JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2013. Vol. **7**(1), p. 303-309

Isolation and Genera Distribution of Hydrogen-oxidizing Bacteria in Loess Plateau *Hippophae rhamnoides* Rhizosphere

Lin Wang[1], Bo Fu[1, 2], Xin Guo[1], Guilin Cao[1] and Weiwei Wang[1]*

[1] Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, Northwest University, Xi'an, China.
[2] Enzyme Engineering Institute, Shaanxi Academy of Sciences, Xi'an, China.

(Received: 20 January 2013; accepted: 02 March 2013)

Rhizosphere of *Hippophae rhamnoides* was designated as research material. 39 bacterial strains were isolated by mineral salt agar (MSA) medium with a continuous H_2 supplying gas-cycling incubating system. Hydrogenase-positive strains were screened by both 2,3,5-triphenyltetrazolium chloride (TTC) and H_2 oxidizing capability tests. 6 strains with positive TTC result and strong H_2 oxidizing capability were determined to be hydrogen-oxidizing bacteria. The 6 strains were classified into genera of *Bacillus* sp, *Aeromonas* sp, *Pseudomonas* sp and *Micrococcus* sp by their cultural, morphology, physiological and biochemical characteristics. 16S rDNA sequence of strain FS2 has a homology of 99% with *Bacillus* sp and they are in the same branch of phylogenetic tree, indicating strain FS2 belongs to *Bacillus* sp.

Key words: *Hippophae rhamnoides*, Symbiotic nitrogen-fixing, Hydrogen-oxidizing bacteria.

Hippophae rhamnoides, a plant belongs to *Elaeagnaceae Hippophae*, is mainly *Hippophae rhamnoides L.subsp.sinensis Rousi* in Loess Plateau (Lian *et al.*, 1997; Yang *et al.*, 2008). It's also an actinorhizal plant that symbioses with *Frankia* and forms actinorhizas for nitrogen-fixing (Torrey, 1987; Zhang *et al.*, 1995; Qu *et al.*, 1998; Liu *et al.*, 2010) and an important species for reclamation of the three northern regions (northwest, north and northeast) of China (Wei *et al.*, 2002; Zhang, 2007; Yang *et al.*, 2008). Hydrogen-oxidizing bacteria have been demonstrated to be a special taxon of plant growth promoting rhizobacteria (PGPR) (Hu *et al.*, 2004; Chen *et al.*, 2008; Fu *et al.*, 2009). The growth of hydrogen-oxidizing bacteria can be promoted by H_2 released in the process of nitrogen-fixation by rhizobium without hydrogenase (Dong *et al.*, 2001; Irvine *et al.*, 2004). The existence and activity of hydrogenase in *Frankia* is evolutionarily different (Mohapatra *et al.*, 2004; Lin *et al.*, 2006), which may cause H_2 content in *Hippophae rhamnoides* rhizosphere soil varied by *Frankia* species. Thus, the growth of hydrogen-oxidizing bacteria may be affected and the growth of *Hippophae rhamnoides* be affected consequently.

To our knowledge, hydrogen-oxidizing bacteria in legume rhizosphere together with their genera distribution and plant promoting effect have been widely discussed (Dong *et al.*, 2001; Madigan *et al.*, 2001; Sangok *et al.*, 2001; Chen *et al.*, 2007; Fu *et al.*, 2009). *Frankia-Hippophae* symbiotic nitrogen-fixing system has also been deeply studied (Benson *et al.*, 1993; Gong *et al.*, 1997). However, reports on hydrogen-oxidizing bacteria in *Hippophae rhamnoides* rhizosphere are few, not to mention the growth promoting effect on *Hippophae*. Thus, in this study we adopted a

* To whom all correspondence should be addressed. Tel.: + 86 29 88302411; Fax: +86 29 88303572; E-mail: wwwang@nwu.edu.cn

gas-cycling incubating system for screening and isolating hydrogen-oxidizing bacteria from *Hippophae rhamnoides* rhizosphere and made a preliminary identification and genera distribution according to their morphological, physiological and biochemical characteristics in hope of providing a basis for analyzing the hydrogen-oxidizing bacterial population in *Hippophae rhamnoides* rhizosphere. Besides, we made a phylogenetic analysis of a strain with strong H_2 oxidization ability in hope of optimizing its incubation conditions, making it into biofertilizer and assessing its specific growth promoting effect on *Hippophae rhamnoides* in further study.

MATERIALS AND METHODS

Sample soil

Vigorous *Hippophae rhamnoides* rhizosphere soil with root nodules was collected at Yongshou (Shaanxi, China).

Enrichment, isolation and purification of hydrogen-oxidizing bacteria

Enrichment of sample soils was applied in a gas-cycling incubating system at room temperature for a month (Chen *et al.*, 2007; Chen *et al.*, 2008; Fu *et al.*, 2009). Gas mixture used in the system was formed by mixing H_2 generated by water electrolysis with fresh air. To isolate and purify hydrogen-oxidizing bacteria, MSA (Chen *et al.*, 2007) plates were coated with gradient dilutions of enriched soil and put in the system. Single colonies were picked followed by streaking on fresh MSA plates which were incubated in the system later. Purified strains were judged by morphology characteristics and microscopy and were inoculated on MSA test-tube slants for preservation and later use. Another copy of sample soils without enrichment was also used for hydrogen-oxidizing bacteria isolation and purification by the same procedures above.

Screening of hydrogenase-positive strain

TTC test (Hans *et al.*, 1995; Dong *et al.*, 2001; Welbaum *et al.*, 2004) was widely used for hydrogenase-positive bacteria screening. In this study, TTC test was slightly modified for purpose of its availability in the gas-cycling incubating system. A sterile nylon membrane point-planted with strains was placed on MSA plates which were later put in the incubating system until colonies grew. The nylon membrane was then placed on a filter paper presoaked with 0.1% TTC solution and was incubated in darkness with air ventilated for 10 min and then incubated in darkness with mixed gas (H_2/O_2 = 9/1) ventilated for 15 min. Hydrogenase-positive colonies were those of no color change in air ventilated and red in mixed gas ventilated.

H_2 oxidizing capability test was based on H_2 concentration decrement assessment by gas chromatography (GC) (Cunningham *et al.*, 1986). Purified strains were inoculated into MSA test-tube slants and incubated in the gas-cycling system. A MSA test-tube slant without inoculant was used as negative control. The test-tubes were sealed with rubber stopper when colonies grew and were injected with 1 mL of pure H_2 by syringe. Initial H_2 concentration was assessed by GC (SP-6890, Lunan Ruihong chemical equipment Co., Ltd., China) when the gases were mixed thoroughly and final H_2 concentration was detected under the same GC conditions (Column, detector and gasification temperatures were 80, 100 and 120 ! respectively; Carrier gas was argon with a flow rate of 40 mL/min) after 3 days' incubation.

Identification of hydrogen-oxidizing bacteria

Conventional physiological and biochemical tests were applied according to "Microbiology Experiment" (Shen *et al.*, 2003). Bacteria strains were classified by comparing their cultural, morphology, physiological and biochemical characteristics with "Common Bacteria Identification Manual" (Dong and Cai, 2001) and "Bergey's Manual of Determinative Bacteriology" (Buchanan *et al.*, 1984).

16S rDNA sequencing and phylogenetic tree construction

A hydrogenase-positive strain with strong H_2 oxidizing ability was designated for phylogenetic analysis. 16S rDNA sequencing was applied by TaKaRa Biotechnology (Dalian) Co., Ltd. 16S rDNA sequence of the strain was uploaded to GenBank and its homology was assessed by BLAST. Phylogenetic tree was constructed by neighbor-joining method with MEGA 3.1 and DNASTAR (Fu *et al.*, 2009; Pan *et al.*, 2009).

RESULTS

Enrichment, isolation and purification of hydrogen-oxidizing bacteria

Single colonies were clearly visible after 1-2 weeks' cultivation on MSA plates in the incubating system regardless of sample soils being enriched or not. Totally 39 purified strains were obtained, among which 18 (WSY1-WSY9 and WS1-WS9) and 21 (FSY1-FSY14 and FS1-FS7) strains were purified from non-enriched and enriched sample soils respectively.

Screening of hydrogenase-positive strain

Totally 18 strains were preliminarily identified to be hydrogenase-positive through TTC test. They were WSY2, WSY3, WSY5, WS4, WS6, WS9, FSY3, FSY4, FSY5, FSY7, FSY8, FSY9, FSY11, FSY14, FSY15, FS1, FS2 and FS6.

H2 oxidizing capability test results showed that H_2 uptake amount of FS20WS40FSY140FSY7 and WS6 were above 10 μmol/L and that of FSY50FSY110W SY50F SY150FSY90FSY3 and FSY8 were between 5 μmol/L and 10 μmol/L and that of FSY40FS 10WS90FS 60WSY3 and WSY2 were between 1 μmol/L and 5 μmol/L (Table 1).

Morphological characteristics of hydrogen-oxidizing bacteria strains

6 hydrogen-oxidizing bacteria strains (FS2, WS4, FSY14, FSY7, WS6 and FSY5) with strong H_2 oxidizing ability (H_2 concentration decrement > 9 μmol/L) were cultured on MSA plates in the incubating system for 7 days before clearly visible round single colonies with diameters of 1-2 mm formed. Gram stain, spore stain and cell

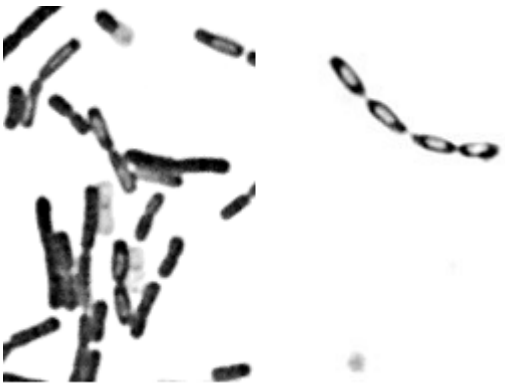

Fig. 1. Microscopy of strain FS2 (left) and WS6 (right) after spore stain (1000×)

Table 1. H_2 oxidizing capability of TTC-positive strains

Strain	Initial H_2 concentration (μmol/L)	Final H_2 concentration (μmol/L)	H_2 concentration decrement (μmol/L)
FS2	46.21	10.78	35.43
WS4	23.01	1.42	21.59
FSY14	24.44	9.98	14.46
FSY7	19.87	5.82	14.06
WS6	16.77	3.44	13.33
FSY5	17.96	8.19	9.77
FSY11	15.56	8.19	7.37
WSY5	19.53	12.31	7.22
FSY15	16.32	9.77	6.55
FSY9	19.00	13.27	5.72
FSY3	15.99	10.66	5.33
FSY8	15.01	10.01	5.00
FSY4	14.55	9.70	4.85
FS1	16.39	12.33	4.06
WS9	17.02	14.57	2.45
FS6	18.40	16.28	2.12
WSY3	17.25	15.47	1.79
WSY2	19.72	18.08	1.65
Control	19.82	19.21	0.61

Table 2. Morphological characteristics of strains with strong H_2 oxidizing ability

Strain	Gram stain	Spore	Cell shape	Cell size	Colony characteristic
FS2	-	+	rod	0.3-1 μm × 0.7-5 μm	large, round, flat, ivory, ragged edge
WS4	-	-	near-cocci	0.5-1.2 μm	large, round, yellow, smooth edge
FSY14	-	-	rod	0.5-1 μm × 2.5-5 μm	small, round, white, semitransparent, ragged edge
FSY7	+	-	cocci	0.5-2 μm	small, round, light yellow, smooth edge
WS6	-	+	rod	0.5-1 μm × 1.5-3 μm	small, round, white, ragged edge
FSY5	+	-	cocci	0.5-1 μm	small, round, yellow, smooth edge

size assessment were applied by routine procedures. Individual and group morphological characteristics of these strains were shown in Table 2. Oval-shaped spores were observed at or near the end of FS2 and WS6 cells after spore stain (Fig. 1).

Physiological and biochemical characteristics of hydrogen-oxidizing bacteria

Physiological and biochemical characteristics of the above 6 strains were assessed according to "Common bacteria identification manual". The results (Table 3) showed that all 6 strains were catalase-positive but test results of cellulose decomposition, Voges-Proskauer (VP), ammonia production and phenylalanine deaminase were all negative. Test results of glucose oxidation/ fermentation, methyl red (MR), starch hydrolysis, gelatin liquefaction, nitrate reduction, indole

Table 3. Physiological and biochemical characteristics of hydrogen-oxidizing bacteria

Characteristics		FS2	WS4	WS6	FSY14	FSY7	FSY5
Catalase		+	+	+	+	+	+
Oxidation/fermentation of glucose		-	-	+	+	+	+
MR		+	-	-	-	-	-
VP		-	-	-	-	-	-
V.P cultures' pH < 6		+	-	-	-	-	-
V.P cultures' pH > 7		-	-	-	-	-	-
Cellulose decomposition		-	-	-	-	-	-
Starch hydrolysis		+	+	-	+	+	+
Gelatin liquefaction		-	-	-	+	-	-
Nitrate reduction		+	+	-	+	-	-
Ammonia production		-	-	-	-	-	-
Indole production		-	+	-	-	-	-
Phenylalanine deaminase		-	-	-	-	-	-
Citrate utilization		+	-	-	-	+	+
Growth with NaCl concentration	2%	+	+	+	+	+	+
	5%	+	+	+	+	+	+
	7%	+	+	+	+	+	+
	10%	+	+	-	+	-	+
Growth pH	5.7	-	+	-	+	+	+
	6.8	+	+	+	+	+	+
	8	+	+	+	+	+	+
	9	+	+	+	+	+	+
	10	+	+	+	+	+	+

"+" and "-" in row of "Oxidation/fermentation of glucose" represent fermentative and oxidative acid production respectively while in the rest rows represent positive and negative result respectively.

production and citrate utilization were varied by strains as shown in Table 3. Salt tolerance and alkaline tolerance of the strains was also assessed with results showed in Table 3, indicating all 6 strains could tolerant salt and alkaline in a certain extent.

Genera distribution of hydrogen-oxidizing bacteria

The above 6 strains were preliminarily classified into 4 genera based on their morphological features and physiological and biochemical characteristics as specified in Table 4.

Phylogenetic analysis

With the help of TaKaRa, a 1465 bp fragment of 16S rDNA sequences of strain FS2 was obtained. The GenBank accession number was GU084156. Through BLAST, the sequence had a homology over 99% with *Bacillus* sp. It could be seen from Figure 2 that strain FS2 and *Bacillus* sp. were in the same branch. Associated with the morphological features and physiological and biochemical characteristics, strain FS2 should be *Bacillus* sp.

Table 4. Distribution of hydrogen-oxidizing bacteria

Strain	Genus
FS2, WS6	*Bacillus* sp.
WS4	*Aeromonas* sp.
FSY14	*Pseudomonas* sp.
FSY7, FSY5	*Micrococcus* sp.

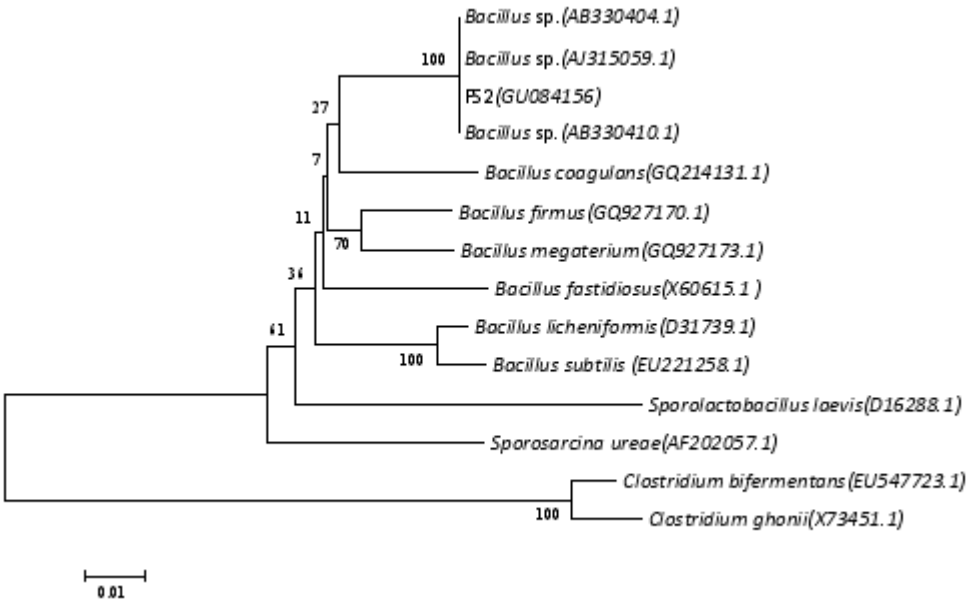

Fig. 2. Phylogenic tree based on 16S rDNA sequences of strain FS2. Numbers at each branch points indicate the percentage supported by bootstrap based on 1000 resampled data sets. The scale bar corresponds to 0.01substitutions per nucleotide position

DISCUSSIONS

Hydrogen-oxidizing bacteria as a member of PGPR have attracted attentions of scholars worldwide as their plant growth promoting effect. The plant promoting effect may lie in the fact that the synthesis of ethylene is regulated by ACC-deaminase produced by those rhizobacteria (Liu *et al.*, 2006; Chen *et al.*, 2008; Fu *et al.*, 2009). To our knowledge, over 20 genera of rhizobacteria including *Pseudomonas* sp., *Bacillus* sp., *Agrobacterium* sp, *Erwinia* sp, *Flavobacterium* sp, *Pasteuria* sp and *Serratia* sp etc are found to be potential in plant disease prevention and crop yield promotion (Burdman *et al.*, 2000). However, to demonstrate definite mechanism of growth promoting effect and make practical applications available, large scale of basic studies still have to

be done. Thus, it's in great need to develop a convenient and reliable way for screening and isolating hydrogen-oxidizing bacteria from various environments with diverse bacterial populations. Hydrogen-oxidizing bacteria could oxidize H_2 into water to obtain energy with the help of hydrogenase and assimilate CO_2 to synthesize cellular material for autotrophic growth. The utilization of H_2 by hydrogen-oxidizing bacteria lies in the fact that the cells contain hydrogenase or hydrogenase genes which could be expressed on certain conditions (Friedrich *et al.*, 1993). Based the facts above, the following four methods have been developed for screening of hydrogen-oxidizing bacteria: H_2 oxidizing capability assessment by GC, autotrophic growth test with H_2 as sole energy source, DNA-DNA hybridization and TTC test. Among the four methods, the first one is more reliable with respect to the rest as they may either leave out positive strains or provide false-positive result (Klüber *et al.*, 1995). Although TTC test may provide false-positive result, it's more cost-effective and less cumbersome. Consequently, in this study, both TTC test and H_2 oxidizing capability assessment by GC were adopted for screening of hydrogen-oxidizing bacteria as for the efficiency of the former and the preciseness of the latter. That is to say, H_2 oxidizing capability assessment carried out after TTC test can authenticate the results of TTC test. The significant difference of H_2 concentration decrement between experimental and control group in Table 1 just corroborated the positive results of TTC test. It gives us a hint that it would be a good choice to adopt TTC test complemented with H_2 oxidizing test for hydrogen-oxidizing bacteria screening in large scales in future.

In this study, 6 hydrogen-oxidizing bacteria strains were isolated from Hippophae rhamnoides rhizosphere in Loess Plateau and were classified into genera of *Bacillus* sp, *Aeromonas* sp, *Pseudomonas* sp and *Micrococcus* sp. As far as we know, hydrogen-oxidizing bacteria have been formerly identified to be genera of *Hydrogenophaga* sp, *Paracoccus* sp, *Xanthobacter* sp, *Mycobacterium* sp, *Alcaligenes* sp, *Nocardia* sp, *Corynebacter* sp, *Spirilla* sp and *Bacillus* sp Etc(Madigan *et al.*, 2001; Chen *et al.*, 2007; Fu *et al.*, 2009). It turns out that *Bacillus* sp and *Pseudomonas* sp are commonplace while *Aeromonas* sp and *Micrococcus* sp are novel, indicating hydrogen-oxidizing bacteria is a huge physiological group widely distributes in nature and relative research is still in its infancy.

Strain FS2 with the strongest H_2 oxidizing ability in the isolates was selected for phylogenetic analysis as it has great potential in the reclamation of the three northern regions of China. However, it's sad that FS2 was merely identified to the level of genus rather species as all strains in the same cluster of FS2 cited in the database are Bacillus sp.

Our future work would be focused on the cultural conditions optimization of strain FS2, the biofertilizer preparations and the specific growth promoting effect on Hippophae rhamnoides based strains with strong H_2 oxidizing ability.

ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China and the Natural Science Foundation of Shaanxi, China. The 16S rDNA sequencing was undertaken with the help of TaKaRa Biotechnology (Dalian) Co., Ltd. The GC analysis was undertaken with the help of institute of analytical science, northwest university.

REFERENCES

1. Bae S., Kwak K., Kim S.G., Chung S.Y., and Igarashi Y. Isolation and characterization of CO_2-Fixing hydrogen-Oxidizing *Marine Bacteria. J. Biosci. Bioeng*. 2001; **91**: 442-448.
2. Benson D.R. and Silvester W.B., Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbio Mol Biol* R. 1993; **57**: 293-319.
3. Buchanan R.E. and Gibbons N.E., Bergey's manual of determinative bacteriology, 8th Edition, the translation and editing group of Bergey's manual of determinative bacteriology of microbiological iInstitute of Chinese academy of sciences translated edition. Science Press, Beijing, 1984.
4. Burdman S., Jurkevitch E., and Okon Y., Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. Calicut, India: Science Publishers Inc. 2000; 229-250.
5. Chen X.D., Wang W.W., Fu B., Xiao L.M., and Guo Y., Growth promoting effect of hydrogen-oxidizing bacteria in soybean rhizosphere. Acta

Bot. Boreal. -Occident. Sin. 2008; **28**, 136-140.
6. Chen X.D., Wang W.W., Guo L.W., Xiong B.T., and Fu B., Isolation, screening and characterization of hydrogen-oxidizing bacteria in soybean rhizosphere. *Chinese J Appl Ecol.* 2007; **18**: 2069-2074.
7. Cunningham S.D., Kapulnik Y., and Phillips D.A., Distribution of hydrogen-metabolizing bacteria in *Alfalfa* field soil. *Appl Environ Microbiol.* 1986; **52**: 1091-1095.
8. Dong X.Z. and Cai M.Y., Common bacteria identification manual, 1st Edition. Science Press, Beijing, 2001.
9. Dong Z. and Layzell D.B., H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. *Plant Soil*. 2001; **229**: 1-12.
10. Friedrich B. and Schwartz E., Molecular biology of hydrogen utilization in aerobic chemolithotrophs. *Annu Rev Microbiol.* 1993; **47**: 351-383.
11. Fu B., Wang W.W., Tang M., and Chen X.D., Isolation and identification of hydrogen-oxidizing bacteria producing 1-aminocyclopropane-1-carboxylate deaminase and the determination of enzymatic activity. *Acta Microbiologica Sinica.* 2009; **49**: 395-399.
12. Gong M.Q., ChenY.L., and Zhong C.L., Research and application of mycorrhiza. China Forestry Publishing House, Beijing, 1997; pp. 37-41.
13. Hu J.C., Xue D.L., Ma C.X., and Wang S.J., Research advances in plant growth-promoting rhizobacteria and its application prospects. *Chin J Appl Ecol*. 2004; **15**: 1963-1966.
14. Irvine P., Smith M., and Dong Z., Hydrogen fertilizer: bacteria or fungi?. *ISHS Acta Horticulturae.* 2004; **631**: 239-242.
15. Klüber H.D., Lechner S., and Conrad R., Characterization of populations of aerobic hydrogen-oxidizing soil bacteria. *FEMS Microbiol Ecol*. 1995; **16**: 167-176.
16. Lian Y.S., Chen X.L., Yu Z.D., and Ao F., Study on origin of the genus Hippophae. Seabuckthorn. 1997; **10**: 1-7.
17. Lin M.Z., Zhang J.K., Zhang G.W., and Zhang X.M., On *Hippophae* roots and functions b! - symbiotic nitrogen-fixing mechanism, conditions and primary nitrogen metabolism. *HippophaeI.* 2006; **19**: 7-14.
18. Liu W.H., Yan S.Z., Yang Q.Y., and Wen X.J., Screening of ACC deaminase positive bacteria and the impact on the growth of tomato seedlings. *Jiangsu Agri Sci*. 2006; **2**: 80-84.
19. Liu Z.Q., and Zhang L.P., Study on species diversity of nitrogen-fixing actinomycetes in *Hippophae* and *Elaeagnus* root nodules. *J Anhui Agri. Sci.* 2010; **38**, 5533-5534, 5536.
20. Madigan M.T., Martinko J.M., and Parker J., Brock biology of microorganisms, 8th Edition, Yang W.B. translated edition. Science Press, Beijing, 2001; pp. 825-608.
21. Mohapatra A., Leul M., Mattsson U., and Sellstedt A., A hydrogen-evolving enzyme is present in *Frankia* sp. R43. *FEMS Microbiol Lett*. 2004; **236**: 235-240.
22. Pan K.C., Feng X., Cui H.M., and Zhang Y.L., Identification of two species of Bacillus using 16S ribosomal DNA sequence. *Chinese Veterinary Science*. 2009; **39**: 550-554.
23. Qu D.M., Wang S., and Han S.H., Nitrogen fixing root nodules and Its *Frankia* Endophyte of *Hippophae rhamnoides* L.. *Acta Bot. Boreal. - Occident. Sin*. 1998; **18**: 60-65.
24. Shen P., Fan X.R., and Li G.W., Microbiology experiment, 3rd Edition. Higher Education Press, Beijing, 2003.
25. Torrey J.G., Endophyte sporulation in root nodules of actinorhizal plants. *Physiol plantarum*. 1987; **70**: 279-288.
26. Wei Y.K. and Wan Y.K., Pioneer vegetation restoration plant on the Loess Plateau - seabuckthorn. *Plants*. 2002; 24-25.
27. Welbaum G.E., Sturz A.V., Dong Z., and Nowak J., Managing soil microorganisms to improve productivity of agro-ecosystems. *Crit Rev in Plant Sci*. 2004; **23**: 175-193.
28. Yang M.B., Xiong Y.C., Wang H.F., Wu X.H., Xue Z.B., Mou S., Liu Z.C., and Ge J.P., Genetic diversity of *Hippophae rhamnoides* L. sub-populations on Loess Plateau under effects of varying meteorologic conditions. *Chin J Appl Ecol*. 2008; **19**: 1-7.
29. Zhang J.K. and Lin W., Nodulation and nitrogen-fixation by *Hippophae*. *Seabuckthorn*. 1995; **8**: 3-9.
30. Zhang R.J), Pioneer desertification plant species - *Seabuckthorn*. *The J Hebei For Sci Tech*. 2007; **S1**: 74-75.