

Molecular Biological Identification and Determination of Growth Conditions of Facultative CO₂ and N₂ Fixing Bacterium BHJ

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Abstract: Currently, the greenhouse effect is becoming increasingly serious, and biological carbon-fixation, especially microbial biological carbon-fixation, can play a unique role in curbing the greenhouse effect. CO₂ and N₂ are the major greenhouse gases. In this study, a facultative CO₂ and N₂ fixing bacterium named BHJ (which can fix CO₂ and N₂ simultaneously) was identified using molecular biology, and its growth conditions were determined. Total DNA from BHJ was extracted and then the 16S rDNA was amplified using polymerase chain reaction. The 16S rDNA sequence was identified and analyzed by alignment with sequences in the GenBank database. Single variables in medium without carbon and nitrogen (KH₂PO₄ 0.2 g, CaCl₂ formula: 0.2 g, NaCl 0.2 g, MgSO₄ 0.3 g, VB 0.0625 g, trace element solution 0.5 mL, distilled water: 1000 mL) was performed. Spectrophotometry was used to measure the optical density changes to determine the optimum temperature, CO₂ and N₂ ratio, and pH for the growth of BHJ. The 16S rDNA sequence of BHJ showed more than 99% homology with that of *Burkholderia cepacia*. For the growth of BHJ, the optimum temperature was 34°C, the best concentration ratio of CO₂ and N₂ was 1:99, and the optimum pH was 5.

Introduction

At present, countries worldwide are facing energy, food, population, and environmental issues. Biological nitrogen fixation has the advantage of a large capacity for nitrogen fixation and causes no damage to the environment. Therefore, the study of biological nitrogen fixation has attracted much attention. Biological nitrogen fixation is regarded as a key scientific and technological project by all governments.

In additional, global CO₂ production is also rising sharply with the rapid development of the economy. According to the findings of the World Energy Commission, the contribution to global CO₂ emissions from China is 13.5%. China is the world's second largest emitter of CO₂ after the USA [1]. If the current economic development trend continues, CO₂ production from China will continue to rise. CO₂ production from China may exceed that of the USA by 2020 [2]. CO₂ is the largest contributor to the greenhouse effect, accounting for about 50% of the total effect. The concentration of CO₂ in the atmosphere has increased by 31% since the Industrial Revolution [3]. The greenhouse effect caused by the accumulation of CO₂ on earth has had serious consequences for human life. The direct consequence of the greenhouse effect is global warming, which is considered potentially disastrous for humankind [4].

Biological nitrogen and carbon fixation are important. Since 2012, bacteria that can fix CO₂ and N₂ at the same time (the facultative CO₂ and N₂ fixing bacterium) [5] have been identified. In the present study, we aimed to use molecular biology to identify the facultative CO₂ and N₂ fixing

bacterium BHJ and further determine its optimal growth conditions. The results of this study will help to maximize the carbon and nitrogen fixation of BHJ from the air, allowing it to effectively utilize this rich carbon and nitrogen resource.

Materials and methods

Three soil samples were collected from a peanut rhizosphere at Guangxi Yulin Normal University (15 cm from the surface of the soil) in Yulin District, Yulin City, Guangxi Province, China, in January 2013. The samples were collected and stored in bags for transport to the laboratory for separation and screening.

The screening medium (i.e. solid medium without carbon and nitrogen sources) was adapted from *Azotobacter* culture medium and culture medium of carbon sequestering bacteria. To optimize the composition the following basic medium was used: KH_2PO_4 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g, CaCl_2 0.2 g, NaCl 0.2 g, agar 18 g, trace element solution 2 mL, and distilled water to 1000 mL. The medium was sterilized at 121 °C for 20 min. The trace element solution, comprising $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.15 g, ZnSO_4 0.14 g, CoCl_2 0.2 g, and distilled water to 1000 mL, was filter sterilized.

Culture isolation

The three different soil samples were mixed. The mixed soil (1 g) was weighed and added to glass beads in a triangular flask with 9 mL of sterile water. The flask was shaken for about 20 min at 30 °C and 150 rpm on a shaking table to fully dissolve the soil suspension. Thereafter, gradient dilution was performed to obtain 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} dilutions. Three concentration (10^{-4} , 10^{-5} and 10^{-6}) were selected under aseptic conditions and 1 mL these soil suspension were plated on solid culture medium without carbon and nitrogen. After multiple purification, a single colony was picked and preserved at 4 °C.

16S rDNA gene identification

Using DNA from the single colony as a template, polymerase chain reaction (PCR) amplification was performed using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTT GTTACGACTT-3'). The amplification system comprised 1 × PCR buffer (MBI Fermentas) 2.5 μL, 2.5 mmol·L⁻¹ MgCl_2 (MBI) 3 μL, 0.2 μmol⁻¹·L⁻¹ of positive primer and reverse primer (1 μL each), 1 U Taq DNA polymerase (MBI) 0.2 μL, and sterile double distilled water to 25 μL. The amplification conditions comprised: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1.5 min; followed by a final extension at 72 °C for 10 min. The PCR products were detected by 6% agarose gel electrophoresis. 16S rDNA sequencing was completed by the Shanghai Mei Ji biology company. The 16S rDNA gene sequences were subject to BLAST searching (<http://www.ncbi.nlm.gov/blast/blast.cgi>) to identify similar sequences.

Determination of the Optimum Growth Temperature of the facultative CO₂ and N₂ fixing bacterium BHJ

Determination of microbial growth (turbidimetric method)

The principle of turbidimetry is that over a certain range, the cell concentration in the suspension is proportional to the turbidity; i.e., it is proportional to the light density, and the more bacteria, the greater the light density. Therefore, we used a spectrophotometer to determine the light density of the bacterial suspension at 620 nm. The amount of bacteria is assessed based on the optical density (OD). Note that the experiment should be controlled within the linear range where the cell density is directly proportional to the light density, otherwise it is not accurate.

Liquid culture of BHJ to identify the optimum temperature

BHJ grown on an agar plate was scraped off and inoculated into liquid medium under aseptic

conditions. Then, 4 mL of bacteria solution was transferred into a culture bottle containing 40 mL of C and N-free culture medium, which was mixed and divided into seven further bottles. The bottles were cultured at on a constant temperature rocker 17, 20, 24, 28, 30, 33, and 36 °C and the OD value was measured by spectrophotometry every 3 hours. Graphs were constructed for the OD at each temperature plotted against time.

Liquid culture of the facultative CO₂ and N₂ fixing bacterium BHJ to determine the optimum CO₂/ N₂ ratio(volume ratio)

Six 300-mL shaking bottles were used and numbered as 1, 2, 3, 4, 5, and 6. No. 1 was filled with air (CO₂ content of 0.03 %, N₂ content of 78 %) and the CO₂/ N₂ ratio was 1:2600. No. 2 was filled with nitrogen and then injected with 3 mL of CO₂, such that the CO₂ content was 1% and the CO₂/ N₂ ratio was 1:99. No. 3 was filled with N₂ and then injected with 15 mL of CO₂, such that the CO₂ content is 5% and the CO₂/ N₂ ratio was 1:19. No. 4 was filled with N₂ and then injected with 30 mL of CO₂, such that the CO₂ content was 10%, and the CO₂/ N₂ ratio was 1:9. No. 5 was filled with N₂ and then injected with 45 mL of CO₂, such that the CO₂ content was 15%, and the CO₂/ N₂ ratio was 3:17. No. 6 was filled with nitrogen only. Then, 4 mL bacteria liquid in 40 mL culture solution was added into the six flasks, which were then incubated on a shaker at 28 °C. The OD of the flasks with CO₂ contents of 0.03%, 1%, 5%, 10%, 15%, 0 % were measured using a spectrophotometer every 3 hours and the growth curves were constructed.

Determination of the optimum growth pH of the facultative CO₂ and N₂ fixing bacterium BHJ

Bacteria from a culture dish that had been cultured for 3–5 days were scraped into liquid culture medium. The pH of the medium in sterile conical flasks was adjusted to various pH values using 1 mol/L HCl and saturated Na₂SO₃, and inoculated with the bacterial suspension. The shaking flasks were cultured at the optimum temperature and the OD of the bacterial suspension was measured every four hours. The growth pH curve of the bacteria was made according to the obtained data.

Results

Identification of the 16S rDNA of strain BHJ

Agarose gel electrophoresis was carried out for the PCR product of strain BHJ. Fig. 1 shows that a single PCR product was obtained, without any other fragments, which suggested that strain BHJ was a pure strain.

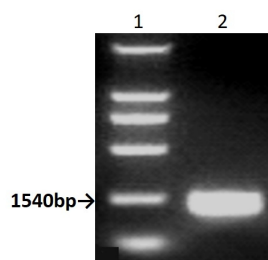


Fig. 1 16S rDNA-PAGE profile of strain BHJ.
Lane 1: DNA Marker; lane 2: BHJ PCR product.

Sequencing of the amplification products showed that the 16S rDNA from strain BHJ comprised 1431 bp. The 16S rDNA sequence is shown in Fig. 2.

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ACCTTAATCTACGGTGGTGACGTCTCCTTGGCGTTAGACTAGCCACTTCTGGTAAACCCACTCCCATGGTGTGACGGGCGGTGTGTACA
AGACCCGGGAACGTATTACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGCACTCGAGTTGCAGAGTGCAATCCGGAC
TACGATCGGTTTTCTGGGATTAGCTCCCCCTCGCGGGTTGGCAACCTCTGTTCCGACCATTGTATGACGTGTGAAGCCCTACCCATAAGG
GCCATGAGGACTTGACGTATCCCCACCTTCTCCGGTTTGTACCGGCAGTCTCCTTAGAGTGCTCTTGGCTAGCAACTAAGGACAAGGG
TTGCGCTCGTTGCGGGACTTAACCCAAACATCTCACGACAGAGCTGACGACAGCCATGCAGCACCTGTGCGCCGTTCTCTTTCGAGCACT
CCCGCTCTCAGCAGGATTCCGACCATTGTAAGGGTAGGTAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTGTGCG
GGTCCCCGTCAATTCTTTGAGTTTTAATCTTGCAGCCGTACTCCCGAGGCGGTCAACTTCACGCGTTAGCTACGTTACTAAGGAAATGAA
TCCCCAACAACTAGTTGACATCGTTTAGGGCGTGGACTACCAGGTATCTAATCCTGTTTGTCTCCACGCTTTCGTGCATGAGCGTCAGT
ATTGGCCAGGGGGCTGCCTTCGCCATCGGTATTCTCCACATCTCTACGCATTTCACTGCTACACGTGGAATTCTACCCCTCTGCCAT
ACTTAGCCTGCCAGTCACCAATGCAGTTCAGGTTGAGCCCGGGGATTTCACATCGGTCTTAGCAAACCGCCTGCGCACGCTTTACGCC
CAGTAATTCCGATTAAACGCTTGACCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTATTCTTCCGGTACCGTCATCCC
CCGACTGTATTAGAGCCAAGGATTTCTTCCGGACAAAAGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATTGCTGGATCAGG
CTTTCGCCCATTTGTCCAAATTCCTCCACTGCTGCCTCCCGTAGGAGTCTGGGCGGTGTCTCAGTCCAGTGTGGCTGGTCTCTCTCAGA
CCAGCTACTGATCGTCGCTTGGTAGGCCCTTACCCCACTAGCTAATCAGCCATCGGCCAACCCCTATAGCGGAGGCCCAAGGTCC
CCCGCTTTCATCCGTGGATCGTATGCGGTATTAATCCGGCTTTCGCGGGCTATCCCCACTACAGGACATGTTCCGATGTATTACTCACC
CGTTCGCCACTCGCCACCAGGTGCAAGCACCCGTGCTGCCGTTGACTGCATGTTAAGCAGCCCCAACGCC

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Fig. 2 The sequence of the 16S rDNA of BHJ

The 16S rDNA sequence of BHJ was compared with 16S rDNA sequences in GenBank using the BLAST algorithm. The results showed that the sequence of 16S rDNA from BHJ was more than 99% similar to a partial 16S rDNA sequence from strain JCM 5506 of *Burkholderia cepacia*.

The optimum growth temperature of strain BHJ

The OD value of BHJ growing in liquid culture at various temperatures was measured and plotted against time to determine the optimum growth temperature. Fig. 3 shows that the optimum growth temperature was 32 °C; with good growth also being observed at 34 °C. Growth was poor at the highest and lowest temperatures tested.

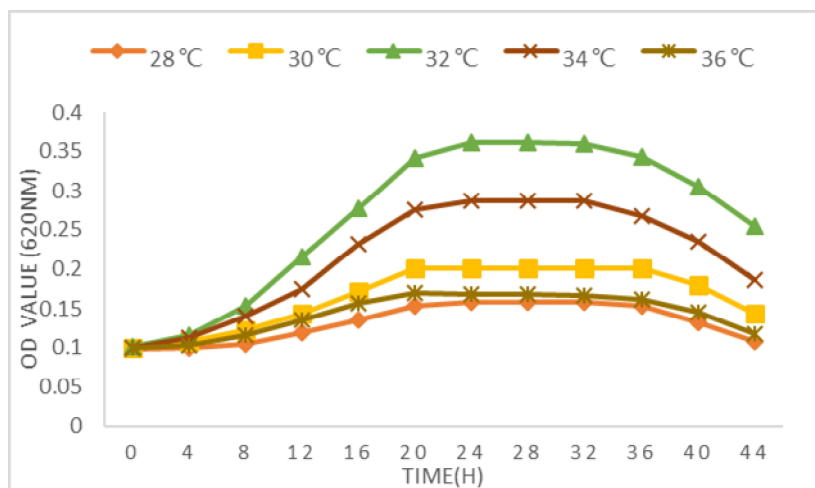


Fig. 3 Determination of the optimum growth temperature of BHJ

Determination of optimum concentration ratio of CO₂ / N₂ (volume ratio) for the growth of BHJ

The growth curves for BHJ at different ratios of CO₂ / N₂ are shown in Fig. 4.

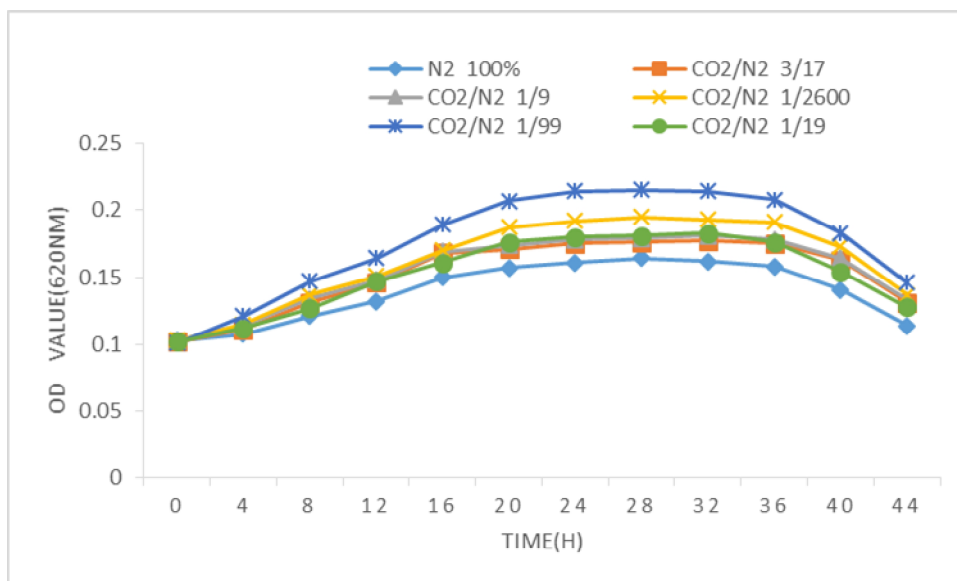


Fig. 4 The growth of BHJ at different CO₂/N₂ ratios

After six times repeated experiments, we concluded that the optimum ratio of CO₂/N₂ was 1:99. High concentrations of CO₂ inhibited the growth of the bacteria BHJ, and the bacteria did not grow well in 0% CO₂.

Determination of optimum pH for the growth of BHJ

The growth of BHJ at different pH values is shown in Fig. 5. The data are from six times repeated experiments.

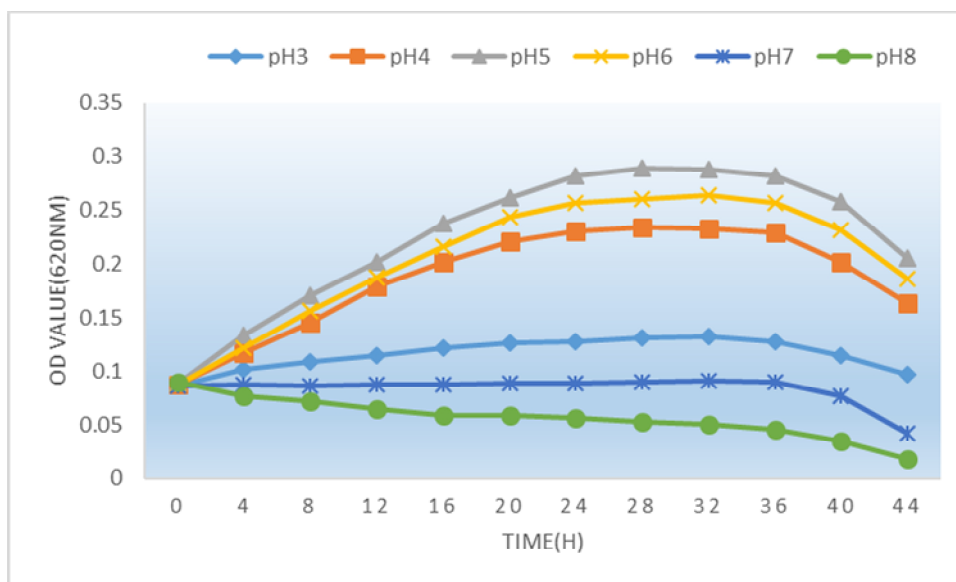


Fig. 5 The determination of optimum growth pH for BHJ

BHJ could grow in culture at pH 3–6. BHJ showed the best growth in culture medium at pH 5, but grew poorly in medium at pH 8. The growth of BHJ at pH 4–6 was significantly superior to that in other pH conditions. Therefore, the optimum growth pH for BHJ is pH 5–6. BHJ could be preliminarily determined to belong to the acidity bacteria. Stable growth of BHJ lasted for about 36 hours under acidity conditions.

Discussion

Since the discovery of biological carbon-fixing bacteria and azotobacter, many scholars have studied them. However, few strains are reported to fix CO₂ and N₂ simultaneously, using them as carbon and nitrogen sources. Since 2012, our team has discovered and verified the existence of these kinds of microorganisms in nature. We have screened several different species of facultative CO₂ and N₂ fixing microorganisms.

In this study, the facultative CO₂ and N₂ fixing bacterium BHJ was obtained from soil. BHJ was identified using molecular biology. In addition, the optimum temperature, CO₂ / N₂ concentration ratio, and pH of BHJ were determined. The results showed that the 16s rDNA sequence of BHJ was very similar (more than 99%) to that of *Burkholderia cepacia*. The optimum growth temperature was 34 °C, the optimum ratio of CO₂ / N₂ concentration was 1:99, and the best growth was obtained at pH 5.

The study of facultative CO₂ and N₂ fixing bacterium has important scientific and theoretical significance. Their study will be an important supplement to the traditional theory of nutrition and metabolism in microbiology. In addition, facultative CO₂ and N₂ fixing bacterium have potential practical applications. Currently, the microbes used for carbon fixing all need a nitrogen source, while the facultative CO₂ and N₂ fixing bacterium do not, indicating a potential to reduce the cost of carbon fixation. They could also reduce greenhouse gas emissions, providing an important and effective way to realize CO₂ microbial immobilization and recycling. Thus, facultative CO₂ and N₂ fixing bacterium also provide an effective way to solve the dual crisis of low cost plant nitrogen, and carbon fixation and resource recovery. The use of facultative CO₂ and N₂ fixing bacterium represents a method of renewing green resources, with great development potential and broad application prospect. To achieve these, the mechanism and efficiency of carbon and nitrogen fixation and their influencing factors should be further studied.

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