Screening of Phosphate-Resolving Bacteria in Rhizosphere of Cold Sunflower and Physiological and Biochemical Study

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Abstract. The purpose of the experiment is to obtain high-efficiency microbial phosphorus dephosphorization fertilizer, increase the soluble phosphorus content of the soil, and promote plant growth. The phosphorus detoxifying bacteria in sunflower rhizosphere were studied through dilution and coating plate method, molybdenum-antimony anti-spectrophotometry, plate culture, physiological and biochemical detection. 10 strains were isolated and purified to form bright rings, D/d value is between 1.10-4.35. The quantitative analysis of the pressure showed that BX2 had the best effect in dissolving phosphorus. The available phosphorus increment was 38.59μg/mL, BX7 had the lowest phosphorus dissolving effect, and the effective phosphorus increase was only 1.77μg/mL. These strains of BX2, BX3, BX5, BX9 and BX10 have excellent phosphorus-dissolving properties, providing necessary conditions for the subsequent development of microbial fertilizer.

1. Introduction
Phosphorus is a component of important organic compounds in plants, participates in plant metabolism in many forms, promotes plant growth. It is one of the primary nutrients essential for plant growth [1]. But most of the phosphorus in the soil is in the form of organic or inorganic phosphides, and this is difficult to be directly absorbed by plants [2]. In actual production, to increase crop yield, people apply a lot of phosphate fertilizer, it leads to problems such as soil compaction, soil function degradation, and micro-ecological environment imbalances, which prevent the soil from being recycled [3]. There is an urgent need to improve this problem through the development and utilization of insoluble phosphorus in the earth [4]. Studies show that there are many phosphate-dissolving bacteria in soil and plant Rhizosphere; they can convert insoluble phosphorus in soil into available phosphorus that plants can absorb and use [5]. Phosphate-solubilizing bacteria secrete organic acids to dissolve insoluble inorganic phosphates in the soil environment or use the secretion of extracellular phosphatase to decompose the insoluble organic phosphorus compounds in the soil, release the available phosphorus, increase the soluble phosphorus content in the ground, and promote plant growth [6]. One of the crucial ways to improve the utilization rate of phosphorus in soil is to screen high-efficiency organic phosphorus bacteria and use it to produce microbial fertilizer or inoculant [7]. In the 1950s, researchers isolated Bacillus sp from the black soil of Northeast China. In recent years, research on the screening and identification of phosphorus-resolving bacteria in plant Rhizosphere soil has increased. However, there are few reports on the detection of organophosphate-dissolving bacteria...
in the Rhizosphere of cold regions [8]. In this study, a bacterial strain with good phosphorus dissolving effect was isolated from healthy sunflower Rhizosphere soil of the Kangjinjing Experimental Base of Heilongjiang Academy of Agricultural Sciences; the molybdenum antimony anti-spectrophotometry method was used to study the phosphorus dissolving ability of the strains. Finally, high-efficiency strains of organophosphate were screened, which laid a solid foundation for the production of microbial fertilizer.

2. Materials and methods

2.1. Materials

2.1.1. Test materials. Soil sample: Healthy sunflower rhizosphere soil select from the Kangjinjing Experimental Base of Heilongjiang Academy of Agricultural Sciences. When sampling, 12 plots set along the diagonal of each scenario. Each parcel sample from the set plots, and take a total of 4 plants. The sunflower roots store in nylon bags.

2.1.2. Medium. Beef extract peptone medium: Beef Extract 5.0 g, Peptone 10.0 g, NaCl 5.0 g, Agar 18.0 g, Distilled water 1000 mL, pH7.4-7.6.

LB liquid medium: Peptone 10.0 g, Beef Extract 5.0 g, NaCl 5.0 g, Distilled water 1000 mL.

Organic phosphorus liquid culture medium: glucose 10.0 g, Ca₃(PO₄)₂ 3.0 g, (NH₄)₂SO₄ 0.5 g, NaCl 0.3 g, MgSO₄·7H₂O 0.3 g, KCl 0.3 g, MnSO₄ 0.03 g, FeSO₄·7H₂O 0.03 g, Egg yolk lecithin 2.0 g, Distilled water 1000 mL, pH7.2-7.5.

Organophosphorus solid medium: 18.0 g of agar add to the organophosphorus liquid medium.

Strain identification medium: Refer to Berger Bacterial Identification Manual.

2.1.3. Main reagents of molybdenum antimony anti-spectrophotometry. For the preparation method of phosphorus standard stock solution, molybdenum antimony storage solution, molybdenum antimony color developer, and indicator, refer to molybdenum antimony colorimetry.

2.2. Method

2.2.1. Isolation and purification of bacteria. Dilute coating plate method was used to isolate sunflower rhizosphere bacteria. Accurately weigh 5.0 g soil sample, put in a 250 mL Erlenmeyer flask with 45mL of sterile water, and put an appropriate amount of glass beads into the bottle. Shake in a 150 r/min shaker for 30 min. Let stand for 30s, which is 10⁻¹ soil dilution bacteria solution, take 1.0 mL of 10⁻¹ supernatant into a test tube filled with 9.0 mL of sterile water. Mix evenly to make a 10⁻² diluted bacteria solution; prepare 10⁻⁴, 10⁻⁵and 10⁻⁶ dilutions with pure water in sequence. Use a sterile syringe to take 0.1 mL of the dilution and inject it into the center of the plate, take the applicator and evenly spread the bacterial solution on the beef extract peptone medium, three replicates per concentration, let stand for 30 min. Invert and incubate for 3d in a 28 ℃ incubator. Pick colonies of different forms for streak culture; the pure bacteria store behind the beef peptone oblique slope (4 ℃) for future use.

2.2.2. Primary Screening of Phosphate-Resolving Bacteria. The initial screening uses the organic phosphorus plate culture method. Activate the bacteria stored on the slanted surface and inoculate them into the natural phosphorus plate. Invert and incubate for 3d in a 28°C incubator. Observe and measure the size of the colonies and their transparent circles, Strains with a sizeable open circle diameter D (mm) and colony diameter d (mm) and a bright open circle, inoculated on slanted medium. After two days of incubation in a 28 ℃ constant temperature incubator, it store at low temperature for later use.
2.2.3. Re-screening of Phosphate-Resolving Bacteria. Take a ring of single colonies into a 50 mL liquid beef paste medium conical flask, after shaking for 24 hours, measure the suspension of bacteria. Using a sterile needle, take 1.0 mL of the bacteria solution and insert it into a triangular flask containing 50 mL of organic phosphorus liquid culture medium. Take the same amount of sterile water as the control (CK), and make three parallels for each treatment, shake at 180 r/min and 30℃. After five days, the culture was transferred to a 10 mL sterile centrifuge tube and centrifuged at a speed of 4000 r/min for 30 minutes. Take 1.0 mL of the supernatant and determine the phosphorus content by molybdenum antimony anti-spectrophotometry.

2.3. Preliminary identification of strains

2.3.1. Morphological identification of phosphate-decomposing bacteria. Strains selected for high-efficiency phosphate-removing effects streak into beef extract peptone plate medium, incubate in a 28℃ incubator for two days. Observe the morphological characteristics of a single colony, and take a separate province with an inoculating loop for Gram staining. Observe its morphological characteristics under a microscope.

2.3.2. Physiological and Biochemical Identification. Refer to the method in Berger Bacterial Identification Manual for VP reaction, contact enzyme reaction, methyl red test, citrate utilization reaction, denitrification reaction, fluorescent pigment reaction, gelatin liquefaction, glucose oxidation and other physiology and biochemistry analysis.

3. Results and analysis

3.1. Screening results of phosphate-dissolving bacteria

3.1.1. Preliminary screening results of phosphate-resolving bacteria. Ten strains were selected from the rhizosphere soil of sunflowers with obvious transparent circles. The ratio of the diameter D (mm) to the colony diameter d (mm) of the phosphorus dissolving circle is between 1.10-4.35, 70% of the strains have a D/d value of more than 2 and 10% of the strains have a D/d value of more than 3. Only strain BX2 with a maximum D/d ratio of 4 or more had the most apparent effect of dephosphorization. The results show in Table 1.

| Numbering | Colony diameter D(mm) | Phosphorus ring diameter D(mm) | D/d | Numbering | Colony diameter D(mm) | Phosphorus ring diameter D(mm) | D/d |
|-----------|------------------------|-------------------------------|-----|-----------|------------------------|-------------------------------|-----|
| BX1       | 6.0                    | 8.5                           | 1.41| BX6       | 11.0                  | 12.6                         | 1.15|
| BX2       | 6.3                    | 27.4                          | 4.35| BX7       | 9.0                   | 10.5                          | 1.17|
| BX3       | 4.2                    | 15.6                          | 3.71| BX8       | 9.8                   | 22.3                          | 2.28|
| BX4       | 9.1                    | 19.5                          | 2.14| BX9       | 10.1                  | 26.4                          | 2.61|
| BX5       | 3.0                    | 7.8                           | 2.60| BX10      | 8.3                   | 24.7                          | 2.98|

3.1.2. Re-screening results of phosphate-resolving bacteria. Analysis of the results showed that the effective phosphorus increase of BX2 strain was 38.59 μg / mL, and that of the BX7 pressure was only 1.77 μg / mL. By comparing Table 1, the ratio of the diameter of the transparent circle and the colony diameter is related to the soluble phosphorus content of the anxiety. The larger the D / d value, the higher the soluble phosphorus content of the strain (in Figure 1).
3.2. Identification results of strains

3.2.1. Morphological identification results. By observing the morphological characteristics of BX2, BX9 and BX10 strains, the colonies were all round. The tension streak on beef extract medium and cultured at 28℃ for two days. A single settlement takes for Gram staining and spore staining. Microscopic examination of strain BX10 showed gram-positive bacteria with spores; Strain BX2 is a Gram-negative bacteria with spores; strain BX9 is a Gram-positive bacteria without spores. The results show in Table 2.

3.2.2. Identification results of physiological and biochemical indicators. Phosphate-decomposing bacteria V-P reaction, contact enzyme reaction, methyl red test, citrate utilization reaction, denitrification reaction, fluorescent pigment reaction, gelatine liquefaction, glucose oxidation physiological and biochemical tests. The gelatine liquefaction reaction of the strains were all positive, and the V-P test, methyl red reaction, and citrate reaction were all negative. It can be seen from Table 3.

Table 2. Morphological characteristics of BX2, BX9 and BX10 Strains

| Numbering | Shape   | Color   | Transparency | Surface   | Gloss | Dry humidity | Edge shape |
|-----------|---------|---------|--------------|-----------|-------|--------------|------------|
| BX2       | circle  | white   | Transparent  | smooth    | Shiny | wet          | neat       |
| BX9       | circle  | white   | opaque       | Grain     | Matte | dry          | Wavy       |
| BX10      | circle  | white   | opaque       | Folds     | Matte | dry          | neat       |

Table 3. Physiological and biochemical identification results of BX2, BX9 and BX10 strains

| Project | V-P reaction | Contact enzyme | Methyl red | Citrate | Denitrification | Fluorescent pigment | Gelatin liquefaction | Glucose oxidation |
|---------|--------------|----------------|------------|---------|-----------------|---------------------|----------------------|-------------------|
| BX2     | -            | -              | -          | -       | -               | +                   | +                    | -                 |
| BX9     | -            | +              | -          | -       | -               | -                   | +                    | +                 |
| BX10    | -            | -              | -          | -       | +               | -                   | +                    | +                 |

Note: +" Means positive, "-" Negative.

4. Discussion and conclusion
The phosphorus cycle in the soil needs to be promoted by phosphate-dissolving bacteria, which can convert insoluble phosphorus into available phosphorus that can be absorbed and used by plants, thereby improving the phosphorus utilization rate in the soil. Phosphate-dissolving bacteria have also been used in actual production for a long time. In 1958, Russian researchers first applied strains with phosphorus-decomposing ability to make microbial fertilizer into farmland [9], the crop has made clear gains. The researchers then investigated the effects of different types of phosphate-resolving bacteria on crop growth and yield; the results show that the phosphate-dissolving bactericide can...
significantly increase crop yield. Phosphate-decomposing microorganisms not only increase the yield of crop, but also have high fertilizer efficiency and can reduce environmental pollution. But overall, the development and application of phosphate decomposing bacteria fertilizer in China is not deep enough. There are few species, so it is essential to develop a new type of sustainable phosphate-resolving microbial fertilizer. In the study of screening of phosphate-decomposing bacteria was sunflower rhizosphere soil. Initially obtained ten strains with the ability of phosphorus solution, the ratio of the diameter D (mm) of the phosphorus dissolving circle to the diameter d (mm) of the colony is between 1.10-4.35. The D / d value of 7 strains were more significant than 2, and the D / d value of the BX2 stress was the largest, which was 4.35. The liquid shake flask re-screening used to determine the strain's ability to decompose the insoluble organic phosphorus in the solution. The results showed that the strain's phosphorus-dissolving effect and the ratio of the diameter of the phosphate-dissolving circle D (mm) to the colony diameter d (mm) were the same. If the D / d value is significant, the phosphorus dissolving effect is also high. The effective phosphorus increase of strain BX2 reached 38.59 μg / mL; it judge to be a high-quality organophosphate-degrading bacteria by its phosphate-decomposing activity. Based on the above results, a total of 5 excellent strains were obtained, including strains BX2, BX3, BX5, BX9 and BX10. They have high application value and can be used as research objects of phosphate-decomposing microorganisms.

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