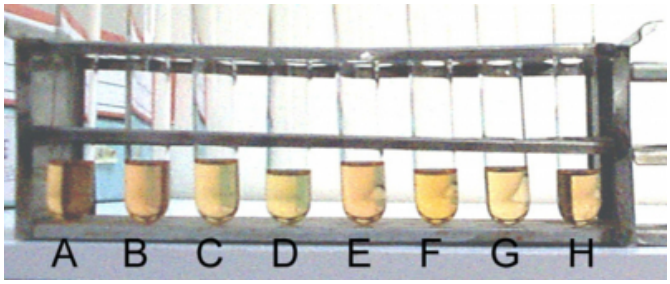


Figure 2

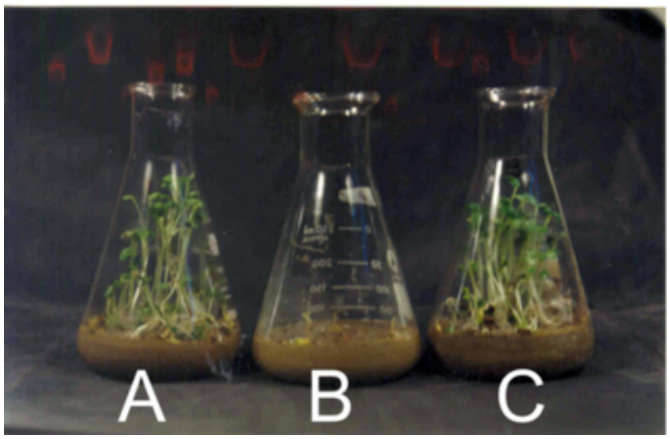
Figure 2: Auxins Production Abilities of PSBs via Standard Bioassay Method



Keys: A = CMG851 B = CMG852 C = CMG854 D = CMG855 E = CMG857 F = Control G = CMG860

Figure 3

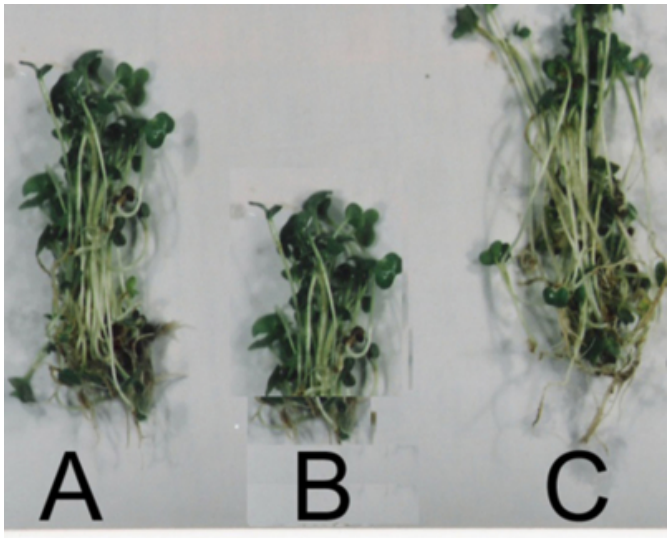
Figure3: Effect of CMG860 on Rye Seedling in Flask (Pilot Scale Experiment)



Keys: A = Only garden soil B = Garden soil and zinc phosphate C = CMG860 + zinc phosphate + garden soil

Figure 4

Figure4: Effect of CMG860 on Rye Seedling (Pilot Scale Experiment)



Keys: A = Only garden soil B = Garden soil and zinc phosphate C = CMG860 + zinc phosphate + garden soil

Figure 5

Table1: Identification of Selected Bacterial strains via 16srRNA

Strain code	Identification	Accession no
CMG851	*Acinetobacter lwoffi*	EU697389
CMG857	*Bacillus thuringiensis*	EU697391
CMG860	*Pseudomonas aeruginosa*	EU037096

Figure 6

Table2: Effect Of PSBs On Plant Growth Promotion

Rye	CMG851	CMG 857	CMG 860	C1	C2
Shoot length	6.75	8	11	5	7
% Increase over control	35%	60%	120		
Root length	7.7	8.7	12	4.9	4.6
% Increase over control	57.1%	77.5%	144.8%		

DISCUSSION

The widespread application of single element fertilizers such as Nitrogen, Phosphorus, Potasium (especially in South East Asian countries) in the cultivation of major crops has led to accelerated exhaustion of other major and minor nutrients leading to nutrient imbalances and poor soil fertility (Karthikeyan et al., 2006). Therefore, an urgent need has been felt to deploy microbial consortia/biofertilizers which are multifaceted i.e. besides being phosphate solubilizers they can promote plant growth through production of auxins, produce secondary metabolites linked to biocontrol of bacterial/fungal diseases or improve soil structure/porosity through secretion of mucilage/polysaccharides aiding in soil aggregation. Present study was aimed to screen soil bacteria which can solubilize complex form of metal compounds (with special emphasis on phosphate solubilization) such as zinc phosphate and calcium tri phosphate.

The highest amount of of soluble phosphate in the culture broth of CMG851 was 2580mg/l at 4 th day of incubation then there is a decline in the amount of free phosphate, after 6 th day the amount of free phosphate was 1078 mg/l and this decline continued without any further revival (Figure 1). It was noted that the highest amount of free phosphate was released from zinc phosphate by CMG860, which released maximum amount of soluble phosphate in the culture broth with 4709 mg/l concentration after 6 th day of incubation (Figure 1) while CMG857 released maximum highest amount of soluble phosphate in the liquid culture after 8 th day of incubation with concentration of 1646mg/l (Figure1).

Many phosphate solubilizing bacteria are reported as plant growth promoter (Hafeez 2004; Katiyar and Goel., 2003; Rodríguez and Fraga, 1999).Recent reports by Ryu et al., (2004) have identified several organic compounds produced by a variety of bacteria that promote plant growth. It is well established that introduction of plant growth promoting bacteria (PGPB) in soil improves the plant growth. PGPB promote plant growth through the production of plant growth hormones (Patten and Glick, 2002; Bottini et al., 2004). Present study was conducted to screen the potential PGPRs and their use as a biofertilizer.CMG851, CMG857 and CMG860 were selected for pot scale trial because of their positive effect on the germination of mung beans, their efficient phosphate solubilization abilities on plate assay as well as their release of free P in liquid culture and their auxin production abilities to enhance the growth of three different crops.

All three PGPRs, improves plant growth by various mechanisms which could not be mutually exclusive. It is likely that phosphate solubilizing strains might have helped in plant root proliferation and production of plant growth regulators by the bacteria at root interface which resulted in better water absorption and nutrients such as P by host plant (Barea et al., 2005; Gupta et al., 2002; Lambrecht et al., 2000).

The above experiments were carried out in complete absence of any synthetic plant growth regulators. Increase in shoot and root length of the tested plants may be attributed to the production of growth promoting substances that carry out an important role in the stem expansion process of lettuce and corn plants with Enterobacter spp. and Pseudomonas spp. due to the auxins produced by bacteria as reported by other workers(Chabot, 1994). Inoculation of rye with CMG860 P .aeruginosa markedly enhanced root and shoot length especially the root system of rye was stimulated and significant increased in the root length (144%) and the shoot length (120%) of rye over control was observed. CMG857 showed 77% increase in the root length over control and 60% increase in shoot length of Chick pea. CMG851 also showed 57% increase in root length over control and 35% increase in shoot length of rye. (Table 2; Figure 3)

Control experiment revealed that deficiency of available phosphate retard plant growth in various parameters such as root and shoot length of rye as compared to test. From these results we conclude that inoculation of rye with efficient PGPRs significantly enhance plant growth in pot scale experiment. The pronounced plant growth by PGPRs observed in the present study can be attributed to the production of IAA, IBA and solubilization of phosphate. Such findings are in agreement with many authors who reported phytohormones production by Pseudomonas (Glick, 1995; Persello-Cartieaux et al., 2003; Tsavkelova et al., 2007). Therefore, it is attractive to speculate that coordination of above mentioned mechanisms may act to stimulate plant growth. Because of their efficient phosphate solubilization and auxin production ability, it is justifiable to propose here that these bacterial strains have plant growth potentials and could be exploited as biofertilizer or bioinoculant.

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