Designing an Open and Comprehensive Microbiological Experiment for Undergraduates

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Abstract. To stimulate the depressed curiosity and enhance the low interest in studying fundamental microbiological theory and skills of undergraduates, an open experiment "Screening and Identification of Biocontrol Bacteria Against Plant Pathogenic Fungus and Preliminary Exploration of the Antibacterial Activity "was designed and implemented. The research focuses on screening and identification of biocontrol bacteria that could inhibit plant pathogens. Biocontrol bacteria with significant inhibition ability of plant pathogens were screened and purified from soil samples. They were identified by morphological observation, physiological and biochemical testing, and also by 16S rDNA gene sequencing analysis. Then, their antibacterial activity was preliminarily explored based on growth curves. Good teaching effect was obtained.

Keywords: Plant pathogen, Biocontrol bacteria, Screening, Identification, Open experiment

1 INTRODUCTION

Microorganisms with small size and simple structure are not only important model materials for life science research, but also closely related to human life [1]. For example, microorganisms that cause plant diseases (also known as plant pathogens, including fungi, bacteria, viruses, etc.) not only directly affect crop yields and cause economic losses, but also may affect the sustainable cultivation of crops and affect human health and development [2-4]. At present, the prevention and control of crop diseases mostly relies on chemical control [5-6], but the use of a large number of chemical pesticides has caused a series of environmental pollution and human health problems. Therefore, it is

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pathogen [7]. With the development of microbial technology, biological control has been increasingly emphasized as an effective means of plant disease control [8].

Cultivating innovative talents with solid professional foundation, strong scientific research ability and high comprehensive quality has become a realistic and urgent issue facing the current teaching reform and sustainable development of colleges and universities. Although students majoring in biotechnology in their third year have a certain foundation in microbiology knowledge and experimental skills, their ability to flexibly apply the knowledge and skills to solve practical problems still needs improvement. Practice has shown that open experimental teaching plays a very important role in cultivating students' innovative spirit and practical ability, and is an effective way to cultivate innovative talents in colleges and universities.

In summary, the microbiology laboratory relies on the "Open Experimental Compulsory Topic" course offered by the center (1 credit, 32 class hours), with the topic of research hotspots in the biological control of plant pathogens, and offers an open experimental project on " Screening and Identification of Biocontrol Bacteria Against Plant Pathogenic Fungus and Preliminary Exploration of Antibacterial Activity ". The research focuses on obtaining biocontrol bacteria that can inhibit plant pathogens. After isolation, purification, and screening, biocontrol bacterial strains with significant inhibition of plant pathogen growth were selected from the soil. Classification and identification of the screened biocontrol bacteria was done through morphological observation, physiological and biochemical detection, 16S rDNA gene sequence analysis, etc. Preliminary exploration of the antibacterial activity of the biocontrol bacteria was performed based on growth curves. This project is very similar to a small scientific research topic, which is systematic and comprehensive, with uncertain experimental results and challenging steps and exploratory throughout the process, which is conductive to improving students' scientific inquiry ability and comprehensive quality.

2 INSTRUMENTS AND EXPERIMENTAL MATERIALS

2.1 Main Instruments and Equipments

Biosafety cabinet, constant temperature incubator, high-pressure steam sterilization pot, ultra clean workbench, optical microscope, high-speed freezing centrifuge, spectrophotometer, etc.

2.2 Experimental Materials

(1) Plant pathogens: Phytophthora infestans donated by Hebei Agricultural University, Hebei, China.

(2) Soil sample: collected from 10~15 cm below the surface of Liutuan Town, Yanshou County, Harbin City, Heilongjiang Province, China.

2.3 Main Reagents

(1) Czapek Dox Agar（Solarbio）and Beef Extract Peptone Broth (Solarbio) were purchased from Tianjin Haos Biotechnology Co. , Ltd.

(2) Improved Beef Extract Peptone Broth and Czapek Dox Agar (hereinafter referred to as improved medium): The above czapek dox agar and beef paste peptone broth are mixed in a 1:1 ratio and high pressure sterilized at 0.1 MPa, 121 ℃, 20 minutes.

(3) Bacteria Genomic DNA Kit was obtained from Tiangen, Biotech (Beijing), China.

3 EXPERIMENTAL METHODS

Before students conduct experiments, design suitable experimental steps (Figure 1) and guide them to conduct experimental operations in a certain order after class. During the experiment, encourage students to think and ask questions, and guide them to solve problems through the experiment; organize students to discuss and answer questions regularly; To guide the students to sum up the experimental results, through reviewing the experimental process, analyzing the experimental data and summarizing the experimental results, to help the students understand the research objectives of the experimental items in depth, looking for more suitable experimental methods.

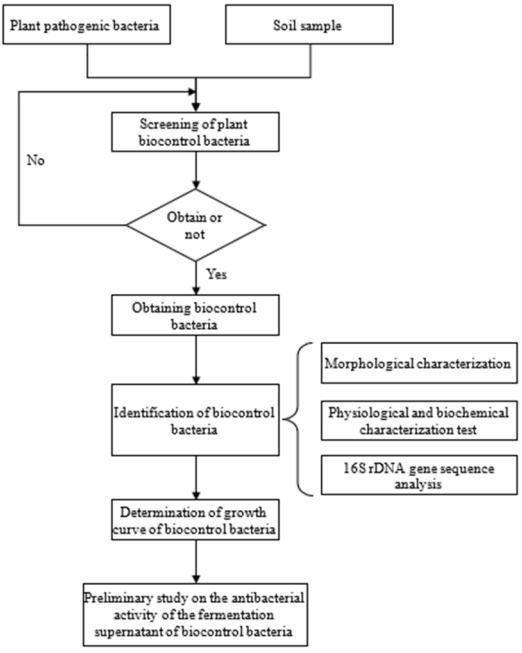

Fig. 1. Flow chart of the experimental design.

3.1 Preparation of Spore Suspension of Plant Pathogenic Fungi

The spore suspension was prepared by adding 5ml sterile water into czapek dox agar with *P. infestans* vigorously growing for one week. The mycelium was rinsed thoroughly, then suspension was transferred into 95ml of sterile water to obtain diluted spore suspension.

3.2 Screening Biocontrol Bacteria

Preliminary Screening of Biocontrol Bacteria. Suspension of Plant pathogens spores and soils were co-inoculated using double-layer plate method and cultivated for 5-7 days until clear antibacterial zone was observed. Biocontrol bacteria with obvious antibacterial circles were selected and separated by multiple plate streaking method. The diameter of bacteriostatic zone was measured by cross method.

Rescreening Biocontrol Bacteria. Spore suspension (100 ul) prepared according to method 2.1 was taken out and spread onto the improved medium plate. The activated biocontrol bacterial strains were inoculated 2 cm away from the center point in a triangular distribution pattern. There were three replicates for each treatment. For negative control, only the spore suspension of P. infestans was coated. The cultures were incubated at 28 ℃ for 5~7 days until clear bacteriostatic zone was observed.

3.3 Identification of the Biocontrol Bacteria

Morphological Characterization. The strains of biocontrol bacteria were inoculated onto beef extract peptone solid culture medium through plate streaking, and incubated overnight at 37 ℃. The characteristics of these colonies were observed and recorded. The morphological characteristics of the biological bacteria were observed by Gram staining.

Physiological and Biochemical Characterization. According to the methods in the Bergey's Manual of Determinative Bacteriology, some characteristics of the biocontrol bacteria were identified, including the M.R test, the V. P test, the indole test, the glucose fermentation test, the lactose fermentation test, and the hydrogen sulfide production test [9].

16S rDNA Gene Sequence Analysis. Bacterial DNA extraction kit (Tiangen) was used to extract the DNA of biocontrol strains, and 16S rDNA sequencing was performed by An Shengda (Tianjin) Biotechnology Co., LTD. The sequencing result was compared with NCBI Blast database, and the phylogenetic tree of these biocontrol strains was constructed by using MEGA 7.0 software through Neighbor-Joining method (Bootstrap value was 1000 times) to verify the classification status of biocontrol strains.

3.4 Determination of Growth Curve of Biocontrol Bacteria and Preliminary Study on Antibacterial Mechanism of Fermentation Supernatant

Determination of Growth Curve of the Biocontrol Bacteria. Sterile water (5 mL) was added into a biocontrol bacterial slope cultured overnight at 37 ℃, and the bacteria lawn was washed to obtain a uniform bacteria suspension. 1 ml of the above bacterial suspension was inoculated into 100 mL beef paste protein liquid medium and cultured at 37 ℃ and 150 r/min overnight. Take 2ml of overnight culture medium for biocontrol bacteria, add it to 100 ml of beef extract peptone liquid culture medium, and mix well. Take 5 ml of the mixed solution and transfer it into sterile test tubes for cultivation at 37 ℃ and 150 r/min.3 ml of the above bacteria solution (sterile operation) was taken out every 1 h, the absorbance value at a wavelength of 600 nm was measured. The bacterial growth curve using cultivation time as the x-axis and absorbance OD value as the y-axis was obtained.

Preliminary Study on the Antibacterial Activity of the Fermentation Supernatant of Biocontrol Bacteria. 100 μ l of the suspension of pathogenic fungal spores prepared according to method 2.1 was taken out and spread onto Czapek Dox Agar. Holes with a triangular distribution at a distance of 2 cm from the center point of the culture medium were Drilled with a diameter of approximately 5 mm.

1 ml of the bacterial solution in 2.5.1 (3) every 1 hour was taken out and centrifuged at 12000 rpm for 1 minute, and the supernatant filter-sterilized through a 0.22 μm filter to obtain cell-free fermentation broth. 200μl of cell-free fermentation broth was added into the holes and the plates were cultured it in an upright position at 28 ℃ to observe the antibacterial effect of supernatant.

4 RESULTS AND ANALYSIS

4.1 Isolation, Purification, and Screening of Biocontrol Bacteria

The results of double-layer plate culture revealed 12 biocontrol bacteria with certain inhibitory effects on the growth of *P. infestans*. Among them, a biocontrol bacterium with number H-12 was selected with the most obvious inhibition zone (Tab.1, Fig. 2), and then this bacterium was further analyzed and identified.

Table 1. Antifungal action of biocontrol bacteria liquid

Stain	Diameter of bacteriostatic circle (cm)
H-1	-
H-2	2.04±0.08 b
H-3	2.04±0.10 b
H-5	2.47±0.05 ab
H-12	2.87±0.44 a

Note: Data with the different lowercases letters in same column indicated significantly different ($P<0.05$).

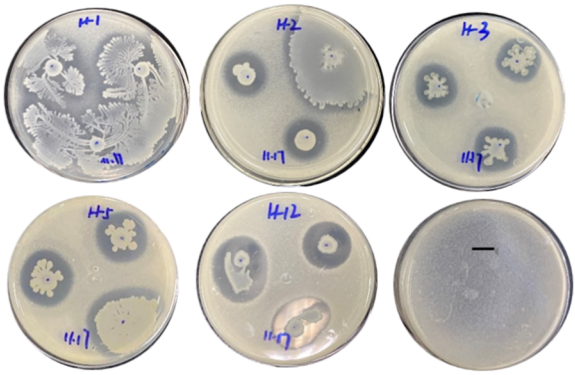

Fig. 2. Inhibition effect of re-screening biocontrol bacteria on *P. infestans*

4.2 Identification of Biocontrol Bacteria Strains

Morphological Features of H-12. The biocontrol bacteria H-12 grows well on the beef extract peptone medium, the bacterial colony is translucent, with smooth surface, and irregular edges (Fig. 3A). It was proved to be Gram-positive by Gram staining, and cells of the bacteria are rod-shaped with endospores (Fig. 3B).

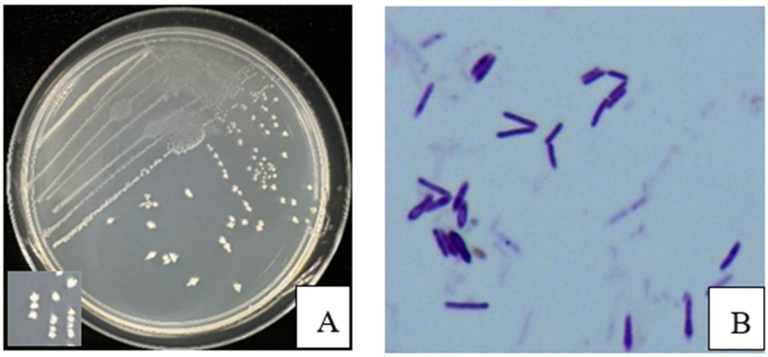

Fig. 3. H-12 colonies, cells of the bacteria and endospores.

Physiological and Biochemical Characteristics of H-12. Six physiological and biochemical characteristic tests were performed on H-12 bacterial strain. The results of indole test, M.R test, V.P test, and H2S test were all negative, indicating that H-12 bacterial strain cannot utilize tryptophan and sulfur-containing amino acids. It can ferment glucose without producing pyruvate. Acetyl methyl methanol is not produced in glucose metabolism. The results of glucose fermentation test and lactose test showed that H-12 produced acid during sugar fermentation with no gas (Fig. 4).

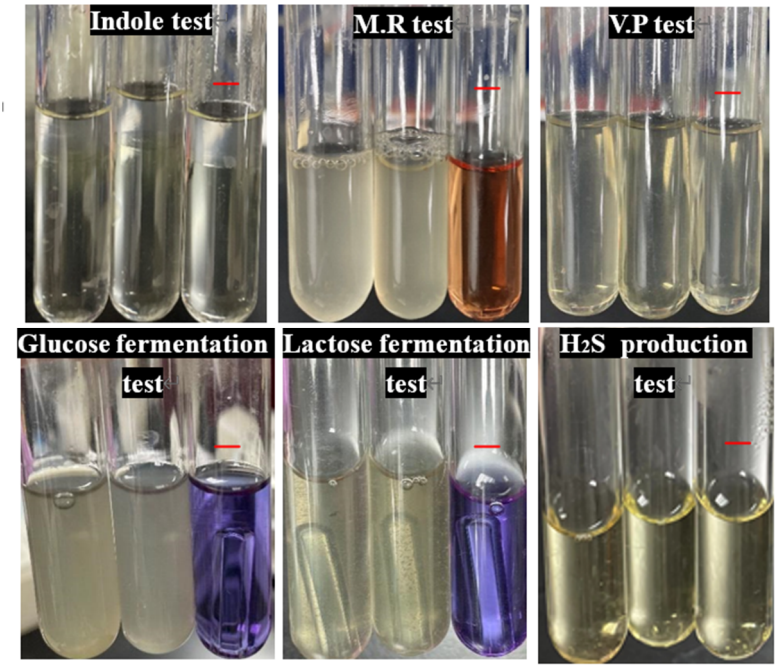

Fig. 4. Physiological and biochemical characteristics of H-12.

16S rDNA Gene Sequence Analysis. 16S rDNA sequence of H-12 bacterial strain was submitted to GenBank for blast comparison in the database. The results showed that it has the highest similarity (98.74% similarity) with Paenibacillus polymyxa. Therefore, it is speculated that this strain belongs to genus Bacillus polymyxa. MEGA 7.0 software was further used to analyze its phylogenetic relationship, the results showed that H-12 strain and Paenibacillus polymyxa were on the same branch with high homology (Fig. 5). By combining colony morphology observation, physiological and biochemical detection, and 16S rDNA gene sequence analysis, H-12 strain was identified as P. polymyxa H-12.

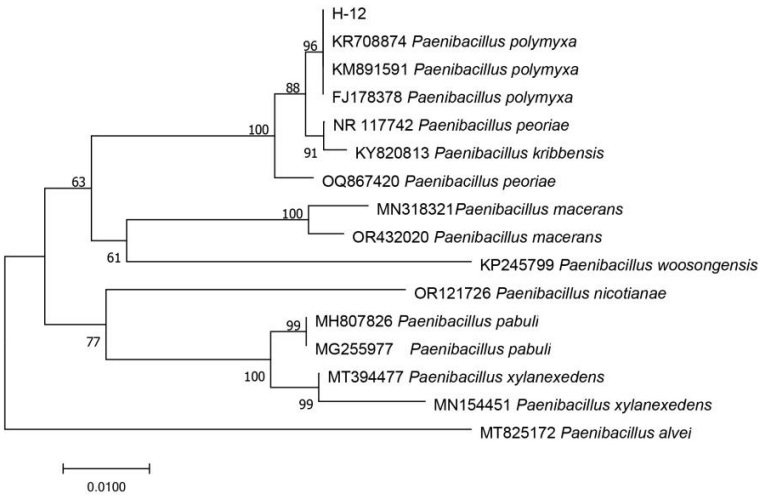

Fig. 5. The phylogenetic tree of H-12 strain constructed using 16S rDNA gene sequences.

4.3 Growth Curve of H-12 and Preliminary Study on its Antibacterial Activity

It can be seen that the logarithmic growth phase of H-12 strain occurs at approximately 2-9 hours, the plateau phase occurs at approximately 9-11 hours, and the decline phase occurs at 11-30 hours (Fig. 6). Through antibacterial experiment of the fermentation broth, it was found that fermentation broth cultured for 26 hours produced the largest antibacterial circle (Fig. 7), indicating that the antibacterial substance was produced during this time period.

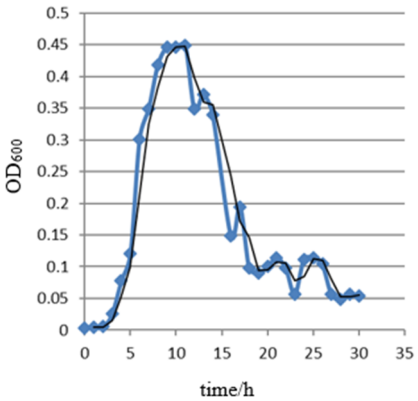

Fig. 6. Growth curve of H-12 bacteria strain.

Fig. 7. Antibacterial effect of fermentation broth of H-12 bacteria strain.

5 TEACHING EFFECTIVENSS

5.1 Consolidating the Foundation and Expand New Skills

Although the junior students have mastered certain basic microbiology experimental skills, it was found that they still lack a solid grasp of some basic operations in actual practices, resulting in several failed experiments and slow project progress. Microbiological experimental techniques have been widely integrated into various fields of modern life sciences, especially aseptic technique, preparation and sterilization of culture media, pure strain isolation technique and other technologies, which are very important basic operations in molecular biology, biochemistry and other research fields. Through project training, students can strengthen their basic skills and lay a solid foundation for their future academic development. In addition, students also need to constantly develop new skills, turn some theories into practical practice, and master methods and training skills in practice, such as: ① expanding experimental skills that cannot be practical in basic courses due to class time limitations: measuring growth curves, testing the antibacterial effect of fermentation broth; ② Molecular biological experimental techniques such as bacterial genome extraction, agarose gel electrophoresis, primer design, PCR, etc. in species identification experiments based on 16S rDNA; ③ Using bioinformatics methods such as sequence alignment and phylogenetic tree construction using GenBank database.

5.2 Improving Students' Scientific Exploration Ability

Continuously improving students' scientific exploration ability is the core of improving their independent innovation. The content of this experimental project is closely related, and the experimental results are uncertain. Students should learn to scientifically explain the experimental phenomena in a timely manner, comprehensively evaluate the

experimental results, and scientifically analyze the problems. For example, in the process of screening biocontrol bacteria, how to judge the general classification of bacteria according to the morphological characteristics of colonies? how to flexibly select and design culture media suitable for mixed cultivation of different bacterial strains? How to set the time interval scientifically when the growth curve is measured? Why do filters need to be used for sterilization in bacteriostatic experiment of fermentation solution? Research has found that the fermentation broth of biocontrol bacteria has a certain antibacterial effect. If the experiment continues, how can subsequent experiments be designed? Through independent thinking, discussions with others, and extensive literature research on these practical problems, students have been able to flexibly adjust and design solutions, effectively exercising their scientific thinking and exploration abilities, laying a solid foundation for their future scientific research.

5.3 Cultivating Comprehensive Qualities of Students

The experimental project provides students with a platform for independent learning. Students can utilize laboratory resources, fully exert their subjective initiative, and continuously improve their comprehensive quality through "learning by doing" and "doing while learning" in practice. For example: ① Through writing a research paper, students can not only master the general ideas and methods of scientific research paper writing, but also establish a rigorous writing attitude through continuous modification and improvement, learn to express scientific viewpoints clearly and accurately with logical writing, improve the writing level of scientific research papers and summarizing ability; ② In the final report section, students describe the experiment in a complete and clear manner, express their opinions flexibly when facing questions, and improve their academic communication skills; ③By collaborating and exchanging ideas with other classmates in the group, exploring and learning, and improving team collaboration skills.

6 TEACHING DISCUSSIONS

6.1 Project Implementation

It mainly consists of four steps: independent topic selection, project implementation, report writing, and final report. Students can choose their own topic within the framework of " Screening and Identification of Biocontrol Bacteria Against Plant Pathogenic Fungus and Preliminary Exploration of Antibacterial Activity ", and determine specific implementation plans by referring to literature. After the feasibility evaluation of the plan by the teacher, the students arrange the experimental progress independently, adopt the form of advance appointment, and use their spare time to enter the laboratory to carry out the experiment. In mid-December, students began to sort out the experimental results and data, write the experimental report and presentation PPT in the format of scientific and technological papers, and make the final report at the end of December, 5 min/ person. In principle, each student should use different soil samples to

screen biocontrol bacteria, with one person per topic. Students lead and participate in the entire experiment, while teachers participate in guiding and controlling the quality of project implementation.

6.2 Performance Evaluation

Course grade=70% average report grade+30% experimental report grade. Pay attention to the assessment of students' experimental process, scientific paper writing, and academic communication abilities.

6.3 Project Types

This open experimental project has been included in the center's "Open Experimental Compulsory Topic" project library, and its type is elective. The number of course takers should be controlled between 2-3 people, and the selection of projects should be determined annually based on the number of course takers.

7 CONCLUSION

In response to the insufficient ability of junior students to flexibly apply their knowledge and skills to solve practical problems, this open experimental project has been established. The experiment not only covers the basic theories and experimental skills of microbiology, such as preparation and sterilization of culture media, aseptic technique, using microscope, staining of bacterial bodies and special structures, pure strain isolation technique, but also expands experimental skills that cannot be practical in basic courses due to class time limitation, such as measuring growth curves, testing the antibacterial effect of fermentation broth .In addition, it also involves molecular biology experimental technologies such as bacterial genome extraction, agarose gel electrophoresis, primer design, PCR and other molecular biological experimental technologies. Bioinformatics methods such as sequence alignment and phylogenetic tree construction using GenBank database. It reflects the intersection and integration of disciplines, fully mobilizes students' subjective initiative, and improves their scientific exploration ability and comprehensive quality. This design would cultivate innovative and exploratory professional talents in the new era.

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