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Identification and Analysis of Preservation Conditions for Soil-Isolated *Priestia megaterium* Subspecies

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Abstract: This study aimed to isolate and identify a subspecies of Priestia megaterium with potential probiotic properties from soil and evaluate its cryopreservation conditions. The bacterium was isolated using dilution and plate spreading methods, and identified through 16S rDNA sequencing and physiological and biochemical experiments. Different cryoprotectants (glycerol, DMSO, and starch) were also assessed. The results showed that the strain is a Gram-positive spore-forming bacterium, with 8% DMSO providing the best protective effect, glycerol showing a relatively poor effect, and starch having a detrimental impact. This study provides a theoretical basis for the development and application of probiotics.

Keywords: Microbes; Probiotics; Protease; Bacillus; Freezing

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1. Introduction

Proteases have significant application value in various industries, including food processing, leather treatment, and detergent manufacturing [1–3]. However, traditional proteases extracted from plants or animals suffer from high production costs, low yield, and lengthy production cycles, gradually becoming insufficient for meeting industrial demands. Microbial-derived proteases have attracted considerable research attention due to their advantages such as high-efficiency production, low cost, and ease of operation [4,5]. In probiotic research, microorganisms with proteolytic abilities have also drawn wide attention [6–9]. Screening and isolating such microorganisms have significant application potential.

For the long-term preservation of microorganisms, selecting appropriate storage conditions is crucial. Cryopreservation technology has become a commonly used preservation method due to its effectiveness in reducing cell damage and maintaining microbial viability. Compared to other preservation methods, cryopreservation is simpler to operate and can preserve the biological characteristics of microorganisms for an extended period [10].

In this study, a protease-producing strain, *Priestia megaterium*, was isolated and identified from soil. Its survival rates under different cryopreservation conditions were evaluated, aiming to provide references for industrial and pharmaceutical applications.

2. Materials and methods

2.1. Materials and reagents

Glucose and magnesium sulfate were purchased from Sinopharm Chemical Reagent Co., Ltd. Yeast extract was purchased from Servicebio, and casein was obtained from Biosharp. Skim milk powder (brand: BTNature) originated from New Zealand. Potassium dihydrogen phosphate was purchased from Tianjin Kaitong Chemical Reagent Co., Ltd. Agar was acquired from BioFroxx, and bacterial culture dishes were purchased from Beijing Labgic Technology Co., Ltd.

2.2. Instruments and equipment

Biochemical incubator: Jintan Medical Instrument Factory; constant-temperature shaker incubator: Shanghai Xinmiao Medical Equipment Manufacturing Co., Ltd.; autoclave: Zhiwei (Xiamen) Instrument Co., Ltd.; double-person single-sided clean bench: Suzhou Purification Equipment Co., Ltd.; refrigerator: Hefei Meiling Co., Ltd.

2.3. Experimental methods

2.3.1. Protease identification medium

The medium contained 0.25% glucose, 0.5% yeast extract, 0.5% casein, 0.5% skim milk powder, 0.1% potassium dihydrogen phosphate, 0.04% magnesium sulfate, and 1.5% agar, prepared with deionized water and adjusted to pH 7.4. The medium was sterilized at 108 ℃ for 30 min by autoclaving, poured into plates at approximately 60 ℃, and used once solidified.

2.3.2. Preparation of bacterial cryopreservation solutions

Solutions containing 50% glycerol and 50% glycerol + 2% potato starch were prepared with glycerol, potato starch, and deionized water, autoclaved at 108 ℃ for 30 min before use. Solutions of 24%, 16%, and 8% DMSO were prepared by mixing sterile liquid medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 0.5% glucose, pH 7.4, with deionized water) directly with DMSO without further sterilization. For preparing 16% DMSO solutions with starch, 8.4 mL of liquid medium was placed in Erlenmeyer flasks, mixed with 0.1 g, 0.2 g, or 0.3 g potato starch, sterilized at 108 ℃ for 30 min, cooled, then mixed thoroughly with 1.6 mL DMSO to obtain 16% DMSO solutions with 1%, 2%, or 3% starch.

2.3.3. Dilution and spread-plate method

Approximately 1 g of soil samples randomly collected from the field were homogenized in 1 mL of sterile water. A 100 μL suspension was diluted 10-fold repeatedly up to 10^6 times by transferring into 900 μL sterile water. Subsequently, 50 μL aliquots from dilutions of 10^4, 10^5, and 10^6 were plated onto solid protease identification medium containing 15–20 mL agar. The samples were evenly spread using sterilized glass spreaders and incubated invertedly at 37 ℃ in a biochemical incubator for 24–48 h. Colonies were counted, and bacteria capable of protein hydrolysis were isolated by the formation of clear zones. These colonies underwent streak cultivation for single colonies, followed by a second protease screening on identification medium to confirm protease production (clear zone formation). Positive isolates were preserved.

2.3.4. Gram staining

Microscope slides were cleaned with alcohol and dried. One drop of saline was placed on a slide, inoculated with colonies grown for 48 h via streak cultivation, evenly dispersed, smeared, and gently heated over an alcohol flame to inactivate and fix. One or two drops of crystal violet were applied for 1 min and rinsed gently with deionized water. After slight drying, Lugol's iodine solution was added for 1 min and gently rinsed with deionized water. Decolorization was performed by gently rinsing with 95% alcohol until no more violet color was released, followed by washing with deionized water. After drying, safranin was added to counterstain for 30 sec, gently rinsed again with deionized water, and dried. Microscopic observation and photography were performed using a 100 × oil immersion lens.

2.3.5. Spore staining

One to two drops of sterile water were added to a small test tube, inoculated with bacterial colonies using a sterile loop, and mixed thoroughly. Then, 2–3 drops of 5% malachite green solution were added. The test tube was heated in a boiling water bath for 15–20 min. From the bottom of the tube, 2–30 μL bacterial suspension was spread onto a slide, air-dried, and fixed over an alcohol flame. Excess stain was gently rinsed off with deionized water until no green color was released. One drop of safranin was added for counterstaining for 5 min, washed with water, blotted dry with absorbent paper, and air-dried. Spore staining results were examined and photographed under a 100 × oil immersion microscope.

2.3.6. Strain identification

(1) 16S rDNA sequencing

A single colony of the purified bacterial strain was inoculated into LB liquid medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) and incubated to obtain a pure bacterial culture. The fermentation broth was then sent to Sangon Biotech (Shanghai) Co., Ltd. for 16S rDNA sequencing, using the following primers:

27F 5’-AGAGTTTGATCCTGGCTCAGGATGA-3’

1492R 5’-TACGGCTACCTTGTTACGACTTAGC-3’

(2) Homology analysis

The sequencing results were aligned using the Standard Nucleotide BLAST on the NCBI website to assess the homology between the 16S rDNA nucleotide sequence of the strain and those of known bacterial species.

(3) Physiological and biochemical tests

Physiological and biochemical tests, including catalase reaction and methylene blue reduction, were carried out according to the methods described in Bergey’s Manual of Determinative Bacteriology (8th edition) and the Manual for Systematic Identification of Common Bacteria.

2.3.7. Study on cryopreservation conditions

Bacterial strains were retrieved from storage and inoculated into liquid culture medium (containing 1% tryptone, 1% NaCl, 0.5% yeast extract, 0.5% glucose, prepared with deionized water, pH adjusted to 7.4), then incubated at 37 °C for 24 hours with shaking at 200 rpm. Viable counts were obtained by the dilution plate method. Aliquots of bacterial suspension were mixed at a 1:1 ratio with different cryoprotectants including 50% sterile glycerol, 16% DMSO, 50% sterile glycerol plus 2% starch, 24%, 16%, or 8% DMSO alone, or combinations of 16% DMSO plus 3%, 2%, or 1% starch, reaching a final volume of 1 mL. Samples were frozen at −20 °C, and viability was assessed by the dilution plate method after 1 day, 2 days, and 7 days to determine bacterial survival rates.

3. Results and analysis

3.1. Colony morphology and protein degradation capability

Bacterial isolates from soil samples formed individual colonies on selective media supplemented with casein and skim milk (protein sources). Colonies appeared creamy-yellow in colour, had smooth edges, moist surfaces, and were easily picked. The average colony diameter was 1.55 cm. A clear zone indicating protein degradation formed around the colonies, averaging 2.55 cm in diameter (**Figure 1**). The ratio between the clear zone diameter and colony diameter was 1.65. The bacterial isolate was designated as strain QG-P-S20.

Figure 1. Colony morphology and protein degradation transparent circle of bacterial strain QG-P-S20.

3.2. Gram staining and spore staining

After Gram staining and observation under an oil immersion lens, strain QG-P-S20 was identified as a Gram-positive bacillus. Spore staining revealed the presence of large central endospores within the bacterial cells (**Figure 2**), rather than terminal spores. The spores appeared green, while the vegetative cells were stained red. These results indicate that QG-P-S20 is a spore-forming bacillus.

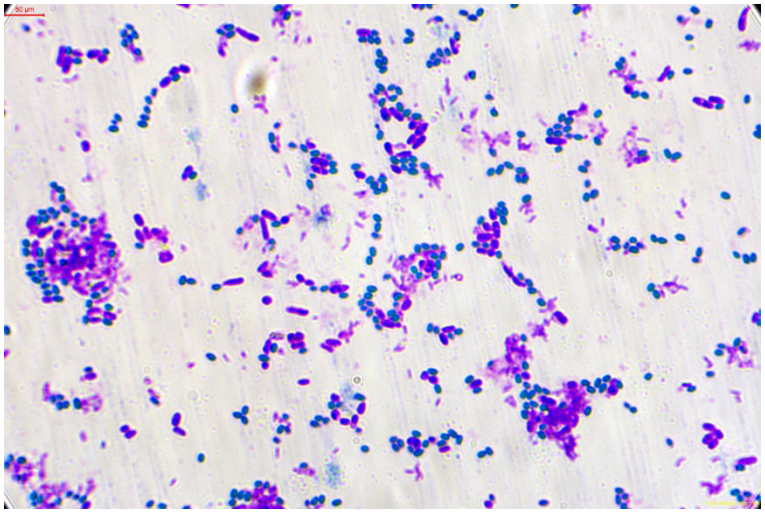

Figure 2. Spore staining of bacterial strain QG-P-S20.

3.3. Strain identification

3.3.1. Sequence determination

The nucleotide sequence of the 16S rDNA gene of strain QG-P-S20 was obtained through sequencing, and is as follows:

GGGGGGGGGGGCTATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGG

ATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTG
ATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAG
CGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGG
TCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCT
GGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTC
CCCTTCGGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT
GGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAG
GCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGAGTAACCGTAAGG
AGCTAGCCGCGTAAGGGGA

3.3.2. Homology analysis

The 16S rDNA nucleotide sequence of strain QG-P-S20 was analyzed using the BLAST tool on the NCBI website. As shown in **Figure 3**, the results indicate that QG-P-S20 shares high sequence similarity with *Priestia megaterium* and is classified as a member of this subspecies.

Priestia megaterium strain 153-1 16S ribosomal RNA gene, partial sequence

Sequence ID: OQ560352.1

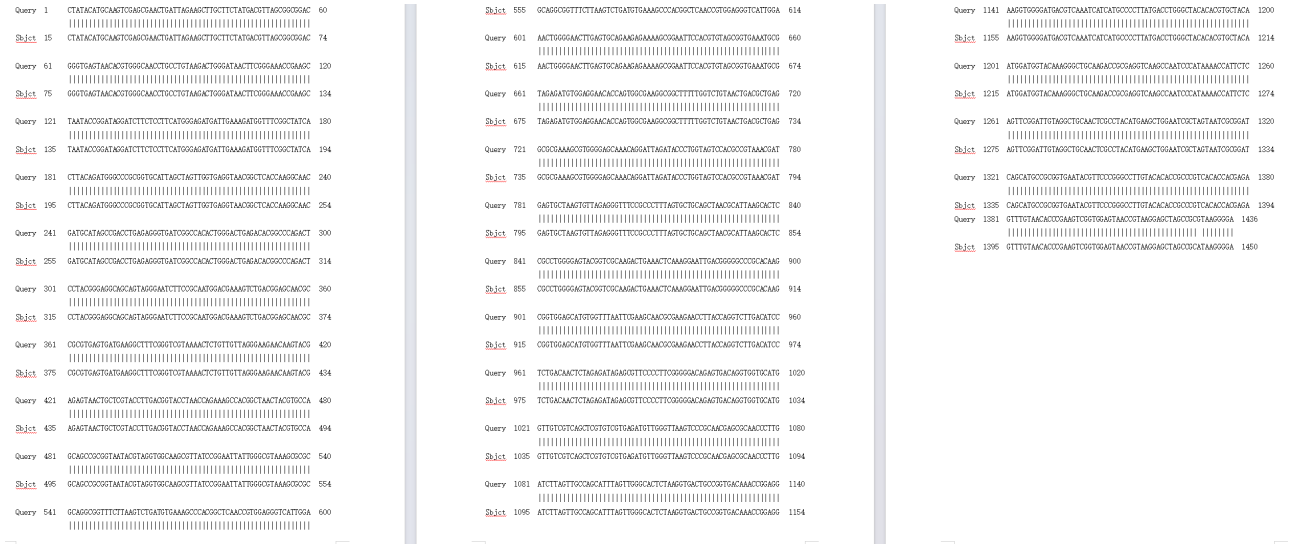

Figure 3. Comparison of 16S rDNA sequences.

3.3.3. Physiological and Biochemical Tests

Physiological and biochemical tests showed that the strain is catalase-positive, indicating the presence of catalase within the bacterial cells. This enzyme is capable of decomposing 3% hydrogen peroxide into oxygen, which is then released. In

the photograph, the colony on the right displays bubble formation due to the reaction with hydrogen peroxide, whereas the colony on the left, which was not treated with 3% hydrogen peroxide, shows no bubble formation (**Figure 4**).

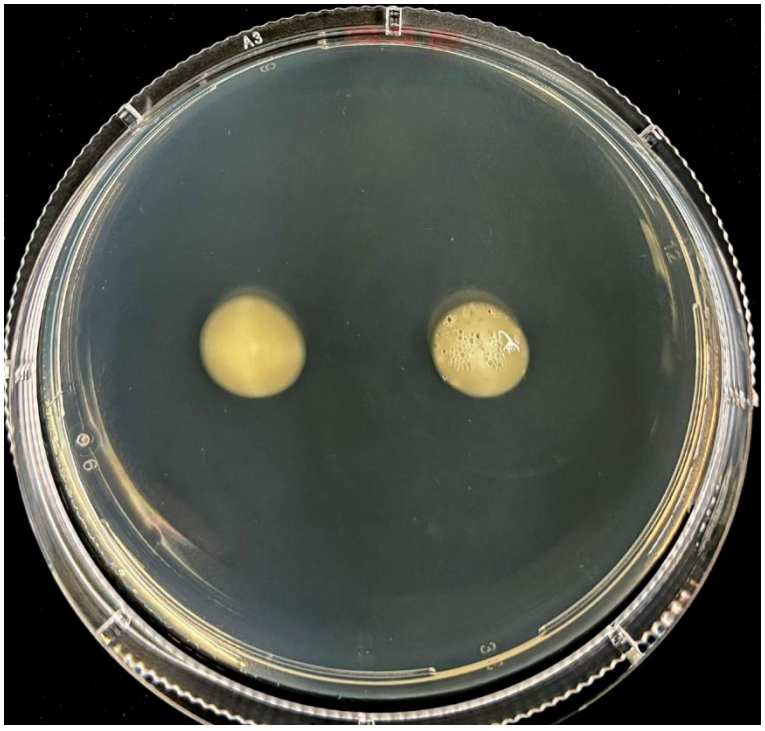

Figure 4. Reaction of catalase.

3.4. Analysis of cryopreservation conditions

3.4.1. Effect of different chemical reagents on bacterial cryopreservation

As shown in **Figure 5**, the experimental results indicate that dimethyl sulfoxide (DMSO) provided the best protective effect for the spore-forming bacterium P-S20. After freezing at −20 ℃ for one day, the survival rate reached 65.5%, and even after one week of storage, 8.1% of the bacterial cells remained viable. In contrast, glycerol and starch offered less effective protection for bacterial cells under the same conditions.

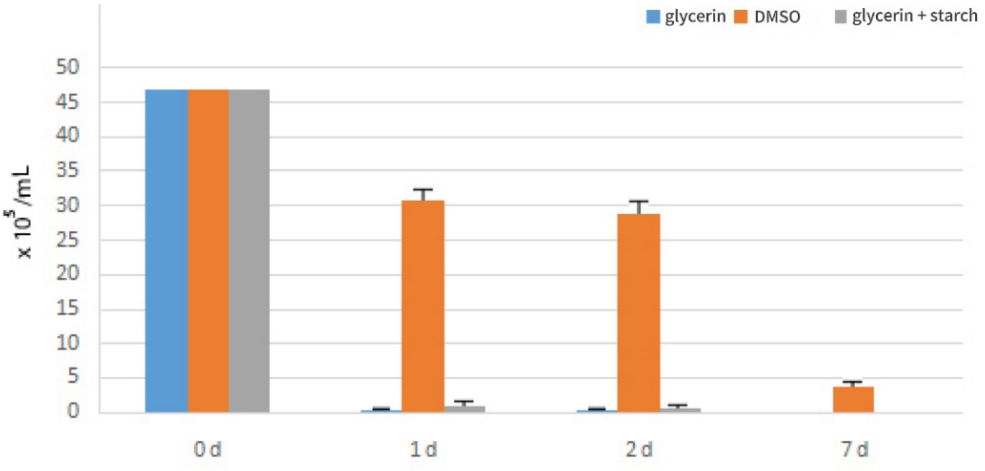

Figure 5. Effects of glycerol, dimethyl sulphoxide, and starch on freezing of bacterial strain QG-P-S20.

3.4.2. Effect of different DMSO concentrations on bacterial cryopreservation

As shown in **Figure 6**, 8% DMSO was sufficient to effectively protect bacterial cells from damage caused by low temperature and freezing. A concentration around 4% provided slightly weaker protection. However, concentrations higher than 8% were detrimental to bacterial viability, as high levels of DMSO can destabilize bacterial cell membranes, leading to cell lysis and death.

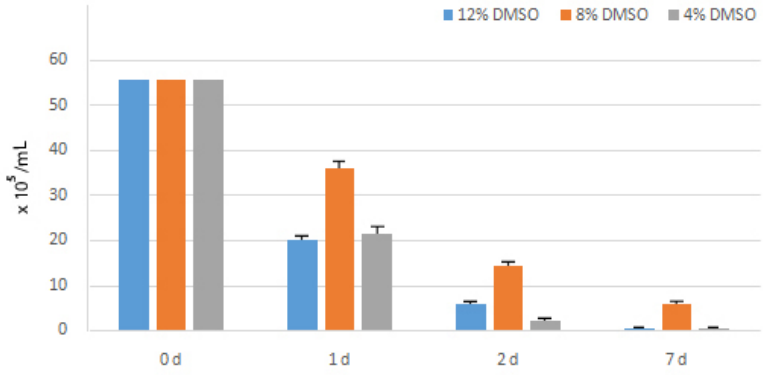

Figure 6. Effects of different concentrations of DMSO on the freezing of bacterial strain QG-P-S20.

3.4.3. Effect of starch on bacterial cryopreservation

At a concentration of 8% DMSO, the addition of starch had a detrimental effect on the cryopreservation of bacterial cells, as shown in **Figure 7**. The presence of starch significantly reduced the survival rate of the bacteria. Therefore, the use of starch or similar substances is not recommended in bacterial cryopreservation solutions.

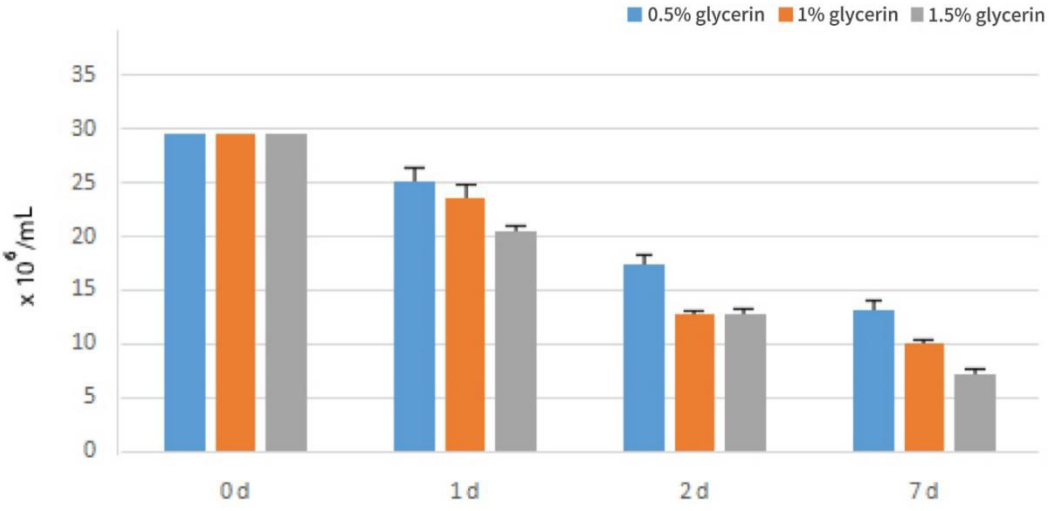

Figure 7. Effects of different concentrations of starch on the freezing of bacterial strain QG-P-S20.

4. Discussion and conclusion

In this study, a Gram-positive spore-forming bacterium was isolated from soil. Based on 16S rDNA sequencing and BLAST analysis, the strain was identified as a subspecies of *Priestia megaterium*. Under cryopreservation conditions, 8% DMSO provided the most effective protection for the strain, while glycerol showed a limited protective effect, and starch was detrimental to bacterial survival.

The strain demonstrated efficient casein degradation, indicating high protease activity. By optimizing culture medium composition, fermentation processes, or applying physical, chemical, or biological mutagenesis, as well as genetic engineering approaches, it is possible to obtain strains with enhanced protease production or activity.

This study provides a new perspective for the screening and application of protein-degrading bacteria and lays a foundation for future industrial applications. Further research should explore the performance of this strain under various industrial conditions and conduct large-scale production trials to evaluate its feasibility and economic potential for commercialization.

Disclosure statement

The author declares no conflict of interest.

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