A Type of Microorganism That Can Fix Greenhouse Gas (CO_2 and N_2)Simultaneously-Isolation and Validation Experiment of Facultative CO_2 and N_2-fixing Bacteria

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Keywords: facultative CO_2 and N_2-fixing bacteria, carbon and nitrogen-free medium, carbon fixation enzymes, nitrogenase.

Abstract. The N_2-fixing microbial azotobacter and the CO_2-fixing bacteria have been discovered and researched. However, few has been reported so far that can fix CO_2, N_2 simultaneously, while taking CO_2, N_2 as microbial carbon and nitrogen sources, respectively. This has been called Facultative CO_2 and N_2-fixing bacteria in this research. A carbon and nitrogen-free medium has been optimized by carbon-fixing medium and nitrogen-fixing medium in this research (airborne CO_2, N_2 were taken as the carbon and nitrogen sources, respectively). A strain of CO_2 and N_2 as the carbon source and nitrogen source, respectively, was isolated by a nitrogen and carbon-free culture medium and has been called *HSJ*. In addition, the *HSJ*'s characteristics of carbon fixation enzyme activity and nitrogenase activity have been determined. The *HSJ* with a carbon fixation enzyme(1,5-two ribulose monophosphate carboxylase) specificity of *cbbL* gene stripe (*cbbL* in RubisCO genes) and nitrogen-fixing enzyme specificity of *nifH* gene stripe was detected by PCR and agarose gel electrophoresis. To testify fixing CO_2 and N_2 in the air simultaneously, and CO_2, N_2 as the carbon and nitrogen sources, respectively, validation testing of the bacterium *HSJ* was carried out. The sequence analysis of 16SrDNA of the *HSJ* is subordinate to the strains of *Streptomyces.*

Introduction

In the academic and traditional theory of the N_2-fixing microbial azotobacter and fixed CO_2 microbial, carbon-fixing bacteria has been studied and found. According to the existing literature, a large number of studies show that in nature, and especially in the soil, there are many microbials with carbon fixation functions, but not necessarily with nitrogen-fixing functions. From microbial evolution theory, microbial responses to environmental adaptability include fast variation, metabolic pathway and nutrition type diversification[1]. Microorganisms that can fix N_2 and CO_2 simultaneously may exist, especially their distribution in leguminous plants (no presence of nitrogen fertilizer was the best choice) root soil (other habitats may also have this kind of microbial). Considerable nitrogen-fixing bacteria are likely to exist, as some microorganisms can have a carbon fixation function of microbial at the same time. Therefore, a hypothesis has been put forward in this paper: there exists certain microorganisms that can fix CO_2, N_2 simultaneously in nature, and CO_2, N_2 have been taken as carbon and nitrogen sources, respectively. They have been called facultative CO_2 and N_2-fixing bacteria, as shown in Fig.1.

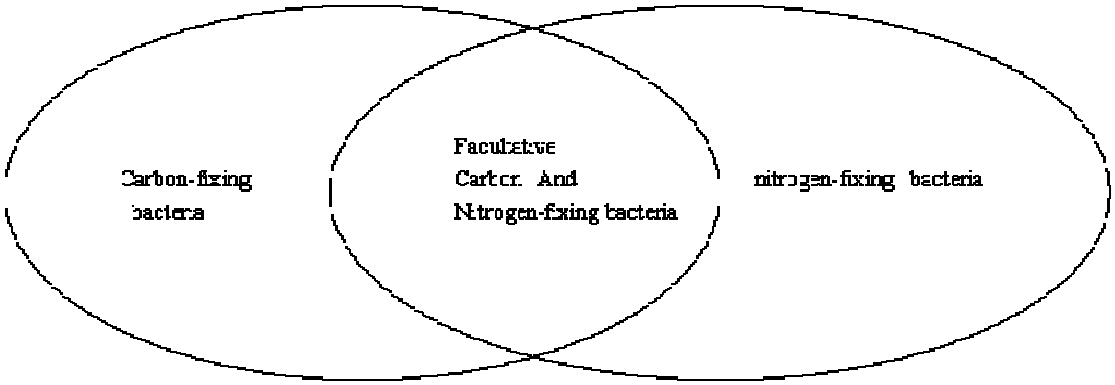

Fig. 1 Relationship hypothesis diagram of carbon-fixing bacteria, nitrogen-fixing bacteria, and facultative carbon- and nitrogen-fixing bacteria. The right, left, and overlapping circles represent the carbon-fixing bacteria, nitrogen-fixing bacteria, and facultative carbon- and nitrogen-fixing bacteria, respectively.

Based on this, the study attempts to screen if one can simultaneously fix CO_2 and N_2 bacteria from a Guangxi Yulin city Yulin Normal University peanut. Its 16SrDNA would be identified, and the strains of carbon fixation enzyme activity, nitrogenase activity would be studied. At the same time, fixing carbon enzymes RubisCO *cbbL* gene and nitrogenase gene *nifH* fragment specific band would be detected by using PCR and agarose gel electrophoresis. Finally, the bacteria control experiment would be proved that whether the bacteria could simultaneously fixed CO_2, N_2 in air, where CO_2, N_2 would be taken as carbon and nitrogen sources, respectively. If the hypothesis is correct, its confirmation could lead to the realization of CO_2 microbial immobilization and recycling, which may provide an important and efficient way to achieve waste and recycling. The utilization of microbials to fix CO_2 and N_2 could be a more rational and effective approach to fix CO_2 and N_2 which is a scientific basis for reducing greenhouse gas emissions.

Materials and methods

A soil sample was collected according to the conventional methods of acquisition of the Guangxi Yulin city Yulin Normal University Guangxi peanut root border soil (distance from the depth of soil surface, 15cm) in December, 2011. Samples were preserved in a laboratory bag, to ensure separation and ease screening.

Isolation and culture of strain HSJ.

Three different soil samples were mixed fully. One gram of which was transferred into a glass bead triangle bottle, to which 9mL of sterile water was added. Then, the sample was oscillated in 30℃, at 150r/min for about 20 min to ensure full dissolution of solid phases. The gradient dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} (six concentration gradients) were prepared, and three concentration gradients of 10^{-4}, 10^{-5}, 10^{-6} were selected. One milliliter of soil solution was absorbed from the soil suspension and injected into screening of culture medium(carbon and nitrogen-free solid medium)(KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.2g, NaCl 0.2g, agar 18g, trace element solution 2mL, distilled water added to the 1000mL. Trace element solution: $FeCl_3$ 0.3g, $FeSO_4$ $7H_2O$ 0.3g, $MnSO_4$ H_2O 0.15g, $ZnSO_4$ 0.14g, $CoCl_2$ 0.2g, constant volume to 1000mL). It was crossed purified 3 times, with a single colony picked out and stored at 4˚C.

16SrDNA gene identification.

A universal primer 8F(5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R(5'-GGT TAC CTT GTT ACG ACT T-3') were amplified. Amplification system: 1×PCR buffer solution (MBI) 2.5 μL, 2.5 $mmol·L^{-1}$ $MgCl_2$ (MBI) 3μL, 0.2$μmol·L^{-1}$ forward and reverse primer 1μL, respectively; 0.2 $mmol·L^{-1}$dNTP 0.5μL, 1UTaq DNA polymerase (MBI) 0.2 μL, adding sterile double distilled water

16.8μL to 25μL, using purified single colony DNA as a template. Amplification conditions: 94˚C predegenerated 5 min, 94˚C degeneration in 1min, 58˚C annealing 1min, 72˚C extended 1.5min, with a total of 30 cycles, 72˚C 10min. The PCR product was tested in 6% agarose gel electrophoresis. 16S rDNA sequencing was conducted by the Shanghai Songon biotechnology company. The 16SrDNA gene sequence was compared by Blasted (http: / /www.ncbi.nlm.nib.gov /blast /blast.cgi) through a sequence similarity search.

Nitrogenase activity and 1,5-two ribulose monophosphate carboxylase activity determination.

Details of nitrogenase activity determination method can be referred to reference[2]. Purified *HSJ* was inoculated into a 5mL semi-solid culture medium in a tube. When HSJ grew to the logarithmic phase, acetylene gas (a final concentration of 1%) was injected into the tube and cultured under the same conditions for 24h; then the nitrogenase activity was determined. Regarding the determination apparatus, SP-2100 gas chromatography (Beijing North Temple Instrument Technology Limited) was used and the column , injector, and FID (flame ionization detector) was set to 50˚C, 150˚C and 180˚C, respectively. N_2, H_2 and drying air velocity were 30, 30 and 300mL/min, respectively. According to the formula: N =hx×C×V/×24.9 HS×t (hx, sample peak area; HS, standard peak area of C_2H_4 ; C, standard concentration of C_2H_4 (μ mol/mL); V, sample tube volume; t,C_2H_2 reaction time (H); N, C_2H_4 concentration ($\mu mol \cdot mL^{-1} \cdot H^{-1}$)). Finally, the nitrogenase activity of the strain's *HSJ* was calculated[3].

The sample and 50mmolL^{-1} Hepes buffer (pH7.8) that had a ratio of sample weight to buffer volume at 1:5 . The Hepes buffer (pH7.8) was mixed with 1mmolL^{-1} EDTA and 0.15 mmolL^{-1}DTT. A small amount of quartz sand was added in the mortar in the ground. After centrifuged at 20000g for 15 min, its supernatant was a crude enzyme solution. These operations were performed at 4˚C,and regarding the RuBP carboxylase activity determination, refer to reference [4] spectrophotometric method.

The cbbL gene of RubisCO detection.

Microorganisms including photosynthetic bacteria, chemoautotrophic bacteria and microalgae ecological system are the main primary producers. These microbes mainly fix CO_2 and synthesis of organic matter by the Calvin cycle. The RubisCO Ⅰ , RubisCO Ⅱ subunit coding gene *cbbL*, *cbbM* length was 1400bp [5]. Various groups of *cbbL* gene sequence are highly conserved, while research has proven its existence by RubisCO *cbbL*, and *cbbM* gene amplification. The study selected six representative gene sequences *cbbL* in RubisCO from the NCBI Genebank (ACCESSION:AF416672, AF416676 , HM440031, HM440033, HM440030, HM440032, HM440036) to design the PCR degenerate primer : F148:CC (G/A) TG (G/C) TAGTAGTTCGG (C/G) TTC (G/A) A (C/G) CC; R445:GACCA (G/T) CT (G/A) CC (A/G) AT (G/A) CAGC (G/C) (G/A) CC (A/G) GG. Amplification of *cbbL* gene fragment of the conserved sequences was 298bp. The PCR was performed in a Biometra ®TGradient Cycler (Biolabo, Chaˆtel-St-Denis, Switzerland) according to the following protocol: initial denaturation at 95°C for 3 min followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 50°C, and 30 s replication at 72°C. A final replication was performed at 72°C for 6 min. The reaction was subsequently cooled to 4 °C .

The nifH gene of nitrogenase detection.

The research proved the nitrogenase existence by amplification of the *nifH* gene of nitrogenase. Nitrogenase *nifH* gene amplification was by Zehr et al.'s design of degenerate primers[6], leading to a positive Zehrf1 (5′-TGYGAYCCNAARGCNGA-3′) and the reverse primer Zehrr2 (5′-NDGCCATCATYTCNCC-3′) to amplify the *nifH* gene fragments. The PCR was performed in a Biometra ®TGradient Cycler (Biolabo, Chaˆtel-St-Denis, Switzerland) according to the following protocol: initial denaturation at 95°C for 3 min followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 50°C, and 40s replication at 72°C. A final replication was performed at 72°C for 5 min. The reaction was subsequently cooled to 4 °C .

The test and verify experiment of facultative carbon and nitrogen-fixing bacteria .

The four known species of bacteria: *Ecoli* bacteria, carbon-fixing bacteria, nitrogen-fixing bacteria, facultative carbon and nitrogen-fixing bacteria *HSJ* were plate streaking on LB Medium(peptone 10g,

yeast extract 5g , sodium chloride 10g, agar 18g, 1000mL distilled water, pH7.0,121.3˚C for 20min.), the CO_2-fixing medium(KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.2g, NaCl 0.2g, NH_4NO_3 0.3g, agar 18g, trace element solution 2mL, distilled water added to 1000mL, 121˚C for 20min.),the N_2-fixing medium (glucose 10g, KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.2g, NaCl 0.2g, $CaSO_4.2H_2O$ 0.2g, $CaCO_3$ 5g, 2mL trace element solution, distilled water 1000mL, pH 7.0-7.2, 113˚C for 30min.)and the C, N-free medium(KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.2g, NaCl 0.2g, agar 18g, trace element solution 2mL, distilled water added to 1000mL, 121˚C for 20min.),respectively, and simultaneously. They were inoculated on 30℃, 48h observations.

Results

16SrDNA identification of HSJ.

The amplification products of *HSJ* 16SrDNA sequenced results has been input into the GenBank nucleic acid sequence databases to be compared, where there have twelve Streptomyces genus (Streptomyces sp.) homology in 99%.

The nitrogenase enzyme activity of facultative carbon and nitrogen-fixing bacteria determination.

The nitrogenase activity of the facultative carbon and nitrogen-fixing bacteria *HSJ* were determined. 2%, l%, 0.1%, 0.05%, 0.01% and 0% (*HSJ* using N_2 as the nitrogen source in the air) peptone (main nitrogen source) were added into experiment group1, 2, 3, 4, 5, 6[autotrophic C, N-free liquid medium(KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.2g, NaCl 0.2g, trace element solution 2mL, distilled water added to the 1000mL. Trace element solution: $FeCl_3$ 0.3g, $FeSO_4$ $7H_2O$ 0.3g, $MnSO_4$ H_2O 0.15g, $ZnSO_4$ 0.14g, $CoCl_2$ 0.2g, constant volume to 1000mL)], respectively. As shown in the measurement results (Fig.2), after adding 2% and l% yeast powder into experiment group1, 2, the nitrogenase activity became extremely low(0.8μmolC_2H_4/ml/h and 1.5μmolC_2H_4/ml/h respectively). In comparison, after adding 0.05% and 0.01% into experiment group4, 5,nitrogenase, the enzyme activity was high(16.8μmolC_2H_4/ml/h and 36.8μmolC2H4/ml/h respectively); while under the absence of protein pepton in experiment group6, the nitrogenase enzyme activity was at its highest(42.6μmolC_2H_4/ml/h). The experiment results indicate When the peptone content decreased, the nitrogenase enzyme activity increased gradually.

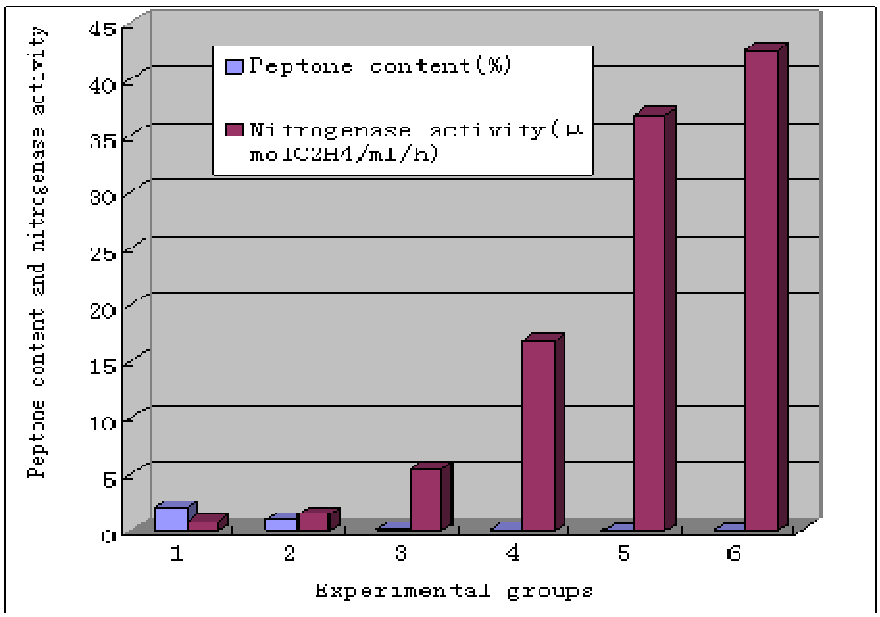

Fig.2 Peptone content and nitrogenase activity. The nitrogenase activity of *HSJ* was determined. Different concentrations of peptone (main nitrogen source) were added to experiment groups 1, 2, 3, 4, 5, and 6 to determine and

measure the nitrogenase activity of *HSJ* The setups were cultured at 30 °C. All the experiments were performed in triplicate. Error bars indicate the standard deviations from the mean values. $P < 0.01$.

The RubisCO enzyme activity of the facultative carbon and nitrogen-fixing bacteria determination.

In order to identify further the presence or absence of Calvin cycle enzymes, RubisCO of the facultative carbon and nitrogen-fixing bacteria *HSJ*, the bacterial RubisCO enzyme activity were determined. In the study, 2%, l%, 0.1%, 0.05%, 0.01%, 0% (*HSJ* using the CO_2 in the air as the carbon source) of yeast powder (mainly to provide the carbon source) were added into experiment group1, 2, 3, 4, 5, 6[an autotrophic C,N-free liquid medium(KH_2PO_4 0.2g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.2g, NaCl 0.2g, trace element solution 2mL, distilled water added to the 1000mL. Trace element solution: $FeCl_3$ 0.3g, $FeSO_4$ $7H_2O$ 0.3g, $MnSO_4$ H_2O 0.15g, $ZnSO_4$ 0.14g, $CoCl_2$ 0.2g, constant volume to 1000mL)], respectively, and cultured at 30˚C. Then, the RubisCO enzyme activity was measured. As shown in the measurement results(Fig.3), through adding 2% and l% yeast powder into experiment group1, 2, the RubisCO enzyme activity was extremely low(0.8 μmol/min/mg and 1.2μmol/min/mg respectively) . The addition of 0.05% and 0.01% RubisCO into experiment group 4, 5, enzyme activity was higher(2.5 μmol/min/mg and 3.3μmol/min/mg respectively). Under the absence of yeast powder RubisCO in experiment group6, enzyme activity was the highest(4.0μmol/min/mg). The experiment results indicate when the yeast powder content decreased, the RubisCO enzyme activity gradually increased.

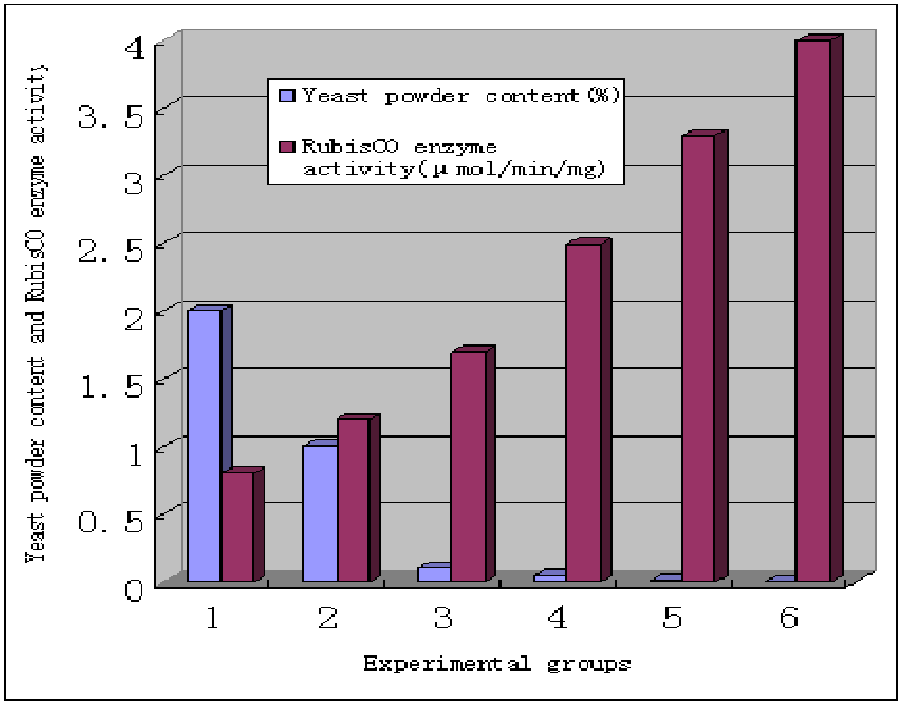

Fig. 3 Yeast powder content and RubisCO enzyme activity. In the determination of The RubisCO enzyme activity of facultative carbon and nitrogen-fixing bacteria *HSJ* experiments, different concentrations of yeast powder (main carbon source) were added into experiment groups 1, 2, 3, 4, 5, and 6 and cultured at 30 ºC. The RubisCO enzyme activity was then measured. All the experiments were performed in triplicates. Error bars indicate the standard deviations from the mean values. $P < 0.01$.

Detection results of nitrogenase enzyme nifH gene and RubisCO cbbL gene.

In order to further prove the nitrogenase *nifH* gene of known strains, and RubisCO *cbbL* gene of the target bacteria's *HSJ* existence from the molecular level, about 360bp of nitrogenase gene fragment *nifH* (Fig.4) and 298bp of RubisCO *cbbL* gene conservative sequence (primers designed amplicon length) (Fig.5) were amplified in this research using PCR.

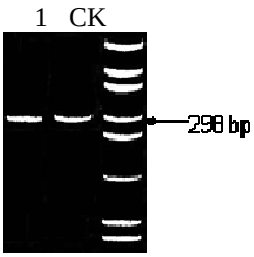

Fig. 4 *HSJ* RubisCO *cbbL* PCR gene product. Lane 1: strain PCR gene product of *HSJ RubisCO cbbL*; CK: *Thiobacillus versutus RubisCO cbbL* PCR gene product. The RubisCO *cbbL* gene of the *HSJ* PCR product and 298 bp of the RubisCO *cbbL* gene conservative sequence (primers with a designed amplicon length) were amplified by PCR under the same conditions.

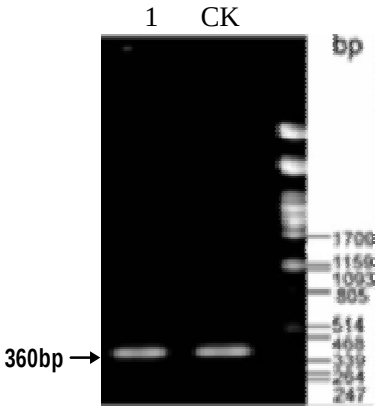

Fig. 5 PCR product of *HSJ nifH* gene. Lane 1: *HSJ nifH* gene PCR product, CK: *Azospirillum lipoferum DSM1691T nifH*. The nitrogenase *nifH* gene of known strains (*Azospirillum. lipoferum DSM1691T nifH*; 360 bp) and the *HSJ* PCR product(360 bp) were amplified using PCR under the same conditions and with the same primers.

The test and verify plate experiment results of the facultative carbon and nitrogen-fixing bacteria.

The four known species of bacteria: *Ecoli* bacteria, carbon -fixing bacteria, nitrogen-fixing bacteria,

facultative carbon and nitrogen-fixing bacteria *HSJ* were plate streaking on LB medium(Fig.6A), the CO_2-fixing medium(Fig.6B), the N_2-fixing medium(Fig.6C) and the C, N-free medium.(facultative fixed

CO_2, N_2 solid Medium)(Fig.6D), respectively, and simultaneously. They were inoculated on 30℃, 48h observations. (A)Four species of bacteria grow well in the LB medium, du to LB medium with adequate

nutrition element. (B)Only carbon-fixing bacteria and facultative CO_2, N_2-fixing of *HSJ* grow in the carbon-fixing medium(the CO_2-fixing medium) while Escherichia *coli,* and the nitrogen-fixing bacteria cannot

grow on the medium because of the lack of a carbon source: nitrogen-fixing bacteria and Escherichia *coli*

have no carbon-fixing capacity .(C)Only nitrogen-fixing bacteria and facultative CO_2, N_2-fixing of *HSJ* grow

in the Azotobacter medium(the N_2 -fixing medium) and Escherichia *coli*,carbon-fixing bacteria cannot grow
because of the fixed N_2 culture medium without a nitrogen source:carbon bacteria-fixing and Escherichia *coli*
have no N_2 fixation ability.(D)Only facultative CO_2, N_2-fixingof *HSJ* grow in the C, N-free medium, and
Escherichia coli, nitrogen-fixing bacteria without carbon fixation ability and carbon-fixing bacteria without
nitrogen fixation capacity, due to the C, N-free medium being without nitrogen and carbon sources, so only
facultative CO_2, N_2-fixing of *HSJ* have the ability to fix CO_2, N_2 in the air at the same time, they can grow in
the C, N-free medium.

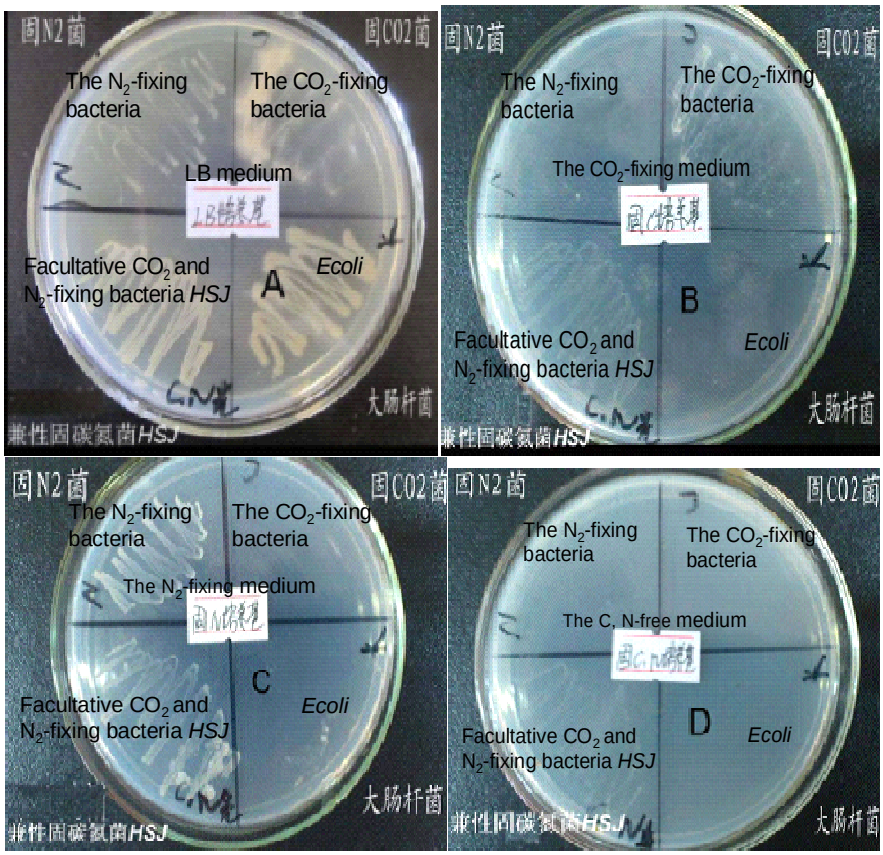

Fig. 6 Growth test of four bacterial types in four kinds of medium. The four species of bacteria tested, namely, *Escherichia coli*, carbon-fixing bacteria, nitrogen-fixing bacteria, facultative carbon- and nitrogen-fixing bacteria *HSJ* were simultaneously streak-plated on LB medium (Fig. 6A), CO_2-fixing medium (Fig. 6B), N_2-fixing medium (Fig. 6C), and C, N-free medium (Fig. 6D). The inoculations were done at 30 °C and were observed after 48 h. (A) All four species of bacteria grew well in the LB medium. (B) Carbon-fixing bacteria and facultative CO_2- and N_2-fixing of *HSJ* grew in the CO_2-fixing medium; *E. coli* and nitrogen-fixing bacteria did not. (C) Nitrogen-fixing bacteria and facultative CO_2- and N_2-fixing *HSJ* grew in the *Azotobacter* or N_2-fixing medium; *E. coli* and carbon-fixing bacteria did not. (D) Only facultative CO_2- and N_2-fixing *HSJ* grew in the C, N-free medium.

Discussion

Since the carbon-fixing and nitrogenfixing bacteria have been found, many scholars have studied these microorganisms. Nevertheless, microorganisms fixing CO_2 and N_2 simultaneously have been

found for the first time. In this study, a strain of bacteria was isolated from the peanut soil which could also fix CO_2 and N_2 simultaneously, and from which the CO_2 and N_2 were taken as the carbon and nitrogen sources, respectively. The nitrogenase activities were the highest, as determined by the facultative fixed carbon and nitrogen strain *HSJ* nitrogenase activity in N free medium (not added peptone). The results display that the facultative fixed carbon and nitrogen strain *HSJ* has nitrogenase activity. At the same time, the facultative fixed carbon and nitrogen strain *HSJ* fixing carbon enzyme (RubisCO) activity assay has been carried out in a C free medium (without yeast powder). When there was no C source in the C free medium (without yeast powder), the carbon fixation enzyme activity was at its highest. The results show that the facultative fixed carbon and nitrogen strain *HSJ* also has fixing carbon enzyme (RubisCO) activity. Secondly, this study also reveals that the nitrogenase enzyme *nifH* gene and RubisCO *cbbL* gene have been detected by the PCR further from the DNA molecular level. Finally, the test and verification experiments were carried out by inoculating the facultative carbon and nitrogen-fixing bacteria strains *HSJ*, carbon-fixing bacteria, nitrogen-fixing bacteria and Escherichia *coli* on the LB culture medium, carbon-fixing medium, nitrogen-fixing medium, facultative carbon and nitrogen-fixing medium(C,N-free medium), simultaneously, and respectively. The results show four species of bacteria grew well in the LB culture medium, because the LB medium has plenty of nutritional elements. There were only carbon-fixing bacteria and facultative CO_2, N_2-fixing strain of *HSJ* growth in the carbon-fixing culture medium; while escherichia coli and nitrogen-fixing bacteria could not grow in the carbon-fixing culture medium due to the lack of a carbon source and nitrogen-fixing bacteria, as Escherichia coli have no carbon fixation capacity. There were only nitrogen-fixing bacteria and facultative CO_2, N_2-fixing strain *HSJ* growth in the Azotobacter medium(nitrogen-fixing medium). Escherichia *coli* and carbon-fixing bacteria could not grow. Because the nitrogen-fixing medium was without a nitrogen source, carbon-fixing bacteria, Escherichia *coli* had no N_2 fixation ability. There was only facultative CO_2, N_2-fixing(carbon and nitrogen-fixing) strain *HSJ* growth in the CO_2, N_2-fixing medium(C,N-free medium), because there was without nitrogen and carbon sources in the facultative CO_2, N_2-fixing medium. However,the Escherichia coli, the nitrogen-fixing bacteria, the carbon-fixing bacteria have no carbon fixation and nitrogen fixation ability,carbon-fixing capacity, nitrogen-fixing capacity respectively, So they could not grow in the facultative CO_2, N_2-fixing medium. In summary, the facultative carbon and nitrogen-fixing bacteria *HSJ* could fix CO_2 and N_2 simultaneously in the air, where CO_2 ,N_2 were taken as the carbon and nitrogen sources.

Facultative CO_2 and N_2-fixing bacteria have been found, which has important theoretical significance, as it will supplement traditional microbiological nutritional and metabolic theory, leading to two significant and potential application values. The carbon-fixing microorganisms need an external source of nitrogen, and the facultative fixed carbon and nitrogen bacteria does not demand an external carbon source in carbon fixation, thus reducing the carbon-fixing costs and reducing emissions of greenhouse gases. This will provide an important and efficient way for CO_2 microbial immobilization and recycling as a resource, and provides an efficient way to solve the dual crisis of the environment and resources depletion. In addition, if the facultative carbon and nitrogen-fixingbacteria could be successfully applied to biological nitrogen fixation, cost could also be reduced. This has great development potential and broad application prospects in the green resources reproducible technology route. Things remains to be further investigated, such as the facultative fixed carbon and nitrogen on fixed carbon and nitrogen, the carbon fixation mechanism of nitrogen efficiency and its influencing factors.

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