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Optimization of culture conditions for phosphate solubilization by a thermo-tolerant phosphate-solubilizing bacterium *Brevibacillus* sp. BISR-HY65 isolated from phosphate mines

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ABSTRACT

In the present study we isolated and characterized thermo-tolerant phosphate solubilizing bacteria (PSB) having high ferric phosphate (Fe-P) and aluminum phosphate (Al-P) solubilizing abilities for the first time from rock phosphate mines of Jhamarkotra. Optimization for phosphate (P) solubilization by the isolate BISR-HY65 was performed. Different insoluble P sources viz. hydroxyapatite (H-Ap), Al-P and Fe-P along with rock phosphate (RP) from two different mines of India were used to characterize phosphate solubilizing (PSE) abilities. Optimum conditions found were: temperature 50 °C, pH 7.5, xylose as carbon source, ammonium oxalate as nitrogen source and potassium sulfate as potassium source. Phosphate solubilization was found to be associated with the release of organic acids in culture. HPLC analysis of the culture broth at 96 h of incubation detected four known acids (citric, gluconic, malic and formic acid) along with three unknown acids. Molecular characterization showed our strain to be of *Brevibacillus* sp.
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1. Introduction

Phosphorus (P) is the second key nutrient for plants and affects several characteristics of plant growth. Though P, both in organic and inorganic forms is abundant, but due to its ability to form complexes with other soil constituents, it is not easily available for uptake by the plants (Vassilev and Vassileva, 2003). To fulfill the P requirement of plants, chemical fertilizers are commercially available in the market which can help in increasing the productivity of crops. The production of these fertilizers is based on chemical processing of insoluble mineral phosphate (high-grade ore) by treating them with sulfuric acid at high temperature thus, the process is environmentally undesirable and a costly affair (Vassilev et al., 2006). Furthermore, the current developments in sustainability require a strong reduction in agrochemical inputs and their replacement by more ecological, efficient and cheap natural products to fulfill the requirements of essential nutrients for plant growth.

In natural environments, i.e. the rhizosphere of different plant species, phosphate-solubilizing bacteria (PSB) are considered to play an important ecophysiological role: indeed, PSB mobilize insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots. In turn, the plants supply root-borne carbon compounds, mainly sugars, which can be metabolized for bacterial growth (Goldstein, 1995; Deubel et al., 2000). The discovery of this mutual relationship between plants and PSB encouraged the development of new technologies, such as the use of phosphate solubilizing microorganisms (PSM) as biofertilizers to improve plant yield (Richardson, 2001; Gothwal et al., 2006). The phosphate solubilizing ability of PSB has been traditionally associated with the production of low-molecular-weight organic acids (Rodriguez and Fraga, 1999; Goldstein, 2000). These acids diffuse freely outside the cells and facilitate solubilization of P from mineral phosphates in high amounts by supplying both protons and metal complexing organic acid anions (Gadd, 1999).

In India and other developing countries the economy being predominantly based on agriculture, fertilizer production plays a pivotal role. In case of raw materials required for producing phosphatic fertilizers, only about 35% is being met through indigenous sources and rest is through import in the form of rock phosphate. In such a situation, Jhamarkotra rock phosphate (RP-J) mines of India play an important role by contributing about 98% of total rock phosphate production of the country.

Moreover, the development of commercial bioinoculant and the large-scale bioprocessing of rock phosphate ores through the action of PSB have resulted in the highly efficient, cost effective and successful commercial technologies now used by the

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agro-industries worldwide (Goldstein et al., 1993; Goldstein, 2000; Hamdali et al., 2010). Hence, isolation and application of novel and potential PSB are significant and necessary. An extensive research have been done on isolation and characterization of mesophilic PSB having varied degree of phosphate solubilizing efficiencies (PSE) from different ecological niches (Richardson, 2001; Chen et al., 2006; Gothwal et al., 2006; Perez et al., 2007; Hamdali et al., 2010) but thermo-tolerant bacteria having ability to solubilize different forms of insoluble phosphate sources efficiently such as TCP, Fe-P and Al-P have been given little emphasis. Also, the rock phosphate (RP) mines have not been explored for such purposes which might yield some novel strains having comparatively better P solubilizing abilities. Thermo-tolerant bacteria possess novel mechanisms to survive under adverse conditions like high temperature, and its ability to solubilize Fe-P and Al-P efficiently, can be exploited in bioactivation of rock phosphate having high Fe and Al content, and using them as bioinoculant having longer shelf-life.

Therefore, efforts were made in the present study to isolate thermo-tolerant bacteria from soil samples of Jhamarkotra rock phosphate mines having ability to solubilize different insoluble phosphates. Further, growth of the prominent strain was optimized in order to increase P solubilization and characterize its mineral phosphate solubilizing ability.

2. Materials and methods

2.1. Sampling site and sample collection

Soil samples were collected from different blocks of mines from 6 in. beneath the surface, in the month of October, 2010. All the samples were packed in sterile plastic bags and vials, and were transported immediately to the laboratory, stored at 4 °C and finally processed within 96 h. Physico-chemical analysis of the collected soil samples was performed at the soil testing laboratory, Durgapura, Jaipur.

2.2. Isolation of phosphate solubilizing bacteria

The collected samples were enriched by taking 1 g soil in Pikovskaya (PVK; Pikovskaya, 1948) broth containing the following ingredients per litre: glucose 10 g, tri-calcium phosphate 5 g, $(NH_4)_2SO_4$ 1 g, KCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, NaCl 0.2 g, yeast extract 0.5 g and $FeSO_4$ in traces. One millilitre of enriched broth was serially diluted in 0.85% (w/v) saline solution and planted on the PVK agar plates and finally incubated at 50 °C for 48 h. Colonies exhibiting different morphological appearances were picked up individually and further purified by sub-culturing. Once purified, each isolate was processed to find out its PSE in PVK broth and was stored as a glycerol stock at −80 °C.

2.3. Quantification of soluble phosphate

Quantification of soluble P was done by molybdenum method (Bray and Kurtz, 1945) by taking KH_2PO_4 as a standard. The soluble P was expressed in terms of milligram per litre P released in the culture medium and was used as phosphate solubilizing efficiency (PSE) of the isolates in culture broth (Gothwal et al., 2006).

2.4. Screening of isolates

More than 80 bacterial strains were obtained and were further screened for their PSE in 250 ml conical flasks containing 50 ml of PVK medium by inoculating with 200 μl of 24 h old culture grown in Luria–Bertani (LB) broth whose cell OD was adjusted to 0.6 OD_{600} final. The flasks were incubated in an orbital shaker (180 rpm) at 50 °C for 96 h. Three millilitre of sample was withdrawn after every 24 h of incubation and centrifuged at 10,000*g* (Mikro-200, Hettich Zentrifugen, Germany) for 15 min and used for estimating the amount of soluble P and pH. One among these 80 strains, designated as BISR-HY65 exhibiting high P solubilization, isolated from A-Extension 560 block of rock phosphate mines, was used for further studies.

2.5. Optimization of P solubilization by BISR-HY65

For optimizing the media conditions all the experiments were performed taking tri-calcium phosphate (5.0 g/l) as the sole P source in PVK media. Strain BISR-HY65 was inoculated in LB broth at 50 °C temperature and 180 rpm, after 24 h of incubation OD of culture broth was adjusted to 0.6 at 600 nm and was used to perform different optimization and analytical experiments. Sampling was done after every 24 h to determine the PSE up to 96 h. The optimal results of each experiment were retained and then carried forward in further experiments. Finally, characterization of mineral phosphate solubilization was performed under optimal growth conditions.

2.5.1. Effect of temperature and initial media pH

The effect of temperature on PSE of strain BISR-HY65 was investigated by inoculating the PVK broth sets at different temperatures of incubation from 37 °C to 60 °C up to 96 h of incubation. Similarly, effect of pH on PSE was monitored by inoculating the PVK broth sets at different initial pH viz. 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 up to 96 h of incubation.

2.5.2. Effect of carbon source

Strain BISR-HY65 was grown in different carbon sources (10 g/l) to observe PSE in PVK medium. The carbon sources used were glucose, xylose, glycerol, mannitol and fructose. In this experiment xylose was found to show maximum PSE therefore, its different concentrations (5.0, 7.5, 10.0, 12.5 and 15.0 g/l) were tested to find out optimum xylose concentration in PVK media.

2.5.3. Effect of nitrogen source

For studying the effect of nitrogen source on PSE, BISR-HY65 was inoculated in media containing different nitrogen sources viz. ammonium oxalate, ammonium nitrate, ammonium chloride, calcium nitrate, ammonium tartrate, sodium nitrate, potassium nitrate, urea and ammonium acetate by replacing ammonium sulfate in the PVK medium at 0.1 g/l of equivalent nitrogen weight.

2.5.4. Effect of potassium source

Effect of potassium sources was determined by growing the bacterial strain on various potassium sources such as potassium sulfate, potassium nitrate, potassium iodide, potassium gluconate by replacing potassium chloride in the PVK medium at 0.1 g/l of equivalent potassium weight.

2.5.5. Effect of phosphorus source

For determining ability of the strain to solubilize different P sources, various P sources like hydroxyapatite, aluminum phosphate, ferric phosphate and two rock phosphate samples (Jhamarkotra rock phosphate [RP-J], Rajasthan and Purulia rock phosphate [RP-P], West Bengal) were added by replacing TCP in the PVK medium at 0.5 g/l equivalent phosphorus weight by using the optimal growth conditions after performing experiments. The chemical composition of both the RP samples has been presented in Table 1.

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