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Full Length Research Paper

Investigation of biological behavior of Iranian indigenous phosphate solubilizing bacteria and determinant of colonization ability of potato roots by these bacteria isolation

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The ability of a few soil microorganisms to convert insoluble forms of phosphorus (P) to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants. In this study, isolation and characterization of 3 strains of phosphate solubilizing bacteria (PSB) from Central province of Iran were carried out. Identification of three isolates was carried out by 16S rDNA sequencing. Three strains namely *Bacillus lentus* strain PS5, *Bacillus Licheniformis* strain PS7 and *Pseoudomonas putida* strain PS13 were isolated from the rhizospere of *Beta vulgaris* and *solanum tuberosom.* All of the three strains were selected *in vitro* for their phosphate solubilizing abilities from alkaline soils. Among the three strains, PS5 and PS7 were the most efficient strains in terms of their capabilities to grow and solubilize phosphorus in the presence of 5% NaCl and 42°C. Root colonization analyses were performed to determine the distribution and metabolic activity of the strains in the potato rhizosper. The soil containing potato seedling was treated with all strains marked with *lux* genes for bioluminescence, as well as resistance to kanamycin and rifampin prior to planting in non-sterile natural soil. The introduced bacteria were quantified on roots by dilution plating on antibiotic media along with observation of bioluminescence. Results demonstrated the strains could survive in the potato root system under stress conditions. The significance of this study lies in the fact that the bacterial strains isolated from alkaline soils have ability to solubilize phosphate in high salt, pH and temperature conditions.

Key words: Phosphate solubilization, potato, *Bacillus*, *Pseudomonas*, Iran.

INTRODUCTION

Phosphorous is one of the major plant nutrients. It exists in soil environment in both inorganic and organic forms. A large portion of inorganic phosphate applied as fertilizer is rapidly immobilized after application and becomes unavailable for plants. Thus the release of insoluble and fixed forms of phosphorus is an important factor in increasing soil phosphorus availability (Chabot, 1996; Shekhar, 2000) and it could be achieved through phosphate solubilizing bacteria (PSB). These bacteria have the ability to solubilize inorganic and/or organic P from soil after their inoculation in soil or on plant seeds (Sundara, 2002; Cabello, 2005; Rodriguez, 1999; Chung, 2005; Yong, 1998; Peix, 2004; Rudresh, 2005; Yang, 2003).

Solubilization of fixed soil P is a mechanism of organic acid production like production of oxalates and carbonic acids and production of enzymes. However, in the process of solubilization, some certain conditions should be taken into consideration, as bacteria could be affected under stress conditions such as high salt, pH and temperature prevalent in alkaline soil ecosystems with a tendency to fixing phosphorus. In this study, biological behavior of

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Abbreviations: PSB, phosphate solubilizing bacteria; rDNA, ribosomal deoxyribonucleic acid; **P,** phosphorus; **SDS,** sodium dodecyl sulfate; **EDTA,** ethylene diamine tetraacetic acid; **PCR,** polymerase chain reaction; **CFU,** colony forming unit.

these bacteria were studied under the environmental conditions prevalent in Iran.

In alkaline soils of the arid and semiarid in Iran, salt concentrations may be as high as 2%, pH as high as 10 and temperature may range between 3 and 42°C (Givi and Abtahi, 1985), which may result in poor growth and survival of PSB.

The successful use of these three strains as biofertilizer in Iran stress conditions requires that these strains survive and colonize roots of the introduced strains in soil and compete with other soil microorganisms. To distinguish the introduced strains from natural flora, a marking system is necessary. The bioluminescent marker *luxAB* combined with resistance to kanamycin and rifampin have been used to differentiate inoculated strains from indigenous bacteria (Beauchamp et al., 1993).

The objective of this investigation was to study biological behavior of Iranian Indigenous phosphate, solubilizing bacteria under stressed conditions (higher salt, pH and temperature) in alkaline soils and study of colonization with plant roots.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains were isolated in the Central province of Iran using soil surrounding the roots of *Beta vulgaris* and *solanum tuberosom* as they are the main products of this province. The soil was a silty loam with an organic matter content of 1.8 and 0.4% lime (pH 8.5 to 9). Available P and mineral NH_4^+-N and NO_3^--N contents were 13.2, 9.8 and 8.1 mg/kg, respectively. Available Fe, Mn, Zn and Cu contents were 4.1, 3.2, 2.4 and 1.6 ppm, respectively.

Isolation of phosphate solubilizing bacteria

The plants were grown in a plantation under natural light which provided a 12 h photoperiod, temperatures of 35°C and relative humidity 35%.

To isolate rhizosphere bacteria, the adhering soil on the root was gently shaken to collect the rhizospere soil. Roots were thoroughly washed with tap water for 2 min to remove all of the loosely adhering soil particles, followed by washing with sterile 0.85% (w/v) saline MiLLi Q water (MQW), to isolate rhizosplane bacteria. The roots were then macerated in 0.85% saline MQW with a mortar and pestle. Serial dilution of the root homogenate and soil (10% soil in 0.85% saline MQW) samples were individually plated on Sperber culture media (Shekhar et al., 2000). Among 100 phosphate solubilizing bacteria isolated from rhizosphere of *B. vulgaris* and *S. tuberosome*, *Bacillus lentus* strain PS5, *Bacillus Licheniformis* strain PS7 and *Pseoudomonas putida* strain PS13 were selected because their ability to solubilize inorganic P in Sperber culture media (Fiske and Subbarow, 1925) was detected more than the other bacteria.

Identification of phosphate solubilizing isolates

The phosphate solubilizing isolates were identified on the basis of morphology, gram stain, endospore stain, catalase and oxidase reaction. The identity of isolates was further confirmed by amplifying and sequencing approximately 1400 bp of the 16S ribosomal DNA (rDNA) for bacteria (Kuhnert et al., 1996, 2000). Amplification of isolates was performed using the universal primers UNI-L (5-AGAGTTTGATCATGGCTCAG-30) and UNI-R (50-GTGTGACGGGCGGTGTGTAC-30) (Kuhnert et al., 1996, 2000). Bacterial cells were cultured overnight in 2 ml of TSB at 35°C and then centrifuged at 8000 rpm for 10 min. The cell pellet was washed and resuspended in 0.5 ml of TE-buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) and then lysed by 20% sodium dodecyl sulfate (SDS). After the solution was boiled for 20 min and the cellular debris was discarded following centrifugation at 13000 g for 3 min, the total DNA in the supernatant was precipitated with 70% ethanol and used as template DNA for PCR.

PCR amplification was performed in 20 µl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, a 0.2 mM concentration for each of the four deoxynucleotide triphosphates, 0.5 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and template DNA (10 ng). Amplications were carried out for 35 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s) in a GeneAmp PCR 2400 Thermal Cycler (Applied Biosystems) with an initial denaturation at 94°C for 4 min and a .nal extension at 72°C for 7 min (Kuhnert et al., 1996, 2000). Amplicons were detected by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) eluted in Tris–HCl (10 mM, pH 8.5) prior to sequencing. The amplified DNA was directly sequenced with the ABI Taq-Dye Deoxy Terminator Cycle sequencing kit and ABI Model 377 automated DNA sequencer (Applied Biosystems). The sequences were analyzed with the BLAST (NCBI) for identification of Phosphate Solubilizing Bacteria.

Marking bacteria with *lux* AB genes

Escherichia coli WA803 (PDLB 30), resistant to chloramphenicol and carrying the Tn5-LuxAB genes of *Vibrio harveyi* with kanamycin resistance (Beauchamp et al., 1993), was used as the plasmid donor by conjugating with the three above mentioned recipient strains. The plasmid donor was grown on TSA medium supplemented with 30 µg of chloramphenicol per ml and 30 µg of kanamycin per ml. The recipient strains were grown on TSA medium supplement with 100 mg/ml of rifampin (TSA-R). After overnight growth at 30°C, the cells were harvested and suspended in sterile 0.85% NaCl solution (normal saline). The optical density at 590 nm was adjusted to 1.1 and 100 µg volumes of both donor and recipient cell suspensions were filtered through 0.22 µm- pore-size sterile nitrocellulose filter (25 mm-diameter) with 2 ml of additional sterile saline. Filters harboring bacteria were placed onto TSA medium for overnight incubation at 25°C. The cells from the filters were resuspended in 10 ml of sterile saline and the resulting suspension was serially diluted and plated on to TSA supplemented with 100 µg of rifampin and kanamycin per ml (TSA-RK), which did not permit growth of recipient strains or the *E. coli* donor but permitted growth of all recombinant bacteria. The dilutions were also plated on TSA-R which permitted growth of all recipient cells but not of the *E. coil* donor, to calculated transconjugant bacteria's frequency. The transconjugant bacteria were selected from TSA-RK using luminescence in the dark. Also the retention of P solubilization abilities was checked on tricalcium phosphate medium (Sundara et al., 2002).

Colonization assays

Pots (10-cm-diameter) containing the soil, which was previously described, were provided without sterilization to prepare a competitive condition for inoculated strains. The soil in each pot was uniformly mixed with suspension (107 CFU/g soil) of 3 bacterial strains PS5, PS7 and PS13. Micropropagated potato plantlets grown

Table 1. Identification of phosphate solubilizing bacteria isolated from the Iranian soil by 16S rDNA, basing on the output results from NCBI database analysis.

Strain	Organism identified	Percentage identity (%)	Gene bank accession number
PS5	*B. lentus*	96	EU008573
PS7	*B. licheniformis*	96	EU008574
PS13	*P. putida*	99	EU008575

in a greenhouse under natural light and average temperature of 25°C were transplanted into the pots containing the selected strains. Plants were irrigated daily with tap water as required. The experiment was set up as a completely randomized design with three replications in a factorial arrangement. Factors considered were 3 strains of bacteria and sampling distance from the crown (2 and 5 cm) and sampling depth (5 cm). Two way analysis of variance (ANOVA) was used to analyze the variance of tree strains of bacteria under two different conditions.

Plants were allowed to grow for 4 weeks in the greenhouse before the sampling process was started. Samples of 1 g rhizosphere soils were taken for serial dilution and plating was performed on TSA medium plus 100 µg of rifampin, kanamycin per ml (TSA-RK) for lux+ bacteria population and on 10% TSA for total bacterial populations. The plates were incubated at 25°C for 36 h. Bioluminescence was used to confirm that isolated colonies were the marked strains, which initially were added to the soil. Bioluminscence was observed by adding one drop N-decyl aldehyde to the covers of inverted Petri plates in the dark. The bacterial counts were expressed as CFU per gr.

Phosphate solubilization capacity and bacterial growth estimation

Quantitative estimation of phosphate solubilization in broth was carried out in Erlenmeyer flasks (150 ml) containing 15 ml NBRIP medium inoculated in three replicates with the above 3 bacterial strains (100 µl inoculums with approximately 15 x 10^8CFU ml^{-1}). The absolute value of the control refers to the amount of each strain (PS7, PS5 and PS13), when individually grown in NBRIP at 30°C without (NaCl) at pH 7. The effect of salt (NaCl), pH and temperature on phosphate solubilization and bacterial growth were tested for each stain on NBRIP containing various amounts of NaCl (0.5, 1, 2.5 and 5%), pH (Nautiyal, 2000; Hilda, 1999; Chung, 2005; Yong, 1998; Kuhnert, 1996) and temperature (25, 35 and 42°C) .The flasks were incubated for 3 days at 30°C on an orbital shaker (ORBITAL MIXER Model 4230 Jal, Iran), at 180 rpm. Sampling was performed at 3, 6, 12, 24, 48 and 72 h for quantitative estimation of bacterial growth. This was done by taking a 100 µl sample from each Erlenmeyer flask and transferring to LB medium for colony counting after serial dilution. For quantitative estimation of phosphate solubilizing capacity the strains were harvested by centrifugation at 19950 g for 10 min using a sigma 3K30 centrifuge (Germany). The concentration of phosphate in culture supernatant was estimated using the Fiske and Subbarow method (Fiske and Subbarow, 1925).

Presence of Lux^+ bacteria on potato roots

Lux strains of PS5, PS7 and PS13 were added to nonsterile potting soil containing potato plants (as described above) in a greenhouse, to observe the extent of colonization on root surface. Two weeks after inoculation, potato roots were harvested, shaked, washed in tap water, air-dried for 5 to 10 min and either complete root systems or segments of it were placed on TSA-RKC plates and incubated for 24 h at room temperature. Bioluminscence bacteria were observed by adding one drop of N-decyl aldehyde to the covers of inverted petri plates in the darkness.

RESULTS

Identification of phosphate solubilizing isolates

Table 1 listed the identity of phosphate solubilizing bacteria as determined by 16S rDNA sequences, following comparison to reference strains, using NCBI database analysis. The PCR amplicons from strains PS5, PS7 and PS13 had 96, 96 and 100% homology with *B. lentus* strain NCIMB8773, *B. licheniformis* strain S2 and *P. putida* strain 5 zhy, respectively. The result showed all three bacteria are new strains.

Lux$^+$ recombinant bacteria

All recipient strains conjugated with *E. coli* W803 (pDLB30) resulted in production of lux bioluminescent mutants, which emitted visual light in the dark after the addition of N-decanel aldehyde into the plate. The frequency of conjugants ranged from 1/106 recipient cells for strain PS13 to 5/106 and 1/104 for strains PS5 and PS7, respectively.

The frequency of transconjugants ranged from 1/103 recipient cells for strains PS5 and PS7 to 1/500 for strain PS13. The introduced luxAB genes were stable on the basis of several repeated transfers on dicalcium phosphate-RK and TSA-RK plates. Phosphate solubilization by the mutants was similar to that of the wild types on dicalcium phosphate plates.

Colonization assay

Results of the present study demonstrated (Figure 1) that strains PS5, PS7 and PS13 could survive in the rhizospheres of potato in a no sterile soil. Although the growth rate (log CFU) of all strains at 2 cm from the crown was greater than 5 cm but the PS13 was the only strain which was significantly ($p < 0.01$) greater. The growth rate (log CFU) of PS13 was significantly ($p < 0.01$) greater than PS5 and PS7 at 2 cm from the crown.

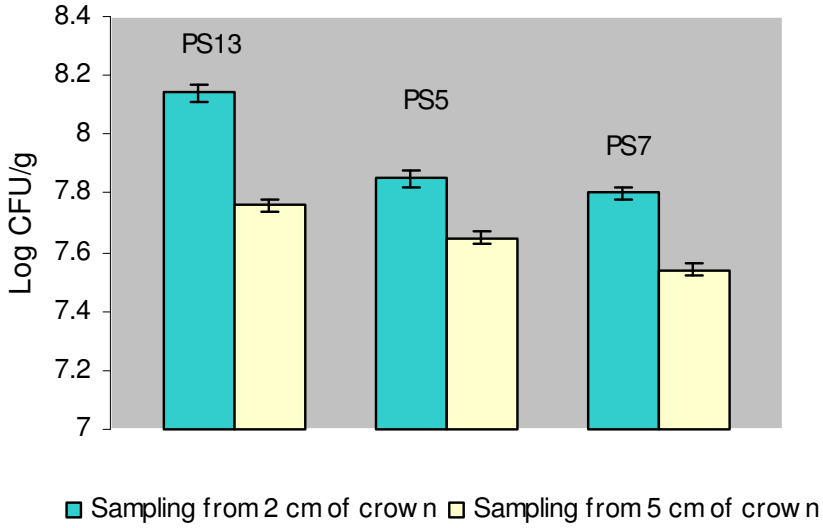

Figure 1. Colonization of roots of potato planted in pots containing soil by bioluminescence of mutant of *Bacillus lentus* strain PS5, *Bacillus licheniformis* strain PS7 and *pseoudomonas putida* strain PS13.

Presence of *Lux*$^+$ bacteria on potato roots

The result of bacterial growth in the prescence of N-decyl aldehyde resolved high luminescence which encompassed the whole root system for strain PS13. This was an indication of higher colony number around the root system for this strain. On the other hand, luminescence was not as extensive for the other two strains and was localized in parts of rhizosphere. Results demonstrated that strain PS13 could significantly colonize in potato roots.

Phosphate solubilization capacity and bacterial growth estimation

Quantitative estimation of phosphate solubilization and bacterial growth in the presence of various salts demonstrated that as salt concentrations in the media increased, bacterial growth and solublized P decreased accordingly. This was more apparent at 2.5 and 5% salt in the media (Figure 2). Results indicated that strains PS5 and PS7 were tolerant to high salt concentration (5%), but strain PS13 was very sensitive. The results of bacterial growth in the presence of various pH (Nautiyal, 2000; Hilda, 1999; Chung, 2005; Yong, 1998; Kuhnert, 1996) demonstrated (Figure 3) that although all strains were able to grow on NBIP broth with varying pH, the optimal growth media pH for these bacteria was between 7 to 10. Phosphate solubilization results (Figure 3) demonstrated that solubilized phosphate was lower for strains PS5 and PS7 at pH 5 and 6 but did not change for stain PS13 considerably.

The results of solubilized phosphate and bacterial growth at various temperatures demonstrated (Figure 4) that 25°C was the optimal temperature and by increasing temperature solubilized phosphate and bacterial growth were decreased rather than plant growth and biomass.

DISCUSSION

Bioluminescence is used in genetic studies as a genetic marker, which occurs with *luxAB* genes. For studying bacterial colonization, these strains were genetically tagged with *luxAB* genes. The result of conjugation of bacteria with *luxAB* genes in all strains (PS5, PS7 and PS13) indicated that PDB30 plasmid was easily hosted by many strains and it should also be noted that the hosting by strains was permanent for the duration of experiment. The evaluation of phosphate solubilizing ability in sperber medium in all strains was tested and it was indicated that there was no change in solubilized phosphorous after conjugation.

Due to the fact that activity of phosphate solubilizing bacteria is necessary to compete with deleterious and pathogenic organisms in soil, root colonization and survival in the rhizosphere play an important role for introduced bacteria. The present results clearly demonstrated that strains PS5, PS7 and PS13 could survive in the rhizosphere of potato in a nonsterile soil and PS13 significantly ($p < 0.01$) could colonize roots and compete with other organisms in soil greater than PS5 and PS7. The result of Bioluminscence bacteria growth in the rhizosphere area on TSA-RKC medium and observation of luminscence in the presence of decanal indicated that the number of colony counting unit (in strain PS13) in the rhizosphere area was greater than PS5 and PS7.

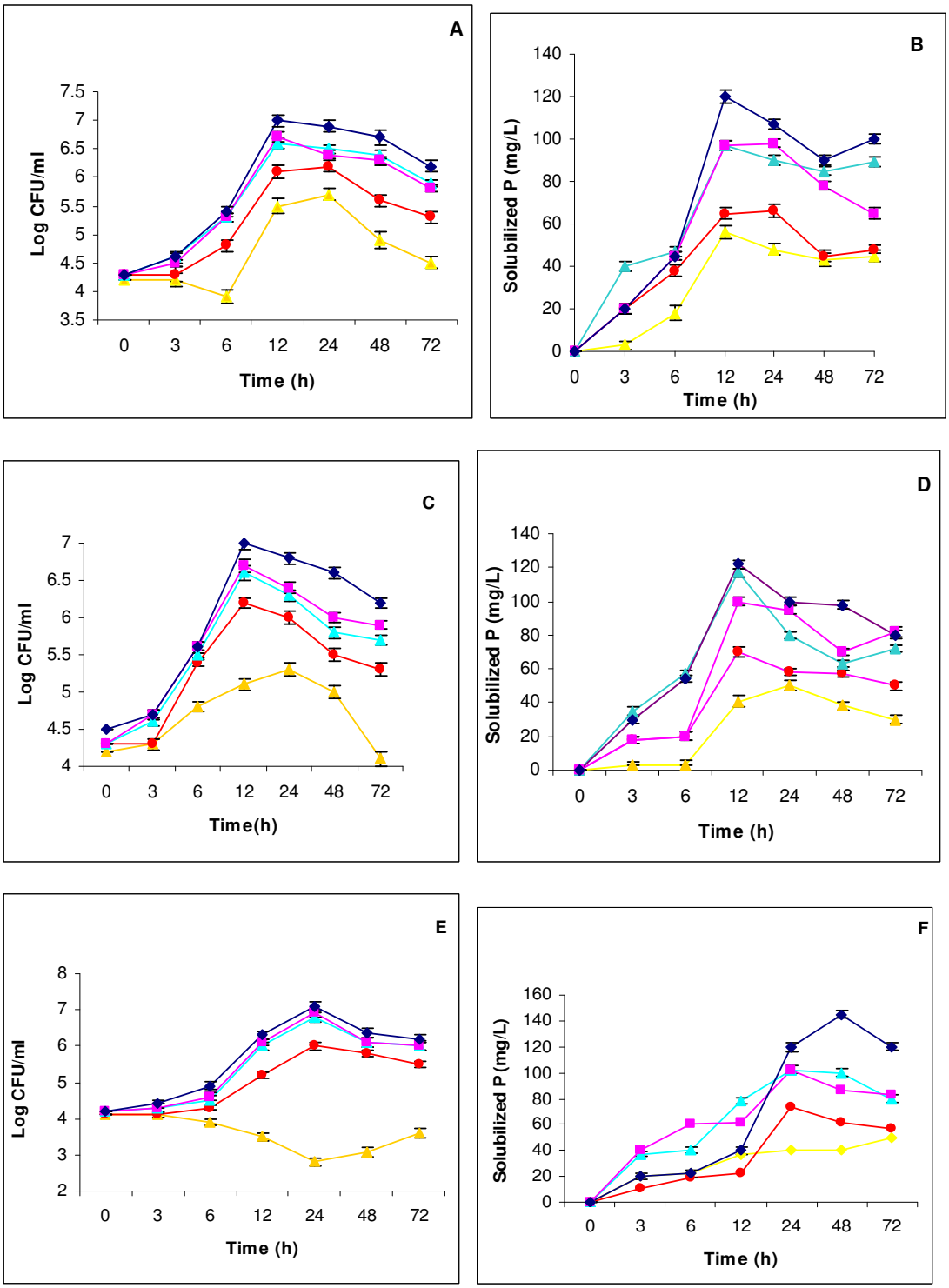

Figure 2. Effect of different concentration of salt (5▲, 2.5 ■, 1▲, 0.5 ■ and without salt ◆) on growth and solubilized P (mg/l) of strains PS5, PS7, PS13.

It is generally known that tolerance to stress such as high salt, pH and temperature may play an important role in the survival, multiplication and spread of bacterial strains in alkaline soils. Stress tolerant bacterial are likely to be found in environments which are affected by salt, pH and temperature.

The significance of this study lies in the fact that the bacterial strains isolated from alkaline soils have ability to

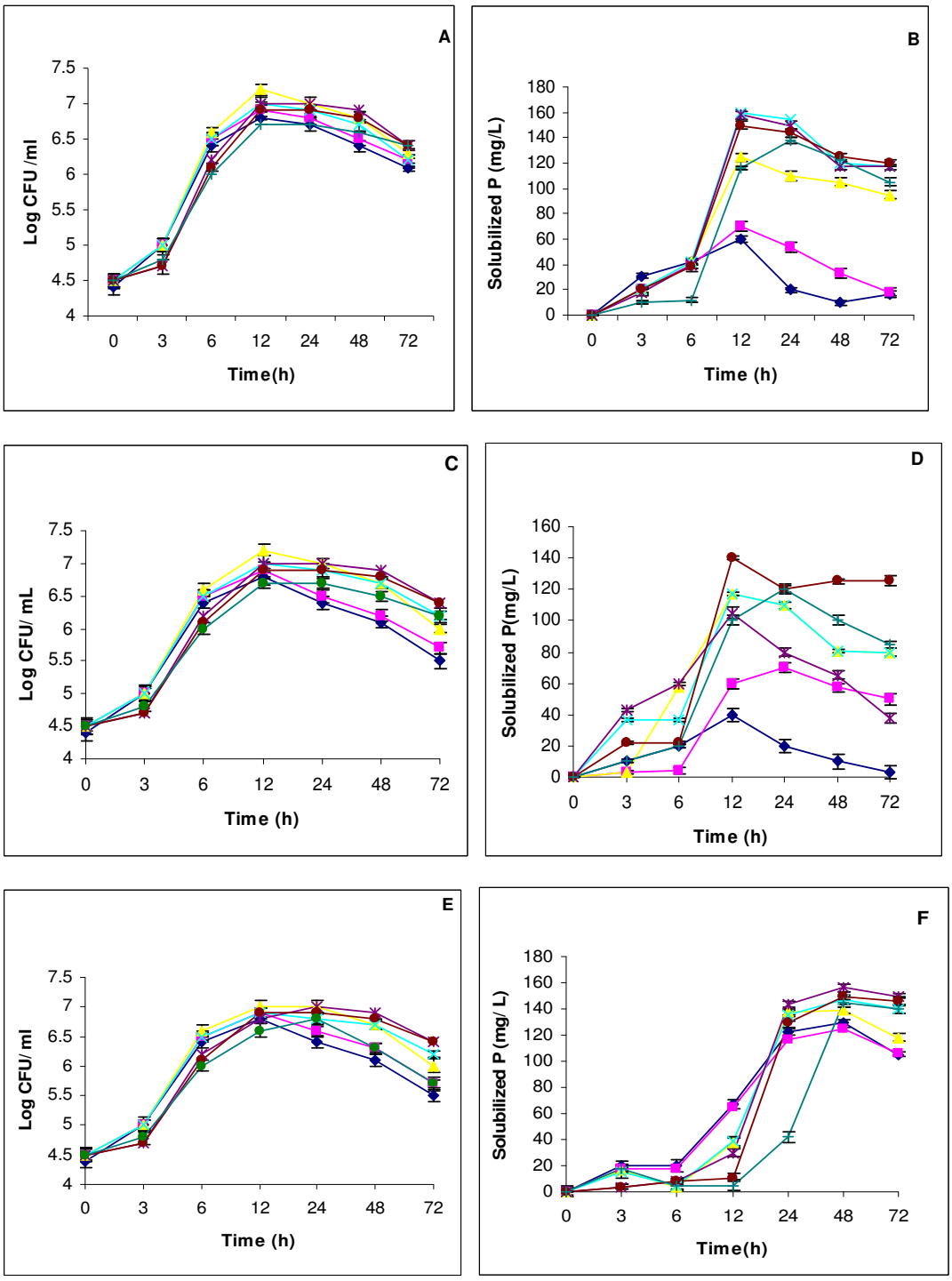

Figure 3. Effect of different concentration of pH (5 ◆, 6■, 7▲, 8 ×, 9*, 10●and 11 +) on growth and solubilized P (mg/L) of strains PS5, PS7, PS13.

solubilize phosphate in high salt, pH and temperature conditions. The growth of strains PS5, PS7 and PS13 in the presence of various concentrations of salt, pH and temperature (Sundara, 2002; Cabello, 2005; Chabot, 1996) demonstrated that PS5 and PS7 strains could grow at temperature as high as 40°C, while PS13 bacterium could not survive above 35°C (Figure 2). Furthermore, PS5 and PS7 strains tolerate high salt concentration up to 13 to 15%, while PS13 bacterium continue to grow when salt concentration was less or equal to 2.5% (Sundara et al.,

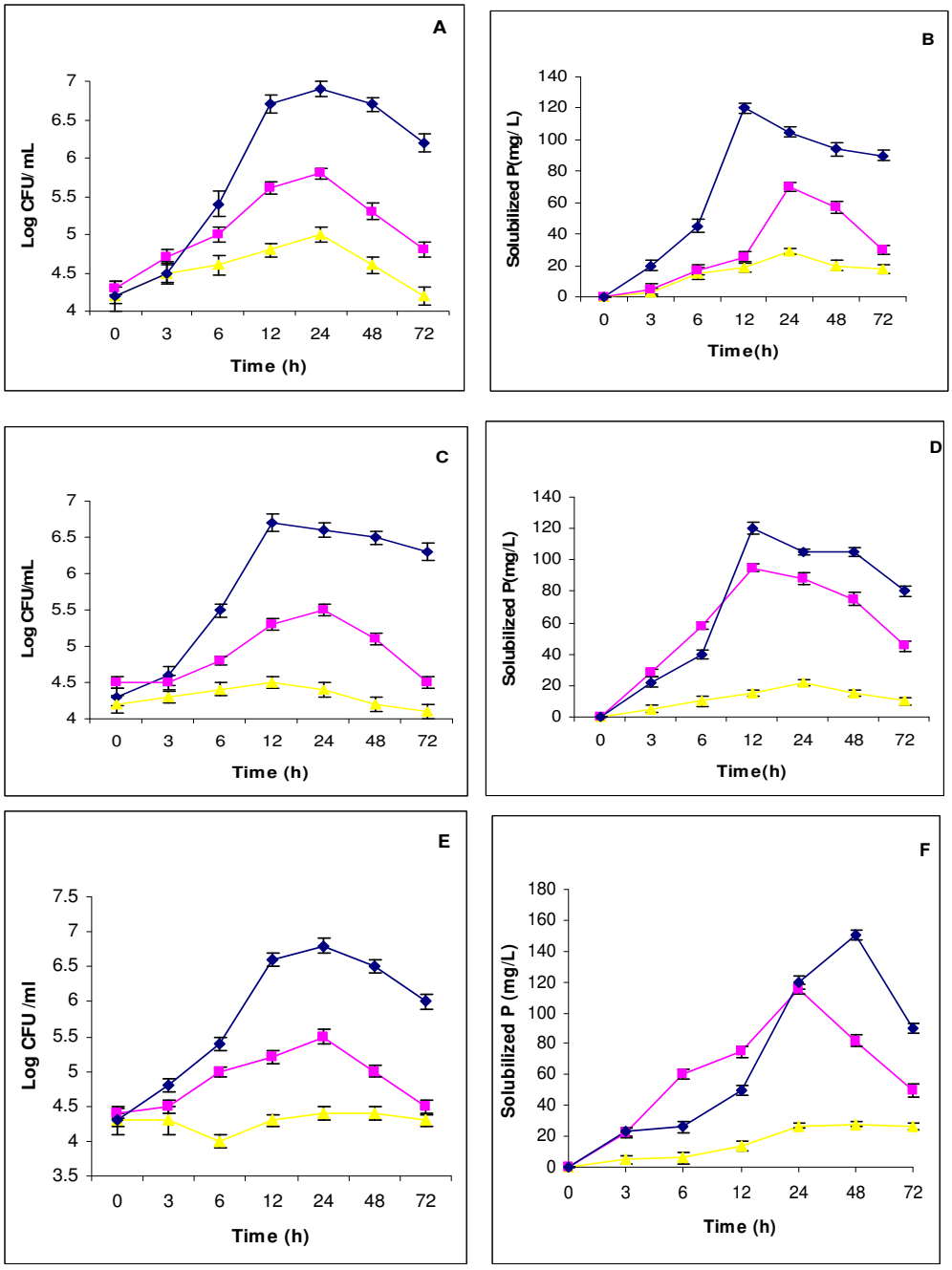

Figure 4. Effect of different temperature (25ºC ◆, 35 ºC ■, 42 ºC ◆) on growth and solubilized P (mg/l) of strains PS5, PS7, PS13.

2002). It could be concluded that these bacteria especially PS13 could be studied more to be used as biofertilizer under stress conditions of Iran plantation soil.

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