

Phosphate solubilization characteristics of efficient nitrogen fixing soil *Azotobacter* strains

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ABSTRACT

Background and Objectives: *Azotobacter* is a diazotroph bacterium reported to possess various plant growth-promoting characteristics. The aim of this study was to isolate *Azotobacter* strains capable of fixing nitrogen and effectively hydrolyzing both organic and inorganic P_i compounds.

Materials and Methods: In this study, soil samples collected from a diverse range of slightly alkaline soil types were screened for *Azotobacter* isolates. The inorganic and organic phosphate solubilization potentials of twenty competent phosphate solubilizing *Azotobacter* isolates were assessed. Variations were noted in the solubilization potentials.

Result: Three isolates, identified as *Azotobacter vinelandii* strains O2, O4 and O6, were able to fix atmospheric N₂ effectively. The nitrogenase activity of these isolates ranged between 158.6 and 326.4 C₂H₄h⁻¹vial⁻¹ (ethylene). Bacterial growth rates and phosphate solubilization activities were measured quantitatively under various environmental conditions. A close association was evident between phosphate solubilizing ability and growth rate as an indicator of active metabolism. All three phosphate solubilizing bacteria (PSB) were able to withstand temperature as high as 45°C, high concentration of NaCl (upto 5%) and a wide range of initial pH from 5 to 10 while hydrolyzing phosphate compounds actively.

Conclusion: *Azotobacter vinelandii* strains O2, O4 and O6 are superior candidates for biofertilizers that may result in the reduction of chemical nitrogen and phosphate fertilizers leading to increase crop production.

Keywords: *Azotobacter*, Biofertilizer, Nitrogen fixation, P_isolubilizing

INTRODUCTION

Nitrogen (N) is the most important nutrient and occupies the highest requirement position in plant nutrient compared to the other essential nutrients (1). N is widely distributed throughout the lithosphere, atmosphere, hydrosphere and biosphere. Only a very small part of this element is presents in the soil, as

both inorganic and organic forms. The inorganic N in the soil is only a small fraction of the total soil N (2). The total N contents of surface mineral soils normally ranges between 0.05 and 0.2 percent, in most cases less than five percent, is directly available to plants, mainly as nitrate (NO₃²⁻) and ammonium (NH₄⁺). Organic N slowly becomes available through mineralization as well (1). Most of the N is to form of dinitrogen (N₂) in the earth's atmosphere. N₂ cannot be used directly by plants and animals; therefore, N₂ must be first 'fixed' or converted into a form such as ammonia before being consumed (2-4). N enters the biosphere through biological nitrogen fixation (BNF) by microorganisms (3,5). N fixation is a process of reducing atmospheric nitrogen to ammonia, catalyzed by the nitrogenase enzyme complex (6-8).

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In the fields, phosphorus (P) is the second most essential macro nutrients for plant growth and development that is absorbed only in soluble forms of phosphate ion (P_i), HPO_4^{2-} or $H_2PO_4^-$. In fact, total soil phosphorus content is well beyond plant needs (400–1,200 mg/kg); however, it is mostly immobilized in the forms of organic and inorganic compounds such that the only a small fraction of P (1 mg kg^{-1} or less) is available for plant growth (for reviews see (3, 9)). Even, a large portion of added chemical P_i fertilizer is immobilized rapidly in soil to its insoluble forms (10, 11). Therefore, the availability of P_i is highly dependent on chemical compositions and biological processes occurring in the soil, particularly within the rhizosphere micro environment (10). It is generally accepted that the release of insoluble and fixed forms of P carried out by the action of phosphate-solubilizing bacteria (PSB) via the secretion of low molecular weight organic acids mainly gluconic and keto-gluconic acids and phosphatases (9, 12-15). These acids are produced in the periplasm of many Gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation), consequently, the organic acids diffuse freely outside the cells and may release high amounts of soluble P from mineral phosphates, by supplying both protons and metal complex organic acid anions (14, 15).

It has already been shown that the use of nitrogen fixing bacteria with high effective ability in solubilizing organic and inorganic phosphates is one of the most feasible strategies for the development of sustainable agriculture. This can supply sufficient N_2 and assist the hydrolysis of a wide range of P compounds leading to increased crop production (3, 9, 16). Among the nitrogen-fixing bacteria those belonging to genus *Azotobacter*, play a significant role (17, 18). *Azotobacter* is a gamma-proteobacterium belonging to the family *Pseudomonadaceae*. It is an obligate aerobic, free-living Gram-negative, broadly dispersed in diverse environments, such as soil, water and sediments (19). In fact, *Azotobacter* has beneficial effects on plant yields, due to their ability of fixing nitrogen (6, 18, 20, 21), solubilizing phosphates (4, 16, 22) and to the microbial secretion of stimulating phytohormones, like gibberellins, auxins and cytokinins (4, 22). An additional reason which justifies the interest on this microorganism is that the species *A. vinelandii* and *A. chroococcum* produces metabolically dormant cysts. The cyst is formed under unfavorable environmental conditions

and is the most important basis of application of *Azotobacter* strains in various environments (20, 23, 24). The use of native soils *Azotobacter* is an important aspect for biofertilizer applications as it is well adapted to the natural climates of their habitat and particularly with respect to applications under harsh environmental conditions.

The aim of this research was to isolate *Azotobacter* strains capable of fixing nitrogen and effectively hydrolyzing both organic and inorganic P_i compounds. As a new approach, we have set up a method for screening of *Azotobacter* with high phosphatase activity. The effects of several environmental conditions on propagations and P_i solubilizing activities of the isolates were assessed too.

MATERIALS AND METHODS

Bacterial isolation and identification. Sixty-four different soil samples from the rhizosphere of agricultural crop of Iran were transferred to laboratory (Tehran, Qazvin, Guilan, Mazandaran, Ardabil and Isfahan). Strategies used for isolation were: (i) Enrichment of *Azotobacter* strains, one gram from each of the soil samples were added into 100 ml Erlenmeyer flasks containing 20 ml of Azotobacter broth of the following composition; mannitol 20g, K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 6H_2O$ 0.10 g, $CaCO_3$ 20 g, $NaMoO_4 \cdot 2H_2O$ 0.05 g supplemented with $ZnSO_4 \cdot 7H_2O$ 10 mg, $MnSO_4 \cdot 4H_2O$ 1.0 mg and cycloheximide (100 μ g/ml) per liter (Adjust to pH 7.2). Incubation was at 28°C for 2-5 days. (ii) Isolation was carried out by preparing serial dilutions from enrichment culture followed by streaking and incubation at 28°C for 2-5 days. All the isolates were subcultured on selective nitrogen-free specific medium Azotobacter Agar plates.

Physiological and biochemical characteristics were performed according to Bergey's Manual of Systematic Bacteriology instructions (19), including colony morphology, the Gram staining, cyst and PHB granules staining as well as production of pigment. Molecular identification was performed with PCR using selective *nifH-gI* primers fD1 (5'-GGTTGTGACCCGAAAGCTGA-3') and rP1 (5'-GCGTACATGGCCATCATCTC-3') (5).

Three selected isolates with the highest nitrogen fixation properties were identified by 16S rDNA amplification and sequencing. Amplifications of the rDNA gene of bacterial strains were carried

out using universal bacterial primers (fD1) 27F; (AGAGTTTGTATCCTGGCTCAG) and (rP1)1492R; (GGTTACCTTGTTACGACTT) to amplify ~1.5 kb product from 16S rDNA (25). Sequence similarities were analyzed at NCBI GenBank database and BLAST program at <http://www.ncbi.nih.gov/blast>.

Phosphate solubilization. To examine P_i solubilization capabilities, 20 μ l of the bacterial suspensions ($\sim 10^4$ CFU/ml) was spotted on the center of Sperber medium plate containing insoluble P_i . Sperber medium composed of 10 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.25 g $MgSO_4 \cdot 7H_2O$ was supplemented with 2.5 g $Ca_3(PO_4)_2$ (TCP) and 15 g agar (in solid medium) per liter at pH: 7.2 (26). The inoculated plates were incubated at 28°C. The diameter of zone of clearance (halo) surrounding the bacterial colony as well as the diameter of colony were measured after 2, 4 and 7 days in triplicates. P_i solubilizing index (PSI) was calculated as the ratio of diameter of halo (mm)/diameter of colony (mm) (27).

All the isolates were also assayed for phosphatase activity according to the method described by Malboobi *et al.* (26). The isolates were cultured on the Sperber agar medium containing 50 mg/l BCIP (5-bromo-4-chloro-3-indolyl phosphate; Sigma, St. Louis, Mo) in the presence of soluble phosphate (KH_2PO_4) or insoluble phosphate (TCP) and incubated at 28°C. Phosphatase activities in isolates were compared based on scoring the intensities of blue stained colonies at 24, 48 and 72 hours.

Nitrogen fixation and Nitrogenase activity. The isolates able to solubilize phosphate on Sperber agar were used for nitrogen fixation capability. The N-fixing efficiency of these isolates was assessed by the acetylene reduction assay (ARA) as described by Hardy *et al.* (28). Briefly, 5 ml of the Azotobacter broth in 12 ml vials was inoculated with $\sim 10^4$ CFU/ml of each isolate and incubated at 28 °C for 48-96 h. Once visible growth was observed, the vials were plugged with rubber. 10% of the head space (7 ml) was injected with pure acetylene gas (C_2H_2) after removing an equal amount of air from vials by means of a disposable plastic syringe (29-31). The gas samples (0.7 μ l) were removed after 24 h incubation, and were assayed for ethylene production with a gas chromatograph (GOW MAC - GM 816 model). The chromatograph was fitted with Poropak N column and a H2-FID detector. The rate of nitrogen fixation

was calculated (30, 31) and values were expressed as nanomoles of ethylene produced per hour per vial ($nmoles C_2H_4 h^{-1} vial^{-1}$) (31, 32).

Bacterial growth and phosphate solubilization. Two indices, including growth index (GI) which is the logarithm of CFU/ml of culture and P_i solubilizing index (PSI) that represents the amounts of hydrolyzed P_i from TCP, were monitored. Time-coursed quantitative measurements were carried out for three isolates with the highest nitrogen fixation (O2, O4 and O6) in 100 ml Erlenmeyer flasks containing 25 ml of Sperber broth medium. 100 μ l of bacterial suspension (with $\sim 10^4$ CFU/ml of each isolate) were inoculated into medium and incubated at 28°C and 120 rpm. The effect of temperature was examined by incubation at 15, 28, 37 and 45°C. For high-salt treatments, the extra amounts of salt (1, 2.5, 5 and 7%; w/v) were added to Sperber medium. Initial pH values of medium ranging from 5 to 9 were adjusted by the addition of either KOH or HCl accordingly. In general, no added NaCl, pH: 7 and temperature of 28°C was used as control conditions. Sampling was carried out within 96 h. For estimation of the growth rate, 100 μ l of medium was removed after 3, 6, 12, 18, 24, 36, 48, 72 and 96 h. Serial dilution were prepared and the growth curve was drawn, while simultaneous released P_i in culture supernatant was measured. In brief, 400 μ l of the medium were removed and centrifuged for 10 min at 5000 rpm. 200 μ l from supernatant were mixed with an equal amount of vanadate-molybdate reagent and diluted to 2 ml with ddH₂O. After 10 min, the optical densities of the samples were measured at 450 nm against a blank. PSI was estimated using standard curve. All the experiments were performed in triplicates.

Data analysis and graph drawing were done using statistics software Graph Pad Prism (v5.0.4) and Microsoft Excel program.

RESULTS

Bacterial isolation and identification. A total of 43 isolates, named O1 to O43, were selected after enrichment, isolation and screening from 64 soil samples. According to biochemical characteristics and molecular method, three species of *Azotobacter*, viz., *A. chroococcum*, *A. virelandii* and *A. beijerinckii* were identified. Three isolates with the highest

nitrogenase activity, O2, O4 and O6 were chosen for further characterization. Several biochemical and biological indicators as well as 16S rDNA sequence were inspected to figure out the taxonomy of these isolates. The compiled data (not shown) revealed that O2, O4 and O6 isolates must be strains of *Azotobacter vinelandii*. 16S rDNA sequences of these isolates (GenBank accession Nos. EU935082, EF620439 and EF634040) were all in agreement with the biochemical tests.

Phosphate solubilization activity. Phosphate solubilization was measured in all isolates; as shown in Table 1, twenty isolates were able to produce clear zone or blue color in minimal medium containing insoluble P_i or synthetic substrate, respectively, in the course of plating compared to the other isolates. In a comparative experiment set up, the largest clear zone was observed for O4 (21.5 mm) followed by O25 (20.6 mm) within the first 2 days whereas O1, O3 and O32 isolate did not show any clear zone. The PSI values were increased in the time course of culture of each isolate. The highest level of PSI was observed for O4 (3.5 ± 0.1) during 7 days while the lowest rate was for O24 (-1.2) during the same period of time (Table 1). The strongest blue color was developed by O4, O17 and O25 within the 24 h and 48h while the others stained weakly (Table 1). Phosphatase activity was not detected in O9 and O24.

Nitrogen fixation and nitrogenase activity. Nitrogenase activities were determined by checking the acetylene reduction activity for phosphate solubilizing isolates. Rates obtained in samples were in the range of 12.1 to 326.4 nmol C_2H_4 /h/vial while cell numbers were adjusted to 10^7 CFU/ml. Five isolates showed the highest N_2 -fixing activity. Three isolates (O2, O4 and O6) were identified as *A. vinelandii* and the other two strains (O1 and O3) were *A. chroococcum*. The acetylene reduction activity (ARA) result of pure cultures of these strains is shown in Table 2. *A. vinelandii* O6 showed the highest activity (326.4nmol C_2H_4 /h/vial) followed by strain O2 with an activity of 265.5nmol C_2H_4 /h/vial. Strain O5 had the lowest nitrogenase activity (12.1nmol C_2H_4 /h/vial).

Correlation between bacterial growth and phosphate solubilization. Three isolates with the highest nitrogen fixation (*A. vinelandii* O2, *A.*

vinelandii O4 and *A. vinelandii* O6) were chosen for further investigations. In a series of preliminary experiments, the effects of various growth conditions on the survival of these isolates were assessed. *A. vinelandii* O2 was capable of growing and P_i solubilization on solid medium with the added salt of up to 2.5% between 25 to 45°C and initial pH 6-8. *A. vinelandii* O4 was capable of P_i solubilization at 15 to 40°C, pH 5-9 and salt as high as 7% when grown on solid medium. In contrast, *A. vinelandii* O6 was able to form colonies at a range of 15-45°C, pH 5-9 and 5% salt. P_i solubilization were observed only at 20-40°C, pH 5-8 and salt concentration of 2.5% for this strain.

This study was further carried out in liquid minimal medium for GI and PSI. In all cases, a close association between GI and PSI was noticeable (Figs. 1-3). Under such conditions, the maximum levels of both GI and PSI for *A. vinelandii* O2 reached after 48 h of incubation at 28°C while exponential growth phase of *A. vinelandii* O6 delayed for 72 h and arrived at stationary phase and maximum PSI (\sim 230mg/l) after 72 h. Generally, bacterial growth and consequently PSI of both bacteria were reduced at both high and low temperatures (Fig. 1). In comparison; the most PSI was obtained for *A. vinelandii* O4 after 24 h at 37°C (225 mg/l) (Fig. 1). This was gradually decreased when incubation was extended beyond 24 h. Bacterial growth and consequently PSI of this strain were reduced at 15°C and 45°C (Fig. 1). This was more pronounced for O4 strain such that it never entered exponential growth phase when incubated at 45°C, although they were alive.

As shown in Fig. 2, increased NaCl concentration in medium was associated with reduced bacterial growth and PSI values. In other words, the least amount of both GI and PSI values were detected at 7% NaCl. A different pattern was observed for O4 strain; P_i solubilization was delayed at low salt medium and the highest PSI value at 24 h in samples obtained from medium with 1% salt. PSI values for O4 were inversely proportional to the GI, particularly when the added salt was higher than 1%.

Medium inoculation at various initial pH values resulted in reduced bacterial growth and PSI of O2 and O6 isolates in both alkaline and acidic media. However, the PSI values for both isolates at acidic pH were higher than the alkaline pH (Fig. 3). While the growth range of O4 was different at various pH levels, the initial pH did not affect the PSI values of

Table 1. The solubilization efficiency of *Azotobacter* strains in plate assay for which Pi solubilizing index (PSI) and presence or absences of phosphatase activity are given

Bacterial strain	Pi solubilizing index (PSI) ^a			Phosphatase activity ^{b,c}					
	2days	4days	7days	With soluble Pi			With insoluble Pi		
				24 h	48 h	72h	24 h	48 h	72h
O1	0	1.7±0.1	2.1±0.1	+	+	+	+	+	+
O2	1.4	1.5±0.1	2±0.2	+	+	+	+	+	+
O3	0	1.7±0.3	2.1±0.1	+	+	+	-	+	+
O4	2.7±0.2	3.3±0.1	3.5±0.1	++	++	++	++	++	++
O5	1.6±0.1	1.7±0.1	1.8±0.3	+	+	+	+	+	+
O6	1.6±0.2	1.7±0.1	1.9±0.1	+	+	+	+	+	+
O8	1.7±0.3	2±0.1	2.4±0.1	+	+	+	-	+	+
O9	1	1.1±0.1	1.3±0.1	-	-	-	-	-	-
O11	1.1	1.4±0.2	1.6±0.3	+	+	+	-	+	+
O14	2.6±0.4	2.8±0.2	3.2±0.1	-	-	+	-	-	+
O15	1.2±0.1	1.3	1.4±0.1	+	+	+	+	+	+
O16	1.4	1.7±0.1	1.8±0.2	+	+	+	+	+	+
O17	2.4	3±0.3	3.4±0.3	++	++	++	+	+	++
O21	1.5	1.6±0.2	1.7±0.2	+	+	+	+	+	+
O24	1	1±0.1	1.2±0.1	-	-	-	-	-	-
O25	2.2±0.2	2.6±0.2	3	++	++	++	++	++	++
O32	0	1.3±0.2	1.4±0.2	+	+	+	+	+	+
O33	1.1	1.4±0.2	1.5±0.2	-	+	+	-	-	-
O34	1.4	1.4	1.6±0.1	+	+	+	+	+	+
O39	1.7	1.8±0.1	1.8±0.1	+	+	+	+	+	+

^aResults presented are means of three replicates ± standard errors

^bSoluble phosphate is KH₂PO₄ and insoluble phosphate is TCP

^c + Light blue stain and ++ dark blue stain

O4 isolate, except for alkaline broth. Maximum PSI was obtained when incubation was extending for more than 72 h. The highest PSI values in acidic and alkaline pH were for O4 strain (Fig. 3).

DISCUSSION

A diverse group of rhizobacteria, collectively called plant growth promoting rhizobacteria (PGPR), has been reported to enhance the growth of plants

directly and/or indirectly. As one of the most known groups of PGPR, nitrogen fixing bacteria have ability of phosphate solubilization considered to main constituents of biofertilizers (3, 9, 16). Of the most widely used method to study N₂ fixation in asymbiotic systems, the ARA is simpler and faster than the other methods because it is easily measured by gas chromatography (20). The results presented in this paper are in agreement with the previous works. Rodelas *et al.*(33) reported the ARA results of pure

Table 2. Acetylene reduction activities in the selected isolates with maximum nitrogenase activity.Values are means of triplicates followed by the standard deviation.

Isolates	Nitrogenase activity (nmol C ₂ H ₄ h ⁻¹ vial ⁻¹)	Identified strain
O1	87.2 ± 4.6	<i>A.chroococcum</i>
O2	265.5 ± 7.2	<i>A. vinelandii</i>
O3	135.7 ± 3.6	<i>A.chroococcum</i>
O4	158.6 ± 4.7	<i>A. vinelandii</i>
O6	326.4 ± 8.7	<i>A. vinelandii</i>

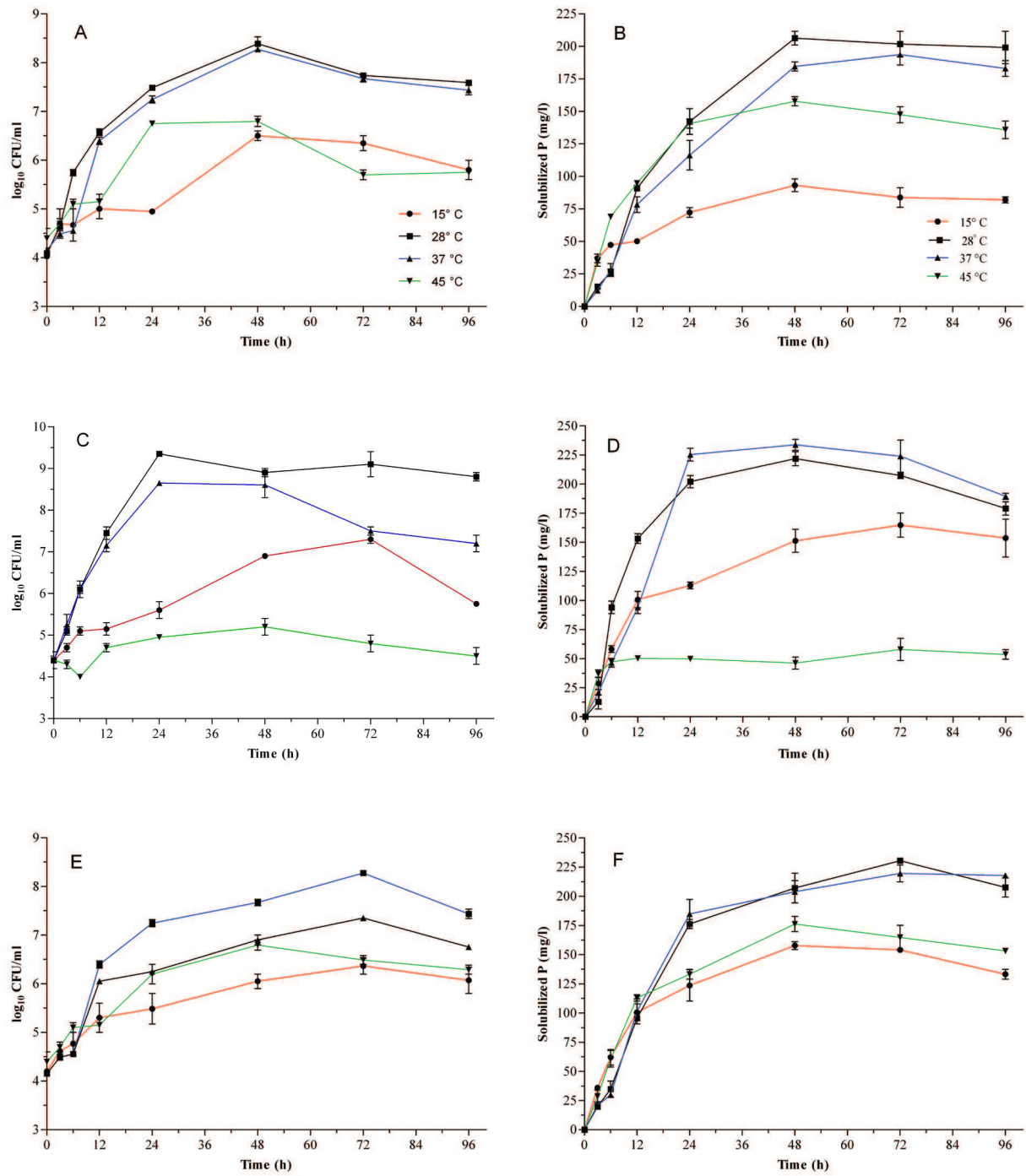


Fig 1. Bacterial strains O2 (panels A and B), O4 (panels C and D) and O6 (panels E and F) were grown in liquid medium containing tricalcium phosphate. All data points are the means of three replicates. Standard errors are shown by vertical bars.

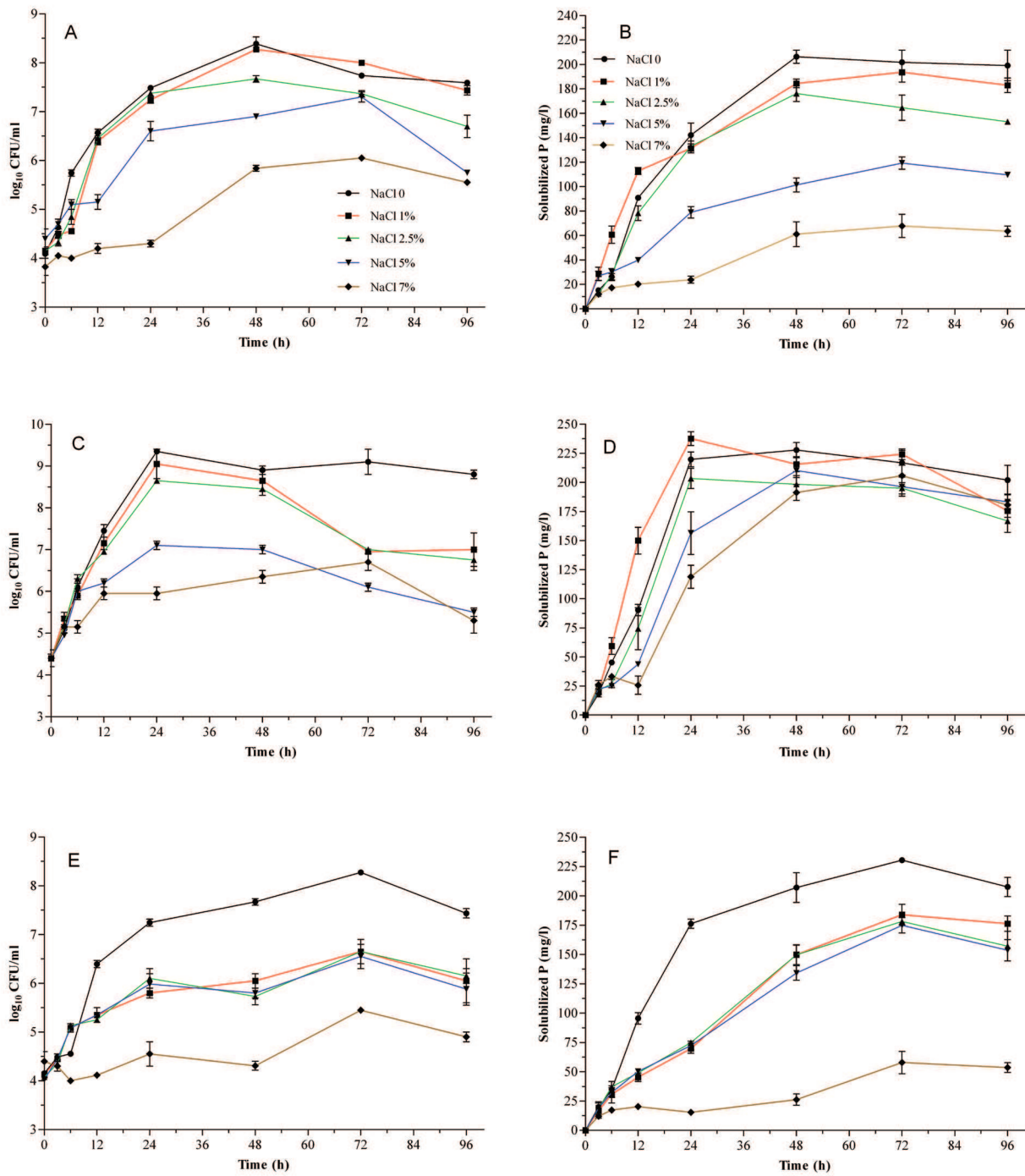


Fig 2. Panels are arranged as Fig. 1. All data points are the means of three replicates. Standard errors are shown by vertical bars.

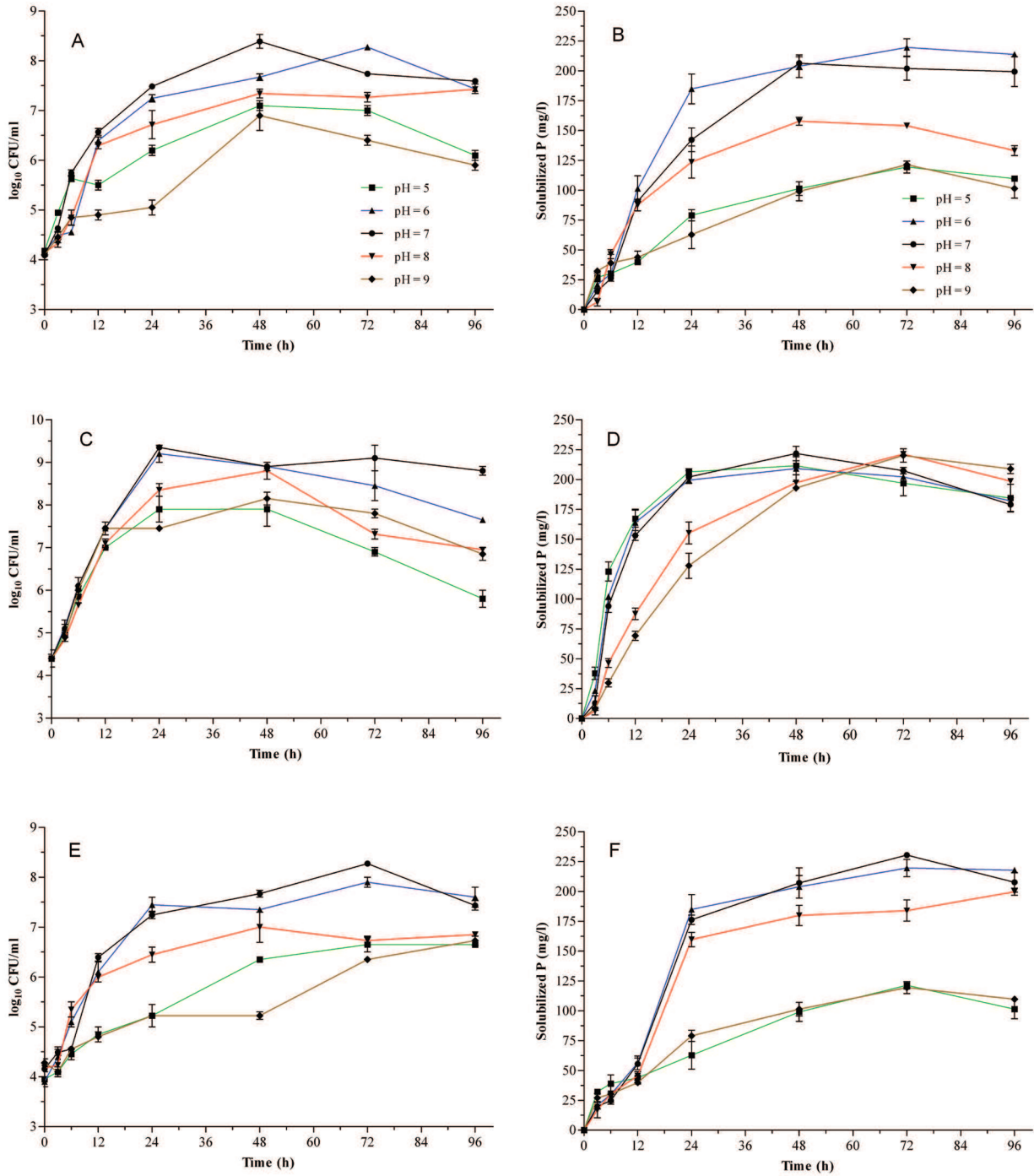


Fig 3. Panels are arranged as Fig. 1. All data points are the means of three replicates. Standard errors are shown by vertical bars.

cultures of strains were between 9.70 to 257.73 nmol C₂H₄ h⁻¹ vial⁻¹. It is worth noting that *A. chroococcum* isolates gave high values of ARA. In another study, Tejera *et al.* (21) surveyed efficient nitrogen fixation *A. chroococcum* isolates from soil or rhizosphere samples with the range of 79.6 to 329.5 nmol C₂H₄ h⁻¹ culture.

Although, most studies have focused on biological nitrogen fixation of *Azotobacter* but several researchers paid attention to other characteristic termed as PGPR. In order to minimize the use of expensive chemical fertilizers, in this study we investigated the potential of phosphate solubilization in these strains of *Azotobacter*. As previously reported by Kumar *et al.* (27), Garg *et al.* (16) and Farajzadeh *et al.* (22), the *Azotobacter* isolates were able to dissolve inorganic and organic phosphate compounds. The PSI results obtained in the solid medium was significantly higher than other observations and corroborated with Farajzadeh *et al.* (22), indicating that indigenous strains of *Azotobacter* isolated from alkaline soils are effective P_i solubilizers. The presented results showed that isolated P solubilizing and N-fixation *Azotobacter* could serve as efficient biofertilizer candidates for improving the N and P nutrition of crop plants simultaneously.

Nevertheless, the performance of PGPR as biofertilizers is severely influenced by both biotic and abiotic environmental conditions of the targeted regions. This has lead researchers to seek out isolation of PGPR from native soil and to explore methods for screening PGPR strains and subsequent evaluations for specific criteria particularly with respect to applications under various environmental conditions. Tolerances to extreme climates are of special interests for bacteria to be used as biofertilizer in arid and semi-arid regions. It has been evidenced that the members of the genus *Azotobacter* produce copious amount of capsular slime. This property allows adaptation of these species to the stressful environments. Besides, it is suggested that the cyst formation under unfavorable environmental conditions reduces oxygen transferring into the cell and consequently increases the nitrogenase activity (23, 34). Therefore, the isolates were intensively examined for tolerance toward high temperature, high concentration of NaCl and a wide range of pH (Figs. 1-3). All PSB strains survived under the examined conditions. Decreased GI and PSI at 45°C for the isolate are acceptable as

in natural situation these bacteria survive hot noons in summer while continue growing on the rest of the times. All isolate tolerated the added NaCl to more than 2.5% indicating that these isolates would functionally be active in most cultivated lands in which the salinity of soil is usually below 2.5%. As a key feature of cultivation soils, we measured GI and PSI of bacteria grown in medium with various initial pH ranging from 5 to 9. The fact that the highest GI and PSI values for isolates were obtained at high pH suggests that these bacteria favor alkaline condition. These results indicate that the isolated *Azotobacter* could be used as biofertilizer in agricultural soils in Iran which are predominately calcareous and are characterized by a high pH and low amounts of plant available phosphorus (35).

Islam *et al.* (36) investigated the effects of pH, temperature and salt on growth of *Azotobacter spp.* isolated from crop rhizosphere. Even though no data was presented for PSI, they however showed tolerance for a number of their isolated strains toward different environmental factors such as NaCl (0-0.8%) and temperature (20– 40°C) while no isolate survived at 50°C. Rajeswari *et al.* (37) isolated *Azotobacter* species which showed tolerance toward the extra amounts of salt (3.5%) in an optimized medium. Tejera *et al.* (21) assessed growth rates at different initial pH values (4- 9) and showed that a lower number of isolates grew on N-free media at pH value as high as 8.7. Ravikumar *et al.* (31) investigated the effects of salinity on nitrogen fixation and also indole acetic acid (IAA) production. They found a maximum level of IAA production and nitrogenase activity at the salinity of 20 g/l and 30 g/l NaCl, respectively.

A noticeable reduction in PSI during stationary phase of growth found in all cases supports the dependence of PSI on bacterial metabolism. It was also shown that the phosphatase activity of bacterial strain could synergistically enhance the release of Pi in the acidified medium (Fig. 3). The advantage of bacteria capable of phosphate solubilizing with simultaneous secretion of organic acids and phosphatase activity on production and yield were shown in both green house and field trials (38).

In conclusion, we have isolated several *Azotobacter* strains, three of which were more promising for being used as N and P biofertilizer. These bacteria are well adapted to various environmental conditions.

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