ORIGINAL ARTICLE

Effect of salt on survival and P-solubilization potential of phosphate solubilizing microorganisms from salt affected soils

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Abstract A total of 23 phosphate solubilizing bacteria (PSB) and 35 phosphate solubilizing fungi (PSF) were isolated from 19 samples of salt affected soils. The ability of 12 selected PSB and PSF to grow and solubilize tricalcium phosphate in the presence of different concentrations of NaCl was examined. Among 12 PSB, Aerococcus sp. strain PSBCRG1-1 recorded the highest (12.15) log viable cell count at 0.4 M NaCl concentration after 7 days after incubation (DAI) and the lowest log cell count (1.39) was recorded by Pseudomonas aeruginosa strain PSBI3-1 at 2.0 M NaCl concentration after 24 h of incubation. Highest mycelial dry weight irrespective of NaCl concentrations was recorded by the Aspergillus terreus strain PSFCRG2-1 (0.567 g). The percent P release, in general, was found to increase with increase in NaCl concentration up to 0.8 M for bacterial solubilization and declined thereafter. At 15 DAI, strain Aerococcus sp. strain PSBCRG1-1 irrespective of NaCl concentrations showed the maximum P-solubilization (12.12%) which was significantly superior over all other isolates. The amount of P released in general among PSF was found to decrease with increase in NaCl concentration at all the incubation periods. Aspergillus sp. strain PSFNRH-2 (20.81%) recorded the maximum P release irrespective of the NaCl concentrations and was significantly superior over all other PSF at 7 DAI.

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1. Introduction

Microorganisms have the ability to solubilize the insoluble phosphates and maintain the nutrient status of soil (Richardson, 2001). Microorganisms are central to the soil P cycle and play a significant role in mediating the transfer of P between different inorganic and organic soil P fractions, subsequently...
releasing available P for plant acquisition (McLaughlin et al., 1988; Oberson et al., 2011). There are two aspects in microbial P solubilization: firstly, P_i released by solubilization processes (Rodriguez and Fraga, 1999) and secondly, P released from accumulated P in biomass of microorganisms (Oehl et al., 2001). Inorganic phosphate solubilizing microorganisms constitute various portions of the soil microbial population and vary from soil with soil (Banik and Dey, 1982; Kucey et al., 1989). The populations of the PSM are reportedly varied and ranged from very low (less than 10^5 CFU g^-1 of soil) in a soil in Northern Spain to very high (3 x 10^8 CFU g^-1 of soil) in Quebec, Canada (Chabot et al., 1993; Piew et al., 2001). Phosphate solubilizing microorganisms were isolated from rhizosphere soils of different crops. The establishment and performance of these microbes are affected severely under stress such as high salt, pH, and temperature prevalent in degraded ecosystems such as alkaline soils with a tendency to fix phosphorus (Johri et al., 1999). In the saline-alkaline soils of the tropics, salt concentrations may be as 2%, pH as high as 10.5, and temperature may range between 35 and 45 °C, which may result in poor growth and survival of PSMs. However, scanty information is available on the occurrence of PSMs in salt affected soils. The present investigation was aimed to isolate PSMs from salt affected soils that could survive and solubilize insoluble phosphate efficiently in the presence of higher salt concentration so as to obtain efficient isolates for application as a potential biofertilizer in saline or problematics soils.

2. Materials and methods

2.1. Collection of soil samples and their characterization

A total of 19 rhizosphere and non-rhizosphere soil samples (0–15 cm depth) from the salt-affected areas of Koppal (Gangavathri, Marathli, Herur, Vaddarahaatti), Belgaum (Hooli) and Dharwad (Alagawadi and Benakoppa villages) districts of Karnataka were collected and four soil samples of Indore from AICRP on saline water, College of Agriculture were also used for isolation of PSMs. After collection samples were stored in plastic bags at low (4 °C) temperature until further processing. The soil samples were analyzed for pH, EC_e, ESP and calcium and magnesium contents (Supplementary Table 1). The pH of the soils ranged from 7.7 to 8.5.

2.2. Isolation, characterization and identification of PSMs from salt affected soils

The mineral phosphate solubilizing microorganisms (PSMs) were isolated from all the soil samples by dilution plating on Pikovskaya’s agar medium (Pikovskaya, 1948) containing tricalcium phosphate (TCP). The plates were incubated at 28 ± 2 °C for two to seven days and colonies with clear zones around were counted. The representative colonies of each type of bacteria and fungi with a clear halo around were purified, subcultured and maintained on the slants of Pikovskaya’s agar for further use. The PSBs isolated from salt affected soils were identified up to generic level based on their colony morphology and microscopic examination as outlined in the manual of Gilman (1957). Molecular identification of bacterial isolates was carried out using 16S rRNA gene amplification using a universal primer pair PA (5’AGAGTTGATCCTTGCCGACTC3’) and PH (5’GGGAGGTGATCCACGCGCA3’) (Edwards et al., 1989). Similarly, fungal 18S rRNA was amplified using universal primer pair NS1 (5’GTAGTCTATGCTTGTCCTC3’) and NS8 (5’TCCGACGGTTACCTACGGA3’) (White et al., 1990). The amplified rDNA was sequenced and subjected to identification using BLAST homology search. The reference culture Pseudomonas striata was obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India and Aspergillus awamori MTCC 6486 was procured from a culture collection of Institute of Microbial Technology, Chandigarh, India.

2.3. Influence of NaCl on Growth and P-solubilizing ability of the isolates

The ability of 12 selected isolates each of bacteria and fungi to grow and solubilize TCP in the presence of different concentrations of NaCl was examined by estimating growth, P_i release from TCP.

2.3.1. Estimation of growth of PSM isolates

The overnight cultures of bacterial isolates were inoculated into 50 ml Luria broth containing 0.09 (control), 0.4, 0.8, 1.2, 1.6 and 2.0 M NaCl and the growth was observed at an interval of 24 h up to eight days by determining the viable cell counts by serial dilution plate count method using Pikovskaya’s agar medium.

Three days old homogenized cultures fungal isolates were inoculated into 50 ml of potato dextrose broth containing 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl and incubated at 28 ± 2 °C for seven days. After incubation the cultures were filtered through pre-weighed Whatman No. 1 filter papers and dried to constant weight at 60 °C in a hot air oven. The dry mycelial weight was then recorded for each culture.

2.4. Estimation of Pi released from solubilization of TCP

All the selected bacterial and fungal isolates were inoculated into 50 ml of Pikovskaya’s broth containing 0.002 (control), 0.4, 0.8, 1.2, 1.6, and 2.0 M NaCl as above. Six replicate flasks for bacteria and eight replicate flasks for fungi were maintained at each concentration of NaCl along with an equal number of replicates as uninoculated controls. The flasks were incubated at 28 ± 2 °C on a rotary shaker for 15 days in case of bacteria and nine days in the case of fungi. The amount of P_i released by the bacterial isolates was estimated at 5, 10 and 15 days after inoculation using two flasks at each period and that by fungal isolates at 3, 5, 7 and 9 days after inoculation. P_i released in the broth was estimated by phosphomolybdic blue method (Jackson, 1973).

3. Results

3.1. Characteristics of soils used for isolation of P-solubilizers

The soil samples used for isolation of PSMs were also analyzed for pH, EC_e, ESP and calcium and magnesium contents (Supplementary Table 1). The pH of the soils ranged from 7.7 to 8.5.
9.6, electrical conductivity from 3.0 to 51.5 dS m⁻¹, ESP from 12.6 to 58.5 percent, calcium from 6.6 to 61.2 meq 100 g⁻¹ soil and magnesium from 1.25 to 22.80 meq 100 g⁻¹ soil. The rhizosphere and non-rhizosphere soil samples from various patches of salt-affected areas were used for enumeration of total bacteria, fungi and actinomycetes as well as phosphate solubilizers (Supplementary Table 2). The population of total bacteria ranged from 4.0 × 10⁵ to 34.5 × 10⁵ CFU g⁻¹ soil, that of total fungi ranged from 1.5 × 10³ to 30.5 × 10³ and actinomycete population ranged from 7.5 × 10³ to 75.5 × 10³ CFU g⁻¹ soil⁻¹. While all the 19 samples showed the presence of PSB, only 15 samples showed the presence of the PSF.

### 3.2. Identification of PSMs from salt affected soils

By using the microscope, morphological and biochemical tests alone, we were able to achieve generic identification of more than 80% of the isolates, which include several frequently occurring genera such as 10 isolates of Aspergillus, 6 isolates of Pseudomonas, two isolates of Penicillium, one each of Enterobacter and Bacillus. Further reliable generic identification was carried out using 16S and 18S rRNA gene amplification and BLAST homology search. No discrepancy was found between the generic identities determined by the conventional and molecular approaches. A total of 24 partial 16S rDNA and 18S rDNA sequences have been submitted to the GenBank under the accession numbers HQ393855-HQ393878 (Supplementary Table 3).

### 3.3. Effect of NaCl on bacterial growth and viable cell counts

The growth was recorded by counting the viable cell population over a period of eight days at an interval of 24 h by serial dilution plate count method. In general, the population counts (log viable cell counts) of majority of the isolates were found to be higher than that of the reference strain (P. striata), particularly at higher NaCl concentrations. Among 12 PSB isolates, strain Aerococcus sp. strain PSBCRG1-1 recorded the highest (12.15) log viable cell count at 0.4 M NaCl concentration after eight days of incubation and the lowest log cell count (1.39) was recorded by Pseudomonas aeruginosa strain PSBI3-1 at 2.0 M NaCl concentration after 24 h of incubation.

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Effect of salt on survival and P-solubilization potential of phosphate solubilizing microorganisms from salt affected soils 429
### Table 3

Per cent $P_l$ release from TCP by bacterial isolates as influenced by NaCl concentrations in Pikovskaya’s broth.

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<th>PSB I$_2$-1</th>
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**Sources**

Strains (A) 
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**Sources**

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Salt conc. (B) 
A × B

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*a* Figures in parenthesis indicate the pH values of broth medium.
Table 4  Per cent P<sub>i</sub> release from TCP by fungal isolates as influenced by NaCl concentrations in Pikovskaya’s broth.

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<th>PSF WRB-2</th>
<th>PSF W&lt;sub&gt;1&lt;/sub&gt; RH-1</th>
<th>PSF NRM-1</th>
<th>PSF CRG&lt;sub&gt;1&lt;/sub&gt;-1</th>
<th>PSF NRO-2</th>
<th>PSF W&lt;sub&gt;1&lt;/sub&gt; RH-2</th>
<th>PSF ORB-4</th>
<th>PSF CRG&lt;sub&gt;2&lt;/sub&gt;-1</th>
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<th>Mean</th>
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**Sources**
- Strains (A)
- Salt concentration (B)
- A × B

**CD at 1%**
- S. Em ± 0.0453
- 0.196
- 0.0306
- 0.160
- 0.1103
- 0.402

| 9                      | 0.002                  | 15.36     | 15.56                 | 17.35    | 16.02               | 17.14    | 15.39               | 15.99    | 15.90               | 14.56   | 15.91               | 14.97   | 14.51                 | 15.88   | 13.00                 | 15.73  |                       |
| 1.2                    | 16.92                  | 12.75     | 14.38                 | 11.00    | 15.17               | 12.30    | 13.55               | 13.00    | 1.72                | 11.53   | 10.69               | 11.44   | 7.57                  | 12.46  |                       |
| Mean                   | 16.13                  | 13.31     | 15.00                 | 12.31    | 15.50               | 13.17    | 13.92               | 13.70    | 12.29               | 12.60   | 11.89               | 12.10   | 10.06                 |       |                       |

**Sources**
- Strains (A)
- Salt concentration (B)
- A × B

**CD at 1%**
- S. Em ± 0.0664
- 0.197
- 0.0361
- 0.189
- 0.1300
- 0.474
The interaction effect between isolates and NaCl concentrations was found to be significant. The isolates in general showed decrease in population to increase in NaCl concentration after 0.4 M. Among the isolates, *Alteromonas* sp. strain PSBI2-1 (7.64) showed a maximum mean population followed by *Pseudomonas mendocina* strain PSBW-RH-1 (7.54), *Enterobacter* sp. strain PSBWRB-1 (7.35) and *Pseudomonas* sp. strain PSBORB-1 (7.13) irrespective of salt concentrations and was significantly superior over all other strains.

The interaction between the strains and incubation periods was also significant. The population in general was found to increase up to 6 DAI with a slight decline thereafter in majority of the isolates. Among the isolates, *Alteromonas* sp. strain PSBI2-1 recorded maximum population (8.06) on 4 days of incubation and was significantly higher than all other strains at all other incubation periods. The interaction effect between the salt concentrations and incubation period, irrespective of strains, was also significant, wherein the maximum population (10.38) was recorded in the control treatment at eight DAI (Table 1).

### 3.4. Effect of NaCl on fungal growth

The data on mycelial mat weight (dry) of the 12 selected fungal isolates as influenced by different concentrations of NaCl are presented in Table 2. Among the PSF isolates, the highest mycelial dry weight irrespective of NaCl concentrations, was recorded by the isolate *Aspergillus terreus* strain PSFCRG2-1 (0.567 g) followed by *Aspergillus flavus* strain PSFW/RH-4 (0.537 g) both of which were significantly superior over all other isolates as well as over the reference strain, *A. awamori* (0.379 g). However, the mycelial dry weight of different strains varied from 0.140 g to 0.567 g. With increase in concentration of NaCl, there was a reduction in mycelial dry weight of fungi and the reduction was significant at 0.8 and 1.0 M concentrations. The interaction effect between strains and NaCl concentrations on mycelial dry weight was also significant wherein the strain *Aspergillus terreus* strain PSFCRG2-1 at 0.2 M NaCl recorded the highest mycelial weight (0.705 g) which was significantly superior over all other combinations except for the combinations involving the same strain with control and at 0.4 M NaCl concentration (Table 2).

### 3.5. Influence of NaCl on P-solubilization by PSB isolates

The selected efficient PSB isolates were also tested for their ability to solubilize TCP in the presence of different concentrations of NaCl in Pikovskaya’s broth. The data on percent P release in broth medium in 10 and 15 DAI are presented in Table 3. The amount of P release was found to increase with increase in incubation period irrespective of strains and salt concentrations. The percent P release, in general, was found to increase with increase in NaCl concentration up to 0.8 M and declined thereafter at all the three incubation periods. However, *Alteromonas* sp. strain PSBCRG2-1 showed the increased P release even up to 1.0 M NaCl at all the stages of incubation whereas *Pseudomonas aeruginosa* strain PSBCRG2-1 showed a similar trend at 10 and 15 DAI.

After five days of incubation, *Alteromonas* sp. strain PSBCRG2-1 showed the highest percent P release (7.18) followed by *Pseudomonas* sp. strain PSNRM-1 (7.07), *Aerococcus* sp. strain PSBCRG1-1 (6.99), *Pseudomonas* sp. strain PSBORB-1 (6.89) and *Alteromonas* sp. strain PSBI2-1 (6.72) all of which were significantly superior over other isolates including the reference strain, *P. striata* (3.47). Among different salt concentrations tested, the maximum solubilization was found at 0.8 M NaCl (6.93%) which was significantly superior over all other concentrations including control.

At 10 DAI, the highest percent P release was recorded by the *Aerococcus* sp. strain PSBCRG1-1 (9.79) followed by *Alteromonas* sp. strain PSBCRG2-1 (9.52), *Pseudomonas* sp. strain PSBORB-1 (9.50) and *Pseudomonas fluorescens* strain PSBNRM-1 (8.27) all of which were significantly superior over other strains including the reference strain, *P. striata* (4.80). Among the NaCl concentrations tested, 0.8 M NaCl recorded the highest level of solubilization (8.79%) which was significantly superior over all other concentrations and control. The isolate *Aerococcus* sp. strain PSBCRG1-1 recorded the highest solubilization (12.75%) at 0.8 M NaCl concentration, which was significantly superior over all other combinations.

At 15 DAI, strain *Aerococcus* sp. strain PSBCRG1-1 irrespective of NaCl concentration showed the maximum P-solubilization (12.12%) followed by *Alteromonas* sp. strain PSBCRG2-1 (11.08%), *Pseudomonas* sp. strain PSBORB-1 (11.04%) which were significantly superior over all other isolates including the reference strain, *P. striata* (6.62%). Among the NaCl concentrations, 0.8 M NaCl showed the highest P release (10.75%) followed by 0.4 M NaCl (10.64%) which were significantly superior over all other concentrations. The interaction effect between NaCl concentrations and strains was also significant wherein the *Alteromonas* sp. strain PSBCRG2-1 at 1.0 M NaCl recorded the highest release of P (14.18%), which was significantly higher than any of the other combinations.

### 3.6. Role of NaCl on P-solubilization by fungal isolates

The selected PSF isolates were also tested for their ability to solubilize TCP in the presence of different concentrations of NaCl in Pikovskaya’s broth. The data regarding the percent P release in broth medium at 7 and 9 DAI are presented in Table 4. The amount of P released in general was found to decrease with increase in NaCl concentration at all the incubation periods. While the control treatment showed maximum P release, 2.0 M NaCl recorded the lowest P content in broth at all incubation periods. Solubilization of TCP was increased with increase in incubation period up to 7 DAI irrespective of strains and salt concentrations and declined slightly thereafter. At 3 DAI, *Aspergillus* sp. strain PSFNKR-2 recorded the highest solubilization (9.32%) and was significantly superior over all other isolates including the reference strain, *A. awamori* (4.88%). The interaction effect between strains and NaCl concentrations was significant at 3 DAI wherein the *Aspergillus* sp. strain PSFNKR-2 at 1.0 M NaCl recorded the highest P release (10.52%) and was significantly higher than the P release in all other combinations. The same trend was observed at 5, 7 and 9 DAI. Among the incubation periods, maximum solubilization was observed at 7 DAI. At 7 DAI, *Aspergillus* sp. strain PSFNKR-2 (20.81%) recorded the maximum P release irrespective of the NaCl concentrations and was significantly superior over all other isolates including the reference strain, *A. awamori* (11.02%). The *Aspergillus* sp. strain PSFNKR-2 at 1.0 M NaCl followed by the same strain at 800 mM NaCl showed highest release of P in broth at 7
bacteria, fungi and actinomycetes were isolated. As in our earlier study we observed somewhat similar trends in populations of total bacteria, fungi and actinomycetes from five soils of northern Karnataka including a sodic soil whereas, Bhardwaj (1974) recorded similar population of total bacteria in saline-alkali soils. Since the P-solubilizers in the present study were isolated from salt-affected soils, an attempt was also made to study the ability of selected efficient isolates to grow and solubilize tricalcium phosphate in the presence of different concentrations of NaCl in broth medium. Among PSB isolates, the population counts of a majority of the isolates were found to be higher than that of the reference strain (P. striata) particularly at higher NaCl concentrations. This could be due to less stress adaptability of the reference strain where as the isolates could tolerate more stress as they were isolated from salt-affected soils. When the isolates were exposed to hyper osmotic conditions, the tolerant ones could have followed some Osmo-adaptation pathways such as synthesis of compatible solutes or accumulation of potassium against NaCl to overcome the Na+ ion toxicity. Many non-halophilic bacteria are also reported to accumulate potassium in response to stress by sodium (Oren et al., 2002). The isolates in general showed decrease in population to increase in NaCl concentration after 0.4 M. The strain *Aerococcus* sp. PSBCRG1-1 recorded the highest log viable cell count (12.15) at 400 mM NaCl concentration after eight days of incubation. The decrease in population with increasing concentration of NaCl can be attributed to the exposure of organisms to the conditions of hyper-osmolality resulting in a decrease in their cytoplasmic water activities. Solute (NaCl) increase the osmolarity of the medium which in turn causes the loss of intracellular water with a concomitant increase in the osmolarity of the intracellular contents (Botsford, 1984). It appears likely that proteins (enzymes) and other biological macromolecules have evolved to function only within certain normal ranges of water activities, outside which some essential cellular functions become impaired (Csonka, 1989). Surange et al., (1997) had reported that the *Rhizobium* strains isolated from alkaline soils tolerated salt concentrations up to 860 mM (5.0%), but a concentration of 1290 mM (7.5%) was inhibitory to the growth of *Rhizobium* strains.

However, the amount of P_i released from TCP by the PSB isolates was found to increase with an increase in salt concentration up to 800 mM NaCl and declined thereafter, whereas the reference strain (P. striata) showed a significant decrease in P-solubilization at all NaCl concentrations. These results indicate better performance of PSB isolates of salt-affected soils in releasing P_i from TCP even under salt stress condition. While one of the isolates, *Aerococcus* sp. PSBCRG1-1 showed maximum solubilization (12.12%) at 800 mM NaCl concentration, *Alteromonas* sp. PSBCRG2-1 showed an increase in solubilization up to 1.0 M. Kumar et al. (2010) also found increased phosphate solubilization with an increase in NaCl concentration. The pH of the broth medium at 15 DAI was found to decrease substantially by all the PSB isolates at all concentrations of NaCl in the present study indicating that the mechanism of P-solubilization i.e., organic acid production was effective even in the presence of salt stress. It is therefore clear that the strains isolated from salt-affected soils are able to tolerate salt stress. All PSB isolates demonstrated recognizable levels of phosphate solubilization in the presence of high salt. It seems therefore, that the strains isolated from salt-affected soils have the genetic potential to solubilize phosphates at much higher salt stress.

The growth of selected PSF in the presence of NaCl was also determined in terms of mycelial dry weight. The mycelial dry weight in general was found to decrease with increasing concentrations of NaCl. However, a few isolates recorded increased mycelial mat weight at 200 and 400 mM NaCl concentrations. The reference strain, *A. awamori* also recorded increased mycelial mat weight up to 400 mM NaCl. The reduced growth of fungi in the presence of NaCl could be due to hindrance in the normal functioning of the synthesis pathways. It has been shown that some mineral ions affect growth because they inhibit the activity of specific enzymes (Botsford, 1984). The presence of Na+ could have affected the enzymes involved in the normal growth process of fungi. Proline stabilizes the enzymes that would otherwise be inhibited by high concentrations of sodium (Demir and Kocaciliskan, 2001).

Similarly, the amount of P_i released in general was found to decrease with increase in NaCl concentration at all incubation periods. However, *Aspergillus* sp. PSFNRH-2 showed significant increase in P-solubilization with increase in NaCl concentration up to 1.0 M and decreased thereafter. This could be due to either hindrance of the normal functioning of the metabolic pathways involved in solubilization by enhanced salt stress or comparatively lower growth at salt stress. Yokoyama et al. (1992) also observed decrease in growth of fungi isolated from salinized soils with increase in NaCl concentration in liquid medium. They reported that the cell yield of the *Aspergillus* spp. at 90 g NaCl L^-1 was almost half compared to the culture without NaCl. All the PSF isolates recorded higher P-solubilization than the reference strain irrespective of NaCl concentrations. *Aspergillus* sp. PSFNRH-2 released P_i almost double (20.81%) the amount that was released by the reference strain, *A. awamori* (11.02%). This indicates better adaptability of these isolates to solubilize TCP in the presence of salt stress. The pH of the broth medium at 9 DAI was found to decrease substantially in different treatments indicating the production of organic acids by PSF isolates in the presence of salt stress.

### 5. Conclusion

A total of 23 PSB and 35 PSF were isolated from 19 samples of salt affected soils. The ability of the selected PSB and PSF to grow and solubilize tricalcium phosphate under salt stress was examined. Under similar conditions *Aerococcus* sp. strain PSBCRG1–1 and *Pseudomonas aeruginosa* strain PSB1–1 scored better during the viable cell population count, while *Pseudomonas aeruginosa* strain PSB1–1 was the least performer. Highest mycelial dry weight irrespective of NaCl concentrations was recorded for the *Aspergillus terreus* strain PSFRCRG2–1. The amount of P_i release increased with incubation period irrespective of strains and salt concentrations.
The per cent P$i$ release, in general, was found to increase with increase in NaCl concentration up to 0.8 M for bacterial solubilization and declined thereafter. At 15 DAI, strain *Aerococcus* sp. strain PSBCRG$_1$–1 irrespective of NaCl concentrations showed the maximum P-solubilization which was significantly superior over all other isolates. The amount of P$i$ released in general among PSF was found to decrease with increase in NaCl concentration at all the incubation periods. *Aspergillus* sp. strain PSFNRH-2 recorded the maximum P$i$ release irrespective of the NaCl concentrations and was significantly superior over all other PSF at 7 DAI.

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**Appendix Supplementary. data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.sjbs.2012.05.004](http://dx.doi.org/10.1016/j.sjbs.2012.05.004).

**References**


