Viability and efficiency of Pantoea brenneri strains as phosphate solubilizing bacteria

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Abstract. Phytate-degrading microorganisms with the ability to solubilize inorganic soil phosphates can serve as the basis of bio-fertilizer. Four low-cost liquid compositions were examined for viability of Pantoea brenneri strains 3.2 and 3.5.2 stored at room temperature and at 4°C for six months. By the end of the experiment, the highest cell survival rate for both P. brenneri strains 3.2 and 3.5.2 at 4°C and at 25°C was ensured by a LB broth supplemented with glycerol. Moreover, strains maintained the highest phosphate solubilizing index in this composition as well.

1 Introduction

One of the main problems of the new millennium is getting more and more agricultural products from fertile arable land annually reducing per capita. The increasing public health concern, the growing organic food industry, environmental pollution with pesticides, eutrophication of water bodies due to the massive use of mineral fertilizers, as well as a significant increase in their expense have played a huge role in the development of the global bio-fertilizer industry. It is expected that by 2026 the global market for bio-fertilizers will reach USD 4.5 billion [1]. The state policy in relation to agriculture today is changing in the direction of its greening and stimulation of biodynamic and organic farming systems [2]. Thus, biological fertilizers and biopesticides occupy a separate unique place in the market of agricultural products all over the world.

A significant improvement in the growth and yield of crops in response to the processing of beneficial microorganisms is noted by many authors [3-6]. The diversity of the genetic resources of microorganisms responsible for plant-growth-promoting activity is large: associative nitrogen fixation, ammonia production, the formation of growth-promoting substances, providing the plant with bioavailable forms of iron, phosphorus, secretion of cellulases and proteases, excretion of bactericidal and fungicidal metabolites. Although phosphorus is present in soil as a mixture of inorganic and organic compounds, and many microorganisms show to break down inorganic soil phosphates, only a few studies have identified P-solubilizing microorganisms capable of solubilizing both organic and mineral P [7]. In soil, the inaccessible organic form of phosphorus is represented mainly in the form of phytic acid salts - phytate. Enzymes that break down phytate are actively secreted into the soil by some microorganisms, and play an important role in the release of bioavailable

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phosphorus from phytates [8]. Since there is no extracellular phytase of plant origin, phytase of microbial origin is the most important link in the cycle of organic phosphorus in soil. Phytate-degrading microorganisms with the ability to solubilize inorganic soil phosphates can serve as the basis of bio-fertilizer.

Bioformulations are present in liquid or solid forms, but liquid is considered better in comparison to solid, as they can easily colonize on the root or plant surface, perform well in pots or in field conditions, and have low cost [9].

The present study was focused on the development of liquid bioformulation to increase the shelf life of two phosphate solubilizing and phytate degrading soil isolates – Pantoea brenneri 3.2 and 3.5.2, as well as maintain the high levels of their biological activities while being stored.

2 Materials and methods

2.1 Bacterial strains and culture media

Four bacterial strains – Pantoea brenneri 3.2 and 3.5.2 – were isolated earlier from the soil samples of the Republic of Tatarstan based on their phytate hydrolyzing activity [10]. Strains were cultivated and stored on Luria-Bertani (LB) medium, containing 10 g tryptone, 5 g yeast extract, 5 g NaCl per 1 L distilled water, pH 7.0. Solid agar medium (LA) includes an additional 2% agar [11].

2.2 Long-term storage formulations

In order to evaluate the viability of P. brenneri cell during long-term storage, 4 different compositions were selected according to the previously published studies [12, 13]: 1) saline solution (0.9% NaCl); 2) 0.2% solution of KNO₃ in 0.9% NaCl; 3) 0.5% solution of KNO₃ in 0.9% NaCl; 4) LB nutrient medium with the addition of 4% glycerol. The solutions were prepared in 50 ml polypropylene falcon tubes and sterilized.

2.3 Bioinoculum preparation

P. brenneri strains 3.2 and 3.5.2 were inoculated in LB nutrient broth at 30 °C, 200 rpm for 18 hours. Cells were pelleted by centrifugation at 5000 rpm for 15 min and washed twice with sterile saline solution (0.9% NaCl). Then bacterial cells were added to each liquid compositions under sterile conditions in order to adjust the cell number to 1 × 10⁷ Cfu/ml, which corresponds to the optical density (OD₅₉₀) of 0.5. The samples were sealed and stored at 25 °C in dark conditions and in 4 °C.

2.4 Evaluation of Cfu

The number of colony forming units (Cfu) per ml of suspension (concentration of living cells) was calculated by the method of standard serial dilutions followed by seeding on LB agar. The evaluation of viable populations of the two strains was made after 1, 3 and 6 months. Serial ten-fold dilutions in sterile tap water were made from each sample. Then 100 μl from each dilution was transferred to LA agar plates and incubated for 24 hours at 30°C. The number of colony forming units (Cfu) was determined using the equation:

\[ N = \frac{c}{(n_1 + 0.1 \cdot n_2) \cdot d} \]
Where:
c – the number of colonies on all plates;
n₁ - the number of plates of the first dilution;
n₂ – the number of plates of the second dilution;
d - the first dilution factor;
0.1 – coefficient taking into account the dilution fold.

2.5 Determination of phosphate solubilizing activity

Phosphate solubilizing ability of the P. brenneri strains in bioinoculums was evaluated after three months of storage. 25 μl of each bioinoculant was spot inoculated onto plates with NBRIP agar medium: glucose 10 g, Ca₃(PO₄)₂ 5 g, MgCl₂ x 6H₂O 5 g, MgSO₄ x 7H₂O 0.25 g, KCl 0.2 g, (NH₄)₂SO₄ 0.1 g, distilled water 1 L; the pH was adjusted to 7.0 ± 0.2 before sterilization [13]. After 5-7 days of incubation at 30 °C tricalcium phosphate hydrolysis was observed as formation of halo-zones around the bacterial colonies. Colony diameter and clear zone diameter were measured to calculate phosphate solubilization index (PSI) [15]:

\[
PSI = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}
\]

3 Results and discussion

One of the goals of microbial fertilizer technology is to maximize the accumulation of viable cells and preserve their viability [16]. Liquid forms of such fertilizers have advantages over the solid forms such as simple application, high efficiency, resistance to a wide range of ambient temperatures, maintenance of viability and bioactivity of bacterial cells. All these characteristics also determine the quality and cost-effectiveness of liquid biofertilizers [17].

We assessed the cell viability of P. brenneri 3.2 and 3.5.2 strains during storage at various temperatures and liquid compositions (Table 1). It was shown that storage of the bacterial suspension at 4 °C was better for maintaining higher number of viable cells in all studied liquid formulation, in contrast to 25°C. Interestingly, at 4°C throughout the entire period of the experiment, a simple saline solution tended to retain the larger number of cells compared to saline containing potassium nitrate. This may be due to the fact that potassium nitrate is a widely used preservative agent that prevents the development of microorganisms [18]. However, no similar effect was observed at 25°C. Goljanian-Tabrizi et al. [12] studied the survival rates of Pseudomonas sp. P15 and Pantoea agglomerans P13 at room temperature on similar formulations. It was shown that the highest number of CFU for Pseudomonas sp. P15 was achieved using a simple phosphate buffer, while a high number of CFU for P. agglomerans P13, on the contrary, was provided by phosphate buffers containing potassium nitrate.

Table 1. Effect of storage at different temperatures on population size of strains P.brenneri 3.2 and 3.5.1 (initially containing 1 x 10⁸ cfu/ml) in liquid formulations.

<table>
<thead>
<tr>
<th>Liquid composition</th>
<th>P. brenneri 3.2</th>
<th>P. brenneri 3.5.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>25°C</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>3.0 ± 0.2 x 10⁶</td>
<td>2.25 ± 0.1</td>
</tr>
<tr>
<td>3%</td>
<td>3.0 ± 0.2 x 10⁶</td>
<td>2.25 ± 0.1</td>
</tr>
</tbody>
</table>
Comparing the survival of bacterial cells at a temperature of 25°C and their content in physiological buffer and fresh LB broth with the addition of glycerol, the highest CFU number was observed in the last one. The LB medium contains nutritional components that support the growth and development of microorganisms, and glycerol plays the role of an osmoprotector, retaining a large amount of water and enhancing the protection of cells during drying [19]. By the end of the experiment, the highest cell survival rate for both P. brenneri strains 3.2 and 3.5.3 at 4°C and at 25°C was also ensured by a nutrient medium with glycerol.

Phosphate solubilizing activity of P. brenneri strains 3.2 and 3.5.2 was assessed after three months of storage (Fig. 1) on NBRIP agar plates, containing tricalcium phosphate as a sole source of phosphorus. All studied liquid compositions allowed to maintain high P-solubilizing activity of P. brenneri strains 3.2 and 3.5.2. However, the highest PSI was observed when strains were inoculated from LB broth supplemented with 4% glycerol and stored at 4°C.

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<table>
<thead>
<tr>
<th></th>
<th>0.2% KNO₃ in 0.9% NaCl</th>
<th>0.5% KNO₃ in 0.9% NaCl</th>
<th>LB+4% glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU (×10⁶)</td>
<td>2.42±0.1 ×10⁶</td>
<td>2.15±0.1 ×10⁶</td>
<td>7.1±0.4 ×10⁶</td>
</tr>
<tr>
<td></td>
<td>5.1±0.2 ×10⁵</td>
<td>4.7±0.2 ×10⁶</td>
<td>5.1±0.3 ×10⁶</td>
</tr>
<tr>
<td></td>
<td>3.5±0.2 ×10⁴</td>
<td>5.1±0.2 ×10⁶</td>
<td>5.1±0.3 ×10⁵</td>
</tr>
<tr>
<td></td>
<td>5.9±0.3 ×10³</td>
<td>8.4±0.1 ×10⁶</td>
<td>7.6±0.4 ×10⁵</td>
</tr>
<tr>
<td></td>
<td>2.1±0.1 ×10⁶</td>
<td>1.2±0.05 ×10⁶</td>
<td>3.3±0.03 ×10⁶</td>
</tr>
<tr>
<td></td>
<td>3.3±0.04 ×10⁶</td>
<td>5.1±0.4 ×10⁴</td>
<td>6.7±0.03 ×10⁶</td>
</tr>
<tr>
<td></td>
<td>4.2±0.03 ×10⁴</td>
<td>5.3±0.03 ×10⁴</td>
<td>4.1±0.03 ×10⁵</td>
</tr>
</tbody>
</table>

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Fig. 1. P-solubilization activity of P. brenneri strains on NBRIP agar plates.
Table 2. P-solubilization index (PSI) of the bacterial isolates stored at different temperatures in various liquid compositions. The assay was done in triplicates, the error bars indicate the standard deviation from the mean.

<table>
<thead>
<tr>
<th>Liquid composition</th>
<th>P. brenneri 3.2</th>
<th></th>
<th>P. brenneri 3.5.2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>25°C</td>
<td>4°C</td>
<td>25°C</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>1.85</td>
<td>1.991</td>
<td>1.85</td>
<td>2.192</td>
</tr>
<tr>
<td>0.2% KNO₃ in 0.9% NaCl</td>
<td>2.8</td>
<td>3.122</td>
<td>1.875</td>
<td>2.273</td>
</tr>
<tr>
<td>0.5% KNO₃ in 0.9% NaCl</td>
<td>2</td>
<td>2.187</td>
<td>2.63</td>
<td>2.434</td>
</tr>
<tr>
<td>LB+4% glycerol</td>
<td>4.38</td>
<td>2.199</td>
<td>3.48</td>
<td>3.338</td>
</tr>
</tbody>
</table>

Liquid formulations of various microorganisms or even microbial consortia have been already applied and allowed to increase yields of agricultural production. Thus, a liquid bioinoculum based on sugar and coconut water, included consortium of Pseudomonas spp., Bacillus spp., Klebsiella spp., Aspergillus spp. and Azotobacter spp. has been used to inoculate soybean plants and resulted in improved plant nutrient and higher yields [20]. It was also shown that the ability to dissolve phosphates and the survival rate of Pseudomonas sp. and Pantoea sp. strains are preserved in liquid preparations, containing diluted concentrations of phosphate buffer and glycerol nutrient broth, up to three months [12]. In addition, Camelo-Rusinque et al. [21] evaluated the population dynamics of the Azotobacter chroococum AC1 strain under bioreactor conditions after 105 days and found that both cell viability and biological properties of the strain were maintained regardless of storage temperature. This indicates that some liquid formulations can be used for a long time storage, while the microorganisms retain their activity and viability when used as bioinoculants.

Proper use of bacterial preparations on the basis of growth-promoting rhizobacteria as an element of ecological farming in the cultivation of various crops allows significantly reducing the chemical load on the ecosystem by reducing the amount of mineral fertilizers and chemical means of plant protection, leads to improved yields, health and quality, and improved quality.

Acknowledgments

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