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ISOLATION AND CHARACTERIZATION OF H_2 OXIDIZING BACTERIA IN H_2 TREATED SOIL AND SOIL ADJACENT TO Hup^- SOYBEAN NODULES

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF H_2 OXIDIZING BACTERIA IN H_2 TREATED SOIL AND SOIL ADJACENT TO Hup^- SOYBEAN NODULES

By Jiamila Maimaiti

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Previous studies have shown that the hydrogen (H_2) gas evolved from legume nodules inoculated by hydrogen releasing (Hup^-) rhizobium strains contributes to growth promotion of subsequent crop plants by altering the populations of soil H_2 oxidizing bacteria; this may be responsible for the major beneficial effect of legumes in crop rotation. To further study the plant growth promoting bacteria in H_2 treated soil and soil adjacent to the Hup^- legume nodules, the H_2 oxidizing bacteria were isolated from H_2 treated soil and soil adjacent to Hup^- soybean nodules grown in greenhouse and field. A much higher bacterial density was found in soils with a higher H_2 uptake rate. Twenty isolates of H_2 oxidizing bacteria were obtained. They are slow growing bacteria on mineral salt medium incubated under H_2 - enriched air. All H_2 oxidizing bacterial strains exhibited one high K_m for H_2 at 1000ppm. The isolates of H_2 oxidizing bacteria were identified as *Variovorax paradoxus*, *Flavobacterium johnsonae* and *Burkholderia sordidicola* by conventional microbiological tests and 16S rRNA gene sequence analysis. All isolated H_2 oxidizing bacteria increased the root elongation of spring wheat seedlings ranging from 21 % to 254% compared to controls. Seventeen of them had a significantly positive effect on root elongation ($p< 0.005$). The strains of *V. paradoxus* found in both H_2 treated soil and soil adjacent to Hup^- soybean nodules had the highest direct plant growth effect. The results confirm the hypothesis that the microorganisms responsible for the oxidation of H_2 released by Hup^- legume nodules are bacteria and they act as plant growth promoting rhizobacteria (PGPR).

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I wish I would be able to live it up to all blessings I have been receiving and offer my best to return it to the others.

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LIST OF ABBREVATIONS

A	ampere
bp	base pairs
g	Gram
hr	hour
L	litre
µL	microlitre
mL	millilitre
mM	millimole
µM	micromole
mm	millimetre
mg	milliGram
min	minute
MSA	mineral salt agar
OD	optical density
ppm	parts per million
°C	degrees Celsius
n	sample size
NA	nutrient agar
SE	standard error
SD	standard deviation
V	voltage
W	weight
NS	nutrient agar slant
NB	nutrient broth
BA	blood agar

1. GENERAL INTRODUCTION

1.1 OVERVIEW OF THE PROBLEMS

Nitrogen-fixing legume plants, including beans, peas, clovers and alfalfa have been used in crop rotation with cereals in agriculture since ancient times (MacRae and Mehuys, 1985). Many experiments show that the yield of crops rotated with legumes increases by 20% or more (Bullock, 1992). It is generally believed that residual nitrogen from legume was responsible for the beneficial effect of the rotation with legumes. However, studies on legumes and nitrogen fixation show that residual nitrogen in legume soils from nitrogen fixation provide only minor not major benefits to the rotation (Bullock, 1992). This finding stimulated many researchers to look for the factor, which is responsible for the major benefit seen in crop rotation with legumes.

Several factors other than nitrogen fixation have been proposed to explain the beneficial effect of legumes in rotation:

- Legumes may decrease the population of the pathogenic microorganisms and increase the growth of beneficial rhizobacteria (Schippers *et al.*, 1987).
- Legumes may help the distribution of nutrients like phosphorous (P) and Potassium (K) in soils (Bullock, 1992).
- The benefit of legumes in crop rotation and intercropping practices may indirectly involve the beneficial rhizospheric fungi, arbuscular mycorrhizal (AM) infection (Fyson and Oaks, 1990).

Besides these proposed factors of the beneficial effect of legumes, hydrogen (H_2) gas released by legume nodules has been suggested recently as an important

"fertilization" benefits plants in rotation with legume crops (Dong and Layzell, 2002; Dong *et al.*, 2003).

1.1.1 Hydrogen gas (H_2) and plant growth promotion

Legume root nodules are very important environments not only for biological nitrogen (N_2) fixation, where rhizobia reduce N_2 to NH_3, but also for H_2 evolution, where nitrogenase reduce protons to H_2 (Hoch *et al.*, 1957; Schuler and Conrad, 1991). Dixon (1967, 1968) reported that some *Rhizobium* species are able to recycle H_2 through enzyme uptake hydrogenase within the legume nodules. These kinds of legume-*Rhizobium* symbioses possess uptake-hydrogenase activity called "positive uptake hydrogenase" (Hup^+) that can extract chemical energy from H_2 and use it for cell metabolism (Baginsky *et al.*, 2002). However, some legume-*Rhizobium* symbioses do not have uptake hydrogenase, and these are termed "negative uptake hydrogenase"(Hup^-), where the rhizobia cannot recycle the H_2 generated by N_2 fixation and releases H_2 from nodules into the soil (Uratsu *et al.*, 1982). From a metabolic point of view, loss of H_2 from the nodules to the soil is a wasteful process (Eisbrenner and Evans, 1983). Also, from an evolutionary perspective, the Hup^+ symbiosis should have more advantages on legume growth than Hup^- symbiosis since the former can use H_2 to make energy. However, the facts show that the majority (75%) of commercial soybean in US contains Hup^- rhizobia in their nodules (Uratsu *et al.*, 1982). Many other leguminous crops such as alfalfa are also Hup^- (Dong and Layzell, 2002). This suggested that H_2 evolved from Hup^- nodules may have a positive effect on certain plant characteristics which are selected by breeders and perhaps in the evolution of these plants (Dong and Layzell, 2001; Dong *et al.*, 2003).

After study of the evolution and breeding of legume crops and N_2 fixation bacteria, Dr. Dong and his colleagues proposed a "hydrogen fertilization theory" (Dong and Layzell, 2002). A series of plant growth experiments on H_2 treated soil and soil after growth of Hup^- soybean nodules was conducted under laboratory and field conditions. The results of plant growth studies showed that H_2 release from legume nodules had a significant positive effect on growth of non-leguminous plants (Dong *et al.*, 2003). The dry weights of seven week-old soybean, spring wheat, canola and barley were 15% to 48% greater in the H_2 treated soil compared to the controls (Dong *et al.*, 2003). The tiller number of seven-week old barley and spring wheat grown in the field were increased 36% and 48%, when H_2 enriched soil as applied to seedlings (Dong *et al.*, 2003). Also, the dry weight of barley was increased when rotated with soybean inoculated by JH47 (Hup^-) strain of *Bradyrhizobium japonicum* in field condition, compared to JH (Hup^+) strain of *B. japonicum* (Dean *et al.*, 2005). These studies indicate that H_2 evolved from Hup^- nodules plays a significant role in the benefits of crop rotation with legumes. Unfortunately, the mechanism by which H_2 evolved from Hup^- nodules affects growth is not understood. A possible explanation may be the influence of H_2 metabolism on soil microorganism populations (Dong *et al.*, 2003).

1.1.2 The influence of H_2 metabolisms on soil microorganisms

ATP-dependent H_2 evolution is an obligate by-product of nitrogenase activity during N_2 fixation which accounts for about 35% of the energy consumed in the overall nitrogenase reaction (Hunt and Layzell, 1993). The production of H_2 costs the plant about 5% of net energy yield from photosynthesis (Dong and Layzell, 2001). It is known that H_2 produced from Hup^- legume nodule during N_2 fixation is released into the soil

adjacent to legume nodules (Uratsu *et al.*, 1982). However, the H_2 that evolved from nodules was not able to be detected in legume soil (La Favre and Focht, 1983). It has been found that both soil H_2 oxidizing bacteria and free soil enzymes were responsible for the oxidation of H_2 in the soil (Conrad and Seiler, 1979). Also the rhizobacterial populations are increased in H_2 rich soils around Hup$^-$ nodules on pigeon pea, soybean and alfalfa (LaFavre and Focht, 1983; Popelier *et al.*, 1985; Cunningham *et al.*, 1986).

To better understand the metabolic fate of H_2 evolved from Hup$^-$ legume nodules in soil, Dr. Dong and his colleague developed a H_2 treatment system (Dong and Layzell, 2001) (Figure 1), which mimics the natural conditions around the H_2 evolving rhizobial nodules. In this system, soil was treated with H_2 gas produced by electrolysis at the same rate as in a legume field (Dong and Layzell, 2001). The oxygen (O_2), carbon dioxide (CO_2) and H_2 exchange rate of H_2 treated soil were measured simultaneously under different partial H_2 concentrations. The results showed that O_2 uptake increased and the evolution of CO_2 decreased with an increase in the H_2 concentration. It was calculated that 40% of the reducing power from H_2 (evolved from N_2 fixing nodules) was used for CO_2 fixation, and 60% of the electrons from H_2 were transferred to O_2 (Dong and Layzell, 2001). This observation showed that the H_2 released from Hup$^-$ legume nodules is utilized by some agent, which can fix CO_2 chemoautolithotrophically (Dong and Layzell, 2001, 2002). Also, the curve of H_2 uptake rate against time of H_2 treated soil resembled a growth curve over 3 weeks of H_2 treatment (Dong and Layzell, 2001). The soil H_2 uptake rate was increased very slowly over the first week, and then increased rapidly until it leveled off when all the H_2 supplied to the soil was used up (Dong and Layzell, 2001). This phenomenon suggested that microbial populations could be increasing in H_2 treated

soil and soil adjacent to Hup$^-$ legume nodules which caused the increase in soil H_2 uptake activity (McLearn and Dong, 2002; Irvine *et al.*, 2004; Dean, 2004). To assess whether this increased H_2 uptake activity was due to bacteria or fungi, an antibiotic and fungicide treatments was used on the H_2 treated soil (McLearn and Dong, 2002). The results revealed that the agent responsible for the increase of H_2 uptake rate in H_2-treated soil or soil around Hup$^-$ nodules was bacterial rather than fungal (McLearn and Dong, 2002; Irvine *et al.*, 2004). It was observed that the population of insect, springtails (O. Collembola) increased in H_2 treated soil (Dong and Layzell, 2002). Therefore, it is hypothesized that increased springtail density in H_2 treated soil might be due to soil bacteria that nourished in H_2-treated soil (Dong and Layzell, 2002). It was also found that the H_2 uptake ability of H_2 treated soil was lost when the physical structure of soil was destroyed, and the H_2 uptake ability of soil recovered after H_2 gas provided after soil disturbance (McLearn and Dong, 2002). This suggests that soil bacteria responsible for the H_2 uptake need a special colonial structure to function or might consist of long filamentous bacterial cells like Actinomycetes (McLearn and Dong, 2002). Interestingly, Dean (2004) found that diverse white spot with a group of bacterial colonies was increased in H_2 treated soil, and the soil which had white spot had high H_2 uptake ability compared to control. These studies strongly confirmed that the H_2 oxidation rate in H_2 treated soil was increased by soil H_2 oxidizing bacterial activity (McLearn and Dong, 2002; Irvine *et al.*, 2004).

In summary, the past studies have shown that the plant growth promotion effect of H_2 observed in H_2 treated soil and Hup$^-$ legume soils are positively correlated with H_2 utilizing bacterial activity (Irvine *et al.*, 2004). However, we still do not know what

bacterial species or bacterial community are responsible for H_2 oxidization in H_2 treated soil and soil adjacent to Hup^- soybean nodules.

1.1.3 Hydrogen oxidizing bacteria and hydrogen oxidation activity

The aerobic hydrogen oxidizing bacteria do not form a taxonomic unit, and comprise species from phylogenetically diverse taxa of H_2 (Knallgas) bacteria, N_2- fixing bacteria, and photosynthetic microorganisms (Lechner and Conrad, 1997, Evans *et al.*, 1987). The group of aerobic H_2 oxidizing bacteria is physiologically defined by the ability to oxidize H_2 gas aerobically or to grow chemolithoautotrophically on H_2 (Aragno and Schlegel, 1981). They include the representatives of the alpha, beta and gamma subclasses of Proteobacteria (Friedrich and Schwartz, 1993; Lechner and Conrad, 1997). Based on the characteristics of aerobic H_2 oxidizing bacteria, they may occur in those habitats in which both H_2 and O_2 are available (Aragno and Schelgel, 1981).

Soils are predominant sink for atmospheric H_2 oxidation activity (Conrad and Seiler, 1979; 1983). There are two different H_2 oxidizing activities exist in soils, which are caused by different affinities of hydrogenase for H_2. The two different H_2 oxidation activities can be distinguished in soils based on their K_m values for H_2 (Schuler and Conrad, 1990). The activities with high-affinity, low K_m is believed to be due to the cell free soil hydrogenase, which can effectively oxidize atmospheric H_2 (0.55ppm). The H_2 oxidation activity with low-affinity, high K_m, is attributed to aerobic H_2 oxidizing bacteria (Conrad *et al.*, 1983; Schuler and Conrad, 1990; Häring and Conrad, 1994).

When Dong and Layzell (2001) examined the H_2 uptake kinetics of H_2 treated soil which had similar properties to soil adjacent to Hup^- legume nodules, they observed that the K_m (H_2) value of H_2 treated soil was to 1038ppm after 3 weeks of H_2 treatment (Dong

and Layzell, 2001). This high K_m value was similar to the one obtained from H_2 (Knallgas) bacterial strains GK1of *Pseudomonas palleronii* (Schuler and Conrad, 1991). This indicates that the high K_m value for H_2 in H_2 treated soil and soil adjacent to the Hup^- legume nodules was caused by the H_2 oxidizing bacterial activity (Dong and Layzell, 2001). Unfortunately, it is not known what kinds of H_2 oxidizing bacteria are responsible for this high K_m value for H_2 in H_2 treated soil and soil adjacent to the Hup^- legume nodules. It is necessary to first isolate and characterize these specific H_2 oxidizing bacteria to better understand their role in plant-soil systems.

1.1.4 The isolation and characterization of H_2 oxidizing bacteria

1.1.4.1 Isolation of H_2 oxidizing bacteria

The H_2 oxidizing bacteria studied so far were isolated by direct plate counting and liquid enrichment cultures with soil, mud, or water samples as inocula (Aragno and Schlegel, 1981). German microbiologists have investigated the isolation condition of H_2 oxidizing bacteria from lake and soil for over 30 years, and isolated some species of H_2 oxidizing bacteria belonging to 5 Gram-positive and more than 15 Gram negative genera (Aragno and Schlegel, 1981; Lechner and Conrad, 1997). However the bacteria responsible for H_2 oxidization in H_2 treated soil and legume soil have not been isolated (Dean, 2004) due to the following reasons:

- Many of the resident soil bacteria are "not culturable" in current type of media (Felske *et al.*, 1999)
- Soil is a rich reservoir of many microorganisms, especially soil adjacent to the roots, the bacterial cells range from from10^{10} to 10^{12} in per gram soil, and fungal cells are from 10^4 to 10^6 (Lazarovits and Nowak, 1997)

- The information of the ecological and physiological characteristics of soil H_2 oxidizing bacteria is scarce (Kluber *et al.*, 1995)
- The growing characteristics of soil H_2 oxidizing bacteria are very unique compared to other bacteria. i.e., they grow chemolithoautotrophically by utilizing H_2 as an electron donor with O_2 and fixing CO_2 (Aragno and Schlegel, 1981).

These factors have affected the isolation of soil H_2 oxidizing bacteria successfully. The mineral agar and mineral salt medium have been employed to isolate H_2 oxidizing bacteria from soils and lake (Schuler and Conrad, 1990; Aragno and Seiler, 1983; Aragno and Schlegel, 1978; Conrad and Seiler, 1980; Dugnanai *et al.*, 1986; Schuler and Conrad, 1991). It is known that the H_2 oxidizing bacteria can grow on low nutrient media in the presence of an atmosphere containing H_2, O_2 and CO_2 (Aragno and Schlegel, 1981). Unfortunately choosing the right composition of gas mixture for culturing the H_2 oxidizing bacteria has been a problem (Aragno and Schlegel, 1981). The requirements of gas composition for the N_2-fixing strains, denitrifying strains, oxygen-tolerant strains of H_2 oxidizing bacteria are different based on the metabolic properties (Aragno and Schlegel, 1981). Some species of the H_2 oxidizing bacteria in lake and soil were cultured in a closed jar under a gas atmosphere consisting of 80% H_2, 10% CO_2 and 10% O_2 (Kluber *et al.*, 1995; Conrad and Seiler, 1979; Aragno and Schlegel, 1981; Schuler and Conrad, 1990). The N_2 fixing strains of H_2 oxidizing bacteria (*Bradyrhizobium japonicum* C33 and *Azospirillium brasilense* Sp7) were isolated in a sealed incubation chamber with gas mixture of 91% N_2, 5% O_2, 3% CO_2 and 1 % H_2 (Cunningham *et al.*, 1986).

Although the isolation condition of H_2 oxidizing bacteria used in previous study such as, low carbon liquid medium and closed system is only suitable for isolating those specific metabolic properties known H_2 oxidizing bacteria, but it is not suitable for the unknown soil H_2 oxidizing bacteria in legume field. In the natural habitat of soil H_2 oxidizing bacteria, the H_2 concentration is unlikely to be very high since the H_2 diffuses from Hup^- legume nodules into the soil and is rapidly oxidized by H_2 oxidizing bacteria within a few cm of the legume nodules (La Favre and Focht, 1983). At the same time, the partial pressure of O_2 and CO_2 around the nodules are close to the atmospheric levels (Dong and Layzell, 2001), and the concentration of all the gases is constant all the time. However, the closed incubation system under a gas mixture consisting of 80%H_2, 10% CO_2 and 10% O_2 employed in previous studies did not meet this natural growing condition of H_2 oxidizing bacteria (Conrad and Seiler, 1979; Schuler and Conrad, 1990; Kluber *et al.*, 1995).

With the difficulty of isolating H_2 oxidizing bacteria using conventional cultivation methods, some researchers tried to detect the H_2 oxidizing bacteria in soil samples using molecular methods, such as, hybridization, extraction of total DNA from soil and PCR amplification of the structural gene of the standard type of dimeric NiFe hydrogenases (Lechner and Conrad, 1997; Dean, 2004). Lechner and Conrad (1997) were able to detect and quantify the aerobic H_2 oxidizing bacteria from environmental samples that are closely related to the *Alcaligenes eutrophus* H 16 by using hybridization and PCR primers targeted to the hydrogenase. Although the detection and quantification of aerobic H_2 oxidizing bacteria in environmental samples were possible by using molecular techniques without prior cultivation of bacteria, there are still limitations of these

techniques for broad-range detection of aerobic H_2 oxidizing bacteria because of no universal hydrogenase probe or primers (Lechner and Conrad, 1997). Also, it is impossible to further study the physiological mechanisms of bacteria in plant interactions without pure culture. The failure in isolation of pure culture of H_2 oxidizing bacteria slowed the study on interactions between legume plants and soil microflora as well as the effect on the subsequent non-legume plants.

1.1.4.2 Identification of H_2 oxidizing bacteria

Classical and molecular characteristics have been employed in taxonomic identification of bacteria. The classical characteristics include morphological, physiological, metabolic and ecological analysis. And molecular characteristics include nucleic acid hybridization, comparisons of proteins, nucleic acid base composition and nucleic acid sequencing. The ribosomal RNA sequencing has been used more extensively in bacterial taxonomy in which the 5S and 16S rRNA isolated from 50S and 30S subunits of ribosomes are sequenced (Knox *et al.*, 1998). The PCR amplification of 16S rRNA genes using universal primers has been widely used for bacterial taxonomy, since the 16S rRNA contains highly conserved with highly variable or divergent sequences that can differentiate one species from another (Knox *et al.*, 1998). Also, the 16S rRNA molecule is much longer than the 5S rRNA, and thus, the ~1500bp sequence, amplified by universal primers contains considerably more information (Head *et al.*, 1998).

As we know, the aerobic H_2 oxidizing bacteria do not form a taxonomic unit, and comprise species from phylogenetically diverse taxa of H_2 (Knallgas) bacteria, N_2- fixing bacteria, and photosynthetic microorganisms (Lechner and Conrad, 1997). Therefore, there is a limited program of simple tests to facilitate the determination of newly isolated

strains. The traditional microbiological methods have been used to identify the isolated unknown H_2 oxidizing bacteria (Aragno and Schlegel, 1981). Aragno and his coworkers (1981) outlined the rapid tests for primary identification of H_2 oxidizing bacteria based on the characteristics of the morphology, motility, Gram staining, pigmentation, denitrification, N_2 fixation, carbon source utilization and tetrathionate reductase. However, these identification tests are only suitable for those well-described strains and they do not fit many sorts of H_2 oxidizing bacteria, which are not known or been described as yet.

Therefore for the identification of newly isolated H_2 oxidizing bacteria, the combination of conventional microbiological identification tests and molecular identification methods are essential to understand the physiological and metabolic properties of isolated H_2 oxidizing bacteria as well as the taxonomic position.

1.2 THE OBJECTIVES OF THE PRESENT STUDY

The H_2 released from Hup^- legume nodules into the soil play a significant role in contributing to the benefits of legumes in crop rotations by altering the population of H_2 oxidizing bacteria. The present study investigates the H_2 oxidizing bacteria and their effect on plant growth promotion.

The aims of the current study are:

- To develop an optimum cultivation condition for isolating aerobic H_2 oxidizing bacteria from legume soil
- To isolate the H_2 oxidizing bacteria from H_2 treated soil, soil adjacent to Hup^- soybean nodules grown in greenhouse and field.

- To characterize the newly isolated H_2 oxidizing bacteria using conventional microbiological identification tests and 16S rRNA sequence analysis
- To study the effect of isolated H_2 oxidizing bacteria on plant growth promotion

2. CULTURE AND ISOLATION OF H_2 OXIDIZING BACTERIA

2.1 INTRODUCTION

2.1.1 Medium for isolating H_2 oxidizing bacteria

Finding an appropriate medium for promoting the growth of H_2 oxidizing bacteria from soil is a key step for isolating them. The previous experiment showed that the H_2 uptake rate of soil was increased in 7-10 days of H_2 treatment (Dong and Layzell, 2001). This indicated that H_2 oxidizing bacteria in H_2 treated soil and soil adjacent to Hup^- legume nodules might be slow growing bacteria. If a nutrient rich medium were used, in the course of incubation, the fast growing bacteria would take over H_2 oxidizing bacteria. Since the 40% of the electrons from H_2 evolved from Hup^- legume nodule are used to reduce CO_2 into organic materials, 60% of the electrons from H_2 go to O_2 to provide energy for cell reaction (Dong and Layzell, 2001), the H_2 oxidizing bacteria can grow on organic free medium as long as in the presence of H_2, and CO_2 and O_2. Also, in the natural habitat, the soil H_2 oxidizing bacteria need growing surface to form colony. Based on these characteristics of H_2 oxidizing bacteria, the solid organic free medium is suitable for isolating H_2 oxidizing bacteria, since it can create not only the growing opportunity for slow growing bacterial strains but also the surface for the bacteria to form colonies just as natural condition.

2.1.2 Incubation condition

Besides the proper culturing medium, the optimum incubation condition with right gas atmosphere is another critical condition for isolating soil H_2 oxidizing bacteria.

The H_2 oxidizing bacteria can grow on low nutrient medium in the presence of a gas atmosphere containing H_2, O_2 and CO_2 (Maire *et al.*, 1978; Aragno and Schlegel,

1981; Dong and Layzell, 2001). The attempts to isolate H_2 oxidizing bacteria from legume field has been tried in a closed jar under an atmosphere consisting of 80%H_2, 10% CO_2 and 10% O_2 (Kluber *et al.*, 1995; Conrad and Seiler, 1979; Aragno and Schlegel, 1981; Schuler and Conrad, 1990). Unfortunately, this closed incubation condition did not meet the natural growth condition of soil H_2 oxidizing bacteria. According to Dong and Layzell (2001), the H_2 exposure rate of soil adjacent to N_2-fixing nodules is 30-250nmoles cm^{-3} h^{-1}. The H_2 concentration in natural habitat of H_2 oxidizing bacteria could not be very high. The concentration of O_2 and CO_2 are close to atmospheric level. Also, all gases are constant all the time. Therefore an optimum incubation condition which is similar to the natural growing habitat of H_2 oxidizing bacteria needs to be developed. Also, the high dilution of soil samples under long incubation is necessary for the slow growing bacteria.

Once the optimum culturing medium and incubation condition with the right gas atmosphere composition set up for isolating soil H_2 oxidizing bacteria, recognizing grown colonies as a H_2 oxidizing bacteria is another main concern.

2.1.3 Detection of H_2 uptake capacity of isolates

The verification of H_2 uptake ability of isolates is very important to determine the H_2 oxidizing bacteria. The TTC (2, 3, 5-triphenyl-2H-tetazoliumchloride) reduction test, DNA-DNA-hybridization techniques and the H_2 oxidizing ability assay of colonies using gas chromatography (GC) were employed for detecting the H_2 oxidizing bacteria in the past (Kluber *et al.*, 1995, Cunningham *et al.*, 1986; Conrad and Seiler, 1979; Schuler and Conrad, 1990). Although the TTC test was simple and quick, it was not reliable for mixed cultures or unknown cultures (Kluber *et al.*, 1995). Despite the DNA probes in DNA-

DNA-hybridization technique would detect hydrogenase gene of bacteria whether it is active or not, but this technique were not able to detect all the bacteria containing hydrogenase gene (Kluber *et al.*, 1995).

So far, the gas chromotography (GC) has been used successfully to detect the hydrogenase activity for the H_2 oxidation.

2.1.4 The H_2 uptake kinetic characteristics of hydrogenase in H_2 oxidizing bacteria

As was mentioned in the general introduction, the H_2 oxidizing bacteria are responsible for the oxidization of H_2 released from Hup^- legume nodules into the soil (Conrad *et al.*, 1983). The hydrogenase in these H_2 oxidizing bacteria catalyzing the H_2 oxidation (Friedrich and Schwartz, 1993) have been reported to have a low affinity for H_2 (apparent K_m 1-60μM H_2) (22.4-1344ppm) (Schuler and Conrad, 1990).

When Dong and Layzell, (2001) examined the kinetics for H_2 oxidation of H_2 treated soil which had similar properties to soil adjacent to Hup^- legume nodules in field, they observed that the K_m value of the H_2 treated soil for H_2 was 1028ppm, while the air treatment soil was 40ppm. This high K_m (H_2) value (1028ppm) found in H_2 treated soil was similar to the one observed in H_2 bacteria (Knallgas bacteria) in Schuler and Conrad (1991). It is known that the H_2 oxidation activities with low affinity and high K_m are attributed to aerobic H_2 oxidizing bacteria (Conrad *et al.*, 1983; Schuler and Conrad, 1990; Häring and Conrad, 1994). Dong and Layzell (2001) suggested that the high K_m in H_2 treated soil and soil adjacent to Hup^- legume nodules was caused by soil H_2 oxidizing bacterial activity (Dong *et al.*, 2003). However, it is not known whether the isolates of H_2 oxidizing bacteria are responsible for the high K_m (H_2) observed in H_2 treated soil and

around the Hup$^-$ soybean nodule. It needs to determine the K_m value for H_2 of our isolates of H_2 oxidizing bacteria.

2.2 OBJECTIVES

- To further investigate the optimum conditions for isolating soil H_2 oxidizing bacteria
- To isolate H_2 oxidizing bacteria from different soil samples
- To determine the H_2 oxidizing capacity of isolates
- To confirm the isolates of H_2 oxidizing bacteria as the ones responsible for the soil H_2 uptake activity

2.3 MATERIALS AND METHODS

2.3.1 Samples for isolating H_2 oxidizing bacteria

The different soil samples used for isolating H_2 oxidizing bacteria were prepared in laboratory, field and green house condition.

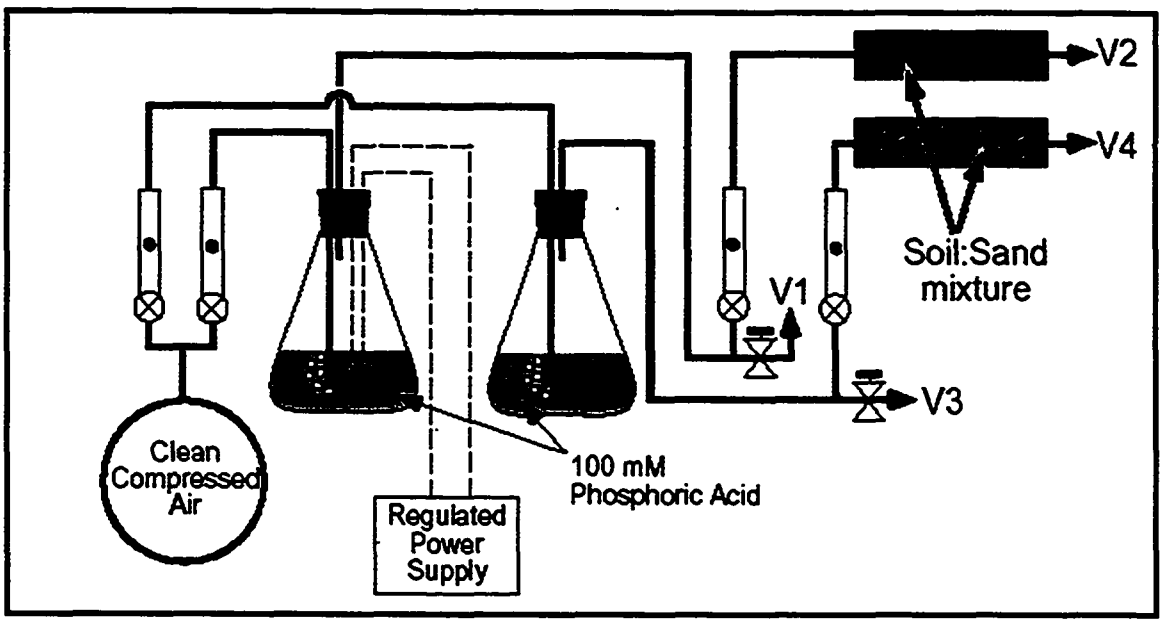
2.3.1.1 Laboratory condition (H_2 treated soil)

The H_2 treated soil used to isolate H_2 oxidizing bacteria was prepared by following procedures: Soil collected from fallow field in Lawrencetown, Nova Scotia, Canada was used. Fifty mL of a 2:1(v/v ratio) soil and fine sand mixture were placed in 1000 mL beaker and autoclaved for 20min. One-gram pre H_2 treated soil was placed into the testing tube containing 2.5 mL autoclaved distilled water (dH_2O) to allow them to mix. 50mL prepared soil was inoculated with 1mL supernatant from test tube and transferred to 60mL syringes. Then the syringe containing soil was treated with H_2 in air

for 3 weeks using H_2 treatment system (Figure 1) as described by Dong and Layzell (2001).

The H_2 treatment system produces H_2 through electrolysis. Two one-liter flasks are added with 300 mL of a 100 mM phosphoric acid solution. Clean compressed air is pumped into the two flasks at a steady rate. One of the flasks is equipped with a power supply that provides a direct electrical current through electrodes. The passing of an electrical current through an electrolyte solution (100 mM phosphoric acid) produces H_2 gas. The H_2-enriched gas (V1 in Figure 1) is conveyed through a syringe that contains 60 mL of a soil sand mixture and is vented out the other end of the syringe (V2 in Figure 1). The second flask without electrolysis used for air treatment. The non- H_2-enriched air (V3 in Figure 1) is conveyed through a syringe that contains 60 mL of the soil sand mixture without inoculation and is vented out the other end of the syringe (V4 in Figure 1).

Figure 1: A simplified diagram of hydrogen treatment system (Dong and Layzell, 2001). The H_2 gas is produced by first flask equipped with a regulated power supply to provide a direct electric current. The second flask acts as a control. Air is provided at a stable rate to both flasks. The H_2 enriched gas stream (V1) were connected with the soil column before venting to the atmosphere at (V2).


V2
V4
Soil:Sand
mixture
V1
V3
Clean
Compressed
Air
100 mM
Phosphoric Acid
Regulated
Power
Supply

2.3.1.2 Field condition

Soil samples adjacent to the Hup$^-$ soybean nodules used to isolate H_2 oxidizing bacteria was prepared by following procedure:

Soybean seeds (OAC vision seeds, Nova Scotia Agriculture College, Bible Hill, Nova Scotia) inoculated with a commercial Hup$^-$ strain of *Bradyrhizobium japonicum* (532C) were planted in field (Dr. Dong's garden) in spring of 2004.

The determination of hydrogenase activity (Hup status) of soybean nodules

After 12 weeks of growth, the soybean nodules were collected to determine the hydrogenase activity (Hup status). The Hup status of nodules was determined by the methylene blue reduction assay following Lambert *et al.* (1985) and Dean (2004).

Nodules were washed and placed on filter paper moistened with 3mL methlyne blue reduction dye solution {(mM): iodoacetic acid, 200; malonic acid, 200; methylene blue, 10; potassium phosphate, 50; magnesium chloride, 2.5; adjust to pH 5.6 with KOH } in petri dishes. Three dishes (six nodules per dishes) were prepared. Then nodules were squashed with a flat surface and expose to the air for 15 minutes for letting solution and inhibitors (iodoacetic acid and malonic acid) to equilibrate with nodule contents and tissue. After 15 minutes exposure in the air, nodules in petri dishes were covered and placed in a metal gas-tight chamber. Gas chamber was vacuumed and flushed in H_2 gas. Large plastic gas bag (Quibit system Inc) and GAST motor (Motor Division St. Louis, Mo. U. S. A) were utilized for vacuuming and flushing H_2. After 36 hours incubation of nodules in H_2, picture of nodules were taken immediately by digital camera (Canon, Power Shot 2G) upon removal of plates from the incubation gas chamber. Since nodules

containing Hup$^-$ inoculated rhizobia are not able to reduce methylene blue dye, nodules without color change around them, are Hup$^-$.

Soil collection

After the determination of Hup status of nodules, the soil within 10mm from soybean nodules was collected for testing the H_2 uptake ability and isolating H_2 oxidizing bacteria. Bulk soil, over 100mm away from nodules, was used as control.

2.3.1.3 Green house condition

The preparation of soil samples adjacent to Hup$^-$ soybean nodules grown in a green house used to isolate H_2 oxidizing bacteria was described in Dean (2004). Two isogenetic laboratory strains of *B. japonicum* JH and JH47, were used to inoculate o soybean seeds (Cat.3 2601R, First Line seeds Ltd., Guelph, Ontario). After 12 weeks of growth in pots, the soybean nodules were collected to determine if the nodules were infected with applied inoculants. The inoculation process and the determination of the Hup status are described in Dean (2004).

After the determination of nodules Hup status, soil samples were collected within 10 mm from the nodules inoculated with Hup$^-$ strain (JH47) and Hup$^+$ (JH) of *B. japonicum* for testing H_2 uptake ability and isolating H_2 oxidizing bacteria.

2.3.2 Measurement of soil H_2 uptake rates

The H_2 uptake rates of soil samples prepared in laboratory, field and green house condition (Table 1) were measured prior to isolation of bacteria. A H_2 sensor (Model S121, Quibit Systems Inc) and Data Logger program were used in the H_2 uptake analysis described by Dong and Layzell (2002). After the measurement of H_2 uptake rate, 0.1g soil from each soil sample was used for isolating H_2 oxidizing bacteria. The rest of the

soil samples were transferred to the 50mL polypropylene centrifuge tube (Fisher brand) and stored at -80°C degree for further investigation.

2.3.3 Isolation of H_2 oxidizing bacteria

2.3.3.1 Cultivation condition

In present study, the mineral salt agar medium (MSA) ($NaNO_3$, 2.0 g; K_2HPO_4, 1.2 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; KH_2PO_4, 0.14 g; Yeast Extract, 0.02 g; $Fe_2(SO_4)_3$. H_2O, 0.01 g; Agar, 15 g; Water, 1 L; pH 7.2) (Schlegel and Meyer, 1985) was employed for isolation. Cycloheximide (10mg/L) was added to the medium to prevent the growth of fungi. The open gas flow-through system was used for bacterial incubation at room temperature. The H_2 gas (~3000ppm) generated by electrolysis in atmospheric air was delivered to the incubation system.

2.3.3.2 General observation and measurement

In present study, grown bacterial colonies were observed under dissecting microscope (Stem SIII APO Zeiss) and pictures were taken with corresponding camera. Colonies shape and color on different medium were recorded by digital camera (Canon, Power Shot 2G). Two days old cultures were used for Gram staining according to the protocol of Benson (1994). The Gram staining slides were examined under an Olympus microscope in 100x magnification and recorded with corresponding camera.

2.3.3.3 Bacterial isolation from soil samples

Soil samples (0.1g) from H_2 treated soil, soil adjacent to Hup^- soybean nodules grown in field and green house were serially diluted (10^{-3}-10^{-12}). The dilutions were plated onto MSA plates. Autoclaved soil (0.05g / plate) was spread over the inoculated plates for creating the growing surface for bacteria. The plates were incubated for 21 days

at room temperature under ~3000ppm H_2 gas in air. Gases were delivered to plates in an open flow through system. Air treated soil, soil adjacent to Hup^+ soybean nodules grown in green house, and bulk soil from field were used as controls.

After two days growth, the colony color and shape were examined. The visible bacterial colonies were picked daily and transferred to new MSA plates. The number of transfers was dependent on the number and kind of accompanying bacteria and the consistency of the colonies. Bacterial culture from new plates were inoculated to the MSA slant tubes and incubated for 5-7 days before testing the H_2 uptake capacity of isolates.

2.3.4 The detection of H_2 oxidizing capacity of isolates

2.3.4.1 Gas chromatography and standard curves

The H_2 oxidizing ability of isolates was determined using gas chromatography (GC) system equipped with H_2 sensor. The principle and calibration of GC system were described in Chen (2000). Since the gas samples analyzed by H_2 sensor is in voltages instead of H_2 concentration, a standard curve is needed to interpret the voltages to H_2 concentration.

A 2256 mL glass jar with a stopper equipped with an injector for gas inputs and withdraws, and glass beads in the jar is used to generate different concentration standard H_2 gas from 100% commercial gas (Paraxair product Inc., UN 1049). An appropriate volume of pure H_2 gas was injected in to the jar with a gas-tight syringe and shaken several times to mix gas. Then 0.5mL of gas samples of mix gas was taken from jar with gas-tight syringe at different H_2 concentrations and injected to GC system. The data collected on the GC system were recorded for each H_2 peak in voltage. Three replications

were obtained from each H_2 concentration for getting average. Standard curve (Appendix 1) was developed by plotting known H_2 concentration (ppm) versus H_2 voltage (V). Three standard curves (Appendix 1: A, B, and C) were obtained since temperature was different for each measuring time. From each generated standard curve, an equation (1) was competed to calculate H_2 concentration (ppm) of a sample when the voltage (V) is known.

$$[H_2]ppm = ae^{bV} \quad (1);\ (e = 2.718282)$$

2.3.4.2 The test of the H_2 oxidizing capacity of isolates

All isolates were grown on 5mL MSA slant in 17mL tubes at room temperature under same H_2 treatment condition for 5~7 days before being tested. A test tube with each isolate under air treatment and the second test tube with non-inoculation under H_2 treatment served as controls. Before testing the H_2 oxidization ability of isolates, caps of bacterial grown slant tubes as well as control tubes were replaced with gas-tight rubber caps, and were flushed with H_2 (<3000ppm) for 30 second. As soon as H_2 was flushed into test tubes, gas samples (0.5mL) from each test tube were taken with a gas-tight syringe and analyzed in GC system for the initial H_2 concentration. Then these test tubes were incubated for 30 minutes at room temperature, and the change of H_2 concentration of each test tube was analyzed. If the bacterial cultures were utilizing H_2, there would be a change in H_2 concentration in closed test tubes over time.

2.3.5 The purification of isolates of H_2 oxidizing bacteria

The morphological characteristics of H_2 oxidizing isolates

The morphological characteristics (Gram specificity, cell shape, arrangement and size) of isolates with H_2 uptake capacity were examined after Gram staining.

The test of H_2 uptake ability of sub-colonies of H_2 oxidizing isolates

After checked the morphology of H_2 oxidizing isolates, the bacterial culture were diluted from 10^{-5} to 10^{-7} and then plated on MSA medium. Three plates were inoculated for each dilution and incubated under H_2 treatment for more than 7 days. After incubation, 30 sub-colonies (ten colonies from each plate) of each dilution were streaked on MSA and incubated for another 4 days. Altogether ninety sub-colonies from original H_2 oxidizing bacteria were transferred to MSA slant tubes and incubated under H_2 treatment for 4 days to further testing their H_2 oxidation capacity. If all sub-colonies have same morphological characteristics and same H_2 uptake ability as original isolate, this isolate is regarded as pure culture.

2.3.6 The confirmation of H_2 oxidizing isolates as the ones responsible for the soil H_2 uptake activity

2.3.6.1 The H_2 uptake kinetics of H_2 oxidizing isolates

The K_m measurement in closed system

To characterize H_2 uptake kinetics of H_2 oxidizing bacteria, the culture grown on 5mL MSA slant in test tubes (17mL) were flushed with H_2 in air (~3000ppm) for 30 second to give desired initial H_2 concentration in closed system. The decrease of H_2 was subsequently determined and analyzed by extracting gas samples (0.5mL) at about 4~5 minutes intervals during the 30 minutes incubation using GC equipped with H_2 sensor. However, the H_2 sensor gives voltages instead of H_2 concentration. The H_2 concentration was interpreted using standard curves (Appendix 1A, B and C).

The H_2 uptake rate of isolated H_2 oxidizing bacteria was determined at different H_2 concentration. The value of V_{max} and K_m of hydrogenase catalyzing the oxidation of

H_2 were determined by plotting the data pairs in Michealis-Menton and Lineweaver-Burk plot.

The K_m measurement in open system

The method to characterize H_2 uptake kinetics of hydrogenase in open system was similar to that employed by Dong and Layzell (2001). The bacterial cultural grown on 5mL MSA slant in test tubes (17mL) were connected with H_2 treatment system (Figure 1), then the input and effluent H_2 gas from test tubes were detected by a H_2 sensor. A series of known [H_2] concentrations (ppm) was acquired by changing the current through the electrolyte, and from these data a standard curve was produced (Appendix 1 D).

Based on standard curve, the H_2 concentration of average and difference of inlet and outlet gas in test tubes was calculated and plotted. The K_m and V_{max} of hydrogenase catalyzing the oxidation of H_2 was determined using Lineweaver-Burke plot.

2.3.6.2 Soil re-inoculation

Five mL of sterile nutrient broth (NB) (Difco) were inoculated with isolates of H_2 oxidizing bacteria and incubated for 28 hours at 28~30°C on a rotated shaker at speed 200rpm. One mL of bacterial suspension from each isolates when $O.D_{600}$ at 0.7 was used to inoculate with 50mL autoclaved soil sand mixture. Re-inoculated soils were transferred to 60 mL syringe and connected with H_2 treatments system (Figure 1) for 3 weeks. Inoculated soils treated with air, and non-inoculated soils treated with H_2 were used as control. After three weeks of H_2 treatment, the H_2 uptake rates of soil re-inoculated with isolated H_2 oxidizing bacterial strains and as controls were measured by using H_2 sensor. The measurement and calculation of H_2 uptake rate of soil were same as Dean (2004).

Re-isolation of the H_2 oxidizing bacterial strains from inoculated soil

The media and cultural conditions of the re-isolated H_2 oxidizing bacteria from inoculated soil were the same as described 2.3.3.1.

Soil dilutions (10^{-3}-10^{-8}) from each soil column were plated on MSA medium and incubated for 4 days. After 4 days of incubation, plates were examined for the growth of bacteria. Single colonies from each plate were streaked on MSA medium and incubated for 2 days under H_2 treatment. After 2 days incubation, each colony from plate was transferred to the MSA slant tubes for the testing of the H_2 uptake ability. The H_2 uptake capacity of re-isolates was analyzed with gas chromatographic (GC) equipped with a H_2 sensor system described in 2.3.4. The morphology of isolates with H_2 uptake ability was examined with Gram staining. The colonial and morphological characteristics of re-isolates were compared with the original isolates. If the re-isolates increases soil H_2 uptake rate and possess identical colony and morphology as original isolates, we regard that our isolates are not contaminated in the course of isolation.

2.4 RESULTS

2.4.1 The hydrogenase (Hup) status of soybean nodules

The Hup status of soybean nodules were tested using methylene blue reduction assay (Lambert *et al.*, 1995; Dean, 2004) to make sure whether the nodules were infected with applied inoculants (Hup^- and Hup^+ strains of *B. japonicum*). Nodules containing inoculated Hup^- rhizobial strain of *B. japonicum* (532C) and (JH47) had no hydrogenase uptake activity, and the methylne blue dye surrounding nodules were not reduced resulting no color change around nodules (blue). However, nodules containing Hup^+ inoculated rhizobial strain of *B. japonicum* (JH) had hydrogenase uptake activity and the

methylene blue dye surrounding of nodules was reduced to form clear zone around the nodules.

2.4.2 The soil H_2 uptake rates

As showed previously, the H_2 treated soil had significantly higher H_2 uptake rate than that of control (air treatment) ($n= 3$, $p<0.001$) (Table 1). Soil adjacent to nodules containing inoculated Hup^- rhizobial strain of *B. japonicum* (532C) had higher H_2 uptake rate than that of the control (bulk soil) (Table 1). The H_2 uptake rate of soil adjacent to Hup^- soybean nodules in green house was significantly higher compared to the soil adjacent to nodules that recycle H_2 (Hup^+) ($n= 4$, $p<0.0001$) (Table 1) (Dean, 2004).

Table 1: Soil H_2 uptake rates
Values are given as means ± standard deviation (SD)

Soil sources		H_2 uptake rate ($\mu molhr^{-1}cm^{-3}$)
Laboratory	H_2 treated	0.697± 0.200 (n=3)
	Air treated	0.031± 0.006 (n=3)
Green house	Soil adjacent to Hup^- nodules	0.085 ± 0.032 (n=14)
	Soil adjacent to Hup^+ nodules	0.017 ± 0.009 (n=13)
Field	Soil adjacent to Hup^- nodules	0.081 ± 0.012 (n=3)
	Bulk soil	0.040 ± 0.005 (n=3)

2.4.3 Bacterial isolation

2.4.3.1 Isolates from H_2 treated soil

Plates inoculated by 10^{-2} to 10^{-12} soil dilutions were incubated for 21 days and examined daily. All plates inoculated by 10^{-2} to 10^{-12} soil dilutions from H_2 treated soil under H_2 treatment had different bacterial colonies in two days. They are snow flake hairy, small white pin point, small yellowish circle and white filamentous colonies. However, the control plates (soil dilution from air treated soil under air treatment) had bacterial colonies only on 10^{-2} to 10^{-9} dilution. These bacterial colonies were similar to those fast growing ones appeared in H_2 treated plates (Figure 2A).

After 4 days incubation, round-diffused translucent bacterial colonies (Figure 2A) began to appear on H_2 treated plates, and their number was increased the following days. However, this kind of colony did not appear on air treated plates (Figure 2B).

The different bacterial colonies grown on plates from H_2 (10^{-9} to 10^{-12}) and air treated plates (10^{-7} to 10^{-9}) were streaked to new MSA plates and given name from JM01 to JM113. After 4 days incubation, MSA slant test tube was isolate inoculated with isolate and incubated for 4-7 days to test the H_2 uptake ability.

2.4.3.2 Isolates from soil adjacent to Hup^- soybean nodules grown in the field

Soil samples within 10mm from Hup^- nodules were used for isolating H_2 oxidizing bacteria. Plates inoculated by 10^{-3}- 10^{-8} soil dilutions were incubated for 21 days and examined daily. In two days, plates inoculated with 10^{-3} to 10^{-4} dilutions had different bacterial colonies. From day 4, the slow growing bacterial colonies such as, creamy white dot like small colonies (diameter <1mm), light brownish diffused colonies

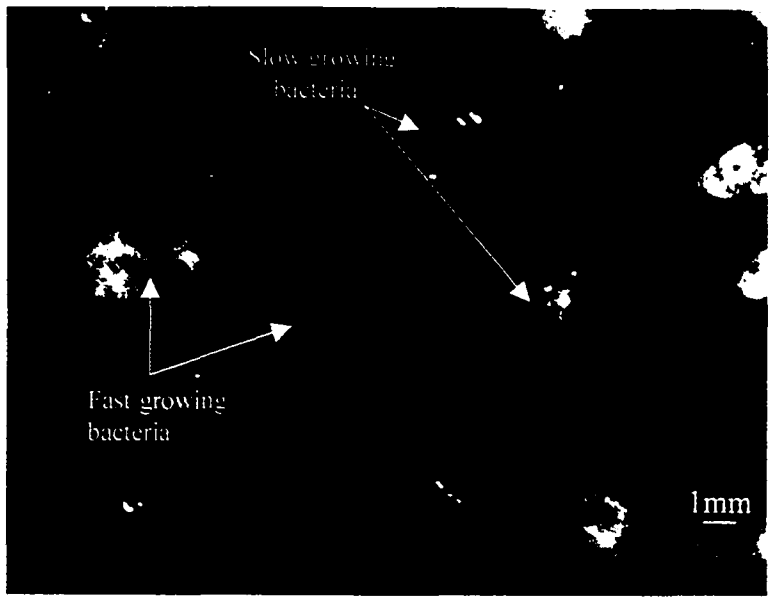
(diameter >3mm) and transparent round colonies were appeared (Figure 2C). Altogether, forty nine colonies (JM126-175) were picked to test H_2 uptake capacity.

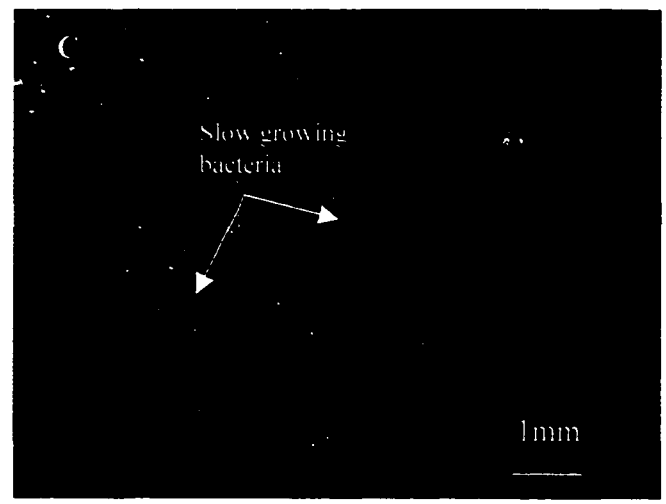
2.4.3.3 Isolates from soil adjacent to the Hup$^-$ soybean nodules grown in the green house

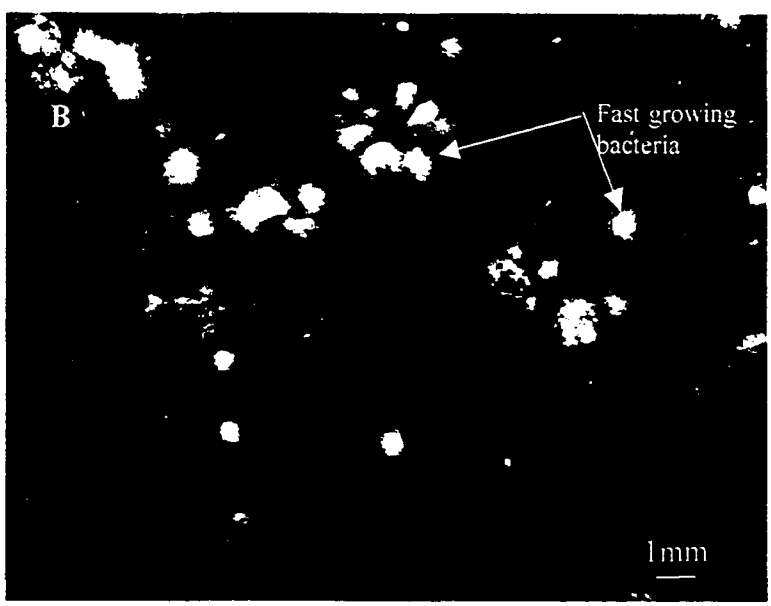
Plates inoculated by 10^{-3} to 10^{-8} soil dilutions from soil samples adjacent to Hup$^-$ and Hup$^+$ nodules were incubated for 21 days and examined daily. After 6 days incubation, three different colonies were dominant on all plates. They were white powdery filamentous colonies, very small dot like colonies and small round white creamy colonies. After 21-day incubation, plates inoculated with 10^{-4} and 10^{-5} dilution of Hup$^-$ soil had new colonies which were different from the previous ones. They were round, transculant, and dencer in the centre. The diameter of them was about 3 ~ 4mm (Figure 2D). Altogether 32 colonies from plates inoculated by soil adjacent to Hup$^-$ nodules (JM119-JM125) and 3 colonies from plates inoculated by soil adjacent to Hup$^+$ nodules were picked to make streak plates, and then transferred to MSA slant test tubes for testing the H_2 uptake ability.

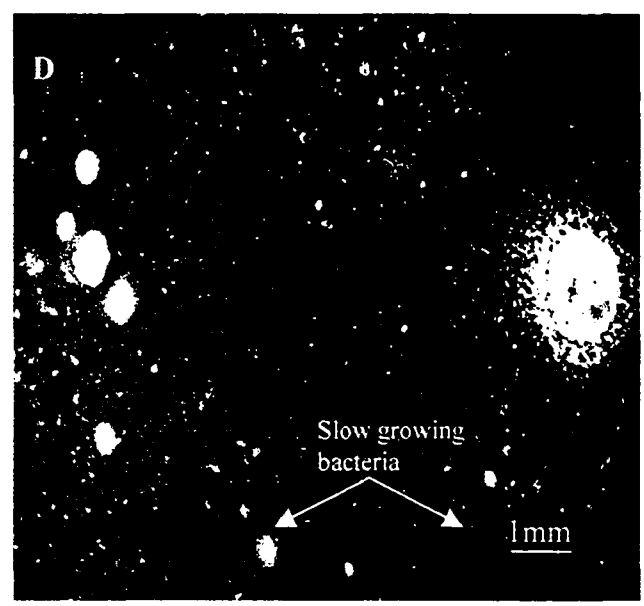
Figure 2: Bacterial colonies from different soil samples

A: Bacterial colonies from H_2 treated soil. B: Bacterial colonies from air treated soil. C: Bacterial colonies from soil adjacent to Hup$^-$soybean nodules grown in the field. D: Bacterial colonies from soil adjacent to Hup$^-$soybean nodules grown in green house.


Slow growing
bacteria
Fast growing
bacteria
1mm


C
Slow growing
bacteria
1mm


B
Fast growing
bacteria
1mm


D
Slow growing
bacteria
1mm

2.4.4 H_2 oxidizing capacity of isolates

Altogether 175 isolates from H_2 treated soil, and soil adjacent to the Hup$^-$ nodules soybean nodules grown in field and green house as well as controls were tested for H_2 uptake capacity. Twenty isolates exhibited H_2 uptake ability (Table 2). These H_2 oxidizing bacterial strains were obtained from H_2 treated soil (twelve strains), soil adjacent to the Hup$^-$ nodules in field (four strains), and green house (four strains). All these H_2 oxidizing bacterial strains were picked from those slow growing colonies on MSA plates from 4-21 days of incubation. Typically, the H_2 concentration in test tubes with H_2 oxidizing isolates decreased from 3000 ppm to ~31ppm within 30 minutes (Appendix 2). The other isolates as well as controls did not have H_2 uptake ability; the H_2 concentration in test tubes with these had little or no changes.

2.4.5 Purification of isolates of H_2 oxidizing bacteria

Cultural and morphological characteristics of H_2 oxidizing bacteria

Isolates that had H_2 oxidizing ability were chosen for Gram staining. Each isolate of H_2 oxidizing bacteria was checked for the Gram specificity and the consistency of cell morphology. The result of Gram stained slide showed that the Gram specificity and the cell morphology each sub colony were consistent with the original H_2 oxidizing isolate (Figures not shown).

The H_2 uptake ability of sub-colonies of H_2 oxidizing bacteria

Plates inoculated by 10^{-5} to 10^{-8} dilutions of the bacterial cultures of twenty isolates of H_2 oxidizing bacteria were incubated for one week. Three plates were prepared for each dilution. After incubation, thirty sub-colonies (10 colonies/ plate) of each

dilution were transferred to new MSA plates. Altogether ninety sub-colonies of original isolate were inoculated with MSA slant to test the H_2 uptake ability. All sub-colonies from each original isolate had H_2 uptake ability (data not shown). All sub-colonies had H_2 uptake ability and possess same morphology as the original colonies (Data not shown).

2.4.6 Confirmation of H_2 oxidizing isolates as the ones responsible for the H_2 uptake activity in legume soil

2.4.6.1 The K_m of hydrogenase of isolated H_2 oxidizing bacterial strain

In closed system

The bacterial cultures grown on MSA slant test tubes (17mL) were flushed with 3000ppm H_2 in air before analyzing the H_2 uptake kinetics of hydrogenase in bacteria.

The H_2 uptake rate of H_2 oxidizing bacteria was subsequently determined and analyzed by extracting gas samples (0.5mL) at about 4-5 minute intervals over 30 minute incubation. The H_2 uptake rates of all isolates of H_2 oxidizing bacteria plotted against the H_2 concentration were following the Michealis-Menton kinetics (Figure 3A). The K_m value of hydrogenase catalyzing the oxidation of H_2 was obtained using Linweaver-Burk plot (Figure 3B). All isolates of H_2 oxidizing bacteria exhibited one K_m (H_2) at about 1000 ppm (Table 3). The apparent parameters of V_{max} and K_m value were shown in table 4.

In open system

Only three stains of H_2 oxidizing bacteria (JM01, JM55 and JM84) were tested for their H_2 uptake activity. The H_2 concentration of average and difference of inlet and outlet gas in test tubes was calculated and plotted. The H_2 uptake rates of these three strains were plotted against the H_2 concentration following the Michealis-Menton kinetics

(Figure not shown). The K_m values for H_2 of three isolates were around 1000ppm, which is consistent with ones obtained in closed system.

2.4.6.2 Soil re-inoculation

Soil re-inoculation and the H_2 uptake rate of re-inoculated soil

After the purification, the isolates were used to inoculate sterile soil. Nineteen H_2 oxidizing bacterial isolates were used to inoculate soil. These inoculated soil columns (nineteen) were connected with H_2 treatment system and treated with H_2 for 3 weeks. The H_2 uptake rates of each inoculated soil column were measured in 3 weeks. All inoculated soil column showed higher H_2 uptake rate than control (Table 4).

Re-isolation H_2 oxidizing bacteria

Soil sample from each inoculated soil column which exhibited high H_2 uptake rate serially diluted. The plates inoculated by 10^{-3}-10^{-8} soil dilutions were incubated under H_2 treatment for 3-4 days. In 3 days, all inoculated plates had colonies, which were similar to the original isolates. Ten colonies from each plate were picked to make streak plates. Pure culture from each streak plate was transferred to MSA slant tubes to test the H_2 uptake ability. The H_2 uptake rate of each test tube of re-isolate was analyzed by GC equipped with H_2 sensor. Nineteen re-isolates from inoculated soils exhibited H_2 uptake ability (data not shown). The colonies and morphology of all re-isolates were identical with the original isolates (Figures not shown).

Table 2: Hydrogen oxidizing isolates from different soil samples

Samples		Isolates had H_2 uptake ability
Laboratory	H_2 treated soil	JM01, JM54, JM55, JM63, JM71, JM81, JM84, JM85, JM87, JM110, JM111 and JM113.
	Air treated soil	None
Field	Soil adjacent to Hup^- soybean nodules	JM 162a, JM162, JM155 and JM169
Greenhouse	Soil adjacent to Hup^- soybean nodules	JM120, JM121, JM122 and JM123
	Soil adjacent to Hup^+ soybean nodules	None

Table 3: The H_2 uptake kinetics of H_2 oxidizing isolates in closed system

Isolates	K_m (H_2) (ppm)	V_{max} (ppm/min)
JM01	828.4	226.1
JM54	990.9	333.3
JM55	862.3	120.1
JM63	944.7	280
JM71	967.3	190.6
JM81	1024.9	216.3
JM84	831.5	243.9
JM85	746.6	222.2
JM87	1277.8	303.03
JM110	1100.5	206.2
JM111	804.3	236
JM113	931.5	250.0
JM169	927.7	99.7
JM155	850	160
JM162a	1059.5	200
JM120	813.1	96.2
JM121	951.8	112.1
JM122	1043.6	135.4
JM123	1125.7	109.2

Table 4: The H_2 uptake rate of re-inoculated soil

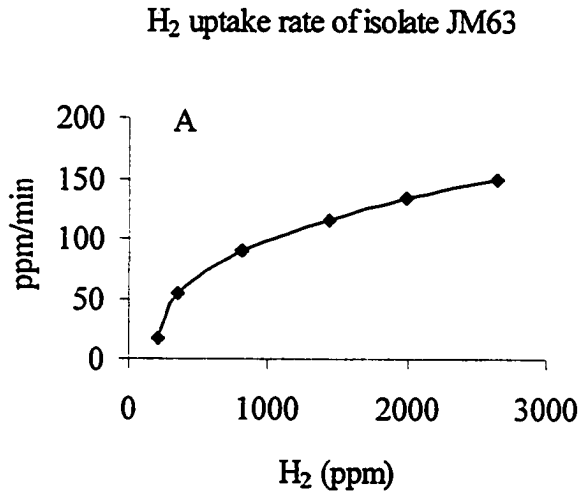
Values are given as mean±standard deviation (SD)

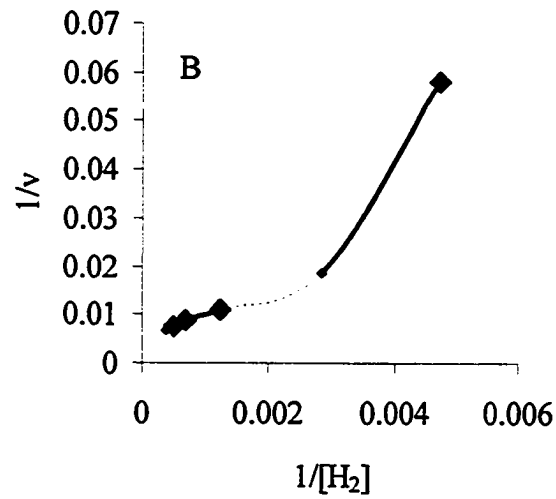
Inoculants	The change of H_2 concentration (ppm) In [H_2] n=3	Out [H_2] n=3	H_2 uptake rate $\mu mol.hr^{-1}.cm^{-3}$
JM01	113.2±7.1	38.5±8.2	0.15
JM54	94.06±7.3	24.2±4.0	0.141
JM55	83.0±9.7	21.4±3.2	0.125
JM63	97.4±7.5	23.9±5.23	0.149
JM71	99.1±3.44	26.1±6.3	0.147
JM81	78.7±7.13	20.6±7.02	0.123
JM84	85.4±8.02	15.0±5.6	0.142
JM85	90.5±6.72	13.1±5.54	0.156
JM87	93.2±3.54	10.4±3.4	0.167
JM110	131.4±4.3	16.3±3.8	0.230
JM111	82.1±5.6	7.6±2.5	0.150
JM113	88.0±7.55	14.3±3.8	0.148
JM120	109.9±4.3	14.05±2.0	0.191
JM121	161.8±8.0	26.4±3.8	0.273
JM122	98.1±4.1	16.3±3.76	0.165
JM123	98.4±7.0	15.6±5.1	0.166
JM155	91.8±0.22	15.5±4.5	0.154
JM162a	91.7±3.7	10.4±2.7	0.164
JM169	98.9±5.3	23.1±2.6	0.163
Control 1	89.0±4.36	87.5±5.89	0.004
Control 2	87.1±6.0	75.1±5.7	0.024

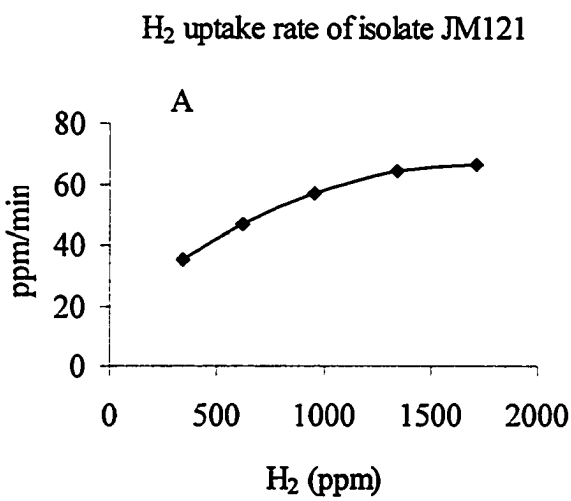
Note: Control 1-non inoculated soil treated with H_2; control 2: inoculated soil treated with air.

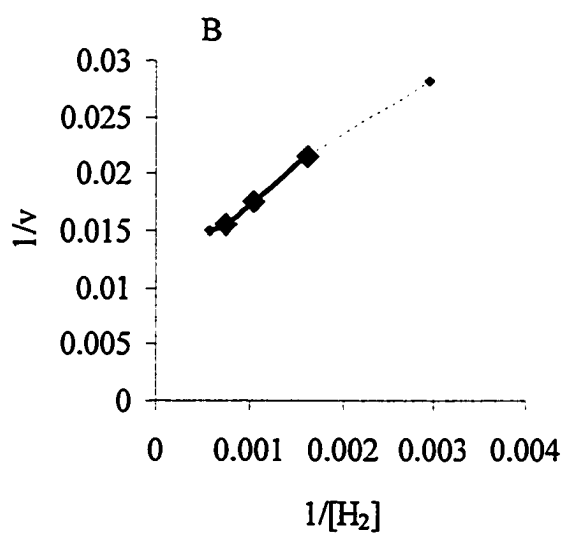
Figure 3: H_2 uptake activitiy of H_2 oxidizing isolates.

A. H_2 uptake rate plotted against H_2 concentration. B. Linweaver-Burke plots.


H_2 uptake rate of isolate JM63
A
ppm/min
200
150
100
50
0
0
1000
2000
3000
H_2 (ppm)


B
1/v
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
0.002
0.004
0.006
1/[H_2]


H_2 uptake rate of isolate JM121
A
ppm/min
80
60
40
20
0
0
500
1000
1500
2000
H_2 (ppm)


B
1/v
0.03
0.025
0.02
0.015
0.01
0.005
0
0
0.001
0.002
0.003
0.004
1/[H_2]

2.5 DISCUSSIONS

2.5.1 Soil H_2 treatment and the H_2 uptake rates

Our results show that nodules containing Hup^- strain of *B. japonicum* (532C) grown in the field and a Hup^- strain of *B. japonicum* (JH47) in green house were negative to the methylene blue reduction assay. Nodules containing Hup^+ strain of *B. japonicum* (JH) were positive on the methylene blue reduction assay (Dean, 2004). This indicated that nodules were inoculated with applied inoculants. Nodules containing the Hup^- strain of *B. japonicum* that lack uptake hydrogenase released H_2 evolved from nodules into soil. Soils surrounding Hup^- nodules are able to receive H_2 during the growing season, which can stimulate the increase of H_2 oxidizing bacterial population. However, nodules containing Hup^+ strain of *B. japonicum* have uptake hydrogenase activity that can recycle H_2 produced by nitrogenase activity within the nodules for cell reaction. The soils around the Hup^+ soybean nodules were not exposured to H_2 gas.

It has been suggested that the high H_2 uptake rate in H_2-treated soil and soil adjacent to the Hup^- nodules is promoted by bacterial activity (McLearn and Dong, 2002; Irvine *et al.*, 2004). As previously reported, the H_2 uptake rate of H_2 treated soil and soil from Hup^- soybean nodules were greater than that of air treated soil, soil from Hup^+ soybean nodules and bulk soil (Table 1). Our results show that the bacterial density in H_2 treated soil and soil adjacent to Hup^-soybean nodules were higher than those of control. The H_2 oxidizing bacteria were found only in soils, which had high H_2 uptake rate, such as, H_2 treated soil and soil adjacent to Hup^- soybean nodules. This indicates that H_2 treated soil and soil adjacent to Hup^- soybean nodules received H_2 treatment, and the H_2 oxidizing bacterial populations increased. The air treated soil, soil adjacent to

Hup^+ soybean nodules and bulk soil did not receive H_2 treatment. Correspondingly the H_2 oxidizing bacterial populations in these soils were not increased. Neither was the soil H_2 uptake activity.

2.5.2 Optimal cultivation condition for H_2 oxidizing bacteria

The results show that H_2 oxidizing bacteria can be isolated from soil if optimal cultivation conditions are applied. As we mentioned earlier, in natural legume field, the H_2 exposure rate of soil adjacent to N_2-fixing nodules has been calculated to be 30-250 nmoles cm^{-3} h^{-1} (Dong and Layzell 2001). The H_2 diffuses from Hup^- legume nodules into the soil and is rapidly oxidized by H_2 oxidizing bacteria within a few cm of the legume nodules (La Favre and Focht, 1983). In this natural habitat of soil H_2 oxidizing bacteria, the H_2 concentration is unlikely to be very high. At the same time the partial pressure of O_2 and CO_2 around the nodules should be close to the atmospheric levels, and the concentration of all the gases should be are constant. However, the closed incubation system under a gas mixture consisting of 80% H_2, 10% CO_2 and10% O_2 employed in previous studies did not meet this natural growing condition of H_2 oxidizing bacteria (Conrad and Seiler, 1979; Schuler and Conrad, 1990; Kluber *et al.*, 1995). In those previous studies, the starting concentration of H_2, CO_2 and O_2 are not even close to that of natural environment for the soil H_2 oxidizing bacteria, and they also decreases in the course of incubation resulting high H_2, CO_2 and low O_2. The open gas flow through incubation system was developed in the current study to mimic the natural growing environment of H_2 oxidizing bacteria. In this system, the H_2 generated by electrolysis of atmospheric air at a concentration was around 3000ppm. Both the [CO_2] and [O_2] were

close to the atmospheric levels. Moreover the concentrations of CO_2, O_2 and H_2 were constant.

Our results show that isolates of H_2 oxidizing bacteria are slow growing bacteria on mineral salt medium incubated under air enriched with H_2. They need more than one week to form visible colonies. This suggests that these isolates are similar to the H_2 oxidizing bacteria in H_2 treated soil in terms of growth rate. Also, the autoclaved soil was used to spread over the inoculated plates. This can create a recognizable growing surface of H_2 oxidizing bacteria similar to the natural habitat where they grow as colony form attached to the soil particles. According to our results, it is likely that more H_2 oxidizing bacteria will be found using the cultivation methods developed in the current study.

The success of isolating the H_2 oxidizing bacteria from soils with fungicide confirmed that the microbial nature of the H_2 oxidizing microorganisms in H_2 treated soil and soil adjacent to the Hup^- legume nodules is bacterial rather than fungal (McLearn and Dong, 2002; Irvine *et al.*, 2004).

In the current study, for analyzing the H_2 uptake ability of bacterial isolates, the bacterial culture was inoculated to MSA slant tubes and incubated 4-7 days under open gas flow through an incubation system with H_2 treatment. Then the H_2 uptake ability of isolates was analyzed using GC within 30 minutes. The advantages of this method are that it may not only keep the shape of the colony perfect but also provide the constant gas atmosphere for the growth of the H_2 oxidizing bacteria. The hydrogenase of H_2 oxidizing bacterial cells is induced easily under H_2 treatment, the H_2 uptake ability can be tested in a very short time (30 minutes). However, this was not met in previous studies (Cunningham *et al.*, 1983).

2.5.3 The H_2 oxidizing isolates are the ones responsible for the H_2 uptake activity in H_2 treated soil and soil adjacent to Hup^- soybean nodules

Dong and Layzell (2001) found that the apparent K_m (H_2) of H_2 treated soil was 1038ppm. Correspondingly, the K_m (H_2) of our isolated H_2 oxidizing bacterial strains from H_2 treated soil and soil adjacent to the soybean nodules was around 1000ppm, which is consistence with the one obtained from H_2 treated soil. Also these H_2 oxidizing isolates increased the soil H_2 uptake ability again when they re-inoculated with autoclaved soil. From these results we can conclude that our isolates are the ones responsible for the H_2 uptake in H_2 treated soil and soil after the growth of Hup^- legume nodules.

In our experiment, H_2 utilizing isolates grown under air treatments did not show H_2 uptake ability indicating that H_2 is needed to induce the expression of hydrogenase.

3. IDENTIFICATION AND CHARACTERIZATION OF H_2 OXIDIZING ISOLATES

3.1 INTRODUCTION

The plant growth studies conducted on H_2 treated soil and soil after growth of H_2 releasing (Hup^-) legume nodules significantly increased the dry weight and tiller number of tested crops (Dong and Layzell, 2002). Therefore it has been speculated that the presence of H_2 in soil may alter certain H_2 utilizing bacteria which in turn improve plant growth, in some way acting like plant growth-promoting rhizobacteria (PGPR) (Dong and Layzell, 2002). To test this hypothesis, the bacteria responsible for the H_2 oxidation in H_2 treated soil and soil from H_2 releasing soybean nodules were isolated. Taxonomic position, physiological and metabolic properties of these H_2 oxidizing bacteria can provide very important information for better understanding the mutual relation between H_2 oxidizing bacteria and plants.

H_2 oxidizing bacteria comprise from diverse taxa (Lechner and Conrad, 1997), and many of them are not described in Bergey's Manual of Determinative Bacteriology (1994) manual as a taxonomic group. In identification of H_2 oxidizing bacteria both the combinations of the conventional microbiological and molecular methods are necessary. It is necessary to get more information of our isolates by determining the morphological, cultural and multi physiological characteristics as well as the 16S rRNA gene sequence.

3.2 OBJECTIVES

To identify the taxonomic position of H_2 oxidizing isolates using conventional microbiological identification tests and 16S rRNA gene sequence analysis.

3.3 MATERIALS AND METHODS

3.3.1 Conventional microbiological identification tests

The conventional microbiological identification tests (Table 5, 6 and 7) were used for morphological, cultural and physiological identification of H_2 oxidizing isolates. All tests such as, Gram stain, spore and acid-fast staining; cell shape and size; motility; colony shape and pigmentation; growth in liquid medium; oxygen requirement; fermentation of glucose, mannitol, lactose and mix acid (methyl red and Voges-Proskauer test); hydrolysis of gelatine, starch, casein, fat, indole and urea; production of oxidase, catalase, hydrogen sulfide (H_2S) and phenylalanine deaminase; reduction of nitrate and litmis milk; utilization of citrate were determined based on the Laboratory Manual in Microbiological Applications (Benson, 1994). The Bergey's Manual Determinative Bacteriology (1994) and the Bergey's Manual of Systematic Bacteriology (1994, 2001) were used to determine the taxonomic position of H_2 oxidizing isolates. The observation method of cultural and morphological characteristics of isolates was the same as isolation part described 2.3.3.2.

3.3.2 16S rRNA gene sequence analysis

Genomic DNA was extracted from H_2 oxidizing isolates following the protocol described by Lechner and Conrad (1997). For H_2 oxidizing bacterial strains JM01, JM111 and JM63, DNA amplification were carried using the primers Com1 (forward) (5'-CAGCAGCCGCGGTAATAC-3', positions 519 to 536) and Com2 (5'-CCGTCAATTCCTTTGAGTTT-3', positions 907 to 926). For H_2 oxidizing bacterial strains JM01, JM111, JM120, JM162, JM162a and JM169, DNA amplification was carried out by using the following universal primers: bacterial 16S forward primer: BSF8/20 5'-AGAGTTTGATCCTGGCTCAG – 3'; bacterial 16S reverse primer:

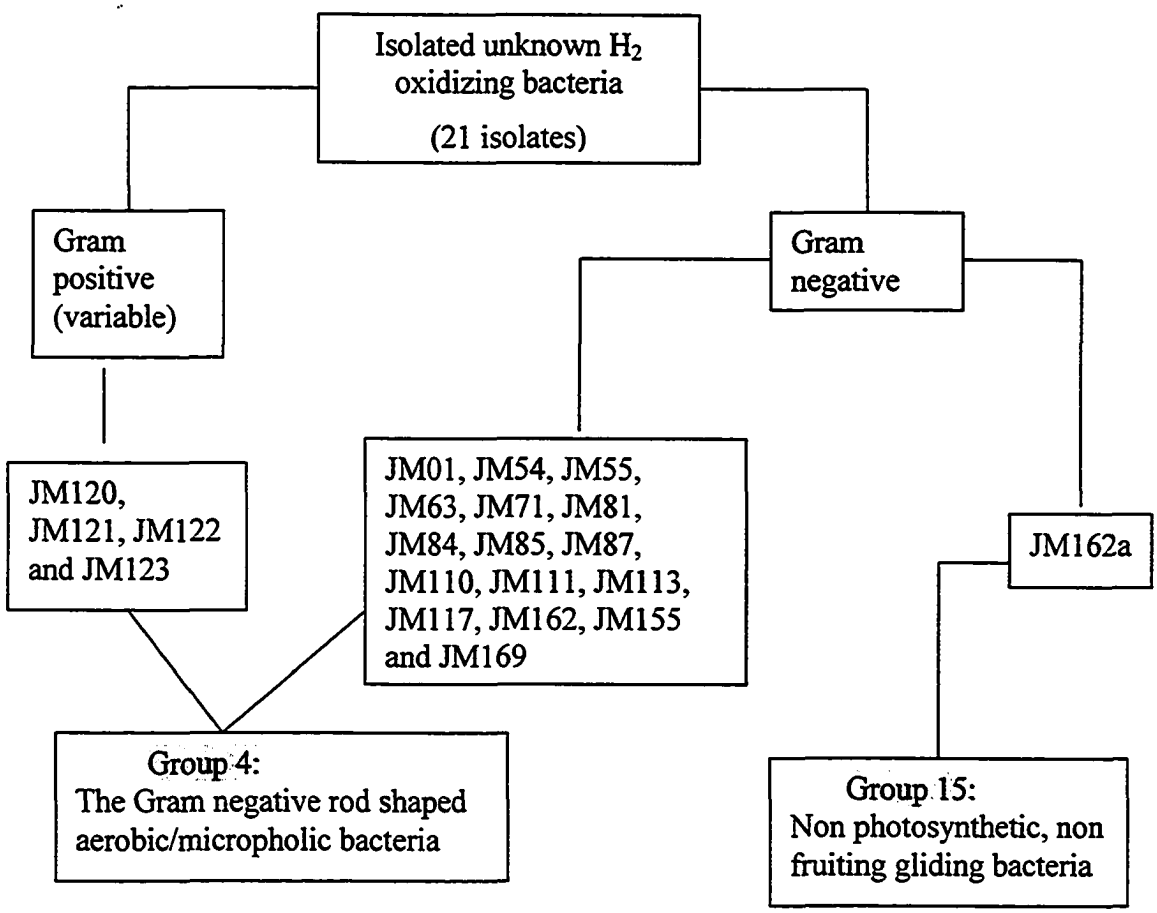
BSR1541/20 5′ - AAGGAGGTGATCCAGCCGCA – 3′. The PCR product was excised from an agarose gel and purified using Gel Extraction Kit (Qiagen). The purified fragment was cloned into pDrive cloning-vector (Qiagen). Plasmid DNA was sequenced using M13 forward (5'-CAGGAAACAGCTATGAC-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. The obtained sequences were compared with similar sequences in the Genbank database using the Blast program (NCBI).

3.4 RESULTS

3.4.1 The identification of H_2 oxidizing bacterial strains using conventional microbiological identification tests

Altogether twenty H_2 oxidizing isolates from H_2 treated soil and soil adjacent to Hup^- soybean nodules were isolated. Seventeen strains were found to be Gram negative rods (Table 5). The rest remaining four of them were Gram-variable rods (Table 5). These 20 isolates were pooled into 2 groups based on Bergey's Manual Determinative Bacteriology (1994) and Bergey's Manual of Systematic Bacteriology (2001): The Group 4 "The Gram negative rod shaped aerobic/micropholic bacteria" and Group 15 "non photosynthetic, non fruiting gliding bacteria" (see chart 1).

Chart 1: Separation outline for isolated H_2 oxidizing bacteria



Fifteen Gram negative isolates belonging to Group 4 (see chart 1) are identical in morphological, cultural and biochemical characteristics (Table 5, 6 and 7) (Figure 4). The presumptive genus of these fifteen Gram negative isolates is *Variovorax* (formerly, *Alcaligenes*).

However, one isolate (JM155) belonging to Group 4 (see chart 1) is different in cultural and some biochemical characteristics from those fifteen strains (presumptive genus *Variovorax*) (Table 5, 6 and 7) (Figure 5). The presumptive genus of JM155 isolate was *Pseudomonas*.

The one Gram negative isolate (JM162a) was pooled into Group 15 "non photosynthetic, non fruiting gliding bacteria" on the basis of its unique colony and cell morphology which is different from other isolate (Table 5, 6 and 7) (Figure 6). The presumptive genus of this strain is *Flavobacterium.*

Four Gram variable strains (JM120, JM121, JM122 and JM123) belonging to Group 4 bacteria (chart 1) are identical in morphological, cultural and biochemical properties (Table 5, 6 and 7) (Figure 7). The presumptive genus of them is *Burkholderia* (formerly *Pseudomonous*).

3.4.2 The identification of H_2 oxidizing bacterial strains using 16S rRNA gene sequence analysis

Based on the conventional tests, twenty isolates of H_2 oxidizing bacteria were identified as three genera, *Variovorax*, *Flavobacterium* and *Burkholderia* (Table 8). This identification result of the conventional tests was supported by the 16S rRNA gene sequence. Five strains (JM01, JM111, JM63, JM162 and JM169) from fifteen isolates of *Variovorax*, JM162a of *Flavobacterium* and JM120 of *Burkholderia* were for 16S rDNA sequence analysis.

The DNA amplification of tested H_2 oxidizing bacterial strains (JM01, JM111 and JM63) using universal primers Com1 and Com2 had distinct clear band at about 400bp (Figure 8 A). The DNA amplification of tested H_2 oxidizing bacterial strains (JM01, JM111, JM120, JM162, JM162a and JM169) using BSF8/20 and BSR1541/20 had distinct clear bands at approximately 1500 bp (Figure 8B). Clear bands of all tested H_2 oxidizing bacteria were cut from the gel, purified and cloned. After cloning, plasmid DNA was sent for sequencing. All samples were successfully sequenced. Obtained

sequences (Appendix 3) of tested H_2 oxidizing bacterial strains were compared with similar sequences in the Genebank database using the Blast program (NCBI).

A blast search of GeneBank showed that the three strains (JM01, JM63 and JM111) had the 99 % similarity to *Variovorax paradoxus* IsoI; JM162 had 99% similarity to uncultured *Variovorax* sp. clone Cl-56-TB2-I (Table 8). Also, the results of physiological tests of them are good fit to the other strain of *V. paradoxus* JMP116 (Fisher, 1978). Isolates of *V. paradoxus* was classified as Bacteria, phylum Proteobacteria, class β-proteobacteria, family Comamonadaceae and genus *Variovorax* (William *et al.*, 1991) (Table 9).

The 16 S rRNA sequence analysis showed that JM162a had 97% similarity to *Flavobacterium johnsonae* IFO 15970 (# 1352) (Table 8), and the conventional characteristics of it match well with the *F. johnsonae* species described in Bergey's Manual of Determinative Bacteriology (1994) and Bergey's Manual of Systematic Bacteriology (1989). The isolate of *F. johnsonae* species was classified as Bacteria, Cytophage/Flexibactor/Bacteroides (CFB) group, Bacteroidetes, Flavobacteria, Flavobacteriales, Flavobacteriaceae, *Flavobacterium* (Table 9).

Based on the 16S rRNA gene sequence, strain JM120 had 98% similarity to *B. sordidicola* strain SNU 020123 (#2678) (Table 8). Isolates of *Burkholderia* sordidicola was classified as Bacteria, phylum Proteobacteria, class β- proteobacteria, order Burkholderiales, family Burkholderiaceae, genus *Burkholderia* (Table 9).

Table 5: The morphological characteristics of H_2 oxidizing isolates

Isolates	Morphological characteristics					
	Gram reaction	Cell shape	Cell size (μm)	Motility	Spore	Capsule
JM01	-	Rod	0.5-0.6x 0.9-2.27	+	-	-
JM54	-	Rod	0.45x1.2	+	-	-
JM55	-	Rod	0.55x1.3	+	-	-
JM63	-	Rod	1.1-1.48	+	-	-
JM71	-	Rod	0.6x1.8	+	-	-
JM81	-	Rod	0.45x2.6	+	-	-
JM84	-	Rod	0.5x1.3	+	-	-
JM85	-	Rod	0.5x1.3	+	-	-
JM87	-	Rod	0.6x1.3	+	-	-
JM110	-	Rod	0.5x2.2	+	-	-
JM111	-	Rod	0.5x1.8	+	-	-
JM113	-	Rod	0.48x2.5	+	-	-
JM169	-	Rod	0.47-0.59x 0.78-1.39	+	-	-
JM162	-	Rod	0.6x1.1	+	-	-
JM162a	-	Flexible rods	2.27-5.9 (long) 0.3-0.45 (short)	+	-	-
JM155	-	Rod	0.47-0.76 x1.0-1.46	+	-	-
JM120	v	Rod	0.38-0.40x 1.15-3.46	+	-	+
JM121	v	Rod	0.39-0.40x 1.7-3.02	-	-	+
JM122	v	Rod	0.42-0.53x 2.07-3.43	+	-	+
JM123	v	Rod	0.4-0.57x 1.6-2.18	-	-	+

+: positive; -: negative; v: variable.

Table 6: Cultural characteristics of isolated H_2 oxidizing bacterial strains

Isolates	Cultural characteristics					
	MSA	NA	BA	NS	NB	FTM
JM01	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM54	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM55	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM63	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Arboracent	Cloudy	Aerobic/ microphilic
JM71	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM81	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM84	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM85	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM87	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM110	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM111	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM113	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM169	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic

JM162	Colony round transparent	Yellow Circular glistening	Yellow circular glistening	Filiform	Cloudy	Aerobic/ microphilic
JM162a	Colony diffused and brownish	Round orange	Yellow circular glistening	Arborecent	Turbid	Aerobic
JM155	Colony circular white dot	White pinhead colony No figment	Yellow circular glistening	Effuse	Granular	Aerobic
JM120	Colony white irregular	Colony round white dry	Colony dry no figment	Erchinulate	Granular	Aerobic microphilic
JM121	Colony white irregular	Colony round white dry	Colony dry no figment	Erchinulate	Granular	Aerobic/ microphilic
JM122	Colony white irregular	Colony round white dry	Colony dry no figment	Erchinulate	Granular	Aerobic microphilic
JM123	Colony irregular	Colony round white dry	Colony dry no figment	Erchinulate	Granular	Aerobic/ microphilic

MSA, mineral salt agar medium; NA, nutrient agar; BA, blood agar; NS, nutrient slant; NB, nutrient broth; FTM, fluid thioglycollate medium.

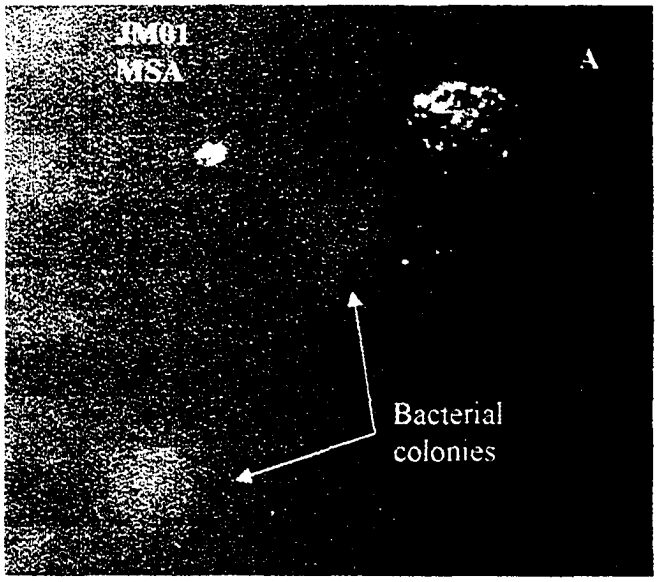
Table 7: Biochemical characteristic of isolated hydrogen oxidizing bacterial strains

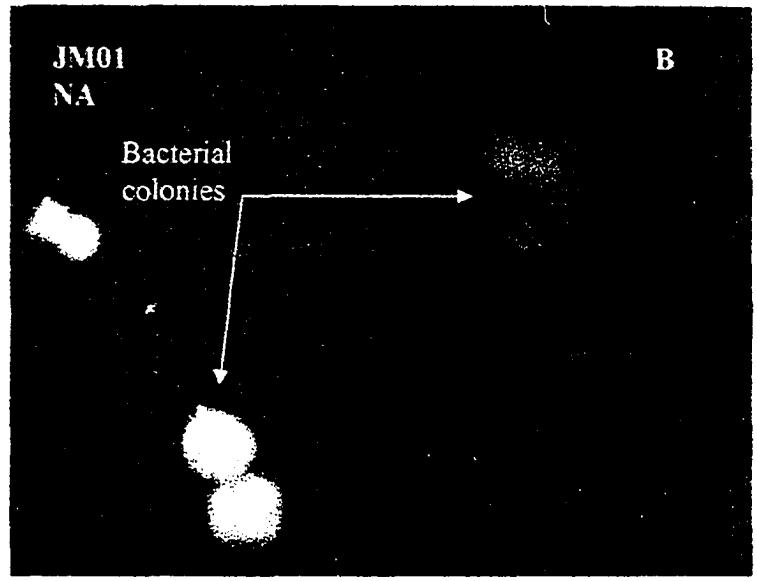
	Tests														
	Fermentation			Hydrolysis					Production					Reduction	
Isolates	Glucose	Lactose	Mannitol	Starch	Casein	Fat	Geletine	Urea	Catalase	Oxidase	H_2S	PPA	IMVi	Nitrate	Litmis
JM01	[illegible]														
JM54	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM55	[illegible]														
JM63	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM70	[illegible]														
JM81	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM84	[illegible]														
JM85	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM87	[illegible]														
JM110	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM111	[illegible]														
JM113	–	–	–	–	–	–	–	–	+	+	–	–	–	+	+
JM162	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
JM169	–	–	–	–	+	–	–	–	+	+	–	–	–	+	+
JM156	[illegible]														
JM162a	–	–	–	+	+	–	+	–	+	+	–	–	–	+	+
JM120	[illegible]														
JM121	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+
JM122	[illegible]														
JM123	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+

Note: +: positive reaction; -: negative reaction; * weak reaction; IMVi: indole, methylne blue and voges-proskauer tests. NT: not tested. PPA: phenylalaine deaminase.

Figure 4: Colonial and morphological characteristics of H_2 oxidizing isolateJM63 of *Variovorax*.

A: Colonies on mineral salt agar (MSA); B: Colonies on Nutrient agar (NA): C: gram staining for two days old culture. Cells are Gram-negative rods.


JM01
MSA
A
Bacterial
colonies


JM01
NA
B
Bacterial
colonies

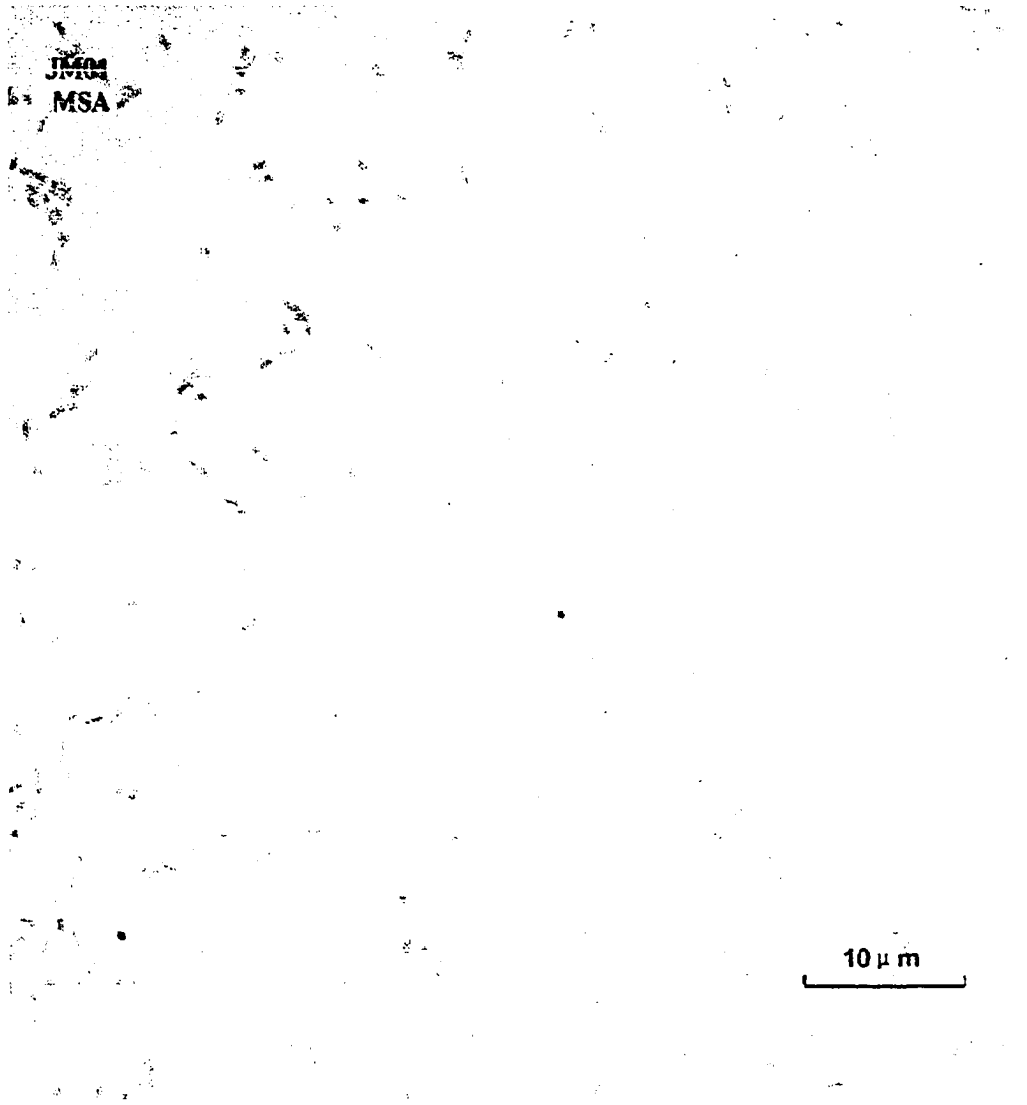
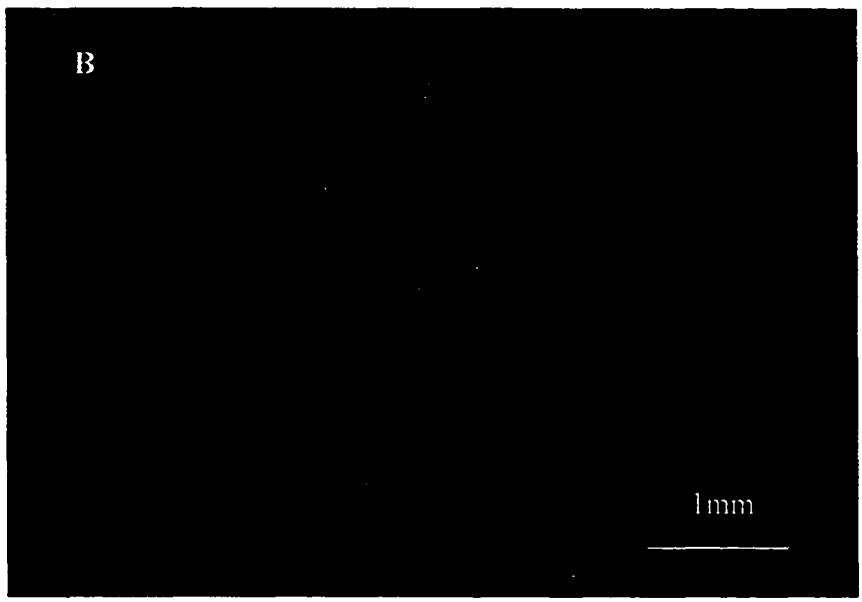

JM01
MSA
10 µ m

Figure 5: Characteristics of H_2 oxidizing isolate JM155 of *Pseudomonas*.
A: Gram stain of 2 days old culture. Cells are Gram-negative rods. B: colonies on mineral salt agar (MSA). C: colonies on nutrient agar (NA).


A


B
1mm

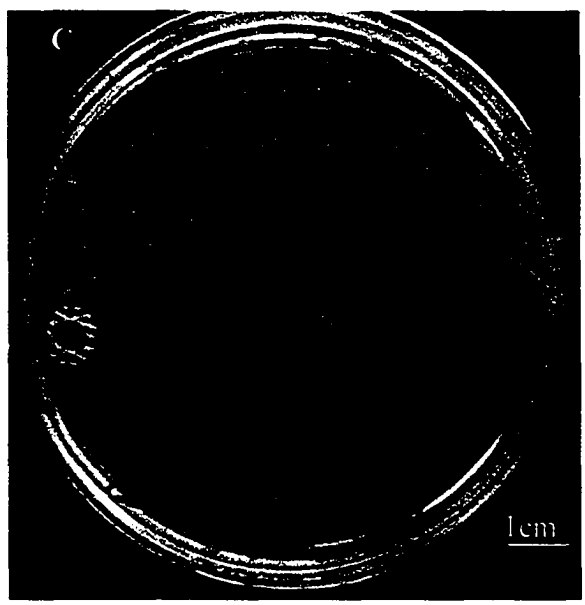
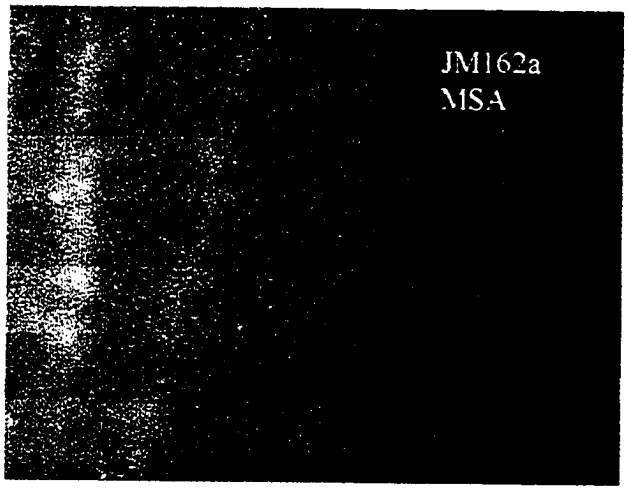
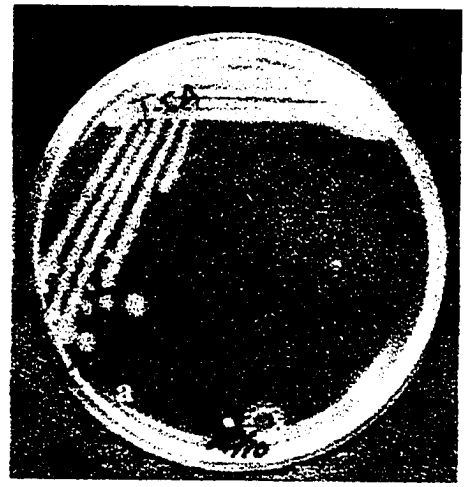

C
1cm

Figure 6: Characteristics of H_2 oxidizing isolate JM162a of *Flavobacterium*.
A. mineral salt agar (MSA). Colonies are diffused. B. streak plate on tryptic soy agar (TSA). C. Gram staining of two days old culture. a, long rods; b, short rods.


JM162a
MSA


TSA
a

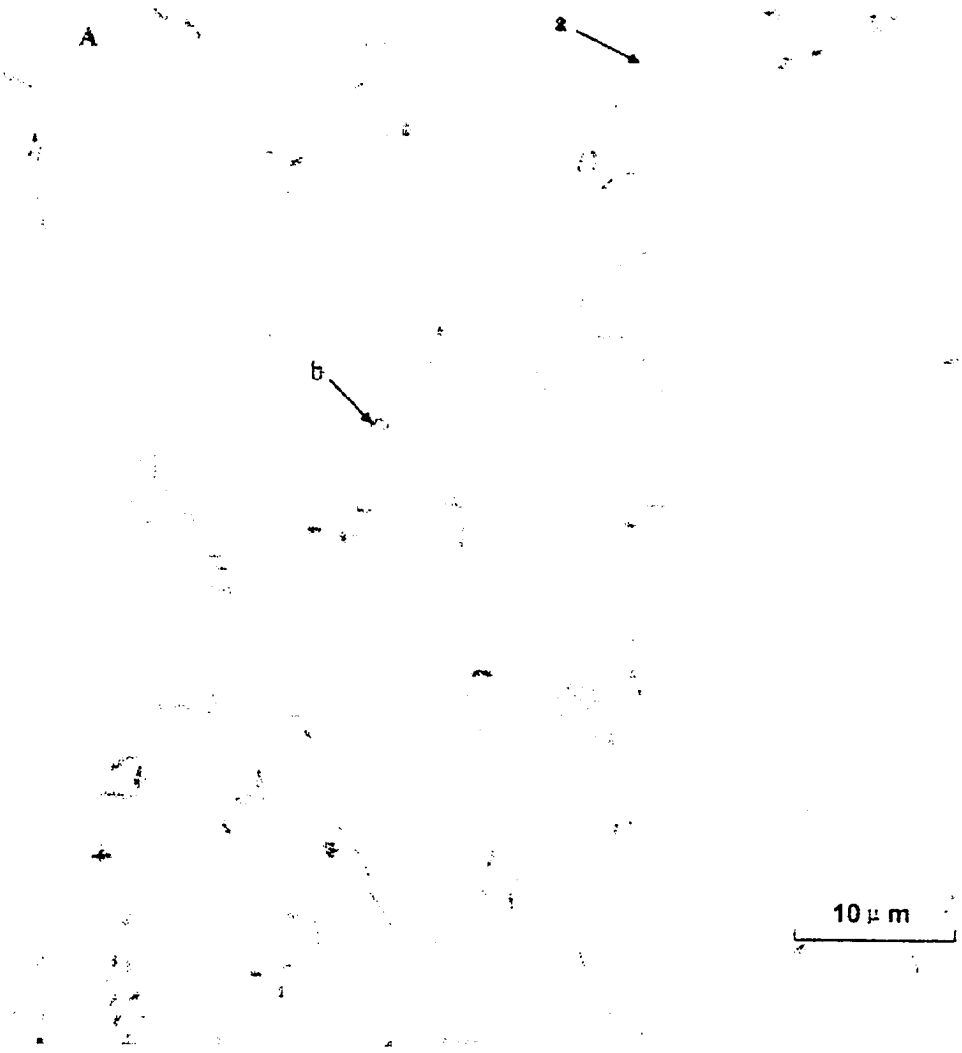
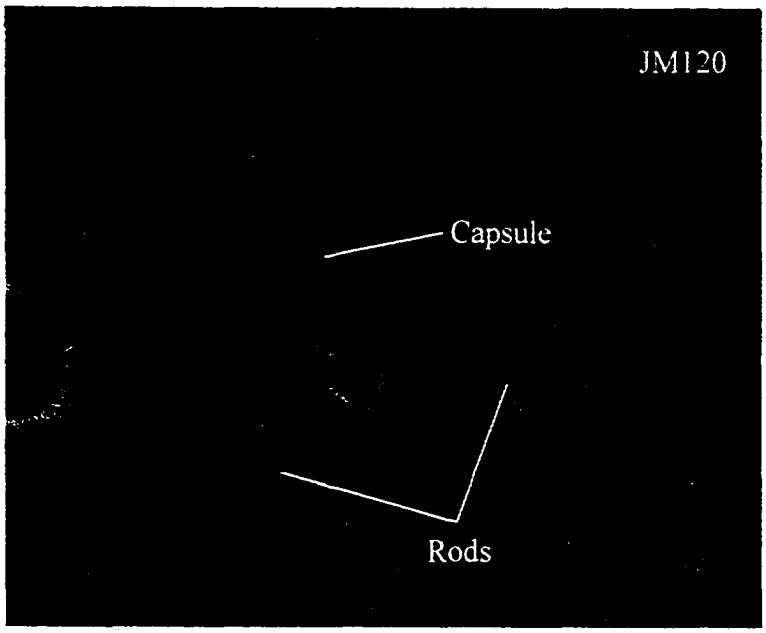

A
a
b
10 μ m

Figure 7: Cultural and morphological characteristics of H_2 oxidizing isolate JM120 of *Burkholderia*.

A: Gram staining of two days old culture. B: Colonies on mineral salt agar (MSA).


JM120
Capsule
Rods

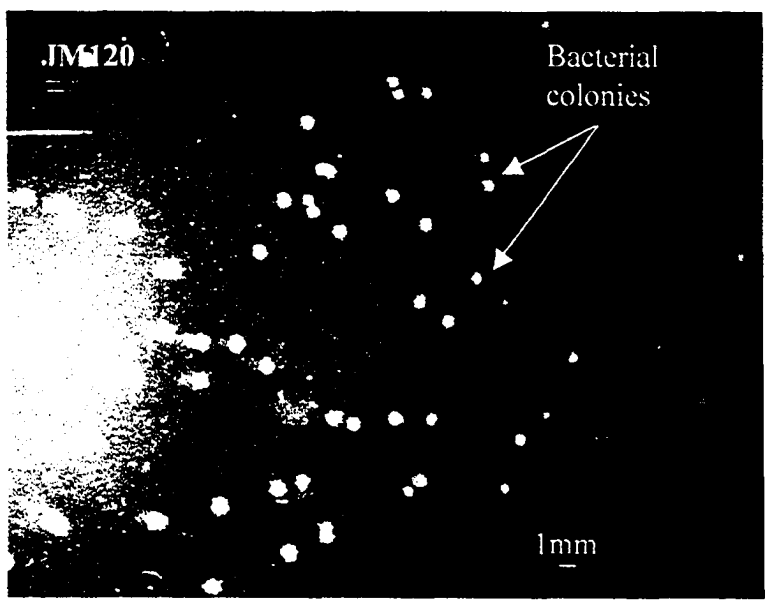
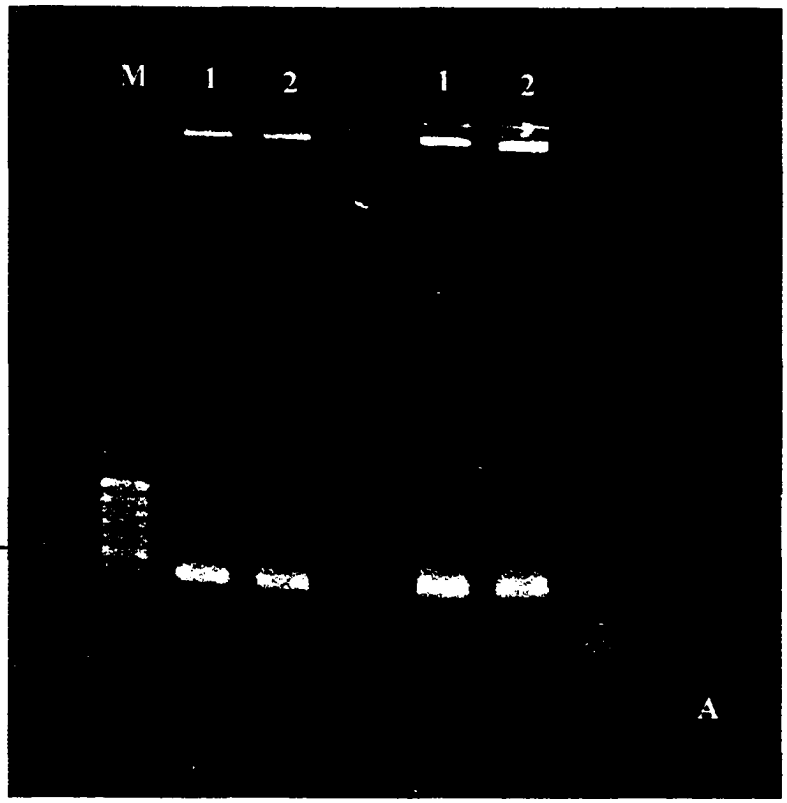

JM120
Bacterial
colonies
1mm

Figure 8: PCR amplification of DNA from H_2 oxidizing isolates JM01 and JM111
A: using universal primers Com1 and Com2. B: universal primers BSF8/20 and BSR1541/20. M: DNA marker. 1: JM01. 2: JM111.


M
1
2
1
2
500bp
A

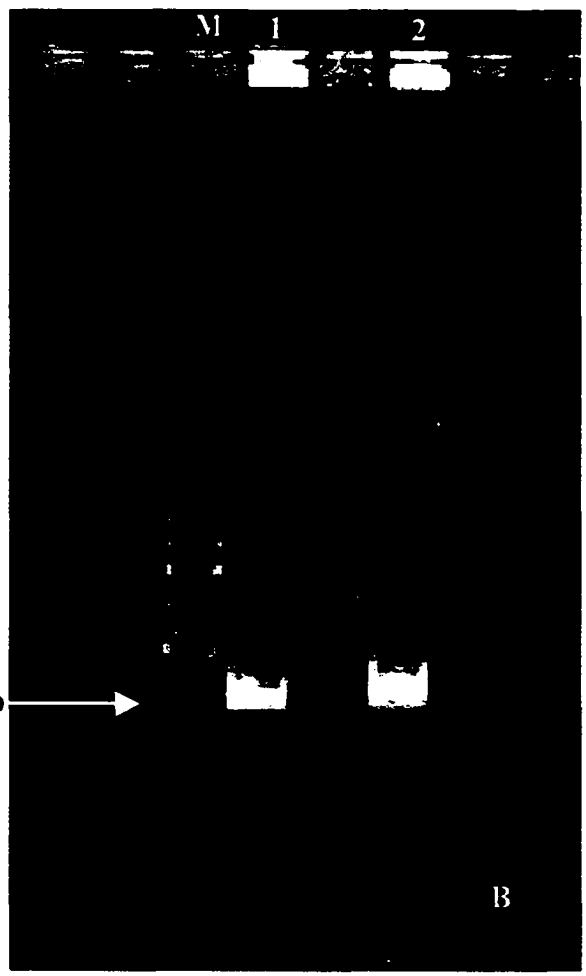
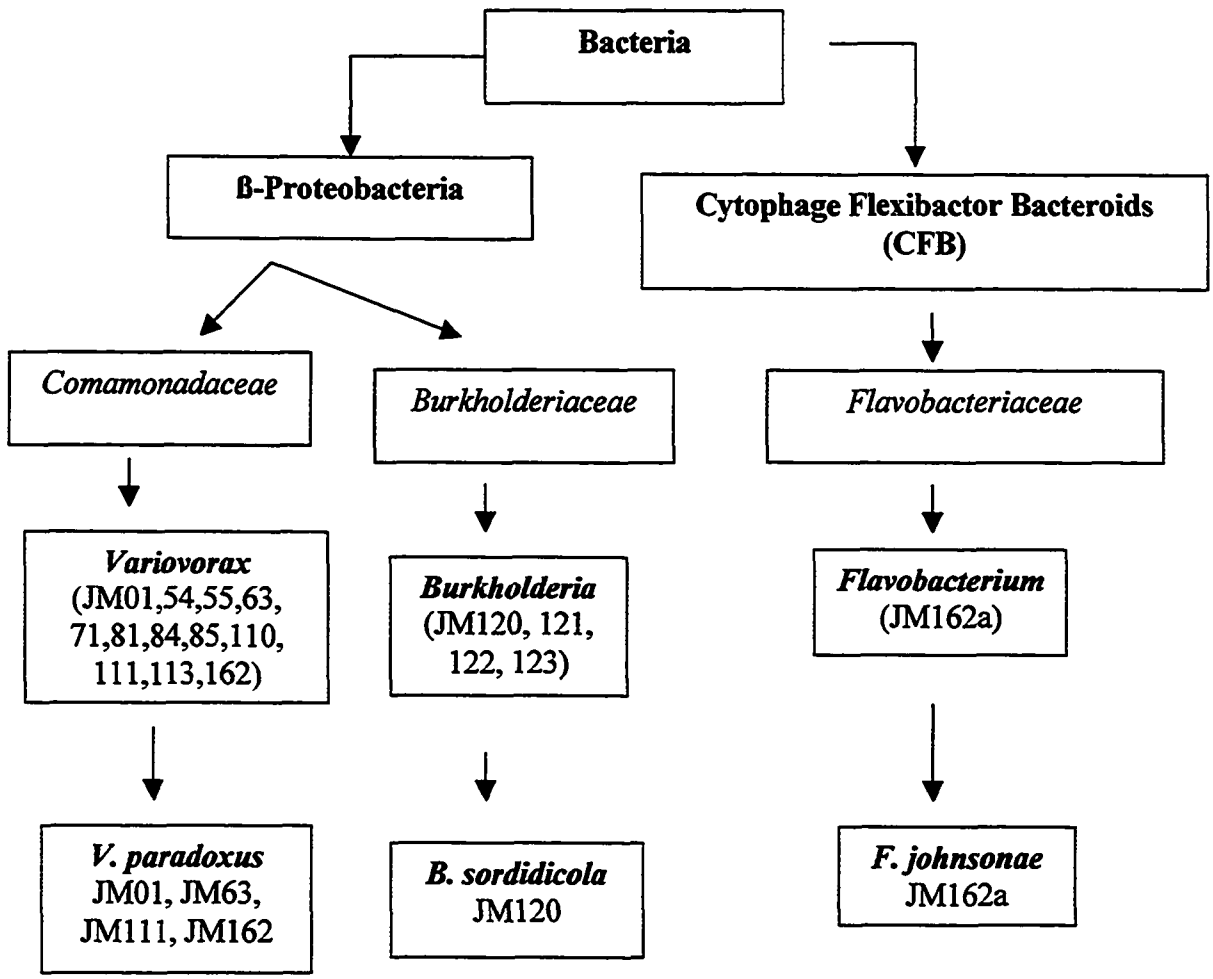

M
1
2
1500bp
B

Table 8: Taxonomy of isolated H_2 oxidizing bacterial strains

Soil sources	Isolates	Identification results	
		Conventional identification test	16S rRNA sequence analysis[a]
H_2 treated soil	JM01	*Variovorax*	99% to *V. paradoxus* Isol
	JM54	*Variovorax*	NT
	JM55	*Variovorax*	NT
	JM63	*Variovorax*	99% to *V. paradoxus* Isol
	JM71	*Variovorax*	NT
	JM81	*Variovorax*	NT
	JM84	*Variovorax*	NT
	JM85	*Variovorax*	NT
	JM87	*Variovorax*	NT
	JM110	*Variovorax*	NT
	JM111	*Variovorax*	99 % to *V. paradoxus* Isol
	JM113	*Variovorax*	NT
Soil adjacent to Hup$^-$ soybean nodules in field	JM169	*Variovorax*	99% to *V. paradoxus* Isol
	JM155	*Pseudomonos*	NT
	JM162	*Variovorax*	99% to Uncultured *Variovorax* sp. clone CI-56-TB2-II
	JM162a	*Flavobacterium*	96% to *F. johnsonae* IFO 15970
Soil adjacent to Hup$^-$ soybean nodules in green house	JM120	*Burkholderia*	98% to *B. sordidicola* strain SNU 020123
	JM121	*Burkholderia*	NT
	JM122	*Burkholderia*	NT
	JM123	*Burkholderia*	NT

[a] Similarities are based on the comparison with the GenBank database using the BLAST proGram. NT: not tested.

Table 9: Classification outlines of isolated H_2 oxidizing bacterial strains



3.5 DISCUSSIONS

The taxonomic study showed that twenty one strains of H_2 oxidizing bacteria isolated from H_2 treated soil and soil adjacent to Hup$^-$ legume nodules belong to three genus, *Variovorax*, *Flavobacterium* and *Burkholderia*. The majority of our isolates are Gram negative bacteria. They are pooled into Beta proteobacteria and Cytophage/Flexibactor/Bacteroides (CFB) groups. The different taxonomy of our isolates of H_2 oxidizing bacteria found in H_2 treated soil and soil adjacent to Hup$^-$ soybean nodules confirmed again that the H_2 oxidizing bacteria in legume soil can be many species from different phylogenetically diverse taxa, such as, H_2 bacteria (Knallgas), nitrogen fixation bacteria and photosynthetic microorganisms (Lechner and Conrad, 1997, Evans *et al.*, 1987).

We found that many isolates of *V. paradoxus* exist both in H_2 treated soil and soil adjacent to the Hup$^-$ soybean nodules in field. It was reported that *V. paradoxus* is H_2 bacterium and its cells contain the membrane-bound hydrogenase (Aragno and Schlegel, 1981). This indicated that H_2 oxidizing bacterial strains of *V. paradoxus* species isolated from H_2 treated soil caused the high K_m value for H_2 in H_2 treated soil. And also, the high K_m (H_2) value of our isolated H_2 oxidizing bacteria is similar to the strain of *Variovorax paradoxus* SA 29 (K_m > 1 μM H_2) (> 22.4ppm) (formerly *Alcaligenes paradoxus*) isolated from eutrophic lake (Conrad *et al.*, 1983) and other H_2 bacterial strains GK1of *Pesudomonas palleronii* species(K_m for H_2 >1310ppm) (Schuler and Conrad, 1991). The results obtained support the concepts that the high K_m for H_2 are caused by H_2 oxidizing bacterial activity (Schuler and Conrad, 1990; Häring and Conrad, 1994; Dong and Layzell, 2001).

V. paradoxus exist in many habitats such as, soil, mud and water (Davis *et al.*, 1969; 1970). The metabolic property of *V. paradoxus* as H_2 bacteria (Knallgas) that grow chemolithoautotrophically using H_2 as an electron donor is well known (Aragno and Schlegel 1981; Conrad *et al.*, 1983). However the bacterial strains of *V. paradorax* species as H_2 oxidizing bacteria in past studies were isolated from eutrophic lake (Conrad *et al.*, 1983). In current studies, the *V. paradorax* isolated from H_2 treated soil and soil adjacent to the Hup^- soybean nodules is firstly reported as H_2 released by legume nodules.

Burkholderia sordidicola was firstly isolated from the white-rot fungus *Phanerochaete sordida* and identified by Lim *et al.* (2003). This is the first report of them to have ability to grow chemolithoautotrophically on H_2, CO_2 and O_2. This new finding in this study expands the list of metabolic traits of *Burkholderia* sp.

Flavobacterium johnsonae (formerly, *Cytophaga johnsonae*) are common soil bacteria. Aragno and Schlegel (1981) reported that other species of *Flavobacterium* (*F. autothermophilum*) had H_2 oxidizing ability and named it as a H_2 oxidizing bacteria. The finding of H_2 oxidizing property of *F. johnsoniae* is a first report, and which added new information of the metabolic characteristics of this species.

Previous research conducted in our lab showed that the yield of plants growing in H_2 treated soil and soil after the growth of Hup^- soybean nodules were significantly increased (Dong *et al.*, 2003; Dean *et al.*, 2005). It was hypothesized that the H_2 gas in soil may influence the populations of certain H_2 oxidizing bacteria, which in turn may improve the plant growth in some way acting as PGPR. Our results proved that H_2 gas in soil increased the populations of H_2 oxidizing bacteria of *V. paradoxus*, *B. sordidicola*

and *F. johnsonae*. Very likely these bacterial strains are responsible for plant growth promotion and soil fertility.

4. THE PLANT GROWTH PROMOTING EFFECT OF ISOLATED H_2 OXIDIZING BACTERIAL STRAINS

4.1 INTRODUCTION

4.1.1 PGPR and their effects on plant growth

The mechanisms behind the effects of PGPR on plants have been classified into two groups. One is the stimulation of plant growth indirectly by preventing phytopathogenic organisms in soils from inhibiting plant growth (Glick, 1995). The other is to increase plant growth directly by providing plants with essential compounds, phytohormones, and/or enyzmes and facilitating the nutrient uptake from the environment (Glick, 1995, 1998). For example, PGPR including the nitrogen fixing, phosphate-solubilizing and siderophore-producing bacteria in the rhizosephere have positive effects on plant growth by providing nitrogen, phosphorous and iron to host plants through fixing atmospheric N_2, changing the insoluble form of phosphorous and iron in soil to soluble forms (Vessey, 2003). Also, PGPR may facilitate plant growth by altering the hormonal levels within the affected plants (Glick, 1995). It has been identified that some PGPR produce phytohormones, such as, indole –3-acetic acid (IAA), cytokinins, gibberellins (gibberllic acid; GA) to improve root growth and morphology by increasing cell division, cell enlargements and root length (Vessey, 2003). It is known that ethylene is an important phytohormone which has key regulatory functions not only in almost every stage of plant life but also in the development of the legume- rhizobia association (Ma *et al.*, 2002). However the high levels of ethylene in roots have negative effect on plant growth and nodule formation (Ma *et al.*, 2002). It was discovered that some PGPR contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase

and rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-trans-but-3-enoic acid] which can reduce the ethylene synthesis in plant roots to stimulate plant growth and root nodulation (Glick, 1995; Glick *et al.*, 1998; Ma *et al.*, 2002).

4.1.2 The assessment of plant growth promoting activity of bacteria

Studies on PGPR have been growing in the last 30 years (Vessey, 2003). A number of PGPR have been already used as inoculants in agriculture to increase the yield of grain and plant biomass (Sindhu *et al.*, 1997). The effect of PGPR inoculants on plant yield applied to crops (plants) has been studied under field, green house and laboratory conditions (Kloepper *et al.*, 1989; Glick, 1995; Lazarovits and Nowak, 1997; Trân Van *et al.*, 2000; Belimov *et al.*, 2001).

In both field and greenhouse, the biomass or yield has been used to assess the PGPR effect (Lazarovits and Nowak, 1997; Trân Van *et al.*, 2000). The disadvantages of this method are that it is time-consuming and there are many variables. In laboratory condition, the root elongation assay has been used to determine the PGPR activity of bacteria. In this assay, the experimental condition is carefully controlled. Therefore, it can overcome any confounding effects that may arise as a result of the soil composition or the presence of other soil microorganisms (Glick, 1995). Also, the plant growth result can be obtained within a very short time compared to that of field or green house conditions.

The yield of legume and non-legume crops were increased when they were planted in H_2 treated soil or soil after growth of Hup^- legume. It has been suggested that the H_2 increase the population of soil H_2 oxidizing bacteria, which in turn improve the plant growth and in some way acts as a PGPR (Dong *et al.*, 2003). However, it is not

known whether our H_2 oxidizing isolates of *Variovoraxus*, *Flavobacterium* and *Burkholderia* are PGRR, although they are believed that they have some link with plant growth promotion. The answer to these questions might help to explain the major benefit of legume in crop rotation and the yield of subsequent crops. Even more it could answer the evolutionary question why the Hup^- symbioses have more advantages than Hup^+ symbiosis (Dong and Layzell, 2002).

4. 2 OBJECTIVES

To investigate the plant growth promoting effect of isolated H_2 oxidizing bacterial strains using the root elongation assay.

4.3 MATERIALS AND METHODS

4.3.1 Root elongation effect of isolated H_2 oxidizing bacteria

The effect of isolated H_2 oxidizing bacteria on root elongation of spring wheat seedlings were tested according to the methods of Belimov *et al.* (2001) and Trân Van *et al.* (2000).

4.3.1.1 Bacterial culture preparation

Sterile NB broth (10mL) (Difco) was inoculated with isolates of H_2 oxidizing bacteria and incubated overnight at 32 °C on a rotating shaker at 200rpm. Bacterial cells were harvested by centrifugation when the $O.D_{600}$ of culture reads 0.6 (Thermo IEC. USA) at 7000g x 20minutes. The bacterial pellets were rinsed in sterile 100μM $MgSO_4$ and centrifuged again at 7000g x 20minutes. The bacterial cells then were re-suspended up to $5x10^7$cells/mL in sterile assay solution (μM): Ca $(NO_3)_2$, 800; KH_2PO_4, 400; $MgSO_4$, 400; $CaCl_2$, 100.

4.3.1.2 Seed sterilization and pre-germination

Before inoculation, spring wheat seeds (cv. AC Barrie) were surface sterilized with a mixture of equal volume of 70% ethanol and 30% hydrogen peroxide (H_2O_2) (1:1) for 20 minutes and rinsed with sterile distilled water (dH_2O). The sterilized seeds were pre-germinated for 2 days at 28°C on damp filter paper in petri dishes in the dark.

4.3.1.3 Seed inoculation

Seeds at the same germination stage were selected and transferred to petri dishes with filter paper. For testing isolates JM01, JM54, JM55, JM81, JM84, JM87, JM110, JM113, JM120, JM121, JM122, JM123, JM162a, JM155 and JM169, three dishes were prepared with twenty seeds for per dish. For JM63, three dishes were prepared with 10 seeds for per dish. Five mL of assay solution containing bacterial suspension of $5x10^7$cells/mL was added to each dish. The dishes were covered and sealed with para-film and incubated for another two days at 28°C in the dark. *E. coli* B and non-inoculated assay solution were used as controls.

After two-days of incubation, the primary root length, the length and number of lateral roots and the amount of root hairs of seedlings in each treatment were measured and recorded. Pictures of the root were taken with a digital camera.

4.3.2 Statistical analysis

All data were analyzed with StatView program 5.0 Version. One way ANOVA was used to compare variance between treatments. Fisher's least significant difference (LSD) was performed.

4.4 RESULTS

4.4.1 The effect of H_2 oxidizing bacterial strains on root elongation

Before inoculation, the 2-days-old pre-germinated seeds were examined. Seeds in each plate were not contaminated and most of them were germinated to 1mm long. In tested plates, the roots of inoculated seeds were grown 7~27mm long from the original 1mm. Most seeds in inoculated plates had 3-4 lateral roots.

All nineteen strains increased the primary root length of spring wheat seedlings by 19~254% compared to the controls. Seventeen of them increased the primary root elongation significantly ($n = 20$, $p < 0.001$), ranging from 57% to 254 % (Table 10; Figure 9 A, B and C). Besides the primary root length, the number and length of lateral roots and the amount of root hair were also increased when the seedlings were inoculated with isolates of H_2 oxidizing bacteria (data not shown).

All isolates of *Variovorax* had significant positive root elongation effect (Table 10). Especially, strain (JM63) of *V. paradoxus* had the highest root elongation effect (Table 10, Figure 9 C and 10). Except for one isolate (JM121), the other three isolates (JM120, JM122 and JM123) of *Burkholderia* sp. significantly increased the primary root length (Figure 9A) (Table 10). The isolate JM155 had no significant effect on primary root elongation (Figure 9 B), but root hair was increased (data not shown). The primary root length was increased by JM162a of *Flavobacterium johnsonae* by 95-100% (Figure 9B).

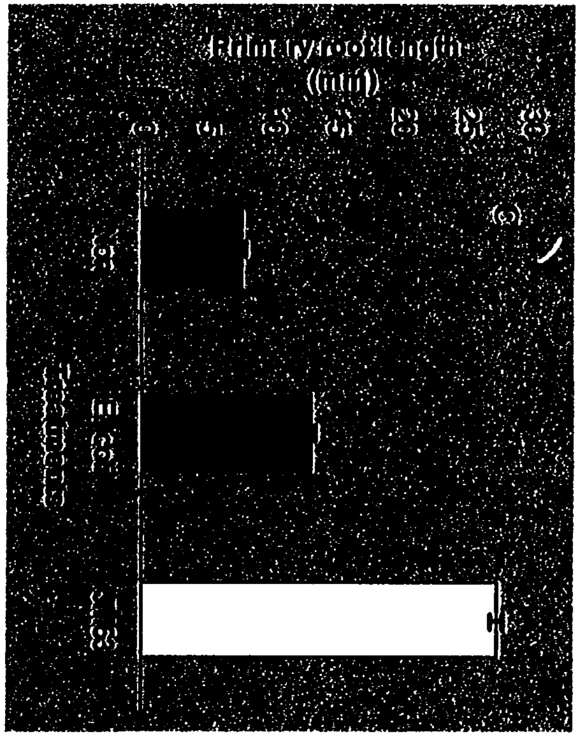
Table 10: The effect of H_2 oxidizing bacterial strains on root elongation of spring wheat seedlings. Mean values are given ±standard error (SE).

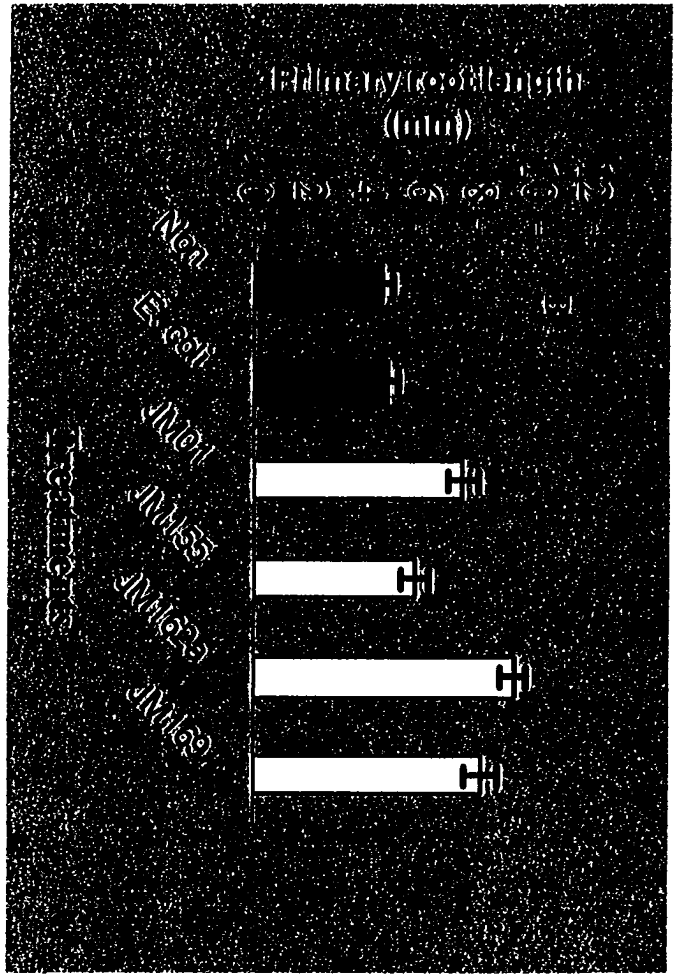
Isolates	Taxon	Root elongation (mm)			Root elongation effect (%)
		Control 1	Control 2	Inoculated	
JM01	*Variovorax paradoxus*	4.5±0.40	4.7±0.44	7.4±0.53	57.4*, 64.4*
JM111	*Variovorax paradoxus*	8.2±0.52	8.5±0.63	17.2±0.72	103.0*, 111.0*
JM63	*Variovorax paradoxus*	7.7±0.28	13.1±0.23	27.3±0.44	108.0*, 254.0*
JM169	*Variovorax paradoxus*	4.5±0.40	4.7±0.44	8.05±0.62	71.2*, 78.8*
JM54	*Variovorax*	8.2±0.52	8.5±0.63	14.9±0.81	82.8*, 76.3*
JM71	*Variovorax*	8.2±0.52	8.5±0.63	13.0±0.72	59.5*, 53.8*
JM81	*Variovorax*	8.2±0.52	8.5±0.63	14.3±0.98	79.0*, 74.8*
JM84	*Variovorax*	8.2±0.52	8.5±0.63	15.5±0.56	82.8*, 89.5*
JM85	*Variovorax*	8.2±0.52	8.5±0.63	15.4±0.82	82.2*, 88.9*
JM87	*Variovorax*	8.2±0.52	8.5±0.63	15.4±0.68	82.2*, 89.5*
JM110	*Variovorax*	8.2±0.52	8.5±0.63	15.7±0.73	85.6*, 92.6*
JM55	*Variovorax*	8.2±0.52	8.5±0.63	12.0±0.71	91.7*, 98.7*
JM113	*Variovorax*	8.2±0.52	8.5±0.63	15.2±0.96	79.8*, 86.5*
JM120	*Burkholderia sordidicola*	8.2±0.52	8.5±0.63	16.2±0.91	91.7*, 98.8*
JM121	*Burkholderia* sp.	8.2±0.52	8.5±0.63	10.1±0.63	19.5, 23.9
JM122	*Burkholderia* sp.	8.2±0.52	8.5±0.63	13.8±0.64	62.7*, 68.7*
JM123	*Burkholderia* sp.	8.2±0.52	8.5±0.63	13.9±0.88	64.5*, 70.5*
JM155	*Pseudomonas*	4.5±0.40	4.7±0.44	5.7±0.53	21.2, 26.6
JM162a	*Falvobacterium johnsoniae*	4.5±0.40	4.7±0.44	9.2±0.44	95.7*, 104.0*

Primary root elongation effect of spring wheat seedlings (%) = (inoculated-controls) x 100/controls. The first number is the % of increase compared with non-inoculated controls and the second number is the % of increase compared with *E. coli* B inoculated controls. * Statistically significant difference between inoculated samples and the controls, based on the Fisher's LSD test ($P \leq 0.05$).

Figure 9: Average primary root length with standard error bars of spring wheat seedlings inoculated with H_2 oxidizing bacterial strains.

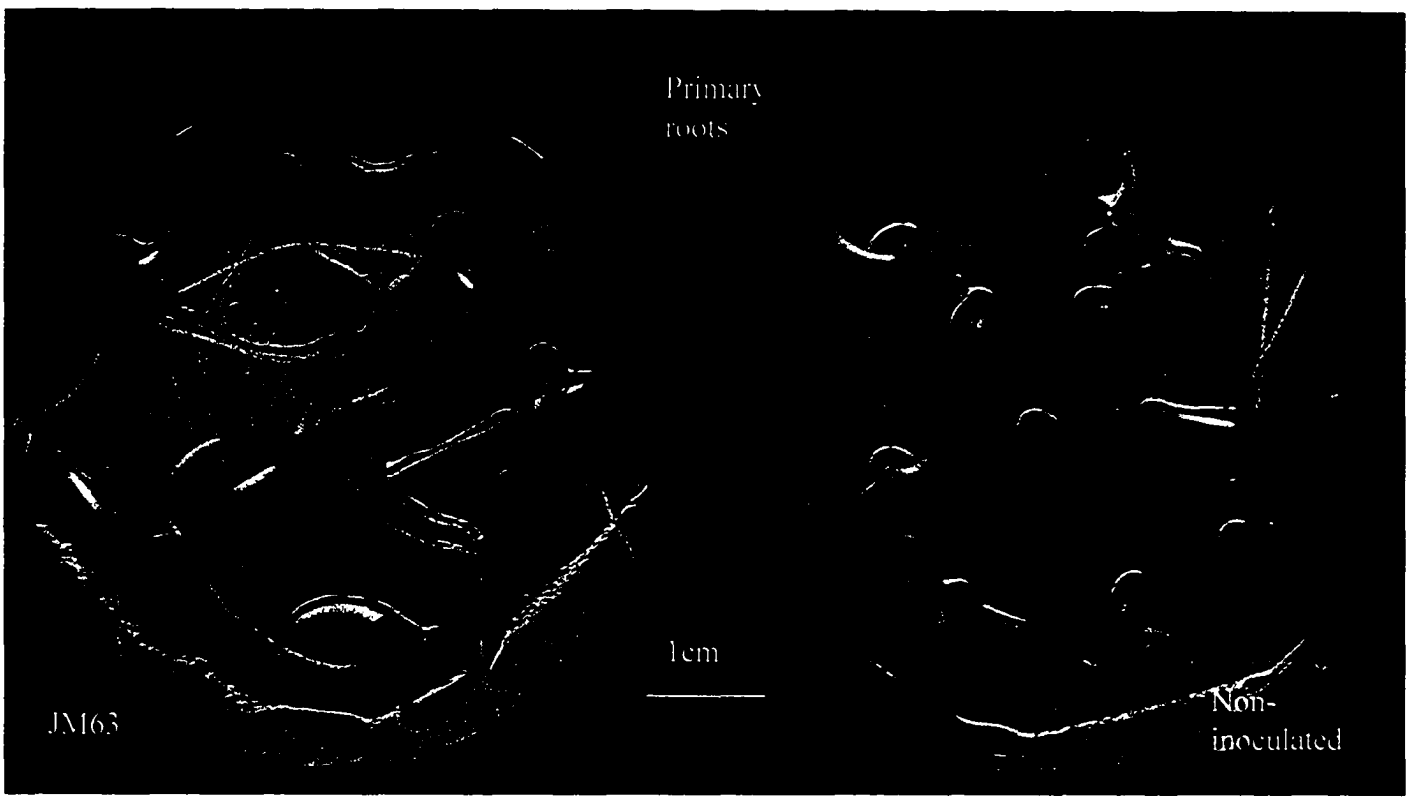
A. Isolates JM54, JM55, JM71, JM81, JM84, JM85, JM87, JM110, JM111, JM113, JM120, JM121, JM122 and JM123. B. Isolates JM01, JM155, JM162a and JM169. C. JM63.Vertical lines represent standard error. Asterisks indicate a statistically significant difference between inoculated samples and controls (*E. coli* B and non-inoculated), according to Fisher's LSD test ($p<0.05$).


Primary root length (mm)


Primary root length (mm)
Non
E. coli


Primary root length (mm)
Non
E. coli
JM54
JM55
JM71
JM81
JM84
JM85
JM87
JM110
JM111
JM113
JM120
JM121
JM122
JM123

Figure 10: Root elongation effect of H_2 oxidizing bacterial strain JM63 of *Variovorax paradoxus* sp. on spring wheat seedlings.


Primary
roots
1cm
JM163
Non-
inoculated

4.5 DISCUSSIONS

Previous studies have shown that the root dry weight and tiller number of several crops were increased when planted in H_2 treated soil and soil after the growth of Hup$^-$ soybean (Dong *et al.*, 2003; Dean *et al.*, 2005). It was reported that the increase of plant growth in H_2 treated soil and soil after the growth of Hup$^-$ legume nodules was caused by the soil bacterial activity (Irvine *et al.*, 2004). Correspondingly, the H_2 oxidizing bacterial strains of *Variovarox*, *Flavobacterium* and *Burkholderia* found in H_2 treated soil and soil adjacent to the Hup$^-$ legume nodules significantly increased the primary root length of spring wheat seedlings directly by 57-254% (Table 10) in two days when compared to the controls. This result strongly suggests that the H_2 evolved from Hup$^-$ nodules increases the growth of H_2-oxidizing bacteria, which in turn improves the plant growth acting as plant growth promoting rhizobacteria (Dong and Layzell, 2001; Dong *et al.*, 2003).

Our results show that the isolates of *V. paradoxus* found both in H_2 treated soil and soil adjacent to Hup$^-$ soybean nodules in the field had the highest direct PGPR effect, increasing the primary root length by up to 254%. The intensity of this PGPR effect is much higher than the effect observed with other strains of *V. paradoxus* (Betrand *et al.*, 2001; Belimov *et al.*, 2001). The results suggest that *V. paradoxus* may be the predominant H_2 oxidizing bacteria in the legume rhizosphere and responsible for most parts of plant growth promotion.

Except for one isolate of *Burkholderia*, the other three isolates had significant PGPR effect. Most species of *Burkhoderia* sp. are predominant soil bacteria which have been reported as human pathogens (Holmes *et al.*, 1998). It has been found recently that some species of *Burkholderia* have nitrogen fixation ability (Mellado *et al.*, 2004). Also

some species of *Burkhoderia* have been used in agriculture as plant growth promoting rhizobacteria (Trân Van *et al.*, 2000). For example, the grain yield of rice was increased by 13 to 22% when it was inoculated with *B. vietnamiensis* TVV75 (Trân Van *et al.*, 2000). This is the first report of *Burkholderia* to increase plant growth with the H_2 oxidizing ability.

Our results show that one strain JM162a of *Flavobacterium johnsonae* also had high PGPR effect. Sindhu *et al.* 1997 described that the free-living soil PGPR include *Flavobacterium* bacteria. Although it was reported that *Flavobacterium* are PGPR, there is no other report about what extent of its effect on plant growth promotion. This is the first report of *Flavobacterium* to increase the root elongation of spring wheat seedlings (97-100%).

Although our isolates of H_2 oxidizing bacteria had PRGR effect, we still do not know the exact direct mechanisms of them on plant growth promotion. But the current and previous studies on plant growth conducted in our lab show that H_2 has effect on the increase of root dry weight, the tiller number and the primary root elongation of plants (Dong *et al.*, 2003; Dean *et al.*, 2005). These results suggest that the soil bacterial activity might change the plant-hormonal metabolism to influence the morphological growth pattern of plants. According to Belimove *et al.* (2001), some *V. paradorax* strains contain (ACC) deaminase. *Burkholderia* could produce rhizobitoxine which inhibit ACC synthase activity (Yasuta *et al.*, 1999). It was observed that seven bacterial strains positive for ACC deaminase production promoted canola (*Brassica napus* L.) seedling root elongation under gnotobiotic condition (Glick, 1995). It is known that ACC is the precursor of ethylene, and ACC synthase is the rate limiting enzyme in the biosynthesis

of ethylene in higher plants (Yang and Hoffman, 1984). So, reducing ACC concentration is one of the most effective ways to reduce ethylene production in plant roots. ACC deaminase might stimulate plant growth and root elongation by hydrolyzing ACC from germinated seeds (Glick *et al.*, 1998). For many plant species, ethylene stimulates germination and breaks the dormancy of seeds. However if the ethylene concentration remains high after germination, root elongation (as well as symbiotic N fixation in legume plants) is inhibited (Jackson, 1991).

Ethylene plays an important role in controlling the rhizobial infection of legumes. Nodule formation can be stimulated by the ethylene inhibition (Yuhashi *et al.*, 2000; Ma *et al.*, 2002). The evolutionary driving force to release H_2 gas into soil for rhizobia may just be to lower ethylene concentration that could lead to more nodule formation. For soil H_2 oxidizing bacteria this means possibly more H_2 gas from nodules. So, reducing ACC concentration and consequently reducing ethylene level gives any legume H_2 oxidizing rhizobacteria a competitive advantage.

Besides the production of ACC deaminase and rhizobitoxin, some strains of *Burkholderia* (*Burkholderia vietnamiensis* TVV75) increased the plant growth by inhibiting phytopathogenic fungi and producing a new and efficient siderophore (Trân Van *et al.*, 2000). It was reported that inoculation of chickpea and soybean seed with siderophore-producing bacteria resulted in increased seed germination, growth, and yield of the plants (Kumar and Dube, 1992).

Cattelan *et al.* (1999) reported that the isolates of *Flavobacterium indologenes* GW2103 and LC1118 from the rhizosphere of soybean produced indoleacetic acid (IAA). It is known that IAA is one of the plant phytohormones which can increase the plant

growth by stimulating cell division, cell enlargements and root length (Vessey, 2003). Tien *et al.* (1979) reported that *Azospirillum brasilense* could increase the number of lateral roots and root hairs in pearl millet under gnotobiotic conditions by producing IAA.

Our results show that H_2 oxidizing bacteria from soil adjacent to Hup^- nodules promote root elongation, the number of lateral roots and the amount of root hairs of young spring wheat seedlings. It is very likely that our H_2 oxidizing isolates improve plant growth by producing ACC deaminase, rhizobiotoxin, IAA or siderophores. Further research to test whether our H_2 oxidizing isolates produce any of substances mentioned above needs to continue.

Another interesting activity of these H_2 oxidizing bacteria that has been reported is the ability to degrade N-acyl homoserine-lactone (HSL or NAHL) which is a quorum-sensing signal and a key regulator of the community behavior of many Proteobacteria (Leadbetter and Greenberg, 2000). Some rhizosphere bacteria need coordinated activation of diverse bacterial functions. The HSL allows the induction of the bacterial function(s) in a synchronous way and when the "appropriate" cell density is reached (Leadbetter and Greenberg, 2000). This quorum-sensing regulates many Gram-negative bacteria. Very likely the NHSL degrading activity of H_2 oxidizing bacteria around roots gives these bacteria potential to compete with Gram-negative bacteria in natural ecosystems and attenuate the plant pathogenicity of other soil bacteria as an "antivirulent" agent to counteract the pathogen.

Legume crops have many long lasting effects on soil. One of them is the change of rhizobacterial population structure and an influence on plant growth. It is known that the soil microflora can improve plant growth through providing 'plant-available' nutrients,

increasing the uptake of mineral nutrients and protecting plants against pests and diseases. Our study has investigated the effect of hydrogen released from nodules on the H_2 oxidizing bacterial population in soil and showed that these organisms act as PGPR, possibly regulating plant growth regulator balance to promote plant growth.

5. GENERAL CONCLUSIONS

It has been hypothesized that the H_2 evolved by Hup$^-$ legume nodules influence the populations of soil H_2 oxidizing bacteria, which improve plant growth acting as plant growth promoting rhizobacteria (PGPR). However, the attempts to isolate and characterize the H_2 oxidizing bacteria from legume soils have not been successful. The present study focused on the culture, isolation and characterization of H_2 oxidizing bacteria and their effect on plant growth.

Twenty strains of H_2 oxidizing bacteria were successfully isolated from H_2 treated soil and soil adjacent to Hup$^-$ soybean nodules by utilizing improved methods in this study. According to Dong and Layzell (2001), the H_2 exposure rate of soil within a few cm of N_2-fixing nodules is 30-250 nmoles cm^{-3} h^{-1}. In the natural habitat of H_2 oxidizing bacteria, the H_2 concentration is low. The gas concentration of O_2 and CO_2 around the nodules is close to the atmospheric level and all gases are constant all the time. Based on this, the open gas flow through incubation system, in which the H_2 generated by electrolysis was developed. The success of isolating H_2 oxidizing bacteria on organic free Mineral Salts Agar medium under open gas flow incubation system with H_2 proved that our cultivation methods fit the natural growing habitat of H_2 oxidizing bacteria in legume field. This finding solves the difficulty that has been a problem for many years in isolation of soil H_2 oxidizing bacteria.

The isolates of H_2 oxidizing bacteria have been shown to be responsible for the H_2 uptake activity and the high K_m in H_2 treated soil and soil adjacent to Hup$^-$ soybean nodules. The visible colonies of our isolated H_2 oxidizing bacterial strains on MSA enriched with H_2 are formed in 7-21 days, and the hydrogenase K_m of H_2 oxidizing

bacterial isolates is around 1000ppm. These findings provide evidence that soil H_2 oxidizing bacteria in legume field are slow growing bacteria, and they are responsible for the high soil H_2 uptake activity.

All isolates of H_2 oxidizing bacteria have been identified as three genera and three species, *Variovorax* (*V. paradoxus*), *Flavobacterium* (*F. johnsoniae*) and *Burkholderia* (*B. sordidicola*) using conventional microbiological identification and 16S rRNA gene sequence analysis. The findings suggest that the majority of isolated H_2 oxidizing bacteria in legume field are Gram negative, β-proteobacteria. The data of morphological, cultural and biochemical characteristics of isolated H_2 oxidizing bacteria provide valuable information that is scarce on the ecology and physiology of H_2 oxidizing soil bacteria. Also, obtaining pure culture of H_2 oxidizing bacteria opened avenues for physiological and molecular study of bacteria in plant root interaction.

It has been shown that all isolates of H_2 oxidizing bacteria tested have been shown that H_2 oxidizing bacteria are plant growth promoting rhizobacteria (PGPR). It was found that isolated H_2 oxidizing bacteria significantly increased the root elongation of spring wheat seedlings ranging from 57% to 254%. These findings shown for the first time the hypothesis that H_2 evolved from Hup^- legume nodules influence the population of H_2 oxidizing bacteria, which in turn improve plant growth acting as PGPR. We suggest that our isolates of H_2 oxidizing bacteria (*V. paradoxus*, *F. johnsonae* and *B. sordidicola*) could be responsible for the major benefit of legumes in crop rotation and intercropping. Regarding the exact PGPR mechanisms of these bacteria, we need to conduct further study.

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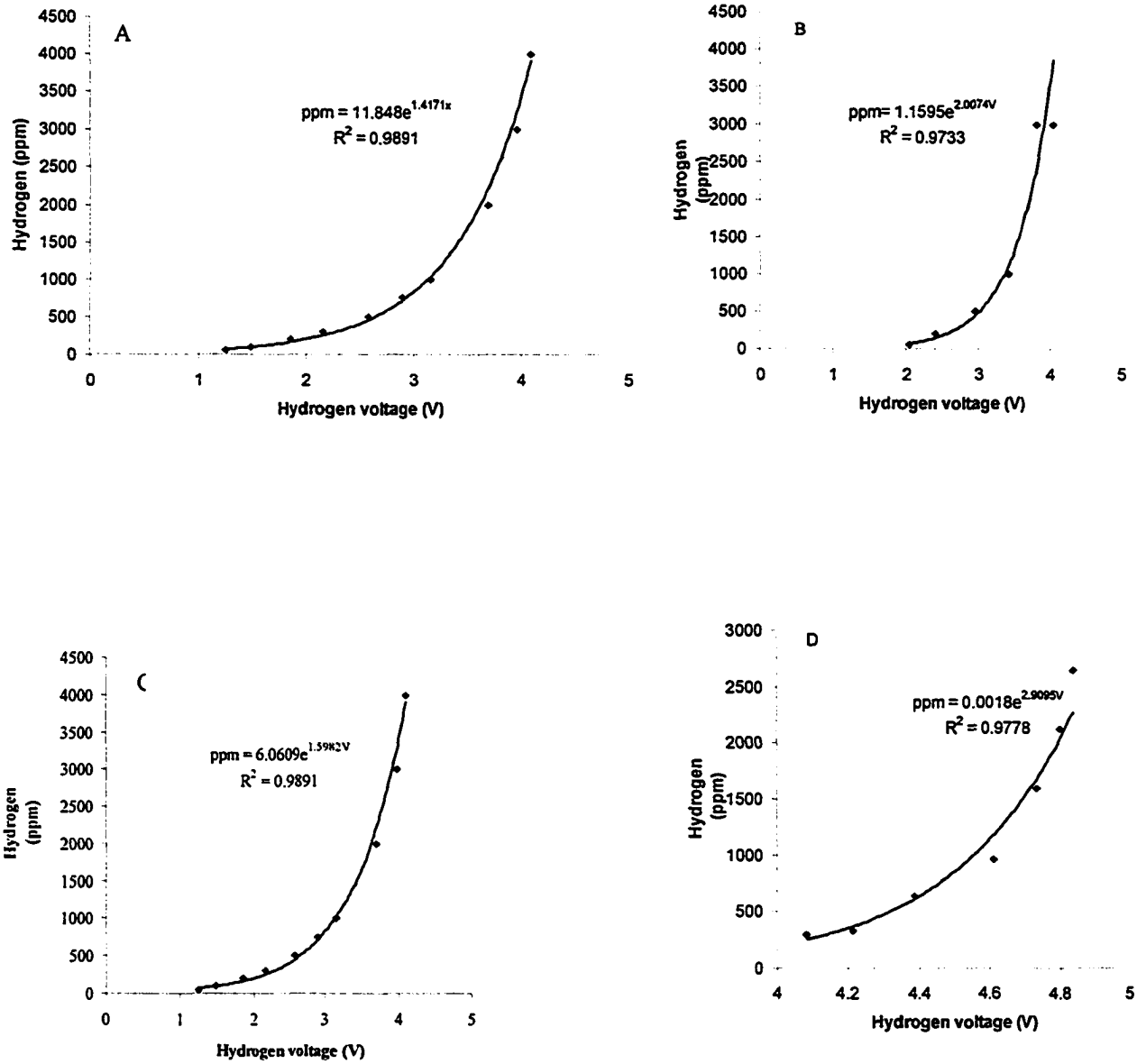
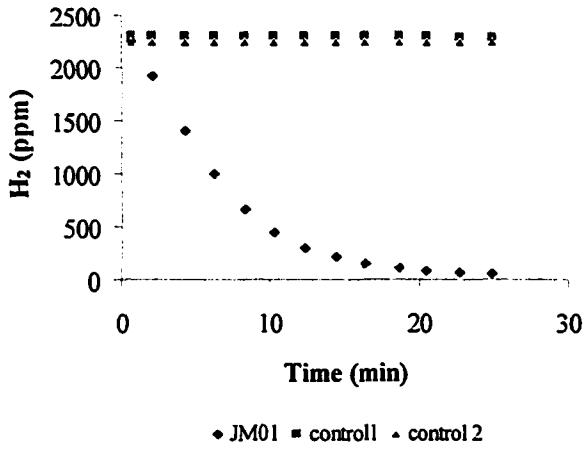
7. APPENDICES

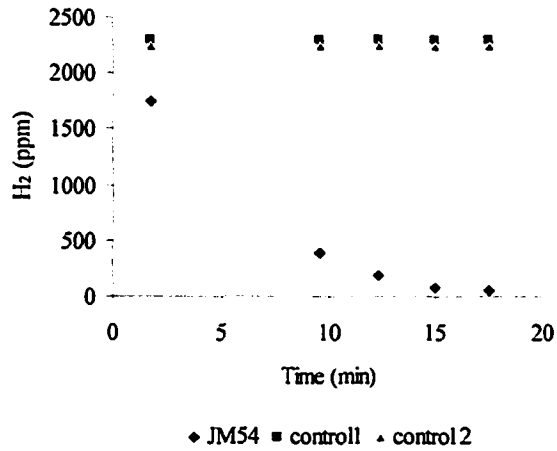
7.1 Appendix 1. Standard curves for testing H_2 uptake activity of isolates

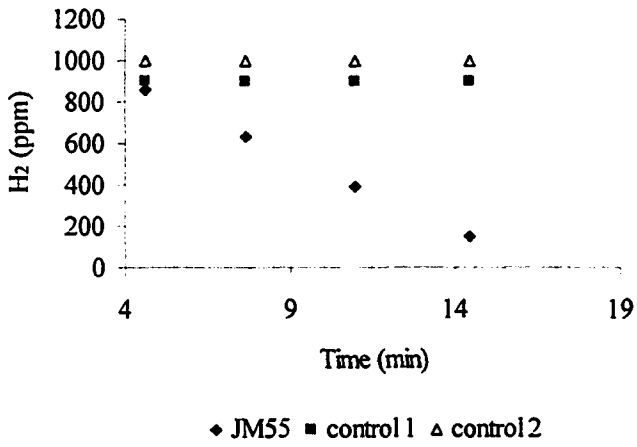


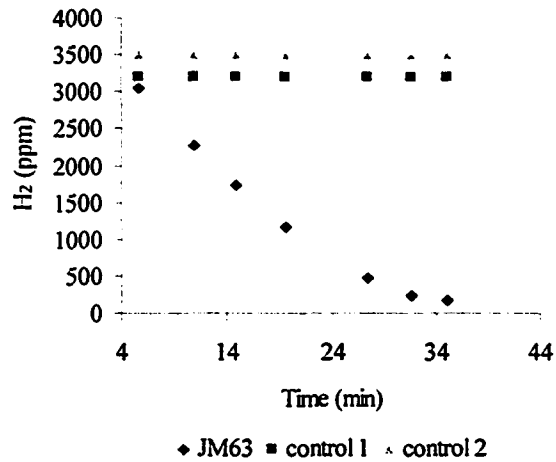
A, B and C were generated in closed system. D was generated in open system. A, used for isolates JM01, JM54, JM55, JM63, JM71, JM81, JM84, JM87. B, used for isolates JM120, JM121, JM122, JM123, JM155, JM162a and JM169. C, used for isolates JM110, JM111 and JM113. D used for isolates JM01, JM55 and JM84.

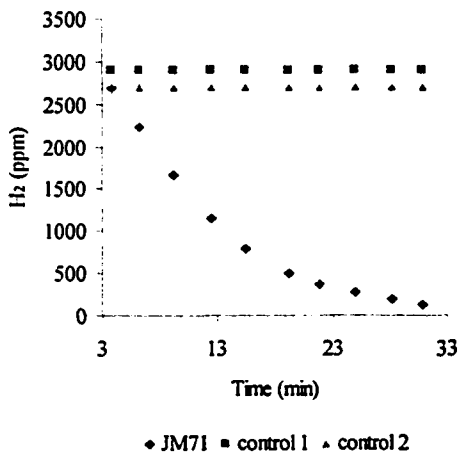
7.2 Appendix 2. Time-course of changes of H_2 concentration (ppm) in test tubes with H_2 oxidizing isolates

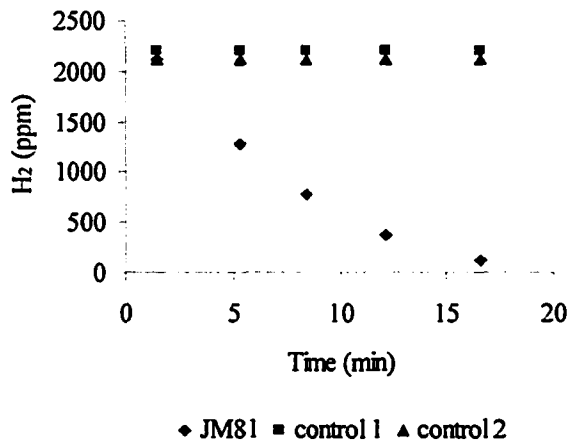


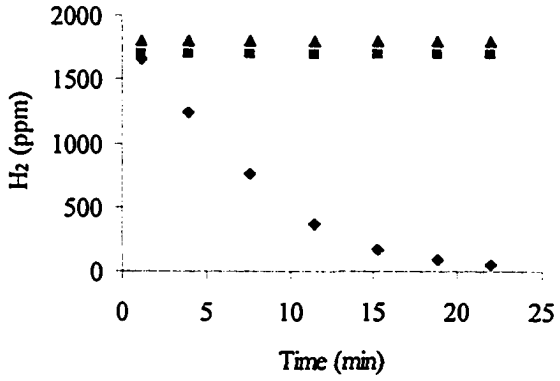




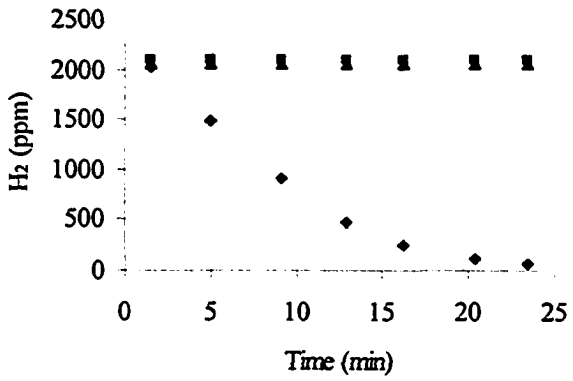




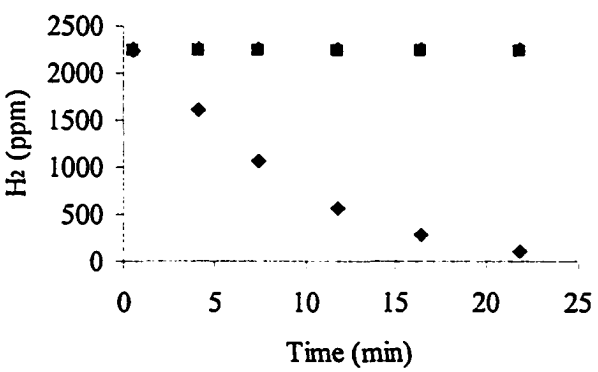




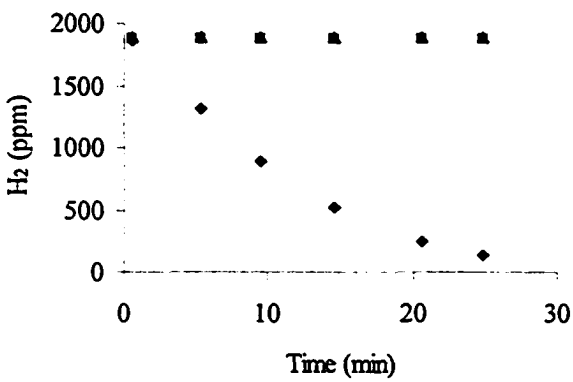
◆ JM84 ■ control 1 ▲ control 2



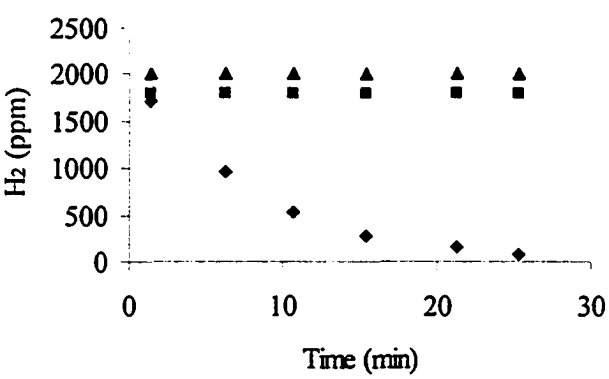
◆ JM85 ■ control 1 ▲ control 2



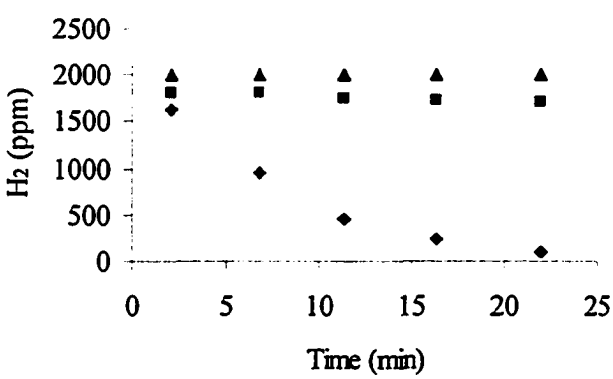
◆ JM87 ■ control 1 ▲ control 2



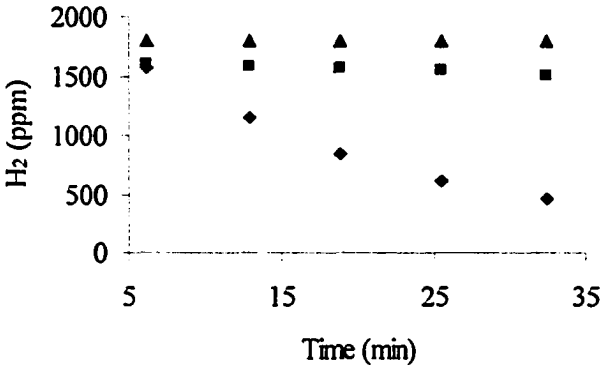
◆ JM110 ■ control 1 ▲ control 2



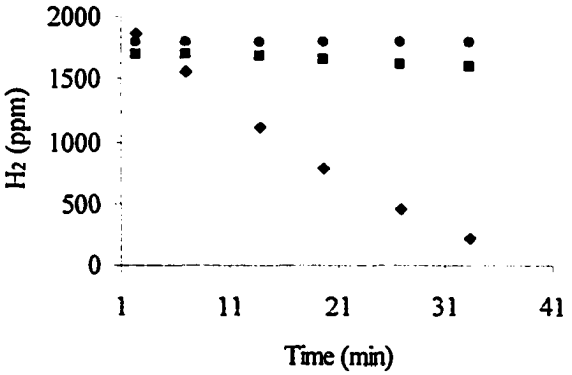
◆ JM111 ■ control 1 ▲ control 2



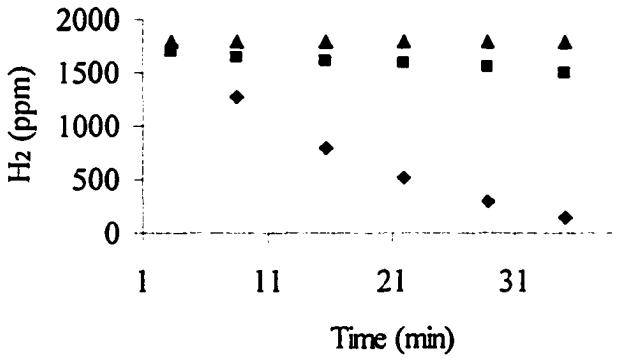
◆ JM113 ■ control 1 ▲ control 2



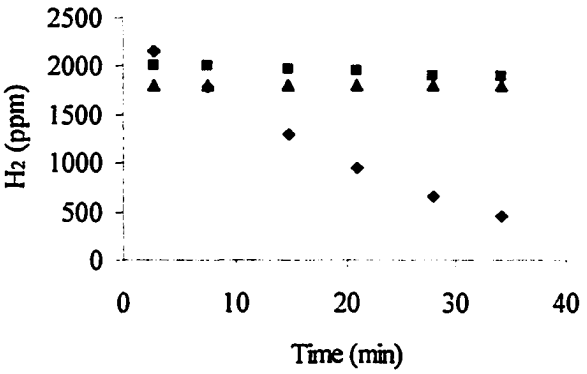
◆ JM120 ■ control 1 ▲ control 2



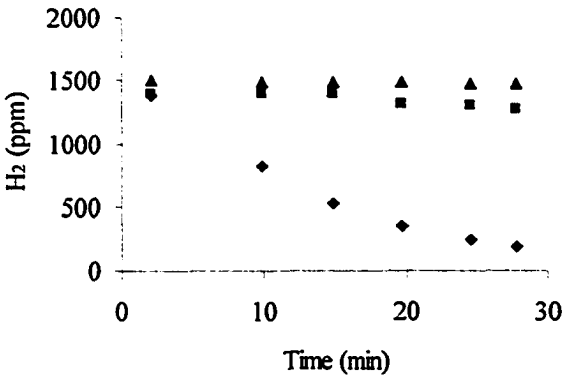
◆ JM121 ■ control 1 ● control 2



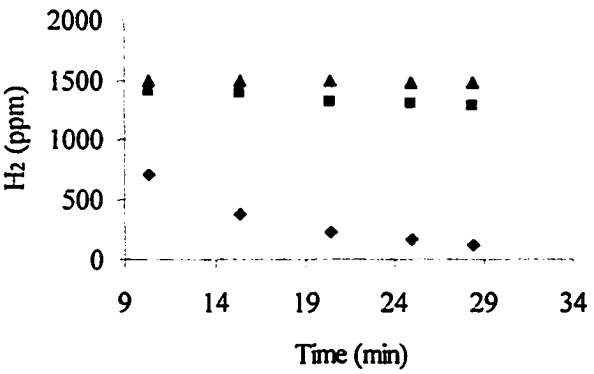
◆ JM122 ■ control 1 ▲ control 2



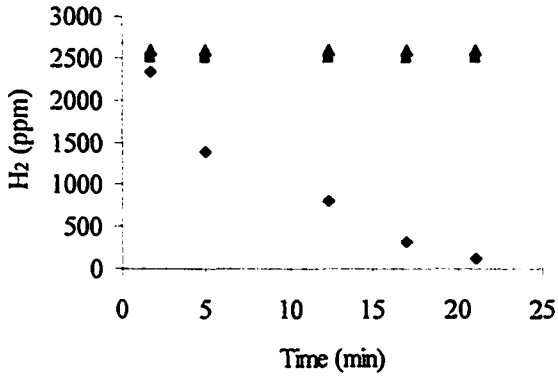
◆ JM123 ■ control 1 ▲ control 2



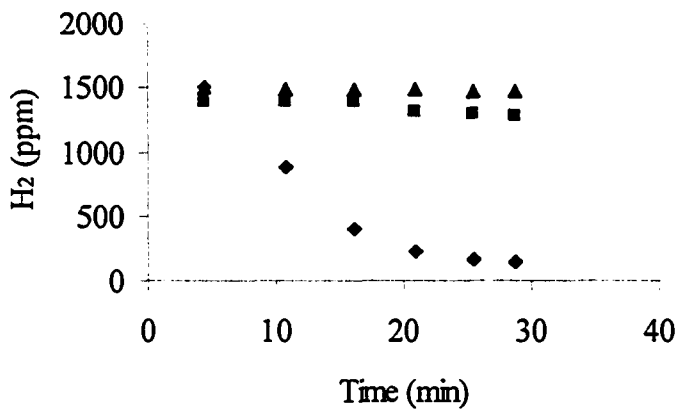
◆ JM155 ■ control 1 ▲ control 2



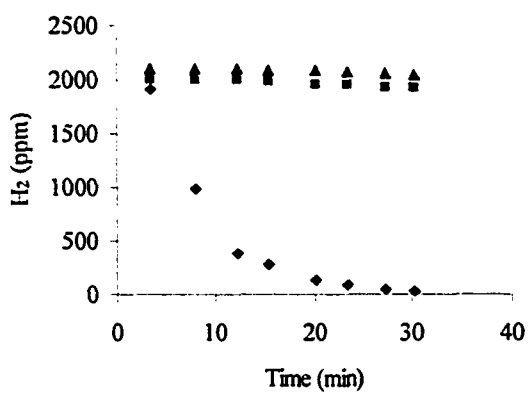
◆ JM162a ■ control 1 ▲ control 2



◆ JM162 ■ control 1 ▲ control 2



◆ JM169 ■ control 1 ▲ control 2



◆ JM117 (white spot) ■ control 1 ▲ control 2

7.3 Appendix 3. 16 S rRNA gene sequences of isolated H_2 oxidizing bacterial strains

JM 63

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AGTCGAACGGCAGCGCGGGAGCAATCCTGGCGGCGAGTGGCGAACGGGTGA
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CGGGTTCTGCCAGAAGTAGTTAGCTTAACCGCAAGGAGGGCGATTACCACGG
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JM01

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JM111

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JM120

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JM162a

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JM169

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