Isolation, screening and characteristics of hydrogen-oxidizing bacteria in soybean rhizosphere

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1. Abstract

40 strains were isolated by incubation system with 1.25×10^{-4}mol·L^{-1} H_2 mixture gas and with mineral salt agar (MSA) medium. The capabilities of uptake hydrogen of these strains were measured. The results showed that the volume of uptake H_2 of 22 strains more than 1.25×10^{-4}mol·L^{-1} and 9 strains in the 22 strains were consumed completely. The results of autotrophic growth tests showed that 22 strains had the ability to grow chemolithoautotrophically. 20 strains were preliminarily identified as hydrogen-oxidizing bacteria. The ability of heterotrophy and the capability of utilizing different carbon resources were studied through cultivating bacteria in liquid MSA medium with different carbon resources without H_2 mixture gas. Bacteria colony form and physiological-biochemical characteristics were also studied. The growth-promoting capability of 20 strains was tested. 10 strains were tested that they could markedly promote root growth of wheat and corn. Increasing amplitude of rootage was from 117% to 397%.

2. Introduction

Rotated crop, intercrop especially for applying rotated crop or intercrop of legumina can enhance the fertility of soil and increase the output. But new researches indicate that nitrogenous fertilizer can't completely replace the effect of rotated crop of legumina, and it is still can't explain the 75% increased production effect by the already known knowledge. Legumina rhizobium can produce H_2 during the nitrogen fixation. Dong and Layzell is the first time to bring a theory forward that H_2 released from root nodule can promote the growth of the microbe around the root, as well as promote the growth of plants. This theory is called hydrogen fertilizer theory. Because the special characteristic of hydrogen - oxidizing bacteria and most soil bacteria unable to be cultured, it's very difficult to separate hydrogen-oxidizing bacteria from soil and there are still not many results at present. This article adopts gas circulating culture system, which can continuously inflow H_2 at an adjustable airflow. Using H_2 as the only energy to separate hydrogen-oxidizing bacteria from the soil around legumes and carry through the pilot study on basic characteristic of hydrogen-oxidizing bacteria that have been separated.

3. Methods

3.1 Material

3.1.1 Bacteria source

Soybean rhizosphere from Sanyuan county, Lantian county and suburb of Tongchuan in Shannxi.

3.1.2 Medium

Mineral salt agar medium (MSA): $NaNO_3$, 2.0g; K_2HPO_4, 1.2g; $MgSO_4$, 0.5g; KCl, 0.5g; KH_2PO_4, 0.14g; $Fe_2(SO_4)_3.H_2O$, 0.01g;Yeast Extract, 0.02g;Agar, 15g; Water, 1L; pH 7.2.

3.1.3 H_2 Treatment System

A H_2 treatment system was set up as shown in Figure 1.

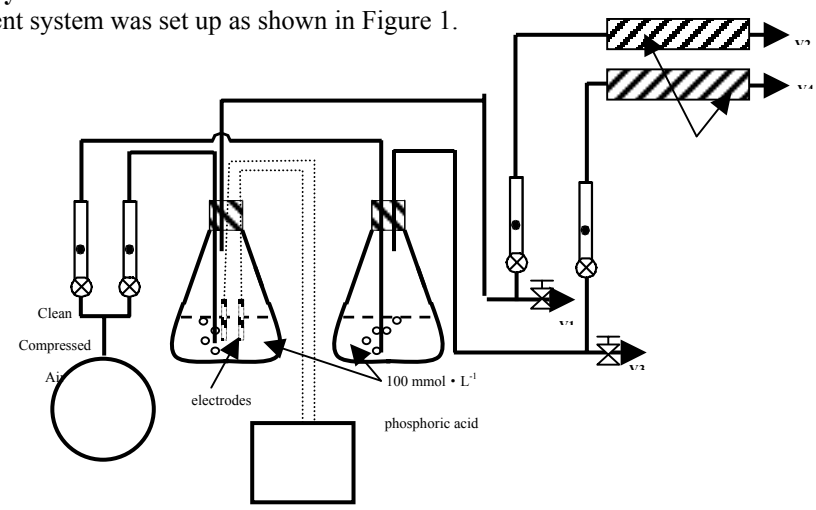

Figure 1 Apparatus of exposing soil: sand mixtures to air or to elevated levels of H_2 gas in air

3.2 Soil enrichment

Soil sample collected from legume rhizosphere were sifted through a sieve (16 mesh) and then mixed with quartz sand (16 mesh, ratio of 2 soil: 1 sand) to prevent from clumping. Soil mixture (60mL) was filled to the glass tubes (2.5cm×60cm) and then did series connection of the 5 tubes and exposed 4.16×10^{5} mol·L^{-1} H_2 concentration, gas flow rate 280mL·min^{-1}. Soil mixture were incubated about 1 month at room temperature. Using gas chromatogram to measure the input and effluent of H_2 concentration everyday to test the H_2 oxidizing ability in soil, and draw the graph of soil uptaking H_2.

3.3 isolation and culture

Soil sample were filled to the glass tubes and exposed 4.16×10^{5} mol·L^{-1} H_2 concentration, gas flow rate 280mL/min and were incubated about 1 month. After soil mixture enrichment, 1g of soil was suspended in 100mL autoclaved water and shaken for 5min on a rotatory shaker at 30□. The supernatants were appropriately diluted and spread in duplicate on MSA plates The plates were put into airtight container which should be left gas vent and culture for 1 month at room temperature under 1.25×10^{-4} mol·L^{-1} H_2 concentration, gas flow rate 280mL·min^{-1} until colony outgrowth.

3.4 Tests of H_2 uptake capacity and autotrophic growth of isolates

The H_2 uptake capacity of isolates was test by gas chromatogram (GC6890). To find out which of the isolated bacteria were capable of chemolithoautotrophic growth, the MSA medium was slightly modified that Yeast Extract was taken out; replicas were made onto MSA medium plates which not include yeast extract and incubated in the gas-tight container under 1.25×10^{-4} mol·L^{-1} H_2 concentration, gas flow rate 280mL/min. The other group that treated with non-H_2-enriched air was controlled as contrast.

3.5 The mensuration of promotive effect of hydrogen-oxidizing bacteria that act on wheat and corn.

Twenty hydrogen-oxidizing bacteria had been selected and separately inoculate. There should be 10 wheat seeds or 4 corn seeds in each plate which was filled with 10mL bacterial water and 5 times repeated for everyone. Water with *Escherichia coli* was the contrast which was put into the plate with seeds of wheat or corn in the same way. Put them into the constant illuminating temperature apparatus and cultured at 26□ for 3-5 days. Observe the growth circs of wheat and corn seeds that were in the plate and record the results of the measurement.

4 Results

4.1 H_2 treatment system

At present, although there have been schemed out some kinds of H_2 treatment systems, they are not ideal . For example Litchfield, Schlegel etc have designed liquid culture system, which is the gas ration equipment of the common, however, it must pay more attention to safety, so as to prevent the H_2 blast (it's prone to blast when the concentration of $H_2\geq5\%$). Repaske and Mayer etc have designed obturating fermenter which has higher rate of utilization and the gas can be used repeated, but when it is used in large scale culture, there should be more big gas storage tank. By many repeated experiments, our lab has designed a H_2 treatment system and founded a culture method of the special weather condition in lab. This system has more strongpoint than the traditional ones: simple system to produce H_2 and cheap; lower concentration of H_2 from this gas-cycle incubation system, stable, easy to control and safe; culture condition is close to the nature those hydrogen-oxidizing bacteria in and it's easy to screen out them. So it has very important significance for the study on the physiological and biochemical characteristic of hydrogen-oxidizing bacteria in natural condition. At the same time, use this gas-cycle incubation system to separate hydrogen-oxidizing bacteria in soybean rhizosphere is also very important for the foundation and optimization of studying system of hydrogen-oxidizing bacteria.

4.2 Soil enrichment culture

Rhizosphere soil of Hup^{-} legumina root nodules indicated that hydrogen-oxidizing bacteria grow slowly and the evidence can be got in our experiments. If the soil was sterilized it will lose the ability to absorb H_2, which indicated that the ability to absorb was independent for the factor of physics or chemistry.

When separating and purifying, because of the slowly growth of hydrogen-oxidizing bacteria, we used MSA culture medium with a little sterilized soil (0.05g/each plate) to enlarge adhering area of the growth of hydrogen-oxidizing bacteria. According to the mixed gas, the velocity of flow was 280 mL·min^{-1} and the content of H_2 was 1.25×10^{-4}mol·L^{-1}, these conditions offered sufficient H_2 energy for hydrogen-oxidizing bacteria as well as restrained the growth of other bacteria, so to promote the rapid growth of themselves. The tests of ability to oxidize H_2 and chemolithoautotrophy experiment showed that it was a feasible method to culture hydrogen-oxidizing bacteria at lower content of H_2.

4.3 The change of H_2 uptake capacity during soil enrichment

When soil was provided with 4.16×10^{-5} mol·L^{-1} H_2 concentration, gas flow rate 280mL/min, only a small

proportion of the H_2 was consumed by examining the H_2 concentration of the effluent gas stream. The H_2 concentration decreased slowly during the subsequent 7 days, but then decreased sharply in 7-12 days and the H_2 concentration downtrend disappeared in the following 2 weeks(Figure 2), reflecting a much greater H_2 uptake rate. When H_2 concentration provided to the glass tubes was decreased from 4.08×10^{-5} mol·L^{-1} to 5.40×10^{-6} mol·L^{-1} by 30 days after the start of the H_2 treatment. According to air-treated soils, the H_2 concentration in the supply air was approximately 2.29×10^{-8} mol·L^{-1}, but the H_2 concentration left in the export after the enrichment was much lower than that in air and nearly to be 0. The change of the H_2 uptake rate of 300mL soil: sand mixture showed that the quantity of the hydrogen-oxidizing bacteria in soil had increased largely, and the soil enrichment was finished.

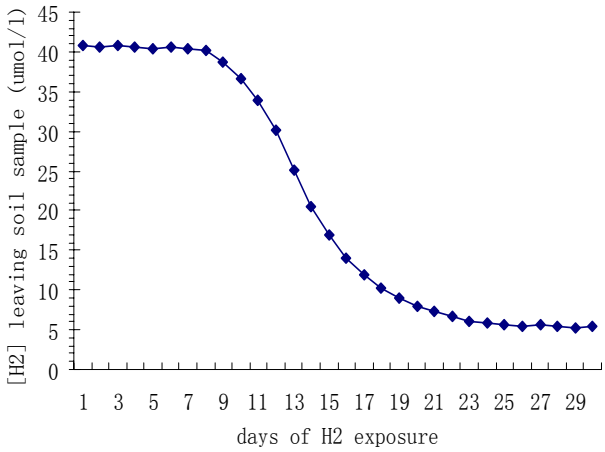

Figure 2 Timecourse of changes in the H_2 left soil: sand mixture

4.4 The results of isolation and screening

The results of 40 strains uptaking H_2 were shown in Table 1. The results showed that the volume of uptaking H_2 of 22 bacterium strains more than 1.25×10^{-4}mol·L^{-1}, account for 55% in total. Uptaking H_2 of A_{04} and A_{19} were over 1.66×10^{-4}mol·L^{-1}, which showed that these two had the strongest ability to oxidize H_2.

Table 1 The result of oxidize hydrogen and the capability of chemolithoautotrophy

strains	value of oxidize H_2	Chemolitho-autotrophy	strains	value of oxidize H_2	Chemolitho-autotrophy
A_{01}	0	n	A_{21}	Ⅲ	+
A_{02}	Ⅱ	n	A_{22}	Ⅱ	n
A_{03}	Ⅲ	+	A_{23}	Ⅲ	+
A_{04}	Ⅳ	+	A_{24}	Ⅲ	+
A_{05}	Ⅲ	+	A_{25}	Ⅱ	n
A_{06}	Ⅲ	+	A_{26}	Ⅲ	+
A_{07}	Ⅱ	n	A_{27}	Ⅲ	+
A_{08}	Ⅱ	n	A_{28}	Ⅱ	n
A_{09}	Ⅲ	+	A_{29}	Ⅱ	n
A_{10}	Ⅲ	+	A_{30}	Ⅱ	n
A_{11}	Ⅲ	+	A_{31}	Ⅲ	+
A_{12}	Ⅱ	n	A_{32}	Ⅲ	n
A_{13}	Ⅱ	n	A_{33}	0	n
A_{14}	Ⅲ	+	A_{34}	0	n
A_{15}	Ⅱ	n	A_{35}	Ⅲ	+
A_{16}	0	n	A_{36}	Ⅱ	n
A_{17}	Ⅱ	n	A_{37}	0	n
A_{18}	Ⅲ	+	A_{38}	Ⅲ	n
A_{19}	Ⅳ	+	A_{39}	Ⅲ	+
A_{20}	Ⅲ	+	A_{40}	Ⅲ	+

0:<4.16×10^{-5}mol·L^{-1},□:4.16×10^{-5}-8.31×10^{-5}mol·L^{-1}, Ⅱ:8.31×10^{-5}-1.25×10^{-4}mol·L^{-1},Ⅲ:1.25×10^{-4}-1.66×10^{-4}mol·L^{-1}; Ⅳ:1.66×10^{-4}-2.08×10^{-4}mol·L^{-1}. +: have the capability of chemolithoautotrophy; n: the capability of the chemolithoautotrophs not be tested

4.5 The result of autotrophic growth

After 2 weeks of H_2 treatment, the H_2 treated 20 bacteria strains grew one after another, the colony form was shown in Table 2. While the non-H_2 treated 20 bacterium strains had not obviously formed colony. The result showed that the 20 strains had the capability to utilize gas hydrogen and to fix carbon dioxide as carbon source, i.e. to grow chemolithoautotrophically (Table 1). 20 strains were preliminarily identified as hydrogen-oxidizing bacteria.

4.6 The results of promoting the growth of wheat and corn roots

A_{03}、A_{06}、A_{10}、A_{11}、A_{14}、A_{20}、A_{21}、$A_{2}6$、A_{31}、A_{35}、A_{39} strains that separately add to the plates can be more evidently promote the growth of wheat roots than *Escherichia coli* and the growth extent was 151%-397% (Table 2). A_{06} and A_{11} strain had the best effect and the growth extent separately was 361% and 397.6% comparing to the contrast. A_{04} and A_{09} strain also had a better effect but the toots growth extent was 102%-111%, which was not obvious. *Escherichia coli*、A_{05}、A_{18}、A_{19}、A_{23}、A_{24}、A_{27} and A_{40} strains could not promote the growth of wheat roots and the length of the wheat roots were little shorter than the contrast.

Table 2 The effect of hydrogen-oxidizing bacteria on wheat /corn growth

Strains	Average root length（mm） wheat/ corn	Corresponding growth (%) wheat/ corn	Strains	Average root length（mm） wheat/ corn	Corresponding growth（%） wheat/ corn
CK	8.2/15.2	100/100	A_{19}	3.1/14.2	37.8/93.4
E.coli	8.1/14.7	98.7/96.7	A_{20}	20.2**/15.3	246.3/100.7
A_{06}	29.6**/34.4*	361/226.3	A_{26}	16.5**/26.3*	201.2/173
A_{10}	12.4**/17.8	151.2/117	A_{31}	24.8**/29.2*	302.4/192
A_{11}	32.6**/33.4*	397.6/219.7	A_{35}	23.7**/24.9*	289/163.8
A_{14}	19.9**/20*	242.7/131.6	A_{39}	15.7**/19.8*	191.5/130.2

* Means significant difference ($p<0.05$), ** Means extremely difference ($p<0.01$). Data in the table were the average of 5 repetitions.

Experiment of promoting the growth of corn roots was in Table 2. A_{03}、A_{06}、A_{10}、A_{11}、A_{14}、A_{26}、A_{31}、A_{35}、A_{39} strains can extremely promote the growth of corn roots 117%-226%, while A06 and A11 strain had the best effect. Corn treated with A_{05}、A_{18}、A_{20} or A_{21} strain can have longer roots tan the contrast and the growth extent was 100%-117%, but it was not obvious. A_{19}、A_{24}、A_{27} and A_{40} strains could not promote the growth of wheat roots and the length of the wheat roots were little shorter than the contrast.

Therefore, from the results all above we can see that A_{03}、A_{06}、A_{10}、A_{11}、A_{14}、A_{26}、A_{31}、A_{35} and A_{39} can obviously promote the root growth of wheat and corn. So these hydrogen-oxidizing bacteria trains were chosen to be the object for continue study.

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